Human intestinal fluid factors affecting intestinal drug permeation *in vitro*

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Graphical abstract

Abstract

Intestinal permeability assessment is an important aspect of drug development, which strongly depends on the solvent system used in the intestinal donor compartment. For this purpose, human intestinal fluids (HIF) can be considered as the golden standard. A recent study demonstrated a reduced apparent permeation across rat intestinal tissue from fed versus fasted state HIF for 9 out of 16 compounds tested. Commercially available fed and fasted state simulated fluids (FeSSIF and FaSSIF) reproduced this food effect for only 3 out of 16 compounds. To elucidate this observed difference, the current study assessed the impact of relevant intestinal fluid factors and 2-factor interactions at a fixed pH of 6.5 (bile salt, phospholipids, cholesterol, free fatty acids (FFA), monoacylglycerides (MAG)) on drug permeation across both rat tissue (Ussing chambers setup) and an artificial membrane (dialysis setup). Four representative compounds were selected for the permeation experiments: for propranolol and indomethacin, a food-induced permeation reduction was previously seen in both HIF and SIF; for metoprolol and darunavir, a reduction was only seen in HIF. Using a fractional 5-1 design of experiments (DoE) approach, 16 SIF combinations were used as donor media for permeation studies.

In the Ussing chambers (rat tissue), FFA and MAG reduced the permeation for all 4 compounds. Only for propranolol and indomethacin, permeation was further reduced by bile salts and phospholipids. This explains why the use of FeSSIF vs FaSSIF, lacking FFA and MAG, still predicted a negative food effect for propranolol and indomethacin but not for metoprolol and darunavir. In the dialysis set-up using an artificial membrane, significantly higher permeation rates compared to the Ussing chambers were observed. Under those conditions, FFA and MAG no longer reduced permeation, while bile salts and phospholipids still did. This may indicate that lipidic structures can provide depot release in the case of a dynamic equilibrium between free and entrapped drug.

Introduction

The majority of new chemical entities are lipophilic, compromising their intestinal absorption potential (Augustijns et al., 2014; Wuyts et al., 2014). Interaction of lipophilic compounds with the colloids in human intestinal fluids (HIF) may increase their apparent solubility but also reduce its apparent permeability by entrapment (Stappaerts et al., 2014; Wuyts et al., 2014, 2015). In this respect, the altered intraluminal composition and ultrastructure upon food intake (Clarysse et al., 2009a; Clarysse et al., 2009b; Mullertz et al., 2012; Mullertz et al., 2013; Mullertz et al., 2015; Riethorst et al., 2016; Riethorst et al., 2015) may exert a positive or negative food-effect on intraluminal drug absorption (Geboers et al., 2016; Wuyts et al., 2014).

To predict food effects on intestinal drug permeation, *in vitro* absorption models can be useful. While the Caco-2 model is often considered as the first choice in predicting intestinal drug permeability, it falls short to assess food effects since fed state media (both human and simulated fluids) are detrimental to Caco-2 monolayers (Wuyts et al., 2015). As such, more robust assays have to be used to study food-induced alterations in drug permeation *in vitro* (Wuyts et al., 2014). The Ussing chambers system using rat intestinal tissue has shown to be compatible with human and simulated fed state media. A recent study using human intestinal fluids demonstrated a reduced apparent permeation from fed versus fasted state fluids for 9 out of 16 compounds tested (Wuyts et al., 2014). The reduced permeation of lipophilic drugs from more complex media is most likely caused by entrapment of drug molecules in the colloidal structures. According to the classic model of passive drug absorption (i.e., permeation across an absorptive barrier is driven by the free drug concentration), the drug fraction solubilized by colloids is not readily available for uptake (Miller et al., 2011). Using fasted and fed state simulated fluids (FaSSIF and FeSSIF) based on the commercially available FaSSIF/FeSSIF/FaSSGF powder, however, this negative food effect on permeation was only seen for 3 out of 16 compounds. Considering the formation of colloidal structures, simulated fluids are comprised of bile salt and phospholipids that only form micelles. In contrast, fed state human intestinal fluids are more complex

with additional food derived components; in particular, lipid degradation products may contribute to the formation of larger colloids such as mixed micelles, vesicles and lipid droplets (Riethorst et al., 2016; Riethorst et al., 2015).

