Characterisation of fasted state gastric and intestinal fluids collected from children

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Abstract

Fundamental knowledge about the composition of intestinal fluids in paediatric populations is currently unavailable. This study aimed to characterise gastric and intestinal fluid from paediatric populations.

Gastric and intestinal fluid samples were obtained during routine clinical endoscopy from paediatric patients at a large teaching hospital. These fluids were characterised to measure the pH; buffer capacity; osmolality; bile acid concentration and composition.

A total of 55 children were recruited to the study aged from 11 months to 15 years of age where 53 gastric fluid samples and 40 intestinal fluid samples were obtained. pH values recorded ranged from pH 0.57 to 11.05 (median: 2.50) in gastric fluids and from 0.89 to 8.97 (median: 3.27) in intestinal fluids. The buffer capacity did not change significantly between gastric and intestinal fluids with median values of 12 mM/L/ΔpH for both fluids. Gastric fluid osmolality values ranged from 1 to 615 mOsm/kg, while intestinal fluid values ranged from 35 to 631 mOsm/kg.

Gastric fluid bile acid concentrations ranged from 0.002 to 2.3mM with a median value of 0.017mM whilst intestinal fluid bile acid concentrations ranged from 0.0008 to 3.3mM with a median value of 0.178mM. Glycocholate; taurocholic acid; glycochenodeoxycholate and taurochenodeoxycholate were the most commonly identified bile acids within paediatric intestinal fluids.

All compositional components were associated with large inter-individual variability. Further work is required to develop simulated paediatric media and to explore the impact of these media on drug solubility and dissolution.

Keywords: gastrointestinal fluid; paediatric; bile acid; buffer capacity; pH; osmolality

Introduction

Drug solubility within the gastrointestinal (GI) tract is key to oral biopharmaceutics parameters including calculation of the maximum absorbable dose [1, 2] and biopharmaceutics classification system [3]. Inadequate solubility can limit absorption of certain active pharmaceutical ingredients (APIs) so it is important to accurately measure solubility in GI fluids. However, GI fluid is a complex media known to exhibit high inter-individual variability. Critical to the prediction of the oral absorption of drugs in children is knowledge of the physical environment within the paediatric intestinal tract. Fundamental knowledge about the composition of intestinal fluids in neonates and children is currently unavailable.

There are several studies where GI fluids have been collected and characterised in the biopharmaceutics arena. The majority of studies have been conducted on adult populations in the fasted state [4-12] yet there are also studies exploring the fed state [13, 14]. Previous studies conducted in adults and children [15] where fasted fluid was collected and characterised are listed in Supplementary Table 1. Methodology associated with the measurement of GI fluid have varied and there has been recent work published to standardise methods of assessment [16].

The development of simulated adult intestinal fluids based on aspirated intestinal fluids has shown superiority in predicting *in vivo* performance compared to simple buffers [17]. Currently used simulated intestinal fluids: fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF) are based on adult data sets [18]. However, it is recognised that the GI environment in children may be different to that in adults [19]. There have been reports that the differences in volumes of fluid present may affect the classification of APIs in children according to the adult biopharmaceutics classification system [20-24].

A comprehensive review [25] on paediatric GI fluids and the component materials revealed several differences in paediatric fluids compared to adult data. The findings included a relatively higher gastric osmolality (of 253 mOsm/L in infants at 8 months) compared to values reported in adults, no reports of bile concentrations or buffer capacity from paediatric intestinal fluids were found in this review. Based on this review, recipes for paediatric fasted state simulated gastric and intestinal fluids were proposed for both neonates and infants, these reflected worse case scenarios rather than informed compositional content [25]. Subsequent to the Maharaj et al (2016) review [25], a study investigating the composition of gastric fluid in a paediatric population was published [15]. This gastric fluid study reported pH values ranging from 1.2-8.3 in neonates up to 20 days old (a similar pH range is observed for infants although details are not listed); 0.93-8.15 in children (2-12 years) and 1.24-6.96 in adolescents (12-18 years). The majority of the osmolality values measured in gastric fluids were between 200-350 $mOsmKg^{-1}$ for neonates and infants, with lower mean values of 152 ±74 $mOsmKg^{-1}$ in children and 196 ±73 $mOsmKg^{-1}$ in adolescents. Bile salt concentrations in gastric fluids were also measured and large variability was shown: for neonates the concentration ranged from 0 to 5.6 mM (mean 0.19mM); infants ranged from 0-1.6 mM (mean 0.24 mM); children 0-1.1 mM (mean 0.10 mM) and for adolescents ranged from 0-6.3 mM (mean 0.76 mM) [15].

Bile acids are chemically similar compounds based on a steroid nucleus, when these acids are conjugated to sodium they are termed bile salts and often the terms bile acid and bile salt are used interchangeably. Differences in the number and position of hydroxyl groups in relation to the steroid structure dictate the specific bile acid present. The structure informs the balance between

hydrophobic and hydrophilic components within the bile acid which in turn affects how the bile interacts with other chemicals, including how this may affect the solubility of an API [26]. Thus knowledge of bile acid composition within the intestinal fluid is critical and has previously been shown to have very large effects on API solubility [27, 28]. Primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDC) are produced by the liver and conjugated to the amino acids glycine or taurine [29].

Several studies have reported the bile acid concentration in adult human intestinal fluids, a review of this literature suggested an overall mean bile acid concentration in the fasted duodenum of 3.3mM and 3.0mM in the fasted jejunum [30]. With regard to bile acid composition, adult studies have shown discrepancies in the bile acids detected and all data shows large inter subject variability that could explain these differences [9, 12, 31]. There is limited data available on bile acids from paediatric populations. Studies reporting bile acid concentrations in the GI fluids of children are listed in Table 1. One study [15] reported relative bile acid compositions of gastric fluid with slight differences found between neonate and infant populations to that of children and adolescents, further details are provided in Table 1.

Drug solubility in the intestine drives absorption for certain APIs and small changes in solubility can have large effects on the absorbed dose and therefore subsequent therapy. The composition of GI fluid, therefore, influences drug product performance and may differ between children and adults. In the paediatric population, knowledge of GI fluid composition is essential to develop and build biorelevant physical and *in silico* models with the potential to minimise the burden of clinical trials in children. This study seeks to characterise gastric and intestinal fluid from paediatric populations to include reports on bile acid concentration and composition and to compare these fluids to data from adult populations as well as gastric data from paediatric populations [15].

Reference	Population age	Fed/Fasted	Fluid	Bile acid/salt concentration	Bile acids present
[32]	8 healthy neonates (3-15 days)	4 hours after last meal	Duodenal	0.50-5.29 mM (fasting values) Mean value = 2.09 mM	Taurine and glycine conjugates of cholic and CDC acids (TC; TCDC; GC; GCDC). The total concentrations of cholic and CDC acids combined during the test meal ranged from 0.41 to 1.48 mM. The ratio typically showed a greater concentration of cholic acid compared to CDC acid
[33]	18 healthy preterm neonates (32-39 weeks)	2 hours after last feed	Duodenal	0.44-23.3 mM Mean value = 6.32 mM	Not stated.
[34]	34 neonates/infants (birth-7 months)	2 hours after last meal	Duodenal	1.65 ±1.1 mM	Glycine/taurine conjugates 0.09 (±0.03) mM Trihydroxy/dihydroxy bile acids 1.8 (±1.3) mM TC acid 0.78 (±0.36) mM TCDC acid 0.68 (±0.40) mM TLC acid 0.32 (±0.17) mM GC acid 0.25 (±0.15) mM GCDC acid 0.55 mM (1 sample)
[35]	20 low birth weight neonates (12-22 days)	3 hours after last meal	Duodenal	3.2-6.9 mM	Glycine conjugates 1.2-4.6 mM Taurine conjugates 0.9-2.3 mM
[36]	36 neonates (34 ±2.6 weeks) 16 infants/children (25 ±21 months)	Pre-prandial sample	Duodenal	~3-4 mM (neonates) ~5-7 mM (infants/children) Data sets read from graph and exact values not available	GCDC formed 11% of total bile salts in neonates TLC was detected in higher frequency in the infant/children group compared to neonates

[37]	66 healthy preterm neonates (33-36 weeks)	1-3 hours after last feed	Duodenal	Median value 3.63 mM in formula fed neonates Median value 7.56 mM in breast fed neonates	High levels of 2-OH cholate bile acids; CDC
[38]	42 low birthweight neonate/infants (15-51 days)	Pre-prandial sample	Duodenal	4.60 ±2.51 mM	No details on composition of bile acids
[39]	41 healthy preterm neonates/infants (8-58 days)	3-4 hours after last meal	Duodenal	27-28 gestational weeks: 4.25 ±2.07 mM 33-34 gestational weeks: 4.47 ±2.10 mM	Secondary bile acids were not detectable Cholic acid; CDC acid; DC acid and LC acid were present
[15]	11 neonates (0-28 days) 3 infants (28 days- 2 years) 30 children (2-12 years) 10 adolescents (12-18 years)	Pre-prandial	Gastric	0.0-5.60 mM 0.0-1.61 mM 0.0-1.11 mM 0.0-6.28 mM	In neonate and infant populations the relative order GC > TC > TCDC > GCDC In children and adolescents where the order was GC > GCDC > TC > TCDC > GDC > TDC > GUDC

Table 1. Summary of cohort details from studies reported where bile acid concentrations were measured in paediatric population. Glycocholic acid (GC); glycochenodeoxycholic acid (GCDC); glycodeoxycholic acid (GDC); glycoursodeoxycholic acid (GUDC); taurocholic acid (TC); taurochenodeoxycholic acid (TCDC); taurodeoxycholic acid (TDC); tauroursodeoxycholic acid (TUDC); taurolithocholic acid (TLC); deoxycholic acid (DC), lithocholic acid (LC), and ursodeoxycholic acid (UDC).

