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1 Developing a predictive *in vitro* dissolution model based on gastrointestinal 2 fluid characterisation in rats

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9

10 *Keywords*

11 *Biorelevant media; gastrointestinal fluids; rats; in vitro in vivo correlation.*

12

13 *Abbreviations*

14 ASSF, amorphous sodium salt of furosemide; GI, Gastrointestinal; HEPES, 4-(2-hydroxyethyl)-1-
15 piperazineethanesulfonic acid; IVIVC, in vitro in vivo correlation; MALDI, matrix-assisted laser desorption ionisation;
16 MSI, mass spectrometry imaging; RGM, Rat gastric medium; RIM, Rat intestinal medium.

17

18 **Abstract**

19 The influence of physiological factors on the solubility of drug compounds has been thoroughly
20 investigated in humans. However, as these factors vary between species and since many *in vivo*
21 studies are carried out in rats or mice, it has been difficult to establish sufficient *in vitro in vivo*
22 relations. The aim of this study was to develop a physiologically relevant *in vitro* dissolution model
23 simulating the gastrointestinal (GI) fluids of fasted rats and compare it to previously published *in*
24 *vitro* and *in vivo* data. To develop the *in vitro* model, the pH was measured *in situ* in six segments of
25 the GI tract of anaesthetised rats, then the fluids from the stomach, the proximal and the distal small
26 intestine were collected and characterized with regard to osmolality, and bile acid and phospholipid

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concentration. The pH and osmolality were found to increase throughout the GI tract. The bile acids and phospholipids were present in high concentrations in the proximal small intestine, and the bile acid concentration doubled in the distal part, where the phospholipid concentration decreased. Matrix-assisted laser desorption ionisation mass spectrometry imaging was applied on a cross section of the small intestine, to study which bile acids and phospholipid classes were present in the small intestine of rats. Both cholic acid, taurocholic acid and glycocholic acid were detected, and phosphatidylcholine (34:2) was found to be mainly present in the intestinal wall or mucus, whereas lysophosphatidylcholine (16:0) was also detected in the lumen. Based on these observations, biorelevant media were developed to simulate fluids in the stomach and the proximal part of the small intestine in fasted rats. The media were implemented in a two-step *in vitro* dissolution model, which was found to better predict the *in vivo* performance of furosemide, when compared to previously published *in vitro* and *in vivo* data.

1. Introduction

During the past years, there has been an intensified focus on how to improve the solubility of poorly water-soluble compounds to optimise their oral bioavailability. Many models are available to evaluate dissolution and solubility of poorly water-soluble compounds *in vitro* [1, 2]. However, currently available *in vitro* models have several times shown inability to predict oral preclinical *in vivo* pharmacokinetics of compounds with poor aqueous solubility [3-5]. For example, it was found in a recent study, that the *in vitro* dissolution of amorphous sodium salt of furosemide (ASSF) had a higher solubility and 20-fold faster dissolution rate compared to the crystalline acid form of furosemide. However, this did not translate into differences in the pharmacokinetic profile after oral dosing to rats [5].

As a step towards improvement of the IVIVC, great effort has been put into investigation of the conditions in the gastrointestinal (GI) tract of humans, to be able to perform the *in vitro* studies under conditions as close as possible to the *in vivo* situation [6-8]. Volume, pH, osmolality, surface tension and surfactant concentration are some of the parameters known to affect the dissolution and solubility of a compound in the GI tract. For both fasted and fed conditions, several fluid compositions have been suggested as biorelevant media to simulate conditions in the human GI tract, and hence, improve IVIVC [9-11]. Multiple biorelevant media are also commercially available, based on the composition of GI fluids in humans and dogs [12]. However, since the composition of the GI fluids vary greatly between species, it is challenging to obtain good IVIVC for *in vivo* studies performed in commonly used laboratory animals, *e.g.* rats and mice. Thus, an approach to improve IVIVC is to obtain knowledge about the fundamental aspects of the GI tract of common preclinical species (*e.g.* rats), which can be translated into novel *in vitro* models.

Warnken *et al.* [13] investigated the capability of an *in vitro* dissolution/absorption method using simulated media based on human data to predict *in vivo* performance in mice. The authors found that especially the change in pH to a value relevant for the preclinical species improved the IVIVC [13]. Besides the pH, also the concentration of bile acids and phospholipids has been found to vary between fasted GI fluids from rats and humans (Table 1). Values as pH and levels of bile acids and phospholipids not only vary from humans to rats, but also between studies of GI fluids from different breeds of fasted rats (Table 1). The current study will focus exclusively on values from fasted Sprague-Dawley rats, since fasted rats are commonly used for pharmacokinetic studies.

Table 1: Overview of studies evaluating pH and levels of bile acids, phospholipids and osmolality in the GI tract of fasted rats. Human values as recently reviewed by Bergström *et al.* [14] are included for comparison.

Parameter		Rat (fasted)	Human (fasted) ^d
pH	Stomach	1.4 – 1.9 ^a , 3.9 ^b , 4.3 (forestomach) ^c , 4.0 (glandular stomach) ^c , germ free rats: 3.8 (forestomach) ^c , 5.7 (glandular stomach) ^c .	1.7 – 3.3
	Proximal small intestine	6.7 – 7.0 ^a , 5.9 – 6.1 ^b , 7.1 ^c	5.6 – 7.8
	Distal small intestine	6.9 – 7.4 ^a , 5.9 ^b , 8.0 ^c	-

Bile acids (mM)	Stomach	< 5 ^a	0 – 0.8
	Proximal small intestine	51 ^a	1.4 – 5.9
	Distal small intestine	102 ^a	-
Phospholipids (mM)	Stomach	hardly detected ^a	0.029
	Proximal small intestine	1.5 – 5.5 ^a	0.1 – 0.6
	Distal small intestine	hardly detected ^a	-
Osmolality (mOsm/kg)	Stomach	-	119 – 221
	Proximal small intestine	-	137 – 300
	Distal small intestine	-	-

References: a: [15], b: [16], c: [17] and d: [14].