Considering the observed differences in food effect on permeation from human versus simulated intestinal fluids, the present study pursued to identify the factors in fluid composition that determine the permeation effect. Since the complex and highly variable human intestinal fluid composition (Bergstrom et al., 2014; Clarysse et al., 2009b; Fuchs and Dressman, 2014; Perez de la Cruz Moreno et al., 2006; Riethorst et al., 2016; Riethorst et al., 2015; Wuyts et al., 2014) makes it difficult to pinpoint those factors, the present study applied a design of experiment (DoE) approach using simulated fluids with variable composition. DoE is widely employed for identifying significant factors to optimize a certain process. However, its capability to identify relevant factors is useful in many other cases as shown, for instance, by Zou and Khadra et al. who used DoE to analyse the effect of SIF composition on solubility (Khadra et al., 2015; Zhou et al., 2017).

To determine the impact of fluid composition on drug permeation, we specifically focused on four compounds from the above-mentioned study (Wuyts et al., 2014) that all exhibited a negative food effect using fed versus fasted state human intestinal fluids (FeHIF versus FaHIF) but showed a different behaviour using FeSSIF versus FaSSIF. Group 1 (propranolol and indomethacin) exhibited a fed state permeability reduction in both HIF and SIF, while group 2 (metoprolol and darunavir) only displayed this negative fed state effect in HIF. To prepare simulated fluids with varying composition, the bile salt taurocholate, phospholipids, cholesterol, monoacylglycerides (MAG), and free fatty acids (FFA) were included as factors of a fractional 2^{5-1} DoE design, which enables the identification of all single and two-factor interactions. Permeation was tested in two set-ups: the Ussing chambers model using rat intestinal tissue and a simpler set-up using an artificial membrane across which colloids are excluded through size, allowing only free drug to permeate (Holmstock et al., 2013).

Materials & methods

Chemicals

Metoprolol tartrate was purchased from AB Hässle (Mölndal, Sweden), propranolol hydrochloride from Pharmachemic (Antwerp, Belgium), darunavir from Cilag AG (Schaffhausen, Switzerland) and indomethacin from Alpha Pharma (Braine-l'Alleud, Belgium). Cholesterol and palmitic acid were bought from Sigma-Aldrich (St. Louis, MO). 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine were purchased from Avanti (Alabama, USA). Taurocholate was obtained from MP Biomedicals (Ohio, USA), 1-palmitoyl-rac-glycerol from Bachem (Dübendorf, Germany) and PBS from Fisher Scientific (New Jersey, USA). Water was purified by an Elgastat Maxima system (Elga, Wycombe Bucks, UK).

Buffer preparation

Blank FaSSIF buffer was made by adding 0.42 g NaOH (pellets), 3.95 g NaH₂PO₄·H₂O and 6.19 g of NaCl to 1 L of ultrapure water. The pH, which was measured using a Portamess 911 pH-meter (Knick, Berlin, Germany) was adjusted to 6.5 with either 1 M NaOH or 1 M HCl. Modified Krebs Bicarbonate Ringer's buffer (KRB) contained 1.25 mM CaSO₄, 1.1 mM MgSO₄, 5 mM KCl, 1.15 mM Na₂HPO₄, 0.3 mM NaH₂PO₄, 25 mM NaHCO₃, and 110 mM NaCl supplemented with 25 mM glucose and adjusted to pH 7.4 with 1 M HCl after saturation with carbogen (O₂/CO₂ 95%/5%).

Design of experiments to define simulated media

To evaluate the impact of simulated fluid components (bile salt, phospholipids, cholesterol, FFA, MAG) on drug permeation, a DoE approach was applied. Design-Expert 7 (Stat-Ease, Inc.) was used to create a fractional 2⁵⁻¹ design with a resolution of five. This resolution enables to determine two-factor interactions without aliasing of other two-factor interactions. The 2⁵⁻¹ design resulted in 16 unique SIF combinations that were tested in triplicate, both in the Ussing chambers and an artificial membrane set-up. The upper (+1) and lower (-1) levels for each factor are displayed in Table 1. These

