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Degradation kinetics of metronidazole and olsalazine by bacteria in ascending colon and in feces of healthy adults

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ABSTRACT

Purpose: To compare the degradation kinetics of metronidazole and olsalazine by the bacteria of ascending colon and the bacteria of feces of healthy adults.
Methods: Contents of the ascending colon of seven healthy adults were collected under conditions simulating the bioavailability/bioequivalence studies in the fasted and in the fed states on a crossover basis. Material from the contents of the ascending colon was prepared by ultracentrifuging and diluting the precipitate with a volume of normal saline equivalent to that of the supernatant. Fecal material was prepared from feces of three healthy adults collected at two occasions that were separated by at least 6 months. *Ex vivo* drug degradation kinetics were evaluated under anaerobic conditions.
Results: Mean half-lives of metronidazole degradation in material from the contents of the ascending colon collected in the fasted state and in fecal material were 16.1 and 2.4 min, respectively ($p < 0.001$). The corresponding numbers for olsalazine were 57.8 and 9.2 min, respectively ($p < 0.001$). Both compounds were stable in material from the contents of ascending colon collected in the fed state.
Conclusions: Compared with data in fecal material, degradation of metronidazole and olsalazine in material from the contents of the ascending colon is significantly slower and it becomes non-significant during the arrival of fresh food remnants in the region.

1. Introduction

Degradation in the lumen of the lower gut is one of the factors limiting colonic drug absorption (Tannergren et al., 2009) and may also dictate the activity of the drug against colonic bacteria. To date, the effects of colonic microbiota on drug degradation in the lower gut are almost exclusively evaluated with experiments in fecal material (Sousa et al., 2008). Reasons for not using a more representative sample (collected from the proximal colon) include the difficulty in sampling from the colon and the assumption that fecal bacteria provide adequate information, especially when considering the huge inter- and intra-variability of bacteria colonies in the human colon (Edmiston et al., 1982; Rowland, 1988; Neut et al., 1989).

Quantitative sampling from the proximal colon requires prior cleaning of the entire large intestine. Mechanical preparation of the colon prior to colonoscopy has non-significant influence on colonic microflora (Arabi et al., 1978; Morotomi et al., 1989). However, a procedure for collecting contents from the ascending colon of healthy adults, so, that the effects of prior cleaning of the colon on the physicochemical characteristics of the colonic environment are fully reversed, was reported only few years ago (Diakidou et al., 2009).

The aim of this study was to evaluate the degradation kinetics of metronidazole and olsalazine by bacteria in the fasting and fed ascending colon with those by bacteria in fecal material. Although a distinction between fasted and fed ascending colon is difficult to be made in clinical practice, the environment in the ascending colon during bioavailability/bioequivalence (BA/BE) studies performed under fasting conditions is clearly different from that performed under fed state conditions (Diakidou et al., 2009). Metronidazole is a 5-nitroimidazole derivative with activity against protozoa and anaerobic bacteria. As a result of the activity of anaerobic bacteria, metronidazole decomposes to N-(2-hydroxyethyl)-oxamic acid and acetamide in fecal material (Sousa et al., 2008) (Fig. 1). It has been shown that relevant metabolites are formed after incubation of metronidazole with either *Clostridium. perfringens* or rat caecal contents and have also been measured in the urine of patients taking the drug (Koch et al., 1979). Access of the drug to

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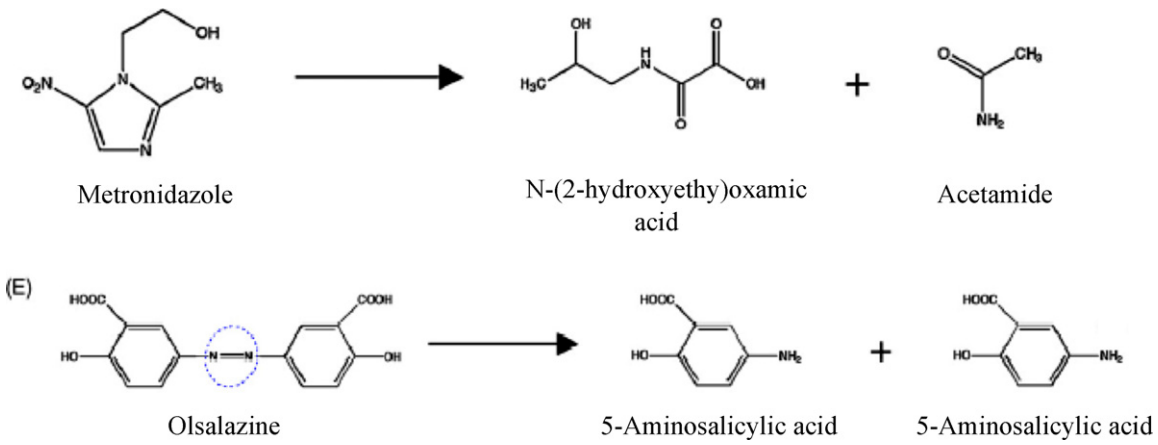

