Pharmaceutical Research, Vol. 26, No. 9, September 2009 (© 2009)
DOI: 10.1007/s11095-009-9927-x

Research Paper

Characterization of the Contents of Ascending Colon to Which Drugs are Exposed After Oral Administration to Healthy Adults

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Received April 14, 2009; accepted June 11, 2009; published online July 2, 2009

Purpose. To characterize the contents of the ascending colon in healthy adults under fasting and fed state conditions, with a view to designing *in vitro* studies to explain/predict dosage form performance in the lower gut.
Methods. Twelve healthy adults participated in a two-phase crossover study. In Phase A, subjects were fasted (water allowed) overnight plus 5 h in the morning prior to colonoscopy (fasted state). In Phase B, subjects were fasted overnight, consumed a standard breakfast (960 kcal) in the morning, and were offered a light lunch 4.5 h later. In this phase, colonoscopy was performed 1 h after lunch (fed state). Volume, pH, and buffer capacity of colonic contents were measured immediately upon collection. After ultracentrifugation, the supernatant was further characterized.
Results. Free water content, pH, surface tension, and isobutyrate levels were lower in fed than in fasted subjects. On the other hand, buffer capacity, osmolality, acetate, butyrate, cholate, and chenodeoxycholate levels were higher in fed subjects. Carbohydrate content; protein content; and levels of long chain fatty acids, phosphatidylcholine, and cholesterol were not affected significantly by prandial state.
Conclusion. Composition of fluids in the ascending colon is affected by feeding. This may affect the performance of products designed to deliver drug to the colon.

KEY WORDS: ascending colon; charged aerosol detector (CAD); fasted state; fed state; lumenal composition.

INTRODUCTION

To date, the performance of orally administered dosage forms has been assessed primarily with bioavailability (BA) / bioequivalence (BE) studies in healthy adults, which are time-consuming and costly. It would, therefore, be of considerable advantage to reduce the likelihood of failing a BE study, or, even more, to avoid performing an *in vivo* BE study. One way to achieve this is to screen dosage form performance *in vitro* in a simulated lumenal environment. In recent years, our knowledge of the environment in the healthy adult gastrointestinal (GI) tract under conditions simulating BA/BE studies has improved substantially (1-3), and, as a consequence, updated compositions of biorelevant media have been proposed (4,5). However, due to the fact that oral drug absorption is usually complete in the small intestine, the emphasis to date has been on conditions in the upper GI lumen.

In cases where the drug has low permeability in the small intestine but can be absorbed to some extent through the colonic mucosa, where an extended release dosage form is administered, or where the dosage form targets the drug to the colonic mucosa for local action, conditions in the lower GI lumen will influence drug/dosage form performance. Due to the substantial residence time in the ascending colon (6,7) and the limited free water volume in the transverse colon (8), the primary region of interest in regard to drug/dosage form performance in the lower gut is the ascending colon.

Due to difficulties in directly sampling from the lumen of the ascending colon, conditions in this region have been studied primarily using imaging techniques or by analyzing samples collected from ileostomy patients or from individuals who have died suddenly. Imaging techniques, although valuable, do not allow for quantifying certain physicochemical characteristics that are of prime importance for the performance of dosage forms, e.g., buffer capacity, surface tension, osmolality, and concentration of potentially solubilizing agents. On the other hand, data from ileostomy patients and/or from individuals who have died suddenly may not be representative of healthy individuals and do not distinguish

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between fasted and fed state conditions. Although in clinical practice such distinctions may not be relevant, in BA/BE studies food effects are of primary concern.

A key characteristic of the ascending colon is the large population of (predominantly anaerobic) bacteria. Microbial densities increase dramatically in the distal small intestine (approximately 10^8 bacteria/ml of luminal contents) and colon (10^{11}–10^{12}/g), with anaerobic species outnumbering aerobic by a factor of about 1,000 (9). At least 500 bacterial species colonize the adult intestine, with 3–40 species comprising up to 99% of the total population (10). The adult (fecal) microflora is influenced by environmental factors as well as by host genotype. The basic set of biochemical reactions carried out by microbes includes degradation of carbohydrates (and to a lesser extent of proteins and peptides) and fermentation of these degradation products to short chain fatty acids (and ammonia) (11,12). Although bacteria may also play a significant role in the intracolonic fate of drugs and dosage forms, in the present study we chose to characterize the contents of the ascending colon in healthy adults in the fasted and fed states on a physicochemical basis, without specifically considering the microbes. Sampling from the region was performed anaerobically via colonoscope.

MATERIALS AND METHODS

Human Studies

Two studies (pilot and main) were performed. Both studies were conducted 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). The same inclusion and exclusion criteria applied to both studies, following the tenets of the Declarations of Helsinki for biomedical research involving human subjects.

Inclusion criteria were willingness 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 3 days prior to the 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 deemed healthy in all these examinations to qualify.

Exclusion criteria were 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.

Pilot Study

Colonoscopy requires a prior cleaning of the colon (especially the distal colon). In order for the environment of the ascending colon to reflect the environment during a BA/BE study, any effect(s) of colon preparation procedure should have been reversed by the time sampling started. The colon preparation and sample handling procedures necessary to enable this reversal were determined in a pilot study performed in the fasted state. The pilot study also provided the material (colonic content) required for the development/optimization of analytical methods.

Six fasted healthy adults (3 males and 3 females) participated in the single phase pilot study. Subjects were between 20 and 36 years old and deviated by not more than 11% from the ideal body weight. Colon preparation procedures involved the ingestion of varying volumes of polyethylene glycol 3350 (PEG) solution in non-carbonated water (59 g PEG/L, Klean-Prep®, 59 g PEG per sachet, Helsinn Birex Pharmaceuticals Ltd., Ireland) or bisacodyl tablets (5 mg/tab, Dulcolax®, Boerhinger Ingelheim, Athens, Greece). Klean-Prep® is a balanced mixture of PEG and electrolytes which, added to water, produces a clear, colourless, iso-osmotic solution (www.klean-prep.net, accessed 5 June 2009). It acts as bowel cleansing agent via osmotic effects of the non-absorbed PEG. Bisacodyl [bis(p-acetoxyphenyl)-2-pyridylmethane] (BIS) is a stimulant laxative. After oral administration it is rapidly converted to the active metabolite bis(p-hydroxyphenyl)-2-pyridylmethane (BHPM) and its laxative action is initiated through a direct interaction of the drug in the intestine by activating protein kinase C, releasing prostaglandin E2 and, thereby, inducing net fluid secretion (13,14). About 50% of the dose is eliminated in the faeces as BHPM (14). Bisacodyl has been used in the past for similar sampling procedures (15), and its effects on the intralumenal physiology have been shown to be reversible (16,17). Table I shows the exact colon preparation procedures applied in the pilot study, together with food consumption details prior to each colonoscopy.