concentrations were selected based on our previously published work regarding the characterization of fed and fasted state human duodenal fluids (Riethorst et al., 2015). The results of the permeation experiments with the different SIF were analysed using the same software. ANOVA was used to determine the statistically relevant limits for each model compound. A half-normal plot was used to identify significant factors and to separate them from the sum of error ($p < 0.05$). Standard effects were calculated by the Design Expert software as well as by hand. A single factor standard effect equals to the average value obtained for the +1 level minus the average value obtained for the -1 level. Two-factor interaction effects were calculated in the same way as single factor effects, considering that the +1 interaction level corresponds to the combinations of -1/-1 and +1/+1, while the -1 interaction level corresponds to the combinations of -1/+1 and +1/-1.

Preparation of simulated media

Blank FaSSIF was heated to 37°C before using it in further preparations. To this buffer, different amounts of lysophosphatidylcholine (LPC), phosphatidylcholine (PC), taurocholate (TC), cholesterol, FFA and MAG were added to create the different DoE-based SIFs. The concentrations used in the 16 different SIFs are presented in Table 2. The used phospholipids (PL) consisted of 80% LPC and 20% PC. For each SIF, the required amounts of LPC and PC were diluted in 1.5 mL of methanol. TC, cholesterol, FFA and MAG were weighted separately. Cholesterol, FFA and MAG were solubilized in 700 μ L, 2 mL and 2 mL of chloroform, respectively. TC was solubilized in warm blank FaSSIF (pH 6.5) buffer (50% of the final volume of the SIF). Then, the mixture of LPC and PC in methanol was added to the TC solution. After 2 minutes, the solutions of cholesterol, FFA and MAG were added drop by drop. Next, the organic solvents were evaporated between 55-65°C after which the volume was adjusted to the final volume with blank FaSSIF buffer and the pH was set to 6.5. The final SIFs were stirred overnight at 37°C.

Permeation across rat tissue (Ussing chambers setup)

Approval for the experiments was granted by the Institutional Ethical Committee for Animal Experimentation of the KU Leuven. Transport studies were based on a previously described method

(Deferme et al., 2002). Briefly, male Wistar rats of 250–300 g (Janvier, Le Genest Saint-Isle, France) were fasted overnight until anaesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, 60 mg/kg, Sanofi Santé Animale, Brussels, Belgium). After laparotomy, a segment of the ileum (0–20 cm proximal to the ileocaecal junction) was rapidly removed, rinsed with ice-cold KRB and put into a beaker with KRB solution on ice that was continuously bubbled with carbogen O₂/CO₂ (95%/5%). Animals were sacrificed immediately after removal of the intestinal segment. After an equilibration period of 15 min, pieces of approximately 1 cm long were cut along the mesenteric border. The longitudinal muscle layer was carefully removed in ice-cold oxygenated KRB and the tissue was mounted in the Ussing chambers setup (Harvard Navicyle, Holliston, MA, USA) with an exposed tissue area of 0.66 cm². Each half-cell was filled with 5 mL KRB, oxygenated with carbogen and kept at a temperature of 37°C using a heating block, after which the tissue was equilibrated for 15 min. Meanwhile, a donor solution of the model compounds (final concentration 100 µM each) was prepared by spiking stock solutions into the preheated DoE SIF medium of interest. After tissue equilibration, the medium in the acceptor (serosal) compartment was replaced by fresh KRB pH 7.4. The medium at the mucosal compartment was replaced by the donor solution with model compounds. Samples of 0.2 mL were taken from the acceptor side every 15 min and replaced by fresh KRB up to 2 h after the start of the experiment. Donor samples (10 µL) were collected after 2 min, 1 h, and at the end of the experiment. Following 2 h incubation with the donor solution, donor and acceptor media were replaced with fresh KRB buffer; at the mucosal side, 10 µM sodium fluorescein was included. The serosal concentration of sodium fluorescein was measured after 1 h using an Infinite M200 plate reader (Tecan, Austria) at an excitation and emission wavelength of 494 and 521 nm, respectively. If permeated concentrations of fluorescein remained below 1% of the donor concentration, the rat intestinal tissue was considered intact. The apparent permeability (P_{app}) of rat intestinal mucosa for the model compounds was calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt} * \frac{1}{A * C_{donor}}$$

where Q is the amount of drug appearing in the acceptor compartment as a function of time (t), C_{donor} the initial drug concentration in the donor compartment, and A the surface area across which transport occurred (0.66 cm^2). The P_{app} calculation was based only on the linear part of the transport curve (steady state). It should be noted that in the colloid-containing SIF media, P_{app} values were calculated using apparent donor concentrations (i.e. both freely dissolved drug and drug solubilized in the colloidal structures).

