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Paracetamol Metabolism in Postoperative Patients

Philip Gerard MacHale Murphy

B Pharm, PG Cert Clin Pharm, MRPharmS

A thesis submitted for the award of the degree of Doctor of Philosophy

School of Pharmacy, National University of Ireland, Cork

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Head of School: Professor Catriona O'Driscoll

Supervisors: Prof Julia Kennedy, Dr Stephen Byrne, Ms Geraldine Creaton

For my ancestors who left Ireland in the hope of a better future for their family; that they are pleased by the fact that I came back to better mine.

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DECLARATION

This Thesis is my own work and has not been submitted for another degree, either at University College Cork or elsewhere

Philip Murphy

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ABBREVIATIONS

5HT 5-hydroxytryptamine, also known as serotonin, an excitatory neurotransmitter and potent vasoconstrictor

αGST alpha-GSH-S-transferase. A marker of hepatocellular damage

AA Arachidonic acid, the precursor to all prostaglandins. Released from cellular phospholipids by the enzyme phospholipase A2 which is activated by inflammatory cytokines

AAG α_1-Acid-glycoprotein

ACTH Adrenocorticotropic hormone, also known as corticotrophin

ALT Alanine aminotransferase. An enzyme used as marker of hepatocellular damage

Antipyresis A reduction in body temperature

AP Anterior pituitary

ASA American Society of Anesthesiologists classification system of preoperative physical status. It numbers from 1-6, from healthy to brain dead respectively

AST Aspartate aminotransferase. An enzyme used as marker of hepatocellular damage

ATP Adenosine-5'-triphosphate. The main intra-cellular energy transfer molecule

AUC Area under the concentration/time curve

AVP Arginine vasopressin, also known as antidiuretic hormone

CAR Constitutive androstane receptor. A nuclear receptor involved in the induction of several CYP450 isoforms and other drug metabolising enzymes

$\mathbf{c_{max}}$ Maximum plasma concentration

CNS Central nervous system

COX Cyclooxygenase. An enzyme involved in the inflammatory cascade which converts AA to prostaglandins. Two variants exist; COX-1 responsible for physiologic or 'housekeeping' functions and COX-2 responsible for 'as needed' functions relating to pain and inflammation

COX-2 inhibitors A selective inhibitor of the cyclooxygenase 2 enzyme

CRH Corticotrophin-releasing hormone

CRP C-reactive protein

CYP450 The cytochrome P450 superfamily of mixed function oxidases. They are classified further by a number, letter and a further number *e.g.* 1A2

EIC Extracted ion chromatogram

EM Extensive metaboliser. An individual having one or two functional alleles for metabolism

EMEA European Medicines Agency

ESI Electron spray ionisation

FDA United States of America's Food and Drug Administration

G1P Glucose-1-phosphate

G6P Glucose-6-phosphate

GABA Gamma-aminobutyric acid, an inhibitory neurotransmitter

GCS γ-Glutamylcysteine synthetase. Synthesises GSH from cysteine.

GP General Practitioner

GSH Glutathione

GST Glutathione S-transferase, the Phase II enzyme involved in glutathione conjugation

HPA Hypothalamic-pituitary adrenal axis

IASP International Association for the Study of Pain

IL Interleukin. Inflammatory cytokines further classified by a number *e.g.* IL-1

IM Intermediate metaboliser. An individual who either possesses a non-functioning allele or alleles with reduced activity

IMB Irish Medicines Board

INR International normalised ratio. A number used to assess the clotting ability of blood

IV Intravenous

LCMS Liquid chromatogram with mass spectrometer

LPS Lipopolysaccharide. An endotoxin produced from gram negative bacterial cell membrane. When administered it elicits a strong immune response and acts as a global inflammatory stimulus

m/z Mass to charge ratio. A dimensionless value obtained by dividing the mass number of an ion by its charge

M3G Morphine-3-glucuronide

M6G Morphine-6-glucuronide

mRNA Messenger ribonucleic acid

MS Mass spectrometry/spectrometer

Multimodal Utilising more than one treatment modality/method

NADPH Nicotinamide adenine dinucleotide phosphate. A reducing agent required for anaerobic reactions such as CYP450 hydroxylation

NAPQI *N*-acetyl-*p*-benzoquinoneimine. The hepatotoxic intermediate product of the Phase I metabolism of paracetamol

NMDA *N*-methyl-D-aspartic acid

NNT Number needed to treat

Nociceptive Painful or injurious stimuli

Nociceptors A peripheral nerve organ or mechanism for the reception and transmission of painful or injurious stimuli

Noxious Physically harmful

NSAIDs Non-steroidal anti-inflammatory drugs

PAHs Polycyclic aromatic hydrocarbons

PAPS 3'-phosphoadenosine-5' phosphosulphate. Co-factor for Phase II sulphonation

PCA Perchloric acid

PG Prostaglandin. One of four families of eicosanoids which are signalling molecules involved in inflammation. They are derived from arachidonic acid by the COX enzyme. The seven relevant to pain and inflammation are named PGD_2-PGI_2

PGE_2 A prostaglandin involved in hyperalgesia, pyrexia and immunomodulation in addition to many cytoprotective functions such as gastric acid and mucus secretion

PGG_2 An intermediary prostaglandin synthesised from arachidonic acid to PGH_2 by the POX active site on the PGHS enzyme

PGH_2 An intermediary product of COX from the breakdown of PGG_2, itself derived from AA. PGD_2, PGE_2, PGF_2 and PGI_2 are all derived from PGH_2

PGHS Prostaglandin H_2 synthase

PGP P-glycoprotein. A membrane associated transport protein involved in the transport of a variety of molecules across extra and intracellular membranes

PM Poor metaboliser. An individual whose genotype lacks any functional allele for metabolism

POX Peroxidase. An active site on the PGH_2 synthase enzyme

PP Posterior pituitary

Prodrug A pharmacologically inactive substance that is modified into an active drug in the body

PVN Paraventricular nucleus

PXR Pregane X receptor. A nuclear receptor involved in the induction of several CYP450 isoforms

Q-TOF Quadrupole time-of-flight

RSD Relative standard deviation

SCr Serum creatinine. A breakdown product of muscle tissues used as a marker of renal function

SULT Sulphotransferase. The enzymes involved in the Phase II sulphonation reaction on xenobiotics

TIC Total ion chromatogram

t_{max} The time maximum plasma concentration is achieved following a dose

TNF-α Tumour necrosis factor-α

TPN Total parenteral nutrition

UDP-GA Uridine diphosphate-glucuronic acid. A co-factor involved in glucuronidation

UGT UDP-glucuronosyltransferase. A superfamily of enzymes involved in glucuronidation as part of Phase II metabolism

ULRR Upper limit of reference range

UM Ultra rapid metaboliser. An individual possessing more than two functional metabolic alleles

WHO World Health Organisation

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ABSTRACT

Introduction

Despite being available for more than 50 years, there is still much to learn about paracetamol. Postoperative analgesic regimens that maintain good pain control while minimising exposure to opiates are beneficial and paracetamol has had a resurgence in this role since an IV formulation came to market. However there is evidence to suggest currently licensed doses are sub-therapeutic, especially when administered orally or rectally. Higher, unlicensed doses are now being advocated but, prior to this study, there was little evidence of their safety in surgical patients. When assessing drug safety in surgical patients a number of surgery and patient related factors influence results, and these must be considered.

Methods

Major and intermediate surgical patients were recruited from two hospitals in Ireland. They were administered IV paracetamol at either 9g or 4g daily doses. In addition they received daily sub therapeutic doses of four other medicines to indicate the activity of their CYP450 enzymes that are involved in paracetamol metabolism. Urine and blood samples were collected to determine paracetamol pharmacokinetics, CYP450 activity, inflammatory cytokine concentration and for evidence of hepatotoxicity.

Results

There were 33 patients that participated in the study. There was no evidence of clinically significant hepatotoxicity occurring in any patient during the study period, but there could have been changes following this time. Paracetamol disposition was shown to change, however half-life remained relatively constant. There were a number of changes to the way paracetamol was metabolised following surgery that maintained this rate of elimination.

Conclusion

Doses of up to 9g per day given to major surgical patients for up to five days postoperatively produced no evidence of hepatotoxicity. Further research is warranted to determine the clinical utility of these higher doses

AIMS AND OBJECTIVES

Aims

The overall aim was to examine the safety of unlicensed doses of paracetamol in major surgical patients.

Objectives

The objectives of the research were to:

1. Measure markers of hepatotoxicity in surgical patients for evidence of change arising from paracetamol administration;
2. Establish IV paracetamol pharmacokinetics and metabolite concentrations from plasma and urine samples to assess changes to disposition; and
3. Measure activity of CYP450 enzymes and inflammatory cytokines to examine for an association with paracetamol disposition

THESIS STRUCTURE

This Thesis examines the safety of high doses of paracetamol in major surgical patients. In doing so, it presents information concerning the changes in drug metabolism that occur around the time of surgery that could be applied to a wide array of drugs. Previous work by Kennedy *et al* (1996) has demonstrated changes to paracetamol's metabolism as a result of surgery. Although the overall clearance of paracetamol, hepatic blood flow and the volume of distribution were all unaltered, there was an important change to the way paracetamol was metabolised postoperatively, with an increase in the toxic metabolites (Kennedy 1996). These toxic metabolites are produced by CYP450 enzymes, which are well known as the source of many drug-drug interactions and inter-subject variability.

Current literature evidences a renewed interest in the role of paracetamol in postoperative analgesia, with some groups advocating higher, unlicensed doses. When reported alteration to metabolism is considered in light of increased paracetamol doses being used in major surgical patients, there is a clear need for further investigation in this area. What cannot be determined from the earlier work, and what is lacking in the literature, is an understanding of why these changes occur. This is a major focus of this project.

The novel component of this project is to simultaneously assess the effect surgery has on paracetamol metabolism and cytochrome P450 activity in order to understand these changes better. Increasing the understanding of the role the cytochrome P450 enzymes play in the changes to paracetamol metabolism is not only vital to this study, it will also provide information relevant to approximately 80% of clinically utilised medicines that share one of the cytochromeP450 enzymes involved in paracetamol's metabolism.

The context of this project is explained by first defining pain and pain mechanisms and the significance of postoperative pain, methods to treat it and paracetamol's place within that treatment (Chapter 1.1). Chapter 1.2 focuses specifically on paracetamol itself, with particular emphasis on its metabolic pathway. Surgery is a time of great physiological changes which would intuitively be expected to impact on drug metabolism, since the liver is a major player in these changes. Chapter 1.3 discusses these changes to drug disposition in the surgical patient during this crucial time. Many other patient factors can also impact on paracetamol metabolism and these are discussed in Chapter 1.4. The final two sections concern the Phase I enzyme system which is responsible for the production

of paracetamol's toxic metabolite. Chapter 1.5 reviews the enzymes of Phase I metabolism and Chapter 1.6 covers the means of assessing it.

The research is then presented and the paracetamol study is then explained.

Pivotal to the interpretation of the changes to metabolism occurring to patients, was the ability to measure the drugs which were being used either therapeutically or as markers of enzyme activity. As such, this project involved considerable assay development for both paracetamol and its metabolites in plasma and urine and the various parent drugs and their metabolites in plasma and urine. Whilst several separate assays could have been developed, substantial work resulted in the development of one assay with all probes and their metabolites being able to be recovered in one run. Techniques used were HPLC and LCMS.

Physiological responses to surgery were monitored as per usual with the clinical situation, but changes in cytokines were measured using ELISA assays and correlated with alterations in the metabolite patterns.

Clinical pharmacists are seen by many as the "Scientist in the High Street". Pharmacists are trained to pull together all the elements of chemistry, pharmaceutics, pharmacology, biology and physiology to provide individualised patient care. This Thesis represents the development and application of those skills and knowledge to solve a clinical question. It involved the development of a novel assay as an instrument to answer part of this question which spanned the spectrum of chemistry to the patient.

1 INTRODUCTION

1.1 BACKGROUND

1.1.1 DEFINING PAIN

The International Association for the Study of Pain (IASP) defines pain as the "unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage". Pain is therefore not a stimulus, but an individual and subjective perceptual experience (Basbaum *et al.* 2008). This experience is the final product of a complex information-processing network. There are a multitude of genetic, psychological, behavioural and social factors that may increase or decrease an individual's response to, and description of, pain (Schaible *et al.* 2004; Webster 2008). Such factors include: previous pain experiences, cultural background, social supports, the meaning and consequence of the pain (*e.g.* if the pain-causing surgery is curative or palliative), the degree of control felt over the pain and disease, as well as fear, anxiety or depression (Loeser *et al.* 1999). These will all interact to produce what an individual describes as pain. Pain is thus different from nociception, which merely refers to perception of painful stimuli by the brain.

Pain is the most common reason for consultation with a General Practitioner (GP) and is the most frequent symptom in hospitals (Loeser *et al.* 1999). In the acute setting, pain normally has a protective function to encourage healing and prevent on-going tissue damage. It serves as a rapid warning system to the motor neurons to act to minimise detected physical harm and preserve tissue integrity. Those with congenital or acquired insensitivity to pain can suffer many various health problems as a result of impairment to this warning system (Isselbacher 1994). Regardless of its aetiology, health professionals have a duty to help those in pain, not only treating the underlying cause but also using effective strategies to rapidly relieve suffering (Aronoff *et al.* 2005). The IASP and World Health Organisation (WHO) state that "the relief of pain should be a human right" (Schug *et al.* 2005).

Pain can be broadly classified into two main types; acute nociceptive or chronic neuropathic. Each has different clinical features.

1.1.1.1 NOCICEPTIVE PAIN

Nociceptive pain is the most common type of pain observed in acute clinical settings. It usually has a definable cause (Greene *et al.* 1993) and is considered the normal response to a noxious chemical, thermal or mechanical stimuli that arise from surgery, trauma or acute illness (Carr *et al.* 1999; McCaffery *et al.* 1999). Nociceptive pain results from the stimulation of specialised primary sensory nerve fibres (nociceptors) in the skin, periosteum, dental pulp, subcutaneous tissue and joint, visceral and somatic structures. This occurs most often from tissue damage and/or inflammation (Isselbacher 1994; Australian and New Zealand College of Anaesthetists and Faculty of Pain Management 2005).

The stimulation of these nociceptors results in impulses that travel along the peripheral nerve, past the sensory cell bodies in the dorsal root ganglion, along the dorsal roots and into the spinal cord or brain stem (Figure 1.1-1). Within the spinal cord or brain stem, the impulses activate second or third order neurons in the thalamic, limbic and cortical structures in the central nervous system (CNS) which is interpreted as pain by a conscious brain (Basbaum *et al.* 2008).

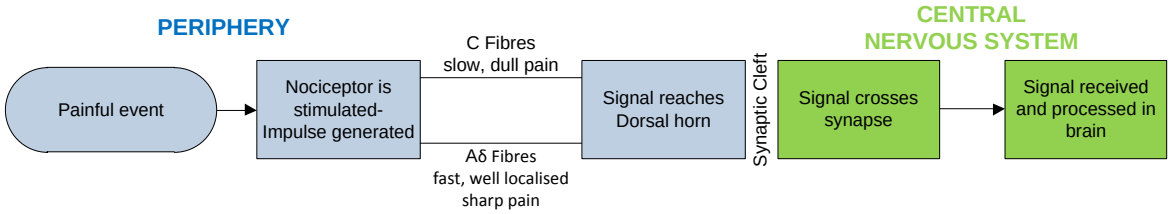


Figure 1.1-1 Pain transmission pathway (Basbaum et al. 2008)

Nociceptors require a high level of stimulation to create an impulse, so that they only transmit stimuli of noxious intensity (Rang *et al.* 2000). Mechanical or thermal receptors transmit much lower intensity stimuli, reflecting the necessity for subtlety in the type of information these receptors convey. Sufficient noxious stimulation causes the membrane of the nociceptor to become permeable to sodium ions, causing depolarisation. As other ions, such as potassium, efflux from the cell, the cell repolarises as it becomes negative again. If sufficient depolarisation and repolarisation occurs, an action potential is created and the stimulus is converted into an impulse, which is transmitted along the nociceptive fibres that extend from the cell body (Bruton *et al.* 2006).

In the absence of any inflammation, acute pain will resolve quickly once the noxious stimulus has been removed. However, in the presence of inflammation, nociceptive

activity changes. Increased levels of prostaglandins (PG) and other inflammatory components, monoamines, cytokines, peptides and other eicosanoids, known collectively as the 'inflammatory soup', bathe the nociceptor (Kasper *et al.* 2008). These inflammatory mediators can promote intense and on-going stimulation of peripheral nociceptors, sustaining the initial response well after the noxious stimulus has been removed. This affects existing nociceptors and also activates dormant ones (Carr *et al.* 1999). Such prolonged stimulation results in the sensitisation of nociceptors. Peripheral and central sensitisation causes the amplification of subsequent pain stimuli (both intensity and area of pain) and a lowered pain threshold. The outcome is that a low level stimulus, such as movement, touch or heat, becomes sufficient to activate nociceptors and result in pain. This is a protective strategy to avoid or minimise further injury and promote healing in the area of the injury (Charlton *et al.* 1999).

Once generated in the periphery, the impulse is transmitted to the dorsal horn of the spinal cord along C or A-δ (delta) fibres. The fibre used for transmission further divides nociceptive pain into two groups, somatic or visceral, each with their own distinguishing features. Visceral pain involves C fibres, which are unmyelinated, small in diameter and slow-conducting, transmit poorly localised, dull, cramping or aching pain, whereas A-δ fibres are partially myelinated, large in diameter and fast-conducting and transmit well-localised, sharp, hot or stinging pain, termed somatic pain (Basbaum *et al.* 2008). C fibres are sensitive to mechanical, thermal and chemical stimuli, whereas A-δ fibres are primarily sensitive to mechanical and thermal stimuli. Visceral pain may also have associated symptoms such as nausea and sweating. Both somatic and visceral pain may be accompanied by other signs of autonomic hyperactivity such as hypertension, tachycardia and pallor (Kasper *et al.* 2008).

At the end of the C or A-δ nociceptive fibre, the impulse travels across the synaptic cleft at the interface (also known as synapse) of the visceral or somatic nociceptors and the dorsal horn, at which site all incoming signals from the periphery are integrated into the CNS (Carr *et al.* 1999). Neurotransmitters, such as substance P, adenosine triphosphate (ATP) and glutamate are released from the presynaptic nerve and diffuse across the synapse, interacting with receptors on the post-synaptic nerve. Similar to impulse generation in the periphery, if enough ion exchange occurs, the impulse is recreated in the post-synaptic nerve. At this stage endogenous opioids act on the opiate receptors on the presynaptic nerve, blocking the release of these neurotransmitters, particularly

substance P, and preventing further transmission of the painful signal (Basbaum *et al.* 2008), thereby dampening the transmission of incoming signals (Carr *et al.* 1999).

Ascending pathways then convey the nociceptive information rostrally to thalamic, limbic and cortical structures responsible for affective and sensory discriminative responses (Carr *et al.* 1999). These cephalad structures are involved in the experience and memory of pain and suffering.

The final process of nociception is the changing or inhibiting of pain impulses known as modulation. Because this involves neurons originating in the brain stem that descend to the dorsal horn of the spinal cord, the pathways of modulation are also known as the descending pain system. By releasing substances such as endogenous opioids (encephalin and endorphins), serotonin (5HT), noradrenalin, γ-aminobutyric acid (GABA) and neurotensin, these descending fibres can inhibit the transmission of noxious stimuli and produce analgesia. This pain modulation is thought to contribute to the wide variation in pain experienced amongst a group of individuals given the same noxious stimuli (Loeser *et al.* 2001).

1.1.1.2 NEUROPATHIC PAIN

Neuropathic pain is associated with injury or disease of the peripheral or central nervous system and can be considered a disease rather than a symptom (Charlton *et al.* 1999). Following such an insult to the nervous system, a number of changes occur, including the development of central sensitisation, the reorganisation of synaptic connections in the spinal cord, and hyper-excitability of damaged peripheral nerves. This can result in normally non-painful stimuli being interpreted as painful, or pain occurring spontaneously without any stimulus. Historically, any type of pain was termed chronic pain if it has lasted longer than six months although the line has become a lot less clear in recent years with increased understanding of pain pathology. As this Thesis is only concerned with acute pain, neuropathic pain will not be discussed further.

1.1.2 BURDEN OF PAIN

It is difficult to determine if pain is a problem for the general population. While accurate statistics from national and international epidemiologic studies are not available, data from a variety of sources suggest that annually 15-20% of the population have acute pain in the United States and many other industrialized nations, and 25-30% have chronic pain.

The appropriate management of pain remains one of the most important pressing issues of society in general, and the scientific community and the health professions in particular. Millions upon millions of people are afflicted with acute and chronic pain each year, and in many patients it is inadequately relieved (Loeser *et al.* 1999). As a result, pain is the most frequent cause of suffering and disability that seriously impairs the quality of life for millions of people throughout the world (Loeser *et al.* 2001).

1.1.3 POSTOPERATIVE PAIN

Acute nociceptive pain is a predictable outcome of surgery due to the tissue damage that is caused (Millen *et al.* 2003). Surgical trauma causes the release of bradykinin, leukotrienes, histamine, substance P and PGs. These inflammatory cytokines lead to sensitisation of nociceptors, transduction from the periphery and the experience of pain (see Section 1.1.1.1).

Maximal postoperative pain occurs for approximately three days following surgery. This time period varies widely, with the site and extent of the surgery being among the best predictors. Pain then decreases as wound healing and overall patient recovery takes place. Epidemiological studies have shown young females are at a higher risk of experiencing severe pain postoperatively than other surgical groups, with other factors, such as level of pre-existing pain, whether the surgery is curative or palliative and previous experiences of postoperative pain, also important contributors, as described in Section 1.1.1.1 (Shipton 2005; Macintyre *et al.* 2007).

The experience of pain itself is a potent stimulus for the autonomic nervous system. Injury causes afferent neural stimuli and activation of the autonomic nervous system, and adds to the stress response of endocrine, metabolic and inflammatory activation contributing to organ dysfunction (Loeser *et al.* 1999; Kehlet *et al.* 2001a). These homeostatic responses occur at the injury site (*e.g.* cytokine release), in the adrenal cortex (release of corticosteroids), in the immune system and in widely distributed areas of the brain (Loeser *et al.* 1999). The stress response is detailed further in Section 1.3.

It is a popular misconception amongst Irish surgical patients that pain after a surgical procedure is indicative of healing (Murphy *et al.* 2007). However, pain is not associated with or required for the healing of surgical wounds. There is evidence that poorly controlled postoperative pain actually impairs wound healing (McGuire *et al.* 2006), with

many of the immune and neuroendocrine functions critical to wound healing being dysregulated by pain.

1.1.3.1 THE IMPORTANCE OF PAIN CONTROL AFTER SURGERY

The need for reduction in pain postoperatively is not only a humanitarian necessity but, as described in the previous section, leads to better outcomes at both system and patient levels.

At the system level, pain can precipitate or increase the duration of hospital stay (Chung 1995), increase the cost of healthcare and reduce patient satisfaction (Sharrock *et al.* 1995; Strassels *et al.* 2002; Shang *et al.* 2003). Prolonged recovery time can also result in delays in return to work (both inside and outside of the home) and the subsequent economic implications that follow these delays (Joshi *et al.* 2005).

At the patient level, inadequate control of postoperative pain causes further preventable morbidity, leading to prolonged recovery time and increased risk of complications (Beauregard *et al.* 1998; Iohom *et al.* 2006). The importance of pain control to surgical patients was shown in a survey of 250 surgical patients across the United States, which found that patients are more concerned about pain after surgery than whether the surgery would actually improve their condition (Apfelbaum *et al.* 2003).

Complications that are thought to increase from inadequately controlled pain include: decreased vital capacity, chest infection, hypoxia, respiratory failure (Ballantyne *et al.* 1998), tachycardia, hypertension, myocardial ischaemia, myocardial infarction, delayed gastric emptying, nausea, vomiting (Anderson *et al.* 1996), neuroendocrine disturbances, metabolic disturbances, thrombus formation, insomnia, anxiety and depression (Apfelbaum *et al.* 2003).

An increasingly prominent postoperative complication, chronic postsurgical pain, can also arise from even brief intervals of acute pain. The experience of pain early on in recovery is thought to shape the way pain evolves at later stage. Changes in gene expression of neurons, the basis of sensitisation, occur within 20 minutes of injury (Crombie *et al.* 1998; Carr *et al.* 1999; Crombie *et al.* 1999; Perkins *et al.* 2000). This can induce long-term neuronal remodelling and sensitisation, leading to chronic postsurgical pain (Australian and New Zealand College of Anaesthetists and Faculty of Pain Management 2005). Some researchers believe acute pain should be viewed as the initiation phase of an extensive,

persistent nociceptive and behavioural cascade triggered by tissue injury, and that adequate analgesia can prevent this cascade (Carr *et al.* 1999).

With the trend toward ambulatory surgery and shorter hospital stays, adequate analgesia and avoidance of the complications listed above are especially important. Patient monitoring that might otherwise identify pain related complications is reduced with shorter inpatient stays (Schug *et al.* 1998).

Since the identification of these long-term negative sequelae arising from poorly controlled postoperative pain, there have been several changes in surgical analgesia. Many of these changes have questioned traditional practices and most of them have involved changes to the use of medicines. There is now an increased focus on the prevention of pain, rather than its treatment because meticulous, perioperative analgesia can lower analgesic requirement and improve functional status months after surgery (Gottschalk *et al.* 1998).

Historically, bolus doses of morphine had been the mainstay of postoperative analgesia. This new philosophy of prevention rather treatment saw the dawn of "multimodal analgesia" in the 1990's (Kehlet *et al.* 1993). This was accompanied by a substantial increase in the prominence of postoperative pain in the research literature (U.S. National Library of Medicine *et al.* 2011)(Figure 1.1-2).

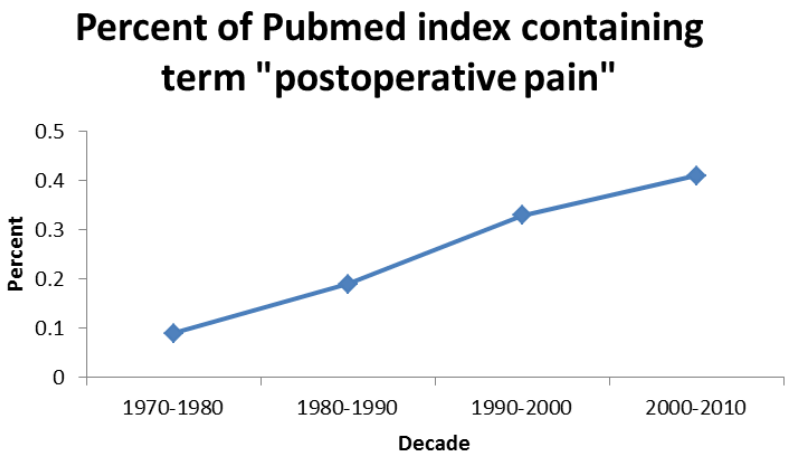


Figure 1.1-2 Per cent of Pubmed titles containing "postoperative pain" (U.S. National Library of Medicine *et al.* 2011)

Despite the evidence for fastidious pain management and analgesic administration, pain following surgery is still reported in the literature as occurring at "an unacceptable frequency and severity" (Rocchi *et al.* 2002; Apfelbaum *et al.* 2003). The designation of pain as the "fifth vital sign" was originally used to increase the visibility of pain assessment in the clinical arena in the early 1990s (American Pain Society Quality of Care

Committee 1995), and while the attention given to pain has increased in recent years, practice patterns are much slower to change (Carr 2002; Dihle *et al.* 2006; Breivik *et al.* 2008; Ene *et al.* 2008).

Patients often report high levels of satisfaction with the effectiveness of their pain medication despite reporting poor satisfaction with overall pain control, indicating patients' low expectations from analgesia or inadequate administration of effective analgesia (Bostrom *et al.* 1997; Rocchi *et al.* 2002).

1.1.3.2 OPTIMISING POSTOPERATIVE PAIN CONTROL

Optimal pain control reduces the intensity of movement-evoked pain and the surgical-stress response, improves postoperative recovery and reduces duration of hospital stay (Hriesik *et al.* 2008).

Research on pain control to discover optimal methods of analgesia is difficult for a number of reasons. Pain research in the clinical setting is complicated by issues of consent, ethics and the subjective nature of pain. Moreover, clinical pain cannot be equated with experimentally induced pain, nor can it be objectively measured. In the clinical setting pain has to be accepted at the level it is reported. Other factors such as anxiety, sleep disruption and illness burden cannot be duplicated in the experimental setting, and conversely results arising from tightly controlled experimental studies may not be achievable or even applicable in a busy ward setting (Carr *et al.* 1999).

Current best practice of postoperative pain management advocates a multimodal approach, targeting different pain pathways with combinations of medication, often in a stepwise approach. The well-known WHO pain ladder (Figure 1.1-3) was designed for management of non-acute pain. The ladder shows recommended progression of analgesics used for treating increasingly severe pain but it can be applied in reverse, in a step-down manner, in the postoperative setting (Shang *et al.* 2003).

The use of multiple analgesics as part of multimodal analgesia acknowledges that there is not one perfect drug for treating all types of postoperative pain and approaches the treatment and prevention of acute pain from several different angles to uncouple tissue injury from the nociceptive and behavioural cascade that normally ensues (Carr *et al.* 1999).

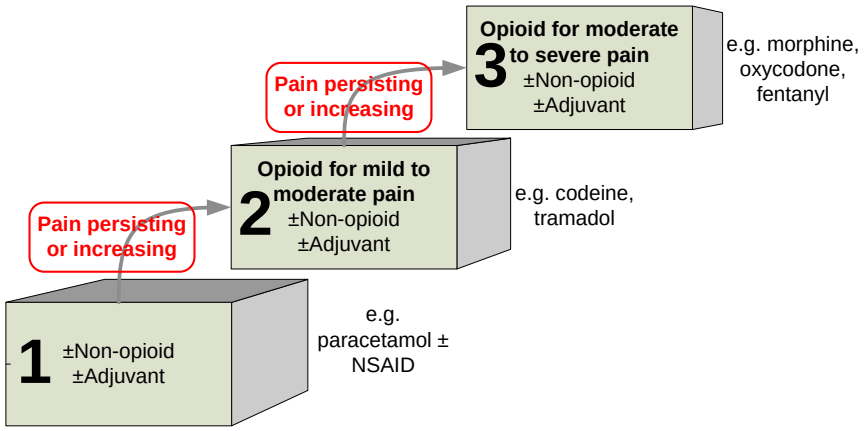


Figure 1.1-3 World Health Organisation analgesic ladder (Crews 2002).

There is good evidence that multimodal approaches not only achieve better analgesia but potentially reduce side effects and analgesic requirements (Park *et al.* 1994; Breivik 2002). By spreading analgesic demand across several different classes, the requirement of more side-effect-prone drugs, such as opiates, can be significantly reduced. Indeed, the avoidance of opiate use has become a key focus of modern analgesia (Cohen 1980; Smith 1989). Analgesics are commonly evaluated in terms of their 'morphine-sparing effect'. This is a measure of the average consumption of morphine of patients who also receive an additional drug under evaluation compared with a similar group of patients receiving morphine alone (Cobby *et al.* 1999). Studies that examine the effect of paracetamol and NSAIDs administration on morphine usage report morphine sparing of $\leq$ 30% (Cobby *et al.* 1999; Fayaz *et al.* 2004; Pettersson *et al.* 2005). This represents a paradigm shift from historic use of one opiate as sole source of postoperative analgesia.

By reducing the quantity of opiate used in exchange for other analgesics, there can be a reduction in nausea, vomiting, respiratory rate depression, constipation, ileus and other side effects associated with opiate use, without any compromise of analgesia.

Improved analgesia can also result from the frequent dosing of different analgesics from standardised prescriptions and protocol (Harmer *et al.* 1998). In multimodal approaches, there is always at least one analgesic in sufficient concentration for therapeutic effect, in contrast to past opiate based practices of waiting for a previous dose to become sub-therapeutic before a subsequent dose could be given, resulting in uncontrolled or "breakthrough" pain.

There are several analgesics used for the treatment of acute pain. Most involve minimising the effects of substances involved in inflammation or transmission of pain signals. For example, a patient who undergoes a laparoscopic bowel resection may

receive paracetamol and parecoxib intravenously, fentanyl and bupivicaine epidurally, fentanyl transdermally and a lidocaine patch applied to the port sites. As the patient improves, oxycodone may be given orally (Harney 2009).

Analgesics currently used in the acute pain setting can be considered in four classes: paracetamol, anti-inflammatories, local anaesthetics and opiates. Each class will be discussed briefly, covering its method of action and role within current pain management strategies. Because of paracetamol's importance to the Thesis, it is mentioned here but covered in greater detail in later sections.

1.1.3.2.1 PARACETAMOL

The WHO has recommended that paracetamol be used as the starting point for all analgesic regimens and it is a major ingredient in numerous prescription analgesics and over-the-counter medications (World Health Organisation 2009). Paracetamol is the most commonly used analgesic in the world with 24 billion doses being sold annually in the US alone (Amar *et al.* 2007).

Compared with other analgesics, paracetamol is favoured as a first-line agent because it is cheap, safe and relatively free from side effects. It is not mood altering and does not cause tolerance, addiction, dependence or withdrawal. Its efficacy has been demonstrated in a wide variety of acute and chronic painful syndromes (Bannwarth *et al.* 2003; Remy *et al.* 2005; Schug *et al.* 2005). It can be used safely during pregnancy and lactation (Prescott 1996).

Due to this safety, paracetamol plays a particularly important role in the treatment of surgical patients on discharge because of its wide commercial availability and lack of legal restrictions associated with potent opiate analgesics (Apfelbaum *et al.* 2003). Fear of addiction to opiates and avoidance of their adverse effects also leads to more patients in this setting using paracetamol.

Paracetamol is also suitable for combining with other analgesics and has been shown to improve the quality of analgesia and reduce a patient's demand for stronger pain relief (Cobby *et al.* 1999). In part due to its wide availability and perceived safety, deliberate or accidental overdose are not uncommon. In overdose, paracetamol can cause potentially fatal damage to the liver. The mechanism of action of paracetamol is discussed in Section 1.2

Paracetamol's role postoperatively has changed dramatically in the last decade. The rise of multimodal analgesia and fall of cyclo-oxygenase-2 selective inhibitors (COX-2 inhibitors) have caused re-examination of the other non-opiates available. Use of non-selective Nonsteroidal anti-inflammatory drugs (NSAIDs) had decreased postoperatively because of their gastrointestinal toxicity and increased bleeding risk, risks COX-2s were specifically designed to avoid (Guindon *et al.* 2007). So with few remaining alternatives, attention refocused on paracetamol. There were several key events around the time of the COX-2 withdrawal in 2004 that gave paracetamol new life in postoperative analgesia:

- Paracetamol in combination NSAIDs was shown to improve postoperative analgesia above NSAID administration alone (Hyllested *et al.* 2002);
- Paracetamol was shown to be "morphine sparing" (Delbos *et al.* 1995; Cobby *et al.* 1999; Korpela *et al.* 1999; Hernandez-Palazon *et al.* 2001; Fayaz *et al.* 2004);
- Licensed rectal doses of paracetamol were shown to be inadequate (Kvalsvik *et al.* 2003);
- Studies exceeding licensed doses were published in the literature, both in loading doses (Juhl *et al.* 2006; Gregoire *et al.* 2007) and repeated postoperative doses (Schug *et al.* 1998);
- A well tolerated IV formulation of paracetamol was bought to the European market and licensed in Ireland in 2003 (Bristol-Myers Squibb 2009).

The last event was the most significant in changing the role of paracetamol postoperatively. This formulation overcame the two most significant barriers to wide spread use of paracetamol postoperative: paracetamol's poor oral absorption postoperatively; and patient distaste for rectal administration (Sinatra *et al.* 2005).

So the situation evolved of the more widespread use of a potentially toxic drug, that previously was poorly absorbed now being given by a route that guaranteed complete absorption. It was these concerns about toxicity that led to the research contained in this thesis being conducted.

1.1.3.2.2 ANTI-INFLAMMATORIES (NSAIDS/COX-2 INHIBITORS/STEROIDS)

NSAIDs and COX-2s are generally considered second-line analgesics in the treatment of acute pain. While corticosteroids share their analgesic activity, corticosteroids are

accompanied by multiple systemic adverse effects that outweigh their usefulness as analgesics.

When considering the mechanism of action of NSAIDs, and that of paracetamol later, it is useful to briefly review the inflammatory response (Figure 1.1-4). Trauma to cells, either by injury or infection, activates phagocytic cells already present in the tissue. These phagocytes digest foreign or injured cells and release inflammatory mediators that augment the inflammatory response (Diaz-Perez *et al.* 1976; Goetzl 1976; McClatchey *et al.* 1976).

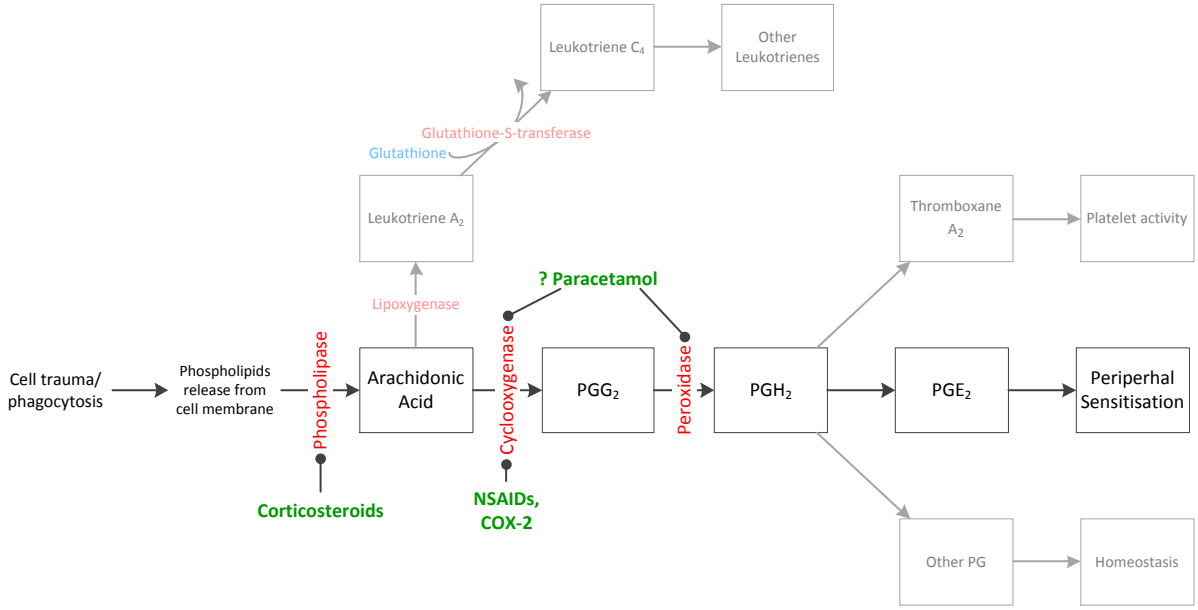


Figure 1.1-4 Eicosanoid synthesis and place of anti-inflammatory drug activity

A major component of cell membrane, phospholipid, is released as foreign or injured cells are digested by phagocytes, or as a direct result or cellular injury. Phospholipids are then broken down by the enzyme phospholipase into arachidonic acid (AA). AA is then enzymatically processed into a group of signalling molecules, collectively called eicosanoids. Eicosanoids are classified into four families, leukotrienes or one of the three families of prostanoids: prostaglandins (PG), prostacyclins and thromboxanes (Soberman *et al.* 2003).

The availability of free AA, which must be liberated from esterified stores in complex lipids, limits the synthesis of eicosanoid, and their subsequent activity (van Dorp *et al.* 1964; Goetzl 1976; McClatchey *et al.* 1976).

PGs are the most relevant eicosanoid to anti-inflammatory activity. AA is converted to PGG_2 by cyclooxygenase (COX). PGG_2 is then subject to further enzymatic activity to form

the other prostanoids, one of which being PGE_2. PGE_2 is most relevant to pain and inflammation as it is responsible for peripheral sensitisation. Although PGs themselves have no intrinsic pain-evoking properties, they act to sensitise nociceptors to bradykinin, serotonin, histamine, adenosine and other components of the 'inflammatory soup', as discussed above, perpetuating the effect of the initial noxious stimulus (Armstrong *et al.* 2008). By inhibiting PG production, NSAIDs prevent a crucial step in the inflammatory response and amplification of pain stimulus (Figure 1.1-5).

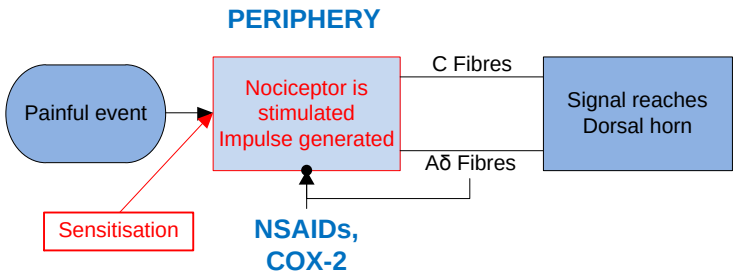


Figure 1.1-5 Site of action of Anti-inflammatories (Extension of fig. 1.1-1)

Corticosteroids target phospholipases, which are required for AA synthesis, whereas NSAIDs and COX-2 inhibitors inhibit COX producing PG. The net result of all three drugs is reduced PG production. Because PGs are also involved in the homeostatic mechanisms of maintaining the gastric mucosa, gastrointestinal erosion is a significant side effect of both corticosteroids and NSAIDs (Macintyre *et al.* 2007). The antiplatelet effect of NSAIDs, derived from the inhibition of thromboxane synthesis, is also well documented. While useful as antithrombotics in cardiovascular medicine, NSAIDs used as antiplatelets may have negative consequences for postoperative bleeding and haemostasis (Niemi *et al.* 1997).

Anti-inflammatory drugs are synergistic with paracetamol, improving analgesia when given in combination (Hyllested *et al.* 2002; Schug *et al.* 2005) and having a significant morphine- sparing effect of up to 30% (Cobby *et al.* 1999; Romsing *et al.* 2002). NSAIDs are available in formulations for oral, rectal and intramuscular use, although the latter route is used infrequently.

1.1.3.2.3 LOCAL ANAESTHETICS

Local anaesthetics have been used since the native South Americans discovered the numbing effects of the coca plant. Its derivative, cocaine, was first used medicinally in the 19th century but has since been replaced with synthetic analogues with more favourable side effect profiles (Yentis *et al.* 1999). Local anaesthetics act by blocking sodium channels

in a nerve cell membrane, preventing it from depolarising, thereby inhibiting generation of an action potential and blocking transmission of the painful stimulus in the spinal cord (Shang *et al.* 2003; Macintyre *et al.* 2007).

Local anaesthetics are utilised increasingly in advanced techniques or 'regional anaesthesia'. These include paravertebral block, used prior to breast surgery, or infiltration of port sites following laparoscopic surgery, in order to minimise postoperative pain (Australian and New Zealand College of Anaesthetists and Faculty of Pain Management 2005). They are more traditionally used topically, prior to cannulation, for anaesthesia during short procedures, such as dental extraction, or in combination with opiates for epidural anaesthesia.

Local anaesthetics are rapidly metabolised and have negligible oral bioavailability. If a prolonged effect is desired, they must be given by infusion or injection into tissue with poor blood flow.

For procedural pain, local anaesthetics may be used as a sole agent (*e.g.* suture insertion/removal, dental procedures), providing analgesia only while the procedure is taking place. For procedures where pain is expected to be more prolonged, an intraoperative dose will provide relief immediately postoperatively, when pain is most severe, or an infusion is used to provide continuing treatment. Transdermal patches of local anaesthetic are currently *en vogue* for continued delivery of anaesthetic to a painful area, such as laparoscopic port sites or new stomas and have the significant advantage of minimising systemic drug administration.

Local anaesthetics are not specifically analgesics and can block all nerve conduction, both motor and sensory. The smaller diameter, unmyelinated C fibres are most responsive to local anaesthetics, and large, myelinated A fibres the least responsive. This allows some specificity with respect to which nerves are blocked. C fibres, which convey pain/temperature and postganglionic autonomic (sympathetic) signals are the first to be affected, followed by B fibres (sympathetic), Aδ (pain, touch, temperature), Aγ (muscle spindle tone), Aβ (touch, pressure) and, finally, Aα (motor, proprioception) (Macintyre *et al.* 2007). Sympathetic blockade is reported first, followed by pain and temperature blockade.

Because local anaesthetics block sympathetic innervation and pain transmission, they are able to prevent 'evoked' pain, the hyperalgesia and allodynia arising from central

sensitisation that can follow surgical trauma. Subsequently, there is increasing evidence of reduction in recovery time and post-surgical chronic pain syndromes with the use of local anaesthetics (Boezaart 2006). The use of these drugs in regional anaesthesia has also been shown to reduce the incidence of nausea and vomiting as well as thromboembolic, pulmonary, cardiovascular and gastrointestinal complications (Guindon *et al.* 2007).

When used by skilled operators, side effects of local anaesthetics are very rare. Common minor-moderate adverse effects from epidural administration are associated with blockade of touch or motor function which can impede the return to mobility and hinder recovery. More serious adverse effects can occur when local anaesthetics are injected or absorbed into the blood stream. The resultant adverse effects also arise from the blockade of sodium channels, leading to interference with the generation of action potentials and conduction in the heart, causing arrhythmias, and in the CNS, causing convulsions (Macintyre *et al.* 2007). Single enantiomer formulations have improved selectivity for sensory over motor blockade and decreased potential for cardiotoxicity (Macintyre *et al.* 2007).

1.1.3.2.4 OPIATES

The term “opiate” refers to a broad range of narcotic drugs that were originally derived from opium. Now synthetically manufactured, opiates share many structural similarities chemically but their analgesic activity varies widely (Shang *et al.* 2003). They are potent analgesics, used mainly for moderate to severe pain. Opiates reduce the distressing, affective component of pain more so than dull the sensation of pain itself (Drolet *et al.* 2001). Unlike paracetamol and anti-inflammatory drugs, the dose/response of opiates varies greatly between individuals, and even within the individual, depending on their state of health. Dose adjustment based on age is more appropriate than doses based on weight (Camu *et al.* 2002). Individualisation of dose is essential because of the wide variation in response; opiates are fatal in overdose and have many common adverse effects. The most common adverse effects due to opiates include nausea and vomiting, sedation, respiratory depression, euphoria and dysphoria, among many, many others (Shang *et al.* 2003).

Exogenous opiates act by mimicking the body’s own endogenous opiates involved in the descending pathways of pain regulation (Schaible *et al.* 2004). Exogenous opioids exert their analgesic activity in the same way, binding to opiate receptors on the presynaptic

nerve, blocking the release of neurotransmitters and impeding nociceptive transmission. They also act supraspinally to activate inhibitory pathways that descend to the spinal segment (Camu *et al.* 2002).

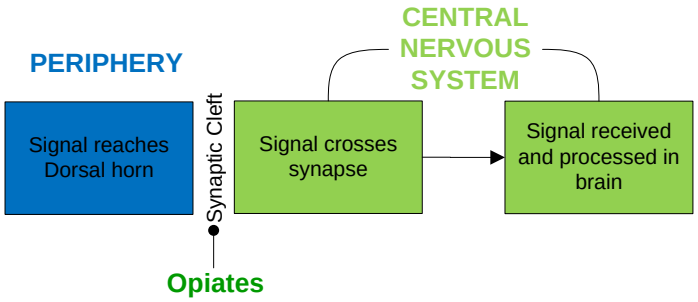


Figure 1.1-6 Site of action of opiates (continuation of Figure 1.1-1)

Opiates are extremely effective analgesics and are the basis of pharmacological management of postoperative pain (Carr *et al.* 1999). The diffuse, visceral pain carried by C fibres is especially sensitive to opiates, whereas the Aδ fibres are less sensitive (Guindon *et al.* 2007). Opiates used most often postoperatively include morphine, fentanyl, oxycodone and tramadol.

From a practical standpoint, most opiates used for the control of acute pain are controlled drugs in Ireland which must be stored securely and face restrictions on their prescribing, dispensing and administration. In a hospital setting these restrictions can hinder or delay their administration. On discharge and at home they may not be prescribed because of these restrictions, or prescribed in insufficient quantity. Paracetamol and anti-inflammatory drugs are not subject to these restrictions, an important advantage to their use.

Morphine is the most commonly used opiate in the acute pain setting, both intra-operatively and postoperatively. It is cheap and widely available in both oral and parenteral formulations. It is considered the gold standard with which other analgesics are compared and has been used as an analgesic for centuries.

Morphine's metabolism is via Phase II glucuronidation (a pathway shared by paracetamol, described in Section 1.2) to two products; predominantly morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in lesser amounts. M6G has increased analgesic potency and fewer side effects compared with morphine. Conversely, M3G has no analgesic properties but is associated with producing several of the adverse effects associated with morphine, including sedation, respiratory rate depression and clinical tolerance. M3G may also antagonise morphine (Wittwer *et al.* 2006). Both metabolites

rely on active renal excretion. Accordingly, morphine should be avoided in patients with end-stage renal disease and its dose reduced in renal impairment, as substantial amounts of M3G can accumulate, causing all the adverse effects with inadequate pain control.

Fentanyl is used both intraoperatively and postoperatively; however, it is not available in an oral formulation due to high first-pass losses. It has a shorter half-life- an advantage given the fluctuating demands for analgesia in the surgical patient- and requires no adjustment for renal impairment. Both fentanyl and morphine are used in combination with local anaesthetics in spinal anaesthesia (Macintyre *et al.* 2007).

Tramadol is a mixed agonist, affecting serotonergic receptors in addition to opiate receptors. It is less potent than morphine but is associated with many of the same adverse effects, particularly changes in consciousness, with the elderly being the most susceptible. Access to the drug is simpler as it is not a controlled drug, although there are reports of change to this status (Macintyre *et al.* 2007). Tramadol's mixed agonism and effect on multiple receptor types is thought to lead to its high percentage of CNS adverse effects. Oxycodone is a more recent addition to the acute pain formulary. It is increasingly used in place of oral morphine, having twice the analgesic potency, but is subject to less frequent adverse effects, particularly nausea, vomiting and changes to consciousness. It is preferred by many patients, but at greater than ten times the cost of an equivalent dose of oral morphine, its cost-utility is debated by pharmacoeconomists (Macintyre *et al.* 2007).

In addition to standard routes of administration, opiates can be used with specialised techniques postoperatively. Intravenous (IV) patient-controlled analgesia with morphine is available in most Irish hospitals and is considered the optimal treatment for postoperative pain in patients who are unable to take oral medication (McCaffery *et al.* 1999). Some specialised centres will also offer patient-controlled epidural analgesia. When compared with intermittent bolus dosing of opiates, patient-controlled analgesia results in better pain control and greater patient satisfaction, without increasing the incidence of opioid-related adverse effects (Macintyre *et al.* 2007). The intensity of pain is rarely constant, but with patient-controlled analgesia, small and frequent IV bolus doses of opioid can be given whenever the patient becomes uncomfortable, enabling rapid titration to the degree of pain the patient is experiencing. This flexibility helps to overcome the wide patient variability (8-10 fold) in opiate requirement and avoids dose-related adverse effects. It can also save medical/nursing time involved in the assessment

and administration of intermittent analgesia (Macintyre *et al.* 2007). One study in patients undergoing abdominal surgery showed significantly improved pain control, but with greater than twice the morphine consumption, in patients with patient-controlled analgesia compared to nurse administered intramuscular *prn* morphine (Everett *et al.* 2005). Despite the greatly increased consumption of morphine, the patient-controlled analgesia group did not have a greater incidence of adverse effects. Nurse administration of *prn* opiates varies greatly between nurses (Gordon *et al.* 2008a), reinforcing the role of the patient as the best person to control their own analgesia. All of these findings: better pain control, increased opiate consumption, no increase in significant adverse effects, are emphasised in a Cochrane Review (Hudcova *et al.* 2006).

Intrathecal and epidural routes of administration opiates are also used. They reduce the drug load to the patient and, potentially, the adverse effects. Adverse effects from opiates are often mediated by peripheral receptors, which are avoided by direct administration in the CNS. This route can also provide sustained analgesia for 12-24 hours after administration due to the sparsity of opiate metabolising enzymes in the CNS.

However, the utility of opiates is often limited by their adverse effects. Respiratory depression is potentially life-threatening, constipation is almost guaranteed, and nausea and vomiting is a common cause of cessation of treatment. Pruritus from opiate induced histamine release is also a significant cause of morbidity. Some of the many other significant adverse effects include urinary retention, hallucinations, drowsiness and confusion, to which the elderly are more prone and more sensitive (Cobby *et al.* 1999). The adverse effects are so considerable that, as described above, the “morphine-sparing effect” is a commonly used indicator of the benefit of other analgesia.

Managing the adverse effects from opiate use adds considerably to the cost of these otherwise cheap drugs. A double-blind randomised controlled trial comparing IV morphine with IV ketorolac for treating pain after limb injury, showed that while the acquisition costs of ketorolac were three times those of morphine, when administration costs, adverse events and admission for adverse events were taken into account, ketorolac was much cheaper (Rainer *et al.* 2000).

Concern over the safety and addiction potential of opiates often results in under-prescribing and inadequate dosing in the hospital setting, even in the presence of severe pain and minimal or no side effects (Gordon *et al.* 2008b). This often leads to inadequate

analgesia (Schug *et al.* 2007). Interpatient variability can alter response to opiates and lead to wide- ranging effects from a similar dose (Schug *et al.* 2007). Factors that affect response include patient and drug characteristics such as prior drug exposure, prior responses to analgesics, age, organ function, pain severity, anticipated pain duration, co-morbid conditions and concomitant drug use (Macintyre *et al.* 2007; Gordon *et al.* 2008b). Due to these factors, titration of opiates to the optimal dose is difficult to achieve, and rarely happens (Bannwarth *et al.* 2003). While opiates are more effective as analgesics, the fear of adverse effects limits their application. Similarly, fear over the renal, haematological and gastrointestinal adverse effects of NSAIDs also limits their usage. Avoidance of these adverse effects is especially important perioperatively. Paracetamol, which has a wide therapeutic index and an enviable safety profile, has many advantages in this way that are reflected in its wide use postoperatively.

1.2 PARACETAMOL

Paracetamol is a white, odourless, crystalline powder with a melting point of 169-171°C and a molecular weight of 151.2 g/mol. The octanol/water partition co-efficient is 0.5 (Moffat *et al.* 2011). The empirical formula is $C_8H_9NO_2$ and structural formula is shown in Figure 1.2-1.

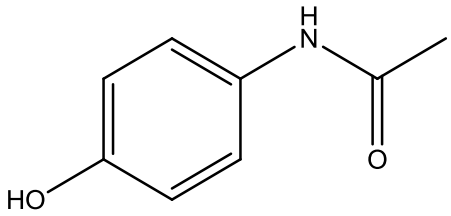


Figure 1.2-1 Paracetamol

Paracetamol is a weak organic acid with a pKa of 9.5. It is moderately soluble in hot water, alkaline aqueous solutions and more polar organic solvents such as methanol and acetone (Prescott *et al.* 1971).

1.2.1 DISCOVERY

Paracetamol was first synthesised in 1878 (Morse 1878). It was developed as an antipyretic agent along with its prodrug, phenacetin, in the second half of the 19th century after its parent drug, acetanilide, was found to cause cyanosis and methaemoglobinemia (Bertolini *et al.* 2006). Phenacetin went to market ahead of paracetamol because it was believed to be less toxic, although the details of this reported toxicity were unclear (Prescott 1996). It was not until 1948 that Brodie and Axelrod showed that paracetamol was the major metabolite of phenacetin and that it was paracetamol that conferred phenacetin's pharmacological activity, thereby 'rediscovering' paracetamol (Brodie *et al.* 1948).

1.2.2 PHARMACEUTICAL DOSAGE FORMS

Paracetamol is unusual in that it is available in a wide variety of formulations. Hampered by poor aqueous solubility (1 in 70) it had been available in tablets, capsules, suppositories and suspensions for many decades (Van Aken *et al.* 2004). Advances in formulation provided an effervescent soluble tablet and more recently IV infusions, initially as a prodrug, proparacetamol (Depre *et al.* 1992), and more recently as paracetamol itself (Van Aken *et al.* 2004). Proparacetamol is no longer in use and is not licensed in Ireland (Irish Pharmaceutical Healthcare Association 2010). While increasing

access to the drug, the variety of formulations and combinations of paracetamol with other medications has been implicated as a cause of unintentional overdose (Albertson *et al.* 2010).

Following single doses in healthy adults, the route of administration does not have a significant effect on metabolism (Clements *et al.* 1984) with identical elimination half-lives following a 1g IV or oral dose (Depre *et al.* 1992), however the pattern of metabolism may be different.

1.2.3 CLINICAL PARTICULARS

1.2.3.1 THERAPEUTIC INDICATIONS

Oral and rectal formulations of paracetamol are licensed in Ireland for the short-term management of headaches, musculoskeletal disorders, menstrual pains, toothache and for symptoms of common colds and flu. It may also be used for the relief of mild to moderate pain associated with osteoarthritis (Glaxo Smith Kline 2008). IV formulations are indicated in the short-term treatment of moderate pain following surgery and short term treatment of fever (Bristol-Myers Squibb 2009).

1.2.3.2 POSOLOGY AND METHOD OF ADMINISTRATION

Licensed doses of paracetamol for adults are 1g repeated if necessary 3-4 times a day, at a minimum four hour interval to a maximum of 4g in any 24 hour period. For children the does is 10-15mg/kg/dose at the same interval (Glaxo Smith Kline 2008; Bristol-Myers Squibb 2009). IV formulations of paracetamol are administered to deliver the same licensed dosage as detailed above by IV infusion over 15 minutes (Bristol-Myers Squibb 2009).

1.2.3.3 DRUG INTERACTIONS

Paracetamol has few clinically significant drug interactions. The most notable are:

1. Prolonged use of paracetamol may enhance the anticoagulant effect of coumarins and may inhibit the metabolism of busulphan;
2. The absorption of paracetamol may be enhanced by metoclopramide and reduced by colestyramine; and

3. Probenecid can almost half paracetamol clearance by inhibiting conjugation with glucuronic acid (Lacy *et al.* 2005; Klasco 2009).

There is also evidence of oral contraceptive hormones increasing the glucuronidation of paracetamol, although the clinical relevance is uncertain (Bock *et al.* 1994).

1.2.3.4 WARNINGS, CONTRAINIDCATIONS AND PRECAUTIONS FOR USE

Doses above those currently licensed are associated with liver damage. Because of the variety of products available that contain paracetamol, it is important that only one source of paracetamol is administered at any one time.

Paracetamol is contraindicated in those hypersensitive to paracetamol, or any of the excipients (Glaxo Smith Kline 2008; Bristol-Myers Squibb 2009). It should be used with caution in those with hepatocellular insufficiency, severe renal insufficiency, chronic alcoholism, chronic malnutrition or dehydration (Bristol-Myers Squibb 2009).

1.2.3.5 PREGNANCY AND LACTATION

Although no comprehensive studies have been conducted, paracetamol is not thought to cause undesirable effects during pregnancy or for the new born infant. One study has shown minor alterations to disposition (Miners *et al.* 1986). Experience with IV formulations is especially limited (Bristol-Myers Squibb 2009).

Following oral administration, paracetamol is excreted in small amounts into the breast milk, however no undesirable effects have been reported and paracetamol may be used in breast-feeding women (Bannwarth *et al.* 2003; Graham *et al.* 2005b).

1.2.3.6 UNDESIRABLE EFFECTS

Considering the quantity of paracetamol consumed annually, it is remarkably safe drug. Undesirable effects are either classified as rare (>1/10000, <1/1000) or very rare (<1/10000) (Bristol-Myers Squibb 2009). Studies have shown paracetamol adverse effects to be comparable with placebo (Moore *et al.* 1997). Most of the reported adverse reactions involve hepatotoxicity. Other rarer adverse effects are those common to most medicines; nausea, vomiting, rash, blood disorders and allergy (Fischereder *et al.* 1984).

Initial IV formulations of proparacetamol were associated with higher rates of allergy, however these have improved since paracetamol replaced proparacetamol.

Proparacetamol is a diethylglycidyl ester of paracetamol and after being cleaved by esterases present in the body, the remaining diethylglycine was a known allergen (Graham *et al.* 2005b). Proparacetamol was also associated with pain at the injection site on infusion, however paracetamol infusions have less than half the incidence, in one study reducing from 33% to 14.7% ($p<0.005$) (Murat *et al.* 2005).

More recently, IV paracetamol has been related to hypothermia and hypotension. These appear to be related to the rate of administration, occurring only in susceptible individuals, such as those hypovolaemic after surgery. Because paracetamol is poorly soluble, mannitol and disodium phosphate are used to increase its solubility. Both of these excipients are known to cause hypotension, and in those affected, reducing the infusion rate minimises this adverse response (Klasco 2009).

1.2.3.6.1 LIVER TOXICITY

The metabolism of paracetamol produces a hepatotoxic metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI). Theoretically, alterations that produce significantly more NAPQI or reduce its conjugation could lead to toxicity, but this does not occur at therapeutic doses.

Prospective studies show that repeated use of a true therapeutic paracetamol dosage may slightly increase the level of serum aminotransferase activity, but hepatic injury, failure or death, have also been reported, although extremely rarely (Kurtovic *et al.* 2003; Moling *et al.* 2006). Some retrospective reports show a higher rate of increased serum aminotransferase levels, and several reported associated liver injury and death in paracetamol users. However, inaccuracies in recording of paracetamol dose in some case-reports suggest that these cases may be inadvertent overdoses, rather than true therapeutic dosages (Dart *et al.* 2007). These reports may also be confounded by aminotransferase increases from other causes. Additionally, a retrospective review of pooled aminotransferase data measured during placebo treatments of nearly 500 patients from 13 Phase I studies showed that 20% of the subjects had at least one aminotransferase value above the upper limit of the reference range (ULRR) and 7.5% had at least one value twice the ULRR (Rosenzweig *et al.* 1999).

Aside from intentional overdoses, toxicity is very rare and confined to case reports mostly in alcoholic adults and children who have been accidentally overdosed by their care-givers (Whitcomb *et al.* 1994).

1.2.3.7 OVERDOSE

Paracetamol overdose is potentially life threatening and can result from single doses of 12g, or ≥150mg/kg of paracetamol (Brok *et al.* 2006). Paracetamol overdose presents as pallor, nausea, vomiting, anorexia and abdominal pain. Those with paracetamol overdose may exhibit liver damage, manifested by liver transaminases elevation eight hours after ingestion of the overdose. Paracetamol overdose presents as pallor, nausea, vomiting, anorexia and abdominal pain. Alterations to glucose metabolism and metabolic acidosis may also occur (James *et al.* 2003). Severe poisoning may progress to hepatic failure, encephalopathy, coma and death (Bartlett 2004).

IV *n*-acetylcysteine is an effective antidote to paracetamol overdose when administered within eight hours of the paracetamol dose. Although its benefit declines after this time, it still may be beneficial up to and beyond 24 hours. Activated charcoal and methionine may also be of benefit in those who have ingested >12g (Clark 2001; Bartlett 2004; Brok *et al.* 2006).

Paracetamol is regarded as a very safe drug in normal doses although in overdose fatalities can occur (Bailey 1980; Shayiq *et al.* 1999; Schmidt *et al.* 2002; Acello 2003; Lee 2004; Holubek *et al.* 2006). Its perception as being such a safe drug has led to toxicity and overdose in those who were taking it with therapeutic intent (Graham *et al.* 2005b). Unfortunately, because of paracetamol's wide availability, it is also frequently used for self-harm. This can result in a prolonged painful death from liver failure. In a study of all 11,092 presentations to Irish hospitals due to deliberate self-harm during 2004, 7,933 related to drug overdose of which 31% involved paracetamol (Ní Mhaoláin *et al.* 2009). The incorporation of *n*-acetylcysteine into paracetamol formulations was attempted to minimise harm, however the additional expense of these products prevented their wide-spread use (Clark 2001). When considering the maximum dose of paracetamol it is important to evaluate both the frequency as well individual dose. There are numerous reports of intentional overdose linked to paracetamol, but these are taken as a single dose (Prescott 2000b; Sheen *et al.* 2002b). Similar amounts may be safe if taken in divided doses throughout the day, and this is one of the hypotheses of this Thesis.

Early observations of toxicity from acute overdoses have shown paracetamol's disposition to be non-linear (Prescott 1996), which has been confirmed in single dose studies of doses ranging from 0.325g to 3g (Slattery *et al.* 1987; Borin *et al.* 1989). This is thought to

due to saturation of the enzymes involved in sulphonation and the depletion of its co-factors (section 0). This finding led to the fear that repeated doses at or slightly above the licensed dose would lead to accumulation of paracetamol and a disproportionate rise in NAPQI, with subsequent toxicity (Gelotte *et al.* 2007). However, a more recent study by Gelotte *et al*. examined the safety and disposition of paracetamol after repeated doses of up to 8g/day for three days. They found paracetamol had linear kinetics after the first dose, which did not change after reaching steady state (Gelotte *et al.* 2007). They also showed that data modelled from initial doses did not predict those of steady state, with the actual concentrations being much lower than those predicted. As discussed above, the authors of this study suggest there is an increase in clearance of paracetamol over time accompanying increased formation clearance of paracetamol glucuronide.

Rats given substantially higher doses of paracetamol IV, up to 600mg/kg, had a prolonged half-life and decreases in clearance. As the dose of paracetamol increased, more of the dose was excreted into the bile with biliary excretion as a proportion of dose increasing from 20 to 49% over the dose range of 37.5 to 600mg/kg. Similarly, over the same dose range the biliary excretion of the glucuronide metabolite increased from 10.5% to 40.2% of the total recovered dose, but the urinary recovery of the glucuronide metabolite remained relatively constant around 20% of the recovered dose. At 600mg/kg the glucuronide conjugate represented over 70% of the recovered dose, however at doses at or above 600mg/kg a significant decline in the rate constant for glucuronide formation was noted. Comparable results were seen for the rate for glutathione (GSH) conjugation at 300 mg/kg, whereas the formation of the sulphate conjugate was decreased at lower dosages (75 mg/kg). The authors concluded that glucuronidation was a high-capacity, high-dose saturable pathway of paracetamol metabolism which preferentially excretes the product in bile after high dosages (Hjelle *et al.* 1984).

1.2.4 PHARMACOLOGICAL PROPERTIES

1.2.4.1 PHARMACODYNAMIC PROPERTIES

There are two systemic effects of paracetamol that are of clinical significance: antipyresis and analgesia (Bruton *et al.* 2006).

1.2.4.1.1 ANTIPYRESIS

Pyresis, the elevation of body temperature, is part of the body's response to eradicate infection. In response to the presence of foreign cell components arising from phagocytosis, cells derive PGs from AA and release them into the circulation. In the CNS, it is thought endothelial cells are responsible for PG synthesis (Cao *et al.* 1996). PGs, especially PGEs, act on the hypothalamus to increase the set point of body temperature. Subsequently body temperature rises through heat generation and conservation measures (Feldberg *et al.* 1972; Kasper *et al.* 2008).

Paracetamol has no effect on pyresis that is not mediated by prostaglandins (Dey *et al.* 1974). It was later discovered that paracetamol prevents PGE_2 synthesis by inhibiting prostaglandin H_2 synthase (PGHS) in the brain (Flower *et al.* 1972), and that endothelial cells are especially sensitive to these actions of paracetamol (Kis *et al.* 2005). Despite considerable research and clinical use for more than a century, the exact mechanism of the other actions and therapeutic concentration required for those actions of paracetamol is still unclear (Graham *et al.* 2005a; Bertolini *et al.* 2006).

1.2.4.1.2 ANALGESIA

The mechanisms of pain and pain transmission are discussed in the previous section, 1.1.1, while the inflammatory process and mechanism of action of NSAIDs is discussed in 1.1.3.2.2. Two pieces of information from these previous sections are important to the discussion of paracetamol's mechanism of analgesia:

1. Nociceptors exposed to PGs are sensitised and require a lower intensity of stimulation to cause pain; and
2. Inhibition of PG synthesis reduces this sensitisation and result in analgesia.

1.2.4.1.2.1 PLACE OF ACTION- PERIPERHAL OR CENTRAL?

Since paracetamol and NSAIDs have similar antipyretic and analgesic effects clinically, much investigation has sought to prove paracetamol inhibits PGHS in a similar way to NSAIDs. While there is little doubt NSAIDs act through the peripheral inhibition of the COX site on PGHS (Vane 1971; Brooks *et al.* 1991), the evidence for where paracetamol works is conflicting and both central and peripheral pathways may be involved. Although paracetamol may have some effect on PGHS, this effect is different from that seen with NSAIDs (Anderson 2008). Paracetamol's lack of any significant anti-inflammatory or

antiplatelet activity *in vivo* are the sentinel differences from NSAIDs, and are consistent with a lack of peripheral inhibition of PG synthesis (Clissold 1986; Bruton *et al.* 2006). Despite this finding, peripheral activity is supported by several experimental studies:

- Lim *et al*. showed in 1964 that greater analgesia could be achieved when paracetamol is administered intra-arterially, proximal to a painful stimulus, rather than IV (Lim *et al.* 1964);
- Paracetamol was shown to block peripheral autonomic response to pain induced by bradykinin injection into the spleen of dogs (Guzman *et al.* 1964);
- In perfused livers, action potentials, either spontaneous or from toxic stimuli, were inhibited in hepatic nerves by paracetamol (Andrews *et al.* 1973);
- Paracetamol was shown to suppress the production of some components of the inflammatory soup such as bradykinin, adenosine triphosphate, slow reacting substance C and AA in the same way as NSAIDs (Vargaftig *et al.* 1973); and
- Paracetamol was also been shown to reduce pain and oedema, widely accepted as a typical inflammatory response, mediated, in part, by PGs' peripheral actions (Vinegar *et al.* 1976).

While these studies indicate a peripheral activity, there is also considerable evidence in favour of a central mechanism of action:

- Paracetamol concentrations in the CSF match the response to fever (Anderson *et al.* 1998) and pain (Anderson *et al.* 2001) more closely than concentrations in the plasma;
- Paracetamol reduces pain following administration directly into the CNS (Pelissier *et al.* 1996); and
- Paracetamol inhibits PG release in the CNS following peripheral noxious stimuli (Muth-Selbach *et al.* 1999).

While these strongly support activity in the CNS, it is the lack of significant anti-inflammatory or antiplatelet activity *in vivo* that are the strongest findings in favour of a central mechanism of action.

1.2.4.1.2.2 NATURE OF ANALGESIC ACTIVITY

As it is central to the understanding of the dose/response relationship, and why paracetamol has no clinically relevant peripheral activity *in vivo*, it is worth reviewing the most popular proposed mechanisms of action of paracetamol.

Prescott believes debate on paracetamol's activity has arisen because of the nature of paracetamol's PG inhibition (Prescott 1996). In humans, paracetamol is generally considered to be a weak inhibitor of PG synthesis, exhibiting a highly variable capacity in different cell and tissue types (Aronoff *et al.* 2006).

The majority of NSAIDs and COX-2 inhibitors inhibit PG synthesis by non-covalently binding to the COX active site, physically obstructing the entry of AA, thereby preventing this oxygenation process from occurring. However, the current wisdom is that this mechanism is not shared by paracetamol which does not work within the COX active site but rather the second site involved in the oxygenation, the peroxidase active site (POX). Here paracetamol prevents the formation of a tyrosine radical by POX, which is essential for COX activity (Figure 1.2-2).

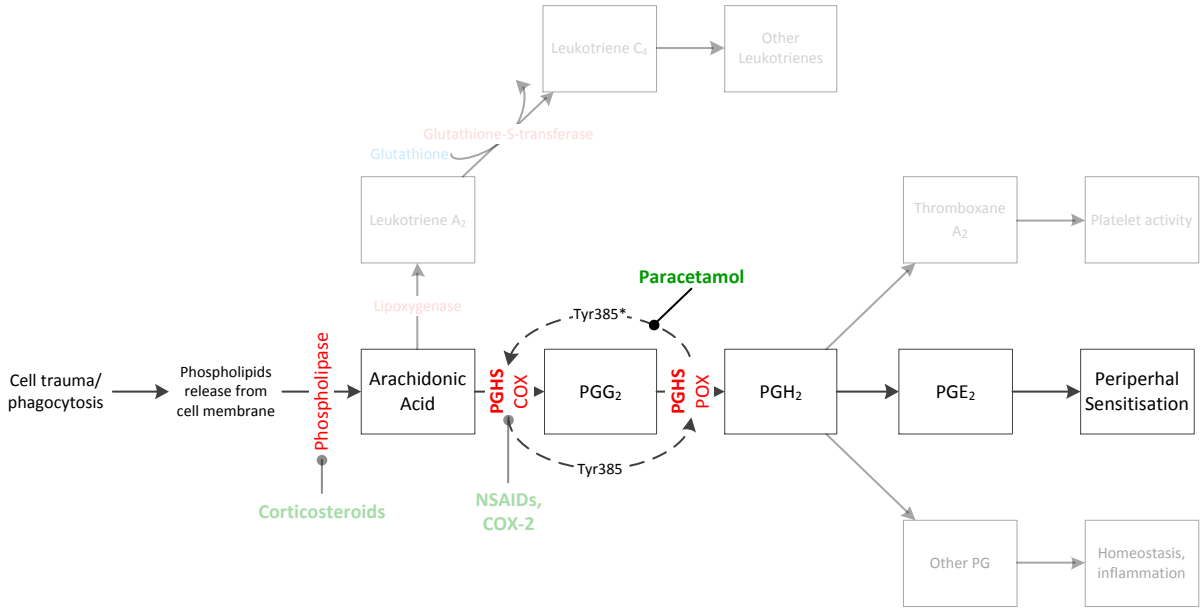


Figure 1.2-2 Inflammatory cascade and location of paracetamol's activity
Paracetamol inhibits the formation of the tyrosine385 radical (Tyr385) by peroxidase (POX). Tyr385*is required by cyclooxygenase (COX) to metabolise arachidonic acid to PGG_2*

PG synthesis begins when PGHS enzymes oxygenate AA to give PGH_2. PGH_2 synthesis from AA occurs in two stages:

1 AA is metabolised to PGG_2 by COX;

2 PGG_2 is metabolised to PGH_2 by POX.

As mentioned previously, both COX and POX are different active sites on the same enzyme PGHS. A more detailed synopsis of the steps is presented in Figure 1.2-3.

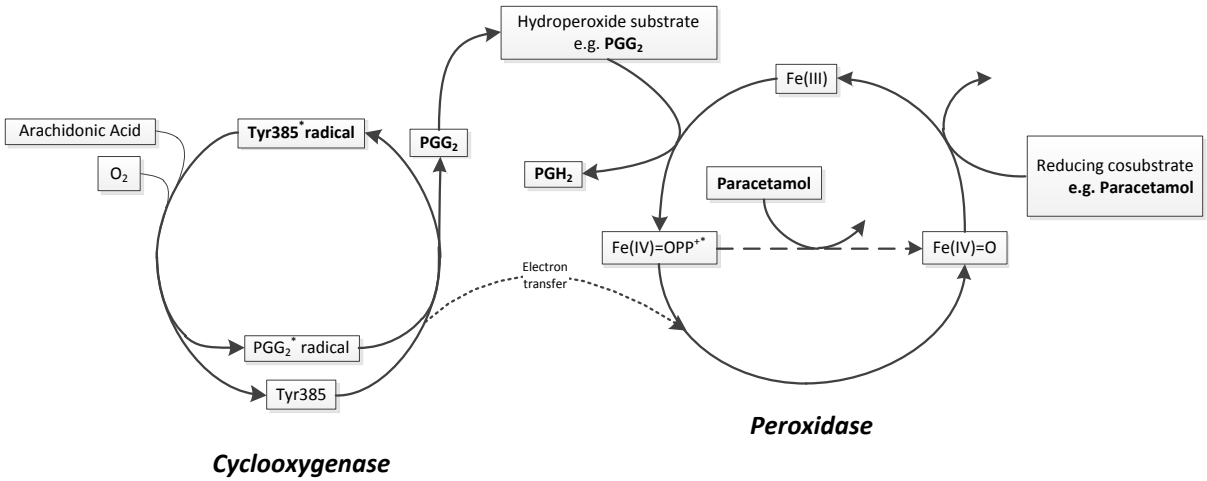


Figure 1.2-3 Schematic of prostaglandin H_2 synthase (PGHS) metabolism of arachidonic acid to PGH_2.
Reduction of the ferryl protoporphyrin IX cation (Fe[IV]=OPP+) at the peroxidase site is necessary for the formation of the tyrosine-385 radical (Tyr385*) at the cyclooxygenase site. Paracetamol partially reduces Fe(IV)=OPP*+ decreasing the amount available for regeneration of Tyr385*, impairing COX activity. In cells with high peroxide concentration greater amounts of Fe(IV)=OPP*+ are produced, overwhelming the actions of paracetamol (adapted from Aronoff et al. 1996 and Anderson 2008).*

Stage 1, the metabolism of AA to PGG_2, occurs in two steps. In the first step a hydrogen atom is taken from AA by a tyrosine 385 free radical (Tyr385*) present in the COX active site. The second step is this arachidonyl radical using oxygen to produces a PGG_2* radical.

In stage 2, PGG_2 is reduced to the equivalent alcohol, PGH_2 and Tyr385* is reformed. This reduction of PGG_2 to PGH_2, in the haem containing POX active site, yields an oxidised haem radical cation, ferryl protoporphyrin IX (Fe[IV]=OPP*$^+$). Fe(IV)=OPP*$^+$ is then used to generate Tyr385* from Tyr385 in the COX active site by intramolecular ion transfer. The Tyr385* is then reused by COX. The newly formed partially reduced haem [Fe(IV)] is further reduced to the resting state [Fe(III)] (Aronoff *et al.* 2006).

POX then reduces hydroperoxides to re-oxidise the haem from the resting Fe(III) state to the catalytic Fe[IV]=OPP*$^+$. A variety of hydroperoxides can be used, not just PGG_2, each with varying efficiency (Markey *et al.* 1987). Hydroperoxides of fatty acids, such as AA, are preferred substrates, as is the case with PGG_2, while hydrogen peroxide is a weak substrate (Ohki *et al.* 1979).

Summarised, COX is dependent on POX to produce the Tyr385* radical for its activity, whereas POX does not rely on COX.

At this molecular level, most NSAIDs and COX-2 inhibitors work by competitively inhibiting the entry of AA and subsequent production of PGG_2, with the exception of aspirin which, uniquely, covalently modifies the COX site, permanently inactivating it. Paracetamol, however, is a reducing co-substrate for the POX active site, like PGG_2, reducing POX haem from the higher oxidative Fe(IV) state back to its resting Fe(III) state. Unlike PGG_2, when paracetamol is the co-substrate, Tyr385 is not reproduced, thus starving COX of its co-substrate Tyr385* (Boyd *et al.* 1981; Moldeus *et al.* 1982; Harvison *et al.* 1986; Markey *et al.* 1987; Potter *et al.* 1987; Harvison *et al.* 1988a). Because the electron is being transferred to paracetamol rather than Tyr385 it would be expected that paracetamol radicals would be produced, and this has indeed been shown (Boyd *et al.* 1981; Moldeus *et al.* 1982).

Peroxides, such as PGG_2, which arises from COX, oxidise the POX haem back to its catalytically active Fe(IV) state, opposing the actions of paracetamol. Experimentally, lowering the concentration of peroxides enhances paracetamol's inhibition of PGHS (Ouellet *et al.* 2001) and conversely, increasing the peroxide concentration reduces it (Boutaud *et al.* 2002). Therefore it can be seen increasing the amount of AA increases the amount of PGG_2 which in turn overwhelms any inhibitory action of paracetamol (Ouellet *et al.* 2001).

Initially paracetamol was thought to have similar activity to NSAIDs action on COX. However, further evidence for paracetamol's activity on POX, as oppose to COX, arises from paracetamol's lack of antagonism of NSAIDs on the COX active site (Catella-Lawson *et al.* 2001; Ouellet *et al.* 2001) and antagonism of paracetamol's activity following addition of exogenous PGG_2, which does not occur with NSAIDs (Boutaud *et al.* 2002). In studies where PGHS concentrations have been increased but AA concentrations have remained constant, paracetamol activity has decreased, in contrast to the activity of NSAIDs.

1.2.4.1.2.3 PARACETAMOL'S LACK OF ANTI-PLATELET AND ANTI-INFLAMMATORY ACTIVITY

The most clinically relevant point of difference between NSAIDs and paracetamol is paracetamol's lack of anti-platelet and anti-inflammatory activity. With respect to anti-platelet activity, there are two factors which make platelets resistant to the actions of paracetamol, which also add to the understanding of paracetamol's mechanism of action in other cells.

Firstly, in inflammation, there is an explosive activation of phospholipase A_2 in platelets by receptor dependent stimuli. This results in an equivalently dramatic increase in PGG_2, which in turn causes resistance to inhibition of PGHS by paracetamol.

Secondly, on activation, a substantial amount of the lipid hydroperoxide 12-hyroperoxyicosatetraenoic acid (12-HpETE) is formed by the platelet 12-lipoxygenase, further increasing the cells peroxide tone (Johnson *et al.* 1998). 12-HpETE acts as a peroxide co-substrate for POX, which is preferred by POX over paracetamol, preventing paracetamol returning POX to its resting state (Calzada *et al.* 1997; Boutaud *et al.* 2002).

This action by platelets, the rapid increase in PGG_2 and production of a preferred co-substrate, also adds to the picture of how different cells respond to paracetamol. When stimulated by the same concentration of exogenous AA, platelets are completely resistant to the actions of paracetamol at concentrations that completely block PGHS activity in human umbilical vein endothelial cells. As discussed in Section 1.2.4.1.1, endothelial cells are particularly sensitive to the PGHS inhibitory actions of paracetamol (Kis *et al.* 2005).

Understanding the importance on the peroxide tone of a cell during activation also explains paracetamol's lack of anti-inflammatory activity. Similar peroxide-producing enzymes, such as platelet 12-lipoxygenase, are highly expressed in inflammatory leukocytes. Their products together with peroxynitrite and hydrogen peroxide generated by activated macrophages, vastly increase the peroxide tone of these cells. This greatly reduces the activity of paracetamol in these cells in inflammatory settings, explaining paracetamol's lack of an anti-inflammatory effect. Certain types of monocyte-marcophage cell lines require over 200 times the concentration of paracetamol to achieve the same block in PGHS activity given the same AA stimulation because of their considerably greater peroxide tone (Fels *et al.* 1982; Martin *et al.* 1984; Sun *et al.* 1996). Indeed, there is evidence that concentrations of paracetamol that completely inhibit PG synthesis in endothelial cells actually stimulated PG synthesis in some types of inflammatory cells (Aronoff *et al.* 2006).

1.2.4.1.2.4 OTHER POSTULATED MECHANISM OF ACTION

COX-3, a splice variant of the constitutive PGHS (COX)-1, has been suggested to be the site of action of paracetamol (Chandrasekharan *et al.* 2002), but genomic and kinetic analysis indicates that this selective interaction is unlikely to be clinically relevant (Bertolini *et al.* 2006).

There are many other theories surrounding the pharmacology of paracetamol including a central serotonergic mechanism through agonism of 5HT-3, supported by the antagonism of paracetamol's analgesia by tropisetron and granisetron, (Alloui *et al.* 2002; Pickering *et al.* 2006; Pickering *et al.* 2007) and activation of cannabinoid receptors (Bertolini *et al.* 2006). Despite initial theories of similar mechanism to salicylates (Woodbury 1965) and *in vivo* effects similar to a COX-2 inhibitor, clinically today, paracetamol is almost unanimously considered to have no peripheral anti-inflammatory activity.

Research into this area is complicated by many factors: major differences in both *in vitro* and *in vivo* effects of paracetamol on PG biosynthesis (Danon *et al.* 1983); variability in PGHS activity depending on source of PGHS; problems controlling co-factor concentrations (Aronoff *et al.* 2006); and *in vivo* effects also being procedure dependent (Gray *et al.* 2005). In a comment still relevant today, Prescott wrote in his 1996 bibliographic review of all studies researching into the mechanism of action of paracetamol, that:

> *"Paracetamol may either inhibit, stimulate or have no effect on prostaglandin synthesis depending on the tissue selectivity, the source of the enzyme, the drug concentration, the presence or absence of cofactors and experimental conditions. The role of prostaglandins in the peripheral analgesic actions of paracetamol remain a subject for debate"* (Prescott 1996).

1.2.4.2 PHARMACOKINETIC PROPERTIES

At concentrations achieved following licensed doses the pharmacokinetics of paracetamol are linear, independent of dose and constant with repeated administration (Bannwarth *et al.* 2003). Each of the four factors that influence drug pharmacokinetics, absorption, distribution, metabolism and elimination are now discussed.

1.2.4.2.1 ABSORBTION OF PARACETAMOL

An advantage of paracetamol is that it is available for oral, IV or rectal administration.

1.2.4.2.1.1 ORAL

Paracetamol is rapidly absorbed following oral administration by passive transport according to first-order kinetics (Heading *et al.* 1973). In humans, the majority of paracetamol is absorbed from the jejunum (Ueno *et al.* 1995). The increased rate of

absorption from the small intestine is thought due to the greater surface area and hence absorptive capacity, compared with the stomach or colon (Prescott 1996). Accordingly, gastric emptying is the rate limiting step of absorption. Delays to gastric emptying have substantial impact on paracetamol absorption (Kennedy 1996). Peak plasma concentrations are obtained 0.5-1.5hrs after intake of standard tablets or capsules. With effervescent tablets, drug absorption and onset of action are more rapid than with conventional tablets. Oral paracetamol has an absorption half-life of 4.5 minutes with no lag time. There are approximately 25% first pass losses which vary with the dose and number of doses and accordingly bioavailability varies from 60 to 98% (Clissold 1986). Food does not affect the extent of absorption, but does reduce the rate by up to 49% in one study of healthy volunteers. The maximum plasma concentrations from 1g of paracetamol reduced from 12.6µg/mL to 6.38µg/mL between the fasted and fed states (Stillings *et al.* 2000).

1.2.4.2.1.2 RECTAL

Paracetamol absorption from the rectum is incomplete and slow when compared with oral absorption. There is wide variation in absorption between individuals and may be greater in children than adults (Hahn *et al.* 2000; Bannwarth *et al.* 2003; Pettersson *et al.* 2006). There is an absorption half-life of 35 minutes, with a 40 minute lag time and a reduced area-under the concentration time curve (Anderson *et al.* 1996). Because of this delay, peak concentrations are reduced and occur up to two hours after administration, resulting in unsatisfactory analgesia (Prescott 1996). The relative bioavailability of a suppository compared with a suspension has been shown to be as low as 30% but is generally considered to range from 60 to 90% (Dange *et al.* 1987). Rectal temperature can also effect the rate and extent of absorption, with a significant effect being shown in infants (van Lingen *et al.* 1999).

1.2.4.2.2 DISTRIBUTION OF PARACETAMOL

Paracetamol is rapidly distributed following IV administration with a half-time of less than 20 minutes (Clements *et al.* 1984) and has been modelled both a non-compartmental and two compartment model (Rawlins *et al.* 1977; Rowland *et al.* 1995; Allegaert *et al.* 2004; Liukas *et al.* 2011). It has a volume of distribution (Vd) 9L/kg (Flouvat *et al.* 2004) in adults and is similar in young and elderly subjects of either sex (Prescott 1996). At therapeutic concentrations, generally considered to be less than 30mg/L, paracetamol's protein

binding is negligible (Clissold 1986) but can increase up to 50% in overdose (Milligan *et al.* 1994; Klasco 2009). In obesity, the weight corrected Vd is reduced (Prescott 1980; Depre *et al.* 1992; Ward *et al.* 1999).

Paracetamol distributes relatively evenly between tissue and plasma with concentrations lowest in the fat and cerebrospinal fluid and highest in the liver and kidney. It is distributed into the brain and cerebrospinal fluid in small and variable amounts by passive diffusion. Here paracetamol has a delayed and sustained peak concentration that occurs two to three hours after administration (Bannwarth *et al.* 1992; Klasco 2009).

Paracetamol is also distributed in the saliva where its concentration is similar to that in plasma. Saliva can be seen as an ultra-filtrate of plasma and for some compounds the saliva: plasma ratio is constant (Fuhr *et al.* 1993; Fuhr *et al.* 1994). Secretion into saliva is suggested to be by passive diffusion, which will vary depending on the solubility of the drug in the saliva and salivary glands. Factors which determine this solubility include: the pH of saliva and lipid solubility, molecular mass, spatial configuration, pKa and extent of plasma protein binding of the drug (Häckel *et al.* 1996; Skopp *et al.* 1999). Small hydrophilic drugs show the best correlations, as the equilibration and equal partitioning between plasma and saliva is rapid enough to be clinically useful for monitoring.

Saliva sampling has been used for determining paracetamol pharmacokinetics, however there are some issues with this technique:

- Only unbound drug can partition into saliva, so alterations to plasma protein binding or plasma protein concentration can invalidate results;
- Saliva more accurately reflects arterial concentrations, rather than venous (Posti 1982), raising questions over the accuracy especially during the absorptive phase of oral formulations (Smith *et al.* 1991);
- Bias towards increased saliva concentrations are seen when compared with plasma values (Borin *et al.* 1989);
- The concentration of paracetamol's metabolites do not correlate, making it unreliable if metabolite patterns are needed (Al-Obaidy *et al.* 1995; Prescott 1996).

Saliva concentration still may have a clinical role in diagnosis and treatment of overdose; one study showed no false negatives, although false positives were seen (Wade *et al.* 2008). While there are obvious advantages over the more invasive plasma sampling

methods, more than one in five patients still reported difficulty with saliva sampling (Wade *et al.* 2008).

1.2.4.2.3 METABOLISM OF PARACETAMOL

Paracetamol's metabolism is complex, involving all of the body's major pathways of drug metabolism. It has a mixed, competitive and sequential biotransformation pattern shown in Figure 1.2-4, Figure 1.2-5 and Figure 1.2-6 that results from Phase I oxidation competing with Phase II conjugation (Clissold 1986; Zamek-Gliszczynski *et al.* 2006). While metabolism is commonly shown as Figure 1.2-5, which accounts for the majority of metabolites, there are also several short-lived metabolites that are shown in Figure 1.2-6.

The liver has the greatest mass of drug metabolising enzymes and is the primary site of paracetamol metabolism, although drug metabolising enzymes are present in many other places in the body (*e.g.* the blood, gut, liver, kidney, lungs). Typical products of metabolism are inactive, detoxified and more likely to be excreted in the urine or faeces (via bile) (Kennedy *et al.* 1998). The same metabolic pathways of paracetamol are used for and regulated by transformation of other substrates that naturally occur in the body *i.e.* cortisol and ketones. The role of Phase I and Phase II pathways in the metabolism of paracetamol is now considered.

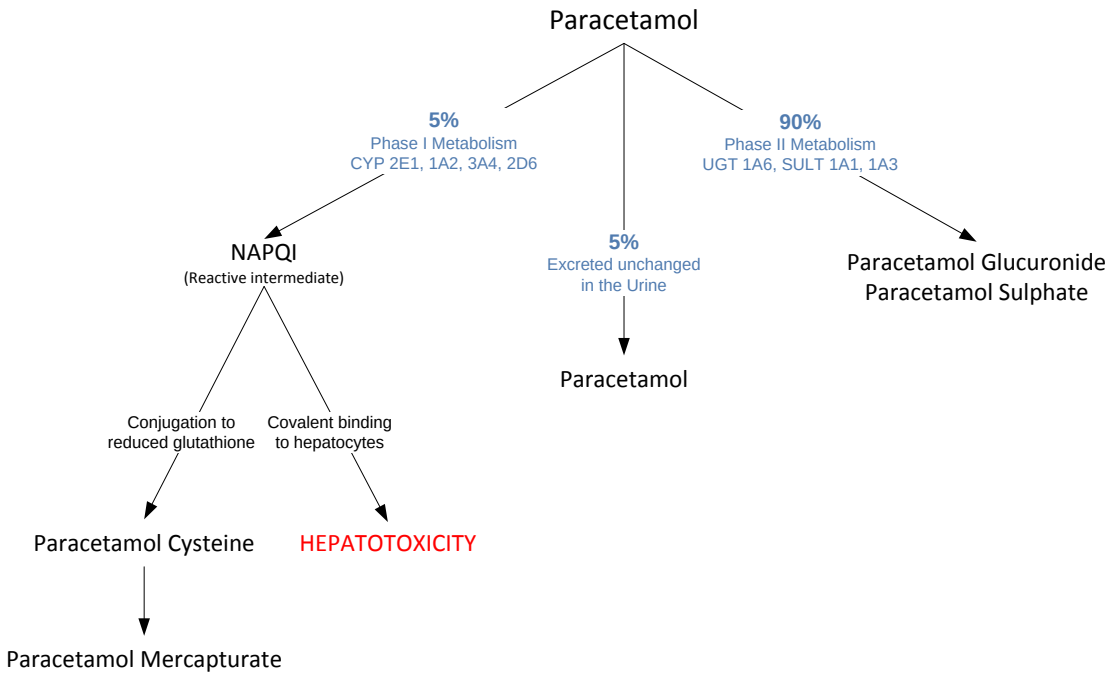


Figure 1.2-4 Metabolism of Paracetamol (Prescott 1996)

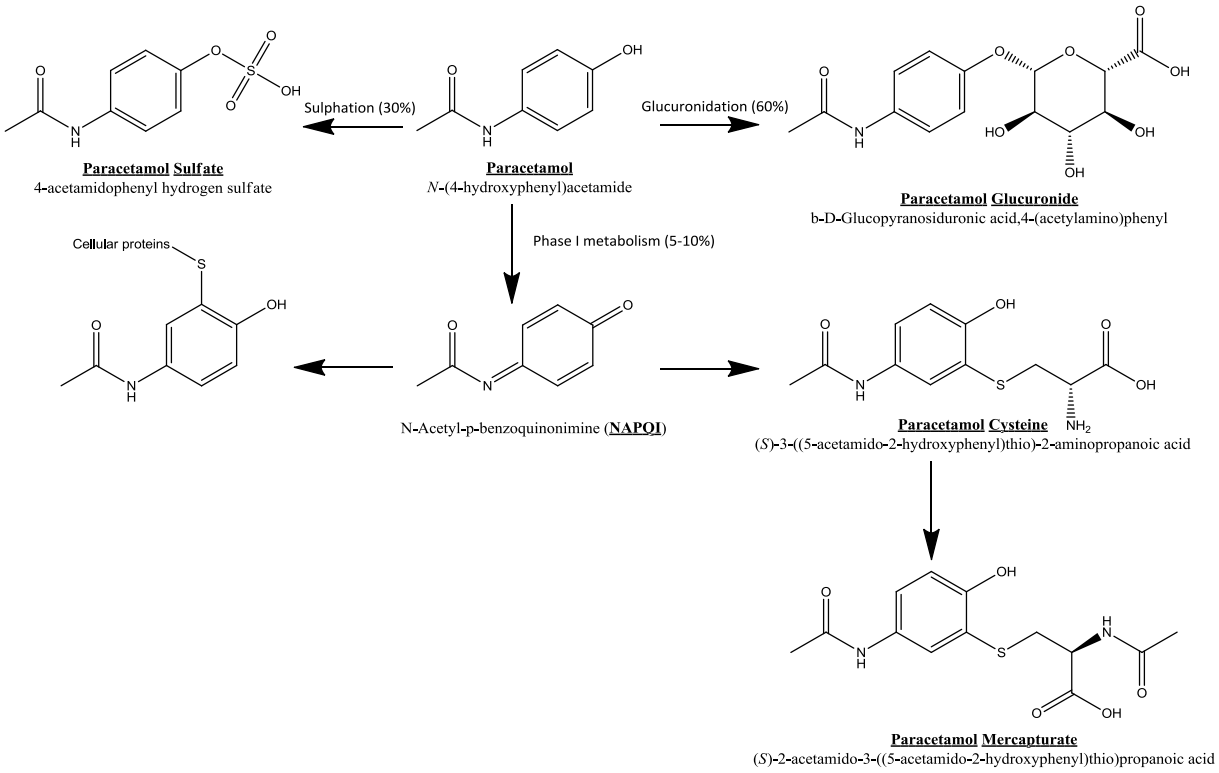


Figure 1.2-5 Paracetamol metabolism shown as structural formulas

1.2.4.2.3.1 PHASE I METABOLISM- THE ROLE OF CYP450 ENZYMES

The Phase I pathway is a minor but important contributor to paracetamol metabolism. Although involving only about 5-10% of a licensed dose, this pathway is important for two reasons:

- The toxicity of its product, NAPQI;
- The inconsistent activity and variable expression of the cytochrome P450 (CYP450) mixed function oxidase enzymes involved.

The production of NAPQI is the only toxic element of paracetamol's metabolism and it is the only dose-limiting factor (Zaher *et al.* 1998). NAPQI is a potent electrophile and is so toxic to the liver that it is often used as a model toxin to understand the mechanisms of hepatic cell injury and death (Davies *et al.* 1991; Elferink *et al.* 2008). It binds covalently to critical cellular proteins in hepatocytes resulting in covalent modification and inhibition of enzyme activity. Mitochondrial proteins are especially sensitive to these changes and ultimately this leads to a loss of mitochondrial energy production. Once intracellular antioxidants are depleted, NAPQI also causes genomic injury and cell death through both necrosis and apoptosis (James *et al.* 2003).

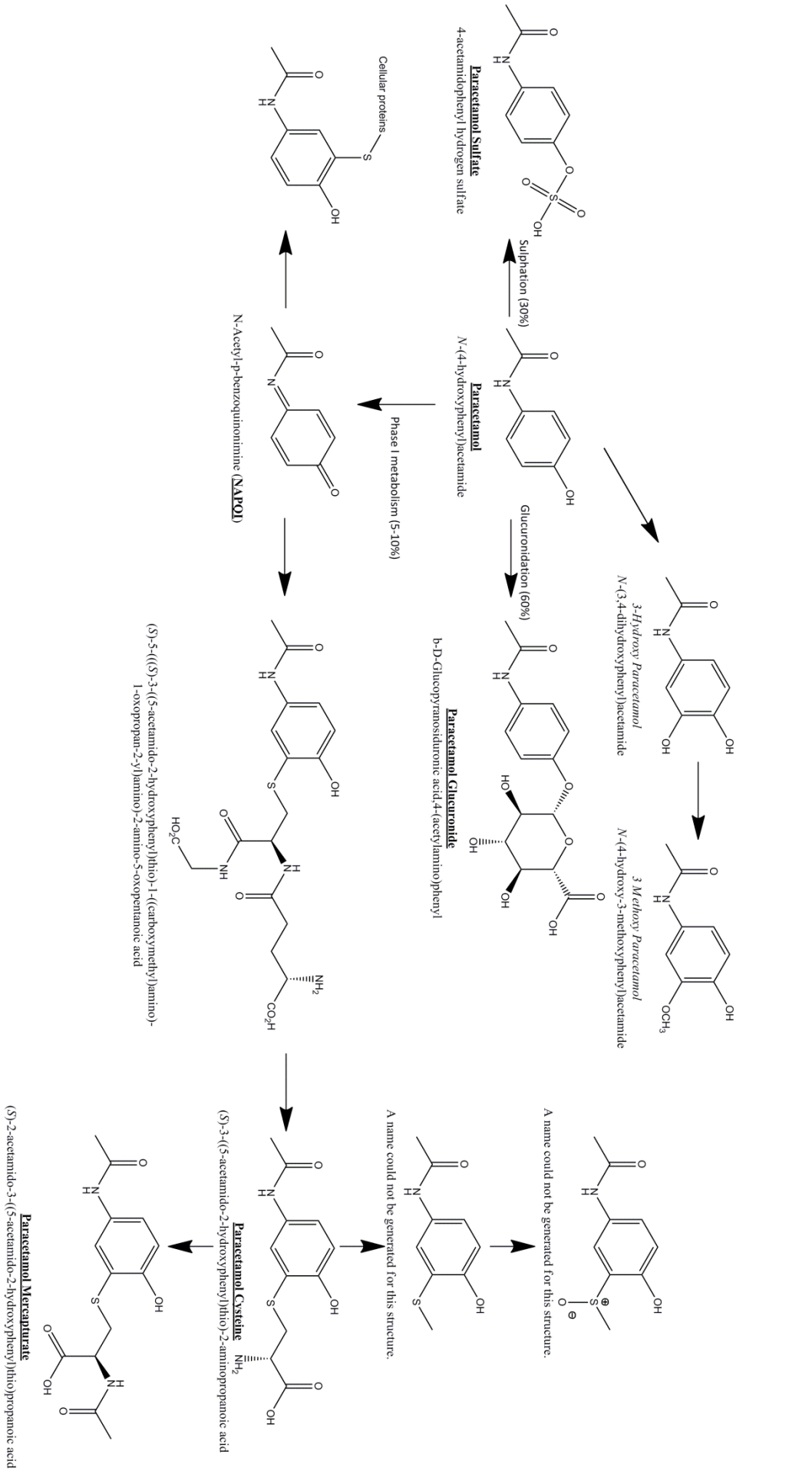


Figure 1.2-6 All elements of paracetamol metabolism showing all short-lived metabolites with major metabolites underlined in bold

Loss of energy production in the cell also compromises Phase II conjugation processes, processes that would otherwise detoxify NAPQI or prevent its formation (Dills *et al.* 1986)(section to 0). At worst, these processes culminate in fulminant liver failure and death (Hinson *et al.* 2004; Grypioti *et al.* 2006). The CYP450 enzymes which produce NAPQI are subject to genetic and environmental influence. These cause induction, inhibition and polymorphisms of these enzymes. Subsequently, CYP450 enzymes are a major source of inter and intra-patient variability in drug response and toxicity of paracetamol and many other drugs. Because of their frequent involvement in drug metabolism, the understanding of these changes is an area of intense research (Zamek-Gliszczynski *et al.* 2006). While the normal role of the CYP450 enzymes to paracetamol metabolism is discussed here, further details of the CYP450 enzymes are given in Section 1.5, while details of CYP450 changes relevant paracetamol metabolism in the surgical patient are given in section 1.3 and 1.4.

The main function of Phase I reactions is to add or reveal sites for Phase II reactions, and usually does not result in large changes to molecular weight or water solubility of the substrate/drug. Other enzyme families do contribute to Phase I metabolism of other drugs, but, as is the case with paracetamol, CYP450 enzymes account for the vast majority of Phase I metabolism.

The CYP450 family is comprised of many isoforms, all of which have differing affinity for substrates. Many isoforms of CYP450 have been shown to oxidise paracetamol to NAPQI *in vitro* and *in vivo*. While there can be considerable variation between individuals, these enzymes exhibit even greater inter-species preferential differences (Hong *et al.* 1987; Johansson *et al.* 1990; Schenker *et al.* 2001; Walubo *et al.* 2004). This is relevant to the study of paracetamol because, as a known toxin, much of the research pertinent to paracetamol use in stressed patients has been done in animal models. In humans, CYP2E1 has been shown consistently to account for over 90% of the Phase I metabolism of paracetamol at licensed doses (Anundi *et al.* 1993; Manyike *et al.* 2000). This has been confirmed by various inhibition and induction studies that have shown paracetamol's preference for CYP2E1. Post-mortem histological studies of patients who have died from paracetamol overdose have also shown that the zonation of hepatocyte death to be consistent with CYP2E1 distribution (Anundi *et al.* 1993; Manyike *et al.* 2000).

CYP2E1 metabolises very few other drugs. Furthermore, no other drugs in common clinical use are known to cause clinically significant induction or inhibition of CYP2E1, resulting in few drug interactions, further contributing to paracetamol's safety (Bannwarth *et al.* 2003).

At doses of paracetamol exceeding 1g in humans other CYP450 enzymes may be involved. CYP2E1 is a low capacity, high affinity enzyme and at higher concentrations of paracetamol it may be overwhelmed leading to the involvement of CYP450 enzymes CYP1A2, 2D6 and particularly 3A4 (Harvison *et al.* 1988b; Anundi *et al.* 1993; Prescott 1996; Frye *et al.* 1997; Chen *et al.* 1998; Tonge *et al.* 1998; Dong *et al.* 2000; Manyike *et al.* 2000; Zhu *et al.* 2001; Tanaka *et al.* 2003; Sharma *et al.* 2004; Zhou *et al.* 2004; Jaeschke *et al.* 2006; Laine *et al.* 2009). The low-capacity nature of CYP2E1 may explain some of the interspecies differences in paracetamol metabolism shown in studies. Doses used in animal studies typically greatly exceed those used in humans and such doses would increase the involvement of other CYP450 enzymes (Prescott 1996). Similarly, the use of supra-therapeutic doses in experimental animals may have also contributed to fears that some CYP450 inducers (*e.g.* rifampicin (Prescott *et al.* 1981), caffeine (Tsutsumi *et al.* 1989)) or inhibitors (*e.g.* cimetidine (Miners *et al.* 1984a)) could increase risk of paracetamol toxicity, all of which have not been shown in humans (Rumack 2004).

The product of the Phase I metabolism of paracetamol, NAPQI, is then subjected to further metabolism by Phase II pathways, along with 80-90% of the remaining, unmetabolised paracetamol.

1.2.4.2.3.2 PHASE II METABOLISM

Unusually, three Phase II reactions contribute to the metabolism of paracetamol: GSH conjugation, sulphonation and glucuronidation. Phase II reactions increase the water solubility and molecular weight of a compound and add a negative charge. Substrates of Phase II metabolism, such as paracetamol are normally lipophilic and diffuse into the intracellular space of the hepatocyte. Conversely, the products of Phase II conjugation are typically too hydrophilic to diffuse across the hepatocyte membrane and require active transport either across the canalicular membrane into bile or basolateral membrane into sinusoidal blood for later excretion into the urine (Zamek-Gliszczynski *et al.* 2006). Phase II metabolism can occur either:

- Directly on a parent drug containing an appropriate metabolic handle; or as more often is the case
- On functional groups added or exposed by Phase I oxidation reactions.

The first case is seen in the esterification of paracetamol's phenolic hydroxyl group with either glucuronic acid or sulphate, while GSH conjugation of the Phase I product NAPQI exemplifies the second case (Zamek-Gliszczynski *et al.* 2006). The relative contributions of these reactions are detailed in Figure 1.2-4 (Gregus *et al.* 1988). Sulphonation and glucuronidation reactions often compete for the same substrate (Mulder 1986).

Glucuronidation involves the transfer of glucuronic acid from its carrier uridine diphosphate-glucuronic acid (UDP-GA) to hydroxyl, amino, carboxyl and sulfhydryl groups resulting in a more acidic, water soluble compound (Kaushik *et al.* 2006). This transfer is undertaken by UDP-glucuronosyltransferase (UGT) enzymes which transfer the glucuronic acid. UDP-GA is synthesised in the cytoplasm and requires glucose-1-phosphate (G1P) conjugation to uridine diphosphate which is finally oxidised to UDP-GA. The preferential source of the G1P, whether endogenous or exogenous, is not known, however impaired glucuronidation in calorie malnutrition and hypoxia have been reported. Although oxygen is not required for this pathway, hypoxia is known to induce glycolysis, which in turn reduces the availability of glucose for UDP-GA formation. *In vitro* this can be overcome by endogenous glucose administration indicating that exogenous and endogenous sources of glucose have a role (Aw *et al.* 1984; Aw *et al.* 1991) (Figure 1.2-7).

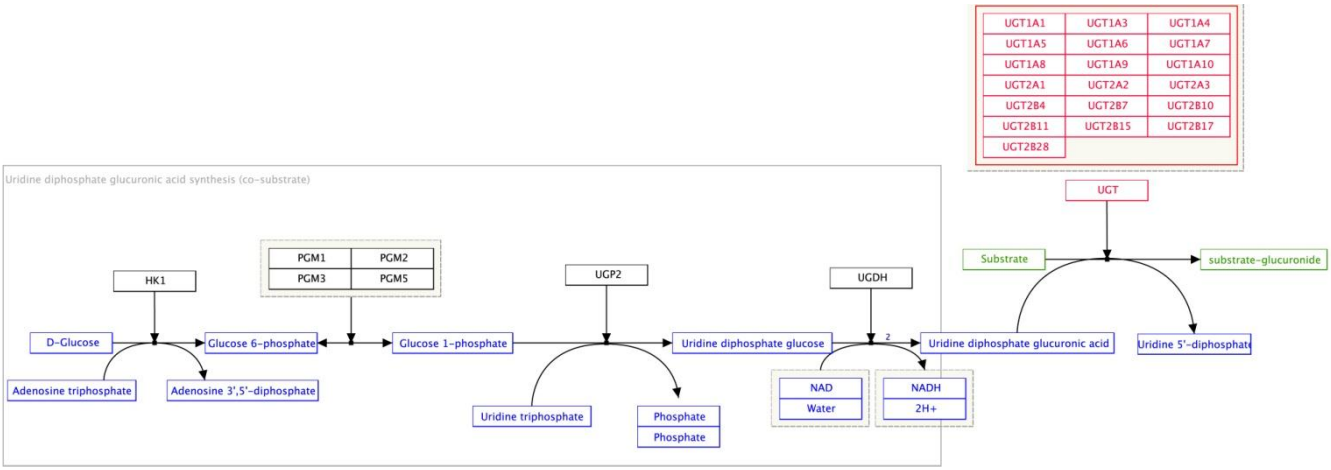


Figure 1.2-7 Glucuronidation pathway in Homo sapiens (Pico *et al.* 2008)

As with CYP450 enzymes, UGT enzymes are also sub-classified, with the isoform UGT1A6 largely responsible for paracetamol glucuronidation. UGT are relatively constitutive and undergo sparse modulation. Induction of UGT enzymes results in only a two to three fold increase in total protein, substantially less than seen with CYP450 enzymes (Zamek-

Gliszczynski *et al.* 2006). Glucuronidated compounds are generally pharmacologically inactive because their lipid solubility is reduced, making crossing cell membranes unlikely, and their structure is altered, which modifies their interaction with receptors (Kaushik *et al.* 2006).

Paracetamol is also a substrate of two sulphotransferases (SULT), 1A1 and 1A3. These enzymes specialise in catalysing sulphonation of hydroxyl groups and monoamine groups on phenolic-type molecules. Sulphonation is governed by availability of inorganic sulphate and the rate of SULT activity; the depletion of the former and the saturation of the latter leads to the sulphonation being overwhelmed (Zamek-Gliszczynski *et al.* 2006). The inorganic sulphur is essential for the synthesis of the co-factor 3'-phosphoadenosine-5' phosphosulphate (PAPS) (Figure 1.2-8) and is absorbed from the diet or produced from oxidation of sulphur containing amino acids. The high affinity, low capacity nature of SULT enzymes and low concentration of co-factor mean that, while sulphonation predominates over other processes at low substrate concentrations, they are saturated readily. Once overwhelmed, glucuronide conjugation predominates as substrate concentrations increase (Zamek-Gliszczynski *et al.* 2006).

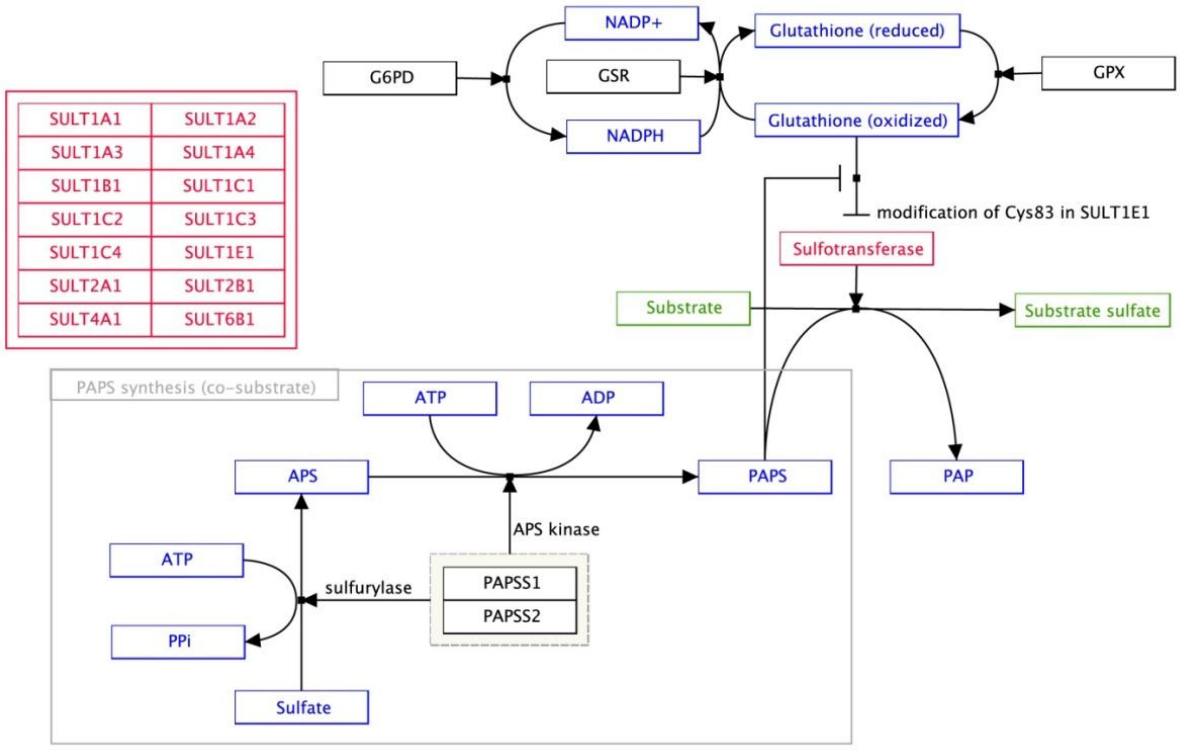


Figure 1.2-8 Sulphonation pathway in Homo sapiens (Pico *et al.* 2008)

Sulphonation often works in tandem with glucuronidation on overlapping substances. In paracetamol metabolism sulphonation plays a smaller role (30-35%) than glucuronidation (50-60%) at licensed doses (Gregus *et al.* 1988). In addition to differing enzyme affinity,

the predominance of glucuronidation over sulphonation may be explained by the concentrations of their respective co-factors. Hepatic PAPS has a concentration in humans of ≈23 nmol/g liver compared with ≈300 nmol/g liver of UDP-GA. The hepatic PAPS concentration is low enough to be depleted rapidly and is at concentrations lower than those required for maximum SULT velocity (Zamek-Gliszczynski *et al.* 2006). PAPS can be synthesised quickly and it is the supply and hepatocellular concentrations of inorganic sulphate, not the hepatic biosynthesis of this co-factor that is thought to be the rate limiting step of sulphonation *in vivo* (Hjelle *et al.* 1985; Zamek-Gliszczynski *et al.* 2006). Inorganic sulphate too is much lower than its glucuronidation counterpart G1P. Whereas G1P is essential for cellular respiration and in high concentrations, the inorganic sulphate is supplied from catabolic processes that supply and breakdown cysteine. Accordingly as inorganic sulphate concentration dwindles, PAPS is depleted rapidly and sulphonation is reduced (Mulder *et al.* 1978; Hjelle *et al.* 1985; Kim *et al.* 1992). While this exhaustion of sulphonation co-factor occurs readily in man, saturation of glucuronidation co-factor is virtually unachievable, requiring plasma paracetamol concentrations 100 fold greater than those needed for sulphonation exhaustion (Reith *et al.* 2009), equating to massive paracetamol doses of several 100mg/kg (Hjelle *et al.* 1985; Zamek-Gliszczynski *et al.* 2006; Lee *et al.* 2007). Despite consistent reductions in sulphonation with increasing doses of paracetamol, the result is that the combined rate of sulphonation and glucuronidation reactions is linear and the hepatic extraction ratio of paracetamol remains relatively constant (Clements *et al.* 1984).

Whereas sulphonation activity is highest in the periportal region of the liver, where xenobiotics are first presented, glucuronidation mostly occurs in the perivenous region, where Phase I oxidation greatest, on whose products Phase II reactions generally depend (Anundi et al. 1993; Zamek-Gliszczynski et al. 2006). As paracetamol doses are increased or repeated, sulphonation processes are unable to cope and paracetamol continues towards the centre of the lobule and into areas of increased glucuronidation (Clements et al. 1984). While this reduction could be due to co-factor exhaustion, it could also occur as a result of enzyme saturation. Complicating this differentiation is the critical differences in sulphonation activity between mice and rats, in which the majority of these studies are based. Whereas sulphonation in rats is limited by depletion of PAPS, in mice it is limited by SULT activity (Dalhoff et al. 1993; Kim et al. 1995a; Liu et al. 1996).

From this discussion it may seem that glucuronidation makes sulphonation redundant, however sulphonation's role in paracetamol metabolism is still an important one in preventing toxicity. This importance of sulphonation is demonstrated in two groups of individuals. Firstly, children have greater expression of SULT enzymes compared with adults and this is thought to confer their tolerance for much greater mg/kg doses of paracetamol (van Lingen *et al.* 1999; van der Marel *et al.* 2003; Allegaert *et al.* 2004). Secondly are those with defective transport of inorganic sulphate into the cell. These individuals have reduced sulphonation capacity and are more susceptible paracetamol-induced hepatotoxicity (Lee *et al.* 2006). Indeed, genetic variations that impair almost every aspect of sulphonation have been identified and linked to increased sensitivity to paracetamol hepatotoxicity (Nowell *et al.* 2006; Dawson *et al.* 2007).

Both glucuronide and sulphate conjugates have much smaller Vd than paracetamol and their rates of formation are much greater than their excretion, both of which lead to their accumulation in plasma (Sahajwalla *et al.* 1991; Miners *et al.* 1992; Haderslev *et al.* 1998).

Another Phase II reaction, GSH conjugation, takes place on the product of the 5-10% of the paracetamol dose metabolised by the Phase I pathway, NAPQI. As a potent electrophile and Phase I metabolite, NAPQI is a typical substrate for GSH conjugation. GSH conjugation results in the detoxification of NAPQI which could otherwise bind covalently to intracellular macromolecules (Corcoran *et al.* 1980; Nelson 1990; Tirmenstein *et al.* 1990). Similarly to its substrate NAPQI, GSH conjugation accounts for only a small percentage of the overall phase II metabolism but without it paracetamol would be universally toxic at licensed doses (Tirmenstein *et al.* 1990).

GSH is a substrate naturally occurring in the body, but the body's ability to synthesise GSH is limited. GSH is synthesised by the formation of a peptide bond between glutamic acid and cysteine, followed by the addition of glycine. GSH is in high concentrations in the liver and conjugation may occur spontaneously, although it is much more efficient when catalysed by glutathione S-transferase (GST). Hepatic concentrations of GSH are by far the highest of the co-factors utilised in the three Phase II reactions discussed. Consequently intracellular GSH is difficult to deplete, although it can be accomplished with extremely high amounts of substrate. Hepatic supplies of reduced GSH begin to be depleted over a range of 0.5 to 3gm of paracetamol (Lane *et al.* 2002). A toxic dose of paracetamol can deplete GSH levels by as much as 90% (James *et al.* 2003; Buzaleh *et al.* 2005). Anaesthetics place an additional demand on GSH in the surgical patient, reducing stores

by as much as 30%. With GSH depleted, severe hepatotoxicity can occur. This is the situation following overdose of paracetamol. Here the large amount of NAPQI overwhelms available stores of GSH. If not bound by GSH, NAPQI is free to bind to hepatocellular proteins as discussed above. Accordingly, it is the body's limited ability to synthesise GSH that determines the safe dose of paracetamol and why single high doses are more toxic that the equivalent divided dose. Any increase in the ratio of NAPQI to GSH generally results in toxicity. This can occur when too much paracetamol is given or when changes to metabolism produce more NAPQI, or less GSH. Potential causes of increased apparent production of NAPQI are discussed further below and in Sections 1.3 and 1.4.

GSH production can be increased with the administration of additional N-acetyl-cysteine, which is used clinically as the antidote to paracetamol toxicity. GSH conjugates are commonly hydrolysed sequentially to form a cysteine metabolite followed by N-acetylation to form a mercapturate metabolite, as is the case with paracetamol (Prescott 1996; Zamek-Gliszczynski *et al.* 2006).

In the Phase II metabolism of paracetamol, the per cent of paracetamol metabolised by sulphonation decreases with increasing doses (Clements *et al.* 1984). This in itself is not of concern as glucuronidation is more than capable of maintaining paracetamol's Phase II metabolism. What is of concern is the complex interaction between sulphonation, GSH conjugation and their respective co-factors (Figure 1.2-9) and if this exhaustion of sulphonation could reflect and even cause deficiency of GSH and reduce conjugation of NAPQI (Mannery *et al.* 2010). This is because cysteine can be used as a precursor for both sulphonation and GSH conjugation.

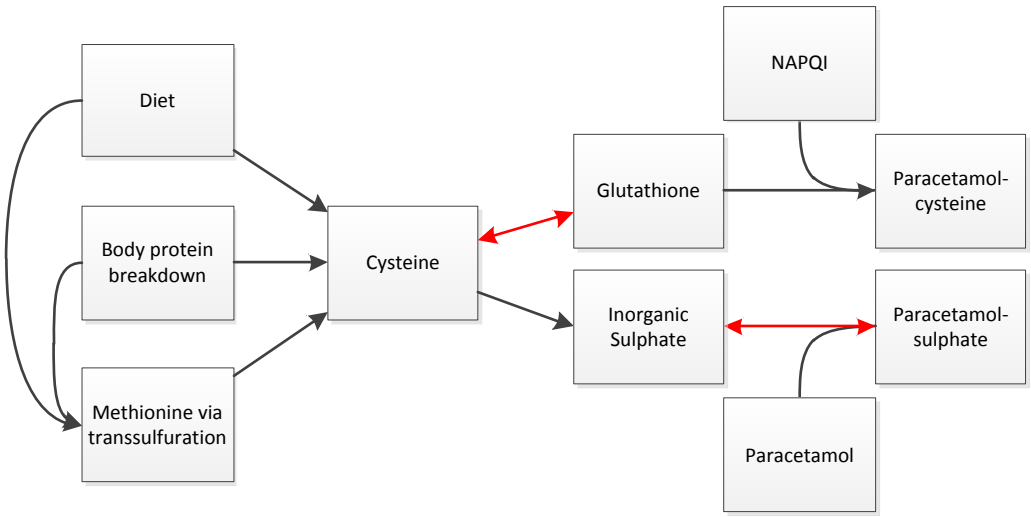


Figure 1.2-9 Sources and fate of cysteine in paracetamol metabolism. GSH is both a source and consumer of cysteine. Inhibition of sulphonation does not increase GSH synthesis (adapted from (Lu 1999))

The complexity of the interaction is demonstrated following administration of the antidote to paracetamol poisoning, n-acetyl cysteine which, as mentioned above, is merely a source of cysteine. This alters the pattern of urine excretion of paracetamol, increasing the GSH conjugates by 10%, but also resulted in a substantial 27% increase in the excretion of the sulphate conjugate (Lauterburg *et al.* 1983; Slattery *et al.* 1987).

The demands for sulphonation *in vivo* are quantitatively greater than that of GSH conjugation, not only for paracetamol metabolism, but for homeostasis in general. When there are demands on both sulphonation and GSH, cysteine is diverted from GSH biosynthesis to synthesis of PAPS in an attempt to preserve sulphonation capacity (Dalhoff *et al.* 1993).

In addition to these limitations on synthesis, dietary sources in fasting, malnourished or elderly patients are normally not sufficient in cysteine for paracetamol metabolism anyway (Reicks *et al.* 1988; Gregus *et al.* 1994a; Gregus *et al.* 1994b; Raguso *et al.* 2000; Joint FAO/WHO/UNU Expert Consultation on Protein and Amino Acid Requirements in Human Nutrition 2007; Nimni *et al.* 2007; Mannery *et al.* 2010). When demands exceed dietary intake, cysteine is produced endogenously by catabolism of one of three sources (in descending order of prominence):

- Methionine and serine (via the transsulphuration pathway);
- GSH stores; and
- Body protein (Di Buono *et al.* 2003; Pujos-Guillot *et al.* 2011).

In those with diets insufficient in cysteine, supplies of methionine are also likely to be lacking as they are generally present in a 1:1 ratio (Nimni *et al.* 2007). If dietary supplies of cysteine or methionine are limited, further cysteine can be supplied from catabolism of GSH. This is limited by the supply of GSH and occurs at the expense of GSH conjugation (Moriarty-Craige *et al.* 2004). This source of cysteine is especially relevant to paracetamol metabolism as it is GSH that detoxifies NAPQI, and the breakdown of GSH for cysteine may harm this process and increase the risk of paracetamol toxicity (Blackledge *et al.* 1991; Wu *et al.* 2004). The depletion of GSH stores for cysteine was shown initially in experimental animals given diets deficient in cysteine and methionine which led to a GSH reduction of up to 60% (Rozman *et al.* 1992) More recent evidence in humans support this observation with anorexic individuals deficient in sulphur amino acid intake shown to have 30% less GSH than controls (Zenger *et al.* 2004). The extent of paracetamol's

demands on this system were shown when therapeutic doses were shown to significantly decrease plasma GSH concentrations above that consumed in GSH conjugation, showing its role as a pool of cysteine (Moriarty-Craige *et al.* 2004; Mannery *et al.* 2010).

The final source, body protein, is catabolised at the expense of muscle growth and repair (McLean *et al.* 1989) and it is this protein catabolism for inorganic sulphate that is thought to determine the rate of obligatory nitrogen loss (Millward 1998). Fasting patients receiving 4g of paracetamol a day are estimated to mobilise up to 50g of skeletal muscle per day to supply the cysteine required for paracetamol's metabolism and homeostasis (Pujos-Guillot *et al.* 2011). The implications of this skeletal muscle catabolism were demonstrated in rats fed a diet of 1% paracetamol devoid of sulphur containing amino acids. These rats failed to grow until cysteine and methionine were replaced in their diet, a phenomena not seen with this dietary restriction alone (McLean *et al.* 1989). A similar study in rats given methionine-deficient diets showed prolonged paracetamol administration caused significant weight loss through protein catabolism (Reicks *et al.* 1988). In humans, colon carcinoma cells have shown similar results with cysteine deficiency inhibiting cell division and decreasing intracellular GSH concentration (Miller *et al.* 2002).

Final hurdles may arise to the sulphonation of paracetamol, even in the presence of adequate plasma concentrations. Inorganic sulphate, once sourced from the catabolic processes detailed above, must be then transported into the cell (Hendrix-Treacy *et al.* 1986). Like many of the transporter molecules in the body the activity of these transporters are affected by stress. Even if the rate of formation was unaffected membrane bound transport molecules must transport the newly sulphonated compound back out of the cell (Buist *et al.* 2003; Chu *et al.* 2004). These transporters are also suspected to be downregulated in times of stress (Chu *et al.* 2004).

Despite the evidence for sulphate depletion, there is also support for saturation of sulphotransferase activity as the major factor restricting the rate of paracetamol sulphonation, not the depletion of inorganic sulphate (Blackledge *et al.* 1991). This would be a preferable situation for the safety of paracetamol as GSH conjugation of NAPQI would be unaffected. In chronic dosing studies, recovery of paracetamol, particularly as the sulphate conjugate, drops along with total urinary sulphate output (Pujos-Guillot *et al.* 2011), but in all cases there was still *some* inorganic sulphur in the urine (Blackledge *et al.* 1991), and plasma concentrations of GSH, plasma sulphates and amino acids were

largely unaffected (Mannery *et al.* 2010; Pujos-Guillot *et al.* 2011). When overdoses of paracetamol have been taken over prolonged periods paracetamol sulphate is produced and inorganic sulphate is still quantifiable in plasma. One alternative explanation for this is that shifts towards glucuronidation occur well before free sulphate stores are depleted (Blackledge *et al.* 1991) and this suggests that plasma levels of inorganic sulphate must be sufficiently high for sulphonation to work effectively. This may be caused by limitations of the transport of cofactors. Cofactors of sulphonation are synthesised intra-cellularly and rely on active transport to maintain intracellular concentrations of their precursors, mainly inorganic sulphate (Lee *et al.* 2006). Demand may exceed the capacity of these sulphate transporters. Alternatively homeostatic inhibition of these transporters may occur given the essential roles sulphonation has. Several critical metabolically active hormones are deactivated by inorganic sulphate including the catecholamines, norepinephrine and dopamine amongst many other (Roth *et al.* 1982; Kauffman 2004), which are increased following surgery (Yoshizumi *et al.* 1998).

While plasma concentrations of these sulphur compounds have been shown to remain steady, what is missing from this story is what is happening to the hepatocellular concentration of these compounds.

There is a substantial difference in plasma concentration of inorganic sulphate during the day, with concentrations exhibiting a circadian rhythm, lowest at 1100 and highest 1900 hours with a 25.8% average difference between the high and low concentrations. In one study repeated administration of 650mg paracetamol four times a day for four day reduced inorganic sulphate by 30.1%. Circadian rhythm still occurred, with the difference between the high and low increasing to 31.3% (Hoffman *et al.* 1990). This study also showed reduced renal excretion and renal clearance of inorganic sulphate by 51 and 33% respectively (Hoffman *et al.* 1990)

Additionally, the increase in catecholamines that arise from the stress response may compete with paracetamol for metabolism by SULTs but also are known to suppress the activity of transporter molecules (Morgan *et al.* 2008). The findings of Slattery *et al,* that administration of n-acetylcysteine dramatically increases the formation clearance of sulphate and GSH conjugates, support this proposition. This also illuminates the different mechanisms behind the toxicity of acute and chronic paracetamol overdose. While acute overdose brings about the immediate exhaustion of GSH stores, chronic usage causes the gradual subjugation of GSH synthesis to NAPQI production (Slattery *et al.* 1987).

Dietary deficiencies of sulphate and cysteine alone has no major effect on paracetamol conjugation but their combined deficiency caused a significant decrease in excretion of the sulphate conjugate (Krijgsheld *et al.* 1981)Serum inorganic sulphate levels were elevated in patients who take paracetamol regularly (Hendrix-Treacy *et al.* 1986), and there is evidence the endogenous synthesis of cysteine is increased.

Finally, this interaction between sulphonation and GSH conjugation raises questions over the reliability of previous assessments of Phase I metabolism of paracetamol which is generally obtained through measures of paracetamol cysteine and its metabolite paracetamol mercapturate in urine. If GSH supply was limited, this may also restrict conjugation of NAPQI and produce less paracetamol cysteine. It would follow that recovery of paracetamol would reduce with chronic and elevated doses and this has indeed been shown in these situations (Pujos-Guillot *et al.* 2011). After 14 days of receiving 3g/day of paracetamol, a group of 10 elderly men and women had overall reductions urinary excretion of paracetamol to only 77% of the daily dose at day fourteen (Pujos-Guillot *et al.* 2011). This value is substantially lower than the 85-95% recovery reported in healthy adults (Forrest *et al.* 1982). Excretion of paracetamol sulphate was particularly effected, reducing to less than 20% of the total paracetamol excreted in the urine, compared to the 30-35% reported in healthy adults (Clements *et al.* 1984). Over the 14 day course substantial amounts of sulphur were excreted in the urine but despite this there was no significant effect on plasma sulphate concentrations or amounts of sulphur containing amino acids. This indicated activity of homeostatic mechanisms to maintain sulphate concentrations and indeed evidence of increased activity of the transulphonation pathway was shown. Curiously these patients spontaneously increased their dietary protein intake (Pujos-Guillot *et al.* 2011).

1.2.4.2.3.3 SATURATION OF PARACETAMOL METABOLISM

As discussed above, sulphonation has a limited capacity in man. A study comparing paracetamol doses of 20mg/kg with 5mg/kg, showed dose-dependent sulphate conjugation of paracetamol after both IV and oral administration (Clements *et al.* 1984). The reduction of the sulphate conjugation at higher doses is matched by increases in glucuronidation. This study also showed much lower renal clearance of paracetamol sulphate at the higher dose, indicating saturation of both the formation and the renal clearance of the sulphate conjugate. It also observed that higher doses of paracetamol lead to a reduction in paracetamol's plasma clearance.

A more recently published study by Gelotte *et al.* (2007) examined the disposition of paracetamol in healthy young adults at doses of 4, 6 and 8g/day for three days. Gelotte *et al.* suggests there is evidence that with increasing doses, paracetamol induces its own glucuronidation, and that the increase exceeds the reduction of sulphonation with a net increase in paracetamol clearance overall (Gelotte *et al.* 2007). The same authors also showed that with increasing dose there were statistically significant increases in recovery of paracetamol and paracetamol glucuronide but reduction in sulphate in the urine. There was no change in the percentage excreted as thiols either in the urine or plasma. The authors considered the increase in glucuronidation explained the less than dose-proportional increase in plasma paracetamol concentration and the absence of a more than dose proportional increase in excretion through NAPQI and GSH conjugation, which would be expected given the saturation of sulphonation. As part of this, these authors postulate paracetamol to be an inducer of UGT1A6.

The reduction in sulphate excretion has also been shown in other studies to occur on the fourth day when patients received lower doses of 650mg every six hours (Hendrix-Treacy *et al.* 1986; Hindmarsh *et al.* 1991). These studies observed a reduction in serum sulphate levels and reduced renal clearance of inorganic sulphate after four days of 650mg paracetamol every six hours (Hoffman *et al.* 1990). However, the study by Hendrix-Treacy *et al.* examining kinetics and disposition of the same dose, 650mg every six hours, after chronic dosing, found an increase in plasma sulphate level, suggesting it is the sulphotransferases activity rather than sulphate depletion that leads to decreased sulphonation in most cases (Hendrix-Treacy *et al.* 1986).

1.2.4.2.4 EXCRETION OF PARACETAMOL

All metabolites of paracetamol are excreted in the urine. Biliary excretion is not an important pathway in man at licensed doses (Jayasinghe *et al.* 1986). Only 2.6% of any oral dose is excreted in bile, mostly as the cysteine conjugate. Renal excretion amounted to 91.3% of the total dose (Siegers *et al.* 1984).

Paracetamol has a clearance of 13.5L/h and a elimination half-life of two-four hours, which is prolonged in children and infants (Prescott 1996).

1.2.4.3 MAXIMUM DOSE OF PARACETAMOL

1.2.4.3.1 DOSE AND CONCENTRATIONS

The current licensed dose of 4g per day in Europe is not well founded on experimental evidence and is based on avoidance of toxicity rather than affecting efficacy(Bristol-Myers Squibb 2009). This may be due, in part, to paracetamol's wide therapeutic index. There are very few drugs in common use with such a wide mg/kg dose where a 45kg adult female would receive the same dose as a 120kg male (22 vs. 8mg/kg), nor are there many where the daily dose is considered in grams and not milligrams or micrograms. With regard to dose, paracetamol is a fairly blunt instrument.

There is no clear indication in the literature of an optimal analgesic dose or maximum safe dose of paracetamol, nor is there a clearly defined analgesic concentration for paracetamol. Ward *et al.* alerted the pain management community to this dearth of information in a prominent journal in 1999, but the question of an analgesic concentration still remains unanswered. Prescott refers to a concentration range for achieving antipyresis as "probably about 5-20mg/L" (Prescott 1996) but he and more recent authors continue to point out that there are few studies in which drug concentrations and effects have been measured simultaneously in man (Gibb *et al.* 2008). It is generally accepted the concentration for analgesia is higher than that for antipyresis, as suggested by Beck *et al*. (Beck *et al.* 2000) and *in vitro* paracetamol has been shown to produce dose-dependent depression of nociceptive activity in rat and human models (Carlsson *et al.* 1987). Replicating these *in vitro* findings in a clinical setting has proven elusive.

While the majority of drugs exhibit concentration-efficacy relationships, some researchers believe that there is no direct correlation between serum concentrations of paracetamol and its analgesic or antipyretic effect. They cite the poor correlation between time of peak plasma concentration and peak analgesia (Prescott 1996; Bannwarth *et al.* 2003). Other groups have shown a good correlation between plasma concentration and effectiveness, once a delayed-response was included (where the maximum effectiveness follows Cmax) (Seymour *et al.* 1981; Nielsen *et al.* 1992). Gibb and Anderson explain this discrepancy as the result of the response not being directly related to the concentration in the plasma, but rather an effect compartment, whose concentrations are proportional to those found in the cerebro-spinal-fluid (CSF) (Gibb *et al.* 2008). These authors support

this theory by suggesting the delay between the peak concentration in the plasma and the peak clinical effect is a result of the partitioning between these compartments. They also point out the concentration/time curve following oral dosing of paracetamol approaches bell shape with the absorption phase resembling the elimination phase. They suggest the symmetry of this curve may confound determining an exact therapeutic concentration as, for example, 10mg/L would occur on both sides of the curve's Cmax making it impossible to determine which one conferred the drug's effectiveness unless plasma concentrations are constantly monitored (Figure 1.2-10). These authors suggest that a significant difference in pain threshold does occur by one hour post-dose in association with a plasma concentration of 12mg/L (Gibb *et al.* 2008). Other authors still relate paracetamol's peak concentration to the central analgesic effect of paracetamol, but without reference to time, finding that intravenous paracetamol is at least as effective as oral formulations (Luthy *et al.* 1993). Although IV and oral formulations have equivalent areas of concentration/time curve, there are reports of greater and prolonged analgesic activity following IV administration ($p<0.01$) (Jarde *et al.* 1997).

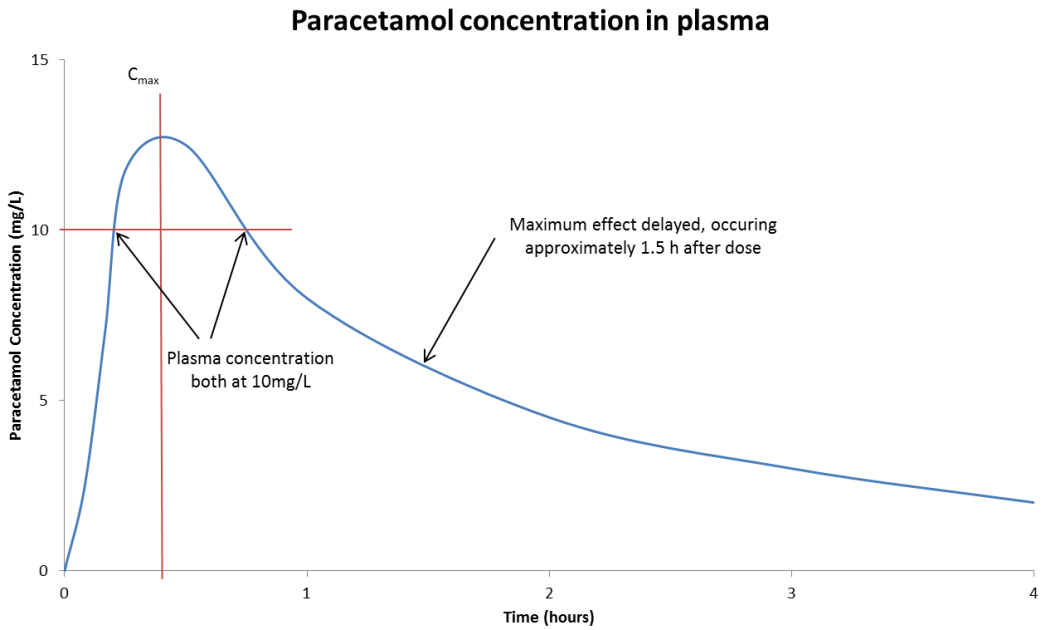


Figure 1.2-10 Time and concentration profile following a theoretical oral paracetamol dose
A concentration of 10mg/L is achieved before (absorption phase) and after (elimination phase) maximum concentration (Cmax). The delay between maximum concentration and peak effect is approximately one hour (adapted from (Gibb et al. 2008)).

Because of the lack of a clear therapeutic concentration and the subjective nature of pain, some research has used antipyresis as a surrogate marker of adequate concentration because it is easier to objectively measure and can be reproduced reliably with the administration of endotoxins (Kis *et al.* 2005). This method is also not without its criticisms:

- Body temperature is subject to circadian variation which may mask or exaggerate temperature reduction;
- The aetiology of the fever may affect the rate of fever reduction; and
- The initial temperature may influence the magnitude of drug response (Gibb *et al.* 2008).

One clinical trial examining the paracetamol concentrations of major surgical patients receiving rectal paracetamol 1g six hourly, concluded the dose to be too low, citing that the concentrations had failed to reach those required for antipyretic effect (10-20mg/L; ≈66-133µmol/L) (Kvalsvik *et al.* 2003). Similar results were shown in another study examining rectal doses of 20 or 40mg/kg in surgical patients. They also concluded that analgesic concentrations may not have been reached, even at the 40mg/kg dose (Beck *et al.* 2000).

Other research has shown no improvement in pain relief with increasing dose from 1g to 2g in a dental pain model (Skoglund *et al.* 1991). Conversely, a systematic review has shown the number of patients who need to receive the drug to achieve a 50% relief of pain (number needed to treat, NNT) reduced from 5 for a 600 or 650mg paracetamol dose to 3.6 for a 1g dose (Moore *et al.* 1997). This review included data from over 1000 patients involved in 29 studies of post-dental, postpartum and postoperative pain.

In paediatric patients, paracetamol is thought to produce dose related analgesia with 15-20mg/kg, reducing pain scores (Bolton *et al.* 2002; Tay *et al.* 2002) compared with 10mg/kg, which has not been shown to be superior to placebo and was associated with high requirements for supplemental analgesia (Watcha *et al.* 1992).

This uncertainty over the maximum dose, withdrawal of alternatives and repeated evidence of benefit, has contributed to an increasingly widespread trend of doses greater than the licensed adult dose (4g/24hr) being used in the perioperative environment. Loading doses of 2g intraoperatively have been shown to be superior to 1g in terms of magnitude and duration of analgesic effect, improving pain control and reducing morphine demand with no additional adverse effect (Juhl *et al.* 2006; Remy *et al.* 2006; Gregoire *et al.* 2007).

Higher doses of up to 6g/24hrs have shown similar results when compared with morphine patient-controlled analgesia usage alone (Schug *et al.* 1998). The usual dose of 1g every

six hours has a less than 10mg sparing effect on 24hr morphine consumption and does not significantly reduce morphine side effects (Sinatra *et al.* 2005).

There is some theoretical rationale for using doses above 4g/24hr. The theory of the "effect compartment" proposed by Gibb and Anderson, required paracetamol to distribute into and accumulate in theoretical third compartment for therapeutic effect. Accordingly, the simplest method to reduce paracetamol's time to effect is to increase the dose, in a similar way to the loading doses used for other drugs.

This has been shown experimentally. A dose of 1g every six hours has been shown to be insufficient even to produce antipyresis, however rectal doses of 40mg/kg did produce concentrations in this antipyretic range, although they were not shown to be clinically superior to 20mg/kg doses (Beck *et al.* 2000). The study by Korpela *et al.* demonstrated a linear relationship between increasing rectal paracetamol dose and analgesia and showed significant differences between 40 and 60mg/kg doses when compared with placebo. Another group measured the anti-nociceptive effect of 0.5, 1 and 2g of IV paracetamol in 11 healthy volunteers and found dose dependent reductions in nociceptive activity of 23, 28 and 40% respectively (Piguet *et al.* 1998). Researchers who showed paracetamol activity related to cannabinoid receptors also found that paracetamol inhibits COX in the brain, but at concentrations not attainable at current analgesic doses (Bertolini *et al.* 2006). A study by Temple *et al*. which gave healthy adults up to 2g every six hours for 3 days, found no clinically important elevations in aminotransferase levels during or for three days after the study (Temple *et al.* 2007)

1.3 CHANGES TO DRUG ABSORPTION, DISTRIBUTION, METABOLISM OR EXCRETION DUE TO SURGERY AND ANAESTHESIA

Surgery is a time when the body faces an extraordinary amount stress over a short period. Systemically, anxiety, pain, hemodynamic changes and cardiovascular instability (Barker *et al.* 1987; Kennedy *et al.* 1998) combine with local proliferation of pro-inflammatory cytokines, complement factors, acute phase proteins and pituitary hormones (Bone 1996). Their combined metabolic effect, referred to as the *'stress response'*, produces a catabolic, immunosuppressed state and activates the sympathetic nervous system (Bessey *et al.* 1993). The effect can persist for several days after surgery (Kehlet 1996) and is even greater in patients who have pre-existing pathology, a genetic predisposition to surgical stress or are of the male gender (Guillou 1993; Pape *et al.* 2000; Ono *et al.* 2005; Giannoudis *et al.* 2006). This stressed, catabolic state has many implications for drug therapy.

While the influence of the stress response can be substantial, surgery exposes the body to a vast array of other factors that can also alter drug disposition, especially when compared with a population of young healthy males, in whom most drug disposition studies are based.

On admission, surgical patients are likely to be of advancing age, have pre-existing pathologies, malnutrition and many will be taking multiple medicines. As a result of surgery they may also be kept from mobilising and may be kept *nil per oral,* fasted for many days before and after surgery. They will be exposed to a wide range of new drugs (patients undergoing major surgery receiving an average of 12 different medications) in addition to having their usual medication with-held (Kennedy *et al.* 2000). Withholding a patient's usual medication can lead to acute abstinence syndromes, such as the tachycardia and hypertension that follows abrupt withdrawal of beta-blockers (Nimmo *et al.* 1988). This, in addition to surgical trauma, places an extraordinary amount of stress on the body and changes the way it deals with medicines. It has been shown the longer patients are without their normal medication, the more non-surgical complications they suffer (Kennedy *et al.* 2000).

This section discusses the stress response experienced by the surgical patient. It is complicated, multifaceted and interlinked (Figure 1.3-1). The impact of the stress response on the various aspects of drug disposition; absorption, distribution, metabolism

and elimination are detailed below. Where available, the effect on paracetamol disposition is given, and where not, examples of drugs that share processes with paracetamol are used.

