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Understanding the impact of lipids on the solubilizing capacity of human intestinal fluids

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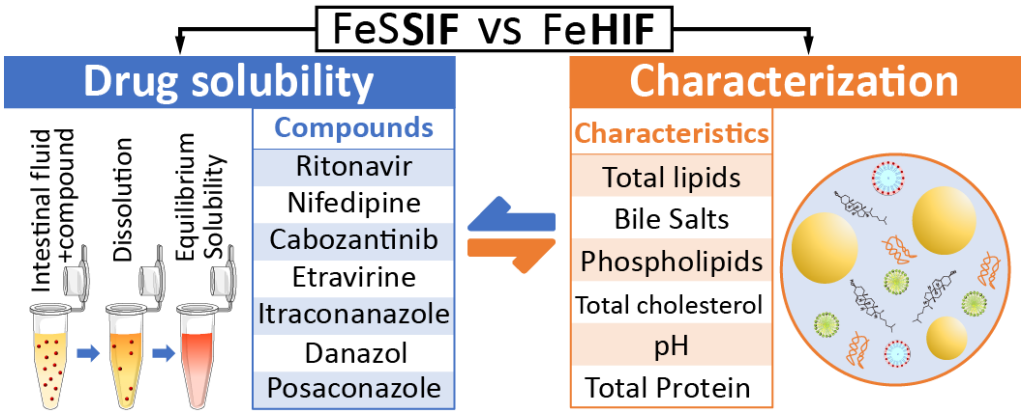
Graphical abstract



Abstract

Lipids in human intestinal fluids (HIF) form various structures, resulting in phase separation in the form of a lipid fraction and a micellar aqueous fraction. Currently used fed state simulated intestinal fluids (SIF) lack phase separation, highlighting the need for a deeper understanding of the effect of these fractions on intestinal drug solubilization in HIF to improve simulation accuracy. In this study, duodenal fluids aspirated from 21 healthy volunteers in fasted, early fed, and late fed states were used to generate 7 HIF pools for each prandial state. The apparent solubility of seven lipophilic model drugs was measured across these HIF pools, differentiating between the micellar fraction and the total sample (including both micellar and lipid fractions). The solubilizing capacities of these fluids were analysed in relation to their composition, including total lipids, bile salts, phospholipids, total cholesterol, pH, and total protein. The solubility data generated in this work demonstrated that current fed state SIF effectively predicted the average solubility in the micellar fraction of HIF but failed to discern the considerable variability between HIF pools. Furthermore, the inclusion of a lipid fraction significantly enhanced the solubility of fed state HIF pools, resulting on average in a 13.9-fold increase in solubilizing capacity across the seven model compounds. Although the average composition of the fluids was consistent with previous studies, substantial variability was observed in micellar lipid concentrations, despite relatively stable total lipid concentrations. This variability is critical, as evidenced by the strong correlations between the solubilizing capacity of the micellar fraction and its micellar lipid concentrations. Additionally, this study identified that fluctuations in bile salt concentrations and pH contributed to the observed variability in micellar lipid concentration. In summary, the influence of the lipid fraction on solubility was twofold: it enhanced the solubility of lipophilic drugs in the total fluid, and contributed to the variability in the solubilizing capacity of the micellar fraction.

Key words: Human intestinal fluids (HIF), food effects, simulated intestinal fluids (SIF), solubility, intestinal fluid characterization, intestinal fluid aspiration, lipids, bile salts, pH

1 Introduction

Over the past few decades, the amount of poorly water-soluble, lipophilic drugs has increased drastically within the pharmaceutical pipeline.[1] To achieve adequate dissolution and subsequent absorption after oral intake, poorly water-soluble drugs often rely on adequate luminal fluid volumes and the presence of solubilizing structures consisting of bile salts, phospholipids and dietary lipids in the gastrointestinal tract. However, these components are present in variable amounts and can differ inter- and intra-individually. This makes poorly water-soluble drugs prone to undesirable variation in intestinal absorption and bioavailability.[2–5]

The intake of food adds to the variability in luminal conditions, potentially affecting drug absorption. Indeed, food causes alterations in the composition of luminal fluids by the intake and digestion of exogenous components such as carbohydrates, lipids and proteins. In addition, food causes functional changes to the gastrointestinal tract, including delayed gastric emptying, increased bile salt secretion and enlarged fluid volumes.[6–8] This interplay can lead to notable changes in the oral bioavailability of poorly water-soluble drugs, resulting in so called "food effects". A positive, negative or no food effect is classified based on the ratio of the systemic exposure between fed and fasted state.[7,8] Food induced changes in systemic concentrations can also have potential implications for the efficacy and safety of a drug.[6,9] Consequently, regulatory bodies have integrated the assessment of food effects on oral bioavailability into the drug registration process,[8] requiring dedicated studies during the clinical phase. In order to understand and possibly mitigate food effects during the drug product development process, early insights into these effects are helpful to pharmaceutical companies for optimal lead compound selection and formulation design and optimization.

Multiple approaches are currently implemented in the pharmaceutical industry to predict food effects in the preclinical phase, including *in vitro*, *in silico* and *in vivo* methodologies.[10] Among these, *in vitro* tools play an important role in the food effect assessment, beginning with the determination of the equilibrium solubility in simulated intestinal conditions as a foundational step for new API and formulations. At later stages, more complex *in vitro* tools such as Tiny-TIM aim to simulate the dissolution and absorption dynamics of a formulated drug. To determine the equilibrium solubility and simulate the dissolution process in both fasted and fed state, simulated intestinal fluids (SIF) are commonly used. These fluids are intended to reflect the composition of human intestinal fluids (HIF) [11]. Yet, current commercially available SIF are rather simple in composition and mainly focus on the inclusion of bile salts and phospholipids.[12]

Other important components of the human luminal fluids in fed state are dietary lipids and their digestion products. Together with bile salts, these lipids form different structures within intestinal fluids (micelles, vesicles and lipid droplets) that have the potential to solubilize lipophilic compounds. As a result, fed state HIF contains both an aqueous or micellar fraction, including mixed micelles, and a lipid fraction,

consisting of larger colloidal structures and lipid droplets, resulting in a two-phase system. Previous studies have emphasized the crucial role of lipids and the lipid fraction on solubility and overall absorption. For instance, a study conducted by Riethorst *et al.* underscored the inadequacy of SIF containing solely bile salts and phospholipids in simulating the full solubilizing capacity of fed state HIF.[12] Another study by Braeckmans *et al*. showed the importance of lipids by measuring the postprandial systemic exposure of fenofibrate with and without the concomitant intake of a lipase inhibitor. Slowing down lipid digestion caused sustained solubilization of fenofibrate in the lipid fraction of intestinal fluids, resulting in increased systemic exposure. This indicated that simulating the interplay between the lipid and micellar fractions of intestinal fluids is necessary to predict the *in vivo* situation.[13] However, the lipid fraction is still poorly represented in SIF and its impact on drug solubilization is largely neglected in current food effect assays.

To better simulate the impact of dietary lipids on intestinal drug solubilization, a thorough investigation in HIF is needed. Previous studies have assessed solubility of various compounds in fed state HIF. However, these studies did not clearly distinguish between solubility in the micellar (aqueous) fraction and the lipid fraction.[14–22] A later study by Riethorst *et al*. demonstrated the effect of the lipid fraction on solubility but did not correlate the solubilizing capacity with fluid characteristics.[12,23]

To improve our understanding of the effect of lipids and their digestion products on intestinal solubilization, the present study aimed to assess solubility data for 7 lipophilic model drugs in a set of variable HIF pools. To this end, we aspirated duodenal fluids from 21 healthy volunteers in the fasted, early fed and late fed state. We analysed the solubilizing capacity of the fluids in relation to their composition in terms of pH, and the concentration of lipids, bile salts, phospholipids and proteins. When assessing the solubilizing capacity and composition of fed state fluids, both the micellar fraction and the total fluid, including the micellar and lipid fraction, were considered.

