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On the usefulness of four *in vitro* methodologies in screening for product related differences in tacrolimus exposure after oral administration of amorphous solid dispersions with modified release characteristics in the fasted state

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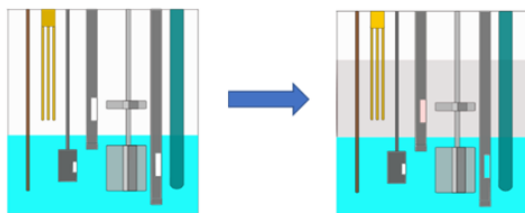
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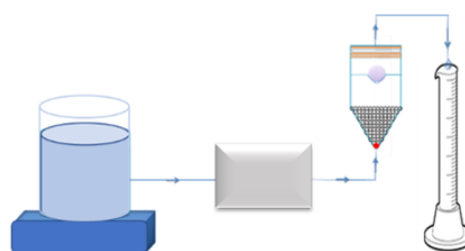
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Small-scale two-stage biphasic system



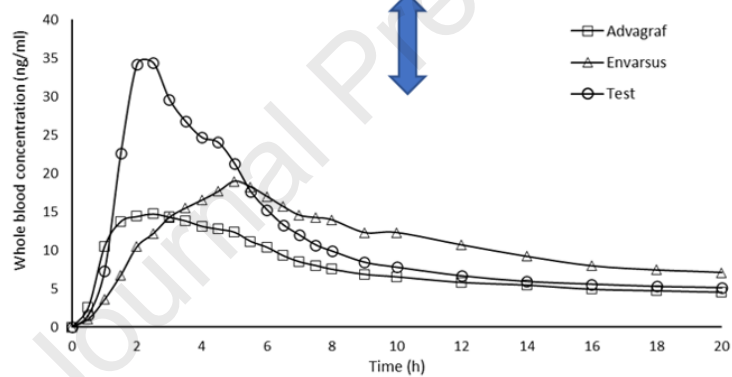
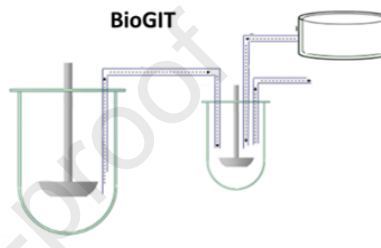
Compendial apparatus IV



Small-scale two-stage D-P system



BioGIT



On the usefulness of four *in vitro* methodologies in screening for product related differences in tacrolimus exposure after oral administration of amorphous solid dispersions with modified release characteristics in the fasted state

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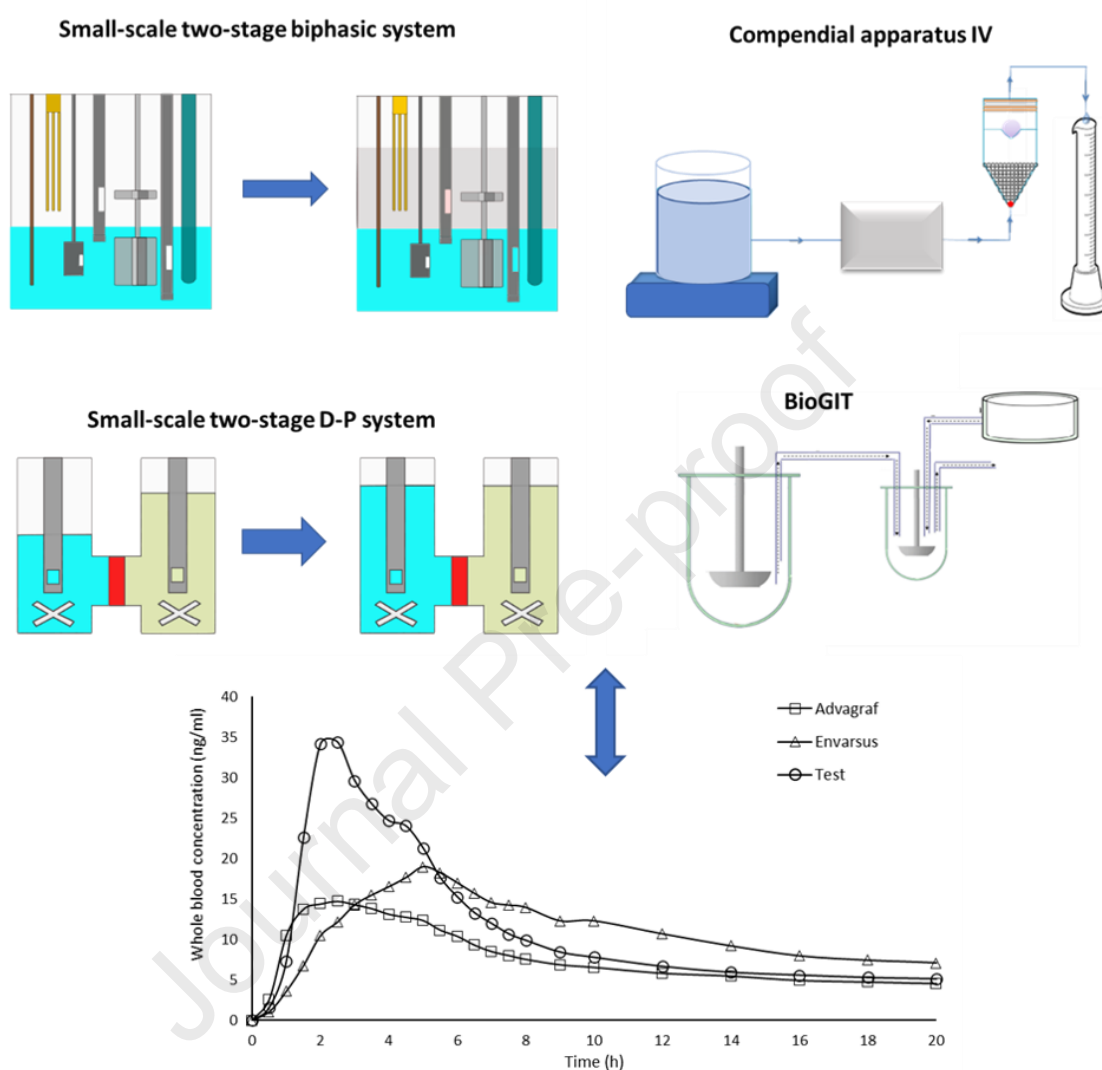
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GRAPHICAL ABSTRACT



ABSTRACT

Objective: To investigate the usefulness of four *in vitro* methodologies in screening for product related differences in tacrolimus exposure after oral administration of amorphous solid dispersions with modified release characteristics in the fasted state.

Methods: Initially, Advagraf® (5mg tacrolimus/capsule), Envarsus® (4mg tacrolimus/tablet) and a modified release Test Tablet (5mg tacrolimus/tablet) were subjected to *in vitro* biorelevant performance testing simulating fasted state conditions using a small-scale two-stage biphasic system, a small-scale two-stage dissolution – permeation (D-P) system, the compendial apparatus IV (open loop mode) and the biorelevant gastrointestinal transfer (BioGIT) system. Early and total exposure, after single dose administrations of the three products to twelve healthy adults in the fasted state on a crossover basis, were then evaluated. Subsequently, the usefulness of *in vitro* data in qualitatively predicting product related differences in early exposure and in total exposure were assessed.

Results: Product related differences in tacrolimus early exposure were successfully predicted by data collected with compendial apparatus IV. The two-stage biphasic system was useful for predicting differences in early exposure between the non-disintegrating Envarsus® and the disintegrating products (Advagraf® or Test Tablet). BioGIT data were useful only for discussing clinical data early after administration of the two disintegrating products. Prediction of product related differences in total exposure was successful only when the compendial apparatus IV was used for comparing the two disintegrating products.

