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Characterization of neonatal and infant enterostomy fluids- Part II: Drug solubility

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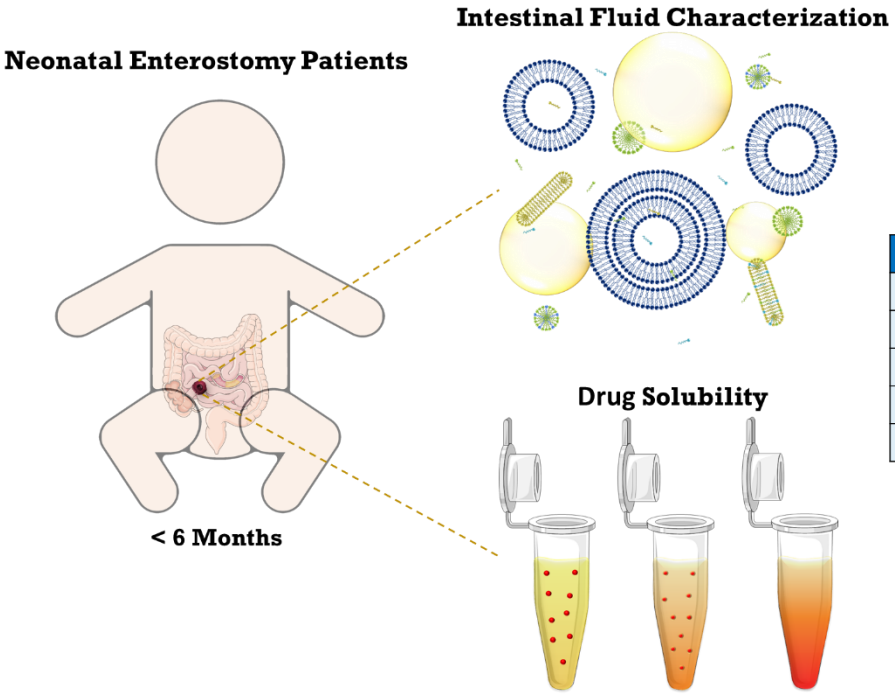

Drugs	Acid/Base
Ibuprofen	Acid
Clopidogrel	Base
Domperidone	Base
Spironolactone	Neutral
Tacrolimus	Neutral

Abstract

Previous research revealed marked differences in the composition of intestinal fluids between infants and adults. To explore the impact on the solubilization of orally administered drugs, the present study assessed the solubility of five poorly water-soluble, lipophilic drugs in intestinal fluid pools from 19 infant enterostomy patients (infant HIF). For some but not all drugs, the average solubilizing capacity of infant HIF was similar to that of HIF obtained from adults (adult HIF) in fed conditions. Commonly used fed state simulated intestinal fluids (FeSSIF(-V2)) predicted fairly well drug solubility in the aqueous fraction of infant HIF, but did not account for the substantial solubilization by the lipid phase of infant HIF. Despite similarities in the average solubilities of some drugs in infant HIF and adult HIF or SIF, the underlying solubilization mechanisms likely differ, considering important compositional differences (e.g., low bile salt levels). Finally, the huge variability in composition of infant HIF pools resulted in a highly variable solubilizing capacity, potentially causing variations in drug bioavailability. The current study warrants future research focusing on (i) understanding the mechanisms underlying drug solubilization in infant HIF and (ii) evaluating the sensitivity of oral drug products to interpatient variations in drug solubilization.

1. Introduction

The lack of oral drug products designed for and tested in the neonatal and pediatric patient populations often forces clinicians to use off-label and unlicensed drugs to treat these patients. Besides ethical concerns, the limited availability of validated preclinical tools to predict intestinal absorption seriously hampers drug development tailored to these populations.

A key factor in drug absorption is the solubility of the drug in the intestinal lumen. Together with the drug's physicochemical properties, the composition of human intestinal fluids (HIF) has been recognized as a main determinant of intestinal drug solubility. For this reason, the intestinal fluid composition has been extensively investigated in healthy adults, resulting in a better understanding of its impact on absorption-related processes such as drug dissolution, solubilization and permeation[1–4]. It also led to the development of simulated intestinal fluids (SIF), greatly improving the biorelevant assessment of oral drug products with *in vitro* solubility and dissolution data[5–8] and their input for physiologically based biopharmaceutics modelling[9–12].

Despite significant progress in the predictive *in vitro* and *in silico* simulation of drug absorption in preclinical drug development, physiological changes related to disease, medication or age are currently poorly implemented due to the lack of reference data[13]. To advance the development of oral drugs tailored to the paediatric population[14,15], for instance, recent studies have explored different absorption-related aspects of the gastrointestinal physiology in children, including fluid volume[16,17], fluid composition[18,19], and drug transporter and metabolic enzyme ontogeny[20,21]. In addition, Maharaj

et al.[22] designed neonatal and pediatric SIF to explore drug solubility in children. However, the composition of these fluids was based on scarcely available data.

In this respect, we recently collected intestinal enterostomy fluids from neonatal and infant patients (further referred to as infant HIF) and characterized these fluids with respect to factors that may affect intestinal drug absorption (i.e., pH, buffer capacity, osmolality, total protein, bile salts, phospholipids and lipids)[23]. For some factors, average values in infant HIF substantially differed from previously reported values in adult HIF[24,25]. Most noticeably, the bile salt concentration was much lower in infant HIF, while the lipid and protein concentrations were relatively high. Since bile salts and lipids are key elements of the colloidal structures in HIF that help to solubilize lipophilic compounds[1,26], the solubilizing capacity for such drugs will likely differ between infant and adult HIF. In addition, the fluid composition appeared highly variable between and within infant patients. This likely translates into variations in solubilizing capacity, as has previously been observed in adults HIF[2,27].

To evaluate the impact of the observed composition and variability in infant HIF on drug solubility, the present study assessed the solubility of five poorly water soluble, lipophilic drugs (i.e., ibuprofen, clopidogrel, domperidone, spironolactone and tacrolimus) in intestinal fluid pools from 19 neonatal/infant enterostomy patients. The composition of the individual infant HIF pools was determined to explore possible drivers for drug solubility. For comparison purposes, solubility was also determined in adult HIF and in commercially available SIF. The selected drugs, of which the physicochemical properties are described in Table 1, are all used in the neonatal and/or paediatric patient population. Because of their lipophilic nature and low intrinsic solubility in water, they are likely sensitive to solubilization by colloidal structures and lipid droplets in intestinal fluids.

2. Material and methods

2.1. Chemicals

Clopidogrel bisulfate, spironolactone, hydrogen chloride (HCl), methanol (MeOH) and acetonitrile (ACN) HPLC grade were purchased from ThermoFisher Scientific (Waltham, MA, US). ACN and formic acid (FA) LC/MS grade were acquired from Biosolve (Valkenswaard, The Netherlands). Sodium and potassium hydrogen phosphate (NaH_2PO_4 and KH_2PO_4) and ibuprofen were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid was bought from Chem-Lab analytical (Zedelgem, Belgium). Domperidone was acquired from VWR International (Radnor, PA, USA). Tacrolimus was procured from Lucerna Chem AG (Lucerne, Switzerland). Internal standard for tacrolimus (13C-D^4) was purchased from Alsachim (Illkirch Graffenstaden, France). All substances used for solubility experiments had a purity above 95 %.

2.2. Intestinal fluid pools

Neonatal and infant intestinal fluids were collected from temporary enterostomy patients during their recovery in the Neonatal Intensive Care Unit at the University Hospitals Leuven, Belgium. The study was approved by the Ethics Committee Research UZ/KU Leuven, Belgium (S56879). In all patients, multiple enterostomy fluid samples were collected during a period of 1 to 16 weeks. The collection and characterization of these distinct samples have been thoroughly discussed in a previous study [23]. To ensure sufficient fluid volume for solubility experiments, a single pool of the collected intestinal fluids was made per patient (infant HIF), except for patient L. For Patient L the samples were pooled separately for samples collected during the first 2.5 months postnatal age (PNA) (L Early) and from 3 to 6 months PNA (L Late). For 19 patient pools, sufficient volume was available to determine the solubility of five model compounds. Relevant information on these patients, including sex, gestational age, age range during collection, stoma location, pathology, medication and enteral feeding, are described in Table 2.