2 Materials and Methods

2.1 Materials

Sodium and potassium dihydrogen phosphate (NaH_2PO_4 and KH_2PO_4), taurochenodeoxycholic acid (TCDC), taurodeoxycholic acid (TDC), glycoursodeoxycholic acid (GUDC), glycochenodeoxycholic acid (GCDC), glycodeoxycholic acid (GDC), glycocholic acid (GC), chenodeoxycholic acid (CDC), deoxycholic acid (DC), lithocholic acid (LC), cholic acid (C), sodium hydroxide (NaOH), cholesterol (Chol), cholesteryl oleate, cholesteryl palmitate (Cholp), tripalmitin (TP), triolein, trilinolein, dipalmitin, diolein, dilinolein (DL), mono-oleate (MO), mono-palmitin, mono-linolein, palmitic acid, oleic acid (OA), linoleic acid, 1-octadecanol, L-tryptophan, ritonavir, danazol, nifedipine and orlistat were purchased from Sigma-Aldrich (St. Louis, MO). Tauroursodeoxycholic acid (TUDC), ursodeoxycholic acid (UDC), and taurocholic acid were acquired from Calbiochem (Darmstadt, Germany). Deuterated cholic acid (d4) was purchased from Cayman chemical (Ann Arbor, Michigan). The internal standards for itraconazole (D5) and chenodeoxycholic acid (d4) were bought from Alsachim (Illkirch Graffenstaden, France). Sodium choride (NaCL) and maleic acid were purchased from VWR chemicals (Leuven, Belgium). Hydrochloric acid (HCl) and acetonitrile (ACN, HPLC gradient grade) were purchased from Fischer scientific (Waltham MA) and methanol (MeOH, HPLC grade) from Acros Organics (Waltham MA). MeOH and formic acid (FA) LC/MS grade were acquired from Biosolve (Valkenswaard, The Netherlands). Isooctane (UV spec grade), ethyl acetate (LC-MS grade) and acetone (LC-MS grade) were purchased from Carl Roth (Karlsruhe, Germany). Acetic acid was bought from Chem-Lab analytical (Zedelgem, Belgium). Cabozantinib was purchased from Bionet Key Organics (Cornwall, UK). Itraconazole and etravirine were kindly provided by Johnson & Johnson Pharmaceutical Research and Development (Beerse, Belgium). Posaconazole was bought from Biosynth Ltd. (Compton, UK). Ensure Plus was purchased from Abbott Laboratories B.V. (Zwolle, The Netherlands). Purified water was produced using a Purelab® Flex water system from Veolia (Paris, F). All substances used for solubility experiments had a purity above 95%.

2.2 Intestinal fluids

Human intestinal fluids were collected at the UZ Leuven as part of a study which was approved by the Ethics Committee Research UZ/KU Leuven, Belgium (S53791). Twenty-one healthy volunteers (HVs) were included in the study. The HVs consisted of 9 women and 12 men, aged between 21 and 56 years with a BMI between 18 and 26 kg/m^2. The volunteers did not have a history of gastrointestinal diseases and did not take any oral medication for two days before participating in the study. The schedule for the aspiration of intestinal fluids is presented in Figure 1. After a fasting period of 12 h, a PVC dual lumen catheter (Salem Sump Tube 14 Ch, external diameter 4.7 mm; Cardinal Health, Dublin, Ohio, U.S.) was placed in the duodenum (D2-D3) through the nose while the position was checked using fluoroscopy. The two separate lumens enable fluid collection without generating negative pressure in the duodenum.

After the positioning of the catheter and a 20-min stabilization period, volunteers drank 240 mL of (still) water, marking the start of the fasted state fluid sampling. Samples were collected every 10 min over a period of 90 min. Immediately after fluid sampling in the fasted state, 400 mL of Ensure Plus was administered in liquid form to simulate fed state conditions. Ensure Plus contains 1.5 kcal/mL, 4.92% m/v of fat and 6.25% m/v of protein. After 20 min, 240 mL of water was consumed, marking the start of the fed state fluid sampling, which was again performed every 10 min over a period of 90 min. On average, 4 mL of fluid was aspirated per timepoint. To avoid lipolysis after sampling of the fluids, a 1 mM stock solution of the lipase inhibitor orlistat in methanol was added to obtain a final concentration of 1 µM orlistat in the samples. This resulted in a minor but negligible addition of methanol to the samples (0.1%, v/v). After sampling, the fluids were kept on dry ice for the remainder of the study and subsequently stored at -26 °C.

To ensure sufficient volumes to characterize composition and solubilizing capacity, intestinal fluid samples were pooled (Figure 1). To this end, the 21 HVs were randomly divided into seven groups with three HVs in each group. In each of these groups, the fluid samples from three HVs were combined to create a fasted state pool, an early fed state pool, and a late fed state pool. These three pools per group consisted of all samples collected in three intervals: from 10 to 90 min after water intake (fasted state pool), from 30 to 60 min after meal intake (early fed state pool), and from 70 to 110 min after meal intake (late fed state pool). Overall, this procedure resulted in a total of 21 HIF pools, comprising of 7 fasted, 7 early fed and 7 late fed states. In Figure 1., the pools are referred to with a letter indicating the prandial state (A: fasted, E: early fed, L: late fed) and a number from 1 to 7 indicating the volunteer group (Figure 1). For example: A1, E1 and L1 refer to the fasted, early fed and late fed intestinal fluid pools comprising samples from the 3 HVs belonging to group 1. Fluid aliquots were stored at -26 °C.

For comparison, solubility experiments were also performed using fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluids (FeSSIF and FeSSIF-V2), prepared according to the manufacturer's protocol (Biorelevant.com, London, UK)