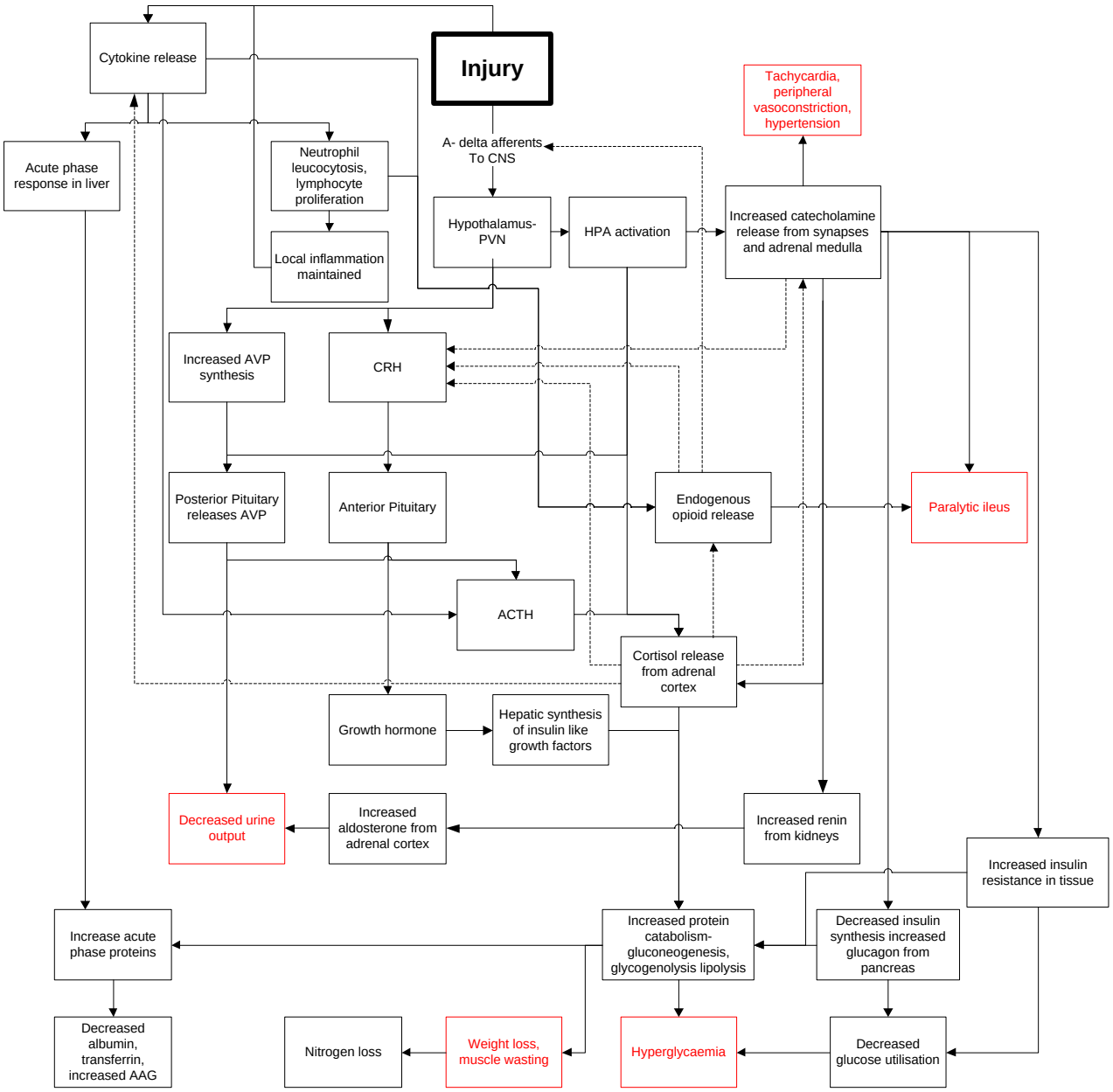


Figure 1.3-1 Stress response schematic showing its complexity of interlinking.

Solid lines indicate direction of stimulus, broken lines represent suppression. Red boxes show physical symptoms of stress response. Abbreviations used: AAG- α1-acid-glycoprotein; ACTH- adrenocorticotropic hormone; AVP- arginine vasopressin; CNS- central nervous system; CRH- corticotrophin releasing hormone; HPA- hypothalamic-pituitary adrenal axis; PVN- paraventricular nucleus;

1.3.1 STRESS RESPONSE

Evolution did not anticipate modern surgery with aseptic techniques and so the body's response to surgical trauma is as if the emergency was infection (Nathan 2002). The stress response is mounted in reply to this perceived microbial threat and, to a much lesser degree, the insult to cardiovascular homeostasis that also arises from surgery. The extent

of inflammatory stress is a key determinant of susceptibility to hepatotoxicity from paracetamol (Roth *et al.* 2010).

The stress response is an integrated hormonal and metabolic response, rather than a series of isolated reflexes. It occurs after injury or trauma and involves both hormonal and metabolic systems (Selye 1976). Creating a catabolic state, it is thought to be an evolutionary adaptation to allow injured animals to utilise their own fuel stores to survive convalescence, sparing glucose for use by neurones (Desborough 2000; Kopp Lugli *et al.* 2010). Other systemic changes include; sharpened attention, increased glucose utilisation and blood flow to the brain and muscle, modulation of the immune response, inhibition of reproductive physiology, decreased feeding and appetite, and water retention (Sapolsky 2000). Although virtually all of the body's systems are eventually affected by surgical trauma, the neuroendocrine, cardiovascular, gastrointestinal and immune systems are the first to show functional change and subsequent changes to drug disposition (Carrasco *et al.* 2003). The initiation of the stress response varies, depending on whether the stressor is an immediate or delayed risk to homeostasis. Stressors that are an immediate risk must be transmitted quickly and are processed via the limbic insensitive pathway. Delayed risks require sensory processing before becoming stressful (limbic stress pathway). An example of this is described in Figure 1.3-2.

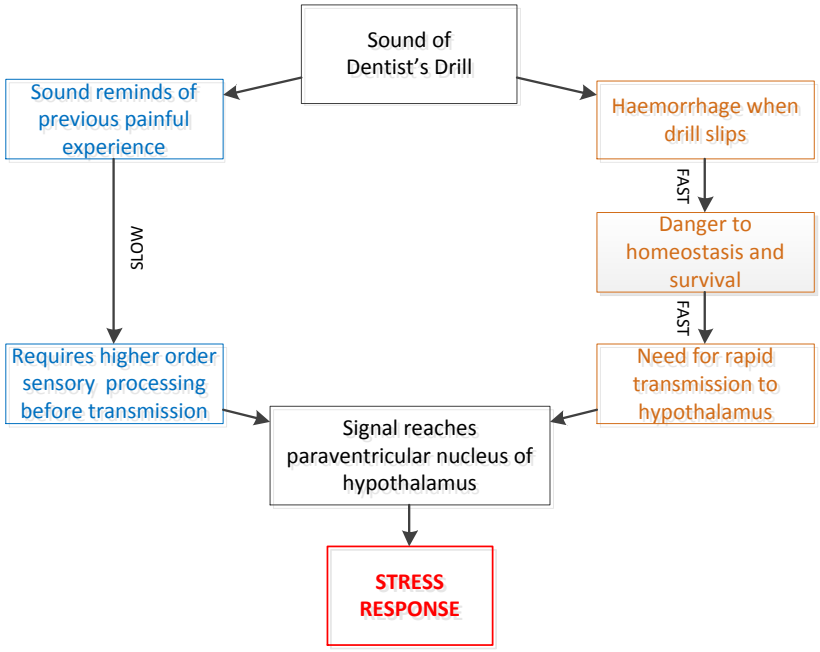


Figure 1.3-2 Initiation of Stress Response
Figure shows two pathways of initiation: limbic stress pathway (left) that is slower and occurs in response to less imminent danger, and limbic insensitive pathway (right) which is rapid and occurs when there is a perceived threat to survival(Drolet et al. 2001).

A slip of a dentist's drill is an immediate danger and requires an immediate response, whereas the sound of a dentist's drill is only stressful when compared with memory of

the drill slipping in the past. For an individual with previous dental trauma the sound of a dentist's drill evokes a stress response; palms sweat, senses are heightened *etc.*, but the response is not as quick as if a new incident had occurred (Herman *et al.* 1997).

Historically, the response to injury was thought to consist of three stages:

- Hypodynamic ebb phase (shock). In the first moments after injury blood flow is redirected to maintain perfusion of vital organs and minimise blood loss;
- Hyperdynamic flow phase. Blood flow is increased to remove waste and provide nutrients to the injured area to encourage repair in the first days after injury; and
- Recuperation phase. Lasting several months, this phase restores the body to pre-injury conditions (Smith *et al.* 1998).

While a useful overview, current opinion is that the response to injury is much more complicated than initially described (Giannoudis *et al.* 2006).

In modern surgical practice, the stress response, whose main function is the eradication of infection, is seen as unnecessary and indeed deleterious to recovery (Giannoudis *et al.* 2006). This is due to the widespread use of aseptic techniques and antibiotics, which have all but eliminated the dangers once posed by microbial invasion (Nathan 2002). Now the need for the surgical stress response has been reduced, progress in surgical care has arisen due to the developments in understanding of this response and, most importantly, techniques to modify or block it (Desborough 2000).

The changes to the endocrine and immune systems that arise during the stress response are complex and highly interactive. The normal physiological function of each of these systems will be briefly described, followed by their role in the stress response.

1.3.1.1 ENDOCRINE RESPONSE

The endocrine system is involved in homeostasis through the secretion of various hormones from endocrine glands throughout the body into the blood stream. The hormones travel in the blood to their target site where they interact with a receptor. The hormone-receptor binding is very specific. This triggers the target site to perform a specific action and can lead to:

- Change in function (ranging from that of a single cell through to a whole organ)
- Alteration of energy usage;

- The triggering of growth and development; or
- In the case of trophic hormones, triggering other endocrine glands to release other hormones.

This enables the endocrine system to regulate many of the other systems throughout the body in response to demands of homeostasis (Kohl *et al.* 2006).

1.3.1.1.1 INITIATION OF THE STRESS RESPONSE

From the time of incision, the activity of the endocrine system changes. Somatic and visceral afferent neuronal signals travel from the point of injury along sensory nerve roots through the dorsal root ganglion, up the spinal cord to the medulla. From there they pass through the amygdala and on to the hypothalamic paraventricular nucleus (PVN), bypassing the limbic system (Figure 1.3-2). These signals are thought to travel along the same fast conducting A-delta fibres as nociceptive signals (Kehlet 1989; Van de Kar *et al.* 1999; Desborough 2000).

Once at the PVN, the signal triggers the hypothalamic-pituitary-adrenal axis (HPA) and increases the production of releasing hormones(Egdahl 1959).

With respect to the stress response, there are two important releasing hormones secreted from the PVN: corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) (Whitnall 1993). These releasing hormones act on the posterior and anterior pituitary (Miner 2008). Following secretion, CRH goes into the pituitary portal circulation whereas AVP is secreted into posterior pituitary for storage and later release into the systemic circulation.

With the involvement of the locus coeruleus, the sympathetic nervous system is also activated by CRH, by increasing release of norepinephrine from presynaptic nerve terminals and increasing secretion of epinephrine from the adrenal medulla (de Kloet *et al.* 2008; Guest 2008).

The majority of these initial processes in the PVN are subject to modulation by endogenous opioids, most commonly enkephalin. Endogenous opiate production may be stimulated by leukocytes or inflammatory cytokines (Glattard *et al.* 2010). Opioids diminish stress-induced changes in the endocrine system and levels of enkephalin mRNA are increased in the PVN after acute stress(Lightman *et al.* 1987). The mechanism by which endogenous opiates act in modulation of the stress response is still uncertain,

although opioids have been shown to reduce peripheral nociceptive impulses, which may reduce stimulation of the hypothalamus (Drolet *et al.* 2001; Miner 2008; Glattard *et al.* 2010; Madbouly *et al.* 2010a).

1.3.1.1.2 EFFECT OF INCREASES IN SYMPATHETIC ACTIVITY

The increase in plasma concentration of norepinephrine and epinephrine is one of the first detectable changes of the stress response, occurring within minutes of incision. The increase in concentration is proportional to the extent of injury, although it is short-lived, returning to baseline concentrations within hours of the initial trauma (Douglas *et al.* 1989).

This increase in sympathetic tone contributes to the characteristic symptoms associated with stress, such as tachycardia, hypertension and peripheral vasoconstriction. The efferent sympathetic stimulation and circulating catecholamines also alter the function of visceral organs including the kidney, pancreas and liver.

In the kidney, increased circulatory concentrations of norepinephrine increase production of renin. Renin promotes the conversion of angiotensin I to angiotensin II. Angiotensin II is a potent vasoconstrictor and also promotes the secretion of aldosterone from the adrenal cortex (Ganong 2001). Aldosterone promotes sodium resorption from the distal convoluted tubule of the kidney, resulting in reduced urine production (Nicholson 2005).

In the pancreas, α-adrenergic inhibition by circulating catecholamines prevents β cells from secreting insulin, whilst glucagon secretion is transiently increased. Insulin is an anabolic hormone normally released from the pancreas after eating, in response to increasing concentrations of glucose and amino acids in the blood. Its function is threefold:

- Promoting glucose uptake and utilisation by cells;
- Promoting glucose storage in the liver as glycogen; and
- Inhibiting gluconeogenesis, the process of *de novo* glucose production by protein catabolism and lipolysis (Desborough 2000).

In the absence of insulin, hyperglycaemia develops.

The effects of inhibiting insulin secretion are further augmented by catecholamine-induced resistance of cells to the remaining insulin in circulation, and the increase in

glucagon release (Thorell *et al.* 1994; Guest 2008). Glucagon is a catabolic hormone that opposes the actions of insulin. It increases hepatic glycogenolysis and gluconeogenesis, although its role in postoperative hyperglycaemia is minor compared with the decreased insulin secretion and sensitivity (Ni Choileain *et al.* 2006). Failure of homeostatic mechanisms to maintain euglycaemia ensues.

Stressed individuals are therefore in a catabolic state, hyperglycaemic, oxidising skeletal muscle protein and fat stores for gluconeogenesis. This resultant hyperglycaemia after surgery may encourage wound infection, further impair wound healing and is a predictor of poor outcome in the critically ill (Bochicchio *et al.* 2005). The weight loss and muscle wasting as a result of the gluconeogenesis and lipolysis causes significant amounts of nitrogen to be excreted into the urine (Wilmore 1991). Urinary nitrogen concentration can be used as a marker of the extent of the stress response as can plasma glucose concentrations and insulin/glucagon ratios (Campbell *et al.* 1984; Anand *et al.* 1987; Bessey *et al.* 1993; Glaser *et al.* 1995).

1.3.1.1.3 EFFECT OF INCREASES IN PITUITARY HORMONE SECRETION

The pituitary is a major endocrine gland of homeostasis. While the pituitary releases the majority of the trophic hormones involved in the stress response, it remains under control of the hypothalamus. The pituitary is divided into two lobes, anterior (AP) and posterior (PP).

In response to the increased level of the trophic hormone CRH and the increased AVP synthesis in the PVN, the AP releases adrenocorticotropic hormone (ACTH). Stress also causes the release of growth hormone from the AP. The PP releases the AVP synthesised in the PVN in response to activation of the HPA. AVP has its own stimulatory paracrine effect on ACTH release, synergistically with CRH, creating a positive feedback loop. However, AVP's main function is as an anti-diuretic. AVP acts on cells that line the distal nephron, causing the translocation of aquaporin water channels from endosomes to the luminal membrane, promoting reabsorption of water. Additionally, constriction of vascular smooth muscle cells occurs (Guest 2008; Costello-Boerrigter *et al.* 2009).

Increasing amounts of ACTH enter the systemic circulation from the AP and travel to the adrenal cortex, stimulating the release of glucocorticoids. Norepinephrine released from the brain stem nuclei is also involved in glucocorticoid secretion but the pathways involved in this control are not clear (Ferreira-Silva *et al.* 2009). Surgery is one of the most

potent stimulators of ACTH, and cortisol release is measurable minutes after the commencement of surgery (Thoren 1974; Nicholson *et al.* 1998; Desborough 2000). Cortisol levels peak four to six hours after surgery, when they can be greater than four times normal. The extent of the increase depends on the severity of surgical trauma and anaesthetic intervention (Traynor *et al.* 1981; Chernow *et al.* 1987; Nicholson *et al.* 1998). This increase in glucocorticoid has a strong catabolic effect. This adds to the catecholamine stimulated changes in the pancreas, further reducing glucose utilisation and promoting catabolism through protein breakdown, lipolysis and gluconeogenesis (Kopp Lugli *et al.* 2010).

Glucocorticoids also have anti-inflammatory activity, inhibiting macrophage and neutrophil accumulation into areas of inflammation and interfering with inflammatory mediator synthesis, particularly PGs. This also has a suppressive effect on cytokine production, and as cytokines promote ACTH release, this forms a negative feedback loop. The cortisol response to surgery is sufficient to depress interleukin-6 (IL-6). IL-6 concentrations and have a suppressive effect on the immune system (Desborough 2000). Cortisol also inhibits the synthesis of CRH and ACTH (Guest 2008). Cortisol is so effective that synthetic analogs of cortisol are used clinically as anti-inflammatories *e.g.* hydrocortisone (Han *et al.* 2002).

The Growth Hormone, also released from the AP, further contributes to catabolism by stimulating the release of somatomedins, also known as insulin-like growth factors, which are mainly synthesised in the liver. Somatomedins normally promote cell growth and division, but also further stimulate glycogenolysis, lipolysis and reduce glucose uptake in the tissue (Guest 2008).

Thyroid function is also affected with reductions to thyroid stimulating hormone and tri-iodothyronine (T_3) secretion after surgery (Edwards 1997). This can lead to changes in both metabolic activity and oxygen consumption of most of the metabolically active tissues in the body, although this is offset by the close functional association with catecholamines, whose concentrations uniformly increase in the stress response (Thoren 1974; Desborough 2000). There are also increases in oxytocin and prolactin synthesis in the PVN, although the importance of these hormones to the stress response is uncertain (Guest 2008).

As a result of the endocrine elements of the stress response the surgical patient is:

- Sympathetically activated (tachycardic, hypertensive, sweating and pale);
- Catabolic;
- Hyperglycaemic;
- Oliguric;
- Peripherally vasoconstricted; and
- Immunosuppressed.

1.3.1.2 IMMUNE RESPONSE

The immune system is typically involved in the eradication of injured or foreign cells by producing an immune response. It is a complex combination of organs, tissues, cells and cell products such as antibodies and cytokines.

Tissue injury following surgical incision causes macrophages to migrate to the damaged area. This increases vascular permeability and generates high circulating concentrations of IL-1β, IL-6 along and tumour necrosis factor-α (TNF-α) at the site of injury (McMahon *et al.* 1993).

Although the major initiation of the stress response is from afferent neural stimuli, a variety of humoral factors are also involved, most of which arise from the immune response (Holte *et al.* 2002). Cytokine production at the site of injury, especially IL-6 and TNF-α, trigger release of subsequent components (Kehlet 1989). TNF-α is released first from activated macrophages and it stimulates the release of more cytokines, especially IL-6, the main cytokine for inducing the systemic changes of the stress response. IL-6 levels peak about 24 hours after surgery and remain elevated for 48-72 hours postoperatively (Sheeran *et al.* 1997). High levels of inflammatory cytokines are also associated with increased mortality and morbidity after surgery (Roumen *et al.* 1993a). The cytokines act locally mediating and maintaining the inflammatory response to tissue injury and initiate some of the systematic changes that occur.

Systemic changes from IL-6 release include the production of acute phase proteins in the liver, such as C-reactive protein (CRP), fibrinogen and other anti-proteinases. These acute phase proteins go on to modulate metabolic pathways and hormonal responses (Giannoudis *et al.* 2006). IL-6 also causes alterations in the synthesis of binding proteins, decreasing albumin and transferrin and, in most cases, increasing α_1-acid-glycoprotein (AAG)(Bourguignat *et al.* 1997). AAG's physiological role is in the regulation of the stress

response as a major steroid and catecholamine binder (Ganguly *et al.* 1967; Sager *et al.* 1987). However, AAG is also adept at binding basic and neutral lipophilic drugs, particularly bupivacaine and anti-retrovirals (Holley *et al.* 1984; Fournier *et al.* 2000; Israili *et al.* 2001). During the acute phase response, IL-6 also acts directly on the pituitary to stimulate ACTH and AVP secretion. This contributes to the initiation of the endocrine response and ultimately increases cortisol concentrations (Roumen *et al.* 1993a). Cortisol in turn has a suppressive effect on cytokine production, impairing the immune response, completing a negative feedback loop (Desborough 2000; Ni Choileain *et al.* 2006).

Studies examining cytokine patterns following surgery report early, transient rises in IL-1β and TNF-α followed by later and more sustained elevations in plasma IL-6 and cortisol concentrations. Peak IL-6 concentrations have been related to the duration of surgery (Cruickshank *et al.* 1990; Baigrie *et al.* 1992; Haas *et al.* 2003). One study examined metabolic responses of 158 patients receiving either hip or knee arthroplasty. They showed cortisol levels peaked within 12 hours of surgery, ahead of IL-6, which peaks on the second postoperative day (Hall *et al.* 2000). Laparoscopic and open bowel resections follow similar patterns, with most reports showing reduced cytokine levels following laparoscopic surgery (Leung *et al.* 2000; Schwenk *et al.* 2000; Veenhof *et al.* 2011).

Due to the immune response elements of the stress response the surgical patient has:

- Large accumulations of inflammatory cells and pro-inflammatory mediators at the site of injury;
- Increased vascular permeability at the site of injury;
- Perpetuation of the endocrine response; and
- Altered plasma protein binding.

1.3.1.3 INFLUENCE OF SURGERY

When performing a surgical procedure, surgeons must have a clear view of the operative field in order to perform their task. Historically, this involved a long and deep incision, the use of clamps to prevent bleeding and retractors to expose the site. As camera and monitor technologies advanced in the latter half of the 20th century their use to give a magnified view of the operative field to the surgeon on a monitor screen provided an alternative to traditional open surgery. The camera and other surgical instruments could be inserted through small incisions, or port sites, and manipulated by the surgeon from

the outside. This laparoscopic, minimally invasive approach is now routine for many diagnostic and surgical procedures. For a cholecystectomy, laparoscopic surgery typically involves three 0.5-1cm incisions for the insertion of instruments, compared with laparotomy, involving a 20-25cm incision through skin, subcutaneous tissue, three layers of muscle and then peritoneum. It is to be expected then that laparoscopic procedures cause less physical trauma to the patient, with less stimulation of the stress response and subsequent alteration to drug disposition.

A recent systematic review in 2009 by Vlug *et al.* compared laparoscopic techniques with open surgery. Identified in this review were a number of studies which had found favourable outcomes for laparoscopic surgery such as shorter hospital stays, and reductions in readmission, mortality and morbidity, but they were of poor design (Vlug *et al.* 2009). Because of the lack of good quality studies the review concluded there was as yet insufficient robust evidence of significant superiority of laparoscopic techniques over open surgery. However, there is little doubt that laparoscopic surgery is accompanied by less extensive tissue damage, painful stimuli, fluid shifts and necessity for opioid analgesia (Mythen 2005). Additionally surgery that minimises tissue dissection and retraction is likely to minimise the stress response (Giannoudis *et al.* 2006; Madbouly *et al.* 2010a). Indeed, a number of studies have shown reductions in some inflammatory markers accompanying laparoscopic techniques (Glaser *et al.* 1995; Kehlet 1999; Desborough 2000; Jess *et al.* 2000; Le Blanc-Louvry *et al.* 2000; Gupta *et al.* 2001).

Despite the predicted reductions in stress response this has not lead to a wholesale reduction in all inflammatory markers, and perioperatively markers of stress are often similar in the two surgical techniques (Le Blanc-Louvry *et al.* 2000). One study showing this compared the inflammatory response following open and laparoscopic inguinal hernia repairs (Jess *et al.* 2000). In terms of the stress response, TNF-α and IL-2 concentrations were not significantly different, although IL-6 concentrations rose significantly rose more sharply following open surgery ($p<0.00$). Additionally there were significantly shorter recovery times in the laparoscopic group (median recovery days for laparoscopic and open surgery 2 vs. 13 respectively). Other studies have reported similar trends (Mansour *et al.* 1992; Bruce *et al.* 1999; Chaudhary *et al.* 1999; Kristiansson *et al.* 1999; Grande *et al.* 2002). Another study found similar inflammatory and metabolic responses between laparoscopic and a “mini-laparotomy” procedures (McMahon *et al.* 1993).

Open surgery is also accompanied by greater increases in endogenous morphine release following surgery, another marker of the stress response (Madbouly *et al.* 2010b). Less invasive surgery does have other benefits for pulmonary function and reduction in hypoxemia than with open surgery (Mimica *et al.* 2000), and is manifested clinically in reductions of pain, morbidity and duration of hospital stay (Kehlet 1999; Le Blanc-Louvry *et al.* 2000). Laparoscopic approaches also lead to reductions in gastrointestinal paralysis and subsequent earlier return to oral nutrition, which has itself been shown to lead to reductions in catabolism and risk of septic complications (Kehlet 1997; Andersen Henning *et al.* 2006).

1.3.1.4 INFLUENCE OF ANAESTHESIA AND ANALGESIA

The method of ventilation, type of pain relief, extent of resuscitation, use of rapid onset, short acting anaesthetic agents, use of muscle relaxants and choice of tubes, drains and catheters, may all have a role in minimisation of the surgical stress through a variety of mechanisms (Kehlet 1989; Wilmore 2002).

The type of anaesthesia itself has not been shown to be a major influence on the stress response to surgery, except for the use of high doses of opioid anaesthesia. This is consistent with the body's own endogenous opiate system having a role in the modulation, mediation and regulation of the stress response and observations of exogenous opiates reducing the emotional and affective response to pain (Drolet *et al.* 2001). High doses of opiates can reduce intra-operative but not postoperative changes to hypothalamic and pituitary hormone secretion (Kehlet 1996). Morphine and fentanyl have been shown to suppress the release of ACTH and thereby cortisol, with a dose of 15ug/kg of fentanyl sufficient to suppress the cortisol and glucose responses in lower abdominal surgery (Lacoumenta *et al.* 1987). However, to achieve meaningful suppression in upper abdominal surgery, doses that result in unacceptable respiratory depression are required. Conventional doses of opiates result in modest reductions in stress response when administered via patient-controlled analgesia, with slightly greater reductions when administered epidurally. While there is evidence of opiates reducing the stress-induced neuroendocrine and autonomic responses, paradoxically opiates stimulate these systems in the non-stressed state, leading to the development of hyperalgesia (Drolet *et al.* 2001).

NSAIDs have been shown to attenuate the endocrine metabolic response to endotoxin *in vitro,* but in studies of surgical patients the effect is less pronounced (Chambrier *et al.* 1996).

Conversely, incisional and regional anaesthesia can reduce both pain and the pituitary stress response by blocking afferent nerve fibres and their contribution to hypothalamic and sympathetic activation (Kehlet 1996). In current practice, regional anaesthesia is commonly used in addition to general anaesthesia because of this effect on the stress response and the subsequent benefits for organ function and postoperative outcome (Rodgers *et al.* 2000; Wilmore 2002). Neural block with local anaesthetics can substantially change the majority of hormonal and metabolic changes, reducing endocrine responses and postoperative catabolism (Vedrinne *et al.* 1989; Holte *et al.* 2002; Schricker *et al.* 2002). Pain relief is achieved, pulmonary is function improved and incidence of ileus is reduced. This provides for earlier oral nutrition and mobility (Kehlet 1996). The same agents administered intravenously, intraperitonealy or intrapleuraly do not significantly alter the stress response (Kehlet 1996).

The approach to postoperative pain control also has clinically significant outcomes. If a "*prn*" (as required) approach is utilised for controlling postoperative pain, higher concentrations of stress hormones, more intense pain, more substantial catabolism and greater immune impairment than with regional anaesthetic blockade can result (Kehlet 1997). Pre-emptive analgesia, that is analgesia administered before the onset of injury, can also minimise the stress response (Miner 2008).

1.3.1.5 OTHER ATTEMPTS TO MODIFY STRESS RESPONSE

Much attention has been devoted to reducing the impact of the stress response in the surgical patient. Being catabolic, the surgical patient is utilising skeletal muscle to produce energy. In attempts to reduce this gluconeogenesis patients have been administered supplemental glucose, which resulted in undesirable hyperglycaemia (Schricker *et al.* 2002). Amino acid infusions have had better outcomes, resulting in positive protein balance and reduction in endogenous glucose production (Donatelli *et al.* 2006).

1.3.1.6 IMPLICATIONS OF THE STRESS RESPONSE

The net result of the stress response is a postsurgical state characterised by fat and muscle breakdown, hyperglycaemia and impaired immune function. There is an increase

in catabolism to provide energy from fuel stores within the body (Desborough 2000). Retention of salt and water to maintain fluid volume and cardiovascular homeostasis can result in fluid overload, tissue oedema and congestive heart failure if fluids are not carefully managed. With tachycardia and hypertension arising from catecholamine stimulation, there is an increased risk of myocardial ischaemia and reduced blood flow to the periphery (Guest 2008). Stimulation of the liver to produce acute phase proteins causes reductions in the synthesis of albumin and increases in AAG. High levels of inflammatory cytokines have also been shown to alter CYP450 activity (Frye *et al.* 2002; Morgan *et al.* 2008), while even mild inflammation has been shown to increase the demand for sulphur containing amino acids, causing increased diversion of cysteine away from GSH production (Mercier *et al.* 2006).

These factors can have significant implications for drug disposition and are discussed in more detail under the headings absorption, distribution, metabolism and elimination below.

1.3.2 ABSORPTION

In the non-surgical patient, drugs taken orally must pass down the oesophagus into the stomach, dissolve from the dosage form and enter the small intestine. Food and medicines taken orally normally pass from the stomach to the small intestine within half an hour (Adelhoj *et al.* 1985). The small intestine is the main site of absorption for any ingested compound, whether it be food, drug or toxin (Chan *et al.* 2004). The bulk of absorption occurs in the upper jejunum. Along with the majority of the small bowel, the jejunum is a highly vascularised tissue, covered in a mucous membrane with internal projections called villi. A villus is coated in an epithelial brush boarder membrane (Figure 1.3-3).

These characteristics give it a high surface area ideal for the efficient absorption of gut contents. Absorption is so efficient that the rate substances leave the stomach into the small intestine is normally the rate limiting step of absorption (Marcos *et al.* 1996). These qualities are clear when considering that the average adult consumes approximately 800-1000g of food and 1200-1500mL of water per day, of which only 50g and 100mL is excreted unabsorbed (Mythen 2005). In addition to absorption, the intestinal epithelium must act as barrier, protecting the body from the outside world, repelling harmful substances and expelling waste for excretion.

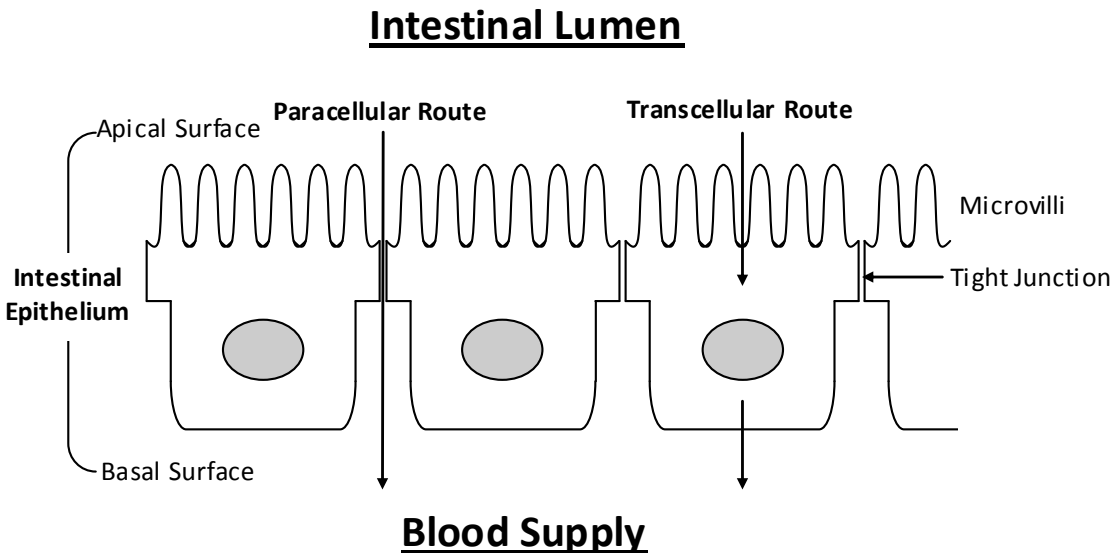


Figure 1.3-3 Routes of Intestinal Absorption (Hayashi et al. 1997)

Substances for absorption are chemically diverse, with substantial variance in size and lipophilicity and this diversity also applies to orally administered drugs. Accordingly, a drugs absorption from the lumen of the intestine into the blood can be quite difficult, and varies with a compound's affinity for specialised membrane transport systems and intracellular metabolising enzymes (Chan *et al.* 2004).

Most compounds cross the intestinal epithelia by one of two routes; paracellular or transcellular. Which route a compound uses is determined by its size and ionic charge (Levine 1970). Small, hydrophilic, ionised drugs can be absorbed by the paracellular pathway, through the tight junctions between epithelial cells. It relies on active transport, utilising specialised transport proteins. Accordingly, absorption by the paracellular pathway is small (Hayashi *et al.* 1997).

The transcellular pathway across the epithelia is almost exclusively accomplished by the partition and passive diffusion of non-ionised, lipophilic drugs. Most oral medicines are lipophilic and rely on this route for absorption (Hunter *et al.* 1997). In most cases it is passive, not requiring any active transport, but some hydrophilic or charged molecules also utilise transcellular transport, exploiting specialised transport mechanisms such as pinocytosis for entry into epithelial cells (Gubbins *et al.* 1991; Chan *et al.* 2004).

Entry into the enterocyte does not assure a compound's passage across the basolateral membrane and into the blood stream. Efflux proteins at the apical membrane transport many compounds back out into the intestinal lumen, and often determine the extent of a compound's absorption. Compounds may also be modified by metabolising enzymes found within the enterocyte. This intracellular metabolism may inactivate a drug or make

it an efflux protein substrate (Chan *et al.* 2004). Once a drug has crossed the basolateral membrane it is transported in the hepatic portal vein to the liver where it will again be exposed to a variety of metabolising enzymes.

In reference to drugs, the fraction of the administered dose that reaches the systemic blood is referred to as a drug's bioavailability. It is determined by two factors:

1. The amount not absorbed; and
2. The amount lost to metabolism either in the intestine or liver as it is absorbed (referred to as first pass metabolism or loss) (Bruton *et al.* 2006; Backes *et al.* 2008).

Of the changes in the stomach and small intestine, the most significant factors that affect absorption are:

- The rate of emptying from the stomach to the small intestine (gastric emptying) (Section 1.3.2.1);
- The ability of the small intestine to absorb the drug (small bowel absorption) (Section 1.3.2.2); and
- The first pass metabolism of the drug (Section 1.3.2.3)

Changes to these factors that arise from surgery mean that oral absorption is, at best, unreliable in postoperative patients (Noble *et al.* 2000). As the first step of drug disposition, changes to drug absorption can severely alter the effectiveness of orally administered medicine.

1.3.2.1 GASTRIC EMPTYING

Gastric emptying can be delayed substantially in the postoperative patient for a variety of reasons. Two of the most significant factors are the:

1. Nature and duration of surgery; and
2. Use of drugs, especially opiates.

As this step is often absorption-rate limiting, understanding changes to gastric emptying is the first and perhaps most crucial step to understanding changes to drug disposition in postoperative patients. Delays to absorption will typically reduce maximum plasma concentration (c_{max}), and extend the time taken to achieve it (t_{max}). This can delay

therapeutic effect, *e.g.* analgesics, or even failure of the therapy if it relies on rapid absorption for effectiveness *e.g.* antibiotics and antiarrhythmics (Nimmo *et al.* 1988).

Changes to the motility pattern of the gastro-intestinal tract as a result of surgery have been known since the early 20th century (Livingston *et al.* 1990), however the implications of this alteration to drug therapy were elucidated in the 1970s (Heading *et al.* 1973). More recently, retropulsive disposition of fluid from the small intestine back into the stomach was observed following surgery, further impacting on the rate of absorption (Kennedy *et al.* 2006).

1.3.2.1.1 INFLUENCE OF SURGERY

Gastric emptying is diminished or absent following any major procedure and ileus, the total paralysis of the gut, may occur, especially when the peritoneum is entered, the small bowel is manipulated or the procedure is long (Graber *et al.* 1982; Livingston *et al.* 1990; Kalff *et al.* 1998; Le Blanc-Louvry *et al.* 2000). In many case series ileus is reported at rates of >90% following intra-abdominal surgery (Kehlet *et al.* 2001b; Mythen 2005). This reduction in motility was initially thought to occur from sympathetic hyperstimulation as a result of surgical stress (Mythen *et al.* 1994). However, as discussed above, metabolic derangement, peritoneal irritation, electrolyte imbalance, hypovolaemic and perioperative medicines are now understood to have a greater contribution (Graber *et al.* 1982; Wallden *et al.* 2006).

1.3.2.1.2 THE ROLE OF OPIATES AND OTHER AGENTS

Perioperative medications most typically associated with delaying gastric emptying are opiate, adrenergic and anticholinergic drugs (Clark *et al.* 1980; Todd *et al.* 1983). These are commonly used as analgesics, anaesthetics, psychotropics, cardiovascular and autonomic drugs around the time of surgery (Kennedy *et al.* 1998).

All other factors are insignificant in the face of opiates (Kehlet *et al.* 2002). They have been shown to diminish propulsive contraction of the gut and delay gastric emptying (Crighton *et al.* 1998). Delayed emptying associated with opiate use has been shown to persist for at least five hours after dosing (Ingram *et al.* 1981) and the time to empty 50% of an ingested solution from the stomach is increased 8 to 10 fold after pethidine or diamorphine administration (Nimmo *et al.* 1975). There is partial reversal of the delay by opiate antagonists naloxone and methylnaltrexone (Frame *et al.* 1984). The efficacy of

methylnaltrexone, which does not cross the blood brain barrier, indicates the delay is peripherally rather than centrally mediated (Murphy *et al.* 1997).

The exact mechanism of opiate delays is unclear, but is thought to arise from agonism of peripheral μ opiate receptors. Opiate receptors are found throughout the gut, but are present in especially high numbers in the gastric antrum and proximal duodenum. Activation of the opioid receptors in the presynaptic nerve terminals of the mesenteric plexus causes increased resting tone and decreased propulsive peristaltic waves of the gut (Fiocchi *et al.* 1982; Manara *et al.* 1985; Manara *et al.* 1986). Agonists of these opiate receptors all cause delays to gastric emptying (Polak *et al.* 1979).

When studies have investigated multiple contributing factors in the cause of delays to gastric emptying, opiate use predominates. A group of 20 patients undergoing elective cholecystectomy were examined for their ability to empty 100ml of water from the stomach before and on the first day after their procedure (Ingram *et al.* 1981). Emptying was grossly retarded following an injection of opiate. No correlation was found between age, sex, duration of procedure or anaesthetics and the authors concluded opiate use was the most significant factor in a patient's inability to tolerate oral fluids and that alternative analgesia should be sought or opiate administration reduced.

The method of administration of the opiate is also influential, with pulse doses of opiates appearing to delay gastric emptying further than continuous infusions of an equivalent dose (Nimmo *et al.* 1988). Long anaesthesia with inhaled anaesthetics may also delay gastric emptying but the changes are relatively small compared with that seen after administration of opiates (Adelhoj *et al.* 1984).

One study attempting to show the influence of these factors used opiate free anaesthesia with sevoflurane in 25 patients undergoing laparoscopic cholecystectomy, but did not show significant improvements to delayed gastric emptying over propofol and remifentanyl based anaesthesia in the same number of patients (Wallden *et al.* 2006). However, two aspects of the method may have reduced the study's sensitivity to changes; the paracetamol they used to measure gastric emptying was 'dissolved' in a large volume of water before administration down a nasogastric tube, which is a poor marker of delay (Kennedy *et al.* 2003), and the study period was only two hours, prohibiting analysis of area under the concentration/time curve (AUC).

Minor delays to gastric emptying have also been shown to occur from alterations to posture. A study of 20 patients prior to cardiac catheterisation examined the difference in oesophageal transit time whilst standing or supine. Also using paracetamol absorption to determine gastric emptying, they found supine patients to have significantly reduced C_{max} from 8.76 ± 1.17 to 5.92 ± 0.95µg/mL ($p<0.04$), with t_{max} 70 minutes longer. Both are strong indicators of delay to gastric emptying (Channer *et al.* 1985).

Other commonly used medications in surgery, benzodiazepines, have no independent effect on gastric emptying (Adelhoj *et al.* 1985), nor does pain or anxiety alone, unless they cause delayed mobilisation or eating or invoke the stress response (Marsh *et al.* 1984; Le Blanc-Louvry *et al.* 2000).

1.3.2.1.3 DURATION OF DELAYED GASTRIC EMPTYING

The duration of the delay to gastric emptying following surgery is highly variable. In most cases, delays to gastric emptying and postoperative ileus resolve spontaneously after two-three days (Livingston *et al.* 1990), but in severe cases, delayed gastric emptying can persist for weeks or even months in those with multiple risk factors (Marcos *et al.* 1996). Emptying can be reduced even in the presence of bowel sounds or the passing of flatus (Kennedy *et al.* 1998), and even if the stomach was not directly involved in the procedure (Condon *et al.* 1986).

1.3.2.1.4 IMPACT OF DELAYED GASTRIC EMPTYING

Delays to gastric emptying are characteristically shown in changes to paracetamol absorption. Paracetamol absorption is frequently used because it is poorly absorbed from the stomach, relying on gastric emptying for absorption (Heading *et al.* 1973; Todd *et al.* 1983; Marsh *et al.* 1984; Nimmo *et al.* 1988). Changes to absorption in the surgical patient are substantial. Kennedy *et al.* used paracetamol syrup to demonstrate reductions of almost 50% in c_{max} (30.2 vs. 16.3mg/L) and greater than doubling in t_{max} (35 vs. 119min) comparing values from before and two days after major abdominal surgery (Kennedy *et al.* 2006).

For drugs that are acid labile, such as penicillin, the extent of absorption can also be reduced if gastric emptying is delayed, as these drugs are degraded by prolonged exposure to the harsh environment in the stomach (Nimmo *et al.* 1988).

In addition to impacting on absorption of medication, the risk of intolerance, regurgitation and aspiration of oral food and fluids may also be a result of delayed gastric emptying. There is also a contribution to constipation and ileus (Mythen 2005). In addition to patient discomfort, ileus is a significant burden on the health system at a minimum through delays to discharge (Livingston *et al.* 1990).

With increasing attention on enhanced recovery programmes to reduce inpatient stays many strategies to minimise changes to gastric emptying have been investigated. Sparing opiates has a major role in this, alongside minimally invasive surgical techniques, regional anaesthesia, early mobilisation and early return to enteral feeding (Kehlet *et al.* 2001b).

1.3.2.2 SMALL BOWEL ABSORPTION

Once the drug is released from the stomach, absorption from the small bowel is itself dependent on three factors relating to the small bowel mucosa:

- The blood supply;
- The motility; and
- The integrity of the mucosa.

Changes to these factors in the perioperative period can cause reductions in absorption and bioavailability, particularly of drugs that are already poorly absorbed (Uhing *et al.* 1995). But for the majority of drugs, changes to the factors listed are insignificant compared with the impact of delayed gastric emptying. Such is the case with paracetamol. As a weak acid, with a pKa of 9.5, it is largely unionised over the physiological pH range (Prescott *et al.* 1971). This, together with its favourable water and lipid solubility, means that it is rapidly and completely absorbed from the small bowel by passive diffusion (Bagnall *et al.* 1979). Therefore, any changes to paracetamol absorption from the small bowel are utterly dwarfed by reductions in gastric emptying.

1.3.2.2.1 BLOOD SUPPLY

The gut normally receives 15 to 20% of the total cardiac output, which can double two-three hours following food ingestion (Lantz *et al.* 1981). Such supply is well in excess of the nutritional demands of the enterocytes alone (Lundgren 1967). A rapid blood flow is required to remove absorbed substances, which maintains the concentration gradient

required for passive diffusion and the health and barrier function of enterocytes (Gubbins *et al.* 1991).

In the very early postoperative period, when haemostasis is most in danger, blood flow is rapidly redistributed away from the gut to maintain circulatory volumes to the more vital organs (Kennedy *et al.* 1998). In addition, spinal and epidural analgesics block autonomic vasoconstrictors, which results in vasodilation of large vessels, further reducing afterload, cardiac output and ultimately blood flow to the bowel (Winne 1979).

Theoretically this reduction in blood flow to the gut could impair absorption of drugs that rely on passive diffusion (Levine 1970). But to significantly influence drug absorption changes to blood flow must be substantial and sustained (Winne 1979). Ischaemia of five minutes or less does not impair absorption; more prolonged periods may, but only during the time of ischaemia (Robinson *et al.* 1965; Bailie *et al.* 1987). Paradoxically, passive absorption may be increased once ischaemia ends, potentially allowing pyrogens to translocate across the gut wall (Crissinger *et al.* 1990).

Active transport of substances is impaired during and after prolonged periods of ischaemia, indicating oxygen deficiency damages active transport mechanisms (Varro *et al.* 1965; Ochsenfahrt *et al.* 1973). However these changes do not have a significant effect on paracetamol absorption due to its passive diffusion.

In the uncomplicated surgical patient, the large blood supply to the gut makes it unlikely that any significant reduction in passive absorption occurs (Lundgren 1967). *In vivo* substantial reductions in splanchnic flow (35% of normal in dog models) must occur before the efficiency of passive diffusion is affected (Crouthamel *et al.* 1975). Only the most critically-ill who have prolonged reduction to cardiac output and splanchnic flow may have some reductions to drug absorption (Kennedy *et al.* 1998).

Paradoxically oedematous patients may also have impaired absorption (Berkowitz *et al.* 1963). While occasionally shown in surgical patients, this is more commonly seen in severely oedematous congestive cardiac failure patients who show resistance to oral furosemide (Berkowitz *et al.* 1963).

1.3.2.2.1.1 ASSESSMENT OF BLOOD FLOW TO THE GUT

It is worth considering how blood flow to the gut and liver is assessed because the assumption that surgery causes no significant alteration to blood flow to the gut, and

subsequently the liver, is important to this Thesis. If blood flow was reduced this could cause alterations to drug metabolism by reducing cofactor and substrate delivery that is not being assessed.

The gut provides 75% of the blood supply to the liver, therefore measurement of the latter provides a good approximation of the former (Desforges *et al.* 1953; Horner Andrews 1957). Two methods are commonly used to approximate hepatic blood flow:

- Measurement of the disappearance of a hepatically excreted compound; or
- Measurement of the appearance of a hepatically produced metabolite.

Either compound or metabolite must possess a number of other favourable characteristics. One such compound, indocyanine green dye, is an example of the first method of approximation and has been used extensively to approximate blood flow to the gut (Caesar *et al.* 1961; Gibaldi 1991; Kanaya *et al.* 1995; Jacob *et al.* 2007).

Because the liver is so efficient at extracting indocyanine green from the plasma and because 75% of the blood supply to the liver is from the portal vein (Desforges *et al.* 1953; Horner Andrews 1957), the plasma disappearance of this compound approaches that of the splanchnic blood flow. Accordingly, it can also be used to assess blood flow to the mesenteric, splenic and hepatic vessels.

Work by Kennedy *et al.* which preceded this research (Kennedy *et al.* 2006) administered indocyanine green and measured the plasma disappearance to approximate blood flow to the gut of 12 major abdominal surgery patients before and after surgery. No statistically significant difference in mean disappearance of indocyanine green was found.

Another study examined indocyanine green clearance in patients before and after undergoing elective surgery with various types of inhaled anaesthetics. Although one inhaled anaesthetic, halothane, did reduce indocyanine clearance, it is not commonly used in current practice. Isoflurane and sevoflurane, two other assessed gases in current use, did not cause any significant change in indocyanine clearance (Kanaya *et al.* 1995). These results have been shown by other authors (Frink *et al.* 1992; Nakaigawa *et al.* 1995), in other surgical groups (Murray *et al.* 1992a; Murray *et al.* 1992b) and confirmed by other, more invasive methods (Gatecel *et al.* 2003). In addition, pigs infected with *Actinobacillus pleuropneumoniae* also did not show any alteration to indocyanine green

clearance, indicating that bacterial sepsis alone also does not alter hepatic blood flow (Monshouwer *et al.* 1995).

1.3.2.2.2 MOTILITY

Small bowel motility is necessary for the mixing of substances in the bowel, moving them into close contact with the brush border membrane, where absorption occurs (Spiller 1994). In surgical patients, co-ordination of small bowel motility is reduced, reducing the efficiency of absorption (Kennedy *et al.* 2006).

As for gastric emptying, opiates are implicated in the reduction of small bowel motility, as are prolonged procedures involving small bowel manipulation, but unlike gastric emptying, small bowel motility returns approximately six hours after surgery in almost all patients (Fiocchi *et al.* 1982). Because small bowel motility is restored quickly in most cases, assuming the absence of ileus, it may be assumed that drug mixing is not significantly altered postoperatively (Kennedy *et al.* 1998).

1.3.2.2.3 INTEGRITY OF THE MUCOSA

The integrity of the mucosa can also influence drug absorption. Changes seen from surgical stress include alteration to cell morphology and loss of normal villus shape. This can result in reductions in absorptive area and potentially reduction in absorption by the transcellular route. These changes even occur in cardiac or orthopaedic surgery when the surgical injury is quite remote from the gut (Mythen 2005).

Changes to pore size in the gut wall can also occur, allowing even large molecules to pass through the mucosa (Crissinger *et al.* 1990) and increased intestinal permeability has been shown after major abdominal surgery (Roumen *et al.* 1993b; Kennedy 1996). Inflammation and infection can also impair the activity of transporter and metabolising enzymes present in the gut wall (Aitken *et al.* 2006).

1.3.2.3 FIRST PASS METABOLISM

First pass metabolism broadly involves enzymes present in the brush border cells and the liver. As drugs pass from the intestinal lumen into the mesenteric capillaries they are exposed to many of the same drug metabolising enzymes that are found in the liver, predominately the Phase II conjugation enzymes glucuronosyltransferases and sulphotransferases (Pacifici *et al.* 1986; Rogers *et al.* 1987; Peters *et al.* 1991), although

Phase I CYP450 enzymes, particularly CYP3A4, are also present (Thummel *et al.* 1997). These enzymes of intestinal metabolism are localised primarily in the mucosal enterocytes.

Similar metabolism can occur again in the parenchymal cells of the liver as absorbed drugs flow through the hepatic portal vein to the liver for the first time on their way to the inferior vena cava and systemic circulation (Thummel 2007).

There are some reports of anaesthesia inhibiting intestinal CYP450 enzymes which may increase the extent of absorption of orally administered drugs that are normally extensively metabolised in the first pass. However, difficulties arising from delays to gastric emptying that occur from most anaesthesia and decoupling the contributions of the intestine from the liver to first pass metabolism complicates this research (Doherty *et al.* 2002; Thelen *et al.* 2009).

Generally changes to first pass metabolism in the surgical patient are of minor importance in comparison to the other factors discussed above, and generally first pass metabolism is not an important contributor to paracetamol metabolism (Clements *et al.* 1984).

1.3.2.4 ABSORPTION FROM OTHER SITES

Intramuscular drug absorption can also be affected by surgery and result in erratic plasma levels and unpredictable clinical effects (Ueno *et al.* 1995). Among other factors, the rate of drug absorption following intramuscular injection depends on blood flow at the site of administration. Anaesthesia and surgery produces a fall in muscle blood flow and this can delay drug absorption. This effect can be minimised by administration into the deltoid rather than the gluteus (Nimmo *et al.* 1988). Absorption following subcutaneous administration is similarly dependent on blood flow and can show comparable fluctuations (Kennedy *et al.* 1998). Absorption from the rectal route is already erratic and highly variable and in the absence of pyrexia, this situation is not improved in the surgical patient (Reddy *et al.* 2002).

1.3.3 DISTRIBUTION

Once a drug has reached the systemic circulation it is distributed throughout the body, firstly in the blood and then the tissues to varying degrees. In this way the body can be

considered to be made up of a number of compartments. The first, or central, compartment represents the blood volume. The second, or peripheral, compartment represents the tissues of the body. Depending on the nature of the drug it may be distributed into other compartments, such as the CNS. The amount of drug found in each compartment represents its distribution, and is the result of several drug and patient related factors. In general terms, drugs that are hydrophilic prefer the predominantly aqueous environment of the plasma in the central compartment while those that are lipophilic distribute more rapidly into the periphery, preferring the more hydrophobic adipose and muscle tissue. The binding of drugs to plasma proteins within the central compartment also influences to the distribution of lipophilic drugs, as bound drugs are retained in the central compartment (Bruton *et al.* 2006).

Patient related factors may prevent drugs from distributing to their full extent. In the surgical patient prolonged operations frequently result in hypovolaemia, vasoconstriction and acidosis. These can alter drug distribution. Many of these factors can be considered as changes to either blood flow, drug binding or extracellular fluid.

The distribution of fentanyl, for example, can be altered in surgical patients. Because of its lipophilicity, it has a high binding capacity to adipose tissue and muscle. As fentanyl is a weak acid, acidosis increases this binding capacity, prolonging fentanyl storage in the periphery. As the acidosis is corrected postoperatively by re-warming, volume replacement and buffers, fentanyl is released from its binding sites. This can result in a second peak, with potential for toxicity (Caspi *et al.* 1988; Klausner *et al.* 1988).

There have been reports of changes to the distribution of paracetamol in surgical patients. The Vd of intravenous paracetamol was found to increase from 69.2L in healthy volunteers (Flouvat *et al.* 2004) to 85.0L in patients undergoing hip arthroplasty (Viscusi *et al.* 2008). Another study examined the intravenous paracetamol kinetics in medium (equivalent to a High Dependency Unit setting) and Intensive Care Unit patients. They found the distribution of paracetamol in Intensive Care Unit patients significantly higher (71.09L) than the medium care unit patients (50.88L p=0.033)(de Maat *et al.* 2010).

Along with these changes, there are also reports about the inhibition and down regulation of a variety of drug transporter proteins, such as P-glycoprotein (PGP), a widely expressed efflux pump. Inhibition is linked with levels of inflammation (Morgan *et al.*

2008). PGP is involved in several areas of drug distribution, including the transport of compounds from the:

- Intestinal epithelia, back out into the lumen, reducing bioavailability;
- Cells back out into the extracellular fluid, conferring multidrug resistance to many drugs, including several chemotherapeutic agents;
- Hepatocytes, into the bile for elimination; and
- CNS, back across the blood brain barrier into the periphery, reducing exposure of the CNS to the drug.

Any reduction in the activity of PGP that occurs during inflammation or infection can therefore be seen to have a wide range of effects on the distribution of drugs that are transporter substrates, such as:

- Increased intestinal absorption and bioavailability;
- Increased sensitivity of cells to drugs;
- Reduced clearance of drugs; and
- Reduction of transport of drugs across the blood-brain barrier. Drugs that are normally transported out of the CNS by PGP can enter, accumulate and potentially cause CNS toxicity.

These changes can have important clinical ramifications. Decreases of 50-70% in hepatic PGP activity have been reported (Piquette-Miller *et al.* 1998). These reductions can reduce drug clearance, prolonging drug exposure and potentially lead to drug accumulation, if multiple doses are given (Goralski *et al.* 2003).

Important changes also occur in the CNS. Here, patients with severe CNS trauma, such as through infection or surgery, can develop an inflammatory response in the brain. When morphine is used in these patients it has been observed that, while it is mostly well tolerated for a few days, some patients go on to become agitated, in a similar manner to those in renal failure who accumulate the CNS irritant metabolite M3G. This metabolite is normally excluded from the CNS by PGP in the blood brain barrier (Letrent *et al.* 1999a; Letrent *et al.* 1999b). Consequently, inhibition of PGP has been shown to lead to morphine metabolite accumulation in the CNS (Morgan *et al.* 2008). This may affect a wide variety of drugs that are PGP substrates (*e.g.* cyclosporine, digoxin and HIV protease inhibitors), which functioning PGPs normally exclude from the CNS (Morgan *et al.* 2008).

Other work has shown that systemic inflammation, not just inflammation in the CNS, can cause changes to PGP activity at the blood-brain barrier (Wang *et al.* 2005).

1.3.3.1 BLOOD FLOW

Anaesthesia usually results in a brief reduction in cardiac output and blood flow to several different areas of the body, particularly the kidneys, liver and muscle (Hickey *et al.* 1980). When this is coupled with a reduction in blood volume for reasons discussed in 1.3.2.2.1, the size of the central compartment into which drugs are delivered can be reduced, especially in the early postoperative period (Kennedy *et al.* 1998). When reductions to cardiac output are large and a drug's target organ is in the central compartment, unexpected toxicity following intravenous drug administration can occur. This effect is more pronounced in the elderly, who have age related reductions in central compartment volume (Moffat *et al.* 1983).

The increase in circulating catecholamines arising from the stress response can also alter blood flow. By causing vasoconstriction, catecholamines cause changes to perfusion pressure and regional blood flow. This can further impact on the rate and extent of drug distribution (Udelsman *et al.* 1994).

1.3.3.2 DRUG BINDING

While paracetamol is not known to be highly protein bound at licensed doses, binding has been shown following overdose. Thus understanding changes to drug binding following surgery is important. Lipophilic drugs may reversibly bind to plasma proteins, increasing their concentration in the central compartment. However, only the unbound drug has pharmacological activity and is available for metabolism. Any alteration to plasma protein binding therefore has important implications to drug therapy. Reductions to drug binding could result in unexpected toxicity and conversely therapeutic failure may arise as a result of increase drug binding (Streat *et al.* 1985).

Serum albumin is the most important plasma protein for drug disposition. Serum albumin turnover increases after surgery and the concentration of albumin falls significantly for a number of days as a result of the stress response, particularly in sepsis (John *et al.* 1969). Surgical patients malnourished or catabolic as a result of their pathology may be hypoalbuminaemic preoperatively (Mann *et al.* 1987). For drugs that are highly bound to albumin a reduction in albumin concentration can increase the volume of distribution

(van Dalen *et al.* 1990). This can have implications for the intensity and duration of the action of the drug as it is only the unbound fraction of the drug which is active. Reductions to fraction of bound drug may result in increased activity of the drug, or even toxicity, as is the case with lidocaine, pethidine and propranolol (Kennedy *et al.* 1998; Kratochwil *et al.* 2002).

The second most important plasma binding protein, AAG, has similar affinity for lipophilic drugs. It is an acute phase protein and may rise as a consequence of stress and surgery (Israili *et al.* 2001). Its synthesis in the liver is stimulated as part of the acute phase response to increasing concentrations of catecholamines and inflammatory cytokines (Fournier *et al.* 2000). Elevated levels of AAG can continue at least 5 days postoperatively and may result in increased binding of highly bound basic and neutral drugs, resulting in reduced unbound fraction and effective drug concentration, and apparent reduction to the volume of distribution (Davies *et al.* 1988). Drugs reported to show this effect include bupivicaine (Wulf *et al.* 1989), lidocaine, propranolol (Sager *et al.* 1989; Davies *et al.* 1991; Hunter *et al.* 1997; Doherty *et al.* 2002), pethidine (Julius *et al.* 1989) and prazosin(Sager *et al.* 1989). For highly bound drugs, if increased plasma binding proteins result in even a small reduction in unbound fraction of the drug, therapeutic failure can result, as only the unbound drug is pharmacologically active (Julius *et al.* 1989).

Theoretically, iso-osmotic fluids administered during surgery can cause haemodilution of plasma proteins, increasing the free fraction of a highly protein bound drug. Conversely, colloidal fluids can result in increased amounts of bound drug, although the clinical relevance of this is uncertain (Svensen *et al.* 1997).

1.3.3.3 EXTRACELLULAR FLUID

Postoperative patients have an increased volume of extracellular fluid (Beckhouse *et al.* 1988). Aldosterone and AVP are secreted as part of the endocrine response to surgery to maintain the circulating blood volume. These substances cause the retention of salt and water, and consequently the enlargement of the extracellular fluid volume.

The distribution of this extracellular fluid can also change in the postoperative patient. Extracellular fluid can leak out of the plasma into a theoretical ‘third space’. The inflammatory cytokines, PGs, kinins and leukotrienes increase capillary permeability causing an accumulation of fluids in the interstitial space, resulting in ‘third spacing’. This

causes a net loss of extracellular fluid from plasma. Fluid sequestered in this way is unavailable to the plasma, which may alter drug distribution, particularly of those drugs with small distribution volumes (Fleck *et al.* 1985). The authors of the study cited above in Section 1.3.3 involving medium and intensive care unit patients (de Maat *et al.* 2010) postulated such a mechanism was responsible for the increase in Vd of paracetamol in the more severely ill intensive care patients.

Postoperative patients with significant third spacing can have substantial changes in observed plasma concentrations of hydrophilic drugs such as aminoglycosides and higher doses may be required to maintain concentrations in the therapeutic range (Dasta *et al.* 1988). As such, aminoglycosides have been frequently used to assess changes to the volume of extracellular fluid. As aminoglycoside antibiotics do not bind to plasma proteins and are very hydrophilic, their distribution volume closely parallels that of the extracellular fluid. There are several studies using gentamicin that show dramatic increases to the volume of extracellular fluid following surgery (Zaske *et al.* 1980; Beckhouse *et al.* 1988; Dasta *et al.* 1988; Reed *et al.* 1989).

One such study by Dasta and Armstrong of 181 critically ill surgical patients found the Vd of the aminoglycoside gentamicin was increased to 0.36 ± 0.1L/kg, nearly 50% above the reference value of 0.25L/kg (Dasta *et al.* 1988). These patients also gained on average 8.4kg in weight and 11.6L of fluid, further indicating changes to volume of distribution, although no direct correlation with Vd and fluid balance could be made.

It is possible that surgery may also alter Vd through changes to total body water, but no clinically significant instances have been reported (Nimmo *et al.* 1988).

1.3.4 METABOLISM

As discussed in Section 1.2, the body's aim for drug metabolism is to a produce a compound excretable in the urine or bile. There are two phases of drug metabolism: Phase I prepares a compound for Phase II metabolism, which involves conjugating the compound to a more aqueous soluble moiety, easing its excretion into the bile and urine. As stated previously compounds do not necessarily proceed through Phase I prior to Phase II, with some using only the Phase II pathway.

While changes to absorption are perhaps the most dramatic change in drug disposition in the surgical patient, these can be surmounted by parenteral administration. Changes to

metabolism are not so easily avoided and represent one of the most clinically relevant alterations to drug disposition for the majority of surgical patients.

Most of the metabolic reactions are catalysed by enzymes and their activity may be altered in surgical patients by one of two ways:

- Alteration of enzyme activity; and
- Lack of enzyme cofactors or substrates.

Enzyme activity can be affected by the acute phase response when the expression of most enzymes is inhibited, although some are induced. This is discussed in greater detail below.

Changes arising from the endocrine aspects of the stress response and alterations to nutrition can affect the availability of co-factors, such as glucose. Glucose is used as a fuel to supply adenosine-5'-triphosphate which is essential for enzyme activity including those involved in drug metabolism (Aw *et al.* 1984).

Changes to respiration and blood flow can also reduce supply of oxygen. All CYP450 enzymes use oxygen as a direct substrate whereas only some Phase II enzymes require oxygen for the generation of essential co-factors. Relatively minor reductions in oxygen supply, such as may arise from surgery and anaesthesia, have been shown *in vitro* to impair these metabolic processes. CYP450 enzymes are especially sensitive to these changes although in situations of poor nutrition or fasting, Phase II processes may also be affected (Aw *et al.* 1984; Angus *et al.* 1990).

Most of the drugs used in anaesthesia are able to reduce drug metabolism whether it occurs by oxidation, reduction or conjugation. Halothane, an inhaled anaesthetic no longer in use, was known to inhibit a vast array of drugs including propranolol, fentanyl, ketamine, pethidine, antipyrine, diazepam and phenytoin. This inhibition of drug metabolism occurred during anaesthesia and persisted well into the postoperative period (Reilly *et al.* 1985; Nimmo *et al.* 1988). Mice anaesthetised with halothane, isoflurane and sevoflurane had reductions to UDP-glucuronic acid, a co-factor required for glucuronidation, of 40-52% as compared with that in unanesthetised control mice (Watkins *et al.* 1990).

Apart from these reports, there is a surprising lack of literature examining these changes to metabolism in the surgical patient. This is perhaps in part due to some of the same

issues mentioned in Section 1.1 concerning consent, ethics and randomisation. The study of these changes may also falsely be seen as lacking clinical relevance for several reasons:

- Changes may have been explained as arising from other sources, such as inter-subject variability normally experienced with many medications, such as morphine;
- Changes may not have been detected because many of the affected patients are seriously ill, making drug toxicity difficult to identify;
- Changes may not be have been detected because most drugs have a wide safety margin and many pathways are not capacity limited;
- The increased monitoring provided while in the surgical unit can adjust the dose to a patient's response, rather than administering a fixed dose, avoiding toxicity;
- Changes to metabolism have been unnoticed due to the lack of pre-operative requirement of the medication or postoperative observation; and
- A lack of interest within the surgical specialties.

However, shorter inpatient stays accompany modern surgical practices and very few surgical patients are cared for in intensive care or high dependency units. Accordingly, surgical patients are receiving less monitoring and are discharged earlier. Given that changes to drug metabolism may persist for several days, even weeks, after surgery, greater understanding is needed to protect the surgical patient.

A number of drugs given to surgical patients have no set dose and must be titrated to the patients' response. This implies a patient must experience periods of inefficacy and toxicity, so that a midpoint can be obtained. Having a greater understanding of changes to drug metabolism that occur perioperatively may also enable a midpoint dose to be predicted more accurately, providing effective doses sooner. While many of the changes to metabolism due to surgery may occur at a subclinical level, they can have serious consequences when they do become apparent; not only when toxicity occurs but also the other potential outcome, lack of efficacy. The area is clearly worthy of study if these outcomes are to be minimised.

1.3.4.1 REPORTS OF CHANGES TO PARACETAMOL'S METABOLISM

Because paracetamol is widely used in surgical patients there have been some studies that have examined changes to its metabolism. Other information regarding changes to

paracetamol metabolism can be gained from studies that were primarily assessing other aspects of drug disposition of surgical patients, such as absorption.

The results of these studies are conflicting. One study examined the pharmacokinetics of paracetamol in seven children/adolescents (median age 13.7 years) during major surgery, four weeks after chemotherapy. Neither major surgery for tumour resection or chemotherapy had a major impact on paracetamol metabolism in this study (Wurthwein *et al.* 2005).

Conversely, Lewis *et al.*(Lewis *et al.* 1991) compared excretion of a single oral dose of 1.5g of paracetamol in a group of volunteers and in general surgical patients on day one postoperatively. They found statistically significant decreases in the unchanged paracetamol and paracetamol sulphate and increased amounts of mercapturic acid recovered in the urine, but found no changes to AUC or $t_{½}$ of paracetamol.

While there are some reports of inhibition of drug metabolism immediately following surgery which persists into the early postoperative period, paradoxically there is also evidence that a period of enzyme induction follows, which may have given rise to the conflicting reports above. Enzyme induction may occur four to eight days following surgery by approximately 30-50%, returning to normal by four weeks (Nimmo *et al.* 1981). This induction can have dangerous ramifications for drugs such as paracetamol which has a toxic metabolite and veritably increased metabolism of paracetamol after surgery has been reported (Ray *et al.* 1985). There are also similar reports of halothane anaesthesia alone inducing paracetamol's metabolism (Ray *et al.* 1986; Lewis *et al.* 1991).

One of the major shortcomings of these studies is the use of saliva paracetamol concentrations. While discussed in greater detail in Section 1.4, of relevance here is the lack of any information on the relative concentration of paracetamol metabolites gained from these samples. Without this information no conclusions can be made about contributions of Phase I or II to the increase of paracetamol's metabolism. Kennedy *et al.*(Kennedy 1996) did examine this in urine samples with HPLC. Using a single oral 1.5g dose of paracetamol, they found significant differences in the pre- and postoperative levels of glucuronide conjugate ($p < 0.01$) and cysteine conjugate ($p < 0.05$) but no significant differences in the pre- and postoperative levels of mercapturate conjugate ($p < 0.01$) and sulphate conjugate ($p < 0.05$) excreted in urine over five hours. The finding was reconfirmed in longer studies lasting for five days postoperatively(Kennedy 2009a)

1.3.4.2 PHASE I

The stress response is considered a major contributor to alteration of Phase I metabolism (Section 1.3.1). Inflammation caused by infection or underlying inflammatory conditions are known to down regulate most drug metabolising enzymes and transporters, both in acute scenarios, such as following surgery, or chronic conditions such as extra-hepatic cancer, inflammatory bowel disease or congestive heart failure(Frye *et al.* 2002; Carcillo *et al.* 2003; Haas *et al.* 2003; Aitken *et al.* 2006; Morgan *et al.* 2008).

1.3.4.2.1 CLINICAL CONSEQUENCE OF CYP450 INHIBITION

This reduction in Phase I activity can lead to a decrease in drug clearance, increasing plasma drug concentrations and potentially may cause drug toxicity or increases in the incidence of adverse effects (Schmith *et al.* 2008). One of the first reports of this arose from an outbreak of influenza-b in the early 1980's when 11 young children receiving theophylline prophylatically for asthma were hospitalised with severe theophylline toxicity. They presented with symptoms as severe as cardiac abnormalities and convulsions. One child's theophylline half-life had increased fivefold, from four to 20 hours, and suffered severe, permanent brain damage as a result of the theophylline-induced convulsions (Kraemer *et al.* 1982). It was suspected inflammation arising from the viral infection inhibited CYP1A2, causing theophylline accumulation and toxicity (Morgan *et al.* 2008). One review stated that infection or inflammation can decrease metabolic clearance of CYP450 substrates by 20-70% (Aitken *et al.* 2006). The impact of this clinically is much greater than is generally reported (Morgan *et al.* 2008) and may be overlooked for reasons discussed in Section 1.3.4.

1.3.4.2.2 MECHANISM OF CYP450 INHIBITION

One of the first experiments to show the effect of inflammation on CYP450 activity in man examined the effect of lipopolysaccharide (LPS) on antipyrine clearance. An artificial stimulant of the immune system, LPS, has shown to reduce clearance of antipyrine, a non-specific marker for CYP450 enzyme activity, in healthy men (Shedlofsky *et al.* 1994). It had been earlier shown in mice that it was the inflammatory cytokines produced in response to the administration of LPS, rather than the LPS itself, which caused this down regulation of CYP450 enzymes (Shedlofsky *et al.* 1987). Further evidence of the involvement of cytokines arises from the finding that inflammation in the CNS, such as that arising from

meningitis, affects drug disposition in the brain and peripheral systems (Garcia Del Busto Cano *et al.* 2003). It is even thought the inflammation-induced inhibition of CYP450 enzymes are involved in the pathogenesis of some types of Parkinson's disease (Goralski *et al.* 2004).

Cytokines IL-1, IL-6, IFN-γ and TNF-α have all been implicated in CYP450 down-regulation. Interestingly, each is thought to have different effects on different CYP450 enzymes and there may also be some overlap between them, with the effects not being additive (Morgan 1997; Renton 2000; Morgan 2001; Renton 2001; Renton 2004; Morgan *et al.* 2008). Given that the concentration, time course and mix of cytokines vary depending on the source of infection and inflammation it is therefore possible that the effect on CYP450 regulation will also vary (Aitken *et al.* 2006; Richardson *et al.* 2006). IL-6 is thought to be key, and is by far the most potent down-regulator of CYP450 with strong correlations between IL-6 concentrations and reductions to CYP450 activity (Gurley *et al.* 1997; Siewert *et al.* 2000; Morgan *et al.* 2008).

In further proof of the involvement of the immune system, pharmacological doses of hydrocortisone, a immunosuppressant, produced an immediate increase in the clearance of antipyrine in human studies (Breckenridge *et al.* 1973). Corticosteroids have also been shown to induce CYP3A4 (Pichard *et al.* 1992; Shimamoto *et al.* 1999).

Research indicates CYP450 enzymes are down regulated during generation of host defences at the level of gene transcription, with a subsequent drop in the corresponding mRNA, protein and enzyme activity (Renton 2004). There may also be post-translational steps on some CYP450s which include enzyme modification and increased degradation (Aitken *et al.* 2006). LPS brings about swift and substantial down-regulation of several hepatic CYP450 mRNAs. Transcriptional suppression is thought to be an important factor in CYP450 mRNA down-regulation and suppression of some CYP450 genes occurs within one hour of LPS injection in rats (Cheng *et al.* 2003).

Despite the speed of this change to transcription, which makes the pace of enzyme induction appear glacial, it cannot explain the rapidity at which mRNA is down-regulated. It is thought that changes to mRNA stability must also occur. Even so, the mRNA suppression is not fast enough to explain the changes in CYP450 protein expression and activity (Morgan *et al.* 2008). Interestingly, nitric oxide, which can arise from neutrophil activation, has been found to have a role in CYP450 enzyme inhibition and protein

degradation (Ferrari *et al.* 2001; Vuppugalla *et al.* 2005), which may provide the final piece in the puzzle.

Oxidative stress may also have a role in CYP450 down-regulation. Antioxidants and inhibitors of xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase have been shown to inhibit LPS-induced CYP3A11 down-regulation, although the mechanism is uncertain (Xu *et al.* 2004; Xu *et al.* 2005).

It is probable that constitutive expression of CYP450 is not significantly affected. Some CYP450 isoforms are induced following exposure to certain drugs, such as occurs with CYP3A4 during treatment with rifampicin or diazepam (Heubel 1969; Pessayre *et al.* 1978). This induction is triggered by Pregane X receptor (PXR) (Lehmann *et al.* 1998; Kliewer *et al.* 2002). It has been found that PXR is also downregulated in hepatocytes during inflammation. However, as downregulation of CYP3A has been shown in the absence of alteration to PXR expression (Sachdeva *et al.* 2003), this is unlikely to be a significant contributor to changes in Phase I activity unless substantial CYP450 induction had previously taken place (Goralski *et al.* 2005).

Roles for the constitutive androstane receptor (CAR) have also been proposed. CAR has a similar role to PXR and inhibition of CAR has been shown to reduce CYP450 response to inducing stimulus such as phenobarbital exposure. CAR itself is positively regulated by glucocorticoids, which, as previously discussed, also inhibit cytokine release, further implicating the stress response in enzyme regulation (Sachdeva *et al.* 2003).

In summary there is still a lot of uncertainty in this area. CYP450 inhibition is thought to occur rapidly following inflammation and it is likely that LPS has a role. This inhibition seems to apply more to enzymes that are induced, rather than those that are constitutively expressed, so that while inflammation may reduce a CYP450 enzyme's activity, it will not eliminate it.

1.3.4.2.3 PATIENT BASED FACTORS AND EFFECTS ON CYP450 ISOENZYMES

Given the relevance of CYP450 to paracetamol metabolism and this Thesis, it is important to consider the impact on the different isoenzymes.

Recent reviews have summarised animal studies investigating the effects of cytokines and inflammation on CYP450 regulation (Morgan 1997; Renton 2001; Aitken *et al.* 2006). Importantly, hepatocyte expression of human and rodent CYP450 enzymes is regulated in

a similar manner (Morgan 2001). In rats, enzymes down-regulated by inflammation include CYPs 1A1, 2A1, 2C6, 2C7, 2C11, 2C33, 2E1, 3A2 and 4F4 and in mice 1A2, 2A5, 2C29, 2E1, 3A11, 4A10, 4A14, 4F15 and 4F16. There is much less research of this type in humans.

In vitro, inflammatory cytokines associated with the stress response, IL-1β, IL-6 and TNF-α, have been shown to reduce the expression of CYP1A2, 2E1 and 3A mRNA by at least 40% in human hepatocyte cultures (Abdel-Razzak *et al.* 1993). Conversely, IL-4, a cytokine involved in T-helper-cell activation, can induce 2E1 up to 5 fold (Abdel-Razzak *et al.* 1993), and is strongly implicated in the pathogenesis of drug-induced liver injury, however IL-4 is not significantly elevated as part of the stress response (Njoku 2010).

One study examined antipyrine metabolism in 57 children with multiple organ failure, critical illness or bacterial sepsis. The half-life of antipyrine was increased up to five fold and clearance was reduced up to threefold, indicating suppression of several CYP450 enzymes. Consistent with findings discussed above, clearance was reduced the most in those with higher levels of IL-6 (Carcillo *et al.* 2003).

As a model of chronic inflammation, Frye *et al.* gave a cocktail of drugs to estimate the activity of CYP1A2, 2C19, 2D6 and 2E1 to patients with congestive heart failure. They showed inflammatory cytokines IL-6 and TNF-α suppressed CYP1A2 and 2C19 activity, but did not appear to affect 2E1 or 2D6 (Frye *et al.* 2002). Similar results were shown in *in vitro* testing of hepatocyte cultures by Muntané-Relat *et al.* (Muntane-Relat *et al.* 1995). These authors examined the effect of known enzyme inducers on the activity of CYP 1A1, 1A2 and 3A4 with and without the presence of inflammatory cytokines IL-1α, IL-6 and TNF-α. By measuring mRNA levels they showed the cytokines substantially reduced the impact of enzyme inducers on protein synthesis, with reductions in both CYP1A1/1A2 and CYP3A4, consistent with the findings of Frye.

Another study examined CYP3A4 in 16 patients following abdominal aortic aneurysm, complete or partial colectomy or peripheral vascular surgery with graft (Haas *et al.* 2003). They found reductions in CYP3A4 activity of between 20-60% with greatest reductions at least 72 hours after surgery. Interestingly, peak IL-6 levels occurring 24-36 hours after surgery. The authors drew attention to the scale of this inhibition by drawing comparisons with the inhibition following administration of ketoconazole, clarithromycin, delavirdine and amprenavir, drugs well known to inhibit CYP3A4 (Lacy *et al.* 2005).

1.3.4.2.3.1 CANCER

Chronic inflammation is a common feature in patients with solid tumours. Cancer patients have been shown to have down regulated CYP3A (Rivory *et al.* 2002; Robertson *et al.* 2008), with functionally relevant alteration to pharmacodynamics/pharmacokinetics of substrates of CYP3A (Charles *et al.* 2006), correlating with plasma concentrations of IL-6 (Rivory *et al.* 2002). Much of the interpatient variation in response to cytotoxic chemotherapy is attributed to differences in CYP3A4 activity as CYP3A4 is responsible for the clearance of several cytotoxic agents: taxanes, vinca alkaloids, cyclophosphamide, tamoxifen, camptothecines, etoposide, imatinib and gefitinib (Morgan *et al.* 2008). Given these observations, the authors of this review recommended that CYP3A4 phenotyping would be a superior means of dose estimation to current crude estimates based on body surface area (Morgan *et al.* 2008).

It has been shown that variation in CYP3A4 genotype alone is unlikely to significantly alter clearance of cytotoxics (Lepper *et al.* 2005), but rather cancer-induced inflammation is thought to be responsible. Inflammation is present in more than 60% of patients with advanced cancer and they have significantly reduced CYP3A4 activity; again there are links with IL-6 concentrations (Charles *et al.* 2006) as well as CRP (Slaviero *et al.* 2003). Of specific relevance to this Thesis is the finding that colorectal cancers, in particular, have shown IL-6 protein actually within the malignant cells of tumours, indicating cytokine-stimulated release from Kupffer cells is not the only source of IL-6 in cancer patients (Brozek *et al.* 2005). Slaviero *et al.* also showed a link between reduced CYP3A4 mediated clearance and greater toxicity of vinorelbine and docetaxel (Slaviero *et al.* 2003).

1.3.4.2.3.2 DEHYDRATION AND STARVATION

Over 25% of hospital inpatients may be malnourished, either occurring before their admission to hospital or subsequently (Thompson 1995). Factors contributing to malnutrition in the surgical patient include increased nutritional requirements associated with their illness, increased losses *e.g.* from malabsorption, stricturing processes limiting nutritional intake, aggressive bowel preparations or stoma output and prolonged periods nil by mouth. All patients are routinely starved before surgery, often commencing the prior evening. Many surgical patients who have undergone major surgery or experienced complications are still not fed on day three postoperatively (Kennedy 1996). Prolonged starvation can contribute to the stress response and insulin resistance. Metabolic pathways more specific to paracetamol, in particular CYP2E1 activity (Nygren 2006), are

well known to be up-regulated in starvation alone (Pessayre *et al.* 1980; Burk *et al.* 1990; Prasad *et al.* 1990; Manyike *et al.* 2000).

Figure 1.3-4 shows the source of energy for CYP450 activity is NADPH. NADPH is derived from glucose-6-phosphate (G6P), which is itself derived from gluconeogenesis or dietary glucose. During gluconeogenesis NADPH production consumes G6P before glucose is produced. Accordingly, during periods of intense CYP450 activity, glucose arising from gluconeogenesis is reduced. Gluconeogenic substances enhance CYP450 activity by increasing NADPH production. In this way oxidation is closely linked to gluconeogenesis (Bánhegyi *et al.* 1988). As CYP450 systems derive energy straight from a source stimulated by starvation (*i.e.* gluconeogenesis), starvation does not cause the reductions in activity seen in other enzyme systems, in fact as described above, activity of CYP2E1 has been shown to be increased by starvation.

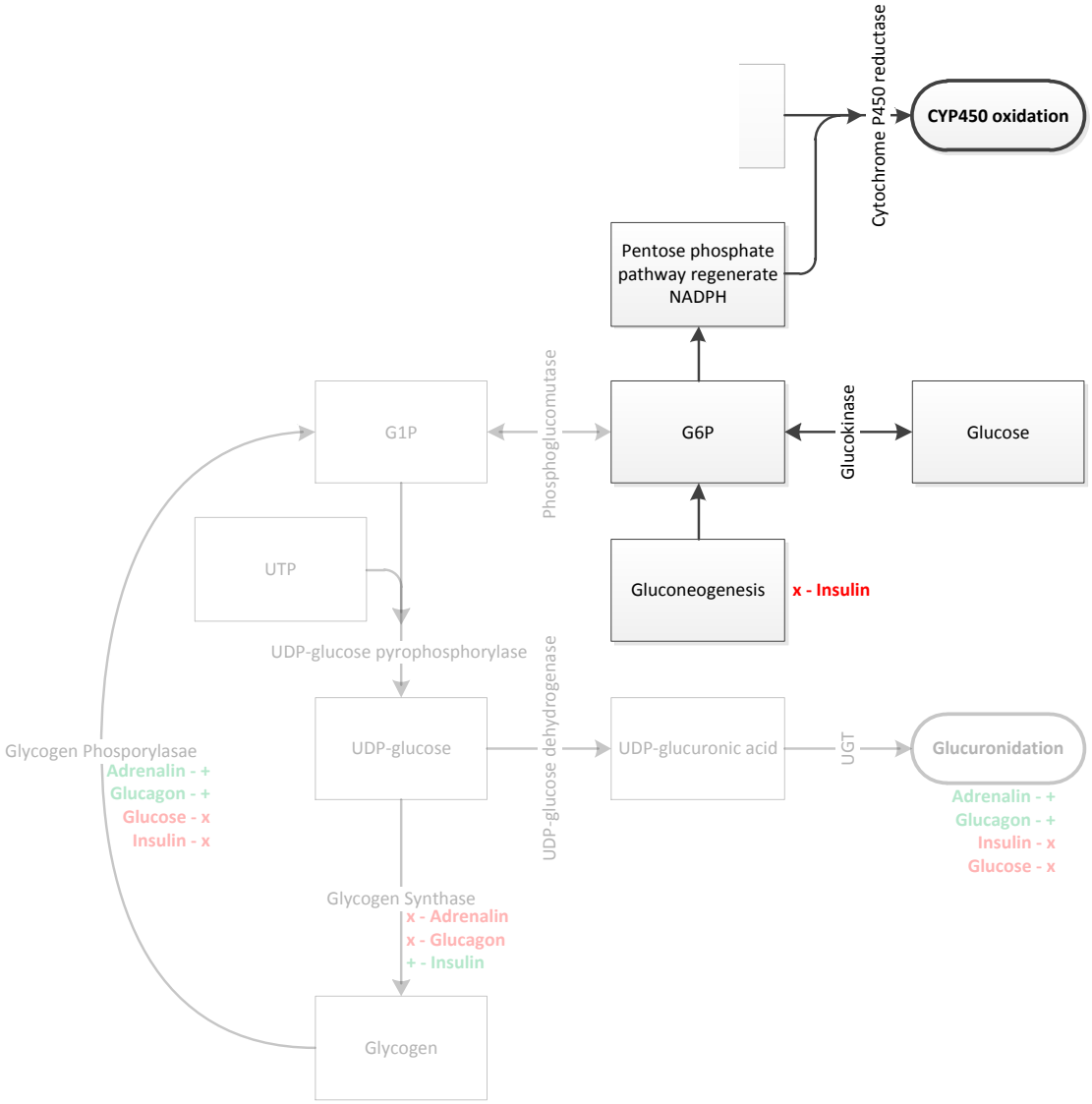


Figure 1.3-4 Intracellular glucose regulation and CYP450

The relationship between to CYP450 activity and intracellular glucose regulation is shown (top right). Abbreviations: G6P- Glucose-6-Phosphate, NADPH- Nicotinamide Adenine Dinucleotide Phosphate. Remainder of diagram is shown in Figure 1.3-5

Protein-containing infusions alone have been shown to prevent elements of the stress response (Kopp Lugli *et al.* 2010) by preserving the activity of drug metabolising enzymes (Pessayre *et al.* 1978). Preoperative glucose infusions have been shown to reduce postoperative insulin resistance, further implying the importance of preoperative nutrition to preventing CYP450 downregulation (Ljungqvist *et al.* 1994).

Dehydration, a common factor in the early postoperative period, is not thought to influence Phase I activity greatly, although there are some dissenters (Lee *et al.* 2008). Zafar *et al.* found that after 96 hours of dehydration the clearance of paracetamol in rats was unaffected, although they did show an increase in the glucuronide conjugate and reductions in the unchanged paracetamol excreted (Zafar *et al.* 1987). The significance of this is uncertain, given metabolism in the rat does not accurately reflect paracetamol metabolism in humans. This may also be a function of changes to excretion, rather than metabolism *per-se*.

Changes in CYP450 activity in dehydrated rats were reported by Kim *et al.* in 2001 who showed CYP2E1 protein levels increased three fold, without effect on CYP1A2, 2B1, 2C11 or 3A4 (Kim *et al.* 2001). However, in their discussion, the authors noted that water deprivation was associated with reduced food intake and they suspected the effect on CYP2E1 arose from the reduction in food intake rather that dehydration as such. Following this work Kim *et al.* went on to again deprive rats of water but give glucose supplementation. They found glucose supplementation ameliorated the changes to CYP2E1 activity, confirming that the effects of starvation were of greater consequence to CYP2E1 activity than water deprivation (Kim *et al.* 2006). These findings were echoed in a study of surgical patients by Jorquera *et al*. By providing an exogenous source of glucose and protein through administration of parenteral nutrition following surgery, Jorquera *et al.* was able to diminish reductions of antipyrine clearance seen in control patients who did not receive the infusion (Jorquera *et al.* 1994).

When considering the complexities of the stress response and other changes experienced by the surgical patient, the involvement of insulin, glucose, corticosteroids and cytokines all probably have a role in the regulation of drug metabolising enzymes.

1.3.4.2.4 PHYSIOLOGICAL ROLE OF CYP450 INHIBITION

The question of why CYP450 enzymes are downregulated during inflammation remains a subject of debate. Some theories include:

- The dependency of the acute phase response on haem. Downregulation of CYP450 would increase the availability of haem and augment the acute phase response;
- The protection of the liver against additional oxidative stress. The inflammatory response causes oxidative stress which contributes to infection-induced hepatic injury. CYP450 enzymes produce reactive oxygen species therefore downregulation of CYP450 could prevent further contributions to this stress; and
- The execution of the inflammatory response. Some CYP450 enzymes generate products with anti-inflammatory and vasodilatory activity. Downregulation of these enzymes would prevent inhibition of the immune response as well as the contribution to hypotension, a significant predictor of morbidity in septic shock (Fleming 2001; Aitken *et al.* 2006).

While it is clear CYP450 is regulated by inflammation, it is a curious finding that the reverse is true. CYP450 inhibitors exacerbate and CYP450 inducers attenuate fever caused by inflammatory stimuli (Aitken *et al.* 2006). The immune response following administration of endotoxin to individuals receiving rifampicin, an enzyme inducer, would be enhanced, whereas the response of an individual receiving the enzyme inhibitor clarithromycin is diminished in comparison.

1.3.4.3 PHASE II

Whilst there is some literature on Phase I metabolism perioperatively, research into alterations in Phase II enzyme activity perioperatively has been almost absent. Compared with the CYP450 system, very little is known about changes to Phase II processes arising from surgery, inflammation or infection (Xin 2002; Aitken *et al.* 2006). This may be due to the perception that there is little variation in this pathway in comparison with Phase I enzyme activity.

1.3.4.3.1 ALTERATION TO ENZYME ACTIVITY

There are two reports relating to changes in Phase II activity postoperatively; both showing reductions in the proportion of Phase II products excreted in the urine. Results were calculated as ratios between the total of Phase II metabolites (paracetamol glucuronide and paracetamol sulphate), to Phase I metabolites (paracetamol cysteine and paracetamol mercapturate). In the first study, where patients acted as their own controls, patients were given a single dose of paracetamol at least one day preoperatively and

again on day two postoperatively. There was a significant reduction in the ratio of Phase II:I metabolites of paracetamol postoperatively (Kennedy 1996). In the second study, patients again acted as their own controls and received a single daily dose, but the duration of administration was extended to five days post-surgery. Twenty seven patients undergoing elective major abdominal surgery completed the study. The ratio of Phase II:I metabolites dropped from 28 preoperatively, to 5 postoperatively, indicating a substantial shift towards the production of the hepatotoxic metabolite (Kennedy 2009a). This confirmed earlier incidental findings showing significant reductions in paracetamol glucuronide excretion and increased in paracetamol cysteine excretion (Kennedy 1996).

Glucuronidation and sulphation often occur on overlapping substrates, and to date, specific probes for these pathways have not been validated. This may explain the difficulty in ascribing alterations to one specific pathway and lack of clinical reports of toxicity arising from changes to Phase II metabolism.

1.3.4.3.1.1 SULPHATION

Sulphation is a high affinity, low capacity reaction, predominating at low substrate concentrations (Section 1.2). Once again there are few studies specifically dealing with sulphate conjugation perioperatively. Reith *et al.* showed that with increasing doses of paracetamol, the proportion of the dose excreted as paracetamol sulphate decreased (Reith *et al.* 2009). This effect may also be seen in single doses as paracetamol sulphate excretion was markedly reduced, approaching significance, in the first study by Kennedy *et al.* in 1996.

Depletion of the inorganic sulphate and saturation of SULT enzymes occur following paracetamol administration (Hjelle *et al.* 1985). SULT enzyme 2A1 and the enzyme that synthesises SULT enzymes' cofactors, PAPS synthase 2, have both been shown to be downregulated by LPS induced inflammation. Similar results have been shown for SULT1A1 and 1C1 with up to 76% suppression of sulphating activity being reported (Shimada *et al.* 1999; Kim *et al.* 2003; Kim *et al.* 2004). As observed for CYP450 enzymes, PXR and CAR are implicated in the down-regulation of SULT enzymes (Kim *et al.* 2003).

1.3.4.3.1.2 GLUCURONIDATION

Glucuronide conjugation predominates at high substrate concentrations, once sulphation has been saturated (Zamek-Gliszczynski *et al.* 2006). The enzymes involved in glucuronidation, UGT, are known to be regulated by factors affected by inflammation

(Aitken *et al.* 2006). Compared with changes in CYP450 enzymes, decreases in hepatic UGT activity are minor, species and stimuli-dependent (Aitken *et al.* 2006). Only one study in human liver biopsies has correlated decreases in mRNA of UGT1A4, 2B4 and 2B7 with inflammation (Congiu *et al.* 2002).

1.3.4.3.1.2.1 GLUCOSE REGULATION AND GLUCURONIDATION

Because glucuronidation contributes substantially more to the metabolism of paracetamol than any other pathway, and is also capable of conjugating greater amounts of parent drug, lack of substrate, G1P, for conjugation could be a reason for this reduction observed postoperatively.

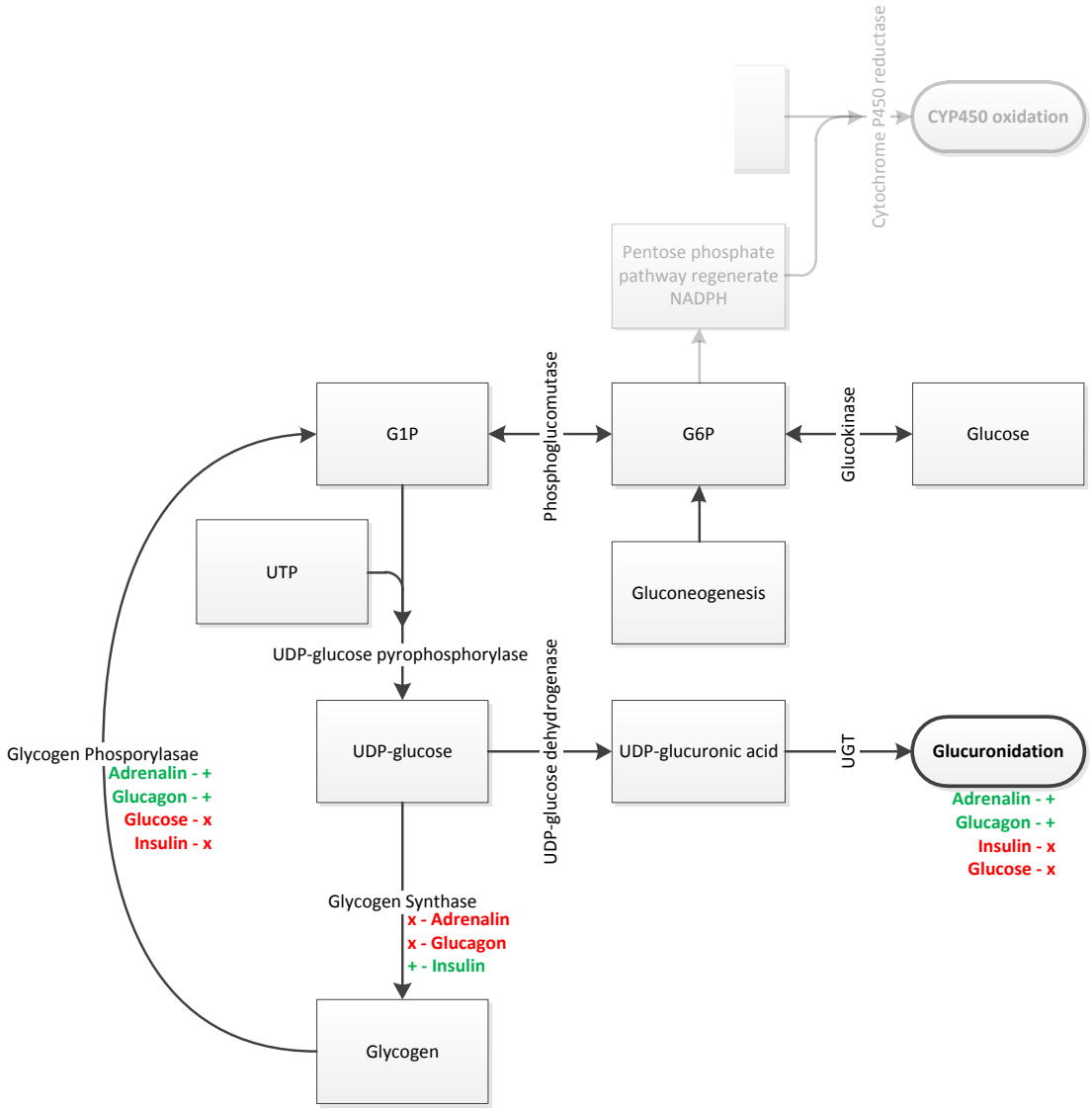


Figure 1.3-5 Intracellular glucose regulation and Glucuronidation
The relationship between to glucuronidation and intracellular glucose regulation is shown. Abbreviations: G6P- Glucose-6-Phosphate; G1P- Glucose-1-Phosphate; UTP- Uridine triphosphate; UDP- Uridine diphosphate; UGT- Uridine 5'-diphospho-glucuronosyltransferase.

Figure 1.3-5 shows G1P is required for glucuronidation and it is thought that the G1P used arises exclusively from glycogenolysis, not from dietary sources *per se*. Inhibition of

glycogenolysis also inhibits glucuronidation and glucose does not reduce glucose production arising from gluconeogeneic substances. Glucuronidation activity is proportional to carbohydrate reserves with maximum rates seen in livers with the largest glycogen reserves. Conversely, depletion of glycogen by starvation inhibits glucuronidation. As a result things that inhibit glycogenolysis may impair glucuronidation (Bánhegyi *et al.* 1988).

Applying these findings to the surgical patient is difficult. Patients are catabolic following surgery; tissues are resistant to insulin and with reduced uptake and utilisation of glucose, are frequently hyperglycaemic. Insulin resistance and perioperative starvation would normally promote glycogenolysis and therefore glucuronidation, but the high ambient glucose levels inhibit glycogenolysis. The net result on glycogenolysis and therefore glucuronidation is uncertain (Schenker *et al.* 2001). There are some *in vivo* studies. One study showed short periods of starvation not to affect glucuronidation in patients(Rumack 2004). Kennedy *et al.*, also did not find an appreciable change in the paracetamol Phase II:I metabolite ratio when patients resuming light diets following major abdominal surgery (Kennedy 2009b).

1.3.4.3.1.3 GSH CONJUGATION

In starved patients, hepatic concentrations of GSH are diminished through lack of dietary cysteine intake. In these patients there is an increased risk of hepatotoxicity from reactive oxygen species such as NAPQI (Figure 1.3-6). Whitcomb found starvation reduced GSH concentration up to 50% in the liver tissue of adult rodents (Whitcomb *et al.* 1994). This is thought to be due to the utilisation of the hepatic and intestinal GSH stores as reservoirs to supply cysteine during cysteine reservoirs during food deprivation (Strubelt 1980; Cho *et al.* 1981; Di Simplicio *et al.* 1997).

No changes to constitutive expression of GST have been found to arise from LPS-induced inflammation; however induction of GST may be prevented by inflammation (Maheo *et al.* 1998). Downregulation of induced expression is thought to be by accelerated degradation of mRNA, in a similar manner to CYP450 downregulation (Maheo *et al.* 1997).

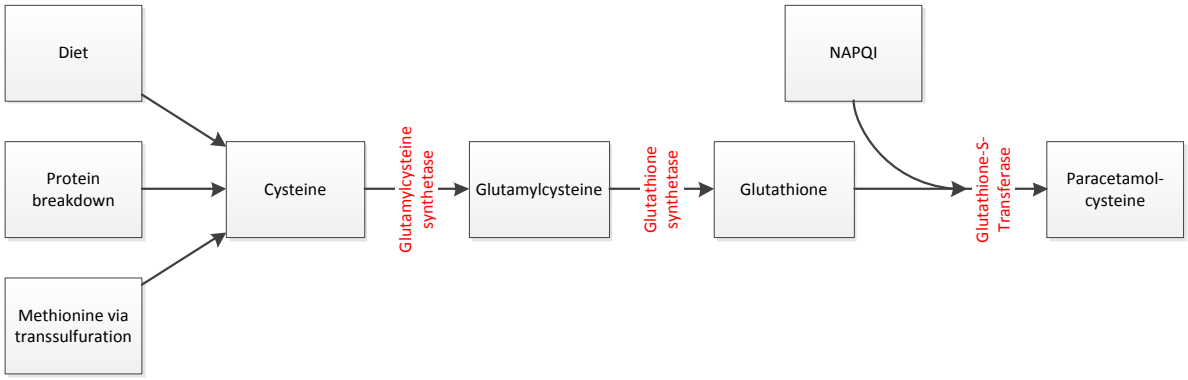


Figure 1.3-6 Role of glucuronidation in paracetamol detoxification and sources of cysteine (Lu 1999)

While no changes to GST activity have been found, GST requires GSH, and the synthesis of this cofactor can be rate limiting. One of the major determinants of GSH synthesis is the availability of cysteine. As already described, exogenous cysteine is generally obtained from the diet and protein breakdown while endogenous cysteine can be synthesised *de novo* in the liver from methionine by the transsulphuration pathway (Takada *et al.* 1984; Colell *et al.* 1997; Fernandez-Checa *et al.* 1997). Cysteine is unusual amongst amino acids as it exists in two forms. Inside the cell it is found in its' reduced, sulph-hydryl form, cysteine. Outside the cell, in the extracellular fluid, it autooxidises to its disulphide form, cystine. Aside from diet, the main factors that determine hepatocellular availability of cysteine are firstly the activity of the membrane transport of cystine, cysteine and methionine and secondly, the activity of the transsulphuration pathway (Fernandez-Checa *et al.* 1997). In normal conditions rat hepatocytes uptake cysteine at a rate about three fold higher than methionine and 13 fold higher than that of cystine. Explaining this, cysteine transport occurs under normal physiological conditions; however, cystine uptake by hepatocytes is poor, but can be stimulated after prolonged exposure to insulin and dexamethasone (Takada *et al.* 1984). This could indicate regulation as part of the stress response. Additionally, the enzyme responsible for GSH synthesis, γ-glutamylcysteine synthetase, (GCS), is modified by factors involved in inflammation and the stress response, being induced by cytokines TNF-α and IL-1β, insulin and glucocorticoids, but inhibited by high glucose concentrations (Urata *et al.* 1996; Lu 1999).

1.3.5 EXCRETION

Through the process of metabolism a drug is more readily excretable, most commonly through the kidneys into the urine, or into the bile from the liver. Once a drug is metabolised there are some factors due to surgery and anaesthesia which may influence its excretion:

- Altering the rate of delivery to the organ of excretion; and
- Changing the activity of enzymes or other active or passive process of excretion (Nimmo *et al.* 1988).

These are discussed below.

1.3.5.1 ALTERING THE RATE OF DELIVERY TO THE ORGAN OF EXCRETION

The excretion of some drugs is so efficient that it is limited by the rate at which they are delivered to their organ of excretion. Such drugs are known to have a high extraction ratio as in the case of volatile anaesthetics, which are excreted by the lungs. They rely on pulmonary blood flow to determine their rate of elimination. Because pulmonary blood flow is determined by cardiac output and, as discussed above, cardiac output can be reduced around the time of surgery, surgery can reduce their clearance (Nimmo *et al.* 1988). These effects are most pronounced immediately after surgery (Haas *et al.* 2003).

Similarly, drugs such as opiates and β-blockers, that have a high hepatic extraction ratio depend on blood flow to the liver to determine rate of elimination (Nies *et al.* 1976). However as paracetamol has only an intermediate hepatic extraction ratio this is not particularly relevant to its disposition (Mather *et al.* 1986; Nimmo *et al.* 1988). (Mather *et al.* 1986; Nimmo *et al.* 1988).

Blood to the kidney can also be reduced and this has been shown to impair renal elimination of gentamicin (Nimmo *et al.* 1988; Kim *et al.* 2001; Kim *et al.* 2006; Lee *et al.* 2008). It may also have implications for other drugs and metabolites which rely on renal excretion. These drugs could accumulate and, if they have pharmacological activity, such as morphine metabolites, potentially cause toxicity (Nimmo *et al.* 1988). In the case of paracetamol, renal failure may cause the glucuronide and sulphate metabolites to accumulate however, because they are pharmacologically inert, they are pharmacologically inert, it does not necessitate dose adjustment.

1.3.5.2 CHANGES TO THE ACTIVITY OF ENZYMES OR OTHER ACTIVE OR PASSIVE PROCESS OF EXCRETION

Other changes due to surgery can lead to alteration of physiological pH and by affecting ionisation states of compounds, this may affect excretion processes. Paracetamol is a weak organic acid with a pKa of 9.5. It is therefore largely unionised in urine over the

physiological pH range and accordingly its excretion is much slower than glomerular filtration rates. Because of its pKa, its clearance is not influenced by changes in urine pH, but is affected by changes in urine flow although the effect is modest. In one study, increases in urine flow rate from 1.6 to 13.7mL/min resulted in less than a doubling of paracetamol clearance(Prescott *et al.* 1973). Reductions to urine flow are frequently seen in surgical patients, especially in the early postoperative period, followed by a period of diuresis, and these changes may alter the excretion of paracetamol. However, paracetamol glucuronide and paracetamol sulphate are extensively ionised in biological fluids, irrespective of pH, and their clearance is therefore unaffected by changes in urinary pH or urine flow (Prescott *et al.* 1973; Prescott 1980). Both paracetamol and its metabolites share similar mechanisms of active renal tubular secretion, and along with other enzymes discussed above, this may be down-regulated during periods of inflammation or infection (Duggin *et al.* 1975).

Although changes to hepatic enzymes involved in excretion of drug metabolites into the bile may occur as a result of surgery, these are not well documented. Reductions in bile flow and bile salt excretion has been reported following surgery, which may arise from impairment of transporter molecules in the canaliculi as discussed above (Herman *et al.* 1971; Prandi 1975), but the implications for drug therapy and paracetamol use is uncertain.

1.4 OTHER INFLUENCES OF PARACETAMOL DISPOSITION

Because of the uncertainty around therapeutic concentrations, literature surrounding changes to paracetamol disposition is primarily concerned with toxicity and its prediction.

Paracetamol is considered an extremely safe drug, especially when it is taken into account that the annual United Kingdom (UK) consumption could be measured in the hundreds of tonnes (Sheen *et al.* 2002a). While safe in almost all individuals, there is evidence of toxicity at licensed doses, although these are generally limited to case reports. Reviewing these case reports reveals a number of common factors for paracetamol toxicity. As discussed in Section 1.2 these can summarised into two types of risk:

- Those that increase NAPQI production (usually through CYP2E1 induction); or
- Those that impair GSH conjugation of NAPQI (usually through depletion or impaired supply of GSH's co-factors).

In the majority of case reports of toxicity where licensed doses have been used, both types of risk factor can be identified and it is unlikely that either one factor alone is sufficient to cause toxicity. The main causes of these risk factors are discussed below.

1.4.1 ALCOHOL

Table 1.4-1 Summary of alterations to paracetamol disposition with alcohol intake

	NAPQI Production	GSH
Acute alcohol	↓	-
Chronic alcohol	↑	↓

Alterations to paracetamol disposition in alcoholism are centred on ethanol-induced changes to CYP2E1 activity, the enzyme primarily responsible for the production of paracetamol's hepatotoxic metabolite, NAPQI. This is compounded by reductions to the supply of GSH (Table 1.4-1).

The understanding of the interaction between CYP2E1, ethanol and paracetamol disposition is complicated by the fact that acute and chronic ethanol intake have opposite effects (Prescott 1996). When administered concurrently, ethanol is preferentially metabolised by CYP2E1 over paracetamol. Thus individuals with suicidal intent who consume a bottle of whisky along with a bottle of paracetamol are provided a large degree of protection from paracetamol toxicity by the ethanol in the whisky (Prescott *et al.* 1983). Providing blood-ethanol concentrations remain sufficiently high, paracetamol

metabolism proceeds along non-toxic Phase II pathways as CYP2E1 remains occupied with ethanol metabolism (Prescott 2000a; Thummel *et al.* 2000; Waring *et al.* 2008).

The detrimental effects of ethanol arise from chronic administration (Liangpunsakul *et al.* 2005). However, the risk arising from ethanol-related induction of CYP2E1 alone may be overstated and requires a substantial and continuous level of ethanol consumption which is reversed quickly by short periods of abstinence.

A significant level of induction is difficult to achieve because, like many CYP450 enzymes, CYP2E1 is a leaky enzyme that, in the absence of substrate, it generates reactive oxygen species that increase its own degradation (Liangpunsakul *et al.* 2005). Because ethanol is a substrate of CYP2E1, this stabilises the CYP2E1 enzyme, reducing the rate of degradation and effectively increasing their amount. CYP2E1 is normally biphasic with half-lives of 7 and 37 hours (Rumack 2002). Ethanol dramatically extends the fast component of degradation so that the half-life appears as a single 37 hour duration without any transcriptional change to mRNA for CYP2E1 (Eliasson *et al.* 1992). A modest (about four-fold) increase in CYP2E1 activity is observed in those individuals who consume substantial amounts of ethanol on a daily basis to maintain blood ethanol concentrations around 250mg/dL. However, this requires the consumption of sufficient ethanol to continuously maintain blood alcohol concentrations at least five times the legal driving limit in UK (Girre *et al.* 1994; Dilger *et al.* 1997; Dupont *et al.* 1998). As consumption increases beyond this there appears to be increased synthesis of CYP2E1 as well in addition to the stabilisation described above (Waring *et al.* 2008). To give further context, Thummel *et al*. projected a doubling of NAPQI formation would require an individual to consume greater than 50 bottles of wine over eight days (Thummel *et al.* 2000). Even once achieved, this induction of CYP2E1 is short-lived, with 50% being lost after 36-48 hours of abstinence, returning to within normal ranges after 8 days (Perrot *et al.* 1989; Girre *et al.* 1994; Lucas *et al.* 1995).

While consuming alcohol, the increased risk presented by this CYP2E1 induction is balanced by the protection afforded by the ethanol present in the blood. Therefore, in those individuals with ethanol-induced CYP2E1 induction, the period of greatest risk from paracetamol toxicity is upon acute withdrawal, such as may occur upon admission to hospital (Perrot *et al.* 1989; Schiodt *et al.* 2002; Graham *et al.* 2005b). At this most susceptible time there can be an increase of 22% in the mean formation of NAPQI (Thummel *et al.* 2000), reducing the average single dose required for toxicity from 15.9g

to 13g according to current treatment guidelines (Sivilotti *et al.* 2005), however, indications of the increased sensitivity to licensed doses cannot be found.

While the role of CYP2E1 induction may be less important, chronic consumption of alcohol does independently increase the mortality of paracetamol overdose, both as single doses (Schiodt *et al.* 2002) and repeated supra-therapeutic doses (Alhelail *et al.* 2011) indicating the effect of other risk factors.

When licensed doses are considered, the role of GSH conjugation may be more important in chronic consumers of alcohol. Following chronic consumption of alcohol, GSH synthesis and transport into mitochondria is reduced (Lu 1999; Lee *et al.* 2004b). This could decrease an alcoholic's capacity to detoxify NAPQI, which as described above may be in increased amounts due to CYP2E1 induction (Fernandez-Checa *et al.* 1987; Lauterburg *et al.* 1988; Sinclair *et al.* 2000a; Sinclair *et al.* 2000b). Additionally, higher energy requirement and subsequent malnutrition seen in chronic alcoholics (often despite adequate nutritional intake) may impair regeneration of GSH, further impeding the detoxification of NAPQI (Lieber 1991; Gloria *et al.* 1997; Bergheim *et al.* 2003). The impact of malnutrition is discussed further below in Section 1.4.5.

Despite an increased sensitivity to paracetamol toxicity and several case reports of alcoholics with severe and even fatal liver damage after therapeutic doses of paracetamol (Whitcomb *et al.* 1994; Moling *et al.* 2006; Krahenbuhl *et al.* 2007; Claridge *et al.* 2010), hepatotoxicity has not been shown in prospective studies. Benson gave 4g paracetamol/day to patients with chronic liver disease including alcoholic cirrhosis for up to 14 days and showed no adverse effects (Benson 1983). A larger placebo controlled study of 385 alcoholics published in 2005 found similar safety of therapeutic doses in this population (Dart *et al.* 2000; Kuffner *et al.* 2005). Critics of the relationship between alcoholism and enhanced toxicity of licensed doses question the validity of the recorded dose in these case reports. Ingestion of paracetamol with therapeutic intent does not always mean therapeutic doses are taken (Whitcomb *et al.* 1994) and extrapolation of measured serum concentrations was indicative of overdose in most cases (Prescott 2000a). Additionally, delayed presentation to care centres and poor recollection of dose taken may also have a role in enhancing cell sensitivity in alcoholics (Whitcomb *et al.* 1994). Delayed presentation or incorrect reporting of time of overdose of one or two hours can make a large difference to the way a patient is treated and their likelihood of developing hepatotoxicity (Rumack 2004). Despite links between alcoholism and

paracetamol toxicity, this has only been shown with paracetamol overdose and there is little evidence that licensed doses carry any additional risk (Rumack 2004; Benson *et al.* 2005; Kuffner *et al.* 2005; Dart *et al.* 2010). Even so the FDA require all paracetamol sold in the United States to be labelled with the warning:

> *"If you consume 3 or more alcoholic drinks every day, you should ask your doctor whether you should take acetaminophen (paracetamol) or other pain relievers/fever reducers. Acetaminophen may cause liver failure"* (Graham et al. 2005b).

1.4.2 SMOKING

Table 1.4-2 Summary of alterations to paracetamol disposition with smoking

	NAPQI Production	GSH	Glucuronidation
Smoking	↑	-	↑

Cigarette smoking's effect on paracetamol disposition is small when compared with alcohol (Mishin *et al.* 1998). Its main effect is to induce CYP1A2, a minor contributor to Phase I metabolism at licensed doses, although some changes to CYP2E1 have also been identified. There may also be induction of glucuronidation of paracetamol, which could potentially oppose these changes, reducing paracetamol's toxicity (Table 1.4-2).

Polycyclic aromatic hydrocarbons (PAHs), the products of incomplete combustion, are some of the major lung carcinogens found in tobacco smoke and are also inducers of UGT1A6, CYP450 1A1, 1A2 and possibly CYP2E1 (Schmidt *et al.* 2003; Kroon 2007). The metabolism of many drug substrates can be induced in smokers resulting in a clinically significant decrease in pharmacological effects. These changes have also been reported in individuals exposed to second hand smoke (Mayo 2001). Effects have been shown to continue for several days after withdrawal of tobacco smoke exposure, with no effect of withdrawal seen in the first 24-36 hours (Kroon 2007), although values normalise to pre-exposure levels within one week of cessation (Eldon *et al.* 1987; Faber *et al.* 2005). Other compounds in tobacco smoke such as acetone, pyridine, heavy metals, benzene, carbon monoxide and nicotine may also interact with hepatic enzymes but their effects appear to be less significant.

The effect of tobacco smoke on CYP1A2 appears to be most pronounced. The induction of CYP1A2 by tobacco smoke and other aromatic hydrocarbons is well established. It is linked to transcriptional events and is probably tissue specific (Zevin *et al.* 1999). The two-fold higher CYP1A2 activity commonly seen in smokers may increase clearance and

necessitate higher doses of drugs that are CYP1A2 substrates (Fuhr *et al.* 1993; de Leon *et al.* 2003; Kroon 2007; Backman *et al.* 2008). Clearance of theophylline is increased by 58-100% (Zevin *et al.* 1999) while caffeine concentrations of smokers can be one half to one third of non-smokers (Benowitz *et al.* 2003).

Increases in CYP2E1 activity and expression due to smoking have been shown in mouse lung, kidney and liver, although most studies in humans have failed to demonstrate significant induction of CYP2E1 in smokers (Miller 1989; Girre *et al.* 1994; Howard *et al.* 2001). Induction may be due to repeated exposure to some of the substrates of CYP2E1 found in tobacco smoke (*i.e.* NMDA, pyridine, benzene, acetone, styrene and vinyl chloride)(Zevin *et al.* 1999), however their effect is small compared with the inducing effects of ethanol, as described in the previous section (Mishin *et al.* 1998). One study of CYP2E1 activity in humans smokers showed intra-individual changes of smokers before and then a week after cessation, although the effect was not seen in all subjects (Benowitz *et al.* 2003). These authors suggested the large inter-individual variation in CYP2E1 activity masks the moderate effects of smoking in studies that use a between subject design, such as the one by Girre *et al*. (Benowitz *et al.* 2003). This wide variation in response may be related to the presence of CYP2E1 gene alleles that are associated with higher or lower susceptibility to induction by cigarette smoking (Hu *et al.* 1999).

Some glucuronide conjugation can also be induced by PAH. Cigarette smoke exerts differential effects on UDP-glucuronosyltransferases; and for many drug substrates clearance is increased. (Ochs *et al.* 1981; Grech-Belanger *et al.* 1985; Fleischmann *et al.* 1986; Walle *et al.* 1987; Bock *et al.* 1994; Zevin *et al.* 1999; Kroon 2007). Paracetamol glucuronidation was found to be increased in some smokers and those exposed to PAH, through induction of UGT1A6. Interestingly the authors noted a correlation with caffeine oxidation (a marker of CYP1A2 activity) in the study group, suggesting an element of co-regulation (Bock *et al.* 1994).

The impact of these changes on paracetamol at licensed doses is minor. At these doses there is no evidence of a significant change in paracetamol's half-life, Vd or clearance due to cigarette smoking (Pantuck *et al.* 1974; Mucklow *et al.* 1980; Miners *et al.* 1984b; Bock *et al.* 1987; Scavone *et al.* 1990; Bock *et al.* 1994; Dong *et al.* 1998). Although increased Phase I metabolism in smokers has been shown, the resultant increase in NAPQI at licensed doses is quantitatively unimportant. Following overdose, however, cigarette smoking has been shown to be an independent risk factor for severe hepatotoxicity and

mortality (Schmidt *et al.* 2003). In overdose situations the role of CYP1A2 may be more important as limited capacity of CYP2E1 is overwhelmed, leading to increased involvement of other CYP450 enzymes. If pre-existing induction of CYP1A2 from smoking was present, the subsequent increase in NAPQI production could explain the poorer outcome of smokers following overdose (Benowitz *et al.* 2003).

It is important to note that these interactions are caused by the PAHs in tobacco smoke with only sparse evidence of a contribution of nicotine *per se*. Therefore nicotine replacement therapy does not cause the same pharmacokinetic drug interactions as tobacco smoke. However, pharmacodynamic drug interactions do result from nicotine, the majority arising from the activation of the sympathetic nervous system which can affect the pharmacological activity of certain drugs, but this is not important for the activity of paracetamol (Kroon 2007).

1.4.3 AGEING

Table 1.4-3 Summary of alterations to paracetamol disposition with aging

	NAPQI Production	GSH
Aging	-	-
Frailty	-	↓

Both pharmacodynamic and pharmacokinetic factors are responsible for age related changes to paracetamol disposition, although the cumulative effect is much smaller than alcohol or smoking (Ochs *et al.* 1981).

Regardless of the small scale of changes, the incidence of all serious adverse drug reactions increase with increasing age, even after controlling for increased medication use (Moore *et al.* 2007). Even though the majority of paracetamol poisoning cases are adolescents and young adults, most paracetamol associated deaths occur in an older population (Schmidt 2005). Additional risk factors that contribute to this poor outcome in the elderly include an increased time to presentation, poly-pharmacy, poor recollection of consumed dose and a greater prevalence of alcohol abuse (Schmidt 2005). Starvation and malnourishment is also more prevalent in the elderly which carry their own risk (see Section 1.4.5 below)(Schwartz 2007) and insufficient dietary intake of sulphur containing amino acids are observed in the elderly (Maher 2005; Mercier *et al.* 2006). However, many studies show no change to disposition of paracetamol at licensed doses in the elderly (Triggs *et al.* 1975; Miners *et al.* 1988) and there is no evidence age should be

considered when deciding the dose of paracetamol in adults (Divoll *et al.* 1982b; Miners *et al.* 1988; Klasco 2009).

As people age they become more sensitive to adverse effects of drugs (Demeure *et al.* 2006), however it may be the frailty that accompanies aging that is more related to changes in drug disposition (Wynne *et al.* 1990). It is frailty that is associated with a loss of reserves and increased state of vulnerability to paracetamol toxicity, not aging *per se* (Mitchell *et al.* 2011b) and frailty has been shown as a better predictor of deranged liver function than age in hospitalised patients receiving regular paracetamol (Mitchell *et al.* 2011a)(Table 1.4-3).

There are some reports of changes to drug absorption in the elderly. These changes are minor and not shown consistently, with most studies showing no change to absorption kinetics at all (Gainsborough *et al.* 1993; Schwartz 2007). Small but statistically significant reductions in the bioavailability of paracetamol in elderly subjects have been shown, but these were lost when co-administered with food (Divoll *et al.* 1982b).

There may also be small reductions to Vd. Physiologically, with increasing age comes increasing body fat, reduction of body size, intravascular volume, organ volume and muscle volume, especially in females (Schwartz 2007). This may lead to reductions in Vd which have been shown in studies, especially in females (Schwartz 2007). been shown in studies using paracetamol (Divoll *et al.* 1982a; Liukas *et al.* 2011).

Typically there are also declines in renal and hepatic function (Klotz 2009), especially in women (Schwartz 2007). Generally, however, half-life of paracetamol is unchanged by aging (Divoll *et al.* 1982a; Galinsky *et al.* 1986; Miners *et al.* 1988; Wynne *et al.* 1990; Liukas *et al.* 2011). Two more recent studies do exhibit some changes: serum paracetamol concentrations increased in elderly after five days of therapy (Mitchell *et al.* 2011a) and in a second, clearance was reduced in the elderly with marked increases in exposure to paracetamol following a 1g dose (Liukas *et al.* 2011).

There are few studies that explain the changes to disposition seen in these last two studies. Generally there is very little age-related change to hepatic clearance, but when occurring, Phase I reactions are more impaired than Phase II in the elderly (Klotz 2009) which only account for a small amount of paracetamol's clearance.

Typically, the role of age in the interindividual variation in Phase I's CYP450 activity is relatively minor (Bebia *et al.* 2004; Liu *et al.* 2005) and has been shown to both increase and decrease (Galinsky *et al.* 1986). Determining CYP450 in the elderly is complicated by the presence of a number of confounders, which increase with age (Johansson *et al.* 1990). Such age-related changes include reductions in creatinine clearance. Once these changes are accounted for, observed age-related differences often disappear (Bebia *et al.* 2004). Specifically there is no evidence of change in the activity of CYP1A2 or CYP2D6 (Simon *et al.* 2001; Bebia *et al.* 2004). There may be a small degree of induction of CYP2E1 (Bebia *et al.* 2004), but this was not seen in an earlier study (Kim *et al.* 1995b). Clearance of CYP3A substrates have been shown to both increase (Bebia *et al.* 2004) and decrease with age (Schwartz 2006) but this is not due to alteration in the activity of the enzyme (Schwartz 2006). Aging-related alteration in the clearance of CYP450 substrates is thought more likely to be secondary to changes in liver blood flow, size, or drug binding and distribution with aging (Hunt *et al.* 1992a; Hunt *et al.* 1992b; Klotz 2009). Again, frailty is more associated with decline in CYP450 function (Wynne *et al.* 1990; Wynne 2005). In the disposition of paracetamol itself, shifts towards Phase I metabolism seen upon chronic dosing to inpatients are more prominent in the elderly, with age related increases to urinary recovery of paracetamol cysteine (Pickering *et al.* 2011).

Changes to Phase II pathways are also minor. Glucuronidation of paracetamol has been shown to reduce in frailty (Wynne *et al.* 1990) and chronic dosing in the elderly (Pickering *et al.* 2011). Reports of increases (Galinsky *et al.* 1986) and no effect due to aging also exist (Miners *et al.* 1988). A small reduction in sulphonation has been reported (Galinsky *et al.* 1986; Miners *et al.* 1988; Pickering *et al.* 2011) although sulphonation was preserved in frailty (Wynne *et al.* 1990). GSH content of the liver has been shown to reduce with age (Liu *et al.* 2004; Maher 2005; Mercier *et al.* 2006) and age related increases in the demand for cysteine may reduce availability of cofactors for GSH formation (Mercier *et al.* 2006). This effect is compounded by the finding that diets containing insufficient inorganic sulphur and sulphur containing amino acids are more common in the elderly (Nimni *et al.* 2007). Despite this, the effect on paracetamol disposition has not been shown in all studies (Galinsky *et al.* 1986; Miners *et al.* 1988).

At the other end of the spectrum, neonates do exhibit changes. The half-life of paracetamol in neonates is prolonged to 3.5 hours, however, this dissimilarity to adult rates reduces by the age of six months (van Lingen *et al.* 1999). This may be in part due to

a lack of glucuronidating enzymes, which have been shown to be deficient in neonates. One study found the expression of some UGT transcripts significantly lower in paediatric liver, leading to substantially reduced glucuronidation activity of up to 24 fold compared to adult controls for one of their study drugs, ibuprofen (Strassburg *et al.* 2002).

1.4.4 OBESITY

Table 1.4-4 Summary of alterations to paracetamol disposition with obesity

	NAPQI Production	GSH activity	GSH reserves
Obesity	↑	↓	↑

There are several changes to drug disposition that relate to obesity, but, in the absence of other accompanying risk factors, namely steatohepatitis and subsequent hepatic impairment, these changes do not confer additional risk of paracetamol toxicity (Table 1.4-4).

A drugs Vd may be increased in obese individuals (Blouin *et al.* 1999). In lipophilic drugs with a Vd greatly beyond that of intravascular volume, there is a risk that such an increase can lead to drug accumulation, escalating the risk of a drug's toxicity (Abernethy *et al.* 1982b). Because paracetamol is only of intermediate lipid solubility and its Vd is only slightly greater than the intravascular volume, obese individuals are not thought to be at a greater risk of paracetamol toxicity due to this factor (Abernethy *et al.* 1982a; Varela *et al.* 2008).

Metabolism of paracetamol has been reported to be increased in obese individuals and those with steatohepatitis (Abernethy *et al.* 1982a). The degree of increased activity of some CYP450 enzymes in obese man is more closely linked to the degree of adiposity of the liver, rather than the degree of obesity itself (Raucy *et al.* 1991; de la Maza *et al.* 2000; Emery *et al.* 2003). This illustrates similarities between the changes in CYP450 activity due to alcoholism and obesity. Similarly, changes to CYP450 activity in obese individuals have been shown to normalise following weight loss (Emery *et al.* 2003).

Of primary importance to paracetamol metabolism, CYP2E1 activity is increased in obese man (O'Shea *et al.* 1994). Kotylar *et al*. reviewed the literature on CYP450 changes as a result of obesity and concluded that CYP2E1 was induced, CYP3A4 was inhibited and the effect on CYP1A2 and CYP2D6 was unclear (Hunt *et al.* 1992b; Kotlyar *et al.* 1999). In addition to increased CYP450 activity, there is also evidence that obesity reduces the activity of GSH peroxidase, the enzyme which detoxifies the CYP450 product NAPQI, adding further to the risk of paracetamol toxicity (Karaouzene *et al.* 2011).

While there is evidence of changes that increase risk from the Phase I system, obesity is not listed as a risk factor for paracetamol toxicity (Bristol-Myers Squibb 2009). Two factors found in obese individuals offer protection against NAPQI toxicity. Generally, obese individuals are well-nourished and are likely to have additional stores of co-factors required for GSH conjugation, which are necessary for NAPQI detoxification, and are subsequently able to detoxify a greater drug load. These additional stores of cofactor may explain why the reduced number of GSH peroxidase shown by Karaouzene *et al* (*ibid*) did not equate to a reduced level of activity. Furthermore, evidence of increased rates of glucuronidation in obese individuals would suggest further protection from paracetamol toxicity as this would draw paracetamol away from the toxic Phase I pathway (Xu *et al.* 2012).

1.4.5 NUTRITION

Table 1.4-5 Summary of alterations to paracetamol disposition with malnutrition

	NAPQI Production	GSH activity	GSH reserves
Malnutrition	↑	↓	↓
TPN	↑		↑

Of all the factors discussed in this section, malnourishment is the most relevant to paracetamol toxicity at licensed dose in the surgical patient, with evidence for both increased NAPQI production and reduced GSH conjugation (Table 1.4-5).

Malnourishment, the intake of a diet insufficient to supply nutritional needs, occurs in over 25% of hospital inpatients, particularly the elderly (Thompson 1995). Nearly a quarter of elderly patients presenting for surgery are malnourished, often a consequence of semistarvation (Schenker *et al.* 2001). Additionally, overnight starvation/fasting is experienced preoperatively by all surgical patients receiving general anaesthesia and is aggravated by the use of aggressive bowel preparations, which usually result in dehydration (Holte *et al.* 2004). Following surgery, many patients are subjected to restricted diets and fasting may be prolonged for several days in those whose surgery requires the bowel to be rested. When allowed to eat, prolonged periods of insufficient energy intake are common, due to nausea, pain or lack of appetite (Nygren 2006)

Poor nutrition or periods of starvation are also common themes in reports of fatalities following “normal doses” of paracetamol (Eriksson *et al.* 1992; Whitcomb *et al.* 1994; Kurtovic *et al.* 2003; Vitols 2003; Moling *et al.* 2006). Hepatotoxicity only arose in patients taking chronic supra-therapeutic doses of paracetamol following impaired nutrition or

fasting (Whitcomb *et al.* 1994). However analysis of the effect of nutrition on paracetamol disposition is complicated by two factors: difficulties in obtaining accurate dietary histories and the differences in the effects of chronic malnutrition, starvation and dehydration (which often accompanies starvation). There is evidence for the role of Phase I and Phase II pathways.

In man, malnourishment has been shown to increase (Krishnawamy *et al.* 1977), decrease (Narang *et al.* 1977; Homeida *et al.* 1979) and not to change CYP450 activity (Sanchez *et al.* 1982). Total fasting has been shown to induce CYP450 (Hong *et al.* 1987) and CYP2E1 activity specifically (Johansson *et al.* 1990; Liu *et al.* 1993). In malnutrition, there seems to be a differentiation between the effect on CYP450 between protein and carbohydrate deprivation, with the relative contribution of these to total calorie content being the most important predictor, not the calorie content itself. As previously discussed, any induction of CYP450 enzymes, particularly CYP2E1 can increase NAPQI production and predispose to toxicity (Schenker *et al.* 2001).

Following on from this, the development of ketosis seems to be an important cause of CYP2E1 induction (Johansson *et al.* 1990) and it is a common factor that links fasting, high fat diets, diabetes and starvation to CYP2E1 induction (Yun *et al.* 1992). In the absence of carbohydrates and insulin, ketones are produced as a by-product from gluconeogenesis and the breakdown of fatty acids. These ketones appear to inhibit the breakdown of CYP2E1 enzymes, effectively causing induction (Eliasson *et al.* 1992). Further evidence is supplied by the reduction of CYP2E1 activity following the administration of glucose (Kim *et al.* 2006), insulin (Truong *et al.* 2005), or diets rich in carbohydrate/poor in protein (Mgbodile *et al.* 1972; Hayes *et al.* 1974; Campbell *et al.* 1976; Campbell 1977; Tranvouez *et al.* 1985; Jorquera *et al.* 1996), which would all reverse ketosis. Carbohydrate rich parenteral nutrition has been shown to reduce CYP450 activity, regardless of the amount of calories provided (Anderson *et al.* 1979; Knodell *et al.* 1984; Knodell *et al.* 1989; Jorquera *et al.* 1996). Specific changes from protein calorie malnutrition have been shown in a 60% suppression of CYP1A2 and 40-50% of CYP2E1 (Lee *et al.* 2004a). This is consistent with findings of the concentrations of theophylline (a CYP1A2 substrate) in asthmatic children being 62% higher in those with high carbohydrate diets indicating reduced activity of CYP1A2 (Feldman *et al.* 1980). Conversely, protein rich diets cause a marked increase to CYP450 activity (Alvares *et al.* 1976; Campbell *et al.* 1976; Jorquera *et al.* 1994; Jorquera *et al.* 1996). Protein rich, hypo-calorific parenteral nutrition given to

surgical patients for five days postoperatively significantly enhanced CYP450 activity, nearly halving antipyrine half-life (Jorquera *et al.* 1994). Conventional amino acid solutions have an identical effect (Pantuck *et al.* 1984). An elegant study demonstrated this by showing a reduction of similar magnitude upon switching from a regular to protein rich diet, which was reversed to original values upon initiation of a carbohydrate rich diet (Kappas *et al.* 1976).

These changes have not borne out alteration to paracetamol disposition or toxicity in humans (Schenker *et al.* 2001), and even inhibition of CYP2E1 metabolism has been shown following a maximum 36 hour fast (O'Shea *et al.* 1994). However, crucially, ketosis did not always develop in this study (Schenker *et al.* 2001). It seems likely that in the presence of prolonged periods of fasting with accompanying development of ketosis some degree of induction of CYP2E1 may also occur in humans, however this is unlikely to increase the risk of adverse effects associated with CYP2E1 (O'Shea *et al.* 1994).

There also seems to be an important role of Phase II metabolism. Malnourished patients presenting with paracetamol toxicity excrete twice the amount of Phase I products as healthy individuals, indicating changes to all three pathways of paracetamol's Phase II metabolism (Davis *et al.* 1976).

For paracetamol disposition, the most important changes to Phase II metabolism in malnutrition and perhaps metabolism in general, are to GSH. As discussed in Section 1.2, GSH conjugation and sulphonation both depend on a supply of cysteine and methionine from the diet: cysteine is used in the synthesis of GSH and supplies inorganic sulphur for the synthesis of the sulphonation cofactor PAPS. Diets require at least 0.9g of cysteine/methionine per day to eliminate 4g/day dose of paracetamol and this equates to on average an additional 50g of protein (Glazenburg *et al.* 1983; Gregus *et al.* 1994a). In the opinion of Nimni *et al.* the diets of lacto-ovo-vegetarians, vegans, those on a "health conscious diet" and those over 75 years were insufficient to metabolise paracetamol without harming homeostatsis (Nimni *et al.* 2007). In those with deficient diets, paracetamol elimination is slower and a greater amount of paracetamol is eliminated via NAPQI (Gregus *et al.* 1994a). In addition to harming sulphonation, these reductions to GSH will impair NAPQI detoxification. Tissue GSH levels are depleted by fasting (Liu *et al.* 1993) and anorexic subjects have been shown to have 30% less GSH than controls (Zenger *et al.* 2004). The effect of starvation may be even greater in surgical patients who are exposed to other drugs, such as propofol, that also deplete GSH during their own

conjugation processes (James *et al.* 2003; Buzaleh *et al.* 2005). Diets restricted in sulphur containing amino acid slow GSH synthesis (Lyons *et al.* 2000) having a marked effect on the redox status of cells (Miller *et al.* 2002). Rats show reduced GSH conjugation of NAPQI following fasting, with reductions to GSH concentration of up to 50% (Whitcomb *et al.* 1994). This is thought to arise from reduced cysteine intake following food deprivation (Cho *et al.* 1981). Supplementation of parenteral nutrition with glutamine, a precursor of GSH, has been shown to maintain GSH concentrations postoperatively and has even been linked to shortened hospital stays (Fan *et al.* 2009).

Lack of glucose or sulphate may also reduce glucuronidation and sulphonation and result in greater amounts being metabolised by the Phase I pathway to NAPQI (Price *et al.* 1987; Price *et al.* 1988). Both the rate and amount of paracetamol glucuronidation depend on hepatic carbohydrate reserves (Price *et al.* 1988). Postoperatively, patients are catabolic, being forced to supply glucose from amino acids through gluconeogenesis contributing to up to a 10-fold difference of glucuronidation activity between fed and fasting states(Price *et al.* 1988). However, short periods of starvation have not been shown to affect glucuronidation in patients (Schenker *et al.* 2001; Rumack 2004). Similarly, sulphonation also relies on inorganic sulphate being retrieved from the diet. With greater demands on sulphur containing amino acids from potentially increased NAPQI production, poor dietary supply can also impair sulphonation.

Dehydration, a common factor in the early post-operative period, is not thought to influence paracetamol metabolism greatly (Zafar *et al.* 1987; Kim *et al.* 2001; Kim *et al.* 2006) but there are no studies on dehydration-related changes to disposition in man.

Adequate nutrition can minimise these risks. Glucose and protein infusions prevent elements of the stress response (Kopp Lugli *et al.* 2010), which in turn have their own impact on drug metabolism (see Section 1.3), and may preserve the activity of drug metabolising enzymes (Jorquera *et al.* 1994). Prolonged use of licensed doses of paracetamol necessitates intake of increased amounts of sulphur containing amino acids (Reicks *et al.* 1988) and curiously these have been shown to spontaneously occur (Pujos-Guillot *et al.* 2011). Without an increase the restricted availability of cysteine and methionine may impair protein synthesis methylation reactions, and drug detoxification (Reicks *et al.* 1988).

1.4.6 DIABETES

Table 1.4-6 Summary of alterations to paracetamol disposition with diabetes

	NAPQI Production	GSH activity
Diabetes	↑	↓

The situation regarding paracetamol metabolism in diabetics is unclear. Work in laboratory animals shows induction of all of the main CYP450 enzymes of paracetamol metabolism and inhibition of GSH conjugation (Lee *et al.* 2010). The effect glucuronidation and sulphation is uncertain although inhibition has been reported (Price *et al.* 1986) . Extrapolation to humans is difficult because a number of the studies arise from rats, which present different metabolite patterns to humans.

Studies in humans demonstrate increased CYP450 activity in type I diabetics, particularly CYP1A2, but this is not seen in type II diabetics (Matzke *et al.* 2000). Conversely, activity of CYP2E1 increases in obese, type II diabetics, but not type I diabetics (Wang *et al.* 2003; Baek *et al.* 2006). However, poorly controlled type I diabetes is often accompanied with high concentration of ketones in the blood. Ketones are substrates of CYP2E1 and these high concentrations may induce this enzyme by prolonging the fast component of degradation, a similar effect to that of ethanol (Lieber 1997; Rumack 2002).

In studies using paracetamol, Dajani showed increased half-life of paracetamol in type I diabetics through changes to volume of distribution, not clearance (Dajani *et al.* 1974). Adithan's work agreed and also showed paracetamol clearance in type II diabetics reduced to less than half of the controls (Adithan *et al.* 1988). A study of Phase II metabolism in type II diabetics showed only lower clearance of paracetamol by sulphation, not glucuronidation, and increases to renal clearance of paracetamol (Kamali *et al.* 1993). While there are reports of induction and inhibition, of note is the induction of GSH synthesis by insulin, which is essential for NAPQI detoxification (Okouchi *et al.* 2006). This could oppose the increase in Phase I activity reported above (Lu 1999).

Conversely, hyperglycaemia and insulin deficiency decrease GSH synthesis to levels that impair anti-oxidant defence (Urata *et al.* 1996). This may theoretically increase the risk of toxicity in overdose, but has not been reported. In otherwise healthy individuals, diabetes possesses no additional risk of paracetamol toxicity at licensed doses.

"Surgical diabetes" in the post-operative patient may arise from the stress response, when the body becomes catabolic and rapidly resistant to the effects of insulin. The duration of diabetes is thought to factor in the nature of alterations to drug metabolism

(Sawant *et al.* 2006). As such, extrapolation of studies in types I and II diabetes to post-operative, surgical diabetes is not possible because of the substantial differences in pathogenesis and time course.

1.4.7 LIVER DISEASE

Table 1.4-7 Summary of alterations to paracetamol disposition with liver disease

	NAPQI Production	GSH activity
Liver disease	↓	↓

Risks of paracetamol toxicity in patients with liver disease are balanced and are not thought to be any greater than the general population at licensed doses (Table 1.4-7). There is a reduction in CYP2E1 in patients with cholestatic forms of cirrhosis compared with unchanged or increased levels of CYP2E1 with non-cholestatic (*e.g.* ethanol) related cirrhosis (Tegeder *et al.* 1999). This would theoretically reduce the risk of toxicity, although in practice the drug is avoided or dose reduced to a maximum of 3g/day (Klasco 2009).

There are other changes to GSH synthesis in cirrhotic patients. GSH is required for the detoxification of NAPQI and GSH itself requires cysteine. In patients with cirrhosis one of the pathways of cysteine production, the transsulfuration pathway, does not function (Mato *et al.* 1994). This may predispose patients with liver disease to paracetamol toxicity, but as discussed above, this has not been shown clinically.

1.4.8 GENDER

Table 1.4-8 Summary of alterations to paracetamol disposition with gender

	NAPQI Production	GSH activity
Male	↑	-

Small gender differences have been found in the pharmacokinetics of beta-blockers, caffeine, selective serotonin reuptake inhibitors, and verapamil (Fletcher *et al.* 1994; Meibohm *et al.* 2002), but most are not clinically significant (O'Shea *et al.* 1994). In the case of paracetamol, minor reductions to clearance are seen in females (Liukas *et al.* 2011)(Table 1.4-8). The activity of some CYP450 enzymes has been shown to be higher in men than women, *e.g.*, CYP1A2 and CYP2E1, with CYP3A4 being higher in women than men (Meibohm *et al.* 2002; Bies *et al.* 2003). However, interindividual differences in CYP450 activity are much greater than those due to gender and these changes are not always seen (Kim *et al.* 1995b; Bebia *et al.* 2004).

Gender differences in paracetamol glucuronidation and the activity of UGT1A6 have also been shown, with increases seen in males (Bock *et al.* 1994), whereas reduced reserves of inorganic sulphate and PAPS in females mean sulphate depletion due to administration of a substrate of sulphation may occur at a lower dose in females than males (Mulder 1986). GSH concentration in the liver is identical between genders as are rates of GSH synthesis (Mulder 1986).

Other gender-related factors causing pharmacokinetic differences include the lower body weight and organ size, higher percentage of body fat, and lower glomerular filtration rate in women than in men (Fletcher *et al.* 1994; Meibohm *et al.* 2002; Bies *et al.* 2003). One review cautioned that the information on the effect of gender is mainly derived from rat studies because they are most pronounced in this species. They quote several studies that have shown the identification of sex-dependent difference in rats do not translate to the same xenobiotic handling differences in humans (Mugford *et al.* 1998).

1.4.9 ETHNICITY

Table 1.4-9 Summary of alterations to paracetamol disposition with ethnicity

	NAPQI Production	GSH activity
African	↑	-

Ethnicity is not considered a significant risk factor for paracetamol toxicity, although some changes to disposition are seen. Critchley *et al*. examined the 24 hour urinary excretion of paracetamol and its metabolites following a single oral dose of 1.5 g in 111 Caucasians (Scotland), 67 West Africans (Ghana) and 20 East Africans (Kenya). They found reduced amounts of the Phase I conjugates of paracetamol amongst the Caucasians compared with Africans ($p < 0.0005$) indicating slightly reduced Phase I activity in the Africans (Table 1.4-9). There were also differences in glucuronide conjugation with Caucasians conjugating significantly less than in the Africans, accounting for the increased amount excreted by the Phase I pathway. The authors conclude that these ethnic differences in paracetamol metabolism may be related to genetic or environmental factors including differences in diet and protein intake (Critchley *et al.* 1986). However, in studies using similar methods, no differences were seen between Caucasian and Chinese males living in South Australia (Osborne *et al.* 1991) or Caucasian and Oriental subjects in Canada (Patel 1992). Prescott warns it may be difficult to distinguish the cause of ethnic variation between the effect of genetic, dietary or environmental factors (Prescott 1996).

1.5 THE CYTOCHROME P450 ENZYMES

Cytochrome P450 (CYP450) enzymes are a very substantial and diverse superfamily of haemoproteins found in eukaryotes, bacteria and archaea (Nelson 2008). The name CYP450 is derived from the fact that these are coloured ('chrome') cellular ('cyto') proteins, with a "pigment at 450 nm", so named for the characteristic Soret peak formed by absorbance of light at wavelengths near 450 nm when the haem iron is reduced and complexed to carbon monoxide (Wijnen *et al.* 2007). They enzymatically metabolise a vast range of both exogenous and endogenous compounds. Usually they form part of multi-component electron transfer chains, called P450-containing mixed function oxidase systems (Wijnen *et al.* 2007).

1.5.1 CLASSIFICATION

CYP450 enzymes have been identified from all lineages of life, including mammals, birds, fish, insects, worms, sea squirts, sea urchins, plants, fungi, slime moulds, bacteria and archaea (Guengerich 1999). In humans the completion of the human genome sequence revealed the presence of 57 active CYP genes (Wijnen *et al.* 2007). These are displayed in Table 1.5-1 based on their major substrate.

Table 1.5-1 Classification of all 57 human cytochrome P450 based on major substrate class (Guengerich *et al.* 2005)

Sterols	Xenobiotics	Fatty Acids	Eicosanoids	Vitamins	Unknown
1B1	1A1	2J2	4F2	2R1	2A7
7A1	1A2	4A11	4F3	24A1	2S1
7B1	2A6	4B1	4F8	26A1	2U1
8B1	2A13	4F12	5A1	26B1	2W1
11A1	2B6		8A1	26C1	3A43
11B1	2C8			27B1	4A22
11B2	2C9				4F11
17A1	2C18				4F22
19A1	2C19				4V2
21A2	2D6				4X1
27A1	2E1				4Z1
39A1	2F1				20A1
46A1	3A4				27C1
51A1	3A5				
	3A7				

CYP450 enzymes are further classified by their amino acid homology. Families are identified by an Arabic number and have at least 40% homology of amino acids, the subfamily is identified by a capital letter and has at least 55% homology and the gene product is identified by a further Arabic number. This classification system has replaced

nomenclature based on the substrate of the enzyme, *e.g.* cyclosporin oxidase and nifedipine oxidase are the same enzyme, P450 3A4 (Bruton *et al.* 2006).

Human CYP450s are primarily membrane-associated proteins, located either in the inner membrane of mitochondria or in the endoplasmic reticulum of cells. While the highest concentrations of CYP450 are in the nose and the adrenal gland, the greatest mass is found in the centrilobular region of the liver (Wijnen *et al.* 2007). Their relative contribution to marketed drug metabolism is shown in Figure 1.5-1.

