Investigating the impact of Crohn's disease on the bioaccessibility of a lipid-based formulation with an *in vitro* dynamic gastrointestinal model

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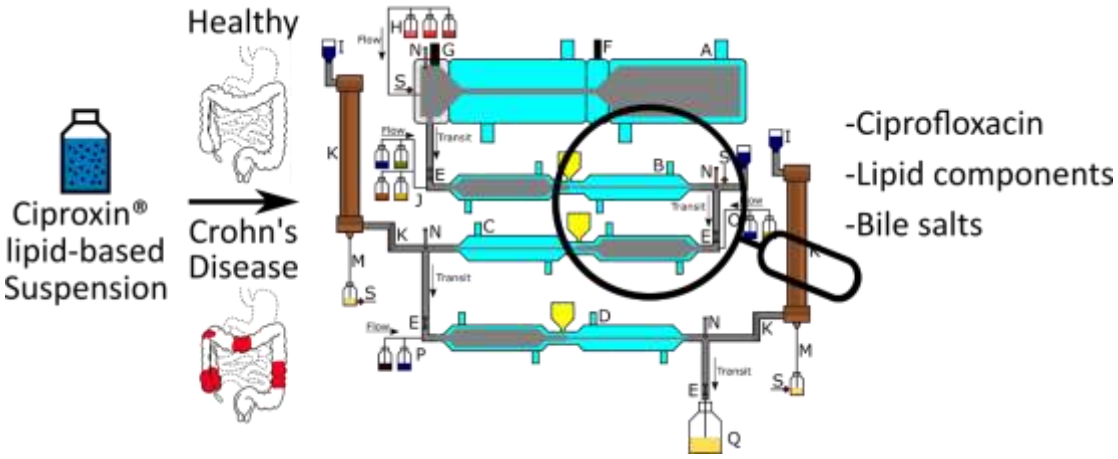
Graphical abstract



Abstract

The aim of the study was to investigate the impact of Crohn's disease (CD) on the performance of a lipid-based formulation of ciprofloxacin in a complex gastrointestinal simulator (TIM-1, TNO) and to compare the luminal environment in terms of bile salt and lipid composition in CD and healthy conditions.

CD conditions were simulated in the TIM-1 system with a reduced concentration of porcine pancreatin and porcine bile. The bioaccessibility of ciprofloxacin was similar in simulated CD and healthy conditions considering its extent as well as its time course in the jejunum and ileum filtrate. Differences were observed in terms of the luminal concentration of triglycerides, monoglycerides and fatty acids in the different TIM-1 compartments, indicating a reduction and delay in the lipolysis of formulation excipients in CD. The quantitative analysis of bile salts revealed higher concentrations for healthy conditions (standard TIM-1 fasted state protocol) in the duodenum and jejunum TIM-1 compartments compared to published data in human intestinal fluids of healthy subjects, while the bile salt concentrations in CD conditions were similar. A lipidomics approach with UPLC/MS has proven to be a time-efficient method to semi-quantitatively analyse differences in fatty acids and bile salts levels between healthy and CD conditions.

The dynamic luminal environment in CD and healthy conditions after administration of a lipid-based formulation can be simulated using the TIM-1 system. For ciprofloxacin, an altered luminal lipid composition had no impact on its performance indicating a low risk of altered performance in CD patients. The reduced concentrations of bile salts in simulated CD conditions correspond to the levels observed in human intestinal fluids of healthy subjects in the fasted state.

Keywords: TIM-1 TNO, Crohn's disease, lipid-based formulation, luminal environment, bile salts, fatty acids**,** lipidomics.

1. Introduction

Crohn's disease (CD), affecting approximately 1.6 million people in Europe, is a chronic auto-inflammatory disorder and one of the main types of inflammatory bowel disease.[1] CD commonly affects the terminal ileum but can be localised in any part of the gastrointestinal (GI) tract. The disease manifests as transmural ulcerations that are discontinuously spread in the GI tract. Additionally, CD patients often present extra-intestinal manifestations such as inflammations of the eyes (uveitis, episcleritis), skin diseases (erythema nodosum, pyoderma gangrenosum), spondyloarthritis or hepato-pancreato-biliary diseases.[2] For the patients, CD results in a lifelong treatment with anti-inflammatory drugs (e.g., mesalamine, steroids, azathioprine, cyclosporine). In addition to this treatment, IBD patients showed a higher use of antidepressants, anxiolytics, oral bisphosphonates, cardiovascular medication, antibiotics, proton pump inhibitors and nonsteroidal anti-inflammatory analgesics compared to the general population.[3]

CD can alter the GI tract in terms of the abundance of metabolising enzymes, GI transit times and the microbiota.[4] Additionally, the composition of GI fluids in CD patients can be affected by pathophysiological changes and hepatobiliary manifestations are a common symptom in CD.[4] A reduced bile acid pool and a decreased pancreatic lipase activity was observed in CD patients compared to healthy subjects.[5-10] These differences can have an impact on drug product performance, especially for drugs that rely on micellar solubilisation and typically belong to BCS class II or IV. Furthermore, this can also be a challenge for drug delivery from complex formulation approaches such as lipid-based formulation (LBF). LBFs can be used to increase drug's bioavailability by circumventing at least partially the drug dissolution step due to the higher drug solubility in the formulation vehicle.[11] For LBFs, drug product performance in CD patients can be a challenge due to an alteration in the digestion of lipid excipients, different excipient concentrations and altered micellar composition along the GI tract. Despite the

affected GI tract of CD patients, formulations are in most cases developed based on healthy GI conditions.

Several LBFs are commercially available and the ever-increasing number of poorly soluble compounds might further increase their number in the future.[11] Upon entering the GI tract, LBFs are subject to a dynamic environment with dispersion and digestion processes. Various excipients of LBFs such as acylglycerols, phospholipids, polysorbates (Tweens), polyethyleneglycol mono- and di-esters can be digested along the GI tract.[12] The enzymes involved in their hydrolysis include gastric lipase (GL) and colipase-dependent pancreatic lipase (PL) hydrolysing mainly triacylglycerols and diacylglycerols.[12] Additionally, several other pancreatic enzymes such as pancreatic carboxyl ester hydrolase, pancreatic lipase-related protein 2 and pancreatic phospholipase A2 act on micellar substrates and possess a phospholipase activity.[12] For the drug, the continuous reorganisation of colloidal structures composed of luminal bile acids, cholesterol, phosphatidylcholine, on the one hand, and excipients and their digestion products, on the other hand, can induce a supersaturated state or precipitation of a drug.[12] This complexity highlights the need for *in vitro* systems considering these dynamic processes to evaluate the formulation performance of LBFs.

Regarding the in vitro testing of LBFs, the digestion and dispersion processes are most often investigated in pH-stat lipolysis models focusing only on the small intestine, the main absorption and digestion area, and using porcine pancreatin as enzymatic source.[13] Therefore, the contribution of gastric lipase, estimated to around 3-37% of triglyceride (TG) digestion, is often neglected.[14-18] This is especially a limitation for the simulation of pathological conditions with a deficiency of exocrine pancreatic enzymes, where gastric lipase is assumed to have a significant role in fat digestion.[17, 19] The complex gastrointestinal simulator TIM-1 (TNO, Zeist, Netherlands) mimics closely the gastrointestinal tract by simulating biliary and pancreatic secretion, controlling luminal pH with bicarbonate secretion, removing

drug/micellar components via ultrafiltration and simulating gastric lipid digestion. The *in vivo* predictive ability of TIM-1 has previously been shown in nutritional sciences and in pharmaceutical formulation performance.[20-25] Due to the high level of biorelevance of the TIM-1 system, its suitability for the evaluation of LBFs has been suggested.[13] However, the high lipophilicity of drugs in LBFs might limit its use due to drug binding to the TIM-1 membranes and filters possibly resulting in a low recovery of the investigated drug.[24]

Ciprofloxacin was chosen as model drug for this study. It is used for the treatment of bacterial infections and belongs to the antibiotic group fluoroquinolones. Antibiotics are often used for CD patients experiencing complications such as fistulas or abscesses.[30] In this case, ciprofloxacin is one of the treatment options and was shown to be beneficial for the treatment of perianal fistulas. In terms of physicochemical characteristics, ciprofloxacin possesses a logP of 0.28, a poor aqueous solubility and is a zwitterionic molecule (high solubility at pH<5, pH>10).[26, 27] Apart from tablets, it is available as lipid-based oral suspension for reconstitution and marketed as Ciproxin® 250 mg/ 5ml oral suspension (Bayer plc, Reading, UK). The lipid excipients are expected to stabilize the suspension over the in-use period for the drug product. Ciprofloxacin tablets have previously been tested in the TIM-1 simulator and shown high levels of drug recovery.[28]

The aim of this study was to investigate the effect of CD on the performance of an oral lipid-based suspension of ciprofloxacin in a complex dynamic simulator of the upper gastrointestinal tract, TIM-1. Differences in the digestion process of excipients of the LBF between healthy and CD conditions were investigated and relevant components (bile acids, cholesterol) of the mixed micelles in the TIM-1 matrix were measured.