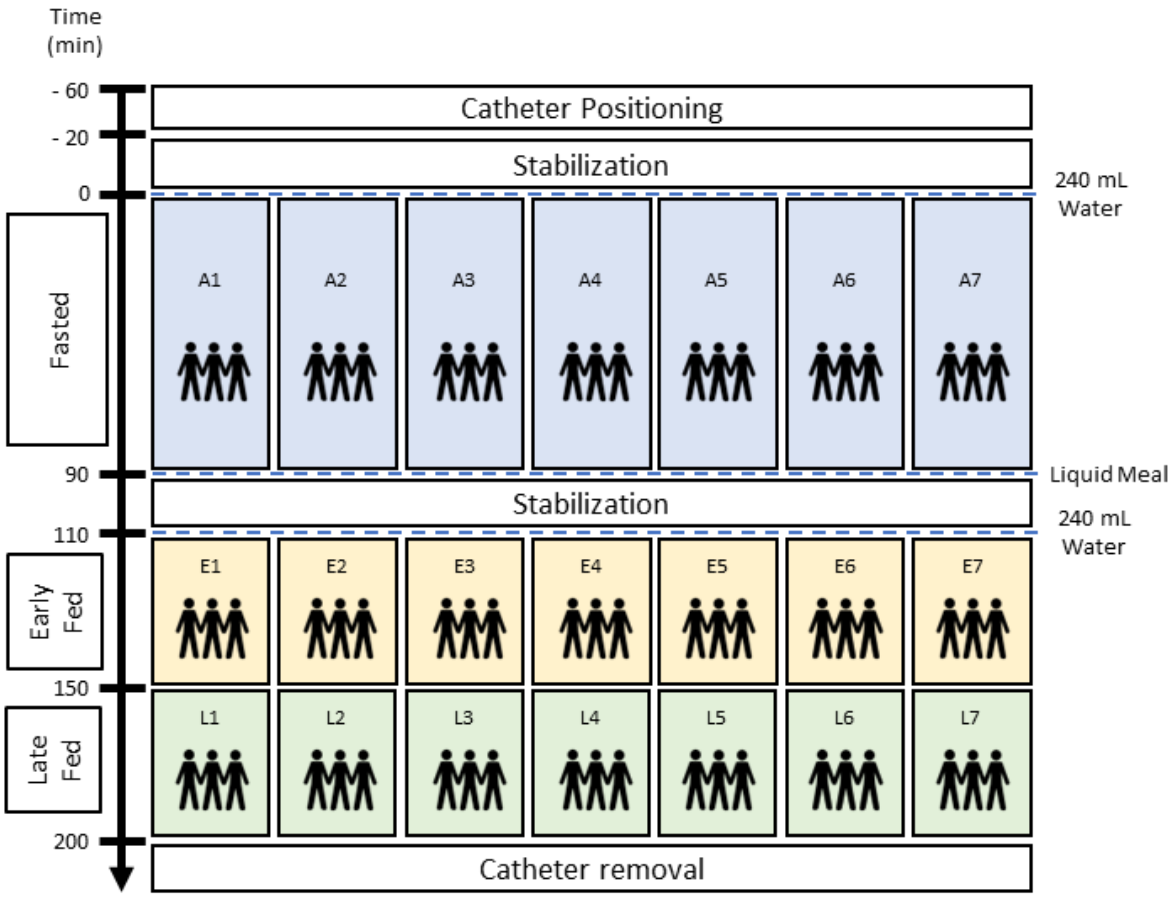


Figure 1*: Protocol for the aspiration and pooling of human intestinal fluids.*

2.3 Apparent solubility of selected model compounds

2.3.1 Solubility assay

Equilibrium solubility was determined in human intestinal fluid pools and simulated intestinal fluids for seven model compounds (*i.e.*, ritonavir, nifedipine, cabozantinib, etravirine, itraconazole, danazol and posaconazole). Compounds were selected based on their poor water solubility, lipophilicity and variable food effect outcomes (physicochemical properties are presented in Table 1). All solubility values reported in this manuscript should be considered apparent, *i.e.*, including both molecules freely dissolved in the aqueous phase and molecules solubilized in colloidal structures and lipid droplets.[24]

In fed state fluids, solubility was determined in the micellar fraction and in the total sample (micellar and lipid fractions combined). In fasted state fluids, the solubility was determined in the total sample only, as these fluids did not contain a lipid fraction. All solubility values were determined in triplicate. A 2.22 mM MES solution in 0.75 M HCl was added to the fasted state pools at a 1/20 ratio to maintain a biorelevant pH of 6.5. This prevented a significant pH increase due to the loss of the bicarbonate buffer following aspiration. In fed state fluid, bacterial degradation was prevented by the addition of a

penicillin/streptomycin mixture in water (both 10 000 U/mL) using a 1/100 dilution ratio (final activity: 100 U/mL).

To an excess of crystalline drug powder (1 mg for all compounds, with the exception of 0.6 mg for cabozantinib), 300 µL of intestinal fluid was added. Subsequently, the suspension was incubated at 37 °C for 24 h under continuous shaking at 175 RPM (IKA KS 4000i control, Staufen, Germany) to reach equilibrium solubility, followed by centrifugation (30 min, 20 000 g, 37 °C) (Centrifuge 5804 R, Eppendorf, Hamburg, Germay).

***Table 1**: Physicochemical properties of the seven model compounds. pKa and intrinsic solubility values were generated using ADMET predictor.*

Compound	***Molecular weight (g/mol)***	***Acid/base/ non-ionizable***	***pKa***	***LogP***	***Intrinsic aqueous solubility (µg/mL)***
Ritonavir	720.9	base	4.46, 2.47	5.6 [25]	9
Nifedipine	346.3	base	1.28	2.50 [26]	85
Cabozantinib	501.5	base	5.3	5.3 [27]	1
Etravirine	435.3	base	2.77	> 5 [28]	0.23
Itraconazole	705.6	base	3.7	6.2 [29]	7
Danazol	337.5	non-ionizable	/	4.53 [30]	2
Posaconazole	700.8	base	3.6, 4.6	5.41 [31]	20

2.3.2 Sample preparation

After centrifugation of HIF, multiple fractions were obtained: the undissolved solid material at the bottom, the aqueous micellar fraction, and the lipid fraction on top, the latter being present in the majority of the fed samples. The micellar fraction was isolated by removing the upper lipid fraction using a suction system. The centrifugation (30 min, 20 000 g, 37 °C) and lipid removal using suction were repeated a second time to account for unwanted mixing during the first suction step. Using separate aliquots, the isolation of the total sample (micellar and lipid fractions combined) required the transfer of both lipid and micellar fractions to a new vial, thus leaving the undissolved solid material behind. The micellar and lipid fractions were subsequently re-homogenized to obtain the total sample, using a vortex. Before quantification, samples were either diluted in 50:50 MeOH/H_2O or with ice cold MeOH 1% FA for protein precipitation. For itraconazole, an internal standard (H^5-Itraconazole) was added to (50:50) MeOH/H_2O at a final concentration of 50 nM.

2.3.3 LC analysis

The diluted samples were analysed using UHPLC with UV absorbance, fluorescence or tandem MS detection, depending on the drug (Table 2).

Itraconazole was determined using a Waters ACQUITY UHPLC H-Class system consisting of a quaternary pump combined with a Waters Xevo TQ-S micro mass spectrometer (Waters, Milford, MA,

USA). Integration was performed using MassLynx 4.2 (Waters, Milford, MA, USA). Separation was performed using a C18 Kinetex column (2.6 µm XB-C18 100 A, 50 × 2.1 mm, Phenomenex, Utrecht, the Netherlands) using an isocratic elution with methanol:0.05% formic acid (70:30) and subsequent wash gradient and re-equilibration (Supplementary table 1). The autosampler was kept at 15 °C and the column temperature was set to 40 °C. Detection was performed by tandem MS, using electrospray ionization as ion source in positive mode. System parameters were: capillary voltage 0.80 kV, cone voltage 20 V, desolvation gas (N_2) flow 800 L/h and temperature 350 °C, cone gas (N_2) flow 40 L/h.

All other compounds were analyzed using an isocratic method on a HPLC system consisting of a Chromaster 5160 pump and a Chromaster 5260 autosampler (Avantor, Leuven, Belgium). Separation was performed using a Nova-Pak C18 column under radial compression (Waters, Milford, MA, USA) and peaks were integrated using Clarity 8.7 (Clarity Software Group, Solihull, UK). Depending on the compound, either a Chromaster 5410 UV detector or a Chromaster 5440 fluo detector was used. Analytical methods were validated according to the ICH M10 guidelines on bioanalytical method validation. At four concentrations in MeOH:H_2O, the accuracy was between 85% - 115% and the relative standard deviation below 10 %. The recovery was above 90% for all compounds.

***Table 2**: Sample preparation and separation of model compounds.*

Compound	*Sample Dilution*	*Mobile Phase*	*Injection volume*	*Flow rate*	*Detection*
Ritonavir	MeOH: H_2O (1:20 v/v)	MeOH:Buffer[1] (80:20)	50µL	1 mL/min	UV: 241 nm
Nifedipine	MeOH:H_2O (1:100 v/v)	ACN:Buffer[1] (60:40)	50µL	1 mL/min	UV: 340 nm
Cabozantinib	MeOH: H_2O (1:100 v/v)	MeOH:Buffer[2] (72:28)	50µL	1 mL/min	UV: 322 nm
Etravirine	MeOH:H_2O (1:100 v/v)	MeOH:Buffer[1] (80:20)	50µL	1 mL/min	UV: 312 nm
Itraconazole	MeOH:H_2O 1:25 v/v (Fasted) 1:250 v/v (Fed)	MeOH:H_2O 0.05% FA (gradient see supplementary table 1)	2µL	0.6 mL/min	MS/MS
Danazol	MeOH:H_2O (1:100 v/v)	MeOH:H_2O (82:18)	50µL	1 mL/min	UV: 285 nm
Posaconazole	MeOH 1% FA (1:10 v/v)	MeOH:Buffer[1] (82:18)	50µL	1 mL/min	Fluo: *ex* 240 nm *em* 385 nm

Buffer[1]: 25 mM Acetic acid in H_2O at pH 3.5
Buffer[2]: 40 mM formic acid in H_2O at pH 2.5

2.4 Characterization of human intestinal fluids

The HIF pools were characterized for total lipids (triglycerides [TAGs], diacylglycerides [DAGs], monoacylglycerides [MAGs], and free fatty acids [FFAs]), bile salts, phospholipids, total cholesterol (cholesterol and cholesteryl esters), pH and total protein. Characterization was performed on (i) the micellar (aqueous) fraction, and (ii) the total sample consisting of both the micellar fraction and the lipid fraction.

pH and buffer capacity

The pH value was determined using a BioTrode glass electrode (Hamilton, Reno, NV, USA), which was calibrated before each use. Buffer capacity (BC) was determined by adding 2 µL 1 M HCL or NaOH to 200 µL of HIF, whereafter the change in pH was determined.[32] Buffer capacity was determined using the equation below.

$$BC = \frac{n}{\Delta pH \times V}$$

with n = amount of mols H^+ or OH^- (2×10^{-3} mmol), ΔpH = change in pH, and V = intestinal fluid volume (200 µL).