Permeation using an artificial membrane set-up

Permeation was tested using the HTD 96b dialysis setup from HTDialysis, LLC (Gales Ferry, CT, US). The donor and acceptor compartments were separated by cellulose membrane strips with a molecular weight cut-off of 3.5 kDa ($< 1\text{ nm}$), which are expected to be impermeable for micelles and vesicles, since these structures were shown to be about 10-50 nm and 20-2000 nm, respectively (Riethorst et al., 2016). The membranes were hydrated according to the manufacturer's instructions. The acceptor compartment was filled with 150 μL of phosphate buffered saline (PBS). The osmolality of PBS was adjusted to the osmolality of the donor media by diluting a 10x concentrated PBS solution. The donor compartment was loaded with an equal volume of DoE SIF spiked with 100 μM of model compound. Samples from the donor and acceptor compartment were taken after 0.5, 2, 4 and 6 h. A single well was used for each time-point. The dialysis setup did not allow for determination of permeability values. Instead, the permeated amount was expressed as the percentage of drug initially present in the donor solution.

HPLC analysis

The HPLC system used to analyse the samples consisted of a Waters 2790 Alliance series separations module and a Novapak C18 column under radial compression (Waters, Milford, MA). UV absorbance was monitored using a Waters 2487 detector and fluorescence emission using an FP-1520 intelligent fluorescence detector (Jasco, Tokyo, Japan). The observed peaks were integrated using Empower Pro (Empower 2) software. Indomethacin, metoprolol, propranolol, and darunavir were analysed by

elution with a gradient using 25 mM acetate buffer (pH 3.5) (A) and methanol (B). A constant flow rate of 1.2 mL/min and a sample injection volume of 95 μ L were used. Gradient elution started at 50% A and 50% B, and changed linearly to 20% A and 80% B over 11 min. Subsequently, the gradient changed to 5% A and 95% B over 4 min. After 16 min, the gradient was changed back to 50% A and 50% B over 0.1 min. The total runtime was 17 min. Metoprolol, propranolol, darunavir and indomethacin peaks eluted after 4.1 min, 7.4 min, 9.1 min, and 14.8 min, respectively. Indomethacin was detected using UV at 260 nm. The other compounds were all detected using fluorescence at the following excitation and emission wavelengths (nm): 271/302 (metoprolol), 249/333 (propranolol), and 268/347 (darunavir). The analysis was linear between 5 μ M and 0.04 μ M. Accuracy, intra- and inter-day repeatability were determined at concentrations of 1.5 μ M, 0.5 μ M and 0.1 μ M for the compounds detected by fluorescence. For indomethacin, concentrations of 7.5 μ M, 2.5 μ M and 0.5 μ M were used. Both intra- and inter-day repeatability showed relative standard deviations below 2%. Accuracy deviations were below 10%.

Results & Discussion

For this study, four compounds were selected based on a negative food effect on their permeation across rat intestine, as observed by Wuyts et al. (Wuyts et al., 2014). As depicted in Table 3, the use of fed compared to fasted state HIF as donor medium in the Ussing chamber system reduced the apparent permeability for these four compounds. Using SIF, such a negative food effect was only observed for propranolol and indomethacin but not for metoprolol and darunavir. To identify the factors determining the observed differences, permeation studies were performed starting from solutions in 16 different SIF (DoE, Table 2). These factors are all believed to play a role in colloids formation. Colloids can affect the free drug concentration, which is the driving force for permeation.