2. Materials and methods

2.1. Materials

The formulation Ciproxin® 250 mg/5 mL granules and solvent for oral suspension (Lot ITA37N0 for API, Lot ITA37N2 for Placebo) from Bayer Plc, Reading, UK was used. The water used was Milli-Q® grade.

For the TIM-1 experiments, potassium chloride, acetic acid and sodium chloride were used from Fisher Scientific, Loughborough, UK. Calcium chloride di-hydrate, hydrochloric acid (37%), pancreatin from porcine pancreas, sodium acetate trihydrate, pepsin from porcine gastric mucosa, sodium citrate, lipase from Rhizopus oryzae, amylase from *Bacillus sp*., (hydroxypropyl)methyl cellulose (HPMC) (2%) in water, porcine bile extract, sodium bicarbonate (1.14 mol/L) in water and trypsin were purchased from Sigma-Aldrich, Gillingham, UK. Sodium hydroxide (1 M) in water was used from Merck KGaA, Darmstadt, Germany. Porcine bile was purchased from Triskelion (Hendrix Slaughter House, Druten, Netherlands).

For the HPLC analysis of ciprofloxacin, formic acid and sodium hydroxide were purchased from Fisher Scientific, Loughborough, UK and ciprofloxacin from USP, Rockville, MD, US.

For the GC-FID (Flame Ionization Detector) analysis, chloroform, octanoic acid, decanoic acid, cholesterol and a Lipid Standard, Mono-, Di-, & Triglyceride Mix containing 1,3-Diolein 10 mg, 1,2-Dioleoyl-rac-glycerol 10 mg, Glyceryl trioleate 10 mg, Monoolein 10 mg were purchased from Sigma Aldrich, Gillingham, UK. Hydrochloric acid 1 M was purchased from Fisher Scientific, Loughborough, UK.

For the HPLC-CAD (Charged Aerosol Detector) analysis, HPLC grade methanol, ammonium formate and formic acid were used from Fisher Scientific, Loughborough, UK. Triethylamine, glycochenodeoxycholic acid (GCDC) sodium salt, glycocholic acid (GC), taurodeoxycholic acid (TDC) sodium salt, taurochenodeoxycholic acid (TCDC) sodium salt and taurocholic acid (TC) sodium salt were purchased from Sigma-Aldrich, Gillingham, UK.

For UPLC-MS analysis, HPLC grade acetonitrile and acetic acid were used from Fisher Scientific, Loughborough, UK and ammonium acetate from Sigma-Aldrich, Gillingham, UK, respectively.

2.2. Methods

2.2.1. TIM-1 experiments

To investigate the effect of CD on the performance of a LBF, a complex *in vitro* gastrointestinal model TIM-1 (TNO, Zeist, Netherlands) was used which has previously been described.[23, 24, 32] The system has been used in a pharmaceutical context to predict drug product performance of formulations [23-25] and in food sciences to investigate e.g., the digestion of lipids.[20-22] An overview of the TIM-1 system is given in Figure 1. The human upper GI tract is simulated with four serial compartments representing the stomach, duodenum, jejunum and ileum. These compartments consist of two connected equal basic units with a glass jacket and a flexible silicone membrane inside. The original standard gastric TIM-1 compartment was used. Mixing of the chyme and control of the luminal temperature is achieved by pumping tempered water around the flexible membranes. Peristaltic valve-pumps connect the different TIM-1 compartments and allow the control of the chyme's flow rate between the different compartments. The volume of the luminal contents is controlled with level sensors and the secretion of buffers. A predetermined pH curve can be programmed for each compartment, monitored with a pH probe in each TIM-1 compartment and controlled by secretion of either water, 1 M hydrochloric acid (only gastric compartment) or 1 M sodium bicarbonate solution. Additionally, secretions of gastric electrolytes, gastric enzymes, pancreatic and biliary juices are included.

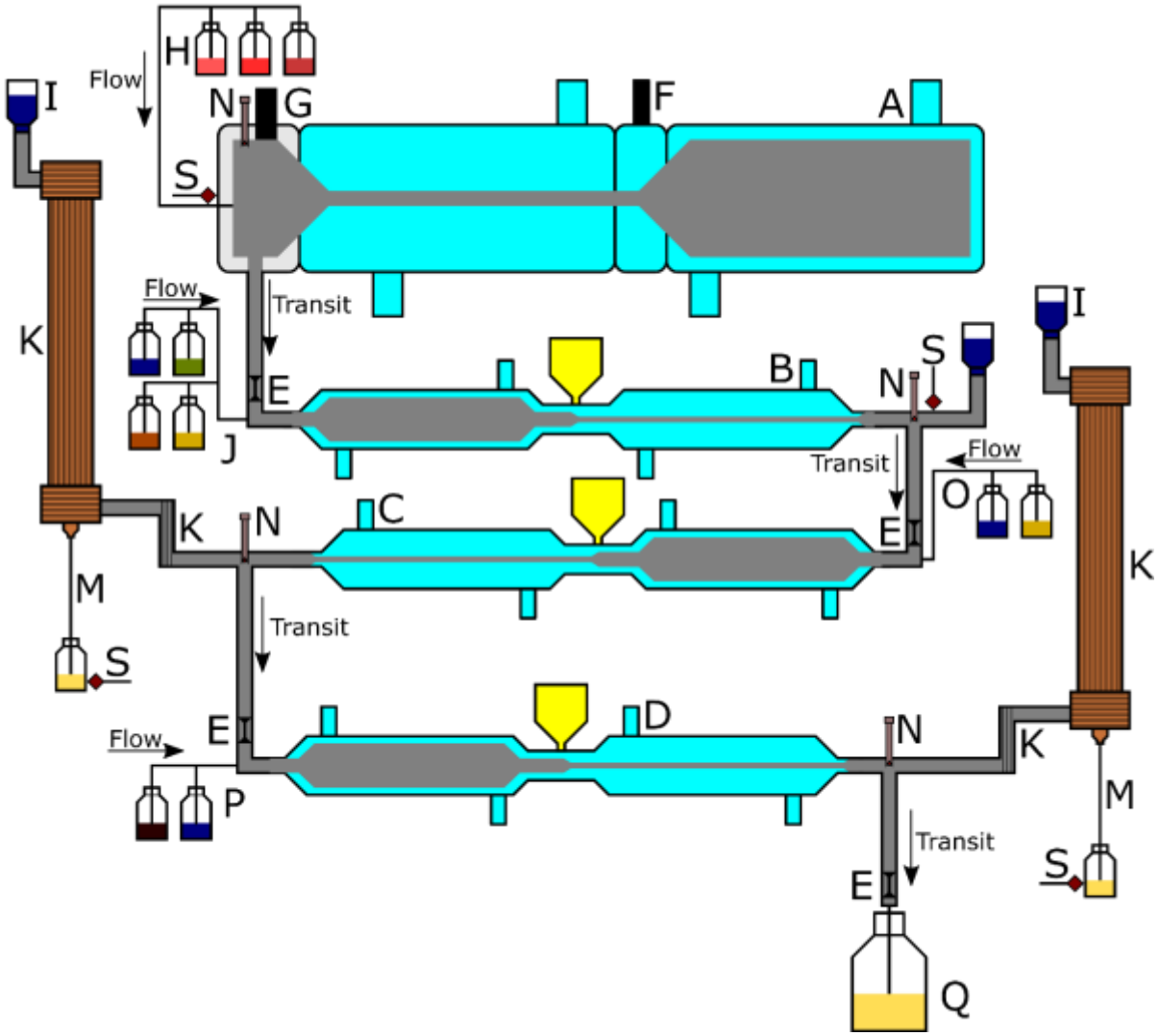


Figure 1: Overview of TIM-1 system (A: Gastric compartment, B: Duodenum compartment, C: Jejunum compartment, D: Ileum compartment, E: Peristaltic valve, F: Dosing port, G: Pressure sensor, H: Gastric secretions, I: Level sensors, K: Filter system, L: prefilter, M: Filtrate (jejunum and ileum), N: pH-electrode, O: jejunum secretions, P: ileum secretions, Q: Ileum efflux, S: sampling points) [adapted and redrawn with permission from [23]. Copyright 2014, Elsevier Inc.]