Materials and Methods

Source of intestinal fluid samples

All samples were collected from patients at Birmingham Children's Hospital, a large teaching hospital that is part of Birmingham Women's and Children's Hospital NHS Foundation Trust, UK. Ethical approval was granted by South Birmingham NRES Committee (IRAS 251909). Gastric and intestinal fluid samples were collected from participants during routine clinical endoscopy. Clinical protocols requested that no fluid was ingested in the 90 minutes prior to the endoscopy procedure. Gastric samples were collected from the gastric antrum and intestinal samples from the duodenum. The samples were stored at -80°C prior to characterisation. The participants were stratified by age into the following groups, based on the International Conference on Harmonization (ICH) E11 classifications: < 2 years: new-born/ infant/ toddler (the term infant is used for this group for the remainder of this manuscript), 2-5 years: pre-school age children, 6-11 years: school age children, 12-16 years: adolescents.

Chemicals

Bile salt standards: Cholic acid (CA); Glycocholic acid (GC); glycochenodeoxycholic acid (GCDC); glycodeoxycholic acid (GDC); glycoursodeoxycholic acid (GUDC); taurocholic acid (TC); taurochenodeoxycholic acid (TCDC); taurodeoxycholic acid (TDC); tauroursodeoxycholic acid (TUDC); taurolithocholic acid (TLC); deoxycholic acid (DC), lithocholic acid (LC), and ursodeoxycholic acid (UDC) were purchased from either Sigma Aldrich (Gillingham, UK) or Acros Organics (Fisher Scientific, Loughborough, UK). Internal standards (IS) were specific isotope labelled standards of cholic acid-D4 (D4-CA) and deoxycholic acid-D4 (D4-DC)**,** purchased from Sigma Aldrich**.** Further details of physchem properties, CAS number, % purity and purchase details of all standards are provided in Table 2 in the supplementary information.

Methodology for characterisation of fluid samples collected

pH: A Hanna HI 2210 pH meter was used for all measurements, calibrated on the day of use. A narrow pH electrode (Hanna HI1331B) was used to enable measurement of the small volumes available.

Buffer Capacity (mmol/L): The buffer capacity was measured by titrating each sample with 0.1M NaOH under constant stirring whilst monitoring the pH to measure the volume required for a change in pH of 1 unit. A calibrated Hanna HI 2210 pH meter was used for all measurements. Previous studies measured buffer capacity using both NaOH and HCl, however due to the small sample volumes available, only titration against HCl was performed for all samples [16]. The buffering capacity (β) was calculated using the following equation:

$$\beta = \frac{\Delta A}{\Delta pH}$$

Where ΔA is the amount of acid added and ΔpH is the change in pH induced by the acid added.

Osmolality (mOsm/ kg): Osmolality was measured using a freezing point Osmomat 3000 that was calibrated prior to use. 50 µl of each fluid sample was placed into the appropriate sample vial using a 20-200 µl Thermoscientific pipette and the osmolality value was recorded.

Quantification and identification of bile salts

A LC-MS/MS method was used based on published literature [12]. Separation of 14 bile acids was achieved using a dual pump Shimadzu LC-20AB Prominence liquid chromatograph equipped with SIL-20A autosampler, a DGU-20A3 vacuum degasser and an Ascentis Express C_{18} column (15 cm x 4.6 mm I.D., 2.7 µm; Sigma Aldrich). A mobile phase program based on (A) 1:1 methanol/water and (B) methanol at a flow rate of 150 µL/min was applied for elution of target analytes. Both solvent's pH were adjusted to 9.0 with 0.1% ammonium hydroxide (25%) and 10 mM/L ammonium acetate. The flow started at 50% B and increased to 100% over 4 minutes, held at 100% B for 5 minutes then reduced to 50% B at 12 minutes.

Mass spectrometric analysis was performed using a Sciex API 2000 triple quadrupole mass spectrometer operated in electrospray negative ionization mode. MS/MS detection operated in the multiple reaction monitoring (MRM) mode was used for quantitative determination based on compound-specific MRM transitions. Full details are provided in Table 3 of the supplementary material.

Sample preparation: A simple protein precipitation method was followed for extraction of all bile acids from gastric and duodenal fluids. An aliquot of 100-250 µL fluid sample was precipitated with 440 µL of acetonitrile:methanol (1:2) solvent containing 10 µL internal standard (IS) (1000 ng/mL) and mixed for 15s on a Vortex Mixer (Fisherbrand, UK). This sample mixture was centrifuged at 14,000 rpm at 10°C for 10 min. Initially the samples were diluted 2 times and then 5, 10, 100, and 200 times depending on the concentrations of each bile salt in the fluid sample. From the diluted supernatant 5 µL was injected onto the LC-MS/MS system for analysis.

A simple protein precipitation extraction technique was sufficient to obtain the best recovery for both the bile acid analytes and internal standards. The results of the comparison of neat standards (methanol: water spiked with bile acids) versus surrogate-matrix extracted standards for all the bile acids and the mean recovery was found to between 95-98% at three concentrations (5, 100 & 2000 ng/ml). The recovery for internal standards at 1000 ng/ml was > 98% in all the recovery samples.

The analysis method was shown to be linear from 2-2000 ng/mL and was capable of accurately and precisely determining bile acid concentrations in GI fluid samples according to the FDA requirements for bioanalytical method validation. Total bile acid concentration in each sample was calculated as the sum of the concentrations of the individual bile acids.

Description of Statistical Methods

Previous work to characterise the gastric and intestinal fluids in adults reported means, medians and range values of pH; buffering capacity; osmolality; viscosity and bile salt concentration [4]. This work aims to characterise the same parameters for paediatric populations and to explore whether the values obtained are statistically similar to those reported in previous studies in both adult and paediatric populations. The differences in mean values between the sub-sets of paediatric populations as well as existing data from adults were compared using ANOVA analysis (with Tukey's post-hoc) to determine any significant differences. Outliers were identified using SPSS, these are presented in figures but were excluded from further analysis (SPSS uses a step of 1.5×IQR (Interquartile range) to identify outliers).

Results and Discussion

Patient demographics

A total of 55 children were recruited to the study ranging in age from 11 months to 15 years old. A total of 53 gastric fluid samples were collected with 2 from infants; 10 from pre-school age children, 20 from school age children and 21 from adolescents. A total of 40 intestinal fluid samples were collected with 2 from neonates-infants; 7 from pre-school age children; 16 from school age children and 15 from adolescents. Demographic data for all participants is provided in Table 2.