Effect of intestinal fluid composition on permeation across rat tissue (Ussing chambers)

The apparent permeabilities (P_{app}) of rat intestinal tissue for the four model compounds using each of the 16 DoE SIF are summarized in Table 4. For each of the model compounds, a relatively broad range in P_{app} values was observed: the fold difference between the lowest and highest P_{app} values amounted to 5.7 for propranolol, 3.6 for indomethacin, 3.3 for metoprolol, and 4.4 for darunavir. The large variation in P_{app} values between the different DoE SIF clearly show the pronounced impact of intestinal media composition on permeation through rat intestine.

Using DoE analysis, the influence of each compositional factor in the DoE SIF was investigated. Figure 1 displays the standard effects of single factors and two-factor interactions, ranked from high to low. Here, the grey zone marks the threshold for statistically significant effects ($p < 0.05$). The pH of all SIF was fixed at 6.5 to exclude any potential pH effect.

The standard effects obtained from DoE show that dietary lipids (MAG & FFA), bile salt and phospholipids all negatively affect the permeation of propranolol and indomethacin (Figure 1a-b). These results are coherent with the previously observed negative food effect in both SIF and HIF for these compounds. In fed state HIF, dietary lipids as well as increased bile secretions may contribute to

the reduction in P_{app} compared to fasted state HIF. Even though FeSSIF only contains bile salt and phospholipids, their increased concentrations compared to FaSSIF may also reduce P_{app} values, as indicated by the DoE results. Thus, the DoE outcome clearly demonstrates why SIF was able to qualitatively predict the negative *in vitro* food effect of HIF on propranolol and indomethacin permeation.

For the majority of compounds investigated by Wuyts et al., the observed negative food effect on permeation from HIF could not be predicted by SIF. Darunavir and metoprolol are clear examples of this behavior. DoE analysis shows a strong negative effect of dietary lipids (MAG and FFA) on permeation of these two compounds (Figure 1c-d), explaining the negative food effect observed in HIF. In contrast, phospholipids and bile salt, the only factors present in FeSSIF, do not reduce the permeation of metoprolol and darunavir (apart from a small negative effect of phospholipids on darunavir permeation). As such, the DoE results explain why the negative food effect on metoprolol and darunavir permeation from HIF could not be predicted by the use of FeSSIF. Interestingly, the higher bile salt concentration in the DoE SIF even increased permeation of metoprolol and darunavir across rat tissue. Although this effect does not seem to determine the observations in HIF for these compounds, it may suggest alternative absorption mechanisms that could be subject of further research (Yeap et al., 2013).

Permeation rate in the Ussing chambers set-up vs. an artificial membrane setup

In all experiments with rat tissue in the Ussing chambers setup, very low relative permeation rates were observed. After 2 h, the permeated amount of each of the four model compounds remained below 2% of the initial donor in all tested media. *In vivo*, it can be expected that the overall permeation is much higher, considering, for instance, the higher surface area available for absorption and the improved sink conditions. The limited permeation in the Ussing chambers implies that the donor solutions, including the dynamics between free and entrapped drug in colloid-rich media, are not significantly altered.

As an alternative, we studied the permeation across an artificial cellulose membrane in a high-throughput dialysis set-up. The dialysis system is designed to determine the free drug concentration, once equilibrium has been reached between donor and acceptor media. Figure 2 depicts the permeation of the model compounds across the artificial membrane as a function of time. For blank buffer, without any colloids, the equilibrium was reached after 4 hours (all compounds).

In colloid-rich media, with high lipid (FFA and MAG) concentrations, permeation was slower and equilibrium was not yet reached after 4 h for certain higher concentrated SIF. Compared to the Ussing chambers experiments, permeation across the artificial membrane in a dialysis set-up was clearly faster: after 4 h, permeated amounts were up to 50% of the initial donor in DoE SIF. Even after 30 min, the permeated amount appeared far higher than in the Ussing chambers after 2 hours. The differences in overall permeation rate between the two models are clearly visible in Figure 3, depicting the permeation averaged over the 16 different DoE SIF.

There are several possible reasons for the increased permeation across the artificial membrane in the dialysis setup. (1) The dialysis membrane, a porous polymer with a Mw cut-off value of 3.5 kDa, is obviously less complex than rat intestinal tissue with mucosa and submucosa. (2) The hydrodynamics are more vigorous in the dialysis setup. Donor and acceptor compartments are shaken at 100 rpm, which is in contrast to the slow mixing in the Ussing chambers by sparged carbogen. (3) The surface to volume ratio is much lower in the Ussing chambers setup (tissue surface 0.66 cm² for 5 mL of donor solution) versus the dialysis system (membrane surface 0.62 cm² for only 0.15 mL of donor solution).