2.2.1.1. Preparation of solutions, reagents and starting residues

Various solutions were prepared to perform the experiments with the TIM-1 system including 0.1 M sodium citrate buffer (pH 7.0) and 1 M sodium acetate buffer (pH 5.0). Gastric electrolyte solution (GES) was prepared by dissolving 8 g/L sodium chloride, 1.7 g/L potassium chloride and 0.16 g/L calcium chloride di-hydrate in water. HPMC 0.4% and bile 0.04% gastric solution was prepared by dissolving 0.4 g/L bile extract in water, subsequently

adding 4.0 g/L HPMC and stirring the solution overnight. Gastric enzymes solution contained 1 mL 1 M sodium acetate buffer, 6000 units lipase, 1440000 units pepsin, 42000 units amylase and 299 mL GES. Small intestinal electrolyte solution (SIES) was prepared by dissolving 7 g/L sodium chloride, 0.35 g/L potassium chloride and 0.1 g/L calcium chloride di-hydrate in water and adjusting the pH to 7.0 with 1 M sodium hydroxide. Pancreatic solution was prepared by dissolving pancreatin powder in water, centrifuging the solution for 20 min at 12.500 G at 4ºC and using the supernatant for the experiment. The bile solution used consisted of prefiltered pig bile in SIES. The concentration of the pancreatic and bile solution varied according to the experimental conditions as detailed below (Section 2.2.1.2).

At the beginning of the experiments, the gastric compartment was filled with 30 g gastric start residue consisting of 15 g gastric enzyme solution and 15 g HPMC 0.4% and bile 0.04% gastric solution. The duodenum compartment was filled with 60 g of a solution consisting of 15 g SIES, 15 g pancreatin solution, 30 g bile solution and 2 mg trypsin in 1 mL SIES. The jejunum compartment was filled with a mixture of 35 g SIES, 35 g pancreatin solution and 70 g bile solution. The ileum compartment was filled with 140 g SIES.

2.2.1.2. Experimental conditions

Ciprofloxacin was selected as model drug for the studies with its lipid-based formulation Ciproxin® oral suspension, since another more lipophilic compound was tested initially but failed in pretesting experiments due to binding to membranes and filters of the TIM-1 system. This limitation is likely to be compound-dependent but may restrict the use of TIM-1 for the evaluation of LBFs of some lipophilic compounds.[23]

The Ciproxin® suspension was prepared according to the patient leaflet (brown bottle with granules was emptied into large white bottle with diluent, turned horizontally and shaken for 15 seconds) and stored in a refrigerator until further use. At the start of each experiment, the

bottle with the formulation was turned horizontally, shaken for 15 seconds and 10 mL of the formulation were added with a syringe to the dosing port of the gastric compartment. According to the patient leaflet, a drink of water may be taken after Ciproxin® administration and therefore, water was additionally added to the gastric compartment according to the experimental conditions shown in Table 1.

The Ciproxin® oral suspension consists of granules dispersed in an oily diluent consisting of miglyol 575, lecithin, sucrose and strawberry flavouring.[33] The medium chain TGs in Miglyol 575 are a mixture of octanoyl and decanoylglycerides. For our study, we selected the fasted state protocol for the TIM-1 experiments since it has been shown that a small quantity of medium chain TGs did not lead to substantial gallbladder contraction and therefore, did not induce concentrations of biliary components representative of a fed state in the intestinal lumen.[34] Therefore, average physiological conditions of the gastrointestinal tract in the fasted state were simulated in terms of pH, temperature, GI transit times and hydrodynamics, GI volumes, electrolyte concentrations and secretions of enzymes, biliary and pancreatic juice.

The pH in the gastric compartment was set to drop from 3.0 to 1.7 within 30 min.[28] The pH of the duodenum, the jejunum and the ileum compartment were 6.3± 0.2, 6.5±0.2 and 7.4±0.2, respectively. The volume of bicarbonate solution secreted to maintain the specified luminal pH in the intestinal compartments was automatically reported by the TIM-1 system. The temperature was maintained at 37 ºC.

Gastric emptying was set according to the equation of Elashoff, et al. [35] with a halftime of 20 min and a b-value (shape factor) of 1.0. To simulate the housekeeper wave, the total content of the gastric compartment was manually emptied and introduced into the duodenum compartment after the first 60 min. GI volumes were 55 mL, 130 mL and 130 mL for the duodenum, jejunum and ileum compartment, respectively.

234 The secretions to the gastric compartment included gastric enzyme solution, hydrochloric acid
235 and water at a total secretion rate of 1.0 mL/min. The duodenal secretion consisted of bile
236 solution, pancreatin solution and SIES. The jejunal secretion consisted of 10% V/V bile
237 solution in SIES and the ileal secretion of SIES, respectively.

238 To mimic the absorption of the dissolved or solubilised drug and digestion products, the "lipid
239 membrane configuration" mode was selected.[24] Therefore, two hollow fibre polysulfone
240 filtration units with a cut-off size of 50 nm and a surface area of 0.3 m^3 (Plasma Flux P1 dry,
241 Fresenius Medical Care, Bad Homburg, Germany) were used. Before the experiment, the filters
242 were saturated with 10 L of water and subsequently preconditioned by filtering a mixture of
243 50 mL porcine bile, 25 mL SIES and 25 mL pancreatic solution. As a next step, the filters were
244 connected to the jejunum and ileum compartment. The drug analysed in both filtrates was
245 considered as the bioaccessible fraction of the drug within a given time period. The
246 bioaccessibile fraction refers to the drug available for absorption through the gut wall.[36] Due
247 to the use of a syringe instead of the supplied measuring spoon to administer the Ciproxin®
248 formulation, the ciprofloxacin dose was slightly higher and the bioaccessible amount of
249 ciprofloxacin was therefore, normalised to the total amount of ciprofloxacin recovered from
250 the TIM-1 system (luminal samples, filtrates, ileal efflux, residues, washing solution).

251 Considering the lipolysis in the TIM-1 system, lipase from *Rhizopus oryzae* was used to
252 simulate human gastric lipase, since human gastric lipase is not commercially available. *In*
253 *vitro* experiments with lipase from *Rhizopus oryzae* showed a significantly higher lipid
254 digestion compared to the *in vivo* lipid digestion by human gastric lipase.[37] Currently, there is
255 still a lack of suitable substitutes for human gastric lipase due to differences in terms of the pH-
256 optimum, the substrate affinity and the stereo selectivity of microbial and animal lipases.[13, 37]
257 To simulate pancreatic lipases, porcine pancreatin was used as enzymatic source, which has
258 previously been shown to be a good substitute for human pancreatic juice.[38]

Three different experimental conditions were used including healthy, CD and healthy blank TIM-1 experiments as shown in Table 1. In healthy conditions, the bile solution consisted of 20.0% v/v pig bile in SIES and the pancreatin solution of 7.0% w/v porcine pancreatin extract in water. The healthy blank run was performed without any formulation and with the same conditions as defined for healthy subjects.

In CD, pathophysiological changes can affect the composition of the GI fluids and hepatobiliary manifestations are common extraintestinal symptoms.[4] In terms of LBFs, differences in lipase activity and bile concentration could impact on drug product performance. In CD patients, the pancreatic lipase activity was decreased to 28-80% of the activity in healthy subjects.[5-7] Additionally, the bile acid pool in CD patients was reduced to 38-58% of the size in healthy subjects.[8-10] To investigate the impact of these differences, CD conditions were simulated in the TIM-1 system (Table 1). The amount of porcine pancreatin was reduced to 28% of the concentration in healthy conditions, assuming a worst-case scenario. The bile concentration was reduced to 43% of the porcine bile concentration in healthy conditions, corresponding to an indirect approach by using the median reduction in studies investigating the bile acid pool.[8-10]

Table 1: Overview over experimental conditions of TIM-1 studies with ciprofloxacin.

Experimental conditions (number of replicates)	**Healthy (n=2)**	**CD (n=2)**	**Healthy blank (n=1)**
Setup	Lipid setup - ultrafiltration		
Prandial state	Fasted state		
Drug product	Ciproxin® oral suspension (10 mL)		-
c (porcine bile)	20.0% v/v pig bile in SIES	8.6% v/v pig bile in SIES	20.0% v/v pig bile in SIES
c (porcine pancreatin)	7% w/v in water	2% w/v in water	7% w/v in water
Experimental time [h]	5.0	5.0	4.0
Water added to the gastric compartment [mL]	230	230	240

SIES: Small intestinal electrolyte solution

2.2.1.3. Sampling and drug analysis

Samples were collected every 30 min for 5 h from the jejunal and ileal filtrate (drug available to permeate the intestinal membrane) and the ileal effluent (drug entering the colon). Additionally, 5 mL samples were taken directly from the gastric compartments at three different time points (0, 30, 60 min) and from the duodenal compartment every 30 min for 5 h. The collected samples were subsampled and stored at -18ºC for further analysis. After completion of the experiment, the residues were collected, the system was cleaned with 0.1 M sodium hydroxide solution and residues in the compartments and washing solution were analysed for remaining ciprofloxacin.

