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2 Assessment of Age-Related Changes in Pediatric Gastrointestinal Solubility

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21   Abstract:

22   Purpose: Compound solubility serves as a surrogate indicator of oral biopharmaceutical performance.

23   Between infancy and adulthood, marked compositional changes in gastrointestinal (GI) fluids occur. This

24   study serves to assess how developmental changes in GI fluid composition affects compound solubility.

25   Methods: Solubility assessments were conducted *in vitro* using biorelevant media reflective of age-

26   specific pediatric cohorts (i.e. neonates and infants). Previously published adult media (i.e. FaSSGF,

27   FeSSGF, FaSSIF.v2, and FeSSIF.v2) were employed as references for pediatric media development.

28   Investigations assessing age-specific changes in GI fluid parameters (i.e. pepsin, bile acids, pH,

29   osmolality, etc.) were collected from the literature and served to define the composition of neonatal

30   and infant media. Solubility assessments at 37°C were conducted for seven BCS Class II compounds

31   within the developed pediatric and reference adult media.

32   Results: For six of the seven compounds investigated, solubility fell outside an 80-125% range from adult

33   values in at least one of the developed pediatric media. This result indicates a potential for age-related

34   alterations in oral drug performance, especially for compounds whose absorption is delimited by

35   solubility (i.e. BCS Class II).

36   Conclusion: Developmental changes in GI fluid composition can result in relevant discrepancies in

37   luminal compound solubility between children and adults.

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42   Abbreviations:

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44   IVIVC – *in vitro – in vivo* correlations

45   GI – gastrointestinal

46   P-BCS – pediatric biopharmaceutics classification systems

47   BCS - biopharmaceutics classification systems

48   FaSSGF- fasted-state simulated gastric fluid

49   FeSSGF- fed-state simulated gastric fluid

50   FaSSIF- fasted-state simulated intestinal fluid

51   FeSSIF- fed-state simulated intestinal fluid

52   US-FDA - United States Food and Drug Administration

53   NaTc – sodium taurocholate

54   PNA – postnatal age

55   GA – gestational age

56   NEC – necrotizing enterocolitis

57   FFA – free fatty acids

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60   Introduction:

61   The use of *in vitro* tests to forecast oral drug performance can serve to identify compounds

62   displaying inadequate or unfavorable absorption profiles during early stages of drug development. To

63   facilitate such *in vitro* – *in vivo* correlations (IVIVC), test media utilized should reflect the complex

64   physiochemical nature of human gastrointestinal (GI) fluids. Accordingly, several formulas of biorelevant

65   media have been developed based on the intraluminal conditions of the GI tract in adults (1-3). For

66   immediate release dosage forms, where drug release is expected to occur within the upper region of the

67   GI tract, biorelevant media depicting the stomach and proximal small intestine are typically formulated.

68   Compared to compendial media, use of biorelevant media within *in vitro* dissolution experiments

69   has been demonstrated to provide IVIVC that better predict oral drug absorption in adults (4, 5). Despite

70   these favorable results, the use of biorelevant media for establishing IVIVC within pediatric populations is

71   contentious. This is because contemporary biorelevant media (1, 2) are formulated based on

72   gastrointestinal conditions of an adult human. Consequently, their applicability towards pediatric

73   populations, who are developmentally distinct in terms of gastrointestinal anatomy/physiology, remains

74   questionable. Of most interest are children belonging to the youngest age groups (i.e. neonates and

75   infants) who display the greatest developmental differences in comparison to adults (6, 7).

76   In recognition of the potential impact that developmental differences in GI anatomy/physiology

77   can exert on oral drug absorption, a Pediatric Biopharmaceutics Classification System (P-BCS) Working

78   Group was assembled to assess whether a similar classification system as utilized in adults could be

79   developed for children (8). The Biopharmaceutics Classification System (BCS) categorizes drugs based on

80   two properties, aqueous solubility and permeability (9). Accordingly, compounds can be classified as

81   either BCS I (high solubility, high permeability), II (low solubility, high permeability), III (high solubility, low

82   permeability), or IV (low solubility, low permeability). The classification system supports several aspects

83   of oral drug development in adults, including assessment of generic biowaiver applicability, lead

84   compound selection, and formulation development (8). Based on their findings in a 2012 publication, the

85   P-BCS Working Group concluded that in order to have merit, substantial knowledge gaps with regards to

86   pediatric GI physiology, intestinal permeability, and ontogeny of drug metabolizing enzymes/transporters

87   needs to be addressed prior to establishing of a pediatric-focused BCS (8). To enhance its applicability

88   towards pediatric populations, it is clear that development of a P-BCS would require considerable

89   modification of the current system. Of interest is how developmental changes in GI fluid composition

90   affects compound solubility in relation to adults.

91   In addition to age-related changes, the composition of GI luminal fluids undergoes positional

92   changes from the stomach to the colon. Changes in composition including bile salt concentration, pH,

93   osmolality, buffer capacity, and presence of fat digestion products, can impart changes in compound

94   specific solubility (10). Therefore, to discern whether relevant differences in luminal solubility exist

95   between pediatrics and adults, quantification of the relationship between age and GI fluid composition is

96   inherently required.

97   This study serves to assess the impacts of growth and maturation on gastrointestinal solubility.

98   Pediatric biorelevant media representative of the stomach and proximal small intestine were developed

99   based on an assessment of the available literature. Developed pediatric media were utilized to perform

100   solubility assessments for seven BCS Class II compounds. To assess the impact of developmental changes

101   in fluid composition, solubility values were compared between the different age-specific media including

102   media representative of adults.

103   Methods:

104   (i)Materials:

105   Acetic acid (>99.7%), acetonitrile, dapsone, fenofibrate, indomethacin, hydrochloric acid 36.5-

106   38%, methanol, pepsin (from porcine), phenytoin acid and sodium oleate were obtained from Sigma-

107   Aldrich Company Ltd., Dorset, England. Griseofulvin, maleic acid, sodium acetate, sodium chloride, sodium

108   hydroxide, orthophosphoric acid and spironolactone were acquired from Fisher Scientific UK Ltd.,

109   Loughborough, England. Ammonium acetate (FSA Laboratory Supplies, Loughborough, UK),

110   carbamazepine (Fagron UK Ltd, Newcastle upon Tyne, England), sodium taurocholate (Prodotti Chimici

111   Alimentari S.P.A., Basaluzzo, Italy), egg lecithin - Lipoid EPCS (Lipoid GmbH, Ludwigshafen, Germany) and

112   glyceryl monooleate - Rylo Mg 19 (Danisco, Brabrand, Denmark) were obtained from the sources

113   specified. Ultra-high-temperature treated whole cow's milk standardized to less than 4% fat was acquired

114   from Sainsbury's, London, England. Two infant formulas manufactured by Cow & Gate, Trowbridge,

115   England were utilized in the study: First Infant Milk (cow's milk-based formula) and Infasoy (soya-based

116   formula). Water was ultra-pure (Milli-Q) laboratory grade. Dialysis tubing (12-14000 Da MWCO) was

117   acquired from Medicell Membranes Ltd., London, England. Equipment utilized in the current investigation

118   included a Buchi R114 Rotavapor (Flawil, Switzerland), a Beckman Coulter J2-MC centrifuge (High

119   Wycombe, England), a Mettler Toledo SevenCompact S210 pH meter (Schwerzenbach, Switzerland), an

120   Advanced Instruments Inc. micro-osmometer Model 3300 (Norwood, MA) and an Agilent Technologies

121   1200 series HPLC system (Santa Clara, CA): binary pump (G1212A), autosampler (G1329A), thermostatted

122   column compartment (G1316A), and diode array detector (G1315D).

123

124   (ii)Media development:

125   Biorelevant media as characterized by Jantratid et al. (2) were selected as the focal points from

126   which subsequent age-specific media were developed. The authors described four separate media

127   reflective of the physiology of the stomach and proximal small intestine in adults in fasting and fed states:

128   Fasted-State Simulated Gastric Fluid (FaSSGF), Fed-State Simulated Gastric Fluid (FeSSGF), Fasted-State

129   Simulated Intestinal Fluid v2 (FaSSIF.v2), and Fed-State Simulated Intestinal Fluid v2 (FeSSIF.v2) (Table I).

130   Based on relative differences between adult and pediatric GI physiology, components of the reference

131   adult media were modified to generate age-specific media.

132   Investigations assessing developmental changes in gastrointestinal fluid composition were collected

133   from the literature. For studies where information was displayed graphically, data was quantified using

134   GetData Graph Digitizer (v2.26). Dependent on the specific media being formulated (i.e. FaSSGF),

135   information pertaining to different physiological parameters were required:

136   (a) FaSSGF – pepsin concentrations, pH, osmolality, and bile salt/lecithin concentrations

137   (b) FeSSGF – feed type(i.e. cow's milk-based vs. soy-based formula), pH, osmolality, and buffering

138   capacity

139   (c) FaSSIF – pH, bile salt/lecithin concentrations, osmolality, and buffering capacity

140   (d) FeSSIF – pH, osmolality, bile salt/lecithin concentrations, fat digestion products, and buffering

141   capacity

142   Parameters values were compiled and, where suitable, graphically displayed as a function of age as

143   an initial evaluation. If changes in GI fluid parameters between pediatric age groups and adults were

144   noted, differences were computed based on a simplistic measure, the arithmetic mean. As the propensity

145   of developmental effects were expected to be most prominent within the earliest stages of life, media

146   reflective of the following age groups were formulated: neonates ($0-28$ days) and infants ($1-12$ months).

147   When data pertaining to specific parameters were unavailable in children, either a default value

148   representative of adult media or an inference based on current physiological knowledge was adopted.

149   Based on this analysis, biorelevant media reflective of pediatric physiology were defined. Media

150   preparation was conducted using the methods depicted in Jantratid et al (2). Measures of osmolality and

151 pH were instituted to ensure prepared media conformed to desired values. Osmolality was measured

152 using freezing point depression (Micro-osmometer - Advanced Instruments Inc.). Discrepancies between

153 measured and desired osmolality were corrected by adjusting media sodium chloride concentrations as

154 described in the literature (2). Media pH was titrated using 1M HCl or 1M NaOH, if necessary. Buffering

155 capacity of pediatric formula (cow's milk-based and soy-based) was determined based on the

156 methodology presented by Hentges et al (11). Values presented represent the amount of acid or base (i.e.

157 mEq) required to induce of pH change of 1 unit per litre of formula (12).

158

159   (iii)Solubility Assessments:

160 (a) Compound Selection

161   Solubility assessments were conducted using BCS class II compounds. Compounds were further

162 restricted to include only those with documented usages, including investigational uses, in both children

163 and adults. Based on the above criteria, seven compounds were selected including carbamazepine,

164 dapsone, fenofibrate, griseofulvin, indomethacin, phenytoin and spironolactone. Compound

165 physicochemical properties are displayed in Table II.

166

167 (b) Solubility Experiments

168   Compound specific solubility assessments were conducted in each of the developed pediatric

169 media as well as in the reference adult media to assess for age-related differences. Experiments were

170 conducted within a shaking water bath set to 37°C and 200 strokes/min.