Furthermore, not only the amount, but also the type of bile acids and phospholipids are varying between species [18]. The most abundant bile acids in humans have recently been determined to be glyco-conjugates [19]. In contrast, Yang *et al.* [20] quantitatively investigated GI fluid from rats and found taurocholic acid to be the most abundant bile acid in the bile duct, whereas the unconjugated cholic acid was mainly present in the small intestine. However, more information is needed about the types of bile acids and phospholipids present in the GI tract of rats. One way to investigate this, is to use matrix-assisted laser desorption ionisation (MALDI) mass spectrometry imaging (MSI). MALDI MSI is a commonly used soft ionisation technique combining information about the molecular structure of an analyte with its location in the sample. It has previously been used to visualise drug distribution in tissue [21-23].

The aim of the current study was to investigate the total bile acid and phospholipid concentration, the osmolality and the pH in the stomach and small intestine of fasted rats, with the overall aim to produce gastric and intestinal biorelevant media simulating the composition of fasted rat GI fluid. In addition, the media were implemented in a setup currently used to study *in vitro* solubility, dissolution and drug release, to create a two-step model, simulating both gastric and intestinal conditions. Furthermore, an aim was to apply MALDI MSI to obtain detailed insight into which bile acid and phospholipid classes are most abundant in the proximal part of the small intestine in rats.

93 **2. Materials and methods**

94 **2.1 Materials**

95 Crystalline furosemide acid, 2,5-dihydroxybenzoic acid (DHB) and taurocholic acid sodium salt
96 hydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, sodium chloride and
97 chloroform were obtained from VWR (Radnor, PA, USA), where 4-(2-hydroxyethyl)-1-
98 piperazineethanesulfonic acid (HEPES) was acquired from Roth (Karlsruhe, Germany).
99 Phosphatidylcholine (purity: 99.1 %) and lysophosphatidylcholine (purity: 80 %), both from soybean
100 oil, were kindly donated from Lipoid (Steinhausen, Switzerland). Purified water was provided from
101 an Ultra Clear water system produced by Siemens (Munich, Germany).

102

103 **2.2 *In situ* pH measurements and sampling in anaesthetised rats**

104 The rat study was carried out at the Department of Pharmacy, University of Copenhagen, under the
105 license number 2016-15-0201-00892, and in compliance with Danish laws regulating experiments on
106 animals and EU Directive 2010/63/EU.

107 Six male Sprague-Dawley rats (295 ± 9 g on the day of the experiment) were purchased from Janvier
108 Labs (Le Genest-Saint-Isle, France) and were acclimatised for a minimum of 7 days on standard feed
109 with free access to water under controlled environmental parameters (temperature: 22.1 °C, relative
110 humidity: 57 %), and with a reversed day/night rhythm (red light during the day and normal light
111 during the night). Before starting the experiment, the animals were fasted for approximately 13 h.

112 The rats were anaesthetised subcutaneously with a mixture of Hypnorm (fentanyl 0.315 mg/mL;
113 fluanisone, 10 mg/mL)/Dormicum (midazolam, 5 mg/mL), with a dose of 236 µg/kg fentanyl, 7.5
114 mg/kg fluanisone and 3.75 mg/kg midazolam and placed on a 37 °C heating pad to keep the body
115 temperature constant. The abdominal wall was incised at *linea alba* and the stomach and the small
116 intestine were located. The pH was measured in four segments of the GI tract; forestomach, glandular

117 stomach, proximal small intestine (5 to 20 cm distal to the stomach) and distal small intestine (5 to
118 20 cm proximal to the caecum) (Fig. 1).

119

120 **Figure 1:** Schematic of the sampling and pH measurements in the GI tract of a rat. The investigated locations are
121 illustrated by the red dots and correspond to; forestomach, glandular stomach, proximal small intestine (5 to 20 cm distal
122 to the stomach) and distal small intestine (5 to 20 cm proximal to the caecum).

123

124 The pH was measured through a small incision in the different segments, using a micro pH electrode
125 (Metrohm, Herisau, Switzerland) connected to a portable pH meter, PHM201 (Radiometer
126 Analytical, Lyon, France). The pH was measured three times in each segment. Hereafter, fluid
127 samples were collected with a syringe from the stomach, the proximal small intestine and the distal
128 small intestine and transferred to polypropylene microtubes. All measurements and sampling were
129 performed within 30 min after anesthesia for each rat.

130

131 2.3 Investigation of osmolality, bile acid and phospholipid levels

132 The osmolality of each sample was measured on the day of sampling on an Osmomat 030-D automatic
133 cryoscopic osmometer (Gonotec, Berlin, Germany), and the samples were stored at -20 °C until
134 further analysis. The total concentrations of bile acids and phospholipids were determined with a
135 fluorometric and colourimetric enzymatic assay kit, respectively (Sigma-Aldrich, MO, USA). The
136 total concentration of bile acids was determined as the combined concentration of the twelve most
137 common conjugated and unconjugated bile acids, whereas the total concentration of phospholipids
138 was measured as the combined concentration of choline containing phospholipids in the samples. The
139 fluids sampled from the GI tract of rats were centrifuged, and the supernatant was diluted with
140 purified water to be within the range of the calibration curve for the kits. All standards and samples
141 were measured on a multimode microplate reader according to the wavelengths in the protocol from
142 the manufacturer (Thermo Fisher Scientific, MA, USA).