For the LC-MS/MS analysis of ciprofloxacin, all TIM-1 samples were diluted with 0.1 M sodium hydroxide solution and filtered through 1.0 µm PTFE syringe filters (Sigma-Aldrich, Gillingham, UK). Ciprofloxacin was quantified according to a published method with a Waters Acquity H-Class Quartenary UPLC equipped with a Waters Xevo TQD Triple Quadrupole Mass Spectrometer (Waters Corporation, Milford, MA, US).[28] A Waters Acquity UPLC BEH300 C18 column (2.1 x 200 mm, 1.7 µm, Waters Corporation, Milford, MA, US) was used

292 and set to a temperature of 40°C. The flow rate was 0.6 mL/min and 3 µL of sample was
293 injected. The mobile phase A consisted of 0.1% Formic acid in water and the mobile phase B
294 of 0.1% Formic acid in acetonitrile. A gradient elution mode was used as shown in Table 2.
295 The mass spectrometer was operated with a cone voltage of 45ºC, a source temperature of
296 500ºC, a desolvation gas flow rate of 800 L/h and a cone gas flow rate of 80 L/h. All samples
297 were measured in positive ion electrospray mode and photodiode array detection was set to
298 210-400 nm (4.8 nm resolution). Multiple reaction monitoring (MRM) was used for the parent
299 and daughter m/z of 332.2 and 288.2, respectively.

300 **Table 2:** Mobile phase gradients used for HPLC-MS analysis of ciprofloxacin, HPLC-CAD
301 analysis of bile salts and UPLC-MS analysis of lipids and bile salts.

	Time [min]	**% Mobile Phase A**	**% Mobile Phase B**
HPLC/MS analysis of ciprofloxacin	0.00	100	0
	12.00	0	100
	12.01	100	0
	15.00	100	0
HPLC-CAD analysis of bile salts	0.00	40	60
	25.00	10	90
	25.10	40	60
	30.00	40	60
UPLC-MS analysis of lipids and bile salts	0.00	65	35
	9.00	5	95
	10.00	5	95
	10.01	65	35
	12.00	65	35

302 2.2.2. Analysis of formulation and matrix components

2.2.2.1. GC-FID for lipid analysis

Lipid components were extracted as previously described.[21] Briefly, 900 µl chloroform and 100 µl of 0.1 M hydrochloric acid were added to 100 µl of sample in a vial, the mixture was vortexed for 1 min and the bottom layer was directly analysed by gas chromatography coupled with a flame ionisation detector (GC-FID). The analysis was performed on an Agilent 6890N network gas chromatograph equipped with an injector from series 7683B and a flame ionisation detector. The column used for the separation was a TG-5MT (Thermo Fisher Scientific, Loughborough, UK) with a length of 15 m, a diameter of 0.25 mm and a film thickness of 0.10 µm. Helium was used as carrier gas. The column was set to a constant pressure of 30.00 psi. Sample injection (1 µl) was performed from the bottom layer of the sample into a split/splitless inlet using split mode with a split ratio of 5:1 with an inlet temperature of 300°C. The initial oven temperature was set to 60°C for 2 min, followed by an increase of 10°C/min during 34 min and a hold time of 2 min at 400°C resulting in a total run time of 38 min. The detector temperature was kept constant at 350°C. Empower® 3 (Waters Corporation, Milford, MA, US) was used for data collection.

For fatty acids (FA) and cholesterol, chromatographic peaks were identified by comparing retention time with those of known standards resulting in a retention time of 3.6 min for octanoic acid, 6.0 min for decanoic acid and 20.6 min for cholesterol. For monoglycerides (MG) and TGs, chromatographic peaks were identified with an Agilent 5975 MS (data not shown) with retention times of 20.0 min, 21.2 min, 22.3 min and 23.4 min for TGs and 9.2 min, 9.5 min and 11.3 min for MGs, respectively. Quantification of TGs was performed against Glyceryltrioleate, MGs against monoolein and for cholesterol, octanoic acid and decanoic acid against their standards.

2.2.2.2. HPLC-CAD for bile salt analysis

For the bile salt analysis, an Agilent 1200 Series (Agilent Technologies, Santa Clara, CA, US) with a degasser (G1379B), binary pumps system (G1312B), autosampler (G1367C), thermostatted column compartment (G1316B) with a Corona Charged Aerosol Detector (CAD) (ESA Biosciences Inc., Chelmsford, MA, US) was used. A modification of a previously published method was applied.[39] A Waters Halo C18 column (150 mm × 3 mm, 2.7 µm) was maintained at 30°C. The mobile phase A consisted of 20 mM ammonium formate with 0.5% formic acid and 0.2% triethylamine. The mobile phase B was methanol. A gradient method was used according to Table 2 with a flow rate of 0.5 mL/min. The TIM-1 samples were appropriately diluted with mobile phase (mobile phase A: mobile phase B 40:60 V/V) and a volume of 20 µL was injected. The charged aerosol detector was used with a response range of 100 pA full scale. As standards TC, GC, TCDC acid and GCDC were used with the retention times 7.4 min, 10.2 min, 10.8 min and 14.0 min, respectively. Linear regression of the calibration curves of the standards TC, GC, TCDC and GCDC was performed and the average slope (difference between the slopes < 2.5%) was used for the quantification of all bile acids present in the TIM-1 samples (identified as peaks between 7.4 and 14.0 min).

2.2.2.3. UPLC-MS for lipid and bile salt analysis

A lipidomics approach with UPLC-MS was used as semi-quantitative tool to identify the magnitude of changes considering FAs and bile salts in CD compared to healthy conditions in a time-efficient way. Therefore, TIM-1 samples from healthy conditions (n=1) and CD conditions (n=1) after administration of the Ciproxin® suspension were analysed. The samples were diluted with acetonitrile in a ratio of 1:3 (sample:acetonitrile). Additionally, a quality control (QC) sample was prepared by mixing 50 µL of each sample and diluting the mixture with acetonitrile in a ratio of 1:3 (sample:acetonitrile). The injection of a QC sample after every 6 TIM-1 samples was used to assure reproducibility. Three dilutions of the resulting QC sample

with acetonitrile (2x, 5x and 10x) served to confirm the linearity of the peaks of interest over the respective range. TIM-1 samples were randomised for the UPLC-MS analysis.

The analysis was performed with a G6550A Agilent Q-TOF LC/MS System (Agilent Technologies, Santa Clara, CA, US) with a 6550 iFunnel Q-TOF equipped with a HiP-ALS autosampler (G4226A), a binary pump (G4220A) and a thermostatted column compartment (G1316C). A previously published method was used with an Acquity UPLC BEH C8 column (1.7 μm, 2.1x100 mm) maintained at 60°C.[40] The mobile phase A consisted of 50 mM ammonium acetate (pH 5.0) and acetonitrile was used as mobile phase B. A gradient according to Table 2 was applied with a flow rate of 0.6 mL/min.

All samples were measured in negative ion electrospray mode with Dual Agilent Jet Stream Electrospray Ionization (Dual AJS ESI). The gas temperature was set to 250°C with a flow rate of drying gas of 15 L/min, a sheath gas temperature of 220°C and a sheath gas flow rate of 10 L/min. The nebulizer was set to 40 psig, the fragmentor to 400 V, the collision energy to 5 V and capillary voltage to 4000 V. A nozzle voltage of 1000 V was applied. Two different reference masses were used for the negative ESI (112.99 and 1033.99).

The data was processed using XCMS online platform (https://xcmsonline.scripps.edu) with a metabolomics workflow including feature detection, retention time correction and alignment.[41] The following parameters were used for data processing. For feature detection, the centWave method was used with a maximal tolerated m/z deviation in consecutive scans of 10 ppm, a signal to noise ratio cut-off of 6, a peak width in the range of 10 to 60 s, a minimum m/z difference for peaks with overlapping retention times set to 0.01, a prefilter intensity of 10000, the prefilter peaks set to 3 and noise to 100. To align the retention time across samples, the method obiwarp was used with a prof step of 1. For the grouping, density was used as method with a bandwidth of 5, a width of overlapping m/z slices of 0.015 and the minimum fraction and minimum number of samples necessary in at least one of the sample groups for it to be

considered as a valid group were set to 0.5 and 1, respectively.