For comparison purposes, drug solubility was also determined in adult intestinal fluid pools (adult HIF). These pools were made from intestinal fluids aspirated following a similar protocol as Riethorst et al[25]. In that study, approved by the Ethics Committee Research UZ/KU Leuven (S53791), duodenal fluids were collected in healthy adults according to a standardized procedure. Volunteers were fasted for 12 hours prior to the aspiration study. Fluid samples were collected for 90 min after receiving 240 mL of water (fasted state) or 400 mL of a liquid meal (Ensure plus, Abbott, IL, USA) and 240 ml of water (fed state). To perform the solubility experiments in the present study, samples were pooled per volunteer. In total, three pools of fasted adult HIF (FaHIF) and three pools of fed adult HIF (FeHIF) were used. Finally, the solubility was also determined in commercially available fasted state SIF (FaSSIF-V1) and

fed state SIF (FeSSIF-V1 and FeSSIF-V2). The SIF were prepared according to the manufacturer's protocol (Biorelevant, London, UK).

Table 1. Physicochemical properties of the investigated drugs.

Drug	Molecular weight (g/mol)	Intrinsic solubility[a] (µg/mL)	Acid/Base	pKa[a]	Log P[a]	Log $D_{pH6.0}$[a]
Ibuprofen	206.3	59	Acid	4.85	3.97	2.36
Clopidogrel[b]	321.8	12	Base	3.89	3.80	4.03
Domperidone	425.9	6	Base	5.83	3.91	2.68
Spironolactone	416.6	2	-	-	2.78	2.78
Tacrolimus	804.0	4	-	-	3.03	3.03

[a] Calculated with Chemaxon Chemicalize [22/03/2023], [b] Clopidogrel bisulfate was used.

Table 2. Demographics of the neonatal/infant enterostomy patients

Patient Pool[a]	Sex	Gestational age (weeks)	PNA range during collection (days)	Stoma type	Pathology	Medication	Feeding type
B	M	34	29 – 120	Ileostomy	SIP and obstruction	FS, ursochol, fentanyl, heparin	HM + IF
C	F	31	35 – 133	Ileostomy	NEC	FS, fentanyl, paracetamol	HM + IF
D	F	25	30 – 100	Ileostomy	SIP	FS, fluconazole, tramadol, vancomycin, hydrocortisone, paracetamol	HM + IF
F	M	24	28 – 70	Ileostomy	SIP	FS, primidone, hydrocortisone, azithromycin, vancomycin, paracetamol	HM
G	F	40	18 – 32	Ileostomy	Meconium ileus, Cystic Fibrosis	FS, erythromycin	HM
H	M	39	29 – 71	Ileostomy	Meconium ileus, Cystic Fibrosis	FS, ursochol, atenolol, omeprazole, ceftazidime	HM + IF
J	M	39	9 - 16	Colostomy	Anal atresia	FS, cefotaxime, vancomycin, acyclovir, tazocin,	HM
K	M	27	33 – 89	Ileostomy	SIP	FS, paracetamol, hydrochlorothiazide, spironolactone, piperacillin, amikacin, vancomycin, furosemide	HM + IF
L Early	M	36	55 – 76	Ileostomy	Gastroschisis and obstruction	FS, clonidine, fentanyl, piritramide, paracetamol, piperacillin, amikacin, midazolam	HM + IF
L Late	M	36	83 – 167	Ileostomy	Gastroschisis and obstruction	FS, clonidine, fentanyl, piritramide, paracetamol, piperacillin, amikacin, midazolam	HM+IF
M	M	27	107 – 219	Ileostomy	NEC	FS, ursochol, loperamide, propranolol, ranitidine, amikacin, vancomycin	HM + IF
N	M	41	24 – 31	Ileostomy	Meconium ileus, Cystic Fibrosis	FS	HM
O	M	33	14 – 98	Colostomy	Imperforatio ani	FS, tazocin, amikacin, vancomycin, cefuroxime, oxybutynin	HM + IF
R	F	26	38 – 80	Ileostomy	NEC	FS, paracetamol, hydrocortisone	IF
S	F	34	35 – 56	Ileostomy	Gastroschisis and ileum atresia	FS, paracetamol, linezolid, amikacin, vancomycin, tazocin	HM
T	M	25	65 – 93	Ileostomy	NEC	FS, hydrocortisone, paracetamol	HM
U	M	40	17 – 31	Colostomy	Imperforatio ani	FS, cefadroxil	HM + IF
V	M	27	87 – 115	Ileostomy	Hirschsprung	FS	IF
W	M	39	40 – 61	Ileostomy	Hirschsprung	FS, ursochol, clonidine,	HM

Abbreviations: female (F), male (M), postnatal age (PNA), ileocecal valve (ICV), necrotizing enterocolitis (NEC), spontaneous intestinal perforation (SIP), food supplements (FS), human milk (HM), infant formula (IF)

[a] The letters assigned to the different patient pools refer to the patient codes in de Waal et al.[23]

2.3. Characterization of intestinal fluid pools

Prior to characterization, aliquots of all adult and infant HIF pools were treated in the same way as during the solubility experiments (see 2.4) but without the addition of drug powder (see scheme in Figure 1). This implied incubation at 37℃ for 24 hours under continuous shaking at 175 RPM (IKA KS 4000i control, Staufen, Germany), followed by centrifugation (30 min, 20000 g, 37°C).

Centrifugation of infant HIF and adult FeHIF resulted in a phase separation between an aqueous layer (possibly with micelles) and a lipid layer. For this reason, two parallel sample preparations were performed to isolate (i) the aqueous fraction, and (ii) the total fluid fraction consisting of both the aqueous layer and the lipid layer. To obtain the aqueous fraction after centrifugation, the top lipid layer was removed using a suction system, upon which the remaining fluid was transferred to a new tube leaving any solid material behind. The aqueous fraction was then thoroughly mixed before a second centrifugation (20 min, 20000 g, 37 ℃). After the second centrifugation, the potentially remaining top layer was again removed via suction, leaving the aqueous fraction. To obtain the total fluid fraction after the initial centrifugation step, both the lipid and aqueous layer were transferred to a new tube, leaving the pellet behind. After thoroughly mixing the total fraction, it was centrifuged a second time (20 min, 20000 g, 37℃). Again, both layers (supernatant) were transferred to a new tube and mixed to form the total fluid fraction. In the event the top lipid layer consisted of a liquid layer and a thick solid layer, the solid top layer was removed, to prevent inhomogeneous samples from entrapping drug powder during solubility tests.

Both the aqueous and total fractions of each adult and infant HIF pool were characterized with respect to pH, buffer capacity, osmolality, total protein, bile salts, phospholipids, and lipid digestion products, following the previously described methodology[23].

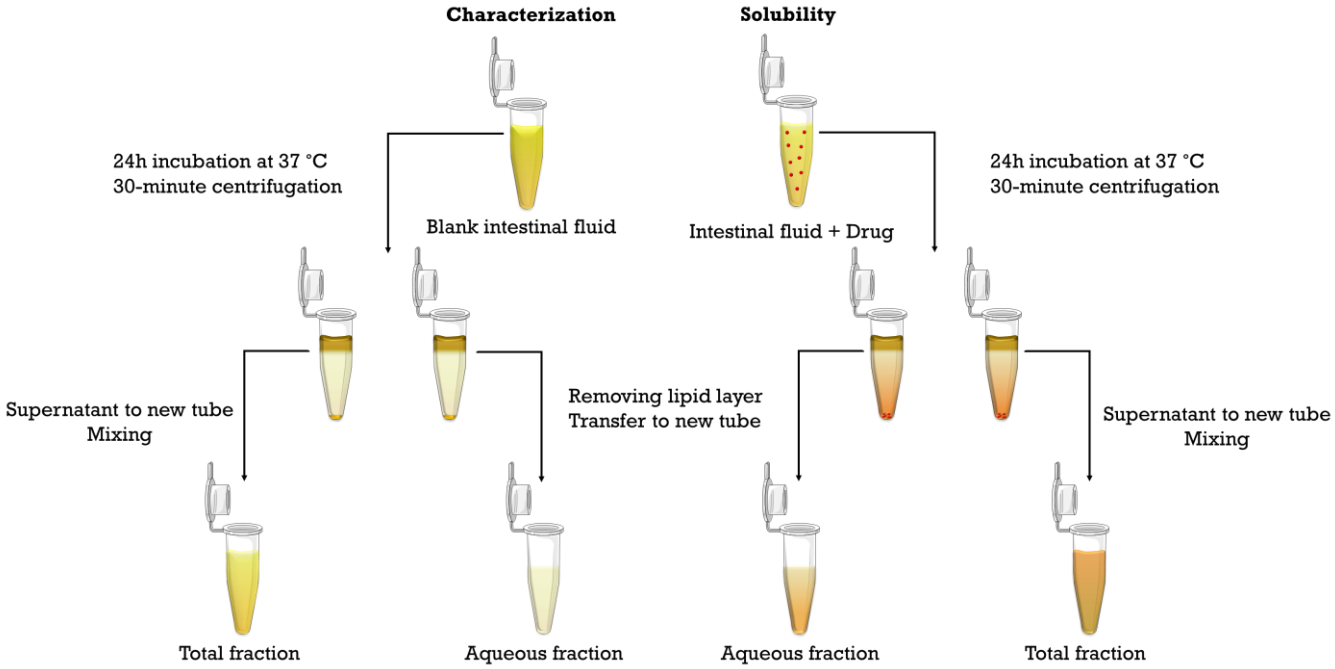

Figure 1. Schematic representation of the intestinal fluid sample preparation for characterization and drug solubility assessment.

