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1 **Influence of feeding regimens on rat gut fluids and colonic metabolism of**
2 **diclofenac-β-cyclodextrin**

3

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20 **KEYWORDS:** fasting, feeding, regimen, gastro-intestinal transit, colonic targeting, prodrug
21 degradation
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23 **Abstract**

24 Feeding states may affect the performance of colonic prodrugs. The aim is to investigate the
25 influence of feeding regimen in Wistar rats on: i) distribution and pH contents along the gut
26 and ii) metabolism of two colonic prodrugs, diclofenac-β-cyclodextrin and a commercially
27 available control, sulfasalazine, within the caecal and colonic contents. Male Wistar rats were
28 subject to four different feeding regimens, the gut contents characterized (mass and pH) and
29 the metabolism of prodrugs investigated.

30 The feeding regimen affects gut contents (mass and pH), more specifically in the stomach
31 and lower intestine, and affects the rate of metabolism of diclofenac-β-cyclodextrin, but not
32 that of sulfasalazine. The latter's degradation is much faster than that of diclofenac-β-
33 cyclodextrin while the metabolism of both prodrugs is faster in colonic (*versus* caecal)
34 contents. Fasting results in most rapid degradation of diclofenac-β-cyclodextrin, possibly due
35 to lack of competition (absence of food) for microbial enzymatic activity.

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41 ## 1. Introduction

42 Colonic drug delivery, for local or systemic action, has many advantages, and can be
43 achieved via different approaches which utilise the properties of the local colonic
44 environment, such as pH and enzymes, for drug release from their carriers (Yang, Chu et al.
45 2002; McConnell, Liu et al. 2009). Cyclodextrins (CDs) have shown promise as colonic
46 carriers. We recently reported the synthesis of diclofenac-β-cyclodextrin conjugate, where the
47 drug is chemically bonded to the CD (Figure 1a) and showed *in vitro* that the drug is released
48 by enzymes present in the colon (Vieira, Serra et al. 2013). The next step is to establish
49 colonic delivery of diclofenac from this conjugate in an *in vivo* model.

50 In early drug development, drug carriers are often tested in rats, most commonly in fasted
51 rats. The extent of fasted/fed state is however critical to the performance of the drug carrier
52 via its influence on gastro-intestinal transit time, pH, contents and availability of water, and
53 microbial enzymatic activity, to mention but a few factors (Varum, Merchant et al. 2010;
54 Scott, Gratz et al. 2013; Varum, Hatton et al. 2013). This is particularly obvious for orally
55 administered colonic prodrugs, whose onset of absorption depends not only on their physico-
56 chemical properties, but also on the time taken for the prodrug to reach the colon, and on the
57 rate of pH/microbial enzyme – controlled drug release.

58 The presence of food in the gastro-intestinal tract reduces gastro-intestinal motility, thereby
59 delaying the arrival of a colonic drug carrier to its site of action (Mittelstadt, Hemenway et al.
60 2005; Varum, Merchant et al. 2010; Varum, Hatton et al. 2013). Food intake also influences
61 the amount of water in the gut that is available for drug dissolution prior to absorption. Food
62 also influences gut contents' pH, which as well as controlling drug release from pH-
63 responsive drug carriers, also influences the ionisation of weakly acidic/basic drugs and thus

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64 their aqueous solubility, stability and absorption (Stella, Borchardt et al. 2007; Varum,
65 Merchant et al. 2010). Food also influences the performance of colonic prodrugs whose
66 conversion to drugs relies on gut bacterial enzymes. Bacterial activity in the colon depends
67 on the quantity and quality of available substrates for fermentation, and determines the
68 intensity and direction of gut bacterial metabolism of prodrugs and thereby drug absorption
69 (Agoram, Woltosz et al. 2001; Mountzouris, Kotzampassi et al. 2009).

70 Given the critical influence of the fasted/fed state on the performance of colonic prodrugs as
71 described above, we determined the influence of different feeding regimens on gastro-
72 intestinal contents, pH and metabolism of the colonic prodrug, in order to establish the most
73 appropriate fasted/fed state that should be employed for its *in vivo* assessment in rats.

74 The rat is an appropriate model for use in early drug development; its mean intestinal transit
75 time is comparable to that in humans despite the different gastro-intestinal lengths, transit
76 time is significantly shorter in the fasted state compared to the fed state, as in man, and its
77 gastrointestinal motility is under the control of the migrating myoelectric complex (MMC),
78 again as in man (Tuleu, Andrieux et al. 1999; Mittelstadt, Hemenway et al. 2005). Rats are
79 thus widely used as an *in vivo* model and have been used to assess different colonic prodrugs,
80 including cyclodextrin-based ones (Minami, Hirayama et al. 1998; Makoto Kamada,
81 Fumitoshi Hirayama et al. 2002).

82 The different feeding regimens tested were: fed *ad libitum*, 12-hour fast, 12-hour fast
83 followed by one hour feeding, which was itself followed by either 30 minutes or 4 hours of
84 fast, prior to the animals being killed, and measurements being taken. These four regimens
85 were selected for a number of reasons: firstly, as stated above, most oral drug delivery
86 experiments are conducted on overnight-fasted rats, secondly, a control experiment for the
87 12-hour fast i.e. animals being fed *ad libitum*, thirdly, a 4-hour fast after feeding, to ensure

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88 complete gastric emptying of food ingested (Booth, Gibson et al. 1986), fourthly, a control
89 experiment for the latter, i.e. 30 minutes fast after feeding assures that the ingested food has
90 not arrived in the lower intestine (caecum and colon) (Brown, Greenburgh et al. 1994).