Irrespective of the underlying mechanism, it is clear that the increased permeation in the dialysis system generates a rapid depletion of free drug concentration in the donor compartment, potentially altering the dynamics between free and entrapped drug in colloid-rich media.

Effect of intestinal fluid composition on drug permeation through an artificial membrane (dialysis setup)

The impact of intraluminal factors on drug permeation across the artificial membrane was investigated using an identical DoE approach as for the Ussing chambers (instead of P_{app} values, the permeated amount after 4 h was used as result). Figure 4 depicts the standard effects of all factors on the permeation of the four model compounds across the artificial membrane. Compared to the Ussing chambers (Figure 1), a vastly different outcome was obtained. For all compounds, the impact of dietary lipids (FFA, MAG, and cholesterol) was greatly reduced and statistically insignificant compared to their major effect on permeation across rat tissue.

For propranolol and indomethacin, Figure 2 demonstrates a substantial impact of SIF composition on permeation across the artificial membrane: the permeated fractions after 6 h ranged between 12-43% for propranolol (3.6-fold difference) and between 21-47% for indomethacin (2.3-fold difference). From the standard effects in Figure 4a-b, it is clear that bile salt is the major factor responsible for reducing permeation in SIF. The bile salt effect is influenced by the interaction with phospholipids. As such, the balance between these two factors appears to play an important role on permeation as well. Review of permeation data in individual SIF showed that entrapment from bile salt and phospholipids is lower if they are in a balanced ratio (i.e. bile salt/phospholipids levels +1/+1 or -1/-1). Combinations of high and low for bile salt and phospholipids (-1/+1 or +1/-1) resulted in the lowest permeation. In contrast to the results obtained in the Ussing chamber (Figure 1a-b), dietary lipids did not affect permeation of propranolol and indomethacin across the artificial membrane.

For metoprolol and darunavir, the permeated values after 6 hours ranged between 42-48% (1.1-fold difference) and between 37%-48% (1.3-fold difference), respectively (Figure 2). This limited spread indicates a minimal impact of SIF composition on permeation across the artificial membrane, which is reflected in the DoE results. While dietary lipids were the main determinants of permeation of these compounds in the Ussing chambers (Figure 1c-d), bile salt concentration is the only significant factor

that could be identified in the dialysis set-up (negative effect on darunavir permeation, influenced by the significant interaction with phospholipids).

Concluding remarks

It has previously been demonstrated that fed state HIF and SIF may reduce the permeation of lipophilic drugs across rat intestinal tissue in the Ussing chambers set-up. For 9 out of 16 compounds, this behavior was observed in HIF but not in SIF (i.e. FeSSIF/FaSSIF combination). In the present study, the intraluminal factors determining the impact on permeation were studied by DoE. For metoprolol and darunavir, two compounds for which a negative food effect was demonstrated using HIF but not SIF; dietary lipids, not present in FeSSIF, were identified as the main factors reducing permeation (Figure 1). Furthermore, permeation of these compounds was not negatively affected by the main constituents of FeSSIF and FaSSIF (bile salt and phospholipids). Clearly these drugs are entrapped by lipidic structures (vesicles, lipid droplets, and mixed-micelles) decreasing the free fraction available for permeation.

In the case that bile salt and phospholipids were identified as reducing factors, as shown for propranolol and indomethacin (Figure 1), a negative food effect could be seen in both HIF and SIF. The fact that dietary lipids further reduce permeation of these compounds, might explain why the observed food effect was more pronounced in HIF than SIF. Overall, the DoE results for the Ussing chamber experiments have shown that dietary lipids are of high importance and should be implemented into simulating fluids for the prediction of food effects on drug permeation. In this respect, FeSSIF-v2, whether or not modified to be compatible with Caco-2 monolayers, may be a good starting point, since it includes both FFA and MAG (Vertzoni et al., 2012; Markopoulos et al., 2014).