171   Compound specific solubility values in aqueous-based media (FaSSGF, FaSSIF and FeSSIF) were

172 determined based on the following procedure. A mass of solid (powdered) compound to saturate 10 mL

173   of biorelevant media was added to borosilicate glass tubes. Next, 10 mL of freshly prepared age-specific

174   media (pediatric or adult) was added. Tubes were covered with parafilm and placed in the shaking water

175   bath. Solubility assessments for all compounds, with the exception of fenofibrate, were conducted

176   following a 24 hour dwell period. For fenofibrate, previous investigations have employed longer dwell

177   periods (i.e. 48-72 hours) in order to achieve equilibrium solubility (13, 14). Correspondingly, a dwell

178   period of 72 hours was utilized in this investigation. Saturated media samples were filtered through 0.45

179   μm regenerated cellulose filters and diluted with fresh media prior to assessment. HPLC-UV was utilized

180   to quantify solubility. Analytical HPLC procedures were based on modifications of methods depicted in

181   the literature and are denoted in Table III. Solubility assessments were conducted in triplicate for each

182   test media (pediatric and adult). Calibration curves were constructed using five standard concentrations.

183   Standards were formulated as mobile phase dilutions of a concentrated stock solution consisting of

184   compound dissolved in an organic solvent (i.e. methanol). All dilutions were conducted using volumetric

185   glassware.

186   Due to the addition of either milk or infant formula, fed-state gastric media exists as a complex

187   multiphase system (15). Proteins within the media deter direct filtration of samples through 0.45 μm

188   filters. As a result, the investigation utilized equilibrium dialysis to assess compound solubility within all

189   fed-state gastric media, which negated the need for sample filtration to remove excess drug. To ensure

190   restrictions in the rate of membrane permeation did not delimit solubility determinations, samples were

191   permitted to dwell for an additional 24 hours compared to aqueous-based media. A mass of solid

192   compound required to saturate 25 mL of media was added to separate 50 mL plastic centrifuge tubes.

193   Twenty mL of freshly prepared media was then added. Next, a dialysis membrane (MWCO 12-14000 Da)

194   containing 5 mL of fresh media was placed in each tube. Tubes were capped and placed in a shaking water

195   bath. Solubility assessments were conducted after a 48 hour dwell period with the exception of

196   fenofibrate, which was assessed after 96 hours. For assessment, tubes were taken from the water bath,

197   the dialysis membrane was removed, and its contents were extracted. One mL of media from the within

198   the membrane was combined with 2 mL of methanol and vortexed for 5 seconds. The mixture was

199   centrifuged at 8000 rpm and 4°C for 15 minutes. The resulting supernatant was filtered through 0.45 μm

200   regenerated cellulose filters (Cronus) and diluted in mobile phase prior to analysis. Solubility values were

201   quantified using HPLC-UV under the conditions specified in Table III. Calibrations curves with five standard

202   concentrations were constructed for each test media. Standards were created by dilution of a stock

203   solution, as described above, with fresh media using volumetric glassware. Solubility assessments were

204   conducted in triplicate.

205   One-way analysis of variance (ANOVA) with a post-hoc Tukey's test was applied to identify

206   statistically significant differences in solubility between various age-specific media (i.e. neonate-infant-

207   adult). All statistical analyses was conducted using R statistical software (v 3.1.2). The investigation

208   utilized a significance level of p≤0.05. Average solubility differences between developed pediatric media

209   and the corresponding reference adult media were expressed as a ratio % ($\mu_{pediatric}$ /μ_{adult} x 100). Values

210   greater than 100% indicate compound solubility within the pediatric media exceeded the solubility

211   observed in adults, whereas values less than 100% conveyed the opposite. To denote relevant

212   discrepancies in solubility, reference points corresponding to ratios of 80% and 125% were used. These

213   values parallel the 80-125% bioequivalence criterion as specified by the US-Food and Drug Administration

214   (US-FDA) (16). Within the analysis, statistically significant mean ratios falling outside the pre-specified

215   boundary range were estimated to be at an increased risk for exhibiting alterations in oral drug

216   performance between children and adults. In contrast, when mean ratios were within the 80-125%,

217   boundary, age-specific solubility differences were not expected to alter oral drug performance.

218   The influence of bile salts (NaTc) on modulating compound solubility within the developed

219   biorelevant media was approximated using the equations presented by Mithani et al. (17),

220 $$logSR = 2.09 + (0.64 \cdot logP) \quad \text{Eq.1}$$

221 $$SC_{bs} = SR \cdot SC_{aq} \qquad \text{Eq.2}$$

222 $$C_{sx} = C_{so} + (SC_{bs}) \cdot (MW) \cdot ([NaTc]) \quad \text{Eq.3}$$

223 where SR is the solubilization ratio, logP is the logarithm of the octanol-water partition coefficient, SC_{bs} is

224 the bile salt solubilization capacity, SC_{aq} is the solubilization capacity of water, C_{sx} is the estimated

225 compound solubility (mcg/mL) in the presence bile salts, C_{so} is the aqueous solubility (mcg/mL), MW is the

226 compound specific molecular weight and [NaTc] is the media concentration (mM) of sodium taurocholate

227 (bile salt). The equations, which describe the quantitative relationship between bile acids and compound

228 solubility within aqueous based systems, incorporated bile salt concentrations for each age-specific media

229 formulated with NaTc (FaSSGF, FaSSIF and FeSSIF). For neutral compounds (griseofulvin, spironolactone,

230 carbamazepine and fenofibrate), experimentally determined aqueous solubility values served as inputs.

231 For ionizable compounds (phenytoin-acid, indomethacin-acid, dapsone-base), pH specific aqueous

232 solubility values, as estimated by the Henderson-Hassalbach equation, were utilized. The ratio of

233 compound solubility, relative to adults, was estimated for each of the developed pediatric media (i.e.

234 *pediatric_pred/adult_pred x 100*). A comparison between these predictions, which solely account for the effect

235 of bile acids, and measured values, which account of the influence of all media components, was instituted

236 using the root mean square error (RMSE).

237 $$RMSE = \sqrt{\frac{1}{n}\sum_{j=1}^{n}\left(\left(\frac{\mu \; pediatric \; solubility(measured)}{\mu \; adult \; solubility(measured)}x100\right) - \left(\frac{pediatric \; solubility(predicted)}{adult \; solubility(predicted)}x100\right)\right)^2}$$

238 Eq.4

239   Here, the RMSE provides a quantitative assessment of the influence of media bile salts on modulating

240   compound solubility. For example, high agreement between predicted and measured solubility ratios, as

241   indicated by lower RMSE values, infers NaTc is primarily responsible for observed solubility changes.

242   Results:

243   Literature data utilized to define age-specific GI parameters were primarily sequestered from studies

244   examining healthy/normal children in order to mitigate the confounding effects of altered health statuses.

245   For example, several investigations examining fasting gastric pH in children focused on pre-operative

246   subjects with no known GI disease undergoing elective surgery. However, due the scarcity of pediatric

247   data, some investigations examining critically ill subjects (i.e. NICU, PICU, or ICU patients) as well as

248   preterm neonates were included in the analysis. Such studies were additionally scrutinized to ensure their

249   appropriateness towards defining GI parameters reflective of normal children. Assessments of gastric pH

250   including critically ill subjects were restricted to studies where acid reducing agents (i.e. H2 antagonists)

251   were withheld (18-20). Two pediatric studies assessing fasting gastric pepsin levels included subjects

252   deemed as critically ill (21, 22). As pepsin concentrations were presented as a percentage of adult values,

253   data from these studies were compared to reference data (23) derived from critically ill adult subjects to

254   normalize for any potential effects of illness. Similar to term neonates, preterm neonates by a gestational

255   age of 34 weeks are expected to possess the ability to suckle and swallow to facilitate oral nutrition (24).

256   Consequently, to minimize the effects of immaturity within the analysis, studies were delimited to those

257   where the average postmenstrual age (gestational age + postnatal age) of subjects was approximately ≥

258   34 weeks.

259   Pediatric Fasted-State Simulated Gastric Fluid (P-FaSSGF):

260   Studies depicting gastric pepsin concentrations in pediatric subjects are presented as a

261   percentage of adult values in Figure1a. Reported concentrations were measured in a fasting state with

262   or without histalog stimulation. Values derived from histalog stimulation tests were compared to adult

263   subjects referenced within the same study (25). For other studies, reference adult values were ascertained

264   from separate investigations by the same research group or investigations utilizing a similar assay

265   technique. A segmented analysis towards neonatal subjects was only conducted in a single study (25).

266   The investigation showed gastric pepsin concentrations approached infantile (1m-12m) levels after the

267   first week of postnatal life. For example, neonates between 1-8 days postnatal age exhibited mean pepsin

268   concentrations of ~15% of adult values while older neonates (10-32 days) and infants (67-110 days) both

269   expressed similar mean concentrations of ~41% of adult values. Neonatal FaSSGF was developed based

270   on the youngest cohort of subjects (i.e. those within the 1st week of life) to depict a state where the effects

271   of development are most pronounced. Infant FaSSGF was formulated using pepsin concentrations

272   summarized over several investigations. Concentrations of 15% and 25% of adult reference values

273   (FaSSGF) were utilized for neonatal and infant FaSSGF, respectively.

274   Investigations depicting fasting gastric pH values in pediatric subjects are summarized in Figure

275   1b. Adult values represented by the mean from separate investigations, as summarized by Di Maio and

276   Carrier (26), are displayed for reference. After the first day of life, fasting gastric pH rapidly normalizes

277   towards adult values. Correspondingly, pediatric media (neonate and infant) representative of the fasted

278   gastric state maintained the same pH as denoted by the reference adult media (i.e. FaSSGF - pH = 1.6).

279   A single pediatric study was identified that investigated fasting gastric osmolality in 40

280   postoperative infants with a mean age of approximately 8 months (27). The investigation depicted an

281   average osmolality of 253 mOsm/L, which is more than twice the value of adult FaSSGF (120 mOsm/L).

282   However, these finding may not be entirely reflective of healthy infants. In postoperative subjects,

283   administered medications and patient induced stress during surgery can effect gastric secretions and,

284   thus, osmolality. Consequently, owing to the lack of appropriate data to establish a relationship between

285   age and fasting gastric osmolality, the pre-established value from adults was employed to develop

286   pediatric media.