The most common reaction catalysed by CYP450 is a mono-oxygenase reaction, *i.e.* insertion of one atom of oxygen into an organic substrate (RH) while the other oxygen atom is reduced to water (Equation 1.5-1).

$$RH + O_2 + 2H + 2e^- \rightarrow ROH + H_2O$$

Equation 1.5-1 Most common reaction catalysed by CYP450 enzymes

Of those whose function is currently known, most CYP450 enzymes present in humans are involved in the metabolism of sterols and vitamins A and D (Table 1.5-1) (Guengerich 2006). About one quarter of the 57 CYP450 enzymes are considered to be involved primarily in the metabolism of "xenobiotic" (not normally in the body) chemical substances (Guengerich 2006).

Five of the CYP450 enzymes carry out 90% of all CYP450 metabolism, making study in this area much simpler than if all 57 human CYP450 were involved (Frye 2004; Guengerich 2006). Furthermore, these enzymes play a vital role in homeostasis (Lewis 2004; Wijnen *et al.* 2007).

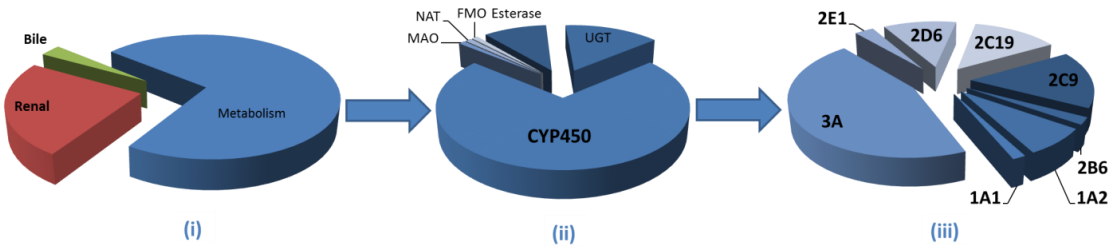


Figure 1.5-1 Contribution of CYP450 to overall drug Clearance

(i) Contribution of each clearance pathway to overall clearance. (ii) Of metabolism as a clearance pathway, the contribution of individual enzyme systems to metabolism of marketed drugs is shown. UGT indicates uridine dinucleotide phosphate (UDP) glucuronosyl transferase; FMO, flavin-containing monooxygenase; NAT, N-acetyltransferase; MAO, monoamine oxidase; CYP, cytochrome P450. (iii) Of CYP 450 mediated metabolism of marketed drugs, the involvement of individual P450s metabolism is shown(Wrighton et al. 1992).

Hepatic CYP450 are the most widely studied of the CYP450 enzymes because of their role in drug metabolism (Bruton *et al.* 2006). Here CYP450 enzymes are probably the most

important element of oxidative drug metabolism in humans (Guengerich 2006). The majority of CYP450 reactions:

- Deactivate drugs;
- Attenuate their biological activity; and
- Accelerate their clearance from the body (Guengerich 2006).

CYP450s can also activate compounds, which may have their own inherent pharmacological activity, or may modify cellular constituents and damage cells, as is the case with paracetamol and its metabolite NAPQI (Guengerich 2006).

1.5.1.1 CAPACITY AND AFFINITY

The metabolic reactions between a CYP450 enzyme and a substrate can be defined as either low affinity/high capacity or high affinity/low capacity. The affinity of an enzyme to a substrate refers to its preference for certain substrates. The rate at which the enzyme can metabolise substrate refers to its capacity. For example, CYP2D6 is a high affinity/low capacity enzyme: it prefers to metabolise specific substrates at low concentrations. As the concentration of a substrate increases, CYP2D6 becomes saturated and other, lower affinity, enzymes become involved in the metabolism of the substrate. Low affinity/high capacity CYP450 enzymes which commonly mop-up the spill over include CYP3A4, and CYP1A2 (Chen *et al.* 1998). While these classifications are true in the main, exceptions do exist; such an example is CYP2E1 which has high affinity/low capacity for some substrates and low/high for others (Kim *et al.* 2007).

1.5.1.2 CYP450 AND DRUG METABOLISM

1.5.1.2.1 CYP450 VARIATION; GENES AND THE ENVIRONMENT

Unlike the CYP450 enzymes involved in homeostasis, the amount of xenobiotic metabolising CYP450 enzymes can vary widely in number and can even be completely absent, depending on an individual's genome (Guengerich 1999). These polymorphisms are an important factor in determining an individual's response to medication (Wijnen *et al.* 2007). The population can be divided into four subgroups based on their genotype:

- Poor metabolisers (PM);
- Intermediate metabolisers (IM);

- Extensive metabolisers (EM) and;
- Ultrarapid metabolisers (UM) (Frank *et al.* 2007).

Those with a PM genotype lack any functional allele, whereas EM subjects have one or two and UM subjects have more than two functional alleles. The IM genotype can arise from heterozygosity from a non-functional allele or the presence of alleles with reduced activity (Frank *et al.* 2007).

The potential impact is shown for a theoretical drug in Figure 1.5-2. In this figure the upper trace represents that of an EM, the majority of the population, and the lower trace that of a PM. EMs metabolise drugs quickly and achieve a much lower steady state concentration. If the metabolite is active, or toxic, this rapid metabolism can lead to toxicity (Gasche *et al.* 2004). Conversely it can prevent therapeutic concentrations of the parent compound being maintained, requiring higher doses (Kirchheiner *et al.* 2004). As EMs constitute the majority of the population, drug dosing is based on their metabolism. In PMs, drug concentration accumulates with each administration as shown in the lower trace. PMs risk toxicity through parent compound accumulation (Lessard *et al.* 1999).

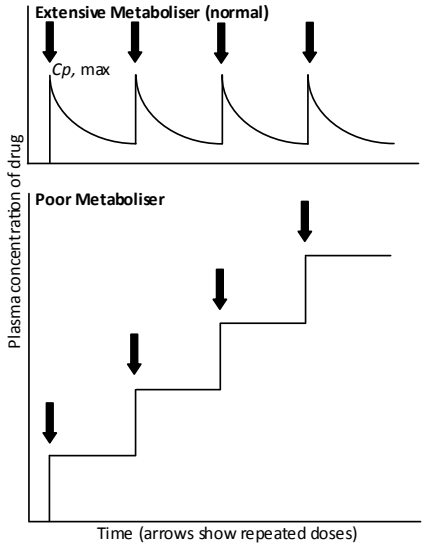


Figure 1.5-2 Effect of variation in human CYP450 activity on plasma concentration.
The effect of different metaboliser status on plasma concentration of a theoretical drug over time is shown. The top trace represents that of an extensive metaboliser and the bottom that of a poor metaboliser AUC indicates area under the curve(Guengerich 2006).

Ethno-specificity is also involved in many cases. As an example the number of CYP2D6 PM ranges from 1-10% across ethnic groups, with the mean prevalence of 8.9% in white British, 3.9% amongst the French and as low as 1% of sub-continental Indians (Mamidi *et al.* 1999).

Although the majority (70-90%) of the variation in function is attributable to this genetic control (He *et al.* 2005), environmental factors, such as disease states, caffeine consumption, smoking, alcohol and concomitant drug use also impact on the enzymes actual activity (Harvison *et al.* 1988b; Prescott 2000a) and these were reviewed in Section 1.4.

Enzyme activity can be either increased or decreased, also referred to as enzyme induction and inhibition, respectively. Many clinically relevant drug interactions arise from this process. For example, a drug that inhibits the CYP450-mediated metabolism of another drug may cause the second drug to accumulate within the body and may result in toxicity. It is especially relevant to medicines with narrow therapeutic indices, such as some anti-arrythmics and anti-epileptics. Some of these factors were also discussed in Section 1.4.

It is the sum of these influences, both genetic and environmental, which determine an individual's actual enzyme activity, or phenotype. The effect of these are seen every day in a clinical environment; why a heavy smoker may need higher doses of benzodiazepine for sedation (Greenblatt *et al.* 1980), why the same dose of theophylline may be toxic for a non-smoker but ineffective for a smoker, or why a heavy alcohol drinker requires greater doses of opiate for pain relief and yet is at a greater risk of paracetamol toxicity (Frye *et al.* 1997; Ozdemir *et al.* 2000). As an explanation for these clinical observations, and the large differences in pharmacokinetics behind them, CYP450 enzymes can show a greater than 30 fold variation in function (Matzke *et al.* 2000; Carcillo *et al.* 2003; Zhou *et al.* 2004). The above examples show how important environmental factors are to determining an individual's CYP450 activity.

Because of the variability of these enzymes and their impact on drug metabolism, reliable measures of their activity play an important role in clinical studies of the current type. More practically, increased understanding of the variability of CYP450 enzymes may lead to the possibility of predicting and managing drug:drug interactions and explaining individual differences in response to or tolerability of medicines (Glue *et al.* 1999).

It is not surprising then that knowledge of an individual's CYP450 activity is becoming increasingly desirable in medical practice. While a genotype can be determined by arrays such as the AmpliChip (Roche Diagnostics, North America) or polymerase chain reaction (PCR) assays, from the examples above, it is clear that the genotype only accounts for a

fraction of the overall variation. Even in the absence of environmental influences, the genotype is inadequate in the majority of cases for predicting enzyme activity. As an example, CYP2D6 shows pronounced overlapping of genotype between IM and EM (Evans *et al.* 1991). For this reason, assessing the sum of genetic and environmental influences, or phenotyping, is the most accurate way to determine enzyme activity at the time of interest.

1.5.2 ASSESSMENT OF CYP450 ACTIVITY

As more is understood about drug metabolism in humans, there is increasing interest in assessing the role of CYP450 enzymes and how they are modulated. An individual's phenotype at any given time is the sum of the effects of an individual's genes and their environmental exposures (Section 1.4). Assessment of either influence alone is insufficient for accurate measurement.

Phenotype can be assessed by three techniques:

- Measuring mRNA or protein levels in liver biopsy for a specific CYP450 enzyme;
- Exposing liver biopsy tissue to a probe drug; or
- Administering a probe drug systemically and taking a blood or urine sample.

The most common approach for assessment of CYP450 phenotype is the use of a probe drug whose clearance is dominated by the CYP450 enzyme of interest. This probe drug can be administered to a patient systemically (*i.e.* orally or intravenously), or can be exposed directly to liver tissue, obtained from a liver biopsy. While liver biopsies are considered the benchmark standard material for this assessment, the ethical and patient safety considerations of obtaining these samples are often insurmountable and systemic administration of probes *in vivo* have been shown to produce equivalent results (Tanaka *et al.* 2003).

1.5.2.1 PROBES FOR CYP450 ACTIVITY

The use of probe compounds to assess CYP450 enzyme activity began with non-specific probes such as antipyrine several decades ago (Breckenridge *et al.* 1973). Antipyrine is extensively metabolised by CYP450 enzymes and measuring its clearance following systemic administration is the benchmark as a general indicator of CYP450 mediated drug metabolism. However, it provides no specific information on individual CYP450 enzymes

(Breimer *et al.* 1990). As this area expanded, several rigorous *in vivo* pharmacokinetic studies found individual probe drugs for specific CYP450 enzymes, such as the clearance of caffeine as a means of assessment of the activity of CYP1A2 (Butler *et al.* 1992).

While this provided huge advances to the understanding of the role of CYP450 enzymes, methods were cumbersome and inexact. Determining the clearance of a probe drug (area under the concentration time curve) ($AUC_{(0-\infty)}$), may be the most accurate way of determining an individual enzyme's phenotype (Frank *et al.* 2007), but it is accompanied by several challenges, *i.e.* the activity of several enzymes other than the CYP450 under investigation contribute to clearance of the drug being measured. The rate of renal excretion may also be a factor which must remain constant for phenotype measurements to be comparable; this may be unachievable in all but the strictest of controlled environments, and all but impossible in the surgical patient.

To avoid these complications, assessment of the partial clearance of the probe to its given CYP450 dependent metabolite can be performed. This approach requires multiple plasma samples and determination of recovery of the drug from the urine; both of which are time and cost intensive strategies, as well as uncomfortable for the test subject.

Simplified metrics, such as those assessing the metabolic ratio of a probe drug to its metabolite at a certain time point, were developed to avoid these problems. Whereas the clearance of caffeine had been used to assess CYP1A2 activity, now the ratio of caffeine to paraxanthine (its primary metabolite) is used. This method was found to compare favourably with the more intensive strategies (Tucker *et al.* 1998), showing bimodal or trimodal distribution, reflecting the PM, IM and EM subgroups of enzyme activity (Frank *et al.* 2007). Moreover, this method is more economically and ethically viable (Frank *et al.* 2007).

Administering several individual probe compounds at the same time in a 'cocktail' was trialled to simplify and improve analysis, in an attempt to eliminate inter-day variation. The feasibility of cocktails was first shown in 1990 (Rogers *et al.* 2003), but, it was later found some of the probes affected the metabolism of other probes, invalidating the assessment. After considerable development, probe cocktails are now common place in drug research. Probes shown not to interact with other probes to provide good approximations of CYP450 activity are used (Fuhr *et al.* 2007).

There are several desirable attributes for the ideal probe for CYP450 activity:

- The probe must have a known, quantifiable metabolic pathway that is principally or exclusively mediated by the CYP450 enzyme under investigation, so no interactions occur;
- The action of the CYP450 enzyme in the clearance or metabolite formation being measured must be the rate limiting step. If other enzymes or processes (such as rate of excretion) are involved they must not be rate limiting;
- If other processes, such as rate of excretion, are rate limiting they must remain constant for the assessment to remain valid; and
- Probes for hepatic CYP450 enzymes should be completely metabolised by the liver and have a low hepatic extraction ratio (E_H) in order to minimise influence of other factors, such as changes to hepatic blood flow or protein binding.

This final point requires further expansion and can be explained by examination of the "well stirred" model of hepatic clearance. The well stirred model assumes that the liver is a single well stirred compartment and that unbound drug in the plasma is in equilibrium with unbound drug in the liver. Hepatic clearance (CL_H) can therefore be described as Equation 1.5-2, where Q_H is hepatic blood flow, Cl_{int} is the intrinsic clearance of the drug and f_u is the fraction of the drug unbound.

$$CL_H = \frac{Q_H f_u CL_{int}}{Q_H + f_u CL_{int}}$$

Equation 1.5-2 Hepatic clearance and influence of hepatic blood flow

Simplifying the model for low (E_H<0.3) and high (E_H>0.7), hepatic extraction ratio drugs will show which factors are the major influences of CL_H. The hepatic extraction can be described as Equation 1.5-3.

$$E_H = \frac{f_u CL_{int}}{Q_H + f_u CL_{int}} = \frac{CL_H}{Q_H}$$

Equation 1.5-3 Hepatic clearance of drugs with high hepatic extraction ratios

The liver is less efficient at clearing drugs with a low E_H from the blood and therefore $Q_H >>> f_u CL_{int}$. As a result for these drugs the denominator can be simplified to Q_H. Applying this to allow further simplification of Equation 1.5-3 shows that for these drugs $CL_H \approx f_u CL_{int}$, revealing that CL_H of drugs with a low E_H depends on both f_u and CL_{int}. Accordingly, they should be minimally bound to plasma proteins to avoid complications from changes in volume of distribution, and be administered intravenously (Frye *et al.*

1997) in order to rely solely on the intrinsic metabolic activity of the responsible enzyme (De Vries *et al.* 1994).

Other, more practical considerations, for the ideal probe include:

- The probe must be safe and relatively pharmacologically benign at doses used;
- The probe should be readily available commercially and registered as a therapeutic drug;
- Analysis of the probe should be as simple as possible;
- The probes should ideally show low intra-individual variability;
- The time between administration of the probe and the taking of the sample should be short to minimise opportunities for introduction of sources of error (*e.g.* caffeine intake when caffeine is a probe) and inconvenience to patient and researcher; and
- Pre-test restrictions should be minimal, *i.e*. fasting, abstaining from alcohol, caffeine or other dietary or lifestyle restrictions (Frye *et al.* 1997; Streetman *et al.* 2000b; Zaigler *et al.* 2000; Frank *et al.* 2007).

Several probes can be administered at the same time in a "cocktail", offering several advantages, whilst also presenting their own set of challenges (Frye *et al.* 1997). When probes are given individually, there is often significant inter-day variability between samples due to a multitude of unquantifiable factors such as the influence of diet, exercise or the body's circadian rhythms. The impact of this can be minimised by the administration of several probes at the same time, providing there is no interaction between them. An ideal cocktail is one that produces the same results as if each probe is administered separately (Frye *et al.* 1997).

Components of a cocktail must not interact metabolically or clinically or cause analytical interference. Further validation is required to ensure that the accuracy of individual probes is not altered by the use of other probes in the cocktail and that independent phenotypic measures of the individual enzymes are still obtained (Berthou *et al.* 1995). The potential for these interactions can be minimised by keeping the doses as small as possible without compromising the assay detection limits. This also minimises any pharmacological activity. For example, chlorzoxazone, a probe for CYP2E1, was found to decrease the metabolism of caffeine, a probe for CYP1A2, by 20% when 500mg doses were given (Frye *et al.* 1997; Zhou *et al.* 2004), however, using a lower 250mg dose of

chlorzoxazone avoided this problem. Chlorzoxazone is metabolised by both CYP2E1 and CYP1A2, but CYP1A2 is only involved when CYP2E1 is saturated by higher doses (Frye *et al.* 1997). Other probes may induce the metabolism of another such as quinidine, a useful substrate of CYP2D6, but inducer of CYP3A4 (Zhou *et al.* 2004).

Ideally sampling schedules should be kept as simple as possible and with multiple probes adjustment in the sampling time can be made without significant change in the accuracy of the exposure estimates (Campbell *et al.* 1987). Analytical interference can also be a problem as with the addition of each drug into the cocktail at least two additional compounds, the parent drug and the metabolite, will appear in the sample. Extraction and chromatographic modifications can overcome this. HPLC mass spectrometry based methods are about 100 times more sensitive and specific than HPLC-UV ones and provide lower limits of detection but the essential equipment is expensive and not often available in laboratories in a clinical setting (Guengerich 2006).

It is important to note that many original studies in this area used non-human CYP450 enzymes as an approximation of human enzymes. These enzymes are similar, but as an example, rat CYP1A2 are only 75% identical to human. For some substrates, rat and human 1A2 have nearly identical catalytic activity, whereas with others, human 1A2 has ≥10 higher catalytic efficiency. There is also a greater variability in 1A2 levels in humans than rats. Many of these differences can be overcome by physiologically based pharmacokinetic modelling techniques that account for these differences (Guengerich 2006).

Probes are used in an attempt to gain knowledge about enzymes involved in drug metabolism so that the behaviour of other drugs can be predicted. The best probe drug for an individual CYP450 enzyme is metabolised exclusively by the CYP450 enzyme under investigation. Probes that have significant alternate pathways of metabolism (*i.e*. are metabolised by more than one CYP450 enzyme) may lead to erroneous conclusions (Swart *et al.* 2004).

By understanding more about these processes there is the possibility of drug therapy being tailored to the individual patient's metabolic parameters, so those who can tolerate a greater dose of a medicine get more and those who are predisposed to toxicity get a dose more appropriate to them.

The probes used to assess the activity of the four CYP450 enzymes relevant to this Thesis and justifications for their choice are discussed in the following section.

1.5.3 PROBES USED IN THIS STUDY

A summary of the CYP450 enzymes associated with Phase I paracetamol metabolism and their chosen probes are shown below in Table 1.5-2. Following this table is a review and evaluation of the enzyme probes for each enzyme.

Table 1.5-2 Summary of CYP450 enzymes being assessed

Enzyme	Test	Time	Sample
CYP1A2	Caffeine PO 100 mg	1 hour post dose	Plasma
CYP2D6	Dextromethorphan PO 30mg	4 hour post dose	Plasma
CYP2E1	Chlorzoxazone PO 250 mg	1 hour post dose	Plasma
CYP3A4	Midazolam IV 0.025mg/kg	4 hour post dose	Plasma

1.5.3.1 CYP1A2

In addition to its role in the Phase I metabolism of paracetamol, CYP1A2 mediates the rate-limiting step in the metabolism of many other drugs including theophylline, clozapine, and tacrine, as well as in the bioactivation of procarcinogens (Simon *et al.* 2001). CYP1A2 activity shows both pronounced intra- and interindividual variability. A major cause of this variability is related to smoking which causes enzyme induction. Other influences include drug intake and diet which can result in either induction or inhibition. In contrast to these exogenous factors, genetic influences on enzyme activity seem to be less pronounced. Therefore, phenotyping of CYP1A2, *i.e.* the determination of the actual activity of the enzyme *in vivo*, represents a useful approach both for scientific and clinical applications. CYP1A2 is almost exclusively expressed in the liver (Faber *et al.* 2005).

1.5.3.1.1 CHOSEN PROBE- CAFFEINE

Caffeine is the drug of choice for phenotyping CYP1A2 (Streetman *et al.* 2000a). It is relatively safe and possesses many favourable pharmacokinetic characteristics as a phenotyping probe. It is rapidly and completely absorbed from the gastrointestinal tract, distributed throughout the total body water, has low plasma binding, a short half-life, negligible first-pass metabolism, minimal renal elimination and first order elimination with almost entirely hepatic biotransformation (Fuhr *et al.* 1993; Kalow *et al.* 1993; Backman *et al.* 2008). Approximately 95% of caffeine's systemic clearance is mediated by CYP1A2 (Kalow *et al.* 1993). The metabolism of caffeine is shown in Figure 1.5-3.

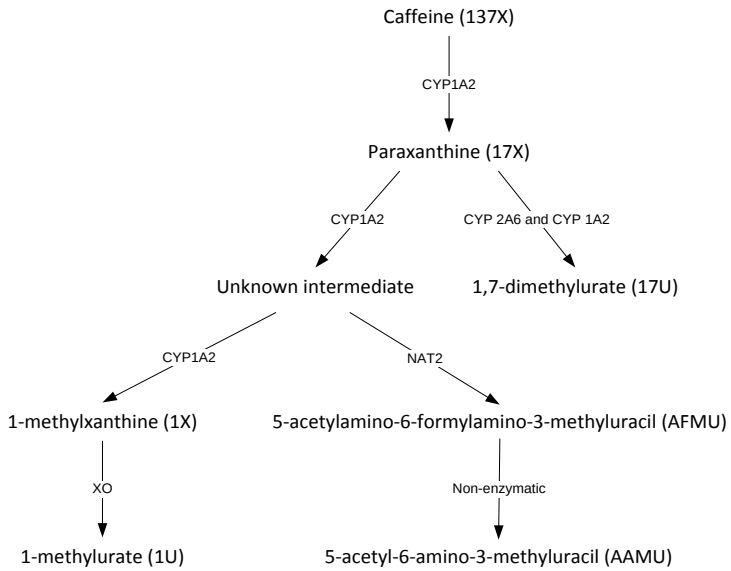


Figure 1.5-3 Predominant routes of caffeine metabolism
(Streetman et al. 2000a)

The caffeine metabolic ratio is also used as a marker for the assessment of liver dysfunction in patients with cirrhosis and is believed by some groups to be better than static determination of standard liver function tests (*e.g.* serum transaminases) (Jodynis-Liebert *et al.* 2004).

Plasma phenotyping compares the ratio of paraxanthine (17X, 1, 7-dimethylxanthine) with caffeine (1, 3, 7-trimethylxantine, 137X). This N-3 demethylation of caffeine accounts for up to 84% of the primary metabolism of caffeine at the doses used *in vivo* (Rost *et al.* 1994; Frye *et al.* 1998b; Holland *et al.* 1998) and quantification of 137X partial clearance by 17X formation is the standard assessment of CYP1A2 activity if liver biopsy samples are not available (Kalow *et al.* 1993).

The metabolite 17X is chosen for this purpose as only CYP1A2 is involved in its formation, whereas the formation of caffeine's other metabolites involve CYP2E1 and CYP3A, in addition to CYP1A2. If these other metabolites are included, the involvement of these other enzymes compromises the accuracy of the assessment of CYP1A2 (Gu *et al.* 1992; Rost *et al.* 1994; Tassaneeyakul *et al.* 1994). Measuring the 137X/17X ratio has been shown to be more sensitive than a direct measurement of caffeine clearance (Jodynis-Liebert *et al.* 2004) and these compounds are only found in plasma (Tanaka *et al.* 2003).

1.5.3.1.2 SAMPLE TIME

The metabolite 17X is both a product and a substrate of CYP1A2 and the goal of the 17X/137X ratio is to measure 17X formation, or 17X as a product, not as a substrate.

Because of this, timing of sample collection is critically important since as time after the caffeine administration increases, 17X concentrations are more likely to reflect 17X degradation.

1.5.3.1.3 DOSE OF CAFFEINE

Most commonly used doses vary from 1-2mg/kg or 100-200mg (Kalow *et al.* 1993) because oral doses of >3mg/kg exhibit saturable pharmacokinetics and involve CYP2E1 and CYP3A4 (Denaro *et al.* 1990). This results in poorer correlation with enzyme activity (Kalow *et al.* 1993; Tassaneeyakul *et al.* 1994).

1.5.3.1.4 PHARMACOKINETIC PARAMETERS OF CAFFEINE

In adults, caffeine has a Vd of 0.6L/kg, is 36% protein binding, has a half-life of five hours and Cmax between half and two hours following oral administration. It is excreted in the urine entirely as metabolised drug (Lacy *et al.* 2005).

1.5.3.1.5 OTHER REPORTED PROBES OF CYP1A2

Plasma samples are most often used for phenotyping CYP1A2, although urine and saliva sampling and a breath test have also been used (Fuhr *et al.* 1994).

1.5.3.1.5.1 URINE SAMPLING

Following plasma sampling, urine sampling is the next most cited method. While urine samples are far easier to obtain than plasma, this method for CYP1A2 assessment is fraught with technical difficulties:

- Less than 2% of the caffeine dose is excreted unchanged so the ratio of caffeine to paraxanthine used for plasma assessment cannot be applied (Butler *et al.* 1992);
- As a consequence, urine samples must be analysed for the ratio of four different metabolites in an eight hour urine collection (Georga *et al.* 2001);
- The four metabolites being measured are amongst a total of 16 metabolites of caffeine found in urine. As a result, they are in low concentrations which may prevent direct analysis (Rostami-Hodjegan *et al.* 1996);
- There are considerable structural similarities between the four metabolites, further hindering their analysis and extraction (Georga *et al.* 2001);

- The metabolites examined are either secondary or tertiary metabolites which are not exclusively formed by CYP1A2 (Fuhr *et al.* 1994) and as discussed above, this can impair the accuracy of the assessment of CYP1A2;
- The validity of urine analysis relies on stable renal function and urine flow. Changes to renal function can invalidate the assessment because the four metabolites analysed are excreted in the urine very slowly, at rates much slower than glomerular filtration. Accordingly, the rate of excretion is the rate limiting step at which the metabolites appear in the urine, not their CYP1A2 mediated synthesis (Kalow *et al.* 1993). The problem arises because changes to renal function can affect the rate of excretion of each metabolite differently, and consequently this may alter their ratio in the urine, invalidating CYP1A2 assessment;
- Stable renal function and urine flow is difficult to achieve in healthy adults, given caffeine induces diuresis (Tang-Liu *et al.* 1982), and virtually impossible in major surgical patients who often have unstable renal function and hydration issues arising from surgery; and
- Urine analysis is also more susceptible to influence by ethnicity and smoking, further contributing to poor correlation of the ratios (Fuhr *et al.* 1994; Tanaka *et al.* 2003).

1.5.3.1.5.2 OTHER TESTS

Saliva has been shown to correlate highly with systemic clearance and has been used for phenotyping CYP1A2. However some of the limitations of saliva testing are discussed in Section 1.2.

Breath testing has also been used. Breath testing involves the administration of a precise dose of 13C-labeled caffeine and the measurement of the amount of 13C labelled carbon dioxide exhaled over two hours (Lambert *et al.* 1983; Lambert *et al.* 1986; Lambert *et al.* 1990). This presents a number of challenges. While the first step in the process, the demethylation of the labelled 3-methyl group from caffeine is mediated by CYP1A2, there are a number of subsequent steps before the labelled carbon atom is exhaled from the lungs as carbon dioxide. All of these steps must remain non-rate-limiting for the assessment of CYP1A2 activity to be valid. This assessment also depends on respiratory quotient and therefore can be influenced by physical activity, smoking and diet (Lambert *et al.* 1983); none of these presumptions have been shown outside of healthy adults.

Additional barriers include sourcing the radio-labelled caffeine and accurately capturing a patients breath for two hours; both quite onerous tasks.

Other, far more invasive tests measure CYP1A2 directly in liver biopsy samples. CYP1A2 occurs in liver but not in blood cells. Hence assessment of its enzymatic presence using Western blot analysis or its formation by way of messenger ribonucleic acid (mRNA) determination is possible only by testing liver tissue, not by testing blood or other accessible tissue (Kalow *et al.* 1993). Accordingly these methods are unsuitable for frequent assessment of enzyme activity as is required in this Thesis.

1.5.3.1.6 PROBLEMS WITH CAFFEINE AS A PROBE FOR CYP1A2

Caffeine's main disadvantage as a probe for CYP1A2 is that it, along with other xanthines, is contained in many food and drinks. Intake of anything containing xanthine will interfere with the phenotyping (Rainska *et al.* 1992). This interference is more prominent in those who habitually drink coffee. It has been reported that habitual coffee drinkers are required to abstain for seven days before blood caffeine concentrations decline below the limit of detection, whereas for those who do not consume caffeine containing products, caffeine concentrations will fall to undetectable levels within three hours of a 500mg oral dose (Warren 1969). Because of the large amounts of caffeine consumed by humans it has also been found in 'blank' human plasma, which may interfere with preparation of control samples. Accordingly, some researchers advocate use of aqueous solutions of bovine albumin for control purposes to produce more accurate results than may be achieved with human plasma (Denaro *et al.* 1990).

1.5.3.2 CYP2D6

Aside from paracetamol, CYP2D6 is involved in the metabolism of about a quarter of all commonly prescribed medicines (Bertz *et al.* 1997) including anti-arrhythmics, antidepressants, neuroleptics, β-blockers and opioids (Lutz *et al.* 2004). It is often quoted for its large inter-individual and interethnic variation in activity and more than 80 allelic variants have been described (Zanger *et al.* 2004). These genetic polymorphisms can result in a 30 to 40 fold difference in the clearance of CYP2D6 substrates. Furthermore, this can cause extremes in drug concentration, both high and low, in the affected group of patients (Frank *et al.* 2007). This can lead to non-response in some and toxicity in others (Bertilsson *et al.* 2002; Kirchheiner *et al.* 2004) and no better example of this is

seen than in the response to codeine. Codeine is metabolised to morphine by CYP2D6 and it is this morphine that confers the majority of codeine's analgesic activity. For the same dose of codeine, CYP2D6 poor metabolisers may receive no analgesic effect, whereas ultra-rapid metabolises may experience life-threatening opioid toxicity (Gasche *et al.* 2004).

CYP2D6 is mainly expressed in the liver but is also found in the lung and heart. CYP2D6 is stable over years. It is thought genetic determinants prevail over environmental factors in determining phenotype as CYP2D6 is not known to be affected by oral contraceptives or enzyme inducers such as rifampicin or ethanol(Bock *et al.* 1994). Neither gender, caffeine consumption, diabetes, menstrual cycle phase nor smoking have been shown to affect it (Matzke *et al.* 2000; Frank *et al.* 2007). However, there is some evidence CYP2D6 activity is modified by infection (Bertilsson *et al.* 2002), but not inflammation alone (Frye *et al.* 2002).

1.5.3.2.1 CHOSEN PROBE- DEXTROMETHORPHAN

Dextromethorphan (DM) is probably the best probe drug for detecting CYP2D6 activity (Streetman *et al.* 2000a; Frank *et al.* 2007). DM metabolism is shown in Figure 1.5-4. Following absorption, DM is *O* and *N*-demethylated to dextrorphan (DX) and 3-methoxymorphinan (3-MM). CYP2D6 mediated *O*-demethylation to DX accounts for more than 90% of DM metabolism and is the rate limiting step (Frank *et al.* 2007).

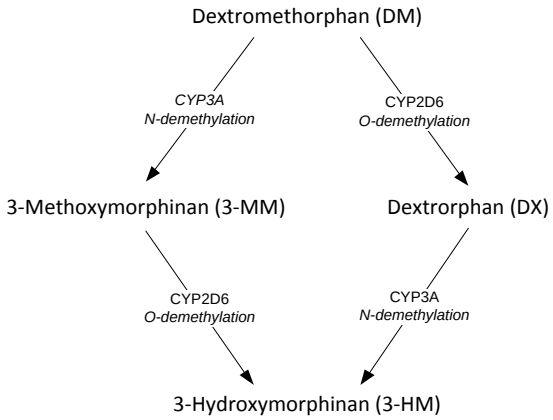


Figure 1.5-4 Predominant routes of dextromethorphan metabolism

1.5.3.2.2 SAMPLE TIME

Oral clearance once again provides the best measure of CYP2D6 activity but this requires multiple samples over a long time to complete an AUC, which is expensive to perform and uncomfortable for the patient.

Single plasma samples can be used but the low analyte concentration requires very sensitive detection methods such as fluorescence detection or mass spectrometry. With these methods, correlations between the metabolic ratio in three or four hour post-dose plasma samples and oral clearance have been reported as high as r=0.74 (Hu *et al.* 1998; Chladek *et al.* 2000; Streetman *et al.* 2000b).

1.5.3.2.3 DOSE OF DEXTROMETHORPHAN

Doses of 20-30mg are most common with adverse effects associated with larger doses(Hu *et al.* 1998). Additionally, CYP2D6 becomes saturated at relatively low levels of substrate concentration, so doses are kept low to avoid the influence of other enzymes(Frank *et al.* 2007). Streetman *et al.* conducted a dose finding study amongst 40 subjects for the purpose of phenotyping with doses ranging from 0.05-1.3mg/kg. They found inaccuracies in the results determined from the higher doses consistent with enzyme saturation. They concluded that 30mg could be used without concern for toxicity or the accuracy of the metabolic ratio to determine phenotype (Streetman *et al.* 1999).

1.5.3.2.4 PHARMACOKINETIC PARAMETERS OF DEXTROMETHORPHAN

DM undergoes rapid absorption with high bioavailability (Pender *et al.* 1991). Maximum concentrations are achieved in two hours. The elimination half-life of dextromethorphan is 2.5 hours and 4 hours for dextrorphan (Hollander *et al.* 1994). It is renally excreted mostly as demethylated metabolites (Pender *et al.* 1991). Absorption may be delayed by reductions to gastric emptying.

1.5.3.2.5 OTHER REPORTED PROBES OF CYP2D6

Saliva and urinary methods have also been used to probe for CYP2D6. The low analyte concentration in saliva makes the analysis difficult to perform and this method also lacks reproducibility (Hu *et al.* 1998; Frank *et al.* 2007). Poor correlations of saliva with established plasma and urinary measures also question the validity of this assessment technique.

Urine collections ranging from 4 to 24 hours in duration have been used and in the literature most phenotyping studies involve an 8 hour urine collection, which does show good correlation with oral clearance (Evans *et al.* 1991; Schadel *et al.* 1995; Hu *et al.* 1998; Kashuba *et al.* 1998b; Chen *et al.* 2003). However, there are some important

problems with urinary measures of the DM:DX ratio as a measure of CYP2D6 activity, which include:

- Changes to urine pH causing up to a 20 fold variation in measured metabolite ratio (Kashuba *et al.* 1998a);
- Excretion of dextrorphan being reduced in patients with impaired renal function (as mentioned in Section 1.3, the stress response temporarily impairs renal function in most surgical patients)(Kevorkian *et al.* 1996; Rostami-Hodjegan *et al.* 1999);
- Poor correlation with oral clearance (Borges *et al.* 2005) and;
- Intra-individual coefficient of variation being reported as 37% on average (Kashuba *et al.* 1998a; Labbe *et al.* 2000).

Debrisoquine and sparteine have also been used extensively and provide the most accurate result, but concerns about their availability and safety, limit their use (Streetman *et al.* 2000a). Tramadol has also been considered but requires further validation before it could be used and has safety concerns of its own. The metabolism of tramadol is also much more complicated than dextromethorphan. Metoprolol also provides a reliable result but the clinical effects of this drug limit its utility (Frank *et al.* 2007).

1.5.3.2.6 PROBLEMS WITH DEXTROMETHORPHAN AS A PROBE FOR CYP2D6

On repeated dosing accumulation may occur to a much greater extent in poor metabolisers (PM) than extensive metabolisers (EM) (Schadel *et al.* 1995). In studies using repeated doses where accumulation is an issue, saliva and serum samples may be more accurate. PM may also exhibit poor glucuronidation ability (Schadel *et al.* 1995). Due to the structural similarities, several opiates may interfere with the assay (Chen *et al.* 1990).

Urinary pH may lead to a 20-fold variation in the measured dextromethorphan/dextrorphan metabolic ratio, however, correction factors have been developed that can adjust for changes in urinary pH (Ozdemir *et al.* 2004). Intra-individual variation in urinary dextromethorphan/dextrorphan metabolic ratios have been investigated (Kashuba *et al.* 1998a). This group found a coefficient of variation of 37% on average; however creatinine clearance and urinary pH were not taken into account which could improve these values.

DM and DX are present in very low concentrations in plasma and saliva samples. The compounds do not absorb UV light and therefore cannot be detected by conventional HPLC instruments with UV detection. The compounds do fluoresce so can be detected by a fluorescence detector in addition to mass spectrometry methods.

1.5.3.3 CYP2E1

The enzyme CYP2E1 is found mostly in the liver but also in several extra-hepatic tissues such as the kidney, lung and lymphocytes (Song *et al.* 1996; Lieber 1997). It exists on the plasma membrane when functionally active (Wu *et al.* 1992). It is involved in the metabolism of more than 70 endogenous and exogenous substrates including suspected carcinogens (Kharasch *et al.* 1993). Aside from paracertamol, the most important substrates are many inhaled anaesthetics, and ethanol (Song *et al.* 1996). There is no evidence of self-induction of CYP2E1 by paracetamol nor is there evidence of cumulative kinetics following prolonged use (Rumack 2002). While not a substrate, azole antifungals have been shown to induce CYP2E1. CYP2E1 is also involved in the metabolism of AA and has a minor role in gluconeogenesis (Song *et al.* 1996).

The metabolites generated by CYP2E1 are often more toxic than the parent compound, commonly resulting in reactive free radical metabolites or oxidative species. These may cause DNA damage, generate protein adducts, cause lipid peroxidation, mobilise iron stores and may result in cytotoxicity (Song *et al.* 1996).

There is considerable inter-individual variability in CYP2E1 activity. Many factors such as fasting, diabetes, obesity, hypophysectomy, alcohol, high fat diets and drug use influence CYP2E1 activity and this may contribute to the variability between individuals (Lieber 1997). These were discussed in Section 1.4.

Genetic factors may also be involved, as variations in the CYP2E1 gene have been described which are associated with altered enzyme activity or expression, although none of the polymorphisms described so far are useful for predicting an individual's CYP2E1 activity (Ernstgard *et al.* 2007). CYP2E1 also exhibits ethnic differences, having greater activity in Asians, particularly Taiwanese, compared with European and African Americans (Stephens *et al.* 1994).

1.5.3.3.1 CHOSEN PROBE- CHLORZOXAZONE

Chlorzoxazone is the only CYP2E1 probe that has been widely studied (Desiraju *et al.* 1983), is the only one currently available and has been proposed to be a nearly ideal probe (Ernstgard *et al.* 2007). Used clinically as a skeletal muscle relaxant for relief of painful musculoskeletal conditions, it is rapidly absorbed when given orally, attaining a peak at 38min ±3.3min after dosing (Frye *et al.* 1996; Ernstgard *et al.* 2004). The concentration then rapidly declines in a mono-exponential manner, suggesting rapid distribution and excretion. It is extensively metabolised and rapidly eliminated. Up to 90% of chlorzoxazone is oxidised by CYP2E1 to 6-hydroxychlorzoxazone which is then glucuronidated and excreted in the urine. Less than 1% of the drug is excreted into the urine unchanged with most (74 +/- 3.4%) of the dose excreted as the glucuronidated metabolite 6-hydroxychlorzoxazone (Desiraju *et al.* 1983). Such a high percentage indicates minimal influence of other elimination pathways. The 6-hydroxylation of chlorzoxazone provides a valid estimate of hepatic CYP2E1 activity and this can be found from a single point plasma ratio (Frye *et al.* 1996). The metabolism of chlorzoxazone was shown not to be affected by concurrent administration of paracetamol in a pharmacokinetic study (Ernstgard *et al.* 2004).

1.5.3.3.2 SAMPLE TIME

Plasma concentrations after 8 hours are generally undetectable indicating rapid elimination(Frye *et al.* 1996) although methods have been developed to detect chlorzoxazone as late as 10 hours after dosing (Desiraju *et al.* 1983). Two hours is considered to be ideal sampling time. High correlation between 2 and 4 hour metabolic ratios indicates 4 hours may also be acceptable but while ratios remain the same, the concentrations drop thereby increasing the influence of analytical errors. Two studies have found crushing the chlorzoxaone tablet improved absorption and similarly improved results (De Vries *et al.* 1994; Ernstgard *et al.* 2007).

1.5.3.3.3 DOSE OF CHLORZOXAZONE

As discussed in Section 1.5.2.1, chlorzoxazone has been found to affect the metabolism of caffeine when given at higher doses of 750mg, indicating its preferred enzyme, CYP2E1, had been saturated. In subjects with low body weight, 500mg is sufficient to saturate CYP2E1 (Frye *et al.* 1998a). This is avoided by using lower doses. However, it is suggested

that intra-individual variation reduces with increasing dose (Tanaka *et al.* 2003). In an attempt to find the ideal dose for phenotyping Frye *et al.* examined the effect of increase dose on kinetics. Their results of increases in the AUC and elimination half-life and reductions in the dose-normalised formation of 6OH-chlorzoxazone indicated saturation of CYP2E1 with higher doses and a non-linear disposition. They concluded that the use of a lower 250mg dose would avoid the complications associated with this non-linear elimination and its effect on phenotypic measures (Frye *et al.* 1998a).

1.5.3.3.4 PHARMACOKINETIC PARAMETERS OF CHLORZOXAZONE

Chlorzoxazone is rapidly absorbed following oral administration with a maximum concentration being achieved at 38min following a 750mg dose. It has a half-life of 66min and clearance 148 mL/min.

1.5.3.3.5 OTHER REPORTED PROBES

Measuring chlorzoxazone in other biological fluids has been examined. The solubility of the drug in the saliva and salivary glands is a crucial factor in the success of salivary monitoring. Chlorzoxazone is a relatively large and hydrophobic molecule and although 6-hydroxychlorzoxazone is mostly glucuronidated or sulphated, the increase in size outweighs this increase in hydrophilicity. Because of these factors, the equilibration and equal partitioning of chlorzoxazone or 6-hydroxychlorzoxazone between plasma and saliva is not rapid enough to be clinically useful for monitoring (Ernstgard *et al.* 2007).

Other methods to measure CYP2E1 activity in lymphocytes by the substrate p-nitrophenol or by flow cytometry, have also been investigated but were shown not to be as useful as chlorzoxazone metabolism (Ernstgard *et al.* 2007).

1.5.3.3.6 PROBLEMS WITH CHLORZOXAZONE AS A PROBE FOR CYP2E1

There may be a small contribution to chlorzoxazone metabolism by other CYP450 enzymes. CYP1A has been shown to metabolise chlorzoxazone but it is of minor importance compared with human CYP2E1 *in vivo* (Desiraju *et al.* 1983).

Chlorzoxazone metabolism may be affected by other confounders such as body weight. Ernstgard *et al.* in their review of chlorzoxazone use, showed that there was a significant correlation between metabolic ratio and body weight, lean body weight, body fat and body mass index (Ernstgard *et al.* 2004). Previous studies have also showed body weight

as a major contributor to inter-individual variability in the oral clearance of chlorzoxazone. The authors predict CYP2E1 activity is related to amount of body fat. In this paper, Ernstgard *et al.* postulated a connection between body fat, increased plasma insulin levels, increased fat catabolism, increased acetone and ketone body formation and, ultimately, increased CYP2E1. Ernstgard *et al.* also found no influence of moderate recent alcohol use, although alcoholics are known to metabolise chlorzoxazone five times more rapidly than healthy subjects. It is unclear if smoking impacts on CYP2E1 activity as it often accompanies alcohol use, although some induction in chlorzoxazone metabolism has been observed (Kroon 2007).

No interfering peaks have been observed with the administration of midazolam and caffeine, as well as many other probes for other CYP450 enzymes. No interference was found in samples from several different patient population including patients with renal or hepatic disease, liver transplants or cancer (De Vries *et al.* 1994).

In vivo 6-hydroxychlorzoxazone is rapidly glucuronidated following its formation and mainly exists as this conjugate in plasma and is entirely glucuronidated in urine. To accurately determine concentrations of 6-hydroxychlorzoxazone, samples must first undergo enzymatic hydrolysis to remove the glucuronide and expose the metabolite for detection (Lucas *et al.* 1993). Lucas showed approximately a threefold increase in the measured concentration of 6-hydroxychlorzoxazone after treatment with *Helix pomatia* juice. Treatment with β-glucuronidase showed yields 12% lower than with *H. pomatia* juice (Lucas *et al.* 1993). Unless exposed to enzyme hydrolysis, 6-hydroxychlorzoxazone in samples is below the limit of detection with some methods (Stiff *et al.* 1993). Incubation for 3 hours at 37°C is sufficient, with hydrolysis being complete after 2hrs of incubation. Longer incubations do not yield increased concentrations of 6OH-chlorzoxazone (Stiff *et al.* 1993).

Chlorzoxazone is a low extraction ratio drug so clearance depends on metabolic activity and not hepatic blood flow (Guengerich 1999). Variation in absorption is thought to be one of the main contributors to inter-individual changes in metabolic ratio seen, with co-efficient of variations commonly reported of 30-40% (Chen *et al.* 2002; Ernstgard *et al.* 2004) and up to 52% in one study (Ernstgard *et al.* 2007). Accordingly changes to gastric emptying in surgical patients may have an impact.

1.5.3.4 CYP3A

CYP3A is the most clinically significant group of enzymes in human drug metabolism, being responsible for the metabolism of 60% of all therapeutic drugs and accounting for up to a quarter of all CYP450 enzymes in the liver. It is the major metabolic enzyme present in the human intestine (Thummel *et al.* 1994; Thummel *et al.* 1996; Lin *et al.* 2001; Chaobal *et al.* 2005; Chung *et al.* 2006). In adults, CYP3A4 and 3A5 are the two most important isoforms with CYP3A7 only being found in foetal liver (Lin *et al.* 2001; McDonnell *et al.* 2005). Of these, CYP3A4 is the major isoform. CYP3A5 is subject to polymorphic expression and is only found in 20-30% of adult human livers, but having greater expression in the small intestine and kidneys (Lin *et al.* 2001). CYP3A4 and 3A5 metabolise many of the same drugs but have different substrate intrinsic clearance and regioselectivity (Lin *et al.* 2001). Variations in CYP3A5 expression have been shown to have no effect on midazolam clearance (He *et al.* 2005) suggesting that CYP3A4 is predominantly responsible for the metabolism of midazolam when administered parenterally.

Wide inter-individual variations in rate and extent of intestinal and hepatic CYP3A activity are seen in humans (Lin *et al.* 2001). Studies have shown up to a 48 fold variation in weight normalised clearance values between individuals (Lin *et al.* 2001). The expression of CYP3A may be affected by environmental, hormonal or genetic influences, however, relatively little is known about these homeostatic influences in comparison with what is known about the effects of drugs and diet (Rogers *et al.* 2002).

1.5.3.4.1 CHOSEN PROBE- MIDAZOLAM

Midazolam is a widely accepted and validated phenotyping probe for CYP3A and is one of the preferred *in vivo* probes by the US Food and Drug Administration (FDA) (Food and Drug Adminstration 1999). It has many of the attributes of an ideal CYP3A probe:

- Its short half-life allows for estimation of the area under the curve (AUC and other pharmacokinetic end-points) within a reasonable time period;
- It is exclusively metabolised by CYP3A4 to a primary metabolite, 1'-hydroxymidazolam when parenterally administered;
- It is not a PGP/multidrug resistant-1 substrate, which itself is subject to inhibition and induction, thereby potentially confounding results (Thummel *et al.* 1996);

- As it is subject to hydroxylation by both intestinal and hepatic CYP3A, oral formulations can be used to assess both intestinal and hepatic CYP3A activity and parenteral formulations to assess only the hepatic component (Tanaka *et al.* 2003);
- It has a low hepatic extraction ratio and clearance that is largely hepatic blood flow independent (Thummel *et al.* 1994); and
- Its clearance correlates with CYP3A activity in liver biopsies and the clearance of other known CYP3A substrates such as cyclosporine (Chen *et al.* 2006; Chung *et al.* 2006). It also reflects changes expected on co-administration with CYP3A inducers and inhibitors and in patients with severe liver disease (Chung *et al.* 2006).

1.5.3.4.2 SAMPLE TIME

Total midazolam clearance is an established *in-vivo* probe to assess the activity of CYP3A, however, accurate quantitation of midazolam clearance after midazolam administration requires the collection of serial blood samples (Lin *et al.* 2001). This is time consuming and costly. Similar to the other CYP450 probes discussed the use of a single time point to predict the integral plasma midazolam exposure (*i.e.* AUC) and indirectly *in-vivo* CYP3A activity has been investigated (Thummel *et al.* 1994; Thummel *et al.* 1996). This study showed that the optimal time for getting the best correlation between a single time point and AUC is the harmonic mean of the mean residence time (MRT). The study of 224 healthy volunteers found the MRT for IV midazolam was 3.5 ± 1.6h and 3.4 ± 1.5h for oral midazolam, concluding that four hours was the best sampling time as the absorption and distribution would be essentially complete. A strong correlation (r=0.80) was shown between the four hour midazolam concentration and the IV midazolam AUC. Similar correlations remained when the study participants were given known CYP3A inducers and inhibitors, showing the usefulness of midazolam in drug interaction studies as an accurate assessment of CYP3A activity in altered states.

Other studies have assessed the use of the ratio of 1`-hydroxymidazolam metabolite/midazolam concentration ratio at 30 minutes and one hour post dose with predicted correlations ranging from r=0.43 to r=0.87 (Dundee *et al.* 1984). However, the study using the four hour midazolam concentration had a much larger cohort. The metabolite 1`-hydroxymidazolam also undergoes rapid, potentially variable

glucuronidation in the liver and may introduce a further source of unpredictability (Food and Drug Adminstration 1999).

1.5.3.4.3 DOSE

The pharmacological effects of midazolam are dose limiting, but also have to be balanced with assay detection limits. Doses range from 0.025mg/kg for MS detection methods and up to 7mg dose for UV detection. Used clinically for sedation with amnesia, the relief of status epilepticus and induction of anaesthesia, the dose for its use as a probe must avoid the potential sedation and respiratory rate depression. The experience of clinical effects seems to have no effect on the validity of the probe. Those with previous exposure to benzodiazepines or alcohol tend not to be as sensitive to the pharmacodynamic effects of the drug.

1.5.3.4.4 PHARMACOKINETIC PARAMETERS OF MIDAZOLAM

Midazolam has a Vd ranging from 0.8-2.5 L/kg, being increased in congestive heart failure and chronic renal failure (Lacy *et al.* 2005). It is extensively protein bound (95%) and has a half-life of elimination of one to four hours, being prolonged in cirrhosis, congestive heart failure, obesity and the elderly. It is excreted in the urine, mainly as glucuronide metabolites (Lacy *et al.* 2005).

1.5.3.4.5 OTHER REPORTED PROBES

Simvastatin has also been used as a probe for CYP3A and was listed as a preferred substrate for the study of CYP3A drug interactions by the FDA (Prueksaritanont *et al.* 2003). Approximately 80% of the metabolism of simvastatin to its metabolite, simvastatin acid, occurs via CYP3A with the remaining 20% via CYP2C8 (Chung *et al.* 2006) making it a less ideal probe for CYP3A. It also requires a similar sampling profile to midazolam. Simvastatin also lacks studies validating it through liver biopsy and there are few randomised controlled studies comparing it with established probes such as midazolam. One recent study showed weak correlations of phenotypic values between the two substrates, with simvastatin having far wider variability (Chung *et al.* 2006). The authors concluded simvastatin was a less accurate measure of CYP3A activity than midazolam and this variability occurred because simvastatin was less specific for CYP3A than midazolam (Chung *et al.* 2006) perhaps due to the influence of CYP2C8, a polymorphic enzyme. Simvastatin showed a double peak on its concentration-time profile indicating it may also

be subject to entero-hepatic recycling, which would also further complicate its analysis (Chung *et al.* 2006). Compared with midazolam, simvastatin is a non-validated, suboptimal CYP3A probe and its use as a CYP3A probe in preference to midazolam cannot be justified (Swart *et al.* 2004).

1.5.3.4.6 PROBLEMS WITH MIDAZOLAM AS A PROBE FOR CYP3A4

Midazolam is a hypnosedative and this does limit its usefulness in some settings and in some populations, with mild to moderate sedation often being reported (Chung *et al.* 2006). There are also some reports of difference in metabolism between sexes (Lin *et al.* 2001).

A history of alcohol abuse and age have also been shown as covariates (Swart *et al.* 2004). Alcoholic cirrhosis can lead to an increased half-life of midazolam due to an increased Vd which is explained by a reduction of plasma protein binding (Swart *et al.* 2004). Compared with the effect of alcohol, the contribution of age is small (Swart *et al.* 2004).

Midazolam has an intermediate to high hepatic extraction ratio which makes its pharmacokinetics susceptible to changes in hepatic blood flow (Swart *et al.* 2004).

Once blood samples have been taken, some midazolam is lost with long periods of storage. El Mahjoub *et al.* showed around 90% of the original concentration of midazolam was measured in whole blood after 1 year of storage at -80°C and approximately 80% if stored at -20°C for the same period. No midazolam was detectable if the same was kept at room temperature (El Mahjoub *et al.* 2000).

1.6 POPULATION STATISTICS

As will be shortly discussed in Section 2.1, this study involved colorectal and breast cancer patients. The population statistics of these groups are outlined below.

1.6.1 COLORECTAL CANCER

1.6.1.1 PREVALENCE IN EUROPE AND IRELAND

Colorectal cancers were the most commonly diagnosed cancer across the European Union (EU) in 2008, representing 13.6% of all cancers diagnosed. Ireland has one of the highest incidence rates of colorectal cancer in Europe with age standardised estimates for 2008 of 66.9 per 100,000 for males and 42.9 for females. This compares with 54.9 and 35.9 for the UK, 54.8 and 36.4 for France and 27.4 and 17.1 for Greece (Ferlay *et al.* 2010).

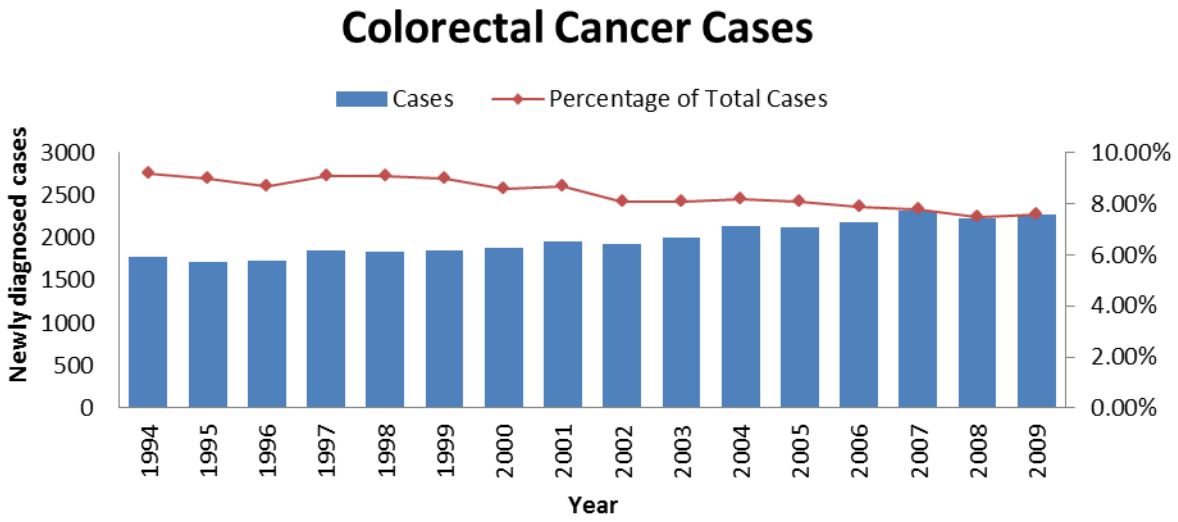


Figure 1.6-1 Newly diagnosed cases of colorectal cancer and percentage of all newly diagnosed cancers
Number of newly diagnosed cases reported each year to the National Cancer Registry of Ireland (solid bar) and this number as a percentage of all cancers diagnosed in that year (dotted line). Source: (National Cancer Registry Ireland 2010).

Figures from the National Cancer Registry of Ireland list 2,271 newly diagnosed cases of colorectal cancers in 2009 (National Cancer Registry Ireland 2010). In Ireland this represents 7.6% of the total number of new cancers diagnosed in 2009, much lower than the 13.6% seen across the EU due to the even higher rates of other cancers in Ireland (National Cancer Registry Ireland 2010).

The number of cases diagnosed with colorectal cancer in Ireland has increased by approximately 35% over the last fifteen years, which may reflect improvements in diagnosis, awareness and screening. The number of cases recorded by the National Cancer Registry of Ireland is shown in Figure 1.6-1

There have also been increases in the population of Ireland over this time which would have some impact, increasing the actual number diagnosed. Despite the increase in numbers diagnosed, the percentage of colorectal cancers of the total number of malignant cancers diagnosed in Ireland, also shown in Figure 1.6-1, has reduced over this time period.

1.6.1.2 PATIENT CHARACTERISTICS

Of the 2,271 newly diagnosed cases of colorectal cancer in Ireland in 2009, 1,342 occurred in males and 929 in females (National Cancer Registry Ireland 2010). The actual number of cases of colorectal cancer diagnosed in 2008 collected by the National Cancer Registry of Ireland differs slightly from the estimates of Ferlay *et al.* given in section 1.6.1.1. When the National Cancer Registry of Ireland rates were adjusted to meet the age of a theoretical European population, colorectal cancer diagnosis rates were 65.1 per 100,000 males and 39.0 per 100,000 females. Both sets of age adjusted rates from Ferlay *et al.* (Ferlay *et al.* 2010) and the National Cancer Registry of Ireland (National Cancer Registry Ireland 2010) indicate that approximately two-thirds of those diagnosed with colorectal cancer are male. There are also important changes in rates of diagnosis in different age group. Data for age at time of diagnosis are shown in Figure 1.6-2.

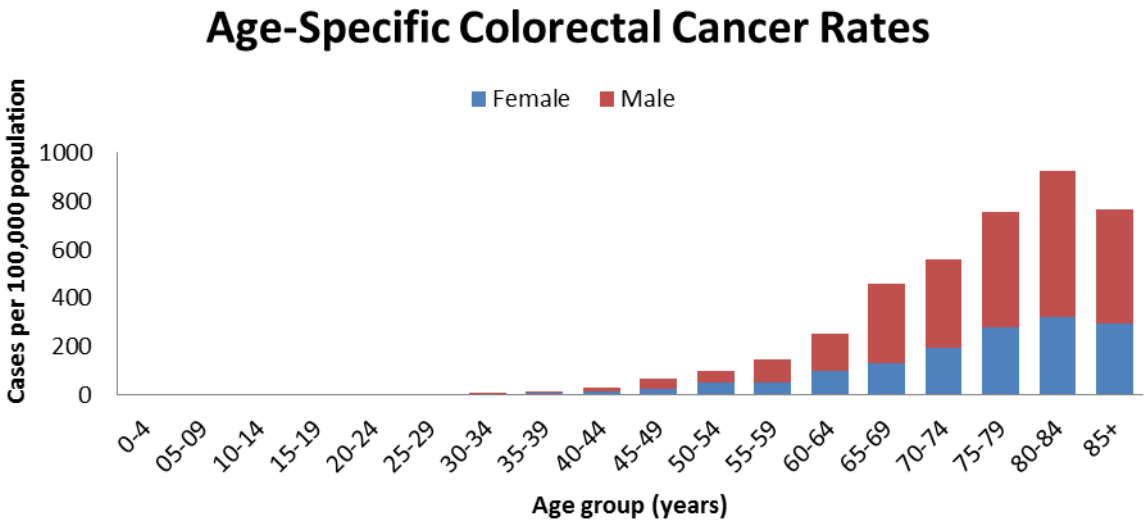


Figure 1.6-2 Age specific rates of newly diagnosed colorectal cancers in Ireland in 2009
Data presented as cases per 100,000 of the population within that age group for each gender. Source: (National Cancer Registry Ireland 2010)

This figure shows that colorectal cancer rates are highest in the 80-84 year old age group, with very few patients being diagnosed under 50 years of age. This has important ramifications for this Thesis in terms of the eligibility criteria for entry into the study, which, to paraphrase, required patients to be otherwise healthy aside from their

indication for surgery. It is to be expected the number of co-morbidities increase with age and this may lead to a greater rate of those who do not meet the entry criteria.

1.6.1.1 DISEASE CHARACTERISTICS

Locations of colorectal cancers newly diagnosed in Ireland in 2009 are displayed in Figure 1.6-3. The National Cancer Registry of Ireland does not publish statistics that distinguish between locations of cancer in colon and, as a result, the majority of colorectal cancers are found within this region.

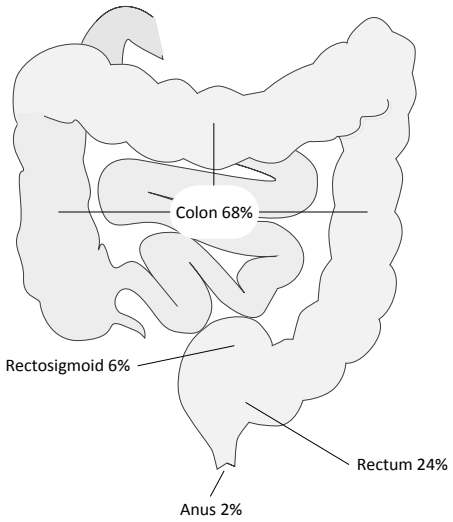


Figure 1.6-3 Location of all newly diagnosed colorectal cancers in Ireland in 2009
Data rounded to the nearest whole number. Source: (National Cancer Registry Ireland 2010)

Locations of colorectal cancers also vary with age. These are displayed in Figure 1.6-4.

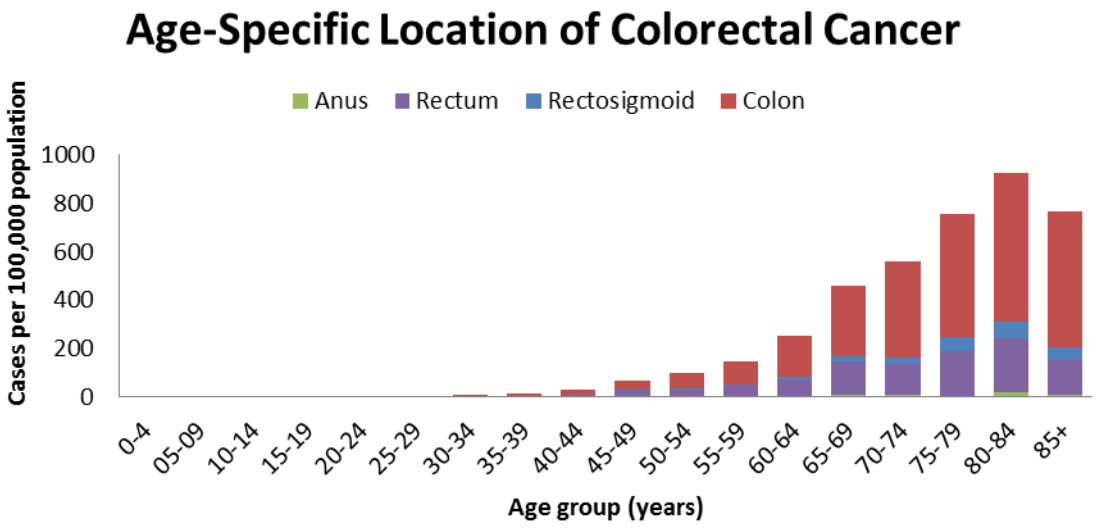


Figure 1.6-4 Age specific location newly diagnosed colorectal cancers in Ireland in 2009
Data presented as cases per 100,000 of the population within that age group for each location. Source: (National Cancer Registry Ireland 2010).

Encompassing the largest area of the bowel, cancers of the colon make up the majority of diagnoses in all age groups, becoming increasingly prevalent in the later stages of life, peaking in the 80-84 year old age group. Rectal cancers are the second largest contributor

to colorectal cancer diagnoses, with relatively few rectosigmoid or anal cancers, however these represent a relatively exiguous anatomical area of the bowel.

1.6.2 BREAST CANCER

1.6.2.1 PREVALENCE IN EUROPE AND IRELAND

Across the EU malignant breast cancer is the second most commonly diagnosed cancer in 2008, representing 13.1% of all cancers diagnosed (Ferlay *et al.* 2010). In a recent report Ireland was listed as having the fourth highest incidence of breast cancer of 27 EU countries, with age standardised rates of 126.5 per 100,000 females. This compares to 119.1 for the UK, 133.8 for France and 61.9 for Greece (Ferlay *et al.* 2010). There were 2,766 newly diagnosed cases of malignant breast cancer reported to the National Cancer Registry of Ireland in 2009 which represented 9.3% of all malignant cancers diagnosed in that year (Figure 1.6-5).

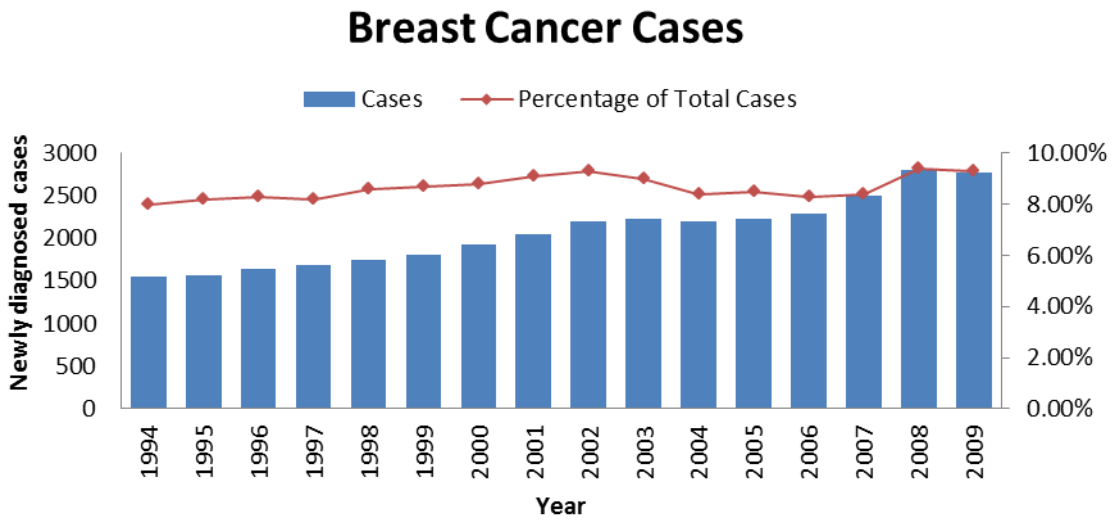


Figure 1.6-5 Newly diagnosed cases of breast cancer and percentage of all newly diagnosed cancers
Number of newly diagnosed cases reported each year to the National Cancer Registry of Ireland (solid bar) and this number as a percentage of all cancers diagnosed in that year (dotted line). Source: (National Cancer Registry Ireland 2010).

Similar to colorectal cancer, the number of breast cancer cases diagnosed has steadily increased in the last fifteen years, although the increase in breast cancer cases has been more marked. There was a dramatic increase in 2008, when numbers of cases diagnosed increased by 12% on the previous year. This may reflect the effect of increased resources and national expansion of the national breast screening program "Breast Check" in Ireland, which commenced screening in December 2007. Unlike colorectal cancers, the percentage of breast cancers diagnoses of all malignant cancer diagnoses has increased from 8.0% in 1994 to 9.3% in 2009 (Figure 1.6-5).

1.6.2.2 PATIENT CHARACTERISTICS

Of the 2766 newly diagnosed cases of breast cancer in Ireland in 2009, 2740 (99.1%) were female. When the Irish population statistics were adjusted to meet the age of a theoretical European population, the rate of malignancy of the breast being diagnosed was 125.23 per 100, 000 population for females and 1.17 per 100,000 males (Figure 1.6-6).

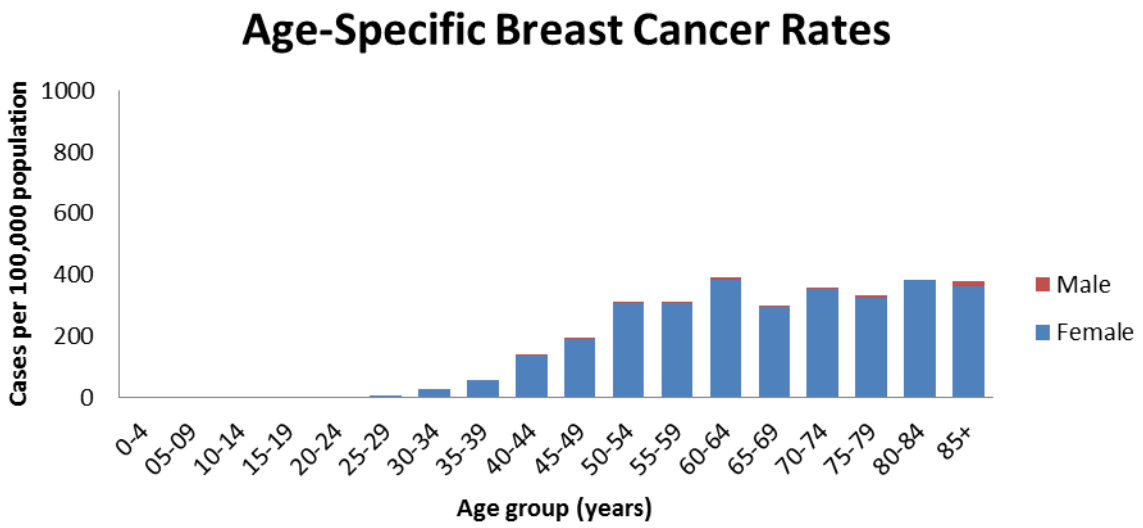


Figure 1.6-6 Age specific rates of newly diagnosed malignant breast cancers in Ireland in 2009
Data presented as cases per 100,000 of the population within that age group for each gender. Source: (National Cancer Registry Ireland 2010)

2 METHODS

2.1 PARACETAMOL METABOLISM STUDY

This study was designed to detect changes to surgical patients' paracetamol metabolism before and after surgery and determine the safety of higher doses of paracetamol. Following review of the literature discussed in this Thesis it was determined that the most effective way of establishing any changes and safety was through the review of:

- Any changes to the kinetics of paracetamol, which would indicate a change in disposition (Section 2.2);
- The detection of the stress response by reviewing the concentration of inflammatory cytokines known to affect drug metabolism (Section 2.3);
- The measurement of αGST concentration, the most sensitive marker of early hepatotoxicity on the market (Section 2.4); and
- The activity of CYP450 enzymes, the enzymes that contribute to paracetamol's Phase 1 metabolism (Section 2.5);

The methods for determining changes to paracetamol metabolism identified above required the collection of both blood and urine samples from patients and to determine the change caused by surgery, patient samples were required, prior to and following surgery. Accordingly, each patient recruited into this study was given paracetamol and had blood and urine samples taken on the evening before and for four days after their surgery (the protocol for which is described below).

Drug metabolism is subject to a vast array of inter-individual differences arising from genetics and environmental influences. As discussed in Sections 1.3 and 1.4 these influences make the comparison between individuals very difficult because they are not applied to all individuals equally, nor are their drug metabolism's affected to the same degree once an exposure to one of these influences has occurred. With this in mind this study compared preoperative to postoperative data from the same patient, using preoperative data as the control to reduce sources of variance. This allowed a more accurate assessment of the effect of surgery on drug metabolism.

2.1.1 THE PATIENTS

Before the details of the study protocol are presented the justification for the patient groups is explained.

The study began collecting patients scheduled for bowel resection into two groups: one group receiving high dose paracetamol (Group A) and the other receiving the licensed dose (Group B).

Bowel resection patients were chosen because of:

- Their need for good pain control postoperatively (to enable coughing, mobilisation *etc.*);
- The effect on drug metabolism arising from the invasive and traumatic nature of their surgery, frequently involving manipulation of the gut and liver;
- The malnourishment and prolonged perioperative fasting regularly seen in these individuals; and
- Their prolonged hospital admission.

Thus, these patients provided a "worst case scenario" for assessing changes to drug metabolism in surgical patients.

Allocation to either group was done sequentially in groups of 10 patients. Doses were not randomised for four reasons:

- This was a study primarily concerned with toxicity, not efficacy. A patient cannot have any influence on their own drug metabolism while in hospital;
- Only one surgeon performed bowel resections at this hospital and he only worked with one anaesthetist. Following surgery all patients returned to the same ICU where they received the same level of nursing care;
- Each patient provided their own control data from preoperative sampling; and
- Randomisation would have necessitated frequent changes to dosing regimen, therefore conducting the study in blocks of patients eliminated the risk of dosing errors.

This recruitment continued until half-way through Group B when reconfiguration of surgical services in the region in 2008 withdrew bowel resection surgery from this hospital (St John's Hospital, Limerick (Hospital 1)).

The next most major surgery still carried out at Hospital 1 was mastectomy. To make the best of the situation they were chosen to give a comparator group (Group C) to bowel resection patients because:

- They also have a need to good pain control postoperatively to allow restoration of function and reduce risk of chronic pain; and
- Their surgery does not involve manipulation of the gut or liver, but is still considered intermediate in nature and can be of similar duration; and
- Patients have short preoperative fasting and an early return to oral nutrition.

However, it became clear that this patient group was unsuitable for this type of research primarily because they had poor IV access making the blood sampling detailed below difficult.

It was decided to abandon this group and collect further bowel resection patients (Group D) in Mercy University Hospital, Cork (Hospital 2). This added a further 14 months to the research and presented significant challenges and some of these are detailed in the discussion.

2.1.2 STUDY APPROVAL

Ethics Committee approval was sought and gained from The Clinical Research Ethics Committee of the Cork Teaching Hospitals (Appendix 1 and 2).

Clinical Trials approval was also sought and gained from the European Medicines Agency (EMEA) and the Irish Medicines Board (IMB) (Appendix 3).

While application for ethics approval was straight forward, involving obtaining written approval from the host hospitals, application, provision of study protocols, presentation at meetings and answering of verbal and written questions, the application to the EMEA was significantly more laborious. This began with the application to apply for application for clinical trial approval, which was assessed on the investigators qualifications and experience. Once this was approved the application for clinical trial approval itself was nearly 100 pages in length which was completed online and then printed. The printed application was then submitted with key references and documents to the authority. This was followed by thorough inspection of every detail of the study by the EMEA and

resulted in further requested for information. Once these requirements were satisfied, approval was finally granted by the IMB as the agency with responsibility for Ireland.

2.1.3 SAMPLE SIZE

It was necessary to determine how many patients were required to enter the study to detect significant differences in paracetamol metabolism. The aspect of paracetamol metabolism of greatest interest was the difference between Phase II: Phase I paracetamol metabolite ratios before and after surgery. Generally, the number of participants required to detect significant differences is related to the size of that difference. Large differences can be detected with fewer participants whereas small differences need many more participants to detect them. A measure used to describe the size of this difference is called the 'effect size'. The effect size can be used to determine sample size necessary to show a significant difference

Changes to urinary metabolite ratio seen in previous work conducted by Kennedy in 1996 were used in the effect size (d) calculation (Kennedy 1996). This was determined using Cohen's d, as shown in Equation 2.1-1:

$$\mathrm{d(the\ effect\ size)} = \frac{\text{average of differences between pre and post op metabolite ratios}}{\text{standard deviation of difference between pre and post op metabolite ratios}}$$

Equation 2.1-1 Application of Cohen's equation for effect size

The effect size (d) was shown to be 0.68. A power calculation was conducted which found that with and effect size of 0.68, 15 patients be required for 95% significance, which was consistent with previous work (Kennedy 1996).

2.1.4 SAFETY

At all times the safety and speedy recovery of the patient took priority over the conduct of the research study. The patients were informed at the time of consenting that they could withdraw at any time without hindrance to their subsequent medical care and this statement was also included in the consent form kept by the patient (Appendix 4).

Low thresholds were set for derangement in liver function tests that were tested and reviewed twice daily. Any alterations resulted in withdrawal of trial doses and alterations to other analgesia were made to ensure continued comfort of the patient.

2.1.5 RECRUITMENT

Prior to admission, all patients scheduled for elective surgery, who fulfilled the inclusion criteria, were identified to the Principal Investigator by the Admissions Co-ordinator (Hospital 1) or Surgeon's Nurse Specialist (Hospital 2). A patient's suitability for recruitment was ascertained by the Principal Investigator prior to consenting. This was done by checking previous laboratory values and medical notes to ensure the patient fulfilled all the inclusion criteria, but none of the exclusion criteria, as defined below. For all suitable patients, this information was presented to the anaesthetist supervising the research for their review and, if they agreed, the patient was eligible for admission into the study.

2.1.5.1 INCLUSION CRITERIA

All patients were eligible for inclusion into the study if they were admitted for any of the following procedures:

- Bowel resection for non-malignant indication (*e.g.* Crohn's disease, diverticulitis);
- Bowel resection for malignancy of the gastro intestinal tract; or
- Mastectomy (with or without axillary node clearance) for malignant indication,

and fulfilled all of the following criteria, which were assessed at preadmission:

- Normal liver and renal function;
- Fitness to consent (age over 18); and
- Medical fitness for inclusion as determined by physician.

2.1.5.2 EXCLUSION CRITERIA

Any patient fulfilling any of the criteria below was excluded from the study:

- Type 1 diabetes;
- Alcoholism (the daily consumption of >2 standard drinks in the previous week was used as a screening tool as definitions of alcoholism vary (Tonnesen *et al.* 1999));
- Deranged liver function greater than the upper limit of normal (INR, ALT, AST);
- Deranged renal function (SCr>110umol/L);
- Intolerance to oral medication;
- Pregnancy;

- Vomiting;
- Porphyria;
- Hypersensitivity to any of the investigational medical products;
- The use of any of the following medicines in the week before surgery:
    - Paracetamol, carbamazepine, hydantoin, imantinib, isoniazid, rifampicin, barbiturate; or
- Those who, in the opinion of a physician, were too frail to be included in the study.

2.1.6 ADMISSION INTO THE STUDY

Patients who were suitable for inclusion into the study were admitted to the ward at least the evening prior to surgery. Patients were visited by the Principal Investigator and the nature of the study was explained to them before they were invited to participate. Patients were informed that complete abstention from caffeine containing foods or drinks was required during the study. The consent form was left with the patient and the Principal Investigator left the patient to review the information and discuss the study with relatives. The anaesthetist supervisor then visited the patient to answer any further questions and to consider their fitness to participate and to consent their participation in the study. If the patient fulfilled all the inclusion criteria but none of the exclusion criteria, the patient either accepted or declined the invitation to enrol in the study.

If the patient agreed to participate in the study the consent form was signed by the patient, the anaesthetist supervising the research and the Principal Investigator (Appendix 4). A copy was given back to the patient for their record. At the time of consenting, the patient was asked to empty their bladder, and this urine was discarded.

2.1.7 PRE-OPERATIVE PHASE OF THE STUDY- CYP450 ACTIVITY AND PARACETAMOL KINETICS

2.1.7.1 CANNULATION AND FIRST BLOOD SAMPLE

A 14 or 16G peripheral line (In-syte® Becton Dickinson, Madrid, Spain) was inserted into a forearm vein of the patient and flushed with 10mL normal saline (B Braun, Dublin, Ireland). One 3.5mL blood sample was then drawn off from the cannula, (being the dead space + 2mL) using a 5mL syringe and discarded to ensure reliability of the sample (Prue-Owens 2006). A further 6mL of blood was immediately taken using a 10mL syringe which

was then transferred to two 2.7mL EDTA blood collection tubes (monovette® Sarstedt, Nümbrecht Germany). This transfer was done without delay to minimise clotting in the syringe and subsequent haemolysis. The blood collection tubes were labelled with the patient number, day of the study, time of the sample and initials of person who took the sample.

Patency and aseptic sampling technique was maintained according to in-house standard operating procedures of each participating hospitals.

2.1.7.2 DRUG ADMINISTRATION

Relevant background information to the 4 drugs accompanying paracetamol which were administered as part of this study is detailed in Section 1.5.

Two doses of paracetamol were used in the study and their allocation is shown in Table 2.1-1.

Table 2.1-1 Paracetamol dosing for each group

Group	Paracetamol Dose		
	Preoperative	Intraoperative	Posoperative
A	1.5g single dose	2g single dose	1.5g every four hours
B, C and D	1g single dose	2g single dose	1g every six hours

As soon as the peripheral line was inserted a standard giving set was primed with the IV paracetamol (Perfalgan®, 1g/100mL, Bristol-Myers-Squibb, Dublin, Ireland). The giving set was connected to the patient's peripheral line and the infusion started at a rate to give the dose over 15min. Due to the poor availability of infusion pumps on the ward for Group A, a drop rate of 30drops/10secs was used to approximate the required flow rate of 10mL/min. The time was noted at the beginning and end of the infusion and recorded on the individualised patient data collection form (Appendix 6).

Caffeine 100mg/2mL (Martindale Pharmaceuticals, Romford, UK) in a 5mL syringe and midazolam 1mg/0.5mL (Hypnovel®, Roche, Basel, Switzerland) in a 2mL syringe were administered *via* the additive port on the giving set while the paracetamol infusion was running. Dextromethorphan 30mg/20mL (Benylin® Non Drowsy, Pfizer, Dublin, Ireland) and Chlorzoxazone 250mg (Paraflex®, AstraZeneca, Södertälje, Sweden) were given by mouth followed by 25mL of water. All five medicines were given as close together as possible, *i.e.* within 1-2 minutes, so that their administration times were considered the

same. After the fifth patient of Group A, the IV caffeine became unavailable and two 50mg caffeine tablets were used in its place (ProPlus®, Bayer, Berkshire, UK).

For those patients scheduled to receive 1.5g of paracetamol (as detailed in Table 2.1-1), the infusion pump was first set to administer 150mL. Once the first vial of paracetamol had been given it was removed from the giving set and the second was attached. In those few doses administered without infusion pumps, once the second vial had run to 5mm below the label of the vial the additional 500mg was deemed to have been given (this level was determined by removing the entire contents of a previous vial and replacing 50mL) and the infusion was stopped. The giving set was detached from the peripheral line, the port on the line was cleaned using an alcohol wipe and the line flushed with 10mL normal saline. Heparinised saline 10 IU/mL (5mL) (Hepsal® CP Pharmaceuticals, Wrexham, UK) was used in those patients thought to have poor venous access by the anaesthetist. The anaesthetist remained in contact with the patients and the researcher for 15-30min until the effect of the midazolam was known.

2.1.7.3 SUBSEQUENT BLOOD SAMPLES

A second blood sample was taken fifteen minutes after the paracetamol infusion. A tourniquet was placed proximally to the peripheral line, the port was cleaned and 3.5mL of blood was collected using a 5mL syringe and discarded. A further 5.4mL was collected into two EDTA blood collection tubes either using a Sarstedt multi-adapter system (Sarstedt, Nümbrecht, Germany) or a 10mL syringe decanted into the EDTA tubes. A syringe was used in those with poor access to reduce the risk of the vein collapsing by using less suction in the vein. The tourniquet was removed and the time of the collection was noted. The port was cleaned and the line was flushed with 10mL saline or heparinised saline as described above. Further samples were collected as shown in Table 2.1-2. The additional CYP450 analysis to be conducted on the 4 hour sample required a further 5.4mL of blood collected at this time point.

Table 2.1-2 Daily sampling times (minutes after administration of monitored dose) and volume of blood needed at each sample (mL)

	Day -1	Day 1	Day 2	Day 3	Day 4	Blood Volume (mL)
Sample time (min)	0	0	0	0	0	**5.4**
	15	15				**5.4**
	30	30				**5.4**
	60	60	60	60	60	**5.4**
	90	90				**5.4**
	240	240	240	240	240	**10.8**

2.1.8 URINE SAMPLES

As patients were not catheterised pre-operatively, they were asked to collect the entire volume of their urine in four hourly aliquots. The importance of collecting the entire volume was stressed to patients. To make this as easy as possible, urine collection containers labelled with the times of collection interval were placed in all toilets accessible to the patient. Patients unable to collect their urine directly into the collection container were offered a disposable bed pan from which urine was decanted into the collection container. Patients using bed pans were asked not to pass faeces into the same container or put toilet paper into the container, however this was unavoidable for some patients. In those cases where faecal matter was present, the urine was still collected into a separate container and the volume recorded. If samples were heavily contaminated with faeces the urine volume was approximated and were discarded as the possibility of contamination arising from biliary excretion of drugs could not be excluded.

It was important the urine collection coincided with the dosing of the study medicines. To facilitate this the first postoperative urine collection was 'rounded up' to the nearest required interval and recorded as pre-operation until that time, even though it may have been longer than four hours.

2.1.9 POSTOPERATIVE PHASE OF STUDY

2.1.9.1 DAY OF SURGERY (DAY 0)

2.1.9.1.1 INTRAOPERATIVELY

The following was performed for all study participants during the intraoperative period:

- IV paracetamol 2g was administered intraoperatively (Table 2.1-1);
- Record of the following was taken:
    - Blood/fluid (Appendix 7);
    - Anaesthetics administered (Appendix 8); and
    - The operation performed, its duration, any liver manipulation that took place and any associated events or complications (Appendix 8).

2.1.9.1.2 POSTOPERATIVELY

The following was performed for all study participants on their return from theatre:

- IV paracetamol was administered according to Table 2.1-1;
- Fluid balance was recorded;
- Pain scores were recorded every hour
- Alternative analgesic usage was recorded; and
- Pharmacodynamic tests (Table 2.1-3) were performed

In addition, urine was collected every 4 hours. All patients returned from theatre with urinary catheters *in situ*. While catheterised, all urine was collected from the catheter bag at four hourly intervals and then processed as described in Section 2.1.10.2

No blood samples were taken for the study on the day of surgery. This was for several reasons:

- Pharmacokinetics could not be accurately predicted from samples obtained on this day. Pharmacokinetic equations exist in either single dose or steady state form. On this day paracetamol concentrations would not have reached steady state, but would have been above those from a single dose. Typically, five drug half-lives must pass before steady state can be assumed.
- Patients who lost large volumes of blood intra-operatively would not have been fit for further blood sampling
- The timing of a patients return to theatre could not be predicted and would have led to inconsistencies between patients.

2.1.9.2 DAYS 1-4 POST OPERATIVELY

For any enrolled patient not fulfilling any of the attrition criteria (Section 2.1.9.3), the study continued for four postoperative days. This length was the maximum period it was considered IV paracetamol use could be justified in clinical practice, given the known problems with postoperative oral absorption. Four postoperative days was also the minimum duration of inpatient stay following major bowel surgery.

The following was performed for all study participants during the postoperative period on days 1–4 inclusively:

- IV paracetamol was administered at regular intervals throughout the day according to Table 2.1-1;

- Plasma samples were drawn usually from the 10pm dose each day according to Table 2.1-2;
- Chlorzoxazone 250mg, caffeine 100mg, dextromethorphan 30mg and midazolam 1mg were co-administered with the monitored 10pm paracetamol dose;
- All urine was collected every 4 hours, the total volume was measured and recorded and two x 25mL aliquots were taken;
- Fluid balance was recorded;
- Pain scores were recorded every hour. Daily best, worst and average pain in last 24hrs, pain now were also recorded;
- Alternative analgesic usage was recorded; and
- Pharmacodynamic tests (Table 2.1-3) were performed and checked daily.

After surgery patients in Groups A, B and D may have had a central line inserted into the vena cava through the jugular vein. When this was present it was used for both drug administration and blood sampling. To avoid contamination of the blood samples, arrangements were made with the nursing staff that paracetamol was only given through the distal line and blood samples were taken from the proximal line. Alternatively, if a peripheral line was still available, the paracetamol was given through that line. When taking samples from the central line, all fluids running through it were stopped and their lines clamped as per local hospital protocols. The allocated port was cleaned and 4 mL of blood withdrawn and discarded. The required sample was then taken, the port cleaned and the line flushed with 10mL of normal saline using pulsatile flushes of 2mL pushes. In patients without a central line, the sampling technique was the same as on day 0, with contra-lateral lines being used for paracetamol administration and blood sampling where available. Again, all fluids were stopped and lines clamped during blood sampling. Urine collection continued as on day 0 until at least 8 hours after the last dose of dextromethorphan on day 4.

Any other non-paracetamol containing analgesia that was administered to the study patients was recorded as per protocol.

2.1.9.3 ATTRITION CRITERIA

During the course of the study, Patients were removed immediately if they fulfilled any of the following criteria:

- An AST or ALT level three times above the upper limit of normal;
- Any other sign of paracetamol toxicity or allergy (*e.g.* thrombocytopaenia);
- Loss of fitness for inclusion as determined by a physician;
- Loss of venous access as to prevent timely blood sampling without excessive discomfort to the patient; or
- The patient withdrew their consent (Section 2.1.13.2)

Any patient who fulfilled any of the attrition criteria did not receive further investigational doses of paracetamol, however, they were monitored for the original proposed study period and their data were included in the final analysis.

2.1.10 PROCESSING OF SAMPLES

2.1.10.1 PLASMA

Within one hour of collection, plasma samples were taken to the laboratory centrifuge and spun at 3000g for 10min. The plasma layer was then pipetted off using a 3mL pasture pipette into labelled 1.25mL plastic screw top collection tubes (Sarstedt, Nümbrecht Germany). The labels contained the patient number, day of study, sample number, time of collection and the initials of the person who took the sample. These plasma samples were frozen in lots according to the sampling day at -20°C until the patient had completed the study, when the samples were transferred to a -80°C freezer in the School of Pharmacy, University College Cork.

2.1.10.2 URINE

The total volume of urine excreted within each four hour time period was measured using a 1L cylindrical flask and recorded (Appendix 6). Two 25mL samples were taken, labelled with the total volume, sample period, day of the study and patient number and frozen along with the preceding days plasma at -20°C until the patient had completed the study, when the samples were transferred to a -80°C freezer in the School of Pharmacy, University College Cork.

2.1.11 OTHER SAMPLING- PHARMACODYNAMIC INFORMATION

The following tests were conducted primarily to ensure the safety of the study participant but to also provide information regarding the onset time of any hepatotoxicity that occurred while it was still subclinical (Table 2.1-3).

To monitor safety during the study, LFT's AST, ALT, INR and bilirubin were done daily during the treatment phase. Monitoring continued on alternate days for the rest of the admission. The daily t=0hr plasma sample was used for αGST and IL-1 and IL-6 levels. Once daily testing of these substances were chosen due to the cost of the sampling kits and on advice from the manufacturer of the kits (Shaw 2009).

Table 2.1-3 Pharmacodynamic testing

Test	Frequency	Rationale
αGST	Daily	An intracellular protein found in hepatocytes in all regions of the liver lobule. Specific marker for the detection of early (yet subclinical) hepatotoxicity
ALT + AST	Daily	Marker of hepatocyte damage, released rapidly, peaks early and returns to normal quickly because of its short half-life once the injury has ceased
INR	Daily	Marker of more substantial hepatocyte damage
Serum creatinine	Daily	Marker of renal function which may impact on the clearance of paracetamol and its metabolites
Interleukin-6 (IL-6)	Daily	Correlates with inflammation and CYP450 activity

2.1.12 DEMOGRAPHIC AND CLINICAL DATA

The following information was also collected from the patient or their medical notes (Appendix 9):

- Nutrition status:
    - Height, weight at time of admission for calcuation of BMI;
    - Previous week's meal plan;
    - Any recent weight loss;
    - Any recent periods of fasting or malnutrition;
- Review of Systems:
    - Note any co-morbidities, ASA status;
    - Pain scores (VNRS) 'best', 'worst', 'average pain in last 24hrs' and 'pain now' scores were recorded daily;
    - Other clinical data:

- Primary diagnosis;
- Any concomitant disease;
- Drug history and any regular medication;
- Smoking history; and
- Preoperative laboratory investigations (serum sodium, potassium, urea, and creatinine, alkaline phosphatase, bilirubin (total), bilirubin (conjugated) and alanine aminotransferase (ALT).

2.1.13 ADVERSE EVENT REPORTING AND HANDLING WITHDRAWALS

2.1.13.1 ADVERSE EVENTS

Adverse events were classified as either an *'adverse event'* or a *'serious adverse event'*. Any patient fulfilling either adverse or serious adverse event criteria did not receive any further paracetamol doses and alteration to other analgesia was made to ensure continuing comfort of the patient.

Additionally, all patient reported adverse events were noted.

2.1.13.1.1 ADVERSE EVENT

- ALT or AST measurements greater than five times the upper limit of normal, or
- Any other event that causes morbidity that was thought to be related to the study.

Adverse events were not reported until after consultation with the anaesthetist supervising the research and further confirmation, by subsequent analysis of paracetamol concentrations and other markers of hepatotoxicity, that the adverse event was a result of the patient's participation in the study. Reporting was delayed due to the possibility of other factors causing alteration to liver function associated with surgery but not related to paracetamol administration.

2.1.13.1.2 SERIOUS ADVERSE EVENT

- ALT or AST measurements greater than ten times the upper limit of normal;
- INR>2; or
- Any other event that is life threatening or fatal thought to be related to the study.

Serious adverse events were reported immediately after consultation with the Consultant directly involved in this research project.

2.1.13.1.3 REPORTING OF EVENTS

If an event occurred it was to be reported to the Irish Medicines Board and host hospital on the adverse event reporting form on the Irish Medicines Board website:

www.imb.ie/EN/SafetyQuality/OnlineForms/ClinicalTrial-SeriousAdverseEvent.aspx

2.1.13.2 WITHDRAWALS

Patients were informed at the time of consenting that they would be withdrawn from the study at any time if they fulfil any of the attrition criteria or if they chose to, for any reason, or for no reason at all. Their data were handled as described in Section 2.1.9.3

2.2 PARACETAMOL AND METABOLITE ASSAY

Paracetamol is a weak organic acid with a pKa of 9.5 (Prescott *et al.* 1971). It is well known for its poor aqueous solubility, being moderately soluble in hot water, aqueous alkaline solutions and polar organic solvents such as methanol and acetone. Because of its small distribution volume and administration in gram doses, the concentration in biological fluid of paracetamol, along with its Phase II metabolites, are high, and remain in the mg/L range for several hours following a therapeutic dose (Rawlins *et al.* 1977).

Along with its metabolites, paracetamol has been analysed by almost every analytical technique and the compounds present few detection problems individually. Common methods used include enzyme linked immunoassay, spectrophotometry and thin layer, gas and high performance liquid chromatography (TLC, GC and HPLC). Because of the complex nature of plasma and urine, some form of chromatographic separation or solvent extraction is necessary to minimise interference by other drugs and endogenous compounds.

HPLC with octadecylsilica as a means of separation was first reported in the late 1970's (Knox *et al.* 1977; Knox *et al.* 1978). Today HPLC is the most cited method in the literature for separating mixtures of analgesic drugs and related compounds (Kazakevich *et al.* 2007). HPLC also meets the rigorous requirements for precision, specificity and sensitivity required by medical regulatory authorities (Moffat *et al.* 2011). While HPLC is useful in research and industrial environments for determining paracetamol concentrations, it is less useful in clinical environments where fast results are necessary for therapeutic decision. Here spectrophotometric and immunoassays are more common. However, these techniques often require costly, highly specialised instruments and do not allow for the simultaneous analysis of compounds, making them unsuitable for the analysis in this Thesis (Bosch *et al.* 2006).

The HPLC methods for analysis of paracetamol and its metabolites have recently been extensively reviewed (Bosch *et al.* 2006; Kaushik *et al.* 2006). HPLC of paracetamol has historically always utilised a gradient method to enable simultaneous analysis of paracetamol and its metabolites in a reasonable time frame. Typically a phosphate or acetate buffer is used in the mobile phase along with a weak organic solvent such as methanol, iso-propanol or acetonitrile. Solvent extraction is rarely performed, but when done commonly utilises ethyl-acetate. In plasma samples, perchloric acid (PCA) and

acetonitrile are the most frequently used for protein removal. Urine samples are often diluted with mobile phase or water up to 50 fold. Internal standards are normally not used, but when they are, theophylline, phenacetin and 3-hydroxyacetanilide are among the most common.

2.2.1 MATERIALS

Paracetamol, potassium phosphate, perchloric acid and formic acid were purchased from Sigma (St. Louis, Missouri, United States of America (USA)). Paracetamol glucuronide, sulphate, cysteine and mercapturate and paraxanthine were purchased from Toronto Research Chemicals (Ontario, Canada). HPLC grade acetonitrile and water were purchased from Fisher Scientific (Leicestershire, UK). All other chemicals were of analytical grade or higher. The mobile phases were filtered through HA filters (0.45µm Millipore, Bedford, Massachusetts, USA) before use.

Polypropylene 1.5mL Eppendorf tubes (Sarstedt, Nümbrecht, Germany) and 100µL low volume inserts (Fisher Scientific, Dublin, Ireland) were used to prevent loss of polar compounds due to adsorption onto the un-silanised glassware available.

Drug free plasma was obtained from the Irish Blood Transfusion Service (St. Finbarr's Hospital, Cork, Ireland) and drug-free urine was obtained in-house.

2.2.2 APARATUS

The HPLC separation was performed using a Waters® 2695 Alliance HPLC system, equipped with a Waters® 2996 Photodiode Array (PDA) Detector and column oven (Waters Corporation, Milford, USA). Chromatograms were captured and processed using Empower Pro interface (Empower 2, Waters Corp., Milford, USA).

Weights of analytical compounds under 5mg were measured using a MX5 Microbalance (Mettler-Toledo Inc., Ohio, USA) while larger weights were measured using an Adventurer Pro AS-214 balance (Ohaus, Nänikon, Switzerland). Centrifugation was undertaken in a Mikro 120 centrifuge (Hettich, Tuttlingen, Germany). Vortex mixing utilised a Lab Dancer S42 at a fixed speed of 2800RPM (VWR, Pennsylvania, USA)

2.2.3 METHODS- PLASMA

2.2.3.1 CHROMATOGRAPHIC METHOD DEVELOPMENT

Initial attempts at analysing paracetamol and its metabolites were based on the method reported by Al-Obaidy *et al.* (Al-Obaidy *et al.* 1995), using 20mM orthophosphoric acid and acetonitrile (4:96, v/v adjusted to pH3.5). However, the Phenomenex Synergi 4μm Hydro-RP 250x4.6mm column with a Phenomenex SecurityGuard precolumn (Phenomenex, Cheshire, UK) available for this research was not suitable for this method as it had a much smaller internal diameter and accordingly, the run times were too long.

After a further review of the literature, the method used by Reith *et al*. (Reith *et al.* 2009) was selected as the column was similar to the one available for this research. The column was heated to 30°C to both reduce the impact of any fluctuation in temperature in the laboratory and improve chromatographic peak shape (Yan *et al.* 2000).

Chromatography was optimised with alterations to the composition of the mobile phases, their gradient and flow rate. The final conditions eluted the compounds of interest with a gradient mobile phase consisting of 0.1M KH_2PO_4 + 1%v/v formic acid (A) and acetonitrile + 1%v/v formic acid (B). The gradient had initial conditions of 95:5 (A:B) for the first 2.5 minutes, which was then reduced to 91:9 by 10 minutes, and decreased linearly to 77:33 by 20 minutes which was then maintained until 21 minutes. Re-equilibration to initial conditions followed for 4 minutes before injection of the next sample. The flow rate remained constant at 1.0mL/min.

These mobile phase ratios resulted from the adaptation of the method of Reith *et al*. (Reith *et al.* 2009), who used two mobile phases containing all three elements: KH_2PO_4, formic acid and acetonitrile in different ratios. The method used in this study split the mobile phases into the aqueous and organic components. This allowed for greater flexibility during method development, taking advantage of the Waters® 2695's quaternary pump. However, KH_2PO_4 is not generally considered compatible with acetonitrile due to salt precipitation (Kazakevich *et al.* 2007). Therefore, rapid changes of organic solvent were avoided to prevent this.

Needle wash and seal wash solution was 5%v/v methanol in water which was kept at 50°C to improve buffer removal. Wash cycles using this solution were incorporated at the end of every run to prevent buffer salt build-up. The sample injection volume was 25μL and

the autosampler compartment was kept at 4°C. All chromatograms were captured using PDA spectra from 220-300nm and the wavelengths of interest later extracted using Empower Pro.

2.2.3.2 UV ABSORBANCE

UV absorbance of a molecule arises from excitation of its electrons by the irradiating light. Excitation of the molecule is dependent on its electronic structure and only usually occurs in the presence of unsaturated compounds or loan pairs of electrons (Shriner *et al.* 1980). Only these molecules have sufficiently stable excited states to give rise to absorption in the near UV range (Silverstein *et al.* 1991).

Traditionally, single wavelength UV detectors were set at the absorbance maxima of the compound of interest, showing an absorbance peak as the compounds pass through the detector. Such a chromatogram of the paracetamol compounds at 242nm is shown in Figure 2.2-1.

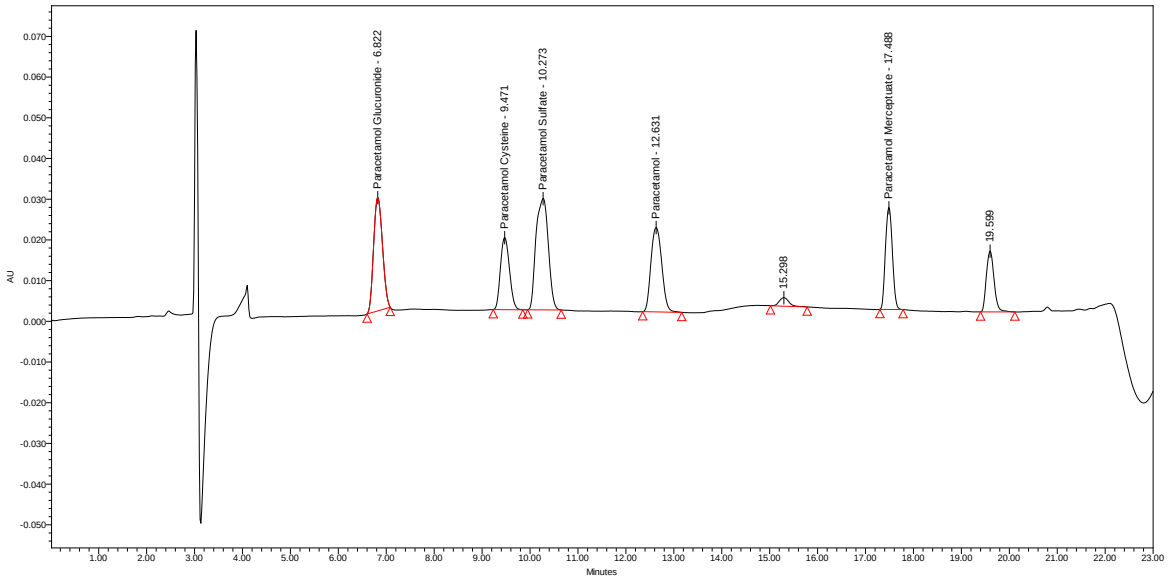


Figure 2.2-1 Sample chromatogram of paracetamol and its metabolites captured at 242nm in human plasma.
The elution order was paracetamol glucuronide, paracetamol cysteine, paracetamol sulphate, paracetamol and paracetamol mercapturate respectively.

Additionally, a PDA detector has the ability to scan across the whole near UV spectrum virtually simultaneously, providing absorbance spectra of the analyte at a range of wavelengths as it passes through the detector (Silverstein *et al.* 1991). It can be seen from Figure 2.2-1 that the only thing distinguishing one peak from another is the elution time, whereas examination of the peak spectra of paracetamol and its metabolites reveals some additional differences (Figure 2.2-2).

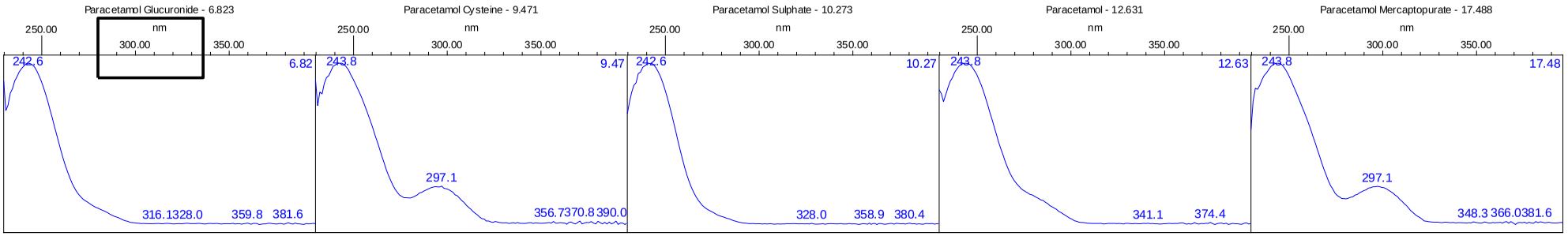


Figure 2.2-2 PDA spectra of peaks from Figure 2.2-1.

From left: Paracetamol Glucuronide; Paracetamol Cysteine; Paracetamol Sulphate; Paracetamol; Paracetamol Mercaptopurate. In addition to peaks at around 242nm, Paracetamol Cysteine and Mercapturate have absorbance peaks at 297nm.

By only absorbing in the presence of some functional groups with free electrons, the PDA spectra can provide some information about constituents of a molecule (Silverstein *et al.* 1991). All paracetamol molecules possess an *N*-acetyl aryl amide group, which produce strong absorption around 240nm. Only the thiol conjugates, paracetamol cysteine and mercapturate showed absorption at 297nm as a result of the aryl thioether and carboxylic acid groups on their side chains (Shriner *et al.* 1980). While it is possible to use molecular features to predict UV absorption, or vice versa, this is more of an art than a science because:

- Most functional groups absorb UV weakly, or not at all;
- Closely positioned functional groups affect the absorbance of one another;
- Many functional groups absorb in the same region; and
- Absorption is affected by the polarity of the solvent.

However, absorbance spectra are useful to confirm the identity of the eluting compound or the presence of co-eluting peaks and were used to determine selectivity and specificity of this assay. Ultimately the wider availability of mass spectrometry and nuclear magnetic resonance have superseded PDA spectra for compound identification (Shriner *et al.* 1980; Silverstein *et al.* 1991).

2.2.3.3 PREPARATION OF STOCK SOLUTIONS

Stock solutions were prepared in 1.5mL Eppendorf tubes by adding a known quantity of compound to water for HPLC to give a final concentration of 1mg/mL. Resultant solutions were then vortex mixed for 30 seconds and sonicated for 5 minutes. Following sonication, samples were dated and stored at -20°C in batches until required for analysis.

2.2.3.4 SAMPLE PREPARATION DEVELOPMENT

2.2.3.4.1 PROTEIN REMOVAL

Biological samples frequently contain proteins. When mixed with mobile phase, proteins still present can precipitate and collect at the head of the HPLC column, leading to its degradation and resulting in poor chromatography. Therefore, samples for HPLC analysis must be free of proteins before injection onto the HPLC column.

Three of the most common methods of separating the compounds of interest from plasma proteins are:

1. Protein precipitation, centrifugation and injection of the resulting supernatant;
2. Addition of solvents into which compounds of interest are miscible but proteins are not, followed by separation and injection of the solvent; or
3. Solid-Phase Extraction.

For paracetamol analysis, protein precipitation was chosen because the compounds of interest were in high concentrations and not bound to plasma proteins. This method was simple, quick and cheap and was adapted from Jensen *et al.* (Jensen *et al.* 2004) and Reith *et al*. (Reith *et al.* 2009).

From the beginning of the method development, efficient use of plasma was of primary importance. Methods above used up to 0.5mL per sample but given the number of planned analyses to be performed on each sample collected for this project, this volume had to be reduced. Additionally, any chemical added for precipitation of proteins diluted the concentration of paracetamol compounds in the sample, making them harder to detect, so it was necessary to keep added volumes low.

2.2.3.4.1.1 CHOICE OF AGENT

Protein precipitation was the first step in all methods reviewed. Chemicals, concentrations and volumes varied amongst papers (Blanchard 1981; Polson *et al.* 2003; Souverain *et al.* 2004; Hendriks *et al.* 2008). PCA and acetonitrile were the most common agents and these were chosen for further investigation. PCA was used at a concentration of 30%v/v to prevent chemical hydrolysis of paracetamol metabolites back to the parent drug. To determine which of the two most commonly cited agents was the most suitable for plasma protein precipitation, the following experiment was undertaken with triplicate samples (Figure 2.2-3):

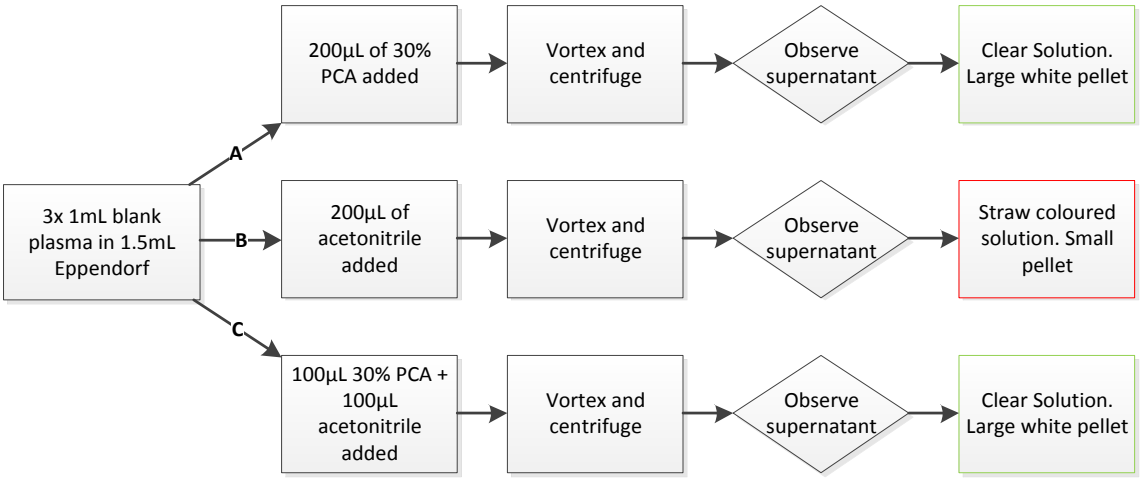


Figure 2.2-3 Experimental design to determine protein precipitant

Each Eppendorf was vortex mixed for 30 seconds and centrifuged at 14000RPM (c.17500G) for 5 minutes. The results of this were:

- Samples (A) produced a clear, colourless solution with a large white pellet of protein precipitant at the bottom of the tube;
- Samples (B) produced a straw coloured solution with a small white pellet; and
- Samples (C) produced the same result as Samples (A).

It was concluded that, volume for volume, PCA was more efficient at precipitating plasma protein. To confirm this finding, further experiments were conducted doubling and then quadrupling the volume of acetonitrile. The same results were found. Given the necessity to restrict volumes of additive, PCA was chosen to precipitate proteins in future samples.

2.2.3.4.1.2 DETERMINATION OF VOLUME

The exact volume of PCA 30%v/v also varied in the literature (Blanchard 1981; Polson *et al.* 2003; Souverain *et al.* 2004; Hendriks *et al.* 2008). An experiment was also designed to determine the optimal volume and is shown in

Figure 2.2-4.

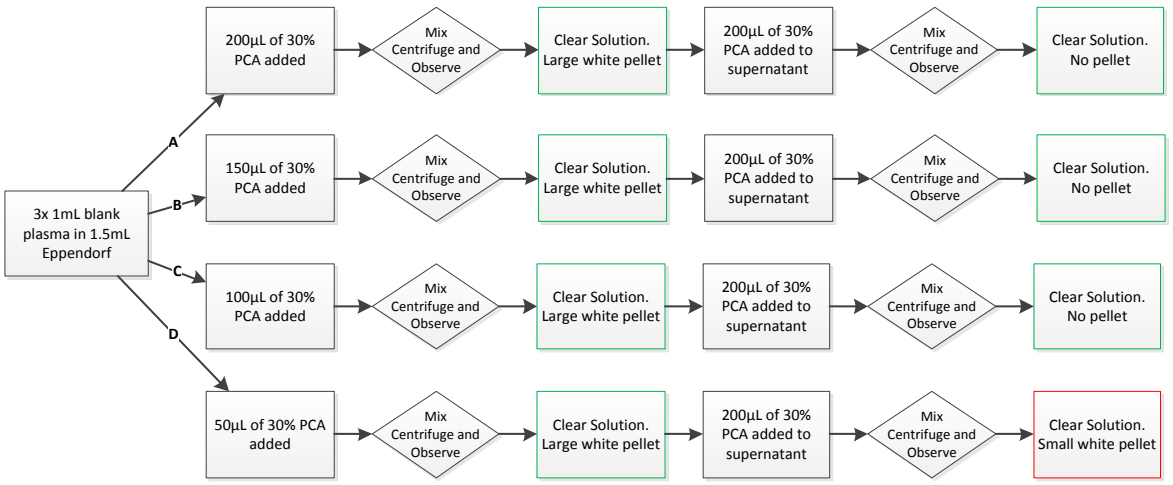


Figure 2.2-4 Experiment design to optimise volume of PCA added

Samples were again vortex mixed for 30 seconds and centrifuged at 14000RPM for 5 minutes. Following the first centrifugation, all samples became a clear colourless solution with a large white pellet of protein precipitant at the bottom of the tube. To ensure complete precipitation of proteins, the supernatant of each sample was removed into a fresh Eppendorf tube and a further 200µL of PCA 30% was added. Samples were mixed and centrifuged as before.

Samples A, B and C returned a clear solution with no visible pellet. The presence of a protein pellet in sample D indicated that protein was still present in solution before the second addition of acid and that 50uL of PCA 30%v/v was insufficient for removal of all proteins from 1mL of human plasma. It was concluded that the volume of PCA 30%v/v must be at least 10% of the volume of the plasma sample for adequate protein removal.

Blank plasma used in these analyses was donated by fit and healthy volunteers, as determined by screening undertaken by the Irish Blood Transfusion Service. This group was likely to have at least the same quantities of plasma protein as study participants who are undergoing surgery for cancer or other disease of the bowel, as the effect disease has on plasma protein concentration, if any, is to lower the total concentration. This would suggest plasma protein precipitation methods developed from blank plasma could be applied to study participants without consuming plasma samples for validation.

2.2.3.4.2 EXTRACTION EFFICIENCY

Although protein removal was optimised, it was necessary to assess if this method altered the concentration of paracetamol measured in the sample and if this effect was consistent across the concentration range. This was determined by the extraction efficiency. Extraction efficiency compares absorption peak areas of a compound in a dilution series suitable for direct injection with a plasma dilution series after the plasma samples have had their proteins removed (Figure 2.2-5). Mobile phase at initial conditions was used as diluent.

2.2.3.4.2.1 PREPARATION OF STANDARDS

Stock solution of paracetamol (1mg/mL) was diluted with blank plasma to give a concentration of 40µg/mL. A seven point concentration range from 40µg/mL to 0.625µg/mL was obtained by diluting 750µL of the sample (paracetamol 40µg/mL) with a further 750µL of blank plasma and vortexing mixing for 30 seconds, followed by five subsequent serial dilutions. The same dilution series was prepared using diluent in place of plasma. All samples were prepared in triplicate.

2.2.3.4.2.2 PREPARATION OF STANDARDS FOR INJECTION

Plasma: 200µL of sample was added to a 1.5mL Eppendorf tube followed by 20µL of 30%v/v PCA. Tubes were vortex mixed for 30 seconds followed by centrifugation at 14000 RPM for five minutes.

Diluent: Samples were prepared as for plasma except that 20µL of diluent was added in place of the perchloric acid (Figure 2.2-5).

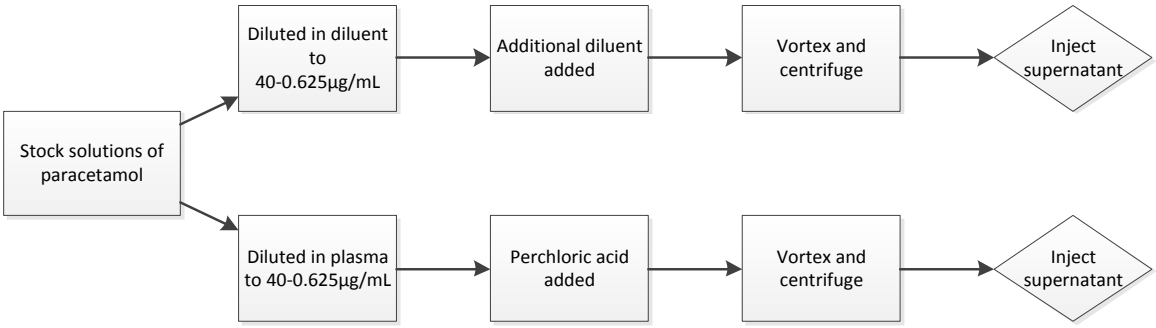


Figure 2.2-5 Extraction efficiency method

The supernatant of each sample was transferred to 100µL low volume inserts (Fisher Scientific, Dublin, Ireland) and then placed inside an autosampler vial (1.5mL screw neck vial, AGB Scientific, Dublin, Ireland) for HPLC analysis. An external standard was not added as no extraction occurred. HPLC analysis was conducted with the conditions described above (Section 2.2.3.1).

2.2.3.5 ASSAY VALIDATION

2.2.3.5.1 LIMITS OF DETECTION AND QUANTIFICATION

Limits of detection (LOD) and limits of quantification (LOQ) were determined from the chromatograms of a dilution series of each compound in blank plasma, prepared and analysed in five replicates according to Section 2.2.3.4. LOD were the lowest concentration at which the signal to background noise ratio was 3:1, based on peak height. LOQ were the lowest concentration at which the signal to background noise ratio was 10:1, tests for precision and accuracy were passed, and the peak area was at least twice that of the LOD peaks (Shah *et al.* 1992; Bressolle *et al.* 1996).

2.2.3.5.2 PRECISION AND ACCURACY

Inter and intra batch variations were determined from low, middle and high concentrations for each compound (Table 2.2-1).

Table 2.2-1 Concentrations used for the determination of precision and accuracy values for each compound

Compound	Low concentration (µg/mL)	Mid concentration (µg/mL)	High concentration (µg/mL)
Paracetamol glucuronide	0.625	10	80
Paracetamol sulphate	0.625	5	40
Paracetamol	0.625	5	40
Paracetamol cysteine	0.3125	5	20
Paracetamol mercapturate	0.625	5	20

Values were determined over five samples of each concentration in the first batch (intra-day variation) and from one sample from a further five batches (inter-day variation). Values were calculated over a batch rather than a day in accordance with accepted validation practices as batches routinely ran over more than one day (Shah *et al.* 1991; Bressolle *et al.* 1996).

2.2.3.5.3 CALIBRATION CURVES

Calibration curves were prepared by plotting the peak area versus the concentration of each compound. These were analysed within each batch during analysis from standards placed after every fifth patient sample. Stock solutions of 1mg/mL were serially diluted with blank plasma to give a calibration curve constructed over the expected range of concentrations in patient samples as shown in Table 2.2-2. Calibration samples had a minimum plasma content of 98%. These were then prepared for analysis as described in Section 2.2.3.4.

Table 2.2-2 Concentrations used to construct calibration curves in plasma (µg/mL)

Paracetamol glucuronide	Paracetamol sulphate	Paracetamol	Paracetamol cysteine	Paracetamol mercapturate
			0.3125	
0.625	0.625	0.625	0.625	0.625
1.25	1.25	1.25	1.25	1.25
2.5	2.5	2.5	2.5	2.5
5	5	5	5	5
10	10	10	10	10
20	20	20	20	20
40	40	40		
80				

The linear regression equations were calculated with:

$$y = mx + c$$

Equation 2.2-1 Linear regression equation

To prove a linear equation was the most appropriate for regression, calibration curves had to be proven to be linear. Calibration curves were accepted as linear if analysis of the validation samples showed the correlation co-efficient was not significantly different from 1, the slope was significantly different from 0 and the intercept was not significantly different from 0. Significant differences were deemed to have occurred if the 95% confidence interval of the mean excluded the value in question (*i.e.* 1 for correlation coefficient and 0 for the other parameters).

2.2.3.5.4 STABILITY

Stock solution of paracetamol was diluted to 10µg/mL with blank human plasma and stored with patient samples in the same polypropylene containers at -80°C and at -20°C, 4°C and bench-top room temperature (≈20°C). These were analysed and compared with calibration curves derived from freshly prepared samples.

2.2.3.6 ANALYSIS

Pharmacokinetic calculations were performed using Microsoft Excel® v12.0.6 (Microsoft, Redmon, Washington, USA) and statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) v15.0 (SPSS, Chicago, Illinois, USA).

2.2.4 METHODS- URINE

2.2.4.1 CHROMATOGRAPHIC METHOD DEVELOPMENT

Using the method developed for plasma samples, clear separation was obtained for paracetamol and its metabolites in spiked samples. However, this method was not optimal for urine analysis because:

1. The concentrations of the paracetamol compounds were much higher in urine so it was possible to identify and quantify paracetamol mercapturate, which was below the LOQ in most plasma samples; and
2. The plasma method used a gradient mobile phase to elute the compounds of interest within a reasonable time of 25 minutes. However, the increasing organic content of the gradient programme altered the chromatographic baseline, rendering identification and integration of absorbance peaks arising from paracetamol mercapturate at physiological concentrations problematic and unreliable (Figure 2.2-6).

Using an isocratic method with different combination of the phosphate buffer and acetonitrile of the plasma method gave a flat baseline. However, on the Phenomenex Synergi column, isocratic combinations would either compromise the separation of the first four compounds (paracetamol glucuronide, paracetamol cysteine, paracetamol sulphate, and paracetamol) or cause the elution time of paracetamol mercapturate to be very long.

Recent developments in HPLC column technology (*e.g.* pore-, particle-size and subsequent increases to the number of theoretical plates), have significantly improved chromatography (Majors 2008). A replacement for the Phenomenex Synergi column was required for this analysis and an Agilent Zorbax Rapid Resolution C_{18} 1.8µm 2.8x56mm column was found suitable (Agilent Technologies, California, USA). It was chosen because the <2.0µm particle size allowed for increased separation and peak resolution of the first four compounds but the shorter column length would reduce the retention time of the paracetamol mercapturate. In addition, the column's reduced particle size increased resolution even at low flow rates with corresponding savings of HPLC solvent.

To develop the method, the mobile phases and initial conditions as used for plasma samples (acidified 0.1M phosphate buffer and acetonitrile) were used. However, paracetamol glucuronide co-eluted with the solvent front (Figure 2.2-6).

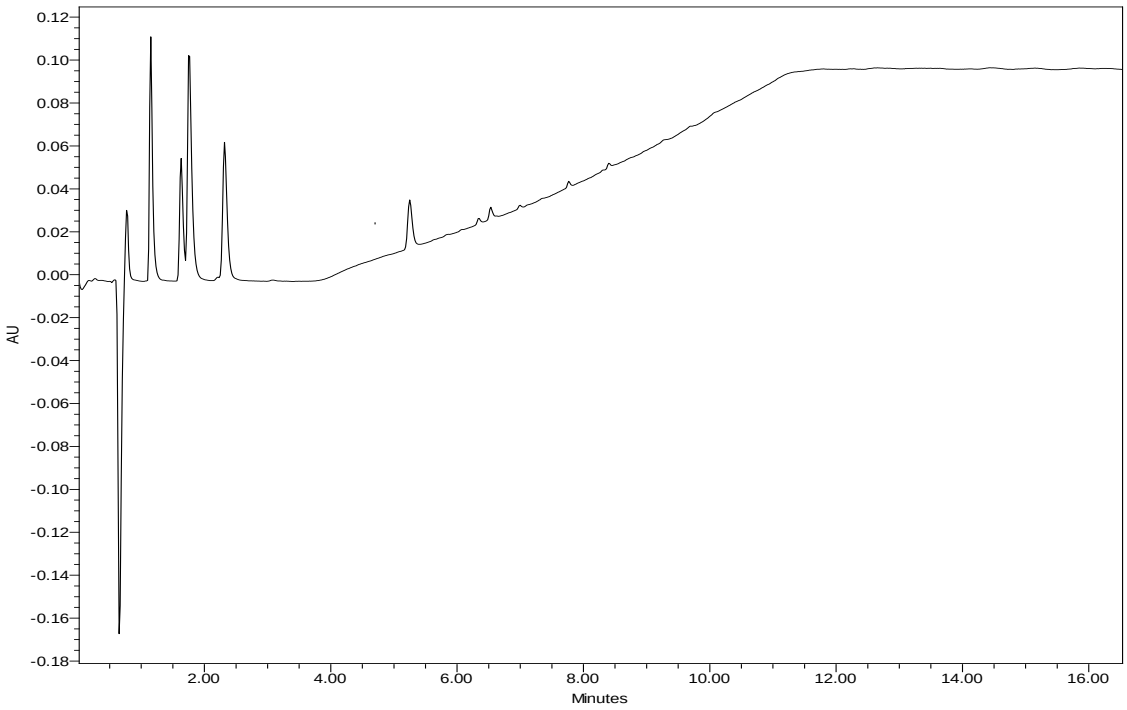


Figure 2.2-6 Chromatogram (242nm) of paracetamol and metabolite on Agilent column with plasma assay conditions
Chromatogram shows effect of gradient mobile phase in changes to baseline. Separation of peaks is poor and elution of paracetamol mercapturate on sloping baseline makes integration difficult.

The proportion of acetonitrile was reduced to 3%, the column manufacturer's stated minimum organic component to prevent collapse of the stationary phase's C_{18} moiety, but this still did not allow sufficient retention of paracetamol glucuronide from the solvent front. The mobile phases were changed to water and methanol, both acidified with formic acid to 1%v/v. Methanol has about 1/3 the elutropic strength of acetonitrile on C_{18} solid phases, and is more polar (Kazakevich *et al.* 2007). These properties increase the retention times of polar analytes relative to mobile phases containing acetonitrile.

The effect of this change is shown in Fig 2.2-7 where the assay using methanol elutes paracetamol glucuronide after the solvent front

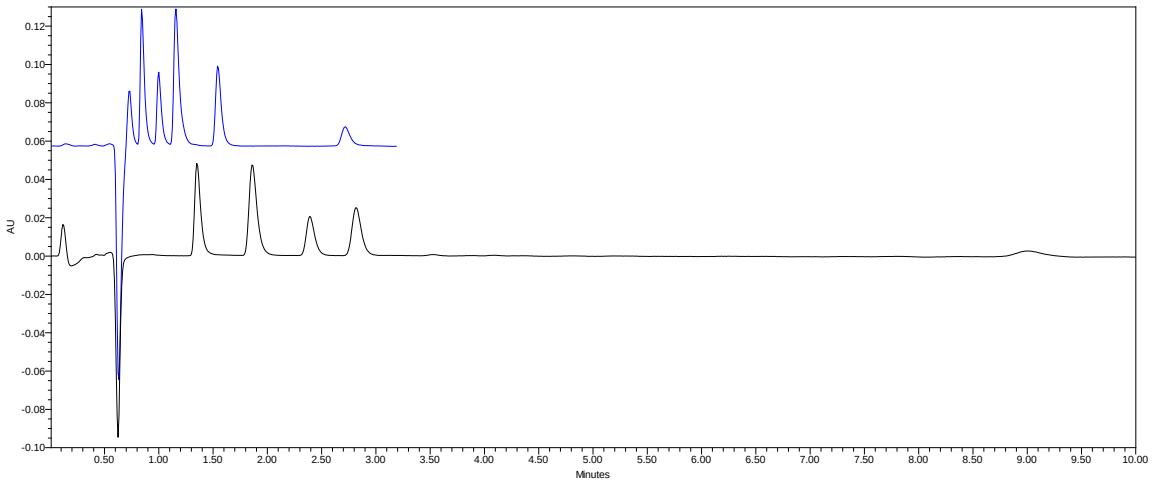


Figure 2.2-7 Chromatogram (242nm) of paracetamol and metabolites using 97% aqueous and 3% methanol (black trace) or 3% acetonitrile (blue trace).
Acetonitrile trace offset on y-axis.

Buffer was not added to the water component as salt precipitation was an issue in the plasma assay, with frequent system washes required to prevent this occurring. Initial method development focussed on optimising paracetamol mercapturate and paracetamol glucuronide retention. Paracetamol mercapturate was retained too long, necessitating a long run-time and paracetamol glucuronide co-eluted with the solvent front. Elution of analytes with the solvent front compromises identification and quantification of compounds. Initial combinations did reduce the retention times of paracetamol mercapturate and improved the peak shape but during the development, the analyte peaks were observed to be fronting. This can signify variable ionisation states of the analyte existing in the mobile phase, causing inconsistent partitioning with the stationary phase. While it was anticipated acidification of the mobile phases was sufficient to prevent this, the re-introduction of the phosphate buffer was necessary to eliminate any variation in ionisation, and this improved peak shape markedly.

Potassium phosphate is soluble in methanol and accordingly, precipitation of the buffer salts was less of an issue than when acetonitrile was used. As a precaution though, to protect the instrument from the corrosive effects of the buffer salts, an end-of-run wash cycle was again incorporated.

While the elution of paracetamol mercapturate had been resolved the separation of the first four compounds now proved troublesome. After several runs it was found a combination of 97%v/v 0.1M phosphate buffer and 3%v/v methanol gave sufficient

separation. This was the minimum organic component of the mobile phase recommended by the manufacturer. Validation was completed as for plasma analysis.

2.2.4.2 SAMPLE PREPARATION

In healthy individuals urine does not contain any proteins. However, neat urine contains high quantities of salts and their concentration can saturate the PDA. Accordingly, 50μL of urine samples was diluted with 950μL of HPLC grade water before injection, directly into the HPLC vial (Goicoechea *et al.* 1995; Di Girolamo *et al.* 1998; Jensen *et al.* 2004; Reith *et al.* 2009). The vial was capped and vortexed for 30 seconds. As no extraction was performed and the quantity of urine in the diluted sample so low, paracetamol standards were diluted with water, rather than urine, to provide calibration curve data.

2.3 CYTOKINE ASSAY

The activation of the stress response in the study patients was determined by the concentration of inflammatory cytokines in their plasma samples. Given their low concentrations and protein nature, a multiplex electrochemiluminescense assay (MSD Gaithersburg; Maryland; USA) was chosen as it is highly sensitive, specific, requires no sample pre-treatment and can process several samples concurrently.

2.3.1 ASSAY PRINCIPLE

The assay used for this analysis uses the same principle as other "sandwich" immunoassays:

1. An antibody for a target protein coats the surface of a well;
2. The target protein is added and binds to the antibody on the well surface;
3. A detection antibody is added which also binds to the target protein forming an antibody sandwich around the target protein; and
4. The detection antibody contains a label (Ruthenium Ru) that emits light when electrochemically stimulated. The amount of light produced is proportional to the amount of secondary antibody captured within the antibody sandwich, and therefore provides a quantitative measure of the amount of the target protein present.

A Meso-Scale-Discovery (MSD) assay was chosen as it provided the most amount of information from the smallest sample volume. There are a few idiosyncrasies of MSD assays that allow this. The antibody for the specific protein target is coated on an electrode (or "spot") in the plate well, rather than the whole well. In the case of the assay used here a multiplex assay was used, which contained four spots in each well. This allowed four cytokines, IFN-γ, IL-1β, IL-6, and TNF-α to be assayed in the same well at the same time from a single small-volume sample without requiring any additional steps over a conventional ELISA (Figure 2.3-1).

The 96-well plates were supplied with the spots pre-coated, each with their specific cytokine capture antibody. The wells were blocked with a supplied diluent to prevent non-specific interactions and then plasma sample was added. The cytokines present in the sample bound to the capture antibodies immobilised on their respective spots. A detection antibody solution was then added that contained a blend of cytokine

antibodies, each labelled with the electrochemiluminescent MSD SULFO-TAG®. Unlike conventional ELISAs that use detection labels that fluoresce when exposed to light, electrochemiluminescence detection uses labels that emit light when electrochemically stimulated. This reduces background signals because the stimulation mechanism (electricity) is decoupled from the signal (light). Multiple excitation cycles of each label amplify the signal to enhance light levels and improve sensitivity.

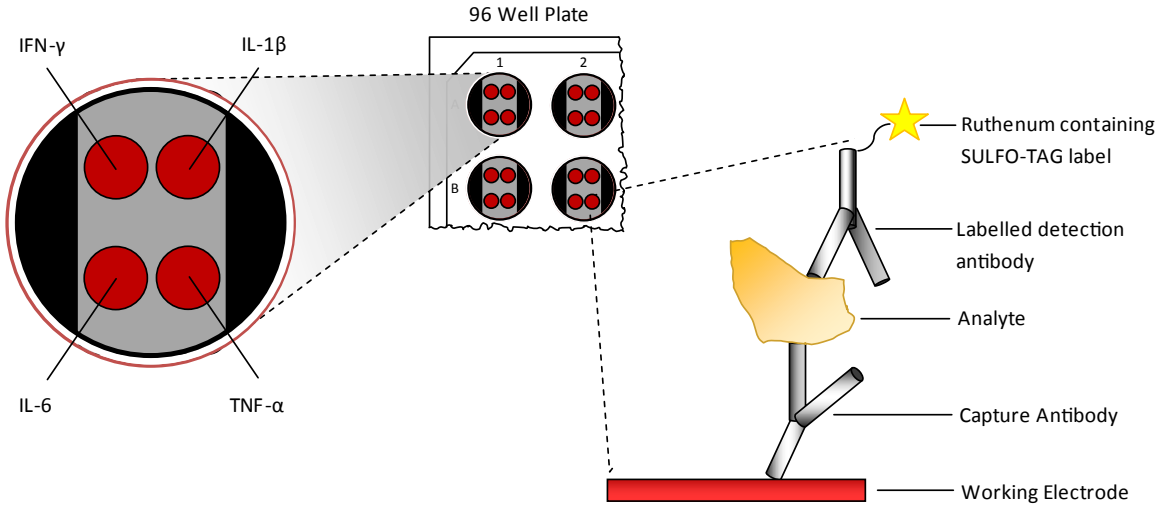


Figure 2.3-1 Spot diagram showing placement of analyte capture antibody and schematic of assay principle.

The labelled detection antibodies then bind to the cytokines, which were already bound to the capture antibody, thus completing the sandwich. MSD read buffer was then added to provide the appropriate chemical environment for electrochemiluminescence. The plate was then loaded into an MSD sector instrument for analysis. Inside the sector instrument, a voltage was applied to the electrodes that caused the labels bound to the electrode surface to emit light. The intensity of emitted light from each spot provided a quantitative measure of IFN-γ, IL-1β, IL-6, and TNF-α present in the sample.

2.3.2 MATERIALS

ELISA kits (Pro-inflammatory Panel 1 (4-plex) catalogue number K15009C-2) were purchased from Meso Scale Delivery Gaithersburg, Maryland, USA. The contents of the kits is detailed in Table 2.3-1. Water for HPLC (Fisher Scientific, Dublin, Ireland) was used as deionised water. Phosphate buffered saline plus 0.05% Tween-20 (PBS-Tween) was purchased from Sigma-Aldridge (St Louis, Missouri, USA) and made according to manufacture's instructions. Tubes for reagent preparation (50mL and 15mL) and 1.5mL micro-centrifuge tubes for preparing serial dilutions were obtained from Sarstedt (Sarstedt, Nümbrecht, Germany).

Table 2.3-1 Contents of Pro-inflammatory panel kit

Item Description	Storage	Number in kit
Multi-Spot 96 well 4 Spot Human Pro-Inflammatory I Plate	2–8°C	4
SULFO-TAG Detection Antibody Blend (50X)	2–8°C	1 vial (375µL)
Human Pro-Inflammatory Calibrator Blend (1µg/mL each)	<-70°C	5 vials (15µL ea)
Diluent 2	<-10°C	1 bottle (40 mL)
Diluent 3	<-10°C	1 bottle (25 mL)
Read Buffer T (4X)	RT	1 bottle (50 mL)

2.3.3 APARATUS

Meso Scale Delivery Sector Imager 2400 was used as the detection device. The imager was attached to computer running Meso Scale Delivery's Discovery Workbench® v3.0.17 software.

2.3.4 ASSAY PROTOCOL

The assay was run as per kit recommendations with one modification on the advice of the manufacturer:

- The assays were incubated with sample overnight to allow the reactions to reach equilibration and achieve better sensitivity.

Otherwise the standard serum assay protocol provided in by the manufacturer was followed. The protocol used is summarised here:

1. **Preparation of calibrator samples:** A serial dilution of Calibrator Blend was performed by diluting calibrators in Diluent 2;
2. **Addition of Diluent 2:** 25µL of Diluent 2 was pipetted to cover the entire bottom of each well. The plate was sealed and incubated for 30 minutes with vigorous shaking (800RPM) at room temperature;
3. **Addition of Sample or Calibrator:** 25µL of each Calibrator or Sample Solution was placed into the wells. The plate was sealed and incubated for 2 hours with vigorous shaking (800RPM) at room temperature;
4. **Washing and Addition of Detection Antibody Solution:** The plate was washed three times with 300µL PBS + 0.05% Tween-20 into each well, tapping out the liquid onto paper towels after each wash. A 25µL aliquot of the 1X Detection Antibody Solution was placed into each well of the MSD plate. The plate was sealed and incubated for 2 hours with vigorous shaking (800RPM) at room temperature; and

5. **Washing and Reading:** The plate was again washed three times with 300µL PBS + 0.05% Tween-20. Then to each well, 150µL of Read Buffer T was added. The plate was analyzed on the sector imager immediately after addition of Read Buffer.

Care was taken to ensure that bubbles were avoided at all stages, including the use of positive pipetting, as bubbles interfere with the reaction and reading.

2.3.5 DATA ANALYSIS

Data were analysed using Meso Scale Delivery's Workbench software and curves fitted using the built-in 4PL fit with $1/y^2$ weighting y = b2 + ((b1 - b2) / (1 + (x / b3)^b4)).

2.3.6 PLATE CONFIGURATION

Each spot was in the same position in every well as shown in Figure 2.3-2. A sample plate layout is shown in Figure 2.3-3

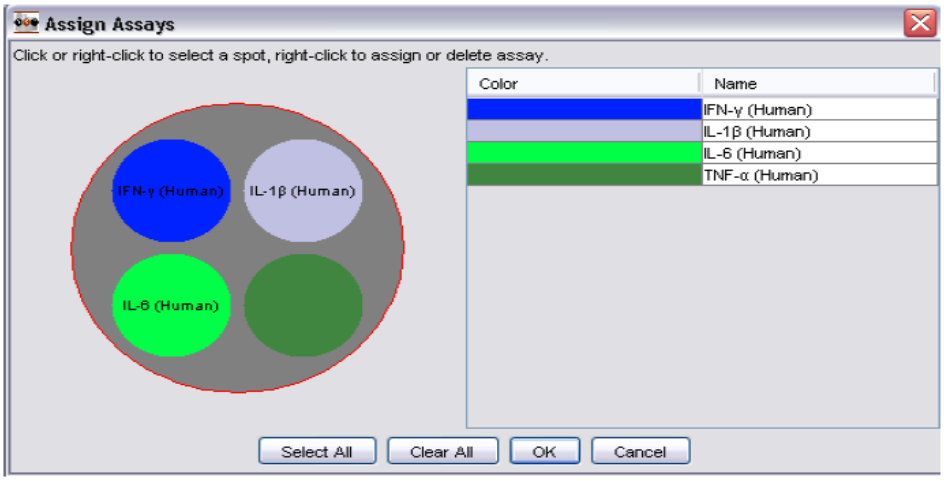


Figure 2.3-2 Layout of assay spots in plate wells

	1	2	3	4	5	6	7	8	9	10	11	12
A	stdA	stdA	1A0	1A0	1A1	1A1	2A0	2A0	2A1	2A1	2A2	2A2
B	stdB	stdB	2A3	2A3	2A4	2A4	3A0	3A0	3A1	3A1	3A2	3A2
C	stdC	stdC	3A3	3A3	3A4	3A4	4A0	4A0	4A1	4A1	4A2	4A2
D	stdD	stdD	4A3	4A3	4A4	4A4	5A0	5A0	5A1	5A1	5A2	5A2
E	stdE	stdE	5A3	5A3	5A4	5A4	6A0	6A0	6A1	6A1	6A2	6A2
F	stdF	stdF	6A3	6A3	6A4	6A4	7A0	7A0	7A1	7A1	7A2	7A2
G	stdG	stdG	7A3	7A3	7A4	7A4	8A0	8A0	8A1	8A1	8A2	8A2
H	stdH	stdH	8A3	8A3	9A0	9A0	9A1	9A1	9A2	9A2	9A3	9A3

Figure 2.3-3 Layout of plate 1 showing standards (red) and patient samples (yellow)
Abbreviations: std= standard. Patient samples shown as patient number, group letter and sample day.

2.3.7 RESULTS

The image of plate 1 captured by the sector imager is shown in Figure 2.3-4 showing the various intensities of light emitted by the calibration standards and samples. As shown in the schematic Figure 2.3-3, the calibration standards are in the wells to the left.

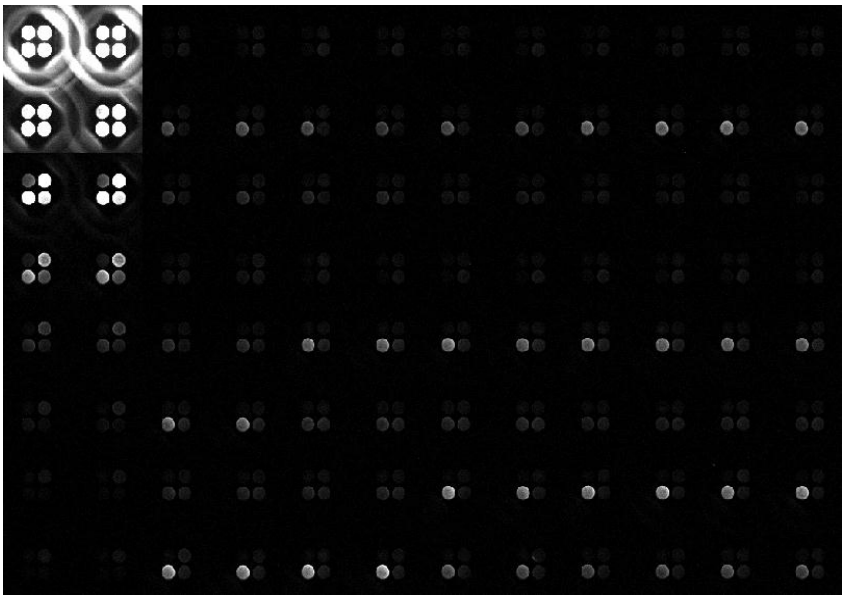


Figure 2.3-4 Image of plate 1 from sector reader from showing light intensities of various assayed samples

2.3.7.1 VALIDATION

Alongside patient samples, standard curves were prepared in duplicate in each plate for all cytokines assayed. Standard curves were constructed based on omitted light intensity (Figure 2.3-5). The concentration of the cytokines in each sample was calculated by plotting its intensity on the standard curve from their respective plates (Figure 2.3-6). Standard curves also provided limit of detections which also provided validation data

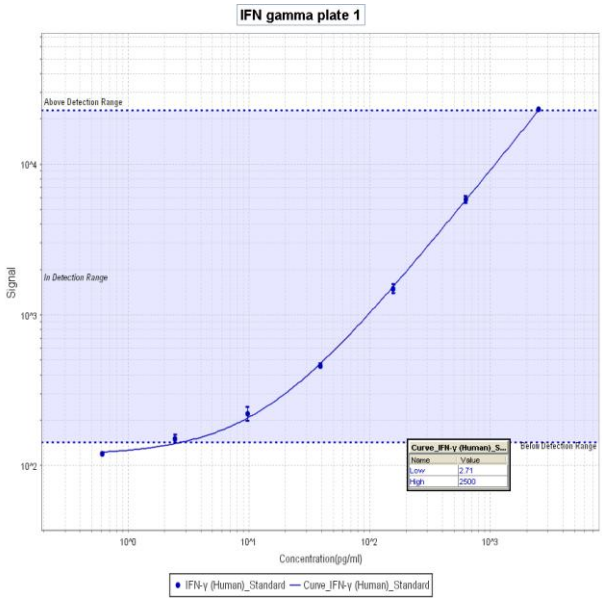


Figure 2.3-5 Standard curve for IFN-γ from plate 1

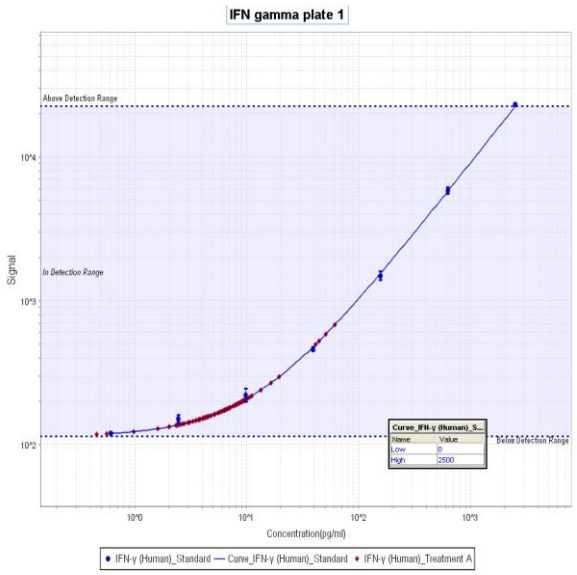


Figure 2.3-6 Standard curve of IFN-γ showing location patient samples from plate 1

Limits of detection (LOD) were calculated and defined as 2.5 standard deviations above the background. They are given in Table 2.3-2 and compared with values published by the manufacturer.