Osmolality

Osmolality was measured using a freeze-point depression osmometer (Advanced Instruments 3250, Norwood, MA, USA). The osmometer was operated in low-range mode with the buzz point set to 3500. The system was verified by measuring standard solutions of 100, 290 and 1500 mOsm/kg before use.

Total protein

Total protein concentration was determined using the tryptophan fluorescence assay described by Wisniewski and colleagues.[33] In short, 2 µL of intestinal fluid was added to 200 µL 8 M urea in 100 mM trisaminomethane pH 7.8. Fluorescence (excitation 295 nm, emission 350 nm) was measured and compared to a calibration curve of different tryptophan concentrations. Total protein content was calculated assuming an average tryptophan content of 1.17%.

Bile salts

Bile salts were quantitatively measured with LC-MS/MS, according to the method described by Riethorst *et al*. [2]. The method was optimized by adding an additional internal standard (CDC-d4) to improve the quantification of the dihydroxy bile salts. Sample preparation consisted of a dilution by 1000 and 10 000 times in 50:50 MeOH/H_2O containing both C-d4 and CDC-d4 as internal standards. The following bile salts could be quantified: C: Cholic acid, GC: Glycocholic acid, TC: Taurocholic acid, CDC: Chenodeoxycholic acid, GCDC: Glycochenodeoxycholic acid, TCDC: Taurochenodeoxycholic acid, UDC: Ursodeoxycholic acid, GUDC: Glycoursodeoxycholic acid, TUDC: Tauroursodeoxycholic acid, DC: Deoxycholic acid, GDC: Glycodeoxycholic acid, TDC: Taurodeoxycholic acid, LC: Lithocholic acid. A C18 Kinetex® column (2.6 µm XB-C18 100 A, 50 × 2.1 mm, Phenomenex, Utrecht, the Netherlands) was kept at 35 °C and 5 µL of sample was injected. Detection was performed using a Xevo™ TQS micro triple quadrupole mass spectrometer (Waters) with electrospray ionization in both positive and negative modes. The LC gradient, MS settings and individual bile salt mass transitions are described in Supplementary table 2 and Supplementary table 3, respectively.

Phospholipids

The concentration of phospholipids was determined using the Labassay Phospholipid kit (Fujifilm, Tokyo, Japan). Phospholipids containing choline were hydrolyzed to choline by phospholipase D. Choline was subsequently oxidized by choline oxidase producing hydrogen peroxide which in turn reacted with N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (DAOS) and 4-aminoantipyrine resulting in a blue colour. The phospholipid concentration was then determined by measuring the absorbance (600 nm) using a Tecan infinite m200 plate reader.

Lipids and cholesterol

Total lipids were determined with high-performance liquid chromatography (HPLC) coupled to a charged aerosol detector (CAD) using a method adapted from Infantes-Garcia and colleagues.[34] Separation was performed using a Vanquish dual pump system (Thermo Fisher Scientific, Waltham, MA) equipped with an Ascentis Express OH5 column (100 × 2.1 mm, 2.7 µm) kept at 35 °C. Lipids and cholesterol were eluted per lipid class using a gradient as specified in Supplementary table 5. The mobile phase composition at the detector was kept stable by employing a secondary pump with an inversed gradient to the analytical gradient to ensure a more uniform analyte response for all analytes. The flow rate was 0.5 mL/min for each of the pumps (*i.e.*, 1 mL/min at the detector). The CAD evaporation temperature was set to 35 °C, data collection at 10 Hz, filter constant to 3.6 and the power function between 1.2 and 1.6, depending on the lipid class.

For quantification, a calibration curve was prepared using one surrogate reference product per lipid class (i.e., TAGs, DAGs, MAGs, FFA, cholesterol, cholesteryl esters) as specified in Supplementary table 4 The method was validated by spiking known concentrations of reference lipids for all classes (*i.e.*, lipids derived from palmitic, oleic or linoleic acid) in FaSSIF and FeSSIF. The average accuracy and precision of three separate runs at different concentrations are given in Supplementary table 6. In addition, recovery in human intestinal fluids was evaluated by spiking a known concentration of one reference product per lipid class in six different pools of human intestinal fluid (*e.g.*, fasted and fed-state human intestinal fluids). During each run, analytical accuracy and precision were monitored using QC samples (0.25, 0.063 and 0.016 mg/mL for FFA and 0.063, 0.016 and 0.004 mg/mL for all other lipid classes); the accuracy was between 85 and 115% and relative standard deviations were below 10%.[35]

2.5 Statistical analysis

Spearman correlation coefficients were calculated to assess the relationships (i) among the compositional factors and (ii) between the solubilizing capacity and the compositional factors of the HIF pools. These analyses were conducted using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). The non-parametric Spearman method was chosen instead of the Pearson method because the data did not follow a normal distribution.

3 Results and discussion

For this study, we collected fasted and fed state (after the ingestion of a liquid meal) duodenal fluids from 21 HVs, who were randomly assigned to seven groups of three HVs. For each group, fluids of the different HVs were combined into three pools based on the sampling time, *i.e.* before meal intake (fasted), 30 to 60 min after meal intake (early fed), and 70 to 110 min after meal intake (late fed). In total, this resulted in 7 pools for each prandial state. This approach allowed us to maintain a significant extent of interindividual and time-dependent variability, while enabling solubility and characterization experiments on adequate fluid volumes.

3.1 Solubility

Figure 2 depicts the equilibrium solubility of seven lipophilic model compounds in the seven fasted, seven early fed, and seven late fed state pools. For the fed state pools, the solubility was determined in both the micellar fraction and the combined micellar and lipid fractions (referred to as the total sample). All solubilities in the HIF pools were determined in triplicate; the relative standard deviations (RSD) averaged at 10.1%, with an interquartile range of 9.8%. For each compound and prandial state, the average solubility in HIF over the 7 pools was calculated. To facilitate comparison, the solubilities of the different compounds in the simulated fluids FaSSIF, FeSSIF, and FeSSIF-V2 were included as well.

Firstly, we investigated the overall differences in solubilizing capacity between fasted and fed state HIF. For all model compounds, the average solubility was consistently lower in the fasted state pools as compared to both the micellar and total samples of the fed state pools. When considering only the micellar fractions, average solubilities were 2.0- to 5.8-fold higher in fed versus fasted state, depending on the compound. When considering the total samples, these fold-differences between fed and fasted state ranged from 2.7 to 42.3. These observations clearly illustrated the effect of food on the solubilizing capacity of intestinal fluids for lipophilic compounds. In addition, this food effect was substantially increased by the lipid fraction of fed state fluids (present in total but not micellar samples).

Secondly, we evaluated the ability of commercially available SIF to predict the average solubilizing capacity of HIF. To this end, a fold error was determined as the ratio between the average solubility in fasted or fed state HIF (solid lines on Figure 2), and the solubility in corresponding SIF (dotted lines on Figure 2). For the fasted state, FaSSIF predicted the mean solubility in HIF with a fold error below 2 for 5 out of 7 compounds. Only for ritonavir and itraconazole, underpredictions were seen of 3.6- and 5.8-fold, respectively. For the fed state, no large difference was observed between the ability of FeSSIF vs. FeSSIF-V2 to predict the average solubility in the micellar fraction of FeHIF. For 6 out of 7 compounds, the fold errors were between 0.5 and 2.5. Only the micellar solubility of itraconazole was greatly underpredicted by both FeSSIF and FeSSIF-V2 with fold errors of 28.6 and 16.0, respectively. The scatterplots in Supplementary figure 2 further depict the relationship between solubility in HIF and SIF for the model compounds. Overall, the ability of commercially available SIF to predict the solubilizing

capacity of fasted state HIF and the micellar fraction of fed state HIF appeared reasonable for most (but not all) of the selected lipophilic compounds.