91 Concomitantly with diclofenac-β-cyclodextrin conjugate (Figure 1a), the effect of the feeding
92 regimens on the metabolism of sulfasalazine (Figure 1b) - a well-known commercially
93 available colonic prodrug of reference - was also studied. Drug release from diclofenac-β-
94 cyclodextrin occurs in the colon by ester hydrolysis and cyclodextrin degradation (Flourié,
95 Molis et al. 1993; Hirayama, Ogata et al. 2000), while sulfasalazine is cleaved in the colon by
96 azoreductase enzymes to 5-aminosalicylic acid (mesalazine) and sulfapyridine (Sousa,
97 Paterson et al. 2008).

98 2. Materials and Methods

99 2.1 Materials

100 Diclofenac sodium (MW = 318.14 g/mol) and sulfasalazine (MW = 398.394 g/mol) were
101 purchased from Sigma Aldrich. Diclofenac-β-cyclodextrin (MW=1411 g/mol) was
102 synthesized according to the method described by Vieira *et al.* (Vieira, Serra et al. 2013).

103 Sodium chloride, potassium hydroxide, sodium hydroxide, potassium dihydrogen phosphate,
104 HPLC grades acetonitrile, methanol and water were purchased from Fisher Scientifics.
105 Peptone water and yeast extract were obtained from Oxoid Limited (Hampshire, UK).
106 Magnesium sulphate heptahydrate and calcium chloride hexahydrate were obtained from
107 VWR (Leicestershire, UK). Trifluoroacetic acid (TFA) and dimethylformamide (DMF),
108 sodium bicarbonate, haemin, l-cysteine HCl, vitamin K and resazurin were obtained from
109 Sigma Aldrich (Dorset, UK). All other chemicals and solvents were of HPLC reagent grade

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110 and were used without further purification. Phosphate buffer saline (PBS) pH 6.0 and pH 6.8
111 were prepared according to the USPXXIV.

112 **2.2 Animals**

113 All the procedures had been approved by the School's Ethical Review Committee and were
114 conducted in accordance with the Home Office standards under the Animals (Scientific Pro-
115 cedures) Act, 1986. Studies were performed using healthy adult male Wistar rats (8 weeks,
116 240-250 g) purchased from Harlan Olac Ltd. (Oxfordshire, UK). All animals were housed in
117 rooms with controlled conditions: 20 ^{o}C, 40-60% humidity, 15-20 air changes per hour. The
118 animals underwent a period of acclimatization, with free access to standard rat chow and wa-
119 ter for 7 days prior the experiment. Twelve hours before the beginning of each experiment,
120 the animals were housed in individual metabolic cages, whose floors were perforated to re-
121 strict the animals' ability to eat their own faeces and allowed the collection of rats' urine and
122 faeces, separately. Water was available *ad libitum* through the experiment.

123 **2.3 Feeding regimens**

124 Four Groups (A, B, C and D) of 5 rats were given different food intake regimens. Rats from
125 Groups A, B and C were fasted for 12 hours overnight. Subsequently, Group A rats were
126 killed. Groups B and C rats were allowed to feed for one hour, after which they were fasted
127 for either 30 minutes (Group B) or 4 hours (Group C), before being killed. Group D rats were
128 not fasted at all, and were given access to food *ad libitum*. The different feeding regimens are
129 shown in Figure 2. The animals were killed by a Schedule One Method (CO_2 asphyxiation),
130 after which the intestinal tract was removed and the pH and the mass of gut contents were
131 determined as follows.

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132 **2.4 Determination of the pH and mass of the gastrointestinal luminal contents**

133 The pH of the contents was measured *in situ* by placing the pH probe (H160 Portable pH Me-
134 ter, Hach, Düsseldorf, Germany) within the luminal contents of each gastrointestinal section.
135 The pH was measured at the anterior (labelled I in Figures) and posterior (except for colon),
136 (labeled II in Figures) of each section of the stomach, small intestine (divided into three sec-
137 tions approximating to the duodenum, jejunum and ileum), caecum and colon before the gut
138 contents were collected into previously weighed vials. The wet masses were recorded, and
139 the vials were stored at – 80 °C. The pH of the distal part of colon contents could not be re-
140 liably measured due to its solid nature.

141 **2.5 Determination of prodrugs' (diclofenac-β-cyclodextrin and sulfasalazine) stability**
142 **in caecal and colonic contents**

143 The stability tests were performed inside an anaerobic workstation (Electrotek 500TG
144 workstation, Electrotek, UK) at 37 °C and 70% RH. The caecum and colonic contents from
145 each Group of rats were mixed with PBS – of differing pHs as explained below - in order to
146 obtain a 40% w/w slurry. The pH of the PBS differed for the different samples, but matched
147 the *in situ* measured pH in the different gastrointestinal sections (section above), in order to
148 maintain the pH of the gut contents. Thus, the gut contents from Groups A and B rats were
149 mixed with PBS pH 6.8, while those from Groups C and D rats were mixed with PBS pH 6.0.
150 The slurries were then homogenized using a glass rod and sieved through an open mesh
151 fabric (Sefar NitexTM, pore size 350 µm) to remove any unhomogenised fibrous material.
152 The sieved faecal slurry was then diluted 50% (w/w) with basal medium containing peptone
153 water, yeast extract, NaCl, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 6H_2O$, $NaHCO_3$, haemin, l-
154 cysteine HCl, bile salts, Tween 80, vitamin K and resazurin (Basit, Newton et al. 2002;
155 Yadav, Gaisford et al. 2013)