287   Literature-based assessments of gastric bile acids and phospholipids (i.e. lecithin) in the fasting

288   state were not available for pediatric subjects. As the gastric mucosa does not contain the capacity to

289   produce or excrete bile, the presence of gastric bile acids are primarily the result of duodenogastric reflux,

290   a normal physiological phenomenon documented in adults (28, 29). Therefore, it was postulated that

291   intestinal bile levels would influence the magnitude of bile acids present within gastric fluids. With

292   frequent feeding schedules, neonates and infants are often maintained within the fed-state during waking

293   hours. As such, bile acid (i.e. NaTc) values within pediatric FaSSGF were derived using fed-state intestinal

294   bile levels. The following formula was used to quantify NaTc concentrations in pediatric FaSSGF,

295        $$pFaSSGF_{[NaTc]}\ (uM) = \frac{pFeSSIF_{[NaTc]}}{FeSSIF_{[NaTc]}} \cdot FaSSGF_{[NaTc]}\quad \text{Eq.5}$$

296   where $pFaSSGF_{[NaTc]}$ is the bile acid (NaTc) concentration in pediatric FaSSGF, $pFeSSIF_{[NaTc]}$ is the NaTc

297   concentration in pediatric FeSSIF, $FeSSIF_{[NaTc]}$ is the NaTC concentration in the reference adult FeSSIF

298   media (10 mM), and $FaSSGF_{[NaTc]}$ is the NaTc concentration in reference adult FaSSGF media (80 uM). Bile

299   acid values within pediatric FeSSIF media are presented in a forthcoming section. For lecithin, pediatric

300   FaSSGF was formulated to maintain the same ratio of [NaTc]/[lecithin] as depicted by adult FaSSGF.

301   Compositions of the developed neonatal and infant FaSSGF media are presented in Table IV.

302   Pediatric Fed-State Simulated Gastric Fluid (P-FeSSGF):

303   The composition of FeSSGF is largely influenced by added meal components. In adult FeSSGF,

304   cow's milk is typically incorporated as it contains similar ratios of carbohydrate/protein/fat as a typical

305   breakfast meal and avoids logistic difficulties associated with the use of homogenized solid meals (30, 31).

306   To institute the most physiologically relevant depiction of gastric contents in children, pediatric media

307   were formulated using two types of commonly marketed infant formula: Cow & Gate First Infant Milk

308   (cow's milk-based formula) and Infasoy (soya-based formula). Development of separate pediatric FeSSGF

309   media comprised of different formulas permitted for forthcoming solubility assessments to investigate

310   the influence of pediatric diet on biorelevant solubility.

311   Gastric pH within the fed-state is dependent of several factors including feed composition and

312   time of measurement (32, 33). Since many pediatric investigations administer various feeds (i.e. breast

313   milk, infant formula, or D5W) and measure postprandial pH at selective time intervals, defining age-

314   specific pH values was quite challenging. Adult FeSSGF represents a snapshot of the 'middle' phase of

315   gastric digestion between 75 and 165 minutes post-meal ingestion (2). The pH of adult FeSSGF was derived

316   from Kalantzi et al.'s study, where a liquid meal consisting of 500mL Ensure plus® was administered to 20

317   healthy subjects (33). The study denoted a pH of 5 as the approximate average over the abovementioned

318   time period. However, in a separate investigation by Dressman et al. (34), where gastric pH was monitored

319   following ingestion of a standard solid meal (1000 Kcal), postprandial pH values differed from the results

320   attained by Kalantzi et al. Following administration of a solid meal, gastric pH decreased towards fasting

321   values at a faster rate compared to subjects administered a liquid meal. For example, median pH values

322   persisted above 3 for approximately 60 minutes vs. > 180 minutes following solid meal vs. liquid meal

323   ingestion, respectively (33, 34). As solid foods are anecdotally the most common form of meals consumed

324   by adults, comparison of postprandial pH changes between children, administered a typical meal (i.e.

325   formula), and adults, administered a solid meal, were used to define pH for the developed pediatric

326   FeSSGF. Sondheimer et al. investigated the influence of postnatal age (PNA) on gastric pH in healthy

327   preterm neonates (35). In-situ pH monitoring was conducted following administration of infant formula

328   in two groups of neonates aged 2-6 days and 7-15 days PNA. Comparing pH values at approximately 120

329   minutes post-meal (i.e, mid-point of the 75-165 minute time frame) between the cohort of older preterm

330   neonates and adults, as reported by Dressman et al., pH was found to be higher (0.7-1.8 units) among

331   neonates. As a result, neonatal FeSSGF was formulated to adopt a slightly higher pH (pH = 5.7) as

332   compared to the reference adult FeSSGF (pH = 5).

333   Osmolality of pediatric FeSSGF was defined by two investigations. The first, conducted by Billeaud

334   et al. (36), characterized gastric osmolality among 15 low birth weight neonates with a mean PNA and

335   gestational age (GA) of 8 days and 35.4 weeks, respectively. Eight test feeds, each differing in osmolality,

336   were administered. The study noted a positive linear relationship between feed and gastric osmolality

337   over the 3 hour study period. In a separate investigation by Thatrimontrichai and Janjindamai (37), three

338   separate expressed breast milk feeds, which ranged in osmolality due to the addition of mineral/vitamin

339   supplements, were tested in 26 neonate/infant subjects with a median PNA and GA of 30 days and 30

340   weeks, respectively. Within the study, meals with higher osmolalities were found to be associated with

341   comparatively higher gastric osmolalities over the 1 hour test period. A linear regression model depicting

342   the degree of association between feed osmolality and 60 minute postprandial gastric osmolality was

343   developed based on the results of Billeaud et al.'s (36) investigation (Figure 2). Although a 60 minute

344   sampling point was not obtained during the original study, the value was estimated as the average

345   between the 45 and 90 minute sampling intervals. The validity of the derived regression equation was

346   tested by comparing gastric osmolality predictions to the data presented within Thatrimontrichai and

347   Janjindamai's study (37). The results of this comparison are depicted in Table V. Estimates from the

348   regression equation were within 8% of measured values. As such, the equation was deemed appropriate

349   for defining osmolality in neonatal FeSSGF. Although a sampling point of 60 minutes was clearly outside

350   the time frame used to define adult FeSSGF (75-165 minutes), children, especially those within the

351   youngest age groups, are typically fed on a more consistent basis during waking hours (i.e. every 2-3

352   hours). Correspondingly, defining gastric osmolality in children based on one hour postprandial values

16

353 may provide an age appropriate representation of the 'middle' phase of gastric digestion, which adult

354 FeSSGF is formulated to mimic.

355 Since basal gastric volumes in infants are minute (38), the composition of gastric fluids

356 postprandially can likely be attributed to the properties of the ingested meal. As such, the buffering

357 capacity of pediatric FeSSGF was determined based on the buffering capacity of infant formula

358 incorporated into the media. Since two separate neonatal media, one based on cow's milk formula and

359 the other based on soy formula, were developed, buffering capacity determinations for each respective

360 formula were required. Determinations were conducted at pH 5.7, the desired pH of neonatal FeSSGF.

361 The buffering capacity (mean ± SD) of cow's milk formula at pH 5.7 was 14.03 ± 0.164. Soy-formula at pH

362 5.7 displayed similar a buffering capacity (14.94 ± 0.318 mEq/L/ΔpH). For simplicity, neonatal FeSSGF

363 based on cow's milk formula and soy formula media were prepared to target a buffering capacity 15

364 mEq/L/ΔpH. Compositions of the developed P-FeSSGF media are depicted in Table VI.

365 Appropriate information to define infantile fed-state gastric fluids (i.e. 1-12m) was not attained

366 from the literature. As a result, an infant FeSSGF media was not developed. However, as the composition

367 of FeSSGF is primarily attributed the contents of the added meal component, assessments conducted in

368 neonatal media which incorporate infant formula should provide a general indication of expected

369 solubility changes in infants consuming similar feeds. In addition, comparisons between neonatal media

370 that are similar in all respects with the exception of the type of meal component added (i.e. cow's milk-

371 based formula vs. soy-based formula) provide an assessment of the impact of feed composition on

372 biorelevant solubility.

373 Pediatric Fasted-State Simulated Intestinal Fluid (P-FaSSIF):

374   Intestinal pH values depicted in the literature are summarized as a function of postnatal age in

375   Figure 3a. The majority of pediatric data was attained from the distal duodenum though a few studies

376   that sampled from the proximal jejunum were also included. Adult pH is depicted as mean values from

377   separate investigations, as summarized by Fuchs and Dressman (39). Studies investigating intestinal pH

378   in children, especially in the youngest age groups, were not widely published in the literature. In addition,

379   data obtained from adults encompassed a large degree of variability. Consequently, no distinct

380   relationship between age and fasted-state intestinal pH was observed. Pediatric media were subsequently

381   formulated using the same pH as denoted for the adult reference media (i.e. FaSSIF – pH = 6.5)

382   Fasting bile salt concentrations from the proximal small intestine are depicted as a function of age

383   in Figure 3b. A large degree of variability was apparent in both children and adults as denoted by the

384   spread of data. Discernable differences between pediatric age groups (i.e. neonates, infants) and adults

385   were not visually evident. Furthermore, the linear association between the logarithm of age and bile acid

386   concentrations was negligible (R^2=0.05) among pediatrics. Due the substantial degree of variability

387   between pediatric studies, P-FaSSIF was developed to assess two potential scenarios. In one media, bile

388   salt concentrations were formulated to be 150% of adult values. In the second media, concentrations

389   were formulated to be 50% lower than adults. A pediatric media where bile salt concentrations were

390   similar to adult values did not necessitate development of a new media as this scenario was already

391   depicted by the adult reference. Developed media represent hypothetical depictions of bile acid

392   concentrations within a biologically plausible range. Correspondingly, the magnitude of compound

393   specific solubility differences denoted in such media provides an indication of whether additional pediatric

394   investigations are required to define bile acids within the fasted-state intestine.

395   No pediatric data pertaining to phospholipids (i.e. lecithin), buffering capacity, and osmolality of

396   intestinal fluids in the fasted-state were ascertained. Pediatric media were therefore formulated to

18

397   maintain the same [NaTc]/[lecithin] ratio as depicted in the adult reference media (FaSSIF). Buffering

398   capacity and osmolality were also defined using adult values. Compositions of the proposed P-FaSSIF

399   media are presented in Table VII.

400   Pediatric Fed-State Simulated Intestinal Fluid (P-FeSSIF):

401   Fed-state duodenal pH values from separate pediatric and adult investigations are presented in

402   Figure 4a. Of the few studies presented amongst pediatrics, pH values appear to overlap with those

403   depicted from adults. Owing to the disparate nature of available data, pH differences between each age

404   group (neonate, infants, and adult) could not be fully elucidated. Pediatric FeSSIF media were therefore

405   formulated using the same pH as the adult reference (i.e. pH = 5.8).

406   Assessments of postprandial intestinal osmolality amongst pediatrics were also scarcely published

407   within the literature. A single study conducted by Billeaud et al. (36), which was also utilized to define

408   pediatric FeSSGF osmolality, was identified. Duodenal osmolality was assessed in 15 low birth weight

409   neonates following administration of a variety of feeds, each varying in osmolality. A positive linear

410   association between feed and duodenal osmolality was found. A regression model was constructed using

411   a congruent approach as previously discussed for defining FeSSGF osmolality (Figure 4b). Although a

412   suitable coefficient of determination (R^2 = 0.92) between feed osmolality and 60 minute postprandial

413   duodenal osmolality was attained, a second study from which the model could be evaluated within

414   pediatrics was unavailable. As an alternative assessment, the model was utilized to estimate to duodenal

415   osmolality in two adult studies. Mean duodenal osmolality values of approximately 405 and 392 mOsm/kg

416   were observed 1 hour following administration of liquid meals containing 610 and 670 mOsm/kg in

417   separate investigations conducted by Kalantzi et al. (33) and Clarysse et al. (40), respectively. The

418   proposed regression model provided duodenal osmolality estimates of 430 (6% over-prediction) and 454

419   (16% over-prediction) mOsm/kg for each respective adult investigation. Although derived from a cohort

420   of neonatal subjects, the model exhibited an adequate predictive capacity in adults. By extension, its use

421   for estimating intestinal osmolality amongst pediatrics (neonates and infants) was considered to be

422   appropriate. The osmolality of neonatal FeSSIF was formulated to reflect two separate feed types, breast

423   milk, with a reported osmolality of ~300 mOsm/kg (36, 41), and cow's milk-based formula with a measured

424   osmolality of 368 mOsm/kg (Cow & Gate First Infant Milk). For FeSSIF reflective of older children (i.e.