2.4. Apparent solubility of selected model drugs

The solubility of five poorly water soluble, lipophilic drugs (i.e., ibuprofen, clopidogrel, domperidone, spironolactone and tacrolimus) was determined in the infant and adult HIF pools, and in FaSSIF-V1, FeSSIF-V1 and FeSSIF-V2. Since the weak base clopidogrel is only administered to neonates and infants as a bisulfate salt, clopidogrel bisulfate was used in the solubility experiments. Since molecularly dissolved drug was not distinguished from drug solubilized in colloidal structures or lipid droplets, the measured solubility should be considered as apparent[28].

Solubility experiments were performed in triplicate, in both the aqueous fraction and the total fraction of each individual fluid pool. To 500 µL of intestinal fluid, an excess of drug powder (i.e., 5 mg ibuprofen, 4 mg clopidogrel bisulfate, 3 mg domperidone, and 1 mg spironolactone and tacrolimus) was added, upon which the suspension was incubated at 37 ℃ for 24 h under continuous shaking at 175 RPM. The aqueous and total fractions were isolated (starting from separate tubes) as described in section 2.3. Prior to quantification of drug in solution, proteins were precipitated by addition of either ice cold MeOH containing 1% FA (ibuprofen) or ACN containing 1% FA (clopidogrel, domperidone, spironolactone and tacrolimus) in a 1 to 10 ratio. For tacrolimus, 5 µM internal standard (tacrolimus-13C-D^4) was added to the ACN mixture. Drug concentrations were determined with liquid chromatography coupled with UV absorbance, fluorescence or MS/MS detection, according to the conditions described in Table 3. The analytical methods were validated according to the ICH M10 guidelines on bioanalytical method validation. Measured at four concentration levels for all

compounds spiked in SIF, the accuracy was between 90 – 110 % and the coefficient of variation was below 5 %, indicating adequate precision. In addition, recovery from infant and adult HIF was determined to be above 90 % for all compounds.

Table 3. Analytical conditions for the liquid chromatography-based quantification of drugs in human and simulated intestinal fluid.

Drug	Column[a]	MP A	MP B	Elution (%A - %B)	Detection
Ibuprofen	1	MeOH	25mM K_2HPO_4; pH 6.5	70-30	Fluo EX: 224 nm EM: 288 nm
Clopidogrel	1	ACN	25mM NaH_2PO_4; pH 2.2	70-30	UV: 250 nm
Domperidone	1	ACN	50 mM NH_4FA; pH 3	60-40	Fluo EX: 285 nm EM: 325 nm
Spironolactone	1	ACN	H_2O	50-50	UV: 237 nm
Tacrolimus	2	ACN	NH_4AC 2mM + 0.1% FA	97.5 - 2.5	MS: $[M + NH_4]^+$ 821.7 → 768.6

[a] Column 1 = Zorbax XBD eclipse c18 (150 * 4.6 mm, 5 µm), column 2 = Kinetex XB-C18 (50 * 2.1 mm, 2.6 µm).
Abbreviations: MP = mobile phase, ACN = acetonitrile, MeOH = methanol, FA = formic acid, Fluo = fluorescence detector, EX = excitation wavelength, EM = emission wavelength, UV = ultraviolet-light spectrometer, MS = mass spectrometry.

2.5. Imaging of microscopic aggregates in infant HIF

The morphology of microscopic aggregates in infant HIF was studied using transmitted polarized light microscopy. Before imaging, the studied HIF sample was thawed at 37 °C for at least 15 min. The sample was homogenized by manual shaking and a small amount was inserted into a glass capillary with a rectangular cross-section (volume 5 µL, length 50 mm, width 1 mm and height 0.1 mm). The capillary was then placed within a custom-made aluminium thermostatic chamber with several cut-out optical windows to enable observation via a microscope[29–31]. A cryo-thermostat JULABO CF30 was used to control the temperature in the metal chamber. The temperature inside the chamber was measured by placing a calibrated thermocouple probe (with an accuracy of ± 0.2 °C and calibrated by a precise mercury thermometer in the relevant temperature range) in an adjacent orifice.

Imaging was performed by an AxioImager.M2m microscope (Zeiss, Germany). Transmitted cross-polarized white light was used, with an included λ-compensator plate, placed after the observed specimen and before the analyzer, oriented at a 45° angle with respect to both the analyzer and the polarizer. At these imaging conditions, both the dispersed fluid objects and the liquid background in the sample have a characteristic magenta color, whereas any crystalline or liquid-crystalline objects appear bright and intensely colored[32].

2.6. Data presentation and statistics

Summarizing data on the characterization of the infant and adult HIF pools are shown as median and range or average ± standard deviation (SD) of the different pools. Apparent solubility values in the aqueous and total fractions of infant HIF are displayed per patient pool as average ± SD of three replicate measurements, unless stated otherwise. Solubility values measured in triplicate in three adult FaHIF and FeHIF pools are depicted as the average ± SD of the three pools combined per prandial state. Solubility values in SIF represent the average ± SD of three replicate measurements. The variability in the data sets is described using a robust version of the coefficient of variance (CV) for non-normal distributed data (RCV_Q), based on median and interquartile range instead of average and standard deviation. The RCV_Q is calculated as: 0.75 × (IQR/Median)[33]. Spearman correlation coefficients were calculated with GraphPad Prism 9.0.2 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

To our knowledge, the current study is the first to explore the solubilizing capacity of multiple infant HIF pools for lipophilic drugs with limited intrinsic solubility and compare them to adult HIF. Infant HIF was collected from 19 infant patients with enterostomy, representing a heterogeneous population with variations in (gestational) age, underlying pathology and medication. As such, the solubility data presented in the following sections can be considered relevant for an actual neonatal patient population.

3.1. Intestinal fluid pool composition

In Table 4, the composition of the 19 infant and 6 adult HIF pools, which were used for solubility testing, is summarized with respect to pH, buffer capacity, osmolality, total protein, bile salts, phospholipids and total lipids (i.e., triacylglycerides, diacylglycerides, monoacylglycerides and free fatty acids). Average values (± SD) are given for infant HIF, adult FaHIF and adult FeHIF. For infant HIF, also median and range are displayed, considering the high variability and skewness of the results. For comparison purposes, the composition of the commercially available simulated fluids FaSSIF-V1, FeSSIF-V1 and FeSSIF-V2 is given as well. The prandial state of the infant HIF pools cannot be clearly defined, as these pools consist of stoma fluids collected three times a day during multiple days in a non-controlled setting. As such, the pools represent a state (i.e., never truly fasted) that is physiologically relevant for infants, who are fed much more frequently than adults. In contrast, the adult HIF pools were collected during a controlled clinical study, with a distinct prandial state. In case of the infant HIF and adult FeHIF pools, values for the lipid concentration in the aqueous and total fraction are reported separately, as a difference was apparent in most pools. For the other variables, only the value in the total fraction is reported since no marked differences were observed between the aqueous and the total fraction.

The comparison in Table 4 highlights some marked differences between the infant and adult HIF pools. While infant HIF characteristics like pH, buffer capacity, osmolality and total protein largely corresponded to the fed state of adult HIF, the average bile salt concentration (712 ± 1105 µM) was far below the concentrations found in fasted or fed adult HIF and the concentrations used in SIF. The average lipid concentration in the total fluid fraction was comparable between infant HIF (6.0 ± 6.7 mg/mL) and adult FeHIF (6.5 ± 1.8 mg/mL), although the variability was more pronounced in infant HIF. In contrast, the average lipid concentration in the aqueous fraction was much lower in infant HIF (0.7 ± 0.5 mg/mL) than in adult FeHIF (4.2 ± 1.0 mg/mL). This discrepancy might be due to solubilization of lipids in bile salt based colloidal structures present in the aqueous fraction of adult HIF, but possibly absent in infant HIF due to the low bile salt concentration. Polarized light microscopy (PLM), able to

detect aggregates (droplets, crystals and liquid crystals) larger than 1 µm, showed remaining small lipid droplets in the aqueous fraction of infant HIF, the amounts of which relatively corresponded to the measured lipid concentrations. This is illustrated in Figure 2, in which PLM images of two infant HIF pools with different lipid concentrations are compared. Possibly, the measured lipids in the aqueous fraction of infant HIF resulted from the incomplete removal of very small lipid droplets with high-speed centrifugation, whereas in adult HIF it is likely to assume that most lipids in the aqueous fraction are solubilized in the colloidal structures.