143

144 **2.4 MALDI MSI on sections from the small intestine**

145 The study was carried out under the license number and in compliance with the directives described
146 in section 2.2. One male Sprague-Dawley rat (305 g on the day of the experiment) was used for
147 studies of the distribution of bile acids and phospholipids in the small intestine. The rat was
148 acclimatised and anaesthetised as described in section 2.2, but with a normal light cycle. The rat was
149 fasted one hour prior to the experiment. The abdominal wall was incised at *linea alba* and the small
150 intestine was located. The small intestine was cut out, immediately transferred to ice and stored at -
151 80 °C until further analysis. The small intestine was cut in four segments (0, 10, 20 and 30 cm distal
152 to the stomach) and pieces of the intestine were mounted on a cryo-microtome specimen plate using
153 water as the only adhesive. The intestine was vertically mounted such that cross sections could be cut
154 and mounted on glass slides. The intestine was cut in 25 µm thin sections using a Leica CM3050S

155 cryo-microtome (Leica Microsystems, Wetzlar, Germany), and the sections were thaw-mounted on
156 glass slides, which were subsequently stored at -80 °C until time of analysis.

157 At time of analysis, the samples were taken directly from -80 °C to a vacuum desiccator where they
158 reached room temperature, thus avoiding condensation of water during thawing of the samples. The
159 sample was coated by MALDI matrix by spraying a 30 mg/mL solution of 2,5-dihydroxybenzoic acid
160 (dissolved in methanol and water, 70:30 v/v) using a setup described in details elsewhere [24].

161 The sample was analysed on a QExactive orbitrap mass spectrometer (Thermo Scientific, Bremen,
162 Germany) equipped with the AP-SMALDI10 ion source (TransMIT GmbH, Giessen, Germany).

163 Imaging was performed in the positive ion mode (scan range m/z 230 – 920) with a pixel size of 10-
164 25 μ m, and a DHB matrix peak was used as lock-mass for internal mass calibration, ensuring a mass
165 accuracy of ± 1 ppm or better. The raw file was converted to imzML using an imzML converter [25],
166 and images were generated in MSiReader 1.01 [26] using a bin width of 6 ppm. To compensate for
167 differences in ionisation efficiency across the sample, the intensities were normalised to the total ion
168 current (TIC) of each individual mass spectrum. For each image, the colour scale was adjusted in
169 order to enable details in the low-intensity regions of the images to appear clearly.

170

171 **2.5 Preparation of biorelevant media**

172 For preparation of the biorelevant media (Table 3), the required amount of a stock solution of
173 lysophosphatidylcholine in chloroform (300 mM) was pipetted and a lipid film was formed by
174 complete evaporation of the solvent under a steady stream of nitrogen. Sodium taurocholate and
175 sodium chloride were added to achieve the desired bile acid concentrations and osmolalities and
176 HCl/HEPES was added according to Table 3. The media were stirred overnight at 37 °C and the next
177 day the pH was adjusted to the desired value followed by addition of solvent to reach volume. The

178 osmolality was measured using an Osmomat 030-D automatic cryoscopic osmometer (Gonotec,
179 Berlin, Germany).

180

181 **2.6 Preparation of amorphous sodium salt of furosemide**

182 An ASSF was prepared as previously described by Nielsen *et al.* [5]. An amount of 0.4 % w/v
183 furosemide and 5 M NaOH (molar ratio of 1:1 with furosemide) was added to a solution of purified
184 water and 96 % ethanol (10:1 v/v). The solution was spray dried and the obtained powder was stored
185 in a glass vial protected from light.

186

187 **2.7 Dissolution of crystalline free acid and amorphous sodium salt of furosemide**

188 The dissolution of crystalline furosemide acid and ASSF in rat gastric medium (RGM) and rat
189 intestinal medium (RIM) was measured on a μ DISS ProfilerTM. The dissolution was measured as
190 intrinsic dissolution as previously described by Nielsen *et al.* [5] in order to compare the dissolution
191 curves. Briefly, experiments were performed at 37 °C with a stirring rate of 100 RPM, and the path
192 length of the UV probes were 1 mm. For the dissolution experiments, small discs of the two
193 furosemide formulations were prepared using a mini-IDR tablet compression system (Heath
194 Scientific, Milton Keynes, U.K.). Each disc comprised of 5 – 8 mg of either of the furosemide forms
195 (compressed at 80 bar for 40 s). The disc loaded with furosemide was inserted into a cylindrical
196 magnetic stirring bar, transferred to a sample vial and covered with 10 mL biorelevant medium (Table
197 3). UV measurements were carried out every 10 s with a total run time of 30 min under gastric
198 conditions and 3 h and 33 min under intestinal conditions. A wavelength range of 310 – 350 nm was
199 used to determine the concentration of dissolved furosemide. Furthermore, the dissolution of
200 crystalline furosemide acid was evaluated in the developed two-step model (Section 3.4). All
201 experiments were performed in 3 – 5 replicates.

202

203 **2.8 Data analysis**

204 Boxplots from the *in situ* rat studies are shown as minimum value, median and maximum value, and
205 25 % and 75 % percentiles were included where $n > 3$. Thus, the osmolality, bile acid and
206 phospholipid levels are reported as medians. For other tables and graphs, the results are shown as
207 mean $\pm$ standard deviation (SD).

208 Visualisation and statistical analysis were carried out in GraphPad Prism version 6.0 (GraphPad
209 software Inc., La Jolla, CA, USA). Statistical differences ($p < 0.05$) were determined using one-way
210 ANOVA with a Tukey's multiple comparison test.