On the Colonoscopy Day (Day 0) each subject reported fasted at the clinic at 8 am and, if female, was tested for pregnancy. All subjects remained fasted (water *ad libitum*) until 1 pm. Prior to colonoscopy (at about 12:55 pm), 0.75 ml of alphentanyl hydrochloride (0.2 mg/ml, Rapifen®) and 0.4 ml of midazolam (5 mg/ml, Dormicum®) were administered intravenously for conscious sedation. 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 removal of the colonoscope, 2 ml of flumazenil (0.1 mg/ml, Anexate®) was administered as antidote to midazolam. At about 2 pm, after a brief examination to ensure that the procedure had not caused any adverse effects, the subject was free to leave.

Sample volume, pH, and buffer capacity were measured immediately upon collection. The remainder of the sample was centrifuged to remove solids (11400 g, 10 min, 4°C), and, after measuring the volume of the supernatant, it was divided in four portions. The first was used for measuring PEG or BHPM, surface tension, osmolality, total protein content,

Table I. Colon Preparation Procedures Applied in the Pilot Study and Relevant Food Consumption Details Until Colonoscopy[1]

Subject number	Agent and Dose	Day and Period of administration	Day and time when consumption of semi-liquid food started[3]	Day and time when fasting (from food but not water) begun
1	3 sachets Klean-Prep® in 3 L of water	Day -1 / 5—9 pm	Day -1 / noon	Day -1 / 5 pm
2	3 sachets Klean-Prep® in 3 L of water	Day -1 / 4—8 pm	Day -1 / noon	Day -1 / 4 pm
3	2 sachet Klean-Prep® in 2 L of water	Day -1 / noon—2 pm	Day -1 / noon	Day -1 / noon
4	1 sachet Klean-Prep® in 1 L of water	Day -1 / 3—5 pm	Day -1 / noon	Day -1 / 3 pm
5	4 Dulcolax® tabs[2]	Day -1 / 2 tabs at 6 pm and 2 tabs at 9 pm	Day -1 / noon	Day -1 / 8 pm
6	4 Dulcolax® tabs[2]	Day -2 / 2 tabs at noon and 2 tabs at 6 pm	Day -2 / noon	Day -1 / 8 pm

[1] Colonoscopy and sample acquisition were performed on Day 0, 1 pm.
[2] Since previous data have shown that 2 tablets (10 mg Bisacodyl) may not be adequate to eliminate the feces from the lower colon (15), it was decided to administer 4 tablets in total.
[3] Semi-liquid food consisted of noodle soups, fish soups, fruit juices, limited amount of milk, refreshments, coffee, and tea.

total carbohydrate content, and 3a-hydroxy bile acids. In the second portion, an aqueous solution containing 8% (w:v) NaN_3 and 10% (w:v) chloramphenicol was added [0.5% (v:v)] to terminate the supernatant's bacterial activity (18), and the same measurements were performed. In the third, a methanolic cocktail of 50 mM diisopropylfluorophosphate, 50 mM diethyl(p-nitrophenyl)phosphate, 50 mM acetophenone, and 250 mM phenylboronic acid was added [2% (v:v)] to terminate the supernatant's lipolytic activity (19), and total protein and 3a-hydroxy bile acid content were measured. In the fourth portion, both bacterial and lipolytic activities were terminated, and total protein and 3a-hydroxy bile acid content were measured. All four portions were stored at −70°C until used.

Main Study

Twelve healthy adults (6 males and 6 females) with a mean age of 23 (range: 19–28 years) participated in this two-phase crossover study. Body weights deviated from ideal by not more than 15%. Subject work-up prior to either Phase involved the administration of 20 mg BIS 50–44 h prior to colonoscopy and consumption of semi-liquid food from 50 h prior to colonoscopy until 8 pm on the evening of the day prior to Day (0) (the Colonoscopy Day), i.e. the procedure was identical to that described for Subject #6 of the pilot study (Table I). Females were tested for pregnancy in the morning of each colonoscopy day. In Phase A, subjects were fasted from food but not water overnight, reported to the clinic at 8:00 am, and then continued fasting for an extra 5 h. Colonoscopy was started at ≈1pm (fasted state). In Phase B, subjects were fasted overnight, reported to the clinic at 8:00 am, and were given a standard breakfast (8:00 am–8:30 am). Four and a half hours after breakfast they were offered a light lunch, and 1 h after lunch, at ≈2 pm, colonoscopy was started (fed state). Breakfast consisted of two eggs fried in butter, two strips of bacon, two slices of toasted bread with butter, four ounces of fried potatos, and eight ounces of milk (960 kcal) (20). Lunch consisted of a sandwich (tuna, chicken, turkey, or ham) and 250 ml of fruit juice (orange, grapefruit, apple and carrot, or pineapple). Two subjects did not eat the lunch. In all cases, colonoscopy lasted for about 1 h in total. Analgesic/antidote administration was the same as described for the pilot study. Contents of the ascending colon were collected anaerobically during each colonoscopy according to the same protocol described for the pilot study. In the fed state, during the 10-min collection period some additional material entered the colon from the terminal ileum. Therefore, measured volumes in the fed state may slightly overestimate actual numbers in the fed state.

Volume, pH, and buffer capacity were measured immediately upon collection. After adding methanolic solution to terminate lipolysis (2% v:v) (19), the rest of the sample was ultracentrifuged (30000 g, 15 min, 25°C) under anaerobic conditions, and the % aqueous content was measured. Ultracentrifugation conditions were appropriate for eliminating both solids and bacteria (e.g. 21,22) without affecting the structure of bacteria, i.e. without liberating intracellular components which could contribute to degradation in the supernatant (unpublished data). The supernatant was divided in ten portions that were kept at −70°C until analysis of one of the following parameters: pH, buffer capacity, BIS, BHPM, surface tension, osmolality, total protein content, total carbohydrate content, short chain fatty acids, bile acids, and lipids.

