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Novel Biphasic Lipolysis Method To Predict *in Vivo* Performance of Lipid-Based Formulations

Patrick J. O'Dwyer,* Karl J. Box,* Niklas J. Koehl, Harriet Bennett-Lenane, Christos Reppas, Rene Holm, Martin Kuentz, and Brendan T. Griffin

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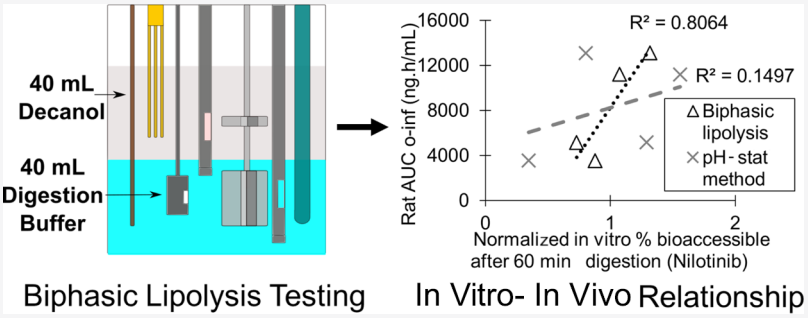

ABSTRACT: The absence of an intestinal absorption sink is a significant weakness of standard *in vitro* lipolysis methods, potentially leading to poor prediction of *in vivo* performance and an overestimation of drug precipitation. In addition, the majority of the described lipolysis methods only attempt to simulate intestinal conditions, thus overlooking any supersaturation or precipitation of ionizable drugs as they transition from the acidic gastric environment to the more neutral conditions of the intestine. The aim of this study was to develop a novel lipolysis method incorporating a two-stage gastric-to-intestinal transition and an absorptive compartment to reliably predict *in vivo* performance of lipid-based formulations (LBFs). Drug absorption was mimicked by *in situ* quantification of drug partitioning into a decanol layer. The method was used to characterize LBFs from four studies described in the literature, involving three model drugs (i.e., nilotinib, fenofibrate, and danazol) where *in vivo* bioavailability data have previously been reported. The results from the novel biphasic lipolysis method were compared to those of the standard pH-stat method in terms of reliability for predicting the *in vivo* performance. For three of the studies, the novel biphasic lipolysis method more reliably predicted the *in vivo* bioavailability compared to the standard pH-stat method. In contrast, the standard pH-stat method was found to produce more predictive results for one study involving a series of LBFs composed of the soybean oil, glyceryl monolinoleate (Maisine CC), Kolliphor EL, and ethanol. This result was surprising and could reflect that increasing concentrations of ethanol (as a cosolvent) in the formulations may have resulted in greater partitioning of the drug into the decanol absorptive compartment. In addition to the improved predictivity for most of the investigated systems, this biphasic lipolysis method also uses *in situ* analysis and avoids time- and resource-intensive sample analysis steps, thereby facilitating a higher throughput capacity and biorelevant approach for characterization of LBFs.

KEYWORDS: *lipolysis, biphasic dissolution, lipid-based formulations, in vitro digestion, absorption, in vitro–in vivo relationship (IVIVR)*

1. INTRODUCTION

The increasing trend toward the development of poorly water-soluble drug candidates has created significant challenges for pharmaceutical scientists to formulate a medicinal product with adequate oral bioavailability. One way to overcome the solubility limitations of emerging drug candidates is through the use of lipid-based formulations (LBFs).[1−5] Although LBFs have become an important bioenabling approach in development, the range of LBF classes and excipient considerations pose many challenges with a high level of uncertainly with respect to the selection of an optimal LBF formulation design.[6−8] Guidelines on the classification of LBFs[9] and risk-based assessments of LBFs[10] have been proposed. However, although these guidelines are useful, there is still a lack of *in vitro* methods that reliably can predict or even rank the *in vivo* performance of LBFs. This lack of predictive and high-throughput *in vitro* tests hampers the efficient development of LBFs. The current *in vitro* setups to assess the performance of

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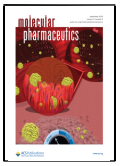

LBFs have many limitations and often produce results which are quite distinct from the *in vivo* performance.[1,11,12]

Commonly, LBFs are tested for their dispersibility, micellar size, and behavior upon digestion, in line with the lipidic formulation classification system proposed by Pouton.[13] The pH-stat lipolysis method is the most widespread standard method for *in vitro* assessment of LBFs.[14] It typically involves the dispersion of an LBF in a medium representing the fasted intestinal environment with the addition of digestive lipases while controlling pH throughout the experiment by the addition of a NaOH solution. After centrifugation of samples, the drug in the different digestive phases (aqueous micellar phase, solid precipitate, and the lipid phase) can be quantified. The aqueous micellar phase contains the solubilized drug, bile salts, phospholipids, cholesterol, and digested lipids. The solid precipitate comprises the precipitated drug and insoluble Ca^{2+}/fatty acid (FA) soaps. The lipid phase consists of the drug and undigested or partially digested lipids. However, the standard pH-stat method has three major limitations. It is lacking an absorptive sink, so the method does not mimic absorption of the drug. As a result, drug concentrations in the digest media are likely to exceed physiological concentrations within the intestine, potentially leading to an overestimation of the risk of luminal precipitation and consequentially leading to poor *in vitro*−*in vivo* relationships (IVIVRs).[11,15] In addition, as the majority of pH-stat experiments are only conducted in conditions mimicking the intestine, they can fail to adequately capture supersaturation or precipitation of the drug upon transition from the stomach into the small intestine. This is especially important for weakly basic drugs, which are much more soluble at gastric pH and prone to precipitate upon entry into the small intestine. Finally, the pH-stat experiments are very laborious involving time-consuming steps, including centrifugation of samples and off-line high-performance liquid chromatography (HPLC) analysis. This low-throughput capacity of the setup is particularly limiting as a screening tool for new LBFs in an industrial development setting, and the sampling with off-line analytics allows only for a limited time resolution to analyze kinetic concentration changes.

Several groups have proposed various modifications in an effort to improve the biorelevance of *in vitro* lipolysis testing. Fernandez *et al.* proposed a two-stage lipolysis procedure with the incorporation of a gastric sector at pH 5.5 using recombinant dog gastric lipase before transitioning to intestinal conditions.[16−18] Sassene *et al.* also proposed the incorporation of a gastric sector using gastric lipase enzymes into the lipolysis setup.[19] The use of an organic layer to mimic absorption in the small intestine during *in vitro* testing has been employed using a compendial dissolution apparatus to assess LBFs.[20,21] As the drug partitions into the organic layer, this helps to maintain sink conditions in the aqueous phase, thus facilitating ongoing dissolution of any undissolved or precipitated drug present. However, these setups did not simulate the digestion process and only examined the release of the drug from the formulation. Therefore, the impact of LBF digestion on supersaturation and precipitation was not considered by these methods. Absorption of the drug in the small intestine has been incorporated into lipolysis testing using both artificial and cellular membranes. Bibi *et al.* employed a PermeaPad system to simulate absorption in the intestine during lipolysis testing using a biomimetic membrane,[22] whereas Keemink *et al.*[23] and Alvebratt *et al.*[24] used a Caco-2 cell system to simulate absorption in the small intestine during lipolysis testing. Finally, by integrating data from a standard pH-stat setup, Stillhart and Kuentz used an *in silico* biopharmaceutical modeling approach to predict the impact of digestion of LBFs under simulated continuous absorption sink conditions.[12]