211

212 **3. Results and discussion**

213 In the present study the rats were fasted during the inactive part of their cycle. Rats are nocturnal
214 animals, i.e. they are active and eating during the night. This was reversed by acclimatising them in
215 normal light during the night and red light during the day. For fasted state pharmacokinetic studies,
216 rats are often fasted during their active period (overnight under normal light conditions), which can
217 lead to stress symptoms and aggressive behaviour during handling. Furthermore, it has previously
218 been observed by *ex vivo* examination, that the stomach of the rats was filled with bedding material
219 after fasting during the active part of their cycle (data not published). All six rats included in the
220 present study appeared healthy and non-stressed after fasting in the inactive part of their cycle, and
221 no content was observed in the stomach at the beginning of the experiment.

222 In the previously published study by Nielsen *et al.* [5], which was used to evaluate the *in vitro in vivo*
223 relation of the developed *in vitro* model, the rats were fasted overnight without a reversed day/night
224 cycle. Although this is different from the fasting method used in the present study, it was decided to
225 apply the reversed day/night cycle for ethical reasons and for the wellbeing of the animals.

226 3.1 *In situ* pH measurements

227 The pH values were measured in the forestomach, glandular stomach, proximal and distal small
228 intestine of the rats (Table 2).

229

230 **Table 2:** pH values measured *in situ* in the GI tract of rats (mean $\pm$ SD, n= 4–6).

GI section	Forestomach	Glandular stomach	Proximal small intestine	Distal small intestine
pH	1.9 ± 0.3	2.9 ± 0.7	7.5 ± 0.3	7.8 ± 0.3

231

232 The pH in the forestomach was measured to be 1.9 ± 0.3 , which was significantly lower ($p < 0.05$)
233 than pH in the glandular stomach (2.9 ± 0.7). For all the individual rats, a similar significant rank
234 order between pH in the forestomach and glandular stomach was seen, implying that the large SD
235 occurs from inter-individual variations. The higher pH in the glandular stomach was not expected
236 since the parietal cells responsible for acid secretion are located in this part of the stomach [27], thus,
237 a more acidic environment would be expected in this area. In literature, only one study has
238 investigated the pH separately in both the forestomach and the glandular stomach of fasted rats (both
239 conventional and germ free), where the gastric pH values in germ free rats (Table 1) followed trends
240 similar to the current study.

241 In the small intestine, the pH was measured to be 7.5 ± 0.3 in the proximal part, whereas it increased
242 to be 7.8 ± 0.3 in the distal part (Table 2). In literature, similar pH values have been found after *in*
243 *situ* measurements in the proximal and distal part of the small intestine in fasted rats (Table 1).

244

245 3.2 Bile acids, phospholipids and osmolality

246 The levels of bile acids, phospholipids and osmolality were determined in the stomach, proximal and
247 distal small intestine (Fig. 2). A bile acid concentration of 1.3 mM was measured in the stomach (Fig.
248 2A). Presence of bile acids in the stomach was not expected, since the bile duct enters the GI tract in

the small intestine [27], but it could be due to reflux from the small intestine to the stomach. This phenomenon has previously been observed in humans [14]. In the small intestine, the bile acid concentration was found to be 24.1 mM in the proximal part and increasing to 54.6 mM in the distal part (Fig. 2A). Phospholipids were hardly detected in the stomach (0.8 mM) (Fig. 2B), resulting in a bile acid/phospholipid ratio of 1.6. The concentration of phospholipids was determined to be 3.7 mM and 0.4 mM in proximal and distal part, respectively. Hence, this results in bile acid/phospholipid ratios of 6.5 in the proximal part and 137 in the distal part of the small intestine. The osmolality in the stomach, proximal and distal small intestine was determined to be 230, 312 and 328 mOsm/kg, respectively (Fig. 2C).

Figure 2: Boxplots showing the concentration of **A:** bile acids, **B:** phospholipids and **C:** the osmolality measured in the stomach and proximal and distal small intestine of rats. Results are shown as individual measurements, minimum value, median and maximum value, and 25 % and 75 % percentiles were included were $n > 3$ ($n = 3-6$).

A similar study found comparable phospholipid concentrations in fasted rats, but two times higher bile acid concentrations (Table 1). Albeit variations in the concentration of bile acids, the overall trend is that the highest concentrations in rats are detected in the distal part of the small intestine. Compared to phospholipid values in the human small intestine (Table 1), the concentration in rats is in a similar concentration range. However, the bile acid concentration in the small intestine of rats is

269 much higher than in the fasted human small intestine (Table 1). An explanation for this difference
270 can be the continuous flow of bile from the bile duct in rats, due to the absence of a gallbladder in
271 rats [15].

272 Compared to the osmolalities detected in the present study (Fig. 2C), the osmolality in human GI
273 fluids has been reported to be slightly lower (Table 1). For fluids simulating the human gastric and
274 intestinal conditions in the fasted state, osmolalities of 120 and 180 mOsm/kg are traditionally applied
275 [9]. These values are notably lower than the values measured in the stomach and proximal small
276 intestine of fasted rats in the current study (230 and 312 mOsm/kg, respectively).

277

278 3.3 MALDI MSI on sections from the small intestine

279 In addition to determination of the total concentrations of bile acids and phospholipids (Section 3.2),
280 the location of different bile acids and phospholipid classes were investigated by MALDI MSI of a
281 cross section of the small intestine (10 cm distal to the stomach) from a fasted rat.

282

283 Cholic acid and its conjugates have shown to be the most dominating bile acid in intestinal fluids in
284 humans and rats [28], and especially sodium taurocholate has been preferred as bile acid for the
285 preparation of biorelevant media [9, 10, 28]. Thus, the presence of cholic acid, taurocholic acid and
286 glycocholic acid, was investigated in a cross section of a rat small intestine 10 cm distal to the stomach
287 (Fig. 3).