Analytical Methods

pH values were measured with a pH electrode (Schott, model CG842, Mainz, Germany). Buffer capacities were measured in both pH directions by dropwise addition of NaOH and HCl solutions and were calculated as described previously (3).

PEG was determined as described previously (3). The limit of quantification (23) varied with sample composition and it was at most 8 mg/ml.

BIS was assayed with HPLC-UV, after liquid-liquid extraction with diethyl ether and by using atazanavir as internal standard. The aqueous layer was evaporated to dryness under a gentle stream of nitrogen, the residue was reconstituted with 300 μl of mobile phase (0.05 M KH_2PO_4 : acetonitrile 67:33 v/v), and 50 μl were injected into the HPLC.

The column was BDS C18 250×4.6 mm (Hypersil), the flow rate of mobile phase was 1.0 ml/min, and the detection wavelength was 230 nm. Retention times for BIS and atazanavir were 25.4 min and 42.6 min, respectively. The limit of quantification (22) was 0.20 μg/ml.

Before assay, BHPM had to be produced, as it is not commercially available. This was achieved by alkaline hydrolysis of BIS according to a well described procedure (24). This procedure ensures 98% conversion of BIS into BHPM with less than 2% of the respective monophenol product due to incomplete hydrolysis (24). The final solution of BHPM was stored in the refrigerator (~4°C) and was found to be stable under these conditions for at least 2 weeks. Assay of BHPM was achieved by modifying a previously published HPLC-UV method (25). The method was identical to that used for assaying BIS, except that mobile phase was slightly modified to 0.05 M KH_2PO_4 : acetonitrile (80:20 v/v), and the internal standard was ethyl paraben. Retention times for BHPM and ethyl paraben were 33.4 min and 40.2 min, respectively. Limit of quantification (23) of BHPM was 0.20 μg/ml.

Surface tension, osmolality, total protein content, total carbohydrate content, and total 3a-hydroxy bile acid content were measured as described previously (3). Limit of quantification for total protein content (using bovine serum albumin as reference), total carbohydrate content (using glucose as reference), and total 3a-hydroxy bile acid content (using sodium taurocholate as reference) was 0.13 mg/ml, 0.37 mg/ml, and 35 μM, respectively.

Short chain fatty acids (SCFAs), i.e. acetic acid (AA), propionic acid (PA), iso-butyric acid (IBA), n-butyric acid (BA), iso-valeric acid (IVA), valeric acid (VA), and caproic acid (CA), in the supernatant after ultracentrifugation were quantified by using a previously developed gas chromatography method (26). Retention times were approximately 2.6, 3.4, 3.9, 4.6, 5.2, 6.4, and 8.5 min for AA, PA, IBA, BA, IVA, VA, and CA, respectively, making the total run time for each sample approximately 28 min. Limits of quantification (23) varied with the SCFA from 33.4 μM (for CA) to 76.3 μM (for PA). The poorest inter-day precision, i.e. the relative standard deviation of data collected from three 400 μM standards, was observed with AA (6.1%). The poorest intra-day precision was again observed with AA (2.5%). With the applied analytical method the mean(SD) % recovery of each SCFA from the supernatant of colonic contents ranged from 90(12) (for CA) to 105(29) (for PA).

Table II. Chromatographic Conditions Applied for the Assay of Individual Bile Acids in Supernatant of Colonic Contents

Pre-column	BDS C18 10×4mm		
Column	BDS C18 250×4.6 mm, 5 μm, Hypersil		
Line A	Methanol : Formate Buffer pH 3, 67:33, v/v		
Line B	Methanol		
Mobile Phase	Time (min)	%A	%B
	0	100	0
	42	100	0
	52	67	33
	90	67	33
	100	100	0
Flow rate	1.0 ml/min		
Injection volume	50 μl		

Table III. Chromatographic Conditions Applied for the Assay of Lipids in Supernatant of Colonic Contents

Pre-columns	BDS C18 10×4 mm		
Column	BDS C18 250×4.6 mm, 5 μm, Hypersil		
Line A	(Methanol : Acetonitrile : Formate Buffer pH 3, 65:25:10, v/v) : 2-propanol 96:4, v/v		
Line B	2-propanol		
Mobile Phase	Time (min)	%A	%B
	0	100	0
	30	100	0
	32	50	50
	52	50	50
	57	30	70
	100	0	100
Flow rate	0.5 ml/min		
Injection volume	50 μl		

Bile acids, i.e. taurocholic acid (TC), glycocholic acid (GC), taurochenodeoxycholic acid (TCDC), ursodeoxycholic acid (UDC), glycochenodeoxycholic acid (GCDC), cholic acid (C), glycodeoxycholic acid (GDC), chenodeoxycholic acid (CDC), deoxycholic acid (DC), and lithocholic acid (LC), in supernatant after ultracentrifugation were quantified by a gradient HPLC method using a Charged Aerosol Detector (Corona CAD, ESA Inc., Chelmsford, MA), and it was based on a recently developed method for the assay of bile salts in the stomach and the small intestine (27). Chromatographic conditions are presented in Table II. In the fasted state, samples were injected directly into the chromatograph. In the fed state, samples were diluted with acetonitrile (1:1), vortexed, centrifuged (11400 g, 10°C, 10 min), and injected into the chromatograph. Retention times were approximately 9.3, 15.0, 16.7, 19.6, 28.8, 31.2, 34.7, 56.3, 57.8, and 75.5 min for TC, GC, TCDC, UDC, GCDC, C, GDC, CDC, DC, and LC, respectively. Limits of quantification (23) varied with the bile acid from 3.9 μM (TCDC) to 12.1 μM (DC).