Despite these recent innovations in lipolysis testing, there is still a need for a rapid and biorelevant *in vitro* lipolysis method to screen LBFs during the formulation development phase to address the change of pH (gastric to intestinal), lipid digestion, and concurrent permeation through the intestinal epithelium. The objective of this study was therefore to develop a new and quick two-stage small-scale biphasic *in vitro* lipolysis protocol, which incorporates dispersion in the gastric sector, gastrointestinal (GI) transit with the absorption of the drug and possible digestion products in the small intestine to act as an effective screening tool for novel LBFs. The addition of a decanol layer for the intestinal sector was intended to mimic absorption as the drug and post digestion products partition into the decanol layer. Biphasic dissolution testing using an inForm instrument (Pion Inc., MA, USA) has previously been employed to test other bioenabling formulations,[25,26] with strong correlations found to *in vivo* data. The high level of automation on the platform, including *in situ* pH control and drug quantification, reduces human experimental errors and makes the setup interesting for lipolysis testing. Evaluation was performed by relating *in vitro* data collected with the novel methodology with four previously published studies that characterized a range of LBFs *in vitro* and *in vivo*.[11,27−29] This included studies involving one weakly basic drug (nilotinib—pK_a 2.1, 5.4)[27] and two neutral drugs (fenofibrate and danazol) with corresponding *in vivo* pharmacokinetic (PK) data from rats,[27] pigs,[11] dogs,[28] and humans.[29] The results from the two-stage biphasic lipolysis test were compared to both the *in vivo* data and the most commonly applied *in vitro* methodology (pH-stat method) of identical LBFs.

2. MATERIALS

Nilotinib and danazol were obtained from Kemprotec Ltd. (Cum, UK). Fenofibrate and decanol were obtained from Acros Organics (Geel, Belgium). Ethanol, olive oil, Tween 85, Kolliphor EL, soybean oil, tris(hydroxymethyl)aminomethane, calcium chloride dihydrate, taurodeoxycholic acid (NaTDC), and pancreatic lipase (8 × USP) were obtained from Sigma-Aldrich (Dorset, UK). Kolliphor RH 40 was obtained from BASF (Ludwigshafen, Germany). Miglyol 812 was sourced from IOI Oleochemical (Witten, Germany). Super refined polysorbate 20-LQ and Tween 80 HP were obtained from Croda Inc. (NJ, USA). Maisine CC and Peceol were kindly donated from Gattefossé (Saint-Priest, France). Capmul MCM and Captex 1000 were kindly donated by ABITEC Corporation (Ohio, USA). SIF powder was obtained from biorelevant.com (London, UK). Milli-Q water (18.2 MΩ cm^{-1}) was used for the experimental work. All other chemicals and solvents were of analytical grade or HPLC grade and purchased from VWR, UK.

3. METHODS

3.1. Preparation of Media. Concentrated intestinal media (10× concentrated) were prepared using SIF powder and 20 mM Tris buffer, containing 1.4 mM calcium chloride and 150 mM NaCl. SIF powder (1.12 g) was added to 25 mL of the buffer and stirred until the powder was dissolved. This was

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Table 1. Overview of the Composition of the (a) Nilotinib LBFs,[27] (b) Fenofibrate LBFs from the Pig Study,[11] (c) Fenofibrate LBFs from the Human Study,[29] and (d) Danazol LBF[28]

(a) nilotinib LBF from the rat study[27]				
formulation name	Peceol	Capmul MCM	Captex 1000	olive oil
formulation category	type I	type I	type I	type I
formulation type	suspension	suspension	suspension	suspension
Peceol (%)	100	-	-	-
Capmul MCM (%)	-	100	-	-
Captex 1000 (%)	-	-	100	-
olive oil (%)	-	-	-	100
drug concentration (mg/mL)	10	10	10	10
LBF dose per experiment (g)	1	1	1	1
nilotinib dose (mg) per lipolysis experiment	10.62	10.97	10	10.64

(b) fenofibrate LBF from the pig study[11]			
formulation name	type IIIA MC	type IIIA LC	type IIIB/IV
formulation category	type IIIA	type IIIA	type IIIB/IV
formulation type	solution	solution	solution
Miglyol (%)	40	-	-
olive oil (%)	-	40	-
Tween 85 (%)	40	40	67
Cremophor RH (%)	20	20	33
fenofibrate concentration (mg/g)	80	80	80
LBF dose per experiment (g)	0.4	0.4	0.4
fenofibrate dose (mg) per lipolysis experiment	32	32	32

(c) fenofibrate LBF from the human study[29]			
formulation name	E5 (80)	E5 (20)	MDS
formulation category	type IIIA	type IIIA	type IIIA
formulation type	solution	solution	suspension
fenofibrate (%)	10	10	20
Myritol 318 (%)	25	25	27.5
TPGS (%)	12	12	9.5
Tween 20 (%)	-	48	38
Tween 80 (%)	48	-	-
H_2O (%)	5	5	5
LBF dose per experiment (g)	0.54	0.54	0.27
fenofibrate dose (mg) per lipolysis experiment	54	54	54

(d) danazol LBF from the dog study[28]				
formulation name	F1	F2	F3	F4
formulation category	type IIIA	type IIIA	type IIIB	type IIIB/IV
formulation type	solution	solution	solution	solution
soybean/Maisine CC (1:1 w/w) (%)	60	37.5	18	-
Kolliphor EL (%)	30	55	64	65
ethanol (%)	10	7.5	18	35
drug load (mg/g) (80% saturated solubility)	11.8	16.1	17.8	18
LBF dose per experiment (g)	1	1	1	1
danazol dose (mg) per lipolysis experiment	11.8	16.1	17.8	18

made up to volume (50 mL) with the buffer and left to stand for 2 h before usage. The gastric media consisted of dilute HCl at pH 2, containing 1.4 mM calcium chloride and 150 mM NaCl.

The porcine pancreatic extract was prepared freshly immediately prior to each lipolysis experiment by adding 1 g of porcine pancreatic enzymes (8 × USP) to 5 mL of blank media (consisting of 1.4 mM calcium chloride and 150 mM NaCl) at 5 °C and vortexed thoroughly. This mixture was then centrifuged at 2800*g* for 15 min, with the supernatant used as the porcine pancreatic extract for the lipolysis experiments.