425   infants) where weaning is commonly instituted, only a single feed type was investigated, cow's milk

426   formula. Using the aforementioned regression equation, osmolality of the developed pediatric FeSSIF was

427   defined as 300 and 330 mOsm/kg post-administration of breast milk and cow's milk-based formula,

428   respectively.

429   Fed-state duodenal bile salt concentrations among pediatrics and adults are summarized in Figure

430   4c. A positive linear association between the logarithm of age and duodenal bile acid concentrations was

431   denoted among children (R^2 = 0.54). Bile acid concentrations among adults displayed variability, but for

432   the most part studies depicted a mean value of approximately 10 mM, corresponding to the concentration

433   of the reference adult media (FeSSIF v2). Mean bile acid concentrations among neonates (0-28 days) and

434   infants (1-12m) were approximately 25% (i.e. 2.5 mM) and 75% (i.e. 7.5 mM) of adult values, respectively.

435   Pediatric FeSSIF were subsequently formulated using these bile acid concentrations.

436   Pediatric studies characterizing concentrations of fat digestion products in the intestinal lumen

437   have not been reported in the literature. However, since the quantity of such products is dependent on

438   the interrelationship between fat digestion and absorption, an examination of these processes was

439   instituted in order to derive age-dependent estimates. In newborns, concentrations of pancreatic

440   colipase-dependent triglyceride lipase, the enzyme primarily responsible for lipid metabolism in adults, is

441   decreased (42). Despite this, the presence of auxiliary enzymes such as human gastric lipase, pancreatic

442   lipase-related protein 2 and bile salt-stimulated lipase, are postulated to provide an efficient means of

443 lipid digestion for newborns (42). In terms of absorption, breast-fed neonates exhibit fat absorption

444 coefficients reminiscent to that of adults despite lower duodenal bile acid concentrations (43). It was

445 therefore inferred that the developmental capacity of both fat digestion and absorption were comparable

446 to adults amongst this pediatric cohort. FeSSIF media reflective of breast-fed neonates were

447 correspondingly formulated using the same concentrations lipid digestion products (glyceryl monooleate

448 and sodium oleate) as defined for the adult reference media (i.e. FeSSIF).

449   However, among formula-fed neonates, fat absorption coefficients are notably lower compared

450 to their breast-fed counterparts as well as adults (43, 44). Unlike breast-fed neonates, intestinal bile

451 concentrations in formula-fed neonates were found to exhibit a positive linear correlation with percent

452 fat absorption (43, 44). To decipher whether a deficiency in lipid absorption or lipid digestion was the

453 primary factor limiting internalization of fats in formula-fed neonates, pathophysiological information

454 pertaining to necrotizing entercolitis (NEC) was used. NEC is a debilitating inflammatory GI condition

455 occurring typically in preterm neonates but also uncommonly in term neonates. In both groups, the

456 incidence of NEC is substantially higher in formula-fed subjects as compared to those receiving enteral

457 feeds with breast milk (45, 46). Though the mechanism of pathogenesis of NEC is not completely

458 understood, one theory as described by the work published by Penn et al. (47) identified the presence of

459 elevated concentrations of free fatty acid (FFA) as the culpable factor. The study found lipase digestion of

460 formula, but not human milk, exhibited a cytotoxic effect in three different cell types. Furthermore,

461 digested formula displayed significantly greater levels of FFA compared to lipase digested human milk.

462 Based on this finding in conjunction the prevalence of NEC amongst the youngest cohort of neonates, it

463 was inferred that the process of lipid digestion was not developmentally impaired in formula-fed

464 neonates. Hence, the decreased capacity for fat internalization was attributed to an inadequate lipid

465 absorptive capacity in such subjects. Correspondingly, formula-fed neonates would be expected to exhibit

466 higher luminal concentrations of lipid digestion products. Using 75% as the average coefficient of fat

467   absorption in formula-fed neonates (42-44), the concentration of lipid digestion products (glyceryl

468   monooleate and sodium oleate) was estimated to be 1.33x (i.e. 1/0.75) greater in the intestinal lumen of

469   neonates that are formula-fed compared those that are breast-milk fed. P-FeSSIF media pertaining to

470   formula-fed neonates was developed based on the above assertion. In infants, where luminal bile acid

471   concentrations are higher, fat absorption is not expected to exhibit developmental impairment. Pediatric

472   FeSSIF reflective of formula fed infants was therefore formulated using the same concentrations of fat

473   digestion products as depicted for the adult reference media.

474   No pediatric studies investigating buffering capacity and concentrations of phospholipids (i.e

475   lecithin) within the fed-state intestinal lumen were obtained. Buffering capacity of the developed

476   pediatric media were consequently formulated using a value of 25 mEq/L/ ΔpH, the adult reference value.

477   Using a similar approach as employed for P-FaSSGF and P-FaSSIF, lecithin concentrations were fixed to

478   provide the same ratio of [NaTc]/[lecithin] as expressed by the reference adult media (i.e. FeSSIF.v2). The

479   compositional details of developed P-FeSSIF media are depicted in Table VIII.

480

481   Solubility Assessments:

482   Solubility determinations for six of the seven compounds (carbamazepine, dapsone, griseofulvin,

483   indomethacin, phenytoin and spironolactione) were conducted in age-specific media representative of all

484   four gastrointestinal states: FaSSGF, FeSSGF, FaSSIF and FeSSIF (Figure 5). For fenofibrate, use of the

485   predefined equilibrium dialysis method did not serve as a suitable technique for solubility determinations

486   in fed-state gastric media. Penetration of fenofibrate through the dialysis membrane was inefficient

487   during the selected study interval (96 hours) and, as a result, solubility could not quantified. Figure 6

488   depicts solubility determinations of fenofibrate in age-specific media reflective of the remaining three

489   gastrointestinal states: FaSSGF, FaSSIF and FeSSIF.

490   For pediatric media representative of the fasted gastric state (i.e. P-FaSSGF), three compounds

491   (carbamazepine, indomethacin and fenofibrate) exhibited mean solubility values below the 80 to 125%

492   reference range, relative to adults. Though for indomethacin, this difference was not statistically

493   significant. Relative solubility changes depicted between neonatal and infant FaSSGF were consistent in

494   terms of direction and magnitude for six of seven compounds. Only one compound, carbamazepine,

495   displayed a statistically significant difference in solubility between neonatal and infant FaSSGF.

496   Solubility assessments in neonatal fed-state gastric media (i.e. P-FeSSGF), developed using cow's

497   milk-based or soy-based formula, were compared to solubilities attained in adult FeSSGF formulated with

498   cow's milk. Five compounds (carbamazepine, dapsone, griseofulvin, phenytoin and indomethacin)

499   exhibited changes in solubility that fell outside the aforementioned reference range in at least one of the

500   developed neonatal media. A trend towards lower solubility values in neonatal media was found for four

501   of the compounds (carbamazepine, dapsone, griseofulvin and phenytoin). For indomethacin, a weak acid

502   (pKa = 4.5), an increase in solubility compared to adult media was observed that was attributed, in part,

503   to the higher pH of neonatal FeSSGF. Statistically significant differences in solubility between neonatal

504   media formulated using either cow's milk-based or soy-based formula was observed in 4/6 compounds.

505   For carbamazepine, solubility values in media comprised with cow's milk formula was greater than that

506   of media comprised with soy formula. For dapsone, phenytoin and indomethacin the opposite was

507   observed. In contrast, for spironolactone and griseofulvin, no statistically significant difference in

508   compound solubility was noted between the respective neonatal media.

509   Since a consensus regarding differences in bile salt concentrations between children and adults

510   within the fasted-state intestine was not achieved, compound solubility was investigated based on two

23

511   theoretical media that incorporated bile salt concentrations of 150% (4.5 mM) and 50% (1.5 mM) of those

512   in adults. For the majority of compounds (6/7), solubility determinations in both media fell within an 80%

513   to 125% range when compared to adult values. However, for fenofibrate (logP = 5.3), solubility in P-FaSSIF-

514   50% media was 56% of the value observed in the adult reference.

515   Solubility determinations conducted in pediatric media reflective of the fed-state intestine (i.e. P-

516   FeSSIF) were compared to values attained in adult FeSSIF. For three of seven compounds (fenofibrate,

517   griseofulvin and phenytoin), mean solubilities of less than 80% of adult values were observed in at least

518   one of the formulated P-FeSSIF. These relevant solubility alterations were exclusively found in neonatal

519   media. In comparison, mean solubility values in infant FeSSIF fell within 80-125% of adult values for all

520   compounds investigated. A general trend towards statistically significant lower solubilities in neonatal

521   media compared to infant FeSSIF was observed in five of seven compounds. Statistically significant

522   solubility differences between neonatal media formulated to depict intestinal fluids following

523   administration of cow's milk-based formula (Pnc-FeSSIF) or breast milk (Pnb-FeSSSIF) were denoted for

524   three compounds (griseofulvin, spironolactone, and phenytoin). A higher solubility was observed for

525   griseofulvin in Pnc-FeSSIF though in both neonatal media, values were below the 80-125% reference

526   range. Solubility was also greater in Pnc-FeSSIF for spironolactone but, in this case, solubility values in

527   both media fell within the 80-125% reference range. In contrast, for phenytoin, a higher solubility was

528   observed in Pnb-FeSSIF. Solubility in Pnb-FeSSIF fell within 80-125% of adult values, but for media

529   depicting formula-fed neonates (Pnc-FeSSIF), the mean solubility was well below the 80% reference point.

530   Changes in compound solubility between pediatric and adult media induced by alterations in bile

531   salt concentrations were estimated according to the equations proposed by Mithani et al (17). These

532   values are displayed in Figures 5 and 6 (red dots) for media formulated with NaTc (i.e. FaSSGF, FaSSIF, and

533   FeSSIF). Table IX displays RMSE values between predicted and measured solubility ratios for the

534   developed pediatric media in order of increasing compound lipophilicity. For the two least lipophilic

535   compounds investigated (dapsone and griseofulvin), predictions made using Mithani el al.'s equations

536   were within a RMSE of 10%. In these cases, the equations provide an acceptable approximation of the

537   direction and magnitude of solubility changes observed in pediatric media. As compound lipophilicity

538   increased, a departure between predicted and measured solubility ratios was observed as indicated by

539   larger RMSE values. For such compounds, the predicted magnitude of solubility changes due to alterations

540   in media NaTc content were typically overstated when compared to measured values.