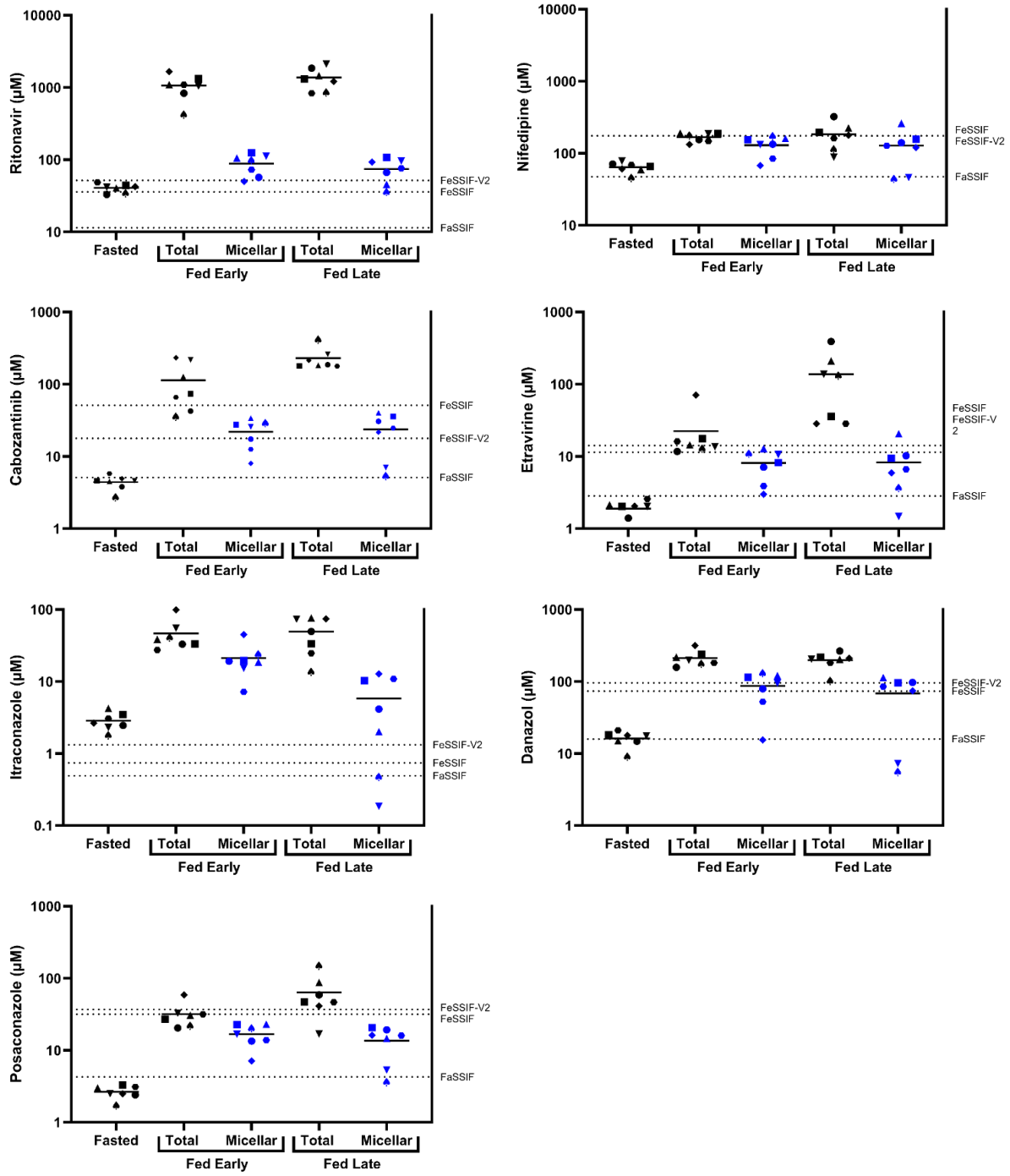


Figure 2*: Equilibrium solubility of the seven poorly water-soluble model compounds in HIF (dots) and SIF (dotted lines). Solubility was assessed in HIF pools representing the fasted state, early fed state and late fed state (7 pools each). In the fed state pools, the total and micellar samples were analysed separately. Each group of HVs was assigned a unique symbol for comparison purposes. Data points represent the mean of an experiment in triplicate, with the solid lines depicting the mean of the solubility values in the different HIF pools. Solubility in SIF (dotted lines) represent the mean of n=6.*

Both FeSSIF and FeSSIF-V2 were developed to mimic the micellar fraction of fed state intestinal fluids and lack a distinct lipid phase.[11] As such, they are unable to predict the total solubilizing capacity of fed state human fluids, which typically do include a lipid fraction. After centrifugation of the fed state HIF pools in the present study, a lipid fraction was indeed visible for all pools, albeit varying in appearance and amount. The presence of this lipid fraction drastically affected the solubility in both the early and late fed state. In the early fed state, the average fold increase in solubility (solubility in the total sample divided by solubility in the micellar fraction) for all model compounds combined was 5.4-fold, with ritonavir exhibiting the largest increase of 14.0-fold. In the late fed state, the impact of the lipid fraction was even more pronounced, with an average increase of 22.3-fold. Nifedipine, having the lowest logP of all model compounds, was consistently the least affected by the lipid fraction, showing an average increase of 1.7-fold in the late fed state. Conversely, ritonavir, cabozantinib, etravirine, itraconazole, danazol and posaconazole showed a significant effect, with fold increases of 20.3, 21.1, 27.2, 69.2, 8.3 and 8.7, respectively. A trend is observed between the fold increase in solubility and logP values for these compounds (see Supplementary Figure 1). These observations underscore the significant influence of the lipid fraction on apparent drug solubilization. Not being able to predict this influence is undeniably a limitation of the current SIF.

Whereas SIF can reasonably predict the average solubility in fasted state fluids and in the micellar fraction of fed state fluids, their standardized compositions do not allow predicting the impact of variability between intestinal fluids on solubility. In the present study, we assessed the solubility in seven intestinal fluid pools per prandial state (symbols in Figure 2), allowing us to assess the sensitivity of the model compounds' solubility to variations between these pools. To quantitatively assess the sensitivity, a maximum to minimum ratio (MMR) was computed per compound by dividing the maximum by the minimum observed solubility of that compound within a set of pools.

In the fasted state, variability was relatively low, exhibiting an average MMR of 2, with all compounds demonstrating similar sensitivity to variation between fasted state pools.

Solubility in the fed state exhibited higher variability for most compounds, despite standardized meal intake. **Figure 3** illustrates the MMRs in the micellar and total samples for the early and late fed states. In the total samples, the average MMR amounted to 3.7 in the early fed state, and 5.6 in the late fed state. In the micellar samples, the variability in solubilizing capacity between intestinal fluid pools was notably higher with an average MMR of 4.5 and 17 in the early and late fed state, respectively. In particular, the solubilities of etravirine, itraconazole and danazol were highly variable in the micellar fraction of the late fed state pools, with MMR-values of 14, 68 and 21, respectively. In this respect, the low solubilizing capacity of the micellar fraction, but not the total sample, of some late fed state HIF pools, was remarkable (see Figure 2).