Conclusions: Biorelevant *in vitro* performance testing with compendial apparatus IV was useful for qualitatively predicting differences in tacrolimus early and total exposure after

administration of disintegrating products with modified release characteristics in the fasted state.

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KEYWORDS

Tacrolimus, Amorphous Solid Dispersions, Modified release, Biphasic system, Dissolution-Permeation system, Compendial apparatus IV, BioGIT, Early exposure, Total Exposure, Adults

1. INTRODUCTION

In recent years, many poorly water-soluble molecules have emerged as drug development candidates and marketed products [1]. Poorly soluble drug substances challenge the drug development process as increased lipophilicity and poor water solubility are linked to problematic dissolution of the dose and poor and/or erratic oral bioavailability [2, 3]. One of the most frequently applied product approaches for overcoming the problem has been based on the conversion of crystalline to amorphous drug particles, allowing for supersaturation of intraluminal contents with the drug [4, 5]. To improve the stability characteristics of the thermodynamically unstable amorphous state, amorphous drug particles are typically dispersed in a polymer matrix so that amorphous solid dispersions (ASDs) are formed [6-8]. However, after ingestion of an ASD, the potential precipitation and/or crystallization of the drug substance may limit its ability to be molecularly dispersed in the aqueous phase of luminal contents and, therefore, to become available for transport through the intestinal mucosa. Evaluation of this potential before entering studies in humans is highly desired not only for ethical reasons (especially when multiple *in vivo* studies are performed until the most optimum product is found) but also for reducing costs associated with the drug product development process.

In the present study, we investigated the usefulness of four *in vitro* methodologies in screening for product related differences in tacrolimus exposure, after administration of amorphous solid dispersions with modified release characteristics in the fasted state.

Tacrolimus is a macrolide lactone (803.5 Da, non-ionizable) with low aqueous solubility (4-12 µg/mL, 25 °C) and is prescribed to graft recipients [9] (Dheer et al., 2018). It is metabolized extensively by the CYP3A enzymes of the intestinal epithelium and the liver, and is a substrate of the P-gp efflux carrier [10, 11].

Three products with modified release characteristics were studied in the present investigation. Advagraf® (5mg tacrolimus/capsule) is a hard gelatin capsule with modified release tacrolimus characteristics and is available at four dose strengths (0.5, 1, 3 or 5 mg tacrolimus/capsule) [12]. Advagraf® is manufactured by the solvent evaporation method; tacrolimus is dissolved together with ethylcellulose (EC) and hydroxypropylmethylcellulose (HPMC) in a volatile solvent which is evaporated to create the tacrolimus solid dispersion. Envarsus® (4mg tacrolimus/tab) is a tablet with modified release characteristics and it is available at three dose strengths (0.75, 1 or 4 mg tacrolimus/tablet) [13]. Envarsus® is based on the MeltDose® technology, where tacrolimus is melted and sprayed on a matrix carrier. As there is no other commercially available tacrolimus amorphous solid dispersion formulation with modified release characteristics, the third product was prepared specifically for the needs of the present study. To better address the objective of the present study, the technology of the third product (Test Tablet, 5mg tacrolimus/tablet) was different than the technology of Advagraf® or Envarsus®. The third product, Test Tablet (5 mg tacrolimus/tablet), was prepared by dissolving tacrolimus together with Eudragit®-S100 (which dissolves at $\text{pH} \geq 7$ [14, 15]) and a surfactant in ethanol and utilizing the spray drying method to remove ethanol, the tacrolimus ASD with modified release characteristics was obtained and was subsequently formulated in a tablet form. Polymer selection was based on its miscibility characteristics with tacrolimus and the stability characteristics of the resultant ASD [16].

Initially, the three modified release tacrolimus products were subjected to *in vitro* biorelevant performance testing simulating the fasted state conditions. For tacrolimus, early exposure, i.e. absorption during the first couple hours post administration, may impact the total bioavailable amount as tacrolimus is extensively metabolized in the upper but not the lower intestinal epithelium [17, 18]. Therefore, the following four *in vitro* setups were employed for screening for product related differences in early and/or total exposure:

- A small-scale two – stage biphasic system [19] for evaluating differences in early and total exposure
- A small – scale two – stage dissolution – permeation (D-P) system for evaluating differences in early and total exposure
- The compendial Apparatus IV (full – scale setup) [20] for evaluating differences in early and total exposure, and
- The biorelevant gastrointestinal transfer (BioGIT) system [21] for evaluating differences in early exposure.

The *in vitro* methodologies were then evaluated for their usefulness in predicting product related differences in tacrolimus early and total exposure. Since linear kinetics seem to operate after single dosing [22], differences in *in vitro* biorelevant performance testing could directly reflect *in vivo* differences in tacrolimus early and/or total exposure in adults. For all three tacrolimus products, *in vivo* data in healthy adults were collected specifically for the needs of the present study.

2. EXPERIMENTAL

2.1 Materials

Crystalline tacrolimus was purchased from APOTEX (Toronto, ON, Canada) and Eudragit®-S100 (methacrylic acid-methyl methacrylate copolymer) was purchased from Evonik Industries AG (Essen, Germany). Solutol® (macrogol 15 Hydroxystearate) was purchased from BASF (Ludwigshafen, Germany). Ethanol was purchased from Honeywell Research Chemicals (Morris Plains, NJ, USA), HPLC grade. Acetonitrile was purchased from Sigma-Aldrich (Saint Lewis, MO, USA) and decanol was purchased from Alfa Aesar (Heysham, UK). Acceptor Sink Buffer (ASB) and GIT (Gastro Intestinal Tract) Lipid Solution (20% lecithin in dodecane lipid solution) were received from Pion Inc. (MA, USA). FaSSIF V2 powder was obtained from biorelevant.com (London, UK). Advagraf® (5 mg/cap, Astellas Pharma, Japan) and Envarsus® (1 mg/tab and 4mg/tab, Chiesi GmbH, Italy) were purchased through Client-Pharma Limited (Staffordshire, UK), from the German market. All other excipients and chemicals were acquired from commercial sources and were used as obtained.

2.2 Preparation of the Test Tablet (5mg tacrolimus/tablet)

2.2.1 Spray drying

8.75 g of tacrolimus, 15.375 g of Eudragit®-S100 and 0.875 g of Solutol® were dissolved in 600 mL of absolute ethanol, forming a clear tacrolimus:polymer:surfactant 35:61.5:3.5 w/w/w solution. A ADL311SA spray drying system (Yamato Scientific America Inc., California, USA), equipped with a GAS410 organic solvent recovery unit (Yamato Scientific America Inc., California, USA) was used for preparation of the spray-dried tacrolimus: Eudragit®-S100:

Solutol® ASD. The clear solution was transferred to a two-way fluid nozzle with a 0.4 mm diameter, using a peristaltic pump and spray dried. The spray-drying conditions were inlet and outlet temperature of 85-100 and 70 °C, respectively; a feed rate of 10 mL/min; and 100% atomization. After drying, ~1-40 µm solid particles were collected by a high efficiency cyclone. Due to the use of a flammable and volatile solvent, the closed loop and the condenser at -20 °C in the solvent recovery unit were enabled along with the use of nitrogen as the atomizing gas with flow rate of ~15 L/h at 0.1 MPa and the blower was set to 80% (~31 m³/h) for circulating nitrogen as the drying gas. Immediately after spray drying, the tacrolimus ASD was placed in glass vials with nitrogen and sealed with a plastic cap and parafilm (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The amorphous state of tacrolimus in the spray-dried powder was confirmed by DSC thermograms and XRPD diffractograms (Supplementary material).