541   Discussion:

542   Based on an assessment of the current literature, biorelevant media simulating the unique

543   intricacies of the upper gastrointestinal tract (stomach and proximal small intestine) in pediatrics were

544   developed and utilized to estimate compound specific solubility. Preferably, solubility comparisons

545   between pediatrics and adults should be conducted using ex-vivo luminal fluid samples, but due to the

546   numerous logistical and ethical constraints associated with obtaining of such samples in pediatrics, the

547   use of biorelevant media was deemed as a suitable approach. In adults the appropriateness of biorelevant

548   media has been established by investigations depicting strong positive correlations in compound solubility

549   between simulated and human intestinal fluids (48, 49).

550   Pediatric fed-state gastric media (P-FeSSGF) was formulated using either cow's milk-based or soy-

551   based formula to assess the impact of feed type on compound solubility. The use of human breast milk

552   within the investigation was precluded due to logistic issues associated sample obtainment and

553   uniformity. In terms of uniformity, the composition human breast milk is well known to exhibit both intra-

554   and inter-subject variability in composition (50). Infant formulas, however, are subject to quality control

555   inspections to ensure batch-to-batch uniformity, ensuring biorelevant media are prepared in a

556   reproducible fashion. Marketed infant formulas are designed to mimic the composition of human breast

557 milk with regards to the proportions of energy provided from protein, fats, and carbohydrates (51). In

558 human breast milk, proteins are predominantly comprised of two forms, whey and casein, in a ratio of

559 60:40, respectively. In contrast, the whey-to-casein ratio of cow's milk is 18:82 (51). To address this

560 discrepancy, many formulas, including Cow & Gate First Infant Milk (cow's milk based), have introduced

561 additional amounts of whey protein in order to mimic ratios observed in human milk (51). The influence

562 of casein on compound solubility has previously been depicted for the anticoagulant dicumarol, where

563 increases in casein concentration corresponded to higher dicumarol solubility values (52). Despite

564 supplementation with vitamins and minerals, infant formula cannot fully reproduce the biological

565 complexity of human breast milk which contains antibodies, enzymes, and growth factors (51). However,

566 cow's milk formula does provide a suitable approximation in terms of macronutrient composition and

567 protein type that is free from the inherent variability associated with breast milk. As such, solubility studies

568 conducted in neonatal FeSSGF comprised with cow's milk formula may encompass applicability towards

569 breast fed neonates.

570   Concentrations of media components were formulated to represent the average tendency over a

571 specific age range. To summarize age-specific data from the literature, the investigation utilized non-

572 weighted arithmetic means. Though simplistic in nature, use of the arithmetic mean was preferred over

573 other more robust computational or statistical analyses based on several considerations. First, there is

574 the relative disparity of literature investigations devoted to defining the composition of luminal fluids in

575 pediatrics compared to adults. For many media components only a handful of studies were available to

576 quantify differences between subsequent age groups. Of studies obtained, high degrees of variability

577 were typically noted. This is likely attributed to the dynamic nature of the developmental process, where

578 the composition of luminal fluids continually change as children mature. Due to this disparity of available

579 data and its inherent variability, employment of statistical tests to identify significant differences in

580 component concentrations between adjacent age groups were not applicable. Similarly, the use of

26

581   regression analyses were typically unable to establish meaningful correlations between parameter values

582   and age. A second consideration for the preferential use of non-weighted arithmetic means was due to

583   the precarious nature of qualifying investigations. A large majority of pediatric studies were completed

584   over three decades ago, where differences in reporting standards and quantitative techniques were wide-

585   ranging. Employment of a non-weighted approach was instituted to simplify the analysis though,

586   understandably, the method lacks the informative capacity of approaches that consider study quality, as

587   frequently adopted by systematic reviews (53). Finally, studies varied in terms of data presentation,

588   making implementation of weighted averages difficult. Reporting of variability associated with luminal

589   fluids components was inconsistent between investigations. For example, separate studies utilized a

590   variety of measures including standard deviation, range, or interquartile range. Additionally, the number

591   of subjects allocated to specific age ranges were not identified by some investigators (21). Due to this lack

592   of consistency between studies, employment of a weighted mean was precluded in favor of a non-

593   weighted approach. As the arithmetic mean does not provide an indication of parameter variability, the

594   analysis is unable to depict expected variations within the population. However, as biorelevant media is

595   developed to represent luminal fluids in an average individual, descriptions of parameter variability were

596   unnecessary.

597   Due to the disparate nature of available pediatric data, the quantitative value of many media

598   components were based on biological inferences or adoption of adult values. For example pediatric

599   investigations pertaining to luminal concentrations of pepsin, phospholipids, fat digestion products, and

600   osmolality were either scarcely reported or lacking within the literature. The proposed age-specific media

601   attempted to approximate the *in vivo* composition of pediatric luminal fluids based on a current state of

602   knowledge. As future investigations are obtained, such formulations should undoubtedly be modified to

603   provide greater degrees of biological relevance.

604   In addition to its primary goal of facilitating suitable IVIVC, biorelevant media should demonstrate

605   a practical degree of stability. Apart from noticeable changes in visual appearance, media stability can be

606   formally evaluated by assessing for alterations in physicochemical parameters under ambient and test

607   conditions. In Jantratid et al.'s original publication (2), which described the reference adult media utilized

608   by this investigation, stability was evaluated through measurements of media pH, buffering capacity and

609   osmolality. For adult FeSSGF and FeSSIF-v2, consistency in physicochemical parameters were observed

610   under ambient conditions over a 72 hour study period. In addition, with the exception of minor changes

611   in osmolality, the abovementioned media demonstrated stability under test conditions of $37^{O}C$ over the

612   same time period. Changes in media physicochemical properties (i.e. poor stability) during solubility

613   assessments may lead to corresponding changes in compound specific saturation solubility. In the current

614   investigation, solubility assessments in fed-state simulated gastric media comprised of cow's milk

615   (FeSSGF) and infant formula (neonatal FeSSGF) were conducted at $37^{o}C$ after 48 hours for most

616   compounds. Though stability studies were not conducted within the developed pediatric FeSSGF, it was

617   inferred that stability would be similar to that of adult FeSSGF. However, if large scale implementation of

618   the depicted pediatric media is desired, future research evaluating media stability will certainly be

619   required.

620   For the majority of study compounds, solubility assessments proceeded without issue. Though for

621   fenofibrate, the most lipophilic compound evaluated (logP = 5.3), logistic issues materialized with

622   solubility determinations in biorelevant media reflective of the fed gastric state. For FeSSGF media, the

623   study employed equilibrium dialysis to assess compound solubility. Though this technique proved

624   effective for most compounds, it was unsuitable for fenofibrate. Following a 96 hour dwell period,

625   fenofibrate concentrations within the membrane were below the limit of quantification. This result may

626   indicate inadequate permeation of the dialysis membrane by fenofibrate in milk or formula-based

627   samples. A congruent example is demonstrated by the *in vivo* pharmacokinetics of fenofibrate. In humans,

628 fenofibrate exhibits extensive protein binding (~99%) and, as such, filtration by hemodialysis is not

629 considered effective (54, 55). Based on this assessment, use of equilibrium dialysis was not considered

630 feasible for determination of fenofibrate solubility in fed state gastric media. These values were

631 correspondingly excluded from the analysis.

632   Solubility assessments were confined to BCS Class II compounds, where limitations in absorption

633 are primarily attributed to inadequate drug solubility. For such compounds, differences in luminal

634 solubility may signify alterations in oral drug performance (56). To maintain a degree of biological

635 relevance, the analysis was further limited to compounds where documented or investigational uses in

636 both children and adults have been depicted. To identify relevant changes in the age-specific solubility,

637 the study utilized the same threshold as depicted by the US-FDA for attainment of *in vivo* bioequivalence

638 (i.e. 80-125%) (16). It should be noted, however, that solubility in only one parameter which can exert an

639 effect on oral compound absorption. Other parameters including gastric emptying time, small intestinal

640 transit time, intestinal permeability, gut metabolism, luminal degradation and presence of intestinal

641 transporters may also impart an influence *in vivo*. In order to fully elucidate the impacts of growth and

642 development on oral compound absorption, a more comprehensive analyses such as physiologically-

643 based pharmacokinetic (PBPK) modeling would be required to integrate age-dependencies in all the

644 aforementioned parameters. The presented analysis which focusses on biorelevant solubility as a

645 surrogate for oral compound performance was therefore an overt simplification. However, this approach

646 was justified based on the cohort of compounds assessed, which was confined to solubility-limited (BCS

647 Class II) drugs.

648   Compared to adult media, solubility in pediatric fasted-state gastric media (i.e. P-FaSSGF) was

649 both statistical different (p≤0.05) and outside the purported bioequivalence criterion for two compounds,

650 fenofibrate and carbamazepine. For fenofibrate, mean solubility values in adult FaSSGF and pure water

651   were comparable at 0.281 and 0.206 mcg/mL, respectively. In contrast, solubility within adult fasted-state

652   intestinal media (i.e. FaSSIF) was considerably greater (2.42 mcg/mL). The discrepancy in solubility values

653   between FaSSGF and FaSSIF provides an indication of the relative influence of each state on modulating

654   oral absorption. In this case, due to its poor solubility in comparison to intestinal fluids, fasted state gastric

655   fluids are unlikely to play an influential role on modulating the extent of fenofibrate absorption. Solubility

656   alterations observed in P-FaSSGF were therefore not postulated to impact the oral performance of

657   fenofibrate in children.   Differences in solubility between neonatal and infant FaSSGF reached a

658   statistically significant threshold for only one compound, carbamazepine. Though for both media, the

659   mean solubility fell outside the bioequivalence threshold when compared to adult values. Based on this

660   analysis, an argument may be formed as to the need for separate pediatric media since solubilities in

661   neonate and infant FaSSGF appear to be similar in most cases. The current investigation focused on

662   solubility, a compound specific property. However, in terms of establishing IVIVC for solid dosage forms,

663   biorelevant media is typically employed within dissolution tests to assess formulation properties (57). In

664   addition to modulating solubility, media components that are age-specific may also exert an influence on

665   the rate of compound release and subsequent dissolution from a formulation. For example, the addition

666   of pepsin into biorelevant media has been demonstrated to decrease surface tension (3). For specific

667   formulations, such changes can exert of an effect on the rate of compound dissolution (58). Also, the

668   presence of pepsin within dissolution media can facilitate effective compound release from cross-linked

669   gelatin capsules (59). Therefore, although comparable solubilities were observed for neonatal and infant

670   FaSSGF media, use of separate age-specific media may be justified for use in dissolution testing.

671   Solubility assessments in age-specific FeSSGF media were conducted for six compounds. For the

672   majority of compounds (5/6), the mean solubility in neonatal media, comprised of either cow's milk-based

673   or soy-based formula, fell outside the 80-125% bioequivalence criterion in relation to adult media

674   comprised of milk. In addition, statistically significant differences in compound solubility between

675 pediatric media comprised with cow's milk-based and soy-based formula were observed in four

676 compounds. These results infer that differences in feed composition between children as well as between

677 children and adults can impart relevant changes in gastric solubility and, potentially, affect oral compound

678 performance.