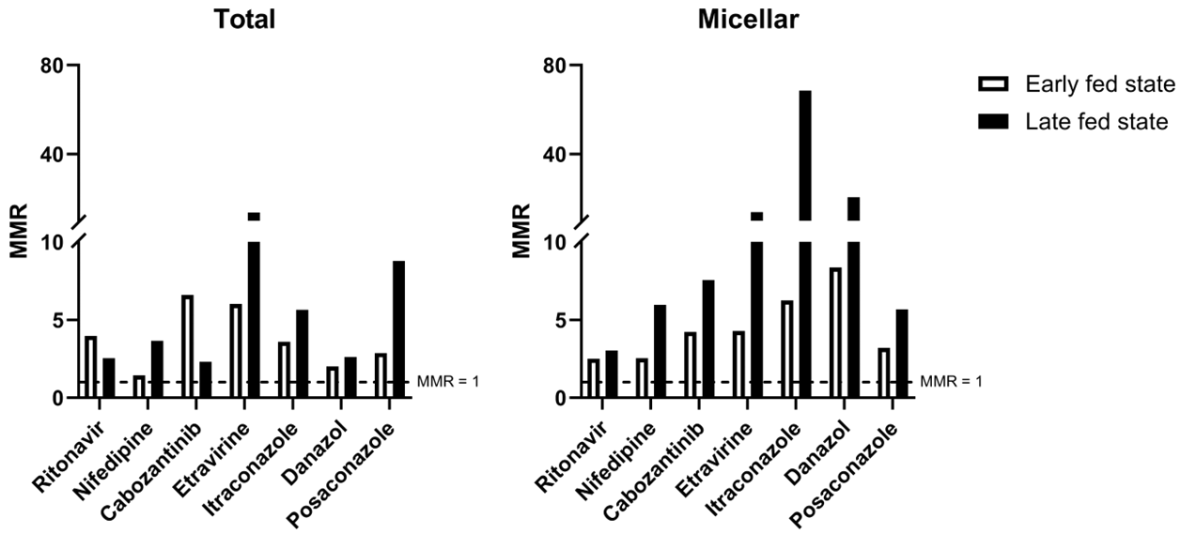


Figure 3: *Variability in the solubility of the seven model compounds in fed state HIF. The bars depict the Maximum to Minimum Ratio (MMR), i.e., the maximum observed solubility divided by the minimum observed solubility of each compound within a set of pools.*

Overall, our data illustrated that the solubilizing capacity of postprandial intestinal fluids for lipophilic model compounds was highly affected by the presence of a lipid fraction, which is absent in current SIF. In addition, the fed state solubility of certain compounds, especially in the micellar fractions, appeared to be highly sensitive to variations between intestinal fluid pools. Even though this sensitivity may contribute to variability in absorption, it cannot be forecasted by standardized SIF. To further elucidate the pronounced variability in the solubilizing capacity of fed state intestinal fluids, we comprehensively characterized the composition of the HIF pools used for the solubility experiments, while specifically distinguishing between micellar and total samples.

3.2 Composition

A comprehensive characterization of the fasted, early fed, and late fed state HIF pools was performed with respect to total lipids (TAGs, DAGs, MAGs and FFAs), bile salts, phospholipids, total cholesterol (cholesterol and cholesteryl esters), pH, and total protein. For the fed state pools, all factors except for pH were quantified in both the micellar fraction and the total sample (which includes the lipid fraction). Results are presented in Figure 4. Average values per prandial state, as well as characteristics of the simulated intestinal fluids FaSSIF, FeSSIF and FeSSIF-v2 are incorporated.

In general, the average composition of the HIF pools aligned with previous research.[2,15] Therefore, we will not extensively discuss the composition itself, but rather focus on the intricacies of the lipid composition, the differences in composition between the total and micellar samples, and the compositional variability among the early and late fed state pools.

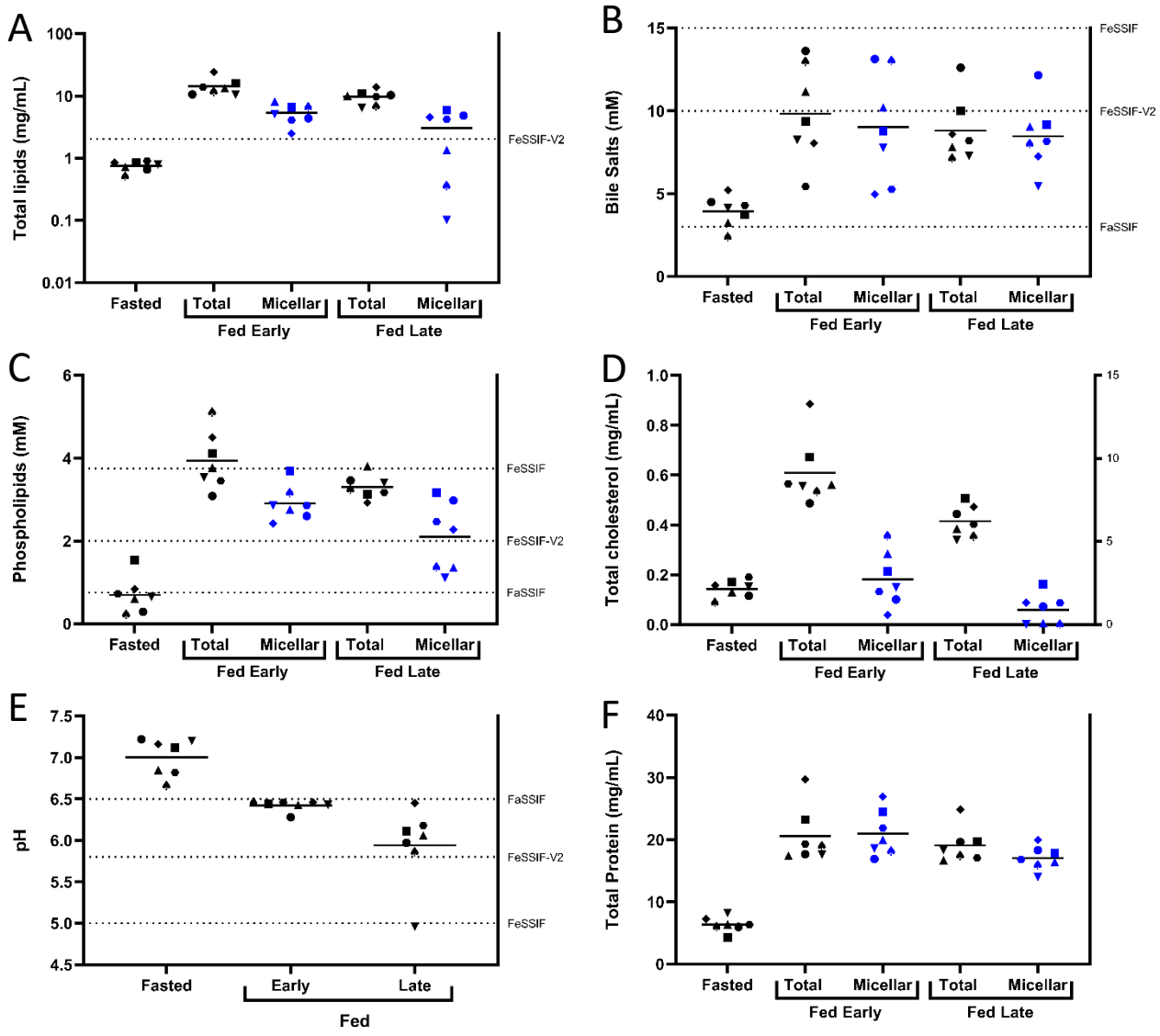


Figure 4*: Composition of HIF (dots) and SIF (dotted lines) with respect to lipids (i.e., TAGs, DAGs, MAGs and FFAs), bile salts, phospholipids, cholesterol, pH, and total protein. Characterization was divided into a fasted state, an early fed state and a late fed state. In the fed state, the total and micellar samples were separated for all compositional characteristics (lipids, bile salts, total protein, phospholipids). Each group of HVs was assigned a unique symbol for comparison purposes. Data points represent a single measurement, with the solid lines depicting the mean of the data points over the different pools.*

Figure 4A depicts the cumulative concentrations of TAGs and all digestion products (DAGs, MAGs, and FFAs). In both the fasted and fed states, FFAs were the predominant lipid class, constituting an average of 88% in the fasted state and 91% in the fed state. This observation suggests a rapid digestion of dietary lipids, which is consistent with previous findings from de Waal *et al*. using a similar aspiration procedure in elderly individuals.[35] The rapid digestion is likely facilitated by the finely emulsified liquid meal, which provides easy access for gastrointestinal lipases.[36]

The average total lipid concentration observed in the fed state HIF pools was 12 mg/mL (total samples), of which a substantial part made up the lipid fraction. However, also in the micellar fraction, a significant concentration of lipids was observed, on average 4.1 mg/mL. In comparison, FeSSIF-V2 contains 2.0

mg/mL of lipids (0.2 mg/mL FFAs and 1.8 mg/mL MAGs). The relatively high concentration of lipids in the micellar fraction highlights a distinct distribution of lipids between the lipid and micellar fractions. Similarly, we observed a specific distribution of other components between the lipid and micellar fraction by comparing the composition of the micellar and total samples. Phospholipids and total cholesterol generally exhibited higher concentrations in the total samples, indicating a preference for the lipid fraction. In contrast, bile salts and total protein showed comparable concentrations in both total and micellar samples, suggesting no specific preference for residence in the lipid fraction.