Besides the clear differences in the average concentrations of bile salts and lipids between the infant and adult HIF pools, the infant HIF composition was marked by huge interpatient variability, as indicated by the RCV_Q values in Table 4. For all explored characteristics, the variability was larger in infant HIF compared to adult HIF. The high variability, in line with previous reports[19,23], could partly be attributed to the non-controlled feeding conditions in the infant patients in contrast to the adult volunteers, who received a standardized meal prior to fluid collection.

Table 4. Composition of the infant and adult human intestinal fluid pools and the simulated intestinal fluids used for solubility experiments. If a layer separation was observed after centrifugation (infant HIF and adult FeHIF), the lipid concentration is reported separately in the aqueous fraction (no lipid layer) and the total fraction (aqueous layer + lipid layer). The bottom part of the table reports the variability for the different characteristics in the infant and adult HIF pools, expressed as RCV_q (0.75 × (IQR/Median)).

	Infant Intestinal Fluids (median; min - max) (average ± SD)		**Adult Intestinal Fluids (average ± SD)**			**Simulated Intestinal Fluids (theoretical value)**		
	Fasted + Fed (n=19 pools)		**Fasted (n=3 pools)**	**Fed (n=3 pools)**		**FaSSIF - V1**	**FeSSIF - V1**	**FeSSIF- V2**
	Aqueous	***Total***	***Aqueous***	***Aqueous***	***Total***	***Aqueous***	***Aqueous***	***Aqueous***
pH	5.67; 4.57 - 7.94 5.72 ± 0.74		7.67 ± 0.34	5.84 ± 0.10		6.5	5	5.8
Buffer capacity (mmol/L/ΔpH)	24; 7 – 97 28 ± 22		5.4 ± 0.1	18.0 ± 0.8		10	75	25
Osmolality (mOsm/kg)	454; 226 - 577 445 ± 97		179 ± 27	345 ± 31		270	635	390
Total protein (mg/mL)	15; 6 – 31 16 ± 7		3 ± 0.4	17 ± 0.2		0	0	0
Bile salts (µM)	495; 0 - 4850 712 ± 1105		2633 ± 893	8453 ± 547		3000	15000	10000
Phospholipids (mM)	0.8; 0.3 - 1.8 0.8 ± 0.4		0.4 ± 0.1	3.5 ± 0.2		0.75	3.75	2
Lipids (mg/mL)	0.5; 0.2 - 2.0 0.7 ± 0.5	3.4; 0.2 - 26 6.0 ± 6.7	0.3 ± 0.03	4.2 ± 1.0	6.5 ± 1.8	0	0	2
pH RCV_Q (%)	7.7		4.1	1.3				
Buffer capacity RCV_Q (%)	75		20	21				
Osmolality RCV_Q (%)	22		9.0	5.6				
Total protein RCV_Q (%)	46		22	8.9				
Bile salts RCV_Q (%)	102		17	5.6				
Phospholipids RCV_Q (%)	53		29	20				
Lipids RCV_Q (%)	89	137	26	30	17			

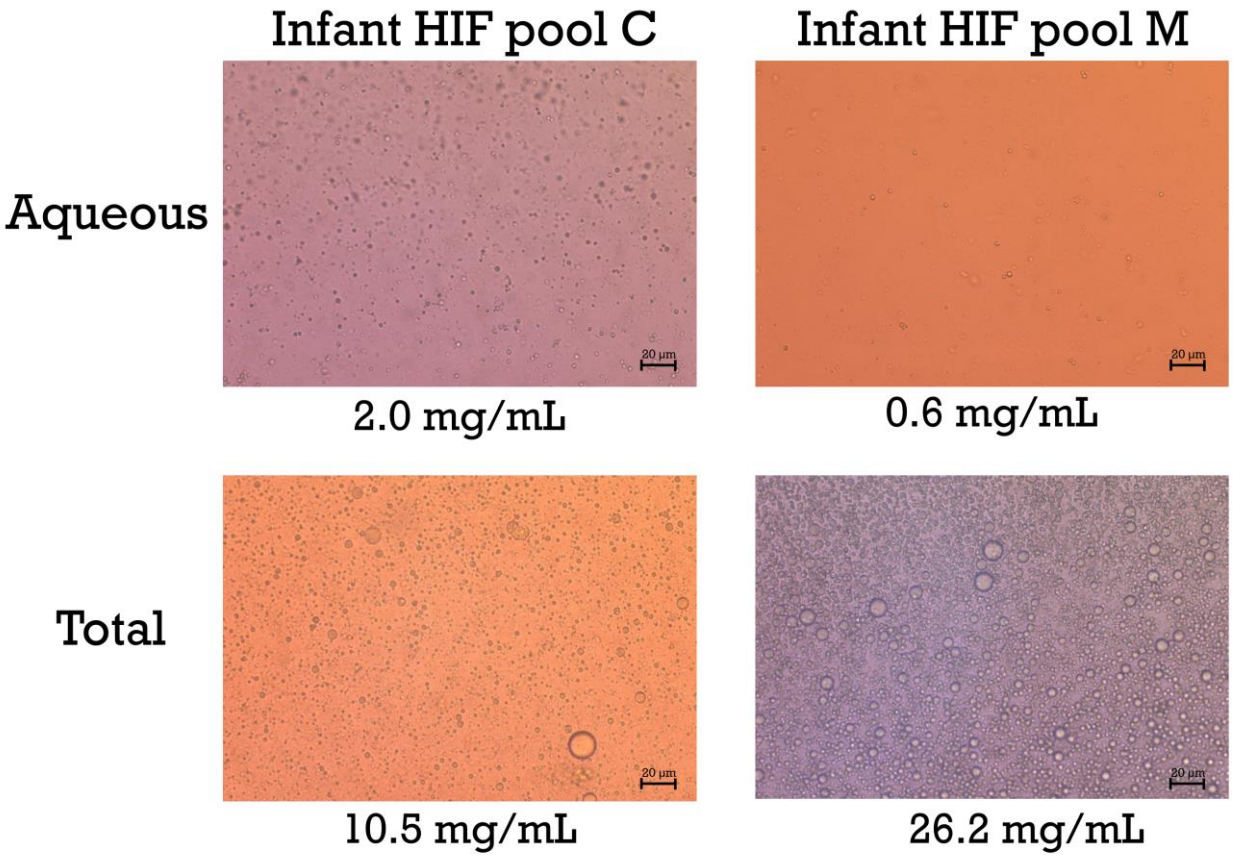

Figure 2. Polarized light microscopy of the aqueous and total fraction for infant intestinal fluid pool C and M, under the pictures the corresponding lipid concentration is depicted.

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3.2. Apparent solubility of selected lipophilic drugs

The apparent solubility of ibuprofen (Figure 3), domperidone (Figure 5) and spironolactone (Figure 6) were determined in 19 infant HIF pools. The solubility of clopidogrel bisulfate (Figure 4) and tacrolimus (Figure 7) could only be determined in 18 infant HIF pools due to insufficient volume of pool S. In addition, solubility for all drugs was determined in adult FaHIF and FeHIF pools and in the simulated fluids FaSSIF-V1, FeSSIF-V1 and FeSSIF-V2. Drug solubility was determined in the aqueous fraction (removed lipid layer) and in the total fraction (aqueous + lipid layer). In Table 5, solubility data are summarized as mean ± SD and as median and range (infant HIF only) over the different pools.

3.2.1. Average solubility in infant HIF compared to adult HIF

The average solubilities (Table 5) of the weak acid ibuprofen and the neutral compounds spironolactone and tacrolimus in infant HIF were comparable (< 2-fold difference) with adult FeHIF in both the aqueous and total fraction. For clopidogrel bisulfate and domperidone, both basic compounds, the average solubility in the total fraction was higher in adult FeHIF compared to infant HIF (3-fold for clopidogrel, 5-fold for domperidone). The reason for these substantial differences is uncertain. Possibly, the basic compounds interact with the negatively charged bile salts that were present in high concentration in adult FeHIF but not in infant HIF. The solubility difference was only apparent in the total but not aqueous fractions of adult FeHIF and infant HIF, suggesting that other mechanisms might be at play, possibly involving both lipids and bile salts.