679 Of the limited pediatric investigations examining luminal fluids within the fasted-state proximal

680 intestine, bile salt concentrations were found to exhibit a high degree of variability without any apparent

681 age dependency. To explore the impact of such variations, two FaSSIF media were developed with bile

682 salt concentrations of 50% (1.5 mM) and 150% (4.5 mM) of adult values. For the majority of compounds

683 (6/7), mean solubility values within the two proposed P-FaSSIF media fell within an 80-125% range from

684 adult values. However for the most lipophilic compound, fenofibrate (logP = 5.3), solubility in P-FaSSIF

685 media containing 1.5 mM NaTc was 56% of adult values. If such a media is reflective of *in vivo* luminal

686 fluids in children, the observed change in solubility may signify an alteration in fenofibrate oral

687 performance compared to adults. Prospectively, hydrophobic compounds are expected to play an

688 increasingly important role in therapeutics as use of drug discovery techniques such a high-throughput

689 screening typically produces candidate compounds of higher lipophilicity (60). To provide an accurate

690 depiction of luminal solubility for such compounds, a consensus regarding intestinal bile salt

691 concentrations in pediatrics is needed. This demonstrates a need for more high quality studies

692 characterizing gastrointestinal physiology in pediatrics.

693 Solubility assessments conducted in fed-state intestinal media representative of infants were

694 within 80-125% of adult values for all 7 compounds tested. Such a result was unsurprising as infant and

695 adult media were compositionally similar, aside from small deviations in bile salt content, lecithin, and

696 osmolality. Two neonatal media were formulated to reflect differences in intestinal fluid composition

697 following administration of breast milk or cow's milk-based formula. Mean compound solubility values in

698   neonatal media fell outside the 80-125% criterion from adult values for 3 of the 7 compounds examined.

699   Statistically significant differences in solubility between media reflective of breast-fed and formula-fed

700   neonates was observed for 3 compounds. The relative magnitude of these differences appeared to be

701   compound specific. For example, spironolactone solubility in intestinal media reflective of breast and

702   formula fed neonates were 83% vs. 91% of adult values, respectively. In contrast, for phenytoin a larger

703   discrepancy between solubility ratios was observed (92% vs. 61% of adult values, respectively). These

704   findings demonstrate the potential impact of different feed types on intestinal compound solubility.

705   The study also included an evaluation the relative importance of bile salts in modulating

706   compound solubility within the developed pediatric media. Predictive equations presented by Mithani et

707   al. (17) were used to estimate the impact of alterations in bile salt content on compound solubility.

708   Measured solubility values, which are influenced by all media components, were compared to estimated

709   values, which only account for differences in media bile salts, using RMSE. The analysis demonstrated a

710   decreased predictive capacity of the aforementioned equations (ie. larger RMSE values) as compound

711   lipophilicity (logP) increased. This indicates that as compound lipophilicity increases, other media

712   components, aside from bile salts, exert a more pronounced role in modulating compound solubility. For

713   example, the capacity of media components such as buffer (sodium phosphate), fat digestion products

714   (sodium oleate) and salt (sodium chloride) to modify compound solubility has previously been

715   demonstrated within the literature (10).

716   Conclusion:

717   The current investigation strove to appropriately depict the *in vivo* composition pediatric luminal

718   fluids based on the current literature and represents an initial foray into the development of pediatric

719   biorelevant media. To increase the biological applicability of future iterations of such media, it is clear

720   prospective studies focused on defining the composition of the pediatric lumen under varying conditions

721   is required. For 6 of the 7 BCS Class II compounds investigated, solubility fell outside an 80-125% range

722   from adult values in at least one of the developed pediatric media. This result demonstrates the impact

723   of age-related alterations in GI fluid composition on compound solubility. Solubility represents an integral

724   component of the BCS, a framework which is extensively utilized by both industry and regulatory bodies

725   to guide drug development in adults. The utility of a similar classification system in pediatrics is in part

726   contingent on our understanding of how developmental differences between children and adults

727   translates to alterations definable properties such compound solubility. The investigation sought to

728   address this concern and, in turn, provides a dialogue surrounding the future development of a pediatric-

729   focused BCS.

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1021   Table I: Composition of adult biorelevant media

Component	FaSSGF	FeSSGF	FaSSIF.v2	FeSSIF.v2
Sodium Taurocholate	80 (uM)	-	3 (mM)	10 (mM)
Lecithin	20 (uM)	-	0.2 (mM)	2 (mM)
Pepsin (mg/mL)	0.1	-	-	-
Sodium Chloride (mM)	34.2	237.02	68.62	125.5
Acetic Acid (mM)	-	17.12	-	-
Sodium Acetate (mM)	-	29.75	-	-
Maleic Acid (mM)	-	-	19.12	55.02
Sodium Hydroxide (mM)	-	-	34.8	81.65
Glyceryl Monooleate (mM)	-	-	-	5
Sodium Oleate (mM)	-	-	-	0.8
Milk:Buffer	-	1:1		-
HCl/NaOH qs	pH 1.6	pH 5	pH 6.5	pH 5.8
pH	1.6	5	6.5	5.8
Osmolarity (mOsm/kg)	120.7	400	180	390
Buffering Capacity (mEq/L/ ΔpH)	-	25	10	25

1023   *adult media compositions as described in Jantratid et al. (2)

1024

1025   Table II: Compound physicochemical properties

Compound	Molecular Weight (g/mol)	LogP	pKa (acid/base)
Dapsone	248	0.97	2.4 (base)
Griseofulvin	353	2.18	-
Carbamazepine	236	2.45	-
Phenytoin	252	2.47	9.5 (acid)
Spironolactone	417	2.78	-
Indomethacin	358	4.27	3.8 (acid)
Fenofibrate	361	5.3	-

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1030   *physicochemical data obtained from DrugBank (61)

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1032

1033   Table III: HPLC-UV analytic conditions

Column	Compound	Mobile	Q^a (ml/ min)	Temp (^{0}C)	Inj Vol (μL)	λ^b (nm)	R_t^c (min)	Reference
1	Carbamazepine	MeOH/ Water (60:40)	1	20	50	285	6.6	(62)
2	Dapsone	Water with ammonium acetate 0.0286M / MeOH (70:30)	1	20	10	295	5.6	(63)
2	Fenofibrate	MeOH/ Acetate buffer 0.010M pH=3.7 (82:18)	1	25	80	286	6.5	(64)
2	Griseofulvin	MeOH/ Water (65:35)	1	20	20	292	4.5	(65)
2	Indomethacin	MeOH/ Water with 1.67% orthophosphoric acid (70:30)	1	23	100	270	9.9	(66)
2	Phenytoin*	Water/ AcN (50:50)	0.5	20	10	210	5.6	(67)
2	Spironolactone	MeOH/ Water (70:30)	1	20	40	237	5.7	(68)

1034

1035   Column 1: Hypersil (Thermo) BDS -C18 250 x 4.6mm - 5 µm

1036   Column 2: Zorbax SB-C18 150 x 4.6mm – 3.5µm

1037   a - Q = flow rate

1038   b - λ = UV wavelength

1039   c - R_t = retention time

1040   * - HPLC conditions altered for solubility assessments with FeSSGF media due to interference with media

1041   components. Mobile phase (Water/AcN – 60:40), Q (1ml/min) and R_t=4.8 mins.

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1044

1045   Table IV: Pediatric Fasted-State Simulated Gastric Fluids (P-FaSSGF)

Component	Pn-FaSSGF[a]	Pi-FaSSGF[b]
Sodium Taurocholate (uM)	20	60
Lecithin (uM)	5	15
Pepsin (mg/mL)	0.015	0.025
Sodium Chloride (mM)	34.2	34.2
HCl qs	pH 1.6	pH 1.6
pH	1.6	1.6
Osmolarity (mOsm/kg)	120.7	120.7
Buffering Capacity (mEq/L/ ΔpH)	-	-

1046

1047   *(a) Pn-FaSSGF – pediatric fasted-state gastric media representative of neonates (0-28 days)*

1048   *(b) Pi-FaSSGF – pediatric fasted-state gastric media representative of infants (1-12 months)*

1049

1050

1051   Table V: Predictive performance of the osmolality regression equation

Feed Osmolality (Median - mOsm/kg)[a]	Measured Gastric Osmolality - 60 min postprandial (Median - mOsm/kg)[a]	Predicted Gastric Osmolality – 60 min postprandial (mOsm/kg)[b]	% Prediction Error ((Pred– Obs) / Obs) x 100
344	354	327	-7.6 %
426	383	368	-3.9 %
315	315	313	-0.6 %

1052

1053   *(a) Values derived from Thatrimontrichai and Janjindamai (37)*

1054   *(b) Predictions based on regression model, as derived from Billeaud et al. (36)*

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1057   Table VI: Pediatric Fed-State Simulated Gastric Fluids (P-FeSSGF)

Component	Pnc-FeSSGF[a]	Pns-FeSSGF[b]
Sodium Chloride (mM)	100.35	94.79
Acetic Acid (mM)	7.25	7.25
Sodium Acetate (mM)	64.65	64.65
Milk:buffer	1:1	1:1
HCl/NaOH qs	pH 5.7	pH 5.7
pH	5.7	5.7
Osmolarity (mOsm/kg)	340	240
Buffering Capacity (mEq/L/ ΔpH)	15	15

1058

1059   *(a) Pnc-FeSSGF – pediatric fed-state gastric media representative of neonates (0-28 days) fed cow's*

1060   *milk-based formula*

1061   *(b) Pns-FeSSGF – pediatric fed-state gastric media representative of neonates (0-28 days) fed soy-*

1062   *based formula*

1063

1064

1065   Table VII: Pediatric Fasted-State Simulated Intestinal Fluids (P-FaSSIF)

Component	P-FaSSIF-50%[a]	P-FaSSIF-150%[b]
Sodium Taurocholate (mM)	1.5	4.5
Lecithin (mM)	0.1	0.3
Maleic acid (mM)	19.12	19.12
Sodium hydroxide (mM)	34.8	34.8
Sodium Chloride (mM)	68.62	68.62
pH	6.5	6.5
Osmolarity (mOsm/kg)	180	180
Buffering Capacity (mEql/L/ ΔpH)	10	10

1066

1067   *(a) P-FaSSIF-50% – pediatric fasted-state intestinal media formulated with bile salt concentrations*

1068   *50% (i.e. 1.5mM) of adult levels*

1069   (b) *P-FaSSIF-150% – pediatric fasted-state intestinal media formulated with bile salt concentrations*

1070   *150% (i.e. 4.5 mM) of adult levels*

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1072

1073   Table VIII: Pediatric Fed-State Simulated Intestinal Fluids (P-FeSSIF)

Component	Pnb-FeSSIF[a]	Pnc-FeSSIF[b]	Pi-FeSSIF[c]
Sodium Taurocholate (mM)	2.5	2.5	7.5
Lecithin (mM)	0.5	0.5	1.5
Glyceryl monooleate (mM)	5	6.65	5
Sodium oleate (mM)	0.8	1.06	0.8
Maleic acid (mM)	55.02	55.02	55.02
Sodium hydroxide (mM)	81.65	81.65	81.65
Sodium Chloride (mM)	95	111.73	107.35
pH	5.8	5.8	5.8
Osmolarity (mOsm/kg)	300	330	330
Buffering Capacity (mEq/L/ ΔpH)	25	25	25

1074

1075   *(a) Pnb-FeSSIF – pediatric fed-state intestinal media representative of neonates (0-28 days) fed breast*

1076   *milk*

1077   *(b) Pnc-FeSSIF – pediatric fed-state intestinal media representative of neonates (0-28 days) fed cow's*

1078   *milk-based formula*

1079   *(c) Pi-FeSSIF – pediatric fed-state intestinal media representative of infants (1-12 months) fed cow's*

1080   *milk-based formula*

1081

1082

1083   Table IX: Predictive performance of Mithani et al.'s (17) equations at characterizing compound specific

1084   solubility changes in pediatric media

Compound	RMSE[a]
Dapsone (logP = 0.97)	8.5%
Griseofulvin (logP = 2.18)	3.7%
Carbamazepine (logP = 2.45)	16.9%
Phenytoin (logP = 2.47)	10.9%
Spironolactone (logP = 2.78)	17.4%
Indomethacin (logP = 4.27)	39.1%
Fenofibrate (logP = 5.3)	28.5%

1085

1086   (a) Root mean square error (RMSE) was tabulated based on Eq 4. Only pediatric media formulated

1087   with bile salts (i.e. P-FaSSGF, P-FaSSIF, and P-FeSSIF) were included in the assessment.