288

289 **Figure 3:** MALDI MSI images of a cross section of a rat small intestine, 10 cm distal to the stomach. **A:** Cholic acid (K^+
 290 adduct, m/z 447.2507), **B:** taurocholic acid (K^+ adduct, m/z 554.2548) and **C:** glycocholic acid (K^+ adduct, m/z 504.2722).
 291

292 Both cholic acid, taurocholic acid and glycocholic acid were present in the lumen of the GI tract (Fig.
 293 3A, B and C). As previously described, Yang *et al.* [20] recently determined taurine conjugated bile
 294 acids to be the most abundant type in bile from rats, whereas the unconjugated cholic acid play a
 295 dominant role in the small intestine.
 296

298 **Figure 4:** MALDI MSI images of a cross section of a rat small intestine, 10 cm distal to the stomach. **A:**
 299 Phosphatidylcholine 34:2, **B:** Lysophosphatidylcholine 16:0 and **C:** Coloured overlay with phosphatidylcholine 34:2
 300 shown in red and lysophosphatidylcholine 16:0 shown in green. The images show the presence of lysophosphatidylcholine
 301 16:0 in the entire cross section and phosphatidylcholine 34:2 primarily present in the intestinal wall. Both compounds
 302 were predominantly ionised, and therefore shown, as their potassium adducts.
 303
 304

305 As seen in Fig. 4A, phosphatidylcholine 34:2 was found to be mainly present in the wall of the GI
 306 tract or in the mucus, and to a less extent in the lumen, whereas lysophosphatidylcholine 16:0 was
 307 quite homogeneously distributed in the entire cross section of the GI tract (Fig. 4B). In the upper
 308 cross sections (0-10 cm distal to the stomach) of the intestine, similar distributions were also observed
 309 for several other phosphatidylcholine and lysophosphatidylcholine species than the
 310 phosphatidylcholine 34:2 and lysophosphatidylcholine 16:0 shown here. However,
 311 lysophosphatidylcholine was not observed in the lumen further down in the small intestine (Appendix
 312 A).
 313

314 These findings illustrated in Fig. 4 are supported by previous investigations [29, 30], where
315 lysophosphatidylcholine was determined to be the most predominant phospholipid class in fasted
316 intestinal fluids from humans. In bile, the most predominant phospholipid class is
317 phosphatidylcholine, however it is hydrolysed to lysophosphatidylcholine by phospholipases in the
318 small intestine, making lysophosphatidylcholine the most abundant phospholipid class in the small
319 intestine [7].

320

321 **3.4 Development of biorelevant media and two-step *in vitro* model**

322 RGM and RIM were designed to simulate the conditions observed in the GI tract of fasted rats,
323 regarding pH and levels of bile acids, phospholipids and osmolality (Sections 3.1 and 3.2). RIM was
324 composed to simulate the conditions in the proximal part of the small intestine. The media were
325 combined in a two-step *in vitro* dissolution model, to study release and dissolution of drug with a
326 μ DISS Profiler™ in both media consecutively. The gastric step was run for 30 min, where after the
327 medium was changed to simulate intestinal conditions, by addition of 5 mL concentrated bile buffer
328 (Table 3).

329 Lysophosphatidylcholine was selected to simulate phospholipids in the developed media, since the
330 MALDI MSI results revealed a more pronounced presence of lysophosphatidylcholine in the lumen
331 of the upper part of the small intestine, compared to phosphatidylcholine (Section 3.3). Traditionally
332 phosphate buffers have been widely used to control pH, but they have low buffering capacity above
333 pH 7.5 and also a tendency to precipitate cations [31]. Thus, HEPES ($pK_a = 7.55$, [31]) was selected
334 as buffering agent in the developed media.

335

336 **Table 3:** Composition of the developed biorelevant media and concentrated bile buffer. The composition of the bile buffer
337 was calculated so that mixing RGM and bile buffer in a ratio of 2 to 1 would result in a biorelevant medium with the
338 composition of RIM.

	RGM	RIM	Bile buffer
Properties			
pH	2.4*	7.5	7.5
Osmolality (mOsm/kg)	230	312	476
BS:PL ratio	1.6	6.5	7.3
Composition			
HCl (mM)	4	-	-
HEPES (mM)	-	100	300
Sodium taurocholate (mM)	1.3	24.1	69.7
Lysophosphatidylcholine (mM)	0.8	3.7	9.5
Sodium chloride (mM)	111.7	119.6	139.4
NaOH/HCl (pH adjustment)	<i>q.s.</i>	<i>q.s.</i>	<i>q.s.</i>

*mean value of the pH in the forestomach and the glandular stomach.

3.5 Evaluation of the biorelevant media and the two-step *in vitro* model

The developed media (Table 3) were applied in a μ DISS Profiler™ to study the dissolution of crystalline furosemide acid and ASSF. The dissolution of the two furosemide forms has previously been evaluated in a biorelevant medium simulating human intestinal conditions (pH 6.5 and 12.5 mM of total surfactant concentration) [5]. However, despite large differences in the *in vitro* dissolution of the furosemide forms, Nielsen *et al.* experienced no significant difference of their performance *in vivo* (Fig. 6) in rats. As an application of the developed media, it was aimed to improve the *in vitro* *in vivo* relation, by performing similar *in vitro* dissolution experiments in the newly developed biorelevant media specifically developed to simulate rats.

First, the dissolution of crystalline furosemide acid and ASSF was investigated individually in RGM and RIM, respectively (Fig. 5A and B).