In general, the average solubility in infant HIF exceeded the solubility in adult FaHIF, except for clopidogrel, which showed a surprisingly high solubility in fasted state conditions (2.7 mg/mL in adult FaHIF, 1.3 mg/mL in FaSSIF-V1). Upon dissolution of the water-soluble salt clopidogrel bisulfate, the release of hydrogen sulphate resulted in a pH reduction in FaHIF (average pH-drop to 2.53) and FaSSIF-V1 (pH 2.80), thereby favoring the solubility of the weak base clopidogrel (pKa 3.9). It is important to note that this pH drop is unlikely to happen in the bulk intestinal environment, as it would be mitigated by continuous bicarbonate secretion into the intestinal lumen. In infant HIF (average pH 5.1), adult FeHIF (average pH 5.8) and FeSSIF-V1 (average pH 5.5), the pH drop was much less apparent due to the higher buffer capacity.

3.2.2. Solubility in SIF compared to average solubility in infant HIF

In addition to infant and adult HIF, the solubility of the selected compounds was determined in fasted and fed state SIF, designed to mimic adult intestinal fluids. A recent industry survey found that these adult SIF are also used to simulate pediatric drug solubility and dissolution, due to the lack of reference data on the intestinal fluid composition in children[14]. The present data shows that the ability of adult SIF to predict average solubility in infant HIF is highly drug dependent. Using FeSSIF-V2, for instance,

the solubility in the total fraction of infant HIF is well predicted for ibuprofen, but underpredicted for clopidogrel, spironolactone, tacrolimus, and, to a lesser extent, domperidone. Such underprediction of the solubility in total HIF samples, i.e., including the lipid fraction, has been described before when comparing the solubilizing capacities of adult FeHIF and FeSSIF(-V2)[27]. The current SIF do not contain fully biorelevant lipid concentrations and therefore lack the distinctive lipid phase present in adult and infant HIF. The solubility in the aqueous fraction of infant HIF was predicted fairly well by using either FeSSIF-V1 or FeSSIF-V2 for all the selected drugs (< 2.3-fold difference). However, it can be expected that the ultrastructure of FeSSIF-V1 and FeSSIF-V2 does not resemble the aqueous fraction of infant HIF, considering the low bile salt levels in infant HIF compared to SIF (Table 4) and the remaining small lipid droplets in the aqueous fraction of infant HIF (Figure 2). Notwithstanding the decent prediction of the average solubilizing capacity of infant HIF's aqueous fraction observed in this study, the underlying mechanisms of solubilization therefore likely differ in FeSSIF(-V2).

3.2.3. Intersubject variability in solubility in infant HIF

By using 19 pools, the present study provides a unique insight into the inter-patient variability in solubilizing capacity of infant HIF. The large variation in solubility between different infant HIF pools is clearly visualized for the five compounds in Figures 3-7. The relative intersubject variability in apparent solubility in the total fraction, expressed as RCV_Q in Table 5, ranged between 60 and 160 % in infants, which is substantially higher than in fed state adults (between 6 and 73 %). In the aqueous fraction, the variability ranged from 46 to 155 % in infants and from 38 to 83% in adult FeHIF. The higher variability in the solubilizing capacity of infant HIF can partly be attributed to the above-mentioned non-standardized but realistic conditions during the fluid collection in infants.

To explore whether the variability in solubilizing capacity across the different infant HIF pools was similar for all tested drugs, Spearman correlation coefficients were determined, comparing the solubility values for different drugs. In the aqueous fraction, only clopidogrel and domperidone solubility significantly correlated (Spearman $r = 0.81$, $P < 0.001$). In the total fraction, however, significant correlations were observed between all drugs, except spironolactone. Relatively weak correlations were seen between domperidone and tacrolimus ($r = 0.48$), and between domperidone and ibuprofen ($r = 0.57$); moderate correlations were observed between domperidone and clopidogrel ($r = 0.68$), clopidogrel and tacrolimus ($r = 0.64$), ibuprofen and tacrolimus ($r = 0.79$), and ibuprofen and clopidogrel ($r = 0.83$). When comparing the individual infant HIF pools for the different drugs (Figures 3-7), it becomes apparent that solubilities are consistently high in pool G, but low in pools L Early and L Late. However, the solubilizing capacity of some other pools (e.g., N, V and K) is highly drug dependent. The same was observed in previous studies on drug solubility in adult HIF[2,24]. Surprisingly,

drug solubility was similar in the two fluid pools of infant L (L early and L late), even though the bile salt concentration increased significant at older age (i.e., 63 µM L early vs. 4850 µM L late).

3.2.4. Physiological factors that affect drug solubilization in infant HIF

In an attempt to identify key factors in determining drug solubility in infant HIF, Spearman correlation coefficients were assessed between drug solubility and infant HIF characteristics (i.e., pH, buffer capacity, osmolality, total protein, bile salts, phospholipids and lipids). Surprisingly, no strong correlations were found in either aqueous or total fraction. Only the total protein concentration correlated significantly, yet weakly to moderately, to the solubility of each of the studied drugs in the total fluid fractions (Spearman r ranging from 0.49 to 0.68, $P < 0.05$). The overall lack of clear correlations between solubility and single intestinal fluid characteristics might indicate that the mechanisms of solubilization vary between the different infant HIF pools. For instance, in some pools, solubility might be mainly driven by lipids or proteins, while in others pH might be the key factor. The present data set, however, does not allow studying such complex mechanisms with multivariate analysis due to the relatively high number of covariates (intestinal fluid characteristics) in relation to the limited number of observations (solubility in infant HIF pools). In addition, it should be noted that the composition of the intestinal fluid pools has been assessed on the level of molecule classes (e.g., total protein and lipid concentrations) rather than individual molecular species. Differences in molecular species could yield different colloidal structures, resulting in altered solubilization of drugs. Thus, a detailed analysis of the molecular species and colloidal structures present in the infant HIF pools might better correlate with drug solubility, although such an analysis is not straightforward.

As mentioned above, drug solubilization effects of the different HIF pools were not always consistent across the selected compounds. However, for all drug combinations, relatively strong correlations were found when comparing the solubility ratios between the total fraction (i.e., aqueous + lipid layer) and the aqueous fraction in the different infant HIF pools (Spearman r ranging from 0.64 to 0.85, $P < 0.001$). This indicates that the additional solubilization resulting from the lipid layers of the different infant HIF pools is fairly consistent among the selected drugs. A significant Spearman correlation was found between the total/aqueous solubility ratios for the individual drugs with the lipid ratio (Spearman r ranging from 0.53 to 0.76, $P < 0.05$). This implies that the lipid concentration plays an important role in the solubilization process.