1088

1089
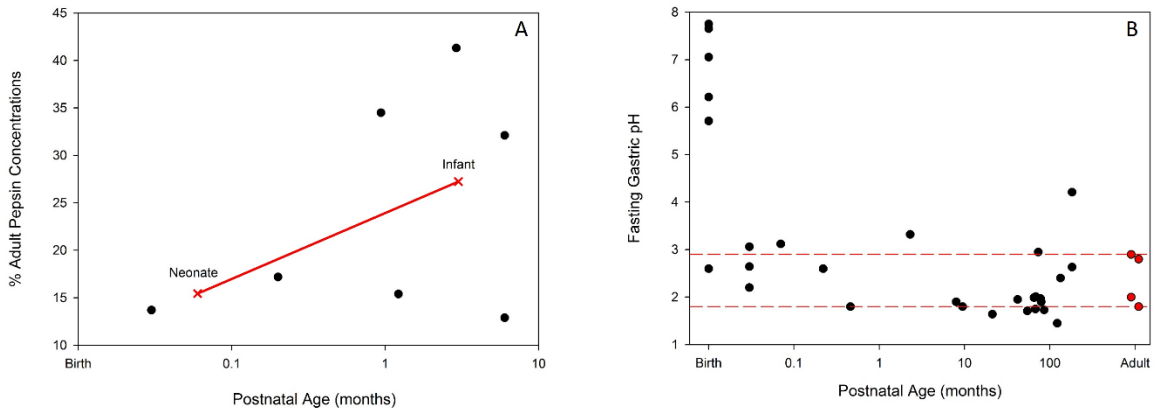
Figure 1: (A) Fasting gastric pepsin concentrations amongst pediatrics (21, 22, 25, 69) is expressed as a percentage of adult values (23, 25, 70). Investigations where pepsin concentrations were quantified over a specific age range without denoting the group's mean age were graphically depicted as the middle of the age range. Average (mean) values pertaining to neonates (0-28days) and infants (1m-12m) are illustrated for reference (red – x's). (B) Fasting gastric pH amongst pediatrics (black circles) is depicted as the central tendency, either mean or median, from separate investigations (18-20, 27, 38, 71-88). Studies where gastric pH values was quantified over specific age range without denoting the group's mean age were graphically depicted using the middle of the age range. Adult data is depicted by mean pH values from separate studies, as summarized by Di Maio and Carrier (26). Dashed reference lines correspond to the maximum and minimum mean pH values observed within the presented adult studies.

$$y = 0.5029x + 154.45$$
$$Rsqr=0.9524$$
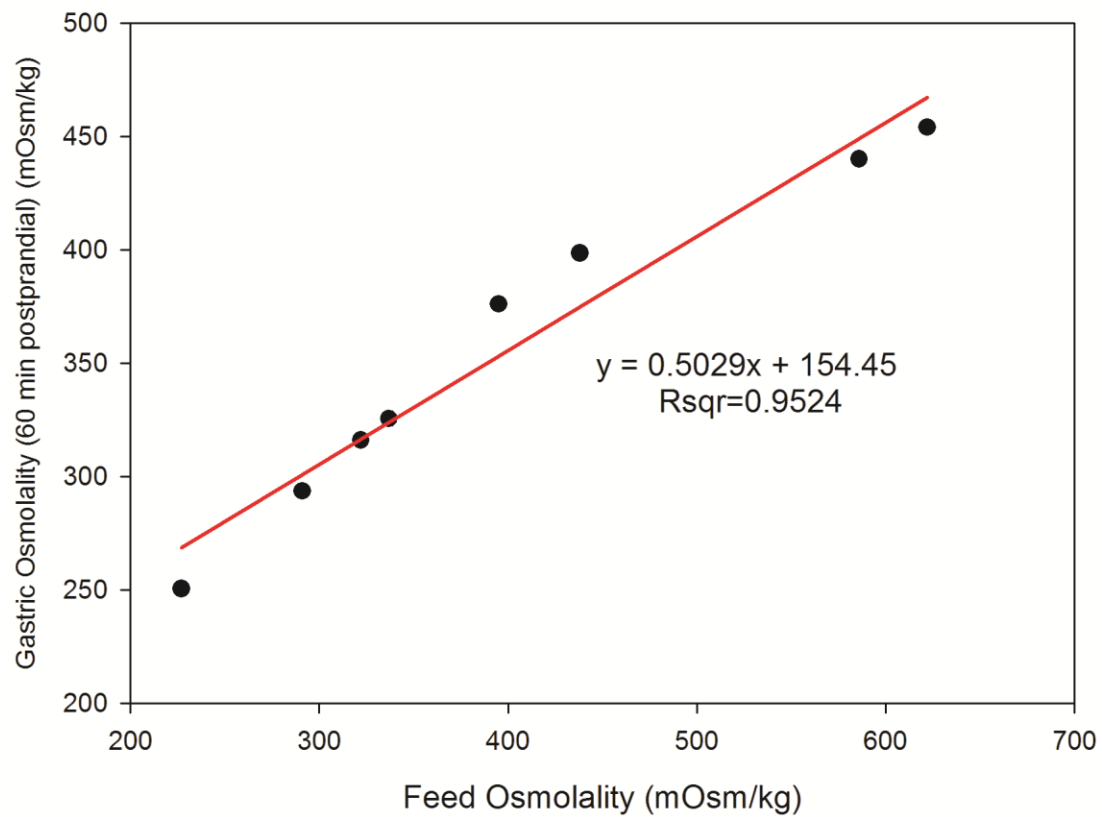
1103

1104   Figure 2: Neonatal gastric osmolality 60 minutes post-meal expressed as a function of feed osmolality.

1105   Data (black circles) represent average gastric osmolality values recorded amongst neonatal subjects as

1106   described by Billeaud et al (36). A linear regression model (red line) was fit to the data.

1107
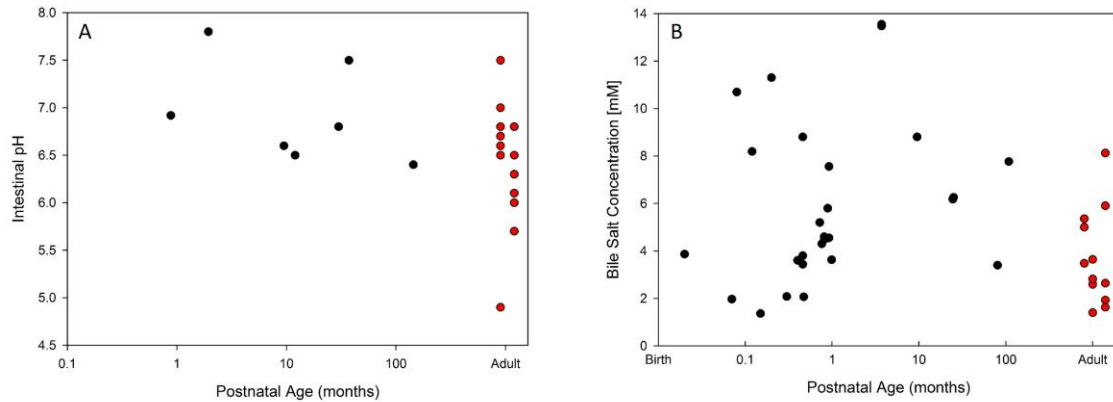
1108

1109   Figure 3: (A) Pediatric fasting intestinal pH (black circles) is depicted as the central tendency, either

1110   mean or median, from separate investigations (20-22, 89-92). Studies where pH was summarized over a

1111   specific age range without denoting the group's mean age were graphically depicted using the middle of

1112   the age range. The majority of data was derived from distal duodenum, though studies which included

1113   sampling sites from the proximal jejunum were also included. Adult duodenal bile acid concentrations

1114   (red circles) are depicted as mean values from separate studies, as summarized by Fuchs and Dressman

1115   (39). (B) Fasting duodenal bile salt concentrations amongst pediatrics (black circles) are depicted as the

1116   central tendency, either mean or median, from separate investigations (43, 44, 93-101). Studies where

1117   bile acids were summarized over a specific age range without denoting the group's mean age were

1118   graphically depicted using the middle of the age range. Adult duodenal bile acid concentrations (red

1119   circles) are depicted as mean values from separate studies, as summarized by Fuchs and Dressman (39).