2.2.2 Preparation and storage of Test Tablets

The spray dried powder along with a small amount of anhydrous lactose was passed through a 0.6 mm sieve and then the rest of lactose was added. The combined powder was mixed *via* a F205 G Series rotary shaker (Falc Instruments, Treviglio, Italy) for 15 minutes. Consequently, magnesium stearate was included, and the excipients were mixed for 3 minutes. The bulk product powder was stored in a glass bottle with a plastic cap, until it was time for tableting. Tableting was performed in a manual hydraulic press (Maassen, Reutlingen, Germany) with the use of a 6.7 × 17 mm dye. The force of tableting was set at 1.4 Tn and the hardness of the tablets was measured at 99.33 ± 5.31 N with the Erweka TBH 220 TD Tablet Hardness Tester (Erweka, Heusenstamm, Germany). The Test Tablet weight was 500 mg and the tacrolimus amount in it was 5 mg (Table 1). Test Tablets were enclosed in PVC aluminium blisters until used.

2.3 In vitro experiments with the small-scale setups

Typically, in relevant experiments the dose is scaled down relative to the administration with a 250 mL glass of water [19, 23, 24]. However, the prolonged release mechanism of Envarsus® matrix tablet i.e., drug diffusion through a swollen polymer matrix, would be disrupted by crushing this dosage form. In addition, previous studies suggested that the use of small-scale methods to test the whole dosage forms of tacrolimus ASD was beneficial to improve the discrimination of the *in vitro* testing, especially for detecting the presence of crystalline drug in the ASD [25]. Therefore, in this study, testing of the tacrolimus products with the small-scale setups was completed by using the whole dosage forms, as applied in full-scale setups and in the subsequent clinical study, resulting in a tested dose of 5 mg tested for each product in each *in vitro* setup. Both of the small-scale setups were able to physically accommodate all the dosage forms tested, i.e. one Advagraf® capsule (~16×6 mm), one Envarsus® 1mg tablet (~8×5 mm) plus one Envarsus® 4 mg tablet (~16×7 mm) or one Test Tablet (~17×6.7 mm).

2.3.1 Two-stage biphasic system experiments

Experiments were carried out using the inForm platform (Pion Inc., MA, USA) as outlined previously [19]. Briefly, the dose was introduced into 36 mL of a 0.01 M acetate phosphate buffer at pH 2 representing the gastric environment. Thirty minutes after the start of an experiment, 10 × concentrated Level II FaSSIF V2 [25] (4 mL) and decanol (40 mL) were added, with the pH of the aqueous media increased to pH 6.8 using 0.5 M NaOH to simulate the transition into the upper small intestine. The stirring speed of the paddles in the vessel was set at 100 rpm. The weak UV absorbance of tacrolimus combined with turbidity present in the aqueous layer meant it was not possible to quantify tacrolimus concentrations in the aqueous

layer using the *in situ* UV dip probes for all three formulations tested. This turbidity was due to the presence of undissolved particles in the medium resulting in a scattering of light recorded in the UV spectra. As tacrolimus weakly absorbs UV light, it is highly sensitive to any scattering of light present in the UV spectrum. This turbidity was not thought to be due to emulsification of decanol droplets in the aqueous medium as the turbidity was present during the gastric sector prior to the addition of decanol into this experiment and previous experiments indicated a minimal impact of decanol emulsification with this setup [19]. The performance of the products in the small-scale two-stage biphasic system were assessed based on the concentration of drug which partitioned into the decanol layer, the amount available for absorption. Due to the turbidity in the aqueous layer, it was not possible to determine if any tacrolimus was released during the gastric sector of the experiment. Concentration in the decanol layer was determined every 120 seconds using *in situ* multi-wavelength fibre optic UV probes. Stirring was temporarily suspended while the decanol was being added to avoid excessive turbulence. The detection wavelength range was 290 - 320 nm for tacrolimus, with a linear relationship established in the range of 2.5 – 130 µg/mL ($R^2 > 0.99$). The duration of the experiment in the intestinal environment was 197 minutes. The pH was monitored throughout using the *in situ* pH probe and was maintained to ± 0.1 pH unit of the target pH, using 0.5 M HCl or 0.5 M NaOH. All experiments were carried out in triplicate at 37°C.

2.3.2 Two-stage D-P system experiments

Experiments with the small-scale two-stage D-P system experiments were carried out using the µFLUX setup (Pion Inc.) as outlined previously [19]. The two chambers were separated by a biomimetic membrane which consists of a 0.45 µm polyvinylidenefluoride membrane coated with 25 µL of the GIT lipid solution. The area of the membrane was 1.54 cm². Stirring was

provided by cross-bar magnetic stirrers in both chambers and was set at 150 rpm throughout the experiment. The acceptor chamber was filled with 20 mL of acceptor sink buffer (ASB) throughout the experiment, to maintain sink conditions during the experiment. For the initial 30 minutes, the donor chamber was initially filled with 15 mL of dilute HCl at pH 2, with the dose manually introduced to begin the experiment. Subsequently, a 4 x concentrated solution of Level II FaSSIF V2 (5 mL) was added to the donor chamber to simulate the transition to intestinal conditions. The pH of the 4 × concentrated Level II FaSSIF V2 solution was adjusted in order to achieve a final pH of 6.8 in the donor chamber. The duration of the experiment in the intestinal environment was 210 minutes. The temperature was controlled to 37°C throughout the experiment. Similar to the two-stage biphasic system experiments, it was not possible to quantify tacrolimus concentration using the *in situ* UV dip probe in the donor chamber due to the weak UV absorption of tacrolimus combined with the light scattering caused by turbidity in the media for all formulations tested. The performance of the products was assessed based on the concentrations in the acceptor chamber of the small-scale two-stage D-P system. The detection wavelength range for tacrolimus was 290 – 320 nm and a linear relationship was established in the range of 1.33 – 64.52 µg/mL ($R^2 > 0.99$).

2.4 In vitro experiments with the full-scale setups

2.4.1 Compendial apparatus IV

Compendial apparatus IV experiments for Advagraf® (5 mg/capsule), Test (5 mg/tablet) and for Envarsus® (4 mg/tablet) were performed with an Erweka apparatus IV model DFZ60 (Heusenstamm, Germany) equipped with 22.6 mm diameter cells and after placing a 5 mm diameter red glass bead in the apex of the cell and 1.7 g of 1 mm sized glass beads on the top

of the red glass bead. Two cellulose filters (MNGF4, 0.4 μm pore size, 25 mm diameter, Machery–Nagel, Germany), 0.1 g glass wool and one cellulose filter were placed on top of the cell, after confirming their filtering efficiency (data not shown).

At time zero, the dosage form was placed on the holder of every cell ($n = 3$) and dissolution medium was introduced in the cells with the use of a piston pump. All experiments were performed at 37 ± 0.5 °C by using the open-loop mode. Level III Fasted State Simulated Gastric Fluid (FaSSGF), Level II Fasted State Simulated Intestinal Fluids (FaSSIF-V2, FaSSIF-V2midgut and SIFileum-V2) and Level II Fasted State Simulated Colonic Fluid (FaSSCoF) [26] were prepared with the FaSSIF/ FeSSIF/ FaSSGF, FaSSIF-V2 and FaSSCoF powders (biorelevant.com, London, United Kingdom), according to the instructions on the biorelevant.com website. Dissolution media, duration of exposure and flow rates during an experiment were as recently described by Reppas et al. (2020) [27]. More specifically, the Level III FaSSGF (pH 1.6) was pumped in the cells for 30 minutes with a flow rate of 8 mL/min. Level II FaSSIF-V2 (pH 6.5), FaSSIF-V2midgut (pH 6.8) and SIFileum-V2 (pH 8.0) were pumped in the cells for 40, 80 and 60 minutes respectively, with with a flow rate of 4 mL/min. Finally, the Level II FaSSCoF was pumped in the cells for 120 minutes with a flow rate of 4 mL/min.