Table 2.3-2 Limits of detection in pg/mL

Cytokine	Typical LOD	Plate 1	Plate 2	Plate 3	Plate 4
IFNγ	0.4	0.91	2.85	2.83	2.41
IL-1β	0.2	0.38	0.89	1.0	0.49
IL-6	0.7	0.42	0.34	0.4	0.36
TNFα	0.5	0.76	0.65	0.64	0.64

All of the LOD's are similar to quoted limits and are reproducible, indicating intra-plate reproducibility as shown in Figure 2.3-7. No sample matrix issues were observed.

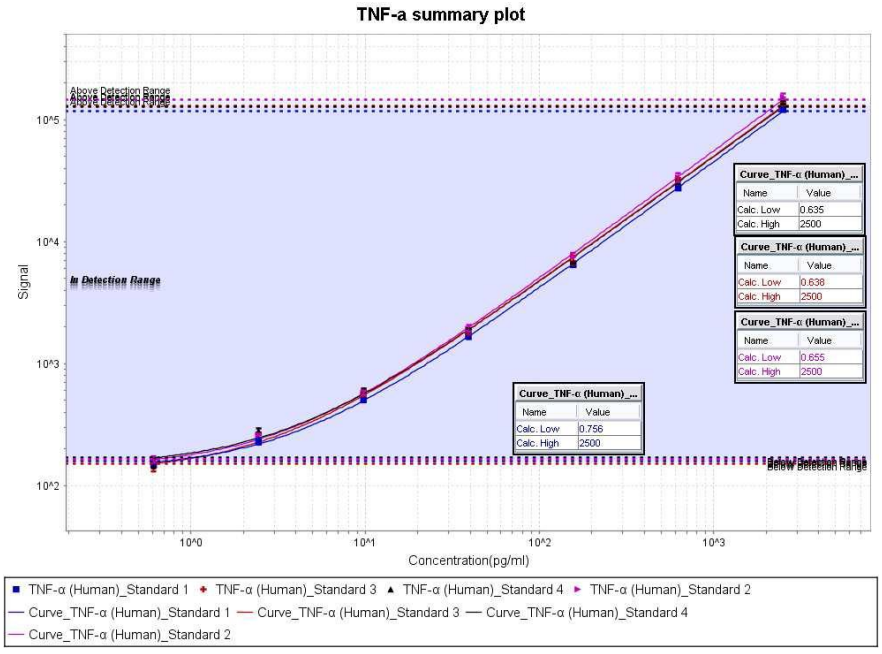


Figure 2.3-7 Plot of standard curve for TNF-α from all plate

2.4 α GLUTATHIONE S-TRANSFERASE ASSAY

Alpha glutathione S-transferase (αGST) is a Phase II detoxification enzyme protein found in high concentration in hepatocytes, where it constitutes 2-5% of all soluble protein (Trull *et al.* 1994; Clarke *et al.* 1997; Nagral *et al.* 1997). Its function in paracetamol metabolism was discussed in Section 1.2. Because it is in such high concentrations in these cells it appears rapidly in the plasma when hepatocytes are injured and become leaky. Measuring the appearance of αGST in plasma is used in drug development and clinical disease management for early detection of liver damage and it is considered the most sensitive parameter of liver tissue damage following paracetamol administration (Beckett *et al.* 1985; Trull *et al.* 1994; Redl *et al.* 1995; Chouker *et al.* 2005)

As a measure of paracetamol induced hepatotoxicity, αGST has several advantages over transaminase enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), conventionally used for this assessment:

- αGST has a half-life in circulation of 90 minutes, significantly shorter than transaminases AST (17±5 hours) and ALT (47±10 hours) (Kasper *et al.* 2008). Consequently, αGST levels return to baseline values quickly when damage to hepatocytes ceases, enabling the detection of the onset and cessation of hepatocyte injury (Beckett *et al.* 1993; Trull *et al.* 1994);
- αGST is more sensitive than transaminases being measurable at low levels of hepatocyte injury and lower concentrations of toxin (Clarke *et al.* 1997);
- Because αGST is found in high concentrations in hepatocytes, statistically significant elevation occurs sooner than other markers of hepatotoxicity(Beckett *et al.* 1993). Accordingly, it may identify preclinical toxicity that is not detected using transaminases (Murray *et al.* 1992a; Sivilotti *et al.* 2002);
- αGST is only released from hepatocytes and is therefore more discriminating than transaminases, which can be released from a variety of tissues (Murray *et al.* 1992a; Kumle *et al.* 2003);
- αGST is equally distributed in both the centrilobular and periportal regions of the liver (Sundberg *et al.* 1993). In contrast, transaminases are not distributed uniformly, with concentrations higher in the periportal region than the centrilobular (Beckett *et al.* 1993). Since the centrilobular hepatocytes are the

most susceptible to damage, αGST is a more sensitive indicator of hepatic damage.

An ELISA αGST assay was chosen as the method of choice for the detection of hepatocyte injury over transaminase enzyme assays due to its advantages outlined above. The upper limit of the reference range for those without hepatic damage is 11.4µg/L (Rees *et al.* 1995).

2.4.1 ASSAY PRINCIPLE

This ELISA used a sandwich assay principle similar to that used for the measurement of cytokines. This ELISA was more conventional than the ELISA used for the cytokine assay as the entire lower surface of each well was pre-coated with anti-αGST IgG antibodies by the manufacturer. A schematic of the assay principle is shown in Figure 2.4-1.

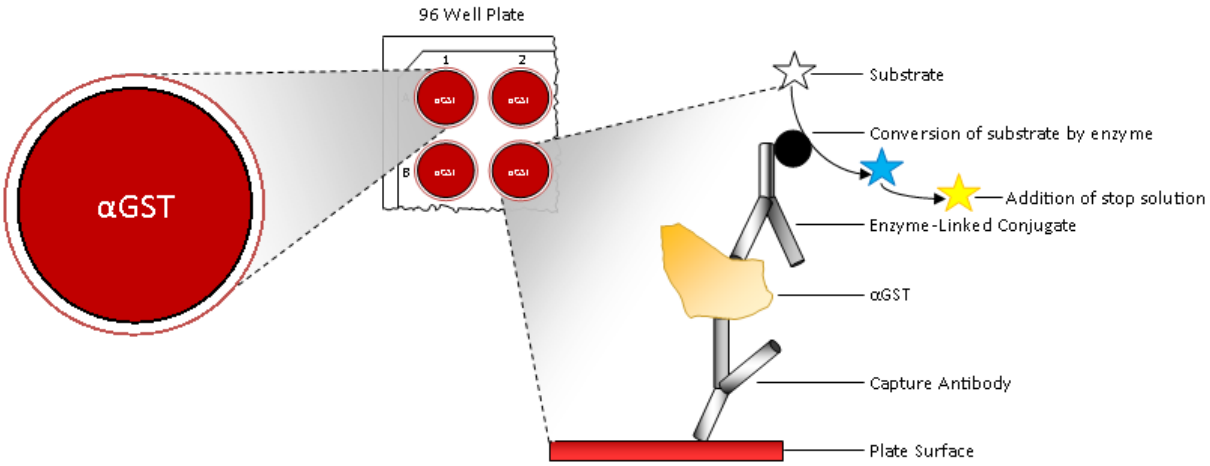


Figure 2.4-1 Spot diagram displaying αGST enzyme-linked immunoassay and schematic of assay principle

Diluted plasma samples were added to each well and any αGST present in the sample bound to the αGST-antibodies coating the well surface. Enzyme-conjugate was added, followed by substrate. The enzyme used was horse-radish peroxidase (HRP), which oxidises the substrate, the chromogen 3,3',5,5'-tetramethylbenzidine (TMB), changing it from colourless to blue (Josephy *et al.* 1982; Josephy *et al.* 1983). TMB is the most popular substrate for HRP detection in ELISA as it is very sensitive and is more quickly oxidised than other HRP substrates, resulting in faster colour development (Liem *et al.* 1979). Upon addition of the stop solution, sulphuric acid, TMB becomes yellow with a maximum absorbance at 450nm (Josephy *et al.* 1982; Josephy *et al.* 1983). Absorbance of the wells was read at 450nm with a reference 655nm. The absorbance intensity was proportional to the amount of αGST present in the sample.

2.4.2 MATERIALS

Four ELISA kits (HEPKIT-Alpha) were purchased with the same lot number (Argutus Medical, Dublin, Ireland). The contents of the kits is detailed in Table 2.3-1. Water for HPLC (Fisher Scientific, Dublin, Ireland) was used as deionised water. Tubes for reagent preparation (50mL and 15mL) and 1.5mL micro-centrifuge tubes for preparing serial dilutions were obtained from Sarstedt (Sarstedt, Nümbrecht, Germany).

Table 2.4-1 Contents of HEPKIT-Alpha

Item Description	Storage	Number in kit
Anti αGST IgG coated 96 well microassay plate	2–8°C	1
GST Calibrator stock solution	2–8°C	1 vial (200µL)
Positive Control	2–8°C	1 bottle (4.5mL)
Conjugate concentrate (anti αGST IgG conjugated to HRP)	2–8°C	1 vial (300µL)
Wash Concentrate (20x PBS Tween-20)	2–8°C	1 bottle (55 mL)
TMB substrate	2–8°C	1 bottle (11 mL)
Stop solution 0.5 mol/L	2–8°C	1 bottle (11 mL)

2.4.3 APARATUS

Plates were read using a Biorad 680 Microplate Reader(Biorad, California, USA) measuring absorbance at 450nm with a reference filter at 655nm. Data were captured using Biorad Microplate Manager Software V5.2.1(Biorad, California, USA).

2.4.4 ASSAY PROTOCOL

The assay was run according to the assay protocol published by the manufacturer with one modification on the advice of the manufacturer's representative:

- The recommended reference filter 630nm was not available. A 655nm filter was used in its place.

2.4.4.1 PREPARATION OF REAGENTS

All reagents were bought to room temperature (20.2°C), mixed well and checked to be free of crystals before use.

2.4.4.1.1 WASH SOLUTION (PBST)

The wash solution was prepared by diluting 50mL of the 20x concentrated solution with 950mL of deionised water. The salt crystals in the concentrated solution were dissolved prior to dilution by gentle warming and agitation of concentrate at 37°C for 30 minutes.

2.4.4.1.2 CALIBRATORS

Calibrators were prepared from the αGST stock solution as follows:

Calibrator A: 25µL Calibrator Stock+ 2500µL Wash Solution. Using labelled micro-centrifuge tubes, Calibrator A was further diluted to prepare the remaining calibrators as shown in Table 2.4-2. Calibrators were used within 30 minutes of preparation.

Table 2.4-2 Concentration and preparation of calibrators

Equivalent Calibrator Concentration	Calibrator Volume (µL)	Wash Solution Volume (µL)
40µg/L (A)	500 (A)	-
20µg/L (B)	500 (A)	500
10µg/L (C)	500 (B)	500
5µg/L (D)	500 (C)	500
2.5µg/L (E)	500 (D)	500
1.25µg/L (F)	500 (E)	500
0µg/L (G)	-	500

2.4.4.1.3 SAMPLE PREPARATION

Samples were diluted 1:5 by adding 50µL of plasma to 200µL of wash solution in a blank microassay plate.

2.4.4.1.4 CONJUGATE

Immediately prior to use the conjugate concentrate was diluted 1:51 by adding 980µL conjugate to 49mL of wash solution.

2.4.4.2 ASSAY PROCEEDURE

All reagents were delivered at the midpoint of the side of the wells with care not to scratch the side with the pipette tip. Positive pipetting was used to prevent introduction of bubbles.

1. **Addition of Sample or Calibrator:** 100µL of each calibrator (A-G) and the control were added in duplicate to each plate in rows 1 and 2 as shown in Figure 2.4-2. Samples were then added, also in duplicate, in the remaining wells. The plates were sealed and incubated at room temperature for 60 minutes with vigorous shaking (800RPM VWR Plate Shaker, VWR Scientific, Cambridge, UK). After 55 minutes of incubation the conjugate was prepared;

2. **Washing and Addition of Enzyme Conjugate Solution:** At the end of the incubation the wells were washed four times with 250µL of the wash solution, tapping out the liquid onto paper towels after each wash. 100µL of the conjugate solution was then added to each well. The plates were incubated again at room temperature for 30 minutes with vigorous shaking (800RPM); and
3. **Washing, Addition of Substrate, Stop Solution and Plate Reading:** The plates were washed again as in step 2. To each well 100µL of substrate was added and the plates were incubated in the dark (inside a drawer) for 15 minutes exactly. Immediately afterwards, 100µL of the stop solution was added to each well. Plates were read immediately with the microplate reader at 450nm with 655nm as reference.

2.4.5 DATA ANALYSIS

Data were captured using Microplate manager software. Mean absorbance was calculated for each calibrator, control and sample. Calibration curves were constructed by plotting $A_{450/655nm}$ on the x axis and [αGST] (µg/L) on the y axis. A polynomial line of best fit was determined using the equation Equation 2.4-1:

$$y = ax^2 + bx - c$$

Equation 2.4-1 Equation for line of best fit for αGST standards

The equations of these lines were then used for determining sample αGST concentration. The actual concentration was then determined by multiplying the calculated concentration by the dilution factor of 5. The concentration of the positive control was read directly from the curve as it was not diluted. The positive control was checked against the reference value specific for the kit.

2.4.6 PLATE CONFIGURATION

A sample plate layout is shown in Figure 2.4-2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	stdA	stdA	5C1	5C1	6C0	6C0	6C1	6C1	6C2	6C2	6C4	6C4
B	stdB	stdB	7C0	7C0	1D0	1D0	1D1	1D1	2D0	2D0	2D1	2D1
C	stdC	stdC	2D2	2D2	2D3	2D3	2D4	2D4	3D0	3D0	3D1	3D1
D	stdD	stdD	3D2	3D2	3D3	3D3	3D4	3D4	4D0	4D0	4D1	4D1
E	stdE	stdE	4D2	4D2	4D3	4D3	4D4	4D4	5D0	5D0	6D0	6D0
F	stdF	stdF	7D0	7D0	8D0	8D0	8D1	8D1	8D2	8D2	8D3	8D3
G	stdG	stdG	8D4	8D4	9D0	9D0	10D0	10D0	10D1	10D1	10D2	10D2
H	CTRL	CTRL	10D3	10D3	10D4	10D4	11D0	11D0	11D3	11D3	11D4	11D4

Figure 2.4-2 Layout of plate 3 showing standard samples (red) and patient samples (yellow)
Abbreviations: std= standard. Ctrl= positive control. Patient samples shown as patient number, group letter and sample day.

2.4.7 RESULTS

A photo of plate 3 was taken immediately after reading and is shown in Figure 2.4-3. Plates developed colour as shown in response to the presence of αGST.

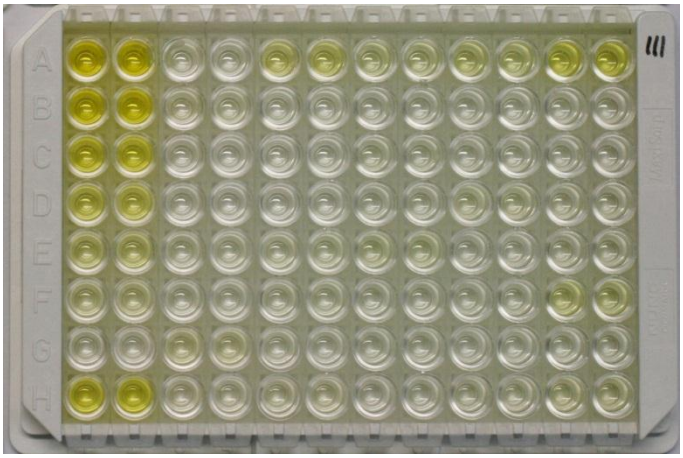


Figure 2.4-3 Photo of αGST ELISA plate 3 immediately after reading.
Development of yellow colour indicates presence of αGST in sample

2.4.7.1 VALIDATION

Alongside patient samples, calibrator samples A-G were assayed in duplicate on each plate to produce a standard curves. Aside from determining αGST in patient samples, standard curves were used to provide the limit of detection and compared across plates to provide validation data. A sample curve is shown in Fig 2.2-4.

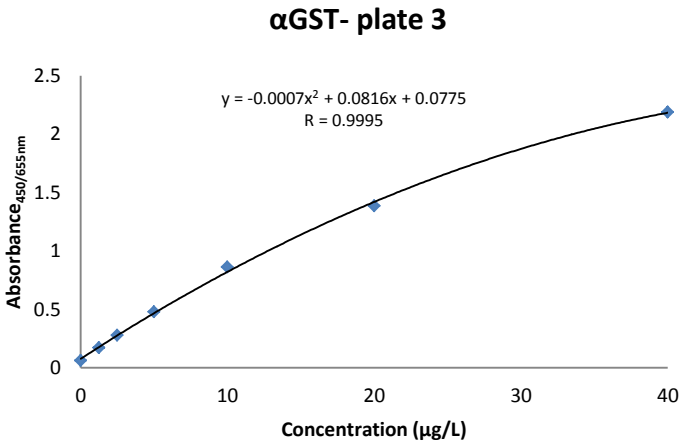


Figure 2.4-4 Standard curve for αGST from plate 3

Limits of detection (LOD) were determined as 2.5 standard deviations above the background. They are given in Table 2.4-3 and compared with values published by the manufacturer.

Table 2.4-3 Limit of detection of αGST (µg/L) on each plate compared to provided reference value

	Typical LOD	Plate 1	Plate 2	Plate 3	Plate 4
αGST (µg/L)	0.25	0.247248	0.261624	0.097255	0.308198

All LOD are similar or better than quoted limits. No sample matrix issues were visually observed. To analyse for inter-plate reproducibility means and standard errors were calculated and are detailed in Table 2.4-4.

Table 2.4-4 Mean and standard error values of standard curve samples

Concentration (µg/L)	Mean absorbance (450/655nm)	Standard deviation	Standard error (%)
1.25	.1775	.01500	0.084507
2.50	.2800	.01414	0.050508
5.00	.4750	.01915	0.040313
10.00	.8450	.03000	0.035503
20.00	1.3775	.03403	0.024707
40.00	2.1800	.02708	0.012422

Graphs of standard curves from all plates were also prepared (Figure 2.4-5).

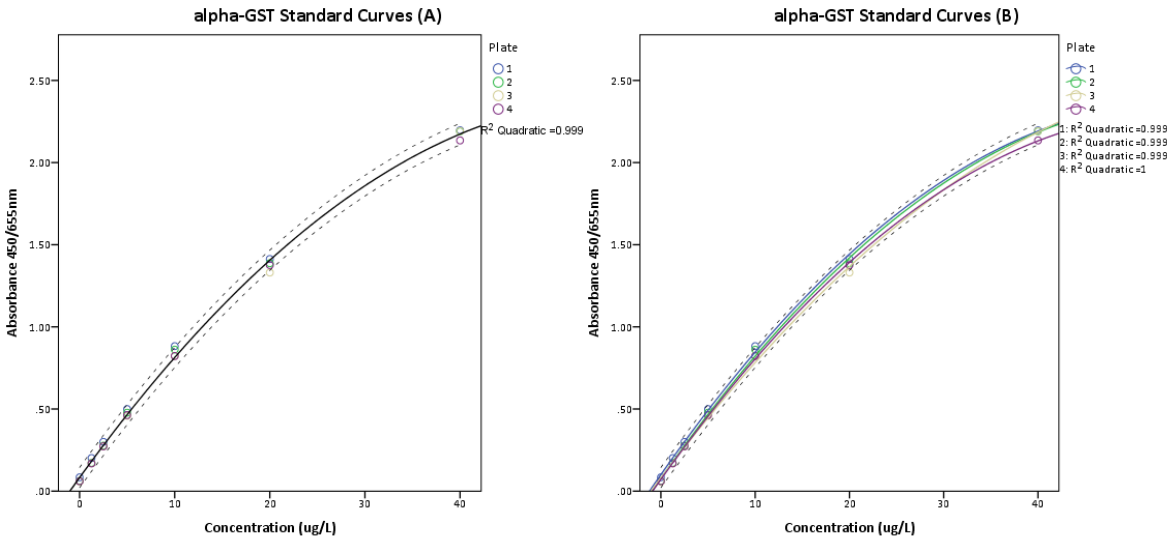


Figure 2.4-5 Standard curve for αGST from all plates.
Graph (A) shows regression line based on mean values from all plates with dotted line representing 95% confidence interval of mean values. No value lies outside of the confidence interval. Graph (B) shows the standard curves for each plate, virtually superimposed, with all curves lying within the confidence interval.

The equation of the lines of best fit were:

Table 2.4-5 Equations of lines of best fit for αGST standard curves

Plate	Equation	Correlation (*r*)
1	$y = -0.0007x^2 + 0.0819x + 0.1009$	0.999649939
2	$y = -0.0007x^2 + 0.0816x + 0.0775$	0.999549899
3	$y = -0.0006x^2 + 0.076x + 0.0813$	0.999449849
4	$y = -0.0007x^2 + 0.0806x + 0.072$	0.999899995

Assessing the mean and standard error values, all are below 10%, indicating there is very little variability between plates. Visually, the lines of best fit for each plate are virtually superimposed. From the equations, the intercepts are also virtually identical. To confirm this, an analysis of covariance was conducted on the absorbance values to test for homogeneity of the regression lines and to test the equality of the error variance. The

results of the Levene's test confirm equal variance between samples F(3, 24)=0.025, p=0.995. Testing concentrations across the plates also gave non-significant results with F(6, 21)=1.647 p=0.184. These results confirm that there was no significant differences between plates. Each observation was independent, variance homogenous (as shown by Levene's tests) and distribution was normal.

Positive control samples were also included with each kit and assayed in duplicate on each plate. Each kit gave the concentration of the quality control sample as a range from 7.6-12.6ug/L. Results of the plate are considered valid if the calculated value of the positive control is within the provided range. The calculated concentrations are provided in Table 2.4-6.

Table 2.4-6 Calculated concentrations of positive control samples determined in each plate (±standard deviation of duplicate samples) compared with provided reference range

Positive Control	Reference range	Plate 1	Plate 2	Plate 3	Plate 4
αGST (µg/L)	7.6-12.6	9.48±1.06	10.19±0.94	10.32±0.54	9.86±0.54

The positive control values were within the reference range on all plates, confirming the validity of the results.

2.5 LCMS ASSAY

Four medicines: caffeine, dextromethorphan, chlorzoxazone and midazolam were used to probe the activity of four CYP450 enzymes, 1A2, 2D6, 2E1 and 3A4, as discussed in Sections 1.5. Separate assays for each of these probes are readily available in the literature but this would have required significant volumes of blood for this study (Tanaka *et al.* 2003; Zhou *et al.* 2004; Fuhr *et al.* 2007). Obtaining such large volumes of blood from very sick patients in the acute stage of recovery was unrealistic as it could impede their recovery and was also difficult from an ethical and practical standpoint. Accordingly, minimising the blood collected from these patients was of paramount importance. As such, an assay that could determine the concentration of all of the compounds of interest in small blood-volume samples, rather than separate assays involving larger blood volumes, was not only an attractive prospect from an efficiency point of view but was also an ethical and clinical necessity.

In determining the activity of the CYP450 enzymes of interest, the concentration of the four probe drugs and their primary metabolites in plasma samples were measured. An internal standard was used resulting in analysis of nine compounds in total. This presented several analytical challenges:

- The expected concentrations of these drugs and metabolites were below the sensitivity limits of HPLC with UV detection;
- Only one of the drugs and metabolites could be detected by fluorescence, rendering fluorescence detection unsuitable;
- ELISA plates were not available for the detection of all of the compounds, simultaneously or as individual assays. ELISAs that were available required too much sample for the separate analysis of all eight compounds arising from the probe drugs (*i.e.* ELISA does not require an addition of an internal standard); and
- Mass spectrometry (MS) is sensitive enough to detect the compounds at the concentration levels expected and is commonly used for this type of research. However, MS methods in the literature either analysed the compounds in separate runs, or in varying combinations with other drugs or used other probe drugs that were purposely avoided in this study (*e.g.* metoprolol). No method was reported at the time of this analysis that examined the concentration of all the compounds of interest simultaneously (Zhu *et al.* 2001; Jerdi *et al.* 2004; Fuhr *et*

al. 2007; Kumar *et al.* 2007; Lahoz *et al.* 2007; Zhang *et al.* 2008; Ghassabian *et al.* 2009; Liu *et al.* 2009).

Unlike UV or fluorescence detection, MS determines the mass to charge ratio of charged particles and determines their abundance as they pass through the detector. MS has two main advantages over photometric detection:

- MS can be several thousand times more sensitive; and (Polettini 2006)
- Co-eluting peaks can be identified by their individual molecular weights. Accordingly, chromatographic separation of compounds is less important than with UV detection and as a result, run times can be shorter (Silverstein *et al.* 1991).

For the reasons stated above a MS method was the most appropriate technique for this particular analysis. Access to a MS was given through the generous support of the Analytical Chemistry Department, UCC. To minimise the time required using the liquid chromatogram/mass spectrometer (LCMS), development of the sample preparation method was completed on the same HPLC instrument used in Section 2.2.

2.5.1 SAMPLE PREPARATION AND EXTRACTION

Sample preparation for LCMS is more demanding than for HPLC. Similar approaches can be taken to those discussed in Section 2.2, utilising one of four techniques: direct injection, protein precipitation before injection, liquid-liquid extraction and solid phase extraction. For this analysis, the aims of the extraction process were to produce a sample suitable for injection that used one of these four methods and, in addition:

- Contained all compounds of interest, unconjugated and extracted in high amounts;
- Was free of proteins and salts;
- Was of reduced volume, concentrated from the original sample;
- Was free of particulate residue;
- Could be produced repeatedly; and
- Was capable of scaling up for the preparation of the several hundred samples taken for this analysis.

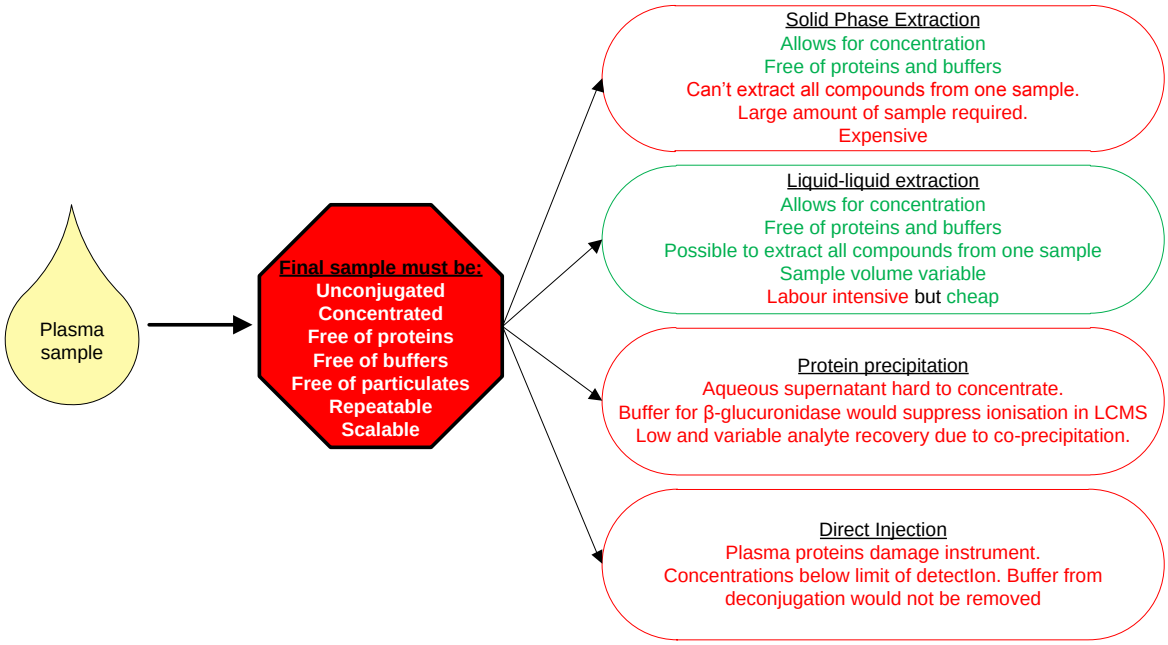


Figure 2.5-1 Aims of sample preparation and factors that influenced choice of extraction method

The important factors that influenced what extraction method was chosen are shown in Figure 2.5-1. The background to these aims listed above are briefly discussed in sequence below:

Contained all compounds of interest, unconjugated, extracted in high amounts;

The first step of sample preparation was to deconjugate the compounds from other molecules added to them during the drug metabolism process.

Drugs are frequently conjugated to other groups during the metabolism process, most commonly to a glucuronide moiety (Daali *et al.* 2008). This was the case with most of the compounds under investigation. Glucuronide conjugation interferes with analysis in two ways:

1. It increases the water solubility of the compound making it harder to extract from plasma with organic solvent; and
2. It increases the molecular weight of the compound, altering its MS signal (which is based on a molecular mass to charge ratio) so that the conjugated compounds would not be seen in their expected mass to charge range.

While the molecular weight could be approximated for a glucuronidated compound (by adding the weight of a glucuronide group to the molecular weight of the compound of interest), and therefore be detected by LCMS, no analytical standards were available for

the glucuronide conjugates to produce standard curves and enable the calculation of their concentration. It was therefore necessary to de-conjugate these compounds from their glucuronide.

There are several methods in the literature that facilitate glucuronide deconjugation, most commonly acid hydrolysis (Daali *et al.* 2008) or the use of a β-glucuronidase enzyme (Kaushik *et al.* 2006). Acid hydrolysis is difficult to limit to the desired deconjugation reaction and may cause degradation of other compounds, whereas β-glucuronidase is specific to the deconjugation of glucuronide. There are several sources of β-glucuronidase but the most commonly used and readily available is derived from the juice of the snail *Helix pomatia*. Optimal activity of this enzyme requires shaking, incubation and a buffered acidic environment; a summary of the conditions used in relevant papers is given in Appendix 14.

The inclusion of buffer salts presented problems for the LCMS, which are discussed further below. Inclusion of a buffer into the sample preparation excluded direct injection of the sample and any simple protein precipitation method of sample preparation, as the buffer would still be present in the final sample. Only solid phase or liquid-liquid extraction methods remained as options. After reviewing the literature and contacting product specialists at various suppliers, there was no one solid phase cartridge that could extract all the compounds being analysed, and sample requirements were too great for separate extractions. This left liquid-liquid extraction as the only option.

Was free of proteins and salts;

The problems arising from the protein content of plasma samples that makes them unsuitable for direct injection in HPLC systems were discussed in Section 2.2. These problems are magnified in LCMS systems. Due to the sensitive nature of detection, proteins present in plasma samples can, at worst, permanently damage the instrument, but more commonly leave 'ghost signals' that indicate the presence of a compound even though none is present in the injected sample. These signals can remain long after the last injection. Salts in samples also cause problems. MS requires the ionisation of the compound being analysed and the presence of salts hampers this ionisation. Therefore samples that contain salts can suppress this ionisation and also permanently damage the instrument, so these also must not be present in samples.

Was of reduced volume, concentrated from original sample;

The concentration of some of the compounds of interest was expected to be so low, it was desirable to increase their concentration by reducing the final volume of the sample. This required the solvents used in the extraction process to be volatile and capable of evaporation to dryness. Following this evaporation, a reduced volume of a solvent could be used to reconstitute the sample's evaporation residue.

Was free of particulate residue;

To protect the column and MS from any residual proteins and particulates, the only way to ensure particulate removal was to filter the samples. Several product specialists were contacted before the final product was chosen.

Could be produced repeatedly;

Results of the extraction process were required to be reproducible across the expected concentration range. Inconsistencies would invalidate the results. An internal standard was required to validate this and account for any variation. Phenacetin was chosen for this purpose as incorporating an internal standard for each compound would have been extremely cumbersome and problematic for the analysis. Phenacetin was widely used in the literature in similar types of analysis as a surrogate internal standard for all compounds of interest.

Was capable of scaling up for the preparation of the several hundred samples taken for this analysis;

The sample preparation process had to be scalable to facilitate analysis of the several hundred samples within the period of access to the LCMS. It also had additional requirements:

1. Preparation must be limited to a 2mL Eppendorf tube to prevent sample loss through adherence to the sides of larger tubes, and to fit available laboratory equipment; and
2. Use a minimum volume of extraction solvent to prevent long evaporation times as these would require large volumes of nitrogen gas to evaporate the samples. The availability of this gas was limited, frequently running out at the weekend.

2.5.2 EXTRACTION DEVELOPMENT

2.5.2.1 MATERIALS

2.5.2.1.1 ANALYTICAL COMPOUNDS

Caffeine, chlorzoxazone, paraxanthine and phenacetin were purchased from Sigma-Aldrich (St. Louis, Missouri, United States of America (USA)). Dextromethorphan, dextrophan, 6-OH-chlorzoxazone and 1-OH-midazolam were purchased from Toronto Research Chemicals (Ontario, Canada). Midazolam was the generous gift of Hoffman-La-Roche AG (Basel, Switzerland). As midazolam is a Schedule Four Controlled Drug, a license to import (Appendix 10) and a license to possess (Appendix 11) midazolam were necessary and these were obtained from the Department of Health and Children.

2.5.2.1.2 SOLVENTS AND BUFFERS AND OTHER MATERIALS

HPLC grade acetonitrile, methanol and water were purchased from Fisher Scientific (Leicestershire, UK). Potassium phosphate (puriss), sodium acetate (puriss), β-glucuronidase powder (type H-1, 1.96MU/g), analytical grade formic acid 88%, glacial acetic acid, diethyl ether, dichloromethane, chloroform and 2-propanol were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The mobile phases were filtered through type HA filters (0.45µm Millipore, Bedford, Massachusetts, USA) to remove particulate matter before use. These filters were chosen as they were compatible with the mobile phases being used.

2.5.2.2 CHROMATOGRAPHIC CONDITIONS

For development of sample preparation, separation of all the above listed analytical compounds in a single run was performed on a Phenomenex Synergi 4µm Hydro-RP 250x4.6mm column with a Phenomenex SecurityGuard precolumn held at 30°C (Phenomenex, Cheshire, UK). A gradient mobile phase combined 0.1M KH_2PO_4 + 1% formic acid (A) with acetonitrile + 1% formic acid (B) (Table 2.5-1).

Table 2.5-1 Chromatographic conditions for HPLC assay for optimisation of LCMS sample preparation

Time	%A	Flow rate (mL/min)
0	85	1
16.75	68 (nonlinear)	1
17	85	1
21	85	1

The gradient had initial conditions of 85:15 (A:B) which declined at a non-linear curve of 5 to 68:32 (A:B) by 16.75 minutes. The initial conditions were then restored by the 17th minute and maintained for a further four minutes. The flow rate remained constant at 1.0mL/min.

2.5.2.3 APPARATUS

Samples were centrifuged using a Mikro 120 Centrifuge (Hettich, Tuttlingen, Germany) at 14000 RPM. Compounds were analysed using an ATI Unicam UV/Vis spectrometer (Unicam Analytical Systems, Madison, USA). Samples were incubated with an Apollo shaker oven HP50 (CLP, Northampton, UK) set at 37°C and 20RPM. Extracted solvents were evaporated using a TurboVap LV concentration workstation (Caliper LifeSciences, Massachusetts, USA) at 40°C under a gentle flow of nitrogen gas until dryness. Final samples were filtered using Multiscreen Solvinert 0.45µm low binding hydrophilic polytetrafluoroethylene filters and Multiscreen vacuum manifold (Millipore, Massachusetts, USA) at 15-20mmHg.

Analysis of samples during the sample preparation method development was conducted using a Waters 2695 Alliance HPLC system (Waters Corporation, Milford, USA) equipped with a 2996 Photodiode Array (PDA) Detector, 2475 Multi Wavelength Fluorescence Detector and column oven. Chromatograms were captured and processed using Empower Pro interface (Empower 2, Waters Corp., Milford, USA).

2.5.2.4 OVERVIEW

A summary of factors relevant to the application of liquid-liquid extraction to this work is shown in Figure 2.5-2.

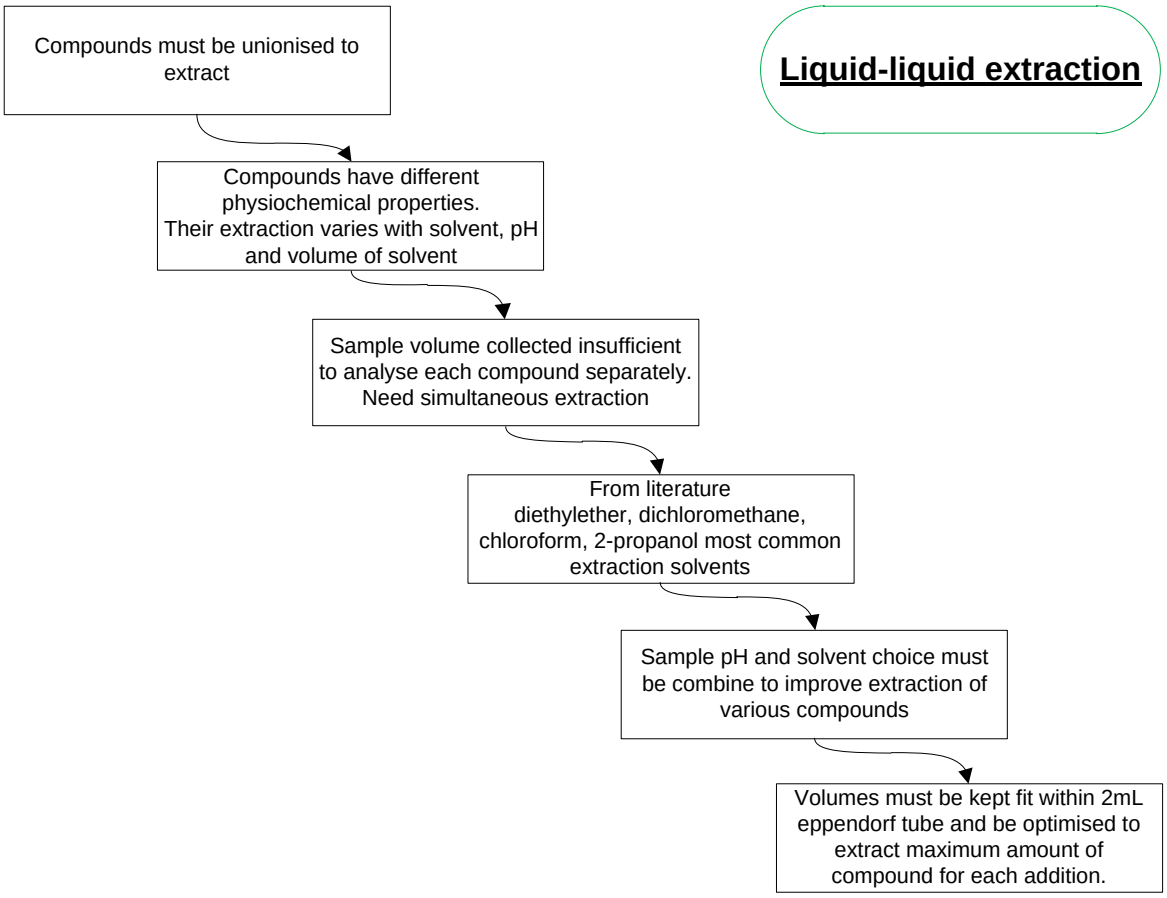


Figure 2.5-2 Summary of factors affecting liquid-liquid extraction

This method of sample preparation relies of the varying solubilities of the compounds of interest and the contaminants present in the plasma sample. Compounds must be ionised to extract and, given the varying physicochemical properties of the compounds being measured, this presented the biggest analytical challenge to this research. The literature was searched for further details of the compounds solubilities and these are summarised in Table 2.5-2 below. Where exact values were unavailable, solubilities are given as freely soluble (1 part in 1–10); insoluble (1 part in more than 10000); or sparingly soluble (1 part in 30–100) (Lund 1994). Molecular weights, necessary for LCMS analysis, were obtained from the Agilent Mass Hunter workstation Acquisition 2.00 software (Agilent Technologies, Massachusetts, USA) based on each molecule's empirical formula.

Several experiments were undertaken to understand, develop and optimise the extraction of the nine compounds of interest. Each of these experiments is summarised with key findings and graphs to support the conclusions where relevant in Table 2.5-3 further below.

Table 2.5-2 Structural and solubility details of compounds under investigation

Abbreviations: MR= molecular weight; (M+H)+= MR of ionised compound; Abs= UV absorbance maxima or fluorescence excitation/emission wavelengths; LogP=octanol:water partition coefficient; H2O=water; EtOH=ethanol; CHCl3=chloroform; (C2H5)2O=diethyl ether; Free=freely soluble; Ins=insoluble; Spr=sparingly soluble. References: 1-(Toronto Research Chemicals 2007), 2-(Sigma-Aldrich 2009); 3-(Moffat et al. 2011); 4-(Lund 1994)

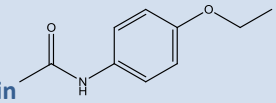
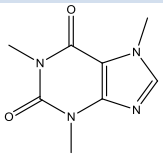
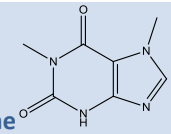
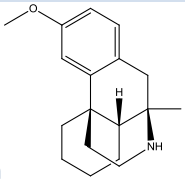
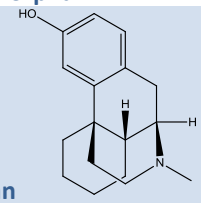
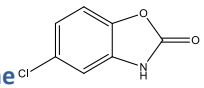
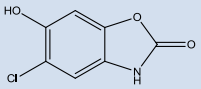
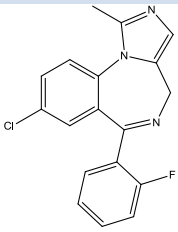
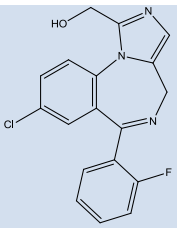
Compound	pKa	M_R $(M+H)^+$	Abs	LogP	Solubility	
 Phenacetin	2.2[3]	179.09 180.1	244nm[3]	1.6[3]	H_2O	1:1300[3]
					EtOH	1:15[3]
					$CHCl_3$	1:14[3]
					$(C_2H_5)_2O$	1:90[3]
 Caffeine	14[3]	194.08 195.09	273nm[3]	-0.07[3]	H_2O	1:46[3]
					EtOH	1:66[3]
					$CHCl_3$	1:5.5[3]
					$(C_2H_5)_2O$	1:530[3]
 Paraxanthine	8.8[2]	180.16 181.07	271nm[2]	-0.2[2]	H_2O	
					EtOH	
					$CHCl_3$	
					$(C_2H_5)_2O$	
 Dextromethorphan	8.3[3]	271.19 272.2	$\lambda_{ex/em}$ 280/ 315nm[3]	3.97[3]	H_2O	1:60[3]
					EtOH	1:10[3]
					$CHCl_3$	Free[3]
					$(C_2H_5)_2O$	Ins[3]
 Dextrorphan		257.18 258.19	$\lambda_{ex/em}$ 280/ 315nm[1]	3.1[1]	H_2O	
					EtOH	
					$CHCl_3$	
					$(C_2H_5)_2O$	
 Chlorzoxazone	8.0[3]	169 170	280nm[3]	1.6[3]	H_2O	Spr[3]
					EtOH	1:20[3]
					$CHCl_3$	1:250[3]
					$(C_2H_5)_2O$	1:60[3]
 6-OH-chlorzoxazone		184.99 186	297nm[1]		H_2O	
					EtOH	
					$CHCl_3$	
					$(C_2H_5)_2O$	
 Midazolam	6.2[3]	325.08 326.09	219nm[3]	4.3[3]	H_2O	Ins[4]
					EtOH	Free[4]
					$CHCl_3$	Free[4]
					$(C_2H_5)_2O$	Free[4]
 1-OH-midazolam		341.07 342.08	246nm[1]		H_2O	
					EtOH	
					$CHCl_3$	
					$(C_2H_5)_2O$	

Table 2.5-3 Summary of experiments in development of liquid-liquid extraction method for CYP450 probe drugs.

Each experiment is summarised in term of Aim, Experiment (a brief summary of the experiment and conditions), Findings (the key findings from the experiment) and Implication (what was learnt from the experiment that was relevant to the assay development and future work). Abbreviations used: DEE- diethyl ether; DCM- dichloromethane; CL- chloroform; P- 2-propanol; CLP- a mixture of chloroform and 2-propanol; N_2- nitrogen gas; HPLC- high pressure liquid chromatography; PDA- photodiode array; AQ- aqueous; ACN- acetonitrile; PCA- perchloric acid; NaAc- Sodium acetate; PH- phenacetin; CA- caffeine; PX- paraxanthine; DM- dextromethorphan; DX- dextrorphan; CZX- chlorzoxazone; 6CZX- 6-hydroxy-chlorzoxazone; MDZ- midazolam; 1MDZ- 1-hydroxy-midazolam; PO_4 potassium phosphate buffer; MeOH- methanol;

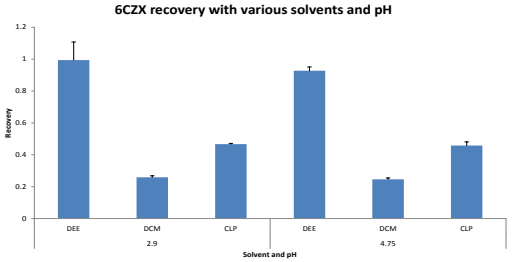
Aim	Experiment	Findings	Implication	Key Graphs
1. Determine evaporation of each solvent	0.5mL of DEE, DCM, CL and P added to separate HPLC vials and timed to evaporate to dryness under gentle stream of N_2.	P very slow to evaporate (45 min), followed by CL (27min) DCM (8min) and DEE (4min).	P component of extraction solvent must be small or offset by other more volatile solvents.	
		DEE evaporated so quickly it is difficult to pipette a volume accurately.	DEE to be stored at -80°C to reduce evaporation.	
2. Determine Solubility of compounds in solvents	Extraction of parent compounds (not metabolites), 20µg/mL, each in 100µl water tested from 500µL of four different solvents: DEE, DCM, CL, P and most common solvent in literature, chloroform and 2-propanol (9:1) mixture (CLP). Sample vortex mixed, centrifuged, organic layer separated and evaporated to dryness under N_2 gas (extracted). Reconstituted 1mL in water. Results compared to standard curves. Analysis by spectrophotometer.	Only DEE forms supernatant, others form subnatant under aqueous layer. Supernatant much easier to work with than subnatant.	HPLC method developed for simultaneous determination. Initial conditions 85:15 AQ:ACN.	
		Analysis of by spectrophotometer took too long, results inconsistent. Simultaneous method needed.	PDA set to scan for UV maxima of each compound.	
		Maximum UV absorbance determined for each compound.		
	Water spiked with all compounds to 10µg/mL. 1mL of each solvent added and extracted Solvent evaporated and reconstituted in mobile phase initial conditions.	Extraction varied with compound and solvent. No one perfect solvent.	Consideration must be given to adjusting pH to have compounds un-ionised.	
		No solvent completely extracted any compound.	Repeated extractions may be necessary.	
		DCM was best overall, but extracted bases poorly.	Consider adding a base during extraction to increase extraction of bases. Given buffering capacity of plasma, experiments to determine base required should be in plasma.	
			Stock solutions containing all compounds at 125µg/mL made. Dilution of 80µL of this solution with 920µL of plasma gives plasma spiked with 10µg/mL of all compounds (spiked plasma).	

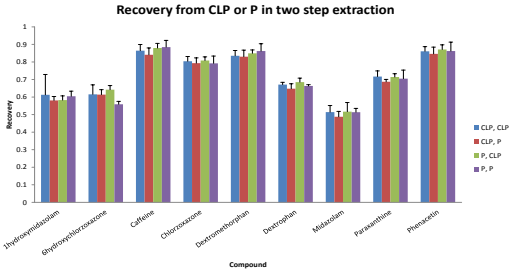
Aim	Experiment	Findings	Implication	Key Graphs
3. Determine extraction from plasma	500µL spiked plasma added to 30µL of 0.33, 0.66 and 1M NaOH. (High concentration used to minimise additional volume). 30µL water added to controls 500µL of DCM added and extracted. Two further 500µL DCM extractions completed. Organic recovered separately, dried and reconstituted in 200µL mobile phase. Analysed separately to see recovery from each step.	CZX and PX extract poorly, 10-20% of controls.	One step extraction not possible.	
		Basic and neutral compounds extract well, >80% of controls.	Adding base unionises and improves extraction of bases.	
		Extraction not significantly affected by base strength.	Second extraction step contributes significantly to recovery, third step less important.	
		The three extraction steps recover approximately 75, 20, 5% respectively of total recovery at each step.	Protein precipitant may be required to form harder protein pellet. Adding acid would do this and also improve extraction of acidic compounds. From paracetamol assay volume of PCA 30% required was 10% of sample volume, but total removal of proteins from plasma is not required, only solidification of precipitating proteins to make organic subnatant easier to access. What is the correct volume for this application? Are there alternative methods?	
		Protein precipitate forms diffuse layer above organic subnatant. Protein sticks to pippette tip making clean extraction of organic layer difficult.		
4. Improve extraction-precipitate protein	25, 50 and 100µL of 30% PCA added to 1mL spiked plasma (5µg/mL) followed by 500µL DCM. Organic extracted and evaporated to dryness and reconstituted in 200µL mobile phase.	After vortexing all samples form a creamy homogenous liquid. Following centrifuging, solid protein pellet formed between aqueous supernatant and organic subnatant of all samples which makes removal of organic much easier.	Smallest volume of PCA sufficient for this purpose.	
		Poor peak shape on chromatogram impairs integration. Significant tailing seen. May be effect of pH.	Introduce phosphate buffer into aqueous mobile phase.	
	Rerun above with 0.01M phosphate buffer as aqueous mobile phase.	Peak shape markedly improved. Recovery of acids >80% but bases <20%. Volume of PCA does not affect recovery.		
		pH of sample with 25µL PCA is 1.	This pH may be too acidic and risk hydrolysing compounds. Also need to account for effect of buffer required for β-glucuronidase activity.	
5. Incorporate buffer for β-glucuronidase activity	NaAc buffer 0.2M pH 4.75 necessary for β-glucuronidase activity. Literature shows buffer volume must be ≥2x sample volume.			
	500µL of spiked plasma added to 1000µL of NaAc and 500µL of DCM. Extract, evaporate and reconstitute.	Extraction of DX decreases with addition of buffer. Adding acid ionises basic compounds which then cannot extract into solvent.	Add base before final extraction to remove ionisation of bases	
6. Improve extraction of bases	500µL of spiked plasma added to 1000µL of NaAc. Add 1M NaOH in 10µL amounts to achieve pH 2 units above pKa of strongest base. Repeat extraction in previous step.	pH9-10 achieved after 120µL addition.		
		Extraction of bases improved.		
		Upon reconstituting dried solution in mobile phase, white supernatant formed and stuck to pipette tips making clean sample transfer to HPLC difficult.		

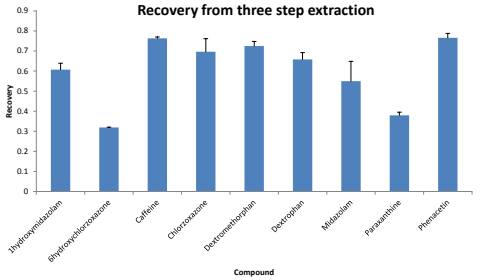
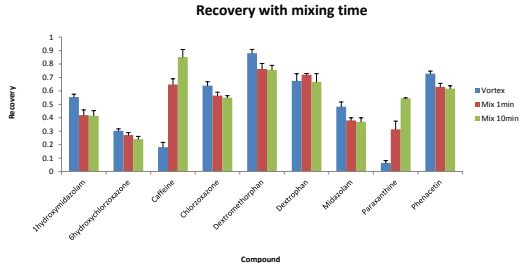
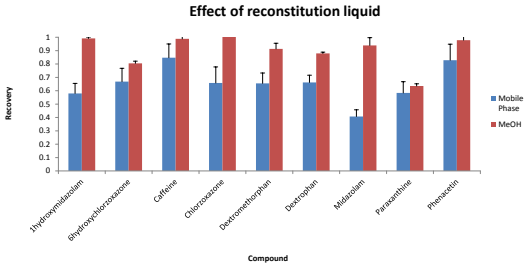
Aim	Experiment	Findings	Implication
7. Clean up solution before injection	Centrifuge samples at 14000RPM for 10min to try and solidify precipitant.	No change to precipipant.	Would using acetonitrile (ACN) or methanol (MeOH) (commonly used for protein precipitation) as reconstituting solvent cause these proteins to precipitate, making them easier to avoid when pipetting?
	Repeated previous experiment using either acetonitrile or methanol as reconstituting solvent in final step.	Both sets of samples free of protein. Reconstitution with ACN gives clearer solution.	ACN chosen as reconstituting solvent.
	Access to Turbovap.	All samples now in triplicate instead of duplicate.	CLP now an option again (took too long to evaporate by hand).
8. Determine superiority of DCM or CLP	500µL of spiked plasma added to 1mL of NaAc buffer. Four samples have 500µL DCM added, another four have 500µL CLP added. Solvents extracted. 120µL NaOH added to remaining aqueous solution and solvents crossed over for the second extraction to give the following combinations (1^{st}-2^{nd}): DCM-CLP, DCM-DCM, CLP-CLP, CLP-DCM. Organics evaporated in Turbovap at 40°C for 45min and reconstituted in ACN.	Peak fronting in chromatograms impairing integration- ?as a result of using ACN to reconstitute evaporation residue.	Reduce injection volume.
		CLP generally superior over DCM at extraction especially for bases following addition of NaOH.	?Some effect of plasma on 6CZX and DX preventing extraction. Go back to aqueous samples to get better assessment of the effect of pH on their extraction- ?buffering capacity of plasma or protein binding making 6CZX and DX extraction difficult to assess.
		Extraction still mostly ≤65%. 6CZX not found in any sample, DX<10%.	
9. Improve extraction of 6CZX and DX	6CZX and DX spiked into buffer 0.5mL pH 4, 7, 10 buffers to give 10µg/mL. Each sample extracted with 0.5mL of one of three solvents, DEE, DCM and CLP. Solvents extracted and evaporated and reconstituted in ACN.	Recovery poor in all samples. Examine left over aqueous samples after extraction- lower 6CZX or DX concentration indicates better extraction.	Compounds in much lower concentrations than controls- compounds are leaving sample- ?Problem with reconstitution.
		6CZX extracted best at pH 4 into DEE.	
		DX extracted best at pH 10 into CLP.	
10. Improve extraction of 6CZX	Reducing pH to 4 in previous experiment increases extraction of 6CZX. Does repeating previous experiment using phosphate buffer of pH2.9 further improve recovery? Method as previous but reconstituted mobile phase.	Reconstitute in mobile phase removes peak fronting.	pH achieved by additional NaAc buffer sufficient for extraction of acids.
		Lowering pH 4.75 to 2.9 does not improve recovery of 6CZX.	
		DEE vastly superior for extraction of acidic compounds.	
		DX extracts best into CLP or DEE at pH 10.	
11. Determine effect of pH	Previous experiment repeated using pH 4, 7 and 10 buffer containing all probes at 10µg/mL. Also included Chloroform/2-Propanol 9:1 mixture (P) also commonly reported.	Chloroform/2-Propanol mixtures have ≈ extraction. 9:1 mix takes longer to evaporate. Recovery still ≤65%.	Stick with chloroform rich mixture.

1MDZ; best at high pH, no strong impact of solvent. Extracting 50-70%
6CZX; strong effect of pH, 4≥7>>10, solvent DEE (70%)>CLP (50%)>>DCM (24%)
CA; Small pH effect, 4<7<10, strong effect of solvent P; (83%)>CLP (81%)>DCM (67%), DEE (10%)
CZX; Strong pH effect 10>>7≈4, some solvent effect P (83%)=CLP (83%)>DCM (76%)>DEE(62%)
DM; solvent effect increases with pH. Best extraction at high pH. CLP=P (83%)> DCM (68%)> DEE (56%)
DX; Strong effect of pH 10>>>7=4. No solvent effect P (86%)≥ CLP (84%)≥ DEE (83%)≥ DCM (81%)
MDZ; pH effect varies with solvent. DEE best at pH 10 (81%), other solvents do not vary with pH (≈60%)
PX; Strong pH effect 4>7>>10. Strong solvent effect P (53%)≥CLP (50%)>>DCM (24%)>>DEE (5%)
PH; Small effect of pH 4≥7≥10. Moderate solvent effect P (82%)≥CLP (81%)>DCM (72%)>DEE (51%).

Key Graphs





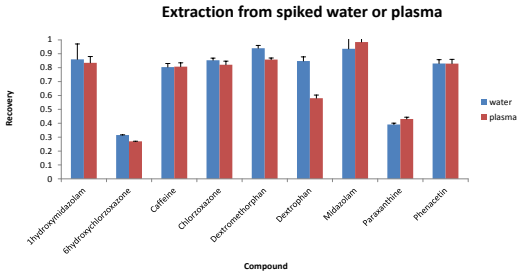
Aim	Experiment	Findings	Implication	Key Graphs
12. Neutralise sample	Advised that pH7 would be optimal for allowing all compounds to simultaneously extract into solvent. What volume of 1M phosphate buffer pH 7.6 (PO_4) would be sufficient to neutralise 500µL of 0.2 NaAc buffer?	240µL required.	Concern over DX extraction and variation of pH after addition of fixed volume of NaOH. Would a buffer help? K_2HPO_4/KH_2PO_4 buffer was used because it also has buffer activity upon addition of NaOH at pH10-12. Add middle extraction for weak acids/bases.	
13. Determine superiority of CLP or DEE	250µL NaAc buffer containing all compounds at 10µg/mL added to 250µL of either CLP or DEE. Extract organic. Add 120µL PO_4 and a further 250µL CLP to all samples. Add 120µL NaOH followed by a further 250µL CLP.	Adding DEE does not improve extraction. Erroneously first extraction with CLP has superior yield to DEE+CLP as if DEE impaired extraction. This experiment was repeated and results confirmed.	DEE does not add to the extraction as the first step.	
		6CZX extracts especially poorly		
14. Improve extraction of bases	Incorporate base prior to final extraction to increase extraction of basic compounds. 250µL NaAc buffer containing all compounds at 10µg/mL added to 250µL of CLP. Extract organic. Add 120µL PO_4 and a further 250µL CLP. Extract organic. Add 50µL of 4M NaOH and 250µL DEE. Extract organic. Evaporate and reconstitute in mobile phase.	Average recovery: 1MDZ; 61% 6CZX; 32% CA; 76% CZX; 69% DM; 72% DX; 66% MDZ; 55% PX; 38% PH; 77%.	Extraction of 6CZX and PX main issues. Only small amounts of DX and DM came out in DEE. No other compound found in thrid extraction. Need to change from DEE? In one set of samples a shaker was used instead of a vortex. This greatly improved extraction.	
15. Compare mixing methods	250µL water containing all probes to 10µg/mL added to 250µL NaAc buffer. 250µL CLP added and extracted. 250µL PO_4 was added followed by addition and extraction of 250µL CLP. 50µL NaOH was added followed by a further addition and extraction of 250µL CLP. Sample were either vortex mixed for 30 seconds or shaken at 1400RPM for either 1min or 10min.	Caffeine and Paraxanthine show strong relationship with mixing time, with greater extraction on longer mixing. Other compounds show no improvement.		
		Reduced variation between replicate samples with longer mixing.	All samples now mixed for 10min at 1400RPM.	
		White precipitate making uncontaminated removal of extraction solvent difficult.	?Precipitant from buffers (note no plasma present in sample unlike earlier experiment that noted this problem). Try adding small volume of PCA.	
			Some compounds have poor aqueous solubility, but all have good solubility in MeOH.	
16. Does using methanol to reconstitute evaporation residue improve extraction?	Reconstitution of evaporation residue in mobile phase compared to reconstitution in MeOH. 125µL water containing all probes 10µg/mL added to 125µL NaAc and PCA 10µL. 250µL CLP added and extracted followed by 120µL of PO_4 and a further 250µL CLP added and extracted. 30µL NaOH then added followed by addition and extraction of another 250µL CLP and lastly the addition and extraction of 250µL DEE. Two sets of triplicate samples produced; 1 set reconstituted in mobile phase, the other in MeOH.	MeOH markedly improves recovery >90%, except for 6CZX , and does not alter peak shape.	Use MeOH to reconstitute evaporation residue.	
		White precipitate still forms between PO_4 and NaOH addition.	Drop PCA from the proceedure (risk of hydrolysis compounds).	
		Negligible amounts found in final DEE extraction.	Remove DEE from final extraction.	
			To scale up method to 500µL of plasma was exceed capacity of 2mL eppendorf. Can volume of additions be reduced?	

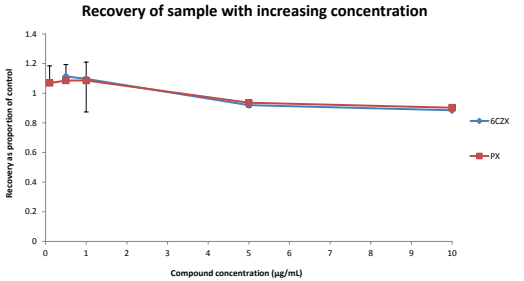
Aim	Experiment	Findings	Implication
17. Role of solvent volume	125µL of water spiked to 10µg/mL of all compounds added to 125µL NaAc. Extraction with either 125, 250 or 500µL of CLP. 120µL PO_4 added followed by repeat extraction using previous volume. 30µL of NaOH added along with a final extraction of CLP with the same volume. Evaporation residue reconstituted in MeOH.	PX proportional to solvent volume, PX most hydrophilic. Others not affected.	Large solvent volume only important when PX extraction occurs (first extraction).
		For 500µL plasma and 500µL NaAc volume of 1M PO_4 required for pH 7-8 = 400µL. To increase pH 10-12 with 4M NaOH volume required 80µL.	Extraction of 6CZX and PX now only problems.
	Volumes of PO4 and NaOH required checked		
18. Examine effect of plasma	Two sets of samples run 1) 500µL plasma spiked to 10µg/mL 2) 500µL water spiked to 10µg/mL. Each added to 500µL NaAc and 30µL PCA followed by addition and extraction of 1mL CLP. 400µL PO_4 added followed by addition and extraction of 500µL CLP. 80µL NaOH then added followed by final addition and extraction of 500µL of CLP. Reconstitute evaporation residue in MeOH.	DX and DM show better extraction from water. All other compounds difference ≤5%.	?DX and DM extraction effected by buffers in plasma- pH not manipulated as much as in water- based samples.
		Plasma reduces recovery to >70%, except for 6CZX and PX at <45%.	Extraction of PX and 6CZX need further work.
	Alter initial HPLC conditions and gradient to improve resolution of 6CZX and DX.	6CZX extraction <60%.	Reintroduce DEE earlier in process for 6CZX and PX extraction.
19. Examine effect of compound concentration	Does concentration of drugs influence their extraction? Prepare 100µL plasma samples containing PX and CZX at concentrations of 10, 5, 1, 0.5, 0.1µg/mL. Add 100µL NaAc, 6µL PCA, 200µL CLP. Extract CLP, evaporate and reconstitute in 100µL MeOH.	No major effect.	As concentrations increase, errors decrease: concentrations derived from chromatograms become more accurate as noise in the chromatogram becomes less of a contributor.
20. Improve PX and 6CZX extraction	3 sets of 250µL plasma containing 10µg/mL PX and 6CZX added to 250µL NaAc and 15µL PCA. Each set had one of the solvent combinations added and extracted: 750µL CLP+250µL DEE; 500µL CLP+500µL DEE; 250µL CLP+750µL DEE.	Organic layer from 750µL and 500µL additions of CLP form subnatant, 250µL addition forms supernatant.	?More as a result of reducing CLP volume.
		Increasing DEE component reduces PX extraction.	?Volume of DEE required >>750µL.
		Increasing DEE component did not alter 6CZX extraction.	CA and PX rely on CLP for extraction and extract poorly into DEE.
	Repeat using plasma containing all probes.	Both CA and PX show strong relationship with CLP volume, with extraction decreasing as CLP volume decreases.	?affinity of DEE for 6CZX strong, needing less than 250µL for extraction.
		Other compounds, do not show any significant effect on changing ratio of solvent.	Changes to mixing and reconstitution procedures have improved recovery.
		Most compounds extracting 65-90%- Only DX extracts poorly at 40% but only one extraction- no added base in this experiment.	Discovered in literature- PCA known to reduce recovery through binding to precipitating protein. ACN does not do this. Try ACN instead.

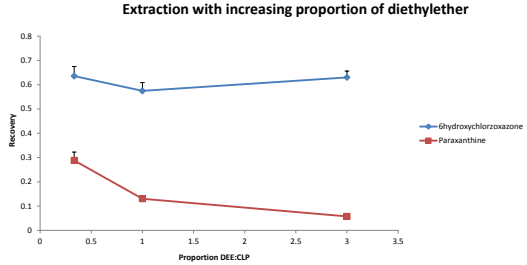
Key Graphs

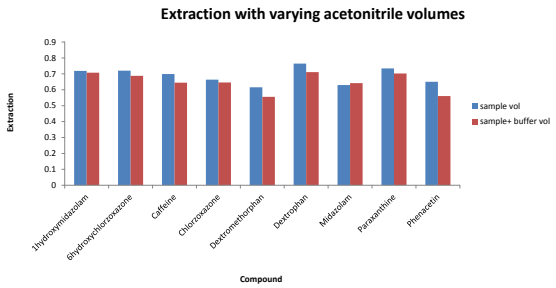
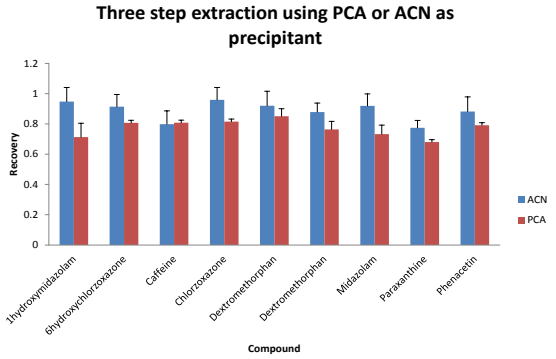
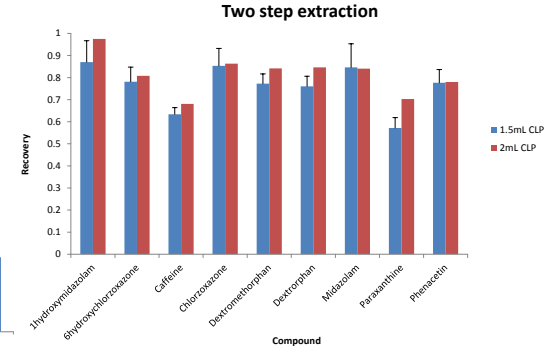
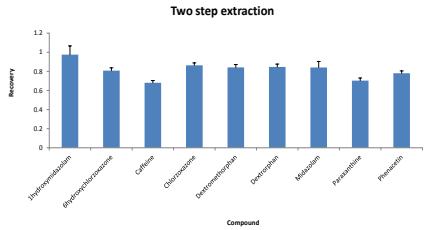


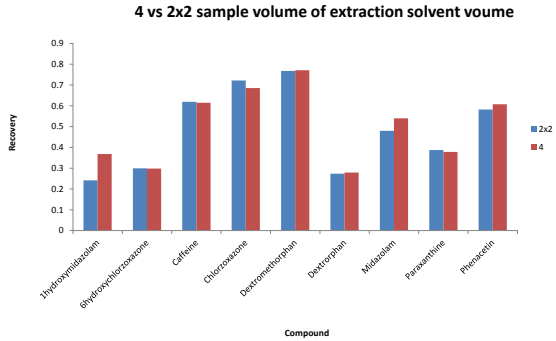
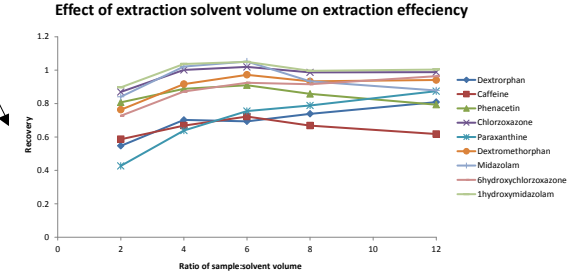
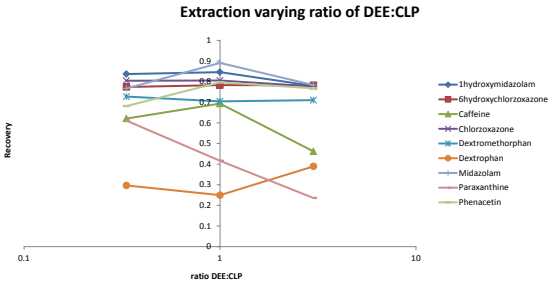
Different steps in extraction from plasma.







Aim	Experiment	Findings	Implication	Key Graphs
21. Improve PX and 6CZX extraction- use ACN as precipitant	125µL of plasma spiked with all compounds to 10µg/mL added to 125µL NaAc in two sets. First set has ACN 250µL added, second has 500µL. Both followed by 250µL CLP which is extracted and evaporated.	Both volumes of ACN provide acceptable protein precipitation. Recovery increases overall by ≈ 10%.	Replace PCA with ACN as precipitant.	
22. Compare ACN to PCA	125µL plasma containing all compounds added to 125µL NaAc followed by 250µL ACN or 10µL PCA. 750µL and 125µL DEE added to both samples and extracted followed by 100µL PO_4. 500µL CLP then added and extracted followed by 25µL NaOH and a final addition and extraction of 500µL CLP.	ACN seems to partition with organics, increasing organic volume to 1100µL from first extraction. Protein precipitation of both precipitants adequate. Following addition of organic for second extraction protein pellet dispersed into aqueous phase- this occurred more prominently in with ACN samples. Following centrifugation immediately prior to removal of second set of organic ?white crystals seen at bottom of sample tube where PCA was used as precipitant. ACN improves extraction 5-20% for all compounds except CA which ≈. Average recovery with ACN as precipitant: 1MDZ; 95% 6CZX; 91% CA; 80% CZX; 96% DM; 92% DX; 88% MDZ; 92% PX; 77% PH; 88%.	?Removal of ACN with organic in first extraction allows proteins to re-suspend. Proteins appear almost dissolved and do not contaminate pippette tip. ?PCA causes buffer salts to precipitate. ?Can this step be removed.	
23. Simplify method	Would a two extraction method without addition of buffer be equivalent? 125µL plasma containing all compounds added to 125µL NaAc followed by 250µL ACN and 750µL CLP and 125µL DEE. Organics extracted. 25µL NaOH added followed by addition and extraction of 750µL CLP.	Average recovery with two extractions: CA; 63% PX; 58% All other compound extraction >75%.	Method does not have same total volume of CLP- did not account for CLP from second extraction in previous method.	
	Repeat above with 1mL of CLP at both extractions.	Average recovery improves: CA; 68% PX; 70% All other compound extraction >75%.		

Aim	Experiment	Findings	Implication	Key Graphs
24. Improve PX and 6CZX extraction- CLP volume	Is there and effect of solvent volume- is 2x250µL extractions superior to 1x500µL? Five sets of 125µL spiked plasma (10µg/mL) were prepared. 125µL NaAc and 250µL ACN added to each. One of the CLP volumes added to each set 250µL, 250µL followed by another 250µL, 500µL, 750µL, 1000µL. Solvents extracted, evaporated and reconstituted in MeOH.	Recovery increases with increasing solvent volume but effect tails off so 750µL≈1000µL, except for PX which shows linear relationship from 20% up to ≈60%.		
		Repeated extraction does not significantly improve recovery over single extraction of equivalent volume at these volumes (2x250µL≈1x500µL).	May not be transferable to larger solvent volumes- ?solvents saturated at these lower volumes. Need to examine effects of larger volumes.	
		In most cases extraction did not improve by increasing solvent volume about 6xsample volume, except for paraxanthine which continued to improve but tailed off after solvent volumes exceed 6xsample volume.		
	Repeat previous experiment with 500µLx2 vs 1000µL and 750µLx2 vs 1500µL volumes of CLP.	Recovery from 1000µL<<2x 500µL recovery.	?Saturation of solvent at lower volumes, ?at larger solvent volumes mixing may be less efficient.	
			Conditions for β-glucuronidase activity checked.	
			Confirms earlier results when mixed with CLP. DEE has strong extraction for 6CZX.	
25. Optimise DEE volume	Four sets of samples increasing to 500µL plasma. Add 100µL 2M NaAc, 500µL ACN, 800µL CLP. To each set add 25, 50 or 100µL DEE. Organic phase extracted and reconstitute in 500µL MeOH.	Increasing DEE does not improve extraction.	PX extraction dependent on CLP volume- is third extraction necessary again?	
		Average recovery reduces: PX; 51%.	Constraints: Turbovap cannot evaporate organic volumes >2mL… ACN partitioning into organic phase so total extraction solvent must be <1.5mL.	
26. Reintroduce middle extraction	Four sets of 500µL plasma containing all compounds 10µg/mL. 100µL 2M NaAc added along with 500µL ACN 450µL CLP and 25µL DEE. Organic phase extracted and a further 550µL CLP added and extracted followed by the addition of 100µL NaOH. To each set of samples a final 500, 250, 100 or 50µL CLP was added and extracted.	Only compounds that extract in basic conditions are 1MDZ and DX. Only very small amount of 6CZX, CA, CZX, DM, MDZ and PH are seen in final extraction.		
		Only 1MDZ and DX affected by reduction in final CLP extraction, DX mostly, reducing only ≈10% across range 500µL to 50µL range.	Use 250µL CLP in final extraction. Add remaining solvent to second extraction (cannot be added to first extraction as the would exceed sample tube capacity).	
		No significant difference between 500µL and 250µL CLP volumes.		
		All compounds extract >80%.		

Aim	Experiment	Findings	Implication	Key Graphs
27. Confirm final method	**Final method:** 500µL plasma added to a 2mL eppendorf tube. 100µL 2M NaAc containing 2000u β-glucuronidase was added, vortex mixed for 30 seconds and incubated overnight in a shaker oven at 37°C and 40RPM. 500µL ACN with 10µg/mL internal standard PH was added at end of incubation 450µL CLP and 25µL DEE was added and mixed for 10minutes at 1400 RPM and then centrifuged at 14000 RPM for 10 minutes. All of organic phase was removed A further 800µL CLP was added and extracted as above. To remaining aqueous sample 100µL NaOH and a final 500µL CLP was added and organic layer extracted. All organics extracted were combined into a 2mL eppendorf and evaporated to dryness in a Turbovap under a gentle stream of nitrogen in a water bath at 40°C To residue 250µL of MeOH was added and sonicated for 10minutes followed by mixing at 1400RPM for a further 10minutes.			
28. Stop peak fronting	Samples prepared as above- different column used in LCMS.	Extraction good by peak fronting impairs integration.	Column on LCMS much shorter than previously used. The shorter column does not allow for the same degree of mixing and suspect the MeOH base of the sample is carrying the compounds through the column- causing the peak fronting. Try reducing MeOH content in solution that dissolves evaporation residue.	
	Repeat method in two sets. Process both sets the same until the final step. Dissolve evaporation residue of one set in MeOH and the second set in MeOH/water 50:50.	Reducing MeOH content reduces recovery by 5-10%. Lower MeOH content samples have symmetrical peaks.	Use MeOH/water 50:50 to dissolve samples.	

2.5.2.5 B-GLUCURONIDASE ACTIVITY

Once the extraction method had been finalised, the last the step in the development of this sample preparation was the optimisation of glucuronide deconjugation. As discussed in Section 2.5.1, some of the analytes are extensively glucuronidated *in vivo* as part of their metabolism and elimination processes. Glucuronide moieties conjugated to analytes alter their *m/z* and this would affect their detection by LCMS. As it was intended to quantitate these analytes it was necessary to ensure complete deconjugation of the glucuronide. Section 2.5.1 also contains the justification for the use of β-glucuronidase for this deconjugation. Like many hydrolytic enzymes, β-glucuronidase requires specific conditions for optimal activity; chiefly amongst these are pH 4.5-5 and temperature of 37°C for molluskan sources (Appendix 14). Control of pH was achieved by dissolving the β-glucuronidase powder in 2M acetate buffer adjusted to pH4.75. Acetate buffer was chosen as it had good buffering capacity around the pH range required for β-glucuronidase activity and, following addition of NaOH, a secondary buffering capacity around pH 10. This alkali environment was desirable in the latter stages of the extraction method described above. Temperature was maintained by incubating the samples in a shaker oven at 37°C. Other factors requiring optimisation included:

- Ratio of buffer: sample volume;
- Effect of substrate concentration;
- Whether activity was dependent on amount of β-glucuronidase or its concentration;
- Amount of β-glucuronidase per sample;
- Temperature of incubation; and
- Length of incubation.

The optimisation of these factors used the only analytical glucuronide conjugate readily available, paracetamol glucuronide, as analytical glucuronide conjugates of the probe drugs/metabolites are not commercially available. The hydrolysis of paracetamol glucuronide back to paracetamol was achieved by incubation with freshly prepared solutions of β-glucuronidase (20000u (10.38mg)/mL 2M acetate buffer, pH 4.75). β-glucuronidase was freshly prepared with each experiment because the hydrolytic activity of the enzyme was not stable (Kaushik *et al.* 2006). Both paracetamol and paracetamol glucuronide were detected using the assay previously described (Section 2.2) and complete conversion was deemed to have occurred when no paracetamol glucuronide was found. All samples were prepared in triplicate along with controls containing acetate buffer without the addition of β-glucuronidase. A summary of the method development is shown in Table 2.5-4. This resulted in the overnight incubation of 1000u β-glucuronidase in 100µL of 2M acetate buffer per 500µL patient plasma sample. Three measures were taken to ensure these deconjugation reaction conditions would achieve complete hydrolysis of the probe drugs/metabolites in the actual patient samples:

- The concentration of paracetamol glucuronide used in this method development was 10-100 fold greater (100µg/mL) than the sum of probe drugs/metabolites under investigation in the patient samples;
- The amount of β-glucuronidase included in the patient samples was at least five times more than was required to fully hydrolyse these more concentrated 100µg/mL of paracetamol glucuronide samples; and
- The period of incubation used (overnight) was at least three times longer than that necessary for the complete deconjugation of the 100µg/mL of paracetamol glucuronide samples.

Table 2.5-4 Summary of experiments to optimise β-glucuronidase activity (abbreviations as in previous figure)

Aim	Experiment	Findings	Implication
1. Optimise conditions for β-glucuronidase activity	Activity determined using conversion of paracetamol glucuronide (PG) to paracetamol (P). 1000u β-glucuronidase dissolved in 50µL NaAC (BG).		
2. Determine effect of NaAc volume	Three samples sets of 100µL plasma containing 10µg/mL PG added to 50µL of BG. To two sets of samples an additional 50 or 150µL of NaAc added. Samples incubated in shaker oven overnight at 37°C. 10µL PCA added, samples vortex mixed for 30seconds and centrifuged. Supernatant injected.	No PG found in samples- No P present in controls.	No influence of NaAc volume on deglucuronidation.
		Concentration of P proportional to dilution by additional NaAc.	2:1 ratio of sample:NaAc sufficient to achieve pH within enzyme activity limits.
3. Determine effect of PG concentration- and duration of incubation	Six sets of 100µL plasma samples spiked with 100, 50, 10, 5, 1 and 0.5µg/mL of PG. 50µL BG added to all samples. Samples vortex mixed and incubated overnight in shaker oven overnight at 37°C. At end of incubation 10µL PCA added, mixed and centrifuged, supernatant injected.	Oven did not heat. As all samples in triplicate run one of three of each set. Incubate other two of each set for a further 8 hours and run.	Temperature critical to β-glucuronidase activity.
		All unincubated samples had poor β-glucuronidase activity.	
		No PG present in all incubated samples regardless of PG concentration.	
4. Determine effect of plasma volume on β-glucuronidase activity	Three sets of 50µL BG and 25µL PG 1mg/mL added to 75, 225 or 475µL plasma. Samples vortex mixed and incubated overnight in shaker oven overnight at 37°C. At end of incubation 10µL PCA added, mixed and centrifuged, supernatant injected.	Volume of sample did not overcome β-glucuronidase activity.	
5. Determine effect of varying amount of β-glucuronidase	100µL of plasma containing PG 100µg/mL. Add BG 50, 40, 30, 20, 10, 0µL made up to 50µL with NaAc. Samples vortex mixed and incubated overnight in shaker oven overnight at 37°C. At end of incubation 10µL PCA added, mixed and centrifuged, supernatant injected.	Even samples with no BG added converted all PG to P. Controls all PG as normal.	BG very potent. Suspect carryover of BG from pipette tip while pipette NaAc sufficient to convert PG to P.
		All samples converted all PG to P.	
6. Determine effect of incubation time on β-glucuronidase activity	Five sets of 100µL plasma containing PG 100µg/mL. 50µL BG added and mixed. Incubate each set in shaker oven at 37°C for 1, 2, 3, 5 and 18hrs. At end of incubation 10µL PCA added, mixed and centrifuged, supernatant injected.	1-3 hour samples have almost complete deconjugation but some variation. 5 and 18 hour sample have complete deconjugation in all samples.	No advantage incubating samples beyond 5 hours.

2.5.3 PATIENT SAMPLES

Once the extraction method was shown to be successful the analysis was transferred to the LCMS for the analysis of patient samples.

2.5.3.1 MATERIALS

Materials used for the method development were also used for the analysis of patient samples. In addition Fisher Optima® LC/MS grade water and acetonitrile were used in combination with Optima® LC/MS grade formic acid (Fisher Scientific, Leicestershire, UK) as the mobile phase for the LC/MS.

2.5.3.2 APPARATUS

Apparatus used for the method development were also used for the analysis of patient samples. In addition, the analysis of patient samples was conducted using an Agilent 1200 HPLC attached to Agilent 1200 series DAD SL detector and a Agilent 6510 Quadrupole time-of-flight (Q-TOF) mass spectrometer, with an electron spray ionisation (ESI) ion source (Agilent Technologies, Massachusetts, USA). Data were acquired on Agilent Mass Hunter workstation Acquisition 2.00, analysed on Agilent Mass Hunter workstation Qualitative Analysis 2.00 and quantitated using Agilent Mass Hunter workstation Quantitative Analysis 2.00.

2.5.3.3 CHROMATOGRAPHIC CONDITIONS

Separation was performed on an Agilent XDB C18, 1.8um, 4.6x50mm column, heated on the heating block to 35°C. A linear gradient method was employed using 95% water 5% acetonitrile 0.1% formic acid (A) and 95% acetonitrile 5% water 0.1% formic acid (B) as shown in Table 2.5-5. Solvents are often premixed in LCMS systems to prevent use of 100% acetonitrile, which can damage and occlude the narrow bore tubing utilised in LCMS systems.

Table 2.5-5 Chromatographic conditions for LCMS assay

Time	%B	Flow rate (mL/min)
0.75	7.0	0.600
3.00	25.0	0.600
5.00	30.0	0.600
7.00	30.0	0.600
7.50	90.0	0.600
9.00	90.0	0.600
9.50	7.0	0.600

2.5.3.4 SAMPLE PREPARATION

Polypropylene 1.5mL and 2.0mL Eppendorf tubes and 50mL sample tubes (Sarstedt, Nümbrecht, Germany) were used for all sample preparation. Samples were inserted into HPLC sample vials containing 100µL low volume inserts (Fisher Scientific, Dublin, Ireland). Reference samples of nominal concentration were prepared in Solvent A. The Injection volume was 10µL.

2.5.3.5 MASS SPECTROMETER PARAMETERS

The parameters of the mass spectrometer were optimised for the detection of the analytes of interest. As the mass of these analytes was known, acquisition was in MS mode only in the *m/z* range of 100-1000. This causes the instrument to behave as a TOF, selecting ions in the desired *m/z* range without fragmentation in the collision cell.

Initially the effects of changes to drying gas flow rate on the detector response were examined. Drying gas flow rate will affect the ionisation of the sample as it leaves the nebuliser. Each analyte was prepared in diluent to 0.1mg/L. 6L was chosen as the ideal flow rate (Figure 2.5-3), however, this caused condensation inside the ESI unit. After attempts to prevent this by reducing the mobile phase flow rate were unsuccessful, a gas flow of 10L/min was chosen as a compromise.

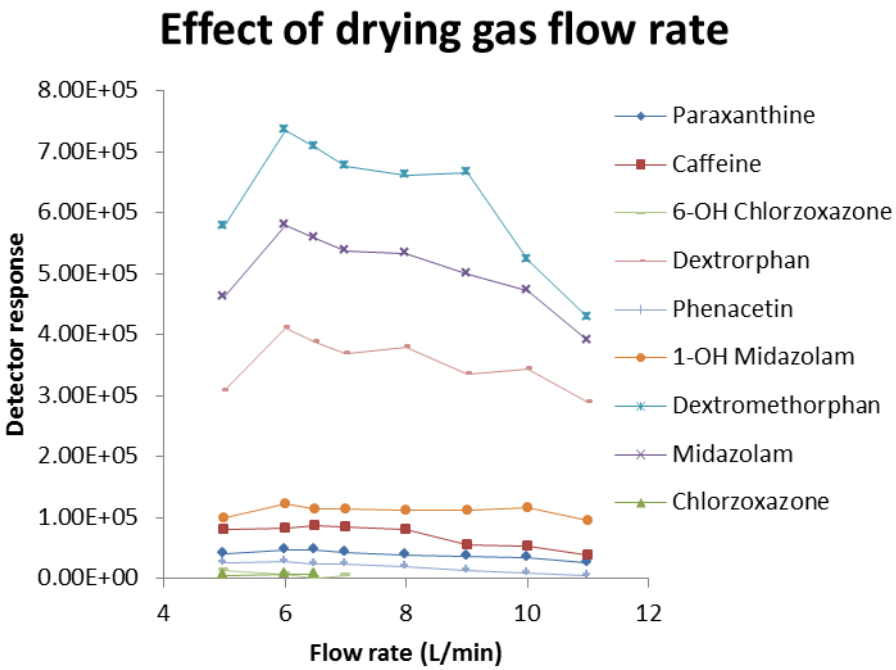


Figure 2.5-3 Effect of drying gas flow rate on LCMS detector response
Shown as mean +standard deviation from 1mg/L sample in diluent

Due to time constraints only midazolam response was optimised further as it was expected to be in the lowest concentration. The remaining conditions role and optimal status were as follows:

- **Gas temperature** effects ionisation in the ESI. There was very little difference across the temperatures assessed with the optimal being 340°C (Figure 2.5-4).

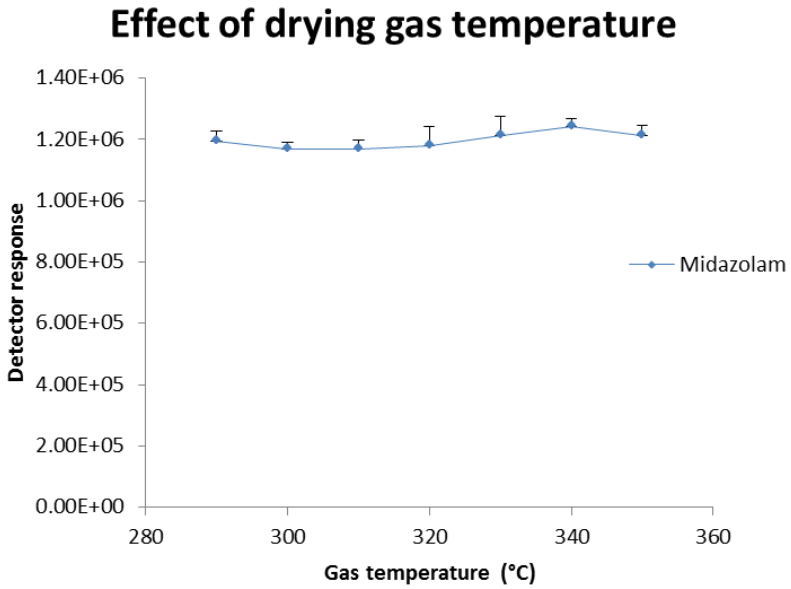


Figure 2.5-4 Effect of drying gas temperature on detector response
Shown as mean +standard deviation from 1mg/L sample in diluent

- **Capillary voltage** provides the pull of charged ions from the earthed ESI unit into the charged MS capillary. There was only minor change and 4000V was chosen (Figure 2.5-5).

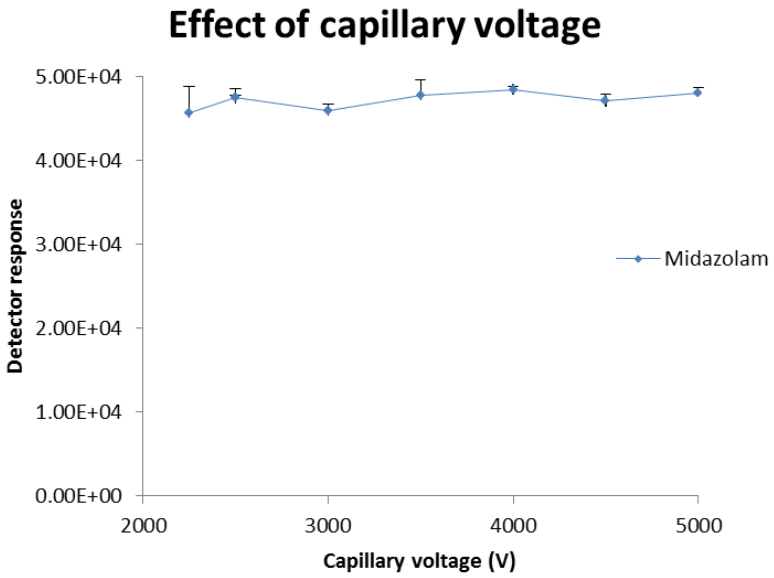


Figure 2.5-5 Effect of capillary voltage on detector response
Shown as mean +standard deviation from 0.1mg/L sample in diluent

- **Fragmentor voltage** in TOF only mode determines the energy applied to the ion stream to exclude ions outside the chosen *m/z* range. It was selected as 210V.

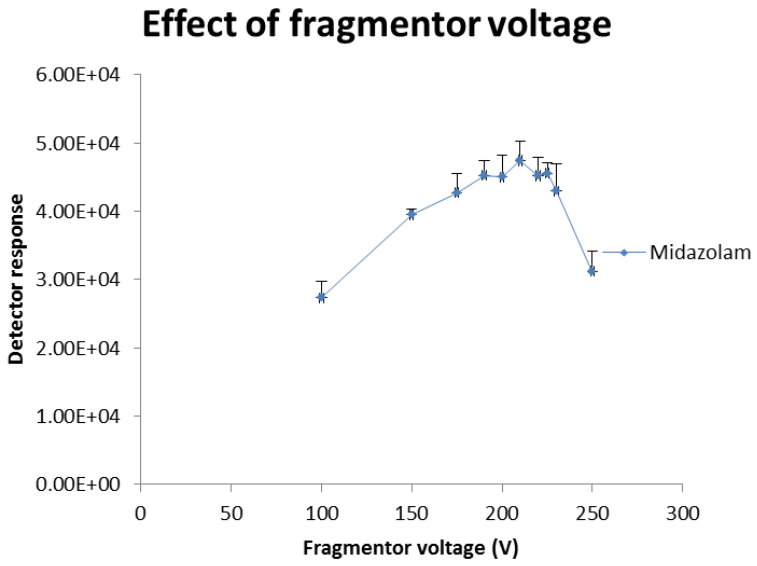


Figure 2.5-6 Effect of fragmentor voltage on detector response
Shown as mean +standard deviation from 0.1mg/L sample in diluent

Additional settings were the rate of acquisition at 2 spectra/second and nebuliser pressure of 50 PSIG.

3 RESULTS

3.1 PATIENT DEMOGRAPHICS

3.1.1 SUMMARY OF PATIENT DEMOGRAPHICS FOR ALL GROUPS

Demographic information was collected from each group. The description of each group was given in Section 2.1.1. This information is summarised below in Table 3.1-1 and continued in Table 3.1-2. All groups were broadly similar in age, although Group A had the highest mean age (63.2±11.0yrs). Group C was the youngest (47.7±8.1yrs), reflecting the different diagnosis of this group. Group D was, on average, the youngest of the groups undergoing bowel surgery although this group had the largest standard deviation (Table 3.1-1).

Table 3.1-1 Summary table of patient demographics

Summary data are presented as mean and standard deviation (Mean (SD)) for continuous data, Count and per cent of group (Count (%)) for categorical data and median and interquartile range (Median (IQR)) for discrete, non-normal data. Other abbreviations: BMI- Body Mass Index; Drinker- broken down by number of self-reported standard drinks consumed per average week- "+" ≥1, <10/week; "++" ≥10, <20/week; "+++"≥20/week; Cancer- if malignancy was main indication for surgery.

Group	N	Age (Years) Mean (SD)	BMI (kg/m^2) Mean (SD)	Male Count (%)	Smoker Count (%)	Drinker Median (IQR)	Cancer Count (%)
A	10	63.2 (11.0)	28.6 (5.6)	8 (80)	0 (0)	+ (0,++)	10 (100)
B	4	57.9 (3.3)	25.4 (2.4)	2 (50)	4 (100)	+ (+,+)	4 (100)
C	8	47.7 (8.1)	23.3 (4.0)	0	1 (12.5)	0 (0,0)	8 (100)
D	11	52.3 (20.9)	27.6 (4.3)	8 (72.7)	3 (27.3)	+ (0,++)	7 (63.6)

Based on the BMI results, all the groups except for Group C could be classified as overweight (*i.e.* BMI>25 kg/m^2). The average BMI of Group C (23.2 kg/m^2) was within the healthy weight range. In many cases the patients in Group C had completed courses of chemo or radiotherapy prior to enrolling in the study which may have resulted in weight loss prior to the study. The majority of the patients in the study were male, except for Group C, again reflecting the diagnosis of those in Group C. There were very few smokers in the study, with the exception of Group B in which all patients smoked. Patients did not smoke during the study itself as this was not permitted by the hospitals involved. There was a wide range of

alcohol consumption amongst the patients, with patients in Groups A and D having the highest average consumption. There were no drinkers in Group C. Unlike all the other groups, many of the patients in Group D had a diagnosis other than cancer as the main reason for surgery.

Table 3.1-2 Summary table of patient demographics (continued)
Other abbreviations (In addition to those used in the previous table) ASA status- (American Society of Anaesthesiologists (ASA) physical status

Group	Duration of Surgery (hr) Median (IQR)	Number of preoperative medicines Median (IQR)	ASA status Median (IQR)	Total Paracetamol dose (g) Median (IQR)
A	3 (2.5, 3.4)	2 (0, 7.5)	1.5 (1, 2)	42.5 (41.4, 43.6)
B	2.25 (1.9, 2.5)	0 (0, 0.25)	1 (1, 1)	21 (21, 21)
C	2.25 (2, 2.6)	0 (0, 0.25)	1 (1, 1.3)	21 (21, 21)
D	5 (4.6,5.6)	0 (0, 2.5)	1 (1, 2)	20 (19.5, 20)

Group D had the longest surgery time (median 5 hrs; IQR 4.6-5.6), reflecting the more time consuming nature of laparoscopic surgery undergone by the majority of this group, whereas Groups A, B and C were broadly similar in duration of surgery (Table 3.1-2). Group A had the highest number of preoperative medicines (median 2; IQR 0-7.5) which is also reflected in their higher median ASA status. No medicines taken preoperatively were known to alter paracetamol metabolism. During the course of the study, Group A had twice the amount of paracetamol than the other groups. This is slightly less than may be anticipated given the dosing schedules for each group, however, the median for Group A (42.5; IQR 41.4-43.6) was brought down by the first patient who was withdrawn from the study early.

As discussed in Section 2.1, reconfiguration of surgical services ended bowel resections at the first hospital. To continue collection bowel resection patients the study was moved to a second hospital (Hospital 2). Groups A, B and C were recruited from Hospital 1, whereas Group D was from Hospital 2.

3.1.2 PATIENT DEMOGRAPHICS OF GROUP A

Group A consisted of 10 patients undergoing major bowel resection. The demographics of this group are detailed in Table 3.1-3.

Table 3.1-3 Group A Patient demographics

Presented as mean and standard deviation (SD), number and per cent (%) of group or median and interquartile range (IQR) as indicated. All other abbreviations are as in previous tables.

Patient	Age	Sex	Weight (kg)	Height (m)	BMI (kg/m^2)	Cancer	Smoker	Drinker	Pre-op medications	ASA status
1A	87	M	96	1.8	29.63	Y	N	N	9	2
2A	65	F	87	1.64	32.35	Y	N	N	3	2
3A	67	M	129	1.78	40.71	Y	N	++	6	2
4A	62	M	83	1.63	31.24	Y	N	+	9	2
5A	52	M	92	1.85	26.88	Y	N	+++	1	2
6A	73	F	58	1.62	22.10	Y	N	N	8	1
7A	58	M	67	1.69	23.46	Y	N	++	0	1
8A	54	M	89	1.71	30.44	Y	N	N	0	1
9A	51	M	68	1.68	24.09	Y	N	+++	0	1
10A	58	M	79	1.79	24.66	Y	N	+	2	1
Mean (SD)	63.2 (11)		84.8 (19.7)	1.71 (0.08)	28.56 (5.57)					
Count (%)						10 (100)	0 (0)			
Median (IQR)								+ (++)	2 (0, 7.5)	1.5 (1, 2)

This group received the high dose of paracetamol, 1.5g every four hours. The majority of all patients were male (80%) and aside from patients 1 and 6, all patients were aged in their 50's and 60's. This is younger than expected from the data given by the National Cancer Registry of Ireland for colorectal cancer patients (National Cancer Registry Ireland 2010). BMI varied widely amongst the group, with six being overweight and four of these being classified obese. All patients in this group had a malignancy as their main indication for surgery. None were smokers but two were heavy drinkers. There was also a wide range in the number of preoperative medicines, which in most cases was reflected in a higher ASA status (Table 3.1-3).

Table 3.1-4 Group A patient demographics (continued)
All abbreviations explained in previous tables

Patient	Main Procedure	Duration of Surgery (hr)	Paracetamol dose (g)	Plasma Samples	Urine Samples
1A	Transverse colectomy	3.5	11	16	16
2A	Extended hemicolectomy	2	44	24	37
3A	Anterior resection	3	39.5	26	30
4A	Anterior resection	5	41	26	33
5A	Anterior resection	3	42.5	26	32
6A	Left hemicolectomy	1.5	41	24	31
7A	Anterior resection	3	41	25	31
8A	Left hemicolectomy	2.5	41	11	27
9A	AP resection	2.5	42.5	21	35
10A	AP resection	4.5	36.5	18	28
Median (IQR)		3 (2.5, 3.4)	42.5 (41.4, 43.6)	24 (19, 26)	31 (29, 33)

The majority of surgery involved an anterior resection, followed by colectomies (Table 3.1-4). The full set of plasma samples was collected from the majority of the patients in this group with the exception of patients 1 and 10 who withdrew from the study early for reasons given in Table 3.1-11. The majority of patients in this group had central lines which were also used for obtaining blood samples. Complete urine collections were also obtained in 9 of the 10 cases in this group from urinary catheters which were present for the duration of the study for all patients. Although not recorded, the majority of these patients were not ambulating until Day 3 or 4, remaining on complete bed-rest until then. Most were fasted until the third day when a light diet of soup and ice cream was commenced. All patients remained in the ICU until at least the evening of Day 3, where they received one-to-one nursing care.

3.1.3 PATIENT DEMOGRAPHICS OF GROUP B

Group B consisted of four patients undergoing the same surgical procedures as Group A, but receiving the licensed dose of intravenous paracetamol, 1g every six hours. The reconfiguration of regional surgical services ended recruitment into this group prematurely and consequently, only four patients were recruited (Table 3.1-5). This group was also younger that the national average (57.9±3.3 yrs) (National Cancer Registry Ireland 2010) and closer to the healthy range of BMI with less variation than Group A. All had a malignancy as their primary indication for surgery and all were smokers. There was a low rate of alcohol consumption amongst all in this group and they had a lower average ASA status than Group A (Table 3.1-5).

Table 3.1-5 Group B Patient demographics
All abbreviations explained in previous tables

Patient	Age	Sex	Weight(Kg)	Height(m)	BMI(kg/m^2)	Cancer	Smoker	Drinker	Pre-op medications	ASA status
1B	60	M	79	1.78	24.93	Y	Y	+	0	1
2B	61	F	64	1.68	22.68	Y	Y	+	1	1
3B	54	M	87	1.85	25.42	Y	Y	+	0	1
4B	55	F	87	1.75	28.41	Y	Y	+	0	1
Mean (SD)	57.9 (3.3)		79.3 (10.8)	1.77 (0.07)	25.36 (2.36)					
Count (%)						4 (100%)	4 (100%)			
Median (IQR)								+ (0)	0 (0, 0.25)	1 (1, 1)

All of Group B required colectomy, with two additionally receiving a cholecystectomy. This was slightly above the national average (National Cancer Registry Ireland 2010) but being a small group it is difficult to apply to population trends. The duration of surgery was also shorter than for Group A. Patient 2 in this group did not consent to give blood samples but complete sets of blood and urine were obtained all other patients (Table 3.1-6)

Table 3.1-6 Group B patient demographics (continued)
All abbreviations explained in previous tables

Patient	Surgery	Duration of Surgery (hr)	Paracetamol dose (g)	Plasma Samples	Urine Samples
1B	Left hemicolectomy, cholecystectomy	1.75	20	19	30
2B	Sigmoid colectomy, cholecystectomy	2	20	-	29
3B	Left hemicolectomy	2.5	20	21	31
4B	Right hemicolectomy	2.5	20	21	30
Median (IQR)		2.25 (1.9, 2.5)	21 (21, 21)	20 (14, 21)	30 (29.7, 30.3)

3.1.4 PATIENT DEMOGRAPHICS OF GROUP C

Group C contained patients undergoing mastectomy who received the licensed dose of paracetamol of 1g every six hours. The mean age (47.7±8.1yrs) of this group was younger than all other groups and younger than the national average of those with breast cancer (Table 3.1-7). They were all female. The mean BMI (23.27kg/m^2) was within the healthy range as were all patients except for one obese patient. Only one patient was a smoker, who was also a heavy drinker. Very few took medications preoperatively and only two were ASA 2. All patients except for patients 4 and 8 received chemotherapy prior to their surgery.

Table 3.1-7 Group C Patient demographics

All abbreviations explained in previous tables

Patient	Age	Sex	Weight (kg)	Height (m)	BMI (kg/m^2)	Cancer	Smoker	Drinker	Pre-op medications	ASA status
1C	41	F	62.3	1.67	22.33	Y	Y	+++	0	1
2C	42	F	67.2	1.74	22.20	Y	N	N	0	1
3C	45	F	72	1.67	25.82	Y	N	N	0	1
4C	52	F	88	1.66	31.93	Y	N	N	1	2
5C	64	F	54	1.67	19.36	Y	N	N	0	1
6C	41	F	62	1.74	20.48	Y	N	N	4	2
7C	52	F	63	1.67	22.59	Y	N	N	0	1
8C	41	F	65	1.74	21.47	Y	N	N	0	1
Mean (SD)	47.7 (8.1)		66.7 (10)	1.7 (0.1)	23.3 (4.0)					
Count (%)						8 (100)	1 (12.5)			
Median (IQR)								0 (0, 0)	0 (0, 0.25)	1 (1, 1.25)

All patients underwent a mastectomy with all but one having concomitant axillary node clearances (Table 3.1-8). The duration of surgery varied from 1.5 to 3 hours. While all patients received the full paracetamol doses, plasma collection was very problematic in this group as none of these patients had central lines postoperatively, and most being post-chemotherapy had poor venous access. Urine samples were also problematic as only a few patients had urinary catheters for longer than the first postoperative day and female patients had trouble collecting all of their own urine.

Table 3.1-8 Group C patient demographics (continued)
All abbreviations explained in previous tables

Patient	Surgery	Duration of Surgery (hr)	Paracetamol dose (g)	Plasma Samples	Urine Samples
1C	Mastectomy, Axillary Node Clearance	2	20	22	13
2C	Mastectomy, Axillary Node Clearance	1.5	20	8	18
3C	Mastectomy, Axillary Node Clearance	2	20	6	19
4C	Mastectomy, Axillary Node Clearance	2	21	19	28
5C	Mastectomy, Axillary Node Clearance	3	20	12	27
6C	Mastectomy, Axillary Node Clearance	2.5	20	15	27
7C	Mastectomy, Axillary Node Clearance	3	20	6	13
8C	Mastectomy	2.5	20	-	7
Median (IQR)		2.3 (2, 2.6)	21 (21, 21)	9 (5, 13)	20 (12, 27)

3.1.5 PATIENT DEMOGRAPHICS OF GROUP D

Group D was a continuation of Group B, being composed of patients undergoing colorectal surgery; however, as noted above, Group D patients were recruited from Hospital 2. Under a different surgeon there was a different approach to colorectal surgery. At Hospital 2 colorectal surgery patients underwent an enhanced recovery programme where they received high protein drinks supplements pre and post operatively and were returned to light diets the morning following surgery, if it was tolerated. Patients were also encouraged to ambulate on the day following surgery and as part of that tubing for IV lines and urinary catheters were removed on this or the second day. In addition, the majority of the procedures were laparoscopic. Similar to Groups A and B, the average age of this group was less than national averages (52.3±20.9 yr). The average BMI for the group was in the overweight range (27.63±11.76 kg/m^2). Unlike all other groups, several of the patients who underwent surgery did not have malignant disease as the main indication for surgery. Few were smokers although several were alcohol drinkers. The number of preoperative medications also varied widely along with ASA status (Table 3.1-9).

Table 3.1-9 Group D Patient demographics
All abbreviations explained in previous tables

Patient	Age	Sex	Weight (kg)	Height (m)	BMI (kg/m^2)	Cancer	Smoker	Drinker	Pre-op medications	ASA status
1D	42	M	58.9	1.67	21.12	N	N	N	0	1
2D	61	M	93	1.74	30.72	N	N	++	9	2
3D	60	M	65	1.68	23.03	N	Y	+++	0	1
4D	52	M	89	1.8	27.47	Y	N	++	3	2
5D	52	F	74	1.68	26.22	Y	N	N	0	1
6D	73	M	93	1.62	35.44	Y	N	N	0	1
7D	33	F	90	1.66	32.66	N	N	+	10	2
8D	65	M	82	1.72	27.72	Y	Y	++	0	1
9D	70	M	69	1.72	23.32	Y	Y	+	2	2
10D	66	M	73	1.67	26.18	Y	N	++	0	1
11D	60	F	79	1.62	30.10	Y	N	N	0	1
Mean (SD)	52.3 (20.9)		78.7 (11.8)	1.69 (0.1)	27.6 (4.3)					
Count (%)						7 (64%)	3 (27%)			
Median (IQR)								+ (0,++)	0 (0, 2.5)	1 (1, 2)

Similar to Group A there were a larger number of rectal cancers than would be expected in the general population although some of those in this group underwent this surgery for inflammatory bowel diseases or congenital malformation (Table 3.1-10). Being laparoscopic, these surgeries were of much longer duration than the open surgeries in Groups A and B (median (IQR) duration hours: Groups A and B 2.5 (2, 3.125), Group D 5 (4.5, 5.75)). Obtaining plasma samples was also problematic as only two had central lines in place, however, for the most part, urine collections were successful.

Table 3.1-10 Group D patient demographics (continued)

All abbreviations explained in previous tables

Patient	Surgery	Duration of Surgery (hr)	Paracetamol dose(g)	Plasma Samples	Urine Samples
1D	Sigmoid colectomy (open)	4.5	19	11	20
2D	Panproctocolectomy, hernia repair (lap)	5.75	18	24	22
3D	Anterior resection (lap)	4.75	19	22	27
4D	Anterior resection (lap)	4.25	19	20	24
5D	Anterior resection (lap)	5.5	19	6	18
6D	Anterior resection (lap)	5	19	6	25
7D	Sub-total colectomy (open)	5.5	19	6	20
8D	Anterior resection (lap)	4.5	19	25	23
9D	Anterior resection (lap)	6	18	6	13
10D	Anterior resection (lap)	6	18	24	21
11D	Anterior resection (lap)	4.75	19	15	24
Median (IQR)	(9 laparascopic, 2 open)	5 (4.5, 5.75)	20 (19.5, 20)	15 (6,23)	22 (20,24)

3.1.6 WITHDRAWALS AND COMPLICATIONS

Table 3.1-11 details withdrawals and complications recorded during the study period. Only one patient withdrew consent for participating in the study due to unpleasant sensations and dreams that occurred following the administration of midazolam. The majority of patients who were withdrawn were as a result of poor venous access for blood sampling. Other patients were withdrawn due to nausea or vomiting that prevented the administration of the oral medicines used in the study and two were withdrawn for reduction in renal function (Table 3.1-11).

Table 3.1-11 Withdrawals or complications

Patient	Complication
1A	Renal failure- withdrawn day 2
8A	Poor IV access- limited sampling post-op, no bloods day 4
10A	Unpleasant dreams following midazolam- withdrew day 4
2B	Consented for urine only- no bloods
1C	Dehydration post-op- urine not produced for some intervals
2C	Poor IV access- no bloods from day 2
3C	Poor IV access- no bloods post op
5C	Poor IV access- no bloods from day 2
7C	Poor IV access- no bloods post op
8C	Poor IV access- no bloods
1D	Poor IV access- no bloods from day 2
5D	Persistent nausea and vomiting- no bloods post op
6D	Renal failure post-op– withdrawn post op, urine collected
7D	Poor IV access- no bloods post op
9D	Poor IV access- no bloods post op
11D	Poor IV access- no bloods days 1 and 2

3.2 PARACETAMOL ANALYSIS VALIDATION

3.2.1 PLASMA

Chromatograms were obtained for all patient plasma samples. A sample chromatogram is shown in Figure 3.2-1.

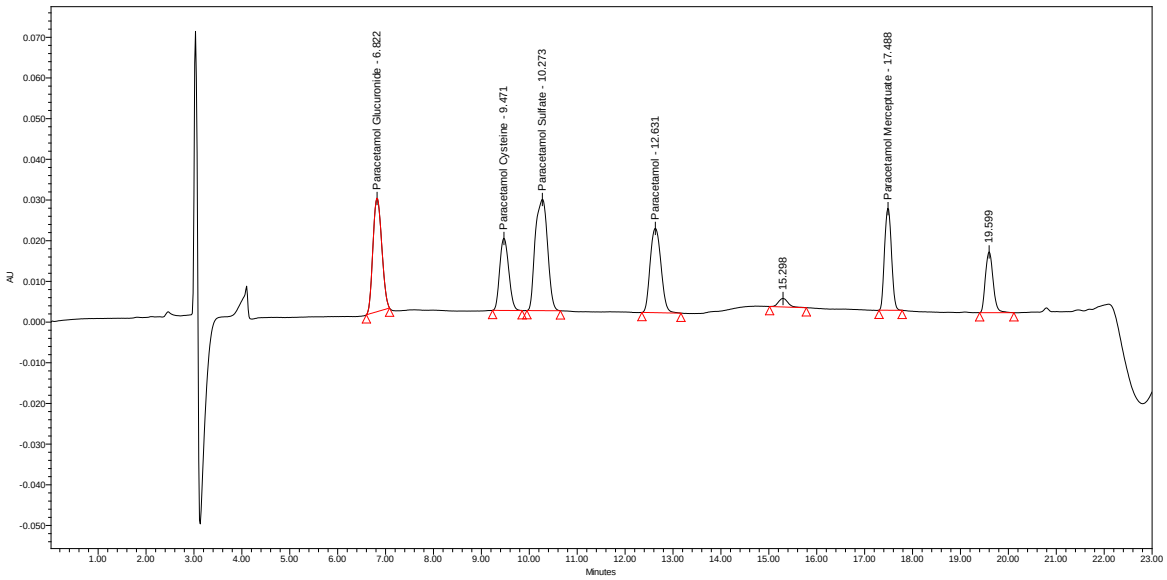


Figure 3.2-1 Sample chromatogram of absorbance of paracetamol and its metabolites in plasma
Chromatogram is from injection of a plasma standard containing 20µg/mL of paracetamol and its metabolites captured at 242nm. The elution order and times (mins) were paracetamol glucuronide (6.82), paracetamol cysteine (9.47), paracetamol sulphate (10.27), paracetamol (12.63) and paracetamol mercapturate (17.49).

3.2.1.1 HPLC VALIDATION

3.2.1.1.1 SPECIFICITY/SELECTIVITY

Specificity of the assay for paracetamol and its metabolites was examined to ensure that endogenous compounds in the patient's plasma samples did not co-elute and interfere with the compounds of interest. This was validated by the absence of interfering chromatographic peaks in blank human plasma and from the pre-dose plasma samples obtained from the first six patients in Group A (Shah *et al.* 1992). Selectivity was confirmed by peak contours from the PDA (Figure 2.2-2) (Bressolle *et al.* 1996).

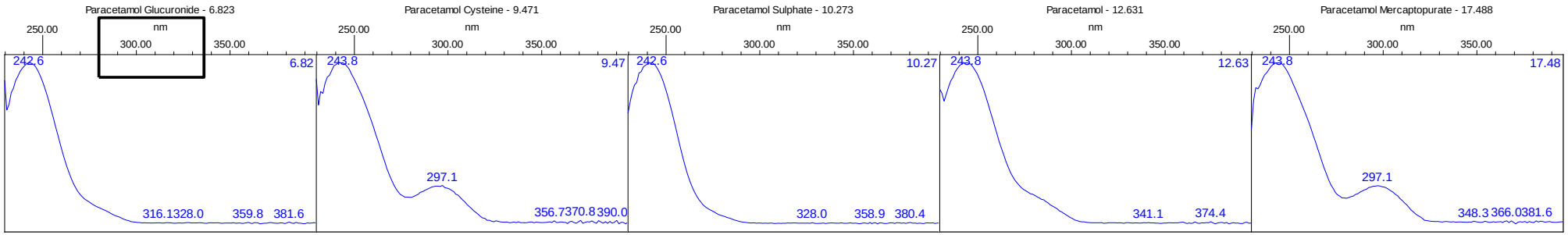


Figure 3.2-2 PDA spectra of peaks from Figure 3.2-1.
From left: paracetamol glucuronide, paracetamol cysteine, paracetamol sulphate, paracetamol and paracetamol mercapturate respectively. In addition to peaks at around 242nm, Paracetamol Cysteine and Mercapturate have absorbance peaks at 297nm

3.2.1.1.2 CALIBRATION CURVES

Calibration curves were prepared as detailed in Section 2.2

Linearity of the calibration curve was shown by:

- The intercept not being statistically different from 0;
- The slope being statistically different from 0; and
- The regression coefficient not being statistically different from 1.

Table 3.2-1 shows all calibration curves passed tests for linearity.

Table 3.2-1 Tests of linearity of calibration curves

Linearity		Intra-batch		Inter-batch	
		Mean	95% CI	Mean	95% CI
Paracetamol glucuronide	Intercept	19616.4	± 26705.31	5074.356	± 24228.202
	Slope	52565.64	± 1633.775	53761.53	± 3070.789
	Correlation	1	± 0.001	1	± 0
Paracetamol sulphate	Intercept	3493.638	± 9192.73	8306.097	± 9772.053
	Slope	39322.89	± 1212.024	40346.56	± 2374.88
	Correlation	1	± 0.001	1	± 0.001
Paracetamol	Intercept	3464.38	± 32134.941	25566.72	± 32763.734
	Slope	90259.51	± 13237.234	79346.08	± 7792.874
	Correlation	1	± 0	1	± 0.001
Paracetamol cysteine	Intercept	4719.723	± 5954.201	4820.502	± 5733.96
	Slope	29537.71	± 1366.425	27697.47	± 2046.41
	Correlation	0.999	± 0.001	0.999	± 0.001
Paracetamol mercapturate	Intercept	1375.735	± 2282.031	2501.135	± 3142.974
	Slope	28157.31	± 406.278	27752.9	± 1151.106
	Correlation	1	± 0	1	± 0

3.2.1.1.3 EXTRACTION EFFICIENCY

A seven point dilution series of paracetamol was prepared in triplicate for both plasma and diluent and analysed according to Section 2.2. Extraction efficiency was determined by dividing plasma sample peak area by the peak area of its respective diluent concentration. Figure 3.2-3 shows extraction from plasma was linear with good correlation (R>0.99) across the concentration range. This indicates extraction of paracetamol from plasma with this method was consistent over concentrations tested.

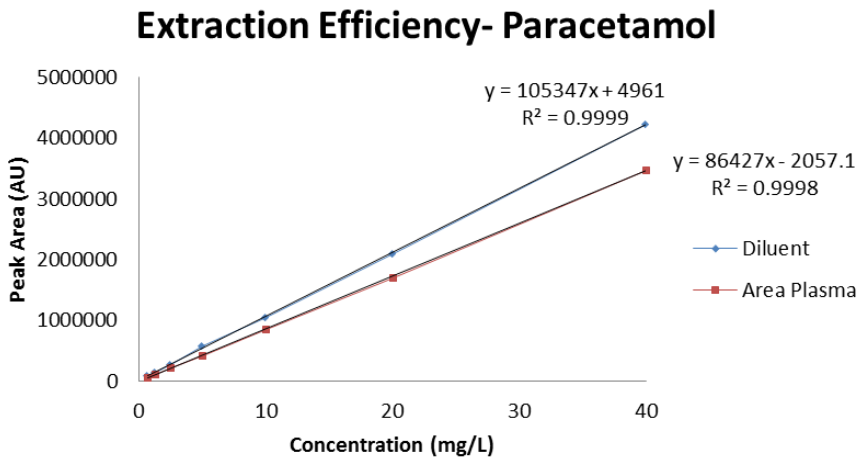


Figure 3.2-3 Paracetamol extraction efficiency

Table 3.2-2 shows extraction efficiency was also high and consistent with an average of 80% extraction efficiency across the concentration range. The extraction method was also proven to be reliable as the per cent relative standard deviation (%RSD) figures was comparable between the plasma and diluent samples with no trends being observed.

Table 3.2-2 Paracetamol extraction efficiency calculation

Concentration (µg/mL)	Diluent Avg peak area	%RSD	Plasma Avg peak area	%RSD	Extraction Efficiency
40	4234101.41	0.256	3472697.13	0.329	82.017
20	2083704.68	1.019	1703640.25	0.333	81.76
10	1051986.77	1.343	837982.57	0.198	79.657
5	528783.63	1.052	417076.94	1.015	78.875
2.5	274278.35	0.736	227213.91	0.426	82.841
1.25	144025.91	0.608	117132.30	2.457	81.327
0.625	84736.31	0.589	67936.76	1.256	80.174
				Average	**80.95**

These findings were considered acceptable as:

1. Extraction efficiency was consistent and repeatable across the concentration range; and
2. Extraction efficiency was above levels where losses due to poor extraction can lead to calibration curves with small slopes and which can produce unreliable results (Bressolle *et al.*) (Table 3.2-2).

3.2.1.1.4 PRECISION AND ACCURACY

Inter and intra batch variations were calculated from high, middle and low concentrations for each compound as discussed in Section 2.2. Precision and accuracy were calculated as the per cent RSD and per cent recovery respectively and are shown in Table 3.2-3. Values were accepted if below 15%, except at the LOQ, when precision and accuracy were accepted if less than 20% (Shah *et al.* 1992; Bressolle *et al.* 1996). All values passed the acceptance criteria. Although still within acceptable limits, lower concentrations generally performed worse than middle and high concentrations as the impact of interference from the sample matrix was proportionally greater. Paracetamol mercapturate preformed worst of all compounds, in keeping with its late elution and poor peak shape.

The lower concentration of 0.3125µg/mL was originally included as the lower value for this compound but this concentration failed tests for precision. Accordingly, the LOQ was raised to 0.625 µg/mL, which fell within acceptable limits (Shah *et al.* 1992). For paracetamol mercapturate the increase in LOQ resulted in only six standards being used to construct the standard curve. As standard curves are required to contain a minimum of five values this was still acceptable (Bressolle *et al.* 1996).

Table 3.2-3 Validation results for plasma HPLC assay

	LOQ	LOD	Conc	Precision (%RSD)		Accuracy (% recovery)	
		µg/mL		Intra-batch	Inter-batch	Intra-batch	Inter-batch
Paracetamol glucuronide	0.3125	0.156	0.625	9.279	8.948	97.945	98.022
			10	3.802	2.858	102.87	107.477
			80	1.104	3.304	100.347	101.229
Paracetamol sulphate	0.625	0.156	0.625	4.881	6.967	102.777	95.622
			5	3.81	5.201	101.533	98.679
			40	1.213	2.335	100.598	99.882
Paracetamol	0.3125	0.156	0.625	6.081	6.466	104.102	94.616
			5	2.069	2.269	97.794	101.692
			40	7.297	7.209	100.114	100.766
Paracetamol cysteine	0.3125	0.156	0.3125	9.922	12.461	90.121	87.439
			5	2.822	4.204	98.415	99.174
			20	2.258	3.287	100.117	100.4
Paracetamol mercapturate	0.625	0.156	0.625	11.055	15.432	88.152	86.197
			5	3.47	2.276	101.871	102.459
			20	0.917	1.912	99.955	99.892

3.2.1.1.5 STABILITY

Stability of paracetamol in human plasma was examined. There was no evidence of degradation of paracetamol in storage for the conditions and durations tested (Table 3.2-4).

Table 3.2-4 Stability of paracetamol

Storage Condition	Duration	Found ±SD (µg/mL)	Precision (RSD %)	Accuracy (%)
Freezer -80°C	18 months	9.907 ±0.423	3.881	99.002
Freezer -20°C	6 months	10.191 ±0.306	2.736	101.907
Fridge 4°C	1 week	10.165 ±0.432	3.876	101.65
Bench top	24 hours	10.104 ±0.347	3.126	101.04

3.2.2 URINE

3.2.2.1 HPLC VALIDATION

The HPLC assay for urine analysis was revalidated as the chromatographic conditions were different to those of the plasma assay. Specifity, LOQ, LOD, precision and accuracy were determined in the same manner as described in Section 3.2.1.1.

3.2.2.1.1 SPECIFICITY/SELECTIVITY

The order of elution was different to the plasma analysis, with paracetamol cysteine and sulphate switching elution order (Figure 3.2-4).

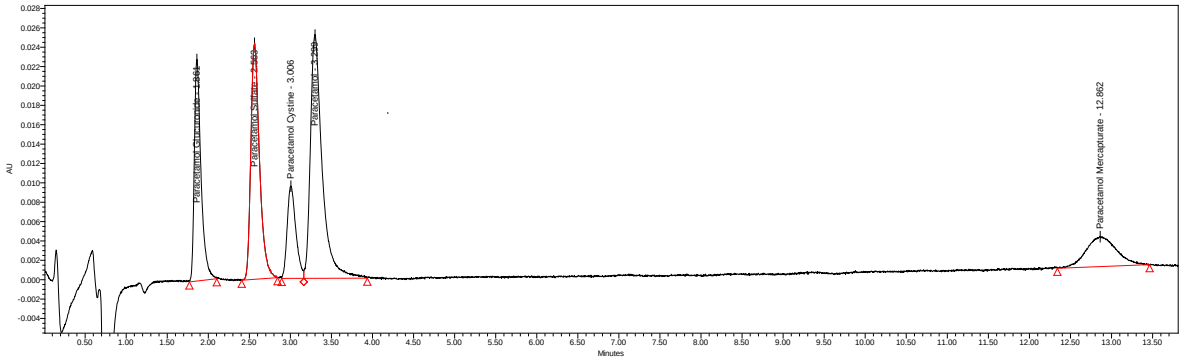


Figure 3.2-4 Sample chromatogram of absorbance of paracetamol and its metabolites
Chromatogram is from injection of a 20µg/mL solution, captured at 242nm. The elution order and times (mins) were paracetamol glucuronide (1.86), paracetamol sulphate (2.56), paracetamol cysteine (3.01), paracetamol (3.30) and paracetamol mercapturate (12.86) respectively.

Identity of the peaks was confirmed by UV spectra, as seen in Figure 3.2-5.

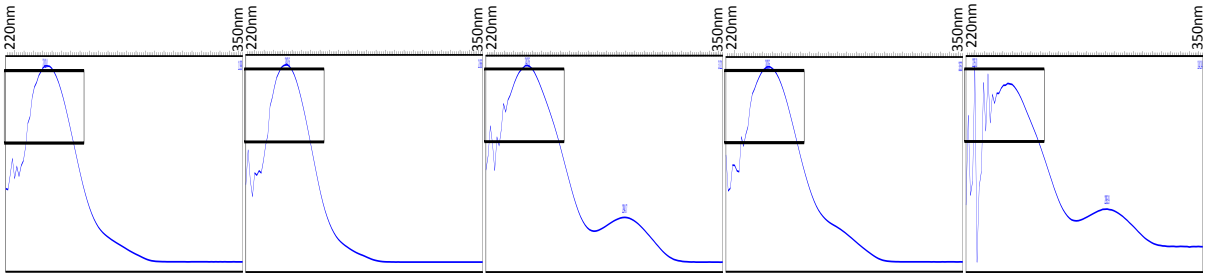


Figure 3.2-5 PDA spectra of compound peaks.
From left: Paracetamol Glucuronide; Paracetamol Sulphate; Paracetamol Cysteine; Paracetamol; Paracetamol Mercaptopurate. The additional absorbance peaks of Paracetamol Cysteine and Mercapturate occur at around 297nm

3.2.2.1.2 CALIBRATION CURVES

Linearity was assessed as detailed in Section 2.2. Table 3.2-5 shows all calibration curves passed tests for linearity.

Table 3.2-5 Tests of linearity of calibration curves

Linearity		Intra-batch		Inter-batch	
		Mean	95% CI	Mean	95% CI
Paracetamol glucuronide	Intercept	6481.754	± 11450.589	4940.385	± 14343.605
	Slope	60309.22	± 1078.379	60195.84	± 1030.175
	Correlation	1	± 0	1	± 0
Paracetamol sulphate	Intercept	-1145.34	± 12817.482	2167.087	± 10390.411
	Slope	108429.9	± 1170.996	107687.5	± 1927.475
	Correlation	1	± 0	1	± 0
Paracetamol	Intercept	-8070.76	± 14594.39	3991.286	± 34133.937
	Slope	147483	± 3405.818	145076.7	± 9248.016
	Correlation	1	± 0	1	± 0
Paracetamol cysteine	Intercept	4798.094	± 6104.336	3959.116	± 7028.147
	Slope	29389.54	± 1451.676	28797.85	± 1718.671
	Correlation	1	± 0	0.998	± 0.004
Paracetamol mercapturate	Intercept	-774.736	± 5550.438	2266.339	± 10125.641
	Slope	32156.99	± 2697.149	32054.07	± 5265.747
	Correlation	0.999	± 0.001	1	± 0.001

3.2.2.1.3 PRECISION AND ACCURACY

Whilst defrosting the first set of urine samples an amount of yellow coloured flocculent precipitate settled at the bottom of the urine sample container. Initially care was taken not to disturb the precipitate and the sample was drawn from the clear, yellow urine. It was assumed that, given their solubility, the paracetamol compounds under investigation would be homogenously dissolved in the urine. During the validation, five of the closed urine sample containers were inadvertently knocked over and agitated just prior to preparation for injection. These samples were then shown to have markedly higher concentrations of paracetamol metabolites than their previous analysis, although all other non-agitated samples and standards absorbed the same (Figure 3.2-6). Graph F of this figure also demonstrated that while the measured concentrations on compounds had reduced, all compounds were affected to approximately the same degree, and the metabolite ratio was not altered. It was suspected the defrosting process created a solvent stratification and a resulting inhomogeneous analyte distribution within the sample container. All subsequent samples were vigorously shaken prior to sampling. Validation then proceeded. Results from this validation are shown in

Table 3.2-6 and were found to be within acceptable limits (Shah *et al.* 1992).

Table 3.2-6 Validation

	LOQ	LOD	Conc	Precision(%RSD)		Accuracy(% recovery)	
	µg/mL			Intra-batch	Inter-batch	Intra-batch	Inter-batch
Paracetamol glucuronide	0.3125	0.156	1.25	5.252	3.788	99.018	112.534
			10	1.079	0.542	99.906	99.616
			80	0.972	1.248	100	100.263
Paracetamol sulphate	0.625	0.156	0.625	7.183	9.642	101.322	96.766
			5	1.531	1.601	99.529	100.337
			40	0.501	1.134	99.997	99.987
Paracetamol	0.3125	0.156	0.625	10.078	10.508	98.746	95.696
			5	0.448	0.373	100.136	100.195
			40	1.065	3.162	100.031	99.976
Paracetamol cysteine	0.3125	0.156	0.3125	7.576	14.447	91.133	118.922
			5	1.058	8.718	97.825	96.429
			20	2.023	2.387	100.464	100.822
Paracetamol mercapturate	0.625	0.3125	0.3125	10.738	17.851	117.801	112.277
			5	3.683	8.544	103.452	102.78
			20	3.614	7.568	99.556	99.512

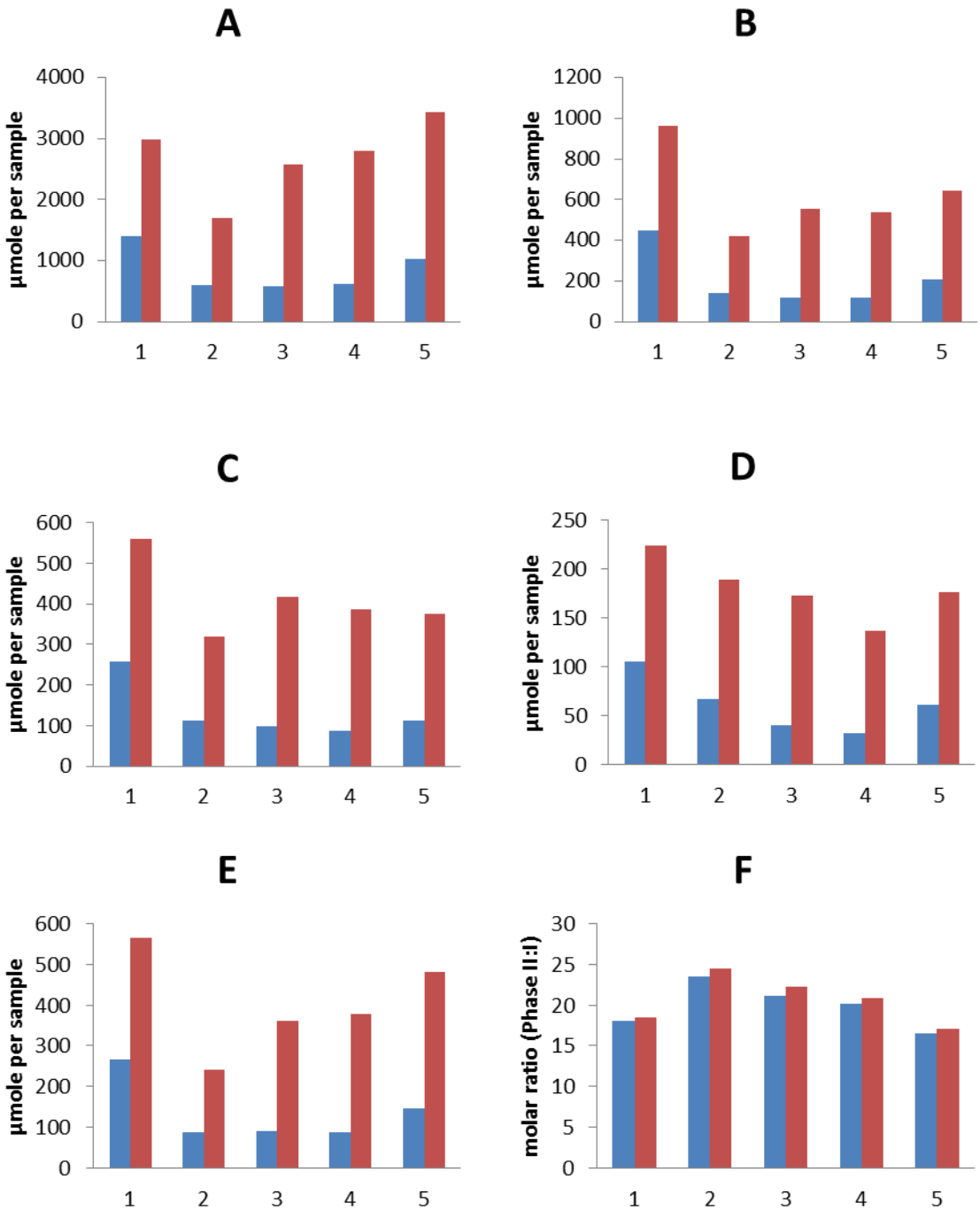


Figure 3.2-6 Effect of shaking urine sample on the amount of analyte measured

Graphs show paracetamol (A) and its metabolites (paracetamol glucuronide (B), paracetamol cysteine (C), paracetamol sulphate (D), paracetamol mercapturate (E)) measured in five different urine samples. First bar (blue) shows the amount in measured in the sample before shaking and the second bar (red) the amount after shaking. While the amount recovered was reduced, graph F shows the ratio of the metabolites and their relative concentration was maintained.

3.2.3 SUMMARY

Two assays for HPLC were developed and validated. Both were found to surpass validation requirements.

3.3 URINE RESULTS

Urine analysis is a useful tool for assessing changes to drug metabolism as the metabolic products of most drugs, including paracetamol, are mainly excreted in the urine (Siegers *et al.* 1984). Metabolites are usually found in high concentrations and sample preparation of urine is usually straight forward. While studies of paracetamol's metabolism and disposition frequently show considerable inter-subject variation, the fractional urinary recovery of each of paracetamol's conjugates remain remarkably constant within each subject (Clements *et al.* 1984; Kietzmann *et al.* 1990). Fractional urinary recovery can be used to prepare metabolic ratios that assess the relationship between drug metabolites and/or parent compounds and provide insight into changes of drug metabolism. These ratios are a composite measure and depend on both metabolic partial clearance and renal partial clearance. For the metabolic ratio to be accurate as a tool to assess changes to metabolic partial clearance of any drug, renal partial clearance must remain relatively constant. When this is achieved, changes to fractional urinary recovery can be attributed to something other than random intra-individual variation (Miners *et al.* 1992).

Paracetamol, although freely filtered at the glomerulus, is subject to substantial, but incomplete, tubular resorption (Miners *et al.* 1992). Tubular reabsorption can potentially cause significant alterations to renal partial clearance of drugs and is dependent on two factors:

- Urine pH (compounds must be unionised to be resorbed); and
- Urine flow rate (resorption efficiency decreases with increased urine flow) (Miners *et al.* 1992).

Paracetamol is a weak acid (pKa 9.5) and is essentially unionised at all urinary pH values; accordingly renal partial clearance of paracetamol is independent of urinary pH (Forrest *et al.* 1982; van der Marel *et al.* 2003). However, during periods of severe dehydration and reduced urine flow, paracetamol's partial renal clearance is reduced (Miners *et al.* 1992). This can have a large effect on metabolic ratios that contain the excretion of unchanged paracetamol and obscure changes to metabolic partial clearances.

Conversely, the increased solubility and active tubular secretion of the major urinary metabolites exempt them from the factors that affect paracetamol's partial renal clearance. As a result their elimination is independent of urine flow and urine pH

(Kietzmann *et al.* 1990; Miners *et al.* 1992). Additionally, excretion of paracetamol and its metabolites are also independent of creatinine clearance (Kietzmann *et al.* 1990).

It is a central hypothesis of this Thesis that paracetamol metabolism changes around the time of surgery. This hypothesis was to be tested in the following manner:

- Quantification of factors known to affect fractional urinary recovery (changes to urine volume and percentage of the dose recovered in the urine);
- Assessment of the contribution of paracetamol and each of the metabolites to overall dose recovery;
- Determination of the contribution of sulphate containing metabolites to the overall dose recovery;
- Preparation of a metabolic ratio to examine changes to the Phase II and Phase I pathways involved in paracetamol's metabolism; and
- Assessment of the factors that potentially impact on this metabolic ratio.

3.3.1 ANALYSIS OF RESULTS- PREAMBLE

Urine was collected every four hours from each patient in the study. This resulted in nearly 1000 urine samples for analysis. Information from each sample included:

1. Patient and patient group;
2. Sample time and day;
3. Volume of urine excreted over collection period; and
4. The concentration of paracetamol and each of its four metabolites of interest.

These values were then used to calculate amounts and ratios of excreted metabolites. For the purposes of analysis of the urine results, concentrations which were determined to be below the LOD were set to 0 and concentrations between the LOQ and LOD were set to half the LOD. (Shah *et al.* 1992)

With at least 30 samples per patient, collected from samples taken every four hours over a five day period, clear trends emerged graphically, however summarising the data for meaningful statistical analysis was difficult. As an example, Group A's urinary paracetamol glucuronide concentrations are presented in Figure 3.3-1.

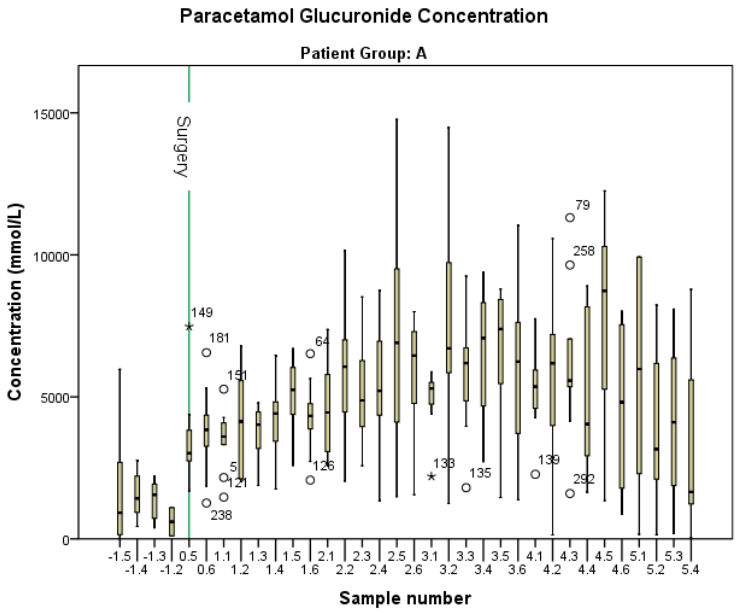


Figure 3.3-1 Box plot of Group A Paracetamol Glucuronide concentration from all samples
Concentration displayed as measured in four hour urine collections. Green line marks first sample obtained following surgery. Samples numbers are given as day.sample

Table 3.3-1 Sampling intervals, corresponding pooled intervals, (used in statistical analysis), and graph intervals

Sample Day Notes on interval timing	**Sample Interval (hr)**	**Pooled Interval**	**Graph Day**	**Graph Sample No.**
Preoperative day Time taken from beginning of preoperative paracetamol dose	0-4hrs	-1	-1	-4
	4-8hrs			-3
	8-12hrs			-2
	12-16hrs			-1
Day of Surgery Times by clock	1400-1800	0	0	5
	1800-2200			6
Day 1 Postop Times by clock	2200-0200	1	1	1
	0200-0600			2
	0600-1000			3
	1000-1400			4
	1400-1800			5
	1800-2200			6
Day 2 Postop Times by clock	2200-0200	2	2	1
	0200-0600			2
	0600-1000			3
	1000-1400			4
	1400-1800			5
	1800-2200			6
Day 3 Postop Times by clock	2200-0200	3	3	1
	0200-0600			2
	0600-1000			3
	1000-1400			4
	1400-1800			5
	1800-2200			6
Day 4 Postop Times by clock	2200-0200	4	4	1
	0200-0600			2
	0600-1000			3
	1000-1400			4
	1400-1800			5
	1800-2200			6

An analysis of variance, such as a Friedman's test, on these data would almost certainly show a significant difference at $p<0.05$ occurred across the study. However, with up to 34 samples per patient, determining where the significant differences occurred in a full *post hoc* test would require nearly 600 Wilcoxon signed rank tests. While useful for displaying graphical trends, the clinical relevance of statistical results from one four hour interval to another was questionable. Consequently, results for descriptive and statistical purposes were pooled. As much of the literature investigating multiple dose paracetamol excretion uses 24 hour urine collections, 24 hour intervals were also chosen for the pooling of samples for this Thesis. The results for each patient's four hourly samples were summed over each 24 hour interval as shown in Table 3.3-1. To coincide with study drug administration, each "day" began at 2200 hours as opposed to midnight. The same data in Figure 3.3-1 are shown after pooling was performed in Figure 3.3-2.

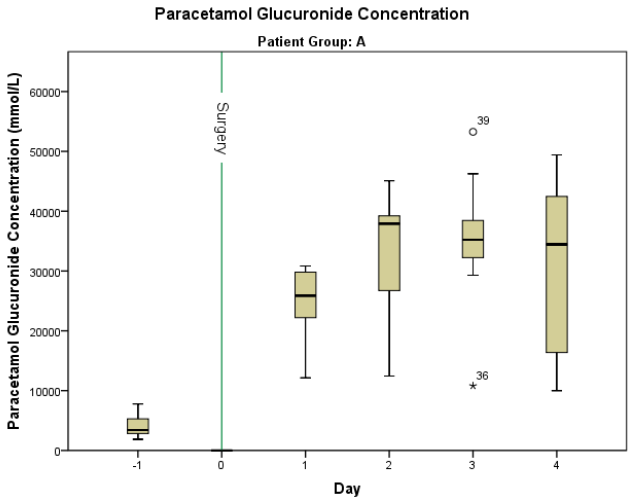


Figure 3.3-2 Box plot of Group A Paracetamol Glucuronide concentration after pooling of sample times
Concentration displayed after samples pooled across day. Green line marks first sample obtained following surgery.

The concern with pooling data in this way is the loss of variance, which can arise from the "smoothing out" of changes to metabolite concentration that occur from one four hour sample to the next. The effect of any loss of variance occurring from the pooling was assessed graphically and examples are shown below (Figure 3.3-3). The left plot shows boxes and whiskers that vary between each other in size and length, demonstrating the differences in paracetamol glucuronide concentrations that occur over the day. This is lost when the values are summed as shown in the right-hand plot. Friedman's tests were conducted to assess if the variation between the four-hour samples within a 24 hour set of samples (such as those in the left plot in Figure 3.3-3) were statistically significant and the majority were.

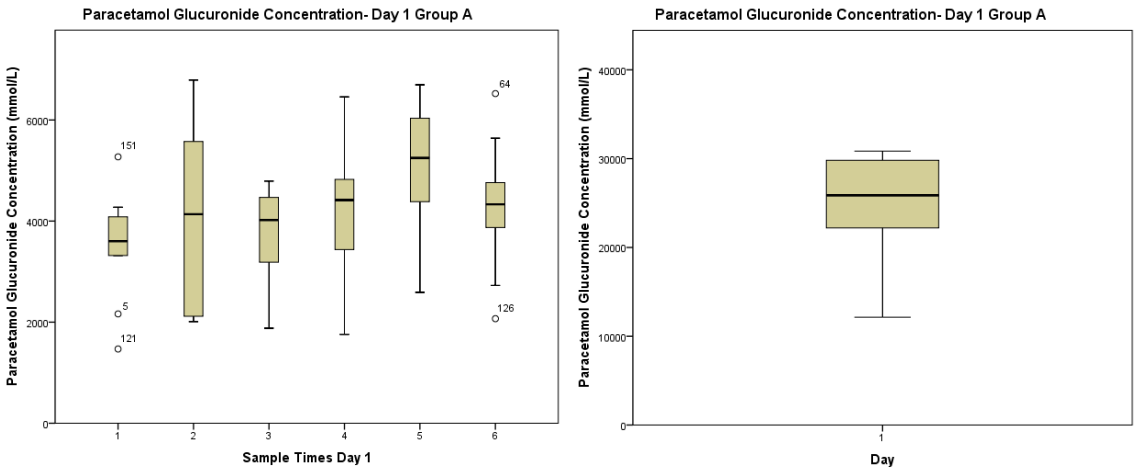


Figure 3.3-3 Box plot of Group A Paracetamol glucuronide Concentration on Day 1 before (left) and after pooling (right)

These revealed that there were significant differences between the sample intervals even within each 24hr period. Accordingly, the statistical results that arise from these pooled values that were used in the analysis below are likely to underestimate the statistical significance of differences. Consequently, data are also presented graphically. While statistical analysis necessitated pooling values, graphical and regression analysis used the full data set (4hr samples). In graphs, sample and day numbers correspond to the times shown in Table 3.3-1. The critical level of significance used in this analysis is $\alpha \leq 0.05$.

3.3.2 DISTRIBUTION OF URINE RESULTS

For each patient group, the summed results for each day were checked for normality visually by preparing a histogram with normal curve superimposed, a Q-Q plot and a box and whisker plot. In addition, formal normality tests were conducted using the Kolmogorov-Smirnov test (D). No result consistently passed normality tests across the groups.

