

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

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ABSTRACT

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

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

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1. Introduction

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

In early drug development, drug carriers are often tested in rats, most commonly in fasted rats. The extent of fasted/fed state is however critical to the performance of the drug carrier *via* its influence on gastro-intestinal transit time, pH, contents and availability of water, and microbial enzymatic activity, to mention but a

few factors (Varum, Merchant, & Basit, 2010; Scott, Gratz, Sheridan, Flint, & Duncan, 2013; Varum, Hatton, & Basit, 2013). This is particularly obvious for orally administered colonic prodrugs, whose onset of absorption depends not only on their physico-chemical properties, but also on the time taken for the prodrug to reach the colon, and on the rate of pH/microbial enzyme-controlled drug release.

The presence of food in the gastro-intestinal tract reduces gastro-intestinal motility, thereby delaying the arrival of a colonic drug carrier to its site of action (Mittelstadt, Hemenway, & Spruell, 2005; Varum et al., 2010; Varum et al., 2013). Food intake also influences the amount of water in the gut that is available for drug dissolution prior to absorption. Food also influences gut contents' pH, which as well as controlling drug release from pH-responsive drug carriers, also influences the ionization of weakly acidic/basic drugs and thus their aqueous solubility, stability and absorption (Stella et al., 2007; Varum et al., 2010). Food also influences the performance of colonic prodrugs whose conversion to drugs relies on gut bacterial enzymes. Bacterial activity in the colon depends on the quantity and quality of available substrates for fermentation, and determines the intensity and direction of gut bacterial metabolism of prodrugs and thereby drug absorption (Agoram, Woltoz, & Bolger, 2001; Mountzouris, Kotzampassi, Tsirtsikos, Kapoutzis, & Fegeros, 2009).

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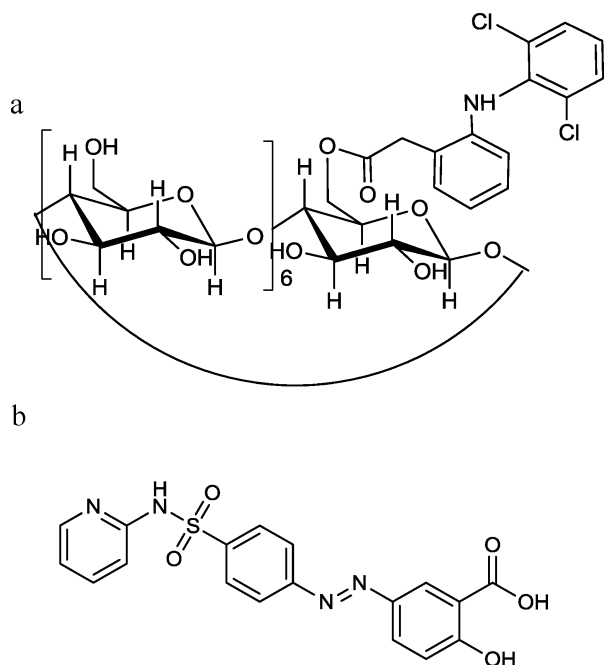


Fig. 1. Structures of diclofenac-β-cyclodextrin (a) and of the control, sulfasalazine (b).

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

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

The different feeding regimens tested were: fed *ad libitum*, 12-h fast, 12-h fast followed by one hour feeding, which was itself followed by either 30 min or 4 h of fast, prior to the animals being killed, and measurements being taken. These four regimens were selected for a number of reasons: first, as stated above, most oral drug delivery experiments are conducted on overnight-fasted rats, second, a control experiment for the 12-h fast *i.e.* animals being fed *ad libitum*, third, a 4-h fast after feeding, to ensure complete gastric emptying of food ingested (Booth, Gibson, & Baker et al., 1986), fourth, a control experiment for the latter, *i.e.* 30 min fast after feeding assures that the ingested food has not arrived in the lower intestine (caecum and colon) (Brown, Greenburgh, & Tomlin, 1994).