2.2.3. Light microscopy

Microscopic images of the TIM-1 samples of different parts of the model and at different time points were taken with a Nikon Labophot 2 microscope (Nikon, Tokyo, Japan) equipped with an Olympus DP12 camera (Olympus, Tokyo, Japan) and a TV lens C-0.45x (Nikon, Tokyo, Japan). After mixing each sample with a pipette, several drops of the TIM-1 sample were transferred onto microscopy slide and a cover slip was placed on top of the preparation. A 40x objective lens was used resulting in a total magnification of 400x. Z-Stacking was used to get a greater depth of field for the resulting images by taking approximately five pictures at different focus distances.

3. Results and discussion

3.1. Bioaccessibility of ciprofloxacin

The bioaccessibility of ciprofloxacin after administration of the Ciproxin® suspension in the TIM-1 system in healthy and CD conditions is presented in Figure 2.

The total bioaccessibility of ciprofloxacin was 82.6% and 86.4% in healthy and CD conditions, respectively, suggesting a similar drug product performance in CD patients compared to healthy subjects. The reduced levels of pancreatic enzymes and bile in CD conditions are therefore, not expected to impact on the performance of Ciproxin® oral suspension, most likely due to the hydrophilic nature of ciprofloxacin. Therefore, the lipid excipients in the Ciproxin® formulation are most likely not needed for solubility enhancement and are expected to stabilize the suspension over the in-use period of the suspension. In the duodenum samples, a slightly higher concentration of ciprofloxacin was observed in CD compared to healthy conditions. Since ciprofloxacin possesses a higher solubility in acidic media, drug precipitation might occur during the transfer to the duodenum with a high pH. Therefore, the higher concentrations of triglycerides present in CD conditions in the duodenum compartment could impede the

precipitation of ciprofloxacin. It should be noted that other transfer experiments did not indicate drug precipitation.[25]

The high ciprofloxacin bioaccessibility was in accordance with previous TIM-1 studies with other formulations (immediate-release and extended-release tablets) and a high human bioavailability of 70-80%.[28, 33] The maximum amount of bioaccessible ciprofloxacin per time period was observed at 0.5-1.0 h with 25.7% in healthy conditions and 23.7% in CD conditions, respectively. Pharmacokinetic studies with the Ciproxin® suspension showed a slightly higher time to maximum plasma concentration (T_{max}) of 1.12-1.50 h.[33] For the first 2.0 h after administration of the formulation, the cumulative bioaccessible amount of ciprofloxacin was high with 68.4% for the oral suspension in healthy conditions and 84.4% for the previously investigated immediate-release tablet, respectively.[28] A similar performance of the oral suspension compared to the immediate-release tablets is in agreement with a clinical study demonstrating their bioequivalence.[33]

Ciprofloxacin behaves as a BCS class I drug *in vivo* as indicated by physiologically-based pharmacokinetic (PBPK) modeling despite its common classification as BCS class II/IV compound.[26] Therefore, a limited effect of differences in simulated GI fluids (e.g., pH) on ciprofloxacin performance was revealed in the same study.

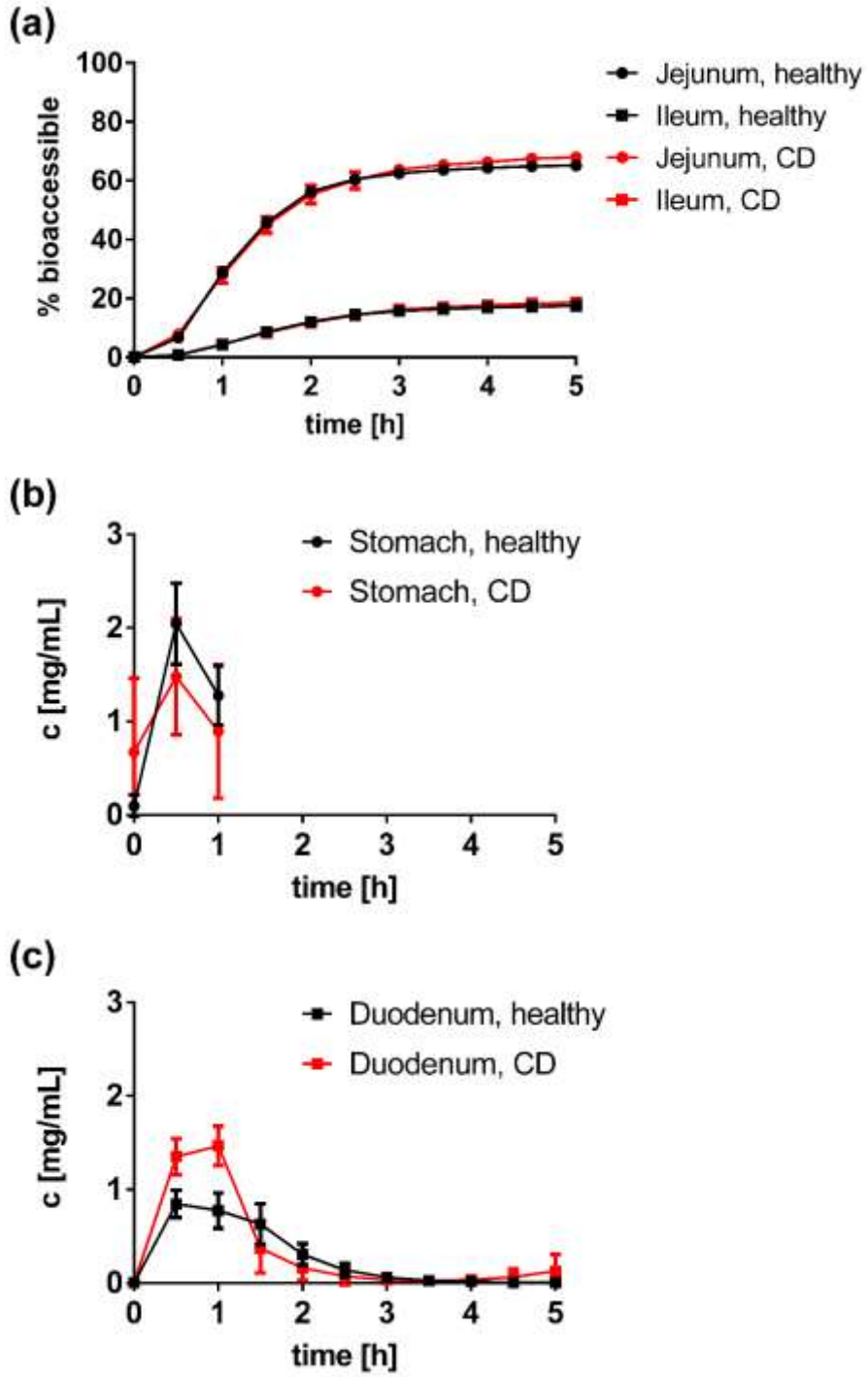


Figure 2: Bioaccessibility of ciprofloxacin in the jejunum and ileum compartment of TIM-1 in healthy and CD conditions (a) and ciprofloxacin concentration in the gastric compartment (b) and duodenum compartment (c) [n=2, mean with SD].

3.2. Formulation and matrix components

3.2.1. Lipids

The digestion of excipients from a LBF can be followed in the different compartments of TIM-1 as shown in Figure 3 by the reduction of TGs and the increase of MGs and FAs over time as measured with GC-FID.