Based on the shape of the histograms, the $\log_{10}$ of the daily values were taken and normality tests repeated. While generally improving normality visually, there was still clear deviation in the histograms and Q-Q plots and the Kolmogorov-Smirnov tests were still significant. For completeness, other transformations were attempted including the natural log, inverse, square and square root of the values, but none improved the distribution beyond that of the $\log_{10}$ transformation. As normal distributions could not be obtained from these transformations, non-parametric methods were used for the description and testing of differences within variables across the samples collected.

3.3.3 URINE VOLUME

The volume of urine was expected to change over the course of recovery as a result of the stress response and the challenges to fluid balance presented by surgery. Crucially, reductions to urine output can affect the excretion of each of paracetamol's metabolites differently. The total volume of urine collected over each four-hour interval was measured and summed for each 24hr interval. This figure was then divided by 24 to give average hourly urine output for each 24hr interval.

Descriptive statistics for the urine output are shown. Exact Friedman's tests were used to assess differences across all days of the study and again over the postoperative days alone. This was followed by one or two tailed Wilcoxon signed rank tests, where appropriate, for *post hoc* analysis.

3.3.3.1 GROUP A

A total of 293 urine samples were collected from patients in Group A. The group median volumes and IQRs of each four-hour sample are shown in Figure 3.3-4. It can be seen that urine output changed considerably over the course of the study. Before and for the day and a half following surgery, median urine volumes were consistent, between 40 and 70mL/hr. Following this oliguria, diuresis occurred with outputs rising swiftly to reach a maximum median of 309mL/hr. Outputs then plateaued (Figure 3.3-4) onto Day 4.

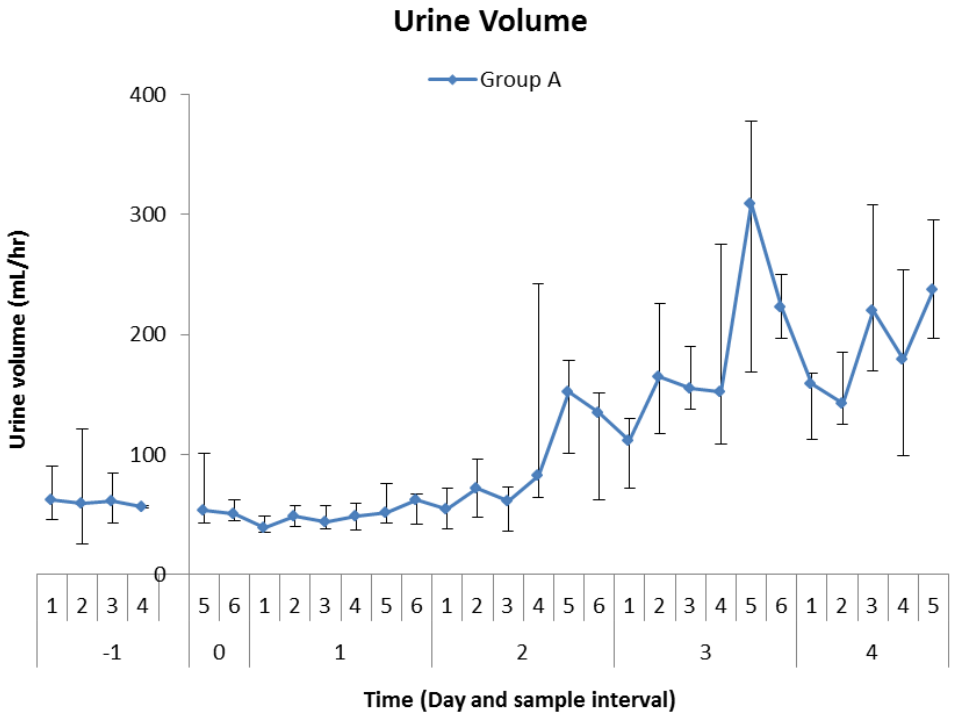


Figure 3.3-4 Four hourly urine volumes collected over study (median±IQR) Group A

Initially IQRs were small, most notably on the day following surgery; however, the increases in median outputs later in the study were accompanied by widening IQRs. The median and IQRs for the summed daily values are presented in Table 3.3-2. Large increases in output were noticed on Day 3 and 4, accompanied by widening IQRs.

Table 3.3-2 Median and interquartile ranges for the summed daily urine volume

mL/hr	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	62, (44, 85)	51 (35, 63)	92 (65, 141)	168 (127, 239)	151 (136, 251)

To examine for changes between the distributions of urine volume over the course of the study exact Friedman's tests were used on summed urine volumes. All values were first examined, followed by only postoperative volumes. The results are shown in Table 3.3-3. These results confirm what was observed graphically, that there were significant differences in urine volumes across the whole study (χ^2=29.067, p<0.000) and also just over the postoperative period (χ^2=20.067, p<0.000).

Table 3.3-3 Friedman's test examining differences in urine volume across study
** = significant difference at p<0.05*

χ2 (p)	24.800 (<0.000)*
χ2 (p)Postop only	20.067 (<0.000)*

As the direction of change of urine volume was unknown, *post hoc* tests were conducted using 2-tailed, exact Wilcoxon signed rank tests to examine on what days significant differences in urine output occurred. Tests between the preoperative day and postoperative days were conducted, along with examining the first postoperative day and all the subsequent postoperative days (Table 3.3-4). To limit the size of the Bonferroni correction and Type I error, comparisons for the following days were not conducted.

Table 3.3-4 Group A, *post hoc* pair-wise analysis (p) of urine volume
** = significant difference at p<0.05*

Group A		
1	0.037*	
2	0.064	0.01*
3	0.008*	0.004*
4	0.008*	0.004*
Day	**-1**	**1**

All comparisons showed highly significant differences. However, as there were 7 comparisons in the *post hoc* analysis, a Bonferroni correction of α/7 needed to be applied. This reduced the critical level of significance to p>0.0072, leaving only the comparisons between Day 1 and Days 3 and 4 below this level. Differences were of

greater significance between the extreme ends of the study, in keeping with the values shown in Figure 3.3-4.

3.3.3.2 GROUP B

Urine output for Group B shows a similar pattern to Group A (Figure 3.3-5). Urine volumes were consistently low (30-40mL/hr) before and until the end of the first postoperative day. Thereafter diuresis developed with median outputs increasing to over 250mL/hr (sample 3.3) with one individual producing 400mL/hr in one four hour period (Patient 4, sample 3.6) and 5.6L that day. The descriptives for the summed values are presented in Table 3.3-5 and Figure 3.3-5.

Table 3.3-5 Median and interquartile ranges for the summed daily urine volume
** = significant difference at p<0.05*

mL/hr	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	23 (18, 45)	32 (26, 77)	117 (57, 192)	173 (84, 221)	150 (102, 209)

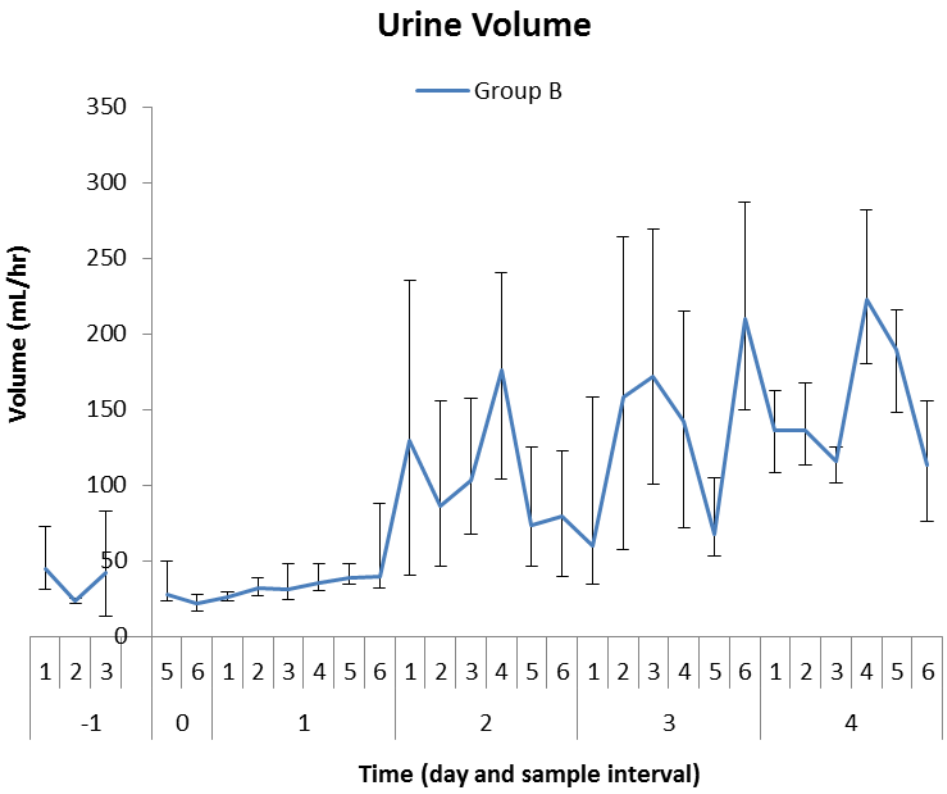


Figure 3.3-5 Four hourly urine volumes collected over study (median±IQR) Group B

Table 3.3-6 shows that there were significant differences in urine volumes across the whole study and within the postoperative period alone.

Table 3.3-6 Friedman's test examining differences in urine volume across study
** = significant difference at p<0.05*

χ2 (p)	10.667 (0.004)
χ2 (p)Postop only	8.100 (0.033)*

To determine the combinations of days that were significantly different, *post hoc* tests were conducted using Wilcoxon signed rank tests. These results are in Table 3.3-7. Despite trends being seen in Figure 3.3-4 and shown in Table 3.3-6, no significant results were found in *post hoc* tests, illustrating the increased sensitivity of a repeated measures test like Friedman's test.

Table 3.3-7 Group B, *post hoc* pair-wise analysis (p) of urine volume
** = significant difference at p<0.05*

Group B		
1	0.109	
2	0.109	0.068
3	0.109	0.068
4	0.109	0.068
Day	-1	1

3.3.3.3 GROUP C

The changes in urine production across the study period were less in Group C than other groups; however, the variation within each sample period was greater (Figure 3.3-6). This group collected their own urine for the majority of the study as they were only catheterised for a brief period following surgery. There was also less distinction between the perioperative and postoperative urine output.

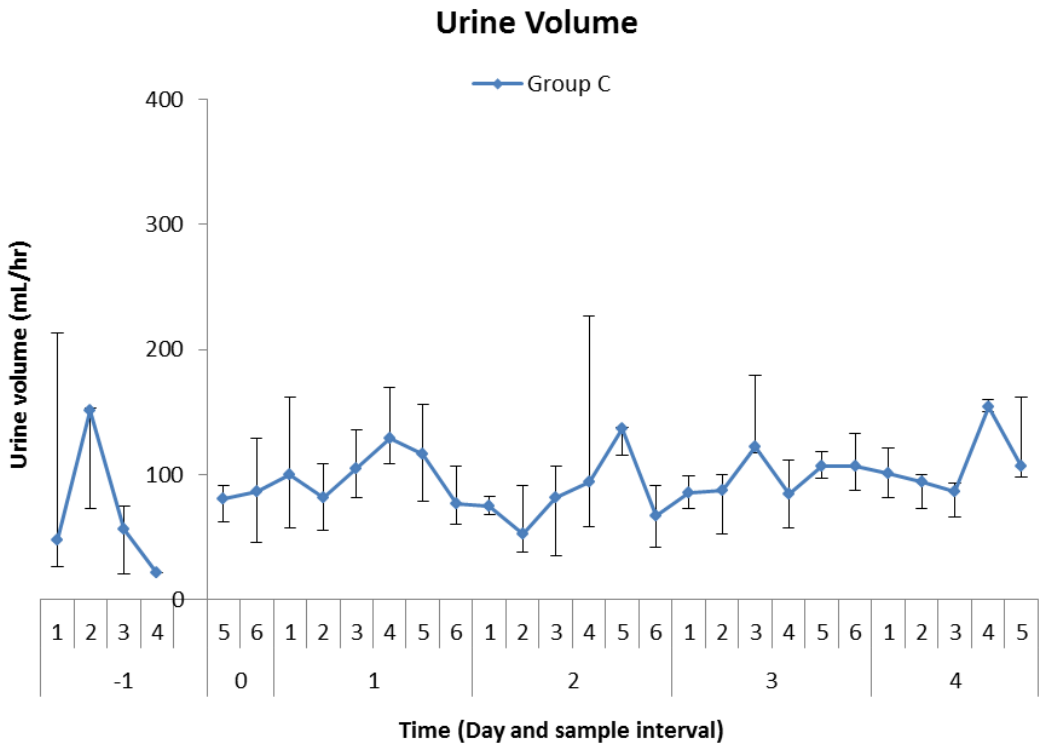


Figure 3.3-6 Four hourly urine volumes collected over study (median± IQR) Group C

Summed daily outputs were also reduced compared with other groups and were comparatively consistent over the duration of the study (Table 3.3-8).

Table 3.3-8 Summed daily urine volume- Median (LQ, UQ)

mL/hr	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	80 (40, 155)	112 (55, 133)	52 (19, 89)	71 (40, 93)	76 (35, 101)

The Friedman's test showed that there were no significant differences in urine output found in either comparison (Table 3.3-9).

Table 3.3-9 Friedman's test examining differences in urine volume across study
** = significant difference at p<0.05*

χ2 (p)	8.480 (0.066)
χ2 (p)Postop only	6.840 (0.075)

Because Friedman's tests exclude cases list-wise, *post hoc* tests were conducted to include the full data set, as these tests exclude pair-wise. This revealed that urine output was significantly higher on the first postoperative day than the rest of the postoperative period, however, once the Bonferroni correction was applied, these values lost their statistical significance (Table 3.3-10). This was the opposite of that seen in the previous two groups of patients.

Table 3.3-10 Group C, *post hoc* pair-wise analysis (p) of urine volume
** = significant difference at p<0.05*

Group C		
1	0.735	
2	0.237	0.05*
3	0.345	0.043*
4	0.345	0.043*
Day	**-1**	**1**

3.3.3.4 GROUP D

Urine outputs in Group D were greater than the other groups undergoing similar surgery types (Groups A and B) until Day 4 when they fell below. Similar to Groups A and B, there was a reduction in urine output and IQRs immediately postoperatively, but for a shorter period of time (Figure 3.3-7). Urine volumes were also more consistent across the study than Groups A+B, not exhibiting the same diuresis seen in these other groups. Variability in urine volume for each four-hour sample was wide, but consistent.

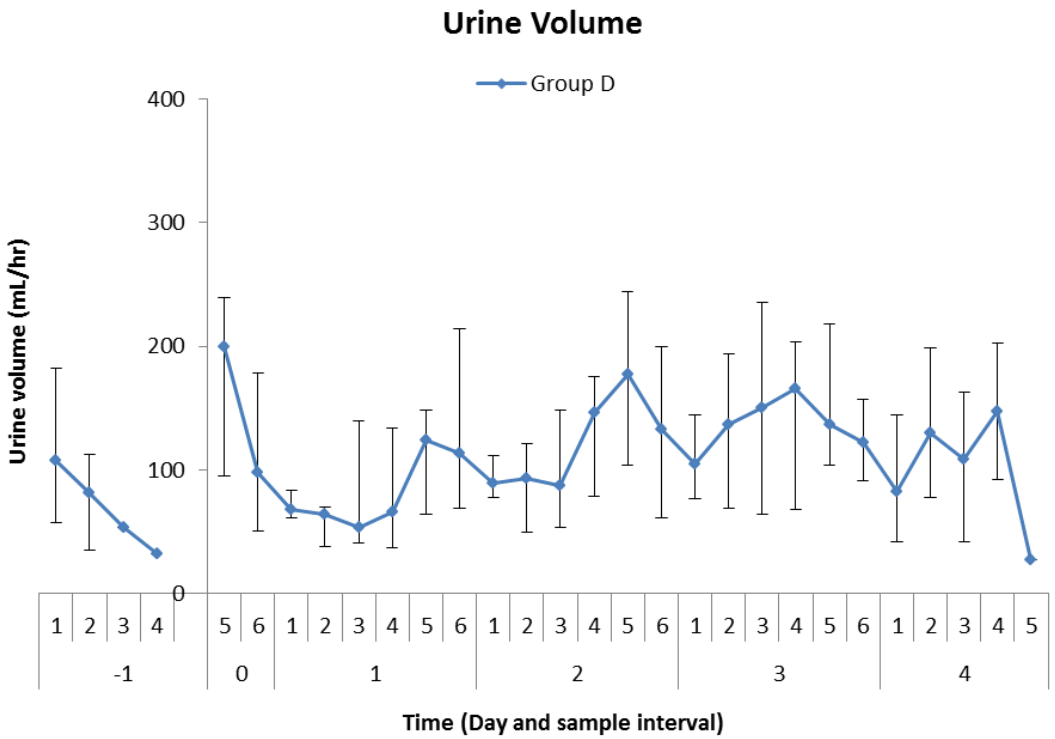


Figure 3.3-7 Four hourly urine volumes collected over study (median±IQR) Group D

The maximum median output of 275mL/hr was achieved earlier than other groups (sample 2.5), and the individual greatest output was from Patient 6D, who produced 467.5mL/hr for sample 4.2 (a total of 1870mL was measured in this four hour sample).

The consistency in urine output was clearly reflected in the daily median outputs shown in Table 3.3-5, which varied very little on Days 1, 2, and 3, and fell away on Day 4. IQRs were large but also remain consistent.

Table 3.3-11 Summed daily urine volume- Median (LQ, UQ)

mL/hr	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	61 (55, 87)	110 (66, 137)	107 (83, 134)	113 (73, 149)	50 (18, 114)

Friedman's tests were again conducted and revealed no significant results (Table 3.3-12).

Table 3.3-12 Friedman's test examining differences in urine volume across study
*= significant difference at p<0.05

χ2 (p)	6.500 (0.167)
χ2 (p)Postop only	4.200 (0.242)

For the reasons mentioned in Section 3.3.3.3, *post hoc* tests were still conducted. These showed significant differences in the comparison of the preoperative urine volume with Day 1, 2 and 3 postoperatively. This is consistent with the values shown in Table 3.3-11, however, as in previous groups, these values were not statistically significant (Table 3.3-13) once the Bonferroni correction had been applied.

Table 3.3-13 Group D, *post hoc* pair-wise analysis (p) of urine volume
*= *significant difference at p<0.05*

Group D		
1	0.019*	
2	0.014*	0.638
3	0.049*	0.695
4	0.641	0.547
Day	-1	1

3.3.3.5 GROUP B+D

As a combination of two groups already discussed, this group exhibited trends similar to that of Group D, who make up the majority of patients. The influence of Group B was to lower median urine outputs preoperative and Day 1 postoperatively, increasing them for subsequent days (Figure 3.3-8). IRQs remained largely similar to those of Group D. Peak median output occurred in interval 3 on Day 3 to just over 200mL/hr. Following the trends seen in Figure 3.3-8, the summed outputs increase steadily from the preoperative day until Day 4 when they decline for the first time. IQRs show accompanying increases and are mostly symmetrical about the median until Day 4 when they show a positive skew (Table 3.3-14).

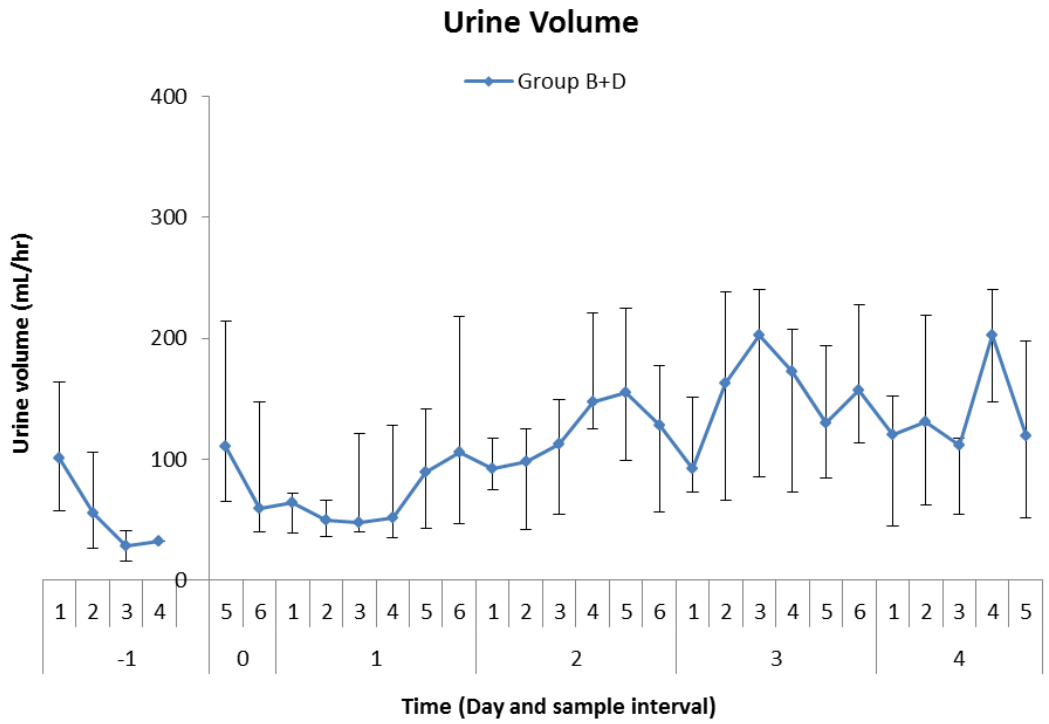


Figure 3.3-8 Four hourly urine volumes collected over study (median±IQR) Group B+D

The skew indicates asymmetry of the distribution derived from the fact that the urine outputs above the median are of greater magnitude, stretching further away from the median, than those beneath it, which are clustered closer to the median (Table 3.3-14).

Table 3.3-14 Summed daily urine volume- Median (LQ, UQ)

mL/hr	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	56 (22, 84)	91 (34, 129)	107 (80, 154)	129 (77, 171)	79 (39, 158)

Once again Freidman's tests were conducted, but did not show significant results (Table 3.3-15). As previously, two tailed *post hoc* tests were conducted.

Table 3.3-15 Friedman's test examining differences in urine volume across study
** = significant difference at p<0.05*

χ2 (p)	8.436 (0.072)
χ2 (p)Postop only	4.900 (0.186)

These show that the preoperative output was significantly lower than that of Days 1, 2 and 3, which was maintained even after the Bonferroni correction had been considered. There was no significant difference at p<0.05 between the outputs of the postoperative days (Table 3.3-16).

Table 3.3-16 Group B+D, *post hoc* pair-wise analysis (p) of urine volume
** = significant difference at p<0.05*

Group B+D		
1	0.005*	
2	0.002*	0.151
3	0.008*	0.135
4	0.365	0.470
Day	**-1**	**1**

3.3.4 PERCENTAGE OF DOSE RECOVERED IN THE URINE

The molar amounts of all metabolites of paracetamol in each urine sample were summed and divided by the paracetamol dose to give the per cent of the dose recovered in the urine. As paracetamol dose and administration intervals varied preoperatively and postoperatively, and between groups, determining what dose to divide the recovered amounts by was calculated as follows:

- For preoperative samples, as they arose from a single dose of paracetamol, the amounts of metabolite in all preoperative urine samples were summed together, divided by the dose of paracetamol administered (paracetamol 1g=6.6mmol, 1.5g=9.9mmol) and are reported as a single preoperative value "*All*"; or
- For postoperative samples, as they arose from steady state, the amounts of metabolite were again summed, but were divided by the dose administered over the four-hour urine collection period. This was determined by summing the total 24hr paracetamol dose and dividing by six, the number of four-hour collection

intervals that occurred within a 24hr period. Therefore, for Group A, the sum amount of metabolite in each sample was divided by 9g/6 = 1.5g = 9.9mmol. For the remaining groups, the sum of metabolites was divided by 4g/6 = 0.67g = 4.4mmol.

For the daily per cent recovered figures, metabolite amounts for the whole day were summed and divided by the 24hr paracetamol dose (59.5mmol for Group A, 26.5mmol for other groups). In this analysis, changes to dose recovery postoperatively could indicate changes to excretion, confounding the interpretation of changes to metabolic ratio.

3.3.4.1 GROUP A

Changes to urinary dose recovery are shown in Figure 3.3-9. Recovery of dose preoperatively is poor with a median of 61.5% for Day-1.

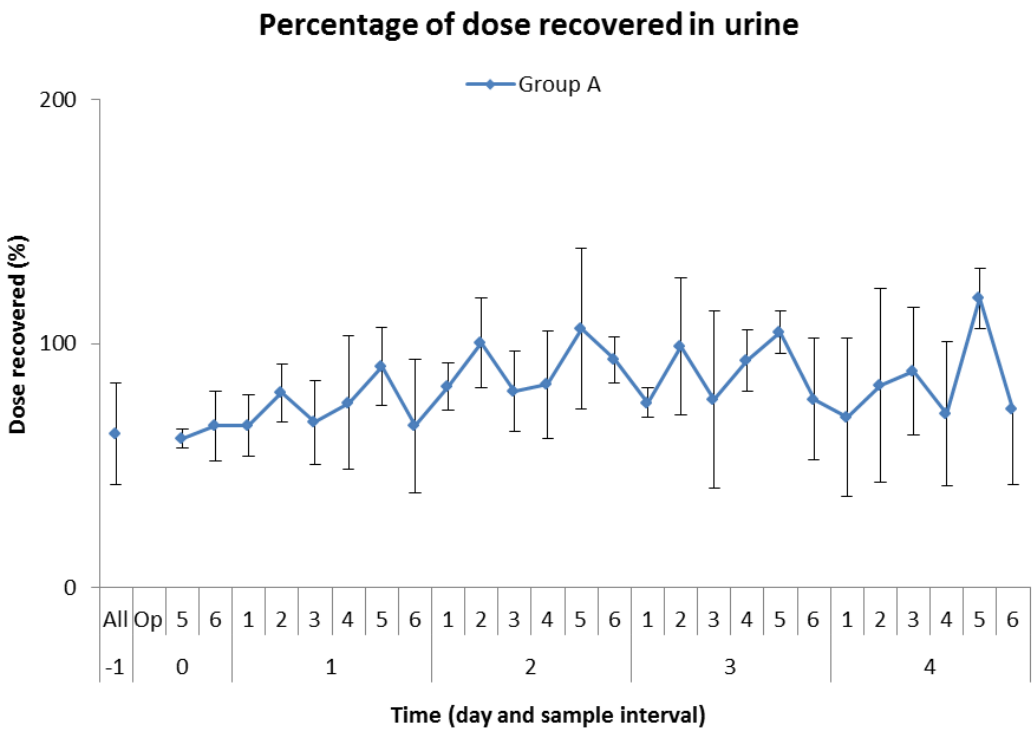


Figure 3.3-9 Per cent of administered paracetamol dose recovered in the urine from four hourly collection
Results shown as median % (±IQR)

Following surgery, recovery increased and remained consistent, with the median peaking on Day 2, at 106% of dose. There was no obvious pattern relating to time of day or time since surgery. IQRs increase in the later days of the study and appear largely symmetric. Daily values exhibited a similar pattern to those seen graphically from the 4hr samples. Median values (Table 3.3-17) remained consistent, up to 94% on Day 2, as did IQRs which stretch beyond the 100% recovery of dose from Day 2 until the end of the study.

Table 3.3-17 Summed daily recovery of dose in the urine - Median (LQ, UQ)

%	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	63.17 (52.02, 83.98)	79.96 (59.03, 86.85)	94.27 (78.59, 107.68)	82.57 (74.73, 106.09)	75.81 (38.89, 106.74)

Analysis of these values using an exact Freidman's tests (Table 3.3-18) showed that the distribution of the postoperative values was significantly different.

Table 3.3-18 Friedman's test examining differences in urinary recovery of dose across study
*= *significant difference at p<0.05*

χ2 (p)	7.378 (0.116)
χ2 (p)Postop only	8.333 (0.036)*

A *post hoc* analysis was undertaken using exact 2-tailed Wilcoxon sign-rank tests (Table 3.3-19). For reasons mentioned in Section 0, a significant difference at p<0.05 was found in increase of Day -1 values to Day 2 values (p=0.027), which was not detected by the Friedman's test, although these failed to reach the level of significance after the Bonferroni correction. The significant result from the Friedman's test in the analysis of the postoperative days arose from the increase from Day 1 to Day 2 which shows a significance of p=0.01. This also failed to reach significance after the Bonferroni correction had been applied

Table 3.3-19 Group A, *post hoc* pair-wise analysis (p) of urinary recovery of dose
*= *significant difference at p<0.05*

Group A		
1	0.232	
2	0.027*	0.01*
3	0.25	0.164
4	0.652	0.734
Day	-1	1

3.3.4.2 GROUP B

Following surgery, urinary recoveries of paracetamol were consistently around the 100% value (Figure 3.3-10). The highest median recovery was 134% on the evening of the day of surgery, when patients would have received a loading dose intraoperatively and lowest in the first sample taken on Day 3. Considering the lower recoveries found in the samples either side of this one, and that it was collected after all patients had had their urinary catheters removed, this may indicate incomplete evacuation of urine within each four hour period and accumulation of the dose in the bladder. This is supported by the fluctuations in urine output seen in Figure 3.3-5. Preoperative recovery was again low at approximately 40% of the administered dose. Further inspection of the values that

contributed to this summed value shown, revealed that, while other patients were excreting 60-80% of the dose in the first preoperative four hour urine collection, patients in Group B excreted approximately 20% of the dose.

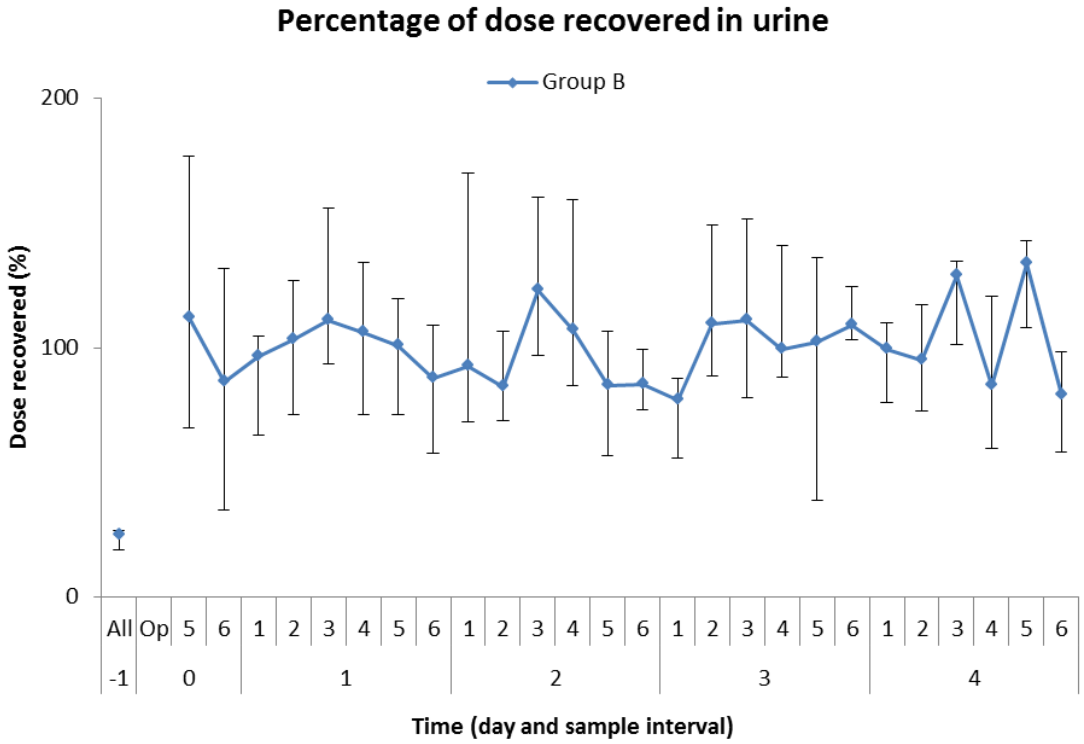


Figure 3.3-10 Per cent of administered paracetamol dose recovered in the urine from four hourly collection
Results shown as median % (±IQR)

While the second urine sample contained a slightly higher metabolite concentration than most other groups, it still only contributed a further 20% to the recovery. The descriptives of the daily values in Table 3.3-20 reflect this consistency in dose recovery, with medians varying less than four per cent over the postoperative period.

Table 3.3-20 Summed daily recovery of dose in the urine - Median (LQ, UQ)

%	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	22.79 (14.20, 31.30)	98.89 (88.94, 110.49)	101.21 (87.95, 116.53)	104.535 (89.361, 115.174)	102.165 (92.49, 109.436)

The clear difference between preoperative and postoperative recovery seen graphically is reflected in the highly significant result of the Friedman's tests reported Table 3.3-21. Also in line with the graphical observations is the lack of significant difference between the postoperative days. *Post hoc* tests were conducted but the combinations used previously failed to show significant differences (data not shown).

Table 3.3-21 Friedman's test examining differences in urinary recovery of dose across study
** = significant difference at p<0.05*

χ2 (p)	11.467 (0.001)
χ2 (p)Postop only	3.000 (0.432)

3.3.4.3 GROUP C

Recovery of the dose in the urine of Group C patients varied widely over the duration of the study (Figure 3.3-11). This group were only briefly catheterised in the immediate postoperative period and spent the majority of the study collecting their own urine. As with the previous group, the greatest dose recovery was from the first postoperative sample, when a median of 176% of the dose was measured. The lowest recovery, 54%, was obtained at the end of Day 2 postoperatively, as was the case with Group B. IQRs of nearly 100% (sample 2.2) were also the greatest of any group and reflected the dose recovery of over 200% of two patients in this sample period.

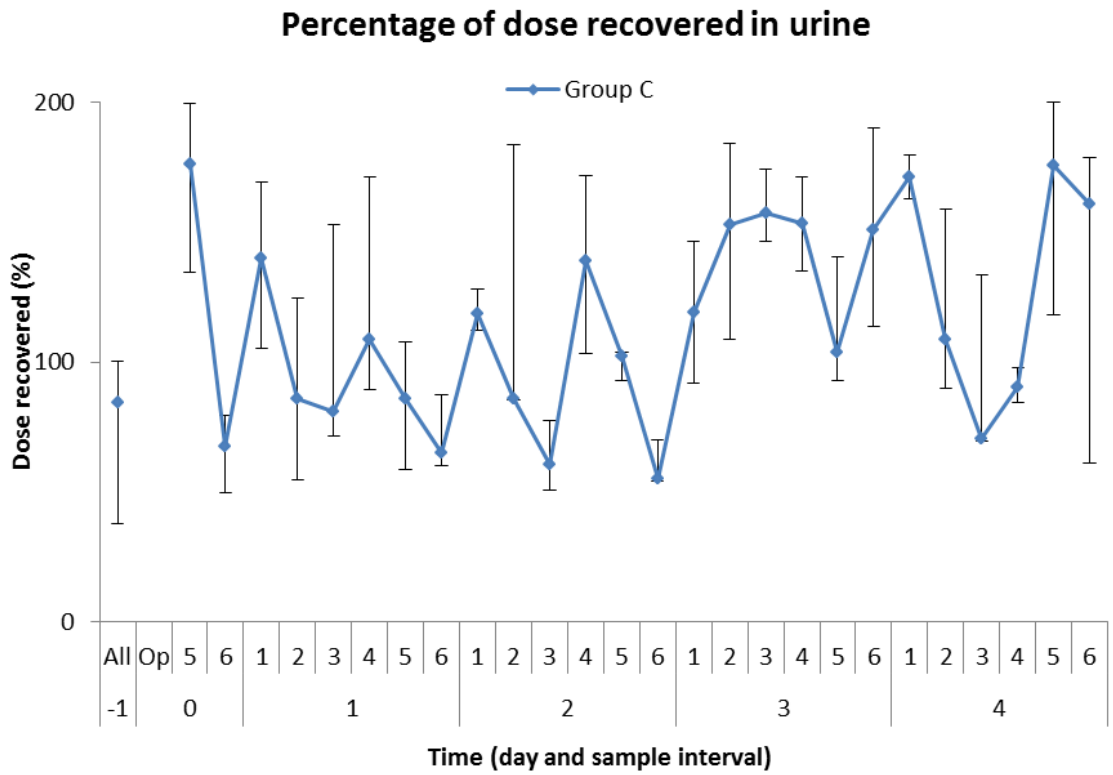


Figure 3.3-11 Per cent of administered paracetamol dose recovered in the urine from four hourly collection
Results shown as median % (±IQR)

Despite the variation described above and seen in Figure 3.3-11, descriptive values obtained from daily recovery were remarkably consistent, however, large IQRs were still seen, most notably on Days-1 and 2 when it was approximately 60% (Table 3.3-22).

Table 3.3-22 Summed daily recovery of dose in the urine- Median (LQ, UQ)

%	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	84.38 (37.91, 100.37)	96.34 (71.46, 114.54)	78.31 (29.29, 96.43)	92.08 (66.63, 104.66)	81.51 (64.00, 114.07)

Freidman's tests were conducted and no significant results were obtained (Table 3.3-23).

Table 3.3-23 Friedman's test examining differences in urinary recovery of dose across study
*= significant difference at p<0.05

χ2 (p)	2.880 (0.613)
χ2 (p)Postop only	2.280 (0.561)

3.3.4.4 GROUP D

As previously, urinary recovery from the preoperative dose was low (median 47%) (Figure 3.3-12). Following surgery, values showed some variation although no pattern occurs in relation to time of day or time since surgery. Values spike in sample 4.2 following a trough in the four previous samples. In most cases, this corresponded with catheter removal.

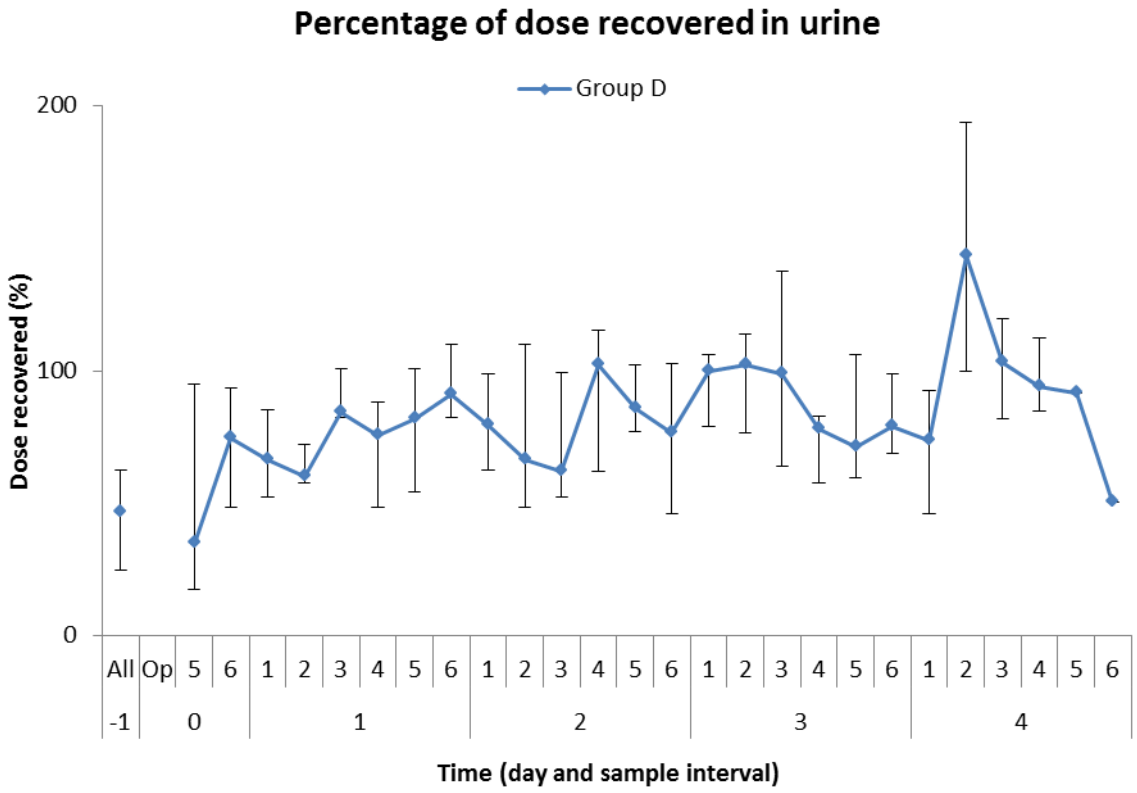


Figure 3.3-12 Per cent of administered paracetamol dose recovered in the urine from four hourly collection
Results shown as median % (±IQR)

Descriptives for the daily values show that median recoveries were less than 100% and were their lowest on the first and last day of the study (Table 3.3-24). However, several individual time points show median recoveries at or near 100% in Figure 3.3-12. IQRs were less than seen in other groups, but failed to extend to 100%.

Table 3.3-24 Summed daily recovery of dose in the urine - Median (LQ, UQ)

%	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	46.648 (24.542, 62.456)	84.807 (70.769, 85.826)	78.695 (48.55, 92.132)	78.163 (57.625, 91.724)	58.357 (25.412, 67.368)

Freidman's tests showed a significant difference at p<0.05 in the comparison including preoperative values, but did not detect a significant difference at p<0.05 within the postoperative period alone (Table 3.3-25).

Table 3.3-25 Friedman's test examining differences in urinary recovery of dose across study
** = significant difference at p<0.05*

χ2 (p)	14.6 (0.02)*
χ2 (p)Postop only	7.050 (0.068)

Post hoc tests were conducted and show the dose recovery on Day -1 was significantly lower than Days 1, 2, and 3 (Table 3.3-26). The comparison with Day 2 was below the critical level of significance after the Bonferroni correction.

Table 3.3-26 Group D, *post hoc* pair-wise analysis (p) of urine volume
** = significant difference at p<0.05*

Group		
1	0.001*	
2	0.019*	0.577
3	0.002*	0.557
4	0.641	0.148
Day	**-1**	**1**

3.3.4.5 GROUP B+D

As previously, the result of combining these two groups is mainly dominated by Group D values, as they make up the larger proportion of the examined values (Figure 3.3-13).

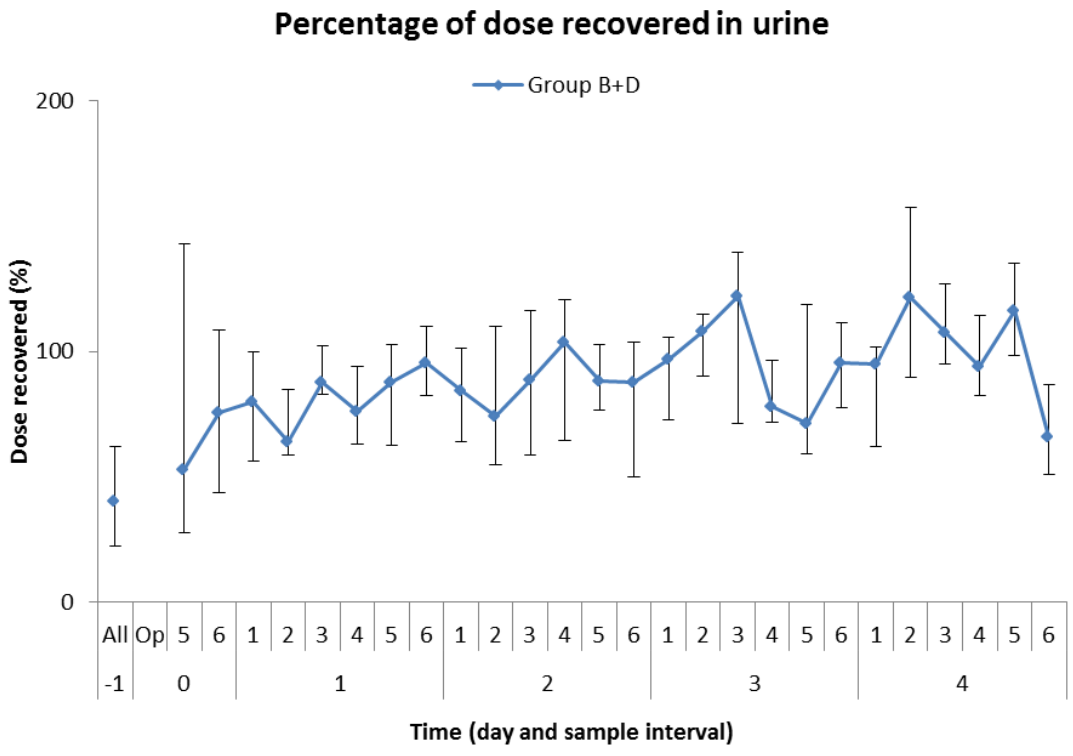


Figure 3.3-13 Per cent of administered paracetamol dose recovered in the urine from four hourly collection
Results shown as median % (±IQR)

The effect of adding Group B was to slightly raise the median recoveries and increase IQRs (Figure 3.3-13). Descriptives of the summed daily values also reflect the influence of Group D, again failing to reach 100%, although upper quartiles now approach or exceed full recovery of the dose (Table 3.3-27). There is no clear trend indicating recovery changes over time.

Table 3.3-27 Summed daily recovery of dose in the urine - Median (LQ, UQ)

%	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	40.4 (22.2, 62.086)	85.316 (76.222, 91.976)	84.702 (55.565, 98.595)	86.816 (66.939, 103.847)	61.164 (56, 107.153)

Freidman's tests showed significant differences across the study when preoperative values were included, but not within postoperative values alone (Table 3.3-28).

Table 3.3-28 Friedman's test examining differences in urinary recovery of dose across study
** = significant difference at p<0.05*

χ2 (p)	16.56 (0.01)*
χ2 (p)Postop only	3.982 (0.277)

As with Group D, *post hoc* tests showed the preoperative dose recovery to be significantly lower than Days 1, 2 and 3, and these all surpassed the required level of significance after the Bonferroni correction had been applied (Table 3.3-29).

Table 3.3-29 Group C, *post hoc* pair-wise analysis (p) of urine volume
** = significant difference at p<0.05*

Group		
1	0.000*	
2	0.005*	0.542
3	0.000*	0.635
4	0.193	0.123
Day	**-1**	**1**

3.3.5 CONTRIBUTION OF METABOLITES TO RECOVERY

Overall, there was very little change shown to the amount of paracetamol dose recovered in the urine during the steady-state period postoperatively. While the total amount of metabolites recovered did not change, the five compounds contributing to this recovery may have varied. Alterations to the pattern of metabolite excretion is as, if not more, important to this Thesis than the amounts of metabolites recovered as it can indicate alterations to the drug metabolism processes. It was therefore import to evaluate the contribution of each of the metabolites to the total urinary recovery. For the purposes of statistical analysis, recoveries of paracetamol cysteine and paracetamol mercapturate were combined into one value, *"Phase I products"*, as both are derived from NAPQI. To

compare the contribution of the metabolites, the amount of each metabolite was divided by the total amount of all metabolites recovered in that urine sample. Accordingly values are reported as *"% contribution to recovery*". Each metabolite was then assessed as with previous sections, with Friedman's tests with and without the day before surgery, and, if appropriate, followed by *post hoc* tests.

3.3.5.1 GROUP A

Several trends appear in the relative contribution of paracetamol metabolites to the overall dose recovery in this group (Figure 3.3-14).

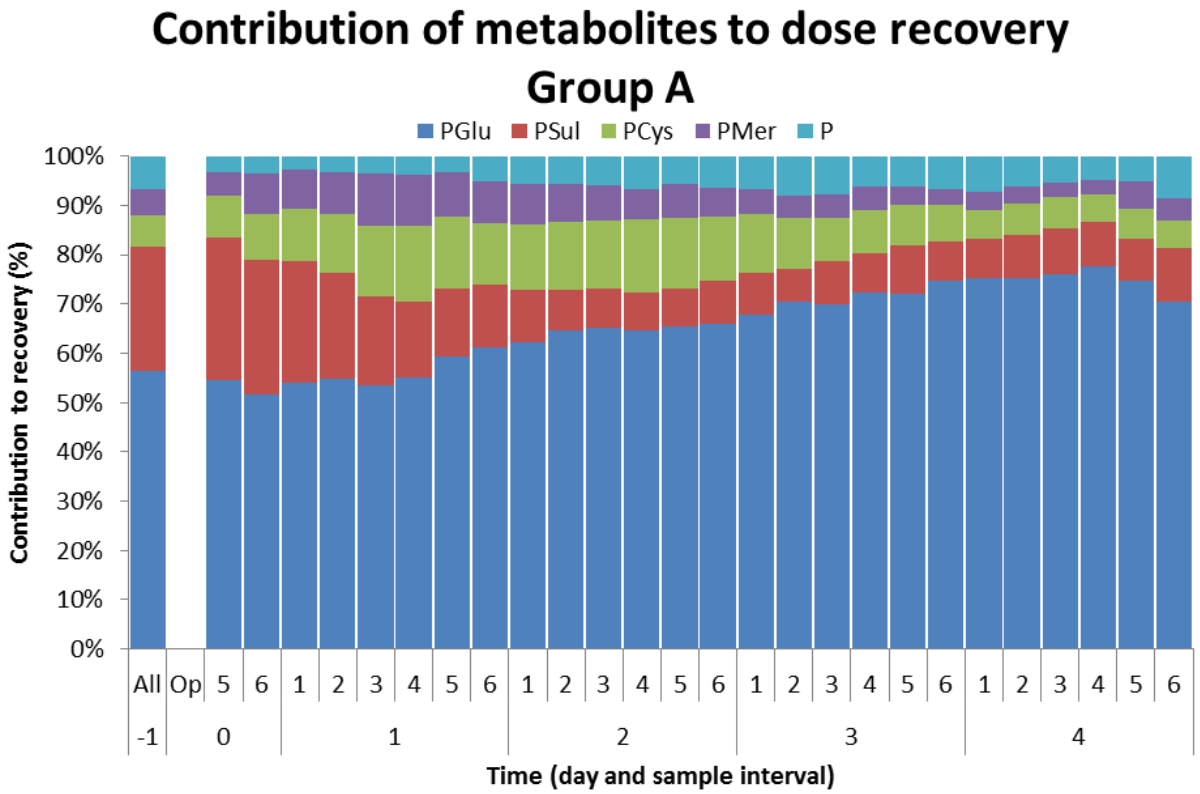


Figure 3.3-14 Contribution of each metabolite to overall recovery
Values reported as group medians. Abbreviations used: PGlu= paracetamol glucuronide; PSul= paracetamol sulphate; PCys= paracetamol cysteine; PMer= paracetamol mercapturate; P= paracetamol

Comparing the first urine sample with the last, noticeable changes included:

- Paracetamol sulphate reduced from 25 to 10%;
- Paracetamol glucuronide increased from 56 to 71%;
- The combined Phase I metabolites decreased slightly from to 12 to 10%, having reached a maximal contribution of 26% in sample 4, Day 1; and
- Unchanged paracetamol remained relatively constant, increasing from to 7 to 8%.

Values were summed as with previous sections. Exact Friedman's tests were used to assess the statistical significance of the resultant values across each day of the study. The results in Table 3.3-30 show that there were highly significant differences in the relative contribution of each metabolite across all days of the study and within the postoperative period alone.

Table 3.3-30 Results of Friedman's test
** = significant difference at p<0.05*

Group A	Paracetamol Glucuronide	Paracetamol Sulphate	Phase I products
χ2 (p)	18.756 (<0.000)*	26.933 (<0.000)*	21.333 (<0.000)*
Postop only χ2 (p)	14.6 (0.001)*	15.8 (<0.000)*	13.667 (0.001)*

To determine the nature of the Friedman's test results, *post hoc* tests were carried out using two-tailed, exact Wilcoxon signed rank tests (Table 3.3-31).

Table 3.3-31 Group A, *post hoc* pair-wise analysis (p) of urine volume
** = significant difference at p<0.05*

Group A	Paracetamol Glucuronide		Paracetamol Sulphate		Phase I Products	
1	0.846		0.027*		0.002*	
2	0.193	0.037*	0.002*	0.002*	0.002*	0.77
3	0.012*	0.004*	0.004*	0.004*	0.02*	0.008*
4	0.02*	0.004*	0.004*	0.027*	0.91	0.012*
Day	**-1**	**1**	**-1**	**1**	**-1**	**1**

For paracetamol glucuronide, the increased contributions from Day-1 to Days 3 and 4 were statistically significant, as was the increase between Day 1 and all other postoperative days. Once the Bonferroni correction had been applied, only the comparison between Day 1 and Day 3 and 4 remained significant. Paracetamol sulphate showed significant reductions between the preoperative and all postoperative days, with all but Day 1 remaining significant after Bonferroni correction. The reduction between Day 1 and Days 2, 3 and 4 postoperatively were also significant with Days 2 and 3 surpassing the rigours of the Bonferroni correction. There were significant increases in Phase I products between the preoperative day and Days 1, 2 and 3; Days 1 and 2 remaining so after Bonferroni correction. There were significant reductions in Phase I contribution between Day 1 and Days 3 and 4, with no comparison remaining significant after Bonferroni correction.

3.3.5.2 GROUP B

Trends in metabolite contribution are less marked in Group B (Figure 3.3-15):

- Paracetamol glucuronide started at 43% of the recovery, peaked at 60% in sample 2, Day 4 and reduced to 51% in the final sample;
- Paracetamol sulphate began by contributing 31%, gradually reducing to 13% in sample 3, Day 3 and increased slightly to 16% at the end of the study; and
- Phase I metabolites began contributing 15% to the recovery, peaked at 36% just before the end of the study in sample 5 Day 4, before reducing to 29% in the last urine sample.

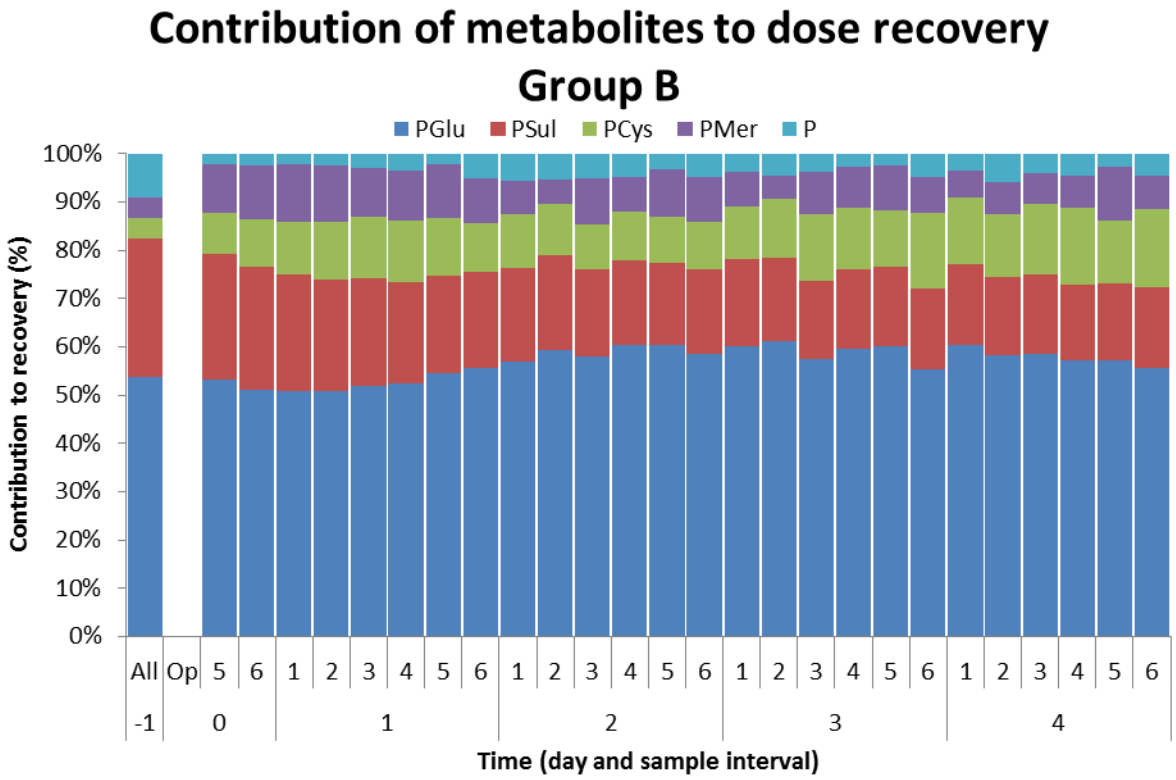


Figure 3.3-15 Contribution of each metabolite to overall recovery
Values reported as group medians. Abbreviations used: PGlu= paracetamol glucuronide; PSul= paracetamol sulphate; PCys= paracetamol cysteine; PMer= paracetamol mercapturate; P= paracetamol

Daily summed values were tested using the Friedman's test (Table 3.3-32). Two significant differences arose in the increase of paracetamol glucuronide and decrease of paracetamol sulphate between preoperative and postoperative values.

Table 3.3-32 Results of Friedman's test
** = significant difference at p<0.05*

Group B	Paracetamol Glucuronide	Paracetamol Sulphate	Phase I products
χ2 (p)	9.067 (0.028)*	10.400 (0.005)*	2.933 (0.469)
Postop only χ2 (p)	6.300 (0.094)	5.8 (0.052)	1.200 (0.753)

However, as this group only contained four patients no significant differences arose in the *post hoc* tests of these values (Table 3.3-33).

Table 3.3-33 Group B, *post hoc* pair-wise analysis (p) of urine volume
*= significant difference at p<0.05

Group B	Paracetamol Glucuronide		Paracetamol Sulphate		Phase I Products	
1	0.5		0.109		0.109	
2	0.25	0.25	0.109	0.068	0.285	0.273
3	0.25	0.125	0.109	0.068	0.285	0.465
4	0.25	0.125	0.109	0.068	0.285	0.715
Day	-1	1	-1	1	-1	1

3.3.5.3 GROUP C

Contributions of metabolites in Group C appeared relatively consistent across the study (Figure 3.3-16). Observations included:

- Contribution of paracetamol sulphate was low from the beginning (19%), and reduced to 12% in the final urine sample;
- Paracetamol glucuronide began at 54% and increased to its maximum contribution at 65% in the last sample on the day of surgery. After falling slightly, contributions remained consistently around the high 50%/low 60% values until finishing at 61%; and
- There was an unusually high contribution of Phase I metabolites from the beginning of the study (19%), which increased to 30% by sample 3 on Day 1 and remained around 25% until the final sample.

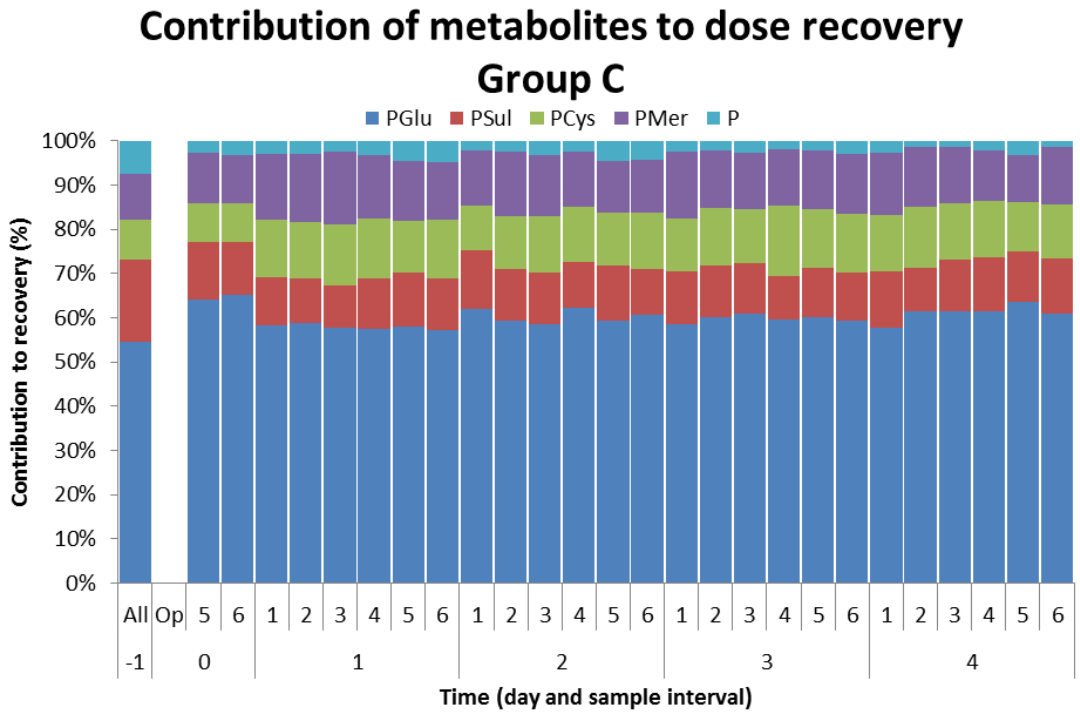


Figure 3.3-16 Contribution of each metabolite to overall recovery
Values reported as group medians. Abbreviations used: PGlu= paracetamol glucuronide; PSul= paracetamol sulphate; PCys= paracetamol cysteine; PMer= paracetamol mercapturate; P= paracetamol

Values were again summed into daily intervals. Table 3.3-34 shows the results of the Friedman's tests that were conducted. This shows that there were statistically significant differences in paracetamol glucuronide and Phase I metabolite contributions across all days of the study and within the postoperative period only. There were no differences seen for paracetamol sulphate.

Table 3.3-34 Results of Friedman's test
** = significant difference at p<0.05*

Group C	Paracetamol Glucuronide	Paracetamol Sulphate	Phase I products
χ2 (p)	15.52 (<0.000)*	7.52 (0.107)	13.6 (0.002)*
Postop only χ2 (p)	10.2 (0.007)*	1.08 (0.857)	9 (0.02)*

Table 3.3-35 shows the results of the *post hoc* tests conducted on these values. For paracetamol glucuronide, the statistical significance of the Friedman's test arose from the decrease between the preoperative day and Days 2, 3 and 4. No comparison remained significant after Bonferroni correction. Significant differences at p<0.05 were seen in the pair-wise tests for paracetamol sulphate in the decrease from the preoperative day to Days 1, 2 and 3, but these also did not remain significant following Bonferroni correction. Further, significant differences were also seen in the increase of Phase I products between the preoperative day and Days 1 and 3 and in the reduction of Phase I products between Day 1 and 4. None of these surpassed the critical level of significance once the Bonferroni correction had been applied.

Table 3.3-35 Group C *post hoc* pair-wise analysis (p) of urine volume
** = significant difference at p<0.05*

Group C	Paracetamol Glucuronide		Paracetamol Sulphate		Phase I Products	
1	0.219		0.031*		0.016*	
2	0.016*	0.078	0.016*	1	0.078	0.195
3	0.043*	0.438	0.125	0.625	0.043*	0.313
4	0.043*	0.043*	0.043*	0.625	0.125	0.043*
Day	**-1**	**1**	**-1**	**1**	**-1**	**1**

3.3.5.4 GROUP D

Clear trends emerged in the relative contributions of metabolites in Group D (Figure 3.3-17). These include:

- Steady reduction in contribution of paracetamol sulphate from 26% on the day of surgery to 9.6% on Day 4 sample 5;
- Consistent contribution of paracetamol glucuronide remaining around 60%; and
- Increased Phase I products from 14% preoperatively, to 25% in sample 6 on Day 3.

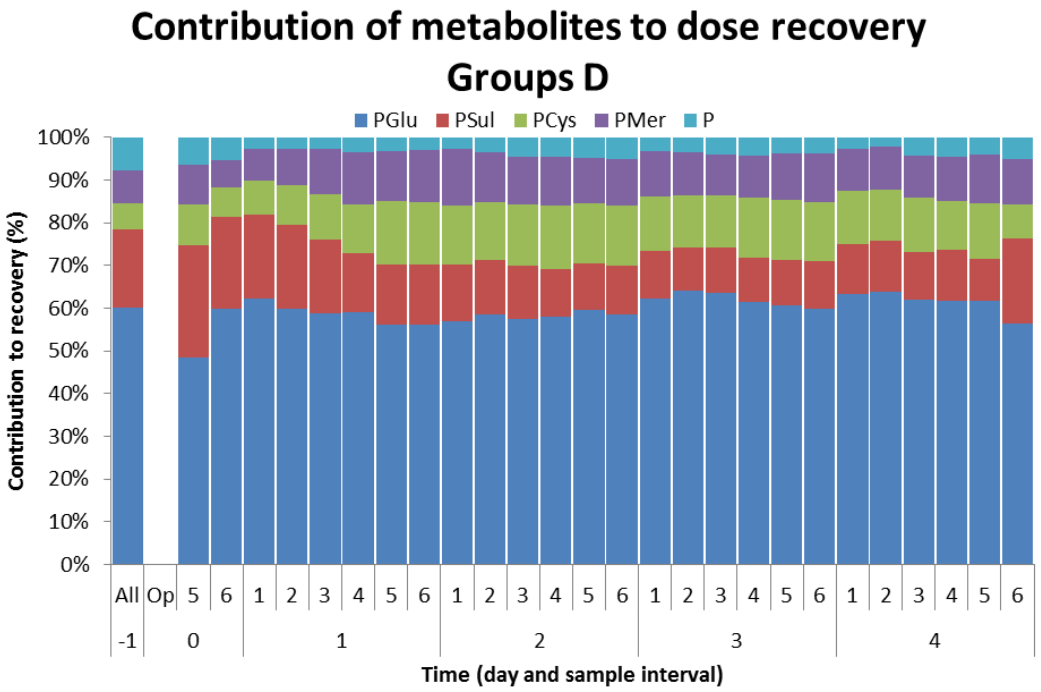


Figure 3.3-17 Contribution of each metabolite to overall recovery
Values reported as group medians. Abbreviations used: PGlu= paracetamol glucuronide; PSul= paracetamol sulphate; PCys= paracetamol cysteine; PMer= paracetamol mercapturate; P= paracetamol

Summed daily values were assessed for variance using Friedman's tests (Table 3.3-32). All metabolites showed significant differences over the entire study and within the postoperative period alone.

Table 3.3-36 Results of Friedman's test
** = significant difference at p<0.05*

Group D	Paracetamol Glucuronide	Paracetamol Sulphate	Phase I products
χ2 (p)	13.5 (0.005)*	28.4 (<0.000)*	18.1 (<0.000)*
Postop only χ2 (p)	12.75 (0.002)*	18.6 (<0.000)*	7.65 (0.049)*

Post hoc tests were also conducted (Table 3.3-37). Significant differences arose from the increase in paracetamol glucuronide contribution between the beginning (preoperative and Day 1) and end of the study (Days 3 and 4), although none remained significant after Bonferroni correction. Conversely, all comparisons of paracetamol sulphate were highly significant; both the reduction from preoperative contributions to all postoperative values and again the further reduction from Day 1 to the following postoperative days. Only the comparisons with Day 4 just failed to meet the requirements of the Bonferroni correction. Phase I products increased significantly between the preoperative and all postoperative days, but do not show a significant change between Day 1 and Days 2, 3 or 4. In these comparisons, all but the increase between Day -1 and 4 remain significant after Bonferroni correction.

Table 3.3-37 Group D *post hoc* pair-wise analysis (p) of urine volume
** = significant difference at p<0.05*

Group D	Paracetamol Glucuronide		Paracetamol Sulphate		Phase I Products	
1	0.898		0.001*		0.003*	
2	0.638	0.52	0.001*	0.001*	0.001*	0.32
3	0.037*	0.047*	0.002*	0.002*	0.002*	0.695
4	0.023*	0.049*	0.008*	0.008*	0.016*	0.461
Day	**-1**	**1**	**-1**	**1**	**-1**	**1**

3.3.5.5 GROUP B+D

Trends emerge once again in this combination group (Figure 3.3-18):

- Paracetamol glucuronide began at 59% and fell to 50% in the first postoperative sample. Gradual increases followed to its peak contribution of 63% in sample 3, Day 3, before it fell again to 52% in the final sample;
- Paracetamol sulphate began at 20%, increased to 26% in the first postoperative sample and then gradually reduced to around 11% by the end of the study; and
- Phase I contributions increased steadily from 13% to around 25% and remained at this level for the remainder of the study.

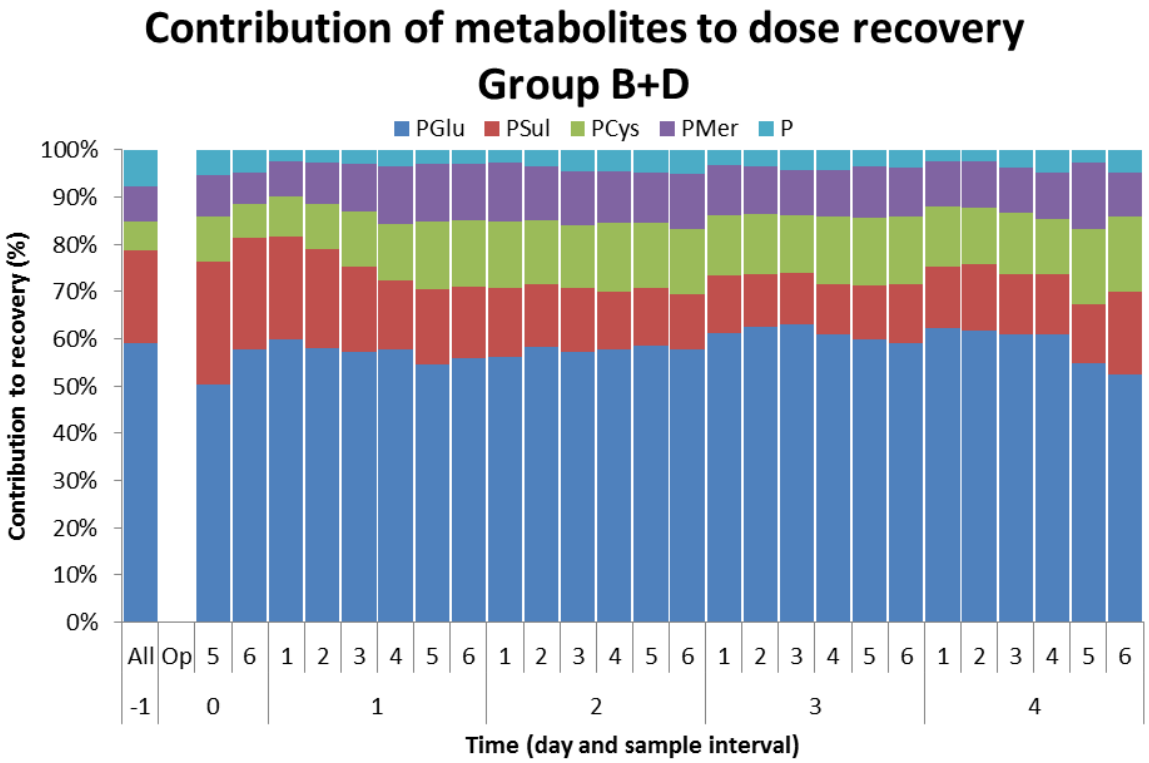


Figure 3.3-18 Contribution of each metabolite to overall recovery
Values reported as group medians. Abbreviations used: PGlu= paracetamol glucuronide; PSul= paracetamol sulphate; PCys= paracetamol cysteine; PMer= paracetamol mercapturate; P= paracetamol

Table 3.3-38 shows the results of the Friedman's tests conducted on the summed daily values. This shows highly significant differences for all comparisons aside from the examination of the Phase I products across the postoperative days.

Table 3.3-38 Results of Friedman's test
** = significant difference at p<0.05*

Group B+D	Paracetamol Glucuronide	Paracetamol Sulphate	Phase I products
χ2 (p)	17.36 (0.001)*	33.68 (<0.000)*	21.12 (<0.000)*
Postop only χ2 (p)	12.709 (0.003)*	23.182 (<0.000)*	4.091 (0.266)

Post hoc test results are also shown (Table 3.3-39). These confirm the observations made from Figure 3.3-18: There were significant increases in the proportion of paracetamol glucuronide between the first two days and the last two. All but the decrease between Day -1 and 4 failed to surpass the requirements of the Bonferroni correction. Paracetamol sulphate showed highly significant reductions between both of the first two days and all other days of the study. There were also highly significant increases in Phase I products between the preoperative day and all other days.

Table 3.3-39 Group B+D, *post hoc* pair-wise analysis (p) of urine volume
** = significant difference at p<0.05*

Group B+D	Paracetamol Glucuronide		Paracetamol Sulphate		Phase I Products	
1	0.735		<0.000*		0.001*	
2	0.305	0.241	<0.000*	<0.000*	<0.000*	0.542
3	0.009*	0.017*	<0.000*	<0.000*	<0.000*	0.685
4	0.006*	0.014*	0.002*	0.001*	0.004*	0.898
Day	**-1**	**1**	**-1**	**1**	**-1**	**1**

3.3.6 CONTRIBUTION OF SULPHATE CONTAINING METABOLITES

As paracetamol sulphate, cysteine and mercapturate rely on inorganic sulphate for their formation, changes to their contribution to urinary recovery across the study were also of interest. Inorganic sulphate is mostly derived from dietary protein and body stores of GSH. GSH is necessary for the conjugation of NAPQI. Reductions to this value in fasting individuals could indicate exhaustion of body stores GSH, and potentially the reduction in NAPQI conjugation.

3.3.6.1 GROUP A

A consistent downward trend emerges in the role of sulphate metabolites in urinary recovery of Group A (Figure 3.3-19). The contribution peaked just after surgery at 45.5% and fell steadily to 17.6% in the second to last sample. Within these results there was a

high degree of variability between participants with wide IQRs noticeable in the last two thirds of the study.

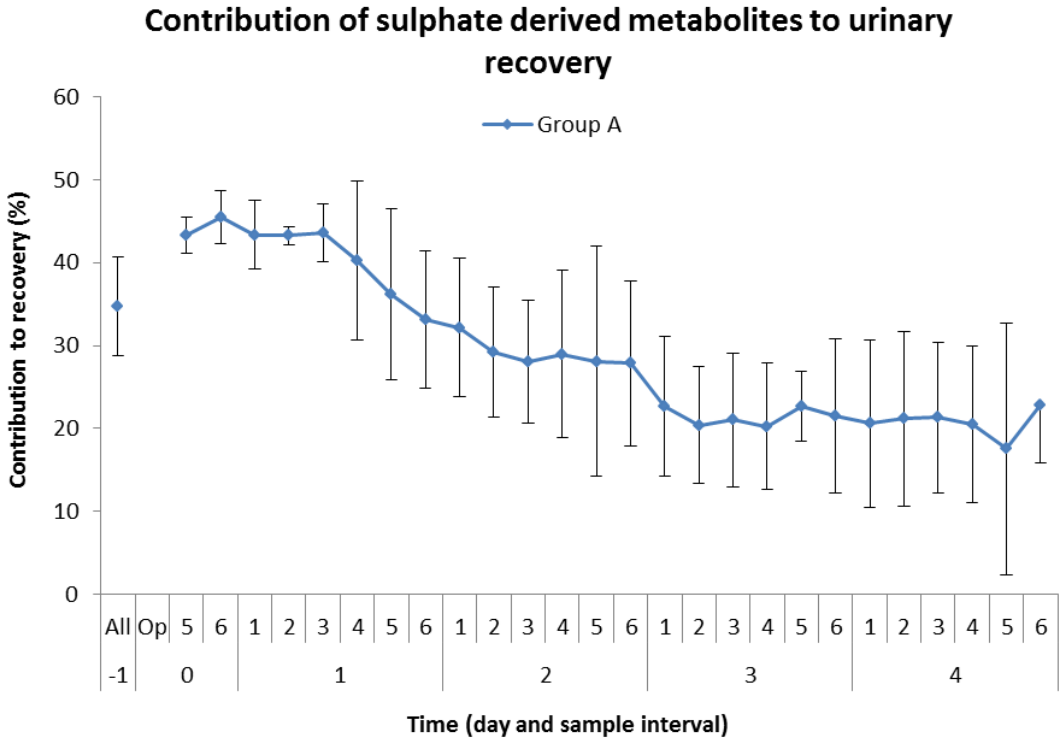


Figure 3.3-19 Per cent of urinary metabolites excreted as sulphate derived compounds.
Results shown as median % (±IQR)

Metabolite amounts were summed into daily values and the percentage recalculated. Descriptives of these values are shown in Table 3.3-40, which also repeat the downward trend seen in Figure 3.3-19.

Table 3.3-40 Summed daily recovery of sulphate derived metabolites in the urine- Median (LQ, UQ)

%	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	36.185 (29.189, 42.704)	39.657 (34.002, 50.449)	28.171 (23.641, 40.135)	21.269 (16.862, 30.455)	21.591 (15.228, 32.218)

Friedman's tests were conducted across the entire study and in the postoperative period only using these daily values (Table 3.3-41). Both comparisons showed highly significant differences across the days tested.

Table 3.3-41 Friedman's test examining differences in urinary recovery of sulphate derived metabolites across study
** = significant difference at p<0.05*

χ2 (p)	23.911 (<0.000)*
χ2 (p)Postop only	17.933 (<0.000)*

Post hoc tests were performed using two tailed, exact Wilcoxon signed rank tests (Table 3.3-42). Highly significant differences were seen in the comparison of the early days of the study with the last two days, confirming the trends observed in Figure 3.3-19. These were the only comparisons to remain significant after Bonferroni correction.

Table 3.3-42 *Post hoc* pair-wise analysis (p) of urinary recovery of sulphate derived metabolites
*= *significant difference at p<0.05*

Group A		
1	0.139	
2	0.093	0.01*
3	0.008*	0.004*
4	0.012*	0.004*
Day	-1	1

3.3.6.2 GROUP B

Figure 3.3-20 shows less of a decline in contribution of sulphate derived metabolites for Group B than with Group A, but still one is apparent, especially on Day 1. From their peak of 46.5% early on the first postoperative day, values fell and remained around 36% for all of the second and most of the third postoperative day. The sulphate contribution then recovers slightly to finish on 40.5%.

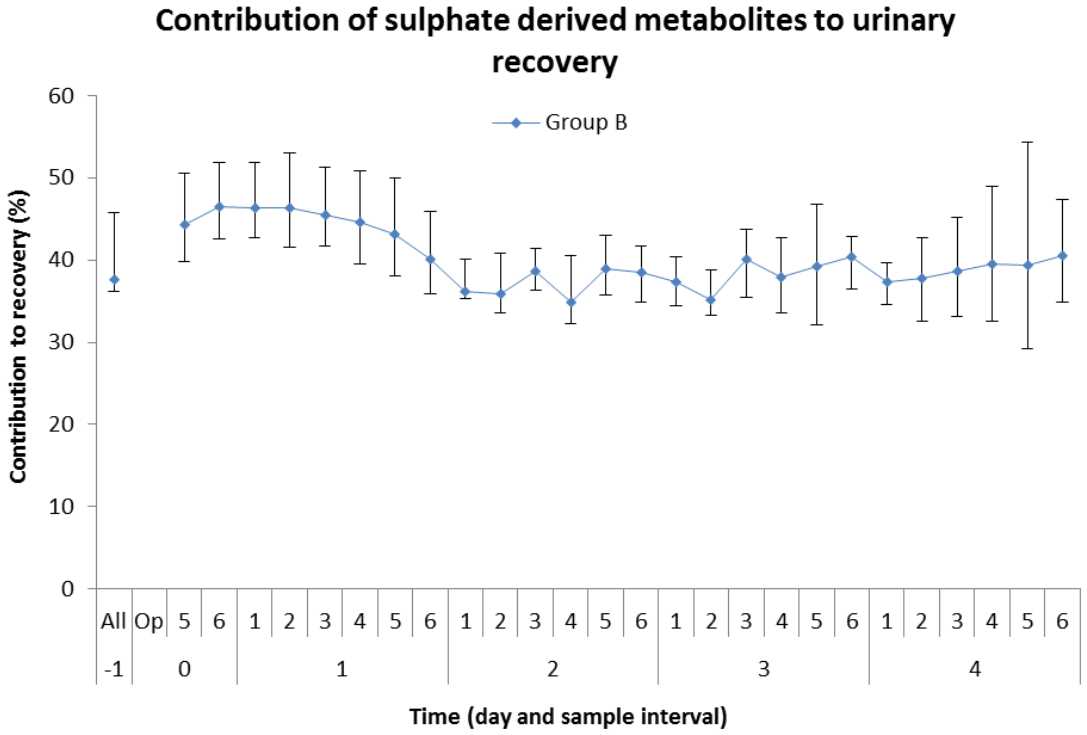


Figure 3.3-20 Per cent of urinary metabolites excreted as sulphate derived compounds.
Results shown as median % (±IQR)

As previously, daily values were calculated and their descriptives are presented in Table 3.3-43. These show the largest reduction from the second postoperative day.

Table 3.3-43 Summed daily recovery of sulphate derived metabolites in the urine- Median (LQ, UQ)

%	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	37.583 (35.866, 53.753)	44.400 (36.761, 57.324)	37.264 (32.868, 46.601)	38.408 (31.999, 44.873)	38.979 (30.499, 52.344)

The statistical significance of the reductions observed in the descriptive values was tested

with Friedman's tests (Table 3.3-44). No significant differences were seen. *Post hoc* tests also did not show significant changes (data not shown).

Table 3.3-44 Friedman's test examining differences in urinary recovery of sulphate derived metabolites across study
** = significant difference at p<0.05*

χ2 (p)	6.133 (0.189)
χ2 (p)Postop only	5.700 (0.127)

3.3.6.3 GROUP C

In comparison with the previous groups, Group C showed very little alteration in the recovery of sulphate metabolites (Figure 3.3-21).

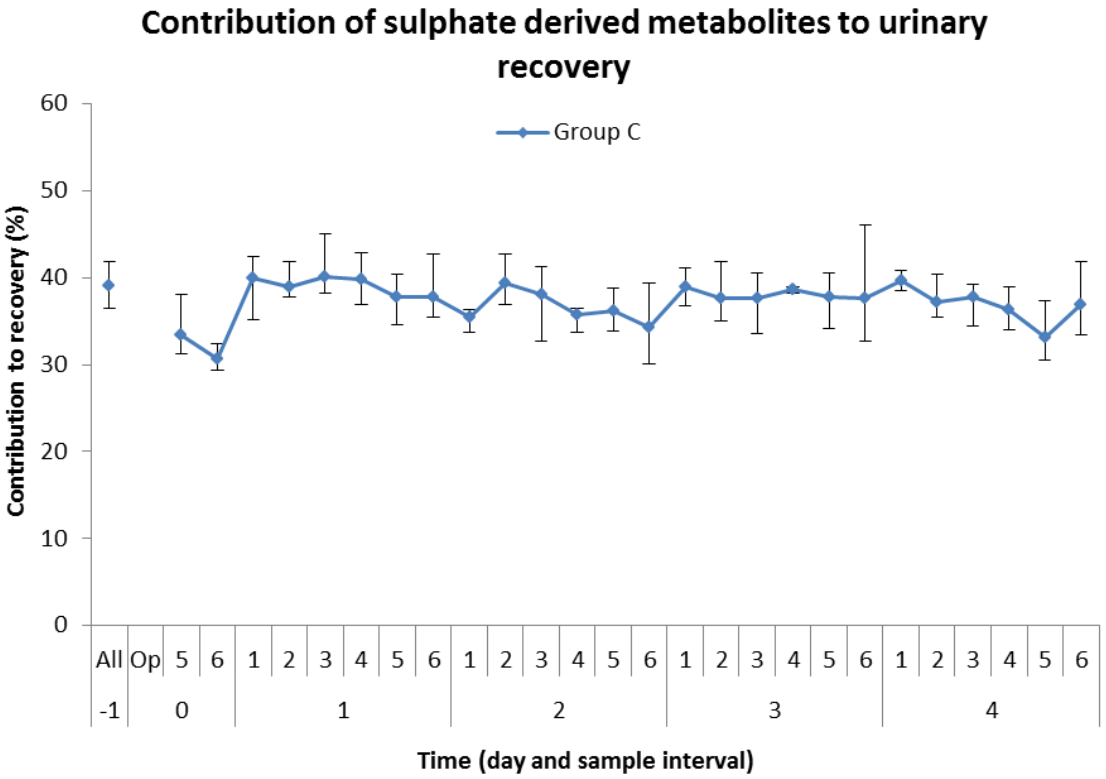


Figure 3.3-21 Per cent of urinary metabolites excreted as sulphate derived compounds.
Results shown as median % (±IQR)

Daily values were prepared as with previous groups. The contribution began at 39.3% and remained within 5% of this value for the remainder of the study. Median values are consistent in the high 30%s but fall slightly on the last day of the study (Table 3.3-45).

Table 3.3-45 Summed daily recovery of sulphate derived metabolites in the urine- Median (LQ, UQ)

%	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	39.293 (31.996, 42.749)	39.873 (35.175, 44.382)	37.349 (33.166, 43.405)	39.381 (33.995, 46.481)	35.953 (33.073, 40.678)

Table 3.3-46 shows the results of Friedman's tests. Only the comparison within the postoperative period showed a significant difference at p<0.05, however, the comparison across all days of the study shows evidence of an association with a p=0.056.

Table 3.3-46 Friedman's test examining differences in urinary recovery of sulphate derived metabolites across study
*= significant difference at p<0.05

χ2 (p)	8.8 (0.056)
χ2 (p)Postop only	9 (0.02)*

As could be expected from the values in Table 3.3-45, the *post hoc* analysis shows only one significant difference at p<0.05, between Day 1 and Day 4 (Table 3.3-47).

Table 3.3-47 *Post hoc* pair-wise analysis (p) of urinary recovery of sulphate derived metabolites
*= significant difference at p<0.05

Group C		
1	0.176	
2	0.398	0.263
3	0.345	0.5
4	0.138	0.043*
Day	**-1**	**1**

3.3.6.4 GROUP D

Figure 3.3-22 shows Group D's gradual decline in the contribution of sulphate containing metabolites to overall urinary recovery. Starting at 35.9% preoperatively, values increased to 44% at the end of Day 1 and then fell to 29.9% by the beginning of the third day. Values then recovered to 34.0% by the end of the study.

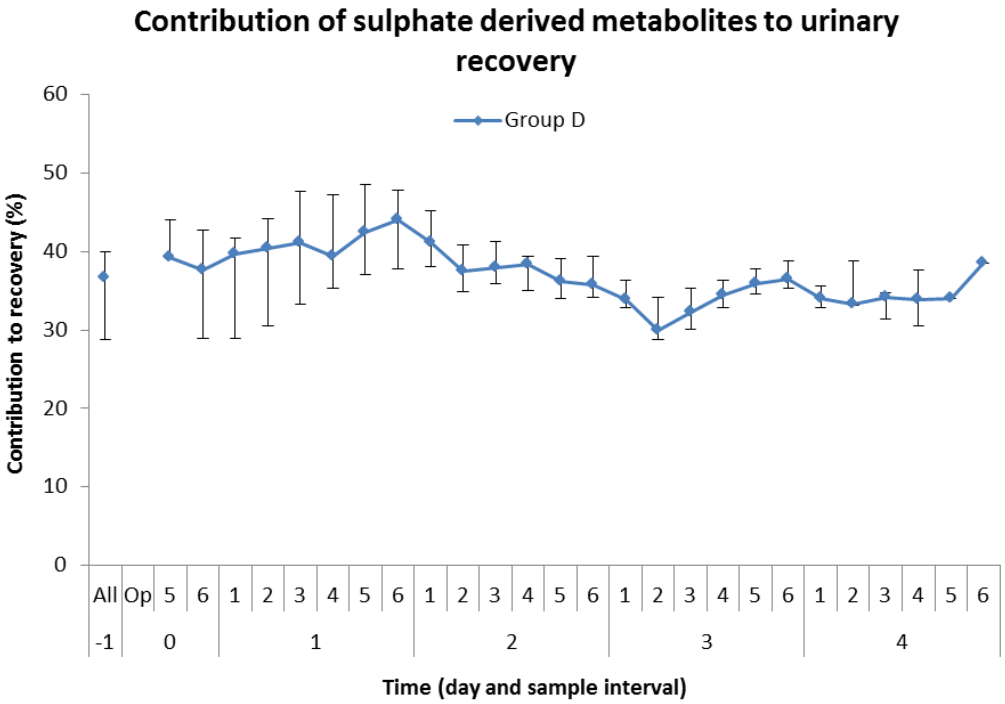


Figure 3.3-22 Per cent of urinary metabolites excreted as sulphate derived compounds.
Results shown as median % (±IQR)

Daily values showed a similar pattern of decline, with median values reducing by 8% between Day 1 and 3 which was then maintained into Day 4 (Table 3.3-48). IQRs were wide at the beginning of the study, but as the contribution of sulphate metabolites reduced so did the IQR.

Table 3.3-48 Summed daily recovery of sulphate derived metabolites in the urine- Median (LQ, UQ)

%	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	35.927 (27.675, 41.189)	41.969 (33.881, 46.898)	36.964 (36.282, 39.085)	33.973 (30.78, 36.962)	33.824 (32.453, 36.915)

Table 3.3-49 shows Friedman's tests results conducted on these values. Significant differences were seen in both comparisons, with the postoperative only comparison showing a highly significant difference.

Table 3.3-49 Friedman's test examining differences in urinary recovery of sulphate derived metabolites across study
** = significant difference at p<0.05*

χ2 (p)	12 (0.011)*
χ2 (p)Postop only	11.55 (0.005)*

Table 3.3-50 shows one significant difference at p<0.05 in the increase between the preoperative day and the first postoperative day. Two more significant differences were seen in the reduction of contribution between Day 1 and Days 3 and 4. However, these p values do not achieve significance after Bonferroni correction.

Table 3.3-50 *Post hoc* pair-wise analysis (p) of urinary recovery of sulphate derived metabolites
** = significant difference at p<0.05*

Group D		
1	0.019*	
2	0.091	0.182
3	0.799	0.027*
4	0.674	0.05*
Day	-1	1

3.3.6.5 GROUP B+D

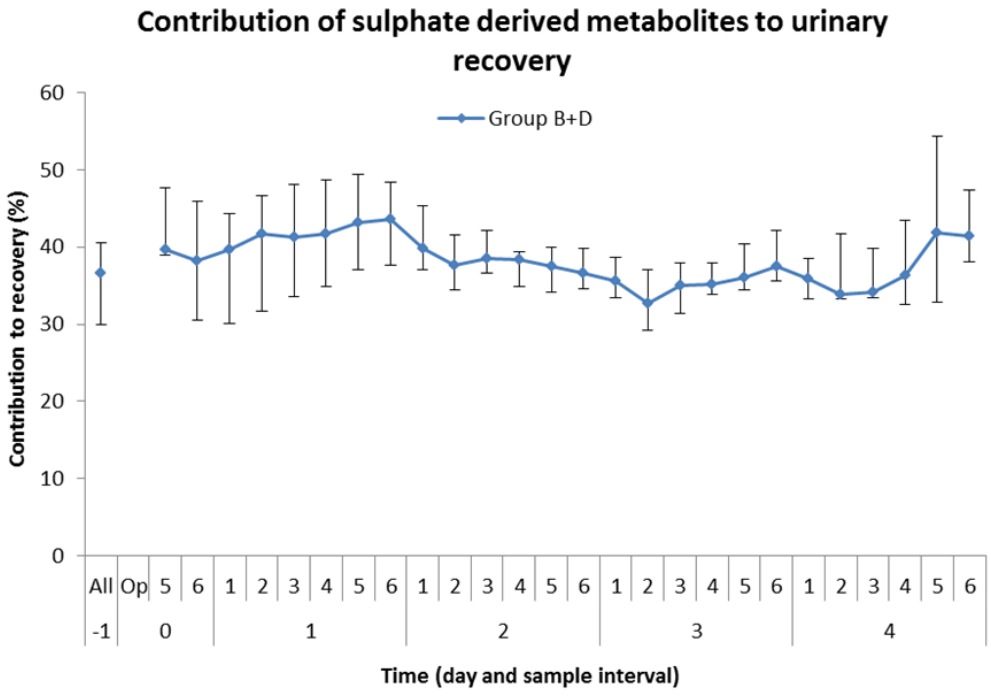


Figure 3.3-23 Per cent of urinary metabolites excreted as sulphate derived compounds.
Results shown as median % (±IQR)

Once again the Group B+D comparison shows trends largely similar to Group D, with a small increase postoperatively, followed by a slight decline in values (Figure 3.3-23). Daily values peaked on Day 1 at 43.2% and fell gradually to Day 4 (34.1%) (Table 3.3-51).

Table 3.3-51 Summed daily recovery of sulphate derived metabolites in the urine- Median (LQ, UQ)

%	Day -1	Day 1	Day 2	Day 3	Day 4
Median (IQR)	35.927 (28.79, 41.671)	43.241 (33.944, 47.394)	37.264 (36.509, 40.553)	35.113 (31.752, 38.975)	34.056 (33.095, 42.332)

Friedman's tests examined for significant differences across all days of the study and across the postoperative period only. Both tests showed significant results (Table 3.3-52).

Table 3.3-52 Friedman's test examining differences in urinary recovery of sulphate derived metabolites across study
** = significant difference at p<0.05*

χ2 (p)	13.92 (0.004)*
χ2 (p)Postop only	12.273 (0.004)*

Table 3.3-53 shows the results of the pos-hoc tests. Significant differences arose in the increase from Day-1 to Day 1 and the decrease from Day 1 to Day 3 and 4. However, none of these results achieve significance after the Bonferroni correction was applied.

Table 3.3-53 *Post hoc* pair-wise analysis (p) of urinary recovery of sulphate derived metabolites
** = significant difference at p<0.05*

Group B+D		
1	0.013*	
2	0.133	0.074
3	0.480	0.008*
4	0.575	0.01*
Day	**-1**	**1**

3.3.7 RATIO OF METABOLITES PHASE II: PHASE I

As discussed in the introduction to this results section, changes to urine flow can have a significant impact on the fractional urinary recovery of paracetamol. As shown in Section 3.3.3, there were significant changes to urine flow across the study and this could potentially invalidate this type of metabolic ratio. Because the fractional urinary recovery of paracetamol's metabolites was comparatively free from the factors that affect the recovery of paracetamol itself, ratios that compared the recovery of Phase II to Phase I products in the urine were prepared. As such, it is the most useful indicator of changes in contribution of each metabolic pathway to the overall clearance of paracetamol.

3.3.7.1 GROUP A

Figure 3.3-24 shows a steep reduction in metabolite ratio from a preoperative value of 8.5 to 2.6 in sample 3, Day 1.

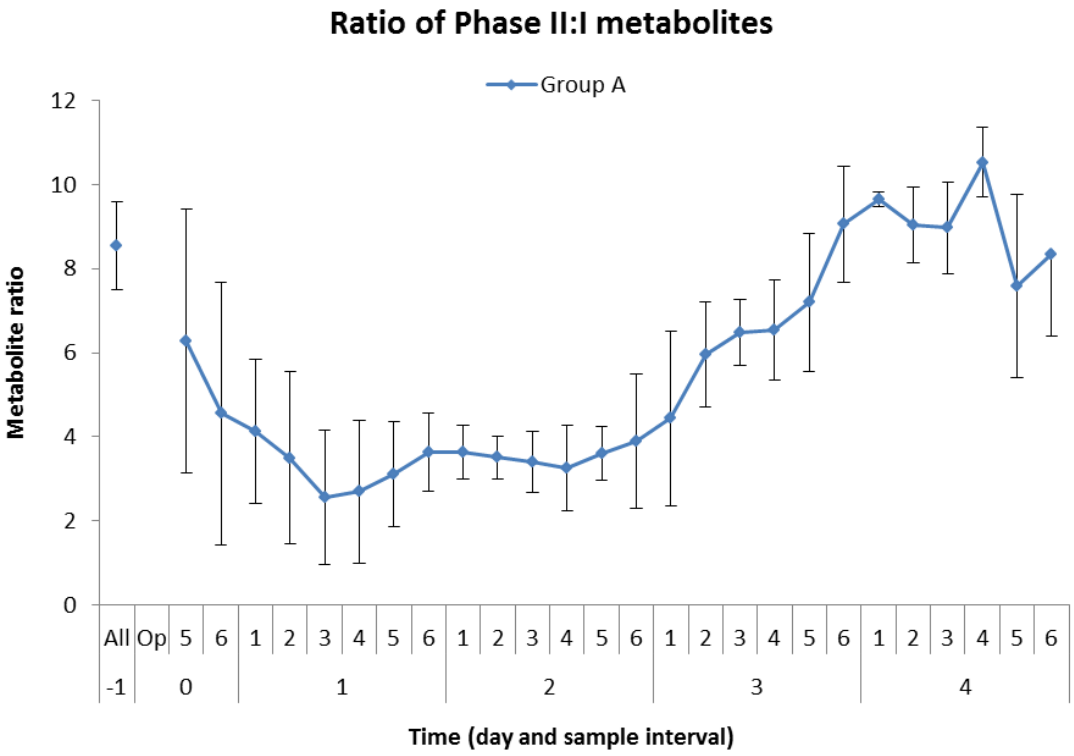


Figure 3.3-24 Ratio of Phase II: Phase I metabolites of paracetamol (median ±IQR)

The ratio then increased gradually to its preoperative value by the end of Day 3, peaking at 10.5 in sample 4 Day 4. IQRs increased sharply from the preoperative to first postoperative sample, and then gradually declined to their narrowest on Day 2. Daily ratios were again calculated. Descriptives are shown in Table 3.3-54 and a box plot derived from these values is shown in Figure 3.3-25.

Table 3.3-54 Ratio of metabolites in daily urine
Shown as median (LQ, UQ)

Ratio	Median (IQR)
Day -1	8.54 (7.12, 11.28)
Day 1	2.99 (2.33, 5.55)
Day 2	3.80 (1.99, 4.23)
Day 3	5.50 (5, 7.63)
Day 4	8.68 (5.52, 10.8)

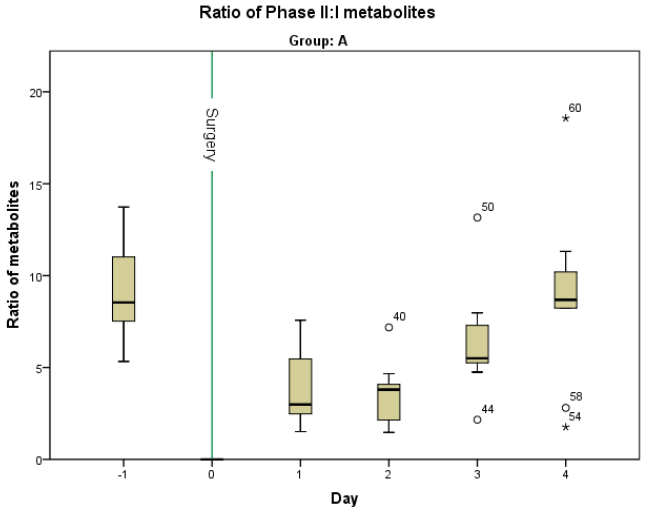


Figure 3.3-25 Box plot of metabolite ratio

Friedman's tests on these values are shown (Table 3.3-55) which shows the differences observed achieved statistical significance.

Table 3.3-55 Friedman's test examining differences in urinary recovery of dose across study
** = significant difference at p<0.05*

χ2 (p)	21.956 (0.000)*
χ2 (p)Postop only	13.4 (0.002)*

Post hoc tests using exact Wilcoxon matched pairs were again performed to compare the preoperative day with all postoperative days and also the first postoperative day to the remaining postoperative days. Prior research indicated the ratio only went down following surgery and accordingly only one tailed tests were used in the comparison of preoperative and postoperative values(Kennedy 2009a). However, as the direction of change from the first postoperative day to the other postoperative days was unknown, two tailed tests were used. Table 3.3-56 shows the results of these tests.

Table 3.3-56 Group A, *post hoc* pair-wise analysis (p) of urine volume
** = significant difference at p<0.05*

Group A		
1	0.001*	
2	0.001*	0.846
3	0.004*	0.012*
4	1.000	0.012*
Day	**-1** (1 tailed)	**1** (2 tailed)

The decrease between the preoperative day and Days 1, 2 and 3 were all highly significant and all surpass Bonferroni correction. The two tailed tests showed the ratio increased significantly between Day 1 and Days 3 and 4 postoperatively, but these are not above the p value required for significance after the Bonferroni correction is considered.

3.3.7.2 GROUP B

There was a gradual reduction in metabolite ratio from 7.9 preoperatively to 3.8 in the last urine sample (Figure 3.3-26). The consistency of this decline was interrupted by a brief increase at the beginning of Day 2, peaking at 5.5, before declining again.

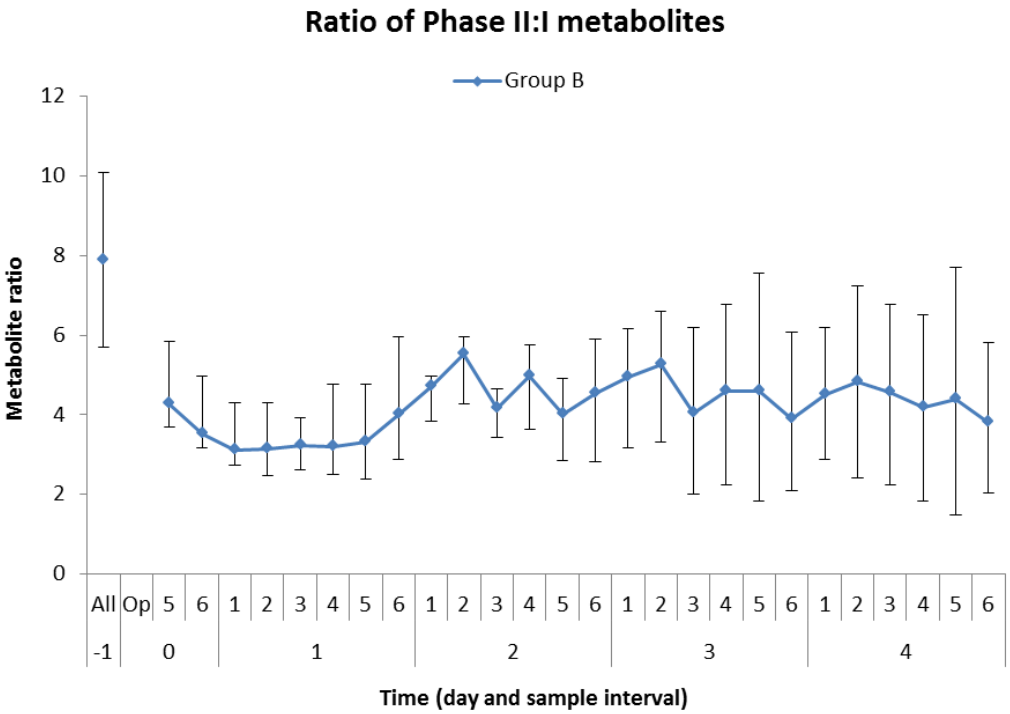


Figure 3.3-26 Ratio of Phase II: Phase I metabolites of paracetamol (median ±IQR)

The daily values were again calculated and the descriptives (Table 3.3-57) and box plot (Figure 3.3-27) arising from these are shown. The median values showed a clear reduction from the preoperative sample. Postoperative ratios remain relatively constant.

Table 3.3-57 Ratio of metabolites in daily urine
Shown as median (LQ, UQ)

Ratio	Median (IQR)
Day -1	5.52 (4.05, 8.89)
Day 1	3.09 (2.22, 4.06)
Day 2	2.44 (2.09, 3.18)
Day 3	3.06 (2.49, 3.23)
Day 4	2.43 (2.03, 2.92)

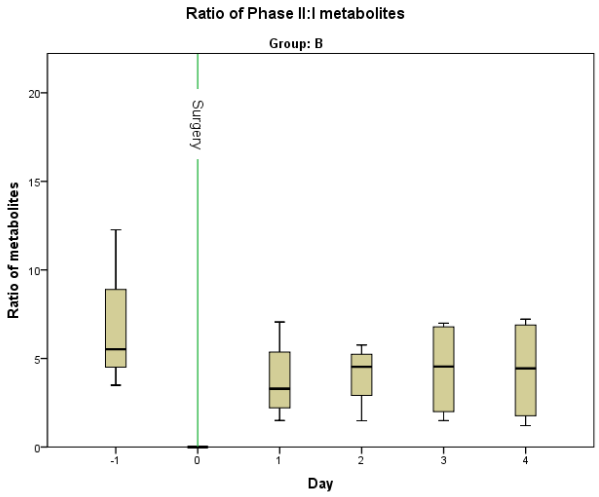


Figure 3.3-27 Box plot of metabolite ratio

Freidman's tests were also conducted (Table 3.3-58). Given the small number of patients in this group, the finding of a significant difference at $p<0.05$ between all days of the study was unexpected. No significant difference at $p<0.05$ was seen in the comparison of the postoperative days. *Post hoc* tests were conducted and did not show any significant difference at $p<0.05$ (data not shown).

Table 3.3-58 Friedman's test examining differences in ratio of Phase II:I metabolites in daily urine
** = significant difference at p<0.05*

χ2 (p)	7.6 (0.042)*
χ2 (p)Postop only	5 (0.207)

3.3.7.3 GROUP C

Group C began with the lowest preoperative ratio of all groups at 4.6 (Figure 3.3-28). This ratio fell gradually to 2.2 halfway through the first postoperative day and remained between 2.5 and 3.0 for the remainder of the study.

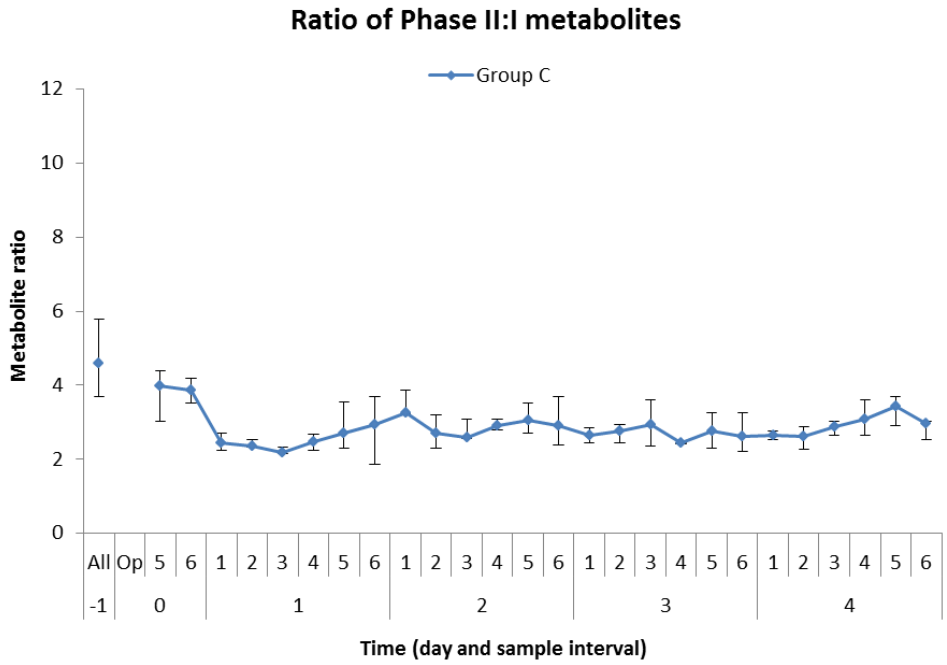


Figure 3.3-28 Ratio of Phase II: Phase I metabolites of paracetamol (median ±IQR)

Daily ratios were determined and descriptives (Table 3.3-59) and a box plot (Figure 3.3-29) were prepared. These show the reduction from preoperative values and also show little variability in the data between patients, as IQRs were narrow.

Table 3.3-59 Ratio of metabolites in daily urine
Shown as median (LQ, UQ)

Ratio	Median (IQR)
Day -1	4.578 (3.387, 6.224)
Day 1	2.425 (2.028, 2.922)
Day 2	3.086 (2.224, 4.055)
Day 3	2.435 (2.092, 3.176)
Day 4	3.058 (2.494, 3.232)

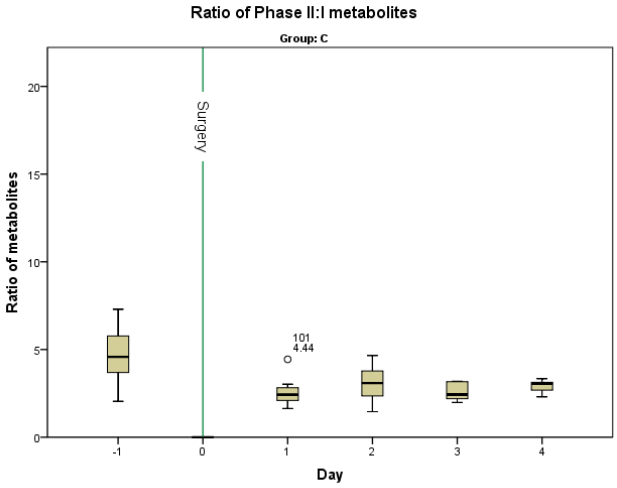


Figure 3.3-29 Box plot of metabolite ratio

Freidman's tests (Table 3.3-60) were conducted on the daily values.

Table 3.3-60 Friedman's test examining differences in urinary recovery of dose across study
** = significant difference at p<0.05*

χ2 (p)	10.72 (0.018)*
χ2 (p)Postop only	8.76 (0.023)*

Significant differences were seen in both comparisons. *Post hoc* tests showed the decrease between the preoperative day and Days 1, 2 and 3 reached significance. In the comparisons of Day 1 and the remaining postoperative days, only the increase between Day 1 and Day 4 reached significance (Table 3.3-61). However, none of these comparisons achieve significance after the Bonferroni correction was applied.

Table 3.3-61 Group C, *post hoc* pair-wise analysis (p) of urine volume
** = significant difference at p<0.05*

Group C		
1	0.016*	
2	0.023*	0.109
3	0.031*	0.313
4	0.063	0.043*
Day	**-1** (1 tailed)	**1** (2 tailed)

3.3.7.4 GROUP D

The ratio in this group began at 6.1 and fell sharply to 2.3 at end of Day 1. The ratio remained consistent from this point, ranging between 3 and 3.5 (Figure 3.3-30).

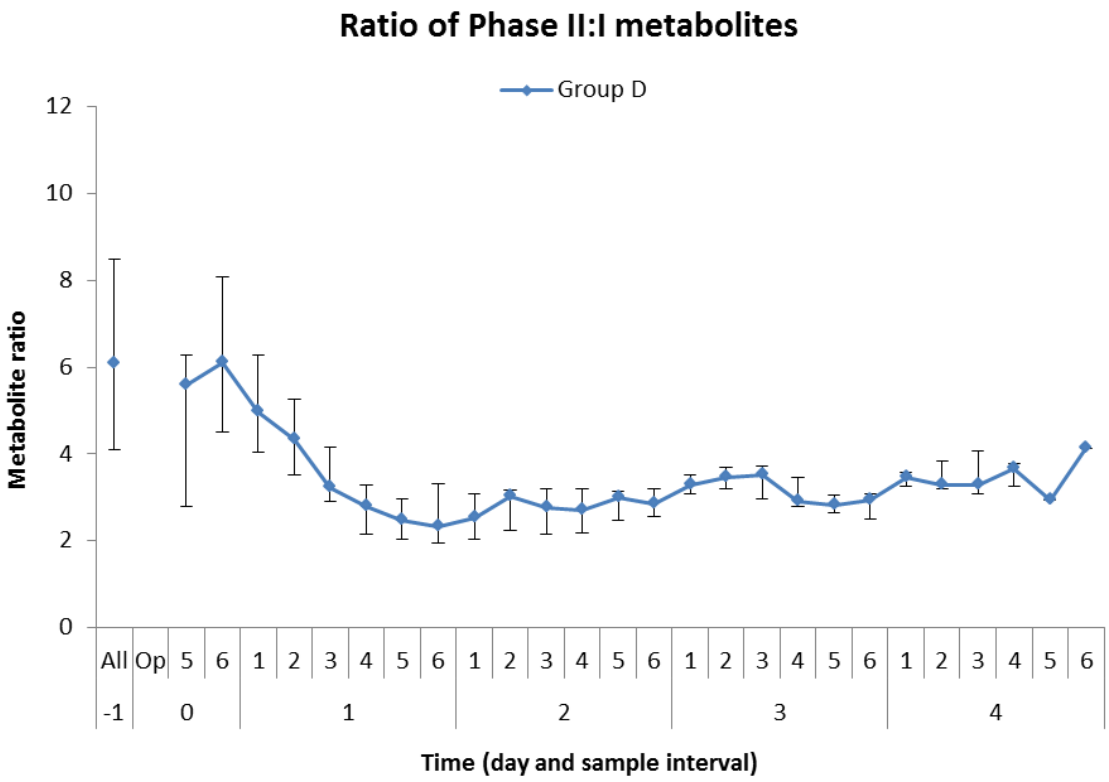


Figure 3.3-30 Ratio of Phase II: Phase I metabolites of paracetamol (median ±IQR)

Four hourly values were summed and used to produce the descriptive statistics (Table 3.3-62) and boxplot (Figure 3.3-31) shown. Median daily values show a marked reduction in ratio and remain consistent postoperatively. Variability between patients also reduced postoperatively as shown by the narrow IQRs.

Table 3.3-62 Ratio of metabolites in daily urine
Shown as median (LQ, UQ)

Ratio	Median (IQR)
Day -1	6.064 (3.877, 9.827)
Day 1	2.967 (2.618, 4.095)
Day 2	2.891 (2.408, 3.067)
Day 3	3.278 (2.859, 3.475)
Day 4	3.322 (2.945, 3.816)

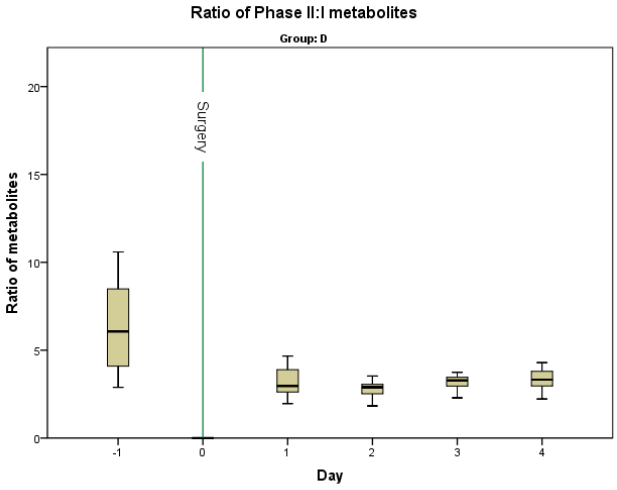


Figure 3.3-31 Box plot of metabolite ratio

Friedman's tests were conducted on these daily values and both comparisons showed statistically significant differences across the days they examined (Table 3.3-63). A greater degree of significance was observed when the preoperative value was included.

Table 3.3-63 Friedman's test examining differences in urinary recovery of dose across study
** = significant difference at p<0.05*

χ2 (p)	17.1 (0.000)*
χ2 (p)Postop only	7.65 (0.049)*

Post hoc tests showed significance in the decrease between the preoperative ratio and all postoperative ratios, with the comparison with all but Day 4 remaining so after Bonferroni correction. However, the postoperative comparisons did not show any evidence of a significant change (Table 3.3-64).

Table 3.3-64 Group D, *post hoc* pair-wise analysis (p) of urine volume
** = significant difference at p<0.05*

Group D		
1	0.002*	
2	0.000*	0.067
3	0.001*	0.922
4	0.02*	0.641
Day	**-1** (1 tailed)	**1** (2 tailed)

3.3.7.5 GROUP B+D

The ratio in this combined group largely reflected the contribution of Group D, with the most notable difference being the increase in IQR, especially in towards the end of the study (Figure 3.3-32). The preoperative ratio was slightly increased to 6.3, but followed a similar trend, decreasing until the end of Day 1 and remaining fairly constant for the remainder of the study.

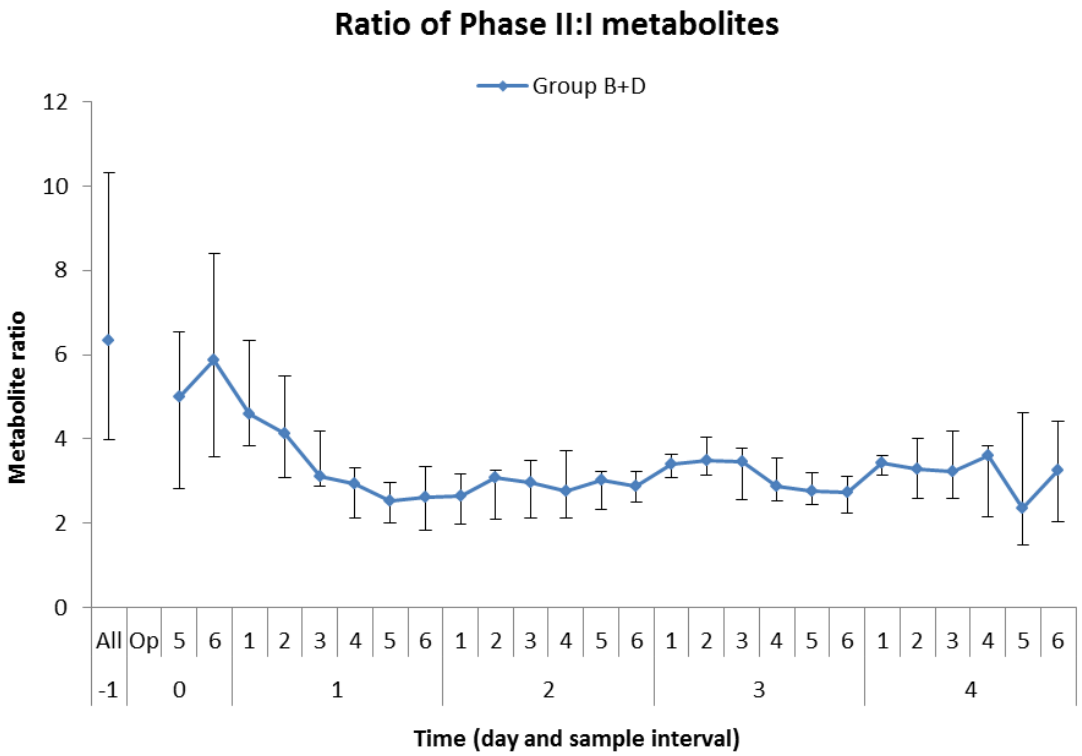


Figure 3.3-32 Ratio of Phase II: Phase I metabolites of paracetamol (median ±IQR)

Values were pooled into daily intervals and used to prepare the descriptives (Table 3.3-65) and box plot shown (Figure 3.3-33). As described above, this is largely similar to Group D with a marked reduction following surgery. The main effect of the addition of Group B is the widening of IQRs and the appearance of outlying values shown in Figure 3.3-33.

Table 3.3-65 Ratio of metabolites in daily urine
Shown as median (LQ, UQ)

Ratio	Median (IQR)
Day -1	6.064 (3.807, 10.154)
Day 1	2.948 (2.537, 4.177)
Day 2	2.945 (2.317, 3.429)
Day 3	3.212 (2.545, 3.49)
Day 4	3.322 (2.325, 3.83)

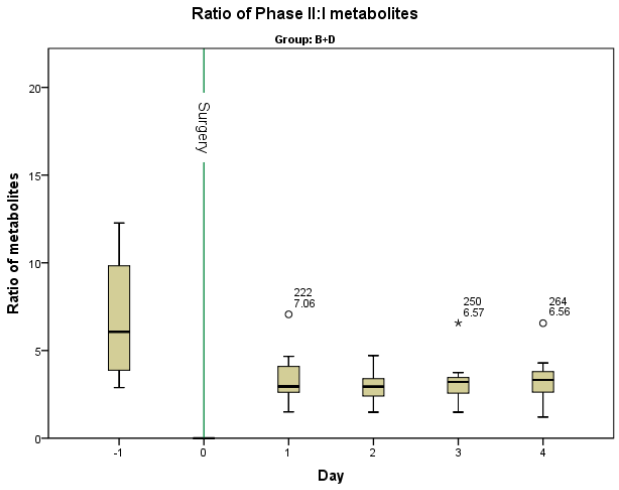


Figure 3.3-33 Box plot of metabolite ratio

Table 3.3-66 shows the results of the Friedman's tests performed on these ratios. As could be predicted from Figure 3.3-33 significant differences were seen in the comparison of all days of the study but not within the postoperative period alone.

Table 3.3-66 Friedman's test examining differences in urinary recovery of dose across study
*= significant difference at p<0.05

χ2 (p)	20.96 (0.000)*
χ2 (p)Postop only	5.509 (0.146)

Post hoc tests (Table 3.3-67) show highly significant reductions between the preoperative ratio and all the postoperative days. As predicted by the Friedman's test, no significant differences were seen in the postoperative comparisons.

Table 3.3-67 Group B+D, *post hoc* pair-wise analysis (p) of urine volume
*= significant difference at p<0.05

Group B+D		
1	0.001*	
2	0.000*	0.135
3	0.000*	1
4	0.005*	0.966
Day	**-1** (1 tailed)	**1** (2 tailed)

3.3.8 FACTORS EFFECTING RATIO OF PHASE II: PHASE I PRODUCTS

From the above results there appeared to be a clear difference in metabolic ratio following surgery. There are, however, a number of other factors amongst surgical patients that could affect the urinary concentration of metabolites, aside from surgery itself. These were examined below to see if they were a strong influence, potentially obscuring what appeared to be the effect of surgery.

In addition to those detailed below, the effects of age and BMI on the metabolic ratio were also assessed using a Spearman's correlation; however the number of patients in each group were too low for this to give meaningful results.

3.3.8.1 GENDER

Graphs of the metabolite ratio for males and females patients were produced for Group A and Groups B+D (Figure 3.3-31). No gender analysis was performed on Group C as all patients were female.

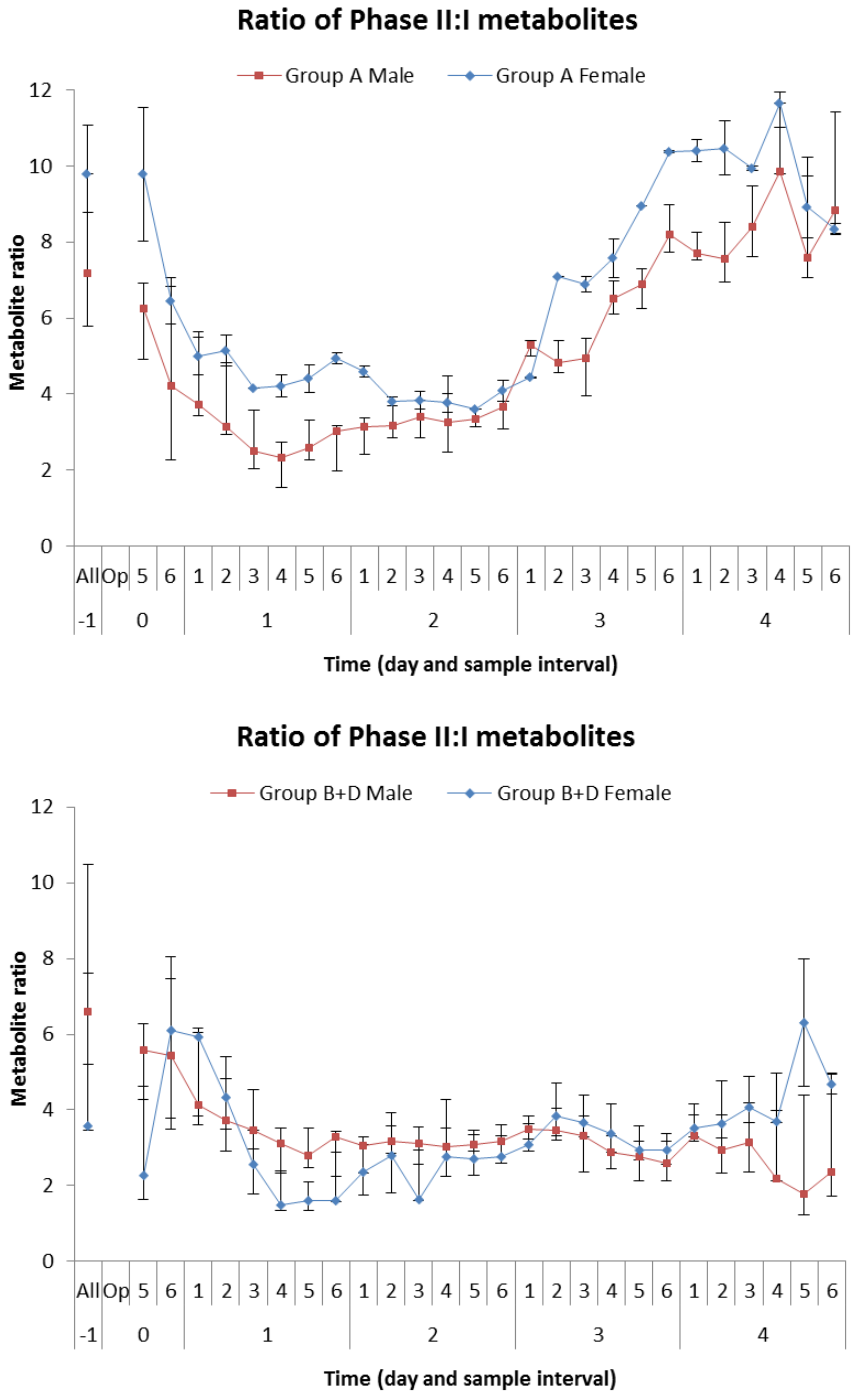


Figure 3.3-34 Changes in metabolite ratio across study period for males and females in Group A (top) and B+D (bottom)

There were no clear differences in metabolite ratio between genders in either figures and both genders followed a very similar course (Figure 3.3-34) although the ratio tended to be higher in Group A females. As an estimate of the relationship between genders, two tailed Spearman's correlations were prepared for the two groups based on the median values for each gender (Table 3.3-68). The metabolic ratio of males in Group A was highly correlated with, and accounted for 68% of the variability of the females' metabolic ratio. In Group B+D, while there was evidence of an association, it was not statistically

significant and therefore a difference in metabolic ratio between genders could not be ruled out.

Table 3.3-68 Results of Spearman's correlation of metabolic ratios between genders
** = significant difference at p<0.05*

	P	ρ^2	Sig (2 tailed)
Group A	0.826	0.682	<0.000
Group B+D	0.319	0.102	<0.148

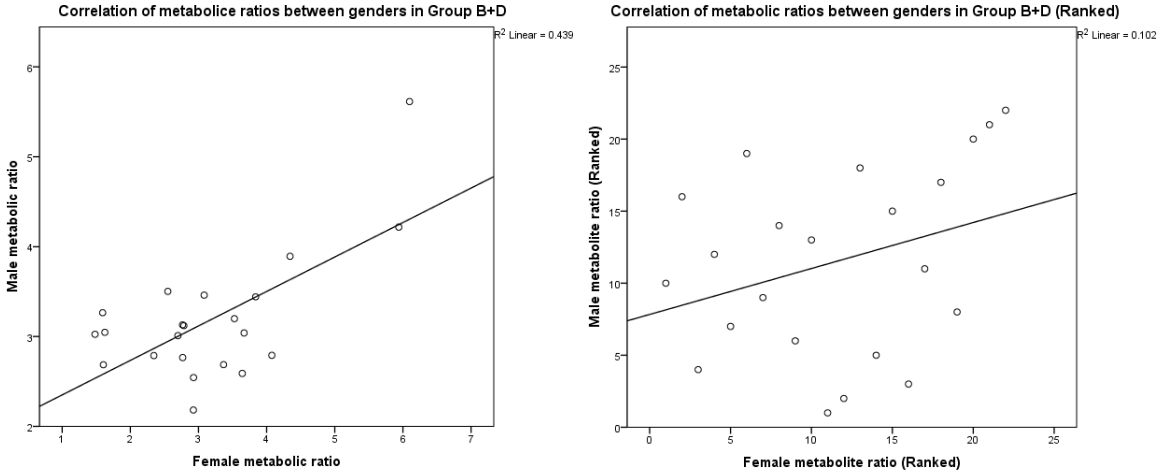


Figure 3.3-35 Correlation of median metabolic ratio between genders in Group B+D before (left) and after (right) ranking
R^2 values relate to Pearson's correlation co-efficients, which is equivalent to the Spearman's correlation co-efficient when based on ranked data (right plot)

Examination of the left graph in Figure 3.3-35 does visually show a relationship. However, because the metabolite ratios were non-parametric, the correct means of assessment was with a Spearman's correlation which uses ranked data. The problem with ranking data in small data sets is weakening of correlations because the magnitude of the values is not taken into account (Figure 3.3-35). A Pearson's correlation on the unranked data did show a significant correlation, but this is not an appropriate test for non-parametric data. Accordingly, a further test of Group B+Ds data was necessary to determine if the genders were significantly different. A Mann-Whitney test was used to determine this and no significant difference between genders at p<0.05 was found (z=0.276, p=0.783).

3.3.8.2 DOSE RECOVERED IN THE URINE

A second potential influence is the amount of the dose recovered in the urine within any given interval. A relationship between these would indicate that some metabolites are more readily excreted in the urine than others causing the metabolic ratio to vary with dose recovery, which itself varied across the study (Section 3.3.4).

To examine the relationship, dot plots were first prepared plotting the four-hourly ratios against the per cent of dose recovered in the same urine samples (Figure 3.3-36). No relationship was apparent in any group.

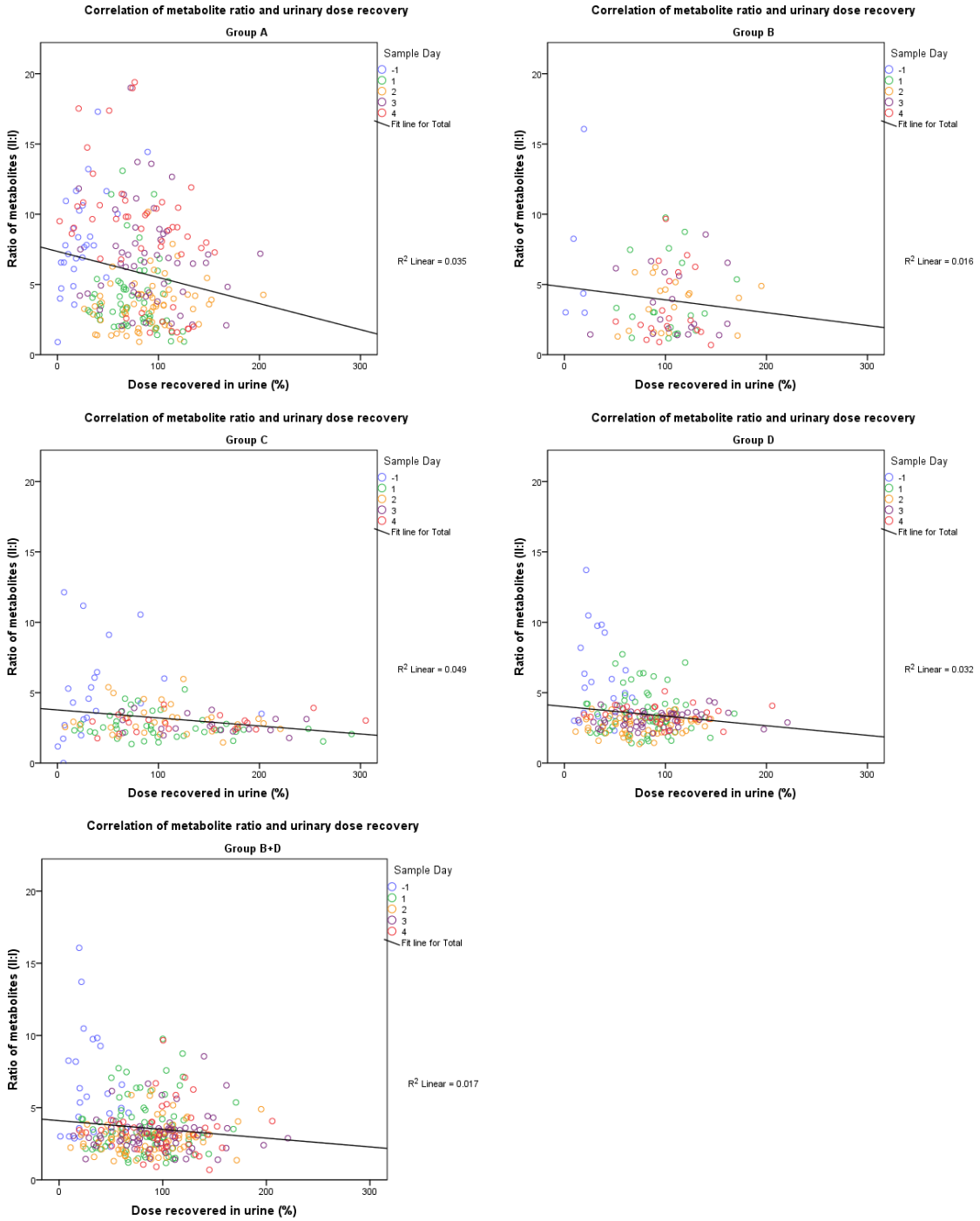


Figure 3.3-36 Dot plots of metabolite ratio and urinary dose recovery, showing values arising from each day

Spearman's correlations were then carried out on these values (Table 3.3-69).

Table 3.3-69 Results of Spearman's correlation between of metabolite ratio and urinary dose recovery
** = significant difference at p<0.05*

	N	P	ρ^2	Sig (2 tailed)
Group A	245	-0.187	0.035	0.003*
Group B	77	-0.042	0.002	0.714
Group C	133	-0.150	0.023	0.086

Group D	219	-0.069	0.005	-0.069
Group B+D	296	-0.063	0.004	0.283

Only Group A showed a significant association, however with only 3.5% of the variability of the metabolic ratio being explained by dose recovery, the influence was very minor.

3.3.8.3 URINARY OUTPUT

As with dose recovery, the possibility of urinary output influencing the metabolic ratio was also investigated. Dot plots were prepared (Figure 3.3-37) followed by Spearman's correlations (Table 3.3-70) as in the previous section. Graphically, no relationship appears, nor does there appear to be any difference in distribution amongst the days of the study.

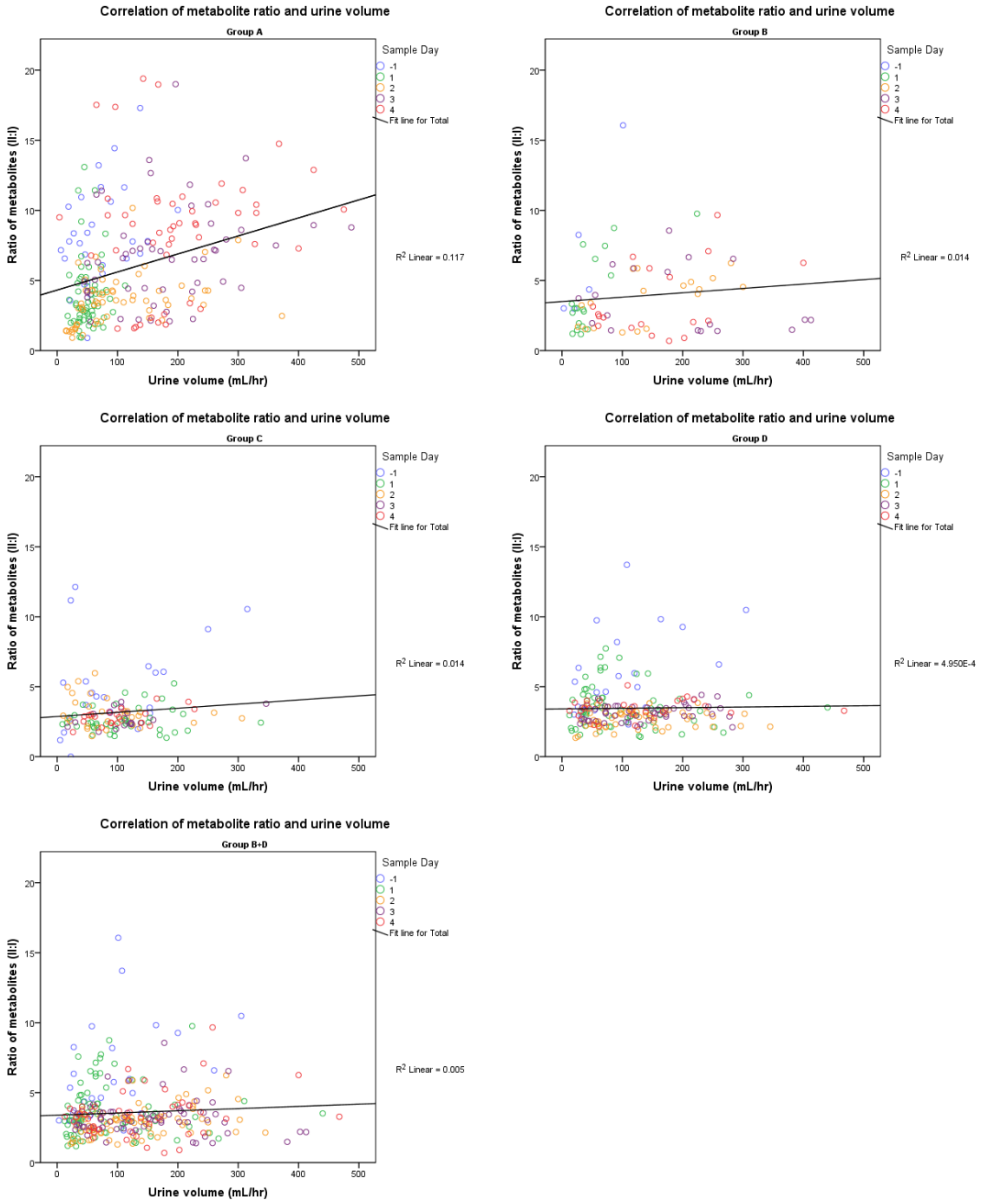


Figure 3.3-37 Dot plots of metabolite ratio and urine volume, showing values arising from each day

The Spearman's correlations revealed a highly significant relationship occurring in Group A, however urine volume only accounted for 16% of the variance of metabolic ratio (leaving the remaining 84% to be explained by other factors). No other group showed a significant relationship or co-efficient of determination.

Table 3.3-70 Results of Spearman's correlation between of metabolite ratio and urinary dose output
*= significant difference at p<0.05

	N	P	ρ^2	Sig (2 tailed)
Group A	245	0.403	0.162	0.000*
Group B	77	0.147	0.022	0.201
Group C	133	0.076	0.006	0.386
Group D	219	0.005	0.000	0.936
Group B+D	296	0.078	0.006	0.183

3.3.8.4 SAMPLE INTERVAL (TIME OF DAY)

The possibility of diurnal differences was also investigated. Dot plots were prepared contrasting the metabolic ratio against the day of the study.

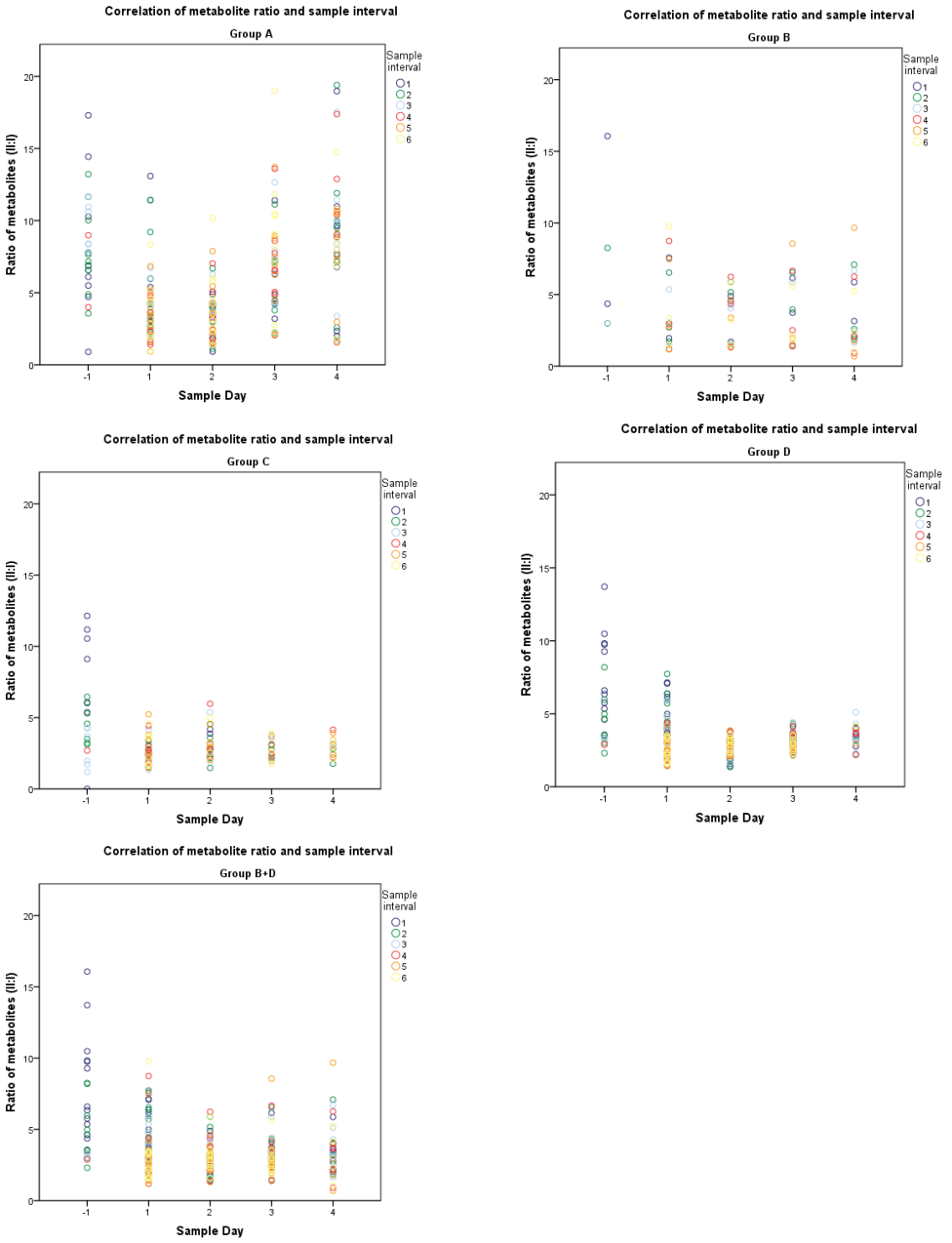


Figure 3.3-38 Dot plots of metabolite ratio and study day showing values arising from each sample interval

For each day of the study the six sample intervals were given a different colour. Banding of the colours in the graph would indicate a circadian variation (Figure 3.3-38). As no clear patterns of colour banding emerged in Groups A, B and C there was no evidence of diurnal variation in metabolic ratio in these groups. In Groups D and B+D some banding did appear to occur, with interval 6 appearing to cluster around the bottom of Days 1, 2 and 3. Further examination of the data revealed this was a product of the order which the data points are applied or "stacked" on the graph by the statistical software, and that all data clustered around this point. To confirm this for Group B+D, day numbers were removed from metabolic ratio results, and a box plot was prepared from these data that contained interval numbers only. This showed that no interval's metabolic ratio sits clearly above any other interval (Figure 3.3-39). Additionally, a Friedman's test was conducted to establish if there was any statistical evidence of difference between the intervals, and none was seen ($\chi 2$ 22.576, p=0.319) (data not shown).

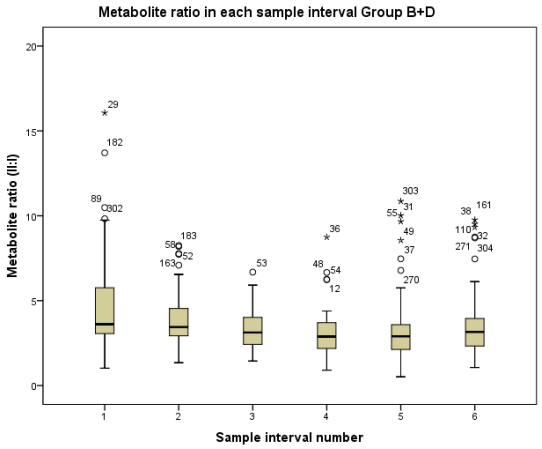


Figure 3.3-39 Box plot of metabolite ratio for each sample interval across all days

3.3.8.5 METABOLITES

Because four compounds contribute to the metabolic ratio, the question arises regarding how changes to a metabolite concentration affect the metabolite ratio, and whether one metabolite had a predominant effect on the ratio. To ascertain this, the metabolic ratio was plotted against the amount of each metabolite in the urine (the two Phase I products were again summed to give a single value as previously discussed). To determine the nature of their interaction a line of best fit was applied. Spearman's correlation coefficients were also determined to assess the strength of the association between each metabolite and the metabolic ratio (Table 3.3-71). While partial correlations may have provided more information about these relationships, they cannot be used on non-parametric data.

Table 3.3-71 Results of Spearman's correlation between metabolite ratio and amount of each metabolite in urine
** = significant difference at p<0.05*

	Paracetamol Glucuronide			Paracetamol Sulphate			Phase I products		
Group	P	ρ^2	Sig (p)	P	ρ^2	Sig (p)	P	ρ^2	Sig (p)
A	0.126	0.016	(0.031)*	-0.047	0.002	(0.422)	-0.665	0.442	(<0.00)*
B	0.406	0.165	(<0.00)*	0.373	0.139	(<0.00)*	-0.739	0.546	(<0.00)*
C	-0.050	0.003	(0.546)	0.011	0.000	(0.894)	-0.418	0.175	(<0.00)*
D	0.074	0.005	(0.258)	0.143	0.020	(0.028)*	-0.450	0.203	(<0.00)*
B+D	0.175	0.031	(0.002)*	0.162	0.026	(0.003)*	-0.540	0.292	(<0.00)*

Figure 3.3-40 shows the dot plot for all groups.