Samples were drawn periodically by collecting the fluid exiting the cell in a volumetric cylinder. The cylinders were exchanged every 20 minutes up to 360 minutes. Samples were immediately diluted with mobile phase in a 1:1 v/v ratio. Diluted samples were injected into a Prominence LC-20A Modular HPLC-UV system (Shimadzu Corporation, Kyoto, Japan). The column used for analyzing the samples was a Zorbax Eclipse-XBD C-18 analytical column (4.6 mm $\times$ 250 mm, 5 μm , 100Å; Agilent Corporation, California, USA). The mobile phase

was an isocratic flow of acetonitrile:water 70:30 (v/v) mixture and the flow rate was set to 1 mL/min. The detection wavelength was set to 210 nm and the temperature of the column was set to 45 °C. The limit of detection was 0.1 µg/mL and tacrolimus eluted at around 15 minutes.

2.4.2 BioGIT system

BioGIT has been useful for evaluating early exposure of disintegrating oral drug products [21, 28]. Due to the non-disintegrating nature of the Envarsus® product, the BioGIT system was not suitable to test this product [21, 28] and relevant experiments were not performed. BioGIT system experiments were performed as outlined previously by Kourentas et al. (2016a) [21]. Advagraf® and the Test Tablet were tested using the same dosage (5 mg) as per the clinical study. The gastric volume of the BioGIT system was initially filled with 250 mL of Level III FaSSGF [26] in a 500 mL capacity mini vessel (Erweka, Heusenstamm, Germany). The duodenal compartment was initially filled with 40 mL of Level II FaSSIF in a mini vessel with 100 mL capacity from Distek (New Brunswick, NJ, USA). The stirring speed was set at 75 rpm in both compartments. A series of phosphate buffer solutions containing sodium chloride, sodium taurocholate, and phosphatidylcholine were employed in the reservoir compartment so that the composition of contents in the duodenal compartment (pH, buffer capacity, osmolality, sodium taurocholate concentration, and phosphatidylcholine concentration) remained unaltered during an experiment. Experiments were performed at 37°C for 45 min using a three-channel peristaltic pump (Reglo ICC pump, part ISM 4308, Ismatec®). Flow rates were changed every 10 min and sampling was performed at the midpoint of these ten-minute intervals, so that emptying of the gastric compartment occurred at a first order rate, with a half-life of 15 minutes. Samples from the duodenal chamber of the BioGIT system were divided into two subsamples. The first part was filtered through a 4-mm, 0.45-mm PTFE filter

(Whatman, Maidstone, United Kingdom) before being diluted using the mobile phase to evaluate dissolved drug concentration. The second part of the sample was used to evaluate the total amount of drug (solid and dissolved) per volume. Drug concentration was quantified by HPLC, as previously outlined above for the experiments with the compendial apparatus IV.

2.5 Determination of crystalline tacrolimus solubility

The equilibrium solubility of crystalline Tacrolimus in water, in Level III FaSSGF and in Level II FaSSIF-V2 was determined in triplicate by using the shake-flask method according to the United States Pharmacopeia (USP) recommended methodology [29]. Excess crystalline tacrolimus (500 mg) was added to 250 mL of medium in stoppered flasks which were continuously agitated at 150 rpm ($37 \pm 0.5^\circ\text{C}$). Samples were collected in triplicate after 24, 36, 48, 60 and 72 hours for water and after 24, 36 and 48 hours for the biorelevant media. Each sample was centrifuged for 10 minutes at 2500 rpm, the supernatant was collected and the drug content was quantified by HPLC, as previously outlined above for the experiments with the compendial apparatus IV. Equilibrium was reached at 24 hours in all experiments.

2.6 Comparative bioavailability study in healthy adults

2.6.1 Design and Approvals

The clinical study was performed in accordance to the requirements of the ICMR-National Ethical Guidelines for Biomedical and Health Research Involving Human Participants-2017, Good Clinical Practices for Clinical Research in India – Amended version - 2005, CDSCO guideline, ICH E6 (R2) 'Guidance on Good Clinical Practice', Declaration of Helsinki (Brazil,

October 2013), New Drugs and Clinical Trials Rules, 2019, EMA guideline, the ethical requirements of Directive 2001/20/EC and with procedures oriented to Good Laboratory Practice (OECD and Schedule L-1 of D&C rule 1945). The study protocol (protocol code: 19-VIN-0210) was approved by the Institutional and Independent Ethics Committee.

Twelve healthy, non-smoking adults volunteered to participate in this Open Label, Balanced, Randomized, Single-Dose, Three-Treatment, Three-Sequence, Three-Period, Three-Way Crossover, comparative bioavailability study of tacrolimus. Subject No. 12 withdrew consent before dosing of period 01, hence withdrawn from the study and replaced with an extra subject. The study subjects were of Asian race (from India), male, aged between 23-43 years of age (both inclusive) and of BMI from 18.80 to 28.90 Kg/m³ (both inclusive). All subjects met all the inclusion and none of the exclusion criteria described in the study protocol and were deemed healthy based on medical and medication history, physical exam, laboratory test results and vital signs. Subjects were instructed to refrain from alcohol, tobacco and caffeine containing beverages 48 hours before dosing as well as during the study. All subjects gave written consent before participating in the study. The total duration of the study was 52 days and a wash-out period of twenty-two days was maintained between each dosing period.

2.6.2 Protocol

At three different occasions, each subject received one Advagraf® capsule (5mg/cap), two Envarsus® tablets (one 1mg/tab and one 4mg/tab) and one Test Tablet (5mg/tab). The dosing units were administered orally to each subject in a sitting posture. The subjects were instructed to swallow the dose with 240 ± 2 mL of water at ambient temperature and not to chew or open the capsule and not to chew or crush the tablet but to consume it as a whole. Blood samples

were drawn at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 9, 10, 12, 14, 16, 18, 20, 24, 48, 72, 96 and 120 and 144 h following administration of each study medication in each study period.

2.6.3 Analytical method

Assay of tacrolimus concentration in whole blood was performed by using a validated Solid Phase Extraction method with an LC-ESI-MS/MS on Shimadzu - LC 30AD and LCMS-8050 mass spectrometer (Shimadzu Corporation, Kyoto, Japan). K3DTA was used as an anti-coagulant. The method showed linearity in the range of 0.05 ng/mL to 75.0 ng/mL, with the lower limit of quantification being 0.05 ng/mL. The chromatographic conditions included a Kinetex EVO C18 analytical column (100 × 4.6mm, 5µm; Phenomenex, California, USA) and the mobile phase buffer consisted of 0.1% Formic Acid (Pump:A) in Methanol (Pump:B), with initial concentration of mobile Phase 60% Pump:B. The flow rate was set at 1.00 mL/minute. The between-run accuracy ranged from 97.22 to 102.00 % with precision less than 5.88 %. The within-run accuracy ranged from 95.00 to 100.67 % with precision less than 4.63 %.

2.6.4 Data treatment

Individual whole blood half-lives ($t_{1/2}$) were estimated using the non-compartment model of Phoenix WinNonlin® 8.0 (Certara USA, Inc., New Jersey, USA). Individual early exposure was assessed by the area under blood concentration vs. time profiles up to 1.5 hours, i.e. by $AUC_{0-1.5h}$ values, and also by AUC_{0-2h} values. Total exposure was assessed by AUC_{0-144} , and $AUC_{0-\infty}$.