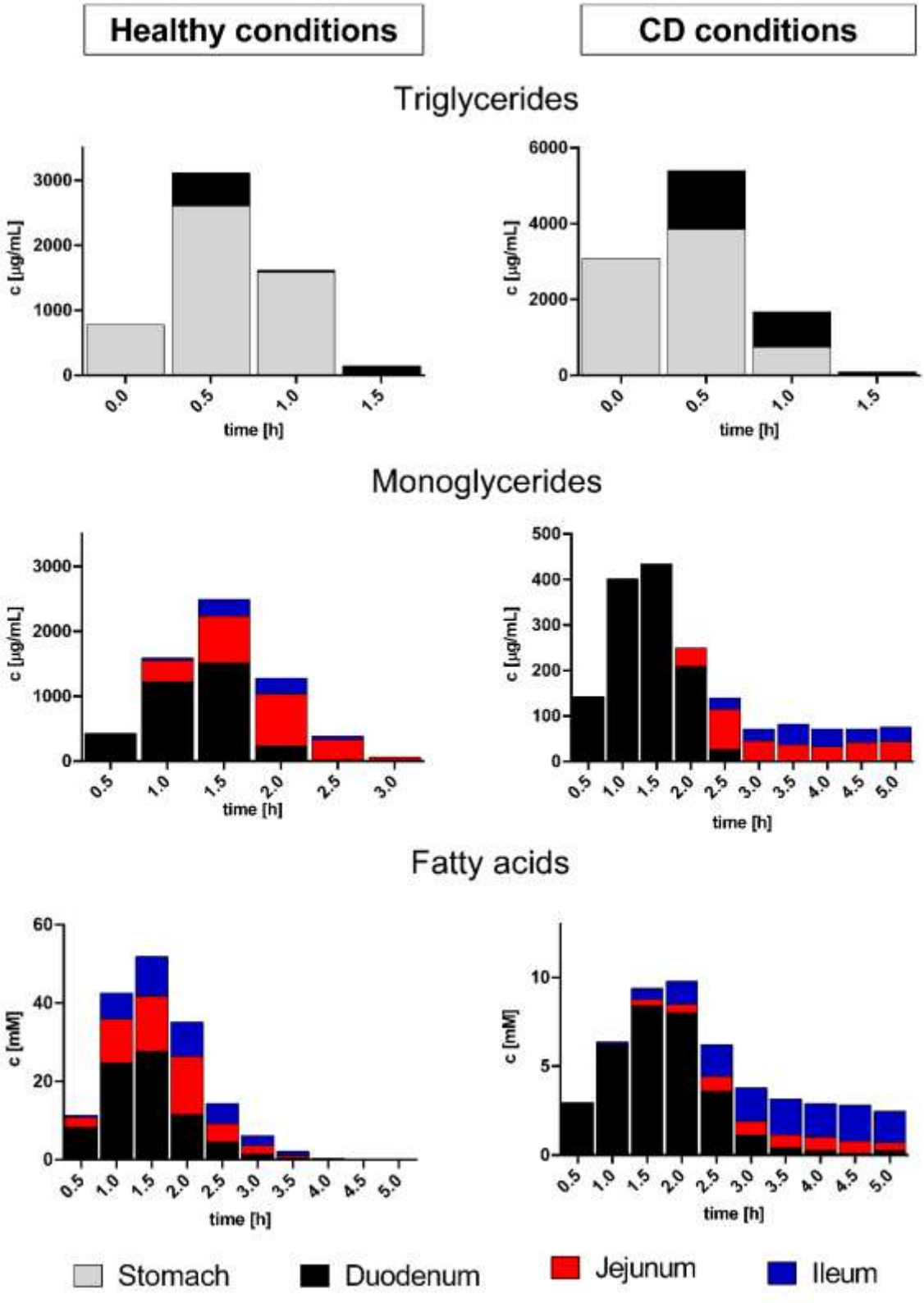
For triglycerides, a higher concentration in the gastric compartment was observed at time point 0.0 h in CD compared to healthy conditions. Since the concentration of gastric lipase is similar in healthy and CD conditions, no difference was expected. The observed difference could possibly be attributed to the gastric content not being well mixed at the start of the experiment and the low number of replicates (n=2). While at 0.5 h the TG concentration is higher in CD compared to healthy conditions, at 1.0 h the opposite is the case. This could be due to variations in the emptying of the gastric content and mixing as suggested also by the high variability with coefficients of variation varying between 12-57%. In the duodenum higher TG concentrations were observed for CD conditions after 0.5 h and 1.0 h, indicating a slower TG hydrolysis due to the reduced concentration of porcine pancreatin. After 2.0 h, no TGs were detected for both experimental conditions in all TIM-1 compartments.

For monoglycerides, the concentration in CD conditions reached only approximately one fifth of the concentrations observed for healthy conditions during the first 2.0 h considering all TIM-1 compartments. In both experimental setups the duodenum compartment showed the highest MG concentrations followed by the jejunum and ileum. While in healthy conditions no MGs were detected after 3.0 h, in CD conditions MGs in the jejunum and ileum compartment were observed from 2.0-2.5 h until the end of the experiment. This indicates that the lipid hydrolysis in healthy conditions is complete after 3.0 h. In contrast, this process is slowed down in CD conditions and not complete within the 5.0 h of the experiment.

In terms of fatty acids, the total FA concentration in all TIM-1 compartments was approximately 5-times higher in healthy conditions compared to CD conditions during the first

2.0 h. Similar to MGs, the highest FA concentrations were observed in the duodenum followed by the jejunum and ileum for both setups. In the healthy setup, no FAs were observed after 3.5 h. Considering CD conditions, FAs in the jejunum and ileum compartment were observed starting from 1.5 h until the end of the experiment. Therefore, in healthy conditions the lipid hydrolysis of the TGs of the formulation is mainly located in the duodenum and jejunum and expected to be complete within 3.5 h. In CD conditions, the lower FA concentrations and their delayed observation indicate a slower and unfinished digestion process.

Consequently, the different concentrations of lipids in CD compared to healthy conditions indicate that the drug is exposed to a different GI luminal environment in CD patients compared to healthy subjects. This is likely to have implications for nutrition as well as drug therapy of CD patients. In terms of nutrition, malabsorption of fat can result in deficiencies of fat-soluble vitamins. In line with this, CD patients were commonly identified to have deficient levels of vitamin A, D and K.[42-44] Additionally, drug absorption can be impeded in CD conditions, in cases where drug release relies on the digestion of lipid excipients. Lipid-based formulations are also used for the treatment of CD, including for example formulations of the immunomodulator cyclosporine. The pharmacokinetics of a lipophilic cyclosporine formulation was investigated in CD patients but no direct control group with healthy subjects was included. The study revealed a similar disposition of cyclosporine in CD patients compared to healthy subjects, while the extent and rate of bioavailability (23.7%) may be lower in CD patients compared to transplant patients (30%).[45, 46] In addition, the pharmacokinetics of cyclosporine after oral administration of a microemulsion formulation were investigated in patients with CD and Ulcerative colitis (UC), revealing a lower maximum plasma concentration for CD compared to UC patients.[47]



471

472 **Figure 3:** Analysis of lipid components in different compartments of TIM-1 in healthy (left)
473 and CD conditions (right) including triglycerides (top), monoglycerides (middle) and fatty
474 acids (bottom).

The ratio of the intensity of the FAs, octanoic and decanoic acid, in CD conditions to healthy conditions in the different compartments of the TIM-1 as assessed with semi-quantitative analysis using UPLC/MS is shown in Figure 4.

For both FAs, a lower concentration was observed in CD conditions compared to healthy conditions in the first two hours with approximately one half of the FA concentration in the duodenum compartment and one quarter in the jejunum and ileum compartment. For octanoic acid, the concentration in CD conditions was higher compared to healthy conditions after 2.5 h in the duodenum compartment and after 3.0 h in the jejunum and ileum compartment. For decanoic acid, higher concentrations in CD conditions were observed after 2.5 h in the duodenum compartment, after 3.5 h in the ileum compartment and after 4.0 h in the jejunum compartment. Consequently, the UPLC/MS results are consistent with a delayed hydrolysis of TGs in CD conditions. Considering the total intensity of the FAs over all time points, in CD conditions only 65% and 61% of the intensity in healthy conditions was observed for octanoic acid and decanoic acid, respectively. This again suggests a lower extent of TG hydrolysis in CD. Therefore, the semi-quantitative UPLC/MS lipidomics approach has proven to be a useful quick tool to assess the differences in luminal FA concentrations.