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156 Two solutions of each prodrug sulfasalazine (5 mg/mL) and diclofenac-β-cyclodextrin (2.4
157 mg/mL) were prepared in PBS at pH 6.8 and pH 6.0, both containing 4% (v/v) of
158 dimethylformamide (DMF). Subsequently, 100 μL of these solutions were mixed with 900
159 μL of caecal or colonic fluids prepared above; the fluids from Groups A and B rats being
160 mixed with prodrug solutions at pH 6.8, while those from Groups C and D rats were mixed
161 with prodrug solutions at pH 6.0 in order to maintain the pH constant. The final
162 concentrations of the conjugate, sulfasalazine and DMF were 0.5 mg/mL, 0.24 mg/mL and
163 0.4% (v/v), respectively.

164 Thereafter, these mixtures were incubated and shaken at 100 rpm (VXRbasic Vibrax®,
165 Leicestershire, UK), with 50 μL aliquots being withdrawn at times 0, 15, 30, 60, 90, 120,
166 180, 240, 360, 400, 600, 720 and 1440 min. The aliquots were immediately mixed with 100
167 μL of methanol and centrifuged at 10 000 rpm for 10 minutes at room temperature, after
168 which the supernatant was removed and analyzed via HPLC to determine the concentration
169 of the prodrugs and diclofenac.

170 **2.6 HPLC analysis**

171 All HPLC runs were performed using an Agilent 1100 series system equipped with a UV de-
172 tector and a XTerra reverse phase C-18 column with 5 μm particle size, 4.6 mm internal di-
173 ameter and 250 mm length. The mobile phase (consisting of acetonitrile and 0.1%
174 trifluoroacetic acid (TFA) in water) was pumped at a flow rate of 1 mL/min. A gradient
175 system of 0.1% TFA in water (A) and acetonitrile (B) was followed: 0-15 min 25-60% B; 15-
176 22 min 60-25% B. The sample injection volume was 20 μl and detection wavelength was 254
177 nm at 30 ºC. Each measurement was performed in triplicate. The results were acquired and
178 processed with the Agilent Chemstation Data System Software 7.

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179 **2.7 Data analysis**

180 Statistically significant differences in the total mass of contents between Groups and in the
181 mass and pH of contents per section among Groups were evaluated using One-way analysis
182 of variance (ANOVA), followed by Tukey test. General linear model (repeated measure-
183 ments) was utilized to assess the differences of pH contents between Groups with different
184 regimens of food intake (A, B, C and D). The relationship between mass and pH was
185 investigated using Pearson Correlation Coefficient. Degradation kinetics of diclofenac-β-
186 cyclodextrin were determined by fitting the percent prodrug remaining *versus* incubation time
187 curves to a first-order kinetic model, and subsequently calculating reaction rate constant (K)
188 and half-life ($t_{1/2}$). Statistically significant differences in the rate constant and half-life of
189 diclofenac-β-cyclodextrin conjugate between Groups were analysed using Kruskal–Wallis
190 test, with Nemenyi's post-hoc analysis. All tests, apart from Nemenyi's test were carried out
191 using SPSS 21.0 for Windows®. Nemenyi's test was conducted as described in (Jones 2002).
192 Results were considered statistically significant when $p < 0.05$.

193 **3. Results and Discussion**

194 **3.1 Influence of feeding regimens on the mass of gastrointestinal contents**

195 As expected, the feeding regimens influenced the total mass of gastro-intestinal contents
196 (Figure 3), and these were statistically different among the four Groups (ANOVA, $p < 0.05$).
197 The total gut content weights of the fed Group D rats were almost twice those of the 12-hour
198 fasted Group A rats (post hoc Tukey, $p < 0.05$). This result in male Wistar rats reflects the
199 previous report in female Wistar rats, where similar values for gut contents are reported
200 (McConnell, Basit et al. 2008). When Group B rats were allowed to feed for 1 hour, they ate
201 sufficiently during the hour, such that their total gut contents masses were similar to those in

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202 Group D rats which were allowed food *ad libitum* throughout the experimental duration ($p > 0.05$). On the other hand, Group C rats which were fed for 1 hour and fasted for 4 hours prior to being killed had similar mass of gut contents as Group A rats ($p > 0.05$).

205 Analysis of the distribution of gastrointestinal contents (Figures 4) shows the influence of feeding/fasting states and timings. Groups A and C rats (which were fasted for substantial durations prior to being killed) have similar profiles to each other (Figure 4). Groups B and D rats (which had short/ no fasting times prior to being killed) also had similar profiles to each other (Figure 4). The major difference in the four profiles is the large stomach contents in Groups B and D, compared to minimal stomach contents in Groups A and C. In contrast, the small intestinal contents measured in the duodenum, jejunum and ileum are low and similar across all the groups.

213 Fasted animals (Group A) have minimal stomach contents, slowly increasing contents in the duodenum, jejunum and ileum, with most of the gut contents being located in the caecum, and the mass of contents dropping in the colon. A 12-hour fast in Group A rats (Figure 2) means that any food eaten prior to the fast has moved down the gastro-intestinal tract to the caecum. In contrast, the profile for the fed Group D rats is very different compared to that of Group A, with larger masses in the stomach and in the colon of the fed animals.