2.7 Statistical evaluation of product differences

One-way analysis of variance and the Tukey post-hoc test was employed for evaluating differences between the three products when using the small-scale setups. Unpaired t-test was employed for comparison of Advagraf® and Test Tablet data collected with the compendial apparatus IV. Repeated measures one-way analysis of variance and the Tukey post-hoc test was employed for comparing *in vivo* AUC values. All comparisons were made at the 0.05 level of significance using Sigmaplot for Windows (Systat Software, Inc. 2008).

3. RESULTS

3.1 Performance of tacrolimus ASDs with modified release characteristics in vitro

3.1.1 Two-stage biphasic system

Appearance in the decanol layer was rapid and greatest when using Advagraf® (> 90 % of the dose within 10 minutes of the switch to intestinal conditions) as shown in Figure 1. The Test Tablet led to the second most complete tacrolimus appearance into the decanol layer, with > 80 % of the dose present in the decanol layer by the end of the experiment. Envarsus® had the lowest % of dose in the decanol layer by the end of the experiment (18.56 ± 2.54 %, $n = 3$, $\pm$ SD). Based on data up to 60 min, early exposure was predicted to be significantly different among the three products ($p < 0.05$ for all ANOVA post-hoc comparisons), i.e. Advagraf® > Test Tablet > Envarsus®. Similarly, based on data up to 227 min total exposure was predicted to be significantly different among the three products ($p < 0.05$ for all ANOVA post-hoc comparisons), i.e. Advagraf® > Test Tablet > Envarsus®.

3.1.2 Two-stage D-P system

For all three products the % of dose in solution in the acceptor chamber was low at the end of the experiment (< 6%, Figure 2), with the lowest for Envarsus®, although scaling the flux/permeability values suggests that tacrolimus is well absorbed. At 30min, the % dose in solution in the acceptor compartment (~0.2%) indicates that concentration is ~0.7 µg/mL i.e., less than the crystalline solubility in water [1.6 (0.73) µg/mL] or Level III FaSSGF [2.0(0.38) µg/mL]. Also, at 4h the % dose in the acceptor compartment (<6% for all products) indicates that concentration is less than ~15 µg/mL, i.e. similar to the crystalline solubility in Level II

FaSSIF. These observations in conjunction with the amorphous solubility of tacrolimus in water (50 µg/mL, [30]) and the continuous ascending profiles indicate that re-crystallization of tacrolimus during the D-P experiments is unlikely. Based on data up to 60 min Advagraf® was predicted to have significantly higher early exposure than the Test Tablet or Envarsus® ($p < 0.05$ for both ANOVA post hoc comparisons), however, the difference between the Test Tablet and Envarsus® was not significant. Based on data up to 240 min Envarsus® was predicted to have significantly lower total exposure than Advagraf® and the Test Tablet ($p < 0.05$ for both ANOVA post hoc comparisons) whereas the difference between Advagraf® and the Test Tablet was not significant.

3.1.3 Compendial apparatus IV

Tacrolimus dissolution from Advagraf® was incomplete and minimally affected by the applied hydrodynamics and composition of the medium (Figure 3). Tacrolimus dissolution from Envarsus® was practically non-existent. The monolithic nature of Envarsus® and its non pH-dependent disintegration which seems to occur in the distal small intestine [31] posed a challenge for the hydrodynamics applied in this apparatus, in line with previous observations [32-34]. Tacrolimus dissolution from the Test Tablet showed a predominantly pH-dependent dissolution behavior, due to the inclusion of a pH-responsive polymer, Eudragit®-S100, which dissolves in pH above 7.0. However, a small portion of tacrolimus (~20% of the dose) is dissolved during the first hour, i.e. when the pH is 1.2 (FaSSGF) and 6.8 (FaSSIF-V2) (Figure 3). Dissolution from the surface of the disintegrated particles, due to incomplete coating may be one reason for this observation, as suggested previously by others [35]. The total volume of FaSSGF supplied during the first 30 min was $8 \times 30 = 240$ mL. The total amount dissolved from the test tablet at 30 min was estimated (by linear extrapolation) to be 0.3 mg, i.e. the average

concentration of tacrolimus in Level II FaSSGF effluent was $0.3 / 240 = 0.00125$ mg/mL. Similarly, the total volume of Level II FaSSIF-V2 and Level II FaSSIF-V2 Midgut supplied during between 30 and 120 min was $4 \times 90 = 360$ mL. The total amount dissolved from the test tablet between 30 and 120 min was 4.15 mg, i.e. the average concentration of tacrolimus in the Level II FaSSIF-V2 and the Level II FaSSIF-V2 Midgut effluents was $4.15 / 360 = 0.0115$ mg/mL. Since average concentrations are lower than crystalline solubilities in relevant media the potential for recrystallization during the experiment with the Test Tablet is unlikely. Based on the data up to 60 min, early exposure after Advagraf® is predicted to be non-significantly different than early exposure after the Test Tablet ($p=0.125$). Based on data at 360 min total exposure after Advagraf® is predicted to be significantly higher than total exposure after the Test Tablet ($p=0.002$).

3.1.4 BioGIT system

In experiments with Advagraf®, tacrolimus concentrations were below the limit of quantification in the duodenal chamber at all times. This was, at least partly, due to the tendency of the Advagraf® product to float on the surface of the gastric media and adhere to the glass walls of the gastric vessel, as the volume decreased when contents of the gastric compartment were being transferred into the duodenal vessel. Similar behavior has been previously described by Kourentas et al. (2016b) [28] with albendazole particles. In that case, quantification was possible and the transfer process was shown to be in line with luminal data in healthy adults. Particles of lipophilic molecules tend to adhere to the gastric mucosa and empty from the gastric compartment much slower than water in the fasted state [28].

Data for the Test Tablet are presented in Figure 4. Since the measured apparent concentrations are lower than the apparent solubility of crystalline tacrolimus in FaSSIF [13.5 (2.52) µg/mL], the lower-than-expected total amount / volume data suggest problematic transfer contents from gastric to the duodenal compartment also when testing the Test Tablet. Mean ratio $[AUC_{0-45\text{min, concentration}} / AUC_{0-45\text{min, theoretical}}] = 57.41 / 338.69 = 0.169$ i.e., 16.9% of total is in solution during the first 45 min of the experiment, in line with the ~20% of dose in solution during the first hour of the experiment with the compendial apparatus IV.

3.2 Performance of tacrolimus ASDs with modified release characteristics in healthy adults

Individual whole blood concentration vs. time profiles after single 5 mg doses of tacrolimus as Advagraf®, Envarsus® and Test Tablet are shown in Figure 5. Elimination half-lives were similar in all three phases (Table 2), thus, allowing for the evaluation of input processes.

Based on partial AUC values up to 1.5 or 2 hours (Table 2), early exposure after Envarsus® was significantly lower than both Advagraf® and Test Tablet ($p < 0.05$ for both ANOVA post-hoc comparisons), however, the difference between Advagraf® and Test Tablet was not significant. Published comparative Envarsus® vs. Advagraf® data from de novo kidney transplant recipients [37] are in line with data of this study. Envarsus® technology involves the atomization of tacrolimus and its inclusion in a complex polymeric matrix which does not allow for substantial release of tacrolimus in the proximal parts of the gastrointestinal lumen with the majority of release happening about 5h post administration [31]. In Advagraf®, HPMC moderately retards the diffusion of water in the formulation, thus, allowing partial release of

tacrolimus in the upper part of the gastrointestinal lumen. In the Test Tablet, excipients nominally prevent absorption early after administration, however early exposure after the Test Tablet was similar to Advagraf®. Differences in gastric emptying rates and/or inadequate coating of disintegrated tacrolimus particles could potentially account for this observation.