3.2. Preparation of the LBFs. The respective LBFs were prepared according to the methods outlined in the original research articles,[11,27−29] with the composition of each formulation shown in Table 1a−d. The nomenclature assigned to each formulation in this study replicates the original research articles. The drug dose-to-lipid ratios were maintained exactly as per the administered LBFs in the *in vivo* testing. The dose of each LBF delivered was scaled down relative to the 40

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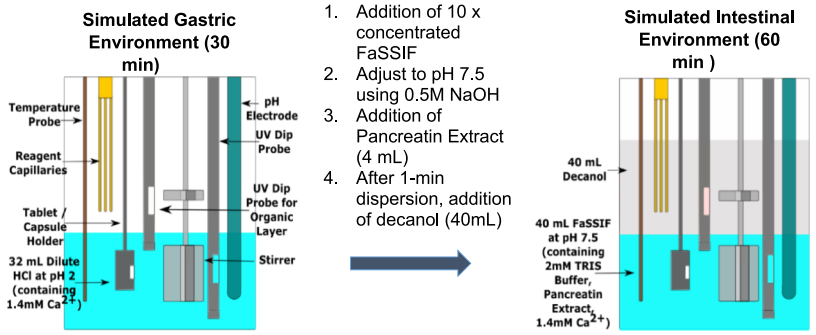

Figure 1. Schematic of the biphasic lipolysis dissolution setup.

Table 2. Comparison of the Different *in Vitro* Lipolysis Experimental Conditions

	biphasic lipolysis	pH stat (this study)	Koehl *et al.* pH stat[27]	Griffin *et al.* pH stat[11]	Cuine *et al.* pH stat[28]
surfactant and bile salt concentration	sodium taurocholate (3 mM), phosphatidylcholine (0.75 mM)	sodium taurodeoxycholate (3 mM), phosphatidylcholine (0.75 mM)	sodium taurodeoxycholate (3 mM), phosphatidylcholine (0.75 mM)	taurocholic acid (5 mM), phosphatidylcholine (1.25 mM)	sodium taurodeoxycholate (5 mM), phosphatidylcholine (1.25 mM)
aqueous volume (mL) after addition of pancreatic enzymes	40	40	60	100	40
intestinal pH	7.5	7.5	7.5	7.5	7.5
buffer	Tris (2 mM)	Tris (2 mM)	Tris (2 mM)	Tris (50 mM)	Tris (50 mM)
sodium chloride concentration (mM)	150	150	150	150	150
calcium chloride concentration (mM)	1.4	1.4	1.4	5	5
absorption component	decanol layer (40 mL)	N/A	N/A	N/A	N/A
gastric sector	dilute HCl	N/A	N/A	N/A	N/A

mL lipolysis volume used in this study, keeping the same lipid formulation-to-volume ratio from any previous pH-stat testing carried out on these LBFs. For example, 1.5 g of nilotinib LBF in 60 mL of digestion media in the pH-stat experiment was scaled down to 1 g of LBF to 40 mL of media in the biphasic lipolysis experiment.

Briefly, the nilotinib suspensions were prepared by adding 10 mg to 1 mL of the lipid excipient with stirring overnight at 37 °C.[27] The fenofibrate lipid solutions based on the pig study were prepared by adding fenofibrate to the lipid excipients and stirring at 50 °C for 30 min before stirring at 37 °C overnight.[11] To prepare the fenofibrate formulations from the human study (two solutions and one suspension), the excipients were added into a glass vial and heated to 80 °C.[29] Fenofibrate for the micronized dispersion system (MDS) formulation was prepared by sieving fenofibrate through a 40 μm analytical sieve. The sieved fenofibrate was added to the lipid blends and stirred at 37 °C overnight. The danazol lipid solutions were prepared by adding the lipid excipients and danazol into a vial, followed by stirring at 50 °C for 10 min.[28] This was subsequently cooled to ambient temperature before ethanol was added and stirred overnight at 37 °C.

3.3. Biphasic Lipolysis. The biphasic lipolysis experiments were carried out using the inForm (Pion Inc.) instrument at 37 °C, with the experimental setup shown in Figure 1 and the dimensions of the setup given in the Supporting Information (Table S1). The LBFs were initially dispersed into 32 mL of simulated gastric media (pH 2), composed of dilute HCl, 1.4 mM calcium chloride, and 150 mM NaCl. Stirring was set to 300 rpm for the gastric sector to ensure adequate dispersion of the LBFs.

After 30 min to simulate the transition into an intestinal environment, concentrated FaSSIF (4 mL) was added. The pH of the media was adjusted to 7.5 using 0.5 M NaOH, with the pH controlled *in situ* throughout the intestinal sector to 7.5 ± 0.1 by the inForm instrument using 0.5 M NaOH. The composition of the final aqueous intestinal fluid contained sodium taurocholate (3 mM), phosphatidylcholine (0.75 mM), Tris base (2 mM), NaCl (150 mM), and calcium chloride (1.4 mM). Digestion was initiated by addition of the pancreatic extract (4 mL), which was dispersed for 1 min prior to the addition of the decanol layer (40 mL). Stirring was temporarily stopped, while decanol was added into the vessel. Stirring was set to 100 rpm for the intestinal sector. After 60 min, the pH was titrated back to pH 9 to determine the release of nonionized free FAs.

The drug concentrations in the decanol layer were quantified using an *in situ* fiber-optic multiwavelength dip probe,[24] with the relevant detection wavelengths shown in the Supporting Information (Table S2). A linear calibration curve was established between drug concentration and UV absorbance (R^2 > 0.99). Calibration standards were analyzed in the same medium as the experimental setup. An excellent fit was found when comparing spectra recorded from the experimental runs with the standard spectra, with a root-mean-square deviation of <0.01 in each case. This confirmed the suitability of the *in situ* fiber-optic multiwavelength dip probe for quantifying the drug concentration under the proposed experimental conditions.

A summary of the biphasic lipolysis experimental conditions and a comparison with the pH-stat experimental conditions from the original research articles[11,27,28] is shown in Table 2.

3.4. pH-Stat Testing of Fenofibrate LBFs Previously Studied in Humans. As pH-stat testing had not previously been carried out on the fenofibrate LBFs previously studied in humans, these were completed to facilitate a comparison

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between all the results from the biphasic lipolysis setup with the pH-stat method. The experimental protocol was adapted from Koehl *et al.*,[27] with the digestion volumes scaled down to match the biphasic lipolysis testing volumes (40 mL). Testing was completed using a pH-stat apparatus (Metrohm AG, Herisau, Switzerland) comprising a Titrando 907 stirrer, an 804 Ti-stand, a pH electrode (Metrohm), and two 800 Dosino dosing units coupled to a 20 mL autoburette. The system was operated by the Tiamo 2.2 software (Metrohm). The composition of the aqueous intestinal media was sodium taurodeoxycholate (3 mM), phosphatidylcholine (0.75 mM), Tris base (2 mM), NaCl (150 mM), and calcium chloride (1.4 mM). The pancreatin extract (USP × 8) for the pH-stat experiments was reconstituted immediately prior to use using the aqueous intestinal media and centrifuged at 2800*g* for 15 min at 5 °C. Initially, the LBF was introduced into 39 mL of the aqueous intestinal media at 37 °C for 10 min to allow adequate dispersion of the formulation, with samples (1 mL) taken at 2.5, 5, and 10 min. To initiate the digestion process, 4 mL of the pancreatin extract was added. Samples (1 mL) were taken at 5, 10, 15, 30, 45, and 60 min during the digestion phase of the experiment. In each sample, enzymes were inhibited by the addition of 1 M 4-bromophenylboronic acid in methanol (5 μL per mL of the sample). The pH of the media was controlled to pH 7.5 throughout the experiment using 0.6 M NaOH. After 60 min of digestion, the pH was titrated back to pH 9 to determine the release of nonionized free FAs.