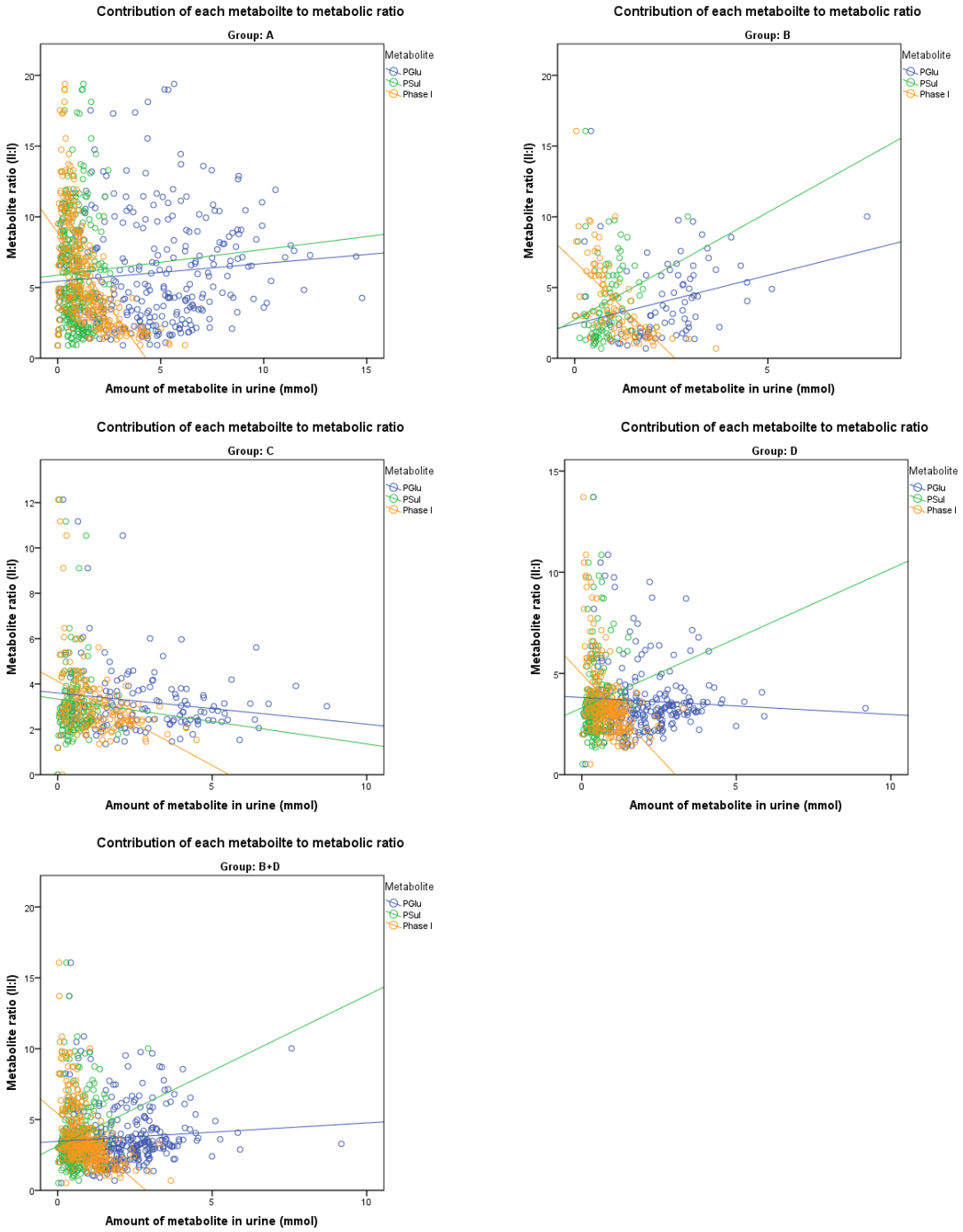


Figure 3.3-40 Contribution of each metabolite to metabolic ratio and lines of best fit for each group
Abbreviations: PGlu= paracetamol glucuronide; PSul= paracetamol sulphate; Phase I: total of paracetamol cysteine and paracetamol mercapturate.

In Group A, the slope of the line of best fit for both Phase II metabolites was fairly flat, indicating very little of the variation in metabolite amount explained the variation in metabolite ratio. There was also a high dispersion on either side of the best fit line, especially in the case of paracetamol glucuronide. This indicated the best fit line itself was a poor reflection of the data. Phase I metabolites cluster to the left of the graph and were represented by a best-fit line with a step negative slope, indicating that as amounts of Phase I values increased, metabolic ratio fell. The results of the correlation in Table 3.3-71 showed highly significant correlations between the metabolite ratio and paracetamol glucuronide and Phase I metabolites. However, while the correlation of the Phase I products appears strong (ρ^2=44.2%), the coefficient of determination for paracetamol glucuronide is small, accounting for just 1.6% of the variation in metabolite ratio.

For Group B the associations appeared stronger, displaying generally the same directional relationship between metabolites and ratio as Group A. Highly significant relationships were seen between all metabolites and metabolic ratio. Coefficients of determination were greater than in Group A, with Phase I metabolites one again showing the strongest relationship, accounting for 54.6% of the variance of metabolic ratio in this group.

Group C metabolites clustered together towards the origin of the graph and did not show any clear relationships with metabolic ratio. As previously, a significant correlation between Phase I metabolites and metabolic ratio was found, but it accounted for only 17.5% of the variation.

A similar clustering of data was seen in Group D with no clear associations emerging visually. Correlation revealed that there were two significant relationships: a weak correlation with paracetamol sulphate; and a stronger correlation with Phase I amounts which explained 20.3% of the variation.

Group B+D showed no relationships between metabolic ratio and any metabolite visually. Correlation showed significant relationships with all three metabolites, however the relationship with the Phase I metabolites was very weak. A stronger relationship was seen with the Phase I metabolites, accounting for 29.2% of the variation.

In summary, the strongest relationship determined by correlation was consistently that between metabolic ratio and Phase I metabolites.

3.3.8.6 REGRESSION

It was intended to use a multivariate method on the data from all samples to examine the relationship between the Phase II to Phase I metabolites ratio and the other variables; initially urine volume, sample number and total amount in the urine. Such analysis was attempted using multiple regression. Although the data going into the multiple regression model do not have to be normally distributed, the residuals from the generated model must. Analysis of the residuals showed that this assumption was violated. This finding applied across all groups and a sample is presented from Group A in Figure 3.3-41. This shows how the distribution of the residuals from Group A is non-normal, with a skewed histogram and deviation in the P-P plot from the normal line. This indicated that the model was invalid and could not be relied on for the description of the data.

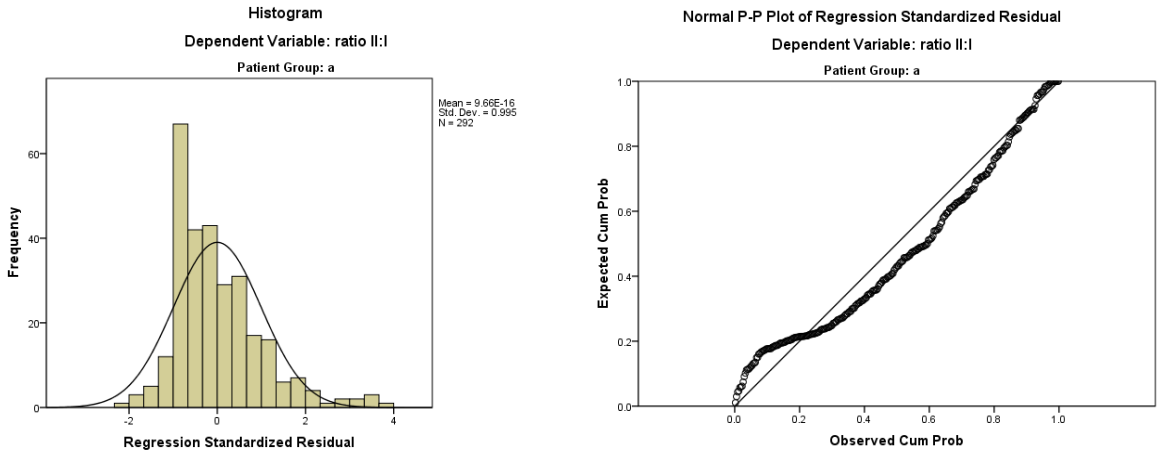


Figure 3.3-41 Histogram (left) and P-P plot (right) testing normality of regression residuals for Group A.
Data failed tests for normality as histogram (left) was well outside imposed normal curve and P-P plot (right) did not fall along the line of normality.

3.3.9 SUMMARY

Several changes were seen to the pattern of excretion of paracetamol metabolites following surgery. These changes were most discernible in the high dose group, Group A, in whom the large metabolic capacity of glucuronide metabolism was seen. Group A also had increased excretions of paracetamol cysteine, one of the metabolites derived from the toxic intermediary NAPQI, in addition to marked reductions in paracetamol sulphate. Similar patterns of change were shown in Group B and D, although to a lesser degree, while the way paracetamol was excreted in Group C did not appear to change noticeably.

3.4 PLASMA RESULTS

Plasma analysis is essential for determining accurate drug pharmacokinetics and most pharmacokinetic parameters are derived from a plasma concentration time series. Paracetamol is found in high concentrations in plasma as it has a low Vd and, as previously discussed in Section 2.2, its extraction from plasma is relatively straight forward. In addition to these pharmacokinetic values, when concentrations are at steady state, comparison of single time-point concentrations of paracetamol in plasma between days can also provide information about changes to drug disposition. For the purposes of this study, chromatograms were obtained, as described in Section 2.2 (Figure 3.4-1).

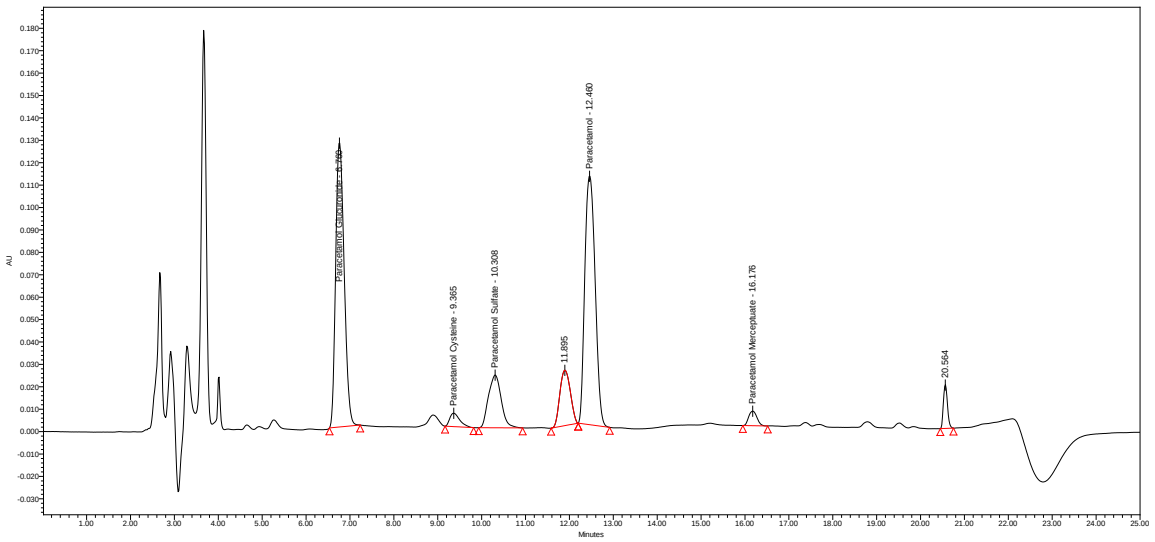


Figure 3.4-1 Sample chromatogram of absorbance of paracetamol and its metabolites in patient plasma
Chromatogram obtained from injection of patient 5A plasma (30min after dose on day 1) at UV 242nm and shows compound and retention time (mins). Retention times (in minutes) for paracetamol and its metabolites were: paracetamol glucuronide 6.78; paracetamol cysteine 9.37; paracetamol sulphate 10.31; paracetamol 12.46; and paracetamol mercapturate 16.18

These were used to determine the concentrations of paracetamol and its metabolites in plasma samples taken at the times shown in Table 2.1-1. As with the urine data, concentrations below the LOD were set to zero and concentrations between the LOQ and LOD were set to half the LOD (Shah *et al.* 1992). Plasma concentrations were then converted to molar concentrations. Although paracetamol was administered at regular intervals throughout each day postoperatively, only one dose interval per day was monitored with plasma sampling, postoperatively, this was usually the 10pm scheduled dose. The following plasma samples were taken as shown in Table 2.1-1:

- A full pharmacokinetic profile following the one preoperative paracetamol dose;
- A second full pharmacokinetic profile following the 10pm paracetamol dose on the first postoperative day; and

- Samples at 0, 1 and 4hrs following the 10pm paracetamol dose on subsequent postoperative days.

Accordingly data presented for Days 2, 3 and 4 arise only from the 0, 1 and 4 hour samples taken on those days.

Concentrations were used to calculate various pharmacokinetic parameters on the preoperative and first postoperative day. On days 2, 3 and 4 the 1 and 4 hour samples were then tested for changes over the duration of the study. Plasma concentrations of a typical patient are presented in Figure 3.4-2.

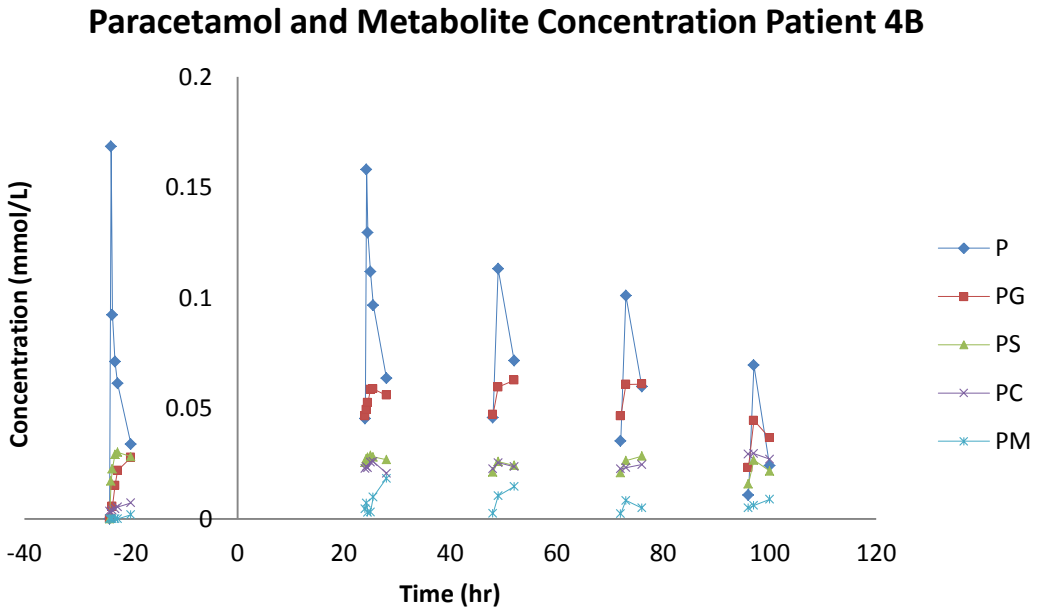


Figure 3.4-2 Plasma and metabolite concentrations for Patient 4B
Concentrations shown as measured over monitored dose interval on each day of the study. Data shown for days 2, 3 and 4 arise from 0, 1 and 4hr samples. Abbreviations: PS- paracetamol sulphate; PM- paracetamol mercapturate; PG- paracetamol glucuronide; PC- paracetamol cysteine; P- paracetamol

Statistical tests were performed using PASW Statistics 18, Release Version 18.0.0 (SPSS, Inc., 2009, Chicago, Illinois), with a critical level of significance of α=0.05. The distribution of paracetamol and metabolite concentrations, and the pharmacokinetic values derived from them, were checked visually for normality and by using the Kolmogorov-Smirnov test (D). Data did not fulfil conditions for normality and accordingly non-parametric tests were used. The critical level of significance was 0.05.

3.4.1 PARACETAMOL HALF-LIFE

Half-lives ($t_{½}$) were determined for the day before and the day after surgery using Equation 3.4-1.

$$t_{1/2} = \frac{0.693}{k}$$

Equation 3.4-1 Plasma half life

$t_{½}$ is a translation of the co-efficient of elimination (*k)* determined from the slope of the elimination phase of the semilogarithmic concentration vs. time plot. It is the relationship between the rate of elimination and the amount of drug in the body (Equation 3.4-2).

$$k = \frac{rate\ of\ elimination\ (Cl \cdot C)}{amount\ in\ body\ (C \cdot Vd)} = \frac{Cl}{Vd}$$

Equation 3.4-2 Elimination rate constant determined by clearance (Cl), concentration (C) and distribution volume (Vd)

The half-lives are displayed in Figure 3.4-3 along with a trend line for each patient.

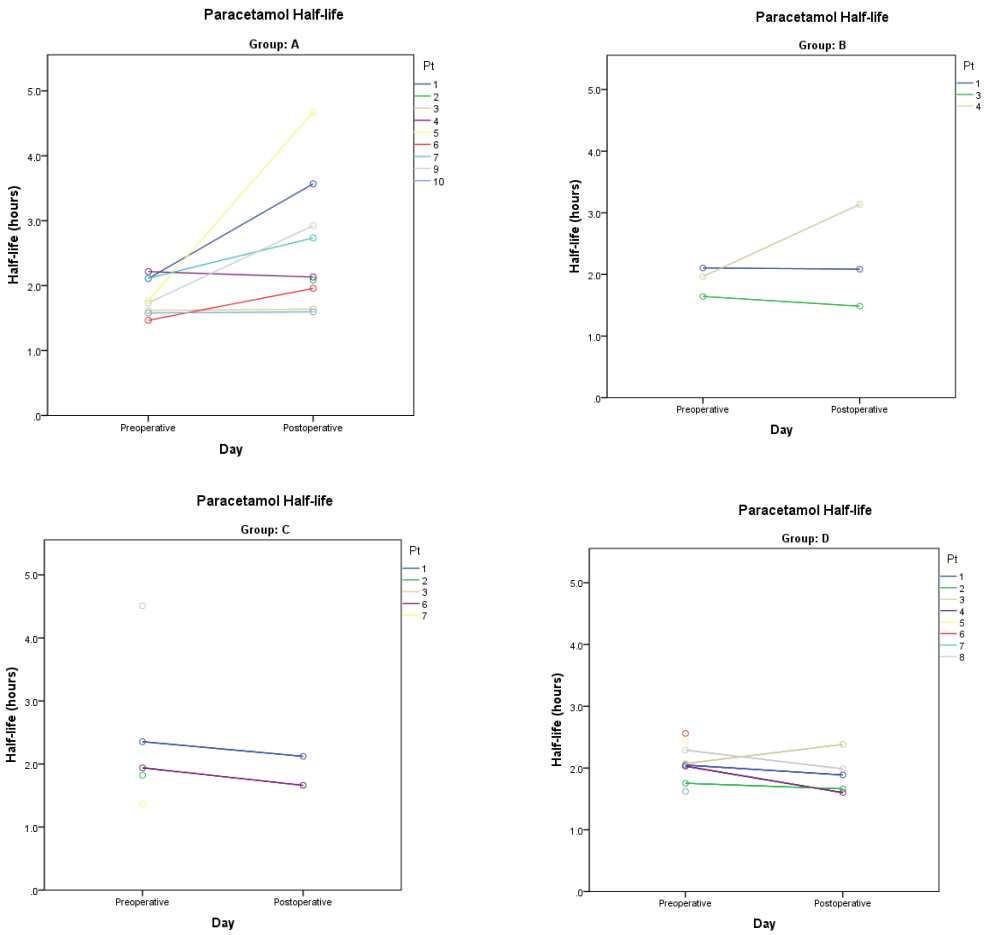


Figure 3.4-3 Pre and postoperative half-lives (hours)
Graphs (clockwise from top left): Group A, B, C and D

The postoperative half-lives for Group A almost uniformly increased, with only patients 3A and 10A exhibiting slight decreases (Pt3A- pre 1.62, post 1.64; Pt10A- pre 1.60, post 1.58). No other group showed such a consistent trend with most other patients' half-lives remaining the same or slightly decreasing. This is also reflected in the descriptive statistics (Table 3.4-1) where again Group A showed an increase in half-life.

One tailed exact Wilcoxon signed-rank tests were conducted to examine the changes to this paired data for significance (Table 3.4-1). This confirmed that Group A was the only group to exhibit a significant change.

Table 3.4-1 Half-life descriptives and statistical analysis
*Descriptives are presented as median (lower quartile, upper quartile) in hours. Wilcoxon matched pair results are presented as Z statistic and p value. * indicates significant result, where p<0.05.*

Group	Day -1	Day 1	Difference between days	Day -1=1 Wilcoxon matched pairs.
A	1.75 (1.59, 2.11)	2.13 (1.80, 3.25)	0.62 (0.02, 1.6)	2.100 p 0.020*
B	1.97 (1.64, 2.11)	2.09 (1.49, 3.14)	0.15 (0.02, 0.16)	0.000 p 0.625
C	1.94 (1.60, 3.43)	1.89 (1.25, 2.27)	0.12 (0.01, 0.23)	1.342 p 0.250
D	2.06 (1.82, 2.40)	1.89 (1.63, 2.19)	0.07 (-0.22, 0.25)	0.944 p 0.219

3.4.2 PARACETAMOL AREA UNDER THE CONCENTRATION VS. TIME CURVE

The area under the concentration versus time curve (AUC) relates both dose and clearance (Equation 3.4-3).

$$AUC = \frac{Dose}{Cl}$$

Equation 3.4-3 Relationship between AUC, dose and clearance (CL)

AUC was calculated over a dose interval (0-τ) at steady state with the trapezoid rule from the concentration time curve (Equation 3.4-4).

$$AUC_{0-\tau} = \sum_{i=0}^{n-1} (t_{i+1} - t_i) \cdot \frac{(C_i + C_{i+1})}{2}$$

Equation 3.4-4 Calculation of AUC using trapezoid rule
0-τ=dose interval, t=time, c=concentration, n=total number of concentration points, i= i^{th} concentration time value

AUC for a single dose was determined out to infinity (0-∞). At steady state:

$$AUC_{0-\tau} = AUC_{0-\infty}$$

Equation 3.4-5 Relationship between single dose and steady state AUC

Determining $AUC_{(0-\infty)}$ for a single dose requires the calculation of the area from the last measured concentration (C_{last}) until it reaches zero. This was achieved by the division of (C_{last}) by the constant *k*, which was then added to $AUC_{(0-\tau)}$

$$AUC_{0-\infty} = AUC_{0-\tau} + \left(\frac{C_{last}}{k}\right)$$

Equation 3.4-6 Calculation of $AUC_{0-\infty}$ for a single dose

The AUC was determined for the day before (Equation 3.4-6) and the day after surgery (Equation 3.4-4) and then divided by dose. These are shown in Figure 3.4-4. The AUC increased for the majority of Group A patients, although there was a marked reduction for patient 1A. For most patients in the other groups the AUC remained constant or reduced slightly. A significant increase in AUC was shown for Group A (Table 3.4-2).

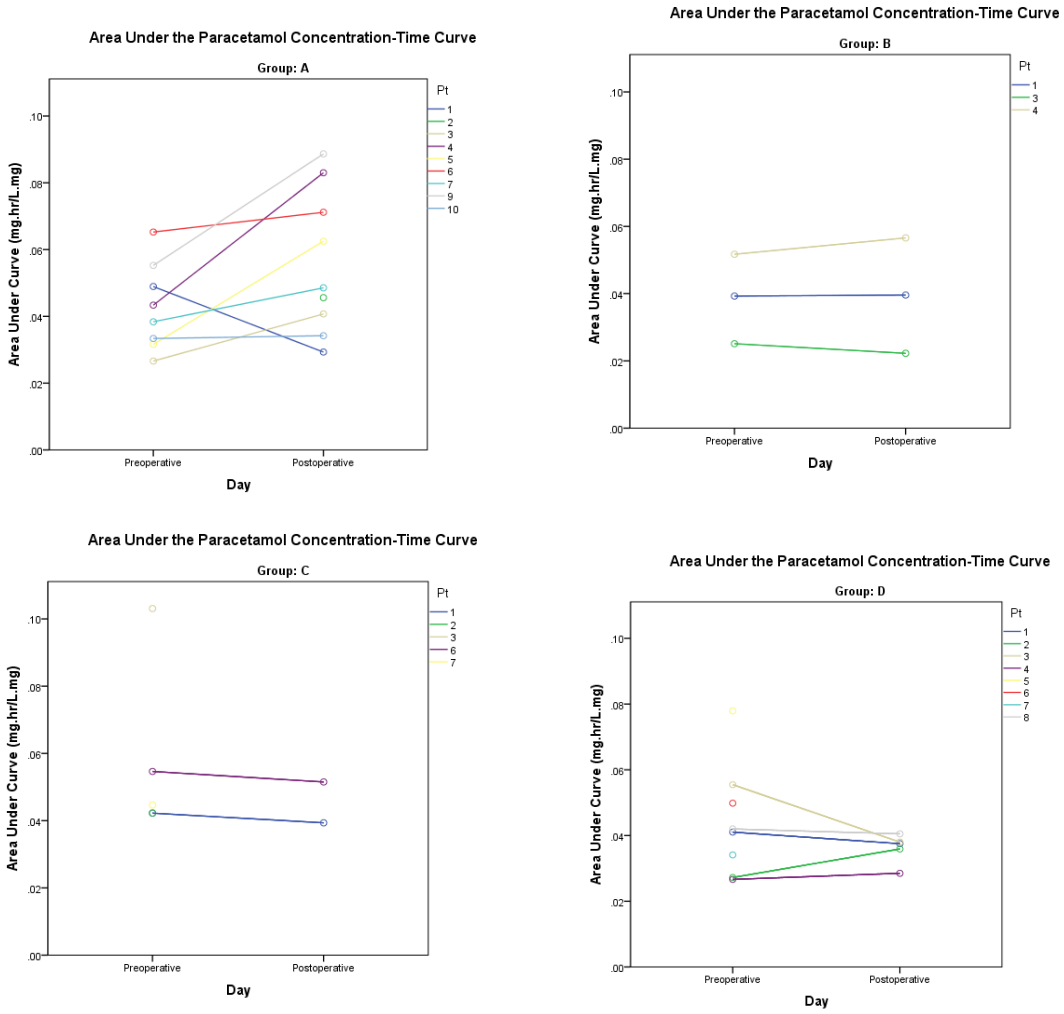


Figure 3.4-4 Pre and postoperative AUC (mg.hr/L.mg)
Graphs (clockwise from top left): Group A, B, C and D

No other significant changes were seen.

Table 3.4-2 AUC values before and after surgery

Group	Day -1 AUC (IQR) mg.hr/L	Day 1 AUC (IQR) mg.hr/L	Wilcoxon matched pairs. Day -1=1, Z
A	0.041 (0.032, 0.054)	0.049 (0.037, 0.077)	1.820 p 0.039*
B	0.039 (0.025, 0.052)	0.040 (0.022, 0.057)	0.535 p 0.375
C	0.045 (0.042, 0.079)	0.045 (0.03, 0.719)	1.342 p 0.250
D	0.042 (0.029, 0.054)	0.038 (0.032, 0.039)	0.405 p 0.406

3.4.3 CLEARANCE

Clearance is a measure of the amount of drug which is removed per unit volume per time period. Equation 3.4-2 shows the relationship between *k* (the co-efficient of elimination),

the *rate* at which a drug is cleared, and Vd (the volume of distribution), the theoretical volume into which a drug is dispersed. It is calculated by the rearrangement of Equation 3.4-3. Clearance values are shown in Figure 3.4-5

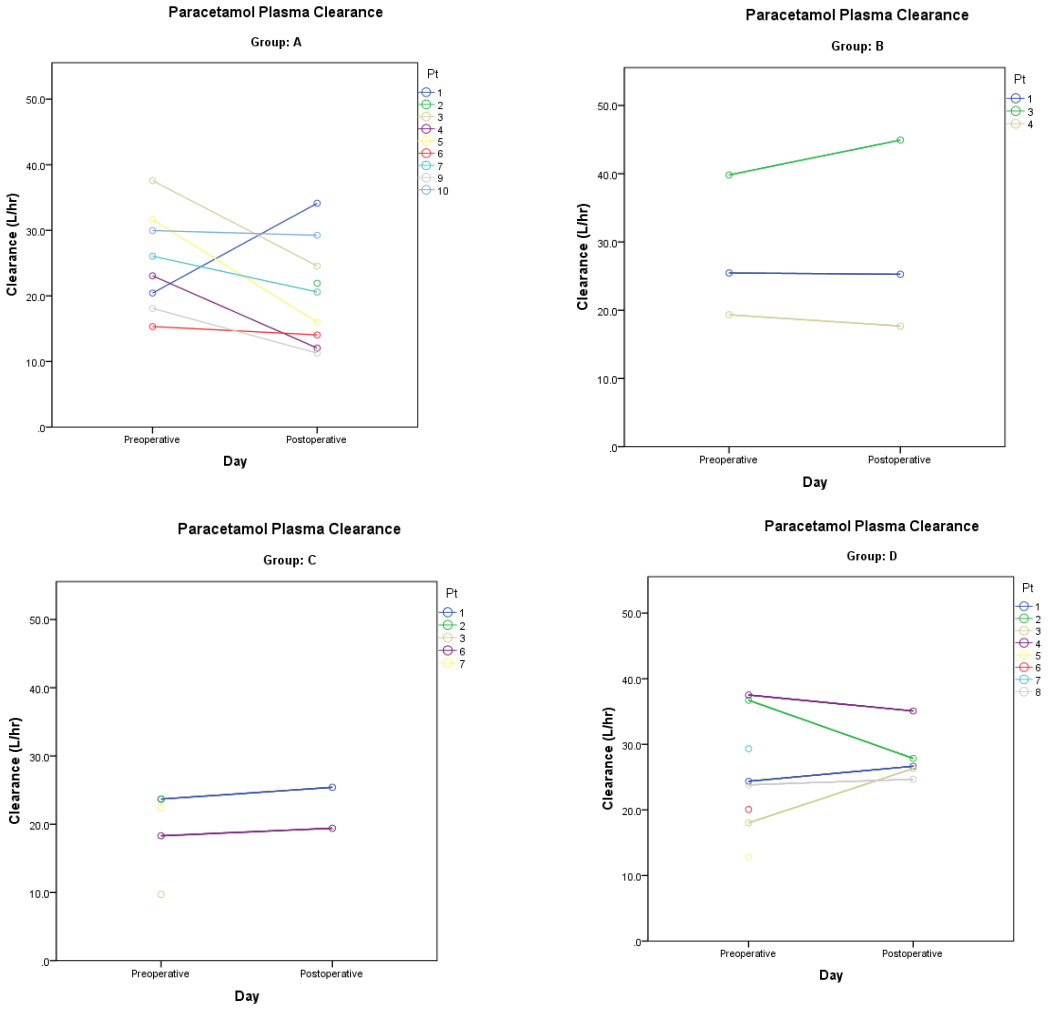


Figure 3.4-5 Pre and postoperative Clearance (L/hr)
Graphs (clockwise from top left): Group A, B, C and D

For Group A all patients showed reduction in clearance, except for patient 1A who showed a large increase. There were no strong trends in the other groups, with most patients showing little or no change at all, aside from patient 2D, who showed a marked reduction, and patient 3D who showed an increase.

Based on these values there were no significant changes exhibited (Table 3.4-3).

Table 3.4-3 Clearance (L/hr) values before and after surgery

Group	Day-1 (IQR)	Day 1 (IQR)	Wilcoxon matched pairs. Day -1=1
A	24.56 (18.68, 31.21)	20.60 (13.05, 26.90)	1.540 p 0.074
B	25.46 (19.34, 39.82)	25.27 (17.67, 44.94)	0.000 p 0.625
C	22.40 (14.00, 23.68)	22.41 (14.56, 19.74)	1.342 p 0.250
D	24.09 (18.54, 34.87)	26.66 (25.49, 31.47)	0.135 p 0.500

Calculating the differences between preoperative and postoperative Cl values showed patient 1A's Cl to lie well outside the distribution of the rest of Group A. Treating patient 1A as an outlier and removing it from the analysis meant that Group A produced a significant difference (2.336 p=0.008).

3.4.4 VOLUME OF DISTRIBUTION

Volume of distribution (Vd) was also determined for the day before (Equation 3.4-7) and the day after surgery (Equation 3.4-8).

$$Vd = \frac{Cl}{k}$$

Equation 3.4-7 Calculation of Volume of Distribution for a single dose

$$Vd_{ss} = \frac{f.d}{\tau.k.C_{ss}}$$

Equation 3.4-8 Calculation of Volume of Distribution at steady state (Vd_{ss})
f=-bioavailability of dose, C_{ss}=concentration at steady state

Where

$$C_{ss} = \frac{f.d}{\tau.Cl}$$

Equation 3.4-9 Calculation of Concentration at steady state

There were reductions shown in Vd in all groups for most patients with the exceptions of patients 1A and 3D. Only the differences in Group A were significant but clear trends were observed in all other groups (Table 3.4-4, Figure 3.4-6).

Table 3.4-4 Vd (L/kg) before and after surgery
**=significant difference*

Group	Day-1 (IQR)	Day 1 (IQR)	Wilcoxon matched pairs. Day -1=1
A	0.77 (0.65, 0.89)	0.48 (0.38, 0.70)	2.240 p 0.012*
B	0.97 (0.63, 1.08)	0.63 (0.52, 0.88)	1.604 p 0.125
C	0.88 (0.76, 1.11)	0.71 (0.42, 1.33)	1.342 p 0.250
D	0.89 (0.77, 1.17)	0.75 (0.58, 0.92)	1.753 p 0.063

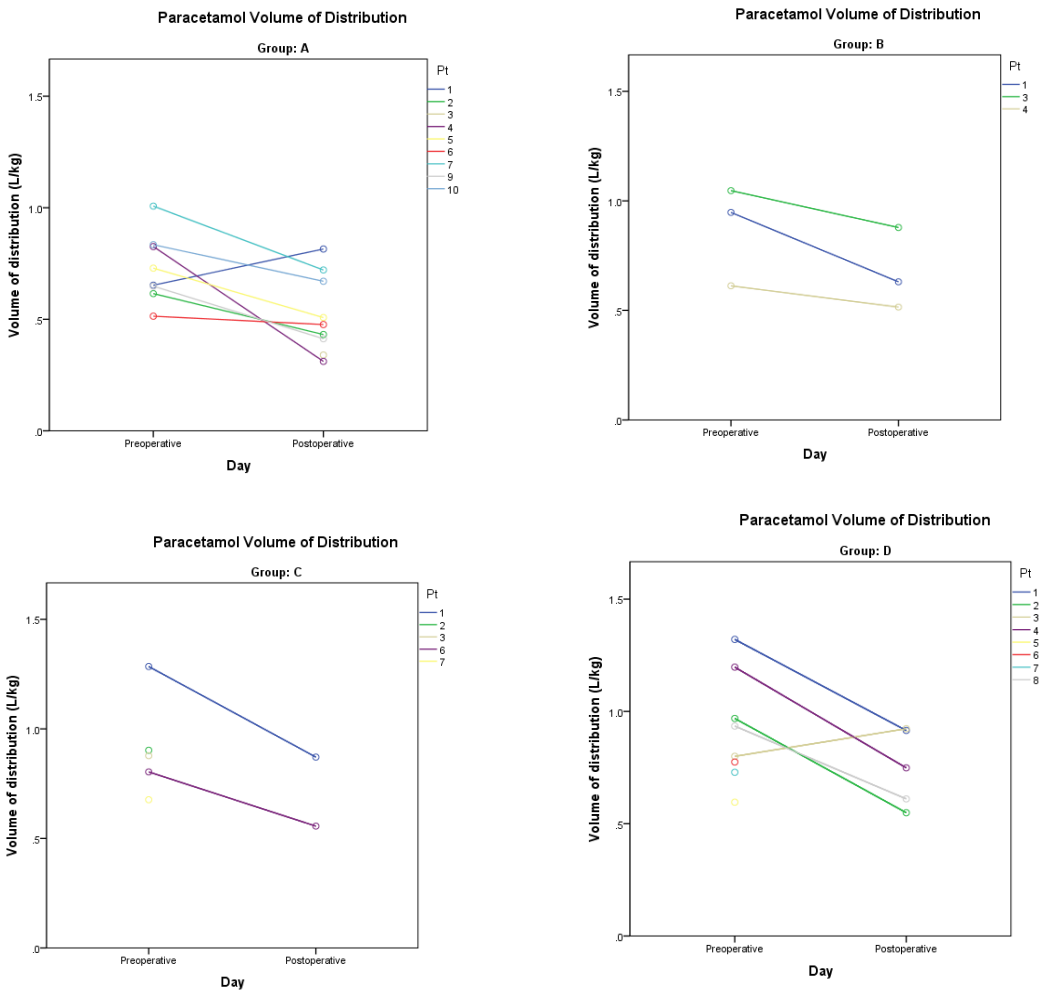


Figure 3.4-6 Pre and postoperative Vd(L/kg)
Graphs (clockwise from top left): Group A, B, C and D

3.4.5 MEAN RESIDENCE TIME

Mean residence time is an approximation of how long an "average" molecule of drug stays in the body. It is determined as shown in Equation 3.4-10.

$$MRT = \frac{AUMC}{AUC}$$

Equation 3.4-10 Calculation of Mean Residence Time (MRT)
AUMC= area under the moment curve, AUC= area under the curve

This value can also be used to approximate half-life.

$$t_{1/2} = 0.693 \times MRT$$

Equation 3.4-11 Calculation of half-life using MRT

There was close agreement between the half-lives calculated using the coefficient of elimination and those using MRT.

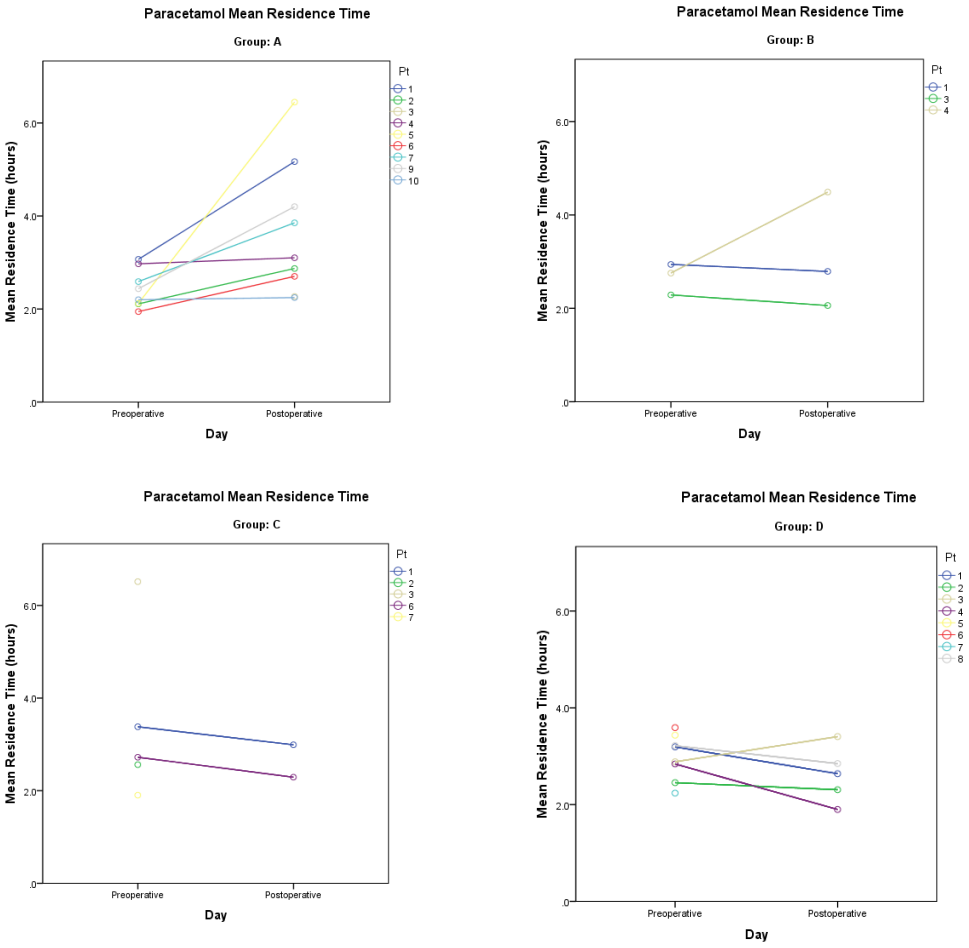


Figure 3.4-7 MRT before and after surgery

This suggests the disposition kinetics of paracetamol can be approximated by a one compartment model. Half-lives derived from the coefficient of elimination that are distinctly greater than those determined using MRT indicate multiple compartments that cannot be predicted using the simple methods here.

All patients in Group A exhibited increases in MRT (Figure 3.4-7).

There were no strong trends in the other groups although there was a tendency for MRT to decrease, as depicted in Table 3.4-5.

Table 3.4-5 Pre and postoperative MRT (hours)
**=significant difference*

Group	Day-1 (IQR)	Day 1 (IQR)	Wilcoxon matched pairs. Day -1=1
A	2.32 (2.11, 2.88)	3.10 (2.49, 4.69)	2.521 p 0.004*
B	2.76 (2.29, 2.94)	2.79 (2.06, 4.49)	0.000 p 0.625
C	2.73 (2.23, 4.95)	2.64 (1.72, 2.92)	1.342 p 0.250
D	3.04 (2.55, 3.38)	2.64 (2.10, 3.13)	1.214 p 0.156

3.4.6 PARACETAMOL CONCENTRATION IN PLASMA ONE HOUR POST DOSE

Plasma samples taken one hour after paracetamol dosing were analysed. They are presented with and without normalisation for dose (Figure 3.4-8). Dose normalised values (*y*) were obtained by multiplying Group A's measured concentration (*x*) by the 24 hourly paracetamol dose the other groups received (4g) divided by Group A's dose (9g), so that $y=(4g/9g)x=0.444x$.

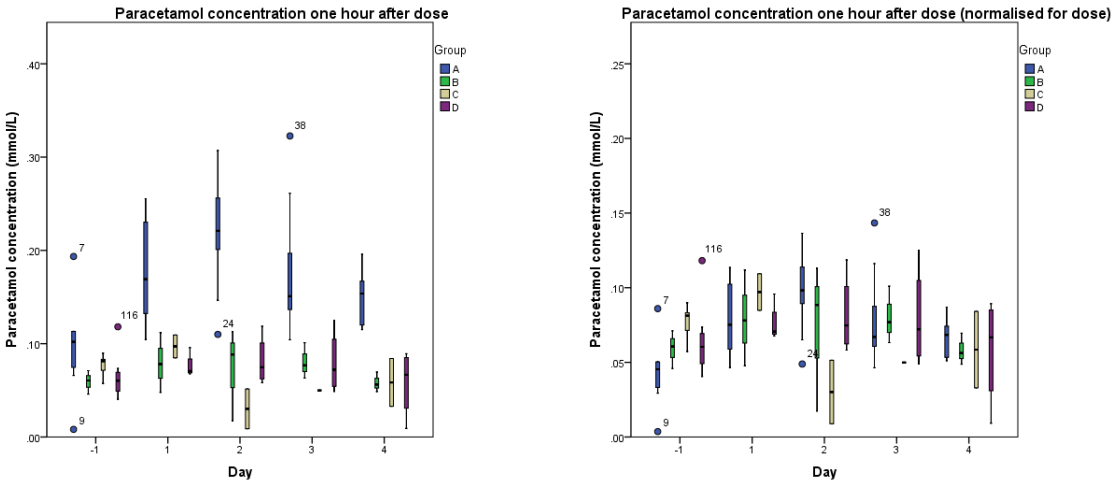


Figure 3.4-8 Plasma paracetamol (mmol/L) at one hour with (left) and without (right) normalisation for dose

The left graph in Figure 3.4-8 shows the extent of accumulation associated with Group A's higher dose. When normalised for dose, trends emerge. For most groups, paracetamol concentrations increased until the middle of the study and then appear to decline.

Differences between distributions were checked with Friedman's two-way analysis of variance by ranks for statistical significance using exact tests (Table 3.4-6).

Table 3.4-6 Plasma paracetamol concentration (mmol/L) 1 hour post dose as median (IQR) and results of Friedman's test

** = significant difference*

Group	Day -1	Day 1	Day 2	Day 3	Day 4	χ^2 (p)
A	0.102 (0.072, 0.113)	0.169 (0.126, 0.232)	0.224 (0.16, 0.272)	0.15 (0.134, 0.243)	0.156 (0.135, 0.174)	11.6 (0.012)*
B	0.061 (0.046, 0.071)	0.078 (0.048, 0.112)	0.088 (0.017, 0.113)	0.077 (0.063, 0.101)	0.056 (0.049, 0.07)	4 (0.475)
C	0.081 (0.064, 0.087)	0.097 (0.085, 0.109)	0.03 (0.009, 0.051)	0.05 (0.05, 0.05)	0.058 (0.033, 0.084)	3 (0.460)
D	0.06 (0.042, 0.074)	0.071 (0.068, 0.09)	0.075 (0.06, 0.11)	0.072 (0.052, 0.115)	0.067 (0.02, 0.087)	5.6 (0.258)

The Friedman's test revealed a significant difference across Group A. A *post hoc* test was conducted with exact Wilcoxon matched pairs tests (Table 3.4-7).

Table 3.4-7 Group A, *post hoc* pair-wise analysis (p)
*= *significant difference*

Group A				
1	0.005*			
2	0.008*	0.173		
3	0.015*	0.594	0.139	
4	0.021*	0.021*	0.028*	0.139
Day	-1	1	2	3

Significant differences were seen between day -1 (preoperative day) and all others days, and Day 4 and Days -1, 1 and 2 (Table 3.4-7). This reflects the parabolic shape of the concentration distribution across the days of the study. While many individual tests exceed the critical α=0.05 level of significance, statistical devotees would insist the Bonferroni correction again be applied to these repeated tests to reduce the chance of a Type I error (*i.e.* chance of a false positive). Because there are 10 comparisons, considering the Bonferroni correction in this test reduces the critical level of significance to 0.005, so that only the increase between Day -1 and 1 remains statistically significant. Wilcoxon matched pairs tests were also conducted for the other three groups. As expected from the Friedman's two-way analysis of variance, only Group A showed any pairwise differences.

3.4.7 PARACETAMOL CONCENTRATION IN PLASMA FOUR HOURS POST DOSE

A similar analysis was conducted for the four hour post dose concentration of paracetamol in plasma (Figure 3.4-9).

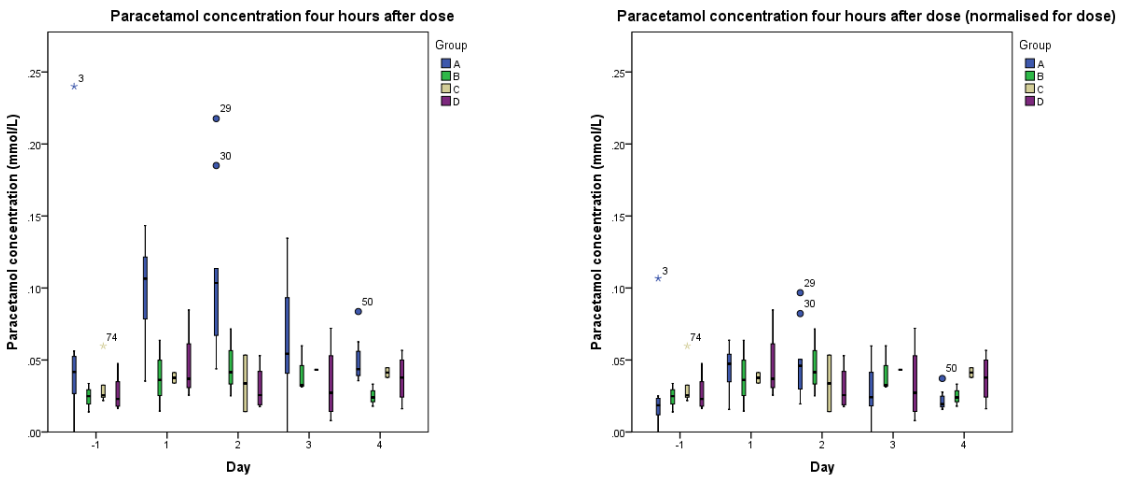


Figure 3.4-9 Plasma paracetamol (mmol/L) at four hours with (left) and without (right) normalisation for dose

Patterns are less clear on analysis of this sample time. The left graph in Figure 3.4-9 shows a stark increase in paracetamol concentration in Group A following surgery which then appears to reduce over the course of the study. Dose normalisation (right graph Figure 3.4-9) does not reveal any obvious patterns; however, a similar shape to the one hour

concentration emerges, with increases after surgery and declining over subsequent days. This effect is most notable in Groups A and B. Once again a significant difference was seen in the distribution of concentrations for Group A (Table 3.4-8).

Table 3.4-8 4 hour plasma paracetamol concentration (mmol/L) as median (IQR) with Friedman's test results
** = significant difference*

Group	Day -1	Day 1	Day 2	Day 3	Day 4	χ^2 (p)
A	0.041 (0.025, 0.052)	0.107 (0.07, 0.127)	0.104 (0.064, 0.149)	0.063 (0.049, 0.108)	0.044 (0.039, 0.063)	18.533 (0.00)*
B	0.025 (0.014, 0.034)	0.036 (0.014, 0.064)	0.042 (0.025, 0.072)	0.032 (0.031, 0.06)	0.024 (0.018, 0.033)	7.467 (0.096)
C	0.026 (0.023, 0.046)	0.038 (0.034, 0.050)	0.034 (0.014, 0.048)	0.043 (0.018, 0.053)	0.041 (0.023, 0.045)	0.406 (0.354)
D	0.023 (0.017, 0.035)	0.037 (0.028, 0.073)	0.026 (0.018, 0.048)	0.027 (0.011, 0.062)	0.038 (0.02, 0.053)	4.267 (0.432)

Another *post hoc* analysis was undertaken (Table 3.4-9)

Table 3.4-9 Group A *Post hoc* pair-wise analysis (p)
** = significant difference*

Group A				
1	0.005*			
2	0.008*	0.314		
3	0.093*	0.123	0.036*	
4	0.050*	0.017*	0.012*	0.161
Day	-1	1	2	3

Similar to the one hour time sample, the preoperative sample from Group A was different from other days'. There were also significant differences between each of Day 1 and Day2 with Day 4. Also similar to the one hour time sample, the application of the Bonferroni correction causes all but the increase between Day -1 and Day 1 to loose significance.

3.4.8 ACCUMULATION OF PARACETAMOL IN GROUP A

Samples were collected at t=0 and t=4hrs for all patients

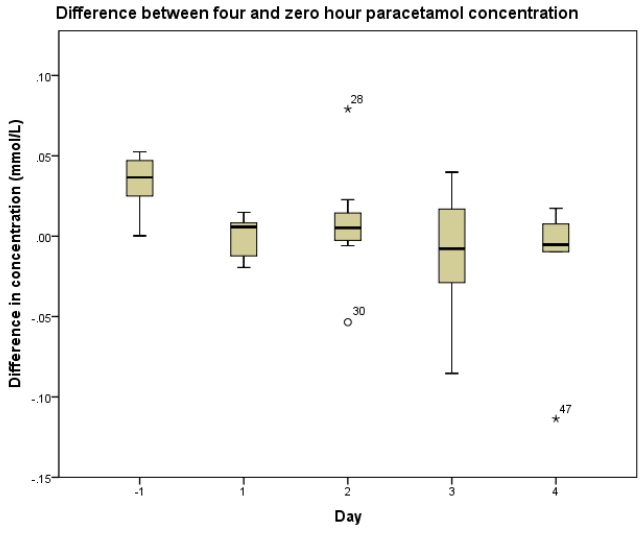


Figure 3.4-10 Difference between t=4 and t=0 paracetamol concentration

For Group A, who received paracetamol every four hours, this represented the beginning and end of the dosage interval and these were compared for evidence of accumulation. Confirming the data above, the largest accumulation across the dose interval occurred on the first dose on the day before surgery (Figure 3.4-10). On the days following surgery, the median value reduced to the point where on Days 3 and 4 the paracetamol concentrations were lower at the end of the dose interval than at the beginning. A Friedman's test was also conducted on these data showing a statistically significant difference.

Table 3.4-10 Difference between t=0 and t=4 concentrations (mmol/L) as median (IQR) and results of Friedman's test
** = significant difference*

Group A	Day -1	Day 1	Day 2	Day 3	Day 4	χ^2 (p)
Median	0.037	0.006	0.005	-0.008	-0.005	10.88
IQR	0.024, 0.049	-0.013, 0.009	-0.004, 0.019	-0.035, 0.025	-0.036, 0.01	(0.015)*

The negative median values indicate concentrations actually reduced over the dosage interval (*i.e.* between the time 0 and time 4 hour samples). A *post hoc* test was again conducted using one tailed exact Wilcoxon signed ranks tests (Table 3.4-11). The significant differences occurred between the preoperative and postoperative days. While none of the postoperative days are significantly different from each other, there is some evidence of a trend between them (p=0.078). However, applying the Bonferroni correction eliminated the statistical significance of all these results.

Table 3.4-11 Results of *post hoc* test of differences between t=0 and t=4 concentrations (p)
** = significant difference*

Group A				
1	0.008*			
2	0.074	0.410		
3	0.008*	0.273	0.527	
4	0.031*	0.281	0.078	0.500
Day	**-1**	**1**	**2**	**3**

3.4.9 PLASMA METABOLITE CONCENTRATIONS

All metabolites studied are readily excreted in the urine. As a result, plasma does not accurately reflect metabolite ratios as the metabolites' rates of formation are different from one another. Glucuronide and sulphate metabolites appear rapidly in plasma as they arise directly from a Phase II reaction on paracetamol. Conversely, paracetamol cysteine and mercapturate arise from a Phase II reaction on NAPQI, itself a Phase I product of paracetamol. Therefore NAPQI formation will delay the rate of paracetamol cysteine and mercapturate formation. The concentrations of paracetamol and these metabolites in plasma over the entire study are presented below for each group (Figure 3.4-11).

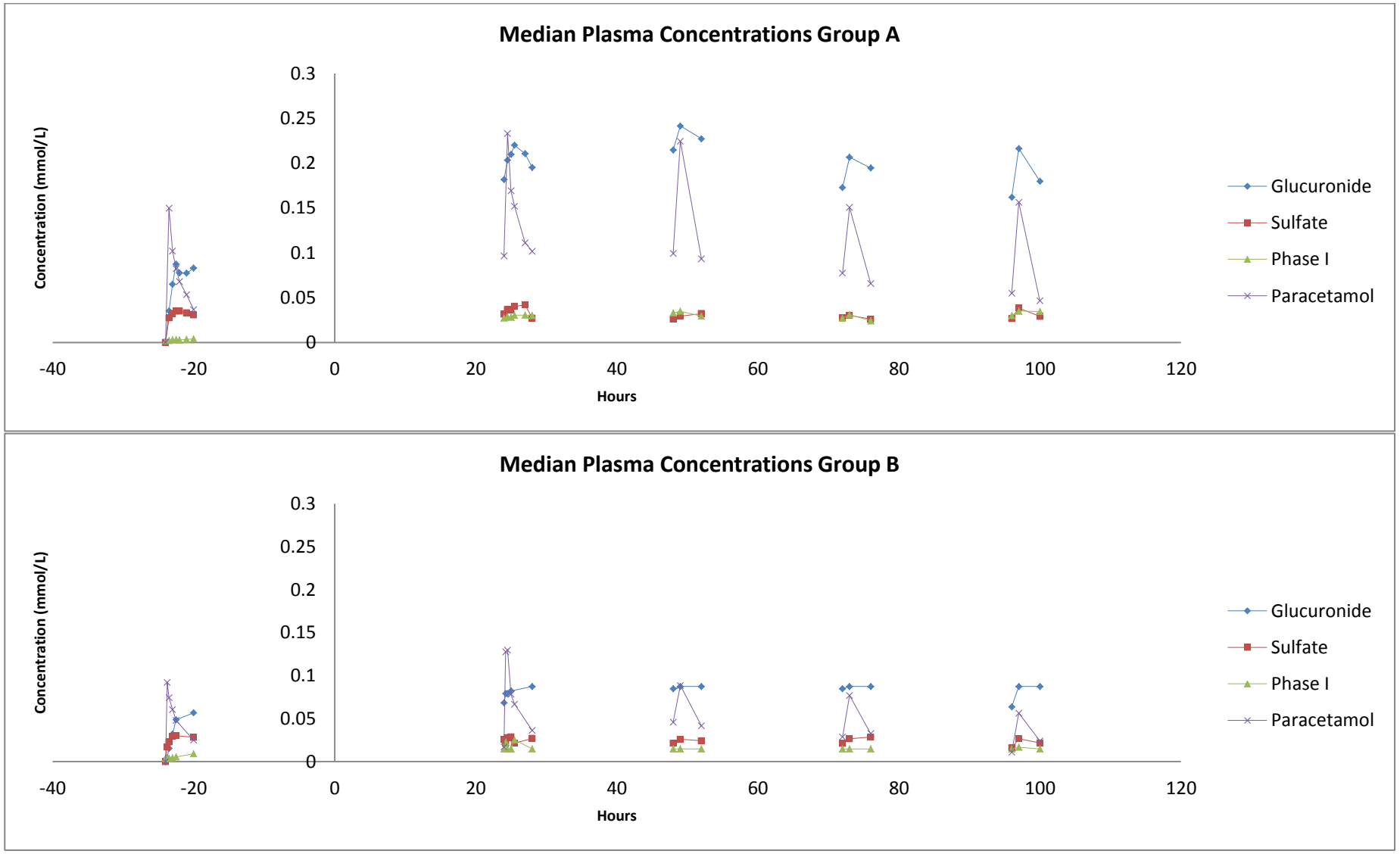


Figure 3.4-11 Paracetamol and metabolite concentrations (mmol/L)

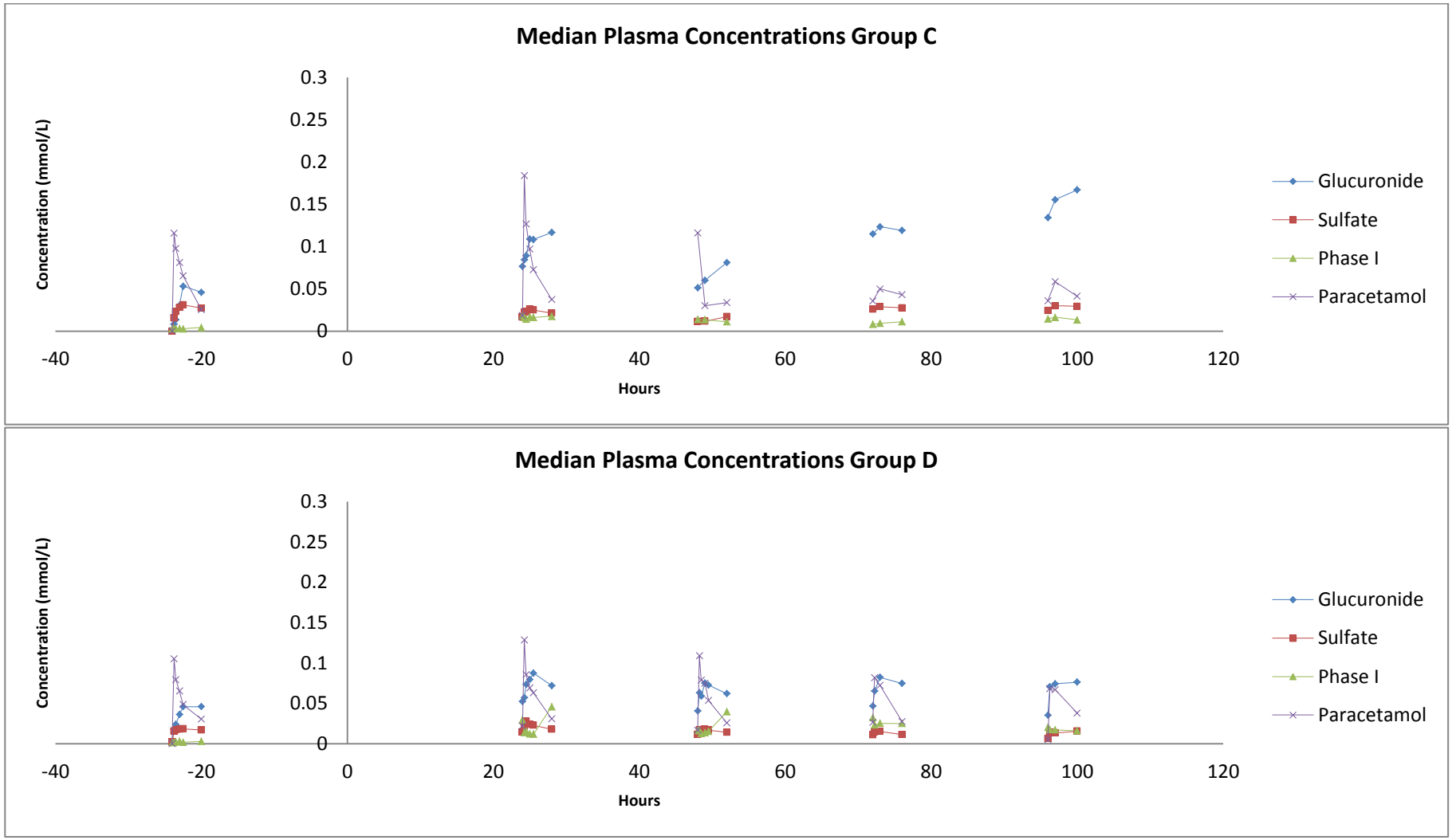


Figure 3.4-11 Continued. Paracetamol and metabolite concentrations (mmol/L)

Some patterns appear from Figure 3.4-11. In Group A paracetamol and paracetamol glucuronide concentrations peak on Days 2 and 3 but there are no accompanying changes in the concentrations of paracetamol sulphate or the Phase I conjugates. Visually, concentrations of paracetamol sulphate or the Phase I conjugates remain relatively consistent across all groups, despite Group A having over twice the daily dose of paracetamol of the other groups.

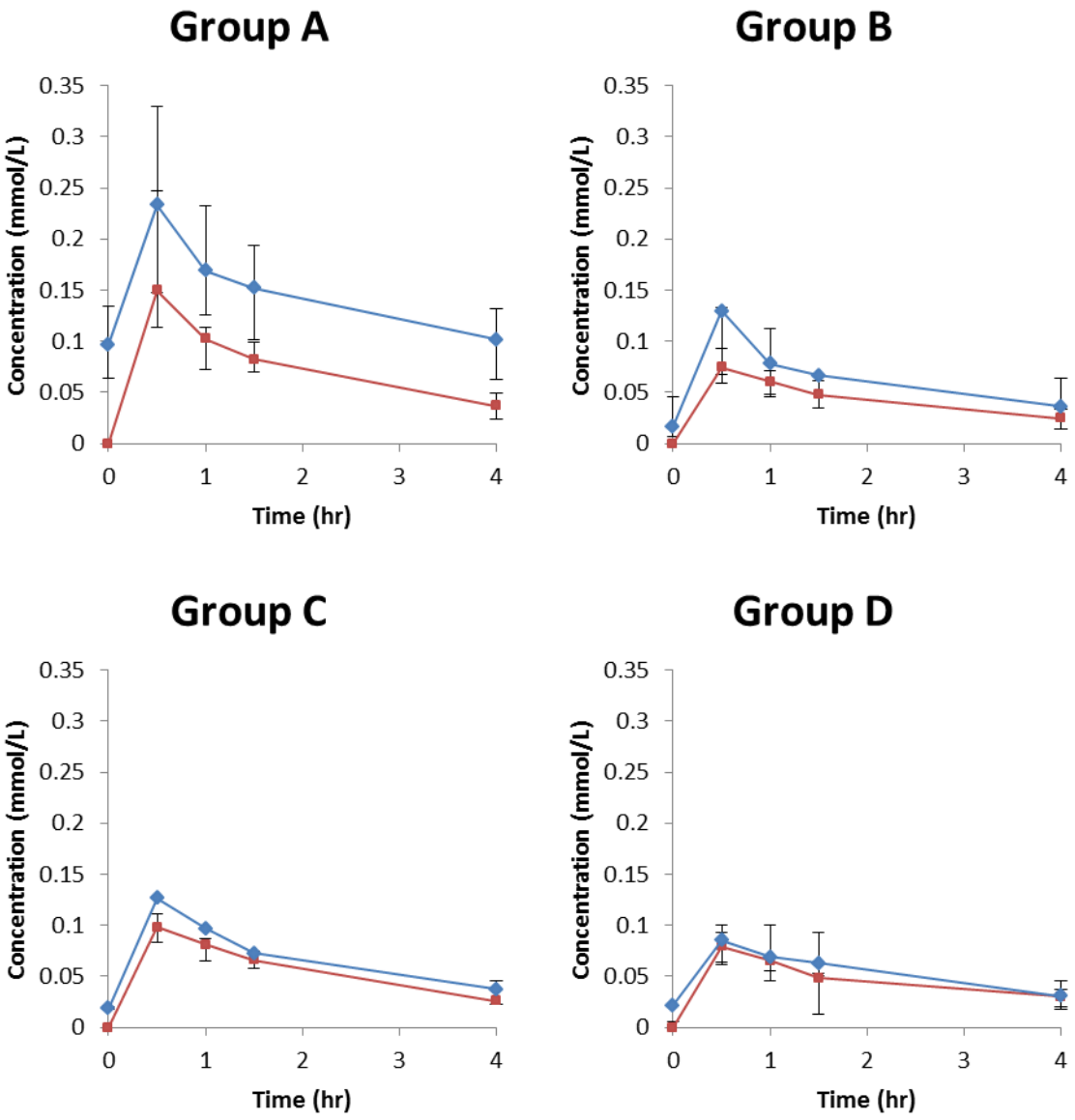


Figure 3.4-12 Median plasma paracetamol concentrations (mmol/L)
Presented as median +/- quartiles preoperatively (red line) and Day 1 postoperatively (blue line).

Paracetamol glucuronide concentrations accumulated in Group A, appearing to reach their highest concentration on Day 2 from which point they appeared to decline. The fact that paracetamol glucuronide concentrations accumulated and in later intervals exceeded those of paracetamol itself, indicates that paracetamol glucuronide rate of formation is greater than its rate of elimination, *i.e.* that its half-life is greater than that of paracetamol.

Further analysis was conducted comparing the kinetics of the preoperative to postoperative days and these graphs are shown in Figure 3.4-12.

For Group A, the median C_{max} of paracetamol increases from 0.254 mmol/L preoperatively to 0.296mmol/L on Day 1 postoperatively, an approximate increase of 17%. The preoperative C_{max} arising from a single dose in other groups is consistent between groups at around 0.1mmol/L. There appeared to be much less accumulation of paracetamol in Groups B, C and D (Figure 3.4-12) although as a percentage of preoperative C_{max}, the increase is greater than of Group A (Table 3.4-12).

Table 3.4-12 Difference between median C_{max} (mmol/L) values

Group	Day-1 C_{max} (mmol/L)	Day 1 C_{max} (mmol/L)	Difference (mmol/L)	% Increase
A	0.140	0.243	0.103	73.4
B	0.092	0.13	0.037	40.6
C	0.116	0.184	0.068	58.8
D	0.105	0.128	0.024	22.4

3.4.10 CONCENTRATIONS WITHIN DOSE INTERVAL

The concentrations of paracetamol and each metabolite were compared to examine the relationship between time of dose and formation of metabolites and the difference between steady state and single dose metabolite concentrations.

3.4.10.1.1 PHASE I PRODUCTS

The same process was followed for paracetamol cysteine and mercapturate. Neither metabolite was found in measurable concentrations following the preoperative paracetamol dose. Paracetamol cysteine was found following the postoperative dose although the concentration did not change over time, showing no increase with the administration of paracetamol. Concentrations in Group A, while still low (0.03mmol/L approximately), were about twice those of the other groups. Slight amounts of paracetamol mercapturate were detected postoperatively but again the concentration was not affected by paracetamol administration. There was no visually apparent difference between groups. These graphs are contained in Appendix 13.

3.4.10.1.2 PARACETAMOL GLUCURONIDE

Concentrations of paracetamol glucuronide are shown on the day before and day after surgery for each group (Figure 3.4-13). Preoperatively, all groups showed increases in paracetamol glucuronide concentration following the dose, until the 1.5 hour sample.

From this point, concentrations appeared to plateau. There was a substantial increase between preoperative and postoperative concentrations in Group A, with accumulations to a lesser degree seen in the other groups. Paracetamol glucuronide was detected prior to the administration of paracetamol postoperatively.

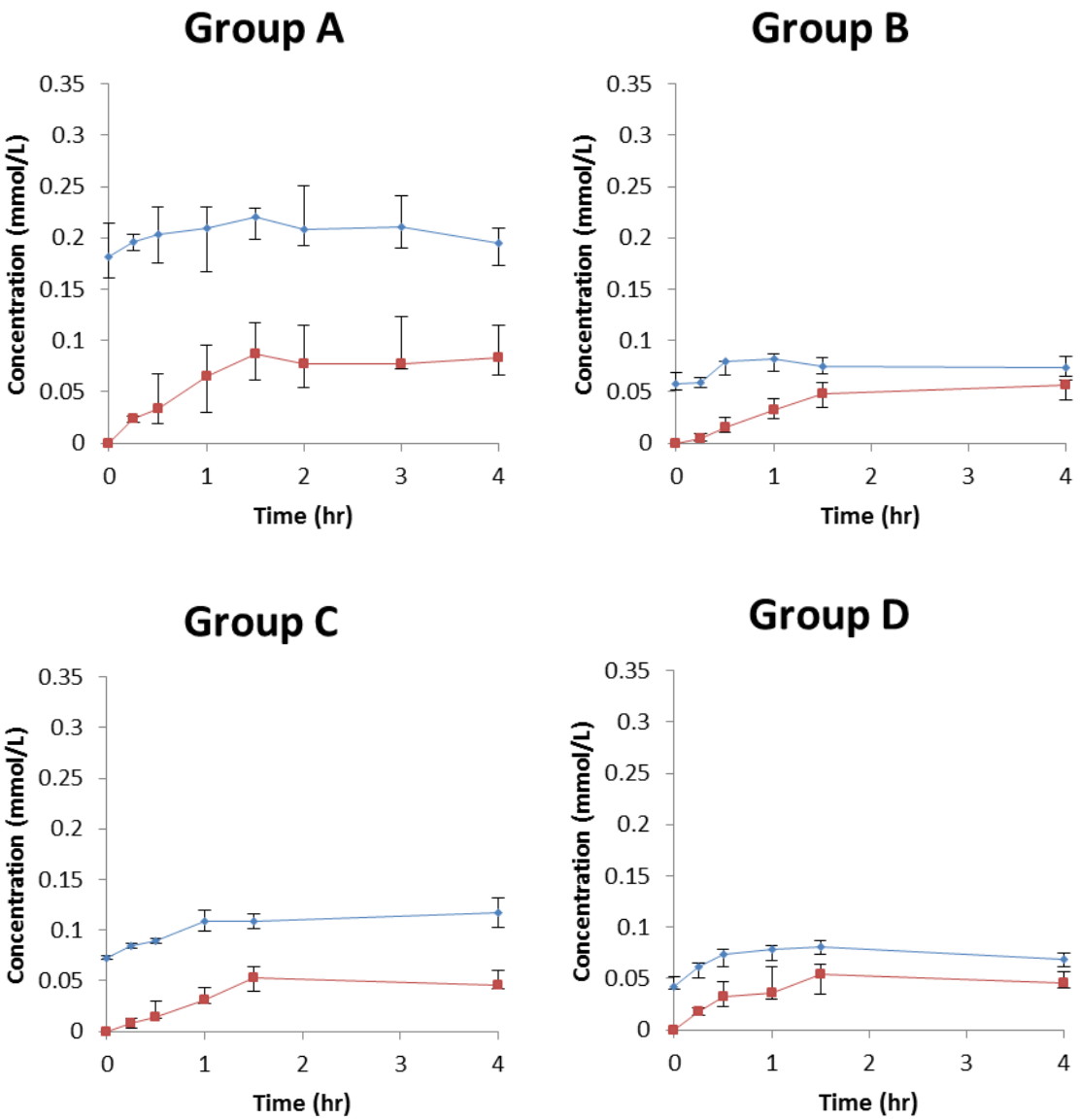


Figure 3.4-13 Median plasma paracetamol glucuronide concentrations (mmol/L)
Presented as median +/- quartiles preoperatively (red line) and day 1 postoperatively (blue line).

3.4.10.1.3 PARACETAMOL SULPHATE

Concentrations of paracetamol sulphate are also shown (Figure 3.4-14). Preoperative and postoperative concentrations are almost identical, not showing any significant increase between the single dose and steady state conditions. There was also very little difference between groups, despite the much higher dose given in Group A. There were slight increases of paracetamol sulphate concentration following the paracetamol dose but

these plateaued within 30 minutes for all groups. Paracetamol sulphate was also detected prior to the administration of paracetamol postoperatively.

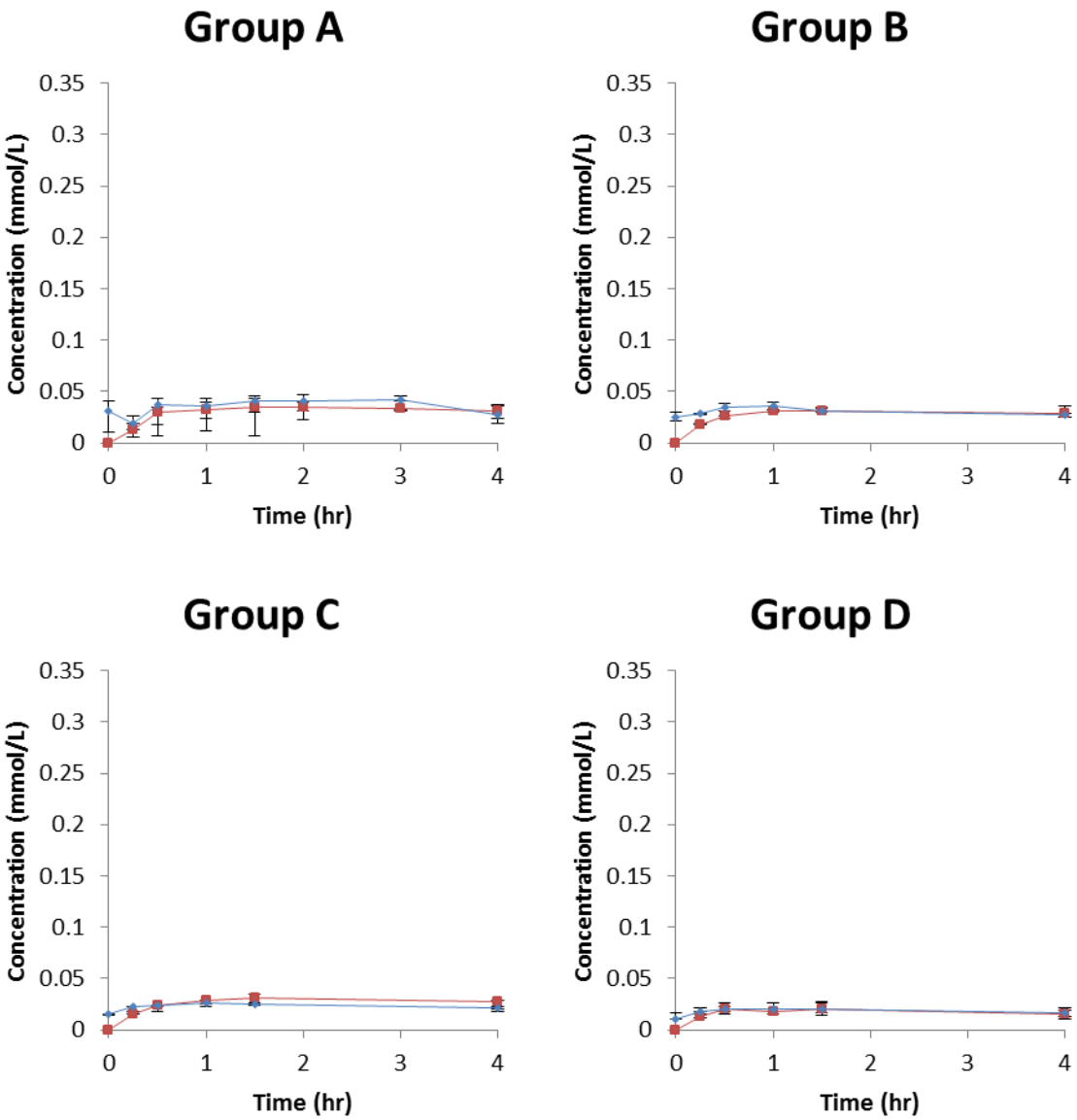


Figure 3.4-14 Median plasma paracetamol sulphate concentrations (mmol/L)
Presented as median +/- quartiles preoperatively (red line) and day 1 postoperatively (blue line).

3.4.11 SUMMARY

Paracetamol's plasma half-lives were seen to increase in Groups A and B which lead to statistically significant increases in exposure to paracetamol in Group A. Clearance and Vd were examined for their contribution to these changes. Reductions in clearance were large in Group A, while all other groups remained static. More noticeable were reductions to Vd which were seen in all groups and achieved statistical significance in Group A. Over the postoperative course one and four hour post-dose concentrations appeared to accumulate in all groups until Day 2, long after steady state was expected, from which time the appeared to decline approaching preoperative concentrations.

3.5 CYTOKINE RESULTS

The concentration of cytokines IL-1β, IL-6, TNFα and IFNγ were measured in daily patient samples by the ELISA methods described in Section 2.3.

Cytokines release and peak at different times following surgical trauma and concentrations following major abdominal surgery have been previously reported (Roumen *et al.* 1993a) (Section 1.3.1.2). TNF-α and IL-1β are key pro-inflammatory cytokines and are amongst the first to be released, and along with IFN-γ they stimulate the release of other cytokines including IL-6 (Dinarello 2000). Together, all four cytokines investigated in this study activate the hypothalamic-pituitary-adrenal axis by stimulating the release of corticotrophin releasing hormone (CRH), suppressing appetite and inducing pyresis. In the liver, they stimulate the acute phase response, leading to an increase in C-reactive protein and a number of other mediators. They also induce insulin resistance in the liver and peripheral tissues. IL-6 is the most important cytokine for this research as it correlates well with surgical trauma, the stress response and the subsequent changes to drug disposition (Roumen *et al.* 1993a; Veenhof *et al.* 2011).

3.5.1 DISTRIBUTION OF CYTOKINE RESULTS

The distributions of the cytokine concentrations for each group were assessed graphically (examples shown in Figure 3.5-1 and 2) and with a formal normality test (Table 3.5-1).

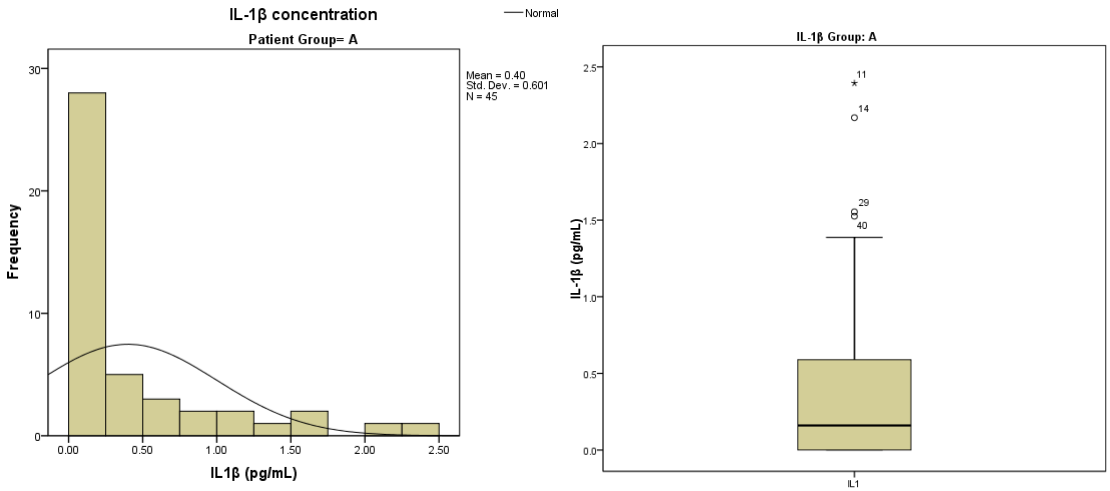


Figure 3.5-1 Graphs depicting distribution of IL-1β values from Group A on the preoperative day.
(Left) Histogram with normal curve superimposed shows distribution skewed to the left; (Right) Box plot shows median bar is not centrally placed in the box and tails do not extend from the box symmetrically. Both graphs indicate the data are non-parametric.

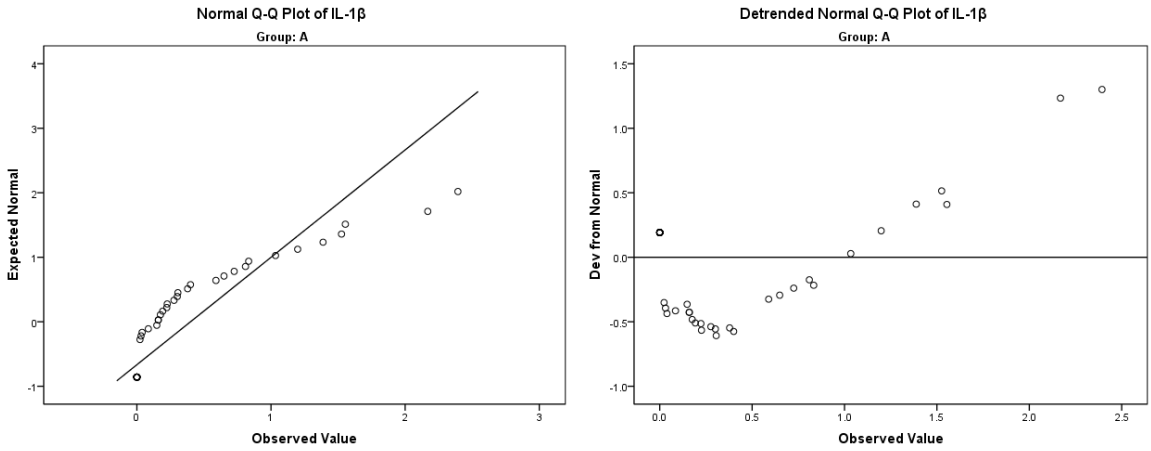


Figure 3.5-2 Q-Q plots of IL-1β distribution
(Left) Q-Q plot shows IL-1β values are not placed along the line of normality; (Right) The de-trended Q-Q plot shows an exaggerated deviation from the normal line. Both graphs indicate the data to be non-parametric.

Kolmogorov-Smirnov tests examine if the data were significantly different from a normal distribution. In Table 3.5-1, a significance value of less than 0.05 indicated a significant variation from a normal distribution *i.e.* that the data were non-parametric. No group of cytokine results consistently showed evidence of normality. Attempts to normalise the data by log, square root and reciprocal transformations did not improve the results of these normality tests.

Table 3.5-1 Results of Kolmogorov-Smirnov test D with (*df*) degrees of freedom for all cytokines.
*Abbreviations: D- Kolmogorov-Smirnov test statistic; df- degrees of freedom; Sig- significance. *=Significance.*

	Group A		Group B		Group C		Group D	
	D (*df*)	Sig.	D (*df*)	Sig.	D (*df*)	Sig.	D (*df*)	Sig.
IL-1β	0.253 (45)	0.000*	0.381 (15)	0.000*	0.236 (23)	0.002*	0.299 (39)	0.000*
IL-6	0.373 (45)	0.000*	0.166 (15)	0.200	0.371 (23)	0.000*	0.332 (39)	0.000*
TNF-α	0.154 (45)	0.009*	0.116 (15)	0.200	0.191 (23)	0.029*	0.148 (39)	0.031*
IFN-γ	0.307 (45)	0.000*	0.118 (15)	0.200	0.347 (22)	0.000*	0.110 (33)	0.200

To assess differences between days of the study, paired tests were used. While it is important to consider the distribution of each cytokine concentration as a group, as shown above, when conducting paired analysis it is more relevant to consider the distribution of the residuals. The residuals are the absolute value of the difference between the values in the paired analysis. Their distribution also failed tests of normality and an example of IL-1β is shown (Table 3.5-2).

Table 3.5-2 Formal normality tests of the residuals for IL-1β

	Group A		Group B		Group C		Group D	
	D (*df*)	Sig	D (*df*)	Sig	D (*df*)	Sig	D (*df*)	Sig
Day -1 v 1	0.277 (10)	0.028	0.439 (4)	0.001	0.387 (8)	0.001	0.353 (11)	0.000
Day -1 v 2	0.516 (10)	0.000	0.439 (4)	0.000	0.325 (8)	0.013	0.353 (11)	0.000
Day -1 v 3	0.515 (10)	0.000	0.439 (4)	0.001	0.455 (8)	0.000	0.351 (11)	0.000
Day -1 v 4	0.428 (10)	0.000	0.449 (4)	0.001	0.391 (8)	0.001	0.352 (11)	0.000

Accordingly, non-parametric tests were used for cytokine analysis. Quartiles were calculated using weighted averages $X_{(n+1)p}$. The results of the analysis of each cytokine are now presented in the following sections.

3.5.2 IL-1β

IL-1β is released from blood monocytes and tissue macrophages upon activation as part of the initial inflammatory response. As a primary inflammatory mediator IL-1β concentrations were expected to peak soon after incision and with a short half-life, resolve quickly in the absence of continuing inflammation (Roumen *et al.* 1993a).

3.5.2.1 DESCRIPTIVES

The median and interquartile ranges of all the groups for each cytokine are presented in the following tables and accompanied by box plots of their distribution. The assay had a lower LOD of 0.19pg/mL and an upper LOD of 2500pg/mL. As can be seen in the graphs that follow, the elevations of IL-1β were comparatively minor, with the highest measured concentration across all groups of 14.29pg/mL. Nearly half (41.8%) of IL-1β concentrations were at or below the LOD (0.19pg/mL).

3.5.3 GROUP A

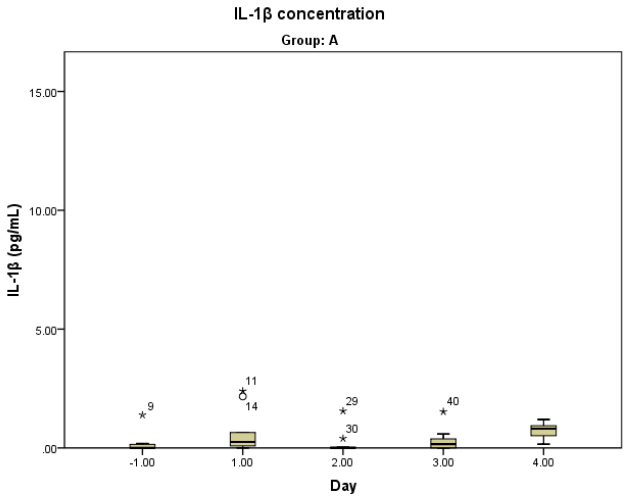


Figure 3.5-3 Box plot for IL-1β concentration- Group A

Table 3.5-3 Descriptives for IL-1β Group A

IL-1β (pg/mL)	Group A	
	N	Median (IQR)
Day -1	10	0 (0 ,0.159)
Day 1	10	0.250 (0.074, 1.029)
Day 2	9	0 (0, 0.216)
Day 3	9	0.160 (0, 0.484)
Day 4	7	0.810 (0.306, 1.034)

Group A began with 10 patients (Figure 3.5-3 and Table 3.5-3). There were only very small fluctuations in IL-1β concentrations, peaking on the day after surgery, falling the following day to baseline values and returning to slightly elevated values towards the end of the study. There was little variation within the study group over the five sampling days.

3.5.3.1.1 GROUP B

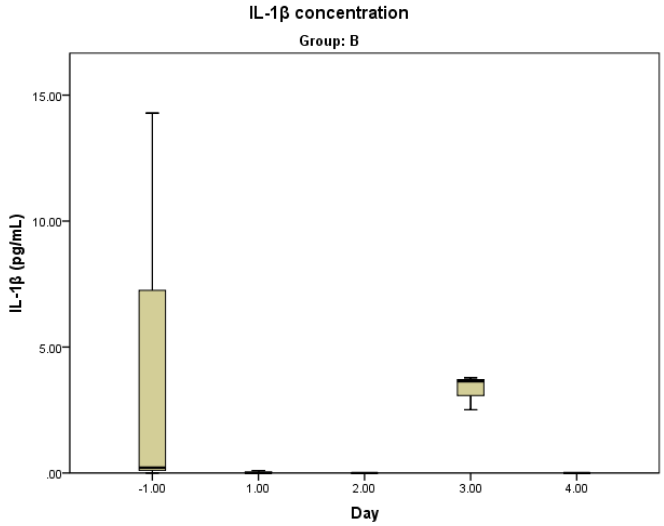


Figure 3.5-4 Box plot for IL-1β concentration- Group B

Table 3.5-4 Descriptives for IL-1β Group B

IL-1β (pg/mL)	Group B	
	N	Median (IQR)
Day -1	3	0.213 (0.106, 7.249)
Day 1	3	0 (0, 0.044)
Day 2	3	0 (0, 0)
Day 3	3	3.636 (2.511, 3.70)
Day 4	3	0 (0, 0)

Group B had three patients who gave blood samples (Figure 3.5-4 and Table 3.5-4). The small sample size limits its statistical inference. Large variability was noticeable on the preoperative day. This was caused by one patient who had elevated IL-1β prior to surgery as shown by the length of the whisker on the first box. This concentration of IL-1β may be expected in the presence of inflammation. Postoperatively, IL-1β concentrations were below the LOD for all days except Day 3 when all patients experience similar elevation in IL-1β concentration.

3.5.3.1.2 GROUP C

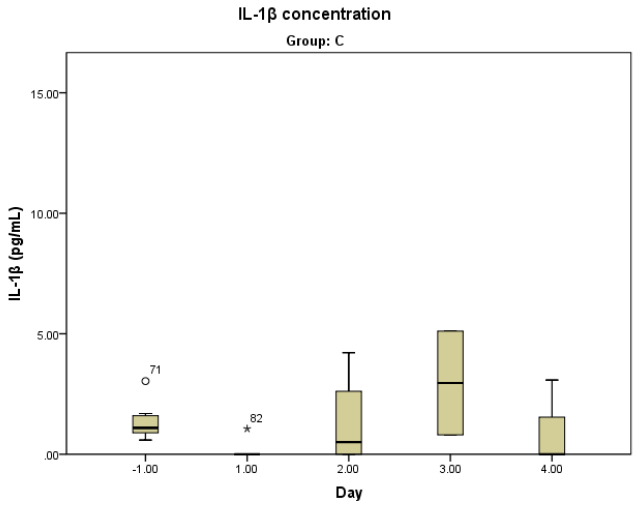


Figure 3.5-5 Box plot for IL-1β concentration- Group C

Table 3.5-5 Descriptives for IL-1β Group C

IL-1β (pg/mL)	Group C	
	N	Median (IQR)
Day -1	7	1.097 (0.890, 1.599)
Day 1	5	0 (0, 0.095)
Day 2	4	0.506 (0, 3.415)
Day 3	2	2.958 (0.807, 4.033)
Day 4	3	0 (0, 1.539)

Group C began with seven patients but complete sample sets were collected from only two patients (Figure 3.5-5 and Table 3.5-5). This group also exhibited detectable IL-1β concentrations preoperatively. Only one patient had detectable concentrations of IL-1β on the first day after surgery. Following this, concentrations rose on Days 2 and 3 and fell to baseline on Day 4. As patients dropped out from this group variability increased.

3.5.3.1.3 GROUP D

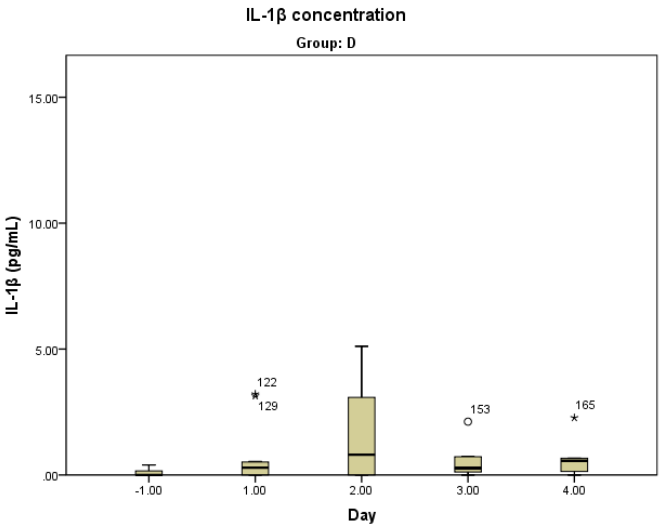


Figure 3.5-6 Box plot for IL-1β concentration- Group D

Table 3.5-6 Descriptives for IL-1β Group D

IL-1β (pg/mL)	Group D	
	N	Median (IQR)
Day -1	11	0 (0, 0.201)
Day 1	6	0.150 (0, 1.030)
Day 2	5	0.492 (0.085, 1.840)
Day 3	6	0.274 (0.091, 1.074)
Day 4	6	0.564 (0.107, 1.066)

Group D began with 11 patients (Figure 3.5-6 and Table 3.5-6). There was negligible inflammation prior to surgery. Concentrations of IL-1β remained low until the second postoperative day falling on Day 3 and 4. There was little variability across the study aside from Day 3.

3.5.3.2 CHANGES TO IL-1β CONCENTRATION

3.5.3.2.1 ALL GROUPS

Visual observation of Figure 3.5-3 through to Figure 3.5-6 does not reveal any obvious trend in IL-1β concentration across the study for any group. To confirm this, a Friedman's test (χ^2) was used to assess if the distribution of each of the days were the same. A significant result indicates differences between distribution of one or more of the study days (Table 3.5-7).

Table 3.5-7 Results of Friedman test for concentration of IL-1β
*Abbreviations: N-number in group; χ^2-Friedman test statistic; df- degrees of freedom; Sig- significance. Significant values are indicated by *.*

	N	χ^2	*df*	Sig.
Group A	7	12.195	4	0.0151*
Group B	3	8.000	4	0.0916
Group C	2	4.445	4	0.3492
Group D	5	4.553	4	0.3363

Group A achieved a significant result indicating that there were significant changes in the distribution of the IL-1β concentrations across the days of the study. A *post hoc* analysis was conducted using a one tailed Wilcoxon's matched pairs test (Z) to determine where

the difference indicated by the significant result in the Friedman's test occurred. One tail was used because IL-1β concentrations were only expected to be increased by surgery.

While no difference occurred in the other groups, a repeated measures test such as Friedman's requires a value to be present on all of the days of the test for the patient to be included in the analysis *i.e.* if one sample out of the five was missing that patient was excluded. This reduced the number of patients included in this analysis considerably giving a poor reflection of the amount of data gathered (Table 3.5-7). To improve this, a paired analysis was also conducted. To minimise the Type 1 error and the size of the Bonferroni correction, only the preoperative day was compared with the other days of the test. This resulted in four comparisons between the five tests, with the Bonferroni correction reducing the critical level of significance from 0.05 to 0.0125 (*i.e.* α/4) (Table 3.5-8).

Table 3.5-8 Results of Wilcoxon matched pairs test for IL-1β concentrations
*Values shown as Z (significance). Significant values are indicated by *.*

IL-1β	Group A		Group B		Group C		Group D	
Day	N	*Z* (sig)	N	*Z* (sig)	N	*Z* (sig)	N	*Z* (sig)
-1 v 1	10	1.836 (0.066)	3	1.069 (0.285)	7	2.366 (0.018)	11	1.779 (0.075)
-1 v 2	9	1.604 (0.109)	3	1.342 (0.18)	4	0.73 (0.465)	5	1.461 (0.144)
-1 v 3	9	1.183 (0.237)	3	0 (1)	2	0.447 (0.655)	6	1.363 (0.173)
-1 v 4	7	1.859 (0.063)	3	1.342 (0.18)	3	0.535 (0.593)	6	2.023 (0.043)

These results show no significant changes in IL-1β concentration between the preoperative and any of the postoperative days of the study. This would indicate the significant result seen in Group A's Friedman's test arose from differences between days within the postoperative phase that were not undertaken in this paired analysis. Further analysis revealed this to be the case with the results of a Wilcoxon matched pairs test between Group A Days 3 and 4 of 2.197 (0.028), showing that the concentration of IL-1β was actually significantly higher on Day 4 than on Day 3, contrary to what would be expected. However once the Bonferroni correction was taken into account, this result lost its significance (Table 3.5-8).

Two other results approached significance during the matched pair analysis summarised in Table 3.5-8, but both lost significance upon incorporation of the Bonferroni correction. In Group C the IL-1β concentration trended to being higher preoperatively than on Day 1 postoperatively, and Group D showed a trend for Day 4 postoperatively to be higher than the preoperative sample.

3.5.3.3 CHANGES TO IL-1β CONCENTRATION AS PERCENTAGE OF PREOPERATIVE VALUE

Some previous reports of cytokine concentrations following surgery have reported values as a percentage of baseline concentration (Veenhof *et al.* 2011). Although interesting, these results are to be interpreted with caution as working with percentages can skew results. However, the advantages of this method are the potential reduction in inter-individual variation as the percentage increase is calculated for each patient. To facilitate this analysis, those with concentrations reported as zero were set at the LOD of the assay.

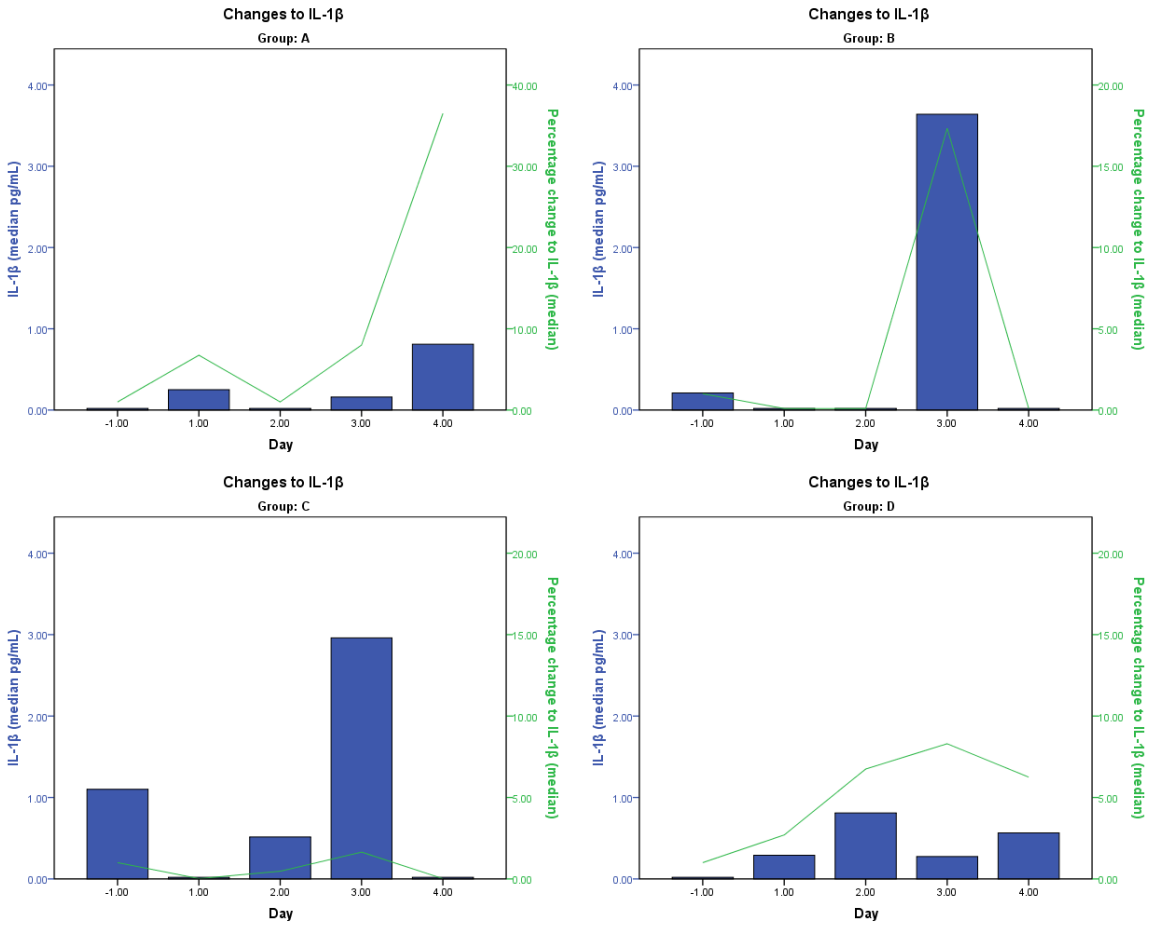


Figure 3.5-7 Dual axis graphs showing median values of IL-1 β concentration (solid bars, left y axis) and median percentage change in IL-1 β concentration when compared to preoperative values.

The graphs above show how a percentage change can exaggerate differences (Figure 3.5-7). In the first graph of Group A, the increases on the final day of the study appear considerably larger than that of the increase to the actual cytokine concentration. Conversely, the increases seen on Day 3 for Group B appear diminished due to the elevated IL-1β concentration measured at the baseline in this group. To assess if this transformation of the data resulted in any change to the statistical analysis, the same analsysis previously undertaken for concentrations of IL-1 β was repeated.

As the transformation was applied to all days of the study, the distribution was unchanged and therefore the results of the Friedman's test were identical to those in Table 3.5-7.

The results of the Wilcoxon matched pairs test are summarised in Table 3.5-9. Some greater differences did arise in these results including the statistically significant increase between the preoperative and first postoperative day in Group A. Groups C and D both had results approaching significance for the same comparison.

Table 3.5-9 Results of Wilcoxon matched pairs test for percentage change of IL-1β concentrations between the preoperative day and consecutive days.
*Values shown as Z (significance). Significant values are indicated by *.*

IL-1β	Group A		Group B		Group C		Group D	
Day	N	*Z* (sig)	N	*Z* (sig)	N	*Z* (sig)	N	*Z* (sig)
-1 v 1	10	2.549 (0.011*)	3	0 (1)	7	2.366 (0.018)	11	1.955 (0.051)
-1 v 2	9	1.604 (0.109)	3	1.342 (0.180)	4	0.365 (0.715)	5	1.461 (0.144)
-1 v 3	9	1.992 (0.046)	3	1.069 (0.285)	2	0.447 (0.655)	6	1.753 (0.080)
-1 v 4	7	2.197 (0.028)	3	1.342 (0.180)	3	1.069 (0.285)	6	2.23 .043)

3.5.4 IL-6

T-cells and macrophages activated by IL-1β and TNF-α secrete IL-6 soon after trauma. IL-6 is also released by unrelated processes such as exercise and obesity, which may affect baseline concentrations (Roumen *et al.* 1993a; Fain 2010). It has both pro and anti-inflammatory activity, promoting IL-1α and IL-10 and inhibiting TNF-α and IL-1 (Heinrich *et al.* 2003).

IL-6 concentrations correlate well with trauma severity following surgery and have been used as a predictor of survival (Gebhard *et al.* 2000). Unlike other cytokines, IL-6 can cross the blood/brain barrier where it initiates PGE_2 synthesis in the hypothalamus, a prostaglandin central to paracetamol pharmacology, causing pyresis (Roumen *et al.* 1993a).

3.5.4.1 DESCRIPTIVES

The median and interquartile ranges of all the groups for each cytokine are presented in the following tables below accompanied by box plots of their distribution. The assay had a lower LOD of 0.42pg/mL and an upper LOD of 2500pg/mL. Much higher levels of IL-6 were measured than of IL-1β, with the highest across all samples of 421.60pg/mL. None of the 121 samples examined were at or below the LOD.

3.5.4.1.1 GROUP A

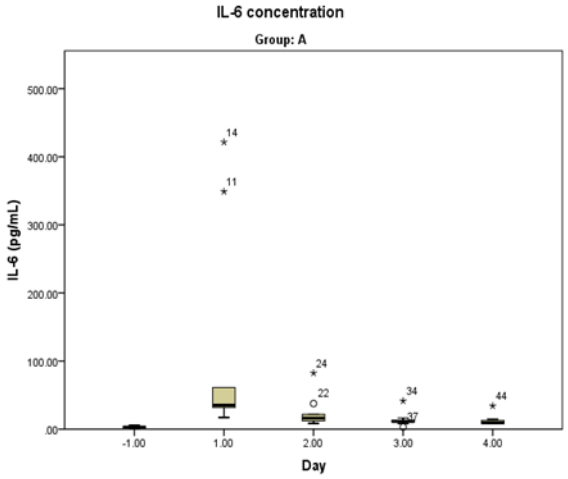


Figure 3.5-8 Box plot for IL-6 concentrations- Group A

Table 3.5-10 Descriptive statistics for IL-6 Group A

IL-6 (pg/mL)	Group A	
	N	Median (IQR)
Day -1	10	3.025 (1.607, 4.339)
Day 1	10	35.192 (29.838, 133.137)
Day 2	9	16.216 (10.602, 29.614)
Day 3	9	11.167 (9.185, 14.797)
Day 4	7	9.298 (7.944, 14.489)

Although there was a small degree of elevation preoperatively, Group A exhibited a marked increase in IL-6 concentration on Day 1 postoperatively (Figure 3.5-8 and Table 3.5-10). The box plot shows two outlying data points on Day 1, shown as 11 and 14, which belong to patient one and four respectively. Patient one went on to develop a leaking anastomosis and sepsis and died two weeks later. Patient four also had complications, also developing a leaking anastomosis, eventually requiring the formation of a colostomy and nearly a month of intensive care.

The median concentration more than halved on Day 2 and continued to decline more gradually on Days 3 and 4. The outlying points 24, 34 and 44 on these later days all belong to patient four described above. Although this patient's IL-6 concentrations declined in a similar manner to those of the rest of the group, they still fell outside of the group's upper quartile. Patient one had been withdrawn from the study due to his complications and accordingly, IL-6 concentrations were not available after Day 1.

The variation on Day 1 was far greater than any other day. On the following days data is clustered much closer around the median.

3.5.4.1.2 GROUP B

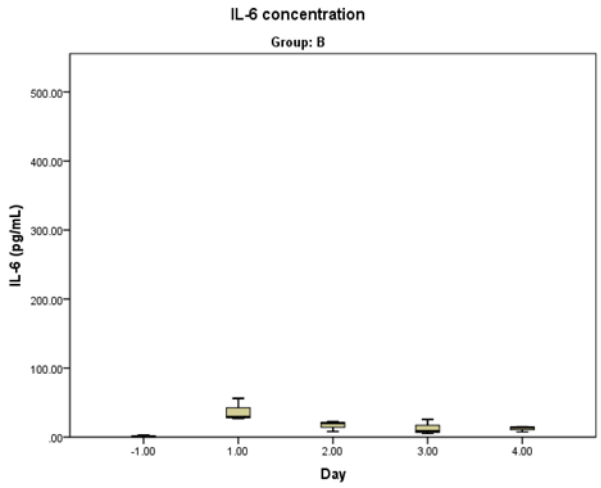


Table 3.5-11 Descriptive statistics for IL-6 Group B

IL-6	Group B	
Day -1	3	1.034 (0.754, 2.915)
Day 1	3	29.100 (26.920, 56.019)
Day 2	3	20.234 (8.179, 22.498)
Day 3	3	8.619 (5.183, 25.602)
Day 4	3	13.995, (7.587, 15.137)
Day -1	3	1.034 (0.754, 2.915)

Figure 3.5-9 Box plot for IL-6 concentration- Group B

As discussed in the results for IL-1β, it is difficult to draw trends because of the size of this group. Similarly to Group A, IL-6 concentrations in Group B increased sharply from amounts just above the LOD preoperatively to concentrations which where thirtyfold higher on Day 1 (Figure 3.5-9 and Table 3.5-11). The concentration reduced gradually on Day 2 and three, increasing slightly on Day 4. The variation was greatest on Day 1 but the size of this group diminishes the usefulness of this measure.

3.5.4.1.3 GROUP C

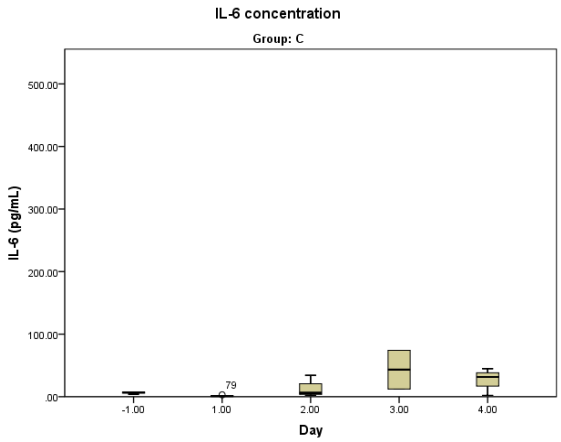


Table 3.5-12 Descriptive statistics for IL-6 Group C

IL-6	Group C	
Day -1	7	1.097 (0.696, 1.692)
Day 1	5	8.127 (3.378, 15.700)
Day 2	4	6.134 (3.004, 27.445)
Day 3	2	43.225 (9.199, 57.410)
Day 4	3	31.546 (2.227, 44.752)
Day -1	7	1.097 (0.696, 1.692)

Figure 3.5-10 Box plot for IL-6 concentration- Group C

The pattern of changes to IL-6 concentration for Group C was unlike any other group (Figure 3.5-10 and Table 3.5-12). There was a high concentration of IL-6 measured preoperatively. Concentrations of IL-6 fell to their lowest levels on Day 1, remaining low on Day 2. On the third day there was a rapid increase in median concentration. Concentrations fell, but still remain markedly elevated on Day 4. Because of the dropout of patients from this group it is difficult to draw conclusions about cytokine trends. On Day 3, samples were obtained from only patients 1 and 4, both of whom had complicated

recoveries, with Patient 4 having the highest concentration of IL-6 of all patients in the study on this day (74.18pg/mL). A sample from an additional patient on Day 4 reduced the median concentration but did not eliminate the effect of these two other patients resulting in another high median concentration.

3.5.4.1.4 GROUP D

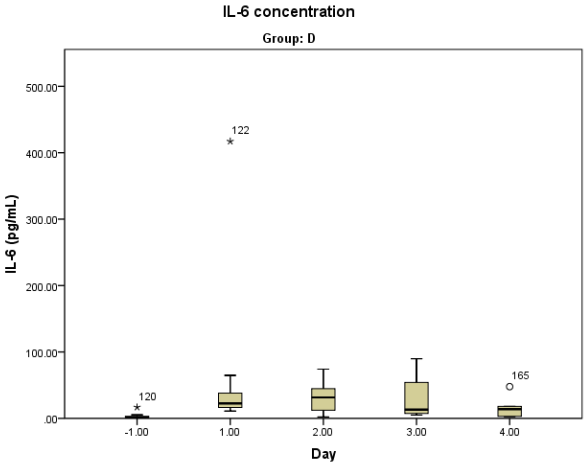


Table 3.5-13 Descriptive statistics for IL-6 Group D

IL-6 (pg/mL)	Group D	
	N	Median (IQR)
Day -1	11	1.350 (0.946, 3.649)
Day 1	6	34.674 (16.464, 152.832)
Day 2	5	19.578 (12.977, 26.223)
Day 3	6	13.239 (6.762, 62.996)
Day 4	6	13.836 (2.960, 25.505)

Figure 3.5-11 Box plot for IL-6 concentration- Group D

Concentrations of IL-6 for Group D began consistently low across the group as shown by the low median and small interquartile range (Figure 3.5-11 and Table 3.5-13). Concentrations increased steeply on Day 1, increasing further on Day 2. On Day 1 an outlier from Patient 1 was seen about 20 times higher than the group median at 417.20pg/mL. This patient underwent more extensive and one of the only two open surgeries in his group to correct a long standing congenital abnormality within his gastro-intestinal tract.

Median concentrations of IL-6 fell by Day 3, and remained relatively constant on Day 4. There was a large reduction in interquartile range from Day 3 through to Day 4. Another outlier appeared on Day 4 from patient 11. At 47.77pg/mL, Patient 11's concentration on this day was four times that of their IL-6 concentration on the previous day (11.81pg/mL) and that of the group median. This patient had an uncomplicated recovery.

3.5.4.2 CHANGES TO IL-6 CONCENTRATION

3.5.4.2.1 ALL GROUPS

Visual observation of the box plots revealed much greater variation in IL-6 concentration across the days of the study than seen with IL-1β. For most groups there was a clear

increase in the IL-6 concentration on the day following surgery. To confirm if this variation was real or due to chance a Friedman's test (χ^2) was used (Table 3.5-14).

Table 3.5-14 Results of Friedman test for concentration of IL-6
*Abbreviations used: N-number in group; χ^2-Friedman test statistic; df- degrees of freedom; Sig- significance. Significant values are indicated by *.*

IL-6	N	χ^2	*df*	Sig.
Group A	7	26.629	4	0.000*
Group B	3	10.400	4	0.034*
Group C	2	7.600	4	0.107
Group D	5	8.480	4	0.075

Groups A and B show significant differences in IL-6 concentration over the course of the study. As with IL-1β, a *post hoc* analysis was conducted using a one tailed Wilcoxon's matched pairs test (Z) to identify on which pair of days statistically significant differences occurred. One tailed tests were again used as inflammation was only expected to increase as a result of surgery, and as before, to minimise the size of the Bonferroni correction only the pre-operative day was compared with each post-operative day. Once the Bonferroni correction was taken into account Group A showed significant differences between pre-operative concentrations and Days 1, 2 and 4 (Table 3.5-15).

Table 3.5-15 Results of Wilcoxon matched pairs test for IL-6 concentrations
*Values shown as Z (significance). Significant values are indicated by *.*

IL-6	Group A		Group B		Group C		Group D	
Day	N	*Z* (sig)	N	*Z* (sig)	N	*Z* (sig)	N	*Z* (sig)
-1 v 1	10	2.803 (0.005)*	3	1.604 (0.109)	7	2.366 (0.018)	11	2.934 (0.003)*
-1 v 2	9	2.666 (0.008)*	3	1.604 (0.109)	4	0.365 (0.715)	5	1.483 (0.138)
-1 v 3	9	2.666 (0.008)*	3	1.604 (0.109)	2	1.342 (0.18)	6	2.201 (0.028)
-1 v 4	7	2.366 (0.018)	3	1.604 (0.109)	3	1.069 (0.285)	6	1.572 (0.116)

As the comparisons of the preoperative day with Day 2 and 3 gave exactly the same *Z* and significance value, this was checked by manual calculation. Like most non-parametric tests, the Wilcoxon matched pairs test is based on ranking of data rather than comparing the data's actual value. In this analysis the same number of positive ranks occurred in both comparisons, resulting in the same *Z* and significance values. This also explained the results shown for Group B, as all postoperative values were greater than the preoperative values.

Group D also showed a significant difference between the Day -1 and Day 1 adding to a general trend across all groups. Ignoring the Bonferroni correction brought Group C into this trend.

3.5.4.3 CHANGES TO IL-6 CONCENTRATION AS PERCENTAGE OF PREOPERATIVE VALUE

As with IL-1β, IL-6 concentrations were transformed to a percentage of preoperative baseline concentration and analysed using the same procedure (Figure 3.5-12).

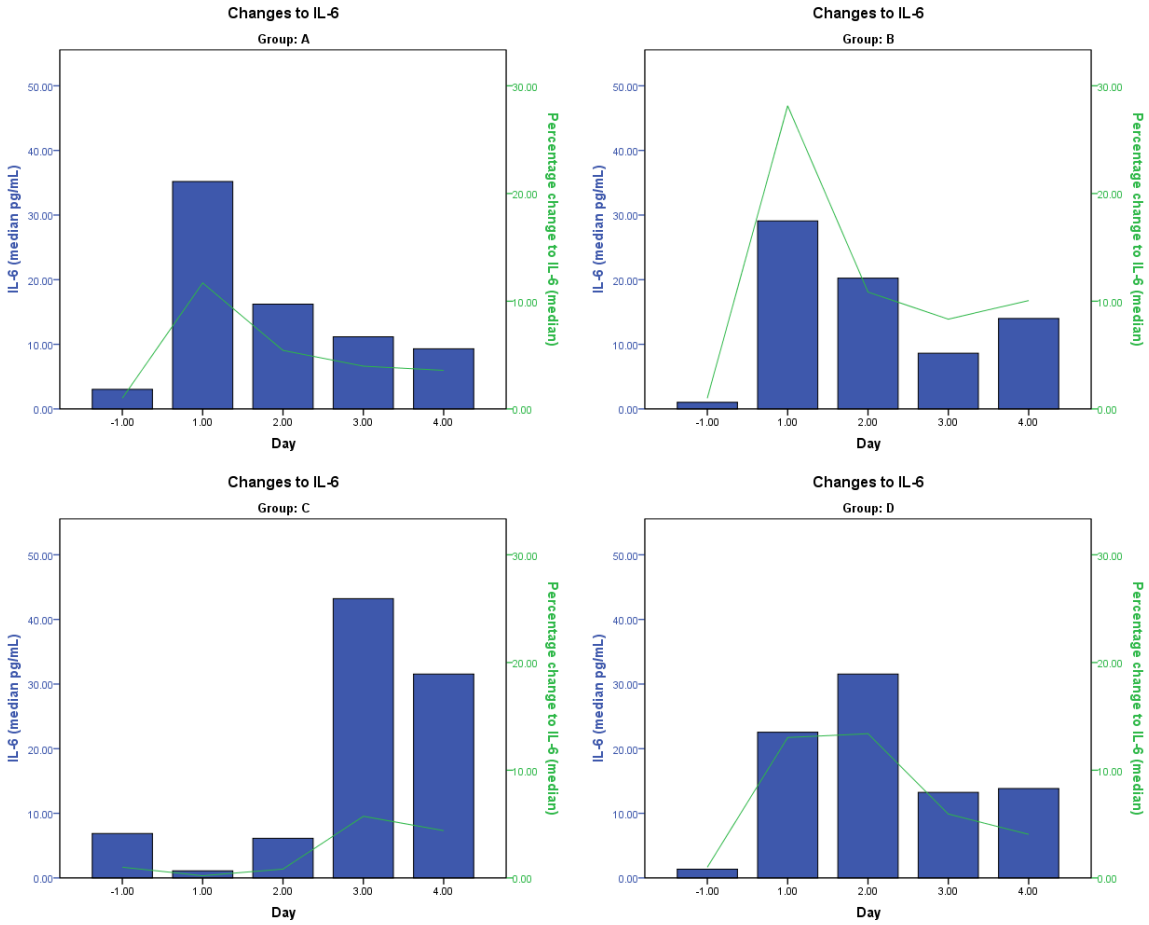


Figure 3.5-12 Dual axis graphs showing median values of IL-6 concentration (solid bars, left y axis) and median percentage change in IL-6 concentration when compared with preoperative values.

Once again the results demonstrate this method's ability to skew data based on preoperative cytokine concentrations and this is demonstrated clearly in the differences between groups. Groups A and C exhibit substantial concentration changes over the course of the study but because of the comparatively high concentration preoperatively, the percentage changes are small. The converse is true for Groups B and D, where a much lower preoperative median concentration results in a large percentage increase postoperatively despite the actual concentration being much less than that of Group A and Group C.

Another Wilcoxon matched pair's test was conducted (Table 3.5-16) to detect difference between days of the study. Once again the Bonferroni correction reduced the critical level of significance to 0.0125 (0.05/4).

Table 3.5-16 Results of Wilcoxon matched pairs test for percentage change of IL-6 concentrations between the preoperative day and consecutive days.
*Values shown as Z (significance). Significant values are indicated by *.*

IL-6	Group A		Group B		Group C		Group D	
Day	N	*Z* (sig)	N	*Z* (sig)	N	*Z* (sig)	N	*Z* (sig)
-1 v 1	10	2.803 (0.005*)	3	1.604 (0.109)	7	2.366 (0.018)	11	2.934 (0.003*)
-1 v 2	9	2.666 (0.008*)	3	1.604 (0.109)	4	0.365 (0.715)	5	1.753 (0.080)
-1 v 3	9	2.666 (0.008*)	3	1.604 (0.109)	2	1.342 (0.180)	6	2.201 (0.028)
-1 v 4	7	2.366 (0.018)	3	1.604 (0.109)	3	1.069 (0.285)	6	1.992 (0.046)

3.5.5 TNF-α

TNF-α is primarily released from activated macrophages and mast cells stimulated by lipopolysaccharides and IL-1β. In the initial stages of the inflammatory response the TNF-α that is released is pre-formed, constitutively expressed in macrophages and mast cells to allow a rapid response to stimulation (Nathan 2002). TNF-α works alongside IL-1β as part of the initial inflammatory response, potentiating the release of IL-6.

3.5.5.1 DESCRIPTIVES

This assay had a lower LOD of 0.65pg/mL and upper LOD of 2500pg/mL. Generally, TNF-α concentration was well above the LOD, with the lowest concentration measured being 3.52pg/mL, which was measured on the first postoperative day from patient 2D. No samples were below the LOD. The highest concentration across all groups was 20.14pg/mL, measured on day 2 from patient 3A. Concentration varied little during the study period and there was no obvious effect of surgery.

3.5.5.1.1 GROUP A

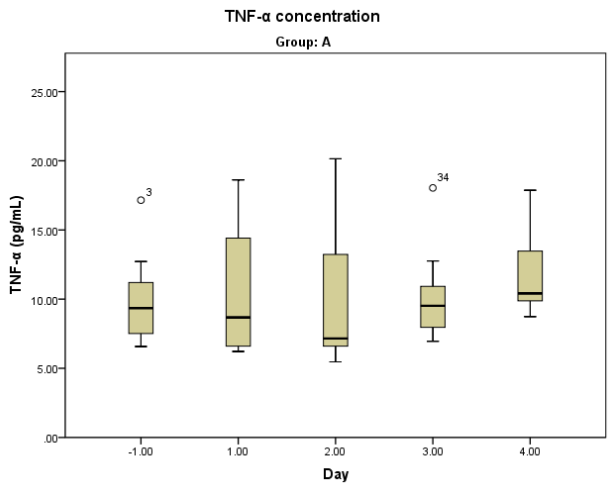


Figure 3.5-13 Box plot for TNF-α concentration- Group A

Table 3.5-17 Descriptive statistics for TNF-α Group A

TNF-α (pg/mL)	Group A	
	N	Median (IQR)
Day -1	10	9.353 (7.323, 11.583)
Day 1	10	8.676 (6.606, 14.545)
Day 2	9	7.159 (6.282, 13.6)
Day 3	9	9.522 (7.645, 11.84)
Day 4	7	10.411 (9.652, 14.341)

There was very little change in TNF-α concentration over the course of the study in Group A and it did not show any expected effect of surgery (Figure 3.5-13 and Table 3.5-17).

TNF-α was measurable in all preoperative samples reflecting the constitutive nature of this cytokine, being expressed even in the absence of inflammation. Median concentrations fell from preoperative concentrations on Day 1 postop although the range increased. A further decrease in median concentration was seen on Day 2 although the IQR covered approximately the same range. On Day 3 there was a slight increase in median concentration and reduction in IQR. A further increase in median concentration was seen on Day 4, finishing slightly above the preoperative median. The first outlying value of 17.15pg/mL belongs to patient 3 on day minus one. As with IL-1β and IL-6 patient 4 gave an outlying value on Day 3 of 18.04pg/mL reflecting his complicated recovery.

3.5.5.1.2 GROUP B

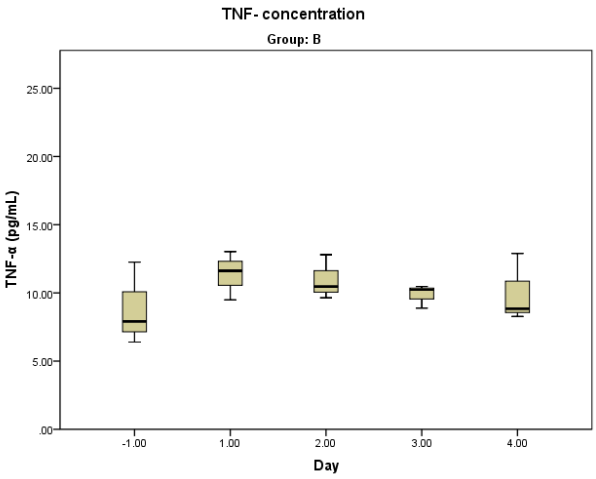


Figure 3.5-14 Box plot for TNF-α concentration- Group B

Table 3.5-18 Descriptive statistics for TNF-α Group B

TNF-α (pg/mL)	Group B	
	N	Median (IQR)
Day -1	3	7.905 (6.393, 12.254)
Day 1	3	11.62 (9.49, 13.025)
Day 2	3	10.466 (9.641, 12.8)
Day 3	3	10.227 (8.879, 10.466)
Day 4	3	8.836 (8.278,12.887)

There were only minor changes in TNF-α concentration in Group B (Figure 3.5-14 and Table 3.5-18). All patients had measurable preoperative concentrations. Median concentrations followed a more expected pattern than other groups, being lowest preoperatively, increasing to a peak Day 1 postoperatively and gradually decreasing as recovery progressed. The median concentration on Day 4 remained elevated above the median preoperative concentration.

3.5.5.1.3 GROUP C

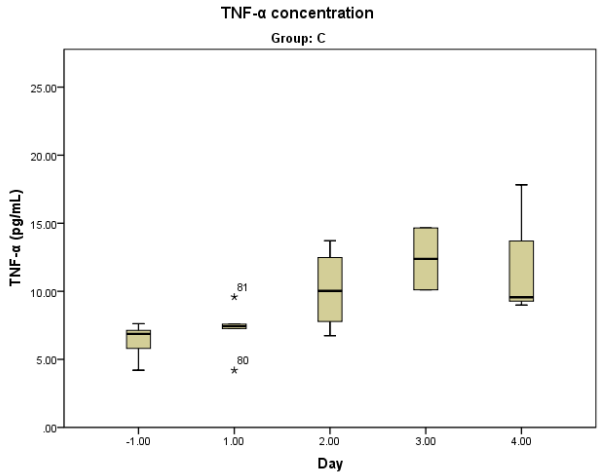


Figure 3.5-15 Box plot for TNF-α concentration- Group C

Table 3.5-19 Descriptive statistics for TNF-α Group C

TNF-α (pg/mL)	Group C	
	N	Median (IQR)
Day -1	7	6.87 (5.424, 7.179)
Day 1	5	7.433 (5.731, 8.587)
Day 2	4	10.02 (7.259, 13.097)
Day 3	2	12.381 (10.105, 13.609)
Day 4	3	9.565 (8.988, 15.982)

TNF-α was once again present in all preoperative samples in Group C (Figure 3.5-15 and Table 3.5-19). The median concentration increased gradually peaking on Day 3. However, as with the other cytokines for this group, the changing numbers of patients make interpretation difficult. Two outlying concentrations occurred on Day 1, of 4.20pg/mL and 9.59pg/mL belonging to patients 2 and 3 respectively.

3.5.5.1.4 GROUP D

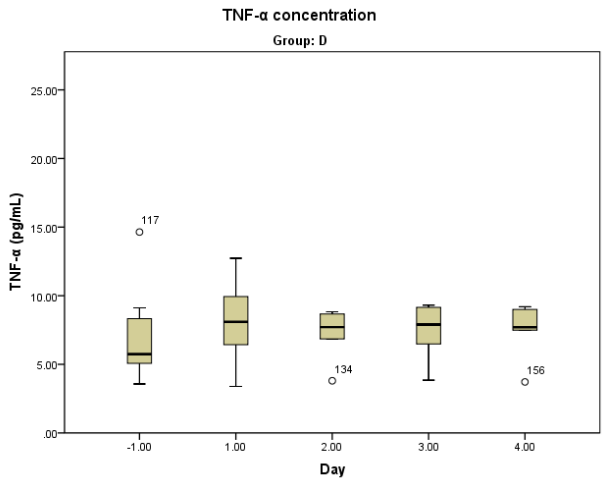


Figure 3.5-16 Box plot for TNF-α concentration- Group D

Table 3.5-20 Descriptive statistics for TNF-α Group D

TNF-α (pg/mL)	Group D	
	N	Median (IQR)
Day -1	11	5.747 (5.059, 8.901)
Day 1	6	8.1 (5.672, 10.635)
Day 2	5	7.75 (5.326, 8.749)
Day 3	6	7.896 (5.828, 9.191)
Day 4	6	7.698 (6.546, 9.05)

Group D started with the lowest preoperative median concentration (Figure 3.5-16 and Table 3.5-20). There was a 41% increase in median concentration following surgery although this was not reflected in changes to the IQR, which remained similar. TNF-α concentrations gradually reduced as the study progressed, with days two, three and four having very similar median concentrations. Outliers were seen on Day 1 (patient 7, 14.64pg/mL), Day 2 (patient 2, 3.80pg/mL), and Day 4 (patient 2, 3.72pg/mL).

3.5.5.2 CHANGES TO TNF-α CONCENTRATION

3.5.5.2.1 ALL GROUPS

Box plots of TNF-α concentrations showed much less variation than IL-6, although they followed a similar pattern. TNF-α concentrations increased to a peak on Day 1 before gradually decreasing as the study progressed. However preoperative TNF-α concentrations were considerably higher than IL-6. While these patterns emerge graphically it was necessary to confirm if these variations were real or due to chance using a Friedman's test (χ^2) (Table 3.5-21).

Table 3.5-21 Results of Friedman test for concentration of TNF-α
*Abbreviations used: N-number in group; χ^2-Friedman test statistic; df- degrees of freedom; Sig- significance. Significant values are indicated by *.*

TNF-α	N	χ^2	*df*	Sig.
Group A	7	5.143	4	0.273
Group B	3	2.400	4	0.663
Group C	2	7.600	4	0.107
Group D	5	2.560	4	0.634

The results of the Friedman's test showed no significant differences for any group over the course of the study. Because this test excludes values case-wise, Wilcoxon matched pairs were used to confirm these results as they include all samples for which there is a matching pair (Table 3.5-22).

Table 3.5-22 Results of Wilcoxon matched pairs test for TNF-α concentrations
*Values shown as Z (significance). Significant values are indicated by *.*

TNF-α	Group A		Group B		Group C		Group D	
Day	N	*Z* (sig)	N	*Z* (sig)	N	*Z* (sig)	N	*Z* (sig)
-1 v 1	10	0.153 (0.878)	3	1.069 (0.285)	7	1.753 (0.080)	11	0.934 (0.345)
-1 v 2	9	0.059 (0.953)	3	0.535 (0.593)	4	1.826 (0.068)	5	0.135 (0.893)
-1 v 3	9	0.770 (0.441)	3	0.535 (0.593)	2	1.342 (0.18)	6	1.572 (0.116)
-1 v 4	7	1.352 (0.176)	3	1.604 (0.109)	3	1.604 (0.109)	6	1.363 (0.173)

Although comparisons in Group C were closest to the critical significance value when preoperative values were compared with Days 1 and 2, they failed to surpass it. This confirms the results of the Friedman's test that no significant changes in TNF-α concentration were shown over the course of the study for any group.

3.5.6 IFN-γ

IFN-γ is produced predominantly by lymphocytes in response to TNF-α induced release of IL-12 from macrophages (Andersson 2005). While concentrations may increase in response to trauma, much greater release occurs from antigen stimulated T-cells as part

of the adaptive immune response. IFN-γ has a main role in antiviral activity and stimulates chemokine production in the early phases of inflammation and suppress it in later stages (Nathan 2002).

3.5.6.1 DESCRIPTIVES

The IFN-γ assay had a lower LOD of 0.91pg/mL and an upper LOD of 2500pg/mL. Six of the 115 samples were at or below the lower LOD, two from patient 10A (Days 1 and 3), two from patient 1B (Days 2 and 4) and one each from Patient 7A (Day -1) and Patient 2C (Day 1), thus all but one patient had measureable concentrations of IFN-γ preoperatively. Generally there were only slight changes in IFN-γ concentration for most patients across the study, and those elevations that did occur appeared later than with other cytokines. The highest measured concentration was 93.81pg/mL from patient 4C on Day 3.

3.5.6.1.1 GROUP A

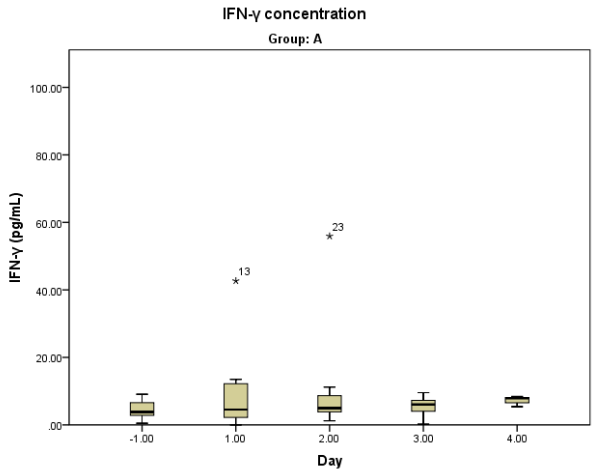


Figure 3.5-17 Box plot for IFN-γ concentration- Group A

Table 3.5-23 Descriptive statistics for IFN-γ Group A

IFN-γ (pg/mL)	Group A	
	N	Median (IQR)
Day -1	10	3.833 (2.513, 6.966)
Day 1	10	4.534 (2.224, 12.509)
Day 2	9	4.987 (3.107, 9.931)
Day 3	9	6.047 (3.555, 8.419)
Day 4	7	7.913 (6.103, 8.178)

There were only small changes to IFN-γ concentration in Group A following surgery and once again most patients (90%) had measurable concentrations of this cytokine prior to surgery (Figure 3.5-17 and Table 3.5-23). Following surgery there was a small increase in median concentration but a larger increase in IQR. Patient 3A produced outlying concentrations on Day 2 and three with concentrations of 42.67 and 55.92pg/mL respectively. The median concentration rose slightly on Day 2 while IQR almost halved. IQR continued to shrink on Day 3 and Day 4 as concentrations became more uniform across the group. Median concentrations continued to increase on Day 3 and Day 4 and finished at more than twice the preoperative value, well above day 1 concentrations.

3.5.6.1.2 GROUP B

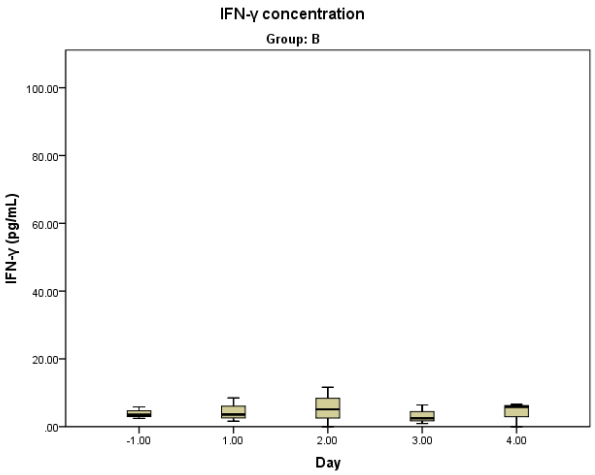


Figure 3.5-18 Box plot for IFN-γ concentration- Group B

Table 3.5-24 Descriptive statistics for IFN-γ Group B

IFN-γ (pg/mL)	Group B	
	N	Median (IQR)
Day -1	3	3.521 (2.418, 6.966)
Day 1	3	3.591 (1.593, 12.509)
Day 2	3	5.103 (0, 9.931)
Day 3	3	2.468 (.939, 8.419)
Day 4	3	5.862 (0, 8.178)

For Group B IFN-γ was found in all preoperative samples. Median concentrations followed an atypical course, increasing slightly on Day 1 followed by a greater increase on Day 2, a reduction on Day 3 and an increase on Day 4 (Figure 3.5-18 and Table 3.5-24). Day 4 had the highest median concentration of this group, although the IQR included 0. There was large variation across all postoperative days in this group with IQRs ranging from 2-3 times the median concentration with the highest upper quartile value being recorded on Day 1.

3.5.6.1.3 GROUP C

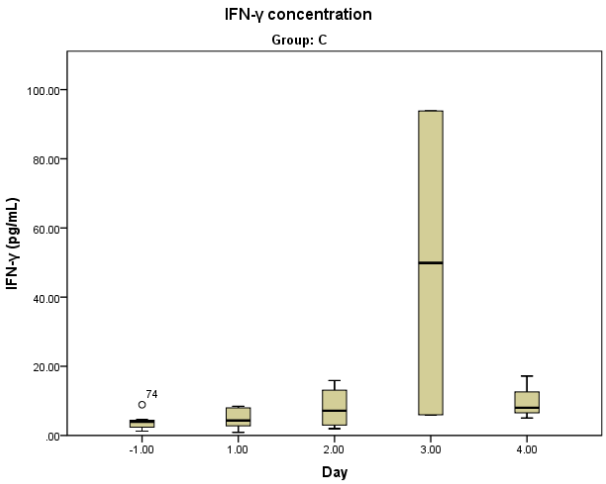


Figure 3.5-19 Box plot for IFN-γ concentration- Group C

Table 3.5-25 Descriptive statistics for IFN-γ Group C

IFN-γ (pg/mL)	Group C	
	N	Median (IQR)
Day -1	7	3.921 (1.967, 4.554)
Day 1	5	4.340 (1.849, 8.211)
Day 2	4	7.172 (2.493, 14.493)
Day 3	2	49.900 (5.987, 8.419)
Day 4	3	8.013 (5.061, 8.178)

Group C began with measurable concentrations of IFN-γ for all patients preoperatively and patient 4C with an outlying concentration of 7.62pg/mL (Figure 3.5-19 and Table 3.5-25). There were increases of median concentration following surgery to a marked peak on Day 3, when samples were only obtained from two patients, one of whom (Patient 4) had the highest concentration across all day for all groups of 93.81pg/mL.

Concentrations fell on Day 4 to give a median approximately twice that of Day 1 values. Patient 4's values also fell on Day 4 to approximately 20% of the Day 3 value. Further interpretation is limited as discussed above.

3.5.6.1.4 GROUP D

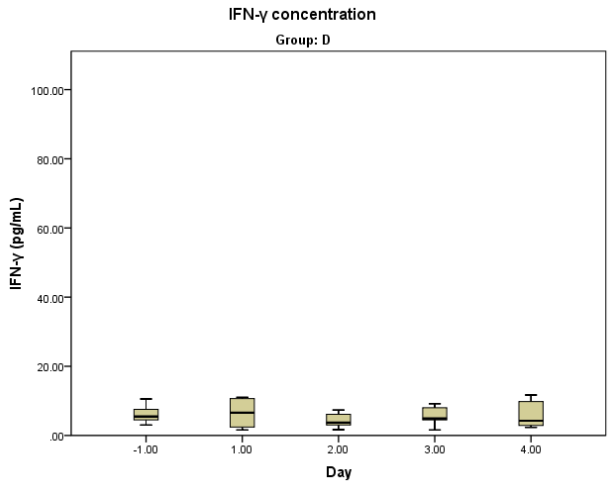


Table 3.5-26 Descriptive statistics for IFN-γ Group D

IFN-γ (pg/mL)	Group D	
	N	Median (IQR)
Day -1	11	5.475 (4.298, 8.008)
Day 1	6	6.589 (2.219, 10.768)
Day 2	5	3.704 (2.366, 6.745
Day 3	6	4.884 (3.79, 8.295)
Day 4	6	4.271 (2.752, 10.273)

Figure 3.5-20 Box plot for IFN-γ concentration- Group D

Once again all patients in Group D began with measurable concentrations of IFN-γ, producing a high median concentration second only to Day 1 postoperatively for this group (Figure 3.5-20 and Table 3.5-26). Following a more typical course, postoperative median concentrations peaked on Day 1 falling markedly on Day 2, increasing slightly on Day 3 before falling again to a value approximately 0.5pg/mL above Day 2 concentrations. IQR was greatest on Day 2 and Day 4.

3.5.6.2 CHANGES TO IFN-γ CONCENTRATION

3.5.6.2.1 ALL GROUPS

Visually from the box plots there were no obvious trends in the changes to IFN-γ. While postoperative concentrations were generally higher than preoperative concentrations, measurable preoperative concentrations were found in all groups, reducing the likelihood of finding significant changes following surgery. Unlike other cytokines, increases to IFN-γ commonly occurred in the later stages of the study, on Day 3 and Day 4. To confirm if these variations were attributable to chance or a significant change a Friedman's test (χ^2) was used (Table 3.5-27). No significant changes in IFN-γ concentration over the course of the study were found using this test. As with other cytokines a *post hoc* analysis was conducted using a one tailed Wilcoxon's matched pairs test (Z) to increase the number of

samples included in the analysis in case this resulted in a statistically significant difference over one of the days.

Table 3.5-27 Results of Friedman test for concentration of IFN-γ
*Abbreviations used: N-number in group; χ^2-Friedman test statistic; df- degrees of freedom; Sig- significance. Significant values are indicated by *.*

IFN-γ	N	χ^2	*df*	Sig.
Group A	7	3.657	4	0.454
Group B	3	2.237	4	0.692
Group C	2	8.000	4	0.092
Group D	5	6.720	4	0.151

One tailed tests were again used and only the pre-operative day was compared with each post-operative day (Table 3.5-28).

Table 3.5-28 Results of Wilcoxon matched pairs test for IFN-γ concentrations
*Values shown as Z (significance). Significant values are indicated by *. ^=The sum of negative ranks equal the sum of positive ranks*

IFN-γ	Group A		Group B		Group C		Group D	
Day	N	*Z* (sig)	N	*Z* (sig)	N	*Z* (sig)	N	*Z* (sig)
-1 v 1	10	1.172 (0.241)	3	0.535 (0.593)	7	0.405 (0.686)	11	0.105 (0.917)
-1 v 2	9	1.125 (0.26)	3	0.535 (0.593)	4	1.826 (0.068)	5	1.753 (0.08)
-1 v 3	9	1.007 (0.314)	3	0.535 (0.593)	2	1.342 (0.18)	6	1.363 (0.173)
-1 v 4	7	1.521 (0.128)	3	0 (1)	3	1.604 (0.109)	6	0.943 (0.345)

These resulted in values consistent with the Friedman test, with no statistically significant difference being shown between the preoperative concentrations and each postoperative day.

3.5.7 SUMMARY

IL-1β, IL-6, TNF-α and IFN-γ were measured in all patients on each day of the study and the results were non-parametric. Most patients had measurable concentrations of IL-1β, TNF-α and IFN-γ in the sample taken before surgery. Significant differences between the preoperative and the post-operative days of the study were seen for IL-1β and IL-6, although these elevations were still small. While these two cytokines were elevated TNF-α and IFN-γ showed no relationship with surgery. The largest differences between preoperative and postoperative values were seen for IL-6. Patients with outlying values of elevated cytokines were those with complicated recoveries.

3.6 α GSH S-TRANSFERASE RESULTS

The concentration of αGST was measured in daily patient samples by the ELISA methods described in Section 2.4.

3.6.1 DISTRIBUTION OF αGST RESULTS

The distribution of the αGST concentrations for each group were checked for normality visually by preparing a histogram with normal curve superimposed, a box plot, a Q-Q and detrended Q-Q plot for each day. A sample is shown below from Group A on the preoperative day (Figure 3.5-1). In addition, formal normality tests were conducted using the Kolmogorov-Smirnov test (D) (Table 3.6-1).

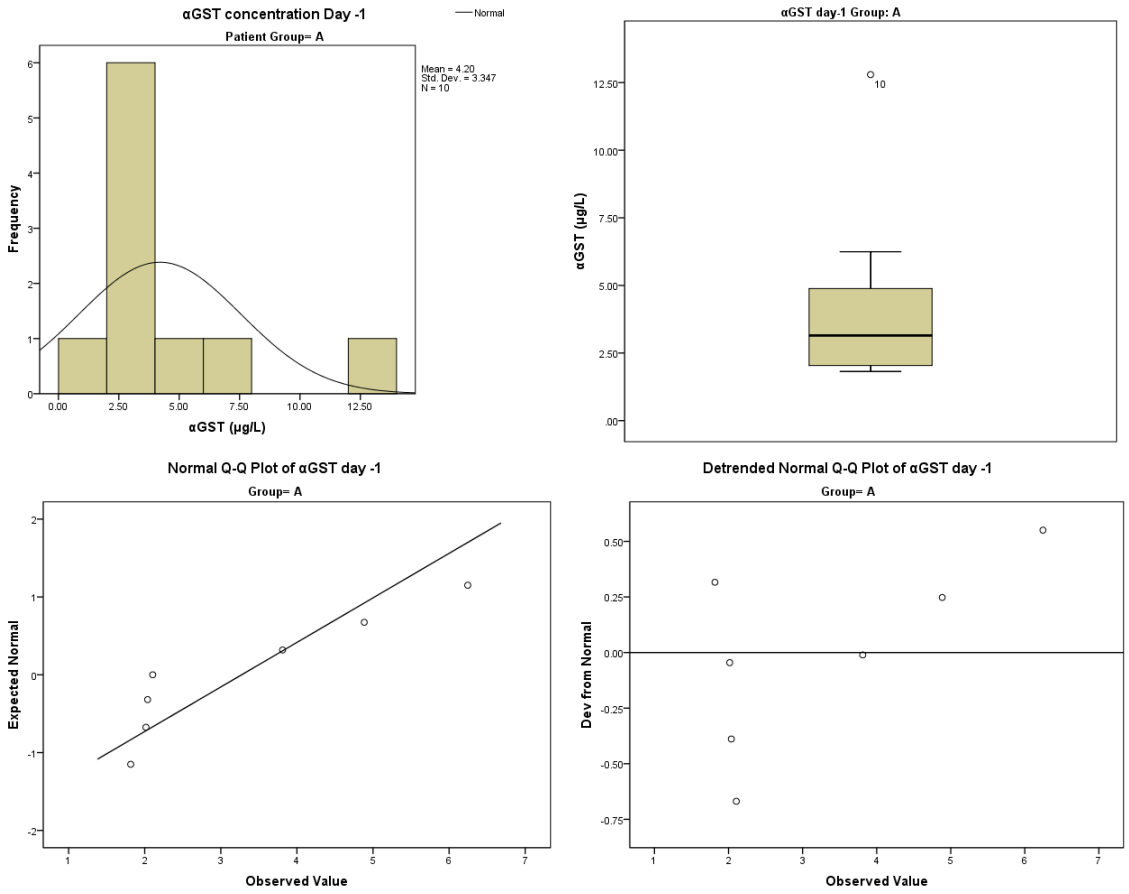


Figure 3.6-1 Graphs depicting distribution of αGST values from Group A on the preoperative day.
(Clockwise from top left) Histogram with normal curve superimposed shows distribution skewed to the left; Box plot shows median bar is not centrally placed in the box and tails do not extend from the box symmetrically; Q-Q plot shows αGST values are not placed along the line of normality; the de-trended Q-Q plot shows an exaggerated deviation from the normal line. All of these graphs indicate the data to be non-parametric.