Figure 5: The percentage of furosemide dissolved as a function of time. The dissolution was measured in **A:** RGM, **B:** RIM and **C:** Two-step model including a 30 min gastric step (RGM) before changing the dissolution medium to RIM (mean $\pm$ SD, $n = 3 - 5$).

In RGM, a dissolution of 87.9 ± 3.0 % was observed for ASSF within the first 5 min of the experiment, followed by a plateau of 95.6 ± 3.7 % dissolved after 15 min (Fig. 5A). In contrast, only 1.1 ± 0.5 % of the loaded amount of crystalline acid was dissolved within 30 min in RGM. This indicates that the crystalline acid is very poorly soluble in RGM, which correlates well with furosemides pK_a -value of 4.2 [6]. Amorphisation and salt formation are both well-known tools to increase the solubility of a compound [32], which appears to be the case for furosemide in RGM as well (Fig. 5A). In RIM, the crystalline acid was almost completely dissolved (94.9 ± 1.1 %) after approximately 90 min in RIM, and this was still slower than for ASSF where 99.3 ± 13.0 % was dissolved after 10 min (Fig. 5B).

To evaluate the two-step dissolution model, the dissolution of crystalline furosemide acid was further investigated in the *in vitro* model with the two media incorporated (Fig. 5C). ASSF was not evaluated in the two-step dissolution model due to the fast dissolution of ASSF in RGM (90 % in 5 min). The dissolution of furosemide crystalline acid in the two-step model showed dissolution trends similar to the dissolution in the individual media. Very limited dissolution was observed in RGM (1.2 ± 0.6 %), but 100.5 ± 6.5 % crystalline furosemide acid was dissolved after 90 min after changing the medium to RIM (120 min in total).

Comparing the *in vitro* dissolution results in the developed media (Fig. 5) with the *in vitro* and *in vivo* results published by Nielsen *et al.* [5] (Fig. 6A and B, respectively) reveals interesting trends. By

376 changing the dissolution medium to RIM, the percentage of dissolved furosemide was increased from
 377 approximately 18 % after 90 min to 95 % after 90 min, for the crystalline acid.

378
 379 **Figure 6:** Previously published furosemide *in vitro* and *in vivo* data. **A:** The percentage of dissolved furosemide as a
 380 function of time (min) studied by dissolution in human simulated intestinal fluid using a μ DISS Profiler™. **B:** Furosemide
 381 concentration in plasma over time studied by oral gavage in rats. Reprinted from [5] with permission.
 382

383 This difference can be explained by the physicochemical properties of furosemide. Increasing the pH
 384 one unit away from the pK_a -value (from pH 6.5 to 7.5) will increase the concentration of the ionised
 385 form of furosemide by a factor 10, resulting in a higher solubility in water. Additionally, increasing
 386 the concentration of bile acids and phospholipids have shown to increase the solubility of many poorly
 387 water-soluble compounds [33, 34]. Hence, the increase in total concentration of bile acids and
 388 phospholipids from 12.5 mM in the human intestinal medium to 27.8 mM in RIM could also
 389 contribute to the increased dissolution.

390
 391 Since both crystalline furosemide acid and ASSF reach more than 90 % dissolved after the first 90
 392 min in RIM (Fig. 5B), it seems more reasonable to predict that there is no significant difference
 393 between the *in vivo* performances of the two compounds. This was also the result from the *in vivo*
 394 study in rats performed by Nielsen *et al.* (Fig. 6B). Based on this comparison, it is, in the present case,
 395 more predictive to apply RIM than human simulated intestinal fluid for *in vitro* dissolution studies
 396 prior to *in vivo* studies in rats.

397

398 **4. Conclusion**

399 The pH and osmolality were found to increase throughout the GI tract of fasted rats and was
400 significant different from human values. Bile acids and phospholipids were present in high
401 concentrations in the proximal small intestine, and the bile acid concentration doubled in the distal
402 part. From MALDI MSI of an intestinal cross section from a fasted rat, the bile acids cholic acid,
403 glycocholic acid and taurocholic acid were found to be present in the lumen of the small intestine.
404 Phosphatidylcholine species were mainly detected in the intestinal wall or mucus of the small
405 intestine, whereas lysophosphatidylcholine species were detected in the whole cross section including
406 the lumen of the upper part of the small intestine. Based on these observations, a two-step *in vitro*
407 dissolution model, with biorelevant media simulating GI fluids of rats, was developed. The
408 incorporation of the media simulating GI fluids in fasted rats provided better prediction of the *in vivo*
409 performance of furosemide in rats compared to human biorelevant media. This indicates that the
410 model is a promising tool to *in vitro* evaluation of a compound before proceeding to *in vivo* studies
411 in rats. However, similar evaluations of other model drugs would be needed to evaluate the full
412 potential of the model.