Participant ID code	Ethnicity	Age (y)	Height (cm)	Weight (Kg)	Reason for the endoscopy	Final diagnosis following endoscopy	Gastric fluid sample	Duodenal fluid sample
UK001	White British	13	158.6	48.5	Bleeding per rectum	Colonic polyp	Yes	
UK002	Any other ethnic group, not specified	10	142	33.2	Abdominal pain	Normal	Yes	Yes
UK003	White- not specified	13	173	56.2	Diarrhoea + Anaemia	Coeliac Disease	Yes	
UK004	Not specified	2	84.1	11.9	Vomiting	Eosinophilic oesophagitis	Yes	
UK005	Asian/Asian British - Indian	11	151	38	Abdominal pain	Normal	Yes	Yes
UK006	White British	8	134.9	44.3	Vomiting	Normal	Yes	Yes
UK007	White British	6	112.8	21	Vomiting	Normal	Yes	Yes
UK008	White- not specified	2	94.2	14.9	Diarrhoea	Normal	Yes	
UK010	White British	12	155.4	45.3	Abdominal pain + constipation	Normal	Yes	Yes
UK011	Black/Black British - any other black background	14	166.6	57.4	Abdominal pain	Normal	Yes	
UK012	White British	15	154	40.7	Abdominal pain	Crohn's Disease	Yes	
UK013	Asian/Asian British - Pakistani	11	147.5	66.6	History of choking episodes	Normal	Yes	Yes
UK014	Mixed White and Black Caribbean	14	169.9	79.9	Abdominal pain	Normal	Yes	Yes
UK015	White British	14	171	62.8	Abdominal pain + Diarrhoea	Normal	Yes	
UK017	White British	14	152.6	52.5	Dyspepsia	Normal	Yes	Yes
UK018	White British	12	169	60.9	Abdominal pain	Normal	Yes	Yes

UK019	Asian/Asian British	15	163.5	37.8	Abdominal pain, diarrhoea + weight loss	Crohn's Disease	Yes	Yes
UK020	Not specified	3	91.8	15.1	Asymptomatic type 1 diabetic patient, screening for Coeliac Disease	Coeliac Disease	Yes	
UK021	Asian/Asian British - Indian	3	94.9	14.3	Hypothyroidism-routine screening for Coeliac Disease	Coeliac Disease	Yes	Yes
UK022	Asian/Asian British - Pakistani	11	139	22.5	Slow weight gain	Coeliac Disease	Yes	Yes
UK023	Not specified	15	177.1	64.2	Abdominal pain	Normal	Yes	Yes
UK024	White British	11	158	38.2	Abdominal pain	Normal	Yes	Yes
UK025	White British	12	162.9	52.2	Abdominal pain + weight loss	Normal	Yes	Yes
UK026	Not specified	13	150.2	42.1	Abdominal pain	Normal	Yes	Yes
UK027	Asian/Asian British - Indian	7	129	22.5	Difficulty in swallowing	Eosinophilic oesophagitis	Yes	
UK028	White British	12	153.7	47.1	Surveillance endoscopy	Eosinophilic oesophagitis	Yes	
UK029	White British	15	179.2	61.8	Abdominal pain	Normal	Yes	Yes
UK030	Asian/Asian British - Pakistani	10	123	21.2	Protein-losing Enteropathy	Normal	Yes	
UK031	White British	9	136.6	25.9	Abdominal pain	Normal	Yes	Yes
UK032	Asian/Asian British - Pakistani	1	77.5	8.5	Part of work up for stem cell transplant	Normal	Yes	Yes
UK033	Asian/Asian British - Indian	15	155.6	39.8	Inflammatory bowel disease surveillance	Normal	Yes	Yes
UK034	White British	15	167.8	47.4	Slow weight gain	Normal	Yes	Yes
UK035	Asian/Asian British - Pakistani	10	130.1	18.6	Abdominal pain, slow weight gain + constipation	Normal	Yes	Yes
UK036	White British	5	117.4	22.6	Suspected Coeliac Disease	Normal	Yes	Yes
UK037	Mixed White and Black Caribbean	6	131.8	35.9	History of diarrhoea	Normal	Yes	Yes
UK038	Asian/Asian British - Indian	8	134	44.1	Surveillance of Reflux Oesophagitis	Reflux Oesophagitis	Yes	Yes
UK039	White British	12	164	57.5	Surveillance of Eosinophilic Oesophagitis	Helicobacter gastritis. Eosinophilic oesophagitis	Yes	Yes
UK040	Mixed other	12	161.9	41.8	Surveillance of Duodenal ulcer	Duodenal ulcer	Yes	Yes

UK041	White British	12	141.3	29.9	Surveillance of Crohn's Disease	Crohn's Disease	Yes	Yes
UK042	White British	3	99.7	14.4	History of blood in vomit	Normal	Yes	Yes
UK043	Asian/Asian British - Indian	11	144.1	31.8	Suspected Inflammatory Bowel Disease	Ulcerative Colitis	Yes	Yes
UK044	White British	8	119.8	21.9	Suspected Coeliac Disease	Coeliac Disease	Yes	Yes
UK045	White- not specified	14	184	57.6	Abdominal pain	Normal	Yes	Yes
UK046	White British	3	97.1	15.4	Complaints of Diarrhoea	Normal	Yes	Yes
UK047	White British	7	112.6	17.6	Surveillance of Crohn's Disease	Crohn's Disease	Yes	Yes
UK048	White British	6	115.2	19.9	History of diarrhoea	Normal	Yes	Yes
UK049	White British	11 months	65	7.4	Suspected Coeliac Disease	Coeliac Disease	Yes	Yes
UK050	White British	10	129.1	25.5	Suspected Coeliac Disease	Coeliac Disease	Yes	Yes
UK051	White British	10	133.6	28.9	Suspected Coeliac Disease	Normal	Yes	Yes
UK052	Asian/Asian British	6	129	24.1	Surveillance of Ulcerative Colitis	Ulcerative Colitis	Yes	Yes
UK053	White- not specified	4	99.7	16.5	Suspected chronic diarrhoea	Constipation	Yes	Yes
UK054	White British	2	88.3	14.1	Bloody diarrhoea, suspected inflammatory bowel disease	Ulcerative Colitis	Yes	Yes
UK055	Asian/Asian British - Indian	4	109.1	18.2	Abdominal pain, suspected coeliac disease	Coeliac Disease	Yes	Yes
UK056	Asian/Asian British - Pakistani	13 months	82	8.6	Not recorded	Not recorded		Yes
UK057	White British	2	83	10.8	Chronic diarrhoea, suspected Coeliac disease	Coeliac Disease	Yes	Yes
UK058	White British	5	110.7	18.4	Chronic diarrhoea	Normal	Yes	Yes
UK059	White British	2	91.8	13.8	Vomiting	Gastro Oesophageal Reflux disease	Yes	Yes
UK060	White British	5	116.9	20.75	Dyspepsia	Functional dyspepsia	Yes	Yes
UK061	Asian/Asian British - Pakistani	3	100.5	14.5	Bloody diarrhoea	Ulcerative Colitis	Yes	
UK062	White British	4	109	19.3	Not recorded	Not recorded	Yes	Yes

Table 2. Demographics of the participants included in the fluid characterisation study

Analysis (visual review of data plus statistical analysis where sample sizes permitted) was undertaken to explore the impact of ethnicity and final diagnosis following endoscopy on all characterisation parameters and no significant correlations were identified. All data was stratified based on age for subsequent data presentation.

pH of gastric and intestinal fluids

The pH values measured are shown stratified by age in Figure 1 from 53 participants' gastric fluid and 41 participants' intestinal fluid.

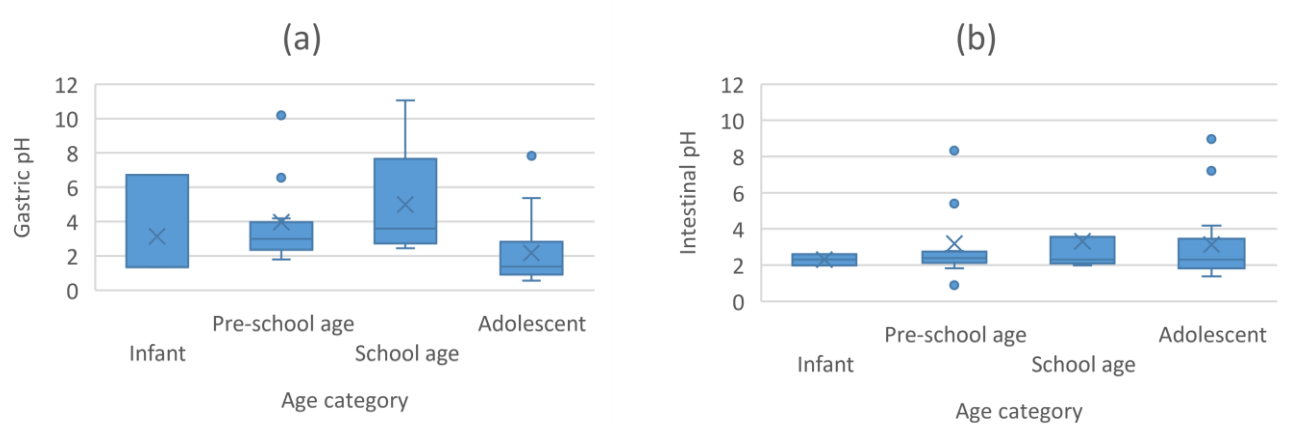


Figure 1. Box and whisker plots showing the pH from (a) gastric and (b) intestinal samples stratified by age group. Boxplots show mean as the x; median as the horizontal line; box as the 1Q and 3Q and the whiskers are the range excluding outliers; outliers are shown as circle datapoints.