Based on AUC_{0-144} or $AUC_{0-\infty}$ values (Table 2), total exposure after Advagraf is significantly lower than total exposure after Envarsus® or Test Tablet ($p < 0.05$ for both ANOVA post-hoc comparisons), however, the difference between Envarsus® and Test Tablet is not significant. Interestingly, the ratio Advagraf®/Envarsus® for the mean AUC_{0-144} and $AUC_{0-\infty}$ is 71% and 70%, respectively, confirming the need for dose adjustment of 1 mg of Advagraf® to 0.7 mg of Envarsus®, when changing from one product to the other [38]. Tacrolimus is more susceptible to metabolism in the epithelium of the upper intestinal tract compared to the lower regions [39]. It has been hypothesized that the slower release of drug from the Envarsus® product results in significant absorption of drug in the colon and, therefore, intestinal metabolism of tacrolimus is reduced [17, 18]. Differences in total exposure between the Test Tablet and Advagraf® may also be attributed to differences in the release mechanisms; although the difference in early exposure between Advagraf® and Test Tablet was not significant, most of the dose after the Test Tablet is dissolved at distal parts of the intestine and, therefore, reduced pre-systemic metabolism is expected.

4. DISCUSSION

Various biorelevant *in vitro* testing methodologies for evaluating the luminal performance of orally administered ASDs have been proposed [40-44]. However, to date, they have been evaluated using ASDs with immediate release characteristics. In addition, the usefulness of relevant methodologies has been investigated after collecting the *in vivo* data, whereas comparative evaluation of proposed methodologies has rarely been performed [43]. In the present investigation we evaluated the usefulness of biorelevant *in vitro* testing methodologies in predicting formulation related differences in tacrolimus exposure after oral administration of ASDs with modified release characteristics in the fasted state.

4.1 Prediction of product related differences in early exposure

In adults, early exposures after Advagraf® and the Test Tablet were found to be non-significantly different and both were significantly higher than after Envarsus®. Based on the % of dose in decanol at 1h, data collected with the two – stage biphasic system correctly predicted the difference between Advagraf® and Envarsus® and the difference between Test Tablet and Envarsus®. Based on % dose in the acceptor chamber at 1h, data collected with the two – stage D-P system predicted correctly the difference between Advagraf® and Envarsus®. Based on the cumulative % dose dissolved at 1h, data collected with the compendial apparatus IV correctly predicted all product related differences in early exposure in adults. Data clearly overpredicted the trend for higher early exposure of the Test Tablet compared to Advagraf®. However, BioGIT data provided a basis for discussing clinical data early after products administration, i.e. the problematic transfer of Advagraf® from the gastric to the duodenal

BioGIT compartment could be related to the observed trend for lower early exposure after Advagraf® than Test Tablet (Table 2) whereas the dissolution of tacrolimus from the Test Tablet in gastric and/or duodenal BioGIT compartment could be related to the higher than expected (by design) early exposure after the Test Tablet (Table 2).

4.2 Prediction of product related differences in total exposure

In adults, total exposures after Envarsus® and the Test Tablet were found to be non-significantly different and both were significantly higher than after Advagraf®. Based on the % of dose in decanol at 4h, data collected with the two – stage biphasic system failed to predict product related differences in total exposure. Practically the entire dose appeared in decanol within a few minutes after transition to the simulated intestinal conditions. Due to the physical characteristics of Advagraf®, particles appeared to float on the surface of the aqueous media leading to the potential ‘non-physiological’ direct mixing of drug floating on the surface of the aqueous media with the decanol layer. Based on the % dose in the acceptor chamber at 4h, data collected with the two-stage D-P system failed to predict product related differences in total exposure. However, the overall rate of flux remains constant for Envarsus® for the duration of the test, whereas the Test and Advagraf® profiles are both slowing down. One potential reason may relate to the simulation of the intestinal transport process both in terms of the type of membrane employed and its surface area, especially versus the surface area assumed when simulating the intragastric conditions [19]. Based on the cumulative % dose dissolved at 6h or 8h, data collected with the compendial apparatus IV correctly predicted the difference in the total exposure between the two disintegrating products, the Test Tablet and Advagraf®. Data collected in this study confirm the need for developing and/or optimizing existing setups [34]

for evaluating the luminal performance of large monolithic non-disintegrating drug products that may be sensitive to intestinal shear forces in vivo, especially products with modified release characteristics that are also subjected to the ileo-cecal valve passage.

5. CONCLUSIONS

Screening for product related differences in tacrolimus early exposure after administration of amorphous solid dispersions with modified release characteristics in the fasted state was successful when using the compendial apparatus IV (open loop mode) with biorelevant media for physiologically relevant residence times. The small scale two-stage biphasic system was useful for the comparison of Envarsus® (non-disintegrating) with Advagraf® or Test Tablet (disintegrating products). The small scale dissolution–permeation system was useful for the comparison of Advagraf® with Envarsus®. BioGIT data provided a basis for discussing clinical data of the two disintegrating products early after administration.

Screening for product related differences in tacrolimus total exposure after administration of amorphous solid dispersions with modified release characteristics in the fasted state was less successful. Compendial apparatus IV (open loop mode) operated with biorelevant media for physiologically relevant residence time was useful for qualitatively predicting differences between the two disintegrating products.

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Table 1: Composition of the Test Tablet

<i>Ingredients</i>	<i>mg per tablet</i>	<i>%</i>
35% Tacrolimus		
61.5% Eudragit [®] -S100	14.29	2.86
3.5% Solutol [®]		
Lactose anhydrous	480.71	96.14
Mg stearate	5.00	1.00
Total	500.00	100.00

Table 2: Mean±SD values of pharmacokinetic parameters after single 5mg doses of tacrolimus as Advagraf®, Envarsus®, and Test Tablet, to twelve healthy non-smoking adult Asians (from India) in the fasted state.

Product	$t_{1/2}$	$AUC_{0-1.5}$	AUC_{0-2}	AUC_{0-144}	$AUC_{0-\infty}$
	h	(ng /mL) × h	(ng /mL) × h	(ng /mL) × h	(ng /mL) × h
Advagraf®	38±6.7	10.0±4.4	17.0±7.1	356±160	382±181
Envarsus®	39.0±5.8	4.0±2.6	8.3±5.3	499±239	540±270
Test Tablet	36.9± 6.7	10.1±5.7	24±11	463±268	495±303

FIGURE CAPTIONS

Figure 1: Tacrolimus cumulative % dose (mean $\pm$ SD, n=3) in the decanol layer of the small-scale two-stage biphasic system for one Advagraf® capsule (5mg/cap) ($\square$), two Envarsus® tablets (4mg/tab + 1 mg/tab) (Δ), and one Test Tablet (5mg/tab) ($\circ$). The dotted line shows the time at which simulated gastric conditions were changed to simulated intestinal conditions. Due to turbidity in the aqueous layer, it was not possible to determine tacrolimus in the aqueous phase of the gastric or the intestinal sector.

Figure 2: Tacrolimus cumulative % dose (mean $\pm$ SD, n=3) in the acceptor chamber of the small-scale two-stage dissolution permeation (D-P) system for one Advagraf® capsule (5mg/cap) ($\square$), two Envarsus® tablets (4 mg/tab + 1 mg/tab) (Δ), and one Test Tablet (5mg/tab) ($\circ$). The dotted line shows the time at which simulated gastric conditions were changed to simulated intestinal conditions.