Samples were centrifuged at 37 °C and 21,000*g* for 30 min using a benchtop centrifuge (Hettich MIKRO 200R). HPLC analysis was carried out using an Agilent 1200 series HPLC system (Agilent, CA, USA) comprising a binary pump, degasser, autosampler, and variable wavelength detector using a Waters Symmetry C18 column (4.6 × 150 mm, 5 μm) with a flow rate of 1 mL/min. Data analysis was done with EZChrom Elite version 3.2 (Agilent). The mobile phase was composed of an 80:20 mixture of acetonitrile/25 mM sodium acetate buffer at pH 5. The detection wavelength was 287 nm. A linear relationship was established between drug concentration and area under the peak (R^2 > 0.999) in the range of 0.1−25 μg/mL.

3.5. Data Extraction from Published Data. To facilitate a comparison of the pH-stat and biphasic lipolysis results for the danazol LBFs, the percentage of drug in the aqueous micellar phase of the pH-stat experiment after 60 min digestion was extracted by digitization of published data[28] using WebPlotDigitizer (version 4.2) (WebPlotDigitizer, CA, USA).

3.6. IVIVR and Data Normalization. To evaluate the results from the biphasic lipolysis experiments and contrast them with the pH-stat results, IVIVR plots were created for each of the data sets. *In vitro* data were evaluated based on the percentage of the dose (w/w) considered to be bioaccessible after 60 min digestion. Therefore, biphasic lipolysis data were evaluated based on the percentage of the dose (w/w) which had partitioned into the decanol layer after 60 min digestion, whereas pH-stat data were evaluated based on the percentage of the dose (w/w) in the aqueous micellar phase of the digestion media after 60 min digestion. The *in vitro* results were compared to the respective *in vivo* area under the curve (AUC) values.

In vitro data were normalized to facilitate a direct comparison of the results from the two experimental setups, that is, biphasic lipolysis or pH stat. Bioaccessibility data from each setup were normalized relative to the mean *in vitro* bioaccessible percentage (w/w) of the LBFs after 60 min digestion from the respective *in vitro* setup. The mean was calculated using all the bioaccessibility results after 60 min digestion from each set of LBFs, with a set of LBFs defined as those which contain the same active pharmaceutical ingredient and have been tested in the same *in vivo* study. The applied normalization equation was as follows

$$X' = X/\mu$$

where X' is the normalized data point, X is the percentage of the dose (w/w) of the tested LBF considered to be bioaccessible after 60 min digestion, and μ is the mean of *in vitro* bioaccessible percentage (w/w) of the LBF set from the respective setup (*i.e.*, the same setup as used to measure X) after 60 min digestion. When a comparison of different *in vitro* setups was not the objective of the IVIVR, non-normalized data would be used to establish predictive relationships (Supporting Information, Figures S1−S4).

4. RESULTS AND DISCUSSION

4.1. Development of the Biphasic Lipolysis Experimental Method. To improve the efficiency of the LBF development process, there is a need for a biopredictive and rapid *in vitro* lipolysis screening method. In this study, a biphasic lipolysis method which incorporates dispersion in the stomach, GI transit, and absorption in the small intestine was developed. This development of a two-stage biphasic test to assess the performance of LBFs presents many challenges. The biphasic method on the inForm instrument was previously used to test amorphous solid dispersions,[25,26] a cyclodextrin solution,[26] and unformulated APIs.[26] These previously validated test conditions were adapted to include the digestion of the LBFs and to account for the properties of the lipid excipients included within the LBF. Stirring is an important factor in biphasic experiments, requiring a balance between sufficient stirring to ensure dispersion of the LBF in the gastric phase while minimizing the risk of emulsification of the decanol phase in the intestinal phase. Therefore, initial method validation focused on the influence of stirring rate on test conditions, starting with stirring at 100 rpm in both the gastric and intestinal sectors, based on previous biphasic experiments.[25,26] Based on observations from these previous studies, stirring speeds of 100 rpm did not induce emulsification of the decanol layer into the aqueous phase. However, at lower speeds (i.e., <100 rpm), there was a risk of formation of air bubbles in the probe windows of the UV fiber-optic dip probes.

In this present study, given the presence of lipid excipients and postdigestion products in the aqueous phase, there was an increased risk of emulsification of the decanol layer. Therefore, initial method validation experiments explored the impact of study speed on experimental setup. Using a stirring speed of 100 rpm, the separation of the aqueous and decanol layers was maintained throughout the 1 h duration of the intestinal sector, with no scattering of light associated with the mixing or emulsification of the two layers detected in the UV spectra taken from the decanol layer. To explore the impact across the range of LBFs involved, a worst-case challenge testing approach was applied. In terms of an LBF that displayed the highest risk of emulsification, a type IV (surfactant-only formulation) was considered the worst case, given that the higher concentration of surfactants may increase emulsification. However, at a stirring speed of 100 rpm using type IV

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formulation, the biphasic layer was maintained through the experiment, with no decanol layer emulsification.

The other worst-case test condition anticipated was an LBF that is a poorly dispersible Lipid Formulation Classification System type I formulation,[9] such as a nilotinib olive oil suspension. An example of the effect of the varying stirring speed in the gastric phase is shown in Figure 2, which shows

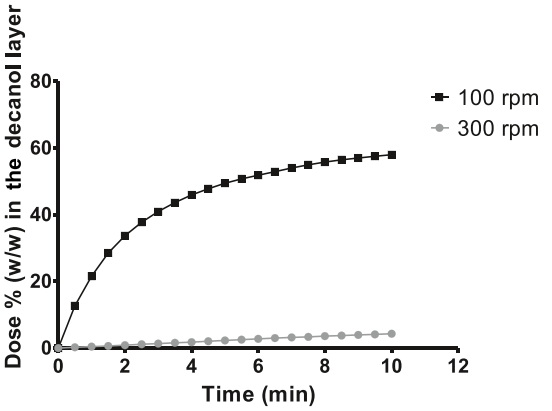

Figure 2. Drug release time profile in the decanol layer for the nilotinib olive oil suspension, with experiments conducted at a single pH (pH 7.5). The profiles using gastric stirring speeds of 100 rpm and 300 rpm are shown by the black squares and gray circles, respectively.

the nilotinib drug concentrations in the decanol phase at both 100 rpm and 300 rpm for an olive oil LFCS type I system. At a lower stirring speed of 100 rpm in the gastric sector, dispersion of the formulation was inadequate and direct mixing of the type I oil into the decanol layer was observed. This was confirmed analytically, with unexpectedly high quantities of the drug (>40% of the administered dose) detected within the decanol layer within 5 min of the transition to the intestinal phase (Figure 2). On increasing the stirring speed to 300 rpm in the gastric phase, complete dispersion of the type I LBF was observed and, quantitatively, the amount of drug appearing in the decanol layer over the first 5 min of switching to the intestinal phase was more gradual (i.e., 2.3% after 5 min) and was therefore considered to more closely mimic the intestinal absorption process. Thus, the finalized stirring conditions were established as 300 rpm during the gastric sector of the experiment. Subsequently, once the decanol layer was added to transition to the intestinal sector, the stirring speed was reduced to 100 rpm. The stirring conditions established were found to be suitable over a range of different types of LBFs used in the study, ranging from poorly dispersible LFCS type I systems to surfactant-only LFCS type IV formulations.