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

2. Materials and methods

2.1. Materials

Diclofenac sodium ($M_w = 318.14$ g/mol) and sulfasalazine ($M_w = 398.394$ g/mol) were purchased from Sigma Aldrich. Diclofenac-β-cyclodextrin ($M_w = 1411$ g/mol) was synthesized according to the method described by Vieira et al. (2013).

Sodium chloride, potassium hydroxide, sodium hydroxide, potassium dihydrogen phosphate, HPLC grades acetonitrile, methanol and water were purchased from Fisher Scientifics. Peptone water and yeast extract were obtained from Oxoid Limited (Hampshire, UK). Magnesium sulphate heptahydrate and calcium chloride hexahydrate were obtained from VWR (Leicestershire, UK). Trifluoroacetic acid (TFA) and dimethylformamide (DMF), sodium bicarbonate, haemin, L-cysteine HCl, vitamin K and resazurin were obtained from Sigma Aldrich (Dorset, UK). All other chemicals and solvents were of HPLC reagent grade and were used without further purification. Phosphate buffer saline (PBS) pH 6.0 and pH 6.8 were prepared according to the USPXXIV.

2.2. Animals

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

2.3. Feeding regimens

Four Groups (A–D) of five rats were given different food intake regimens. Rats from Groups A–C were fasted for 12 h overnight. Subsequently, Group A rats were killed. Groups B and C rats were allowed to feed for 1 h, after which they were fasted for either 30 min (Group B) or 4 h (Group C), before being killed. Group D rats were not fasted at all, and were given access to food *ad libitum*. The different feeding regimens are shown in Fig. 2. The animals were killed by a schedule one method (CO₂ asphyxiation), after which the intestinal tract was removed and the pH and the mass of gut contents were determined as follows.

2.4. Determination of the pH and mass of the gastrointestinal luminal contents

The pH of the contents was measured *in situ* by placing the pH probe (H160 Portable pH Meter, Hach, Düsseldorf, Germany) within the luminal contents of each gastrointestinal section. The pH was measured at the anterior (labelled I in figures) and posterior (except for colon), (labelled II in figures) of each section of the stomach, small intestine (divided into three sections approximating to the duodenum, jejunum and ileum), caecum and colon before the gut contents were collected into previously weighed vials. The wet masses were recorded, and the vials were stored at –80 °C. The pH of the distal part of colon contents could not be reliably measured due to its solid nature.

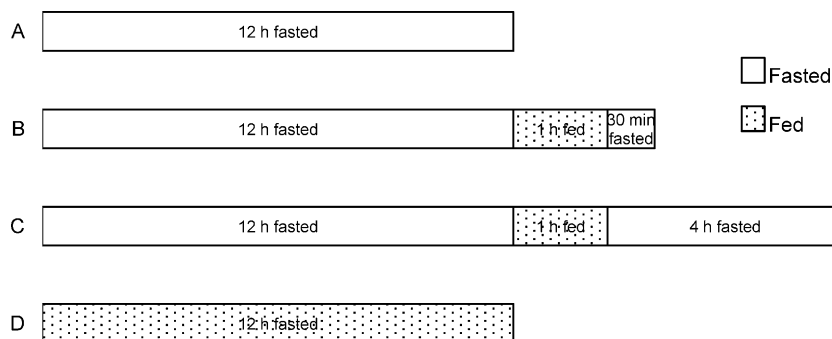


Fig. 2. Schematic representation of the different feeding regimens of the rat Groups A, B, C and D. (A) 12-h fast; (B) 12-h fast followed by 1-h feeding, followed by 30-min fast; (C) 12-h fast, followed by 1-h feeding, followed by 4-h fast; (D) fed *ad libitum*.