Figure 3: Tacrolimus cumulative % dose (mean $\pm$ SD, n=3) dissolved vs. time profiles when using the compendial Apparatus IV (open loop mode) for one Advagraf® capsule (5mg/cap) ($\square$), one Envarsus® tablet (4mg/tab) (Δ), and one Test Tablet (5mg/tab) ($\circ$). Dotted lines show the times at which media and flow rates changed. FaSSGF is the Level III Fasted State Simulated Gastric Fluid, FaSSIF-V2, FaSSIF-V2midgut and SIF-ileum-V2 are the Level II Fasted State Simulated Intestinal Fluids and FaSSCoF is the Level II Fasted State Simulated Colonic Fluid

Figure 4: Mean $\pm$ SD ($n = 3$) data for the total amount per volume (●) and apparent concentration (○) in the duodenal compartment of BioGIT system vs. time profiles for the tacrolimus Test Tablet (5mg/tab). Advagraf® capsule (5mg/cap) data were below the limit of quantification at all timepoints. Envarsus® tablet (4mg/tab) was not evaluated, due to its monolithic nature (please see chapter 2.4.2). The dotted line shows the theoretical total drug amount per volume vs. time when assuming homogeneous transfer of contents and first-order transfer kinetics (Kourentas et al., 2016c) [36].

Figure 5: Individual whole blood tacrolimus concentrations vs. time profiles after (A) one Advagraf® capsule (5mg/cap), (B) two Envarsus® tablets (one 1mg/tab and one 4mg/tab), and (C) one Test Tablet (5mg/tab) to twelve healthy adults in the fasted state. Insert graphs represent magnifications of the profiles at early times after administration.