Table 3.6-1 shows the results of the formal normality tests. A significance value of less than 0.05 indicates a significant variation from a normal distribution *i.e.* that the data are non-parametric. No group consistently passed normality tests.

Table 3.6-1 Results of Kolmogorov-Smirnov test D with (*df*) degrees of freedom.

	Group A		Group B		Group C		Group D	
	D (*df*)	Sig.	D (*df*)	Sig.	D (*df*)	Sig.	D (*df*)	Sig.
Day 0	0.246 (10)	0.087	0.309 (3)	0.038	0.389 (7)	0.002	0.298 (11)	0.007
Day 1	0.332 (10)	0.003	0.342 (3)	0.026	0.297 (5)	0.017	0.362 (6)	0.014
Day 2	0.362 (9)	0.001	0.364 (3)	0.014	0.281 (4)	0.001	0.207 (5)	0.200
Day 3	0.227 (9)	0.200	0.385 (3)	0.000	0.260 (2)	0.057	0.355 (6)	0.018
Day 4	0.295 (7)	0.066	0.349 (3)	0.019	0.333 (3)	00.06	0.189 (6)	0.200

To assess differences between days of the study, paired tests were used. While it is important to consider the distribution of αGST concentrations on each day of the study as shown above, when conducting paired analysis it is more relevant to consider the distribution of the residuals. The residuals are the absolute value of the difference between the values in the paired analysis. Their distribution also failed tests of normality (Table 3.5-2). Accordingly non-parametric tests were used through the analysis of αGST results.

Table 3.6-2 Formal normality tests of the residuals

	Group A		Group B		Group C		Group D	
	D (*df*)	Sig	D (*df*)	Sig	D (*df*)	Sig	D (*df*)	Sig
Day -1v1	0.277 (10)	0.028	0.439 (4)	0.001	0.387 (8)	0.001	0.353 (11)	0.000
Day -1v2	0.516 (10)	0.000	0.439 (4)	0.000	0.325 (8)	0.013	0.353 (11)	0.000
Day -1v3	0.515 (10)	0.000	0.439 (4)	0.001	0.455 (8)	0.000	0.351 (11)	0.000
Day -1v4	0.428 (10)	0.000	0.449 (4)	0.001	0.391 (8)	0.001	0.352 (11)	0.000

3.6.2 DESCRIPTIVES

The median and interquartile ranges of all the groups are presented in Table 3.6-3 below. Additionally a box plot of the distribution of all αGST results is shown in Figure 3.6-2.

Table 3.6-3 Descriptive statistics for αGST

αGST (µg/L)	Group A		Group B		Group C		Group D	
	N	Median (IQR)	N	Median (IQR)	N	Median (IQR)	N	Median (IQR)
Day -1	10	3.15 (2.03, 5.22)	3	1.28 (0.66, 3.57)	7	2.01 (0.82, 2.78)	11	1.71 (1.40, 2.65)
Day 1	10	2.07 (1.70, 2.70)	3	0.15 (0.09, 0.51)	5	0.82 (0.00, 1.95)	6	1.21 (1.16, 2.04)
Day 2	9	2.53 (1.97, 3.33)	3	0.47 (0.46, 0.66)	4	5.97 (0.93, 13.70)	5	1.37 (0.97, 2.25)
Day 3	9	3.20 (2.33, 4.24)	3	1.39 (1.14, 1.39)	2	13.67 (1.47, 25.88)	6	3.19 (1.22, 4.95)
Day 4	7	3.56 (2.76, 10.67)	3	0.95 (0.83, 1.93)	3	26.42 (14.34, 28.61)	6	4.52 (2.31, 5.63)

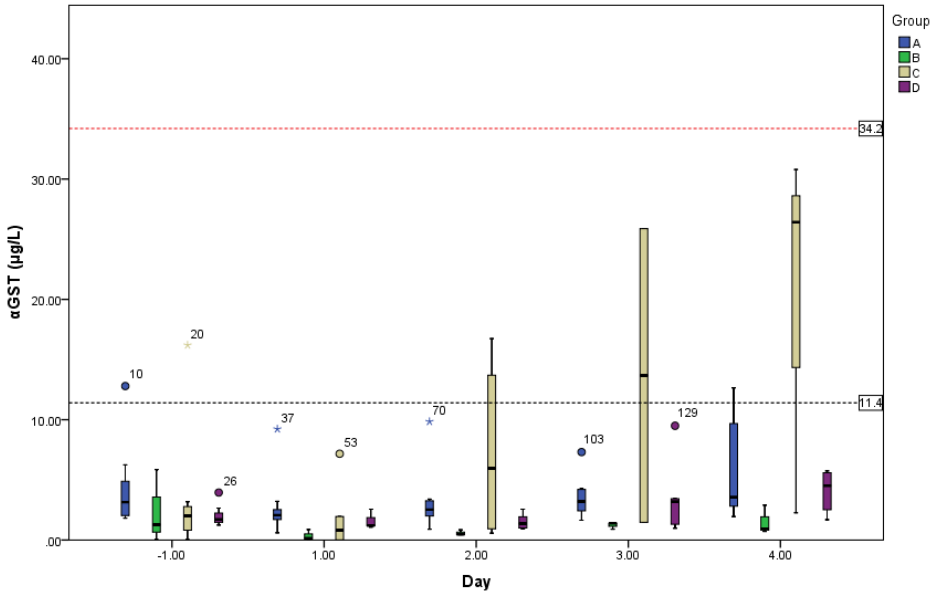


Figure 3.6-2 Boxplot of αGST concentrations in each group on each day
Black dotted line indicates upper limit of reference range in healthy individuals (11.4µg/L) and red dotted line indicated three times this limit (34.2 µg/L), from where hepatotoxicity may be suspected.

Figure 3.6-2 shows Group C as the only group to exceed the upper limit of the reference range even though they received the lower dose of paracetamol (Section 2.1). As noted in Section 3.1, Group C had poor retention in the study and the number of αGST values from this group was small.

Group A also showed an increase in αGST concentrations, with the upper whisker stretching beyond the upper limit of the reference range on the final day of the study. Similar trends were observed in Figure 3.6-2 for both Group D, and to a lesser extent, Group B. However, both fall well short of the upper limit of the reference range (Figure 3.6-2).

It is also notable in Figure 3.6-2 that all groups start the study on day-1 with median αGST concentrations above zero, despite other LFTs being normal, although the median values for all groups were well below the upper limit of the reference range. Also of note for day -1 were the two outlying values from one patient in Group A and one in Group C (Figure 3.6-2). These αGST concentrations exceeded the upper limit of the reference range, but were taken before the study began. These two patients had a αGST of 12.4 and 16.2 µg/L while having a AST concentration of 40 and 28 IU/L, which are both within the normal range. This could indicate an increased sensitivity of αGST, or hepatic damage yet to cause AST to increase.

3.6.3 CHANGES IN αGST OVER THE STUDY PERIOD

While trends can be observed from the box plot in Figure 3.6-2 statistical tests are necessary to determine if the differences were statistically significant. Initially a Friedman's test (χ^2) was used to assess if the distribution for each of the days were the same for each group. A significant result would indicate there was a difference between one or more of the days and all the other days of the study, which was not found to be present in these results, except for Group D (Table 3.5-7).

Table 3.6-4 Results of Friedman test
Abbreviations used: N-number in group; χ^2-Friedman test statistic; df- degrees of freedom; Sig- significance

	N	χ^2	*df*	Sig.
Group A	7	7.314	4	0.12
Group B	3	5.867	4	0.209
Group C	2	5.6	4	0.231
Group D	5	15.520	4	0.004

However, a repeated measures test such as Friedman's requires a value to be present on all of the days of the test for the patient to be included in the analysis *i.e.* if one sample out of the five taken was missing that patient would be excluded. As a result the number of subjects who were included in this analysis was low (Table 3.5-7) and a poor reflection of the amount of data gathered.

To further investigate differences of distribution between the days of the study a paired analysis was conducted using a one tailed Wilcoxon's matched pairs test (Z). One tail was used because αGST was only expected to increase following surgery and IV paracetamol administration. To minimise the Type 1 error and the size of the Bonferroni correction, only the preoperative day was compared with the other days of the test. This resulted in four comparisons between the five tests, with the Bonferroni correction reducing the critical level of significance from 0.05 to 0.0125 (*i.e.* α/4) (Table 3.5-8).

Table 3.6-5 Results of Wilcoxon matched pairs test
*Values shown as Z (significance). Significant values are indicated by *.*

Pair	Group A	*n*	Group B	*n*	Group C	*n*	Group D	*n*
Day -1 vs 1	1.89 (0.059)	10	1.07 (0.285)	3	1.48 (0.138)	5	2.20 (0.028)	6
Day -1 vs 2	1.24 (0.214)	9	1.07 (0.285)	3	0.00 (1.000)	4	2.02 (0.043)	5
Day -1 vs 3	0.18 (0.859)	9	0.00 (1.000)	3	0.45 (0.655)	2	1.15 (0.249)	6
Day -1 vs 4	1.18 (0.237)	7	0.53 (0.593)	3	1.07 (0.285)	3	2.20 (0.028)	6

When the Bonferroni correction is considered there are no significant changes in αGST concentration over the study period.

Ignoring the Bonferroni correction, there are some significant differences in Group D, who received the lower dose of paracetamol. These differences were refelcted in the significant result of Friedman's test above (Table 3.5-7), however, once the Bonferroni correction is considered these results serve only as an indication, and while of interest, cannot be interpreted as actual significant results (Table 3.5-8). Of interest here is that the differences do not show any consistent trend as may have been anticipated; neither increasing in magnitude as more paracetamol was given or reducing as recovery after surgery proceeded.

Group A, who had the highest dose of paracetamol (*i.e.* 9g daily) with invasive surgery, did not show any significant changes in αGST over the course of the study, with the only result from this group approaching significance being from the comparison of the pre-operative with the first postoperative day (Table 3.5-7, Table 3.5-8). There appears to be no relationship with αGST concentration and the total dose of paracetamol received.

Given the major focus of this study was to examine the safety of higher doses of paracetamol and that αGST is such a sensitive marker of hepatotoxicity, it is worth examining Group A, the high paracetamol dose group, in greater detail. From examination of Figure 3.6-2 there are some clear trends that have not been considered in Table 3.5-8 in an attempt to minimise the Type 1 error. To determine if this group's elevated concentrations of αGST on admission may be masking some differences that arise following surgery, further tests were undertaken to compare the differences between the other days of the study. Two comparisons yielded results that required further discussion: Day 1 v Day 2 (Z=2.67 (p=0.008)) and Day 1 v Day 4 (Z=2.03 (p=0.043)). These differences are more in line with what was observed in Figure 3.6-2, however in order to find these, the number of tests that were conducted created a Bonferroni correction that, once applied, prevented these results from achieving significance. Accordingly, they are only indicative and must be interpreted with caution. Even where these trends were observed, all the αGST concentrations fell below a level of clinical significance and there was no evidence of hepatotoxicity occurring during the time αGST was monitored.

3.6.3.1 αGST AND AST CONCENTRATIONS

αGST concentration is not a standard measure of hepatocellular damage clinically. AST is more commonly used in the hospital setting for this purpose, in addition to being part of the assessment following paracetamol overdose (Amar *et al.* 2007). The major drawback

with AST is that its release from cells and appearance in the plasma is reported to be slower than αGST.

To compare and validate αGST with the standard AST test, the correlation of one to the other was checked by pairing each patient's αGST and AST result (Figure 3.6-3). When all patients were combined both αGST and AST were determined to be non-parametric with the Kolmogorov-Smirnov giving highly significant results for both αGST and AST.

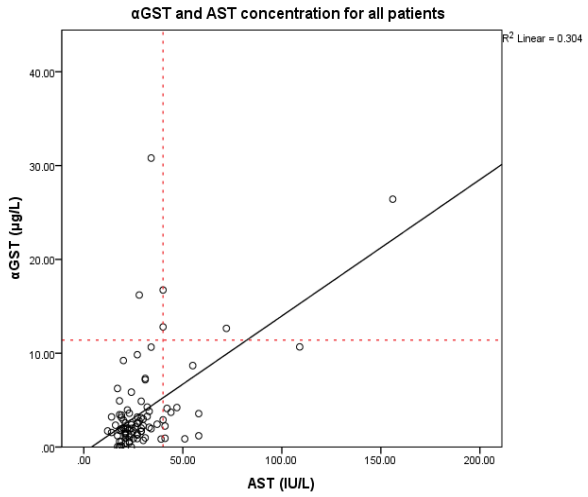


Figure 3.6-3 Correlation of αGST and AST concentrations.
Also shown: Trend line (black) and upper limits of normal range for both αGST and AST (red lines)

Most of the points in Figure 3.6-3 cluster around the origin, however there is some general trend, shown by the trend line, such that higher αGST concentrations were associated with higher AST concentrations. There was one notable exception where the highest αGST concentration was associated with a normal AST value. Additionally, not all elevated AST concentrations were associated with elevations of αGST, nor was the opposite true.

To test the correlation a Spearman's correlation coefficient was determined as the data were non-parametric. As an increase in one test was expected to result in an increase in the other, a one-tailed test was used. The correlation coefficient r_s was 0.372 with p (one-tailed) <0.01 indicating a significant relationship between the αGST and AST concentrations: as AST concentrations increased, so did αGST. The coefficient of determination shown in Figure 3.6-3 indicates αGST accounts for 30.4% of the variance in AST.

3.6.3.1.1 DELAYED AST ELEVATIONS

As discussed above, one of the criticisms of AST is that its elevation after hepatic damage was much slower than αGST. To investigate this Figure 3.6-3 was modified to show the day of each test (Figure 3.6-4).

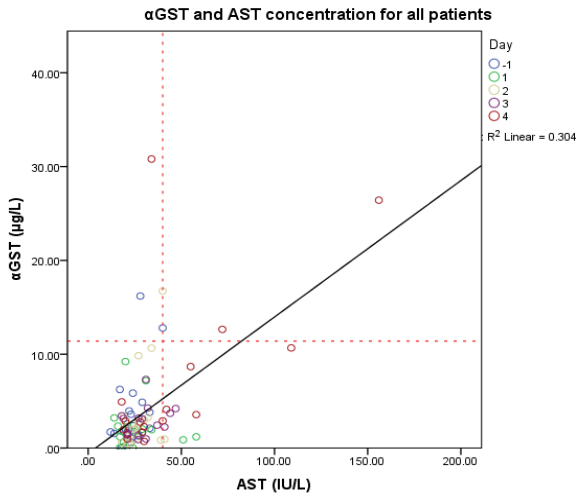


Figure 3.6-4 αGST and AST concentrations showing the day of each test.
Also shown: Trend line (black) and upper limits of normal range for both αGST and AST (red lines)

If AST was delayed, elevations in AST would only occur in the latter stages of the study. Also, elevations in αGST occurring early on in the study would not be matched by elevations in AST, skewing those results to the left of the graph. To some degree these were confirmed in Figure 3.6-4 although too few patients had elevations in αGST or AST to make this possible visually.

In order to confirm this, the day of the test was included into a partial correlation. It was seen the day of the test had a stronger relationship with AST (r= 0.344 p (one tailed) <0.001) than with αGST (r= 0.224 p (one tailed) 0.008), indicating increases of the day of the test were a better predictor of the AST results than that of αGST. Even so, both αGST and AST correlations coefficients were significant.

3.6.4 INDIVIDUAL PATIENT ANALYSIS

To confirm the αGST concentrations measured in the daily samples and to assess any changes that occurred within the dosing interval, three patients had αGST measured in all of their plasma samples taken for the pharmacokinetic analysis (as detailed in Section 2.1). Each of the three patients were from one of the colorectal surgery groups (Groups A, B and D) and were chosen as they were all males with malignancy and were of broadly

similar age and BMI. These patients were chosen before αGST results were known and without reference to other liver-function tests.

3.6.4.1 PATIENT DEMOGRAPHICS

The demographic information of these patients is illustrated below (Table 3.6-6) followed by their paracetamol dose characteristics (Table 3.6-7). The patients were all male and in their early 50s. Their weights and BMIs were similar and all were undergoing surgery for colorectal cancer.

Table 3.6-6 Individual analysis patient demographics

Patient	Age	Gender	Weight	BMI	Surgery	Indication
5A	52	M	92	26.9	Anterior resection	Malignancy
3B	54	M	87	25.4	Hemicolectomy	Malignancy
4D	52	M	89	27.5	Anterior resection	Malignancy

Table 3.6-7 Individual analysis paracetamol dose characteristics

Patient	Paracetamol Dose	Interval (hour)	Daily dose	Total paracetamol given (g)
5A	1.5g (16.3 mg/kg)	4	97.8 mg/kg	42.5
3B	1g (11.5mg/kg)	6	46.0 mg/kg	20
4D	1g (11.2mg/kg)	6	44.9 mg/kg	20

All patients received their full quota of paracetamol. Patients 3B and 4D were very similar in daily dose on an mg/kg basis, with patient 5A receiving just over twice the daily mg/kg dose and total amount of paracetamol. While there were substantial differences between Group A and Groups B and D in the daily and total doses, the quantity received was not greatly different at 16.3mg/kg versus 11.5 and 11.2 mg/kg respectively. These doses were similar to the licensed paediatric dose of 10-15mg/kg with patient 5A's 16.3mg/kg only just outside it (Glaxo Smith Kline 2008).

3.6.4.2 αGST CONCENTRATIONS

αGST concentrations were obtained in the same manner as, and measured alongside, the samples from the beginning of this section. The concentrations are presented below in Figure 3.6-5.

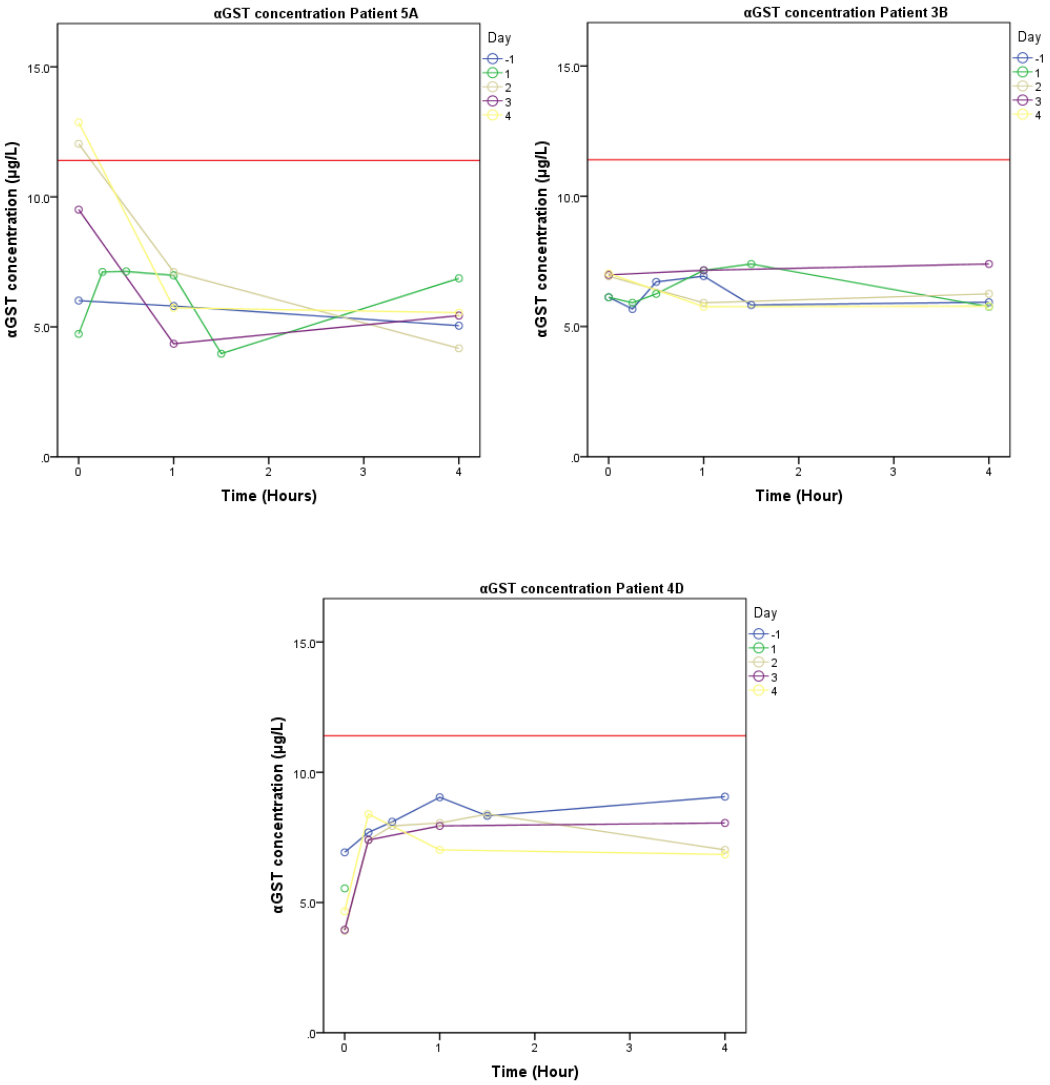


Figure 3.6-5 Patient 5A, 3B and 4D αGST concentrations over time on each day of the study
Graphs show αGST concentrations and red reference line, which indicates upper limit of reference range (11.4µg/L). Circles show measured concentrations. Graphs are (clockwise from top left) 5A; 3B and 4D.

The graph of αGST concentrations for patient 5A shows three interesting trends:

- The concentrations at time zero for Days 2,3 and 4 are the highest, with Days 2 and 4 exceeding the upper limit of the reference range. As time zero samples, the time these were taken was *before* the administration of paracetamol;
- Samples after time zero decrease in αGST concentrations, even though these were taken after the paracetamol had been administered. αGST concentrations appear to reduce during the dosing interval;
- For Group A paracetamol was administered every four hours. As such the four hour time sample shown in the graph was equivalent to the time zero of the next dose, and it would be expected that the time zero and four hour αGST concentrations would be similar. Curiously there was very little similarity between them, with the time zero sample being almost universally higher;

- There was no elevation as a result of the first 1.5g dose of paracetamol on day -1; and
- There does not appear to have been any accumulation of αGST concentrations in patient 5A as the four hour time samples were approximately equivalent on all days of the study. However, the predose αGST concentration on the fourth day was the highest of all this patients concentrations.

Graphs for Patients 3B and 4D were more comparable, as may be expected given they received the same paracetamol dose. The most important difference between these two patients was that Patient 3B underwent open surgery whereas Patient 4B's surgery was laparoscopic. For patient 3B:

- The shape of the curves for Days -1 and 1 indicated a small rise in αGST concentrations following administration of the paracetamol dose, which returned to predose concentrations by the end of the dosing interval. This effect does not seem to continue on subsequent days of the study where one hour αGST concentrations were less than or approximately equal to time zero αGST concentrations; and
- There was no substantial accumulation of αGST over the study period. The one hour αGST concentration was less on Day 4 than Day 1.

For patient 4D:

- Similar patterns emerge as those of patient 3B. There was a more consistent trend of αGST concentrations increasing after the administration of paracetamol. Unlike patient 5A the paracetamol dosing interval of this patient was six hours. Therefore αGST concentrations would have had an additional two hours to reduce before the administration of the next paracetamol dose; and
- There was no evidence of accumulation of αGST over the course of the study with αGST concentrations on the preoperative day amongst the highest of this patients values.

3.6.5 FINDINGS AFTER STUDY PERIOD

In addition to those data collected as part of the study protocol, further information was collected from several patients who remained in hospital after the study had finished. In these patients, results from any liver function tests that were done as part of their usual

care after the study were recorded. These results were not included in the above analysis as they were not part of the study protocol and were not accompanied by measurements of αGST concentrations. While this information was incomplete it nevertheless demonstrates clinical significance because it opposed the findings from the reportedly more accurate measure of hepatic damage, αGST concentrations, and had implications for patient safety. Figure 3.6-6 shows dramatic increases in AST concentration in many patients on Day 5 and following. When Days 1-4 are viewed in isolation there is no evidence of change to liver function tests, however, when the following days are considered, slight elevations that were seen on Days 3 and 4 take on new meaning. These changes were only seen in the high dose group, Group A. Generally these elevations resolved within 3-4 days after the conclusion of the study, with the exception of Patient 9A who showed clinically significant AST elevations for a week after receiving his last investigational dose of paracetamol.

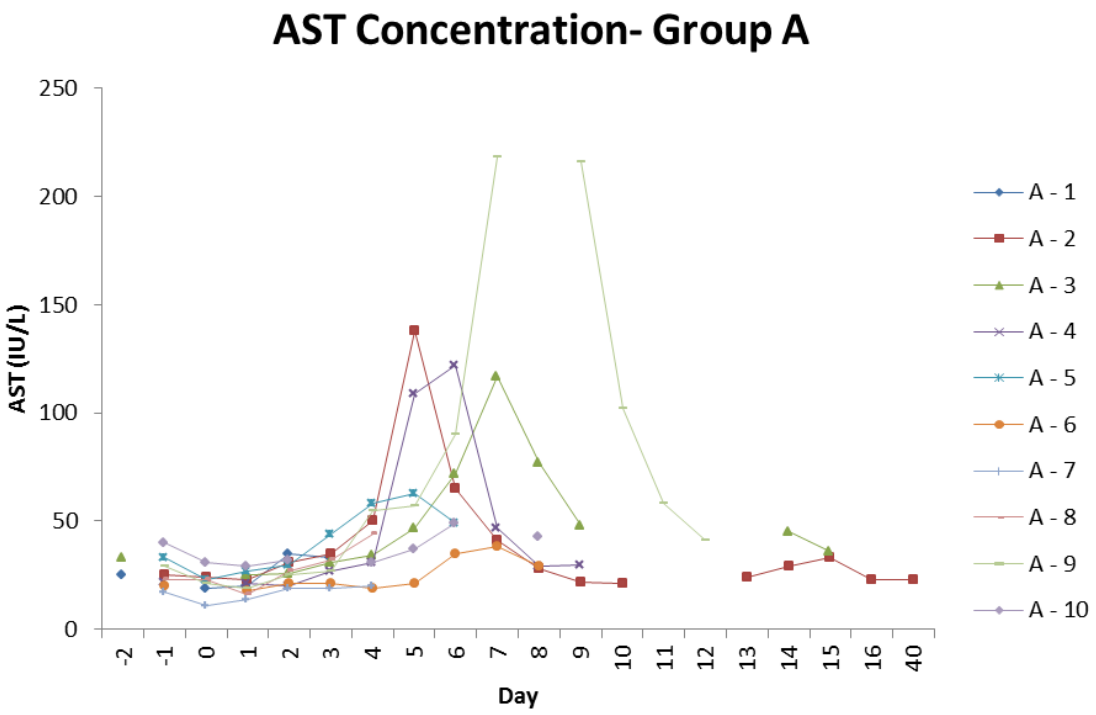


Figure 3.6-6 AST concentrations in Group A for each patient

Comparing those patients who showed the greatest increase in AST concentration after the study finished (Patients 2A, 3A, 4A and 9A), with the remainder of the group, there was only a very slight increase in αGST concentration seen prior to the conclusion of the study (Figure 3.6-7). While elevated above the remainder of the group, these patients' αGST concentrations were still well below the upper limit of the reference range. Considering only Patients 2A, 3A, 4A and 9A, in whom the greatest AST elevations were shown, there was no consistent factor between them that explained why their AST rose.

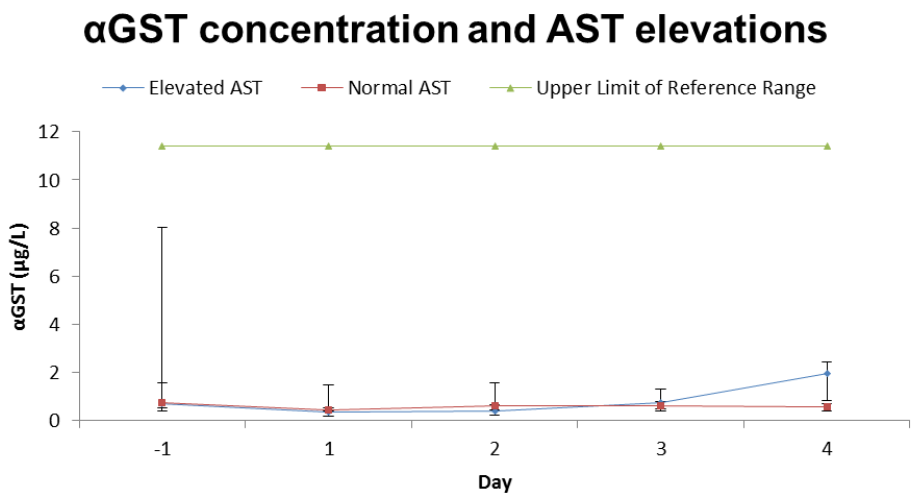


Figure 3.6-7 αGST concentrations of those with large AST elevations following the conclusion of the study compared with the remainder of the group.
Values shown as median (±IQR)

These patients had a wide ranging BMI, were both male and female, had a variety of bowel cancer diagnoses, surgeries of both short and long durations and both drank alcohol and did not. There was also no distinction in their paracetamol plasma kinetics or urinary excretion ratio. In their urinary excretion in particular their Phase I metabolite output was no higher, nor their Phase II:I ratio clearly different to the remainder of their group. However, one clear distinction between these patients did arise; they were admitted to hospital for the longest period of their group, with Patients' 2A, 3A, 4A and 9A admitted for 20, 15, 34 and 15 days respectively, compared with the average of 8 days for the remainder of the group.

3.6.6 SUMMARY

αGST was measured in all patients on each day of the study and the results were non-parametric. Two individuals entered into the study with αGST concentrations above normal but possessing normal AST concentrations. There was no significant difference between the preoperative and any postoperative day of the study. A correlation between increases in αGST and AST was demonstrated. One patient from the high dose group (Group A) showed substantial variation in αGST concentration over the dose period that was not seen in similar patients from Groups B or D, who received the normal paracetamol dose. There was no evidence from the analysis of αGST concentrations that any hepatic damage was done to any patient during the study, however, review of post-study AST concentrations revealed that damage had occurred after the study finished.

3.7 CYP450 PROBE RESULTS

A total of 518 patient plasma samples were analysed for the concentration of 9 compounds of interest as described in Section 2.5 in 10 batches by LCMS in addition to approximately 100 standard samples. Access to the instrument was generously provided by the Department of Chemistry, UCC at limited hours over a six week period.

The compounds eluted in the order and times shown in Table 3.7-1. Following chromatographic separation, samples were ionised by dual electrospray ionisation and a total ion chromatogram (TIC) was acquired using a quadrupole time-of-flight mass spectrometer. From the TIC, extracted ion chromatograms (EIC) were obtained using the mass/charge ratios (*m/z*) shown in Table 3.7-1. Compounds were quantified by the detector response which is proportional to parent ion abundance. Integration of detector response was conducted by Agilent Mass Hunter workstation Quantitative Analysis 2.00 (Agilent Technologies, Massachusetts, USA) after the analysis had taken place.

Table 3.7-1 Analyte, their mass/charge range of the protonate ion $(M+H)^+$, elution order and elution time

Analyte	$(M+H)^+$ range	Elution Order	Elution time (min)
Paraxanthine	181.00-181.10	1	1.41
Caffeine	195.05-195.15	2	2.14
6-OH Chlorzoxazone	185.90-186.10	3	3.85
Dextrorphan	258.15-258.25	4	4.10
Phenacetin	180.05-180.15	5	6.19
1-OH Midazolam	342.00-342.10	6	6.47
Dextromethorphan	272.20-272.30	7	6.55
Midazolam	326.05-326.15	8	6.78
Chlorzoxaone	169.95-170.05	9	7.68

3.7.1 LCMS VALIDATION

Faults in the mass spectrometer resulted in poor chromatography from this instrument.

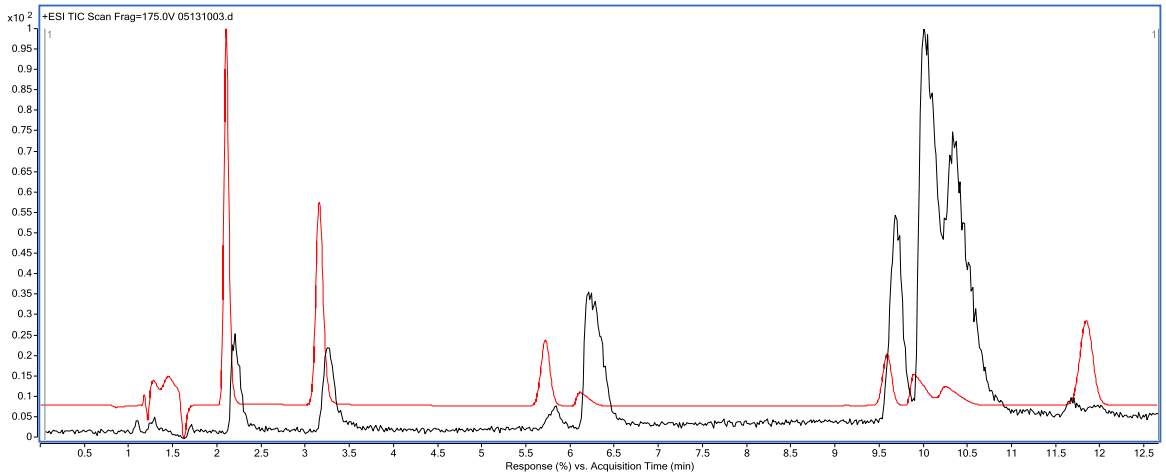


Figure 3.7-1 Chromatogram from a prepared plasma sample containing 10mg/L of all compounds of interest.
Red trace: UV absorbance at 280nm; Black trace: total ion chromatogram.

These complications are discussed in greater detail in the discussion. Figure 3.7-1 was obtained from a 10mg/L plasma standard, prepared as described in Section 2.5. The chromatogram in red displays smooth contours arising from the PDA, whereas the jagged, non-Gaussian peaks in black arose from the mass spectrometer. The extracted ion chromatograms showed more pronounced spiking (Figure 3.7-2) and this had ramifications for peak integration, on which quantitation depend.

The trace arising from the PDA was good (Figure 3.7-1), affirming the sample preparation and chromatographic separation methods. This also indicated that the problems experienced with this process lay with detection, not the sample itself.

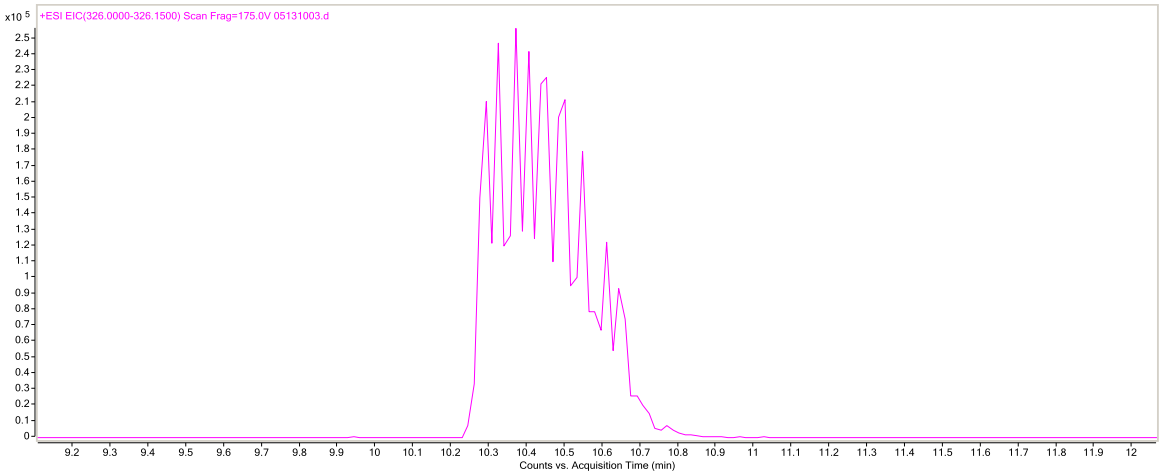


Figure 3.7-2 Extracted ion chromatogram 326-326.15 (midazolam) (2.5x10^5 abundance)

Extensive attempts were made at improving the peak shape shown in Figure 3.7-2 including the revision of all of the MS settings optimised in Section 2.5, but these were unsuccessful. Reassurance was given by the researcher supervising the instrument that the integration software was capable of smoothing over such misshapen peaks and, given the time constraints, analysis should proceed. This turned out to be a flawed advice.

3.7.1.1 SPECIFICITY/SELECTIVITY

Specificity was examined to ensure endogenous co-eluting compounds in the patient's plasma samples did not cause a matrix effect with the compounds of interest. This was confirmed in two ways (Polettini 2006):

- Chromatographic separation of the compounds from the solvent front and other compounds was achieved (Figure 3.7-1); and
- No interfering peaks were identified in blank human plasma samples.

Selectivity was confirmed by examination of EICs. EICs were extracted over a narrow *m/z* range on either side of the compound's known *m/z* value (Polettini 2006). As the quadrupole time-of-flight mass spectrometer used was stated as able to determine *m/z* accurate to four decimal places, this, when combined with the elution time, meant the chances of mistaking compounds were negligible. No peaks with the same *m/z* were identified.

3.7.1.2 INTERNAL STANDARD RECOVERY

As detailed in the sample preparation method, the internal standard, phenacetin, was added before any extraction had taken place (Section 2.5). In robust sample preparation methods, internal standard concentrations vary little between samples, and this is a measure of the consistency of the extraction method. During the validation procedure, large variance in detector response of the internal standard phenacetin was seen. To examine the source of this, repeat injections were performed (Figure 3.7-3).

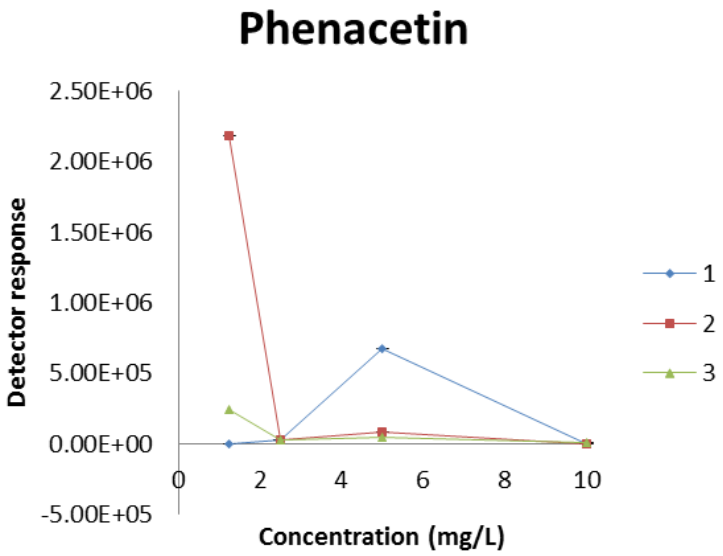


Figure 3.7-3 Detector response for internal standard following repeated injection of plasma standard curve samples

The results are shown of the repeated injection of a standard curve prepared in plasma, which contained the same concentration of internal standard in each sample. With an expected response around $2x10^6$, there was considerable deviation. This triggered the investigation of peak shape described above. Figure 3.7-4 shows the variation in detector response of phenacetin across all patient sample batches by plotting the day of analysis against the detector response (note batches frequently ran over more than one day). Inconsistency in response is especially noticeable in the first few batches.

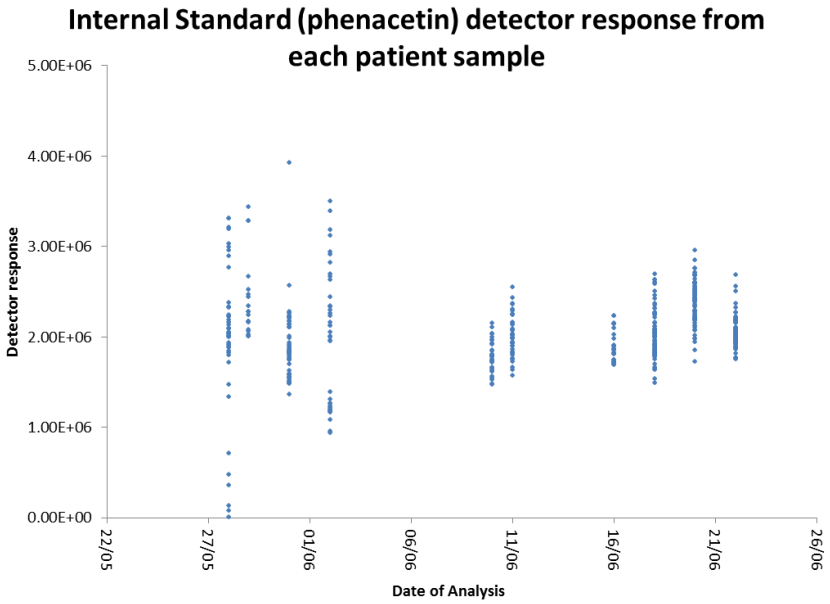


Figure 3.7-4 Internal standard detector response

To quantify what can be seen in Figure 3.7-4 visually, internal standard precision values were determined (Table 3.7-2).

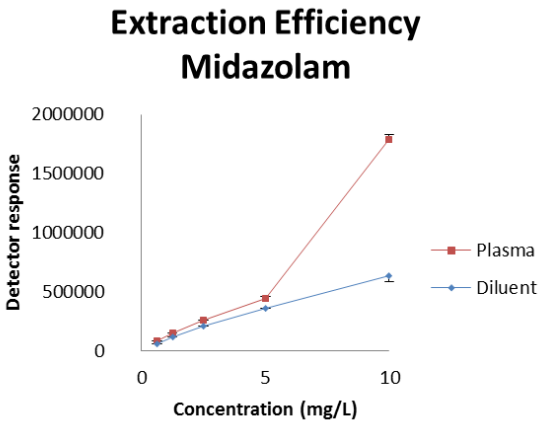
Table 3.7-2 Internal standard precision values

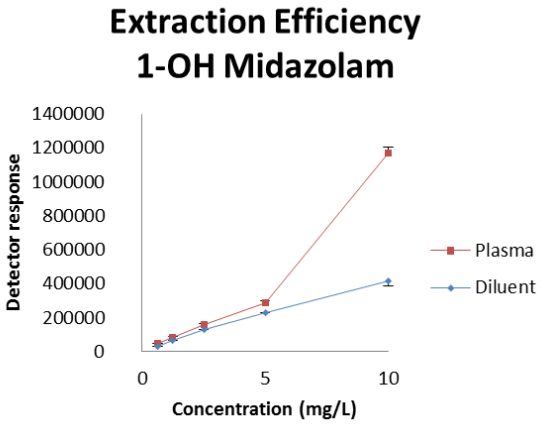
Sample batch	Number of samples	Precision (%RSD)
1	37	22.50
2	20	17.81
3	55	20.74
4	36	34.34
5	35	11.90
6	40	13.36
7	28	12.21
8	72	13.92
9	73	9.68
10	62	8.68
	Inter-batch	**29.98**

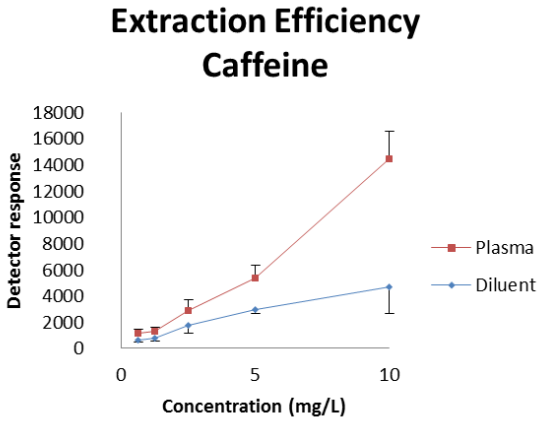
The first four of the ten batches failed standards for validation. Precision improved in later batches and the last six were within the 15% of the acceptable limit (Shah *et al.* 1991; Bressolle *et al.* 1996).

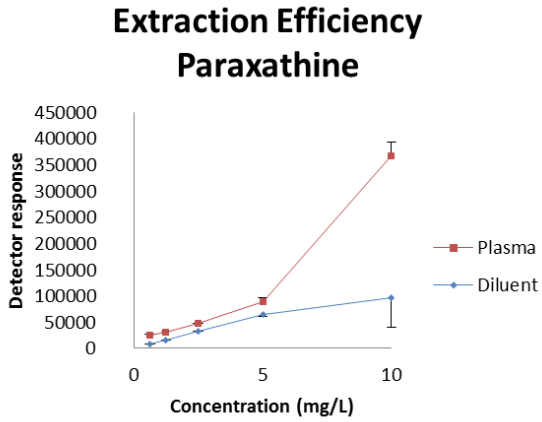
3.7.1.3 EXTRACTION EFFICIENCY

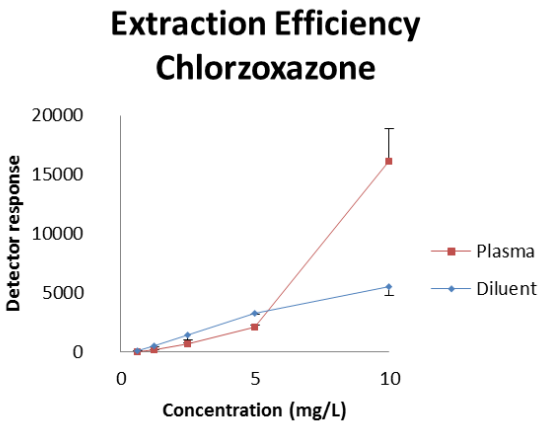
Prior to running patient samples, nominal concentrations of analyte in diluent (water for LCMS) and prepared plasma samples were analysed to determine extraction efficiency. Three sets of samples were prepared and the first of these injected three times. There was a good correlation between plasma and diluent samples for most analytes until the 5mg/L concentration after which the responses deviated (Figure 3.7-5).

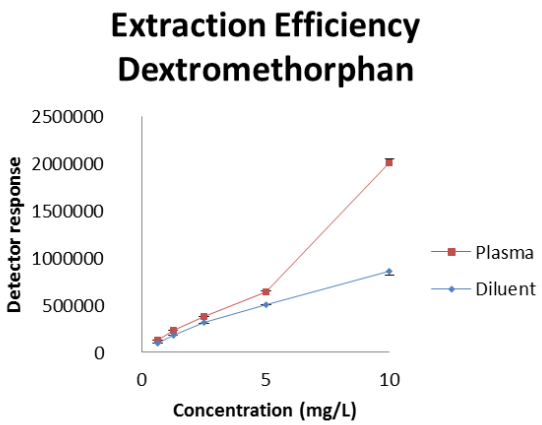












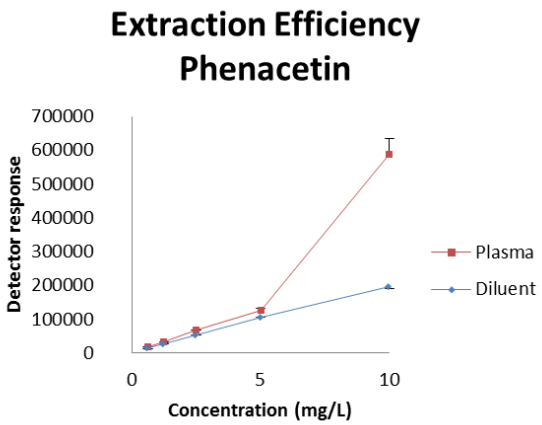


Figure 3.7-5 Extraction efficiency of each compound
Data shown as mean +/- standard deviation.

Five critical problems arose from these calibration curves which were not discovered until the use of the quantification software subsequent to the analysis:

- The plasma derived curves were not linear;
- The relationship between the diluent and plasma curves was not consistent;
- No internal standard data could be obtained as phenacetin concentration was varied along with the other metabolites; and
- The detector response was substantially less than with subsequent samples.

These issues and the ramifications for subsequent samples meant that the analysis could not be accurate. Subsequent processing of the data abandoned validation standards and therefore results that are presented are of interest only and the applicability and precision of these findings is very limited.

3.7.1.4 CALIBRATION CURVES

Calibration curves were constructed using the ratio of the analyte's detector response to that of the internal standard, phenacetin. The problems discussed above also impacted on these values and the intermittent changes to detection shown in Figure 3.7-3 meant that many of the calibration points were unusable. Accordingly calibration points were picked across the entire data collection to provide composite values. These were used to determine plasma concentration.

Despite all of the issue discussed above, plasma concentration profiles in some patients for some probes were in line with those expected (Figure 3.7-6, Figure 3.7-7).

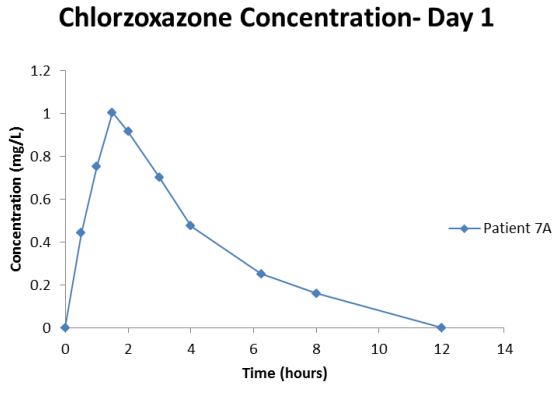


Figure 3.7-6 Plasma concentration/time profile of chlorzoxazone on Day 1 for patient 7A.

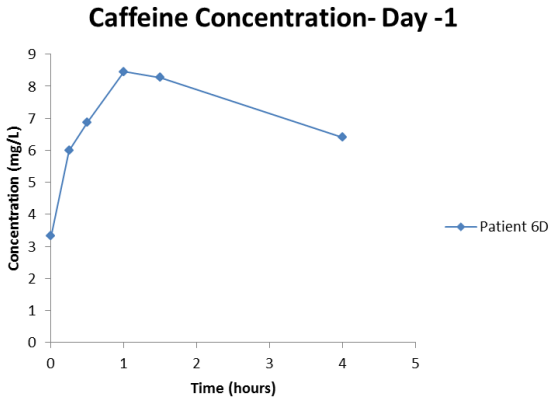


Figure 3.7-7 Plasma concentration/time profile of caffeine on Day-1 for patient 6D.

The concentrations determined were converted to their molar equivalent and the metabolic ratio of each compound was then ascertained as shown in Table 3.7-3.

Table 3.7-3 Summary of CYP450 probe method

Enzyme	Drug, route and dose	Sample matrix	Sample time
CYP1A2	Caffeine PO 100 mg	Plasma	1 hour post dose
CYP2D6	Dextromethorphan PO 30mg	Plasma	4 hour post dose
CYP2E1	Chlorzoxazone PO 250 mg	Plasma	1 hour post dose
CYP3A4	Midazolam IV 0.025mg/kg	Plasma	4 hour post dose

3.7.2 CYP 1A2

The activity of CYP 1A2 was assessed by the molar ratio of paraxanthine to caffeine present in the one hour plasma sample. Despite the best efforts of the Principal Investigator, caffeine was above the LOD in 63/107 time zero samples, indicating caffeine intake in addition to that administered as part of the study, which as referred to in Section 2.1, patients were asked to remove from their diet as part of their involvement in the study. Such an example is shown in Figure 3.7-7, where the time zero sample contained considerable amounts of caffeine.

3.7.2.1 GROUP A

The molar ratio of paraxanthine to caffeine for Group A is shown in Figure 3.7-8 as median and quartiles. Most noticeable was the wide interquartile range, indicating a high variability in the data, but this is not inconsistent with the high inter-individual variation to be expected with this type of data. Despite this variation, a trend does emerge where the ratio falls from the preoperative value and slowly recovers as the study progresses. A reduction in the value of this ratio indicates a fall in the amount of paraxanthine relative to that of caffeine. Amongst many other possibilities, this could indicate a reduction in CYP1A2 activity postoperatively.

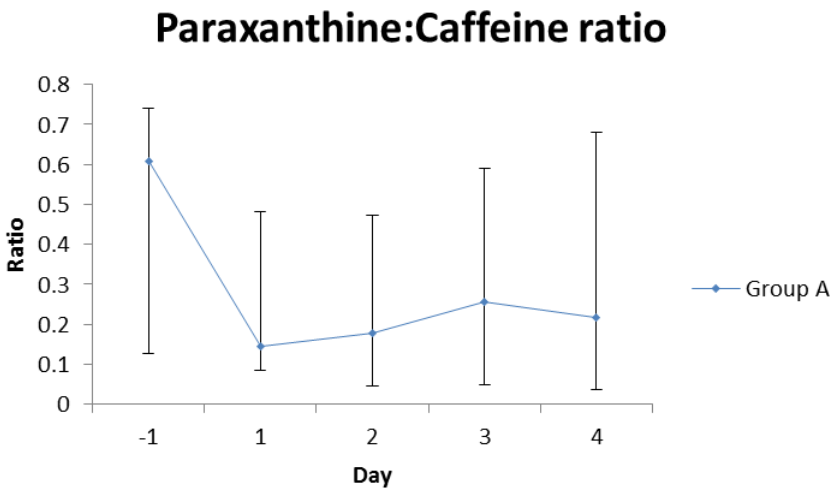


Figure 3.7-8 Median paraxanthine:caffeine ratio for Group A patients
Shown as median ± IQR

3.7.2.2 GROUP B

Group B's paraxanthine: caffeine ratio shows a similar pattern to that of Group A, a reduction from preoperative ratio and gradual return toward the end of the study (Figure 3.7-9). The median ratio begins slightly higher than Group A at 0.87, decreased but then returned to a higher value by the end of the study. This pattern of change could also be explained by reduction in CYP1A2 activity around the time of surgery, but with a small group size and wide IQRs many other factors could also account for the changes.

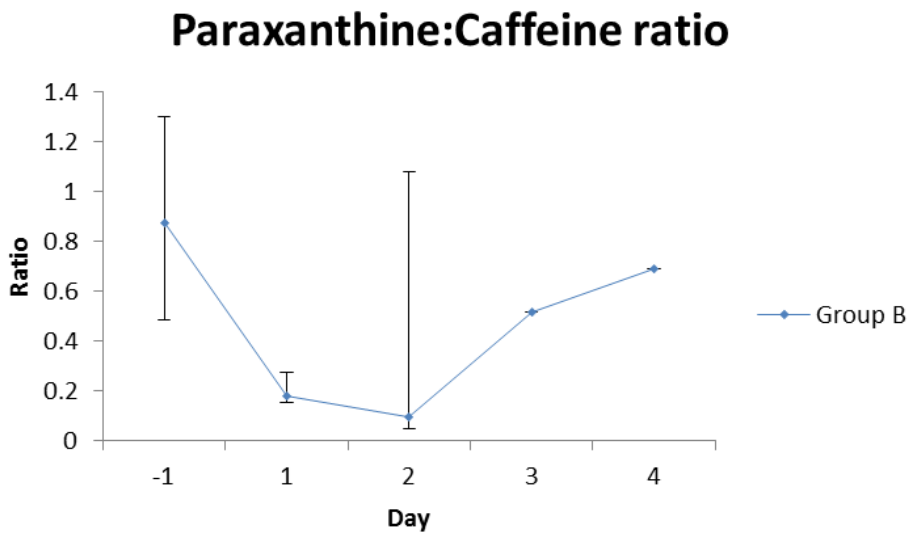


Figure 3.7-9 Median paraxanthine:caffeine ratio for Group B patients
Shown as median ± IQR

3.7.2.3 GROUP C

The paraxanthine:caffeine ratio shows much less variation across the study in Group C than other groups, however, variation within the day still remains high (Figure 3.7-10).

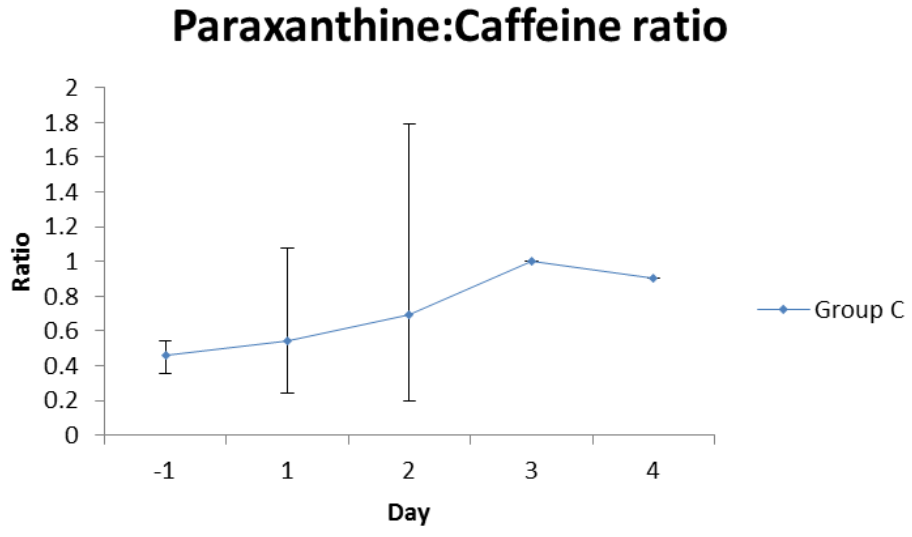


Figure 3.7-10 Median paraxanthine:caffeine ratio for Group C patients
Shown as median ± IQR

The ratio starts lower than with previous groups (0.46) and there is very little change between preoperative and postoperative values until Day 2, when the ratio slightly increases. These changes could be explained by increases to CYP1A2 activity over the postoperative period.

3.7.2.4 GROUP D

The paraxanthine:caffeine ratio in Group D had the lowest starting value from all the groups. The ratio falls slightly towards the middle of the study and then increases sharply between Day 3 and 4.

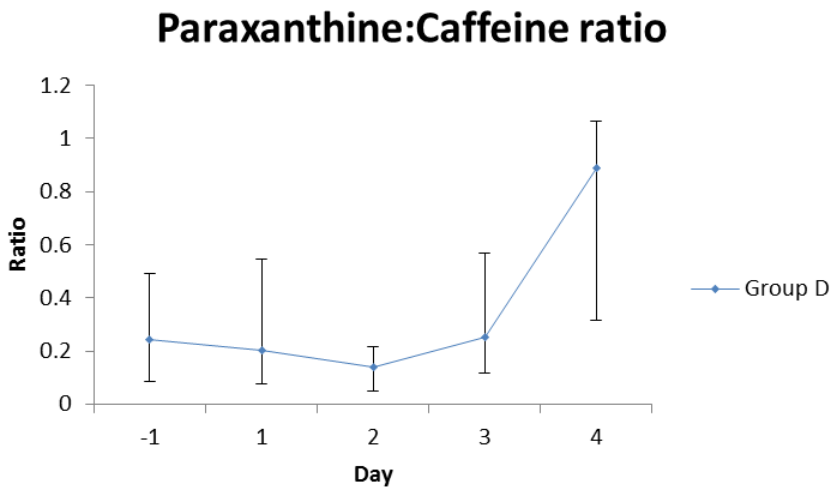


Figure 3.7-11 Median paraxanthine:caffeine ratio for Group D patients
Shown as median ± IQR

3.7.2.5 GROUP B+D

The results from Group B+D have a very similar pattern to those of Group D.

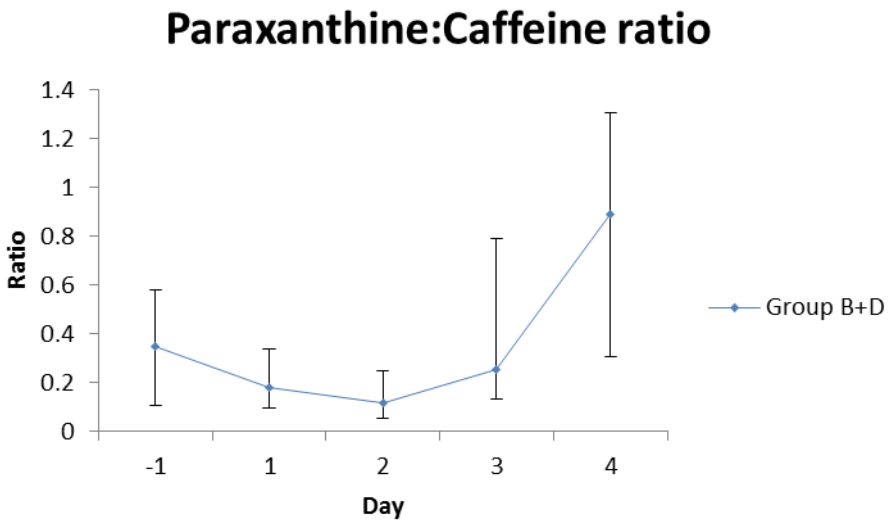


Figure 3.7-12 Median paraxanthine:caffeine ratio for Group B+D patients
Shown as median ± IQR

However, the starting ratio was larger than Group D, there was a greater fall towards the middle of the study and also the dramatic increase between Day 3 and 4.

3.7.3 CYP 2E1

In addition to the problems with this assay discussed above, the CYP2E1 probes chlorzoxazone and 6-hydroxychlorzoxazone are ideally detected in negative mode by the MS. Because the instrument used for this analysis was not capable of switching polarity within runs the detector response of these analytes was especially low.

3.7.3.1 GROUP A

The median 6-hydroxychlorzoxazone:chlorzoxazone ratio for Group A appeared to increase following surgery and then returned to preoperative values (Figure 3.7-13). One possible explanation for the increase in ratio seen could be a brief induction in CYP 2E1 activity following surgery.

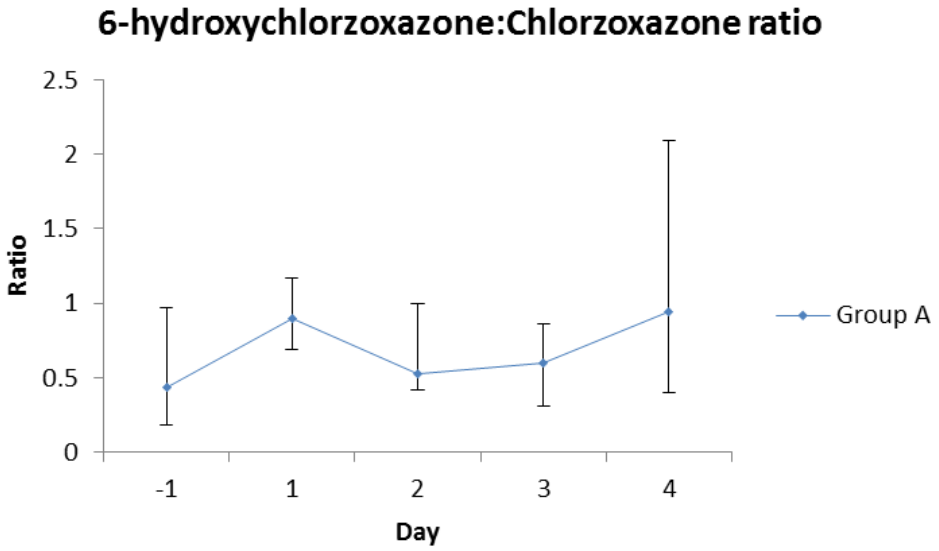


Figure 3.7-13 Median 6-hydroxychlorzoxazone:chlorzoxazone ratio for Group A
Shown as median ± IQR

3.7.3.2 GROUP B

A remarkably similar pattern in 6-hydroxychlorzoxazone:chlorzoxazone ratio to Group A was seen in Group B results (Figure 3.7-14). The main difference between the groups is Group B's values remain around preoperative median ratio for the last three days of the study. Similarly, in Group A, these changes could be explained by short lived induction of CYP2E1 perioperatively.

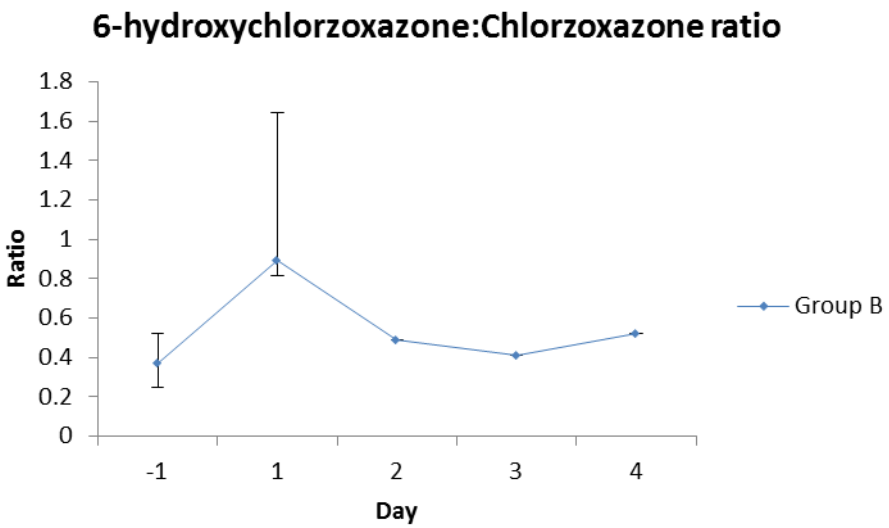


Figure 3.7-14 Median 6-hydroxychlorzoxazone:chlorzoxazone ratio for Group B
Shown as median ± IQR

3.7.3.3 GROUP C

Group C's median 6-hydroxychlorzoxazone:chlorzoxazone ratio showed a slightly different course from the previous two groups. Still increasing following surgery, the increased ratio was maintained at a steady level for three further days postoperatively before returning towards preoperative values (Figure 3.7-15). The possibility of increased CYP2E1 activity could also explain these changes.

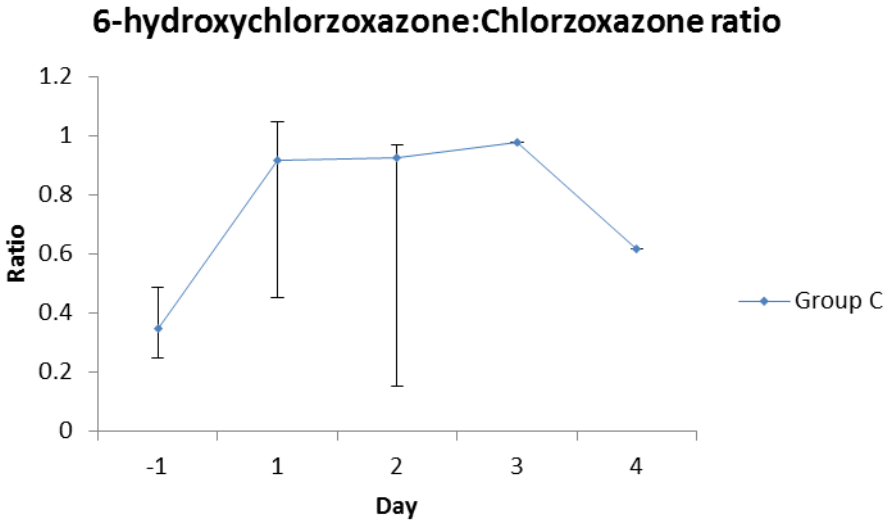


Figure 3.7-15 Median 6-hydroxychlorzoxazone:chlorzoxazone ratio for Group C
Shown as median ± IQR

3.7.3.4 GROUP D

Figure 3.7-16 shows the median 6-hydroxychlorzoxazone:chlorzoxazone ratio in Group D took a similar course to that seen in Groups A and B. There was a wide variation within

each days' values in this group, shown by the large IQR. As above, a short lived induction in CYP2E1 activity could have explained these changes.

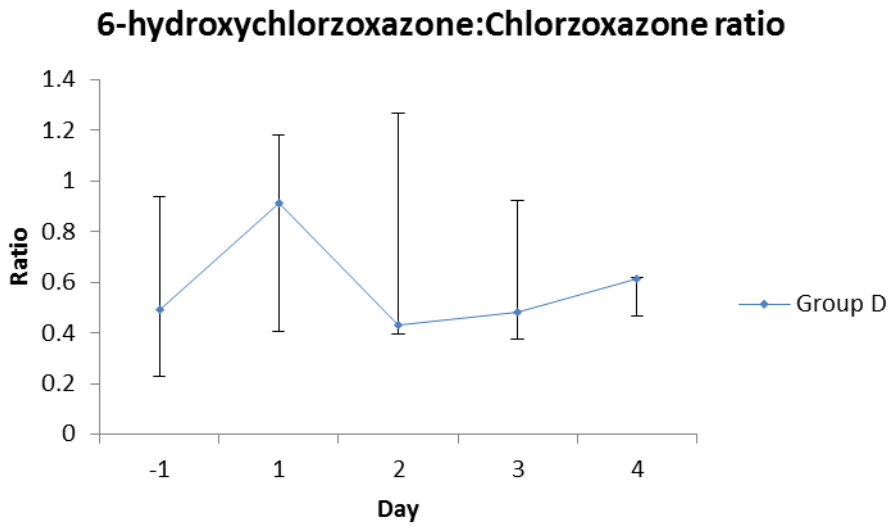


Figure 3.7-16 Median 6-hydroxychlorzoxazone:chlorzoxazone ratio for Group D
Shown as median ± IQR

3.7.3.5 GROUP B+D

As a combination of two groups already shown to follow a similar pattern, the only effect the amalgamation of these two groups had was to reduce the size of the error bars (Figure 3.7-17). Otherwise, the same conclusions of increased CYP2E1 activity could be reached.

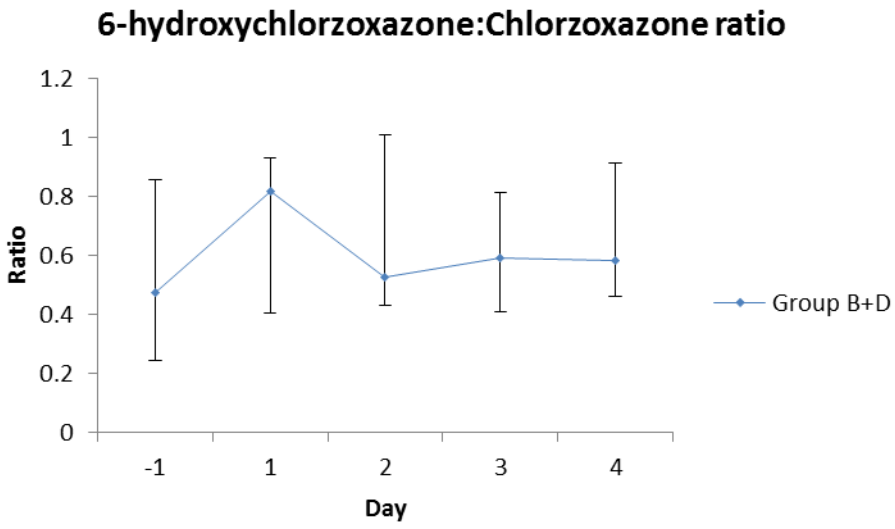


Figure 3.7-17 Median 6-hydroxychlorzoxazone:chlorzoxazone ratio for Group B+D
Shown as median ± IQR

3.7.4 CYP 3A4+2D6

Detection of midazolam was too erratic to provide usable data and 1-hyrdroxymidazolam was below the LOD. Dextrophan was below the LOD and the detection of

dextromethorphan was too inconsistent to provide enough usable data to represent graphically.

3.7.5 SUMMARY

Problems with instrumentation impaired reliability and interpretation of results. As a consequence assessment of CYP2D6 and CYP3A4 was not possible. Results for CYP1A2 for Groups A, B and D suggest an initial induction of enzyme activity immediately following surgery that resolves and recovery progresses. There is no discernible effect on Group C. Conversely assessment of CYP2E1 suggests that inhibition occurs in the immediate postoperative period which then returns towards preoperative activity levels as time passes.

3.8 CASE STUDIES

The majority of the data before this point have been presented as a group. Given the high degree of inter-individual variation to be expected with this type of research, the course of a few individual patients who were involved summarise the various changes observed. A patient was chosen from each group and their case is presented in sections concerning: liver function; inflammatory cytokines; CYP450 probes; plasma paracetamol concentrations and finally the data from their urine analysis.

3.8.1 PATIENT 4A

Patient 4A was a 62 year old male who was scheduled for a low anterior resection following the discovery of a mid-rectal adenocarcinoma for which he had received six weeks of radiotherapy, completing this six weeks previously. He was a retired factory worker who reported consuming eight standard units of alcohol per week and until five years earlier was a heavy smoker, with a 20 pack year history. He was overweight with a BMI of 31.2kg/m^2 and had a history of ischaemic heart disease and Type II diabetes. He was taking the following medicines prior to admission: aspirin, clopidogrel, losartan, lansoprazole, atorvastatin, furosemide/hydrochlorothiazide, bisoprolol, iso-sorbide mononitrate and metformin. He was classified by the anaesthetist as ASA 2.

He was admitted into hospital two days prior to the scheduled procedure and was invited to join the study on that day. He consented the following day and the preoperative medication dosing and sampling took place without any deviation from Section 2.1. That evening he was fasted from 6pm and also received 4L of Klean Prep and a phosphate enema. The following day, his surgery was uneventful but took five hours, the longest of any patient undergoing open colectomy. He lost 2.5L of blood and received three units of whole blood and 3.5L of additional IV fluids. He also received 100µg fentanyl, 4mg ondansetron, 2g paracetamol, 140mg propofol and 60mg rocuronium intravenously, 0.125% levobupivicaine, 100µg fentanyl and 3mg morphine lumbar epidural anaesthesia, in addition to inhalation of 1.5-1% sevoflurane in oxygen. Some manipulation of the liver was reported in the surgeon's notes. He returned to the ICU with a lumbar epidural infusion of bupivicaine 0.125% and fentanyl 2µg/mL running at 15mL/hr, urinary catheter and central venous access. In addition to his preoperative medication he was also prescribed IV paracetamol 1.5g every four hours, cefuroxime 1g twice daily, metronidazole 500mg three times each day, 160mg of gentamicin daily, 5000 units of

subcutaneous heparin twice daily and a 10% glucose/10 unit insulin infusion. The remainder of the study protocol was undertaken without deviation from Section 2.1 and the patient went on to receive a total of 42.5g of paracetamol over the study period. He had a complicated postoperative course. On Day 2 he became pyrexial and gentamicin was increased to 400mg daily. As he had not resumed oral intake, and the surgeon wanted to continue resting the bowel, total parental nutrition (TPN) was initiated at 3pm on day two. He was the only study patient to receive TPN.

Pharmacodynamic parameters obtained during the study are summarised in Table 3.8-1. His pain was well controlled throughout. His renal function and clotting time showed short-lived impairment following surgery. Transaminase concentrations remained within normal limits trending slightly upwards at the end of the study. Following the conclusion of the study his paracetamol dose was reduced to 1g every six hours. By day 7 he had not improved and returned to theatre where a leaking anastomosis was found which precipitated the formation of a stoma. He did not resume any oral nutrition for two and half weeks after surgery, requiring two weeks of TPN.

Table 3.8-1 Pharmacodynamic monitoring values
Abbreviations used: SCr- serum creatinine; AST- aspartate aminotransferase; INR- international normalised ratio; NRS- numerical rating scale- measure of pain experienced in last 24hrs and at time of study.

	Day -1	Day 1	Day 2	Day 3	Day 4
SCr (µmol/L)	94	110	87	74	69
AST (U/L)	22	21	20	27	109
INR	1	1.6	1.7	1.1	1
NRS Best	0	0	0	0	0
NRS Worst	0	3	4	3	5
NRS Now	0	1	1.5	1	1

3.8.1.1 LIVER FUNCTION

Preoperative αGST concentration was high enough to indicate liver injury prior to surgery (Figure 3.8-1). This potential liver injury was not known at the time of the study as AST was not increased. The lack of sustained αGST elevation and absence of AST increase questioned the accuracy of this one elevated αGST. Postoperatively, there was no evidence of hepatotoxicity with αGST and AST remaining within normal limits until Day 4, when AST values spiked to just above three times the upper limit of normal. αGST concentrations, however, remained low, slightly above group concentrations. This increase in AST was minor in comparison with that seen with paracetamol toxicity, which registers in the thousands. As the only patient to experience an elevation outside the upper limit of normal this elevation is notable, but may not be clinically significant.

Transient LFT increases are widely reported following initiation of TPN (Gabe *et al.* 2010) and continued monitoring showed the AST elevation resolved quickly.

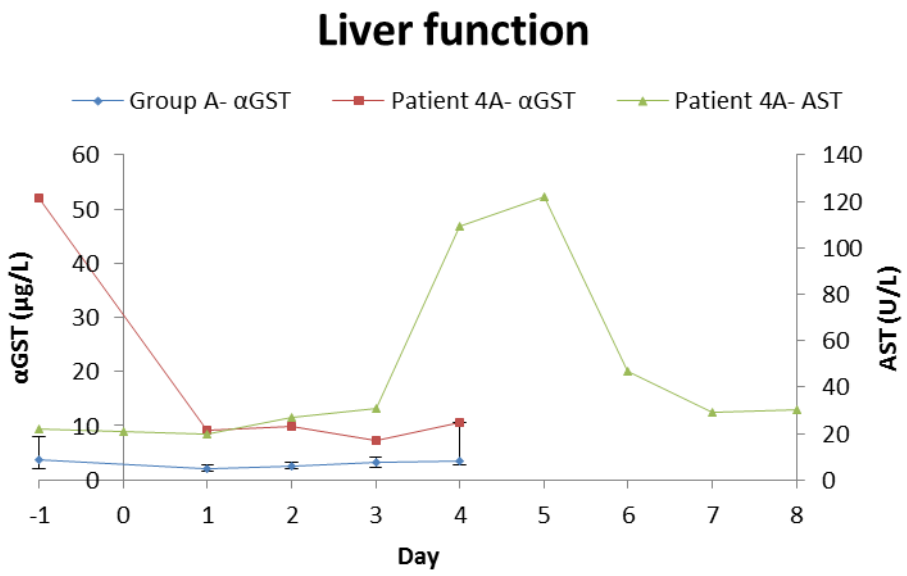


Figure 3.8-1 Liver function tests αGST and AST on each day of the study with group median αGST (±IQR)

3.8.1.2 CYTOKINES

All cytokines were elevated on the postoperative day (Figure 3.8-2-Figure 3.8-5).

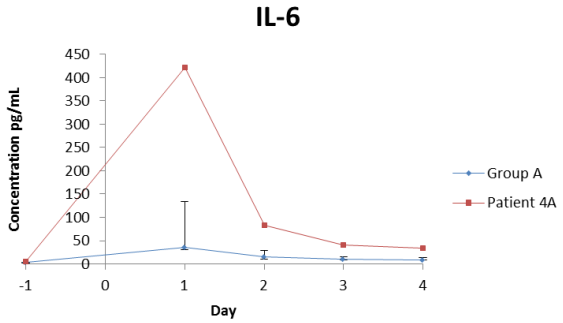


Figure 3.8-2 Individual and group median (±IQR) IL-6 concentration in daily samples

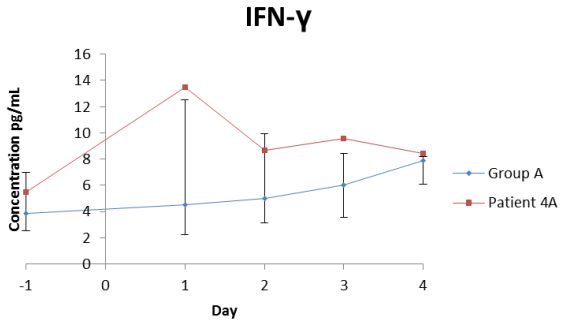


Figure 3.8-3 Individual and group median (±IQR) IFN-γ concentration in daily samples

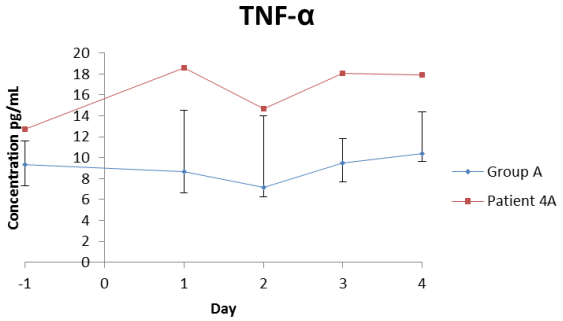


Figure 3.8-4 Individual and group median (±IQR) TNF-α concentration in daily samples

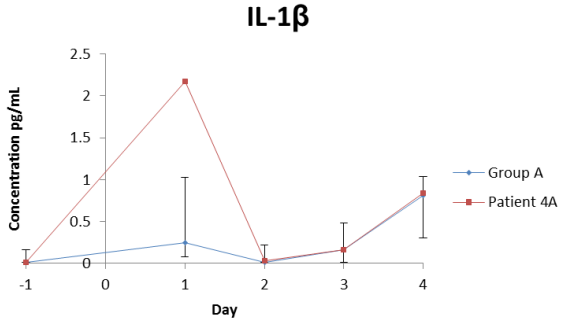


Figure 3.8-5 Individual and group median (±IQR) IL-1β concentration in daily samples

IL-6 increases were especially prominent, rising to the highest level measured in any patient in any group. This was not surprising given that this patient had the longest operation of the most traumatic surgery. Despite the substantial increase in IL-6,

increases in the other cytokines were comparatively minor (Figure 3.8-3 and Figure 3.8-4). Notable was the increase in IL-1β, one of the first cytokines to be released, on the third and fourth day as the patient became increasingly unwell (Figure 3.8-5), however the magnitude of this increase was very minor.

3.8.1.3 CYP450 PROBES

The paraxanthine:caffeine ratio for patient 4A reduced from preoperative values. The ratio continued to fall in a similar pattern to the rest of the group until Day 4 when it showed a sharp increase (Figure 3.8-6). There is nothing in this patient's clinical course that easily explains this induction on Day 4, which indicates CYP1A2 operating at above preoperative levels. While CYP450 activity is known to be reduced by inflammation, considering this patients state health on Day 4 and the indication given by IL-1β that inflammation was, if anything, increasing, the paraxanthine:caffeine ratio should fall, not peak on this day. Given the deteriorating health of this patient, subsequent samples would have been interesting to further examine the role of inflammation.

Figure 3.8-7 shows the 6-hydroxychlorzoxazone:chlorzoxazone: ratio followed a similar course, falling from preoperative values until Day 2, indicating an induction of CYP2E1. A gradual increase in ratio was seen on Days 3 and 4. This coincides with the administration of TPN, which is known to induce CYP450 activity as seen in the last two days. While it is difficult to see relationships with caffeine metabolism, the inhibition of CYP2E1 by inflammation could explain slightly delayed inverse relationship between inflammation (IL-6 concentration) and the 6-hydroxychlorzoxazone:chlorzoxazone ratio.

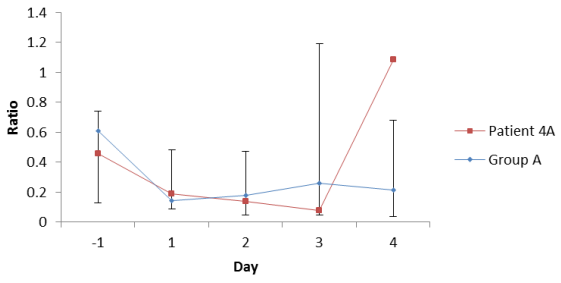


Figure 3.8-6 Individual and group median ratio (±IQR) of paraxanthine to caffeine in plasma

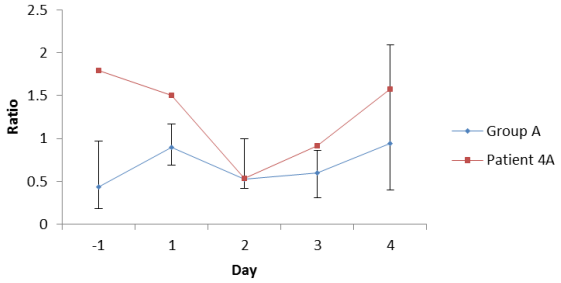


Figure 3.8-7 Individual and group median ratio (±IQR) of 6-hydroxychlorzoxazone to chlorzoxazone in plasma

3.8.1.4 PLASMA

Figure 3.8-10 shows the paracetamol concentrations were used to calculate pharmacokinetic values (Table 3.8-2). This patient's half-life fell postoperatively.

Clearance, Vd and AUC values were all substantially different to the remainder of this patient's group. The large reduction in clearance postoperatively, indicated metabolism was slowed, but this was opposed by a substantial reduction in volume of distribution. Because both clearance and Vd values changed by a similar magnitude, there was little change to the mean residence time or half-life postoperatively. However, the reduction to clearance may have allowed an increase in AUC postoperatively, showing some plasma accumulation of paracetamol, which was not seen in the remainder of the group.

Table 3.8-2 Plasma pharmacokinetics preoperatively and postoperatively with group medians (±IQR)

	Day -1		Day 1	
	Patient 4A	Group A	Patient 4A	Group A
t ½ (hr)	2.22	1.75 (1.59, 2.11)	2.13	2.13 (1.80, 3.25)
AUC (mg.hr/L.mg)	0.043	0.041 (0.032, 0.054)	0.083	0.049 (0.022, 0.057)
Cl (L/hr)	23.07	24.56 (18.68, 31.21)	12.0475	20.60 (13.05, 26.90)
Vd (L/kg)	0.83	0.69 (0.62, 0.83)	0.309	0.48 (0.38, 0.70)
MRT (hr)	2.97	2.32 (2.11, 2.88)	2.13	3.10 (2.49, 4.68)

Because Phase I processes contribute a relatively small amount to paracetamol metabolism overall, it is difficult to link the assessment of CYP1A2 and 2E1 to changes in clearance, however, both enzymes show reduced levels of activity postoperatively in this patient in conjunction with substantial reductions to plasma clearance. The contributions of the more significant pathways, glucuronidation and sulphation, do appear reduced as seen in the reductions to the ratio of metabolites recovered in the urine. It is possible that the Phase II processes are susceptible to inhibition due to inflammation, pharmacokinetic values were not calculated beyond the first postoperative day the relationship between clearance and cytokine concentration cannot be reliably determined.

Daily plasma paracetamol concentrations at one and four hours post dose were used to assess for accumulation (Figure 3.8-8 and Figure 3.8-9). Both one hour and four hour concentrations more than doubled between preoperative and postoperative sampling. Following their peak on Day 1, a steady state concentration did not seem to be achieved and there was a continuous reduction in paracetamol concentration at both one hour and four hour times. The final samples had paracetamol concentrations similar to preoperative values and this fall indicated metabolism of paracetamol was induced.

The increase in plasma concentrations immediately following surgery is greater than the remainder of his group and coincides with increases to plasma cytokine concentrations. Cytokines are known to reduce volume of distribution, which was also lower in this patient than the rest of his group. This could explain the short lived increases in plasma

concentration seen. However, considering the size of the increase in IL-6, the highest of any patient, the plasma concentrations of this patient are too consistent with that of his group for any strong effect of cytokine concentrations to exist.

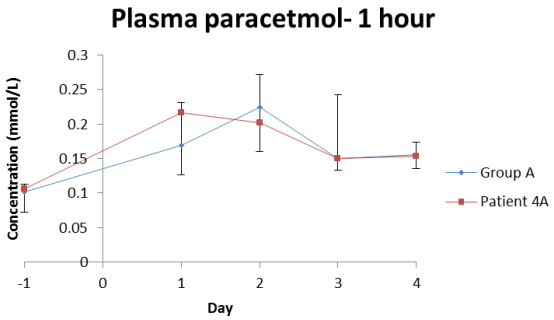


Figure 3.8-8 Individual and group median Plasma concentration of paracetamol- 1hr

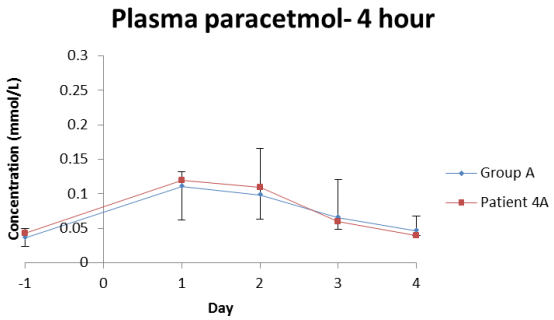


Figure 3.8-9 Individual and group median Plasma concentration of paracetamol- 4hr

Figure 3.8-10 shows a substantial increase in paracetamol glucuronide concentrations postoperatively. Glucuronide concentrations rose to dwarf all other metabolites and even surpass paracetamol itself on the final day. This reflects the considerable reserve capacity of glucuronidation and how its activity can expand rapidly to meet demand. Comparatively, paracetamol sulphate concentrations did not alter from preoperative values, despite the much higher concentrations of paracetamol present, indicating the saturation of sulphation. Furthermore, unlike paracetamol glucuronide, there is very little difference in paracetamol sulphate concentrations over the dose interval postoperatively, despite the much higher concentrations of the substrate paracetamol, indicating saturation of sulphonation processes. Paracetamol cysteine and mercapturate were not detectable preoperatively; however cysteine concentrations were greatly increased postoperatively and by the end of the study had risen above those of paracetamol sulphate. This suggests a rise in the conjugation of potentially toxic of NAPQI; however it is not possible to determine if NAPQI production is increased, or if, as a result of increased cysteine from the TPN, it is simply that GSH conjugation has increased. However, considering the increases in AST in this patient, it may be the former.

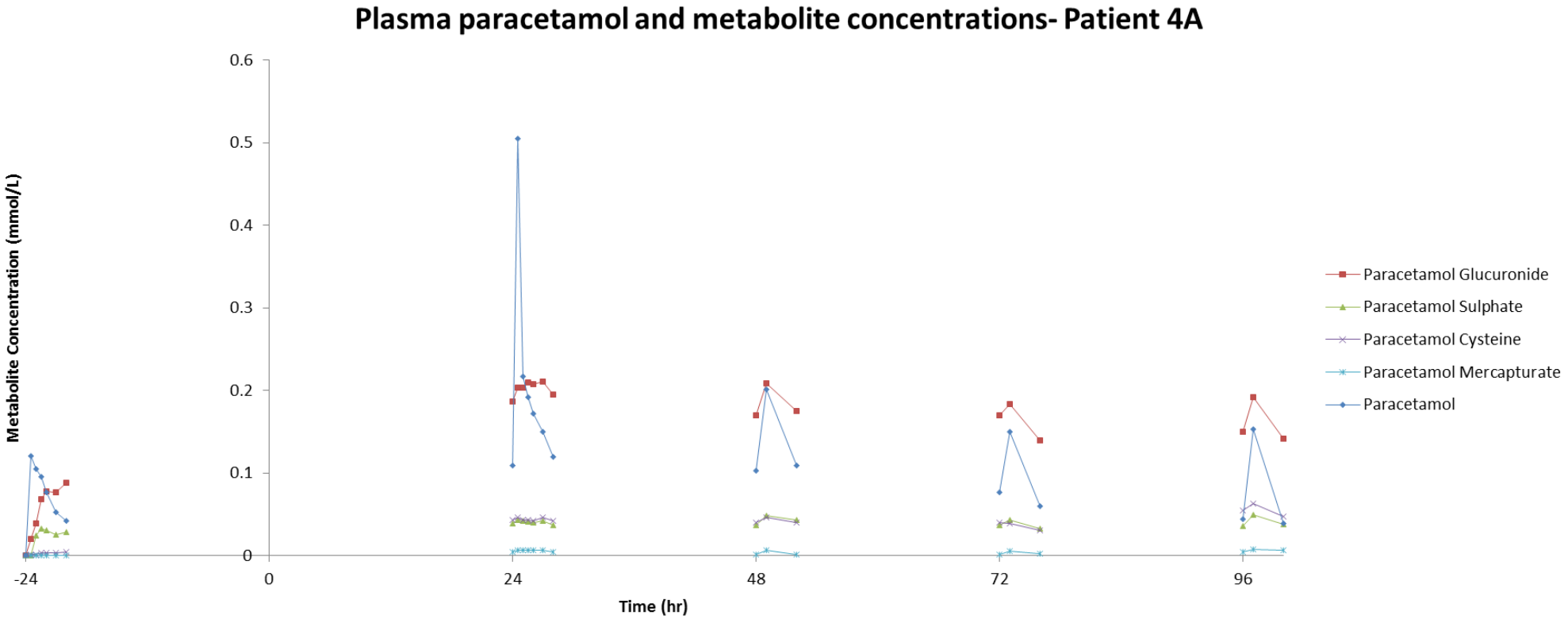


Figure 3.8-10 Concentration of paracetamol and its major metabolites in plasma

3.8.1.5 URINE

The two most important graphs arising from the urine are presented in Figure 3.8-11 Figure 3.8-12 and Figure 3.8-13.

Marked changes were seen in all these figures that coincide with the initiation of TPN. As discussed in Section 1.4, the main changes to disposition of paracetamol following the initiation of TPN were the induction of the CYP450 system and an increase in the supply of sulphur containing amino acids.

3.8.1.5.1 SULPHATE CONTRIBUTION

This patient's contribution of sulphate derived metabolites initially followed a similar track to that of his groups (Figure 3.8-11). The fall seen between Day 1 and Day 2 occurs even though plasma concentrations of paracetamol sulphate and cysteine seen in Figure 3.8-10 remain steady. This could indicate as much a lack of glucuronide increase that was seen in other groups, as an increase in sulphate derived metabolites in this patient.

From sample 4 on Day 2 this patient's values diverge from his group's, when the patient's values appear to spike and then continue to slowly rise while his group's continue to fall. This change coincided precisely with the initiation of TPN in this patient. As a rich source of inorganic sulphur and sulphur containing amino acids this would maintain the urinary output of sulphate derived metabolites and were indicative that the decline seen in the remainder of his group could be attributed to malnutrition. Other possible explanations for this pattern could be the patient's condition, being of poorer health than the remainder of his group with evidence of returning inflammation. However this could not explain the marked increase in the sulphate containing metabolites in the urine seen in exactly the same interval as when TPN was initiated and that his values are virtually identical to his preoperative values, whereas the groups values declined.

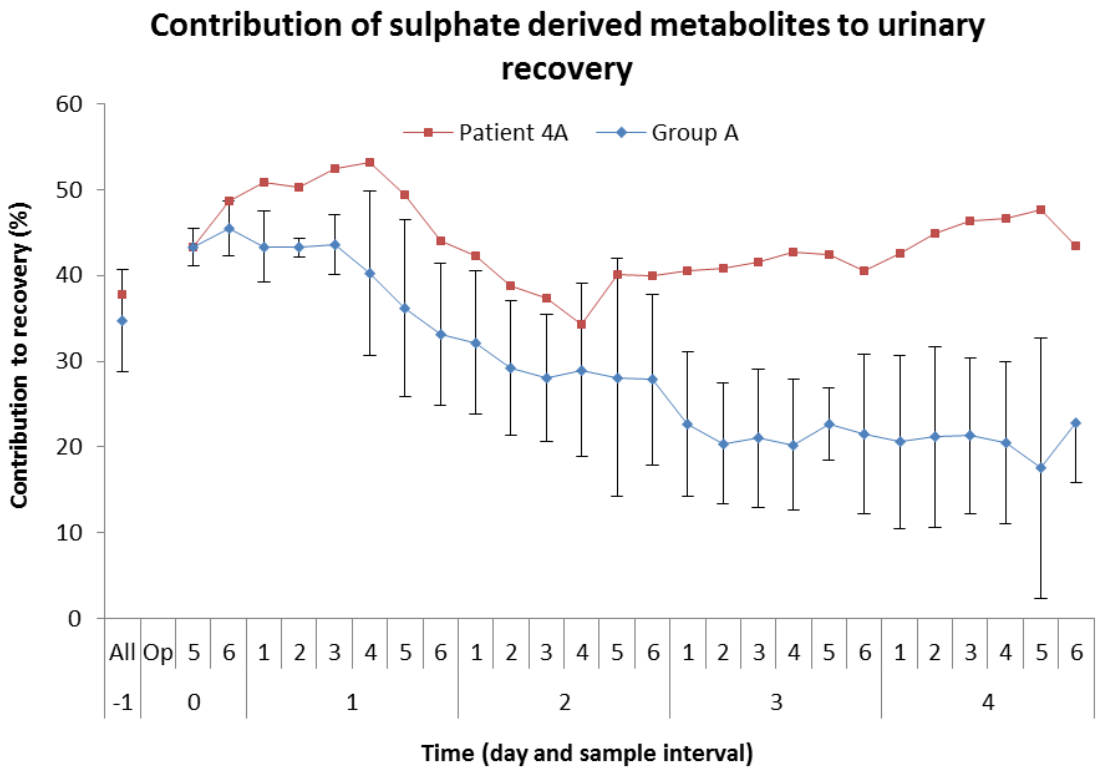


Figure 3.8-11 Per cent of urinary metabolites excreted as sulphate derived compounds with group median (±IQR)

3.8.1.5.2 PHASE II:I METABOLITE RATIO

As in the previous figure, there is a clear divergence of this patient's values from his groups that begin at the same time as TPN initiation (Figure 3.8-12).

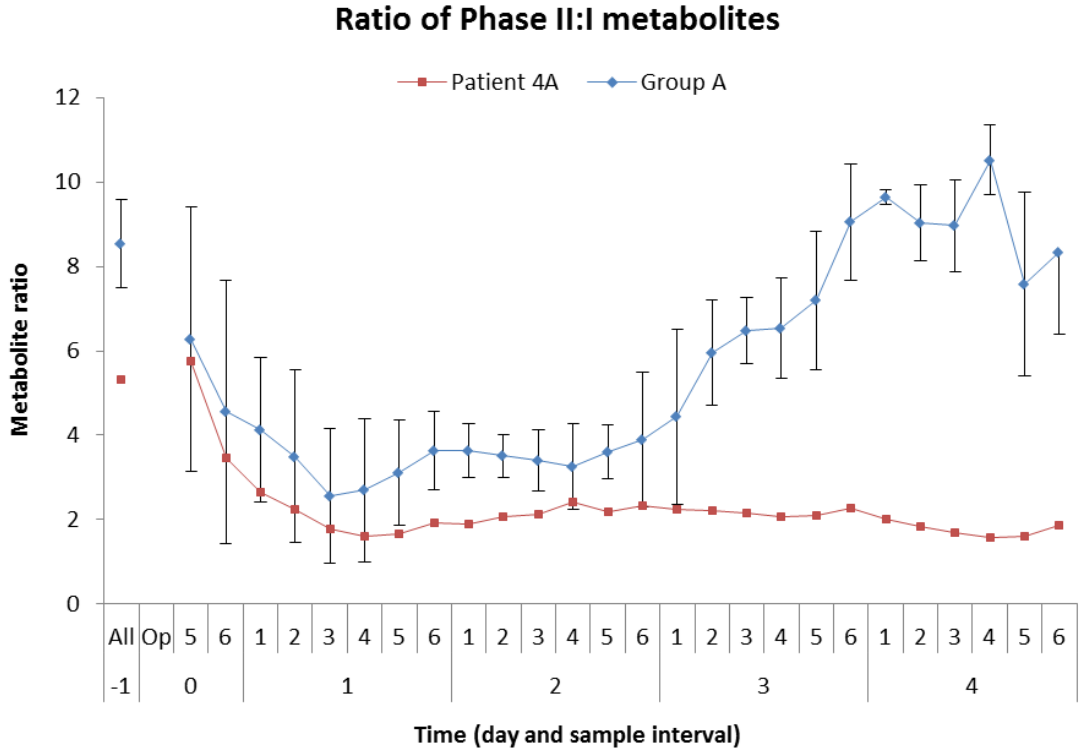


Figure 3.8-12 Ratio of Phase II: Phase I metabolites of paracetamol with group median (±IQR)

The maintained reductions in this ratio could indicate increased CYP450 activity, as has been shown with TPN administration (Section 1.4.5), or at least an increased conjugation of the CYP450 product NAPQI as discussed in Section 3.8.1.4. The lack of change in the ratio of these metabolites in the urine matches what is seen in the plasma concentrations, where the contribution of each of the metabolites remained relatively static, although plasma levels are not good indicators of metabolism due to difference rates of renal excretion. This pattern of Phase II:I ratio is unlike any other patient in his group, as shown by the recovery of group values towards the end of the study, and would suggest that the effect of TPN administration is to favour NAPQI production/recovery, not increase the rate of sulphonation. Further investigation reveals the cause of this altered Phase II:I ratio. Figure 3.8-13 shows there was no alteration to total urinary recovery of the dose or clear influence of TPN, maintaining total urinary recovery around 100%.

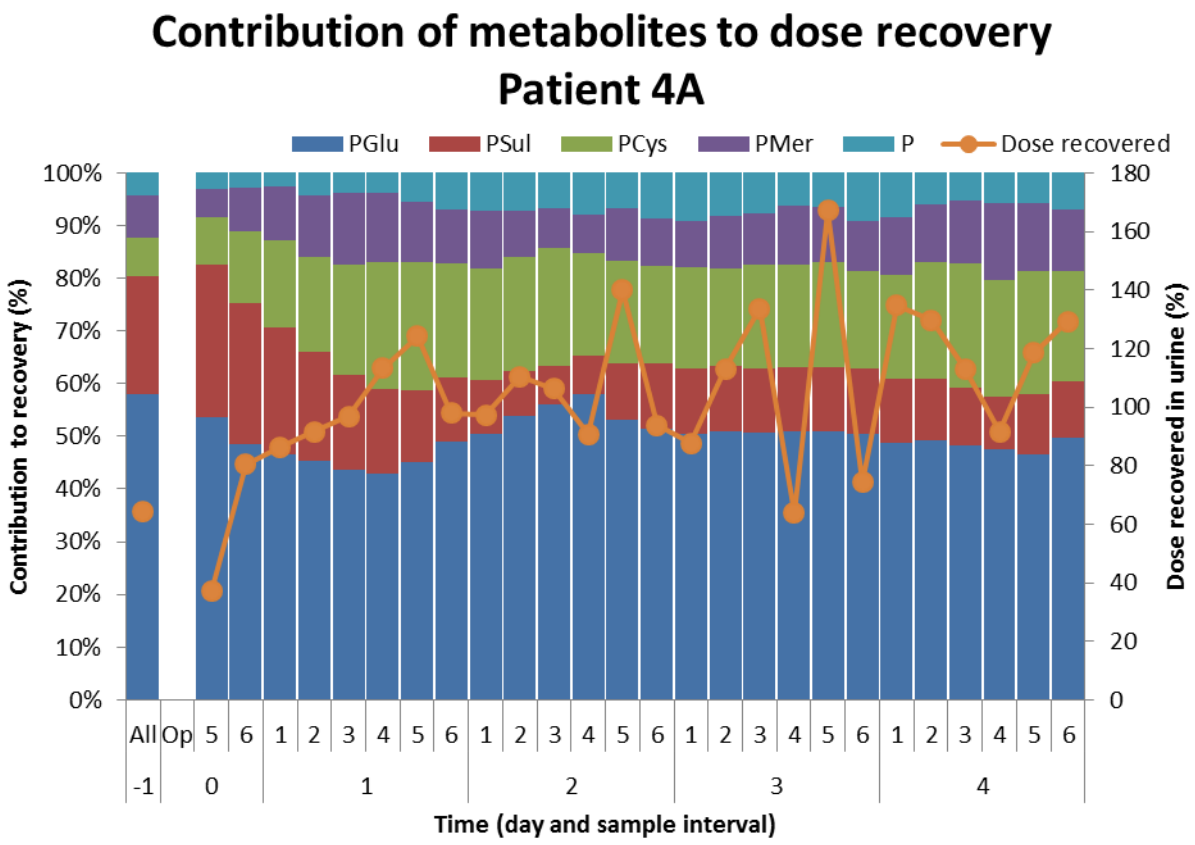


Figure 3.8-13 Contribution of paracetamol metabolites to urinary recovery of dose (left axis) and percentage of total dose recovered (right axis)

This shows that the change in urinary Phase II:I ratio was not due to plasma accumulation or alteration to urinary excretion, but was due to changes in the amount of metabolites produced. In fact, at 103% over the total postoperative course, this patient had the highest dose recovery of any in his group, where the next closest was at 98%. This was

achieved through an unusually high contribution of paracetamol cysteine, not through increases to paracetamol glucuronide as seen in all other patients of his group (Figure 3.3-14). As a conjugate of NAPQI and indicator of CYP450 activity, this patient's high paracetamol cysteine recovery, the highest of his group, may explain why he showed altered liver function towards the end of the study period. While TPN is known to increase CYP450 activity, it would also provide a source of cysteine for detoxification in the same manner as an antidote. However, in this patient, the increased AST concentrations indicate the increased cysteine was not sufficient to completely prevent liver injury.

There were three other notable change in the contribution of each of the metabolites that coincide with the initiation of TPN:

1. Paracetamol sulphate recovery increases;
2. Paracetamol glucuronide recovery reduces; and
3. The relative contribution of each metabolite remains static from the point onwards.

As a source of sulphur containing amino acids (precursors for sulphonation) it is not surprising that paracetamol sulphate contribution increased following TPN administration, however the increase was to values still well below those seen preoperatively and its role remains minor. Assuming the abundance of cofactor supplied by TPN, this could be explained in two ways: SULT enzymes were saturated or Phase I pathways were induced and deprived Phase II pathways of substrate. It seems unlikely SULT enzymes were saturated because an increased capacity is exhibited earlier in the study by the greater recoveries of paracetamol sulphate seen. However, the possibility CYP450 pathways are induced to such a degree as to deprive Phase II pathways also seems remote. As already stated, TPN is known to cause CYP450 induction but this would require unreported levels of induction, in situations where inhibition is more commonly reported, and these are not seen in the CYP450 probe results above. Furthermore, while the increase to AST could be due to increased NAPQI production, given the limited extent of the elevation, it would seem more likely to be associated with TPN (Gabe *et al.* 2010). Additionally, the TPN administered to this patient contains 3.5g of methionine in each daily bag Kabi, 2011 #4630}. While well short of the maximum 300mg/kg infusion of *n*-acetlycysteine given to patients in the 24 hours following overdose, this is still a significant amount which should reduce the risk of toxicity (Brok *et al.* 2006).

There is also something occurring in this patient at the time TPN was initiated that prevents the rise of glucuronidation that occurs in the other members of his group. The absence of this rise may explain the deviations seen in Figure 3.8-11 and Figure 3.8-12. It is possible that this rise in the other group members was not a factor of increased glucuronidation activity, but rather a reduction in the activity of the sulphur containing pathways. As detailed in Section 1.4.5 while malnutrition is known to increase Phase I metabolism, it has a more significant effect in the reduction of GSH conjugation and sulphonation. This would explain the apparent rise in glucuronidation, as it is not affected by starvation. Further evidence of this change was exhibited by the continuing rise of paracetamol glucuronide in the group data shown in Figure 3.3-14, indicating worsening exhaustion of the alternative pathways, whereas, once TPN was initiated and adequate nutrition supplied, this patient's metabolic pattern abruptly stabilised. Further support for this hypothesis is this patients highest urinary recovery of dose, whereas the potential for unconjugated NAPQI remaining in the liver may explain the lower dose recovery recorded in the other patients.

3.8.2 PATIENT 1B

Patient 1B, was a 60 year old mechanical engineer. He had a BMI of 24.9kg/m^2, smoked 15-20 cigarettes a day for 35 pack years and reported drinking only on special occasions, having no regular alcohol consumption. He had no significant medical history or preoperative medications until recent complaints of biliary colic which led to the discovery of a high grade dysplastic polyp near his splenic flexure. He was scheduled for a left hemicolectomy and cholescystectomy and was graded by the anaesthetist as ASA 1.

He was admitted the day prior to the procedure and consented for this study that afternoon. Preoperative study medications and sampling went ahead without deviation from the protocol Section 2.1. That evening he was fasted from 6pm, received four litres of Klean Prep and a phosphate enema the following morning. The following day his surgery went ahead as scheduled taking 1.75 hours. In theatre he received 0.125% levobupivicaine, 100µg fentanyl and 3mg morphine lumbar epidural anaesthesia, 4mg ondansetron, 130mg propofol, 35mg rocuronium, 2g paracetamol, parecoxib 40mg intravenously in addition to 1.5-1% sevoflurane in oxygen. He received 4L of IV fluid intraoperatively and some liver palpation was noted by the surgeon. He returned to the ICU with a lumbar epidural infusion of bupivicaine 0.125% and fentanyl 2µg/mL running

at 15mL/hr, urinary catheter and central venous access. He was also prescribed twice daily parecoxib 40mg, 1g paracetamol every six hours, 5000 units of subcutaneous heparin twice daily, pantoprazole 40mg daily, cefuroxime 1g twice daily, metronidazole 500mg three times each day. Subsequent study procedures went ahead as in Section 2.1 with the omission of the 15min plasma sample on the first postoperative day as no line was free for access. The patient received a total of 21g of paracetamol over the study period. His postoperative course was uncomplicated and he resumed oral intake on the third postoperative day with sips of water, followed by tea and toast on the fifth day. His epidural infusion was reduced to 12mL/hr on the first postoperative day and continued at this rate until midday on the fourth postoperative day when it was stopped. Pharmacodynamic values showed some derangement around the time of surgery, with increased creatinine and INR on Day 1 (Table 3.8-3). Both resolved by Day 3 without intervention. Pain was well controlled throughout the patient's recovery.

Table 3.8-3 Pharmacodynamic monitoring values

Abbreviations used: SCr- serum creatinine; AST- aspartate aminotransferase; INR- international normalised ratio; NRS- numerical rating scale- measure of pain experienced in last 24hrs and at time of study.

	Day -1	Day 1	Day 2	Day 3	Day 4
SCr (µmol/L)	105	114	95	83	74
AST (U/L)	24	51	39	27	21
INR	1	1.5	1.2	1	1
NRS Best	0	0	0	0	0
NRS Worst	0	6	0	0	3
NRS Now	0	4	0	0	0

3.8.2.1 LIVER FUNCTION

Despite the changes seen in the rest of the group, there was no alteration in this patient's liver function seen with either αGST or AST (Figure 3.8-14).

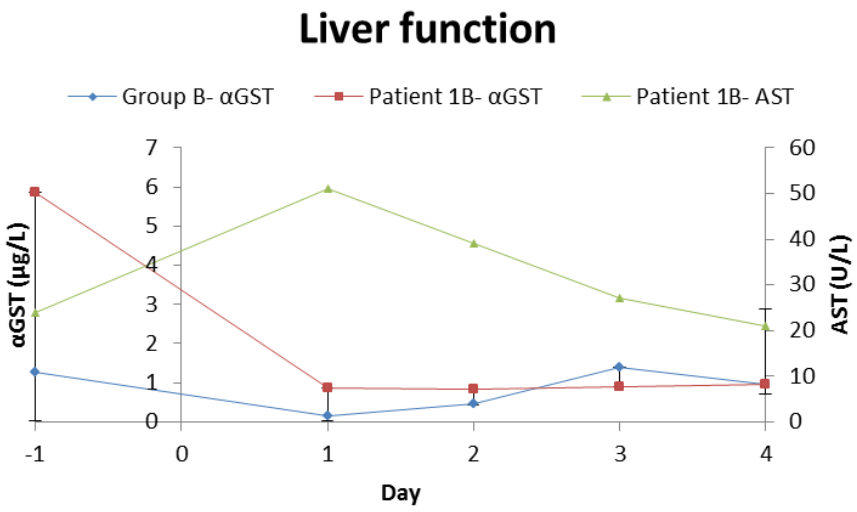


Figure 3.8-14 Liver function tests αGST and AST on each day of the study with group median αGST and IQR

Similar to patient 4A, αGST concentrations appeared to actually decrease following of surgery, although this patient's preoperative αGST concentration was 10 times less than that of patient 4A.

3.8.2.2 CYTOKINES

Elevations in IL-6 were seen in this patient on the day following surgery (Figure 3.8-15) which was accompanied by slight increases to TNF-α (Figure 3.8-17).

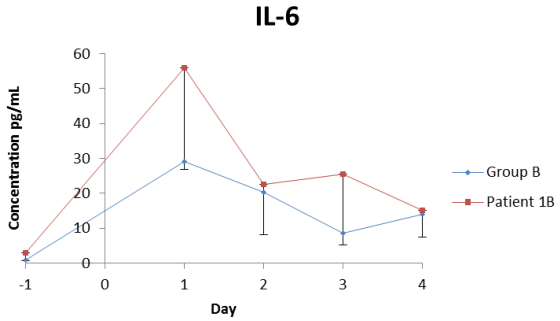


Figure 3.8-15 Individual and group median IL-6 concentration in daily samples

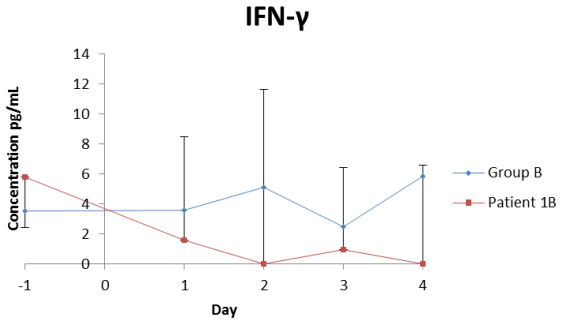


Figure 3.8-16 Individual and group median IFN-γ concentration in daily samples

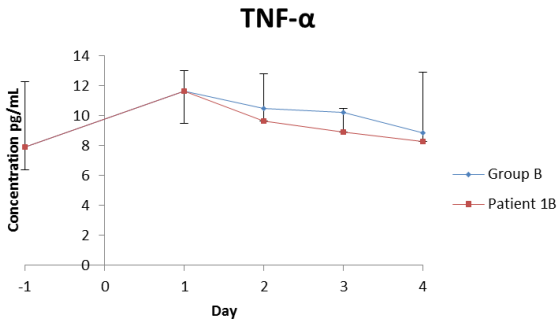


Figure 3.8-17 Individual and group median TNF-α concentration in daily samples

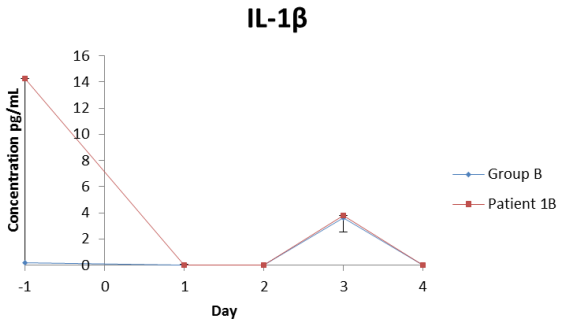


Figure 3.8-18 Individual and group median IL-1β concentration in daily samples

IL-6 fell on the following day but remained above that of the group until the final day of the study. TNF-α remained slightly elevated, but the size of the elevation was very minor. Elevations in IFN-γ (Figure 3.8-16) and IL-1β (Figure 3.8-18) preoperatively were slight and resolved postoperatively with no effect of surgery seen.

3.8.2.3 CYP450 PROBES

Both Figure 3.8-19 and Figure 3.8-20 show changes to the ratio of these probes for this patient were very close to that of the rest of his group. Ratios of caffeine:paraxanthine fell and then remained low for the remainder of the study, indicating induction of CYP1A2 and subsequent production of more paraxanthine following surgery. Conversely, the ratio of 6-hydroxychlorzoxazone to chlorzoxazone increased initially, and then returned to

preoperative values. This indicates brief inhibition of CYP2E1 following surgery, which was quickly corrected by Day 2.

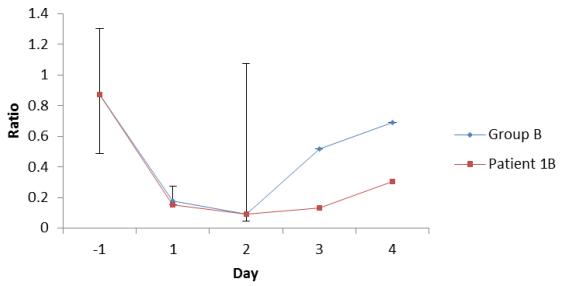


Figure 3.8-19 Individual and group median ratio of caffeine to paraxanthine

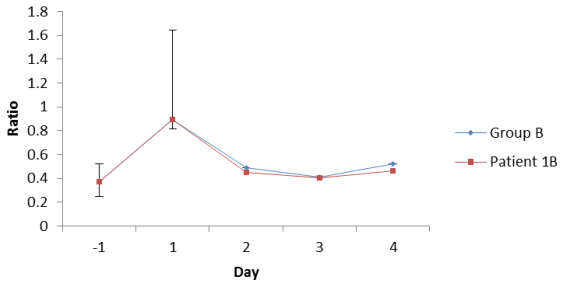


Figure 3.8-20 Individual and group median ratio of chlorzoxazone to 6-hydroxychlorzoxazone

3.8.2.4 PLASMA

Pharmacokinetic values are shown in Table 3.8-4. The half-life of paracetamol in this patient was slightly above that of his group preoperatively but changed very little following surgery to become the group's median value. There was no substantial difference between the half-life of this patient and Patient 4A who received the high dose. The only value to show a noticeable change postoperatively was the reduced Vd, with all of the other values remaining relatively constant.

Table 3.8-4 Plasma pharmacokinetics preoperatively and postoperatively with group medians (±IQR)

	Day -1		Day 1	
	Patient 4	Group	Patient 4	Group
t ½ (hr)	2.11	1.97 (1.64, 2.11)	2.09	2.09 (1.49, 3.14)
AUC (mg.hr/L.mg)	0.039	0.039 (0.025, 0.052)	0.040	0.040 (0.022, 0.057)
Cl (L/hr)	25.46	25.46 (19.34, 39.82)	25.27	25.27 (17.67, 44.94)
Vd (L/kg)	0.95	0.95 (0.61, 1.05)	0.68	0.70 (0.53, 0.93)
MRT (hr)	2.94	2.76 (2.29, 2.94)	2.13	2.19 (1.80, 2.63)

Figure 3.8-21 shows paracetamol concentrations changed very little following surgery and there was only a slight accumulation seen in the t=0 and Cmax samples postoperatively. Following surgery metabolite concentrations remain relatively constant with slight accumulation of paracetamol glucuronide, cysteine and mercapturate and reduction in paracetamol sulphate.

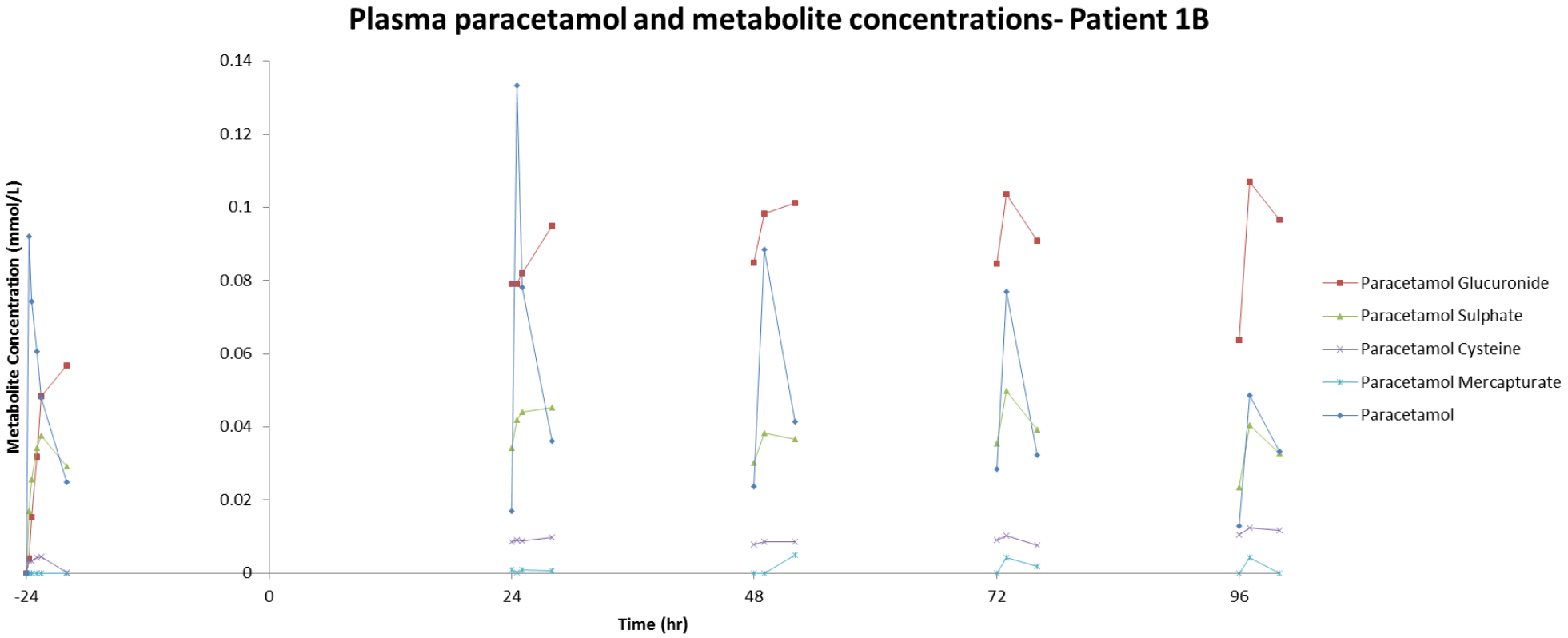


Figure 3.8-21 Concentration of paracetamol and its major metabolites in plasma

One hour and four hour concentrations of paracetamol are shown in Figure 3.8-22 and Figure 3.8-23. Both show a very minor accumulation until Day 2 and then a gradual return to preoperative levels.

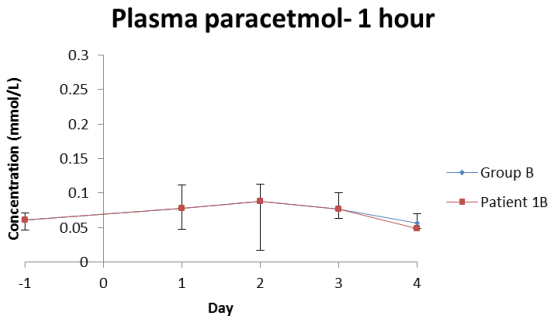


Figure 3.8-22 Individual and group median Plasma concentration of paracetamol- 1hr

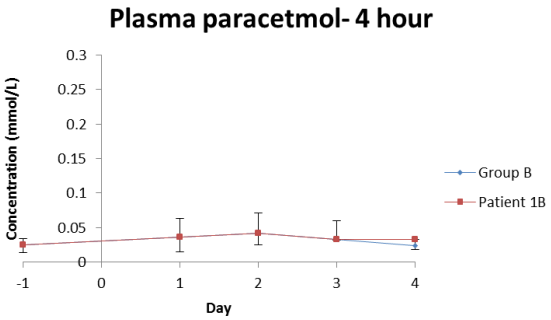


Figure 3.8-23 Individual and group median Plasma concentration of paracetamol- 4hr

3.8.2.5 URINE

3.8.2.5.1 SULPHATE CONTRIBUTION

Patient 1B showed a steady reduction in sulphate derived metabolites being excreted in the urine until half way through Day 2 which plateaued thereafter (Figure 3.8-24). These values were consistently below that of the rest of his group.

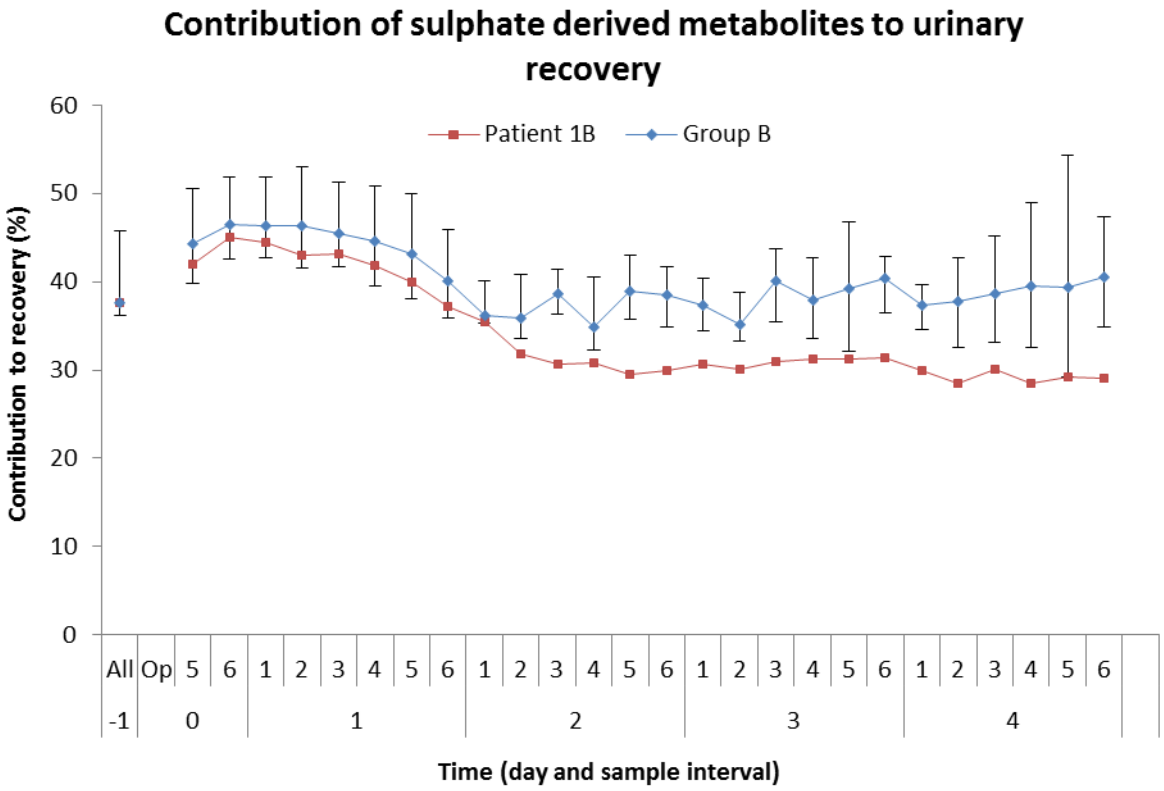


Figure 3.8-24 Per cent of urinary metabolites excreted as sulphate derived compounds with group median (±IQR)

3.8.2.5.2 PHASE II:I METABOLITE RATIO

The ratio of metabolites in the urine from this patient was quite different from the expected course. Although values dipped immediately following surgery they climbed back to preoperative levels quickly, and finished slightly above those seen preoperatively. This was in stark contrast to the results of Patient 4A. In Patient 1B the contribution of paracetamol glucuronide increased postoperatively, exceeding the reductions in paracetamol sulphate recovery, and this accounts for the return of the metabolic ratio to beyond preoperative levels. The dip in paracetamol metabolite ratio seen on Day 1 was in the opposite direction to that seen in chlorzoxazone metabolism, which appears inhibited on this day, confounding the possibility of a role of CYP2E1. A closer relationship could be seen with caffeine metabolism, although this appeared most induced on Day 2, well after paracetamol's metabolic ratio had recovered. A better relationship between metabolite ratio and cytokine concentration could be drawn with IL-6 concentrations peaking at the same time the metabolic ratio was at its lowest.

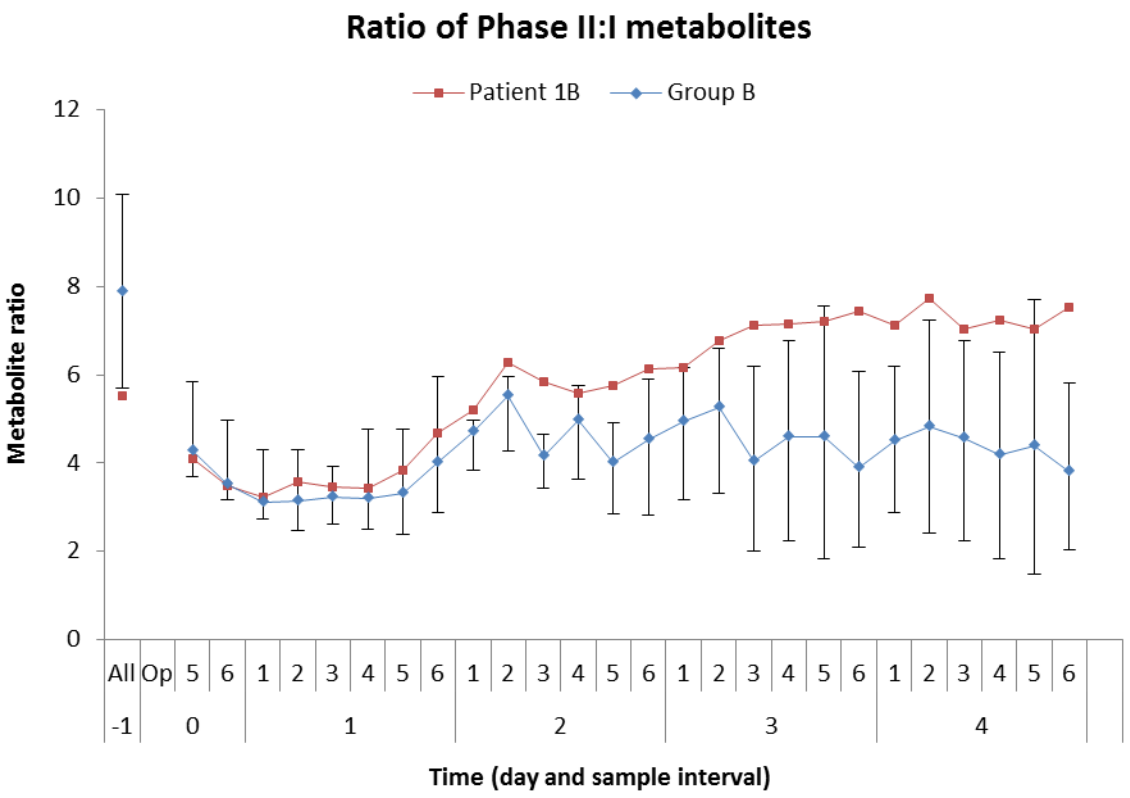


Figure 3.8-25 Ratio of Phase II: Phase I metabolites of paracetamol with group median (±IQR)

3.8.3 PATIENT 1C

Patient 1C was a 41 year old female who was scheduled for a left mastectomy and axillary node clearance following three months of chemotherapy and radiotherapy for an invasive

ductal carcinoma of her left breast. Her main chemotherapy agents, docetaxel, doxorubicin and cyclophosphamide, were given three weekly prior to surgery. She had a BMI 22.3kg/m^2, consumed 10-14 standard units of alcohol per week and smoked 15-20 cigarettes a day for 20 pack years. She was taking no regular medication prior to the procedure and, having no medical history of note, was graded as ASA 1. She was fasted from midnight prior to the procedure and shortly before induction levo-bupivicaine was given to provide a left paravertebral block.

Surgery and anaesthesia were uneventful. In theatre she received remifentanyl and propofol infusions, receiving a total of 1.9mg and 5.6mg of each respectively over the two hour long procedure. In addition, she received paracetamol 2g intravenously, 100mg diclofenac rectally and 4.5L of IV fluids. She returned to the ward with IV access into her external jugular vein, a peripheral line and a urinary catheter. She also had a morphine patient-controlled analgesia and required 32mg of this on demand over the next three days. Additionally, she received twice 75mg diclofenac orally and 5000 units of heparin subcutaneously for the remainder of the study period.

The remainder of her recovery was uncomplicated (Table 3.8-5) and the study followed Section 2.1. Her urinary catheter was removed on the day after surgery and she continued to collect her own urine for the study. Problems on the same day drawing the predose sample from the external jugular line necessitated the use of the peripheral line which remained patent for the rest of the study. In total 21g of paracetamol was administered over the course of the study.

Table 3.8-5 Pharmacodynamic monitoring values
Abbreviations used: SCr- serum creatinine; AST- aspartate aminotransferase; INR- international normalised ratio; NRS- numerical rating scale- measure of pain experienced in last 24hrs and at time of study.

	Day -1	Day 1	Day 2	Day 3	Day 4
SCr (µmol/L)	73	80	94	90	82
AST (U/L)	24	24	21	27	29
INR	1.1	1.1	1	1	1
NRS Best	0	0	0	0	0
NRS Worst	0	1	1	3	4
NRS Now	0	0	0	0	1

3.8.3.1 LIVER FUNCTION

Patient 1C's liver function tests showed very little variation across the study duration, remaining below that of her group for the duration of the study (Figure 3.8-26).

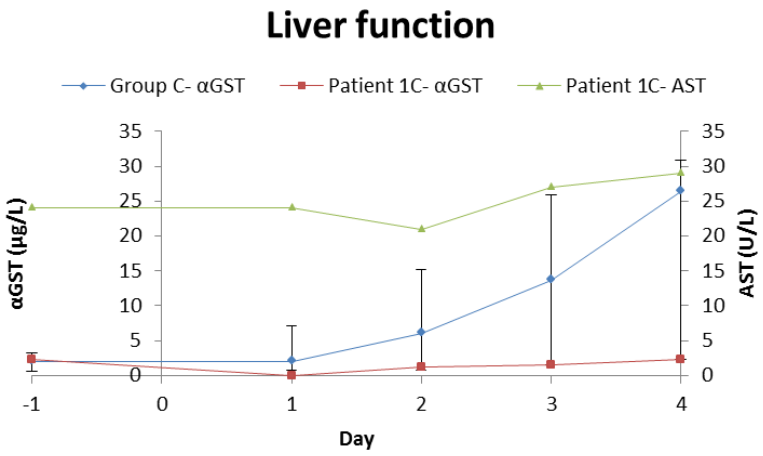


Figure 3.8-26 Liver function tests αGST and AST on each day of the study with group median αGST and IQR

3.8.3.2 CYTOKINES

There were only minor elevations in cytokine concentrations following surgery that were either equivalent to or less than the rest of this group. IFN-γ (Figure 3.8-28) and TNF-α (Figure 3.8-29) concentrations showing barely any change at all over the five days of monitoring. IL-1β (Figure 3.8-30) and IL-6 (Figure 3.8-27) concentrations were elevated towards the end of the monitoring period, but these were very small.

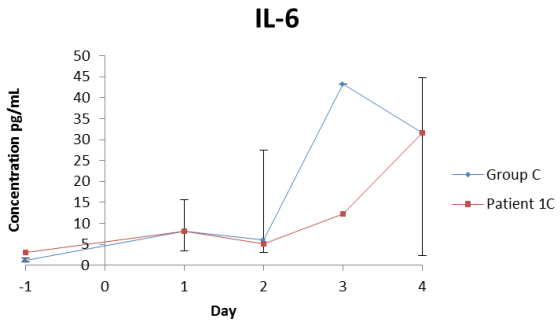


Figure 3.8-27 Individual and group median IL-6 concentration in daily samples

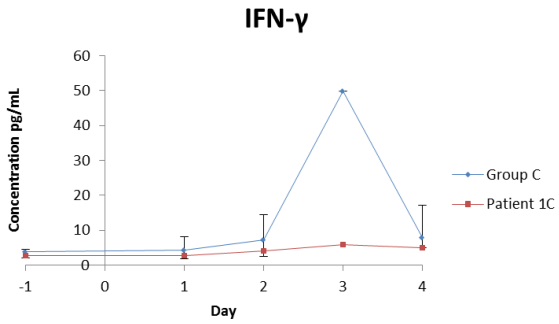


Figure 3.8-28 Individual and group median IFN-γ concentration in daily samples

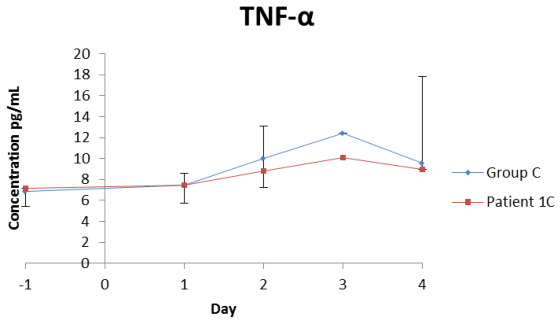


Figure 3.8-29 Individual and group median TNF-α concentration in daily samples

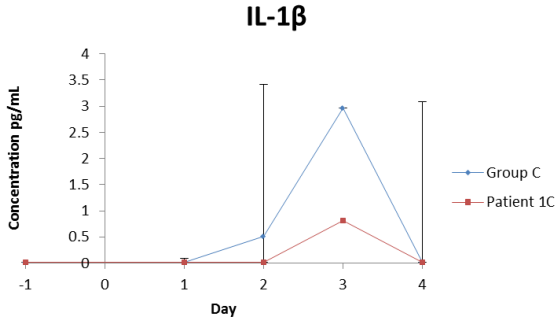


Figure 3.8-30 IL-1β Individual and group median concentration in daily samples

3.8.3.3 CYP450 PROBES

Figure 3.8-31 and Figure 3.8-32 were suggestive of induction of both CYP1A2 and, to a lesser extent, CYP2E1 in this patient, occurring maximally for both enzymes on Day 3.

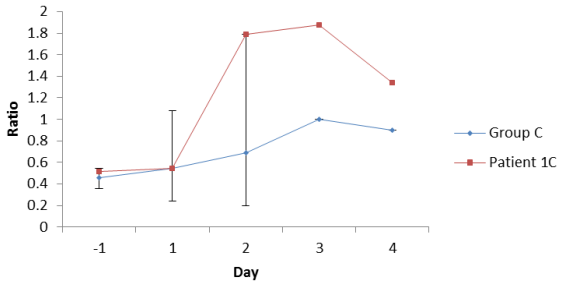


Figure 3.8-31 Individual and group median ratio of paraxanthine to caffeinein plasma

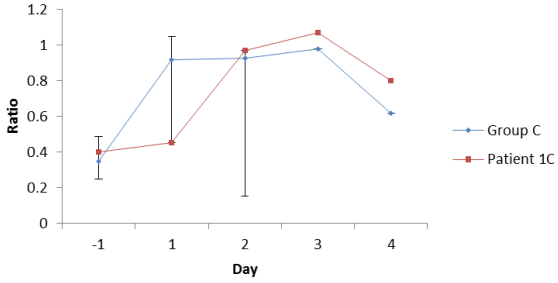


Figure 3.8-32 Individual and group median ratio of 6-hydroxychlorzoxazone to chlorzoxazone in plasma

3.8.3.4 PLASMA

This patient showed a marked accumulation of paracetamol glucuronide and a slight accumulation of paracetamol sulphate, while plasma concentration of paracetamol and the other metabolites remained relatively constant following surgery. Despite the changes seen in chlorzoxazone and caffeine metabolic ratios (Figure 3.8-31 and Figure 3.8-32) there was not significant change in either of the CYP450 products, paracetamol cysteine or mercapturate (Figure 3.8-33).

There was very little evidence of accumulation of paracetamol with preoperative and postoperative profiles being very similar (Figure 3.8-32). Plasma data pharmacokinetic parameters (Table 3.8-6) showed plasma half-life of paracetamol was longer than most of her groups and slightly fell postoperatively. This was accompanied by reductions to AUC. The two determinants of half-life, clearance and volume of distribution, both fell, but the fall of Vd was much larger (≈25%). These changes were reflected in reductions to MRT of about one third.

Table 3.8-6 Plasma pharmacokinetics preoperatively and postoperatively with group medians (±IQR)

	Day -1		Day 1	
	Pt 1C	Group median (±IQR)	Pt 1C	Group median (±IQR)
t ½ (hr)	2.35	1.94 (1.60, 3.43)	2.12	1.89 (1.25, 2.27)
AUC (mg.hr/L.mg)	0.042	0.045 (0.042, 0.079)	0.039	0.045 (0.039, 0.052)
Cl (L/hr)	23.68	22.40 (14.00, 23.68)	25.41	22.41 (19.41, 25.41)
Vd (L/kg)	1.29	0.88 (0.74, 1.094)	0.904	0.758 (0.612, 0.904)
MRT (hr)	3.38	2.72 (2.233, 4.947)	2.216	2.09 (1.95, 2.22)

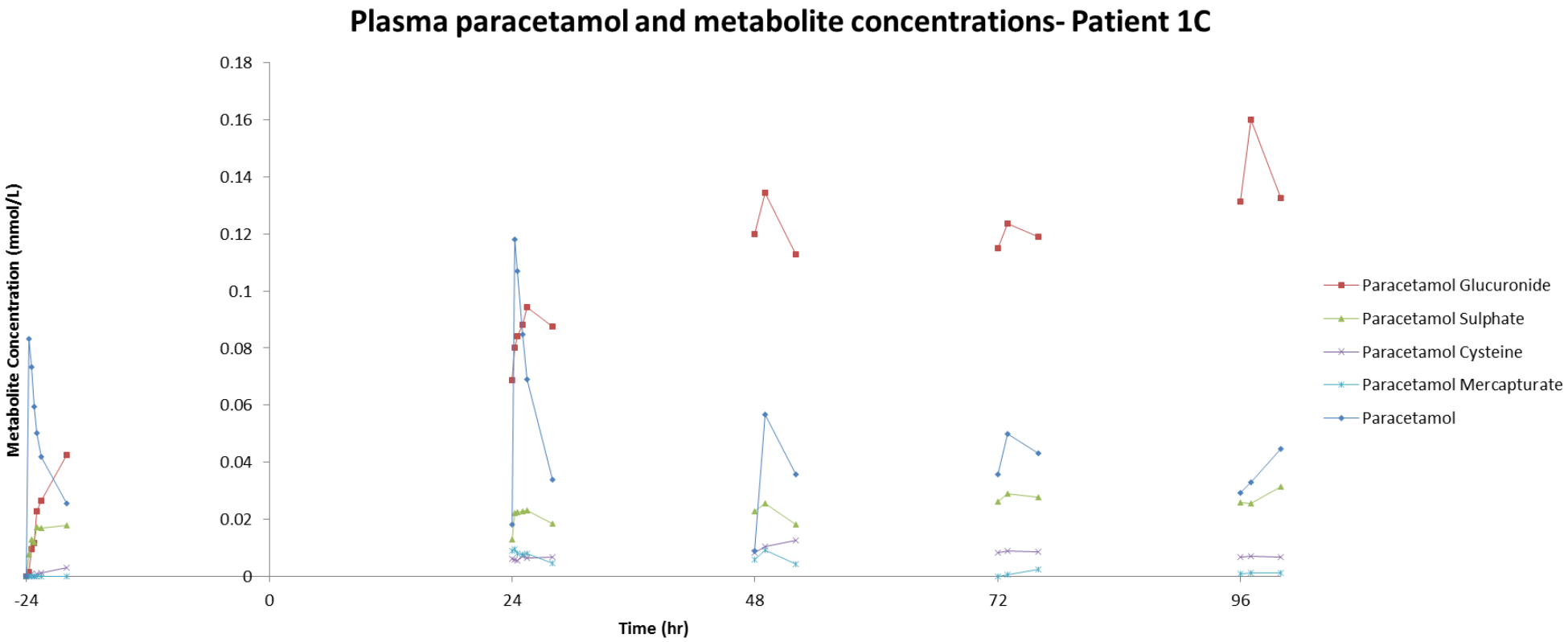


Figure 3.8-33 Concentration of paracetamol and its major metabolites in plasma

There was very little evidence of accumulation in either the one (Figure 3.8-34) or four hour concentration (Figure 3.8-35), with the one hour concentration appearing to decline on the third and fourth day. These show very little relation to either CYP450 enzyme result.

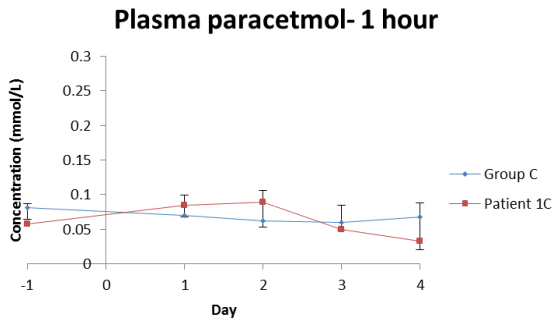


Figure 3.8-34 Plasma concentration of paracetamol- 1hr

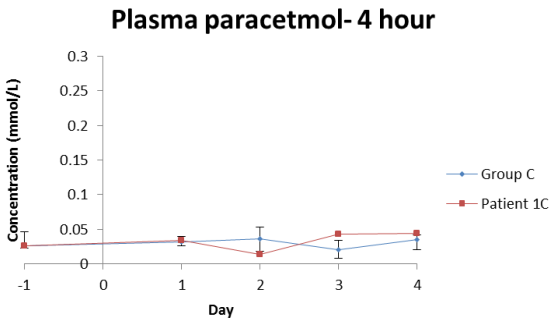


Figure 3.8-35 Plasma concentration of paracetamol- 4hr

3.8.3.5 URINE

3.8.3.5.1 SULPHATE CONTRIBUTION

Unlike the patients undergoing bowel surgery, there was no notable change in sulphate derived metabolite recovery in this patient throughout the study or in their group (Figure 3.8-36).