Lipids, i.e. triglycerides (TGs), diglycerides (DGs), monoglycerides (MG), palmitic acid (PA), linoleic acid (LA), oleic acid (OA), phosphatidylcholine (PC), cholesterol (CHO), and cholesterol ester (CE), in the supernatant after ultracentrifugation were assayed with a gradient HPLC method using a charged aerosol detector. The only parameter that required user input was the response range, which was set to 100 pA full scale. Chromatographic conditions are presented in Table III. Samples were appropriately diluted with 2-propanol (up to 1:100, v/v), vortexed, centrifuged (11400 g, 10°C, 10 min), and injected into the chromatograph. Glycerol trioleate was used for quantifying TGs, dipalmitin was used for quantifying DGs, and 1-monooleyl-rac-glycerol was used for quantifying MGs. Both egg PC and soya PC were used for quantifying colonic PC, and estimated colonic PC concentrations were identical. Cholesterol oleate was

used for quantifying cholesterol esters. Retention times were approximately, 21.2, 23.6, 31.2, 32.9, (46.0–54.0), 56.0, (60.0 & 62.0), 69.5, and 73.3 min for LA, MG, OA, PA, PC, CHO, DG, TG, and CE, respectively. Limits of quantification (23) varied from 0.96 μM (CHO) to 12.0 μM (PC). The poorest inter-day precision, i.e. the relative standard deviation of data collected from four 50 μM standards, was observed with PC (10.6%). The standard addition method was applied for assessing the % recovery of each lipid (in triplicate) from the supernatant of a sample collected in the fed state. For cholesterol, mean(SD) recovery was 113.1(9.30) %. Recovery of PA was only 23(28)%, so its concentration is clearly underestimated (at least in the fed state). For the rest of lipids, recovery varied from 58.3(8.1)% (glycerol trioleate) to 95(23)% (oleic acid).

All chemicals used for the development, optimization, and application of analytical methods were of analytical grade and purchased from Sigma Aldrich Chemie GmbH (Germany), except for BIS, which was donated by Boehringer Ingelheim (Greece), atazanavir, which was donated by Bristol-Myers Squibb (Greece), and egg and soybean PC, which were donated by Lipoid GmbH (Germany). All solvents were of HPLC grade.

Data Analysis

Data are presented as Box-Whisker plots showing the median value; the 10th, 25th, 75th, and 90th percentiles; and the individual outlying data points, with dotted lines indicating the mean value.

For each parameter, differences between fasting and fed state were evaluated with the paired t-test or the Wilcoxon test, depending on the results of normality and equal variance tests. Type I error was set to 0.05 and the desired power of the test was 0.800. All statistical comparisons were performed using Sigmastat 2.03 (SPSS Inc., USA).

RESULTS AND DISCUSSION

Pilot Study (fasted state)

The mean(SD) volume of contents of ascending colon collected was 27.1(6.1) ml. Since incoming contents of the ileum were very limited (if any), these numbers correspond to the volume of contents in the ascending colon. Buffer capacity of colonic contents was lower when measured with NaOH than with HCl, suggesting that in the main study it should be measured in both directions. Mean(SD) values were 6.0(3.6) mmol/l/ΔpH (NaOH) and 11.0(7.8) mmol/l/ΔpH (HCl).

In the four subjects who had been prepared for colonoscopy with PEG, the supernatant after centrifugation of the colonic contents had increased PEG concentrations and it was iso- or hyper-osmotic, regardless of the presence of an antibacterial (Table IV). Therefore, PEG was deemed inappropriate for the purposes of the present study. The concentration of BHPM in the supernatant of contents of Subjects #5 and #6 was 0.56 and 0.63 μg/ml, respectively (Table IV). Based on data in rat colon, mucus secretion occurs only at BHPM concentrations of at least 1 μg/ml, whereas sodium and fluid secretion occurs at intracolonic BHPM concentrations of ≥5 μg/ml (16). Therefore, for both subjects there is little risk that the effects of bisacodyl were still extant at the sampling time. However, based on osmolality data, it seems that for subject #5 the changes on intracolonic environment were not fully reversed by sampling time, as they are hyperosmotic. It was therefore decided that the colon preparation procedure applied to Subject #6 is the most appropriate for achieving the objectives of the present investigation.

Table IV. Characteristics of the Supernatant After Centrifugation (11400 g, 10 min, 4°C) of Contents of the Ascending Colon in the Pilot Study[1]

Subject number	PEG content, mg/ml	BHPM content, μg/ml	Osmolality, mOsmol/kg
1	60.6/64.3	NA	288/309
2	226.7/167.8	NA	342/332
3	144.5/139.4	NA	260/212
4	130.5/134.5	NA	569/421
5	NA	0.56/NM	381/347
6	NA	0.63/NM	133/99

[1] NA=not applicable. NM=not measured. In each cell, the first number is in absence and the second in presence of antibacterial agents.

Termination of bacterial activity did not have substantial effects on surface tension, carbohydrate content, total protein content, or total 3a-hydroxy bile salt content (data not shown). However, it led to decreased osmolality (in 5 out of 6 subjects, Table IV), most likely due to curtailing the production of osmotically active components by the residual bacteria (the bacteria were only partially eliminated by the centrifugation of contents). Based on these data, it was decided not to use antibacterial agents in the main study, but rather to remove both bacteria and solids by ultracentrifuging the contents prior to physicochemical characterization and analysing only the supernatant.

Addition of a cocktail to terminate lipolysis did not affect total protein content and/or total 3a-hydroxy bile salt content, regardless of the presence of antibacterial agents (data not shown). However, the cocktail affected the osmolality; based on data in distilled water, the cocktail (at 2% v:v) increased osmolality by 363.3(2.1) mOsmol/kg [Mean(SD) / n=3]. In the main study, cocktail was added immediately after sample collection, but measured osmolarities were corrected for its presence.

Main Study

Volume and Aqueous Fraction of Contents of Ascending Colon

The volume of contents recovered did not differ significantly with prandial state (p=0.059, power of the test: 0.385). Mean(SD) values were 22.3(7.7) ml and 29.9(10.8) ml in the fasted and in the fed state, respectively (Fig. 1). With a gas volume in the ascending colon measured at up to about 200 ml (12), i.e. roughly equal to the geometric capacity of the ascending colon (28), only 10% of the available lumenal space of the ascending colon contains liquid/solid material. Further, the non-significant difference between volumes in