219 Greater variability in the gut (especially stomach) contents is also seen in Group D rats fed *ad libitum*. Rats with full/partially full stomachs are expected to show variable gastric emptying times, leading to variable drug release from the drug carrier, and hence greater variability in the latter's performance. This explains why most *in vivo* experiments in laboratory animals are conducted in the fasted state when oral drug absorption is assessed.

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224 The similar profiles of Groups B and D rats show that a 30-minutes fast after feeding is obvi-
225 ously not sufficient for gastric emptying. In contrast, the similarity of profiles of Group C rats
226 to those of Group A rats shows that gastric emptying has taken place after 4 hours. The
227 Group A and C profiles indicate that during the extra 5 experimental hours undergone by
228 Group C rats, the gastrointestinal contents moved down the gut, such that more of it was pre-
229 sent in the colon.

230 **3.2 *In situ* pH of the gastrointestinal contents**

231 The pH of the contents measured along the gastrointestinal tract for the different Groups of
232 rats is shown in Figure 5. The profiles are as expected and reflect those previously reported in
233 rats (McConnell, Basit et al. 2008) and man (Dressman, Berardi et al. 1990), the pH value
234 being variable in the stomach depending on food intake and its buffering/dilution effect on
235 gastric HCl, rising in the small intestine due to pancreatic juice secretion, and slightly falling
236 in the large intestine due to the production of short chain fatty acids (SCFA) by bacterial fer-
237 mentation of dietary fibre. The largest differences among the Groups occur in the early sec-
238 tions of the gastrointestinal tract (the stomach) with smaller differences in the caecum and
239 colon (Figure 5). In contrast, the small intestinal pH is immune to the effects of fast-
240 ing/feeding regimens. Similarities between pH profiles for Groups A and C rats (repeated
241 measures ANOVA, $p > 0.05$), and those of Groups B and D rats (repeated measures ANO-
242 VA, $p > 0.05$) shown in Figure 5, reflect the influence of feeding/fasting states and times.

244 Groups A and C rats which have no or limited food in the stomach show a low pH in the first
245 part of the stomach (forestomach) compared to Groups B and D rats which have more food
246 (see Figure 4 for food contents). Indeed, a correlation of 0.7 (Pearson, $p < 0.05$) was found
247 between the mass of the stomach contents and pH in stomach I in the rats, reflecting the

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248 buffering and dilution effects of food in the stomach (Evans, Pye et al. 1988 ; Fallingborg,
249 Christensen et al. 1989). In contrast to Stomach I the pH in the lower part of the stomach
250 (Stomach II) was low in all Groups of rats, and was independent of food presence (Pearson, p
251 > 0.05). This correlates with previous reports that in rats, the non-glandular stomach I
252 (forestomach) is used for the storage and mechanical digestion of food (Ghoshal and Bal
253 1989), whose presence/absence is the principal factor responsible for the local pH (Ward and
254 Coates 1987), while the pH in the glandular HCl-secreting Stomach II is influenced mainly
255 by microbial products, despite HCl-secretion being stimulated by the presence of food (Ward
256 and Coates 1987). It must be noted that in man, the whole stomach is glandular and harbours
257 few bacteria, in contrast to the large bacterial numbers in rats (Kararli 1995).

258 As mentioned above, the pH profiles of the four Groups of rats diverge at the large intestinal
259 caecal and colonic fractions. Although differences are small, the lowest pHs are seen in
260 Groups C and D, with the highest in Groups A and B. Comparison of Figures 4 and 5 suggest
261 a correlation between mass of contents and pH measured. Indeed a strong correlation ($r = -$
262 0.9, Person $p < 0.05$) was found between the content's mass and the pH in the first part of the
263 colon (Colon I) when all the rats' data was analysed, i.e. $n = 20$. This reflects the production
264 of bacterial fermentation products, the short chain fatty acids (acetate, propionate and bu-
265 tyrate) (Scott, Gratz et al. 2013); with a greater mass of dietary fibre leading to greater bacte-
266 rial metabolism and production of short fatty acids (Ferguson, Tasman-Jones et al. 2000;
267 Paturi, Butts et al. 2012).

268 A higher pH in the large intestine of Group A fasted rats (compared to Group D fed rats) re-
269 flects previous reports in rats (McConnell, Basit et al. 2008) and in man (Evans, Pye et al.
270 1988 ; Fallingborg, Christensen et al. 1989) and may be explained by their lower concentra-
271 tions of SCFA compared to rats fed *ad libitum* (Mountzouris, Kotzampassi et al. 2009).

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272 Similar large intestinal pH in Groups A and B suggests that food ingested by Group B rats
273 during the 1 hour feeding has not travelled down the gastro-intestinal tract during the 30
274 minutes fast prior to measurement. Meanwhile, the lower large intestinal pH in Group C rats
275 suggests that food ingested prior to a 4-hour fast has travelled down the gastro-intestinal tract
276 to some extent. This shows the importance of the fasting/fed states and feeding regimens
277 when evaluating colonic drug carriers in the rat *in vivo* model. The fasted rat (most
278 commonly used model) may demonstrate a poor performance of a pH-controlled colonic drug
279 carrier, due to an insufficiently low pH in the colon, rather than due to a poor formulation. On
280 the other hand, while the fed rat may possess the correct (low) colonic pH required for drug
281 release from such a pH-controlled carrier, variable feeding by a Group of animals could lead
282 to variability in gut contents, transit times, drug release and absorption profiles, which could
283 in turn mask the true potential of the colonic drug carrier.