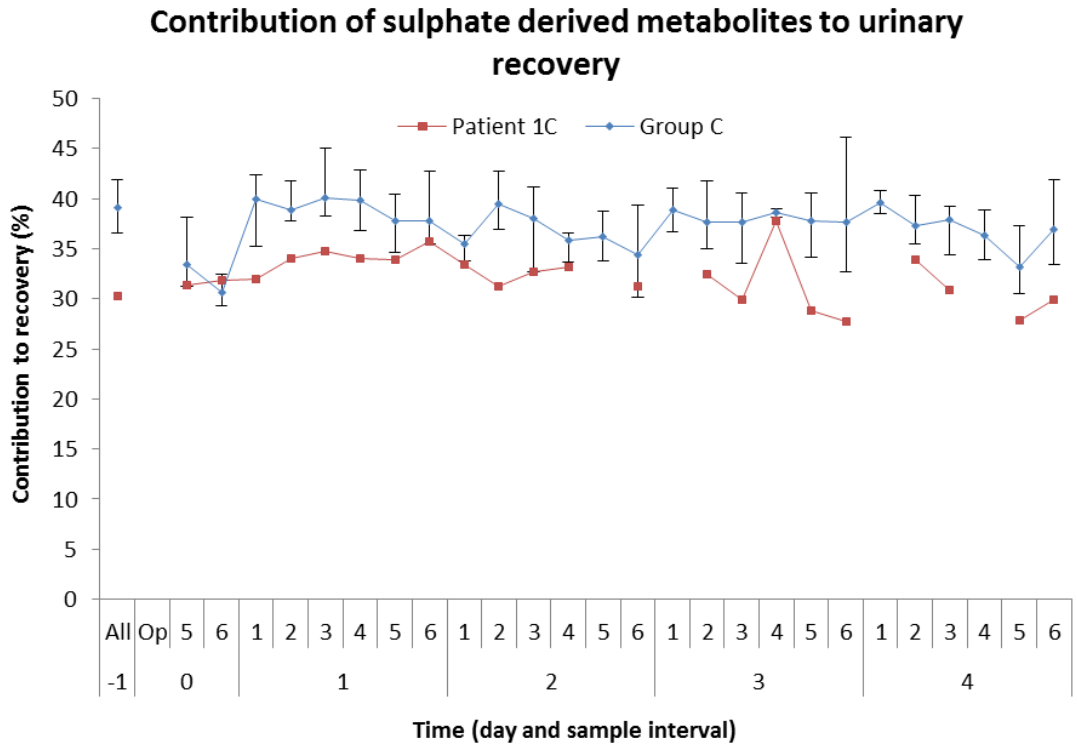


Figure 3.8-36 Per cent of urinary metabolites excreted as sulphate derived compounds with group median (±IQR)

3.8.3.5.2 PHASE II:I METABOLITE RATIO

The metabolic ratio recovered in the urine of this patient fell from preoperative values and remained at this level for the duration of the study (Figure 3.8-37). This was consistent with the lack of change to cytokine concentration and sulphate contribution to recovery.

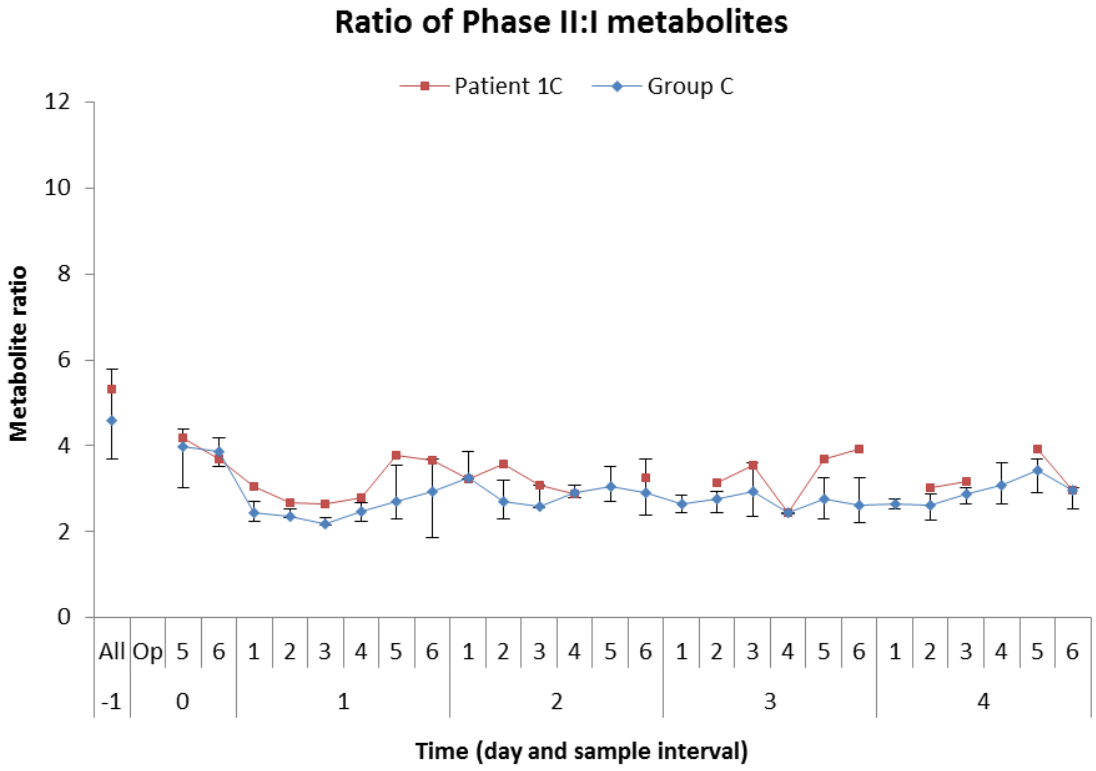


Figure 3.8-37 Ratio of Phase II: Phase I metabolites of paracetamol with group median (±IQR)

3.8.4 PATIENT 8D

Patient 8D was a 65 year retired factory worker. He was scheduled for a laparoscopic low anterior resection to remove a circumferential low rectal cancer. He reported smoking 15-20 cigarettes a day, with a 30 pack year history, and drinking 10 units of alcohol per week. He had a BMI of 27.7kg/m^2 and with no comorbidities was graded as ASA 1.

He was admitted and consented to this research the day prior to surgery and sampling and dosing proceeded as per Section 1.2 that evening. The following day his surgery proceeded routinely taking 4.5 hours. As part of his anaesthesia he received a total of 375µg of fentanyl, 18mg vecuronium, 10mg morphine, 8mg dexamethasone, 8mg ondansetron, 2g of paracetamol and 75mg of diclofenac. He also received sevoflurane 1.5-1% and a total of 4.5L of IV fluids perioperatively. On return to the ward he had

patient-controlled analgesia containing fentanyl 500µg/50mL, of which he used 48mL on demand until 1500hrs of Day 3 when it was removed. He also received 1g paracetamol every six hours and twice daily 75mg diclofenac. He was prescribed 10mg oxycodone (immediate release) up to every four hours after the patient-controlled analgesia was removed but did not require it. There was no clinically significant change in laboratory values and pain remained well controlled, rising slightly when the patient-controlled analgesia was removed (Table 3.8-7).

Table 3.8-7 Pharmacodynamic monitoring values

Abbreviations used: SCr- serum creatinine; AST- aspartate aminotransferase; INR- international normalised ratio; NRS- numerical rating scale- measure of pain experienced in last 24hrs and at time of study..

	Day -1	Day 1	Day 2	Day 3	Day 4
SCr (µmol/L)	74	68	57	60	68
AST (U/L)	9	12	32	28	42
INR	1.0	1.1	1.0	1.0	1.0
NRS Best	0	0	0	0	0
NRS Worst	0	0	0	0	2
NRS Now	0	0	0	0	1

3.8.4.1 LIVER FUNCTION

The concentration of αGST in Patient 8D paralleled that of the group until Day 3 when it rose sharply to 9.4µg/L, still within the upper limit of normal of 11.4µg/L. AST values also remained lower until Day 3 when it rose gradually to finish at 42U/L, slightly above the upper limit normal (40U/L). Neither of these tests indicated clinically meaningful changes to liver function during the study, and the only changes that were observed occurred on the last two days of the study.

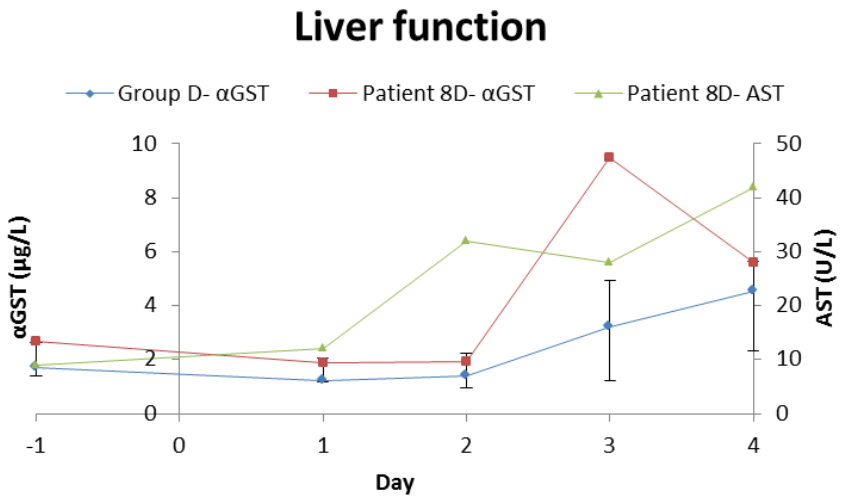


Figure 3.8-38 Liver function tests αGST and AST on each day of the study with group median αGST and IQR

3.8.4.2 PLASMA

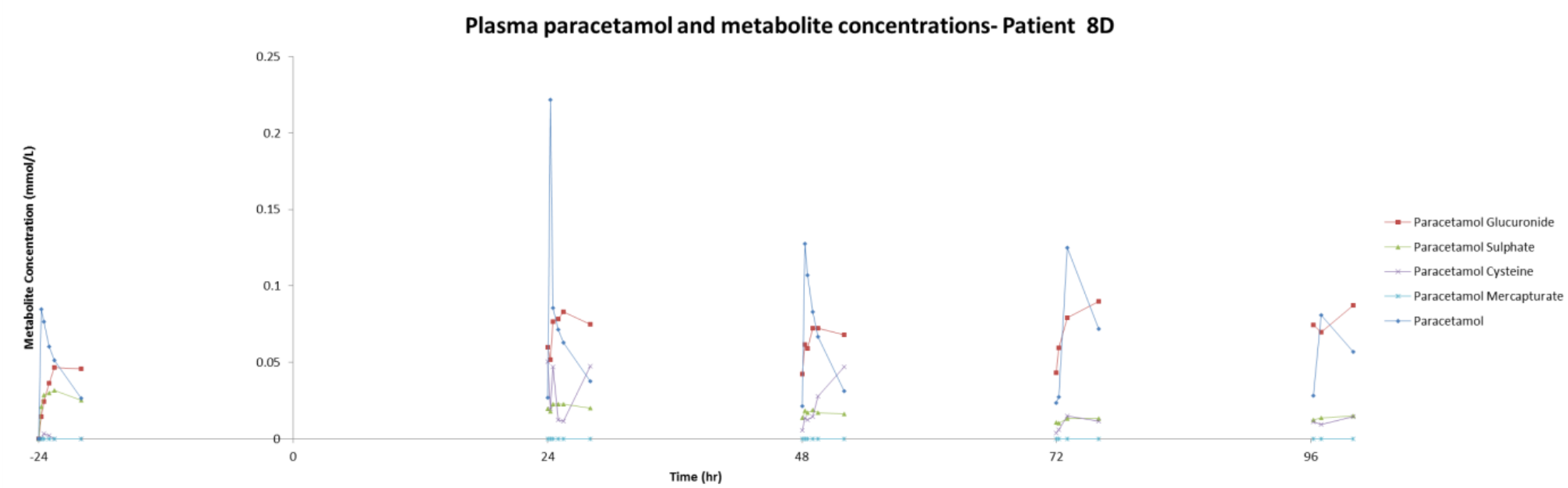


Figure 3.8-39 Concentration of paracetamol and its major metabolites in plasma

Plasma paracetamol concentrations were similar preoperatively to postoperatively although there was a large increase in the Cmax concentration on Day 1. Paracetamol sulphate values fell postoperatively and were matched by a rise in paracetamol cysteine. Paracetamol cysteine concentrations in plasma remained constant despite the increased CYP1A2 and CYP2E1 activity suggested by the changes to caffeine and chlorzoxazone metabolism. Paracetamol mercapturate failed to rise above the limit of detection and there was no evidence of accumulation in paracetamol glucuronide (Figure 3.8-39).

Kinetic values were altered following surgery with a reduction in half-life of over 10%. This reduction mainly arose from a reduction in Vd, although a slight increase in clearance was also seen. Almost the exact same reduction in Vd in this patient was seen in patient 1B who received the same surgery type, however in the case of .

Table 3.8-8 Plasma pharmacokinetics preoperatively and postoperatively with group medians (±IQR)

	Day -1		Day 1	
	Patient 8D	Group	Patient 8D	Group
t ½ (hr)	2.29	2.06 (1.82, 2.40)	1.99	1.94 (1.72, 2.28)
AUC (mg.hr/L)	0.042	0.042 (0.029, 0.054)	0.041	0.038 (0.036, 0.04)
Cl (L/hr)	23.82	24.09 (18.54, 34.87)	24.68	26.48 (25.09, 27.55)
Vd (L/kg)	0.93	0.87 (0.74, 1.14)	0.65	0.8 (0.58, 0.96)
MRT (hr)	3.22	3.04 (2.55, 3.38)	2.16	2.14 (1.91, 2.30)

There was no evidence of accumulation of paracetamol until Day 3 when both one and four hour concentrations increased. This was different from that of his group which showed no evidence of accumulation.

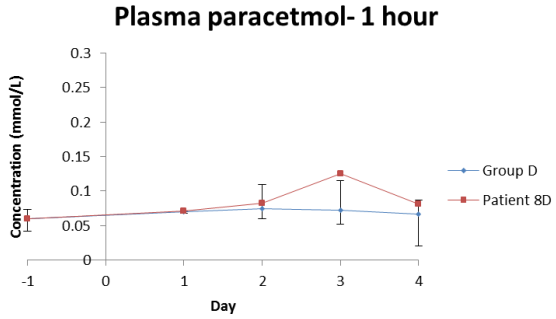


Figure 3.8-40 Plasma concentration of paracetamol- 1hr

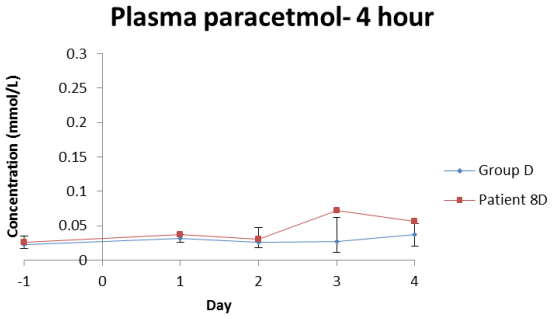


Figure 3.8-41 Plasma concentration of paracetamol- 4hr

3.8.4.3 CYTOKINES

Changes to cytokine levels for Patient 8D were also largely unremarkable. There was a slight increase in IL-6 (Figure 3.8-42), IFN-γ (Figure 3.8-43) and TNF-α (Figure 3.8-44) but no change to IL-1β (Figure 3.8-45).

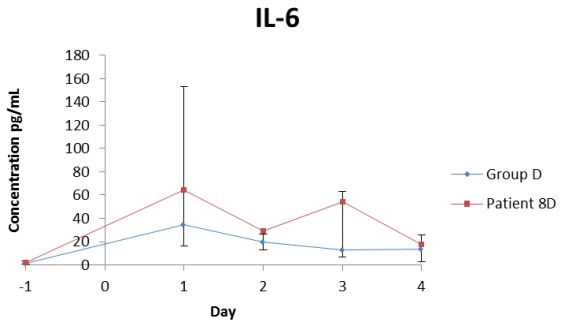


Figure 3.8-42 IL-6 Concentration in daily samples

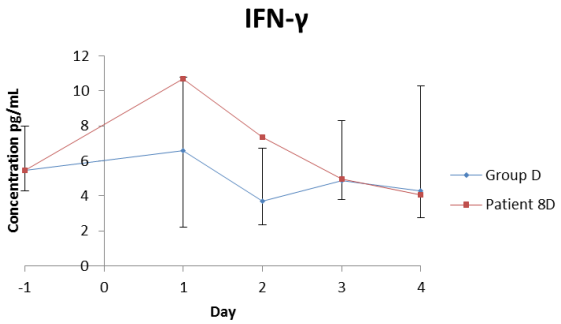


Figure 3.8-43 IFN-γ Concentration in daily samples

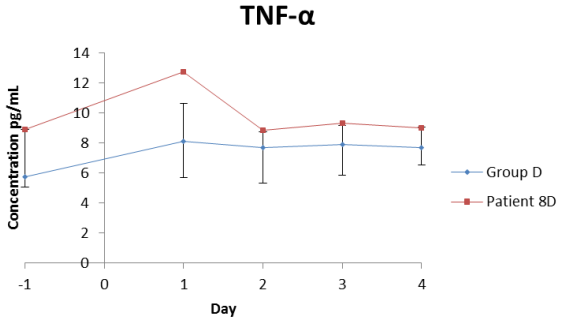


Figure 3.8-44 TNF-α Concentration in daily samples

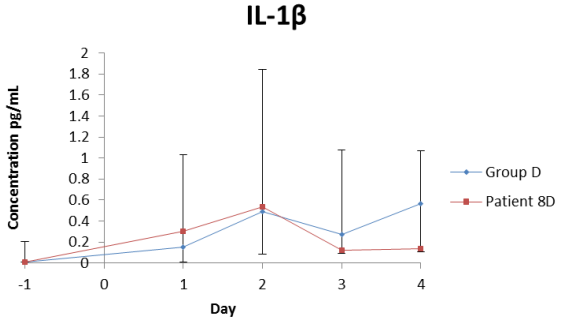


Figure 3.8-45 IL-1β Concentration in daily samples

3.8.4.4 CYP450 PROBES

There was a strong increase in the ratio of paraxanthine to caffeine in the final days of the study (Figure 3.8-46) and the opposite was seen the chlorzoxazone ratio (Figure 3.8-47). The chlorzoxazone ratio opposed that of his group.

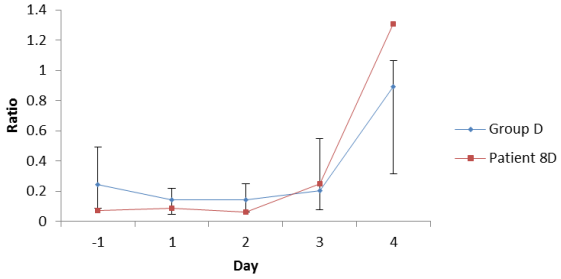


Figure 3.8-46 Individual and group median ratio and IQR of paraxanthine to caffeinein plasma

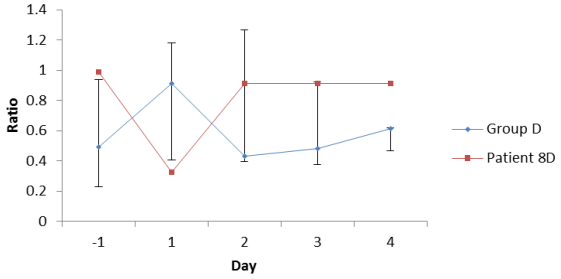


Figure 3.8-47 Individual and group median ratio and IQR of 6-hydroxychlorzoxazone to chlorzoxazone in plasma

3.8.4.5 URINE

3.8.4.5.1 SULPHATE CONTRIBUTION

There was a slight reduction in sulphate derived metabolite contribution immediately postoperatively which briefly recovered. Otherwise there were only minor changes to this value (Figure 3.8-48).

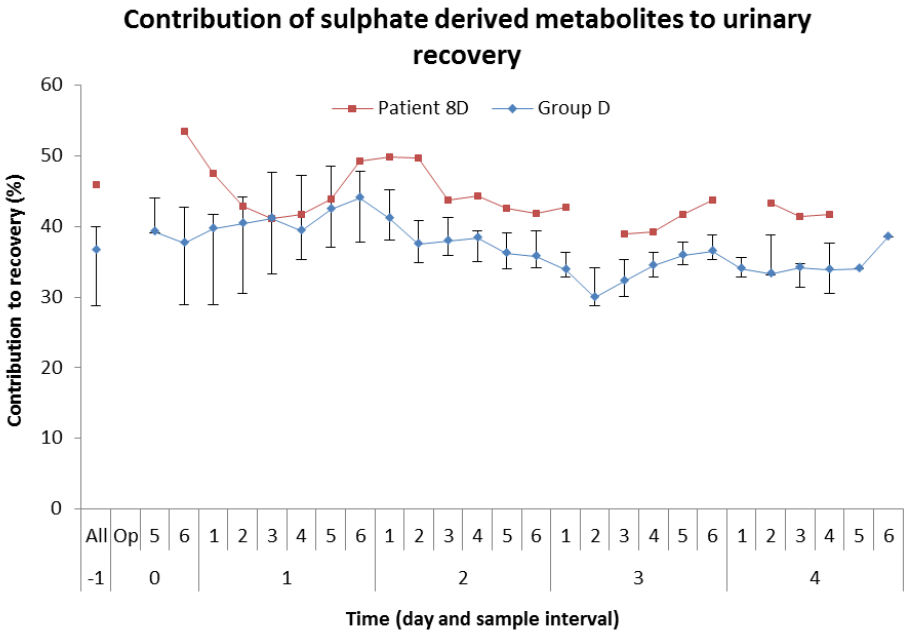


Figure 3.8-48 Per cent of urinary metabolites excreted as sulphate derived compounds with group median (±IQR)

3.8.4.5.2 PHASE II:I METABOLITE RATIO

Figure 3.8-49 shows Patient 8D's metabolite ratio started lower than the rest of his group but still fell further following surgery. This was maintained for the remainder of the of the study period.

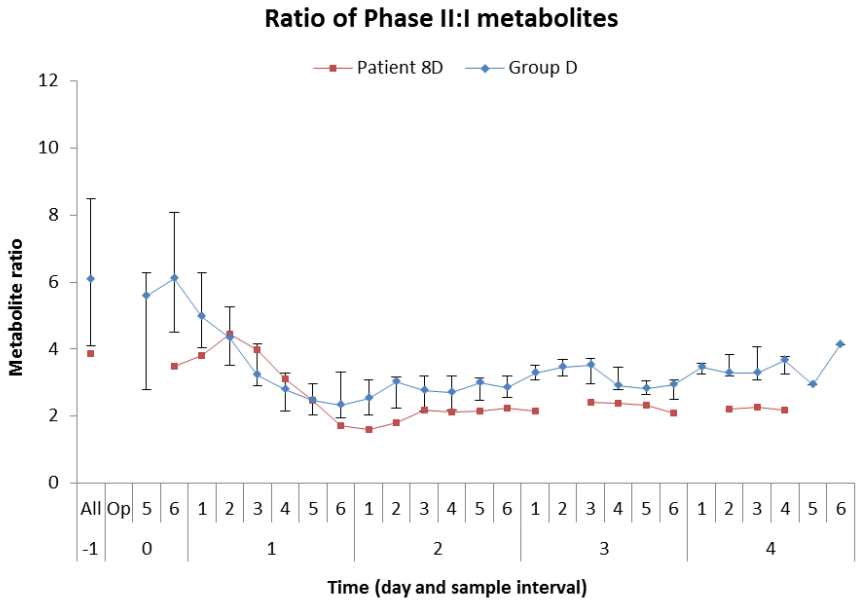


Figure 3.8-49 Ratio of Phase II: Phase I metabolites of paracetamol with group median (±IQR)

Similar, but to a lesser extent that Patient 4A, this ratio arose from increases to paracetamol cysteine contribution in the absence of increases to paracetamol glucuronide. Increases to paracetamol cysteine contributions did not align with either measured CYP450 activity or cytokine concentrations. Paracetamol glucuronide concentrations still remained within expected ranges.

4 DISCUSSION

This study investigated the safety of high doses of IV paracetamol in postoperative patients. There were several factors that resulted in this research:

- Paracetamol had re-emerged as a popular drug perioperatively and, despite it being toxic on overdose, doses exceeding those licensed were being advocated;
- Paracetamol had been used safely for decades in postoperative patients when licensed doses were given by oral or rectal routes. However, the bioavailability of these routes was known to be poor in surgical patients; and
- A new IV formulation of paracetamol became available, however the impact of its greater bioavailability on drug safety in surgical patients was unknown.

These factors, the increased doses and increased bioavailability, were a cause for concern given the toxicity of paracetamol on overdose. However, the safety of IV paracetamol in postoperative patients had been assumed by health-professionals for two reasons:

1. The active ingredient is one of the most consumed, safest drugs on the market when given at licensed doses (Amar *et al.* 2007); and
2. This safety can be applied to the IV formulation because many aspects have been shown to be bioequivalent to enteral products in healthy adults (Depre *et al.* 1992; Flouvat *et al.* 2004; Duggan *et al.* 2009).

Subsequently, the product has been widely used and well tolerated by the vast majority of surgical patients. However, there were a growing number of reports of individuals who have developed toxicity after receiving licensed doses, and malnourishment seems to be a common theme (Vitols 2003; Kaplowitz 2004; Moling *et al.* 2006; Forget *et al.* 2009; Gray *et al.* 2011). A recent determination as to the causes of death of a hospital patient who died following repeated IV paracetamol doses found a “prevailing culture of assumed familiarity” with intravenous paracetamol “…derived from the common use of oral paracetamol”. This “misplaced assumed familiarity” was listed as a cause death (Sheriffdom of Glasgow and Strathkelvin 2011).

Drug safety shown in healthy adults cannot always be applied to surgical patients. As discussed in Section 1.3 there are several differences between these two populations that affect drug disposition. In this specific case, IV and oral paracetamol are far from bioequivalent. In surgical patients the rate and extent of oral absorption of paracetamol is

significantly reduced (Goldhill *et al.* 1995; Kennedy *et al.* 2006; Duggan *et al.* 2009) and overcoming this with IV paracetamol is considered of therapeutic benefit and sufficient to justify its use (Sinatra *et al.* 2005; Gregoire *et al.* 2007; Candiotti *et al.* 2010; van der Westhuizen *et al.* 2011). This leads to substantial differences between the pharmacokinetic profiles of oral and IV paracetamol with a doubling of Cmax and plasma concentrations not equivalent until at least an hour after the dose (Depre *et al.* 1992). It could be possible that this delayed and reduced oral absorption conferred protection from paracetamol toxicity to vulnerable surgical patients, however, just as with efficacy, whether the link with toxicity is between Cmax or AUC is not certain. This would suggest that the IV formulation has hidden risks associated with its different pharmacokinetics and therefore safety established with oral paracetamol doses could not realistically be relied upon.

These concerns and potential issues highlighted above led to the research questions of this clinical project:

1. Was IV paracetamol safe in surgical patients at unlicensed doses?
2. Did surgery change paracetamol disposition?
3. If disposition changed, why?

To answer these, patients scheduled for surgery volunteered to provide blood and urine samples and to receive up to 9.5g of paracetamol daily and daily doses of CYP450 enzyme probes for the day before and the four days after surgery. To analyse these samples, analytical tools were developed and applied to measure clinical and pharmacokinetic observations and address the research questions:

1. αGST concentrations were measured in daily plasma samples to assess safety of paracetamol;
2. Paracetamol pharmacokinetics and metabolite concentrations were determined from plasma and urine samples to assess changes to disposition; and
3. Cytokine concentrations and CYP450 activity were measured in plasma samples to determine why disposition changed.

Once these methods were established and validated analysis of samples collected from patients was carried out. From these, interpretation of parameters became possible, with conclusions finally drawn.

This discussion will be divided into four parts:

1. The development and establishment of the assays;
2. Recruitment and conduct of the study;
3. The disposition of parenteral paracetamol and its metabolites in the various surgical groups and the factors affecting disposition; and finally
4. The strengths, limitations and of future work of the research

4.1 DEVELOPMENT AND ESTABLISHMENT OF THE ASSAYS

4.1.1 PARACETAMOL ANALYSIS- HPLC

The assay used for plasma analysis was adapted from previous reports while a second was developed with novel conditions for urine analysis. Both assays performed well and both surpassed accepted validation standards.

Challenges which presented with the development and application of the assays were of three categories: relating to either sample, mobile phase and run time.

The plasma sample preparation following the method of Reith *et al.* (Section 2.3.3.4) was selected and optimised for this research as it was quick and simple, favourable qualities given the large numbers of samples that needed to be processed (Reith *et al.* 2009). However, by some standards, the prepared sample would have been regarded as relatively "dirty" (Chen *et al.* 2008). Small quantities of contaminant (mostly protein precipitate) were occasionally present in the sample. With the large number of samples analysed, over time, these contaminants accumulated in the guard column and increased back pressure. This collection at the head of the column in turn shifted retention times and caused problems with peak shape and integration. Replacing the guard column cartridge resolved these problems and as long as it was maintained, this facilitated the safe use of this simple and rapid sample preparation method without compromising accuracy.

The mobile phase components of the first assay detailed in Section 2.2.3.1, potassium phosphate buffer and acetonitrile, are considered incompatible in certain conditions (Kromidas 2005). In this assay, precipitation of buffer crystals caused blockages of check values and failure of the HPLC pump. Once the source of the problem was identified, a warmed methanolic solution was used as a wash at the end of the run and the gradient of mobile phase changes between the phosphate buffer and acetonitrile were reduced. The final details of the mobile phase gradient and wash solution are also given in Section 2.2.3.1. This eliminated the pump problems as well as protected the HPLC and the HPLC column from the corrosive effects of the phosphate buffer.

The run time of the method adapted from Reith *et al.* was 25 minutes (Section 2.2.3.1). While reasonable in many settings, the length of this run time meant that a set of samples from a patient would run over several days. Additionally, the detection of paracetamol

mercapturate was poor in the first assay. It was therefore desirable to reduce the run time and improve the detection. This led to the acquisition of another column and redefining the chromatographic conditions from the beginning to develop an isocratic assay with phosphate buffer and methanol. A valuable lesson was learnt though, that it can take longer to do some things quickly, and even though there was the advantage of a shorter run time, this benefit was lost in the additional time it took to determine new conditions and revalidate the assay. However the additional benefit of improved detection of paracetamol mercapturate did justify the time spent on the development of the new assay.

4.1.2 LCMS METHODS

Changes to the disposition of drugs in surgical patients in the first instance, and elderly surgical patients in the second instance, is a much under researched area. Because CYP450 systems and Phase II systems alter quickly and extensively, it is important to be able to quantify those changes to identify those at risk from subsequent inefficacy and toxicity. This is especially relevant given that an excess or a lack of drugs in a patient who is haemodynamically unstable perioperatively can have effects on their postoperative outcome (Kennedy *et al.* 2000).

In doing clinical research, the maxim of *primum non nocere* dictates that minimising discomfort and harm to the patient is the primary objective. Therefore, research undertaken in patients must not interfere with their recovery. Assay's that are developed to support this objective are not only a great advantage to the patient but also to other clinical researchers who want to do this type of research. While determining the activity of the relevant CYP450 was necessary, methods in the literature commonly used one assay per drug, in a similar approach to the analysis of paracetamol described above. Adapting this technique for the analysis of the four CYP450 probe drugs and their metabolites would have necessitated prohibitively large amounts of blood to be taken from patients who had had major blood loss from surgery and difficulty with IV lines and venous access postoperatively.

To avoid this, an assay was developed with novel conditions on HPLC using both UV and fluorescence detection that separated five analytical compounds which, unlike the paracetamol assay, were of a very heterogeneous nature with different pKa, solubility's and functional groups that complicated their extraction, separation and detection. The

difficulty of this assay was further increased by the need to detect the metabolite of each of four of these analytical compounds, which were of very similar nature to their parent. As the ratio of these two entities was the focus of this analysis, their clear separation and peak resolution needed to be obtained, but in conditions that achieved separation of the parent compounds, which were very different in nature. Additional complications arose for the glucuronidation of some of the probe metabolites that required the sample to be hydrolysed with enzyme incubation prior to analysis. This and each of the eight remaining steps in extraction were optimised as shown in Table 2.5-3.

The development of this assay was very positive and proved very successful in terms of the extent and reliability of both extraction and separation methods. All compounds were extracted from one sample, thereby reducing the amount of blood sample required. Extraction was consistently around 80% for all compounds of interest and separation of all nine compounds was achieved in a 25 minute run. However, when the method was transferred to patient samples, it was limited by its inadequate detection of midazolam, which was anticipated given the low doses administered to patients. An LCMS assay was an attractive alternative as this instrument provides a much lower limit of detection. Similar methods were published for the simultaneous detection of CYP450 probe drugs using LCMS but none contained the same cocktail used here. The transfer of the HPLC to LCMS method was complicated by the use of a buffer and column that were not compatible with the LCMS instrument.

Transfer to LCMS analysis encountered additional, unexpected challenges which led to the accuracy of this assay being compromised. As a result, the information on the CYP450 analysis in the level of detail that was anticipated was not able to be retrieved, although from an educational point of view it was extremely useful and had provided much valuable data. The factors which compromised the transfer of this assay were:

1. Changes to supervision of the LCMS;
2. Access to the LCMS; and
3. Problems with the instrument.

The personnel changes for the supervision of the LCMS, which occurred at the time of transfer of the assay led to a loss of expertise in the operation of the equipment, making transfer of the assay to this instrument more of a challenge.

Access to a LCMS instrument was not available in the School of Pharmacy and therefore access to such technology was sought from another department. As a user outside of the parent department owning the instrument, access was only possible for a limited period of time for which this the researcher remains most grateful. This situation though limits the time available to transfer and develop the assay especially in studies where there are large numbers which must be processed.

Given the use of this type of analytical equipment is central to the work of many disciplines, other Universities have overcome access, maintenance and supervision issues and prevented duplication of resources by introducing a network of Platform Technologies. These networks are essentially a central location in the University that hosts the analytical equipment for the use of the entire University and industry community. Such a network is present in Monash University, Australia, who saw benefits in creating *"a one-stop first class technology shop, with access to cutting edge equipment, and a critical mass of leading scientists."* (Monash University 2010). Such a network is currently under development in UCC for researchers whose own departments do not possess the analytical equipment required for their analysis and will be beneficial for this type of research in the future.

There were several problems with the LCMS instrument which were compounded by the two factors already described. Peak shape from the MS was poor and abundance was very low, even in standard samples prepared in mobile phase. As discussed in the Section 3.7, at concentrations sufficient to be detected by UV, the PDA upstream from the LCMS produced Gaussian peaks (Figure 4.1-1), whereas the peaks from the same injection on the MS's TIC were splintered and asymmetrical (Figure 4.1-2). The integration software was capable of smoothing over misshapen peaks, but the calibration curves constructed from the resultant area under the curve of these peaks were poor, with few points along the line of best fit and large changes in area on repeated injection of the same sample.

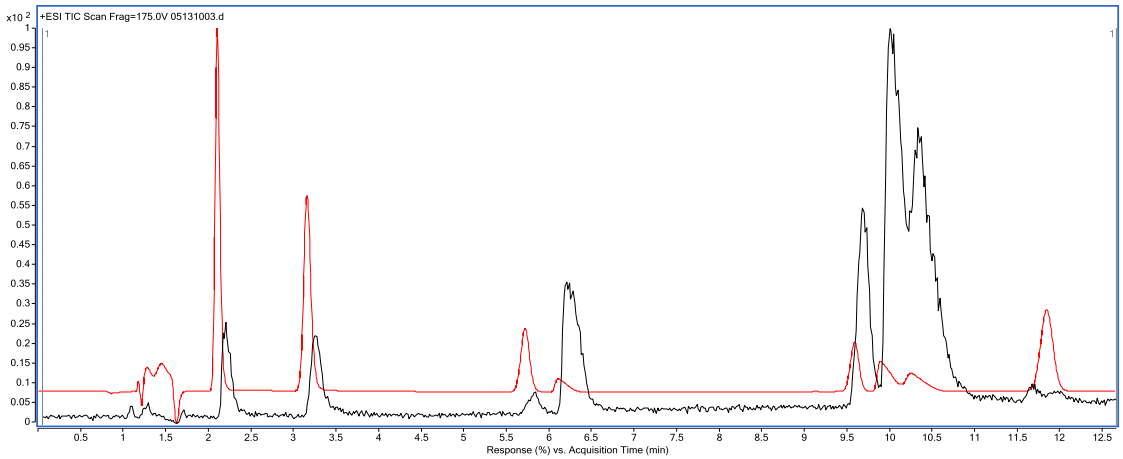


Figure 4.1-1 UV 280nm (red line), and Total Ion Chromatogram (black line) from same injection of 1ug/mL standard.

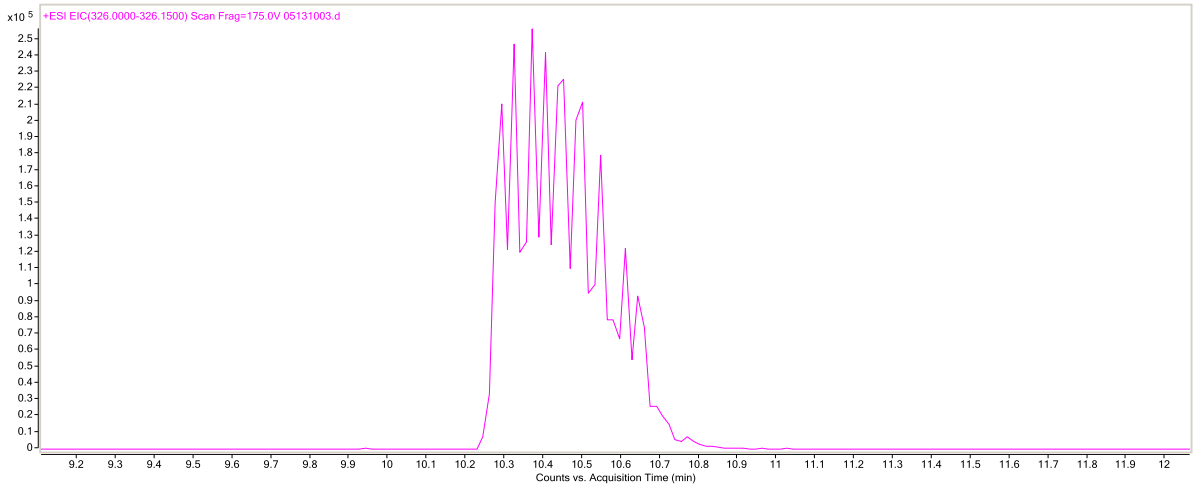


Figure 4.1-2 Extracted Ion Chromatogram (EIC) 326-326.15 (2.5x10^5 abundance) (midazolam)

Much work was done to try to overcome these problems. The symmetry of the PDA peak indicated the problem was not with the sample or chromatography, and this was confirmed firstly by injection of other freshly prepared standards, and subsequently by using a new column of the same specifications. The parameters of the MS were all confirmed and optimised (Section 2.5.3.5) and the ion source was cleaned. During this time it was noticed the calibrant liquid was not being drawn into the ion source and there were problems with the drying of the electrospray plume which were causing condensation on the inside of the ESI unit. Attempts at resolving both these issues were made and thought to be resolved. At this stage there was only enough time left in the period of access to the instrument for it to run the remaining samples. A set of standards were run which appeared no different so the remaining samples were analysed. Further attempts at resolving the ESI problems revealed a piece of lint/fluff across the entrance to the capillary (Figure 4.1-3). Once removed, detector response did improve. It was concluded this was the cause of the low and irregular detector response previously discussed.

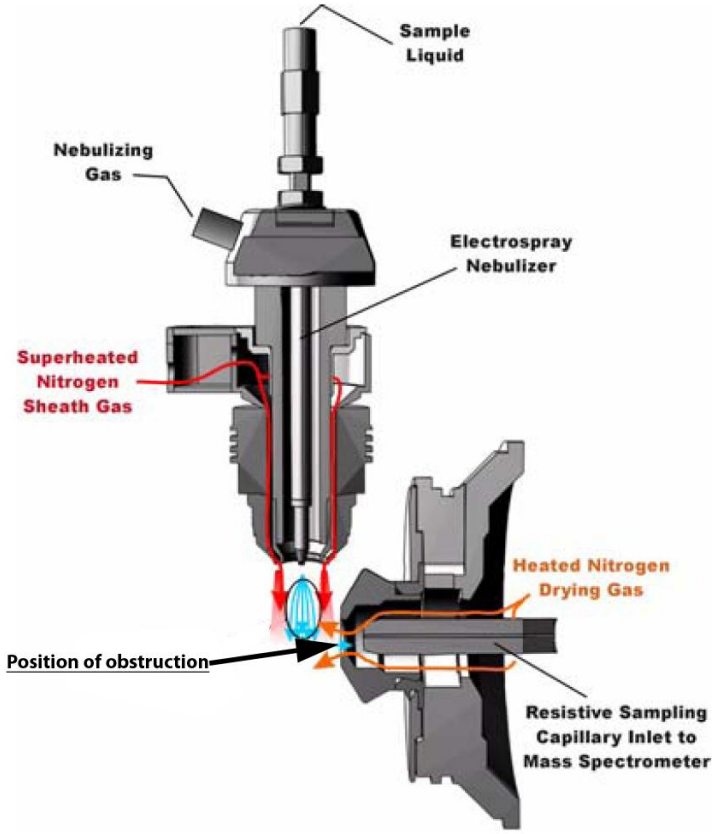


Figure 4.1-3 Electrospray Ion Source of Agilent 6520 mass spectrometer showing position of obstruction
(Agilent Technologies Inc 2009) Used with permission.

Throughout the use of the LCMS there were problems with the hydrogen gas sensor in the laboratory where the LCMS was situated. The alarm frequently detected hydrogen despite there being no source of hydrogen in the laboratory. Upon sensing hydrogen, the alarm system would shut down the supply of all the gases to the laboratory, including nitrogen, and without a supply of nitrogen the MS would shut down, ending the sample set prematurely. Aside from ending the sample set, further samples could not be analysed until gas had been restored and the MS recalibrated. Gas could not be restored to the laboratory until the system was reset, which could only be done by the system's engineers, who were external to the university and unavailable over the weekend. On three separate occasions the system alarmed on a Friday night and as the access period required weekend work, this was a significant set-back.

While delaying the analysis of further samples, the MS shutting down during the run also raised issues regarding the stability of the un-analysed samples sitting in the LC awaiting analysis. This was exacerbated by the LC not having a refrigerated sample drawer. The samples were loaded in the evening and the sample run overnight. On each occasion when the instrument shut down the samples would be removed and refrigerated immediately upon discovery in the morning. Extreme care was taken not to confuse

samples. Samples that had run successfully were re-run to examine for degradation and no significant change in abundance was observed.

There was a further problem around the negative and positive detection mode of the instrument. The MS (an Agilent 6510 QTOF) is a powerful instrument for the determination of accurate mass, capable of giving molecular weights to beyond four decimal places at femtogram concentrations; abilities far beyond the requirements for this analysis, given that the masses of the analytes were known. MS can work in either positive or negative mode. The signal-to-noise (S/N) ratio of an analyte will be greater in one mode or the other and this is just a property of the analyte. The vast majority of compounds are best detected in positive mode and this was the case for all analytes in this analysis, except for two: chlorzoxazone and its 6-hydroxy metabolite. Although the height of the negative-mode peaks can be one-tenth those of the equivalent positive-mode peaks, there is less noise in negative mode so signal peaks are more distinct.

Many MS instruments can detect in both positive and negative mode simultaneously, but this newer technology is mostly available in nominal mass instruments, unlike the accurate mass Agilent 6510 being used. Upon discussing the capabilities of the MS being used with the manufacturer before the analysis began, they provided assurance that detection of both positive and negative modes was possible with this instrument too, but when this option was not available in the software it was revealed that this particular instrument was, in fact, not capable of this function. In this way the extended capabilities of this instrument's accuracy was its downfall in this analysis, preventing simultaneous detection of positive and negative modes. Switching between negative and positive mode on the Agilent 6510 required the analysis to stop and the instrument to be recalibrated with a different calibrant solution from the one used for positive mode. This process cannot be automated and would have required significantly longer periods on the instrument and access to the instrument throughout the night. As can be seen from the results (Section 3.7), detection of chlorzoxazone and its 6-hydroxy metabolite in positive mode was still possible but in very low abundance, which is contrary to what would be expected given that it was administered in the highest dose of all the CYP450 probe drugs (Section 2.1), and a satisfactory extraction was achieved. Ideally the sample would be run in positive and then negative mode. This would have required frequent changing of the calibrant and recalibration of the instrument, which the researcher in control of the MS did not sanction. Additionally, because of the necessity for recalibration, the samples

could not have been analysed in positive and negative mode consecutively. This effectively would have doubled the sample load on the instrument and run times would have gone beyond the allocated access time for this project. It also would have resulted in the samples sitting in the sample drawer at room temperature for twice as long, as they waited to be re-run in negative mode. Further, the volume of the sample following extraction was not always sufficient to allow for two injections and the volume of the plasma sample was not always sufficient for duplicate samples. When these factors were considered in addition to the time constraints, the only option was to run the samples in positive mode in expectation that the anticipated high concentrations of chlorzoxazone and its 6-hydroxy metabolite in the samples would be sufficient to overcome the problems with their detection in positive mode.

The final product of the extraction process was filtered before injection in the LCMS. The researcher supervising the LCMS did not believe a guard column would be sufficient to protect the MS from any contaminant remaining in the sample, even though such methods are reported (Kharasch *et al.* 2004) and there were no changes to backpressure on the HPLC used for method development. This meant that the samples could not be filtered "on-line" with a guard column and that a separate filtering step was necessary. There were some limitations to the type of filtration that could be used. Namely, the product of the final evaporation was reconstituted in 50% methanol, which was not compatible with a number of filters typically used for clean-up of samples before LCMS injection. Further, there was a limited budget and over 600 samples to filter.

When choosing the product, the technical assistance of two suppliers both referred onto a manufacturer, Millipore (Massachusetts, USA). Millipore recommended a 96 well plate format filter that was subsequently purchased. The additional apparatus for the operation of the filter, a vacuum manifold and a vacuum pump, were to be lent by Millipore, this loan did not materialise due to the vacuum pump been irreparably damaged by the previous user imediately prior to its use in this work. No other vacuum pump was available from within the department for the period of time it was required. A water vacuum was used in its place which produced a vacuum just at the lowest end of the usable range specified by the manufacturer. Given the low vacuum, filtration was slow and recoveries were very poor, with 100µL of sample yielding approximately 10-20µL of filtrate, with a small amount of the sample remaining unfiltered but the majority lost to evaporation. This introduced variation arising from the evaporation of the sample,

in addition to a volume that would not pass through the filter at the vacuum used. Attempts to improve the yield included pre-wetting the filter with the methanolic solution used to reconstitute the sample following evaporation, occluding unused wells with adhesive plate films and centrifuging the filtration system in place of using a vacuum, none of which caused significant improvements to the yield. As there was no budget for an alternative filtration product this process was deemed adequate as, with careful handling, there was sufficient volume filtered for a single injection into the LCMS and the internal standard incorporated at the beginning of the extraction could be used to account for any changes due to evaporation of the sample during filtration.

Despite these problems, the extraction procedure was very successful in terms of recovery and developing the method provided much useful experience and knowledge. As discussed above, the problems mainly arose with this method in the detection of the compounds subsequent to their successful extraction from plasma. With additional time on the LCMS this method could be validated, however insufficient patient plasma would prevent its application to this project.

4.2 RECRUITMENT AND CONDUCT OF THE STUDY

4.2.1 SETTING

For the first two groups of patients, the setting of Hospital 1 was as close to ideal as possible for this type of research. Groups A and B patients returned from theatre with a triple lumen central line and urinary catheter to an ICU with one-to-one nursing. Blood and urine samples were obtained on time with comparative ease. In a small hospital, it was possible to establish personal relationships with the various staff and they were generally supportive of the research and would offer help when it was not widely outside their usual practices *i.e.* emptying urinary catheter bags into collection vessel as opposed to into the sluice. Other than this relatively small amount of help, these groups required no other assistance for conducting the study in the postoperative phase at all.

The major drawback of Hospital 1 was that recruitment into the study was slow. Fewer than one colectomy was performed a week and many of these patients were unfit for inclusion. It was difficult finding patients sick enough to require major surgery, but well enough to be involved in the research. Of those eligible, the consent rate of 70% of eligible patients was good, considering the research was demanding for the patient. Similarly high rates have been reported elsewhere in Irish populations (Desmond *et al.* 2011), which are higher than found in other populations (Myles *et al.* 1999; Dorantes *et al.* 2000). Ideal situations for consenting patients for this type of study have been determined by patient surveys as:

1. At preadmission testing;
2. With enough time for patients to consult with their own surgeon, who endorses the research;
3. In private, when the patient is in street clothes
4. With assurances the investigator would also consent if eligible (Mingus *et al.* 1996).

The evening prior to surgery, after being recently diagnosed with potentially life threatening cancer, is a difficult time for anybody without the addition of being asked to consent to a clinical trial. The difficulties in consenting for clinical research at this time have been previously reported and are in part due to the shorter hospital stays that are demanded by economic pressures (Mingus *et al.* 1996). Consequently, the four aspects

listed above were often unobtainable. Frequently, preadmission testing did not occur as the cases were urgent, so consenting was done at the time of admission. This prevented patients having enough time to consult with their own physician and also meant the patient was on the ward and often in a hospital gown. Despite these issues, there was an overwhelming spirit of good will, altruism and willingness to participate in Groups A and B. The most significant hurdle to recruitment was not the chance of being administered large doses of paracetamol or frequent blood tests, but the necessity to abstain from caffeinated drinks. Here a lack of cultural understanding on the researcher's part came into play because it became clear that asking Irish adults to abstain from tea for a week was extremely difficult given that tea is such an integral part of everyday life in this culture. Despite this, in the first two groups of patients, three made totally unsolicited offers to write letters in support of the study to any potential study patient, commending participation in the study to them.

There was no apparent difference between consenters and non-consenters in this study, although other work has shown that the most important influences on consent rate include: age of the patient (those >60 years being *more* likely to consent), trust in the investigator and trust in the institution/hospital (Patel *et al.* 2004). Prospective study participants who were less trusting of or assured by study personnel or who *"felt like a guinea pig"* were shown by Patel *et al.* to be less likely to participate.

Conducting the study became more difficult in the third and fourth groups (Groups C and D). Group C, the mastectomy patients, were frequently post chemo/radiotherapy. Patients consented to an external jugular line being inserted in theatre as a central line could not be justified. However, even these lines would frequently occlude. This lead to a number of missed samples. The external jugular lines proved less effective than peripheral lines and were very positional for obtaining blood samples. Most had occluded by the second day, if not before. Additionally, urinary catheters were removed promptly after surgery and many patients had trouble collecting their own urine postoperatively. It was also not possible to ensure the bladder had been emptied every four hours, which could be assumed while catheterised. Another problem was that, when the IV catheter that was in place occluded, the maintenance of IV access beyond the first postoperative day was difficult to justify to the surgeon as it was only for the purposes of this research. No IV antibiotics were prescribed to these patients and IV fluids did not continue beyond the first postoperative day. With the frequent occlusion of lines and trouble reinserting

them, few patients had complete data sets. As a result of these sampling problems, this group was abandoned.

Reconfiguration of surgical services in Hospital 1 necessitated moving to a second site (Hospital 2) where Group D was collected. At Hospital 2, the Investigator was there as a researcher external to the hospital, compared with Hospital 1, where the Investigator was a member of staff with clinical duties.

4.2.1.1 ADDITIONAL CHALLENGES EXPERIENCED AT HOSPITAL 2

Clinical research, whilst highly rewarding, presents many obstacles which have to be overcome. It became clear on transferring to the second hospital that a large part of those obstacles is actually building relationships with those who are involved on a day to day basis with the patients. This is especially difficult, for instance, when there is shift work involved and frequent changes of personnel that occur in large institutions.

Irregular contact with ward staff meant that they may not be as familiar with the study protocol as would be desired. While the Investigator was primarily responsible for the conduct of the study, the assistance of many individuals was required to varying degrees for the smooth running of the study. At Hospital 1, the necessary relationships had been built up over time because the Investigator was a member of staff. At Hospital 2 the key relationships that needed to be re-established were with:

- Ward management;
- Nursing staff;
- Ancillary staff/healthcare assistants;
- Admissions co-ordinators;
- Medical records;
- Surgical teams; and
- Anaesthetists.

In most cases “assistance” meant not disturbing the conduct of the study and adhering to study protocols, which for the staff part, would be considered standard care. Education was conducted with all ward staff, but due to the size of Hospital 2, there were frequent changes to nursing staff and it was not possible to ensure all staff coming in contact with study patients had received training.

Additionally, staff may not have fully realised the ramifications of their actions to the validity of the study, *i.e.* disposing of a urine collection or inaccurate recording of time of paracetamol dose administration. In Hospital 1, as it was much smaller, it was possible to explain the reasons for a request in the study protocol to all members of staff. In Hospital 2, study patients were admitted to one of three surgical wards, greater than tripling the number of staff involved and on occasion they were outliers on medical wards.

Following their surgery, study patients in Hospital 2 returned to a six bed ward. Here they were cared for by one nurse and one healthcare assistant, who also had responsibility for another room of equal size. In comparison, Hospital 1 returned bowel resection patients to an ICU with one to one nursing. With the additional demands on staff in Hospital 2, the timing of paracetamol administration outside of those given by the Investigator was less certain. There were no paracetamol doses that were recorded as being administered outside of their correct time and this was assumed for the purposes of these results. However, conduct incidentally noted on the ward in relation to the administration of other drugs indicated that this may not have always been the case.

There is no evidence to confirm, but it was presumed by the Investigator, that having capacity as a member of staff with other clinical duties improved adherence to the protocol by other staff because the Investigator would have had: more incidental presence on the ward; been better known to staff; and had the support of a department of other pharmacy staff who could have assisted and alerted the Investigator to issues involving the study. Additionally, the Investigator being familiar to the other staff and part of the normal preoperative routine (in the Investigator's capacity as a pain team member) may have improved the consent rate in Hospital 1, which was much lower in Hospital 2. These presumptions are supported by the findings of Patel *et al.* discussed above that relate a patients likelihood to consent to their trust in study personnel and the host institution (Patel *et al.* 2004).

Anaesthetists at Hospital 2 used different anaesthetics from those used in Hospital 1 for the same surgery and they generally refused to put in central lines. Only two study patients in Hospital 2 had central lines and both were removed before the conclusion of the study. In addition, the Surgeon at Hospital 2 used different surgical techniques, with the majority being laparoscopic bowel resections. He also encouraged early re-feeding and the use of high protein drinks after surgery, which reduced the time of fasting experienced by patients in this group.

4.2.2 PATIENTS

Diagnoses were comparable within groups and between groups A, B and D, with the exception that Group D had some non-malignant indications for surgery. Procedures were also similar between Groups A and B, whereas the majority of the procedures for Group D were laparoscopic. This technique led to Group D having considerably longer duration of surgery.

There were a few patients who were withdrawn from the study, mostly as a result of loss of IV access for blood sampling. Only one patient was withdrawn as the result of a drug administered during the study, but upon review by the anaesthetist it was thought unlikely to be associated with the study.

4.3 CHANGES TO DISPOSITION

As shown in Table 3.4.1 there were alterations to half-life. Median increases were 0.6 and 0.2 hours in Group A and B respectively, while smaller reductions in Group C and D of 0.1 hours were seen in both groups. Half-life is the drug disposition term with the greatest clinical utility because of its tangible nature, however, on its own it describes very little about a drugs disposition. Equation 3.4-2 shows that it is a composite term comprising of both the Vd and clearance of a drug. Clearance itself arises from both a drugs metabolism (in paracetamol's case this is entirely hepatic) and elimination. These three factors: Vd, metabolism and elimination, provide the structure for the discussion of the changes to paracetamol's disposition in surgical patients. Absorption is not considered as paracetamol was administered parenterally.

4.3.1 DISTRIBUTION

Volume of distribution (Vd) was calculated using a non-compartmental method that is commonly used (Rowland *et al.* 1995; Allegaert *et al.* 2004; Liukas *et al.* 2011). As discussed in Section 1.4.4.2.2, the Vd of IV paracetamol is listed as approximately 0.9 L/kg (Flouvat *et al.* 2004). As shown in Section 3.4.4, preoperative volumes of distribution are largely in agreement with this figure aside from Group A, whose preoperative value was slightly lower at 0.77L/kg. At slightly more than 20% different from the reported population values, the difference in paracetamol distribution is substantial however, as a drug with intermediate distribution, the reduction is not clinically meaningful. Indeed, there are several other studies reviewed with similar and even lower volumes of distribution in man (Prescott 1996). Given the wide IQR shown in Table 3.4-75, Group A's reduced preoperative Vd is most likely due to the wide intra-individual variation seen in this value compounded by the use of medians, not means in the analysis. The mean Vd was closer to the listed value, but given the small group size, the use of parametric tests was not statistically warranted. There are two other factors that make Group A stand out from the remaining groups that could explain this slightly smaller distribution preoperatively: their higher age and prevalence of obesity as shown in Table 3.1.1. Age related changes to blood flow and extracellular fluid volumes have been shown to reduce drug Vd as discussed in Section 1.4.3 (Schwartz 2007; Liukas *et al.* 2011), however with a median age of 64 years, only 5 years greater than the closest other group, this explanation would seem unlikely. Secondly, with five out of the 10 in the group being

classified as obese, the finding of reduced weight corrected volumes may be applicable given that there are only four other obese in the entire study (Prescott 1980; Abernethy *et al.* 1982b; Depre *et al.* 1992; Ward *et al.* 1999). Furthermore, there was possibly some impact from the bowel preparations those patients received which may in turn have led to dehydration, but the impact of this on plasma volume has been reported to be negligible and if this was a factor it would also be seen in Group B who underwent the same treatment (Zafar *et al.* 1987; Kim *et al.* 2001; Kim *et al.* 2006).

When preoperative and postoperative Vds are compared there are stark changes. There were reductions to Vd observed in all groups which were statistically significant in Group A. The universality of these changes and their direction was surprising. Generally, increased volumes of distribution are reported in surgical patients, in response to increases to capillary leakiness and reductions in plasma proteins due to the stress response. However there are few studies in adults that report this for paracetamol and none reuse the same patient before and after surgery as has been done here (Schuitmaker *et al.* 1999; Viscusi *et al.* 2008; Fouad *et al.* 2009). Generally gentamicin is used for this purpose. As discussed in Section 1.3.3.3 gentamicin is poorly protein bound and highly lipophilic, making it ideal for the determination of extracellular fluid volume. In surgical patients, amongst vast inter-individual variation, gentamicin's distribution is generally greater than given for healthy adults (Zaske *et al.* 1980; Mann *et al.* 1987; Beckhouse *et al.* 1988; Dasta *et al.* 1988; Reed *et al.* 1989; van Dalen *et al.* 1990; Schuitmaker *et al.* 1999; Viscusi *et al.* 2008; Fouad *et al.* 2009). Additionally, the timing when distribution is estimated in these papers varies considerably, sampling intra- or immediately post-operatively, or not stating the time of sampling in relation to the patient's surgery at all. This has been reported to have a significant impact on the Vd due to changes in haemodynamics and the stress response that wax and wane as recovery progresses (Dasta *et al.* 1988). Furthermore, while generally surgical patients, many of the participant in these studies are critically ill and septic, much worse than the generally expected course. The effect of infection alone on Vd is much greater than routine surgical trauma and therefore the applicability here may be limited (Zaske *et al.* 1980; Mann *et al.* 1987; Beckhouse *et al.* 1988; Dasta *et al.* 1988; Reed *et al.* 1989; van Dalen *et al.* 1990)

Reductions in distribution when compared with the general population have also been reported in major surgical patients (Wurthwein *et al.* 2005) and medium and intensive care unit patients (de Maat *et al.* 2010). The latter group had paracetamol Vds of

approximately 0.66L/kg, similar to what was shown postoperatively in this study (Table 3.4.4). While not assessing it directly, a study using IV paracetamol also indicated reductions to Vd through increases to Cmax following repeated doses postoperatively (Holmer Pettersson *et al.* 2006).

As discussed in Section 1.3.3, there are three factors that can affect the Vd:

1. Blood flow;
2. Drug Binding; and
3. Extracellular fluid.

It is possible that blood flow may have been reduced in these patients, impairing distribution. Reductions to blood flow could arise from three causes: stress induced vasoconstriction; blood loss during surgery and lack of activity and bedrest.

Extensive vasoconstriction seems the most likely explanation for this reduction to Vd and could arise from the increase in circulating catecholamines from the stress response (Udelsman *et al.* 1994). There are two conflicting facts on the role of catecholamines in these patients: cytokine concentrations were low, but oliguria was present. Cytokines stimulate the release of catecholamines and result in vasoconstriction, therefore without cytokines, stimulation of vasoconstriction would be low; however, the presence of oliguria in the majority of patients is a strong indicator of catecholamine release and the activity of the stress response. The low concentrations of cytokines reported in this study in Section 3.5 are questionable, as, given the nature of surgery and complicated postoperative courses of some patients, they were expected to be much higher. The limitations of this assay are discussed further below.

Consistent with the involvement of the stress response in causing the changes to distribution is the finding that those patients with open major surgery (Groups A and B) did have smaller volumes of distribution than the other patients postoperatively. This may have arisen from a greater degree of activation of the stress response and vasoconstriction, although this was not shown in cytokine concentrations, but was supported in changes to urine output. Section 3.3.3 shows diuresis began from Sample 2.2 in both Groups A and B where oliguria was barely seen at all in Group C and only until Sample 1.3 in Group D. When these changes to Vd and urine output are considered together it may give some indication of the extent of stress response experienced by each group. While it is to be expected that Group C's stress response would be the least (due

to the intermediate nature of their surgery), the finding that Group D's is less than Group A or B's is interesting. With similar indications for surgery, the difference between open and laparoscopic surgical techniques may explain this.

The laparoscopic procedures performed on the majority of Group D, while considerably longer with attending increases to administered anaesthetics, seem to have evoked less stress response than the much shorter laparotomy procedures. In short, it appears that the extent of surgical trauma is more important than time in theatre in predicting the extent of the stress response for a group of patients with the same indication for surgery. These findings are widely reported elsewhere (Glaser *et al.* 1995; Kehlet 1999; Desborough 2000; Jess *et al.* 2000; Le Blanc-Louvry *et al.* 2000; Gupta *et al.* 2001; Giannoudis *et al.* 2006; Vlug *et al.* 2009; Madbouly *et al.* 2010b). However, because cytokine levels in this study were so low in all groups, they could not be used to confirm this.

The role of blood loss seems less important. Samples used for the estimation of Vd postoperatively were taken on the evening of the day after surgery. Blood loss arising from surgical trauma is unlikely to have been a factor as late as when these samples were taken, in all but two cases, fluid balance had been restored and patients were haemodynamically stable. Therefore, given intraoperative blood loss alone would have been replaced and paracetamol is not extensively protein bound, reductions to Vd seem unlikely to be explained by this factor alone. However, with pitting oedema observed in many patients loss of fluid from the plasma into the ECF may explain the reduced Vd. As discussed in Section 1.3.3.3 the volume of ECF is increased postoperatively. Fluid sequestered from ECF in plasma during "third spacing" is not available for drug distribution, however this is more associated with infection and sepsis and the effect is mainly on highly hydrophilic drugs

There may have been a minor role played by inactivity. All patients would have remained almost permanently supine until the time Vd was determined. Inactivity, with subsequent reduction in blood flow to adipose and other inactive tissues may reduce drug distribution to these tissues and result in a reduction to Vd being measured (Ylitalo 1991). Without published of similar design it is difficult to compare and validate results.

The paper by Schuitmaker exemplifies some previous barriers to this type of work. Based only on oral dosing, their calculations of Vd required complicated modelling techniques,

and secondly, unreliable postoperative absorption and bioavailability may introduce significant inaccuracies (Hopkins *et al.* 1990; Kennedy *et al.* 2006). Both of these factors could be overcome with the use of IV formulations.

Consequences for the patient arising from a reduced Vd include faster elimination, and potentially better distribution into the CNS (considered as paracetamol's site of action) (Gibb *et al.* 2008). The latter could result in greater efficacy, whereas the former could result in toxicity and is discussed under metabolism below. Considering this potential for toxicity, those with LFT or αGST increases did not show Vds different from the remainder of the group.

Further plasma samples were taken later in the study at one (C1) and four (C4) hours. Unexpectedly, paracetamol concentrations in the one hour samples followed an inverse parabolic pattern across the study period (Figure 3.4-8). C1 concentrations of paracetamol increased from preoperative values until Day 2 and then, rather than maintain a steady concentration as would typically be expected following multiple infusions, they fell to equate to preoperative values by Day 4. Because pharmacokinetic sampling was not carried out on these days a definitive explanation for these changes cannot be determined, however, a C1 concentration is influenced by both the Vd and clearance. In addition to those points discussed above, Vd related changes later in the postoperative period that could explain this phenomenon could include:

- Falling Vd secondary to continuing stress response until Day 2 followed by reductions in the stress response and subsequent vasodilation;
    - Development of diuresis around this time in most patients supports this, although diuresis had begun well before plasma sampling on Day 2 in most patients.
- Further expansion to plasma volume arising from;
    - The consistently positive fluid balances and oedema especially prominent in Group A and B;
    - Reductions to plasma proteins through starvation and the stress response (although paracetamol is not considered heavily bound to plasma proteins).

Reduction to clearance could also explain these changes. The contribution of clearance to the change in these values is now discussed.

4.3.2 METABOLISM/CLEARANCE

As paracetamol is not renally metabolised, nor excreted renally to any great extent, the rate of clearance is dependent on hepatic metabolism. There were several changes to paracetamol's plasma concentration and urinary recovery that indicated paracetamol metabolism had changed following surgery. The first, paracetamol's plasma concentration, provides information about the rate of metabolism, the second, urinary recovery, provides indications on how metabolism is occurring and by which pathways.

The changes to C1 concentrations described in the previous section on Vd (increase until Day 2 and then fall to approximately preoperative concentrations) show variation in elimination across the study and can provide some information about changes to clearance. These changes to C1 were most prominent in Group A, with statistically significant reductions from Days 1 and 2 to Day 4, although a similar pattern was noticeable in Group B and to a lesser extent Group D (Table 3.4.6). Problems with plasma sampling in Group C make their results difficult to compare. The postoperative oliguria developed into polyuria in most patients prior to midday on Day 2. Sampling for this day took place later that night from 10pm. Therefore continuing stress response-induced reductions to Vd on this day seem unlikely as a cause for the peak concentration occurring on this day indicating other factors at play. This continuing increase in paracetamol concentration until Day 2 is anomalous and is explored further.

It seems most likely that in Groups A, B and D patients there was an initial inhibition of paracetamol metabolism on the first two days following surgery. Metabolic capacity was then recovered and may have progressed to the point of induction. Inhibition of metabolism would lead to accumulation of paracetamol from the previous dose and this could explain the increases to C1 apparent in the first two postoperative days. The reduced urinary dose recovery of paracetamol on Days 1 and 2 in Groups A and B support this hypothesis of accumulation in the plasma (Tables 3.3-17 and 3.3-20). Mechanisms of both Phase I and II drug metabolism are known to be inhibited by surgery, anaesthesia or the stress response and these have been shown to return as recovery progresses (Pessayre *et al.* 1978; Nimmo *et al.* 1981; Frye *et al.* 2002; Renton 2005; Aitken *et al.* 2006; Morgan *et al.* 2008). Processes behind these changes are reviewed in Section 1.3.4, however the lack of studies using repeated measures means the sequence of the inhibition followed by induction cannot be compared. While the effect of the stress response on cytokine concentration and urine output had resolved by Day 2 in most cases

in this study, a prolonged effect on drug metabolising enzymes has been shown elsewhere (Nimmo *et al.* 1981). The literature would also support that alterations to clearance in Group D would be less considering the less invasive procedure and reduced stress response arising from laparoscopic surgery (Glaser *et al.* 1995). Following the sequential increases in C1 concentations on Days 1 and 2, the subsequent reduction in concentration is most likely a result of the stress response resolving. However this does not explain the continued reduction in C1 concentations on Days 3 and 4 to approximate preoperative values that arose from a single dose. This could be a factor of induction of metabolism through repeated exposure to the paracetamol, which has been shown elsewhere (Gregoire *et al.* 2007; Kim *et al.* 2007), or through exposure to other drugs or hormones arising from surgery (Kairaluoma *et al.* 1979; Ray *et al.* 1985). Enzyme induction was also shown to occur four to eight days following surgery by approximately 30-50%, returning to normal by four weeks (Nimmo *et al.* 1981), findings which align well with the reduced C1 values shown here. Without full kinetic profiles on Days 2-4, increased clearance cannot definitively be proven in this study, however other factors that could explain this pattern of C1 concentrations can be eliminated:

- Dose - this did not change. An individual's dose was consistent throughout the study;
- Accumulation due to steady state - this was already achieved. In stable environments, steady state is assumed after five half-lives (Dhillon *et al.* 2006). Half-lives shown in Table 3.4-1 estimate steady-state would have been achieved half way through Day 1, assuming disposition factors remained constant. The fact that C1 concentrations still rose on Day 2 invalidates this assumption; and
- Changes to Vd – these have been discussed above.

While indicative, C1 concentrations are difficult to interpret in isolation. As previously mentioned, they are influenced by both distribution and clearance. Full kinetic profiles of paracetamol were taken on Day -1 and Day 1 and provide accurate clearance values. Reported clearance values in healthy males range from 22-27L/hr (Prescott 1996) and the values reported here are in general agreement with this. Some patients do show changes after surgery (Table 3.4-3). Figure 3.4-5 displayed a downward trend in Group A, while values in the other groups stayed remarkably constant. The lack of numbers and small changes in the others groups makes their interpretation and inference to the causes of the changes in Group A difficult. These reductions to clearance are in the face of

reductions to volume of distribution, which, if anything, should improve the efficiency of metabolic processes by increasing the concentration of paracetamol in the plasma available for metabolism. If one was to assume the reduction to Group A's clearance was a short-lived inhibitory effect of surgery, it is supported by the reductions in urinary recovery of paracetamol on Day 1 seen in this group described above. Group A's urinary dose recoveries on Day 1 were the lowest of any postoperative day. Consistent with this reduction in clearance only occurring in Group A is that none of the other groups had their lowest urinary recoveries on Day 1. Additionally, all patients would have been catheterised at this time, reducing the possibility of incomplete urine collections explaining these findings. However, a major confounder of this explanation is that if this reduced clearance seen in Group A was due to inhibition of metabolism, then a similar trend would be expected in Group B as they underwent the same surgery, but this was not seen. This suggests a degree of dose limitation in the clearance of paracetamol. This has been shown with single-high doses (Prescott *et al.* 1973; Prescott 1980; Mutlib *et al.* 2006), but has not been reported with doses similar to those that were used here (Gelotte *et al.* 2007).

Generally clearance of paracetamol is not altered following surgery (Lewis *et al.* 1991; Schuitmaker *et al.* 1999; Wurthwein *et al.* 2005). Slightly increased clearances were seen in another study (Ray *et al.* 1985) but this had methodological flaws, most importantly the use of an oral dose of paracetamol for the calculation of clearance. A more recent study using IV formulations in surgical patients has reported reductions similar to those seen here (Fouad *et al.* 2009).

One patient who was an outlier in Group A with an increased value was Patient 1A, who went on to have a very complicated postoperative course. The processes of induction of metabolism are very slow in comparison to inhibition (Park 1996), taking up to 48 hours (Barry *et al.* 1990). Thus most factors in this patient would lead to the expectation of a reduction in clearance, as seen in the rest of the group, not the increase seen. The increase of Patient 1A is difficult to explain and is most likely due to error, especially when it is considered that his opposing change to Vd resulted in a half-life consistent with the remainder of his group. This would indicate a problem with the administration of the paracetamol dose as both the calculation of clearance and Vd account for dose, whereas k and the equation for half-life do not. The only other factor seen in his clinical scenario on Day 1 which could account for such a divergent clearance figure is his IL-6

concentration, which at 349pg/mL was one of the highest measured. Stress is known to cause vasoconstriction, which can increase hepatic blood flow and therefore drug clearance, however this can be discounted as the patient's distribution volume increased and even if it had not, paracetamol has a low hepatic extraction ratio so changes to hepatic blood flow would not have a major impact on clearance (Lewis *et al.* 1991). Additionally, IL-6, as by far the most potent down-regulator of CYP450, is known to inhibit metabolism, not induce it (Gurley *et al.* 1997; Siewert *et al.* 2000; Morgan *et al.* 2008). Despite this there was evidence of induced CYP2E1 activity in this patient with his ratio for 6-hydroxychlorzoxazone:chlorzoxazone as 2.5:1, whereas the group median was 0.9:1 (Section 3.5.3). However it is possible poor absorption of this orally administered chlorzoxazone influenced this analysis by reducing the amount of parent drug in the ratio. As an outlier, if this patient was removed from the analysis, the consistency of the other values in the group did result in a statistically significant reduction in postoperative clearance in Group A.

In summary there are some changes in paracetamol disposition that could be attributed to high doses and surgery, however reflection on the half-life values (Table 3.4-3) shows that these changes do not lead to accumulation and are unlikely to be clinically important on their own. Further unexpected findings to support this were from the one and four hour plasma concentrations that had been normalised for dose (Figures 3.4-8 and 3.4-9). There were no visual differences occurring between dose groups which had suggested that paracetamol had not accumulated in the high dose group beyond that of the normal dose groups. This would indicate that the metabolic processes that eliminate paracetamol were not overwhelmed at this higher dose.

So the question arises over how drug elimination of paracetamol was maintained following surgery, and to what extent the milieu of reported changes to drug metabolism influence how paracetamol was eliminated. This question is most applicable to the high dose group, with more frequent doses of larger amounts, and if this was influenced by surgery.

There were several changes in the pattern of metabolite excretion in the urine that were of interest. The high dose group (Group A) showed several clear changes to the excretion pattern in the postoperative period. The most significant change was the increased contribution of paracetamol glucuronide to the excretion, which increased by more than 20% over the course of the study (Figure 3.3-14). This was matched with an increasingly

large reduction in paracetamol sulphate, which finished with less than half its preoperative contribution. There was also a bulging of Phase I metabolite contribution on Days 1 and 2, consistent with the possible increase in CYP2E1 activity as evidenced by increases to the ratio of metabolite:parent ratio of the probe drug chlorzoxazone (Section 3.7.3). This was consistent with the literature where identical patterns of change to metabolite excretion have been reported following increased doses (in healthy adults) (Clements *et al.* 1984; Gelotte *et al.* 2007), repeated doses (Hendrix-Treacy *et al.* 1986; Allegaert *et al.* 2005) and repeated increased doses (Gelotte *et al.* 2007). All scenarios exhibit similar increases to the contribution of paracetamol glucuronide, as is the case here in this study (Hendrix-Treacy *et al.* 1986; Allegaert *et al.* 2005). Repeated doses of paracetamol have been suggested to have an inductive effect on UGT enzymes (Allegaert *et al.* 2005) and this has been reflected in increases to amounts of paracetamol glucuronide recovered in the urine. While *in vitro* methods exist for quantification of UGT activity, existing *in vivo* methods depend on morphine administration (de Wildt *et al.* 1999). Morphine could have been used as a marker drug, but hospital policy did not allow fentanyl patient-controlled analgesia that would have been needed to replace morphine patient-controlled analgesia to facilitate this analysis .

The reduction in paracetamol sulphate and Phase I excretion in Group A on Days 3 and 4 of the study are of interest. Sulphate-derived metabolites (paracetamol sulphate, cysteine and mercapturate) share a common precursor, GSH. GSH is itself derived mostly from dietary protein. Because GSH detoxifies NAPQI, it is integral to the safety of paracetamol. Reductions to excretion of sulphate derived metabolites indicated a potential drop in GSH levels which may have led to a drop in NAPQI detoxification. Sulphation is known to saturate (Hendrix-Treacy *et al.* 1986; Martin *et al.* 1991), however, its decreased contribution later in the study is more difficult to explain. Group A were fasted until at least Day 3 postoperatively. The question of the nutritional impact on these patients on inorganic sulphate reserves arises given inorganic sulphate's necessity for the production of GSH and precursors for sulphation. When viewed as a whole for this group, the contribution of sulphate-containing metabolites reduced at the same time as overall dose recovery (Figures 3.3-19 and 3.3-9 respectively). Group A's median sulphate-containing metabolite contribution on the final day was almost half that of their Day 1 postoperative value. If this indicates exhaustion of inorganic sulphate stores, there is a possibility that the unaccounted for paracetamol seen in the reductions to dose recovery remains in the body as unconjugated NAPQI. This would explain the appearance of raised liver function

tests in some Group A patients after the study was concluded. Even if adequate concentrations of inorganic sulphur remained in the blood, reductions to sulphonation could also arise from insufficient sulfotransferase activity to maintain intracellular concentrations after several days of repeated paracetamol administration (Hendrix-Treacy *et al.* 1986; Hindmarsh *et al.* 1991).

Results for Group B in Table 3.3-32 show a similar, but much less marked pattern with statistically significant increases to paracetamol glucuronide excretion and reductions to paracetamol sulphate excretion. In Group C similar changes were seen in comparison with preoperative values although relative contributions postoperatively were more consistent (Table 3.34). There was a large degree of similarity between Group A and D in their metabolite pattern in the early postoperative period with statistically significant reductions to paracetamol sulphate recovery and increases to glucuronide and Phase I products, however this was not to the same extent as Group A.

At the outset of this research, it was believed that changes previously seen to the Phase II:I metabolic ratio were a product of CYP450 induction postoperatively, but there are problems with this assumption:

Using the contribution of paracetamol cysteine and mercapturate to urinary recovery of paracetamol as an approximation of Phase I metabolism assumes that all of their precursor, NAPQI, produced by this pathway is rapidly conjugated and excreted. The culmination of published work and results of this study would now suggest this not to be the case and that the role of nutrition is more significant. Conjugation of NAPQI relies on the presence of sufficient quantities of GSH, which as detailed in the discussion of the urine results, is depleted in fasting postoperative patients and at higher doses (Slattery *et al.* 1987).

The assumption also relies on the change of ratio being due solely to changes in the contribution Phase I metabolites, while Phase II metabolites remain static. As most clearly shown in the contribution of each metabolite to overall recovery for Group A (Figure 3.3-14), the reduction to paracetamol sulphate and increase to paracetamol glucuronide recovery in the urine would suggest this is not the case. To the contrary, both Phase II metabolites are dynamic, as previously reported (Hendrix-Treacy *et al.* 1986), and the metabolic ratio is a combination of both Phase I and II changes.

From a pure mathematical standpoint, because Phase I products contribute a much smaller value to this ratio, a small change in their contribution can cause a disproportionately large change in the ratio. Additionally, as much smaller values, they are more sensitive to analytical errors arising from the instrumentation.

Finally, and perhaps most importantly, the rapid changes seen in Group A's metabolic ratios postoperatively, cannot be explained by CYP450 enzyme induction. CYP450 enzyme induction is a slow process, requiring the up-regulation of transcription and synthesis in the cells that are involved in enzyme production (Takahashi *et al.* 1993) and several aspects of the stress response would oppose these processes (Renton 2005; Morgan *et al.* 2008). Generally inhibition of CYP450 enzymes is reported postoperatively (Monshouwer *et al.* 1996; Morgan 1997; Haas *et al.* 2003). Results from the analysis of the individual CYP450 enzymes are difficult to interpret because of methodological problems with the assay (discussed above) and unfortunately the two drug probes which were assessable had additional confounders. Both were administered orally to patients known to have poor oral absorption. This could explain the variation in chlorzoxazone ratio. In the case of caffeine, additional intake, outside of that administered in the study, could explain the variation seen in its metabolic ratio and despite the best efforts of the Investigator, the possibility of this additional intake could not be excluded.

Similar but less pronounced changes were seen in Group B. Especially prominent in this group was the contribution of paracetamol-cysteine that did not diminish as it did in Group A (Figure 3.3-15). This observation could further suggest exhaustion of GSH in Group A by the high dose, as, in that group, both paracetamol-cysteine and paracetamol-sulphate reduced, whereas in Group B paracetamol-cysteine increased towards the end of surgery. This is supported by the evidence of enzyme induction in this group shown in Section 3.7, which would cause an increase in the production of NAPQI and therefore its metabolite, paracetamol cysteine, when sufficient stores of GSH are present. The lack of change in metabolite ratio in Group C (Figure 3.3-28) also supports this hypothesis of the role of nutrition, more than stress, as these patients were able to eat the day after surgery, but experienced a similar stress response to the surgical patients in Group A and B (Section 3.5). There is further support for role of nutrition from Group D findings, where reductions to sulphate derived metabolites were reversed early on Day 3 and trended back towards preoperative levels. This group were able to eat as soon as they could tolerate it, which was generally the evening of Day 2.

Although this metabolic ratio, comparing Phase II with Phase I products, has not been used previously, applying it to other published results leads to similar findings as those described here. The paper by Pickering *et al.* is one of the only other papers to publish urinary metabolite concentrations in surgical patients over several days. When the ratio is calculated, their data follow a similar form, beginning from the day after surgery with a ratio of 5.2 which reduces to 2.7, 2.6 and 2 on each subsequent postoperative day (Pickering *et al.* 2011). This compares favourably with the ratios shown here for Groups C and D especially, in addition to the Group A Patient 4A who received TPN.

Prior to the review of the results, cytokines were thought to play an important role in determining CYP450 activity (Section 1.3.1). Inflammation caused by infection or underlying inflammatory conditions is known to down regulate most drug metabolising enzymes and transporters, both in acute scenarios, such as following surgery, or chronic conditions such as extra-hepatic cancer, inflammatory bowel disease or congestive heart failure (Frye *et al.* 2002; Carcillo *et al.* 2003; Haas *et al.* 2003; Aitken *et al.* 2006; Morgan *et al.* 2008). This reduction in Phase I activity can lead to a decrease in drug clearance, increasing plasma drug concentrations and potentially may cause drug toxicity or increases in the incidence of adverse effects (Schmith *et al.* 2008). While mostly associated with untoward events, this inhibition can also be protective against toxicity of compounds that are activated by Phase I metabolism (Renton 2000; Morgan 2001; Renton 2001; Renton 2004) such as NAPQI formation. Results of this study (Section 3.5) show cytokine concentrations fell rapidly postoperatively and for the remainder of the study were often close to the lower limit of detection. The cytokine most affected by surgery was IL-6, which showed substantial increases in Day 1 in some patients. However, as a whole, group values showed only minor changes with very large inter-individual variation. As is consistent with the nature of IL-6, its concentration fell rapidly on the following postoperative days. Therefore, it was difficult to draw any relationship with paracetamol concentrations as there was no consistent elevation in this cytokine across a group. Another group used the erythromycin breath test as a measure of CYP3A4 in major surgical patients up to 72 hours after surgery and compared this with their IL-6, IL-1β and TNF-α blood concentrations (Haas *et al.* 2003). CYP3A4 activity progressively declined from preoperative values to the 72 hour sample and the depth of this decline was significantly and negatively correlated with peak IL-6 concentrations only, not subsequent concentrations. In almost all cases the peak IL-6 concentration occurred within the first 24 hours of surgery at sampling times not included in this Thesis' analysis. Similar to this

work by Hass *et al*., Section 3.5 showed the other cytokines measured exhibited even less of an effect from surgery (compared with IL-6) and their elevation was even more inconsistent and minor, but this may be related to sampling times used in this present study.

Previous work that examined cytokine concentration postoperatively began sampling at the time surgical wounds were closed (Leung *et al.* 2000; Haas *et al.* 2003; Catena *et al.* 2009). Therefore, the daily interval used in this study may not have been appropriate for the determination of the peak cytokine elevation in the study patients. These sample times were not used in this study for a culmination of reasons, including that the effect of surgery was thought to cause longer elevation in cytokine levels and determining paracetamol pharmacokinetics was the primary importance, while the assessment of other factors, such as cytokine concentration, were less so. Paracetamol concentrations arising from intraoperative samples would have been meaningless as they would have been between steady state and single dose conditions, therefore calculation of pharmacokinetic values could not be accurately performed.

Other potential confounders to these cytokine results include the use of NSAIDs in some patients which are known to depress cytokine elevations (Chambrier *et al.* 1996) and thus the full extent of cytokine concentrations increases may not have been visible.

There is only one other group that has examined the regular administration of supratherapeutic doses of paracetamol similar to those in Group A. Gelotte *et al.* found that although sulphonation of capacity was exceeded, rapidly increased formation of the glucuronide conjugate prevented an increase in NAPQI synthesis (Gelotte *et al.* 2007). This is at odds with this study where statistically significant increases in Phase I metabolites were seen in Group A towards the end of the study (Figure 3.3-14), findings similar to those of Slattery *et al.* (Slattery *et al.* 1987). The conflict between the findings here and those of Gelotte *et al.* may have arisen because of the difference in subjects and the trial conditions most obviously that their study was conducted in healthy adults in a clinical trials centre where patients were regularly fed and given oral doses of paracetamol. This study highlights the difference between a usually homogeneous, healthy study group used to establish pharmacokinetic parameters and the usual clinical setting of immense individuality and variation which can fluctuate on an hourly basis, depending on the severity of illness or metabolic assault.

With hepatotoxic doses, paracetamol metabolism is impaired and the half-life prolonged. (Prescott 1980). Increased half-lives were shown here when they were measured on the first postoperative day, but these were well within ranges contained in the product literature. However, plasma paracetamol concentrations can also provide information on toxicity. One of the first studies to advocate the use of unlicensed doses of IV paracetamol as a loading dose prior to surgery lists a 150mg/L four hour plasma concentration as the threshold for hepatotoxicity to occur, 200mg/L as a risk of some damage and 300mg/L as a concentration at which liver damage will always occur (Gregoire *et al.* 2007). Gregoire *et al.* cite Prescott and Rumack as their sources (Prescott 1996; Rumack 2002). In this present study the maximum four hour concentration was recorded in Patient 8A on day 2 and at 32.9mg/L, was well short of the 150mg/L threshold for toxicity suggested above. Those patients who went on to developed AST elevations above the upper limit of normal (Patients 2A, 3A, 4A and 9A) all had four hour paracetamol concentrations consistently below this. While empiric, this may suggest that four hour concentrations are not a good predictor of hepatic injury. While thoughts then shift to examining prevalence of the Phase I products in the urine, these are only excreted once they are made safe by GSH. NAPQI causing toxicity is not excreted. With this in mind another measure of hepatotoxicity, αGST, was measured. αGST concentrations are touted as a better predictor of hepatotoxicity than standard laboratory tests (Beckett *et al.* 1985; Rees *et al.* 1995; Clarke *et al.* 1997; Kumle *et al.* 2003; Chouker *et al.* 2005). These concentrations provided one of the biggest surprise of the study. Even in the group of patients receiving an excess of twice the licensed daily dose (Group A), no clinically significant increase in αGST concentrations were seen after four days of this dose. In many cases αGST appeared to decrease following surgery, which was completely opposed to what was expected. αGST is marketed as an early marker of hepatic damage, however, the results of this study question this assertion. While no patient showed AST elevation during the study, several did go on to have elevations once the study had finished. The fact that αGST concentrations did not show these elevations prior to the AST rise was very disappointing. Examining αGST alone would suggest the high doses of paracetamol are completely safe over a four day period and it is only AST concentrations that question this assertion. Similar work administering up to 8g daily to patients also failed to report liver function tests beyond the three days the study dose was administered, despite them being obtained (Temple *et al.* 2007).

There is some explanation for the safety of the high dose given to Group A offered in the literature. Experimental animals have shown changes to the location paracetamol is metabolised in the liver following repeated, high, dosing, which has led to a reduction in bioactivation and production of NAPQI (Shayiq *et al.* 1999). If the percentage of a dose metabolised to NAPQI fell, then the safe dose could theoretically increase. It is not until GSH stores are depleted by 90% that hepatotoxicity can ensue (James *et al.* 2003; Hinson *et al.* 2010). In addition, there are several studies in man that have shown the capacity for glucuronidation exceeds that utilised by currently licensed doses of paracetamol. Clearly the key determinant of safety is the state of an individual's CYP2E1 activity and the opposition provided by their GSH reserves. Both factors are linked to nutrition. This finding is not new, but the example shown by Patient 4A suggests that care must be taken with how nutrition is managed in postoperative patients. In his case, the supplementation of sulphur-containing amino acids by administration of TPN may have been outweighed by the induction in CYP450 enzymes this protein rich solution caused in an undercurrent of induction to his CYP450 enzymes following surgery. It may also be relevant in his case that once paracetamol toxicity has developed, GSH synthesis and conjugation is depressed, so that paracetamol toxicity forms something like a positive feedback mechanism (Lauterburg *et al.* 1982). This may explain why he went on to develop elevated ASTs in the face of receiving methionine in the TPN (Fresenius Kabi 2011), what is equivalent to the low dose of the antidote to paracetamol toxicity. However, the paracetamol-cause of the increased AST could be questioned as LFT elevations are very commonly seen with TPN initiation of administration alone (Gabe *et al.* 2010)

4.3.3 ELIMINATION

Both previous sections on disposition refer to one of the most obvious impacts of the stress response to an external observer: a reduction in urine output following surgery.

The reduction in urine volume following surgery is a well-known phenomenon in response to injury-induced increases of arginine vasopressin and aldosterone concentrations in the circulation (Section 1.3). For this research, a reduction in urine output was not of interest in itself, but was of concern because of its effect on the excretion of paracetamol, which is unreliable when urine volumes are low (Kietzmann *et al.* 1990). This had major ramifications for what was of interest to this research; the change in metabolism. This was because traditional metabolic ratios used to determine

changes to metabolism contain both metabolite and parent concentration, and therefore, because of the unreliability of paracetamol excretion, these could not be used. Accordingly, another ratio was derived that was urine flow independent, but still provided information about changes to metabolism. This ratio compared the concentration of Phase II with Phase I metabolite in urine as the urinary excretion of all of the metabolites of paracetamol are urine flow rate independent (Prescott *et al.* 1973).

While data showing a change in metabolism following surgery were presented in the Section 3.3 of the results, it was necessary to rule out other factors that could have had an undue influence and invalidate these finding. Firstly, recovery of the paracetamol dose in the urine needed to be high and consistent as this confirmed the fact that urine remained the main route of excretion of paracetamol following surgery. In this study, catheterised patients in the steady state period had good recoveries, generally around 90-95% of the administered dose. These values are concordant with those reported elsewhere (Miners *et al.* 1992; van der Marel *et al.* 2003). This confirmed the validity of the assay and that the effect of the reduction in urine flow rate was not substantial enough to make a difference to overall recovery. However, urinary recovery of paracetamol from the single preoperative dose was 40-60% slightly below the 65% that has been reported in other studies (Kietzmann *et al.* 1990). This may have been due to incomplete urine collections, both in terms of the volume collected and duration. Ideally all urine produced within the four hourly interval collection period would have been collected in the relevant collection. While this was requested of patients, enforcing patients to empty their bladder every four hours throughout the night would have seen recruitment drop even further. Therefore, complete evacuation of the bladder could not be confirmed by the Investigator and there were several four hourly samples pre-operatively that contained no urine. In addition, incomplete urine collections could have arisen from patients being unable (or unwilling) to collect the entire volume of their urine, loss of urine through transferring between the collection and storage vessels or the use of an absorbent collection vessels (such as cardboard bedpan). In terms of duration of urine collection, it may have been more appropriate to collect urine right up until the time of surgery. However, asking patients to collect their own urine at this time would produce almost insurmountable issues given the necessity for pre-operative sedation and procedures such as X-ray and ultrasound immediately prior to surgery.

The factors discussed above may also apply to Group C patients who were only briefly catheterised and mostly collected their own urine. This group had the lowest measured urine volumes, although they did not receive as much IV fluid as the other groups. Group C had large variability in dose recovery over the course of the study, even though the net collection was around 95% of the overall administered dose. As drug input was constant, this could be explained by accumulation of the metabolites in the urine in the bladder and the difficulties arising from obtaining complete urine collections from patients, especially females (which constituted the entirety of this group).

Other differences between groups that were noticed included that the recoveries were lowest in the high dose group (Group A), especially towards the end of the study period. This may suggest a rise in extra-renal excretion, plasma accumulation or excretion as metabolites not monitored. Increases to extra-renal excretion cannot be ruled out but these have been shown to be only a minor contributor in previous work (Hjelle *et al.* 1984; Jayasinghe *et al.* 1986; Gregus *et al.* 1988). However, biliary excretion has also been shown to be dose dependant (Siegers *et al.* 1984) so the importance of this route is still a possibility. Plasma accumulation of paracetamol in this group seems to be an unlikely cause of the change in recovery as, while increased paracetamol concentrations were seen until Day 2, they reduced on Days 3 and 4 to pre-operative concentrations. Increased metabolism may have led to accumulation of metabolites in plasma, but these are readily excreted so this would seem to be an unlikely cause. The metabolites not monitored as part of this study are generally considered to make an insignificant contribution to the overall metabolite excretion (Prescott 1996). As shown in Section 1.2 there are many other metabolites of paracetamol not monitored in this study but given the presence of the phenol moiety in their structure, if they were a significant contributor large peaks would be expected on the HPLC traces at 242nm (Silverstein *et al.* 1991).

4.4 STRENGTHS, LIMITATIONS, AND FUTURE WORK

4.4.1 STRENGHTS

This is the first study to examine the effects of surgery on paracetamol pharmacokinetics in the same patient. One of the major strengths of this study is that paired or repeated measures methods were used to detect statistical significance to allow each patient to act as their own control. Previous work of this nature has compared paracetamol's pharmacokinetic values obtained from postoperative patient's and then sought to compare these to published values obtained from normal, healthy individuals. There are some flaws in this method for determining an effect of surgery as it assumes surgical patients' have normal drug disposition prior to their surgery. However, as referred to in Section 1.3, very few people have "normal" values for drug disposition as these are themselves, in fact, an average. For example, very few people would have a Vd of exactly 0.90L/kg and this is borne out of the fact that paracetamol's half-life is given as a range of 2-3 hours. By not using the same patient, research ignores all the genetic and environmental variation that those patients have even prior to surgery and can grossly underestimate the effect size as a consequence.

The high dose used in this study was novel, especially considering the population to which it was administered, however, the fact that it was administered without any evidence of hepatic damage is a significant strength. The dose was chosen because 1g every four hours was well established (Schug *et al.* 1998) and had been shown to "be unlikely to have useful clinical impact" (Schuitmaker *et al.* 1999) as a result of failing to reach 10mg/L concentrations which were considered to be therapeutic. The next logical step was to either 2g six hourly (8g in total) or to the dose used here, 1.5g four hourly. Considering the toxicity to be linked to a maximum concentration, rather than exposure, the latter was chosen. There is a lack of pharmacokinetic studies in the adult surgical populations and the use of an IV formulation adds significant strength as it facilitates the accurate calculation of paracetamol pharmacokinetics.

A further strong point is drawn from the multitude of tests applied to the patient. Simultaneous measurement of plasma and urine concentrations of paracetamol, CYP450 activity, cytokine concentrations and αGST concentrations allows their integration to produce explanations to phenomena that are only otherwise observed. It can also direct future research more precisely when observations do not line up.

4.4.2 LIMITATIONS

The most significant short fall of this study was that it failed to reach its recruitment goals. As a consequence, this study was probably not large enough to detect differences in disposition in the groups that received normal doses that arose due to surgery. However, the effect size of surgery when combined with the larger dose was large enough to result in detection of significant results, even though the recruitment into this group was less than desired. Without powering the study for comparisons between groups, differences due to dose and surgery type can only be discussed phenomenologically, without the strength of statistical rigour. In addition to these comparisons, larger groups may allow the detection or exclusion of the role of other potential influences on disposition, such as smoking, obesity and others discussed in Section 1.4. This study is too small to detect these.

While changes to disposition values, such as clearance, were not detected in the normal dose groups (which was potentially due to under-powering), the possibility cannot be ignored that this was because there weren't any changes to detect. The metabolic capacity of paracetamol is vast, with several overlapping mechanisms competing for whatever paracetamol is around. What this study has contributed is information of the changes in metabolism that arise underneath a constant clearance value. Changes were virtually instantaneous, when sulphonation decreased, glucuronidation's increase was immediate, without a noticeable reduction to total excretion. Even in Group B, with only four participants, statistically significant alterations to the contribution of paracetamol sulphate and glucuronide were seen (Table 3.3-32).

Further limitations arise from the number of plasma samples that were missing due to problems with obtaining blood. These were discussed above under conduct of the study. Urine data could also be flawed by incomplete bladder emptying and partial urine collections. While catheterised, these influences are minimised, however once urinary catheters were removed, these issues re-emerged. Longer urine collections preoperatively may have led to a more complete recovery of paracetamol and given a better indication of metabolism. Although close to reported values, this reduction in recovery may have altered excretion patterns as the proportion of metabolites in the urine changes with time following a single dose. A metabolite ratio of this type is considered to be highly variable and show large interindividual variation. While factors such as gender, sample time and dose recovery could not be excluded as confounding the

metabolic ratios used in this research, their contribution was analysed and found to be minor. However a larger analysis that could control for these variables in a multiple regression would be more appropriate. Given the change in metabolite ratio was seen in Group D, a group that showed very little change in plasma kinetics or concentrations of paracetamol metabolites, the question arises whether the change in urinary ratio was a result of repeated dosing rather than a change in metabolism. However, as no previous published work has used the metabolite ratio used here or reported metabolite concentrations on repeated days, it is impossible to validate this possibility. Another limitation is due to the comparison of single-dose with steady-state paracetamol kinetics. An ideal comparison would have been to have patients at steady state paracetamol concentrations prior to surgery. The only flaw in this type of analysis would be that it could not account for changes in paracetamol metabolism due to repeated dosing.

The most significant limitation of α-GST measurement was the cessation of monitoring at the conclusion of the sampling period. This was done as a number of patients were discharged shortly after the conclusion of the study and due additionally to the cost of the assay, which necessitated limiting the number of samples analysed. Additionally, there was a spuriously high preoperative concentration of αGST seen in many patients. This diluted the significance of any postoperative elevations seen and also could suggest the under-reporting of alcohol consumption. However, all those with high αGST concentrations preoperatively had AST concentrations within the normal range.

The primary limitation of the cytokine analysis, as discussed above, was the comparatively late sampling time. This almost certainly missed peak cytokine concentrations and as such, their effect on paracetamol kinetics could not be determined. Additionally, the number of cytokines that were monitored were superfluous as, although of interest, IL-6 has been shown to have the most significant correlation with drug metabolism (Abdel-Razzak *et al.* 1993; Muntane-Relat *et al.* 1995; Frye *et al.* 2002; Morgan *et al.* 2008). It was on the advice of a company representative that all four pro-inflammatory cytokines were measured, however, retrospectively, this advice may have been self-serving.

There may have also been problems with absorption of the CYP450 probes caffeine and chlorzoxazone that were administered orally. Future work of this kind in major surgical patients should rely solely on the use of probes that can be administered via IV. This is due to the well-known problems with oral absorption in surgical patients. Unfortunately at the date of this study no IV formulation of chlorzoxazone was commercially available,

so alternative probes for CYP2E1 would need to be identified. Additionally, caffeine should be avoided as a probe for CYP1A2 due to the difficulties in preventing incidental intake, discussed above.

Finally, there were some factors that could reduce the generalisability of the data to other groups:

- There was an under-representation of females in Groups A and D. Similarly, there were no male patients in the intermediate surgery Group C;
- The majority of bowel surgery patients underwent anterior resection, followed by colectomies. This may limit the applicability to the general population as it deviates slightly with the colorectal cancer statistics for the Irish population, discussed in Section 1.6, from which more colectomies would be expected. This may reflect the expertise of the hospital where these patients were treated and the comparative ease of diagnosis of rectal cancers. An additional source of deviation in the data may arise from the National Cancer Registry recording the number of diagnoses, not the number of patients with each diagnoses coming forward for surgery, which was recorded in this Thesis.
- Generally the age of patients was younger than expected from the data given by the National Cancer Registry of Ireland for colorectal cancer patients (National Cancer Registry Ireland 2010) and this may also affect the generalisability. This may be due to the problems recruiting older patients due to their comorbidities and a large number of older colorectal cancer patients who were assessed for inclusion into the study were not fit. Additionally, it was observed during the study that older patients were more difficult to cannulate, would often occlude their cannula and were more sensitive to the effects of midazolam.
- All surgery was elective. Applicability of data to emergency surgery could not be assumed as factors relating to fasting and the stress response could be different. However, this would exclude relatively few as the vast majority of these procedures are planned;
- Most indications for surgery were for malignancy, which could reduce to the applicability of data to other diagnoses for surgery;
- Most patients were either overweight or obese. This is known to influence CYP450 enzyme activity;

- Most patients did not smoke or had given up smoking in the last 5 years. As enzyme activity reportedly normalises within one week of smoking cessation (Faber *et al.* 2005) this limits the applicability of these data to surgical patients who do smoke as their induced level of enzyme activity is maintained, even after 36 hours of smoking withdrawal (Eldon *et al.* 1987); and
- Alcohol consumption reported was less than reported in other European populations and may have been the subject of recall bias (Tonnesen *et al.* 1999). Alcohol consumption is a known inducer of CYP2E1 and if significant quantities of alcohol were normally consumed by the patient and then abruptly withdrawn upon admission to hospital, this could have had a significant effect on paracetamol's metabolism. If consumption was under-reported then these patients could not be identified.

4.4.3 FUTURE WORK

Future work could include:

- Conducting a much larger study in patients undergoing various types of surgery who received licensed daily doses, collecting only their urine. A less invasive study such as this would potentially increase patient recruitment. This would finally conclude the relationship between surgery and changes to paracetamol's metabolic ratio;
- Comparing changes of Vd with blood losses and fluid administration. This could determine the effect of IV fluid administration on paracetamol distribution, and if colloidal fluids have an influence;
- Calculation/presentation of values that represent the change within individuals, not within the group. A "per cent change" value could be determined, but this can over-represent small changes seen in individuals with initially low values;
- Combining urine and plasma data to calculate and model formation clearance and apparent oral clearance and predict steady state concentrations from the preoperative single dose with the use of pharmacokinetic software;
- Determination of a correlation between CYP450 activity and the recovery of paracetamol-cysteine and mercapturate. This would hold significant value and could determine the true effect of enzyme induction on paracetamol's metabolic ratio;

- Administering morphine to patients to assess UGT activity in conjunction with IV paracetamol. Fentanyl patient-controlled analgesia/non morphine based analgesia would be necessary to facilitate this;
- The measurement of inorganic sulphate in the urine in surgical patients receiving paracetamol to determine if it matches the reduction of sulphate-containing metabolites in the urine;
- Conducting a larger study in non-surgical patients that compares the effects of repeated IV and oral dosing on paracetamol metabolism;
- Monitoring liver function in a similar study beyond that of the time where the investigational doses were stopped. Examining post study AST concentrations suggest that at least an additional three sampling days would be necessary;
- Obtaining plasma samples closer to the time of surgery to measure peak cytokine concentration appears to be the most significant factor. For reasons of economy, it would seem efficient to examine changes to IL-6 concentration alone and not those of the other cytokines assessed as part of this study;
- Conducting the study in patients with central lines or obtaining an intravenous cannula designed for bloodletting. Postoperative patients are renowned for their poor venous access and failure of venous access was a perennial issue in this study. In addition, many of the study patients had recently received IV chemotherapy which can, through frequent use, reduce the number of veins suitable for cannulation. This will always be a barrier to this type of research and choosing a group of patients who frequently return to the ward with central lines is probably the easiest way around this problem;
- Consider the use of other CYP450 probes. Aside from the problems with the assay, detailed above, there were additional problems with probes chosen for this analysis. Midazolam was a problem from a clinical perspective; its potency as a hypnosedative is its main advantage in clinical use, however, for the purposes of this study it was a substantial drawback. Whereas some studies were able to administer up to 10mg of midazolam, administering this dose to postoperative patients who were receiving narcotics could have led to severe respiratory depression and hypotension. Consequently a compromise dose was chosen, but this lead to the necessity of LCMS detection. Ideally a probe that could be given in higher doses and detected by UV would be used in this research. Secondly, using caffeine as a probe was a significant problem for patients. As discussed above,

requesting patients to abstain from tea and coffee was the source of many refusals to participate. The likelihood of patients taking caffeinated drinks during the study cannot be ignored and this raises questions about the validity of the findings. As already suggested, another probe for CYP1A2 should be considered; and finally

- More Pharmacists conducting clinical research.

The concept of a Clinical Pharmacist leading and conducting research of this nature is unusual in Ireland, if not the world, but it makes a lot of sense. Pharmacists have many advantages in the understanding of pharmacokinetics, why samples must be taken on time and what the best strategy is if they are missed. Clinical Pharmacists are also well used to the hospital ward environment, dealing with hospital patients and working with and around teams of other health professionals. Although Pharmacists do not have "built in" technical skills such as phlebotomy, cannulation or observation recording, these can be and were taught to the Investigator in this study. Aside from the barrier of being unable to prescribe drugs, Pharmacists can be just as able to conduct clinical research as any other health professional and thankfully Hospital 1 were largely of the opinion of "not why?, but why not?" and provided all the extra training that was necessary alongside newly recruited nurses.

Despite this, there was initially a high level of suspicion from other staff, especially surgeons, which took some time to overcome. As mentioned previously, surgeons were aware of the study, were content to have their patients involved, but only as long as it did not cause them any interference. The relationship between the study and both surgeons was more one of non-maleficence than beneficence. Other staff members were also suspicious initially, but after a few patients staff members affected were generally interested in the research and by the end of the study most were happy to assist where they were able to.

While there are few barriers to Pharmacists carrying out this type of research, it is immensely beneficial for Pharmacists to be involved in it. Although Pharmacists talk a lot about drugs, it is rare for Pharmacists to administer them to a patient. It was a revelationary experience to administer a drug, such as midazolam, and see the effect on the patient straight away as well as the varying sensitivities to this drug both between individuals and within individuals over the postoperative course. This variability captured the essence of this research.

5 CONCLUSIONS

A clinical pharmacokinetic study was undertaken in surgical patients in two hospitals. The study examined changes to the disposition of IV paracetamol and the safety of licensed and a higher, unlicensed dose.

The safety of both licensed and unlicensed doses was assessed during the study. Results indicated that there was no evidence that IV doses of up to 9.5g of paracetamol a day caused any clinically relevant liver damage during the study period. However, monitoring beyond the conclusion of the study showed substantial elevations in AST concentrations in some individuals, questioning the assumption of safety. No clear determining factor arose that could identify those individuals that showed AST elevation, other than that they had prolonged hospital stays (over 20 days). Given this evidence of safety, the next step would be to establish if this increased dose improved surgical analgesia.

There were also several changes to paracetamol disposition. In terms of kinetics, the most commonly seen were reductions to paracetamol's Vd postoperatively. Vd changes were frequently offset by smaller increases to clearance and resulted in small reductions to half-life postoperatively. Curiously, paracetamol concentrations did not achieve a steady state, but instead concentrations peaked on the second day and then reduced to nearly preoperative values.

Changes to urinary excretion were also seen. In the high dose group there was a substantial increase in the role of paracetamol glucuronide in the recovery of the dose for most patients. However, along with the other groups, the ratio of Phase II to Phase I metabolites reduced sharply following surgery. The absence of literature reporting paracetamol using this ratio makes interpretation of these changes difficult as it is impossible to determine if the they have arisen from a "push" of increasing Phase I products, or a "pull" of retracting Phase II products. Regardless, these changes return towards preoperative values within the study period in most cases and therefore do not align well with the changes to liver function.

While large enough to show significant differences within tests, this study is too small to be able to draw conclusion about safety or the effect of co-variates between groups of patients. However patient recruitment in this population is difficult. As described above, a study that would examine only paracetamol's urinary metabolic ratio in a larger group of surgical patients undergoing surgery of multiple severities could be a useful next step.

Such a study may benefit from better recruitment rates and confirm the relationship between surgery, the stress response and paracetamol's metabolic ratio.

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APPENDIX 1 ETHICAL APPROVAL

UCC
Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

COISTE EITICE UM THAIGHDE CLINICIÚIL
Clinical Research Ethics Committee
Cork,
Ireland.

Our Ref: ECM 5 (16) 05/09/06

6th September 2006

Professor Julia Kennedy
Professor of Clinical Pharmacy
School of Pharmacy
Cavanagh Building
University College Cork
College Road
Cork

Dr Brendan Conroy
Consultant Anaesthetist
Department of Anaesthesia
St John's Hospital
Limerick

Re: High dose paracetamol metabolism in general surgical patients.

Dear Doctors

The Clinical Research Ethics Committee of the Cork Teaching Hospitals reviewed your correspondence at its recent meeting held on 5th September 2006.

Full approval is granted by the Committee to carry out the above study at the following site:

- St John's Hospital, Limerick

Subject to receipt of further information:

- The Committee found the protocol confusing. High doses of paracetamol are to be used in a series of surgical patients. We note that these does will be higher than 4 g daily. The protocol does not state how high the doses will be

The Committee approved the following documents:

- Protocol Submission Form
- Subject Information & Consent Form

We note the following Co-investigators will be involved:

- Dr Stephen Byrne
- Ms Geraldine Creaton
- Mr Philip Murphy

Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

Clinical Research Ethics Committee

Lancaster Hall
6 Little Hanover Street,
Cork,
Ireland.

The following Committee Members attended the above meeting.

Dr Michael Hyland – (Chairman)
Dr Seamus Hart
Dr John McKiernan
Dr Brendan Buckley
Mary Gilmartin
Finola O'Sullivan
Cormac O'Hanlon (lay person)

The Clinical Research Ethics Committee of the Cork Teaching Hospitals, UCC, is a recognised Ethics Committee under Regulation 7 of the European Communities (Clinical Trials on Medicinal Products for Human Use) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to Ethics Committees and the conditions and principles of Good Clinical Practice.

Yours sincerely

Dr Michael Hyland
Chairman
Clinical Research Ethics Committee
of the Cork Teaching Hospitals

APPENDIX 2 ETHICAL APPROVAL- AMENDED PROTOCOL

Tel: + 353-21-490 1901
Fax: + 353-21-490 1919

Coláiste na hOllscoile Corcaigh, Éire
University College Cork, Ireland

COISTE EITICE UM THAIGHDE CLINICIÚIL
Clinical Research Ethics Committee

Lancaster Hall,
6 Little Hanover Street,
Cork,
Ireland.

Our Ref: ECM 2 (f) 06/01/09

7th January 2009

Professor Julia Kennedy
Professor of Clinical Pharmacy
School of Pharmacy
Cavanagh Building
University College Cork
College Road
Cork

Dr Brendan Conroy
Consultant Anaesthetist
Department of Anaesthesia
St John's Hospital
Limerick

Re: High dose paracetamol metabolism in general surgical patients.

Dear Doctors

The Clinical Research Ethics Committee of the Cork Teaching Hospitals reviewed your correspondence at its recent meeting held on 6th January 2009.

The Committee approved Amendment 1 incorporating the following documents:

- Revised protocol version 2.0 dated 05/12/08.
- Revised Patient Information and Consent Form for Dr Donal Harney and Dr Brendan Conroy (Please put a new electronic version and date on these documents prior to use).

Yours sincerely

Dr Michael Hyland
Chairman
Clinical Research Ethics Committee
of the Cork Teaching Hospitals

APPENDIX 3 CLINICAL TRIALS APPROVAL FROM THE IRISH MEDICINES BOARD (IMB) AND EUROPEAN MEDICINES AGENCY (EMEA)

IRISH MEDICINES BOARD

1st May 2009

University College Cork,
College Road,
Co. Cork.

EUROPEAN COMMUNITIES (CLINICAL TRIALS ON MEDICINAL PRODUCTS FOR HUMAN USE) REGULATIONS, 2004

RE: CT number: CT 900/480/1 - Paracetamol
Case number: 2063053
EudraCT number: 2009-010818-30
Protocol number: 2.0
Title of trial: Paracetamol metabolism in general surgical patients

Dear Sirs,

The Irish Medicines Board has considered the application dated **2nd March 2009** seeking authorisation to conduct the above clinical trial.

On the basis of the evidence available, the application is acceptable.

Please note that the date of this letter is the date of authorisation of the trial.

Yours sincerely,

Sinéad Murphy
A person authorised in that behalf by the said Board

Bord Leigheasra na hÉireann
Kevin O'Malley House, Earlsfort Centre, Earlsfort Terrace, Dublin 2
Tel: 353-1-676 4971 Fax: 353-1-676 7836
Website: www.imb.ie

AUT-F0010-2

CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH

Patient Name: _____

Title of Protocol: Paracetamol metabolism in general surgical patients

Doctor Directing Research: Dr. Brendan Conroy Phone: (061)462222

You are being asked to participate in a research study. The staff at St John's Hospital study the nature of disease and attempt to develop improved methods of diagnosis and treatment. In order to decide whether or not you want to be a part of this research study, you should understand enough about its risks and benefits to make an informed judgement. This process is known as informed consent. This consent form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

NATURE AND DURATION OF PROCEDURE(S):

Paracetamol (Panadol®/Calpol®) is a medication that most people will have taken or have been given at sometime in their lives. It is generally considered to be a safe drug when used at its recommended dose. It is commonly used for minor ailments such as headaches, however it is also extremely useful for patients who have had surgery. Studies have shown that when it is used with stronger painkillers such as morphine, patients need less of these stronger drugs and hence have fewer side effects.

Because there is the general feeling that paracetamol is such a safe drug, some doctors are now using higher doses in patients who have had surgery. Currently there is little published research that this practice is safe and this is what we want to investigate. We expect the findings of this study to be very important for surgical care.

The aims of this study are

1 To see if paracetamol at higher than normal doses achieves better pain control in surgical patients.
2 To see if surgery changes the way the body metabolises paracetamol by sampling the blood and urine
3 To monitor patients liver function from the blood samples that are taken to determine if the higher doses of paracetamol have any effect on the liver.

If you choose to enrol in this study you may receive up to two and a half times the normal daily dose of paracetamol on the day before surgery and for up to 4 days after. We will also be using very low doses of four other medications to look at your liver's ability to metabolise medicines. For the purposes of the study and to ensure your safety up to 26-teaspoon size samples of blood will be collected over these 5 days as well as saliva and all your urine. This is to monitor for the breakdown products of paracetamol, if any of the harmful effects of paracetamol occur and to look at your genetics that relate to your liver's ability to metabolise medicines. We will also be asking you questions about your pain to see if you think your pain has improved

POTENTIAL RISKS AND BENEFITS:

This study may be using higher than normal doses of paracetamol. The current dose recommendations represent a dose that is known to be safe over long periods. The risks of adverse effects from paracetamol are thought to increase over time as the breakdown products build up. Because of this, higher doses over short periods are thought to be safe. However by using these higher doses there may be an increased risk of problems associated with paracetamol toxicity. You will receive regular monitoring for the duration of the study and in the unexpected event that toxicity does occur you will not participate

toxicity. An antidote for paracetamol toxicity is readily available and in the event of physical injury caused by taking part in the research project, compensation shall be available. We will let you know any significant new information that we find out during the course of the study that may relate to your willingness to continue.
We will be asking to take some blood samples. Some potential risks with taking blood include a bruise at the site of vein puncture; inflammation of the vein and possible infection but care will be taken to avoid complications. The blood sample will be used for the sole purpose of this study and the information we receive will not be presented in a form that will identify you.

By participating in this study you may receive higher doses of paracetamol, which has been shown in trials to reduce the need for more potent painkillers such as morphine. This may improve pain management. By using less of these more potent painkillers you are less likely to experience their adverse effects. Medications like morphine, in their usual doses, can often cause nausea, vomiting, constipation and hallucinations.
The information that we will record during this study will help show us whether these higher paracetamol doses are safe so that the benefits you experience can be passed on to the general population

POSSIBLE ALTERNATIVES:
Participation in this study is voluntary. Choosing not to participate in this study will not alter the treatment you receive. You can also choose withdraw from this study at any time without effecting your treatment.

AGREEMENT TO CONSENT

The research project and the treatment procedures associated with it have been fully explained to me. All experimental procedures have been identified and no guarantee has been given about the possible results. I have had the opportunity to ask questions concerning any and all aspects of the project and any procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. When required by law, government agencies and sponsors of the research may review the records of this research.

I understand that the investigators have such insurance as is required by law in the event of injury resulting from this research.

I, the undersigned, hereby consent to participate as a subject in the above described project conducted at St John's Hospital. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the doctor and fellow investigators listed above. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

Doctor/Co-investigator	Signature of Subject
Dr. Brendan Conroy / Philip Murphy (Clinical Research Pharmacist)	

Witness:_____ Date: _____ Time:_____

APPENDIX 5 CONSENT FORM- MERCY UNIVERSITY HOSPITAL

CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH

Patient Name:_____

Title of Protocol: Paracetamol metabolism in general surgical patients

Doctor Directing Research: Dr. Donal Harney Phone: (021)4271971

You are being asked to participate in a research study. The staff at Mercy University Hospital studies the nature of disease and attempt to improve methods of diagnosis and treatment. In order to decide whether or not you want to be a part of this research study, you should understand enough about its risks and benefits to make an informed judgement. This consent form gives detailed information about the research study, which will be discussed with you. Once you understand the study, you will be asked to sign this form if you wish to participate.

NATURE AND DURATION OF PROCEDURE(S):

Paracetamol (Panadol®/Calpol®) is a medication that most people will have taken or have been given at some time in their lives. It is generally considered to be a safe drug when used at its recommended dose. It is commonly used for minor ailments such as headaches; however it is also extremely useful for patients who have had surgery. Studies have shown that when it is used with stronger painkillers such as morphine, patients need less of these stronger drugs and hence have fewer side effects.

Because there is the general feeling that paracetamol is such a safe drug, some doctors are now using higher doses in patients who have had surgery. Currently there is little published information about the way the body handles medicines around the time of surgery and this may affect the safety of medicines, such as paracetamol. This is what we want to find out. We expect the findings of this study to be very important for surgical care.

The aims of this study are

To see if surgery changes the way the body metabolises paracetamol by sampling the blood and urine

To monitor patients liver function from the blood samples that are taken to determine if the higher doses of paracetamol have any effect on the liver.

If you choose to enrol in this study you will receive the standard, normal dose of paracetamol (1g every six hours). We will also be using very low doses of four other medications to look at your liver's ability to metabolise medicines. One of these medicines is caffeine so to be as accurate as possible we ask you not to drink tea, coffee or other caffeinated beverage while you are in the study. Herbal teas are provided.

We will also ask for some blood samples. Up to 24-teaspoon size samples of blood will be collected over the entire length of the study as well as all your urine. This is to monitor for the breakdown products of paracetamol, to ensure the paracetamol isn't unsafe and to look at your genetics that relate to your liver's ability to metabolise medicines. We will also be asking you questions about your pain to see if you think if it is controlled.

POTENTIAL RISKS AND BENEFITS:

You will receive the standard normal dose of paracetamol. This dose is known to be safe over long periods in health adults and is routinely used in surgical patients.

As with any medicine there is a small risk from the harmful effects of paracetamol, although these are extremely rare. You will receive regular monitoring for the duration of the study and in the unexpected event that toxicity does occur you will not receive any further paracetamol. An antidote for paracetamol is readily available. We will let you know any significant new information that we find out during the course of the study that may relate to your willingness to continue.

CONSENT BY SUBJECT FOR PARTICIPATION IN RESEARCH

We will be asking to take some blood samples. Some potential risks with taking blood include a bruise at the site of blood sampling, inflammation of the vein and possible infection but care will be taken to avoid complications. The blood sample will be used for the sole purpose of this study and the information we receive will not be presented in a form that will identify you.

By participating in this study you will receive regular doses of paracetamol, which has been shown in trials to reduce the need for more potent painkillers such as morphine. This may improve pain management. By using less of these more potent painkillers you are less likely to experience their adverse effects. Medications like morphine, in their usual doses, can often cause nausea, vomiting, constipation and hallucinations.

The information from this study will help show us whether paracetamol is safe so that any benefits you experience in pain relief can be passed on to others. By being involved in the study you will be contributing to what we expect to be important research for surgical care.

POSSIBLE ALTERNATIVES:
Participation in this study is voluntary. Choosing not to participate in this study will not alter the treatment you receive. You can also choose to withdraw at any time without effecting your treatment.

AGREEMENT TO CONSENT
The research project has been fully explained to me. All experimental procedures have been identified and no guarantee has been given about the results. I have had the opportunity to ask questions about the project and the procedures involved. I am aware that participation is voluntary and that I may withdraw my consent at any time. I am aware that my decision not to participate or to withdraw will not restrict my access to health care services normally available to me. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. I understand that the investigators have such insurance as is required by law in the event of injury resulting from this research.

I, the undersigned, hereby consent to participate as a subject in the above described project. I have received a copy of this consent form for my records. I understand that if I have any questions concerning this research, I can contact the investigators listed above. If I have further queries concerning my rights in connection with the research, I can contact the Clinical Research Ethics Committee of the Cork Teaching Hospitals, Lancaster Hall, 6 Little Hanover Street, Cork.

After reading the entire consent form, if you have no further questions about giving consent, please sign where indicated.

_____ _____
Signature of Subject Doctor/Co-investigator

Witness:_____ Date: _____ Time:_____

Paracetamol Metabolism Study

Patient Demographics

Hospital Number: Study Number:

Date of Birth: ☐ Male ☐ Female

Surname: First Name:

Study Day

☐ Preop ☐ Day 0 ☐ Day 1 ☐ Day 2 ☐ Day 3 ☐ Day 4

Medicine Administration Notes:

100mg Caffeine

30mg Dextromethorphan

250mg Chlorzoxazone

mg Midazolam 0.025mg/kg

1g Paracetamol

started at finished at

Plasma Sampling	*Due*	*Actual*	*Sample no.*
Predose			*1*
15 min			*2*
30 min			*3 / -*
60 min			*4 / 3*
90 min			*5 / -*
4 hours			*6 / 4*

Previous Urine Samples	*Start*	*Finish*	*Volume (mL)*
0-4h			
-4-8h			
-8-12h			
-12-16h			
-16-20h			
-20-24h			

Previous Paracetamol Dose timing	*Due*	*Actual*
-6		
-12		
-18		

APPENDIX 7 FLUID BALANCE SHEET

PARACETAMOL METABOLISM IN GENERAL SURGICAL PATIENTS FLUID CHART

MRN_____ DOB_____

Day 0	Fluid INTAKE			Fluid OUTPUT		Obs	
IV meds		mL	Urine		mL	Temp	
IV fluids		mL	NG Tube		mL	BP	
Epidural		mL	Drains		mL	NRS	High
Blood		mL	Intraoperative		mL		Low
Oral		mL			mL		Mean
TOTAL			TOTAL				
		NET	+/-				
Epi Rates							

Day 1	Fluid INTAKE			Fluid OUTPUT		Obs	
IV meds		mL	Urine		mL	Temp	
IV fluids		mL	NG Tube		mL	BP	
Epidural		mL	Drains		mL	NRS	High
Blood		mL	Intraoperative		mL		Low
Oral		mL			mL		Mean
TOTAL			TOTAL				
		NET	+/-				
Epi Rates							

Day 2	Fluid INTAKE			Fluid OUTPUT		Obs	
IV meds		mL	Urine		mL	Temp	
IV fluids		mL	NG Tube		mL	BP	
Epidural		mL	Drains		mL	NRS	High
Blood		mL	Intraoperative		mL		Low
Oral		mL			mL		Mean
TOTAL			TOTAL				
		NET	+/-				
Epi Rates							

Day 3	Fluid INTAKE			Fluid OUTPUT		Obs	
IV meds		mL	Urine		mL	Temp	
IV fluids		mL	NG Tube		mL	BP	
Epidural		mL	Drains		mL	NRS	High
Blood		mL	Intraoperative		mL		Low
Oral		mL			mL		Mean
TOTAL			TOTAL				
		NET	+/-				
Epi Rates							

Day 4	Fluid INTAKE			Fluid OUTPUT		Obs	
IV meds		mL	Urine		mL	Temp	
IV fluids		mL	NG Tube		mL	BP	
Epidural		mL	Drains		mL	NRS	High
Blood		mL	Intraoperative		mL		Low
Oral		mL			mL		Mean
TOTAL			TOTAL				
		NET	+/-				
Epi Rates							

APPENDIX 8 SURGICAL NOTES SHEET

PARACETAMOL METABOLISM IN GENERAL SURGICAL PATIENTS
SURGICAL NOTES
MRN_____ DOB_____

Surgeon	Anaesthetist

Preoperative Diagnosis

Operation

Anaesthetic Drugs

Duration	Gut Handling	Minimal Intermediate Extensive

Associated events/complications

APPENDIX 9 BASELINE MEDICAL INFORMATION SHEET

PARACETAMOL METABOLISM IN GENERAL SURGICAL PATIENTS
BASELINE DATA CHART
MRN_____ DOB_____

Patient Name | Date of Birth | Place of Birth
Ethinicity

Occupation
Previous Work history

Weight | Height

Smoking history

Ethanol history

Diet history

Medical History
General/Constitutional

Skin

Eyes/Ears/Nose/Mouth/Throat

Cardiovascular

Respiratory

Gastrointestinal

Genitourinary

Musculoskeletal

Neurologic/Psychiatric

Allergic/Immunologic/Lymphatic/Endocrine

Usual medicines

Name	Dose	Frequency	Since?		

Lab data

LFT's & U&E's	AST			SCr	
	ALT			Urea	
	GGT			Na	
	Bilirubin			K	
Coag	INR			Glucose	
				Fasting?	

Department of Health & Children
AN ROINN SLÁINTE AGUS LEANAÍ

24th April 2009

Philip Murphy
Clinical Research Pharmacist
School of Pharmacy
Cavanagh Pharmacy Building
University College Cork
College Road
Cork

MISUSE OF DRUGS ACTS, 1977 AND 1984

MISUSE OF DRUGS REGULATIONS 1988

Dear Mr. Murphy,

I refer to your letter of 6th April 2009, concerning your proposed importation of reference standards containing **Midazolam (50xMidazolam 200mg Vials)** from ***Roche Diagnostics GmbH, Sandhofer Strasse 116, 68305 Mannheim, Germany.***

I wish to state that **Midazolam** and preparations containing **Midazolam** including medicinal products, are contained in Schedule 4 to the Misuse of Drugs Regulations 1988 (as amended). I wish to state that since your company is registered under Article 9(3) of the above Regulations to enable it to possess drugs specified in Schedule 4 including medicinal products, you have authority to possess and that no licence to **import or export** is currently required.

Accordingly there is no objection to your proposed **importation** of medicinal products containing **Midazolam (50xMidazolam 200mg Vials)** during the period ending **31st July 2009**.

I wish to request however, that, in the importation of **Midazolam**, your company adhere to the assessment limits set out in the Circulars issued to you from time to time by this Department in connection with the international trade in psychotropic substances.

Yours sincerely,

Pátrick Keating
Drugs Section

Cuirfear fáilte roimh chomhfhreagras i nGaeilge
Department of Health & Children
Hawkins House Dublin 2
Teach Haicin Baile Átha Cliath 2
Tel (01) 635 4000
Fax (01) 635 4001
Email info@health.gov.ie
Web www.dohc.ie

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Department of Health & Children
AN ROINN SLÁINTE AGUS LEANAÍ

BD 14/5/411

22nd April 2009

Philip Murphy
Clinical Research Pharmacist
School of Pharmacy,
Cavanagh Pharmacy Building,
University College Cork,
Cork

Form CDR. 3C

Registration No: M17

Misuse of Drugs Acts, 1977 and 1984

Misuse of Drugs Regulations 1988

Dear Mr. Murphy,

I am directed by the Minister for Health and Children to inform you that your name has been entered in the register kept by him for the purpose of Article 9(3) of the Misuse of Drugs Regulations, 1988 (S.I. No. 328 of 1988) in respect of your premises at:-

School of Pharmacy,
Cavanagh Pharmacy Building,
University College Cork,
College Road,
Cork

By virtue of this registration you are entitled to have in your possession such drug(s) as are specified in Condition No. 1 below. Such registration is subject to you complying with the following conditions:-

1. This registration only relates to the following controlled drugs

_____Midazolam_____

including any stereoisomeric forms and the salts thereof and any preparation or other product containing any portion of the drug(s) or its/their salts (hereinafter called "the drug(s)").

Cuirfear fáilte roimh chomhfhreagras i nGaeilge
Department of Health & Children
Hawkins House Dublin 2
Teach Haicin Baile Átha Cliath 2
Tel (01) 635 4000
Fax (01) 635 4001
Email info@health.gov.ie
Web www.dohc.ie
Printed on Recycled Paper

2. The drug(s) shall be used for the sole purpose of **research** conducted at **School of Pharmacy, Cavanagh Pharmacy Building, University College Cork, Cork.**

3. This registration does not authorise the obtaining of the drug(s) for any purpose other than the purpose specified in Condition No. 2.

4. You shall ensure that all stocks of the drug(s) shall at all times be in the charge of a responsible servant appointed by you for this purpose.

5. In addition to the provisions of the Misuse of Drugs Regulations 1988, in regard to the keeping of records, you shall keep a record of each quantity of the drugs imported. Such records shall include the date of each transaction, the necessary qualitative and quantitative details and the country of origin.

6. You shall inform the Minister for Health and Children in the event of ceasing to carry on business in respect of the drug(s) at the registered premises.

As a consequence to this registration, you may from time to time receive requests from the Minister for Health and Children, under Article 21 of the Misuse of Drugs Regulations 1988 for information relating to the amounts of drugs obtained and used at the registered premises. Since such requests must be responded to within fourteen days, the keeping of appropriate records will facilitate compliance.

Yours sincerely,

p.p. C. Brady

Mary O'Reilly
Drugs Section

Roche

Mr Philip Murphy
School of Pharmacy
Cavanagh Pharmacy Building
St John's Hospital
University College Cork
College Rd
CORK
IRELAND

Mannheim, March 23, 2009
Your request for Midazolam

Dear Mr Murphy,

Thank you very much for your enquiry for Midazolam.

As already outlined by e-mail, please fill in the enclosed form "agreement" and return both originals (duly signed and with a stamp of your institution) to me as soon as possible:

Ms Andrea Schuster
Roche Diagnostics GmbH
TG-QCS
Sandhofer Straße 116
68305 MANNHEIM
GERMANY
Tel. +49/621-759-9298
Fax. +49/621-759-1304
mailto:andrea.schuster@roche.com.

In addition, kindly also send us your import permit for the amount of Midazolam you wish to receive from us. When applying for your import permit, please make sure, the enclosed memo is observed. On receipt of the forms and your import permit, we will process your order.

In case of any query, please do not hesitate to contact me.

Yours sincerely,

Andrea Schuster

Andrea Schuster

Enclosure

U:\My Documents\SCHUSTA3\Forscher_externe Anfragen\MTA\Irland\2009\Cork\Uni\Murphy 230309.doc

Roche Diagnostics GmbH
Sandhofer Strasse 116
D-68305 Mannheim
Telefon +49 - 621 - 759 0
Telefax +49 - 621 - 759 28 90

Registergericht Mannheim
HRB 3962
Aufsichtsrat:
Dr. Severin Schwan, Vorsitzender

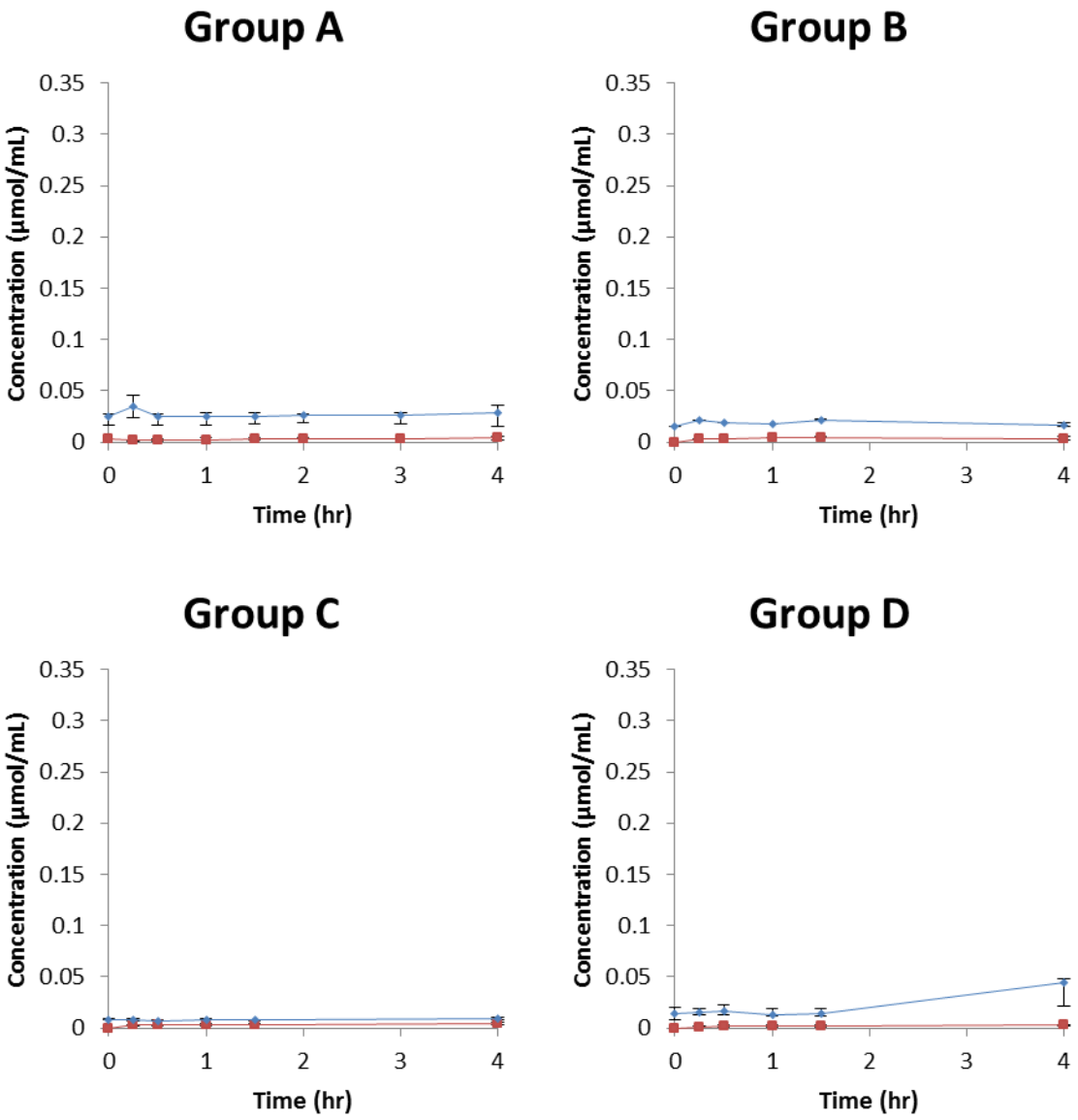
Geschäftsführung:
Thomas Schmid, Sprecher
Jürgen Redmann,
Peter-Claus Schiller,
Prof. Dr. Dr. Klaus Strein,
Franz T. Walt

12111683001-04/2008

APPENDIX 13 ADDITIONAL PLASMA CONCENTRATION GRAPHS

Paracetamol Cysteine

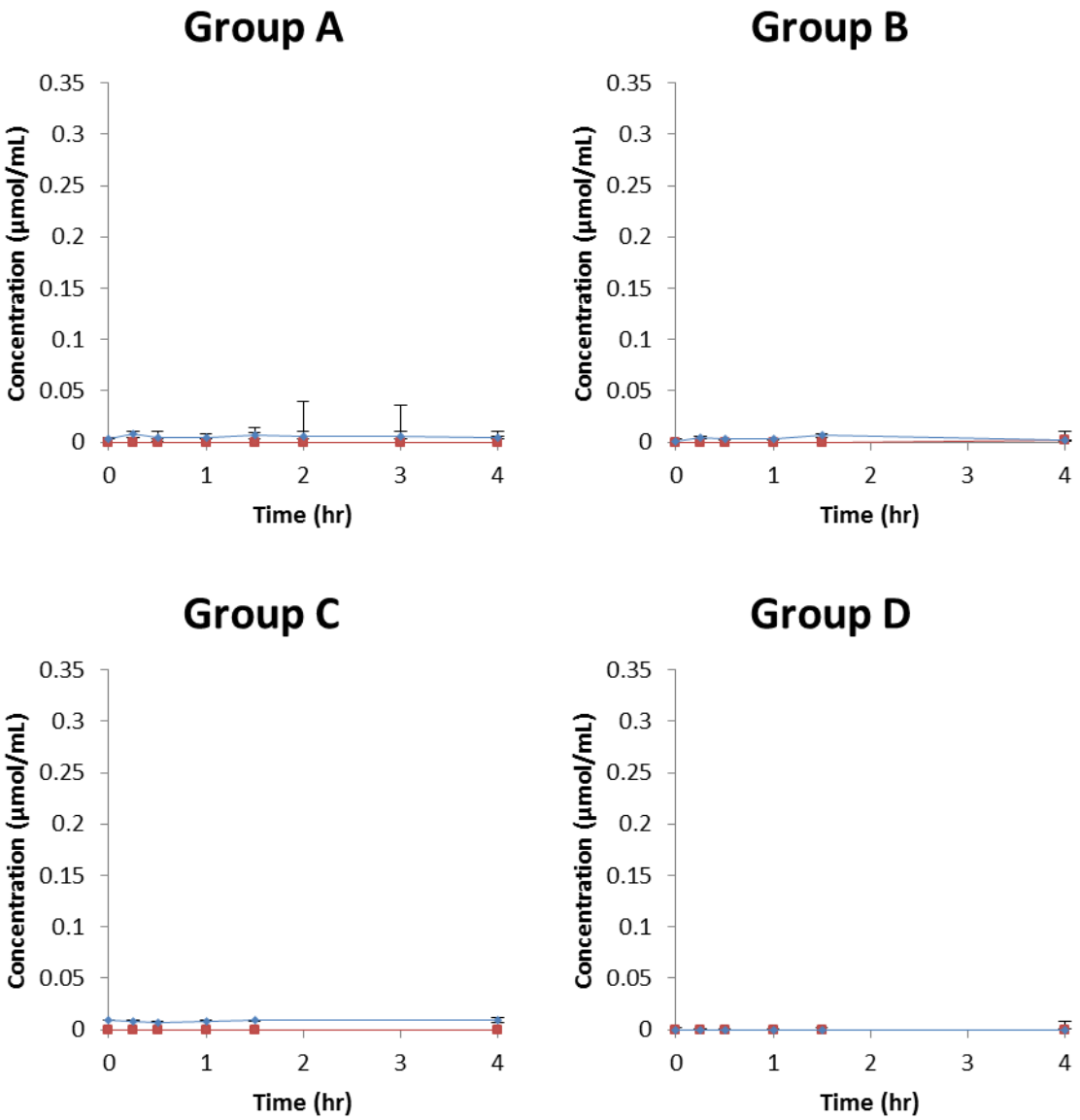
Median plasma paracetamol cysteine concentrations are presented as median +/- quartiles preoperatively (red line) and day 1 postoperatively (blue line).



There was very little change in concentration over the dose intervals. Group A paracetamol cysteine concentrations were noticeably higher following surgery, and in Group D some elevation was seen towards the end of the sampling period on the postoperative day.

Paracetamol Mercapturate

Median plasma paracetamol mercapturate concentrations are also presented as median +/- quartiles preoperatively (red line) and day 1 postoperatively (blue line).



Paracetamol mercapturate was only found in very low concentrations in the plasma. There appears to be no effect of dose or surgery.

APPENDIX 14 REVIEW OF β-GLUCURONIDASE METHODS

Ref	Drug	Sample Volume	Amount β-glu	Vehicle	pH	Temp	Time	IS	Clean Up
(Chen *et al.* 2002)	CZX	0.5mL plasma	1000 1mL	0.2M NaAc	4.75	37	4	Phenacetin 50uL 20umol/L	5ml EE
(Chen *et al.* 2003)	CZX	0.5mL plasma	1000 1mL	NaAc	4.75	37		Phenacetin 50uL 20umol/L	5mL DEE
(Chen *et al.* 2003)	MDZ	1mL plasma	200	NaAc	4.75				DEE
(Daali *et al.* 2008)	DX	0.25mL urine	2000 1mL vs. 85uL 10N HCl	0.1M NaAc	5	37	O/N	5uL Levallorphan 2.3 nmol/mL	↑pH 90uL KOH 10N and 500uL NA2CO3 1M. 5mL Hex:EA (50:50) for 15 min. Spin. Add 0.5mL OPA 50mM to organic
(Desiraju *et al.* 1983)	CZX	0.5mL urine	?	NaAc	4.6	37	O/N	N-butyl-p-aminophenol	↑pH 7.4, 1mL KP, 5mL EA X2. Dry. 50uL MeOH
(Dreisbach *et al.* 1995)	CZX	0.5mL plasma	1200				O/N	6 fluorochlorzoxazone	DEE
(Frye *et al.* 1997)	4'OH mephenytoin	0.5mL urine		500uL NaAc 1M	5	37	3	100uL 0.05mg/mL phensuximide	5mL DEE. Dry 500uL 40% MeOH. Inj 100uL
(Frye *et al.* 1996)	CZX	0.5mL plasma	1000/0.5mL 0.2% NaCl	1mL NaAc 0.2M	4.75	37	3	Incubation completed in 2hours with 1000u	3mL DEE. Dry. 200uL MP inject 50

Ref	Drug	Sample Volume	Amount β-glu	Vehicle	pH	Temp	Time	IS	Clean Up
(Girre *et al.* 1994)	CZX	0.5mL plasma/ urine (dil 1:100)	2000 (20uL)			37	O/N		4mL 0.6N PCA, 2 X 4mL EA
(Gurley *et al.* 2005)	Cocktail	250uL serum	500/250uL	0.2M NaAc	5	37	2.5		
(Haufroid *et al.* 2005)	CZX	500uL plasma	20uL	500uL 2M NaAc	4.5	37	O/N		500uL removed. 1mL 1.2M PCA. Spin. 1mL extracted with 2mL EA. Dry 1.5mL. 500uL MP inj 20uL
(Kashuba *et al.* 1998b)	DEX	3mL urine	100MU			37	18		↑pH 12 SPE
(Liangpunsakul *et al.* 2005)	CZX	0.5mL urine	2000			37	1		
(Lutz *et al.* 2004)	DEX	250uL urine+ 250uL MeOH						5uL Levallorphan 2.3 nmol/mL	
(Lutz *et al.* 2008)	DEX	500uL urine							Column switchin
(Manyike *et al.* 2000)	PA	500uL urine	40uL	500uL NaAc 0.125M	5	37	O/N		Filter
(Mishin *et al.* 1998)	CZX	500uL plasma	200/0.1mL 0.2%NaCl	1mL NaAc 0.2mM	4.75				5mL DEE. Dry 3.5mL. Wet with 250uL 40% ACN. Sonicate

Ref	Drug	Sample Volume	Amount β-glu	Vehicle	pH	Temp	Time	IS	Clean Up
(O'Shea *et al.* 1994)	CZX	2mL plasma+ 0.5mL H2O	200	1mL 1:1 1M NaAc: 1M glacial acetic acid	5	20	O/N		SPE
(Park *et al.* 1984)	DEX	2mL urine	0.2mL	50mM NaAc pH 3.5	5-5.5	37	18		↑pH 12 1M NaOH. 6mL Hex mix 40min. Freeze off aqueous. Back extract with 0.2mL 0.1M HCl mix 30min. Repeat for aqueous layer
(Petsalo *et al.* 2008)	Cocktail	125uL urine	750U in 125uL 0.9% NaCl	250uL 0.1M NaAc	5	37	6	phenacetin	480uL spiked with 20uL of IS in ACN
(Sarich *et al.* 1997)	ME	200uL urine	10uL	1mL Ac 200mM	5	37	O/N		2 X 3mL 2:1 EA:DEE. Dry. 0.5mL 20% ACN inj 5uL
(Scott *et al.* 1999)	Cocktail	0.5mL	1000/0.5mL 0.2% NaCl	1mL 0.2M NaAc	4.75	37	3		SPE
(Stiff *et al.* 1993)	CZX	0.5mL plasma	1000/0.5mL 0.2% NaCl	1mL NaAc 0.2M	4.75	37	3		SPE
(Streetman *et al.* 1999)	DEX	3mL urine	100mU			37	18		↑pH 12, SPE
(Varela *et al.* 2008)	CZX		2000			37	1		
(Wang *et al.* 2003)	CZX	0.5mL urine	2000	0.2mL NaAc 0.2M	5	37	1	Phenacetin	+10uL sodium azide0.6M

Ref	Drug	Sample Volume	Amount β-glu	Vehicle	pH	Temp	Time	IS	Clean Up
(Zhang *et al.* 2008)	Cocktail	0.5mL urine	1250 250uL in 20mM ammonium formate pH 4.75	.25mL 20mM AcA	4.75	37	12		
(Zhu *et al.* 2001)	CZX	0.5mL plasma	?			37	3	10uL 1mM Phenacetin	5mL DEE

Abbreviations used: AcA- Acetic Acid; CA-Caffeine; CZX-Chlorzoxazone; DM-Dextromethorphan; EA-Ethyl acetate; EE-Ethyl ether; DEE-Diethyl ether; KP-Phosphate buffer; MDZ-Midazolam; MeOH-Methanol; ME-Mephenytoin; MP-Mobile Phase; NaAc-Sodium Acetate; O/N-Overnight; OPA-Orthophosphoric Acid; PA-Paracetamol; PCA-Perchloric acid.