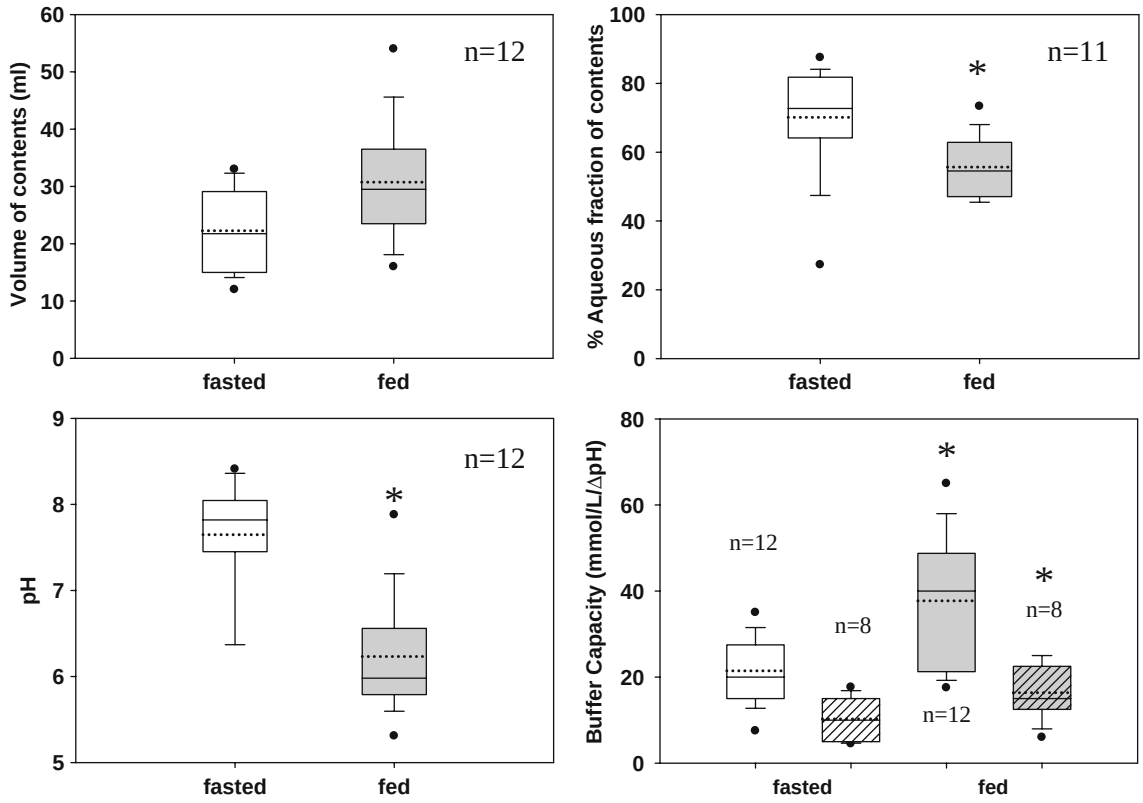

Fig. 1. Volume, aqueous fraction, pH, and buffer capacity [measured with HCl (plain boxes) and with NaOH (lined boxes)] of the contents of ascending colon of healthy adults measured in the fasted state (white boxes) and in the fed state (grey boxes) on a crossover basis. n is the number of subjects contributed to the construction of box plots. For each box plot and from bottom to top continuous horizontal lines indicate the 10th, 25th, 50th (median), 75th, and 90th percentile; black dots indicate the individual outlying data points; and dotted line indicates the mean value. Asterisk (*) indicates that the difference from corresponding fasted state data is significant.

the fasted state and 1 h after meal coincide with previous data (collected using an imaging technique), which indicate that the ascending colon response to a meal in health is characterized by a variable initial change in volume, with ileal chyme passing into the colon in some individuals, and a more consistent reduction in volume from 30 to 105 min postprandially (29).

In agreement with recently reported data (8), the aqueous fraction of contents was significantly lower in the fed state (p=0.022). Mean(SD) values were 70.3(17.0)% in the fasted state and 56.0(9.0)% in the fed state (Fig. 1). These values are lower than previous estimations, according to which the water content of the fluid in the fasted ascending colon is about 90% (29), and somewhat higher than the volumes of free water in the entire large intestine determined by a recently applied imaging technique (8).

pH and Buffer Capacity of Contents of Ascending Colon

pH of contents differed significantly between prandial states (p=0.009). Median values were 7.8 and 6.0 in the fasted and in the fed state, respectively (Fig. 1). To date, most of pH data in the healthy proximal colon have been collected using radiotelemetric capsules (RTC), and average pH values have been reported to be between 5.7 and 6.8 (30). The typical protocol in those studies involves the ingestion of RTC in the morning after an overnight fast with a small quantity of water and eating/drinking *ad libitum*, after gastric emptying of RTC (31-33). Therefore, the data measured with RTC should be closer to fed state conditions and are indeed in line with the fed state data reported in the present study. pH in the fasted state remained unaltered after standing the sample for 10 min on the bench at room temperature. In the present study, pH in the fed state decreased by an average of 3.5% in all 10 tested samples during the same procedure, presumably due to continuation of fermentation of food residues by the bacteria. The latter argument is strengthened by the fact that pH of contents of ascending colon collected at autopsy is typically 6 or lower (33); autopsy is usually carried out at 3–4 h after death (12,34).

When measured with HCl, mean buffer capacity increased from 21.4 mmol/l/ΔpH in the fasted state to 37.7 mmol/l/ΔpH in the fed state (p=0.002) (Fig. 1). Observed values in the fasted state are almost four times higher than that in the fasted small intestine, whereas in the fed state they are only slightly higher than those in the fed small intestine (3). When measured with NaOH, buffer capacity was likewise found to be significantly increased in the fed state (p=0.044), but the mean values were much lower compared to the values estimated with HCl, i.e. 10.3 and 16.4 mmol/l/ΔpH in the fasted and in the fed state, respectively (Fig. 1).

Physicochemical Characteristics of the Supernatant After Ultracentrifugation

Bisacodyl and its Metabolite. Bisacodyl in the supernatant of colonic fluid both in the fasted and in the fed states was either not detected, or measured concentrations were ≤0.02 µg/ml, i.e. lower than the limit of quantification. Similarly, BHPM was either not detected or measured concentrations were lower than the limit of quantification. These data confirm data from the pilot study that 20 mg BIS administered 50–44 h prior to colonoscopy should be adequate for preparing the colon for colonoscopy, without affecting the intracolonic environment at sampling time (16).

pH and Buffer Capacity. Regardless of dosing conditions, there was a trend to slightly lower pH of supernatant after ultracentrifugation, compared to that of total colonic contents measured immediately after sample collection (medians of 2 fasted and 4 fed supernatants (n=6) were 6.5 (supernatants) *vs*. 6.8 (total contents)). Buffer capacity measured with HCl both in the fasted and in the fed state was approximately 50% lower than that of total colonic contents measured immediately after sample collection (n=6, p=0.019). Even so, buffer capacity of supernatant in the fasted state was higher than that in the fasted small intestine (3). In contrast, when measured with NaOH, buffer capacity of supernatant was not significantly different from that of total colonic contents. Since these data are not affected by the cocktail added for termination of lipolysis (data not shown), they may reflect the fact that reactions performed by colonic bacteria [reductions, hydrolyses, dehydroxylations (35)] are typically acid mediated, and, therefore, the HCl added to the total colonic contents (unlike in the supernatant) is primarily spent for assisting relevant reactions.