When comparing the early and late fed states, we observed no apparent differences in average composition, apart from pH. The average pH shifted from pH 6.4 in the early fed state pools to pH 5.9 in the late fed state pools. We hypothesize that prolonged re-acidification in the stomach followed by gastrointestinal transfer contributed to lower pH values in the late fed state.

In addition to assessing the average composition, we investigated the variability in composition across different pools. To quantitatively assess this variability, we calculated maximum to minimum ratios per component and prandial state (MMR) by dividing the maximum by the minimum observed concentration within a set of pools. Variability in the fasted state was relatively low across all characteristics (MMR < 7). However, in the fed state pools, considerable variability was seen for pH, total lipids, and total cholesterol (MMR > 30), with less variability for bile salts, phospholipids, and proteins (MMR < 4). Notably, the variability in lipid and cholesterol concentrations was rather limited in the total samples (MMR < 4), but very high in the micellar samples (MMRs > 70). Interestingly, when examining the early and late fed states separately, a higher variability was observed in the late fed state. In the early fed state, pH, micellar lipid and micellar cholesterol concentrations exhibited MMRs of 1.5, 3.2, and 9.2, respectively. However, in the late fed state, these values increased to 30.9 for pH, 56.9 for total lipids, and 80.6 for total cholesterol.

Overall, fed state HIF pools varied considerably in not only solubilizing capacity but also composition, particularly regarding lipids. Moreover, variability was more pronounced in the micellar samples than in the total samples. These observations suggest that it is worthwhile to explore possible correlations between solubilizing capacity and fluid composition.

3.3 Relation between solubility, composition and lipid distribution

In this section, our objective was to investigate possible correlations between the variability in solubilizing capacity and the variability in the composition of HIF pools. Additionally, we aimed to explore the phase separation in HIF by examining how compositional characteristics influence the distribution of lipids between micellar and lipid fractions. Considering most variability was observed in the micellar fraction, it was unsurprising to find more statistically significant correlations within the micellar samples (**Table 3**) compared to the total samples (Supplementary table 7). As depicted in **Table 3**, the large variability in lipid concentration of the micellar fractions correlated with the variable

solubility within these fractions. Significant correlations were observed for all model compounds, with strong positive correlation coefficients (r values) ranging from 0.59 up to 0.94. This relationship is exemplarily illustrated in **Figure 5** for 3 model compounds.

Additionally, the concentrations of bile salts and cholesterol correlated in a statistically significant way with the solubility of most but not all model compounds. The influence of bile salts on the solubilizing capacity of media is well-established, leading to their incorporation in current SIF.[37] Similarly, the effect of lipids on solubilizing capacity is unsurprising, given their potential to increase the quantity and size of colloidal species, thereby enhancing solubility within the micellar fraction.[23,38-40]. However, their incorporation is largely neglected in current SIF. The influence of cholesterol on solubility is less clear, as its micellar concentration is probably highly dependent on that of the lipids.

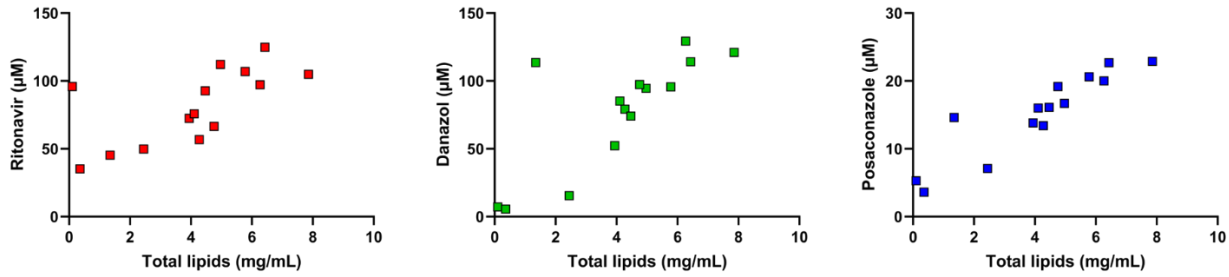


Figure 5: *Relationship between total lipid concentration in the micellar fraction of fed state HIF pools (n = 14) and corresponding apparent solubility of three model compounds (i.e. ritonavir, danazol and posaconazole).*

The strong, positive correlation between lipid concentration in the micellar fractions of the 14 fed state HIF pools and the corresponding solubility of all model compounds, clearly indicated that understanding and simulating the large variability in solubilizing capacity of fed state intestinal fluids requires careful consideration of micellar lipid concentrations.

Table 3: *Spearman correlations examining the relationship between the solubility of model compounds in the micellar fraction of the fed state HIF pools and the characteristics of these pools (n=14). For each correlation, the statistical significance (p-value) is indicated; for each significant correlation, the correlation coefficient (r-value) is indicated as well.*

		Solubility in micellar samples						
Composition micellar samples		**Ritonavir**	**Nifedipine**	**Cabozantinib**	**Etravirine**	**Itraconazole**	**Danazol**	**Posaconazole**
	pH	ns	ns	ns	ns	** (r = 0.78)	ns	ns
	Bile salts	ns	** (r = 0.75)	* (r = 0.62)	** (r = 0.69)	ns	** (r = 0.70)	ns
	Total lipids	** (r = 0.74)	* (r = 0.65)	* (r = 0.63)	* (r =0 .63)	* (r = 0.59)	** (r = 0.79)	**** (r = 0.94)
	Total cholesterol	** (r = 0.74)	* (r = 0.60)	ns	* (r = 0.54)	* (r = 0.64)	** (r = 0.71)	*** (r = 0.82)
	Phospholipids	* (r = 0.64)	ns	ns	ns	ns	* (r = 0.62)	** (r = 0.76)
	Total protein	ns	ns	ns	ns	* (r = 0.67)	ns	ns

**: p < 0.05, ** : p < 0.01, *** : p < 0.001, **** : p < 0.0001, ns: not significant*

3.3.1 Lipid distribution and composition

As mentioned in the previous section (3.2 Composition), the lipids in the fed state HIF pools were distributed across both the micellar and lipid fractions. The variability in lipid concentration was fairly limited when considering the total samples, which is probably related to the standardized meal taken by the volunteers. In contrast, lipid concentrations in the micellar samples appeared highly variable (Figure 4A) and, interestingly, did not correlate with the lipid concentrations in the total samples ($p > 0.05$). This suggests that other factors than the overall lipid concentration in fed state HIF contributed to the distribution of lipids between the lipid and micellar fractions.

Lipid distribution is largely dependent on the incorporation of lipids from the lipid fraction into the micellar fraction, which is facilitated by surfactants such as bile salts, phospholipids or potentially proteins. Therefore, we examined the correlation between the total concentration of bile salts, phospholipids and proteins, and the distribution of lipids between the micellar and lipid fraction. To this end, a lipid distribution factor was calculated per pool as the lipid concentration in the total sample divided by the lipid concentration in the micellar fraction. A higher distribution factor (> 1) will therefore indicate a larger proportion of lipids residing within the lipid fraction.

Total phospholipid ($p=0.59$) and protein concentrations ($p=0.98$) did not exhibit a statistically significant correlation with the lipid distribution factor. For bile salt concentrations, however, a strong, negative correlation was observed ($p= 0.004$; $r= - 0.74$), depicted in Figure 6A. At high bile salt concentrations (> 9 mM), the lipid distribution factor remained relatively low. However, as the concentration dropped, a sharp increase in the lipid distribution factor was observed, indicating a drop in lipid concentration in the micellar fraction. This surge is likely attributable to the critical micellar concentration (CMC) of the bile salt mixture, reported to range between 2 and 10 mM for the most common bile salts in HIF.[39,40] As the bile salt concentration approaches the CMC, fewer micelles are available to solubilize both lipids and lipophilic drugs. Sufficiently high levels of bile salts are thus needed to assist in the shift of lipids towards the micellar fraction of postprandial intestinal fluids. These observations showed that bile salts enhance their impact on the intestinal solubilization of lipophilic drugs by altering the distribution of lipids. It also underscored the inadequacy of current SIF, with fixed bile salt concentrations, to capture the variable food effect on solubilizing capacity.