Experiments were initially trialed at a single pH (pH 7.5), simulating only intestinal conditions, matching the standard pH-stat experiments (Table 2). However, this was found to be poorly predictive of the *in vivo* performance of the nilotinib LBFs. Therefore, a pH shift mimicking the transition from gastric to intestinal conditions was introduced as a part of the setup. Level II FaSSIF[30] was selected as an appropriate intestinal fluid because of both its widespread commercial availability and the ease of preparation. The medium in the intestinal sector was set to pH 7.5 instead of pH 6.8, which had been used in previous biphasic experiments,[25,26] to match the pH-stat experiments and reduce the variables when comparing the results from both the setups.

The performance of the LBFs in the biphasic lipolysis experiments was determined by the drug concentration of the drug in the decanol layer, that is, the "absorbed drug". It was not possible to quantify the drug concentration in the aqueous phase using the fiber-optic UV dip probes because of the turbidity caused by the presence of the lipid formulations and the pancreatic enzyme extract in the aqueous layer. In addition, the digestion of lipids by the pancreatic enzyme extract was measured by the addition of NaOH. The rank order of the addition of NaOH and free FAs liberated in the biphasic lipolysis method was broadly similar to the results available from the pH-stat testing (Supporting Information, Table S3),[11,27] with the extent of digestion during the biphasic lipolysis experiments shown in Table 3. However, some

Table 3. Amount of FAs (mmol) Liberated after 60 min Digestion during the Biphasic Lipolysis Experiment[a]

formulation	titrated FAs released after 60 min digestion (mmol)
Nilotinib LBFs	
Peceol	0.419 ± 0.037
Capmul MCM	2.437 ± 0.087
Captex 1000	0.245 ± 0.042
olive oil	0.034 ± 0.016
fenofibrate LBFs from the pig study	
type IIIA MC	0.929 ± 0.052
type IIIA LC	0.624 ± 0.018
type IIIB/IV	0.282 ± 0.055
fenofibrate LBFs from the human study	
E5 (80)	0.797 ± 0.024
E5 (20)	0.744 ± 0.064
MDS	0.432 ± 0.061
danazol LBFs	
F1	1.814 ± 0.065
F2	1.305 ± 0.048
F3	0.860 ± 0.070
F4	0.193 ± 0.043

[a]The values of titrated FAs represent the values corrected for the level of FA released in background lipolysis (digestion media alone). Each data point represents the mean ± standard deviation (SD).

differences in the rate and extent of digestion of lipids may be due to minor differences in the composition of the intestinal media between the *in vitro* lipolysis tests and potential permeation of some postdigestion products into the decanol layer.

4.2. Nilotinib LBFs. Nilotinib lipid suspensions prepared with Peceol, olive oil, Capmul MCM, or Captex 1000 were prepared (Table 1a)[27] and subsequently evaluated using the optimized biphasic *in vitro* lipolysis method. The results obtained for the percentage of the nilotinib dose detected in the absorptive decanol phase are presented in Figure 3. IVIVRs were created to compare the application of the biphasic lipolysis and pH-stat methods to predict the *in vivo* performance. This comparison of the previously published[27] rat *in vivo* results with the *in vitro* results from both lipolysis setups is shown in Table 4 and Figure 4. A strong positive correlation was established between *in vivo* bioavailability and the biphasic *in vitro* method (R^2 = 0.8064). In contrast, the pH-stat method poorly correlated with *in vivo* data (R^2 = 0.1497).

As nilotinib is a weakly basic drug, supersaturation generated upon transfer from the stomach to the small intestine is likely

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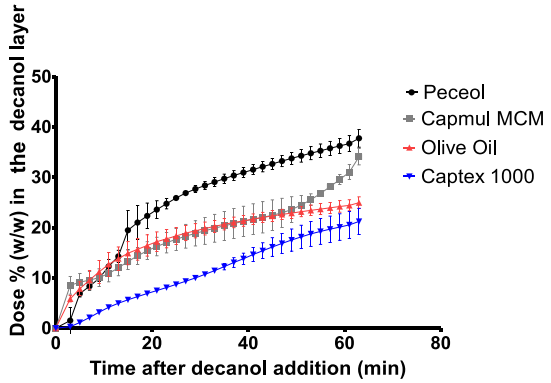

Figure 3. Drug release time profile in the decanol layer for the nilotinib LBFs tested in rats. The Peceol, Capmul MCM, olive oil, and Captex 1000 suspensions are represented by the black circles, gray squares, red triangles, and blue inverted triangles, respectively. Each data point represents the mean ± SD (n = 3).

Table 4. Overview of the *in Vivo* and *in Vitro* Results for the Nilotinib LBFs[a]

	Sprague Dawley rat AUC_{0-inf} [ng·h/mL] (n = 5)[27]	biphasic lipolysis: percentage released in the decanol layer after 60 min digestion (n = 3)	pH-stat method: percentage released in the aqueous micellar phase after 60 min digestion (n = 3)
Peceol	13,103 ± 2557	36.21 ± 1.49	0.51 ± 0.06
Capmul MCM	11,210 ± 5476	29.53 ± 1.01	0.99 ± 0.06
Captex 1000	5168 ± 2197	20.09 ± 2.62	0.82 ± 0.09
olive oil	3548 ± 2711	24.18 ± 1.13	0.22 ± 0.03

[a]Each data point represents the mean ± SD.

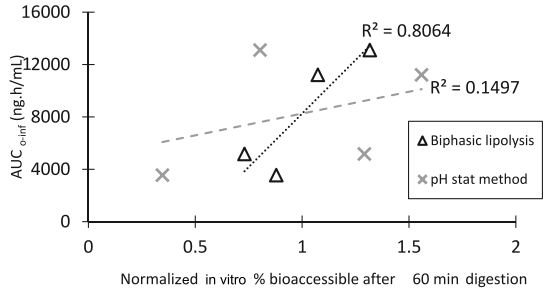

Figure 4. IVIVR for the nilotinib LBFs with PK data in rats available. *In vivo* bioavailability is plotted as AUC_{0-inf} in Sprague Dawley rats.[27] *In vitro* data were normalized to facilitate a direct comparison of the results from different experimental setups. Data were normalized to the mean drug concentration in the respective *in vitro* setup. Biphasic lipolysis data are based on the percentage of the dose partitioned into the decanol layer after 60 min digestion and are represented by the black triangles, R^2 = 0.8064. pH-stat data are based on the percentage of the dose in the aqueous micellar phase after 60 min digestion and are represented by the gray crosses, R^2 = 0.1497.

to be a key factor when considering its oral bioavailability. As the pH-stat approach for nilotinib LBFs was conducted at a single pH simulating intestinal conditions (pH 7.5), any transient supersaturation of weakly basic drugs in the intestine resulting from an increased solubilization capacity of the gastric compartment would be overlooked. As the biphasic lipolysis test incorporates this switch in conditions, the effect of nilotinib supersaturation should be captured based on drug appearance in the decanol layer. This improved biomimetic setup most likely contributed to the improved prediction of the performance of nilotinib LBFs in the biphasic lipolysis setup.