Surface Tension. Surface tension was significantly lower than that of water, regardless the dosing conditions. In the fed state the mean value was significantly lower (p=0.009) than that in the fasted state (means were 39.2 *vs*. 42.7 mN/m, respectively) (Fig. 2). Surface tension in the colon is higher than that measured in the upper small intestine in the fasted state (mean 32.3 mN/m) and in the fed state [mean, 28.1–28.8 mN/m (3)].

Osmolality. Supernatants of ultracentrifuged contents were hypoosmotic in the vast majority of cases, regardless of dosing conditions. In the fed state, osmolality was higher than that in the fasted state (p=0.023). Mean (SD) values were 224 (125) and 81 (102) mOsm/kg, respectively (Fig. 2). These values are lower than those measured in the upper small intestine at corresponding dosing conditions (3). Hypoosmolality of the supernatant of colonic contents further confirms that the effects of administered bisacodyl for colon preparation are already reversed at sampling time.

Protein Content. Mean (SD) protein content was lower in the fed state, but the difference did not reach significance [9.7 (4.6) in the fasted state *vs*. 6.9 (2.3) mg/ml in the fed state,

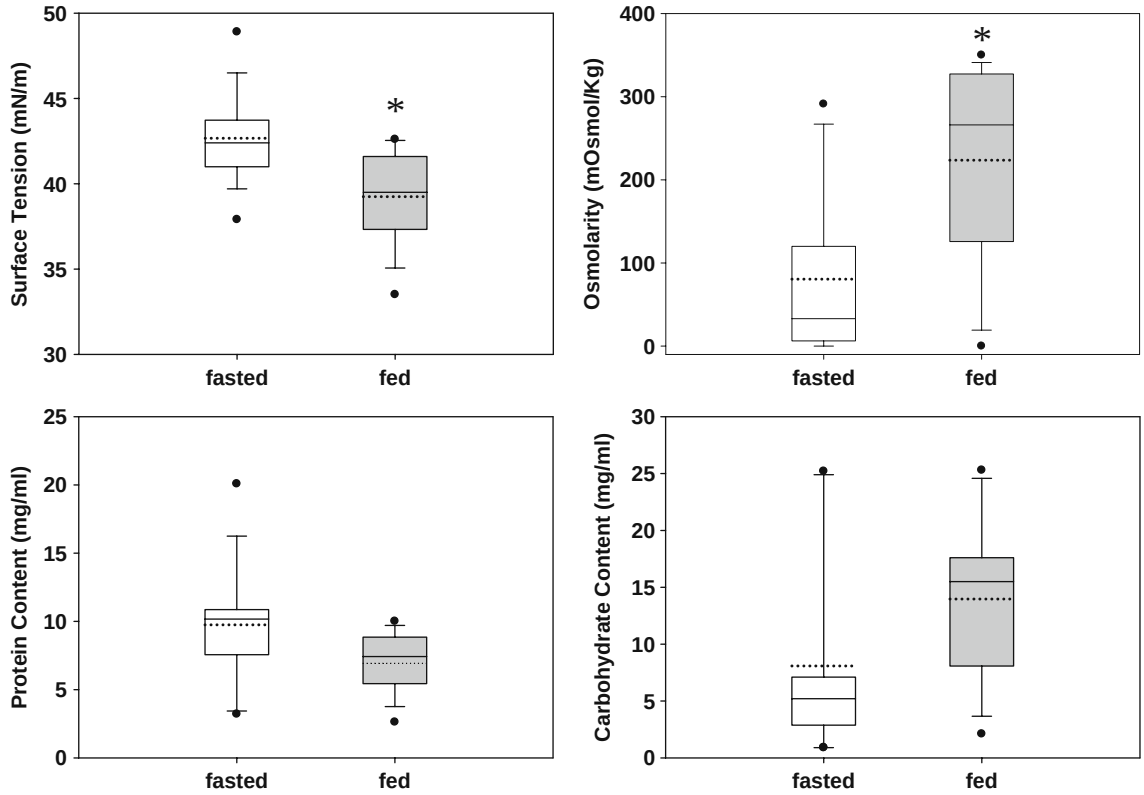

Fig. 2. Surface tension, osmolality, total protein content, and total carbohydrate content of supernatant after ultracentrifugation of contents of ascending colon of 11 healthy adults in the fasted state (white boxes) and in the fed state (grey boxes) on a crossover basis. Asterisk (*) indicates that the difference between fasted and fed state data is significant.

p=0.057, power: 0,398] (Fig. 2). The trend for lower protein content in the fed samples may relate to the fact that in the fed state digestion and absorption of peptide/proteins administered with the meal are almost complete by the end of the small intestine. Therefore, the liquid that arrives in the colon in the fed state primarily dilutes existing proteins in the ascending colon. Total protein content in the fasted upper small intestine (~3 mg/ml) (3) is lower than that in the fasted colon measured in this study, whereas in the fed upper small intestine (~10 mg/ml) (3) it is similar with that in the fed colon. Soluble protein in ascending colon at autopsy has been measured to be between 1 and 17 g/kg wet wt contents (12,36). The high variability may be (at least partly) due to post-mortem change during the short period before samples are removed from the gut. The substantial presence of peptide/proteins in the ascending colon (primarily due to mucosal cell regeneration), in conjunction with the substantial buffer capacity and the different buffer capacities measured with HCl and with NaOH, implies that pH buffering species in the ascending colon, as well as SCFAs, include also peptides/proteins.