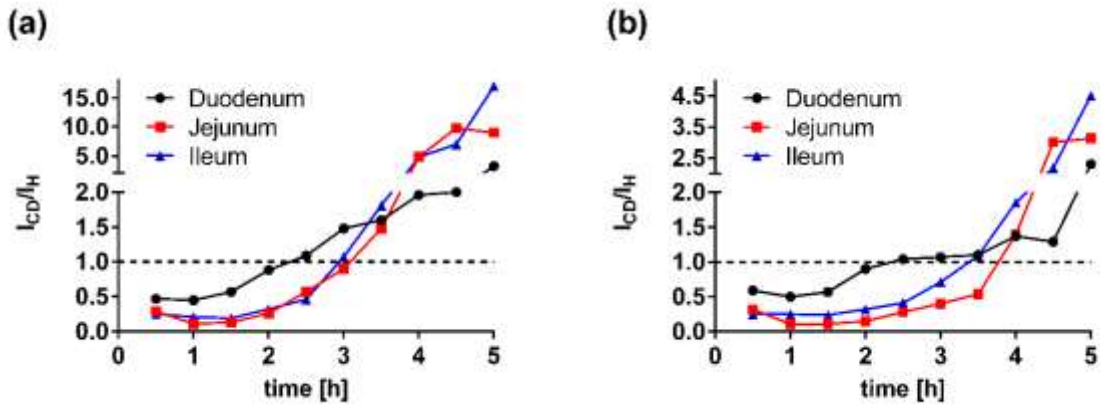
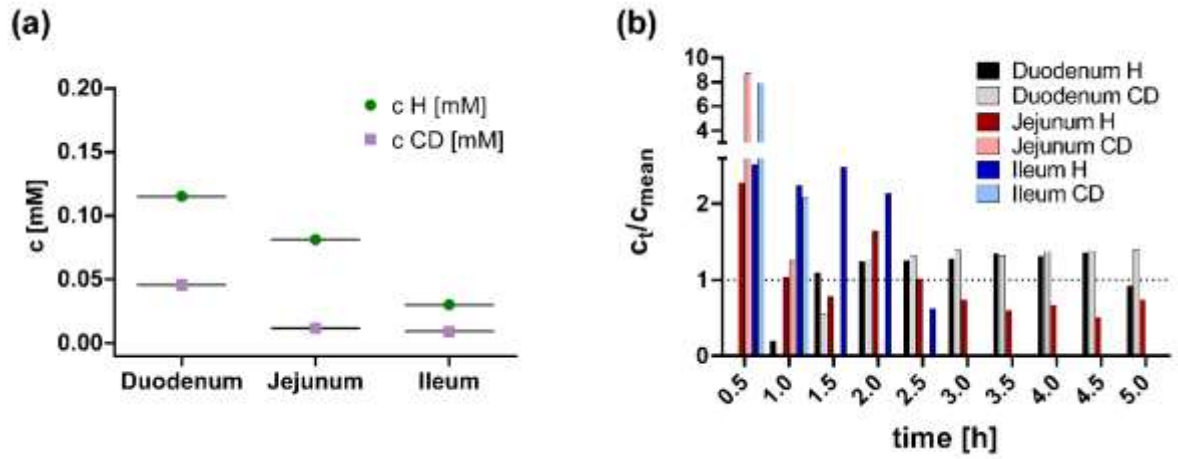


Figure 4: UPLC/MS intensity of fatty acids (n=1) illustrated as ratio of intensity in CD conditions to healthy conditions for octanoic acid (a) and decanoic acid (b).

Cholesterol is a formulation excipient from the Ciproxin® suspension but also a biliary component and therefore, present in the TIM-1 matrix. Since no cholesterol was observed in the gastric compartment, the observed cholesterol in the small intestinal TIM-1 compartments is expected to be mainly from the biliary secretions (porcine bile). In Figure 5a, the mean cholesterol concentration over the 5.0 h time course of the experiment as measured with GC-FID is shown in the different TIM-1 compartments and experimental setups. For the CD conditions, the cholesterol concentration is less than half of the concentration observed for healthy conditions, as expected due to the lower concentration of porcine bile in CD conditions. In terms of the biorelevance of the TIM-1 conditions, the mean duodenal and jejunal cholesterol concentrations in healthy conditions correspond to the range observed in human intestinal fluids that has been reported between 0.08 mM and 1.80 mM (mean cholesterol concentration).[34, 48-51] The time course of the cholesterol concentration in the different TIM-1 compartments is shown in Figure 5b. In the duodenum compartment, a lower concentration of cholesterol is observed in the first hour of the experiment, most likely due to the transfer of the gastric content to the duodenum compartment in the first hour until the housekeeper wave. In contrast, higher concentrations of cholesterol are observed for the first hour in the jejunum and ileum compartment, indicating a higher cholesterol concentration due to the preconditioning of the filter with a solution containing porcine bile or a higher concentration in the starting residues of both compartments.



513

514 **Figure 5:** Concentration of cholesterol in different TIM-1 compartments in healthy and CD
515 conditions shown as mean value over 5 hours (a) and time course (b) [H: Healthy, CD: Crohn's
516 disease].

517 3.2.2. Secretion of bicarbonate solution

518 The volume of bicarbonate solution secreted in the different TIM-1 compartments to maintain
519 the preset pH in the different experimental conditions as reported by the TIM-1 system is shown
520 in Figure 6. In healthy and CD conditions with the Ciproxin® suspension, more bicarbonate
521 solution was secreted compared to the blank TIM-1 run in all compartments, indicating an
522 impact of formulation components on pH. The digestion of TGs results in a release of FAs,
523 which in turn provokes a pH reduction and is therefore, expected to trigger the secretion of
524 bicarbonate solution. In the duodenum and jejunum compartment, more bicarbonate solution
525 was secreted in healthy compared to CD conditions, possibly due to more FAs being released
526 in healthy conditions (Section 3.2.1). In the jejunum compartment, the bicarbonate secretion
527 slightly increased after 3 h in CD conditions, which agreed with increased FA concentrations
528 observed at later time points (Section 3.2.1). Another point for consideration is that there is no
529 direct relationship between the volume of bicarbonate solution secreted and the amount of FAs
530 released in the compartments. For example, the concentration of FAs in the duodenal samples
531 was higher compared to the jejunal samples in healthy conditions, while the total bicarbonate

secretion was slightly higher in the jejunum. This highlights that other formulation factors and TIM-1 matrix components are also influential to the bicarbonate secretion.

The control of the bicarbonate secretion in TIM-1 is comparable to the use of sodium hydroxide in the pH stat method, another *in vitro* method for the evaluation of LBFs. For the pH stat method, the degree of lipid digestion is determined by the sodium hydroxide necessary for the neutralization of the FAs released by enzymatic lipid hydrolysis.[13] In comparison to the pH stat method, additional factors including various secretions and the compartmental transfer of formulation and matrix components can influence the pH in TIM-1 and therefore, the bicarbonate secretion. Additionally, it is difficult to assess the total digestion of the formulation in TIM-1 due to the constant removal of lipids e.g., MGs via filtration. It should be considered that in the case of formulations with long chain FAs possessing a higher pKa, the bicarbonate secretion might not be indicative of their release due to their presence in the undissociated form at luminal pH values of TIM-1.[52]

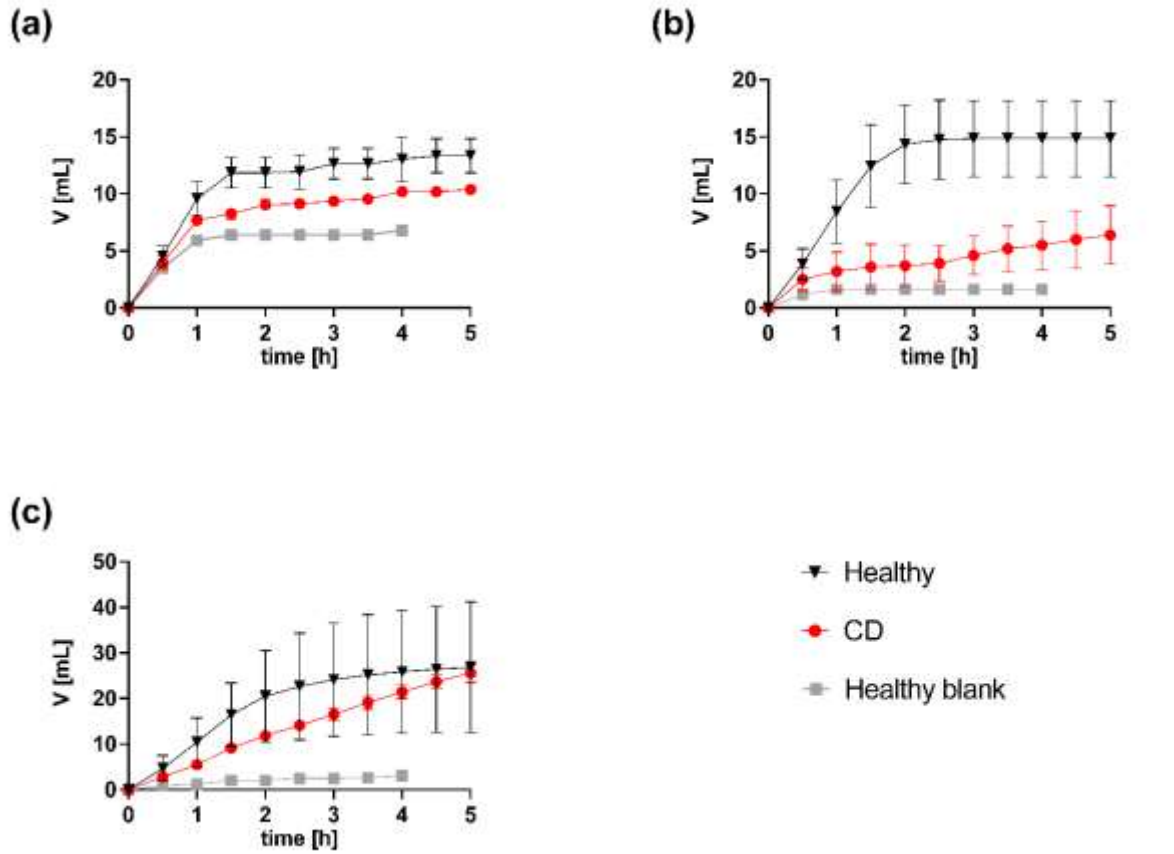


Figure 6: Secretion of bicarbonate solution in the duodenum compartment (a), the jejunum compartment (b) and the ileum compartment (c) in healthy and CD conditions with Ciproxin® suspension and healthy blank conditions.