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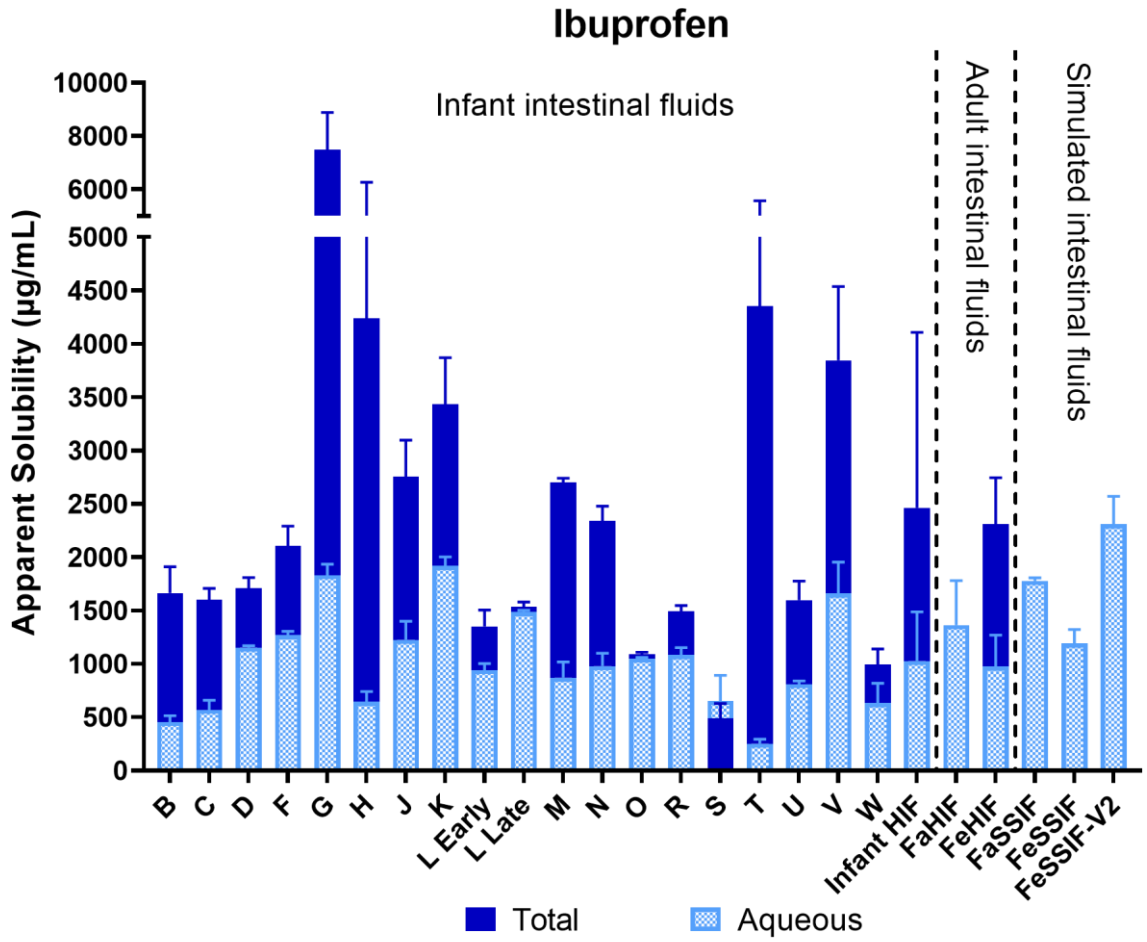

Figure 3. Apparent solubility of ibuprofen in (i) intestinal fluid pools from 19 infant enterostomy patients and the average of all infant HIF pools (Infant HIF), (ii) fasted and fed state intestinal fluid pools from adults (FaHIF and FeHIF), and (iii) simulated intestinal fluid representing fasted (FaSSIF-V1) and fed (FeSSIF-V1 and FeSSIF-V2) state. Bars represent solubility values in either the aqueous fraction (light bars) or the total fraction (dark bars) of the fluids. Data are presented as mean + SD of (i) triplicate measurements in individual infant HIF pools, (ii) measurements in three different adult HIF pools, or (iii) triplicate measurements in SIF.

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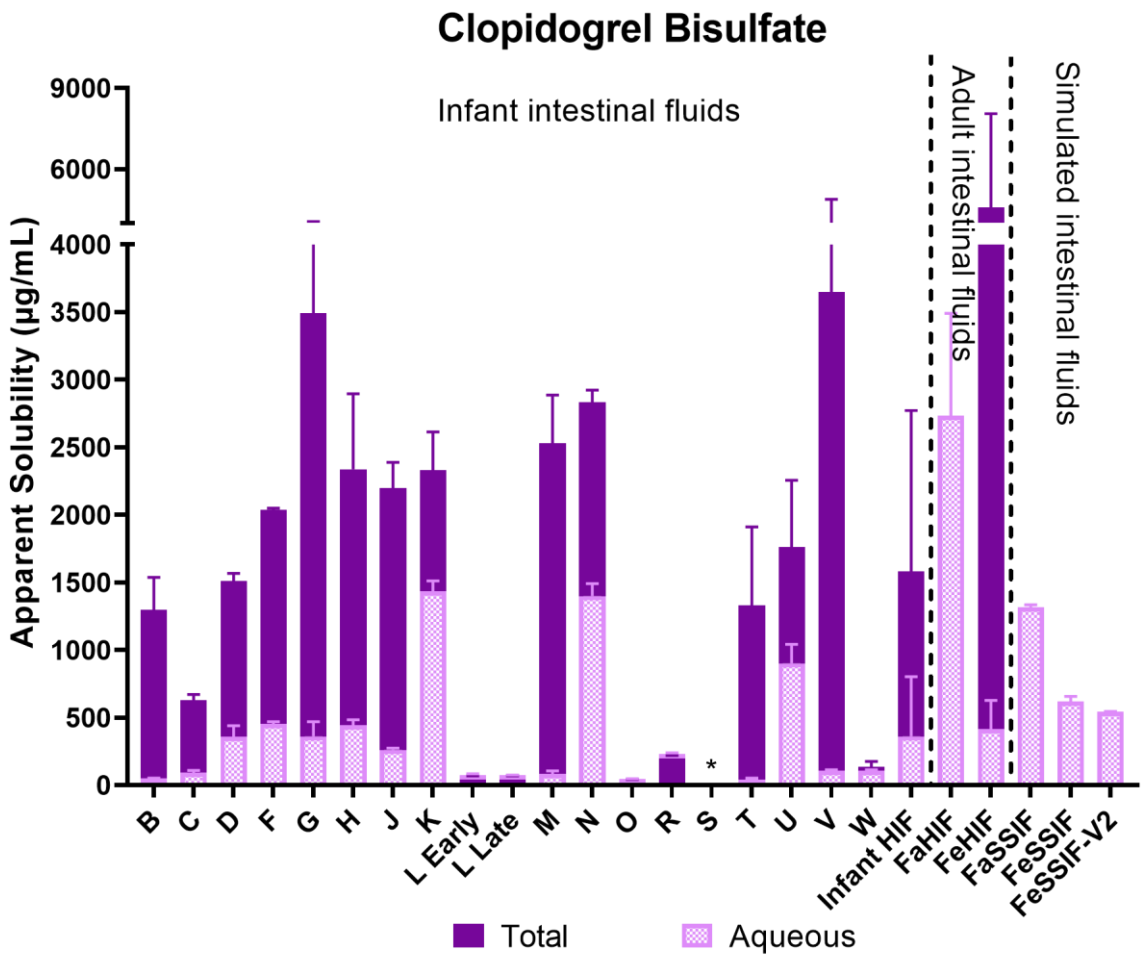

Figure 4. Apparent solubility of clopidogrel bisulfate in (i) intestinal fluid pools from 18 infant enterostomy patients and the average of all infant HIF pools (Infant HIF), (ii) fasted and fed state intestinal fluid pools from adults (FaHIF and FeHIF), and (iii) simulated intestinal fluid representing fasted (FaSSIF-V1) and fed (FeSSIF-V1 and FeSSIF-V2) state. Bars represent solubility values in either the aqueous fraction (light bars) or the total fraction (dark bars) of the fluids. Data are presented as mean + SD of (i) triplicate measurements in individual infant HIF pools, (ii) measurements in three different adult HIF pools, or (iii) triplicate measurements in SIF.