Fig. 1. Metronidazole, olsalazine and their metabolites that are formed by anaerobic bacteria in the large intestine (Wadworth and Fitton, 1991; Sousa et al., 2008).

the flora is probably facilitated by its secretion from the systematic circulation into the gut lumen (Abu Shamat, 1993). Olsalazine (sodium azodisalicylate; azodisal sodium) is a prodrug consisting of two 5-aminosalicylic acid moieties bridged by an azo bond. Olsalazine has been developed as a way of delivering the anti-inflammatory 5-aminosalicylic acid to the large intestine, since very little of the parent molecule is absorbed from the gastrointestinal tract after oral administration whereas in the large intestine, azoreductase-containing anaerobic bacteria split olsalazine into two 5-aminosalicylic acid molecules (Wadworth and Fitton, 1991; Jain et al., 2006) (Fig. 1).

2. Experimental

2.1. Materials

2.1.1. Chemicals

Metronidazole was purchased from Acros, Organics (NJ, USA) and olsalazine from Beijing Huameihli Bio-Chem Trade Centre (Beijing, China). Acetonitrile was of LC–MS grade and all other chemicals were of analytical grade.

2.1.2. Material from the contents of the ascending colon

The contents of the ascending colon were collected under anaerobic conditions from seven healthy adults at two different occasions on a crossover basis.

Inclusion criteria: Willingness of subject to participate as indicated by his/her signed informed consent, age 18–60 years old, within 15% of ideal body weight as determined by Metropolitan Life Tables, verification of suitability by a general physical examination and ability to abstain from cigarette smoking, alcohol, and over-the-counter and prescription medication(s) for three days prior to Colonoscopy Day until the end of the Colonoscopy Day. A blood sample was taken to assess electrolyte balance, kidney and liver function, blood morphologic characteristics, and lipid levels, and the subject had to be found healthy in all these examinations to qualify.

Exclusion criteria: Existence of a major health problem (cardiovascular, pancreatic, hepatic, thyroid etc.) and/or existence of any condition requiring prescription drug therapy, recent history of gastrointestinal symptom regardless of the severity (e.g. heartburn, constipation, haemorrhoids, etc.), women who were pregnant, lactating, or had been on birth control pills for less than 3 months, receipt of an investigational agent (new or generic) within 30 days prior to the initiation of study, presence of antibodies indicating active acute or chronic HIV, HBV, or HCV infection, use of medication which may affect GI function (including antibiotics) within 30 days of the study, and irregular bowel habits.

Colonoscopies were held in the Red Cross Hospital of Athens after receiving approvals by the Scientific and the Executive Committee of the Hospital (AP 23783 and AP 27573).