As shown in Figure 1 there is a lot of variability in the data where the pH values recorded ranged from pH 0.57 to 11.05 (mean: 3.51) in gastric fluids and from 0.89 to 8.97 (mean: 3.15) in intestinal fluids. Statistically significant differences in pH values were only identified for gastric samples between the school age and adolescent aged sub-groups (p=0.013). Previous reports on pH measurements of aspirated fluids from both paediatric and adult populations have also reported large variability [5, 15]. The median, mean and standard deviation are shown by age in Table 3.

Some of the very high pH values measured may be an artefact of the measuring technique where the pH probe was not measuring a homogenous aqueous liquid and there was some form of physical interference by the other components, for example, solid materials or oils within the aspirated fluid that prevented an accurate measurement. Previous data sets have reported higher than expected pH values within gastric fluids in paediatric populations [15]. However, outliers, which included those with a gastric pH>10, were excluded from subsequent analysis (as described in the methods section).

		Infants	Pre-school Children	School age children	Adolescents
Gastric fluid	Number of samples	3	13	12	18

	Median	1.36	2.77	3.24	1.31
	Mean	3.14	2.78	4.06	1.67
	Standard deviation	3.09	0.61	2.05	1.15
Intestinal fluid	Number of samples	2	13	6	13
	Median	-	2.36	2.29	2.26
	Mean	2.30	2.23	2.49	2.38
	Standard deviation	-	0.48	0.58	0.81

Table 3. Median, mean and standard deviation of the pH of gastric and intestinal samples. Note that outliers were excluded from this analysis.

The gastric pH was higher this study than previously reported in adults and paediatric studies. The samples in the current study were taken during endoscopy where the children were under a general anaesthetic. Under anaesthetic there may be relaxation of the pyloric sphincter and thus mixing of gastric and intestinal fluids which can affect the pH. Depression of protective reflexes during anaesthesia and loss of consciousness has been reported to predispose patients to, duodenal-gastric reflux, specifically those with abdominal pain [40]. This factor may explain the increased mixing between intestinal and gastric fluids within this patient population. It is worth noting that previous studies characterising paediatric gastric fluids obtained the fluid samples via indwelling nasogastric tubes rather than under anaesthetic [15].

The mean values for gastric pH in school age children and adolescents matches previously reported values pH<3 in the literature [15, 41, 42]. Although previous studies [41, 42] have reported that children have gastric pH values of less than 3; Van den Abeele et al reported that 3/35 gastric samples from children were greater than pH4 whereas in our study 11/49 were higher than pH4 thus there was an increased frequency of higher pH values in our study compared to previous data. As stated previously this difference may be linked to the methodology associated with collection of fluids.

Previous literature (shown in Table 4) reported mean or median intestinal fluid pH values in children to range from 6-8 which is higher than the values identified in this study. Our values are also lower than reported values from a review of studies conducted in adults where mean values ranged from 5.7-7.5 [30]. These lower than anticipated values are again likely to be a result of the mixing of gastric and duodenal fluids due to the sampling technique where the participants were anaesthetised prior to the endoscopy. The samples were frozen prior to measurement of pH as immediate measurement was not possible. Previous studies have reported that pH values can drift over time when samples are exposed to laboratory conditions due to transformation of bicarbonates to carbon dioxide which may also contribute to the higher than expected values for the gastric and lower for the intestinal fluids [43].

Reference	**Sample details**	**Intestinal pH value recorded**
[44]	In situ pH measurements from 34 children <4 years old	Mean pH of 6.6 Range 5.6-7.3

[45]	Intestinal aspirates from 35 children (8 days – 19 years) In a subset of fasted children (n=25)	Mean pH of 6.8 Range 4.5-12.0 Mean pH of 7.0 Range 5.5-7.6
[46]	Intestinal aspirates from 7 children (0-14 years)	Mean pH 7.5 Range 5.9-8.2
[47]	Intestinal aspirates from 2 infants	Mean pH 7.8 Range 7.39-8.20
[41]	In situ pH measurements from 12 children aged 8-14 years	Mean value of 6.4 (duodenum)

Table 4. Reported intestinal pH values from studies conducted in children

Buffer Capacity

Buffer capacity measurements were obtained from 52 gastric and 38 intestinal fluids and the results are shown in Figure 2. The measured buffer capacity (mmol/L/ΔpH) ranged from 0-189 and 5-150 for gastric and intestinal fluids respectively. There was no effect of age upon buffer capacity, although higher gastric buffer capacity values were recorded for older children.

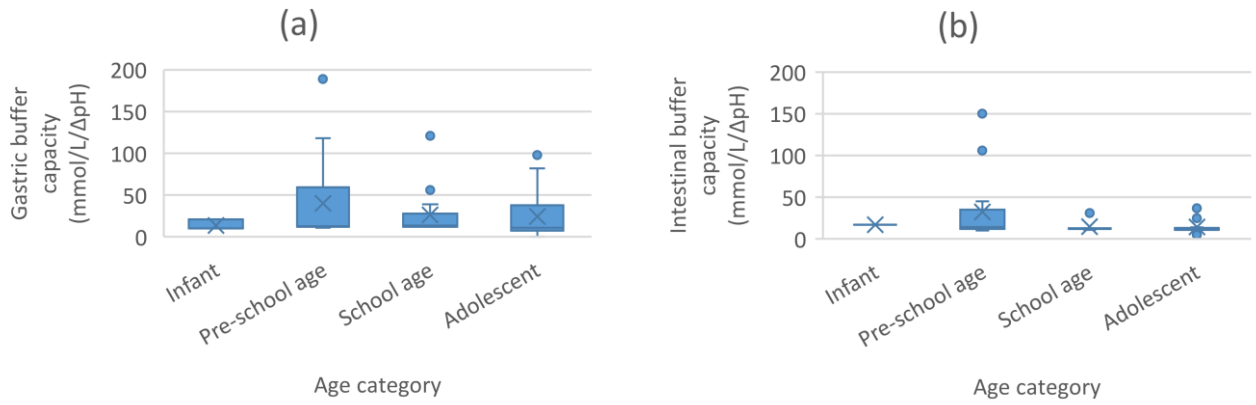


Figure 2. Box and whisker plots showing the buffer capacity (mmol/L/ΔpH) for (a) gastric and (b) intestinal samples by age group. Boxplots show mean as the x; median as the horizontal line; box as the 1Q and 3Q and the whiskers are the range excluding outliers; outliers are shown as circles.

Following removal of outliers the median, mean and standard deviation were calculated and are presented in Table 5. No statistically significant differences were found in buffer capacity based on age sub groups.

	Infants	**Pre-school Children**	**School age children**	**Adolescents**

Gastric fluid	Number of samples	3	16	14	15
	Median	10.00	13.00	13.50	10.00
	Mean	13.67	39.94	26.29	11.71
	Standard deviation	6.35	52.61	30.19	11.12
Intestinal fluid	Number of samples	1	14	5	11
	Median	17.00	13.00	12.00	12.00
	Mean	17.00	18.57	12.20	12.09
	Standard deviation	-	10.86	0.45	0.94

Table 5. Median, mean and standard deviation of the buffer capacity of gastric and intestinal samples. Note that outliers were excluded from this analysis.

The buffer capacity did not change significantly between gastric and intestinal fluids, this could be attributed to the fact that pylorus sphincter is relaxed due to anaesthesia causing the gastric and upper intestinal contents to be mixed.

The buffer capacity of intestinal fluids is of interest as changes in the pH can have dramatic effects on API solubility, thus low buffer capacity can indicate a risk of a change in the pH due to dissolution of an API or excipients. Previous literature has reported buffer capacity values for fasted adult human intestinal fluids ranging from 2.5-13 mM/L/ΔpH [30, 43]. Higher buffer capacity has been reported in the fed state and also following ingestion of water [43]. No data has been identified that reports the buffer capacity of paediatric fluids. The existing proposed paediatric simulated intestinal fluids have a buffer capacity of 10 mM/L/ΔpH [25] which matches the median values from our data as well as (the buffer capacity of adult FaSSIF which is 12 mM/L/ΔpH) [48].

Osmolality

Osmolality measurements were obtained from 52 gastric and 41 intestinal fluids and the results are shown in Figure 3.