284 **3.3 Stability of diclofenac-β-cyclodextrin and sulfasalazine prodrugs in caecum and**
285 **colon contents.**

286 The stability of the prodrugs in caecal and colonic fluids is shown in Figures 6. Moreover
287 Figure 6 indicates that the disappearance of diclofenac-β-cyclodextrin coincides with the ap-
288 pearance of free diclofenac in each release medium; therefore confirming the prodrug is able
289 to liberate the drug in a colonic environment. It can be seen that, in all the animal Groups:

290 1. Degradation of sulfasalazine is much faster than that of diclofenac-β-cyclodextrin in
291 both milieus. Sulfasalazine is degraded by azoreductases, which are produced by many dif-
292 ferent bacterial species in the large intestine. The supply of the enzyme azoreductase being
293 almost unlimited, sulfasalazine's degradation can take place without delay, and does not seem
294 to be influenced by the feeding regimen. In fact, the sulfasalazine degradation was so fast that

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295 the degradation profiles have few time points, and the curves were not fitted for further anal-
296 ysis for reaction rates and half-lives.

297 In contrast, the cyclodextrin conjugate's metabolism is much more complex, and involves
298 two types of enzymes, amylase and esterase. The esterase can only act after the amylase has
299 started degrading the cyclodextrin carrier as reported previously (Hirayama, Ogata et al.
300 2000). Moreover, the compounds formed in the initial stages of amylase-degradation of
301 cyclodextrin - high-membered maltooligomers (maltohexaose, maltopentaose, maltotetraose)
302 - are themselves substrates for the amylases and can therefore act as competitive inhibitors of
303 the enzymatic reaction (Suetsugu, Koyama et al. 1974; Jodal, Kandra et al. 1984). In addition,
304 while the lower-membered maltooligomers (glucose, maltose and maltotriose) formed during
305 the reaction are not substrates for the amylase, they can become "non-competitive" enzyme
306 inhibitors by linking to the enzyme protein (Jodal, Kandra et al. 1984). Thus, the diclofenac-
307 CD conjugate shows potential as a sustained-release formulation.

308 2. The degradation of both prodrugs is faster in colonic contents, compared to caecal contents
309 (Table 1). This could be due to a number of reasons; such as the caecal slurry having a lower
310 bacterial concentration (due to its greater liquid content), or the caecal slurry being more nu-
311 trient-rich (the caecum being the main site of bacterial fermentation), such that less prodrug is
312 metabolized by the bacteria as a source of substrate.

313 3. The rate of CD-drug conjugate's degradation by bacterial enzymes was influenced by the
314 feeding regimen in the colon (Figure 6). Degradation was fastest from Groups A and B fasted
315 rats, followed by Group D, followed by Group C (Table 1). This order of degradation rates K
316 was statistically significant ($p < 0.05$, Kruskal Wallis, followed by Nemenyi's test). The fast
317 prodrug degradation in Group A rats (which had been fasted for 12 hours) could be due to a
318 lack of nutrient in the colon for the bacterial enzymes to act on, which therefore act exclu-

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319 sively on the prodrug, degrading the latter. Similarly, in Group B rats, the colon is nutrient-
320 poor and exclusive enzyme action on the prodrug leads to the latter's fast degradation. Al-
321 though Group B rats were fed for one hour (Figure 2), the food had not had time to move
322 down to the colon by the time the rats were killed 30 minutes after the one-hour feeding time.
323 In contrast, food and nutrient were present in the colon of Group D fed rats, and competition
324 between nutrient and prodrug for enzyme action led to slower prodrug degradation. The
325 slower rate of prodrug degradation in Group C rats (compared to Group D rats) shows an
326 even greater amount of nutrient in the colon of Group C rats, and hence greater competition
327 of enzyme action. It is possible that the bolus intake of food by Group C rats during the one
328 hour feeding arrives in the colon at some point during the four hours of fasting, such that
329 there is overwhelming competition for the enzyme.

330 Overall, it is interesting to note that the rates of degradation of diclofenac-β-cyclodextrin in
331 rats's colonic contents in Group A and B is close to the rate of degradation observed in the
332 human faecal slurries collected from individuals without any food intake control, as reported
333 previously (Vieira, Serra et al. 2013).

334 In contrast to the obvious influence of feeding regimen on prodrug degradation in the colon,
335 the influence was less obvious in the caecum. The degradation curves for all rat Groups have
336 similar profiles, especially at the beginning of the *in vitro* degradation reaction (Figure 6).
337 The caecum has such a high content of material in all rat Groups (Figure 4) that feed-
338 ing/fasting did not seem to alter the nutrient content and subsequently, any competition be-
339 tween nutrient and prodrug for enzyme action. One point to note though is the completion of
340 prodrug degradation in Group A fasted rats' caecal contents at 600 minutes (Figure 6) in con-
341 trast to the other Groups. This correlates with the fact that Group A fasted rats had a lowest
342 amount of nutrient (and hence competition for enzyme action) in their caecum.

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343 **4. Conclusions**
344 This study demonstrates the importance of feeding regimens, specifically the timing of meal
345 ingestion, on the gastrointestinal conditions in rats and how this influences the metabolism of
346 colonic prodrugs. In addition to changes in the distribution of gut contents along the GI tract,
347 which directly affects the gastrointestinal transit time, different feeding regimens are accom-
348 panied by changes in the pH of gut contents, specifically in the stomach and large intestine.
349 Moreover, differential gut contents in the large intestine have an impact on the microbiota
350 activity, which affects the rate of diclofenac-β-cyclodextrin metabolism and hence drug re-
351 lease and absorption. We also show that the different feeding regimens did not seem to im-
352 pact on the metabolism of sulfasalazine, which was rapidly metabolized. Thus, we conclude
353 that while feeding regimen influences the performance of the colonic prodrug, diclofenac-β-
354 cyclodextrin, that influence has to be measured for each prodrug individually, given the dif-
355 ferent metabolic pathways of different colonic carriers.