4.3. Fenofibrate LBFs. The biphasic lipolysis method was subsequently employed to evaluate two sets of LBFs containing fenofibrate. The first set was based on formulations previously evaluated *in vivo* in pigs, consisting of a type IIIA MC, a type IIIA LC, and type IIIB/IV fenofibrate lipid solutions (Table 1b).[11] To examine the ability of the biphasic lipolysis and pH-stat methods to predict the *in vivo* performance, an IVIVR was created. The results from the biphasic lipolysis experiments had a better correlation with the reported *in vivo* bioavailability (R^2 = 0.9785) than the conventional pH-stat experiment setup (R^2 = 0.0608) (Figures 5 and 6, Table 5). A second study involved three LBFs where

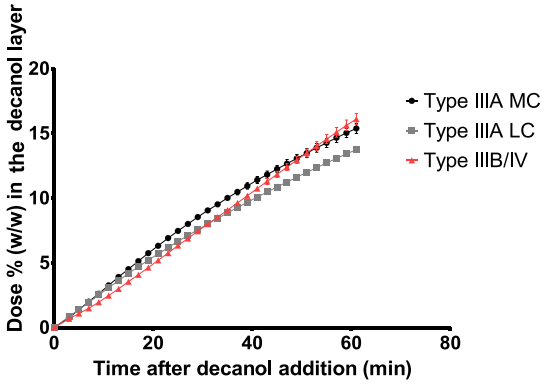

Figure 5. Drug release time profile in the decanol layer for the fenofibrate LBFs tested in pigs. The type IIIA MC, type IIIA LC, and type IIIB/IV formulations are represented by the black circles, gray squares, and red triangles, respectively. Each data point represents the mean ± SD (n = 3).

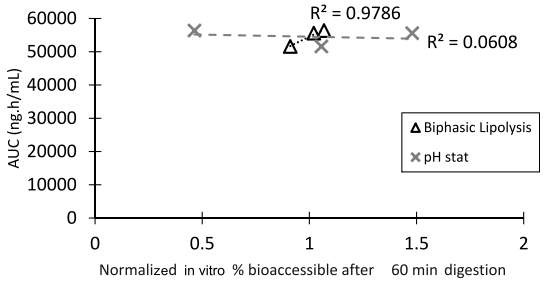

Figure 6. IVIVR for the fenofibrate LBFs with PK data in pigs available.[11] *In vivo* exposure is plotted as Landrace pigs AUC. *In vitro* data have been normalized to the mean drug concentration in the respective *in vitro* setup. Biphasic lipolysis data are based on the percentage of the dose partitioned into the decanol layer after 60 min digestion and are represented by the black triangles, R^2 = 0.9786. pH-stat data are based on the percentage of the dose in the aqueous micellar phase after 60 min digestion and are represented by the gray crosses, R^2 = 0.0608.

bioavailability data have been reported from a human trial.[29] The LBFs were classified as two type IIIA lipid solutions and one type IIIA micronized lipid suspension (Table 1c). The biphasic lipolysis method was more highly correlated with the *in vivo* bioavailability (R^2 = 0.9076) than the pH-stat approach (R^2 = 0.8548) (Figures 7 and 8, Table 6).

As fenofibrate is a neutral compound, it was not anticipated that the change from the gastric to intestinal pH in the biphasic lipolysis would have the predominant impact on the ranking of

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Table 5. Overview of the *in Vivo* and *in Vitro* Results for the Fenofibrate LBFs from the Pig Study[a]

	Landrace pigs AUC [ng·h/mL] (n = 6)[11]	biphasic lipolysis: percentage released in the decanol layer after 60 min digestion (n = 3)	pH-stat method: percentage released in the aqueous micellar phase after 60 min digestion (n = 3)
type IIIA MC	55,593.0 ± 10659.5	15.38 ± 0.64	92.5 ± 12.0
type IIIA LC	51,605.3 ± 8348.7	13.73 ± 0.20	66.0 ± 7.1
type IIIB/IV	56,378.3 ± 15439.5	16.10 ± 0.75	29.0 ± 5.8

[a]Each data point represents the mean ± SD.

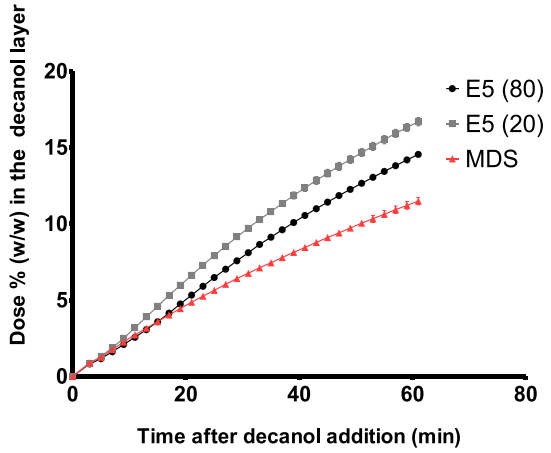

Figure 7. Drug release time profile in the decanol layer for the fenofibrate LBFs tested in humans. The E5 (80), E5 (20), and MDS formulations are represented by the black circles, gray squares, and red triangles, respectively. Each data point represents the mean ± SD (n = 3).

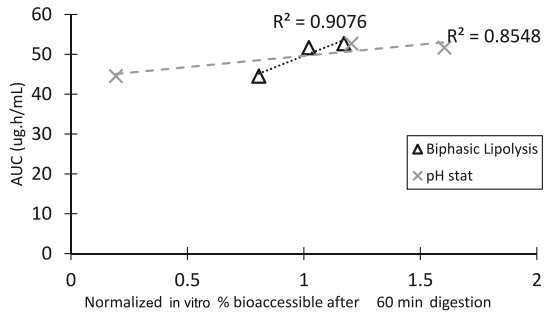

Figure 8. IVIVR for the fenofibrate LBFs with PK data in humans available.[29] *In vivo* exposure is plotted as human AUC. *In vitro* data have been normalized to the mean drug concentration in the respective *in vitro* setup. Biphasic lipolysis data are based on the percentage of the dose partitioned into the decanol layer after 60 min digestion and are shown by the black triangles, R^2 = 0.9076. pH-stat data are based on the percentage of the dose in the aqueous micellar phase after 60 min digestion and are represented by the gray crosses, R^2 = 0.8548.

the LBFs. However, the presence of an absorptive sink was considered to play the more significant role for fenofibrate, given the drug's highly lipophilic characteristics. The pH-stat experiments on the fenofibrate LBFs (tested in pigs) displayed substantial differences between the type IIIA and type IIIB/IV formulations. The type IIIA formulations displayed a limited drug precipitation (<5%), whereas the type IIIB/IV