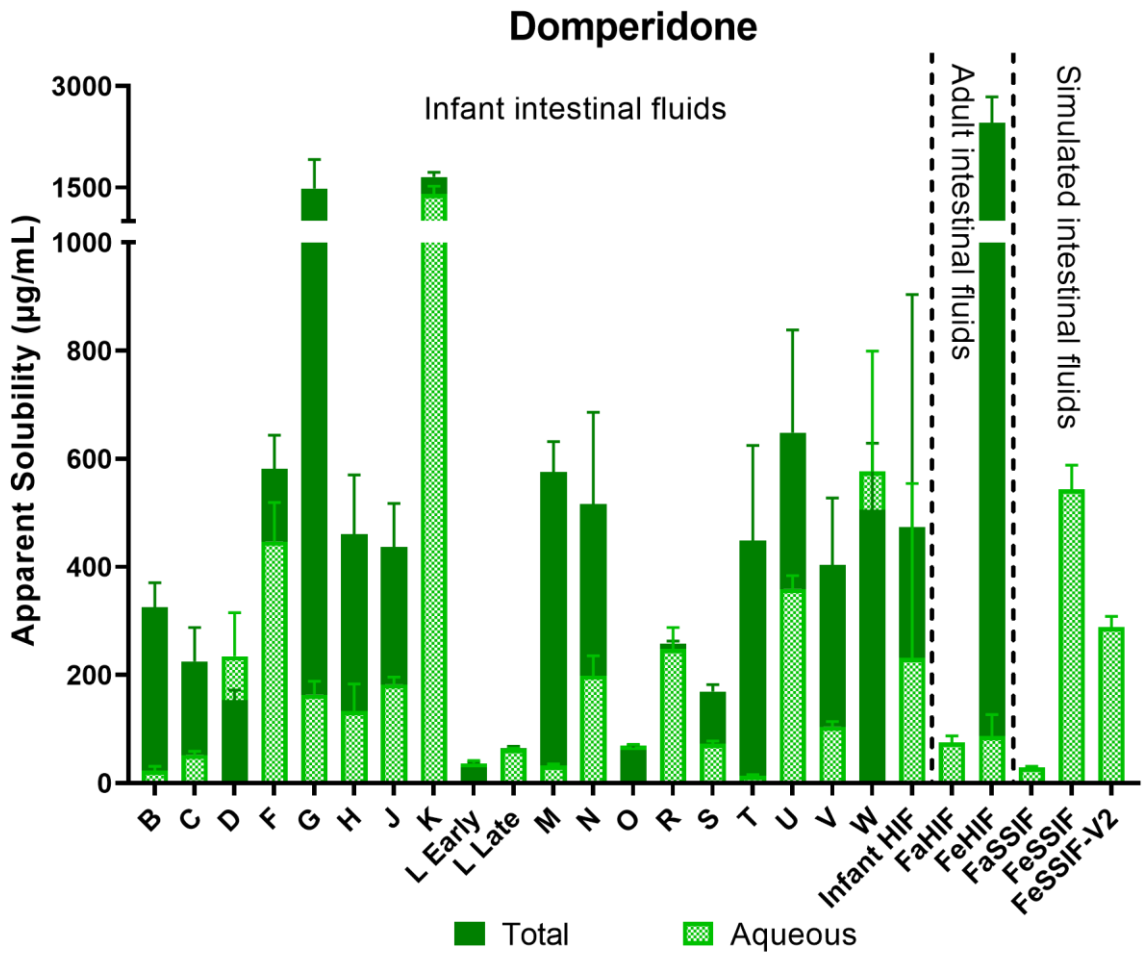

Figure 5. Apparent solubility of domperidone in (i) intestinal fluid pools from 19 infant enterostomy patients and the average of all infant HIF pools (Infant HIF), (ii) fasted and fed state intestinal fluid pools from adults (FaHIF and FeHIF), and (iii) simulated intestinal fluid representing fasted (FaSSIF-V1) and fed (FeSSIF-V1 and FeSSIF-V2) state. Bars represent solubility values in either the aqueous fraction (light bars) or the total fraction (dark bars) of the fluids. Data are presented as mean + SD of (i) triplicate measurements in individual infant HIF pools, (ii) measurements in three different adult HIF pools, or (iii) triplicate measurements in SIF.

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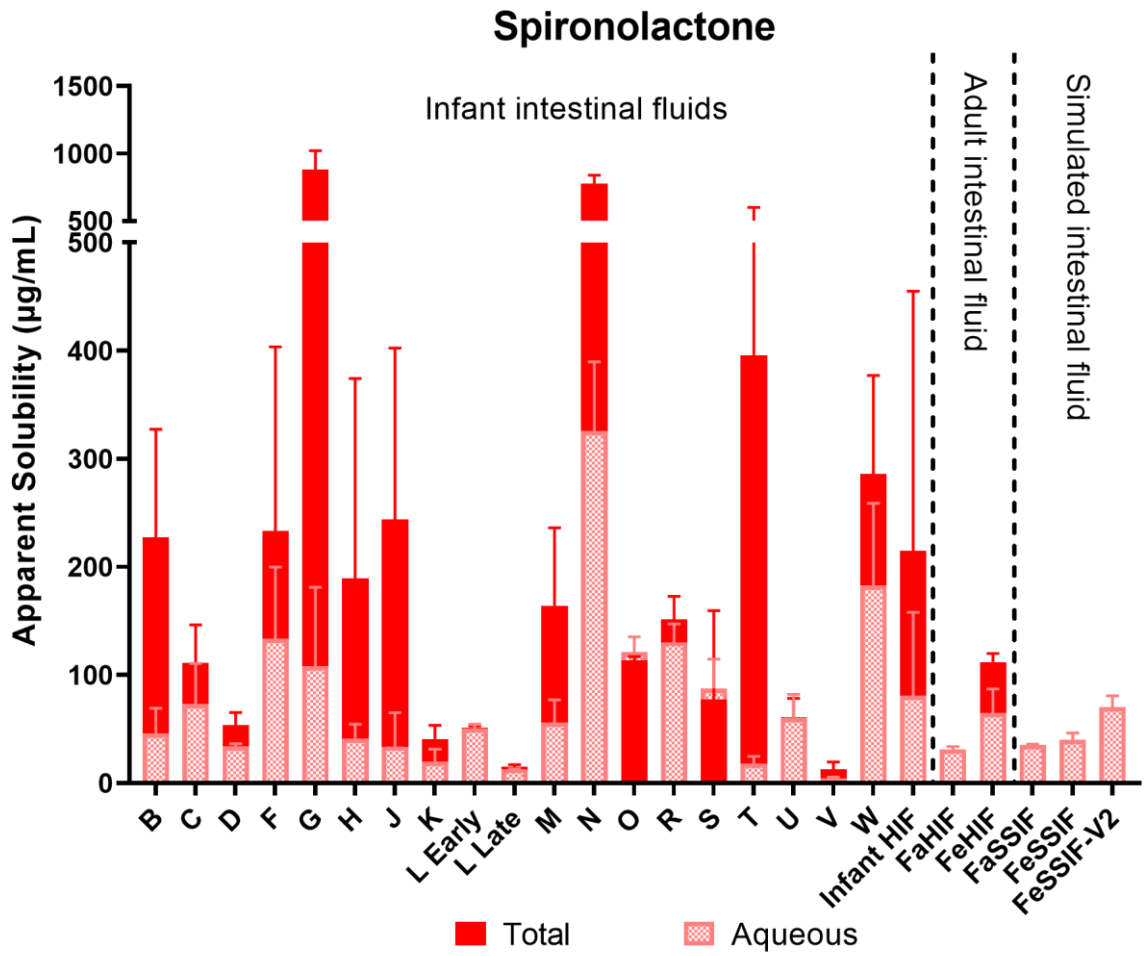

Figure 6. Apparent solubility of spironolactone in (i) intestinal fluid pools from 19 infant enterostomy patients and the average of all infant HIF pools (Infant HIF), (ii) fasted and fed state intestinal fluid pools from adults (FaHIF and FeHIF), and (iii) simulated intestinal fluid representing fasted (FaSSIF-V1) and fed (FeSSIF-V1 and FeSSIF-V2) state. Bars represent solubility values in either the aqueous fraction (light bars) or the total fraction (dark bars) of the fluids. Data are presented as mean + SD of (i) triplicate measurements in individual infant HIF pools, (ii) measurements in three different adult HIF pools, or (iii) triplicate measurements in SIF.

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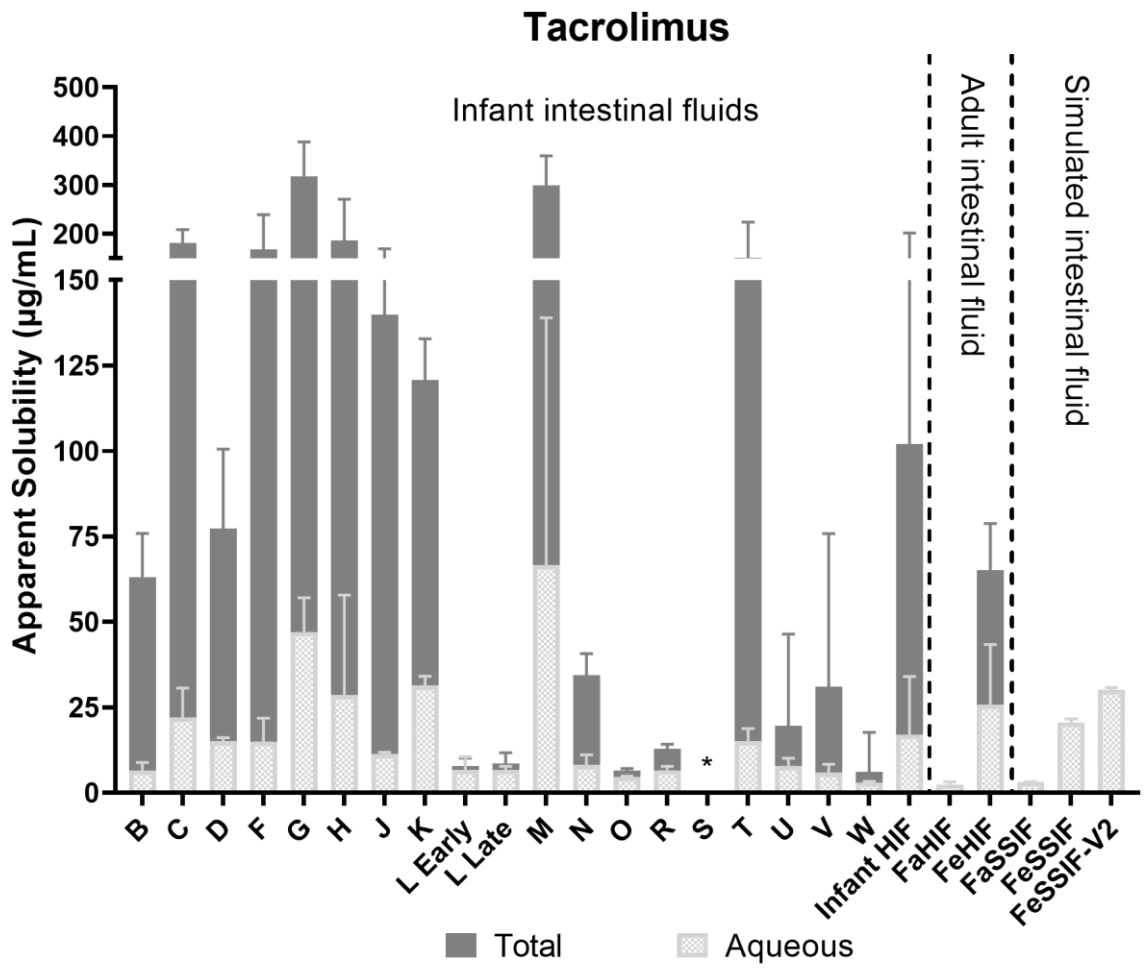