Carbohydrate Content. Mean (SD) carbohydrate levels were 8.1 (8.6) mg/ml and 14.0 (7.4) mg/ml, in the fasted and in the fed states, respectively. The difference was not significant (p=0.101), but the power of the test was low (0.252). In contrast with proteins, carbohydrates administered with the meal may not be digested in the small intestine and, may, therefore, reach the colon. The small (if any) difference between dosing conditions may be related to the fact that the meal administered in this study contains only small amounts of fiber, and, therefore, most carbohydrates are expected to be digested and absorbed during residence in the small intestine. It is interesting to note that, after normalizing for the different input amounts of carbohydrates between the present and a previous study (3), total carbohydrate content in the fed colon is about 30% of that observed in the fed upper small intestine (3).

Short Chain Fatty Acids. All seven SCFAs were quantified in the supernatant but acetate dominated (followed by propionate and butyrate), whereas caproate was found in only trace amounts. Mean (SD) total SCFA levels in the fed state [48.1 (21.7) mM] were not significantly different from total SCFA levels in the fasted state [30.9 (15.4) mM] (p= 0.112; power: 0.244). To date, the effect of food on SCFA concentration has been studied only in animals, and it has been shown that fasting reduces production and concentrations of SCFAs (37). Acetate levels were higher in the fed state; mean(SD) values were 20.8(11.6) mM in the fasted state and 35.9(14.0) mM in the fed state (p=0.046). Similarly butyrate levels were higher in the fed state; median(range) values were 1.4(0.1–4.8) mM in the fasted state and 3.4(1.4–10.6) mM in the fed state (p=0.027). In contrast, isobutyrate levels were lower in the fed state; mean(SD) values were 0.5 (0.2) mM in the fasted state and 0.3(0.1) mM in the fed state (p=0.027) (Fig. 3). In healthy humans, SCFAs have been quantified in colonic contents of adults who died suddenly but with no GI disease. In accordance with the present study, acetate, followed by butyrate and propionate, are the major SCFAs in all parts of the large intestine (12,34). However, in

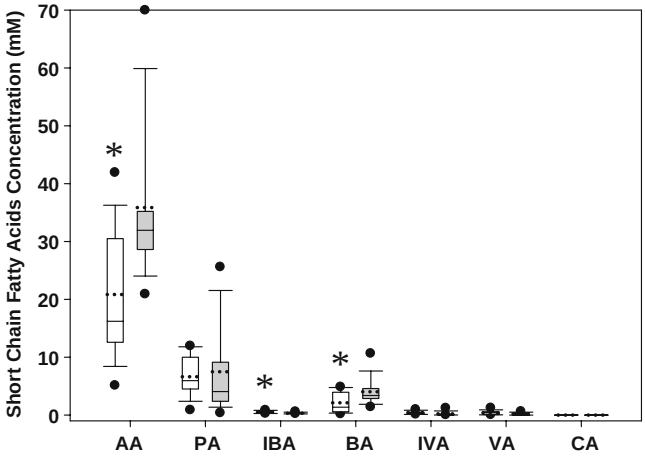

Fig. 3. Concentrations of individual short chain fatty acids in the supernatant after ultracentrifugation of contents of ascending colon of 10 healthy adults in the fasted state (white boxes) and in the fed state (grey boxes) on a crossover basis. Asterisk (*) indicates that the difference from fed state data is significant. Key: AA, acetic acid; PA, propionic acid; IBA, iso-butyric acid; BA, n-butyric acid; IVA, iso-valeric acid; VA, valeric acid; CA, caproic acid.

the ascending colon specifically, total SCFAs were about 130–180 mmol/kg wet wt contents, with acetate being 60–98 mmol/kg wet wt contents, butyrate 20–40, and propionate 25–35 mmol/kg wet wt contents (12,34). It might be argued that the discrepancy is related to the colon cleaning procedure applied in this study prior to collection of colonic contents. However, a similar discrepancy was not observed with total protein content (see above), and literature data suggest that cleaning procedures (more rigorous than the one applied in this study) do not affect the type and number of organisms in the colon (38,39). In addition, based on measurements performed for 3 h during fasting and for 6 h after a 1000 Kcal meal, the fundamental motor profile does not seem to be different than that recorded in the unprepared colon (40). The higher levels of SCFAs measured in the previous studies may be related to the fact that autopsies (i.e. sample collection) had been performed about 4 h after deaths (12,34), and unlike in present study, no specific eating and fasting protocol could have been applied. Given the origin of intracolonic SCFAs and peptide/protein levels, eating and fasting protocols prior to sample collection may be more important for intracolonic SCFA levels than for peptide/protein levels.

Bile Acids. Total bile acid concentration in the fasted state was significantly lower than in the fed state (p=0.011). Mean (SD) total bile acids concentration in the fasted and in the fed state was 115.2 (119.3) μM and 587.4 (412.8) μM, respectively (Fig. 4). Individual bile acid concentrations that were significantly higher in the fed state included C [300.6 (257.9) *vs.* 29.9(35.7) μM, p=0.007] and CDC [108.6(101.6) *vs.* 7.8(5.8) μM, p=0.009], for UDC the difference was of borderline significance [28.5(33.5) *vs.* 7.1(6.1) μM, p=0.071, power: 0.343]. Conjugated bile acids were detected in only 3 subjects in the fasted state and were quantified in only 3 subjects in the fed state, implying that deconjugation of bile acids is almost complete “in” or “by” the ascending colon of

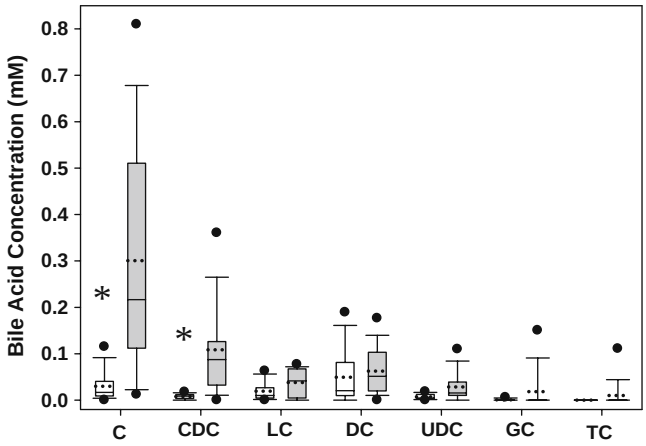

Fig. 4. Concentration of individual bile acids in the supernatant after ultracentrifugation of contents of ascending colon of 11 healthy adults in the fasted state (white boxes) and in the fed state (grey boxes) on a crossover basis. Asterisk (*) indicates that the difference from fed state data is significant. Key: C, cholic acid; CDC, chenodeoxycholic acid; LC, Lithocholic acid; DC, Deoxycholic acid; UDC, Ursodeoxycholic acid; GC, Glycocholic acid; TC, taurocholic acid.