413

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419

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421

422 5. References

- 423 1. C. J. Andreas, J. Rosenberger, J. Butler, P. Augustijns, M. McAllister, B. Abrahamsson, J.
424 Dressman, Introduction to the OrBiTo decision tree to select the most appropriate in vitro
425 methodology for release testing of solid oral dosage forms during development, *Eur. J. Pharm.*
426 *Biopharm.* 130 (2018) p. 207-213.
- 427 2. J. Butler, B. Hens, M. Vertzoni, J. Brouwers, P. Berben, J. Dressman, C. J. Andreas, K. J.
428 Schaefer, J. Mann, M. McAllister, M. Jamei, E. Kostewicz, F. Kesisoglou, P. Langguth, M.
429 Minekus, A. Mullertz, R. Schilderink, M. Koziolk, P. Jedamzik, W. Weitschies, C. Reppas,
430 P. Augustijns, In vitro models for the prediction of in vivo performance of oral dosage forms:
431 Recent progress from partnership through the IMI OrBiTo collaboration, *Eur. J. Pharm.*
432 *Biopharm.* 136 (2019) p. 70-83.
- 433 3. H. Higashino, T. Hasegawa, M. Yamamoto, R. Matsui, Y. Masaoka, M. Kataoka, S. Sakuma,
434 S. Yamashita, In vitro-in vivo correlation of the effect of supersaturation on the intestinal
435 absorption of BCS Class 2 drugs, *Mol. Pharmaceutics* 11 (2014) p. 746-754.
- 436 4. T. Tran, S. D. V. S. Siqueira, H. Amenitsch, A. Müllertz, T. Rades, In vitro and in vivo
437 performance of monoacyl phospholipid-based self-emulsifying drug delivery systems, *J.*
438 *Controlled Release* 255 (2017) p. 45-53.
- 439 5. L. H. Nielsen, S. Gordon, R. Holm, A. Selen, T. Rades, A. Mullertz, Preparation of an
440 amorphous sodium furosemide salt improves solubility and dissolution rate and leads to a
441 faster Tmax after oral dosing to rats, *Eur. J. Pharm. Biopharm.* 85 (2013) p. 942-951.
- 442 6. P. Augustijns, B. Wuyts, B. Hens, P. Annaert, J. Butler, J. Brouwers, A review of drug
443 solubility in human intestinal fluids: implications for the prediction of oral absorption, *Eur. J.*
444 *Pharm. Sci.* 57 (2014) p. 322-332.
- 445 7. A. Fuchs, J. B. Dressman, Composition and Physicochemical Properties of Fasted State
446 Human Duodenal and Jejunal Fluid: A Critical Evaluation of the Available Data, *J. Pharm.*
447 *Sci.* 103 (2014) p. 3398-3411.
- 448 8. P. B. Pedersen, P. Vilmann, D. Bar-Shalom, A. Mullertz, S. Baldursdottir, Characterization
449 of fasted human gastric fluid for relevant rheological parameters and gastric lipase activities,
450 *Eur. J. Pharm. Biopharm.* 85 (2013) p. 958-965.
- 451 9. E. Jantratid, N. Janssen, C. Reppas, J. B. Dressman, Dissolution media simulating conditions
452 in the proximal human gastrointestinal tract: An update, *Pharm. Res.* 25 (2008) p. 1663-1676.
- 453 10. A. Fuchs, M. Leigh, B. Kloefer, J. B. Dressman, Advances in the design of fasted state
454 simulating intestinal fluids: FaSSIF-V3, *Eur. J. Pharm. Biopharm.* 94 (2015) p. 229-240.
- 455 11. C. Markopoulos, C. J. Andreas, M. Vertzoni, J. Dressman, C. Reppas, In-vitro simulation of
456 luminal conditions for evaluation of performance of oral drug products: Choosing the
457 appropriate test media, *Eur. J. Pharm. Biopharm.* 93 (2015) p. 173-182.
- 458 12. Y. Tanaka, T. Baba, K. Tagawa, R. Waki, S. Nagata, Prediction of oral absorption of low-
459 solubility drugs by using rat simulated gastrointestinal fluids: the importance of regional
460 differences in membrane permeability and solubility, *J. Pharm. Pharm. Sci.* 17 (2014) p. 106-
461 120.
- 462 13. Z. Warnken, M. Puppulo, J. Hughey, I. Duarte, S. Jansen-Varnum, In Vitro-In Vivo
463 Correlations of Carbamazepine Nanodispersions for Application in Formulation
464 Development, *J. Pharm. Sci.* 107 (2018) p. 453-465.
- 465 14. C. A. Bergstrom, R. Holm, S. A. Jorgensen, S. B. Andersson, P. Artursson, S. Beato, A. Borde,
466 K. Box, M. Brewster, J. Dressman, K. I. Feng, G. Halbert, E. Kostewicz, M. McAllister, U.
467 Muenster, J. Thinner, R. Taylor, A. Mullertz, Early pharmaceutical profiling to predict oral
468 drug absorption: current status and unmet needs, *Eur. J. Pharm. Sci.* 57 (2014) p. 173-199.