356

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437 Table 1: Degradation rate (k, min^{-1}) and half-life ($t_{1/2}$, min) for diclofenac-β-cyclodextrin in
438 cecal and colonic contents of rat Groups A, B, C and D.

439

	CECUM		COLON	
Groups	k (min^{-1})	$t_{1/2}$ (min)	k (min^{-1})	$t_{1/2}$ (min)
A	0.004 ± 0.001	185 ± 22	0.016 ± 0.001	44 ± 2
B	0.002 ± 0.000	397 ± 56	0.013 ± 0.005	60 ± 22
C	0.001 ± 0.000	490 ± 76	0.003 ± 0.001	250 ± 43
D	0.003 ± 0.000	249 ± 27	0.008 ± 0.002	90 ± 16

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442 **Highlights**

443 - Rats subject to different feeding regimens (4 groups)

444 - Gastrointestinal contents characterized in terms of mass and pH

445 - Stability of diclofenac-β-cyclodextrin versus sulfasalazine: influence of feeding regimen

446 - Feeding state affects diclofenac-β-cyclodextrin but not sulfasalazine metabolism.

447 - Diclofenac-β-cyclodextrin degradation is fastest in fasted state

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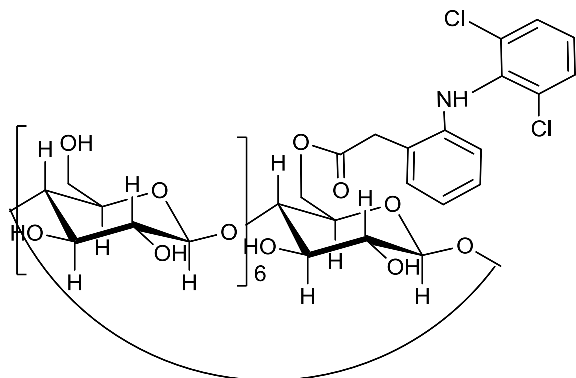
Figure 1

a



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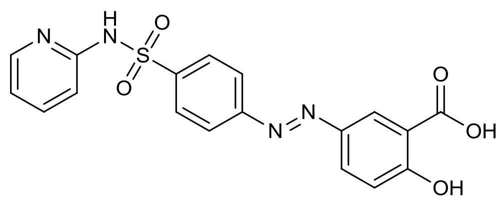

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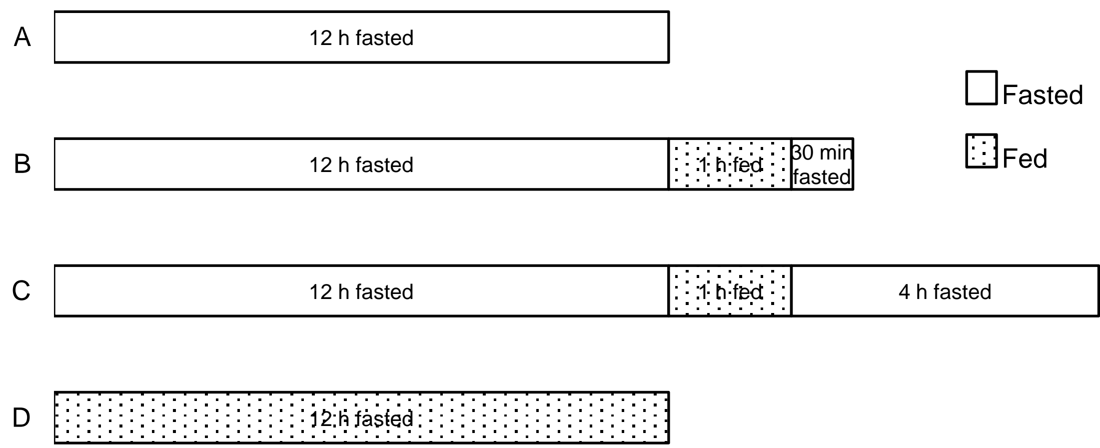

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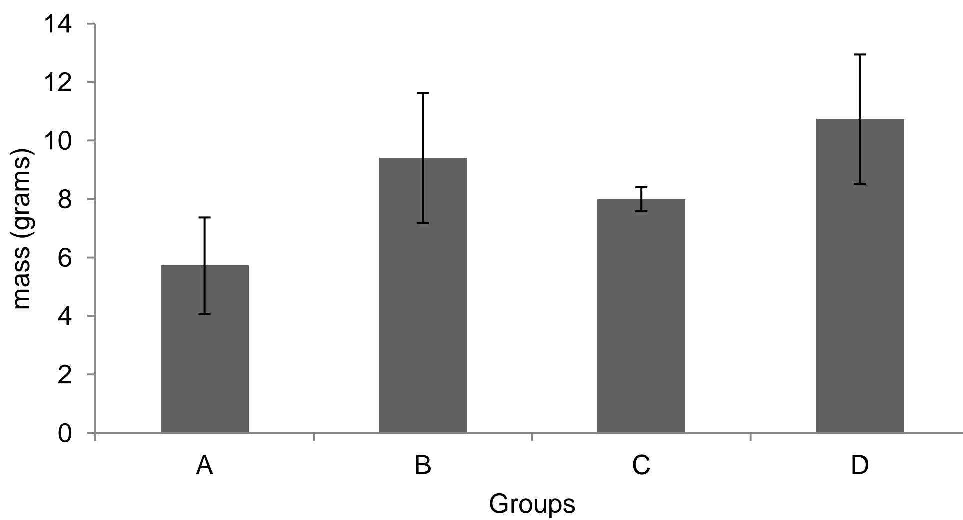