In Phase I, subjects were fasted (water *ad libitum*) from 8 pm on the day prior to colonoscopy until 12 noon the following day when colonoscopy was performed (i.e. they fasted for 16 h). In Phase II, subjects were fasted (water *ad libitum*) from 8 pm on the day prior to colonocscopy until 8 am on colonoscopy day when they consumed a standard breakfast (960 kcal). At noon they were offered a light lunch and at 2 pm colonoscopy was performed. These conditions simulate the typical BA/BE study conditions whereas times, at which colonoscopies were performed and colonic contents were collected, correspond to the times at which orally administered drug products are expected to be in the ascending colon during a BA/BE study. All subjects had been administered 10 mg of bisacodyl 50 h before and 10 mg of bisacodyl 44 h before each colonoscopy (Diakidou et al., 2009). After placement of the colonoscope, collection of contents from the ascending colon was performed under anaerobic conditions. The collection lasted approximately 10 min. This period was adequate for removing almost all of the contents from the region. After each collection, the colonic contents were transferred to appropriate test tubes (capacity: 11 ml) so that the tubes were filled up to the top, a cocktail for inhibiting lipolysis and proteolysis was added (methanolic solution containing 50 mM diisopropylfluorophosphate, 50 mM diethyl(p-nitrophenyl)phosphate, 50 mM acetophenone, and 250 mM phenylboronic acid, 20 μl/ml) (Hernell et al., 1990) and the tube was sealed. This procedure was performed under aerobic conditions and it lasted for up to 5 min. After ultracentrifugation (30,000 × *g*, 20 min, 25 °C) the precipitate was collected under aerobic conditions. This collection lasted for up to 5 min. The precipitate was stored under anaerobic conditions at −70 °C. On the experimental day, the material from the contents of the ascending colon was prepared under anaerobic conditions (10% H_2, 10% CO_2 and 80% N_2, Electrotek anaerobic workstation AW 800 TG, Electrotek Limited, West Yorkshire, UK) by diluting each individual precipitate with a volume of normal saline equivalent to that of the supernatant (after ultracentrifugation of colonic contents from which the specific precipitate had been obtained) and by vortexing for 2 min. Depending on the sample, homogenization of colonic precipitate with normal saline was performed by using 7–10 ml of normal saline for the fasted state samples and 5–7 ml normal saline for the fed state samples. Ultracentrifugation of colonic contents and subsequent regeneration of bacterial content with normal saline were necessary for eliminating potential effects of enzymes that may exist in the fluid of ascending colon.

2.1.3. Fecal material

Freshly voided feces were collected from three healthy adults at two different occasions separated by at least six months ($n = 6$). Feces were transferred into the anaerobic workstation as soon as possible after defecation (<10 min).

Different methods of preparation of the fecal slurry from human feces have been reported in the literature using water (Koch et al., 1979), buffers (Strong et al., 1987; Krook et al., 1981; Basit and Lacey, 2001) or normal saline (Vince et al., 1978; Mortensen et al., 1992) and dilution factors vary from 2 to 10. In this study, feces were homogenized with 3.8 parts normal saline (w/w) until a homogenous material was obtained (Vince et al., 1978) and the resulting fecal material was stored at −70 °C until used. In all cases, the pH of fecal material was almost neutral.

2.2. Degradation experiments

All experiments were performed at 37 °C into the anaerobic workstation.

For metronidazole experiments, a stock solution of 100 μg/ml was prepared in normal saline. 125 μl of the stock solution were added in 1 ml of the medium to be tested, i.e. the initial nominal concentration at $t = 0$ was 11.1 μg/ml. For olsalazine experiments, a stock solution of 140 μg/ml was prepared in normal saline. 125 μl of the stock solution were added in 1 ml of the medium to be tested, i.e. the initial nominal concentration at $t = 0$ was 15.6 μg/ml. Samples (100 μl) were withdrawn at 0, 5, 10, 15, 20, 30, and 80 min (metronidazole) and at 0, 10, 20, 30, 60, 90, and 120 min (olsalazine) after the beginning of incubation at 800 oscillations per minute (Eppendorf Thermomixer comfort, Eppendorf AG, Hamburg, Germany).

2.2.1. Preliminary experiments

The chemical stability of the model compounds was confirmed with experiments in simple buffers. For metronidazole, experiments were performed in phosphate buffers with pH 4.0 and 8.5, to confirm literature data suggesting that metronidazole is stable in the 2–8 pH range (Wang and Yeh, 1993; Erah et al., 1997). As there are no relevant published data, chemical stability of olsalazine was evaluated in phosphate buffers having pH 4.0, 5.0, 6.5, 8.0 and 9.0 to cover a wide range of pH values that may exist in the lower gut (Diakidou et al., 2009).