Table 6. Overview of the *in Vivo* and *in Vitro* Results for the Fenofibrate LBFs from the Human Study[a]

	human AUC [μg·h/mL] (n = 4)[29]	biphasic lipolysis: percentage released in the decanol layer after 60 min digestion (n = 3)	pH-stat method: percentage released in the aqueous micellar phase after 60 min digestion (n = 3)
E5 (80)	51.66 ± 22.65	14.55 ± 0.25	6.22 ± 0.75
E5 (20)	52.66 ± 19.23	16.69 ± 0.48	4.67 ± 2.20
MDS	44.52 ± 9.20	11.49 ± 0.42	0.75 ± 0.46

[a]Each data point represents the mean ± SD.

formulation displayed an extensive drug precipitation (~70% dose) in the pH-stat lipolysis setup. However, this difference was not observed in the *in vivo* study, with all three formulations displaying similar bioavailability in pigs. This higher-than-expected bioavailability for the type IIIB/IV formulation would suggest that *in vivo* there may be a lower degree of precipitation[11,15] and compensatory mechanisms such that the drug precipitate would quickly redissolve as rapid drug permeation would maintain sink conditions in the intestine. Alternatively, the study suggested a key limitation of the pH-stat approach where there may be a tendency to overestimate the potential impact of lipolysis-triggered precipitation because of nonsink conditions.

Thomas *et al.* have similarly observed large differences between fenofibrate LBFs during *in vitro* lipolysis without an absorption sink, which were not evident *in vivo*.[31] Sassene *et al.* studied precipitation from LBFs in the stomach and proximal duodenum of rats,[32] comparing samples from the intestine and *in vitro* pH-stat lipolysis testing. No precipitation of drug was found for fenofibrate in the intestine of the euthanized rats at any timepoint, which contrasted the pH-stat lipolysis results for these formulations which showed precipitation under simulated intestinal conditions. By incorporating an absorptive sink, our new study in the biphasic lipolysis setup therefore confirmed that more reliable predictions of the *in vivo* performance of LBFs can be achieved particularly for high-permeable drugs such as fenofibrate.

4.4. Danazol LBFs. We further studied a set of danazol lipid solutions (2 × type IIIA, 1 × type IIIB, and 1 × type IIIB/IV) using the biphasic lipolysis setup based on formulations which had previously been tested in dogs.[28] The results from the biphasic lipolysis showed that the partitioning of danazol into the decanol layer was in a reversed order to that observed *in vivo* (Figures 9 and 10, Table 7). In contrast, the nonsink pH-stat results from these LBFs achieved a correct rank order and were highly correlated with the *in vivo* findings (R^2 = 0.9846). To determine if the presence of the gastric sector had caused the reverse ranking, the biphasic lipolysis experiments were carried out excluding the gastric sector, thus replicating the pH profile of the pH-stat experiments. As danazol is a neutral drug over the physiological pH range, the presence of the gastric sector was not anticipated to cause the reversed rankings. Indeed, these experiments without the gastric sector resulted in the same ranking as the previous biphasic lipolysis experiments, that is, a reversed order to that reported *in vivo*. Another potential reason for the discrepancy could be the different intestinal media used in the biphasic and pH-stat lipolysis *in vitro* testing. Level II FaSSIF was used as the intestinal medium for biphasic lipolysis testing, which differed in terms of the bile salt and concentration of the endogenous

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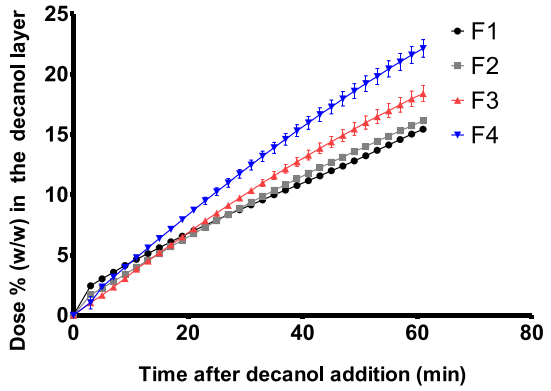

Figure 9. Drug release time profile in the decanol layer for the danazol LBFs tested in dogs. The F1, F2, F3, and F4 formulations are represented by the black circles, gray squares, red triangles, and blue inverted triangles, respectively. Each data point represents the mean ± SD (n = 3).

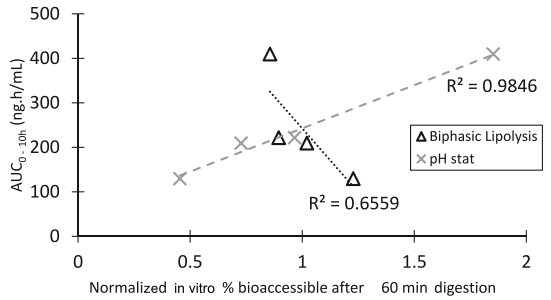

Figure 10. IVIVR for the danazol LBFs with PK data in dogs available.[28] *In vivo* exposure is plotted as beagle dog AUC_{0-10h}. *In vitro* data have been normalized to the mean drug concentration in the respective *in vitro* setup. Biphasic lipolysis data are based on the percentage of the dose partitioned into the decanol layer after 60 min digestion and are shown by the black triangles, R^2 = 0.6559. pH-stat data are based on the percentage of the dose in the aqueous micellar phase after 60 min digestion and are represented by the gray crosses, R^2 = 0.9846.

Table 7. Overview of the *in Vivo* and *in Vitro* Results for the Danazol LBFs from the Dog Study[a]

	beagle dog AUC_{0-10h} [ng·h/mL] (n = 4)[28]	biphasic lipolysis: percentage released in the decanol layer after 60 min digestion (n = 3)	pH-stat method: percentage released in the aqueous micellar phase after 60 min digestion (n = 3)[b]
F1	409.5 ± 92.9	15.44 ± 0.43	~90.9 ± 4.5
F2	221.5 ± 70.5	16.14 ± 0.34	~47.4 ± 1.7
F3	209.4 ± 114.5	18.40 ± 1.14	~35.7 ± 4.7
F4	129.6 ± 22.9	22.14 ± 1.25	~22.3 ± 2.4

[a]Each data point represents the mean ± SD. [b]Values extracted from the research article figure.

surfactants used compared to the pH-stat experiment (Table 2). It may be possible that the rate and extent of digestion of the formulations differed because of the media composition, which could lead to differences in their *in vitro* performance. However, Kilic and Dressman reported a correct ranking of formulations using a simplified *in vitro* lipolysis experiment, without the presence of a sink, using Level II FaSSIF V2 as the intestinal medium.[33] Level II FaSSIF V2 contains the same surfactants as Level II FaSSIF with the same concentration of sodium taurocholate (3 mM) present in both media. This indicated that the difference in media composition and subsequent effects on digestion were likely not the primary reasons causing the difference between the biphasic *in vitro* lipolysis results and the *in vivo* findings.