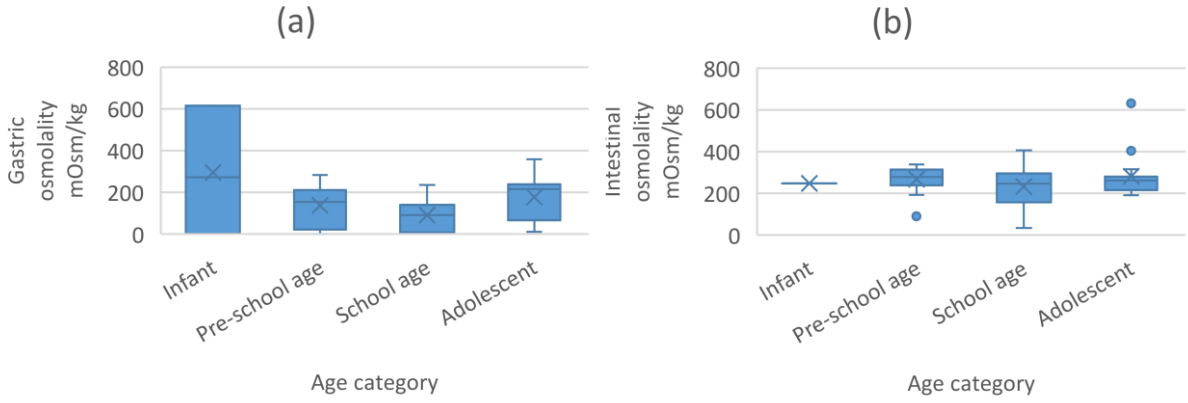


Figure 3. Box and whisker plots showing the osmolality for (a) gastric and (b) intestinal samples by age group. Boxplots show mean as the x; median as the horizontal line; box as the 1Q and 3Q and the whiskers are the range excluding outliers; outliers are shown as circles

The data generated in this study showed that gastric fluid osmolality values ranged from 1 to 615 mOsm/kg, while intestinal fluid values ranged from 35 to 631 mOsm/kg. Outliers, as identified in Figure 3 were excluded prior to statistical analysis and the median, mean and standard deviation for each age group are shown in Table 6. Statistically significant differences were observed between the gastric osmolality in infants compared to that in school age children, however the variability was large for infants and the sample size small thus caution is required with the interpretation of this data.

		Infants	**Pre-school Children**	**School age children**	**Adolescents**
Gastric fluid	Number of samples	3	16	14	20
	Median	272.00	154.50	91.00	216.00
	Mean	296.33	137.50	91.79	176.75
	Standard deviation	307.22	96.65	71.79	100.87
Intestinal fluid	Number of samples	2	15	5	14
	Median	-	283.00	246.00	260.50
	Mean	248.00	280.47	237.60	248.29
	Standard deviation	-	42.24	51.22	36.58

Table 6. Median, mean and standard deviation of the osmolality of gastric and intestinal samples. Note that outliers were excluded from this analysis.

There is limited data available on osmolality in paediatric populations. Previous reports from aspirated gastric fluids from children include mean values of 253 mOsm/L from a sample of 40 children under 2 years of age [49] and median values of 274 mOsm/kg for neonates; 188 mOsm/kg for children aged 2-12 years and 219 mOsm/kg for adolescents (12-18 years) [15]. Note that some studies reported osmolarity (per litre) whereas others report osmolality (per kg), these units can be interchanged if we assume that the density of the carrier fluid is 1kg/L. Thus the data obtained in our study shows relatively low values for gastric osmolality in comparison with previous studies. The presence of food components or digested food is likely to have a major effect on the osmolality of gastric fluids and is acknowledged as a potential limitation by other similar studies [15], our samples were from fasted patients which may explain our lower values.

Published data on osmolality of intestinal fluids in children was not identified, however, previous literature has reported osmolality values of 137-299 mOsm/kg in aspirated intestinal fluid from adults [30]. Thus, the osmolality values measured within this study are within the range previously reported for adults.

The osmolality of the current paediatric fasted state simulated gastric fluid proposed by [25] is 120.7 mOsm/kg and our mean data (stratified by age) ranges from 91-216 mOsm/kg which encompasses

this value. However, paediatric fasted state simulated intestinal fluid has an osmolality of 180 mOsm/kg which is somewhat lower than the values measured in our study [25].

Quantification of bile acids

Bile acid concentrations were determined from 47 samples; both gastric and intestinal samples were bile acids from gastric and intestinal fluids by age c summary data shown in Table 7.

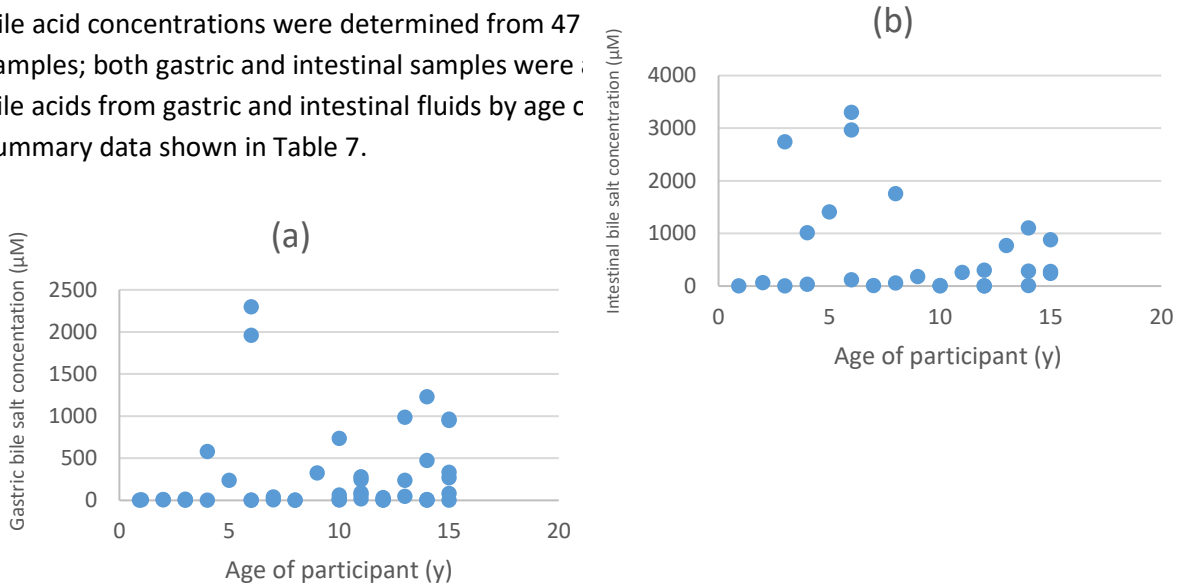


Figure 4. Total bile salt concentration (µM) plotted versus the age of the participant in the (a) gastric and (b) intestinal fluids.

A summary table of the median; mean and standard deviation of the bile salt concentrations stratified by age is shown in Table 7.

		Infants	Pre-school Children	School age children	Adolescents
Gastric fluid	Number of samples	2	8	19	18
	Median	-	9.59	40.23	65.69
	Mean	4.65	108.25	325.24	314.11
	Standard deviation	2.07	207.66	663.87	420.76
Intestinal fluid	Number of samples	1	6	10	10
	Median	0.81	537.37	149.80	277.34
	Mean	0.81	876.81	866.18	386.26
	Standard deviation	-	1088.88	1308.14	393.84

Table 7. Median, mean and standard deviation of the bile salt concentration (µM) of gastric and intestinal samples.

The quantitative analysis of bile salts in both gastric and duodenal fluids (Figure 4) showed large variability among subjects and the summary of median, mean and standard deviation are presented in Table 7. The outliers were not excluded in this analysis as the variability as well as mean data is of interest for this population. These variabilities could be due to the presence of solid remnants in

aspirated fluids. Some of the gastric samples collected were found to be transparent fluid and some contained suspended greenish floccules. It was observed that typically transparent fluids showed lower bile acid concentrations although this was not statistically significant. The total concentration of bile acids in gastric fluid ranged from 0.002-2.3 mM and in intestinal fluid from 0.0008-3.3 mM. These ranges are similar for those previously reported where large variability was also reported (see data in Table 1). However, the mean values are much lower than values in previous studies where mean values were typically greater than 2 mM (see data and references from Table 1). This lower mean value may have implications for the solubilising capacity in paediatric populations as the critical micelle concentration for bile is often reported to be greater than 2mM [50].

There was no statistically significant differences in the bile acid concentrations with the age of the participants although there was a trend towards an increased mean concentration in the gastric fluid with increasing age. The large variability and small age-stratified sample sizes from intestinal fluids limit the interpretation of the data. Higher mean and median bile acid concentrations were found in the intestinal samples compared to the gastric samples. However, as stated previously there may be some mixing of these fluids during the endoscopy thus it was not appropriate to pool samples from the same individual.

The concentration of bile is typically lower in the stomach compared to the small intestine, as reflected in the concentration of bile salts in FaSSGF at 0.08mM whilst the concentration in FaSSIF is 3mM [48]. Proposed paediatric fasted state simulated gastric fluids reported a bile salt concentration of 0.02mM for neonates and 0.06mM for infants which is higher than that reported in our work yet still lower than that in adults which is supported by our data [25]. Due to the large variability in reported data two bile salt concentrations were proposed for paediatric FaSSIF to account for a minimum and maximum. These were 1.5mM and 4.5mM [25]. However, the bile acid concentrations measured in intestinal fluid in this study are lower and <1mM in the majority of samples.