Figure 1

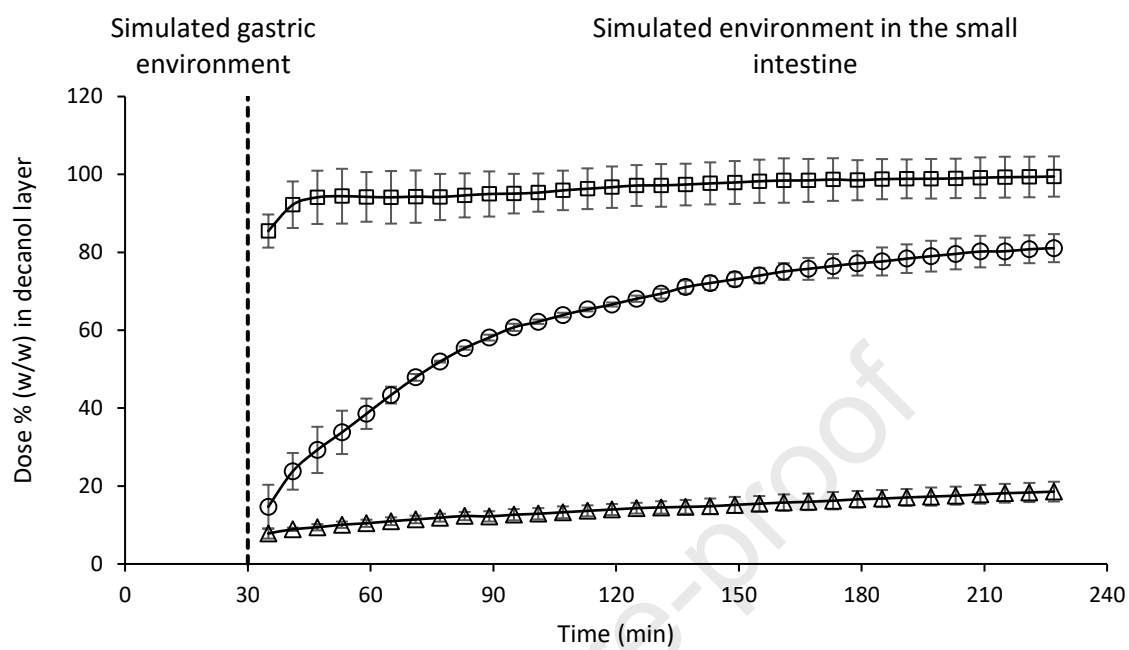


Figure 2

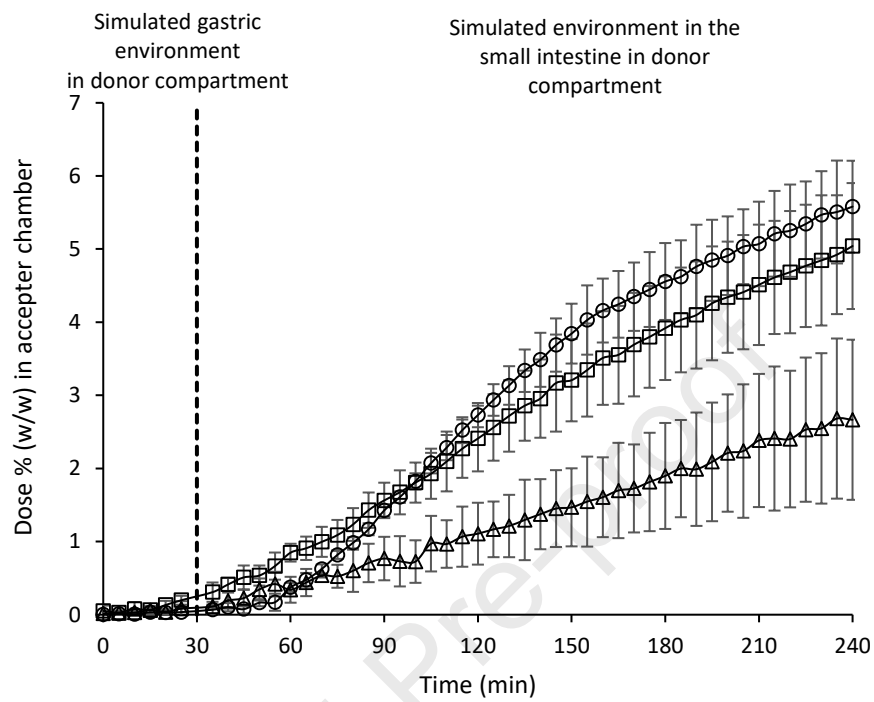


Figure 3

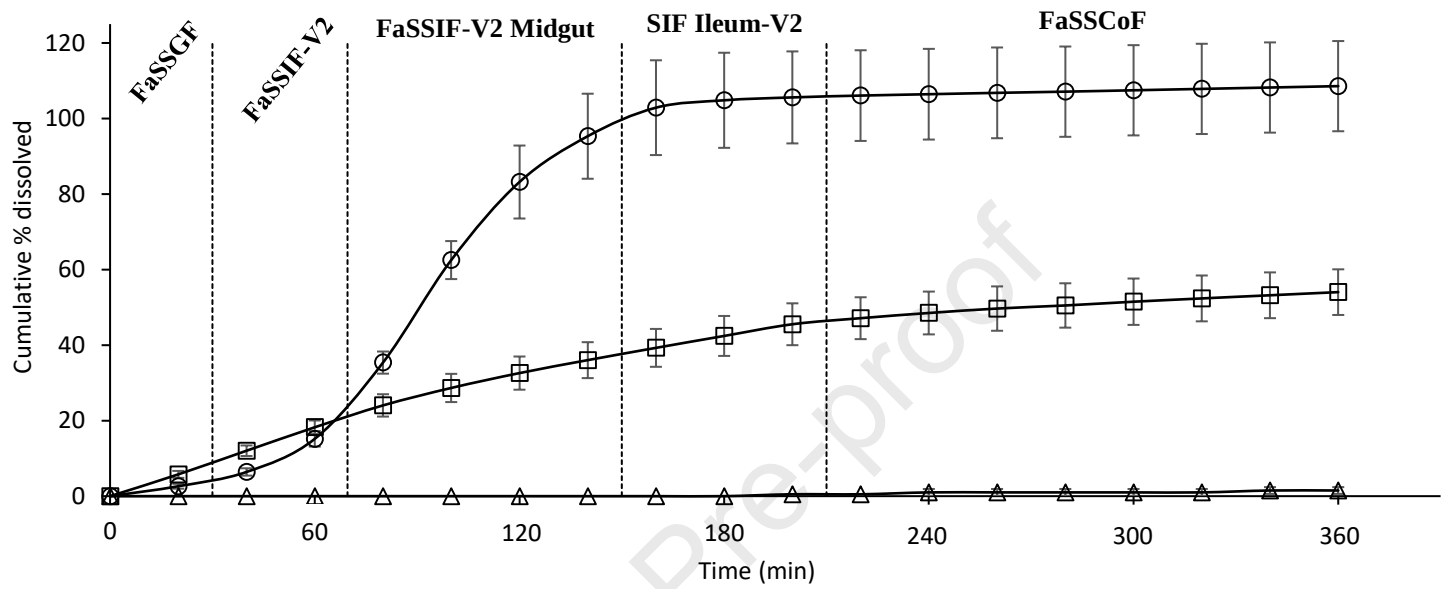


Figure 4

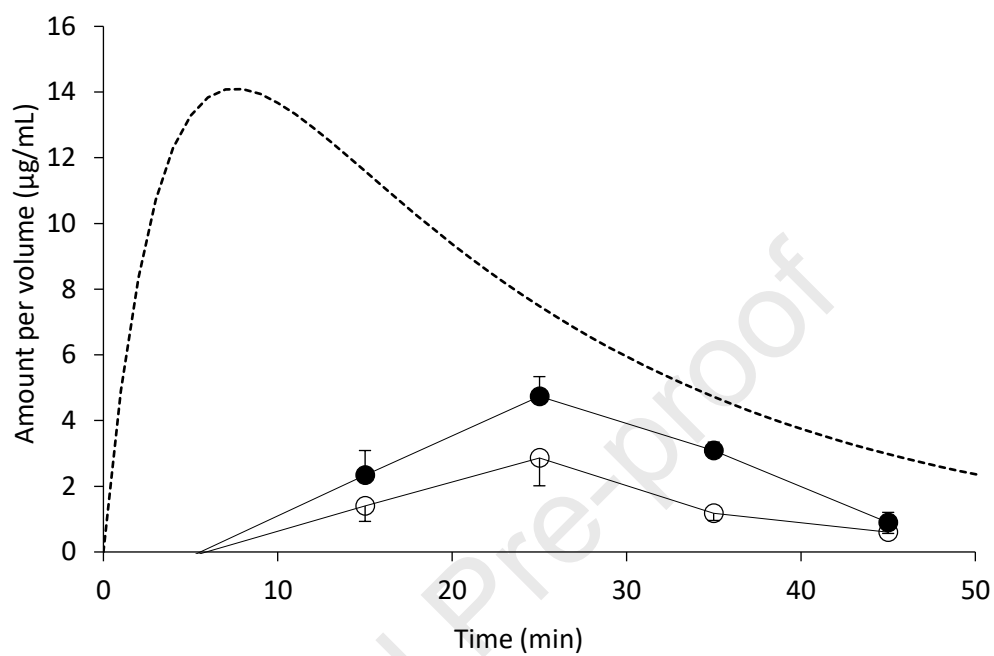
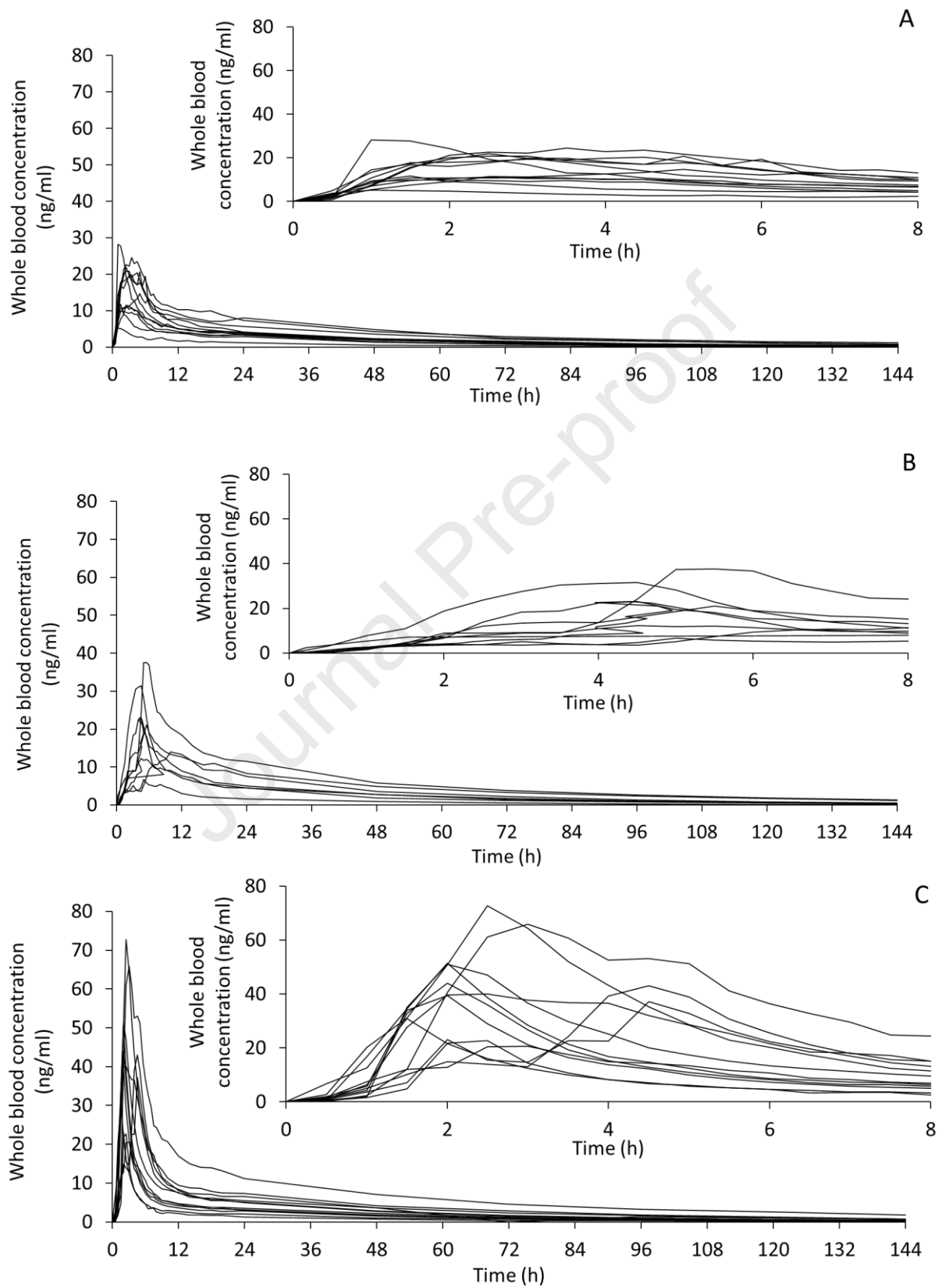


Figure 5



On the usefulness of four in vitro methodologies in screening for product related differences in tacrolimus exposure after oral administration of amorphous solid dispersions with modified release characteristics in the fasted state

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