- 469 15. Y. Tanaka, T. Hara, R. Waki, S. Nagata, Regional differences in the components of luminal
470 water from rat gastrointestinal tract and comparison with other species, *J. Pharm. Pharm. Sci.*
471 15 (2012) p. 510-518.
- 472 16. E. L. McConnell, A. W. Basit, S. Murdan, Measurements of rat and mouse gastrointestinal
473 pH, fluid and lymphoid tissue, and implications for in-vivo experiments, *J. Pharm. Pharmacol.*
474 60 (2008) p. 63-70.
- 475 17. F. W. Ward, M. E. Coates, Gastrointestinal pH measurement in rats: influence of the microbial
476 flora, diet and fasting, *Lab. Anim.* 21 (1987) p. 216-222.
- 477 18. R. Holm, A. Mullertz, H. Mu, Bile salts and their importance for drug absorption, *Int. J.*
478 *Pharm.* 453 (2013) p. 44-55.
- 479 19. D. Riethorst, R. Mols, G. Duchateau, J. Tack, J. Brouwers, P. Augustijns, Characterization of
480 Human Duodenal Fluids in Fasted and Fed State Conditions, *J. Pharm. Sci.* 105 (2016) p. 673-
481 681.
- 482 20. T. Yang, T. Shu, G. Liu, H. Mei, X. Zhu, X. Huang, L. Zhang, Z. Jiang, Quantitative profiling
483 of 19 bile acids in rat plasma, liver, bile and different intestinal section contents to investigate
484 bile acid homeostasis and the application of temporal variation of endogenous bile acids, *J.*
485 *Steroid Biochem. Mol. Biol.* 172 (2017) p. 69-78.
- 486 21. A. Rompp, B. Spengler, Mass spectrometry imaging with high resolution in mass and space,
487 *Histochem. Cell Biol.* 139 (2013) p. 759-783.
- 488 22. E. Marxen, L. Jin, J. Jacobsen, C. Janfelt, B. Hyrup, J. A. Nicolazzo, Effect of Permeation
489 Enhancers on the Buccal Permeability of Nicotine: Ex vivo Transport Studies Complemented
490 by MALDI MS Imaging, *Pharm. Res.* 35 (2018) p. 70.
- 491 23. S. E. Hansen, E. Marxen, C. Janfelt, J. Jacobsen, Buccal delivery of small molecules - Impact
492 of levulinic acid, oleic acid, sodium dodecyl sulfate and hypotonicity on ex vivo permeability
493 and spatial distribution in mucosa, *Eur. J. Pharm. Biopharm.* 133 (2018) p. 250-257.
- 494 24. E. Wenande, U. H. Olesen, M. M. Nielsen, C. Janfelt, S. H. Hansen, R. R. Anderson, M.
495 Haedersdal, Fractional laser-assisted topical delivery leads to enhanced, accelerated and
496 deeper cutaneous 5-fluorouracil uptake, *Expert Opin. Drug Delivery* 14 (2017) p. 307-317.
- 497 25. T. Schramm, A. Hester, I. Klinkert, J. P. Both, R. M. Heeren, A. Brunelle, O. Laprevote, N.
498 Desbenoit, M. F. Robbe, M. Stoeckli, B. Spengler, A. Rompp, imzML--a common data format
499 for the flexible exchange and processing of mass spectrometry imaging data, *J. Proteomics* 75
500 (2012) p. 5106-5110.
- 501 26. G. Robichaud, K. P. Garrard, J. A. Barry, D. C. Muddiman, MSiReader: an open-source
502 interface to view and analyze high resolving power MS imaging files on Matlab platform, *J.*
503 *Am. Soc. Mass Spectrom.* 24 (2013) p. 718-721.
- 504 27. T. T. Kararli, Comparison of the gastrointestinal anatomy, physiology, and biochemistry of
505 humans and commonly used laboratory animals, *Biopharm. Drug Dispos.* 16 (1995) p. 351-
506 380.
- 507 28. K. Kleberg, J. Jacobsen, A. Mullertz, Characterising the behaviour of poorly water soluble
508 drugs in the intestine: application of biorelevant media for solubility, dissolution and transport
509 studies, *J. Pharm. Pharmacol.* 62 (2010) p. 1656-1668.
- 510 29. D. Psachoulis, M. Vertzoni, K. Goumas, V. Kalioras, S. Beato, J. Butler, C. Reppas,
511 Precipitation in and supersaturation of contents of the upper small intestine after
512 administration of two weak bases to fasted adults, *Pharm. Res.* 28 (2011) p. 3145-3158.
- 513 30. E. Persson, L. Lofgren, G. Hansson, B. Abrahamsson, H. Lennernas, R. Nilsson,
514 Simultaneous assessment of lipid classes and bile acids in human intestinal fluid by solid-
515 phase extraction and HPLC methods, *J. Lipid Res.* 48 (2007) p. 242-251.

- 516 31. N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, R. M. M. Singh, Hydrogen
517 Ion Buffers for Biological Research*, Biochemistry 5 (1966) p. 467-477.
- 518 32. Y. Perrie, T. Rades, Pharmaceutics - Drug delivery and targeting, Pharmaceutical Press,
519 London, 2012, p. 28 and 47.
- 520 33. J. H. Fagerberg, O. Tsinman, N. Sun, K. Tsinman, A. Avdeef, C. A. Bergstrom, Dissolution
521 rate and apparent solubility of poorly soluble drugs in biorelevant dissolution media, Mol.
522 Pharmaceutics 7 (2010) p. 1419-1430.
- 523 34. C. M. Madsen, K. I. Feng, A. Leithead, N. Canfield, S. A. Jorgensen, A. Mullertz, T. Rades,
524 Effect of composition of simulated intestinal media on the solubility of poorly soluble
525 compounds investigated by design of experiments, Eur. J. Pharm. Sci. 111 (2018) p. 311-319.
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555 **Figure A.1:** MALDI MSI images showing the location of three bile acids in four cross sections of a rat small intestine;
556 section 1 – 4 (0, 10, 20 and 30 cm distal to the stomach, respectively).). Left column shows cholic acid (K^+ adduct, m/z
557 447.2507), middle column shows glycocholic acid (K^+ adduct, m/z 504.2722) and right column shows taurocholic acid
558 (K^+ adduct, m/z 554.2548).
559

Phosphatidylcholine 34:2

Lysophosphatidylcholine 16:0

Overlay

Section 1
Directly distal
to the stomach

Section 2
10 cm distal to
the stomach

Section 3
20 cm distal to
the stomach

Section 4
30 cm distal to
the stomach

Figure A.2: MALDI MSI images of four cross sections of a rat small intestine; section 1 – 4 (0, 10, 20 and 30 cm distal to the stomach, respectively). Left column shows phosphatidylcholine 34:2 (K^+ adduct, m/z 796.5253), middle column shows lysophosphatidylcholine 16:0 (K^+ adduct, m/z 534.2956) and the right column shows an overlay with phosphatidylcholine 34:2 shown in red and lysophosphatidylcholine 16:0 shown in green.