The relative contribution of individual bile acids to the total bile concentration was determined and the data are shown by individual sample in ascending order of age in Figure 5. No trends were identified that linked bile acid concentration to age, thus the data were pooled for gastric and intestinal samples to determine the median, mean and standard deviation of each component identified, the data are presented in Table 8.

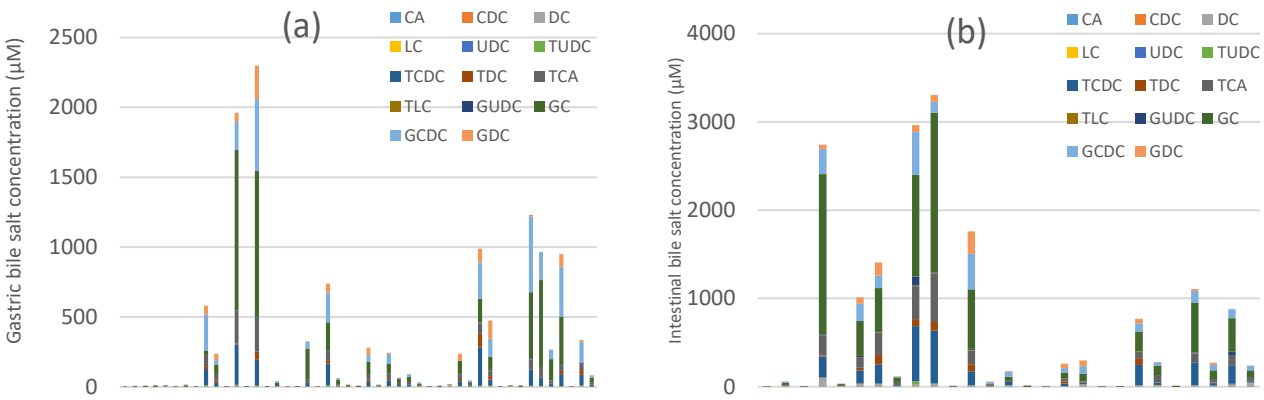


Figure 5. Relative contribution of bile salts to the total bile salt concentration in (a) gastric and (b) intestinal individual samples ordered from youngest to oldest within each population.

		Concentration (µM)													
		Primary bile acids		Secondary bile acids			Taurine Conjugated bile acids					Glycine Conjugated bile acids			
		CA	CDC	DC	LC	UDC	TUDC	TCDC	TDC	TC	TLC	GUDC	GC	GCDC	GDC
Gastric fluid (n=47 samples)	Median	0.125	1.549	1.783	0.510	0.000	0.000	1.041	0.146	1.671	0.032	0.075	5.660	3.449	0.582
	Mean	0.148	1.729	1.918	0.576	0.004	0.937	34.547	7.623	26.283	0.417	0.945	107.006	68.056	20.402
	Standard deviation	0.166	0.745	0.767	0.276	0.029	1.918	67.976	17.643	50.879	1.053	2.317	246.538	130.247	44.797
	Number where BS was present	45	47	47	47	2	23	46	37	47	26	43	46	47	39
Intestinal fluid (n = 27 samples)	Median	0.138	0.167	13.434	0.025	0.844	0.334	19.248	1.473	23.085	0.135	0.907	66.860	48.150	3.194
	Mean	0.307	0.291	15.947	0.033	0.929	2.749	105.934	19.644	81.860	1.380	7.814	299.974	87.519	34.253
	Standard deviation	0.518	0.266	19.994	0.028	0.993	6.838	170.549	34.789	131.602	2.291	20.865	514.438	124.453	57.432
	Number where BS was present	21	27	27	27	22	20	27	24	27	25	25	27	27	24

Table 8. Median, mean and standard deviation of the concentrations (µM) of bile acids present in the gastric or intestinal fluid samples from children.

In both gastric and intestinal fluids the most abundant bile acids were glycine conjugated with glycocholate being the most abundant. Primary bile acids were detected in almost all gastric samples as well as glycine conjugated bile acids. UDC was only present in 2/47 gastric fluid samples and TUDC and TLC were present in approximately half of the gastric samples. The order of magnitude (based on mean values) for the presence of bile acids in gastric fluid was GC> GCDC> TCDC> TC> GDC> TDC> DC> CDC> GUDC> TUDC> LC> TLC> CA> UDC. This relative order matches well to the order previously reported by Riethorst et al (2016) based on adult gastric fluids [12]. There are some differences with the relative order reported by Van den Abeele et al (2018) yet this may be due to the difference in the ages within the paediatric populations or due to some contamination of gastric samples with intestinal fluids due to the method of sample collection [15].

Most bile acids were detected in the 27 intestinal fluid samples analysed. The taurine conjugated bile acids were present in higher levels compared to within the gastric samples. The order of magnitude (based on mean values) for the presence of bile acids in intestinal fluid was GC> TCDC> GCDC> TC> GDC> TDC> DC> GUDC> TUDC> UDC> CA> CDC> LC. Previous literature (highlighted in Table 1) showed similarities to the relative concentrations found in these samples where GC; TC; GCDC and TCDC are the most commonly identified bile acids in paediatric intestinal fluids.

Further work is required to generate understanding on the implications of the range of bile salts present and their relative concentrations on the solubilisation of APIs. Specific work will investigate the impact of bile salt concentration and composition identified within this study on the solubility of a series of drugs previously investigated in adult human intestinal fluids [8, 51] to better understand how solubility may differ in a paediatric population compared to an adult population. Furthermore, the data will be used to drive the development of paediatric relevant fasted state simulated intestinal fluid to integrate into paediatric biopharmaceutics toolkits.

General Discussion

The data generated from the paediatric gastro-intestinal samples showed large inter-individual variability in all parameters characterised. There were no trends identified when the data was interrogated based on the age, ethnicity or disease-state of the participant. The lack of trends identified may have been masked by the variability observed. The similarities in the properties of the gastric and intestinal fluids suggests mixing of these fluids during the endoscopy procedure as this was conducted under anaesthetic. Characterisation from indwelling naso-gastric or naso-duodenal tubes would provide a cleaner data set and will be the target for future research.

However, the variability associated with gastro-intestinal fluids needs is an important finding as this can affect the solubility of drugs within a population. For example, recent work has highlighted that variability in bile acid metabolism as a result of gut microbiota can affect the solubility of a series of drugs [52]. Thus, it is prudent to develop a suite of biorelevant media for a paediatric population to reflect this diversity and better understand the potential variability associated with solubility in vivo based on differences in the gastro-intestinal environment. The use of the median data to develop a mid-point fluid will provide a single point estimate for solubility whereas this in conjunction with extreme variants will provide understanding on the potential sensitivity to solubility within a highly variable paediatric population.

Conclusions

This work provides a comprehensive characterisation of gastric and intestinal fluids from a paediatric population. This provides a useful data set to generate simulated media to represent paediatric populations and to compare to existing simulated fluids based on adult data. The differences noted between paediatric and adult fluids justifies the need for additional experimental research to better understand the implications of these differences on drug solubility. It should be noted that there was large variability within the samples and that there was likely to be mixing of gastric and intestinal fluids. Caution is required for interpretation of the data as the mean values do not represent any single individual, therefore media that represent the extreme individual samples as well as the mean are likely to provide greater insights into the impact of fluid attributes on API solubility and dissolution in paediatric populations.

Acknowledgements

The authors would like to acknowledge all clinical staff involved in the study for their efforts in enrolling patients and collecting samples leading to the generation of the dataset.

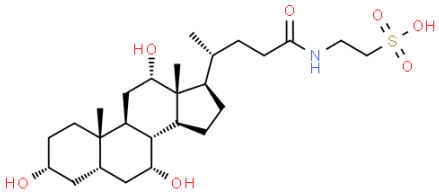
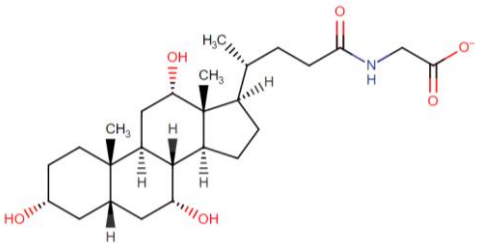
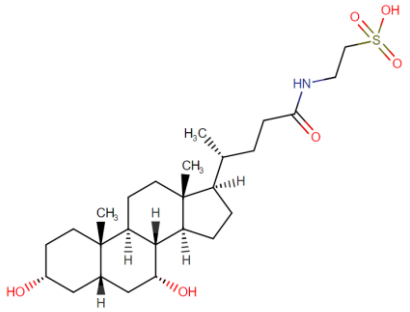
Funding: This work was supported by Janssen Research and Development, Belgium.