In addition to surfactants, pH may also play a pivotal role in altering lipid distribution. In this regard, Figure 4E highlights an interesting observation: pH levels were relatively stable in early fed state pools, but lower and more variable in late fed state pools. These variable pH levels appear to have contributed to variable micellar lipid concentrations. In late fed pool 6 (Figure 4, ▼), for instance, a very low pH of pH 4.96 translated into a remarkably low micellar lipid concentration. Given that the predominant lipids in the fed state are FFAs, of which the charge is pH-dependent (pKa ~ 5), we assume that lower pH values lead to increased lipophilicity of FFAs due to protonation, thereby promoting a shift towards the

lipid fraction. Conversely, at higher pH values, deprotonation of FFAs reduces their lipophilicity, facilitating their incorporation into micelles. Even though no statistically significant correlation was found between pH and the lipid distribution factors (p= 0.37), the scatterplot in Figure 6B depicts a negative trend.

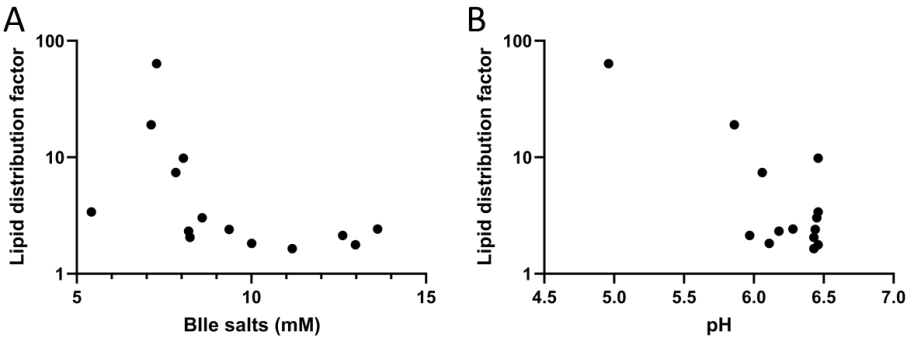


Figure 6: *The effect of bile salts (A) and pH (B) on lipid distribution (lipid concentration in total sample divided by lipid concentration in the micellar sample) in fed state HIF pools (n = 14).*

The effect of pH on lipid distribution may (partly) explain why the solubility of lipophilic compounds without relevant ionization may still correlate with pH. In the present study, for instance, itraconazole lacks a change in ionization in the pH-range between 5 to 7, excluding a direct effect of pH on solubility. Yet, the solubility of itraconazole in the micellar fraction of the fed state HIF pools correlated with pH (r = 0.78; p = 0.002), as illustrated in Figure 7.

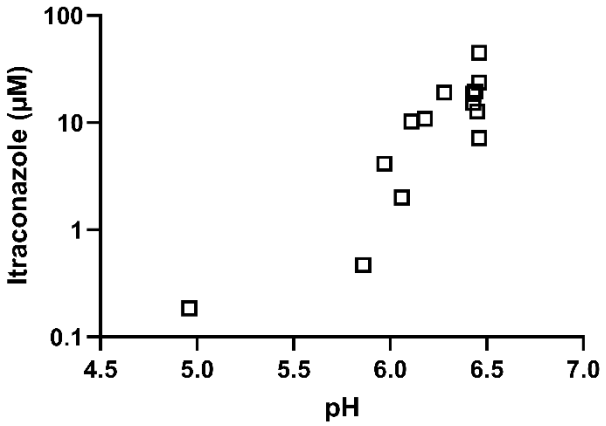


Figure 7: *The effect of pH on the solubility of itraconazole in the micellar fraction of fed state HIF pools (n = 14).*

3.4 Implications and future perspectives

The objective of this study was to perform a detailed assessment of the impact of lipids on drug solubilization in fed state HIF, and to consider the efficacy of the currently used single-phase SIF (FeSSIF and FeSSIF-v2). In this section, we want to reflect on the implications of our observations for

further research aimed at improving the simulation of the role of dietary lipids in absorption-related processes.

Our study demonstrated that the presence of a lipid fraction in the fed state HIF pools significantly enhanced their solubilizing capacity by, on average, 13.9-fold across the seven model compounds. Additionally, the distribution of lipids from the lipid fraction into the micellar fraction appeared to be a major source of variability in solubilizing capacity. An adequate simulation of the behaviour of drugs and drug candidates in the postprandial intestinal environment thus requires the development of multi-phase SIF with biorelevant lipid concentrations (on average 12 mg/mL, consisting mostly of FFAs) instead of the current single-phase SIF. In this regard, we recognize the earlier reported development of multi-phase SIF. However, their utilization was limited due to the challenging handling of these multi-phase systems and the pursuit of obtaining a single-phase, transparent fluid, which can easily be incorporated in a routine workflow.[11,41] In line of the results of the current study, however, new efforts to develop and incorporate such fluids are certainly warranted.

An apparent observation of the present study was the considerable variation observed in the solubilising capacity of different fed state HIF pools, particularly in their micellar fraction. For all model compounds, the micellar lipid concentration, and thus the distribution of lipids between the lipid and micellar fractions, emerged as a major source of this variability. Limiting the sensitivity of drug absorption to such variations in intestinal conditions requires the relevant integration of variability in early food effect assessment. Further research is necessary to find the best strategy for such integration. An interesting approach in this respect is the 9 media system by Pyper et al. as a strategy to simulate variability in the composition of the micellar fraction in fasted and fed state intestinal fluids.[42] This system has been applied by Silva et al. to examine the influence of compositional variability on drug solubility[43]. To further optimize such strategies, our data indicated that, combined with the presence of a distinct lipid fraction, bile salt concentration and pH are two important compositional factors that affect lipid distribution and should be considered to introduce variability in multi-phase SIF. It is also important to note that the procedure to collect HIF in the present study reduced compositional variability by pooling samples, using a standardized meal, and recruiting only healthy volunteers. Most likely, administering formulations to diverse patient populations, across different age groups, and with various (mostly solid) meals, will even enhance variability and requires further investigation.

While this study demonstrated the complex and variable impact of dietary lipids on intestinal drug solubilization, translating these observations to possible effects on absorption, requires the integration of permeation. It is generally accepted that unbound free drug molecules are the driving force for permeation, and that drug entrapped in both colloids and lipid droplets do not contribute extensively [44–47]. However, it is essential to recognize that *in vivo*, entrapment is dynamic as colloids and lipid droplets evolve over time during lipid digestion and absorption.[2,44,48–50] This dynamic process has the potential to sustain or even elevate free drug concentrations when solubilized drug becomes available. With these

processes in mind, we underscore the importance of the lipid fraction due to the accumulation of drugs within lipid droplets and the substantial variability in micellar solubilizing capacity, attributable to the indirect influence of lipid integration into the micellar fraction.

Finally, advanced solubilization data as reported in the present study could serve as relevant input data to improve the prediction of food effects by physiologically based biopharmaceutics modelling (PBBM). This approach would contribute to translating food effects on drug solubilization into food effects on drug absorption, provided that PBBM further evolves in modelling the dynamic processes involved in this translation.

4 Conclusion

This study presents a comprehensive dataset on the solubility of seven lipophilic model compounds in HIF, coupled with a thorough characterization of the utilized fluids, focusing on both the micellar and lipid fraction of fed state HIF. Our results showed that the lipid fraction not only enhanced the apparent solubility of the tested drugs in the total fluid, but also contributed to variations in solubilizing capacity of the micellar fraction, particularly when combined with fluctuations in bile salt concentration and pH. This study underscores the necessity of developing multi-phase SIF with biorelevant lipid concentrations while introducing compositional variability to better simulate the complex solubilizing and permeation behaviour of APIs in the gastrointestinal environment.

5 Associated content

5.1 Supplementary information

In the supplementary information we included analytical method specifications and validation. In addition, graphical relationships between solubility in HIF and the LogP values of model compounds were provided, as well as relationships between solubility values in HIF and SIF. Additional Spearman correlations were also included, exploring the relationship between solubility in HIF and various characteristics of HIF.

6 Acknowledgments

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