It is important to realize that overall permeation across intestinal tissue in Ussing chambers is very limited and possibly not fully reflecting the *in vivo* situation due to a low surface to volume ratio, tissue accumulation, and sub-optimal hydrodynamics. Therefore, we investigated the impact of intraluminal factors in an alternative system, consisting of an artificial membrane in a dialysis set-up. Here, the fraction of drug that permeated the barrier was much higher than in the Ussing chambers. As schematized in Figure 5B, the increased flux may create a more dynamic environment in the donor

solution, where the relatively fast removal of free drug due to permeation might continuously shift the equilibrium between free and entrapped drug. In the Ussing chambers, the donor solution remains more static since the free fraction is almost not affected by the limited permeation (Figure 5A). As demonstrated in the current study, the impact of entrapment on permeation is less pronounced in the dynamic donor compartment of the dialysis setup. Since the DoE results indicated that dietary lipids no longer influence permeation in this setup (Figure 4), it is believed that lipidic colloids (vesicles, lipid droplets, mixed-micelles) can provide depot release of entrapped compound in such a dynamic situation. Apparently, this depot release is less pronounced from bile salt- and phospholipid-based colloids as these factors could still significantly reduce permeation (Figure 4).

In conclusion, this study has shown that lipids increase the biorelevance of SIF in reproducing drug entrapment effects on *in vitro* permeation, as seen using human intestinal fluids. Furthermore, it has demonstrated that the impact of entrapment on drug permeation strongly depends on the dynamics of the donor environment. In this respect, further research is required on how different *in vitro* models for permeation can be used to provide complementary insights. Ussing chambers using rat intestinal tissue may be used to determine the intrinsic link between colloidal entrapment and the permeability for lipophilic drugs. On the other hand, a more dynamic model, such as an artificial membrane in a dialysis setup, may better reflect the impact of entrapment in the human intestine, known for its high surface to volume ratio and sink conditions.

Tables

Table 1. Factors and values used for DoE.

Factor	-1	+1
Bile salt	3.00 mM	20.00 mM
Phospholipids	0.75 mM	5.00 mM
Cholesterol	0.10 mM	1.00 mM
FFA	0.50 mg/ml	7.00 mg/ml
MAG	0.10 mg/ml	3.00 mg/ml

Table 2. All SIF combinations generated in the 2^{5-1} DoE.

SIF #	Bile salt (mM)	Phospholipids (mM)	Cholesterol (mM)	FFA (mg/ml)	MAG (mg/ml)
1	20.00	0.75	0.10	7.00	3.00
2	20.00	5.00	0.10	0.50	3.00
3	20.00	5.00	0.10	7.00	0.10
4	3.00	5.00	1.00	7.00	0.10
5	20.00	5.00	1.00	0.50	0.10
6	20.00	0.75	0.10	0.50	0.10
7	3.00	0.75	0.10	0.50	3.00
8	3.00	0.75	0.10	7.00	0.10
9	3.00	5.00	0.10	0.50	0.10
10	20.00	0.75	1.00	0.50	3.00
11	3.00	0.75	1.00	7.00	3.00
12	20.00	5.00	1.00	7.00	3.00
13	3.00	5.00	0.10	7.00	3.00
14	3.00	5.00	1.00	0.50	3.00
15	3.00	0.75	1.00	0.50	0.10
16	20.00	0.75	1.00	7.00	0.10

Table 3. Food effects on the apparent permeability of rat intestine for the selected model compounds in the Ussing chambers model, starting from a solution in HIF or SIF. - indicates the presence of a negative food effect. / indicates no significant food effect. Data from Wuyts et al., 2014.

	HIF	Ratio	SIF	Ratio
		fed/fast		fed/fast
		P _{app}		P _{app}
Propranolol	-	0.10	-	0.25
Indomethacin	-	0.30	-	0.35
Metoprolol	-	0.55	/	0.85
Darunavir	-	0.45	/	1.00

Table 4. Individual permeability of rat intestinal tissue for the four model compounds, obtained from all 16 DoE SIF (Ussing chambers setup). P_{app} values (10^{-6} cm/s) represent the average and standard deviation (SD) of $n=3$. For each compound, the highest and lowest P_{app} values are indicated in bold and underlined.