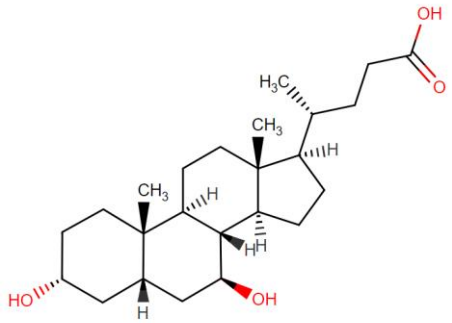
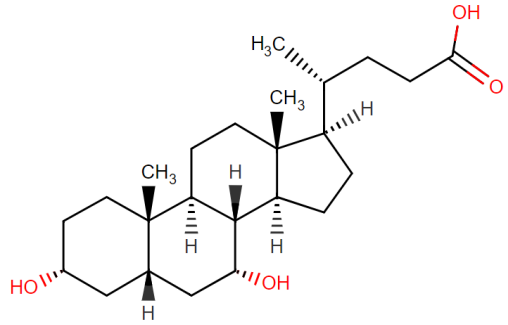
Supplementary Information

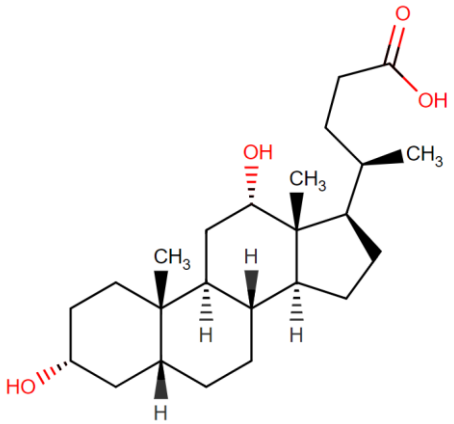
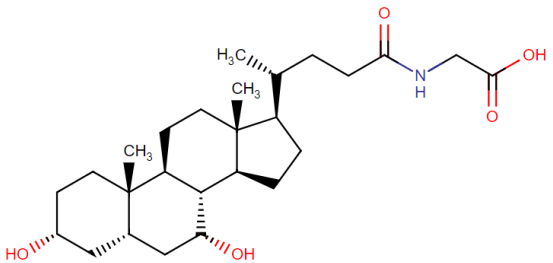
Supplementary Table 1. Summary of cohort details from studies reported where fasted gastrointestinal fluid was collected for characterisation

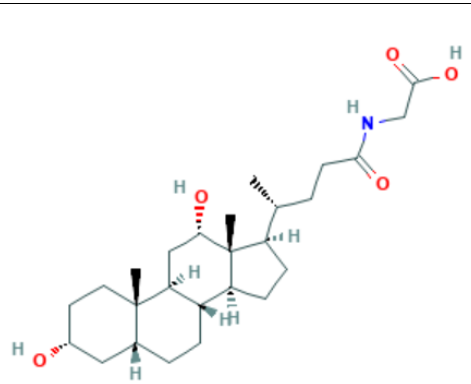
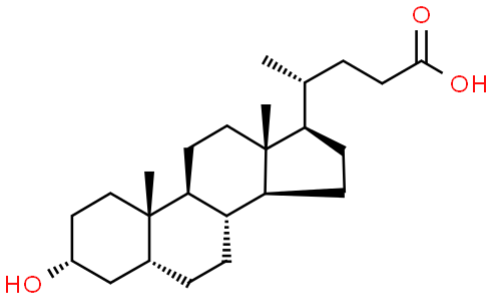
Reference	Population	Fluids characterised	Relevant parameters measured	Co-administration of water
[4]	24 adult healthy volunteers (19-37 years)	Fasted gastric and jejunal fluid	Bile salt content, pH, osmolality	None
[5]	9 healthy volunteers (age not reported)	Fasted jejunal fluid	Bile salt content, pH, osmolality	None
[6]	12 healthy volunteers aged 24-40	Fasted jejunum	Bile salt content, pH, buffer capacity	None
[9]	6 adult volunteers (22-35 years)	Fasted duodenal and jejunal fluid	Bile salt content, pH, osmolality, buffer capacity	None
[7]	5 adult healthy volunteers	Fasted duodenal fluid	Bile salt content, pH, osmolality	None
[8]	5 healthy volunteers (24-39 years)	Fasted duodenal fluid	Bile salt content, pH, osmolality	Sampling followed 15 minutes post ingestion of 250mL water
[11]	4 healthy volunteers (19-35 years)	Fasted duodenal fluid	Bile salt content, pH, osmolality, viscosity	Sampling followed ingestion of 200mL water
[10]	Adult patients undergoing thoracic surgery	Fasted gastric fluid	Bile salt content	None
[12]	20 adult healthy volunteers (18-31 years)	Fasted duodenal fluid	Bile salt content, pH	250mL water administered prior to sampling
[15]	Paediatric (0-18 years)	Fasted gastric fluid	Bile salt content, pH, osmolality	None

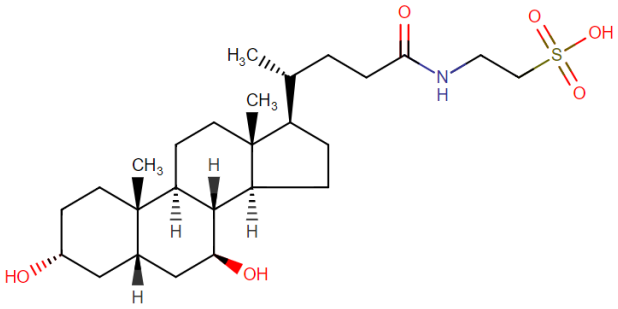
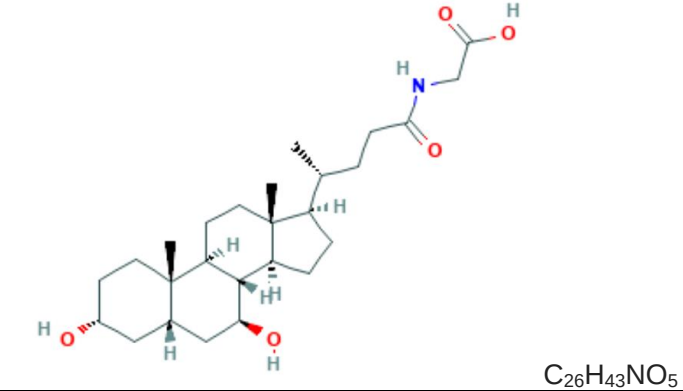
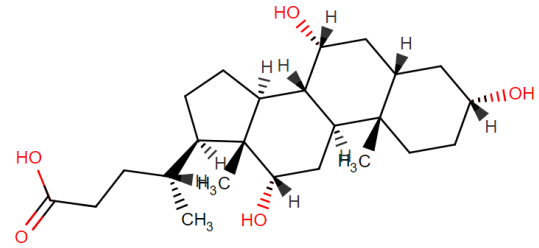
Supplementary Table 2. Structure and properties of the bile acid standard used in the analysis.

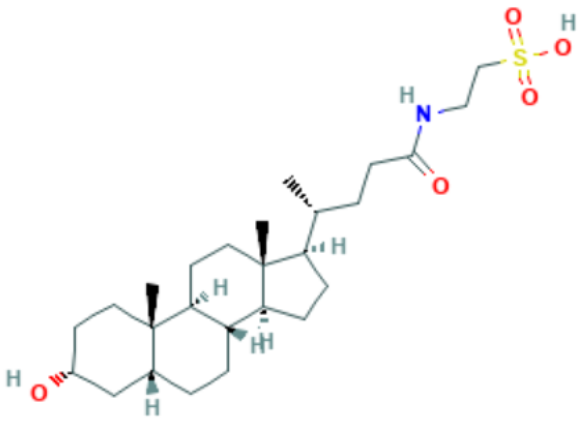
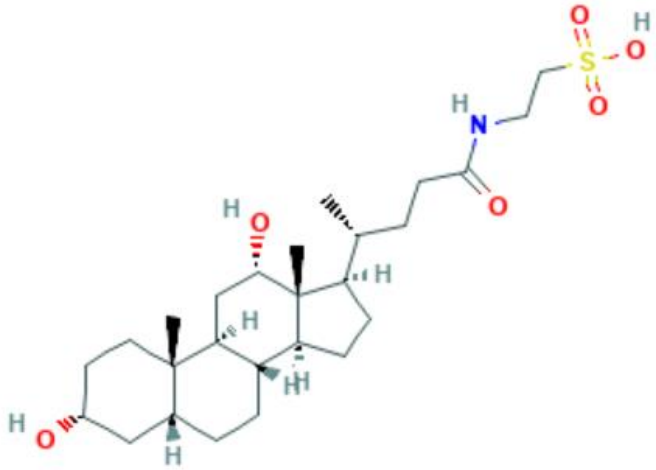
Bile salt	Chemical structure	Molecular weight (g/mol)	LogP	Water solubility	Purchased	% purity; Mol Wt with salt form
Taurocholic acid (TCA)	 $C_{26}H_{45}NO_7S$	515.703	0.79 (ALOGPS)	0.0771 mg/ml (ALOGPS)	Taurocholic acid, sodium salt hydrate, 98%, ACROS Organics (CAS No.-345909-26-4)	98% 555.703
Glycocholic acid (GC)	 $C_{26}H_{42}NO_6$	464.624	1.65	3.3 mg/L (at 20 °C)	Sodium glycocholate Hydrate Sigma-Aldrich (CAS No.-338950-81-5)	≥95% (TLC) 487.60 (anhydrous basis)
Taurocheno deoxycholic acid (TCDC)	 $C_{26}H_{45}NO_6S$	499.704	1.38 (ALOGPS)	0.00748 mg/ml (ALOGPS)	Sodium taurochenodeoxycholate Sigma-Aldrich (CAS No.-6009-98-9)	≥97.0% (TLC) 521.69