Figure 7. Apparent solubility of tacrolimus in (i) intestinal fluid pools from 18 infant enterostomy patients and the average of all infant HIF pools (Infant HIF), (ii) fasted and fed state intestinal fluid pools from adults (FaHIF and FeHIF), and (iii) simulated intestinal fluid representing fasted (FaSSIF-V1) and fed (FeSSIF-V1 and FeSSIF-V2) state. Bars represent solubility values in either the aqueous fraction (light bars) or the total fraction (dark bars) of the fluids. Data are presented as mean + SD of (i) triplicate measurements in individual infant HIF pools, (ii) measurements in three different adult HIF pools, or (iii) triplicate measurements in SIF.

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Table 5. Apparent solubility of the investigated drugs in infant and adult human intestinal fluid pools and simulated intestinal fluid. If a layer separation was observed after centrifugation (infant HIF and adult FeHIF) the apparent solubility is reported separately in the aqueous fraction (no lipid layer) and total fraction (aqueous layer + lipid layer). The bottom part of the table reports the variability for the different apparent solubilities in the infant and adult HIF pools, expressed as RCV_q (0.75 × (IQR/Median)).

	Infant Intestinal Fluids (median; min - max) (average ± SD)		**Adult Intestinal Fluids (average ± SD)**			**Simulated Intestinal Fluids (average ± SD)**		
	Fasted + Fed (n=19 pools)		**Fasted (n=3 pools)**	**Fed (n=3 pools)**		**FaSSIF - V1**	**FeSSIF - V1**	**FeSSIF- V2**
Drug	***Aqueous***	***Total***	***Aqueous***	***Aqueous***	***Total***	***Aqueous***	***Aqueous***	***Aqueous***
Ibuprofen (µg/mL)	978; 251 - 1919	1707; 488 - 7482	1365 ± 416	977 ± 293	2307 ± 437	1779 ± 29	1189 ± 132	2307 ± 262
	1026 ± 461	2462 ± 1645						
Clopidogrel Bisulfate (µg/mL)	167; 39 - 1434	1636; 38 - 3647	2732 ± 757	414 ± 212	4570 ± 3481	1316 ± 18	617 ± 39	544 ± 2
	361 ± 441	1580 ± 1192						
Domperidone (µg/mL)	133; 14 - 1400	437; 36 - 1654	75 ± 12	87 ± 40	2454 ± 387	29 ± 2	544 ± 45	289 ± 20
	232 ± 322	474 ± 430						
Spironolactone (µg/mL)	56; 4 - 326	151; 13 - 882	31 ± 2	65 ± 22	112 ± 8	35 ± 1	40 ± 6	70 ± 11
	81 ± 77	215 ± 240						
Tacrolimus (µg/mL)	10; 3 - 67	70; 6 - 318	2.4 ± 0.8	26 ± 18	65 ± 14	3.1 ± 0.1	21 ± 1	30 ± 1
	17 ± 17	102 ± 100						
Ibuprofen **RCV_Q (%)**	46	70	30	38	26			
Clopidogrel Bisulfate **RCV_Q (%)**	155	92	33	56	73			
Domperidone **RCV_Q (%)**	104	60	21	22	6.1			
Spironolactone **RCV_Q (%)**	108	90	11	42	6.2			
Tacrolimus **RCV_Q (%)**	104	160	27	83	14			

4. Concluding remarks

Based on the apparent solubility assessment of five lipophilic drug compounds in 19 infant HIF pools from enterostomy patients and 6 adult HIF pools, the present study indicates that the average solubilizing capacity of infant HIF may or may not resemble that of fed state adult HIF, depending on the compound. The commonly used simulated fluids FeSSIF-V1 and FeSSIF-V2, mimicking adult fed state HIF, appeared fairly informative for solubility estimation in the aqueous fraction of infant HIF. Yet, the underlying solubilization mechanisms likely differ, considering the differences in composition (in particular, the low bile salt levels in infant HIF). Similar to adult HIF, the lipid phase of infant HIF may contribute substantially to drug solubilization, an effect which cannot be simulated by current SIF.

Since the 19 infant HIF pools were collected from a heterogenous population of enterostomy patients with a realistic but non-standardized diet, the obtained data provide a unique insight into the variability in drug solubilization to be expected in clinical practice. Overall, it is evident that the huge variability in the composition of infant HIF translates into a highly variable solubilizing capacity for lipophilic drugs. The lack of strong monotonic correlations between the solubilizing capacity and single compositional factors of infant HIF highlights the complexity of the solubilizing mechanisms.

Altogether, the observed variability in fluid composition and solubilizing capacity is likely to impact drug bioavailability in the infant patient population, with the risk of erratic therapeutic responses and/or safety issues. Thus, the sensitivity of a drug compound or formulation to the variable GI environment must be considered during the development of safe and effective oral drug products tailored to infants.

Predicting the impact of altered drug solubilization on drug absorption and bioavailability in infants is challenging and should fit in a physiology based simulation strategy, which considers several age-related factors, including dose adjustments, reduced gastrointestinal fluid volumes, an increasing intestinal surface area, and the ontogeny of intestinal transporters and metabolizing enzymes that may affect drug permeation. Considering the extensive solubilization of drug molecules in colloidal structures and/or lipid droplets present in infant HIF, it is important to note that their contribution to absorption is still poorly understood. Indeed, only free drug in the aqueous phase is readily available for permeation[35–37]. Entrapment of drugs in colloidal structures or lipid droplets could hinder permeation, but at the same time function as a reservoir, continuously exchanging with the free drug fraction. As such, the vastly different composition and thus ultrastructure of infant HIF versus adult HIF (e.g., low bile salts and lipids) could have a profound effect on the interplay between solubilization and permeation. In this respect, also the possible role of drug binding to proteins, which are fairly abundant in infant HIF and appear to correlate with drug solubility, requires further research.

Ideally, robust dosage forms are developed that give a reproducible drug bioavailability, irrespective of the variable GI environment. To guide the development of such formulations, effort has been put into designing simulated intestinal fluids for neonates, infants and children. Based on the scarcely available data, Maharaj et al.[22] designed neonatal and pediatric gastric and intestinal SIF, and performed solubility experiments. These infant SIF already incorporated some of the observed differences with adult SIF, including lower bile salt levels (although levels were even lower in most of the infant HIF pools in the present study). The drug solubilizing capacity of these infant SIF appeared lower compared to adult SIF[22,38], which is, based on the current data, not necessarily true for infant versus adult HIF. Other factors, such as relatively high concentrations of lipids and proteins, should be taken into account to accurately predict oral drug solubility in infants. Considering the obvious

interindividual variability of infant HIF, a wider range of SIF might be useful to determine how sensitive a drug compound or formulation is to these variations.

Overall, it is clear that further research is needed, not only to explore the solubilization mechanisms in infant HIF, but also to integrate them into a physiology based simulation strategy that guides the development of robust, effective and safe oral drug products designed for infant patients.

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Data Availability Statement: Data will be made available on request.

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