Preliminary experiments were also performed to confirm that bacterial degradation of model compounds is not be affected by the presence of lipolysis/proteolysis inhibitors. Such inhibitors had been added in the contents of ascending colon upon aspiration in order to maintain their composition unaltered until the time of the experiments (Hernell et al., 1990). In addition, preliminary experiments were performed in order to confirm that precipitated bacteria (following to ultracentrifugation of colonic contents) remain intact and maintain their initial activity. Since the volume of contents in the ascending colon is small (Diakidou et al., 2009) relevant experiments were performed in pooled fecal material (prepared by mixing equal quantities of individual materials from 3 healthy adults) as follows:

i) Degradation experiments of both model compounds in fecal material in absence and in presence of lipolysis/proteolysis inhibitors (20 μl/ml).
ii) Degradation experiments in regenerated fecal material containing or not containing lipolysis/proteolysis inhibitors (20 μl/ml). Regenerated fecal material was obtained after ultracentrifuging fecal material (30,000 × *g*, 20 min, 25 °C), diluting the precipitate with a volume of fresh normal saline equal to the volume of supernatant, and vortexing for 2 min.
iii) Degradation experiments of metronidazole in supernatant obtained after ultracentrifugation of fecal material (30,000 × *g*, 20 min, 25 °C) that contained or did not contain lipolysis/proteolysis inhibitors (20 μl/ml).

Each preliminary experiment was performed once. Variability of degradation data was evaluated by performing olsalazine experiments in regenerated fecal material in triplicate.

2.2.2. Main experiments

Experiments were performed in individual materials from the contents of the ascending colon collected in the fasted and in the fed state on a crossover basis ($n = 7$) and in individual fecal materials ($n = 6$).

2.3. Methods for assaying the drugs

2.3.1. Metronidazole

Each sample (100 μl) was transferred to a microcentrifuge tube containing 300 μl of acetonitrile. After vortexing for 20 s, tubes were centrifuged for 10 min at 10,000 rpm (Hettich Micro 200, Hettich, Tottlingen, Germany). 100 μl of the clear supernatant were diluted with 900 μl of water and, after vortexing, part of the latter solution was injected into the LC–MS system. The exact chromatographic conditions are presented in Table 1. For the quantification of samples, calibration curves were constructed in fecal material (0.02–2 μg/ml).

2.3.2. Olsalazine

Each sample (100 μl) was transferred to a microcentrifuge tube containing 300 μl of acetonitrile. After vortexing for 20 s, tubes were centrifuged for 10 min at 10,000 rpm and part of the supernatant was injected into the HPLC system. The chromatographic conditions are presented in Table 2. For the quantification of samples, calibration curves were constructed in fecal material (0.03–5 μg/ml).

2.4. Data treatment

The zero- and the first-order models with and without lag times were fitted to each experimental data set. Akaike's criterion (Wagner, 1993) was used for selecting the comparatively most appropriate model. Degradation rate constants were compared by using two-tailed unpaired *t*-test, after confirming normality and equal variance.

3. Results and discussion

3.1. Preliminary experiments

Metronidazole was stable for at least 80 min in phosphate buffer (pH 4.0 and 8.5) and olsalazine was stable for at least 2 h in all tested phosphate buffers (pH 4.0, 5, 6.5, 8 and 9) indicating that both model compounds are chemically stable in the range of pH values that could be observed in the contents of the ascending colon.

Based on Akaike's criterion, the first order model was fitted better than the zero-order model to the individual data sets. Based on the estimated first order degradation rate constants, lipolysis/proteolysis inhibitors do not exert any significant effect on degradation of model compounds (Table 3) whereas the regenerated fecal material seems to have a degrading activity similar to that of the initial fecal material regardless of the presence of inhibitors (Table 3). Although most of these data have been collected from experiments performed once, the high reproducibility of the olsalazine data in the regenerated fecal material (Table 3) suggests that relevant conclusions could be generalized safely. In addition, the supernatant after ultracentrifugation of fecal material (regardless of the presence of lipolysis/proteolysis inhibitors)

Table 1
Chromatographic conditions applied in this study for the assay of metronidazole in fecal material and in material from the contents of the ascending colon.