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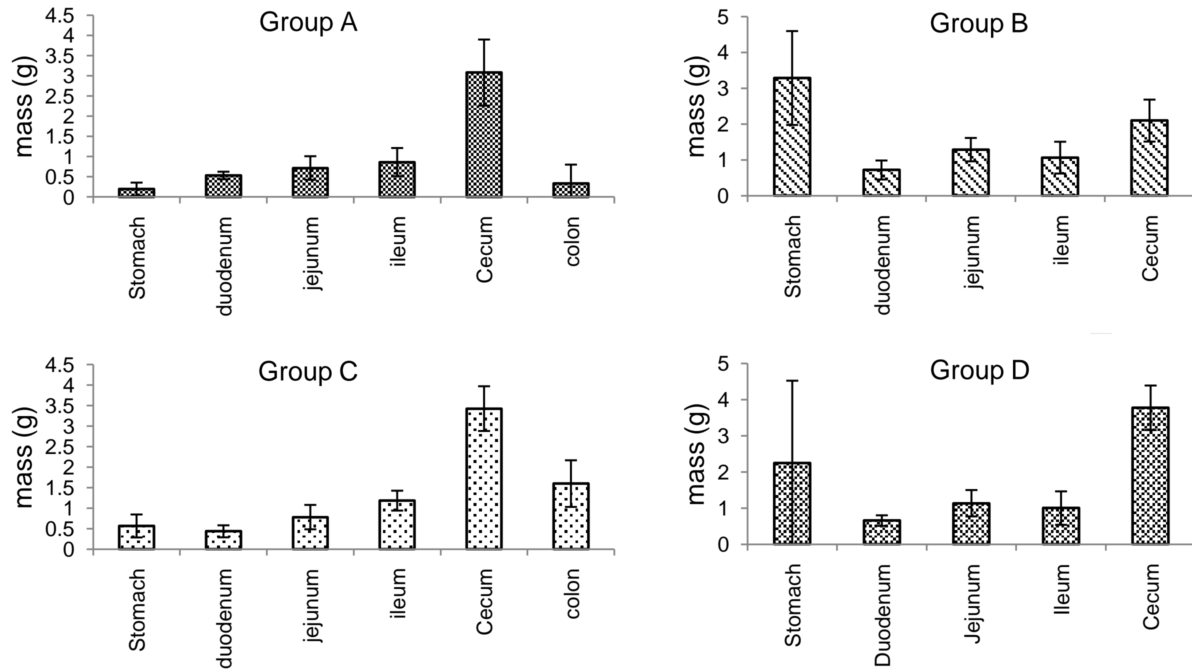

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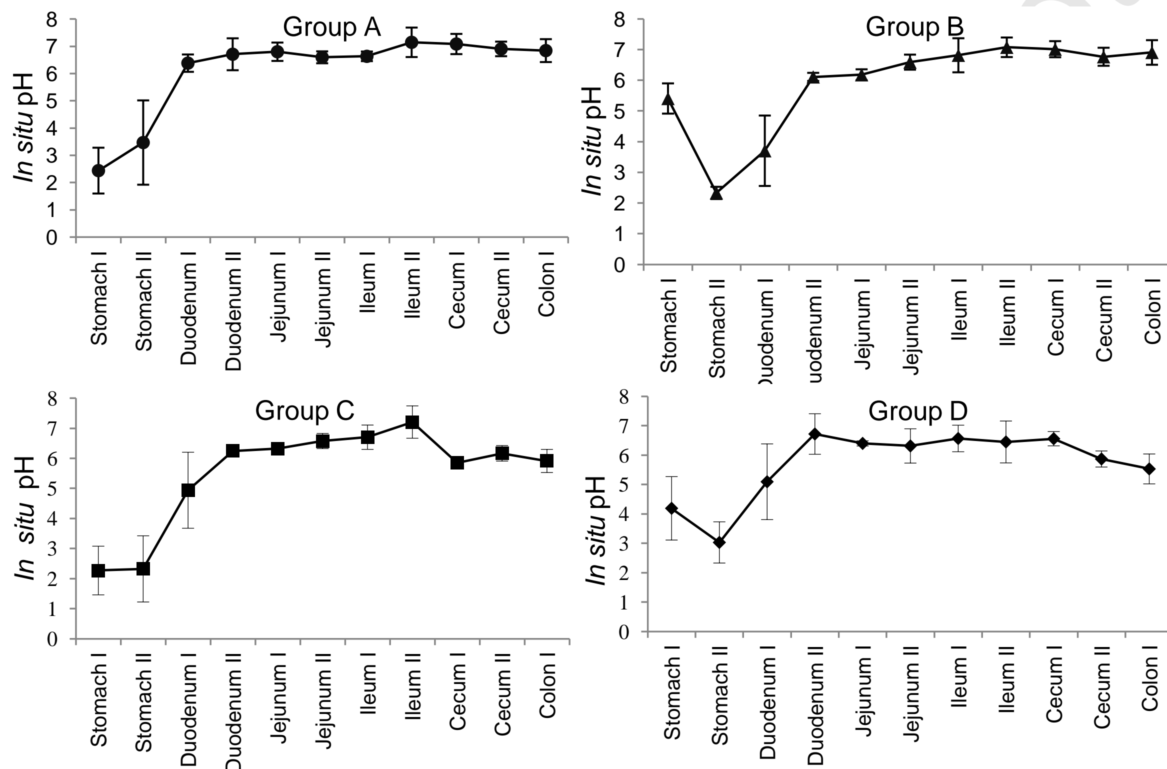