Ursodeoxycholic acid (UDC)	 $C_{24}H_{40}O_4$	392.572	3.00	20 mg/L (at 20 °C)	Sigma-Aldrich (CAS No.-128-13-2)	≥99%
Chenodeoxycholic acid (CDC)	 $C_{24}H_{40}O_4$	392.572	4.15	89.9 mg/L (at 20 °C)	Sigma-Aldrich (CAS No.-474-25-9)	≥96%

Deoxycholic acid (DC)	 $C_{24}H_{40}O_4$	392.572	3.50	43.6 mg/L (at 20 °C)	Sigma-Aldrich (CAS No.-474-25-9)	≥98%
Glycochenodeoxycholic acid (GCDC)	 $C_{26}H_{43}NO_5$	449.6233	2.12	3.15 mg/L (at 20 °C)	Sodium glycochenodeoxycholate Sigma-Aldrich (CAS No.-16564-43-5)	≥97% (HPLC) 471.61

Glycodeoxycholic acid (GDC)	 $C_{26}H_{43}NO_5$	449.6	4.3	Water Solubility at 25 deg C (mg/L): 17.95	Sodium glycocholate hydrate Sigma-Aldrich (CAS No.-338950-81-5)	≥95% (TLC) 487.60 (anhydrous basis)
Lithocholic acid (LC)	 $C_{24}H_{40}O_3$	376.573	8.263	Water <1 mg/ml	Sigma-Aldrich (CAS No.-434-13-9)	≥95%

Tauroursodeoxycholic acid (TUDC)	 $C_{26}H_{45}NO_6S$	499.7	1.38	0.00748 mg/ml	Tauroursodeoxycholic Acid, Sodium Salt Sigma-Aldrich (CAS No- 1180-95-6)	≥95% 521.69
Glycoursodeoxy cholic acid (GUDC)	 $C_{26}H_{43}NO_5$	449.6	4.3 (XLogP3)	0.00135 mg/ml	Sigma-Aldrich (CAS No.-64480-66-6)	≥96.0% (TLC)
Cholic acid (CA)	 $C_{24}H_{40}O_5$	408.5714	-3.37	175 mg/L (at 20 °C)	Sigma-Aldrich (CAS No.-81-25-4)	≥98%

Taurolithocholic acid (TLC)		483.7	4.9 (XLogP3)	25 mg/ml	Sodium taurolithocholate (CAS No.-6042-32-6)	≥97.0% (TLC)
Taurodeoxycholic acid (TDC)		499.7	3.6 (XLogP3-AA)	41 mg/ml	Sodium taurodeoxycholate hydrate (CAS No- 207737-97-1)	≥95% (HPLC) 521.69 (anhydrous basis)

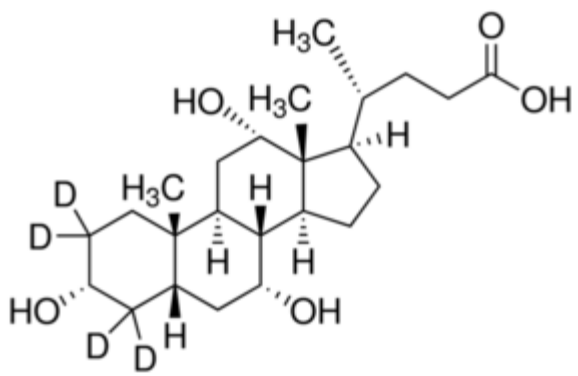
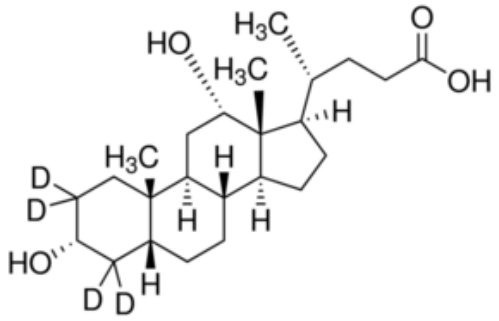
Cholic acid-D4 (Internal standard)		412.60			Sigma Aldrich (CAS No.-116380-66-6) 100 µg/mL in methanol	98 atom % D, 98% (CP)
Deoxycholic acid-D4 (Internal standard)		396.60			Sigma Aldrich (CAS No.-112076-61-6) 100 µg/mL in methanol	≥98 atom % D, ≥98% (CP)

Table 3: Optimised MS/MS parameters for the analysis of bile acids

Instrument- LC-MS/MS API 2000

Method development: The parameters below in table were obtained using direct infusion experiments of the target compounds (1000 ng/µL each, in MeOH: H20) into the MS/MS system via a built-in Harvard syringe pump at a flow rate of 10 µL/min

	TCA	**GC**	**TCDC**	**UDC**	**CDC**	**DC**	**GCDC**	**GDC**	**LC**	**D4C (IS-1)**
Ionization	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Ion spray voltage (V)	-4500	-4500	-4500	-4500	-4500	-4500	-4500	-4500	-4500	-4500
Curtain gas (a.u.)	35	35	35	35	35	35	35	35	35	35
Temperature (°C)	275	275	275	275	275	275	275	275	275	275
C.A.D gas (a.u.)	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Declustering potential (V)	-70	-50	-50	-55	-40	-50	-50	-50	-40	-50
Focusing potential (V)	-365	-365	-365	-365	-365	-365	-365	-365	-365	-365
Collision energy (eV)	-110	-70	-120	-20	-20	-30	-70	-70	-30	-30
Cell entrance potential (V)	-10	-10	-10	-10	-10	-10	-10	-10	-10	-10
Cell exit potential (V)	-6.0	-6.0	-6.0	-6.0	-6.0	-4.0	-6.0	-6.0	-6.0	-6.0
MRM→Frag. m/z	514.2→80	464.3→74	498.3→80	391.3→391.3	391.3→391.3	391.3→391.3	448.3→74	448.3→74	375.3→375.3	411.2→411.2

	TUDC	GUDC	CA	TLC	TDC	D4-DC
Ionization	Negative	Negative	Negative	Negative	Negative	Negative
Ion spray voltage (V)	-4500	-4500	-4500	-4500	-4500	-4500
Curtain gas (a.u.)	35	35	35	35	35	35
Temperature (°C)	275	275	275	275	275	275
C.A.D gas (a.u.)	7.0	7.0	7.0	7.0	7.0	7.0
Declustering potential (V)	-50	-50	-50	-50	-40	-50
Focusing potential (V)	-365	-365	-365	-365	-365	-365
Collision energy (eV)	-110	-70	-30	-108	-116	-30
Cell entrance potential (V)	-10	-10	-10	-10	-10	-10
Cell exit potential (V)	-6.0	-6.0	-6.0	-6.0	-6.0	-6.0
MRM→Frag. m/z	498.3→80.0	448.3→74	407.3→407.3	482.2→80.0	498.3→80.0	395.3→395.3

a.u. – arbitrary units; C.A.D – collisional activated dissociation; TC- Taurocholic acid; GC-Glycocholic acid; TCDC- Taurochenodeoxycholic acid; UDC-Ursodeoxycholic acid; CDC- Chenodeoxycholic acid; DC- Deoxycholic acid; GCDC- Glycochenodeoxycholic acid; GDC- Glycodeoxycholic acid; LC- Lithocholic acid; D4C- Deuterated Cholic acid (Cholic-2,2,4,4-d4); TUDC- Tauroursodeoxycholic acid; GUDC-Glycoursodeoxy cholic acid; CA-Cholic acid; TLC-Taurolithocholic acid; TDC- Taurodeoxycholic acid; D4-DC- Deuterated Deoxycholic acid; IS- Internal standard; MRM-Multi reaction monitoring; m/z-mass/charge number

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