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

The stability tests were performed inside an anaerobic workstation (Electrotek 500TG workstation, Electrotek, UK) at 37 °C and 70% RH. The caecum and colonic contents from each Group of rats were mixed with PBS – of differing pHs as explained below – in order to obtain a 40% w/w slurry. The pH of the PBS differed for the different samples, but matched the *in situ* measured pH in the different gastrointestinal sections (section above), in order to maintain the pH of the gut contents. Thus, the gut contents from Groups A and B rats were mixed with PBS pH 6.8, while those from Groups C and D rats were mixed with PBS pH 6.0. The slurries were then homogenized using a glass rod and sieved through an open mesh fabric (Sefar Nitex™, Heiden, Switzerland, pore size 350 μ m) to remove any unhomogenized fibrous material. The sieved faecal slurry was then diluted 50% (w/w) with basal medium containing peptone water, yeast extract, NaCl, K₂HPO₄, MgSO₄·7H₂O, CaCl₂·6H₂O, NaHCO₃, haemin, L-cysteine HCl, bile salts, Tween 80, vitamin K and resazurin (Basit, Newton, & Lacey, 2002; Yadav, Gaisford, Merchant, & Basit, 2013).

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

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

2.6. HPLC analysis

All HPLC runs were performed using an Agilent 1100 series system equipped with a UV detector and a XTerra reverse phase C-18 column with 5 μ m particle size, 4.6 mm internal diameter and 250 mm length. The mobile phase (consisting of acetonitrile and 0.1% trifluoroacetic acid (TFA) in water) was pumped at a flow rate of 1 mL/min. A gradient system of 0.1% TFA in water (A) and acetonitrile (B) was followed: 0–15 min 25–60% B; 15–22 min 60–25% B. The sample injection volume was 20 μ L and detection wavelength

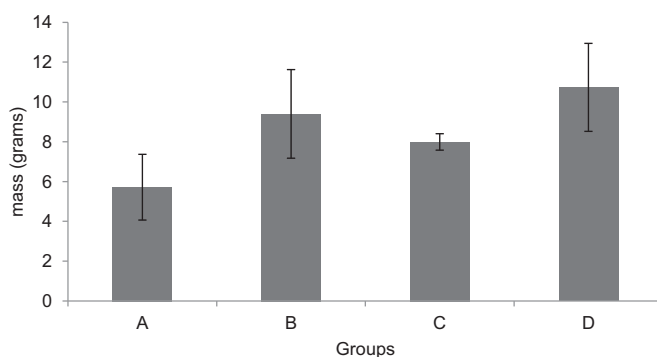


Fig. 3. Total mass of gastrointestinal contents in healthy male rats in the different Groups. (A) 12 h fast; (B) 12 h fast then 1 h fed then 30 min fast; (C) 12 h fast then 1 h fed then 4 h fast; (D) fed. Each bar represents mean $\pm$ S.D, $n = 5$.

was 254 nm at 30 °C. Each measurement was performed in triplicate. The results were acquired and processed with the Agilent Chemstation Data System Software 7.

2.7. Data analysis

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

3. Results and discussion

3.1. Influence of feeding regimens on the mass of gastrointestinal contents

As expected, the feeding regimens influenced the total mass of gastro-intestinal contents (Fig. 3), and these were statistically

different among the four Groups (ANOVA, $p < 0.05$). The total gut content weights of the fed Group D rats were almost twice those of the 12-h fasted Group A rats (post hoc Tukey, $p < 0.05$). This result in male Wistar rats reflects the previous report in female Wistar rats, where similar values for gut contents are reported (McConnell, Basit, & Murdan, 2008). When Group B rats were allowed to feed for 1 h, they ate sufficiently during the hour, such that their total gut contents masses were similar to those in 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 h and fasted for 4 h prior to being killed had similar mass of gut contents as Group A rats ($p > 0.05$).

Analysis of the distribution of gastrointestinal contents (Fig. 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 (Fig. 4). Groups B and D rats (which had short/no fasting times prior to being killed) also had similar profiles to each other (Fig. 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.

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-h fast in Group A rats (Fig. 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.

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.

The similar profiles of Groups B and D rats show that a 30-min fast after feeding is obviously not sufficient for gastric emptying. In contrast, the similarity of profiles of Group C rats to those of Group A rats shows that gastric emptying has taken place after 4 h. The Group A and C profiles indicate that during the extra five experimental hours undergone by Group C rats, the gastrointestinal contents moved down the gut, such that more of it was present in the colon.