The reverse ranking was therefore most likely caused by an excipient-specific effect in the biphasic system. The danazol LBFs used in the study of Cuiné *et al.*[28] used high concentrations of ethanol as a cosolvent in the formulation. The partitioning of the drug into the decanol layer broadly correlated with the concentration of ethanol in the formulations. The high concentration of ethanol in the formulations may therefore have resulted in an altered drug flux into the decanol layer and ethanol-dependent partitioning effects in the biphasic system compared to the *in vivo* situation. Although this seems to be the most likely explanation for the obtained findings, it cannot be excluded that other components of the formulations caused further partitioning effects *in vitro* of danazol that were not relevant for the *in vivo* absorption. It was beyond the scope of this study to evaluate how each formulation component may affect drug partitioning into the decanol layer. However, based on current findings, the biphasic lipolysis test appears highly promising for common LBFs, but there is a caveat regarding *in vivo* predictability when formulations which comprise high levels of cosolvents, such as ethanol, are studied.

4.5. Limitations of the Study. Although the two-stage biphasic lipolysis method was an improvement over the standard pH-stat setup in terms of predicting the effect of GI transit, there were still aspects which were not fully reflective of the *in vivo* process. In the biphasic lipolysis setup, the switch from gastric to intestinal conditions occurs very rapidly, whereas *in vivo* the process of gastric emptying is usually more gradual.[34] This rapid pH transition may lead to an overestimation of the precipitation rate. Also, the 1 min delay after transition to pH 7.5, included to facilitate dispersion of the pancreatic enzyme prior to the addition of decanol, indicates that permeation during the initial transient supersaturation period upon shift to intestinal conditions may not have been fully captured by the decanol concentration profile. In addition, the gastric media used in the biphasic lipolysis experiments did not incorporate gastric lipases. Thus, the effects of gastric lipolysis were overlooked by the setup. Furthermore, the surface area of the interface between the aqueous and decanol layers (approx. 19.63 cm^2) was much lower than the surface area of the small intestine,[35] which limited the *in vitro* "absorption" of the drug during its brief supersaturated state. This concern of a limited surface area and absorptive flux is a more general aspect and also needs consideration when, for example, Caco-2 cells or a biomimetic membrane is used to mimic physiological absorption.[22−24]

Although the biphasic setup was validated using four different *in vivo* studies, it should be acknowledged that there is a paucity of published *in vivo* studies using LBFs where significant *in vivo* differences are observed as a function of different LBF types. Indeed, in two of the four studies in the present study, differing lipid formulation types failed to yield differences in the *in vivo* bioavailability.[11,29] Further mechanistic studies that relate formulation type to *in vivo* performance would facilitate more reliable IVIVRs. In addition, these *in vivo* studies were conducted using four different species (rats, pigs, dogs, and humans), whereas the biphasic setup was not adapted to account for differences between the species. Therefore, it is possible that interspecies differences in

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bioavailability of these drugs may be confounding factors. Although the majority of other studies investigating LBFs did not adapt the *in vitro* lipolysis method depending on the species used in the *in vivo* testing, some groups have successfully adapted methods to match rat lipolysis conditions.[36,37]

The setup was tested with three different drugs and a wide variety of LBFs and therefore demonstrates versatility to test a broad range of LFCS types, including poorly dispersible LFCS type I systems and surfactant-only LFCS type IV formulations.[9] However, testing on the experimental setup was limited to the types of LBFs explored in this paper and the drugs utilized. Therefore, for future studies with different drug/LBF combinations, optimization of the test conditions may need to be revisited to meet particular challenges of the drug/LBF combination.

As mentioned in Section 4.1, the turbidity resulting from the presence of the lipid formulations and the pancreatic enzyme extract meant that it was not possible to quantify the drug in the aqueous layer using the *in situ* fiber-optic dip probes. However, the use of a nylon mesh around the probe and immobilized lipase, instead of the pancreatic extract, would potentially allow quantification of the aqueous drug concentrations using the UV fiber-optic dip probes, as outlined very recently in the work by Alvebratt *et al.*[24] Quantification of the drug concentrations in the aqueous layer may provide additional insights into the kinetics of drug concentrations in this aqueous layer, for example, drug precipitation, and future approaches that provide an option to quantify the aqueous concentrations simultaneously with the concentrations in the absorptive phase are merited.

5. CONCLUSIONS

Overall, the biphasic lipolysis method provided an improved *in vitro* prediction of the relative performance of the LBFs compared to the pH-stat lipolysis method for the majority of the investigated systems. The pH-stat lipolysis method was advanced by incorporating a pH shift to mimic GI transfer, leading to an improvement in the prediction of weak bases, such as nilotinib LBFs. By including a decanol layer as an intestinal absorptive sink, the proposed method more reliably predicted the *in vivo* performance of fenofibrate LBFs both in pigs and in humans. In addition, the biphasic lipolysis method had a superior throughput capacity using fiber-optic dip probes to assess the performance of the LBF, thus eliminating several time-consuming procedures such as centrifugation and HPLC analysis of samples. Because of the automation available on the platform, much of the human error may be removed from the experimental process, resulting in highly reproducible results. Although some further development work is necessary to fully understand any limitations associated with the setup, such as the impact of ethanol/cosolvents on drug appearance in the decanol layer, it has the potential to act as a useful tool in the formulation development space, facilitating a more efficient screening process of novel LBFs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.0c00427.

Dimensions of the vessel and stirrers on the inForm instrument, detection wavelengths used to quantify drugs, digestion rankings from the biphasic lipolysis experiments and pH-stat experiments, and non-normalized IVIVRs for all LBF sets (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Patrick J. O'Dwyer − *Pion Inc. (UK) Ltd., Forest Row RH18 5DW, East Sussex, U.K.; Department of Pharmacy, School of Health Sciences, National and Kapodistrian University of Athens, Zografou 157 72, Greece; School of Pharmacy, University College Cork, Cork T12 YN60, Ireland;* orcid.org/0000-0002-5350-8364; Phone: +353 (0)21 4901676; Email: patrick.odwyer@ucc.ie

Karl J. Box − *Pion Inc. (UK) Ltd., Forest Row RH18 5DW, East Sussex, U.K.*; Phone: +44 (0)1342 820720; Email: kbox@pion-inc.com

Authors

Niklas J. Koehl − *School of Pharmacy, University College Cork, Cork T12 YN60, Ireland;* orcid.org/0000-0002-0565-9192

Harriet Bennett-Lenane − *School of Pharmacy, University College Cork, Cork T12 YN60, Ireland*

Christos Reppas − *Department of Pharmacy, School of Health Sciences, National and Kapodistrian University of Athens, Zografou 157 72, Greece*

Rene Holm − *Drug Product Development, Janssen Research and Development, Johnson & Johnson, Beerse 2340, Belgium; Department of Science and Environment, Roskilde University, Roskilde 4000, Denmark*

Martin Kuentz − *School of Life Sciences, Institute of Pharma Technology, University of Applied Sciences Northwest Switzerland, Muttenz 4132, Switzerland;* orcid.org/0000-0003-2963-2645

Brendan T. Griffin − *School of Pharmacy, University College Cork, Cork T12 YN60, Ireland;* orcid.org/0000-0001-5433-8398

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.molpharmaceut.0c00427

Notes

The authors declare no competing financial interest.

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