Column	Atlantis® dC_{18}, 50 mm × 2.1 mm, 3 μm particle size, Waters (Ireland)		
Mobile phase	Line A	Water:acetonitrile:formic acid 95:5:0.1 (v/v/v)	
	Line B	Acetonitrile:formic acid 100:0.1 (v/v)	
	Time (min)	%A	%B
	0	100	0
	1.5	100	0
	3.5	5	95
	4.0	5	95
	5.0	100	0
	13.0	100	0
Flow rate		0.25 ml/min	
Injection volume		5 μl	
SIR of mass		172.10	
Gas flow (l/h)	Desolvation	400	
	Cone	80	
Temperature (°C)	Source	115	
	Desolvation	370	
Voltages	Capillary (kV)	3.00	
	Cone (V)	25	
	Extractor (V)	3	
	RF Lens (V)	0	

Table 2
Chromatographic conditions applied in this study for the assay of olsalazine in fecal material and in material from the contents of the ascending colon.

Column	Atlantis™ dC_{18}, 150 mm × 4.6 mm, 5 μm particle size, Waters (Ireland)		
Mobile phase	Line A	Water:acetonitrile:trifluoroacetic acid 90:10:0.1 (v/v/v)	
	Line B	Acetonitrile:trifluoroacetic acid 100:0.1 (v/v)	
	Time (min)	%A	%B
	0	100	0
	15	100	0
	16	5	95
	17	5	95
	23	100	0
Flow rate		1 ml/min	
Detection wavelength		365 nm	
Injection volume		5 μl	

had no degrading activity on either model compound [for at least 80 min and 2 h for metronidazole and olsalazine, respectively (data not shown)], i.e. under the applied ultracentrifugation conditions bacteria are quantitatively removed from the supernatant. It could, therefore, be claimed that the material from the contents of the ascending colon used in drug degradation experiments practically contain all bacteria present in colonic contents prior to ultracentrifugation.

3.2. Main experiments

Degradation data are presented in Figs. 2 and 3 for metronidazole and olsalazine, respectively.

The first-order model was found to fit better than the zero-order model in the vast majority of those individual data sets in which degradation was apparent. However, lag times had to be assumed for the degradation data of both model compounds collected in the material from the contents of the ascending colon of subjects #2 and #3 (10 min and 30 min for metronidazole and 5 min and 30 min olsalazine, respectively). It is also interesting to note that regardless of dosing conditions, the material from the contents of the ascending colon from two subjects (#1 and #4) did not have any degrading activity on either compound (Figs. 2 and 3). We have observed that activity of fecal material on metronidazole degradation decreases when pH decreases (data not shown). It is possible, therefore, that bacteria in the ascending colon of subjects #1 and #4 had lower activity than of rest of subjects on metronidazole at collection time, because the pH in the ascending colon of those 2 subjects (6.4 for both) was lower than the pH in ascending colon of rest of subjects (pH ≥ 7.2). No specific comment can be made for the absence of olsalazine degradation in those two subjects.

Table 3
Effect of lipolysis/proteolysis inhibitors on first order degradation rate constant (min^{-1}) of metronidazole and olsalazine in fecal material and efficiency of ultracentrifugation on the quantitative removal of bacteria from fecal material without affecting their activity.[a]

	Metronidazole	Olsalazine
Fecal material	0.277	0.053
Fecal material containing 2% lipolysis/proteolysis inhibitors	0.182	0.056
Regenerated fecal material	0.217	0.042/0.043/0.041
Regenerated fecal material containing 2% lipolysis/proteolysis inhibitors	0.204	0.037/0.037/0.039

[a] All experiments were performed once, apart from those of olsalazine in regenerated fecal material that were performed in triplicate. In all cases, fitting of the first-order model did not require the introduction of a lag time and determination. Determination coefficients ranged from 0.98 to 0.9999.

For both compounds, degradation rate constant in the material from the contents of the ascending colon collected in the fasted state is highly variable but significantly lower than that observed in fecal material (Table 4). For metronidazole, mean degradation half lives were 16.1 min and 2.4 min in the material from the contents of the ascending colon and in fecal material, respectively ($p < 0.001$). Corresponding mean degradation half lives for olsalazine were 57.8 min and 9.2 min, respectively ($p < 0.001$). It is interesting to note that Kellow et al. (1986) demonstrated that after direct instillation of salicylazosulfapyridine in the caecum in the fasted state, the appearance of its metabolite (sulfapyridine) in plasma was rapid (~5 min). This is in agreement with our data according to which olsalazine is degraded up to 20% to 5-ASA within 10 min in the fasted ascending colon. Kellow et al. (1986) however, do not provide data on its rate of appearance in plasma.