3.2. *In situ* pH of the gastrointestinal contents

The pH of the contents measured along the gastrointestinal tract for the different Groups of rats is shown in Fig. 5. The profiles are as expected and reflect those previously reported in rats (McConnell et al., 2008) and man (Dressman et al., 1990), the pH value being variable in the stomach depending on food intake and its buffering/dilution effect on gastric HCl, rising in the small intestine due to pancreatic juice secretion, and slightly falling in the large intestine due to the production of short chain fatty acids (SCFA) by bacterial fermentation of dietary fibre. The largest differences among the Groups occur in the early sections of the gastrointestinal tract (the stomach) with smaller differences in the caecum and colon (Fig. 5). In contrast, the small intestinal pH is immune to the effects of fasting/feeding regimens. Similarities between pH profiles for Groups A and C rats (repeated measures ANOVA, $p > 0.05$), and those of Groups B and D rats (repeated measures ANOVA, $p > 0.05$) shown in Fig. 5, reflect the influence of feeding/fasting states and times.

Groups A and C rats which have no or limited food in the stomach show a low pH in the first part of the stomach (forestomach) compared to Groups B and D rats which have more food (see Fig. 4 for food contents). Indeed, a correlation of 0.7 (Pearson, $p < 0.05$) was found between the mass of the stomach contents and pH in stomach I in the rats, reflecting the buffering and dilution effects of food in the stomach (Evans et al., 1988; Fallingborg et al., 1989). In contrast to Stomach I, the pH in the lower part of the stomach (Stomach II) was low in all Groups of rats, and was independent of food presence (Pearson, $p > 0.05$). This correlates with previous reports that in rats, the non-glandular Stomach I (forestomach) is used for the storage and mechanical digestion of food (Ghoshal & Bal, 1989), whose presence/absence is the principal factor responsible for the local pH (Ward & Coates, 1987), while the pH in the glandular HCl-secreting Stomach II is influenced mainly by microbial products, despite HCl-secretion being stimulated by the presence of food (Ward & Coates, 1987). It must be noted that in man, the whole stomach is glandular and harbours few bacteria, in contrast to the large bacterial numbers in rats (Kararli, 1995).

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

A higher pH in the large intestine of Group A fasted rats (compared to Group D fed rats) reflects previous reports in rats (McConnell et al., 2008) and in man (Evans et al., 1988; Fallingborg et al., 1989) and may be explained by their lower concentrations of SCFA compared to rats fed *ad libitum* (Mountzouris et al., 2009). Similar large intestinal pH in Groups A and B suggests that food ingested by Group B rats during the 1 h feeding has not travelled down the gastro-intestinal tract during the 30 min fast prior to measurement. Meanwhile, the lower large intestinal pH in Group C rats suggests that food ingested prior to a 4-h fast has travelled down the gastro-intestinal tract to some extent. This shows the importance of the fasting/fed states and feeding regimens when evaluating colonic drug carriers in the rat *in vivo* model. The fasted rat (most commonly used model) may demonstrate a poor performance of a pH-controlled colonic drug carrier, due to an insufficiently low pH in the colon, rather than due to a poor formulation. On the other hand, while the fed rat may possess the correct (low) colonic pH required for drug release from such a pH-controlled carrier, variable feeding by a Group of animals could lead to variability in gut contents, transit times, drug release and absorption profiles, which could in turn mask the true potential of the colonic drug carrier.

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

The stability of the prodrugs in caecal and colonic fluids is shown in Fig. 6. Moreover, Fig. 6 indicates that the disappearance of diclofenac- β -cyclodextrin coincides with the appearance of free diclofenac in each release medium; therefore confirming the pro-drug is able to liberate the drug in a colonic environment. It can be seen that, in all the animal groups:

- (1) Degradation of sulfasalazine is much faster than that of diclofenac- β -cyclodextrin in both milieus. Sulfasalazine is