healthy subjects, especially in the fasted state. In the fasted state, in 8 out of 11 subjects, secondary bile acids (i.e. DC and LC) were found in higher % content than primary bile acids (i.e. C and CDC), whereas UDC [tertiary bile acid produced by lumenal bacteria (41)] was similar to C and higher than CDC. In contrast, in the fed state, in 10 out of 12 subjects, primary bile acids were found in higher % content than secondary bile acids, indicating that when more bile acids enter the colon (fed state) the capacity for conversion to secondary bile acids is overwhelmed, resulting in more bile acids quantified in the primary form. Another reason might be that, as *in vitro* data have shown (42), bacterial breakdown of primary to secondary bile acids is inhibited when carbohydrates are simultaneously fermented. Data of the present study are in line with previous data (43,44) showing that, in ileostomy subjects, excreted bile acids consist of C (70%) and CDC (30%). In those studies the analytical methodology (gas chromatography) did not allow for distinguishing between conjugated and unconjugated bile acids. Further, since in those previous studies no LC or DC was found in the ileum, the present study suggests that secondary/tertiary bile acids [that dominate fecal bile acid pool (e.g. 41)] start to form in the ascending colon.

Glycerides, Fatty acids and Cholesterol. Regardless of dosing conditions, triglycerides, diglycerides, and cholesterol esters are below detection levels in the supernatant after ultracentrifugation of the contents of the ascending colon of healthy subjects. Mean (SD) values of palmitic acid, linoleic acid, oleic acid, phosphatidylcholine, and cholesterol in the fasted state were 49.6(43.7), 37.4(29.6), 32.8(36.7), 362(210), and 1703(1764) μM, respectively, whereas the corresponding values in the fed state were 103.8(112.1), 47.8(30.0), 73.4 (81.7), 539(393), and 1882(1325) μM (Fig. 5). For cholesterol, median values in the fasted and in the fed states were 594.2 and 1501.8 μM, respectively. Differences between fasted and fed states were not significant for any of the quantified lipids; however, the power of the performed tests was 0.254 at most. It should be underlined that apart from cholesterol, all other reported concentrations may or actually do underestimate true values (see experimental section). In a few chromatograms, there was one extra peak in the region where monoglycerides and long chain fatty acids elute. Since absorption of ingested fat is typically complete by the middle third of the jejunum (45), most of the fat in the ascending colon must be derived from non-absorbed fats that have been secreted in the lumen and from cellular debris and microorganisms (46,47). Indeed, since PC is converted to lysophospatidylcholine by pancreatic phospholipase A in the upper intestine (48), the absence of lysophosphatidylcholine from the ascending colon (data not shown), suggests that measured PC (at least in the fasted state) is not of pancreatic origin. The comparatively substantial presence of PC in the ascending colon could be attributed to its abundance in the apical epithelial membrane and to its slow transport via the intestinal epithelium (49). For cholesterol, the observed levels are similar to those in the supernatant of fed duodenal contents (19). This finding is consistent with the fact that cholesterol absorption is regulated by influx and efflux transporters in the proximal small intestine that its net absorption is low (range 30–80%, mean 56%), that fluid volumes are much smaller in the lower than in the upper intestine, and that the major route of its elimination is via the feces (8,47,50). In addition, the absence of significant food effect on cholesterol intracolonic levels, is consistent with the fact that dietary cholesterol constitutes only 30% of total intralumenal cholesterol (47). Maintenance of relatively high cholesterol levels in the supernatant might have been possible by the presence of various solubilizing agents and/or micellar structures, whereas part of it could be housed in vesicles, i.e. in bilayers of cholesterol and phospholipids (51), and perhaps other lipid components (4 out of 23 supernatants after ultracentrifugation were turbid).

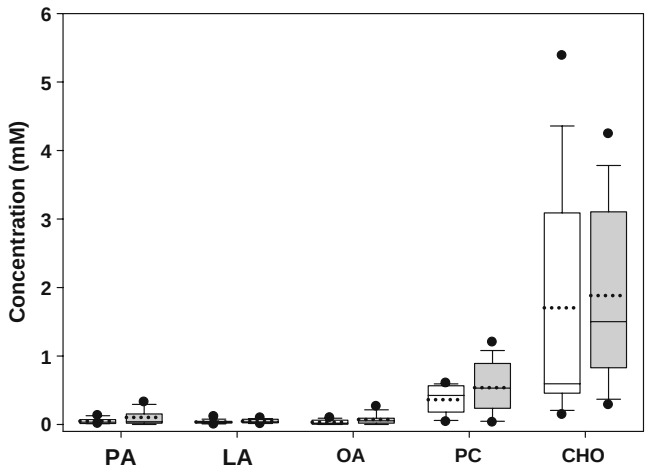

Fig. 5. Concentration of long chain fatty acids, phosphatidylcholine, and cholesterol in the supernatant after ultracentrifugation of contents of ascending colon of 11 healthy adults measured in the fasted state (white background) and in the fed state (grey background) on a crossover basis. Key: PA, palmitic acid; LA, linoleic acid; OA, oleic acid; PC, phosphatidylcholine; CHO, cholesterol.

CONCLUDING REMARKS

To date, the *in vitro* assessment of dosage forms that perform in the colonic region has been based largely on pH considerations, without taking into account potential food effects (e.g. 52). The present study provides a basis for more accurate simulation of colonic environment in regard to parameters such as buffer capacity, osmolality, and solubilizing agents (i.e. cholesterol, bile acids, phospholipids and, perhaps, proteins). Such parameters affect drug release, especially from diffusion layer coatings, diffusion matrices, osmotic pumps, and hydrophilic matrices (53). In addition, this study shows that free water content, pH, buffer capacity, osmolality, surface tension, short chain fatty acid content, and bile acid content are significantly affected by the dosing conditions applied during BA/BE studies. It would be interesting to investigate the degree of variability of the parameters measured in the present study by studying other population groups (e.g. in subjects with inflammed colon) as this should provide an indication of the actual environment in which orally administered dosage forms perform in clinical practice.

ACKNOWLEDGEMENT

Authors would like to thank Ms. Maria Koursari for her excellent technical assistance during colonoscopies.

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