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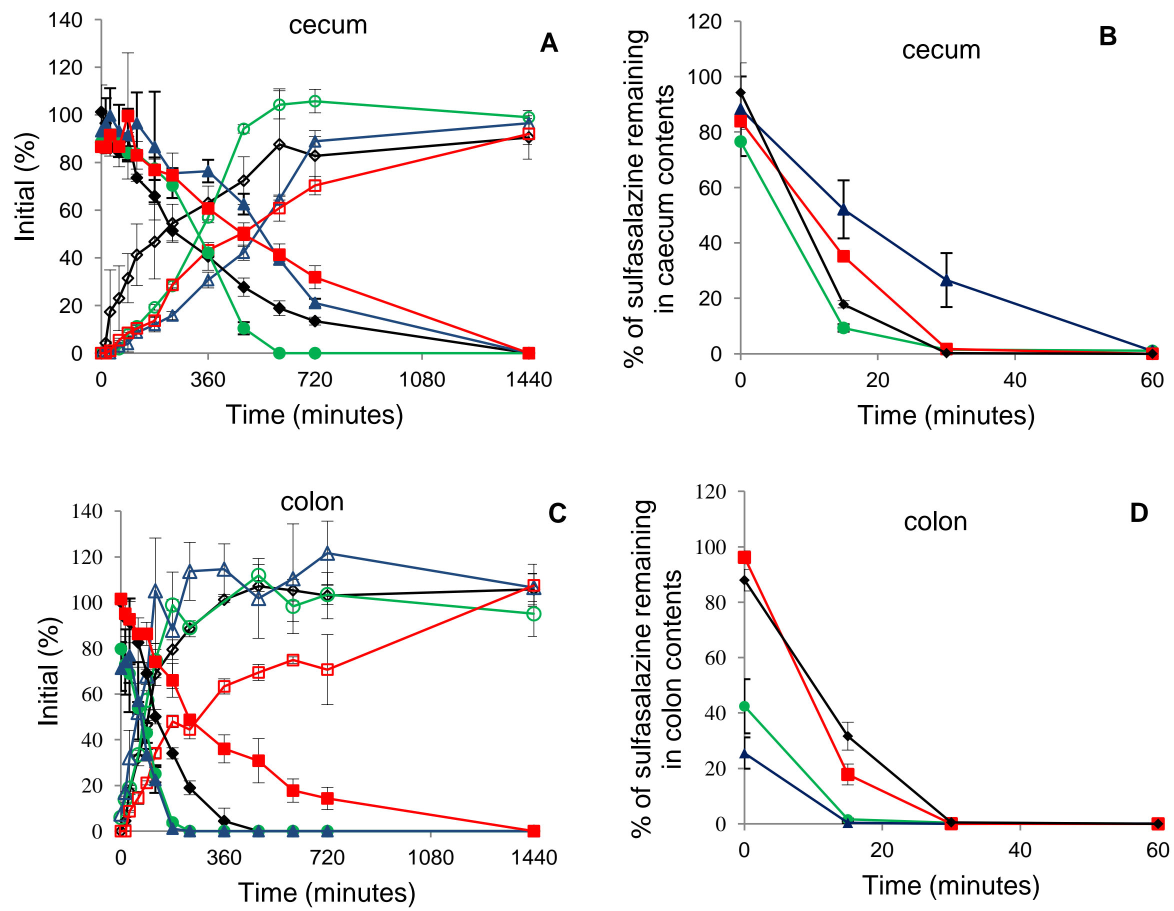

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Figure 1 Structures of diclofenac-β-cyclodextrin (a) and of the control, sulfasalazine (b)

Figure 2 Schematic representation of the different feeding regimens of the rat Groups A, B, C and D. A: 12-hour fast; B: 12-hour fast followed by 1-hour feeding, followed by 30-min fast; C: 12-hour fast, followed by1-hour feeding, followed by 4-hour fast; D: fed *ad libitum*.

Figure 3 Total mass of gastrointestinal contents in healthy male rats in the different Groups. A: 12 hours fast, B: 12 hours fast then 1 hour fed then 30 min fast, C: 12 hours fast then 1 hour fed then 4 hours fast; D; fed. Each bar represents mean ± S.D, n = 5.

Figure 4 Mass of luminal contents in the different gastrointestinal sections in the rat Groups. Each bar represents mean ± S.D. n = 5

Figure 5 *In situ* pH of gastrointestinal contents in the different sections of the gastrointestinal tract in the rat Groups. I and II refer to the anterior and posterior parts respectively. Each point represents mean ± S.D, n = 5.

Figure 6 Mean levels of diclofenac-β-cyclodextrin (A, C) and sulfasalazine (B, D) remaining when prodrugs were incubated in caecal and colonic contents from rats from Group A (●), B (▲), C (■), and D (♦). Means of diclofenac (A, C) appearance when diclofenac-β-cyclodextrin was incubated in caecal and colonic contents from Group A (○), B (Δ), C (□), and D (◊). Each point represents mean ± S.D, n = 3.

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