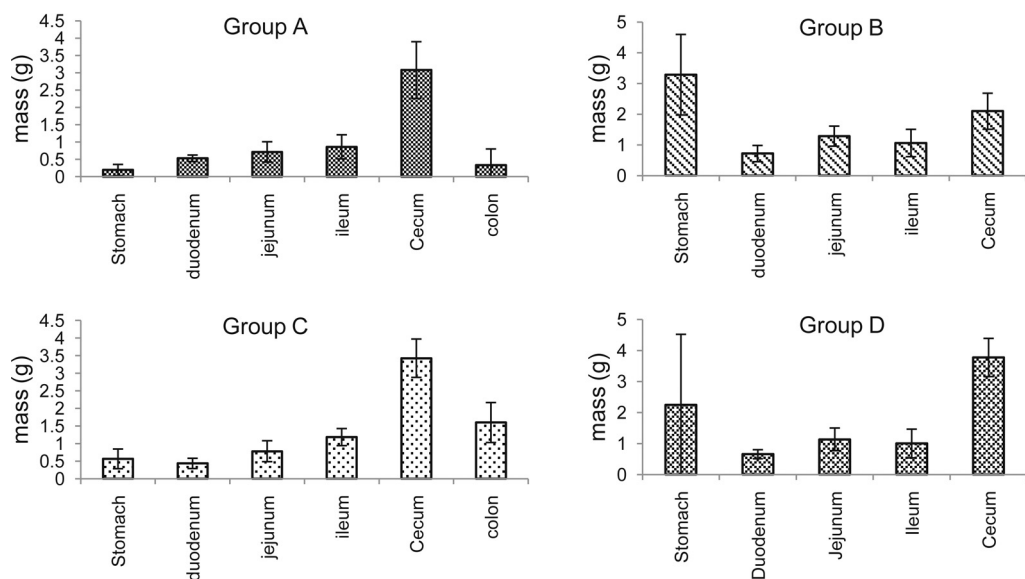


Fig. 4. Mass of luminal contents in the different gastrointestinal sections in the rat Groups. Each bar represents mean $\pm$ S.D. $n = 5$.

degraded by azoreductases, which are produced by many different bacterial species in the large intestine. The supply of the enzyme azoreductase being almost unlimited, sulfasalazine's degradation can take place without delay, and does not seem to be influenced by the feeding regimen. In fact, the sulfasalazine degradation was so fast that the degradation profiles have few time points, and the curves were not fitted for further analysis for reaction rates and half-lives.

In contrast, the cyclodextrin conjugate's metabolism is much more complex, and involves two types of enzymes, amylase and esterase. The esterase can only act after the amylase has started degrading the cyclodextrin carrier as reported previously (Hirayama et al., 2000). Moreover, the compounds formed in the initial stages of amylase-degradation of cyclodextrin

– high-membered maltooligomers (maltohexaose, maltopentaose, maltotetraose) – are themselves substrates for the amylases and can therefore act as competitive inhibitors of the enzymatic reaction (Suetsugu, Koyama, Takeo, & Kuge, 1974; Jodal et al., 1984). In addition, while the lower-membered maltooligomers (glucose, maltose and maltotriose) formed during the reaction are not substrates for the amylase, they can become “non-competitive” enzyme inhibitors by linking to the enzyme protein (Jodal et al., 1984). Thus, the diclofenac-CD conjugate shows potential as a sustained-release formulation.

(2) The degradation of both prodrugs is faster in colonic contents, compared to caecal contents (Table 1). This could be due to a number of reasons; such as the caecal slurry having a lower