	Propranolol		Indomethacin		Metoprolol		Darunavir	
SIF #	P_{app}	SD	P_{app}	SD	P_{app}	SD	P_{app}	SD
1	1.5	0.5	6.2	0.5	5.4	0.5	6.2	0.7
2	3.8	1.1	7.9	1.1	13	3	11	2
3	3.8	1.0	9.3	1.0	12	3	9.3	2.0
4	6.5	1.1	16	1.1	11	2	9.9	1.4
5	4.7	0.8	17	0.8	12	2	11	1.1
6	6.4	0.5	16	0.5	18	2	17	0.7
7	8.6	0.8	18	0.8	9.3	2.2	8.2	1.3
8	5.8	1.0	14	1.0	8.0	1.1	6.4	0.8
9	4.2	0.5	10	0.5	8.6	0.6	6.1	0.3
10	3.1	0.5	8.8	0.5	11	1.2	8.3	1.9
11	5.2	1.4	9.2	1.4	6.7	1.6	5.5	0.8
12	1.8	0.4	6.6	0.4	7.1	1.8	4.9	1.1
13	4.2	0.5	7.9	0.5	5.5	0.3	3.9	1.0
14	4.5	1.2	14	1.2	7.4	2.1	7.7	1.2
15	8.5	1.5	22	1.5	14	3	15	3
16	4.0	1.4	11	1.4	13	1.1	11	3

Figures

Figure 1 Impact of intestinal fluid factors on permeation across rat intestine in the Ussing chamber setup. For each factor and 2-factor interaction, the DoE standard effect on permeation is shown. These effects are ranked from large to small (top to bottom). The grey area indicates the threshold for statistical significance (ANOVA, $p < 0.05$) calculated using Design Expert 7. Graphs a and b depict the effects on propranolol and indomethacin permeation, for which a negative food effect was previously

shown in both HIF and SIF (Wuyts et al., 2014). Graphs c and d depict the effects on metoprolol and darunavir permeation, for which a negative food effect was previously shown in HIF but not in SIF (Wuyts et al., 2014). BS = bile salt, PL = phospholipids, Chol = cholesterol, MAG = monoacylglycerides, FFA = free fatty acids. * stands for the interaction between 2 factors.

Figure 2: Time dependent permeation across the artificial membrane using a dialysis set-up. Permeation is expressed as the permeated amount relative to the initial amount in the donor solution.

The red-line indicates the permeation from colloid-free donor buffer (blank FaSSIF pH 6.5). The different symbols represent permeation from the 16 DoE SIF (shown in Table 2). Each timepoint represents the average of n=3.

Figure 3. Difference in overall permeation between the Ussing chambers with rat intestine and the artificial membrane setup. The results depict the average (+ SD) permeated amount over the 16 different DoE SIF as donor media.

Figure 4 Impact of intestinal fluid factors on permeation in the artificial membrane setup. For each factor and 2-factor interaction, the DoE standard effect on permeation is shown. These effects are ranked from large to small (top to bottom). The grey area indicates the threshold for statistical significant (ANOVA, $p < 0.05$) calculated using Design Expert 7. Graphs a and b depict the effects on propranolol and indomethacin permeation, for which a negative food effect was previously shown in both HIF and SIF (Wuyts et al., 2014). Graphs c and d depict the effects on metoprolol and darunavir

permeation, for which a negative food effect was previously shown in HIF but not SIF (Wuyts et al., 2014). BS = bile salt, PL = phospholipids, Chol = cholesterol, MAG = monoacylglycerides, FFA = free fatty acids. * stands for the interaction between 2 factors.

A: Ussing chambers (rat intestinal segment)

B: Dialysis membrane (artificial membrane)

Figure 5 Low permeation (static) versus high permeation (dynamic) model. Part A depicts the situation as it occurs in the Ussing chambers. Here, only a small fraction of free drug permeates from the donor compartment. As a result, the equilibrium between free and entrapped drug remains practically unaltered, which can be considered as a relatively static environment. Part B depicts the artificial membrane setup, where a much larger fraction of free drug permeates. As a result, the donor balance between entrapped and free drug is continuously shifted, possibly resulting in depot release from certain colloids.

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