1120
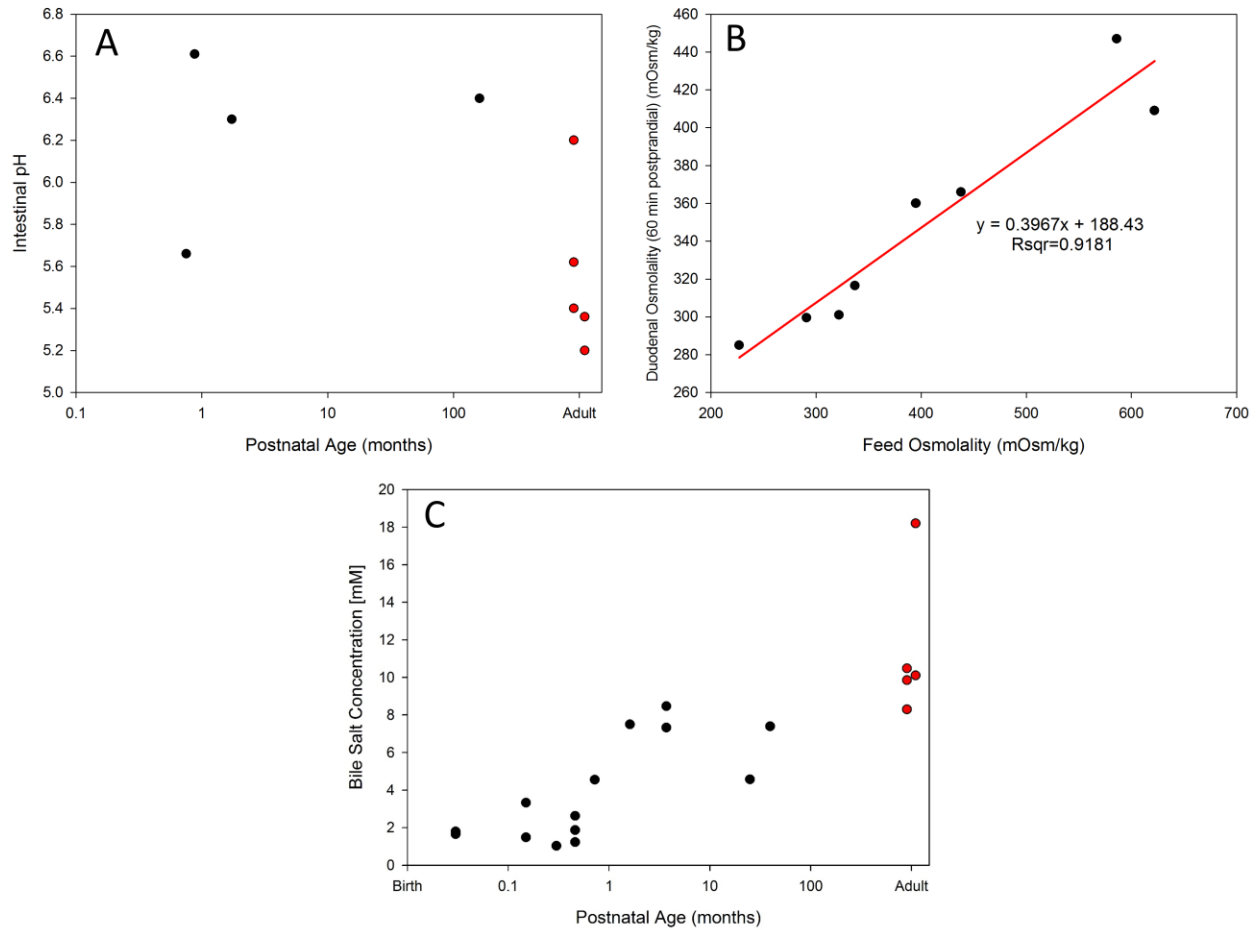
1121

Figure 4: (A) Fed-state duodenal pH from separate pediatric investigations (black circles) is depicted by

the central tendency, either mean or median (90, 102-104). Studies where pH was summarized over a

specific age range without denoting the group's mean age were graphically depicted using the middle of

the age range. Adult pH values (red circles) are presented as the central tendency (mean or median)

from separate investigations (33, 34, 40, 90, 105). (B) Neonatal duodenal osmolality 60 minutes post-

meal expressed as a function of feed osmolality. Data (black circles) represent average duodenal

1128   osmolality values recorded amongst neonatal subjects as described by Billeaud et al (36). A linear

1129   regression model (red line) was fit to the data. (C) Fed-state duodenal bile salt concentrations amongst

1130   pediatrics (black circles) is depicted as the mean from separate investigations (44, 96, 98, 99, 103, 106,

1131   107). Studies where data was summarized over a specific age range without denoting the group's mean

1132   age were graphically depicted using the middle of the age range. Adult duodenal bile acid

1133   concentrations (0.5-1hr postprandially) (red circles) are depicted as the mean value from the various

1134   publications (26, 33, 40, 108, 109).

1135

1136

Dapsone

Griseofulvin

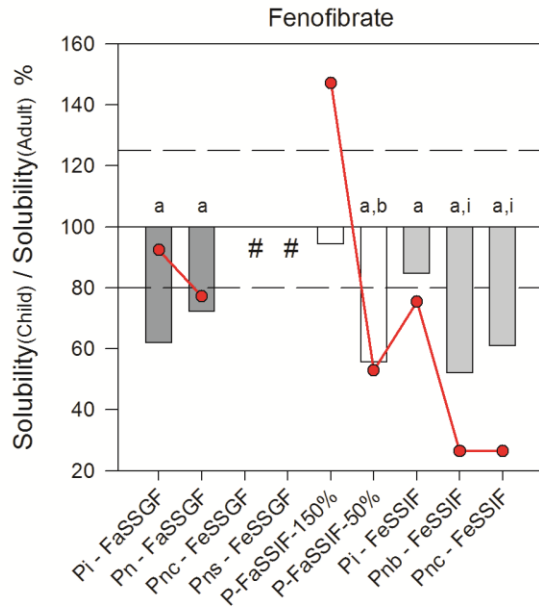Carbamazepine

Phenytoin

Spironolactone

Indomethacin
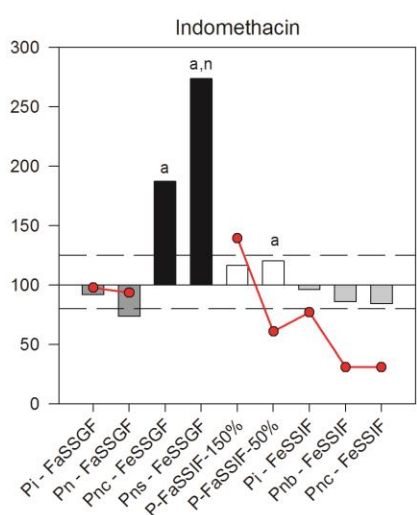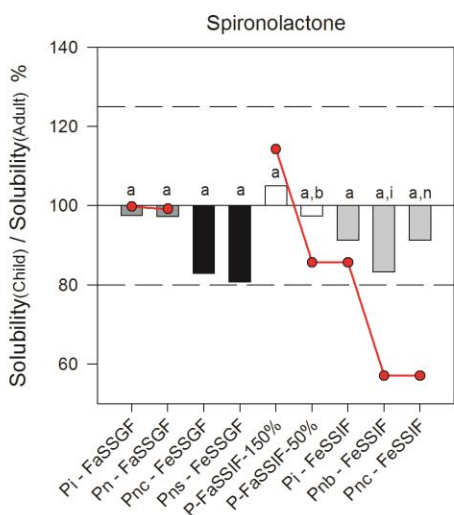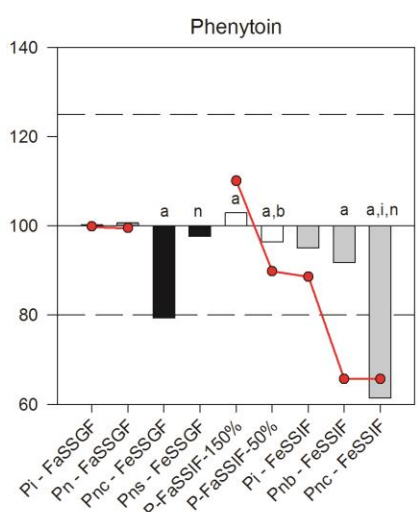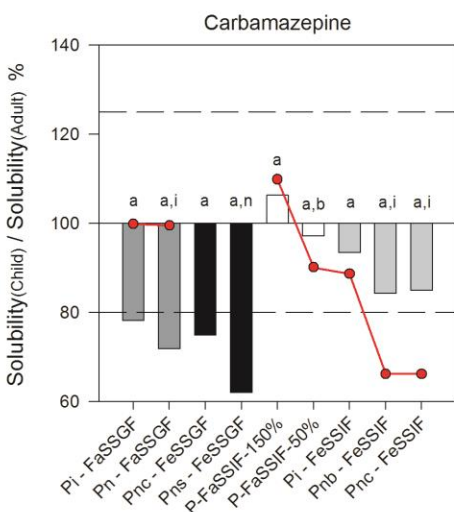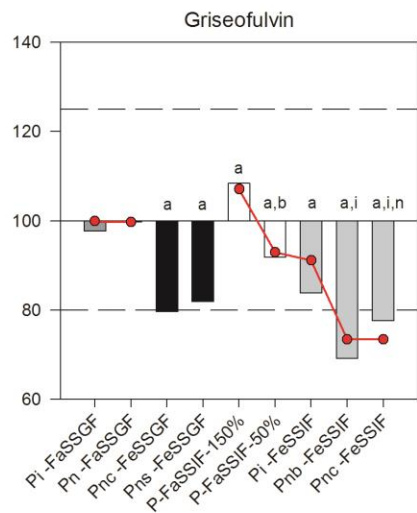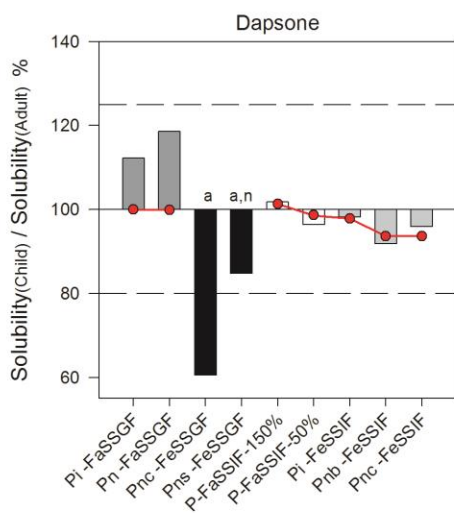
1138 Figure 5: Measured solubility in age-specific biorelevant media expressed as the mean solubility ratio

1139 (bars) between each respective pediatric and adult media (i.e. Pi-FaSSGF / FaSSGF$_{Adult}$). Predicted

1140 solubility ratios due to differences in media bile acid content were calculated for P-FaSSGF, P-FaSSIF, and

1141 P-FeSSIF according Mithani et al.'s equations (red line, dots) (17). Dashed lines (---) characterizing the

1142 bioequivalence criterion (80-125%) are displayed for reference. Media are denoted as follows: Pi-FaSSGF

1143 (Infant FaSSGF), Pn-FaSSGF (Neonate FaSSGF), Pnc-FeSSGF (Neonate FeSSGF comprised of cow's milk-

1144 based formula), Pns-FeSSGF (Neonate FeSSGF comprised of soy-based formula), P-FaSSIF-150% (Pediatric

1145 FaSSIF comprised with 4.5 mM NaTc), P-FaSSIF-50% (Pediatric FaSSIF comprised with 1.5 mM NaTc), Pi-

1146 FeSSIF (Infant FeSSIF), Pnb-FeSSIF (Neonatal breast-fed FeSSIF), and Pnc-FeSSIF (Neonatal formula-fed

1147 FeSSIF). Statistically significant solubility differences (p≤0.05) compared to (a) adult media, (i) infant

1148 media, (n) neonatal media, and (b) P-FaSSIF-150% were depicted using the symbols indicated.

1149

1150

Figure 6: Measured solubility in age-specific biorelevant media expressed as the mean solubility ratio (bars) between each respective pediatric and adult media (i.e. Pi-FaSSGF / FaSSGF$_{Adult}$).   Predicted solubility ratios due to differences in media bile acid content were calculated for P-FaSSGF, P-FaSSIF, and P-FeSSIF according Mithani et al.'s equations (red line, dots) (17). Dashed lines (---) characterizing the bioequivalence criterion (80-125%) are displayed for reference. For a description of media abbreviations and symbols (a,b,n,i), see the footnote to Figure 5.