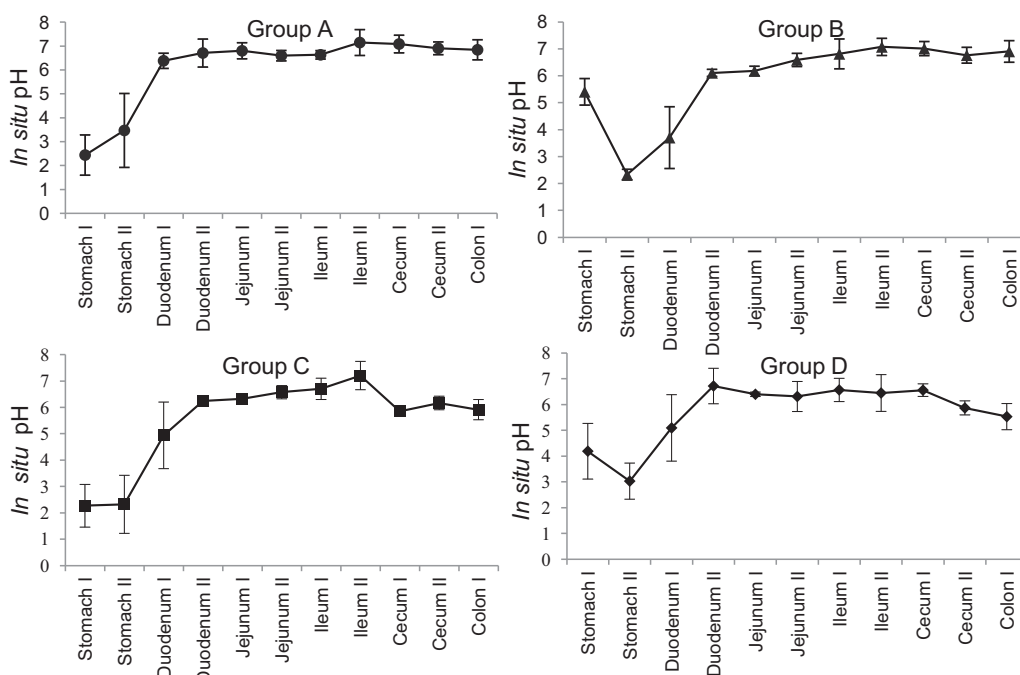


Fig. 5. *In situ* pH of gastrointestinal contents in the different sections of the gastrointestinal tract in the rat Groups. I and II refer to the anterior and posterior parts, respectively. Each point represents mean $\pm$ S.D. $n = 5$.