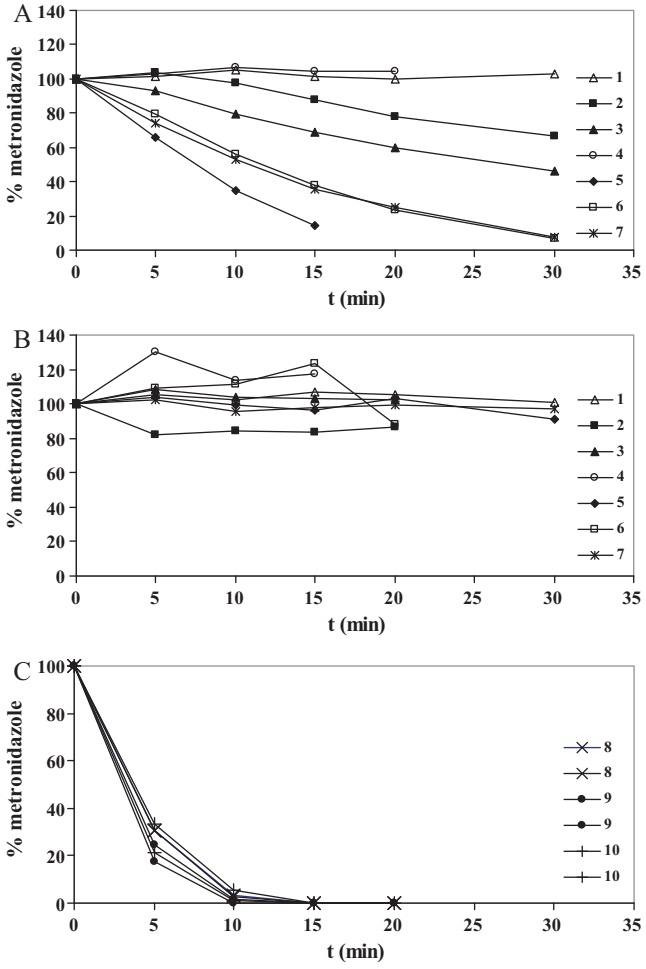

Fig. 2. % metronidazole remaining in (A) individual materials from the contents of the ascending colon collected in the fasted state ($n = 7$), (B) individual materials from the contents of the ascending colon collected in the fed state ($n = 7$), and (C) individual fecal materials [$n = 6$ (3 adults, 2 fecal materials from each adult)].

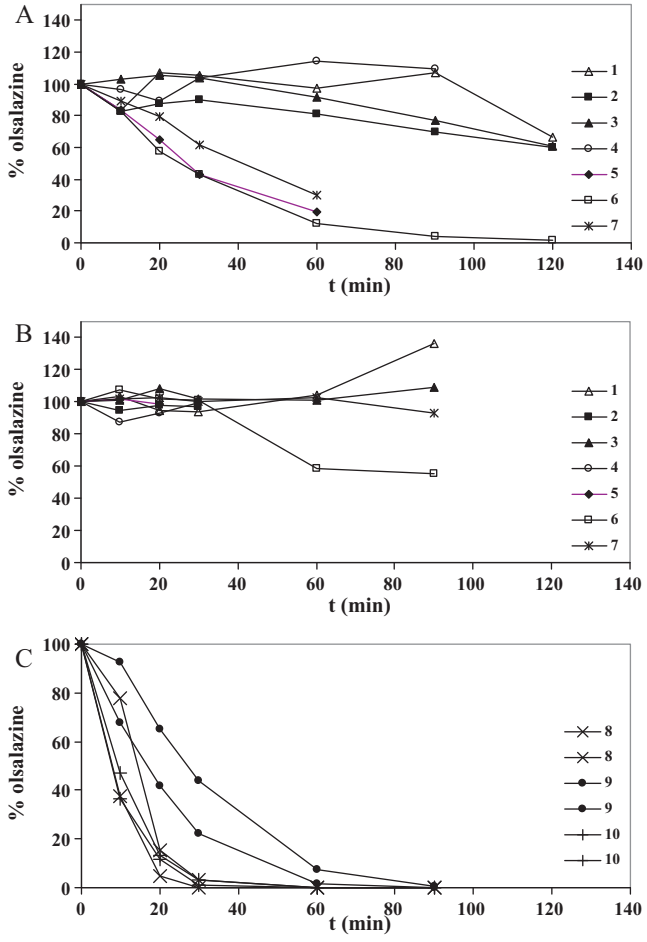

Fig. 3. % olsalazine remaining in (A) individual materials from the contents of the ascending colon collected in the fasted state ($n = 7$), (B) individual materials from the contents of the ascending colon collected in the fed state ($n = 7$), and (C) individual fecal materials [$n = 6$ (3 adults, 2 fecal materials from each adult)].