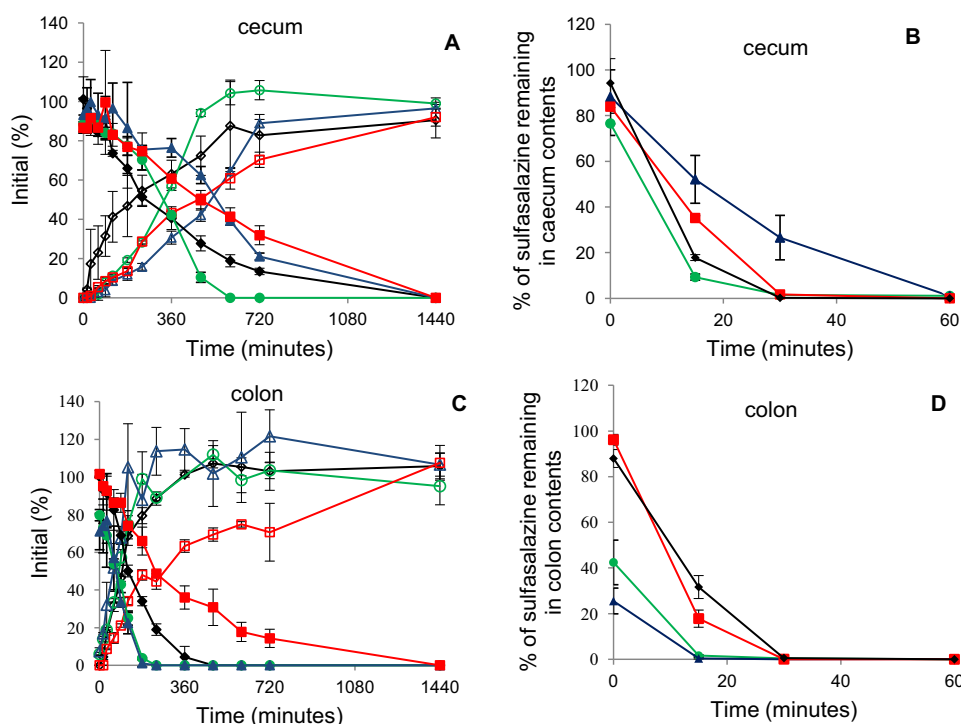


Fig. 6. Mean levels of diclofenac- β -cyclodextrin ((A) and (C)) and sulfasalazine ((B) and (D)) remaining when prodrugs were incubated in caecal and colonic contents from rats from Group A (●), B (▲), C (■), and D (◆). Means of diclofenac ((A) and (C)) appearance when diclofenac- β -cyclodextrin was incubated in caecal and colonic contents from Group A (○), B (△), C (□), and D (◇). Each point represents mean $\pm$ S.D., $n = 3$.

Table 1

Degradation rate (k , min^{-1}) and half-life ($t_{1/2}$, min) for diclofenac- β -cyclodextrin in caecal and colonic contents of rat Groups A–D.

Groups	Caecum		Colon	
	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

bacterial concentration (due to its greater liquid content), or the caecal slurry being more nutrient-rich (the caecum being the main site of bacterial fermentation), such that less prodrug is metabolized by the bacteria as a source of substrate.

- (3) The rate of CD-drug conjugate's degradation by bacterial enzymes was influenced by the feeding regimen in the colon (Fig. 6). Degradation was fastest from Groups A and B fasted rats, followed by Group D, followed by Group C (Table 1). This order of degradation rates k was statistically significant ($p < 0.05$, Kruskal Wallis, followed by Nemenyi's test). The fast prodrug degradation in Group A rats (which had been fasted for 12 h) could be due to a lack of nutrient in the colon for the bacterial enzymes to act on, which therefore act exclusively on the prodrug, degrading the latter. Similarly, in Group B rats, the colon is nutrient-poor and exclusive enzyme action on the prodrug leads to the latter's fast degradation. Although Group B rats were fed for 1 h (Fig. 2), the food had not had time to move down to the colon by the time the rats were killed 30 min after the one-hour feeding time. In contrast, food and nutrient were present in the colon of Group D fed rats, and competition between nutrient and prodrug for enzyme action led to slower prodrug degradation. The slower rate of prodrug degradation in Group C rats (compared to Group D rats) shows an even greater amount of nutrient in the colon of Group C rats, and hence greater competition of enzyme action. It is possible

that the bolus intake of food by Group C rats during the one hour feeding arrives in the colon at some point during the 4 h of fasting, such that there is overwhelming competition for the enzyme.

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

In contrast to the obvious influence of feeding regimen on prodrug degradation in the colon, the influence was less obvious in the caecum. The degradation curves for all rat Groups have similar profiles, especially at the beginning of the *in vitro* degradation reaction (Fig. 6). The caecum has such a high content of material in all rat Groups (Fig. 4) that feeding/fasting did not seem to alter the nutrient content and subsequently, any competition between nutrient and prodrug for enzyme action. One point to note though is the completion of prodrug degradation in Group A fasted rats' caecal contents at 600 min (Fig. 6) in contrast to the other Groups. This correlates with the fact that Group A fasted rats had a lowest amount of nutrient (and hence competition for enzyme action) in their caecum.

4. Conclusions

This study demonstrates the importance of feeding regimens, specifically the timing of meal ingestion, on the gastrointestinal conditions in rats and how this influences the metabolism of colonic prodrugs. In addition to changes in the distribution of gut contents along the GI tract, which directly affects the gastrointestinal transit time, different feeding regimens are accompanied by changes in the pH of gut contents, specifically in the stomach and large intestine. Moreover, differential gut contents in the large intestine have an impact on the microbiota activity, which affects the rate of

diclofenac- β -cyclodextrin metabolism and hence drug release and absorption. We also show that the different feeding regimens did not seem to impact on the metabolism of sulfasalazine, which was rapidly metabolized. Thus, we conclude that while feeding regimen influences the performance of the colonic prodrug, diclofenac- β -cyclodextrin, that influence has to be measured for each prodrug individually, given the different metabolic pathways of different colonic carriers.

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