Both metronidazole and olsalazine were found to be practically stable in the material from the contents of the ascending colon collected in the fed state (Figs. 2 and 3). Since the activity of the flora is a function of availability of fermentable substrates (Tannock, 1999), it could be argued that the slower degradation of both compounds in the fed state relates with the arrival of fermentable substrates from the small intestine, i.e. a competitive inhibition mechanism operates.

Fecal excretion of unchanged olsalazine between 24 and 96 h after oral administration is 4–5% whereas the corresponding total fecal excretion of 5-ASA is 47–50% (Sandborn and Hanauer, 2003).

Table 4
Mean (SD) values for the degradation rate constant (min^{-1}) of metronidazole and olsalazine in fecal material ($n = 6$), and in material from the contents of the ascending colon collected in the fasted and in the fed state on a crossover basis ($n = 7$).[a]

	Metronidazole	Olsalazine
Fecal material	0.289(0.046)	0.075(0.033)
Material from the contents of the ascending colon		
Fasted	0.043(0.040)[b]	0.012(0.013)[b]
Fed	N.A.	N.A.

[a] Fitting was performed to individual data and required the inclusion of lag time in subjects #2 and #3 (see text for details). Normality and constant variance tests passed in all cases. Determination coefficients ranged from 0.97 to 0.999. N.A. means not applicable, due to lack of degradation for at least 80 min (metronidazole) or 2 h (olsalazine).

[b] Significantly different from fecal material ($p < 0.001$).

Incomplete degradation of olsalazine in the colon has been also indicated by the data of Staerk Laursen et al. (1990) according to which, although treatment with olsalazine is expected to double the colonic concentration of 5-ASA when compared with equimolar doses of a slow release or delayed mesalazine preparation, significantly lower systemic absorption of 5-ASA and its acetylated metabolite after olsalazine administration has been observed. Also, the systematic availability of olsalazine is not affected by food (Ryde and Ahnfelt, 1988). These data in conjunction with data of the present study suggest that the degradation of olsalazine is limited in the ascending and becomes more pronounced in the descending colon both in the fasted and in the fed state.

It is worth noting that extrapolation of data from this study to patients should be done cautiously. In patients with active ulcerative colitis, significant decreases in the number of anaerobic bacteria, gram-negatives anaerobes, and Lactobacillus were observed in the colonic mucosa (Fabia et al., 1993). Also, Pathmakanthan et al. (1999) observed a significant quantitative decrease in growth of *Lactobaccilus* spp. in colitic biopsies.

In regard to azoreductase, its activity in feces of 14 patients with active Crohn's disease is also decreased as compared with 12 healthy subjects (Carrette et al., 1995). In patients with active ulcerative colitis under sulfasalazine treatment, the circulating level of its main metabolite, sulfapyridine, was decreased (compared with that in healthy adults) and in part these data could be explained by a decreased azoreductase activity in these patients (Carrette et al.,

1995). It should be mentioned that, azoreductase is not produced by a unique bacterial species and numerous intestinal bacteria are able to produce it.

After administration to patients with Crohn's disease and to healthy volunteers, metronidazole was detected in feces of most patients but not in feces of any healthy individual (Edmiston et al., 1982). Therefore, it is expected that both olsalazine and metronidazole are more stable in patients than in healthy subjects.

In conclusion, for both metronidazole and olsalazine, activity of flora in fecal material was found to be much higher than that in material from contents of the ascending colon during a BA/BE study, especially in the fed state. Performing dissolution/stability studies in presence of fecal material under anaerobic conditions is a sensitive method for evaluating the drug dissolution/stability in the large intestine but data should be used only on a qualitative basis.

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