3.2.3. Bile salts

The total bile salt concentrations, measured quantitatively with HPLC-CAD, are presented in Figure 7 as mean concentrations with range and concentrations over time for the different TIM-1 compartments and experimental conditions.

Apart from the first two time points (0.5 h and 1.0 h), the bile salt concentration in the different TIM-1 compartments is stable over the remaining run time of 4 h. For the duodenum compartment, the difference in the beginning is most likely due to initial transfer of luminal content from the stomach to the duodenum compartment until the housekeeper wave after the first hour. In contrast, the higher bile salt concentration in the beginning in the jejunum and ileum compartment indicates a higher bile salt concentration due to the starting residues or

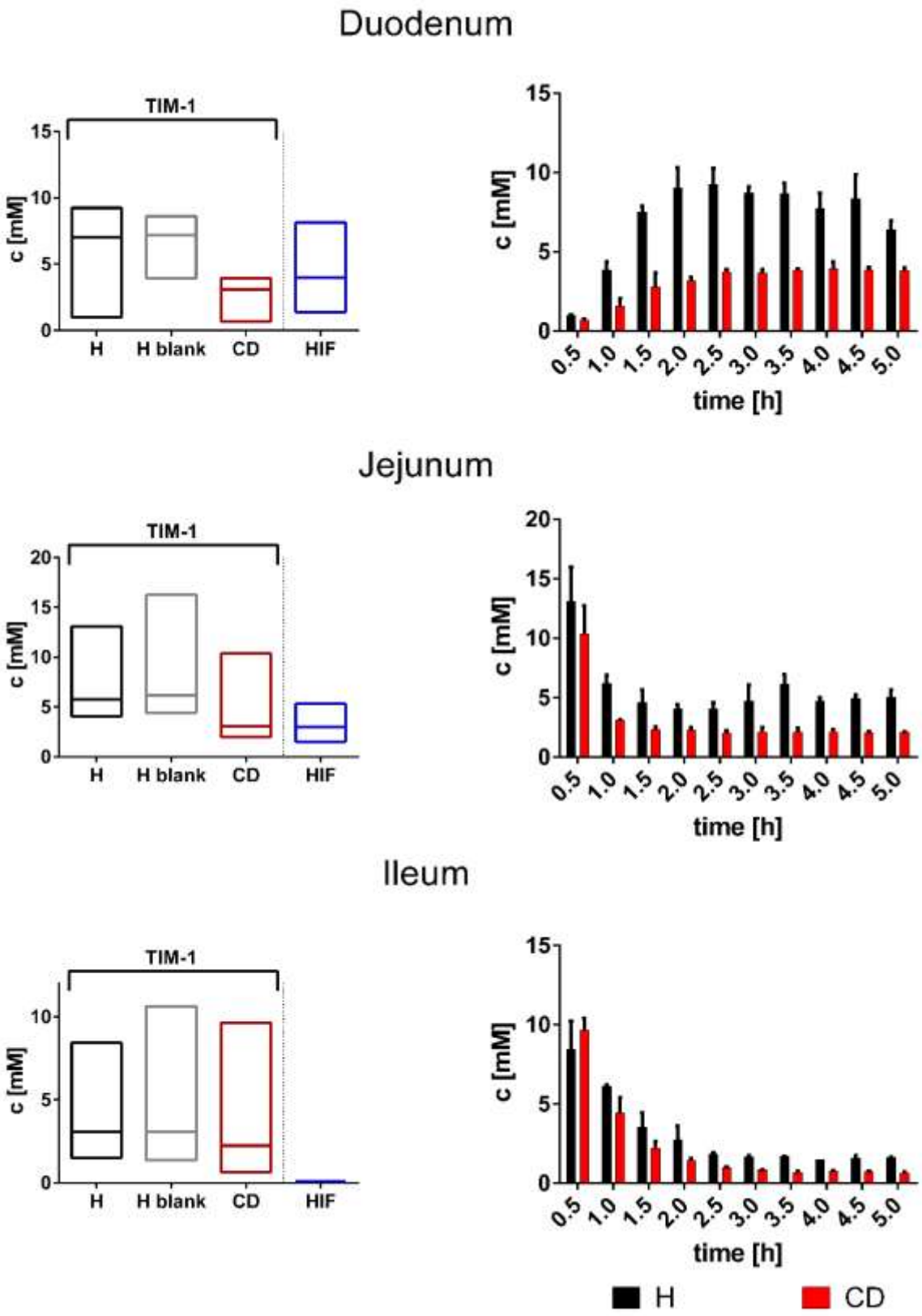
559 initial preconditioning of the filters. For both compartments (jejunum, ileum), it should be
560 noted that our samples were from the filtrate and not directly from the TIM-1 lumen.

561 Similar bile salt concentrations were observed in the different TIM-1 compartments for the
562 healthy run with and without the Ciproxin® formulation.

563 For the healthy conditions, the average duodenal total bile salt concentration was 7.04 mM, the
564 jejunal total bile salt concentration was 5.76 mM and the ileal total bile salt concentration was
565 3.07 mM. For the CD conditions, the average duodenal total bile salt concentration was
566 3.10 mM, the jejunal total bile salt concentration was 3.05 mM and the ileal total bile salt
567 concentration was 2.24 mM. As expected, the reduced bile salt concentration in CD conditions
568 (lower concentration of porcine bile) was reflected in all compartments with a reduced total
569 bile acid concentration.

570 In comparison to human intestinal fluids, the duodenal bile salt concentration of the healthy
571 experimental setup was significantly higher with 179% of the mean observed value in 13
572 different studies in healthy subjects.[18, 48, 51, 53-62] In contrast, the total bile salt concentration of
573 the CD experimental setup was with 78% much closer to the concentration in human duodenal
574 fluid. Similarly in the jejunum compartment, the total bile salt concentration in the healthy
575 experimental setup was almost doubled the mean concentration in human jejunal fluid (192%)
576 as observed in 10 different studies, while the total bile salt concentration in CD conditions was
577 similar (101% of the concentration in human jejunal fluids).[48, 49, 61, 63-71] Considering the ileum
578 compartment, in both experimental setups the total bile salt concentration was 32- to 43-fold
579 higher compared to the mean concentration in the human distal ileum in the fasted state as
580 investigated in one study.[72] It should be taken into account that the high bile salt concentrations
581 during the first hour have a high impact on the mean value of the ileum compartment. For
582 example, when only the last two hours of the experiment are considered, the ileal total bile salt

583 concentration in CD conditions was only 10-fold higher compared to the observed
584 concentration in the human distal ileum. Additionally, bile salt concentrations used for in vivo
585 comparison were measured in the terminal ileum. Since bile salts get reabsorbed in the terminal
586 ileum, this reabsorption process is expected to contribute to the observed low in vivo
587 concentrations. The TIM-1 ileum compartment represents not only the terminal part but the
588 whole ileum. Thus, the higher bile salt concentration is also expected for the in vivo situation.



589

590 **Figure 7:** Overview of total bile salt concentration in TIM-1 in healthy and CD conditions as
591 measured with HPLC-CAD with mean concentrations over time plus range in different
592 compartments of the TIM-1 in comparison to human intestinal fluids (left) and total bile acid

concentrations at different time points during TIM-1 run (right) [H: healthy conditions with Ciproxin®, H blank: healthy conditions without formulation, CD: CD conditions with Ciproxin®, HIF: Human Intestinal Fluids].[18, 48, 49, 51, 53-71]

The ratio of the intensity of specific bile salts in CD conditions to healthy conditions in the different compartments of the TIM-1 as assessed with semi-quantitative analysis using UPLC/MS is shown in Figure 8. The presence of the bile acids TC, TCDC and GCDC was confirmed due to the same retention time of the bile acids in the TIM-1 samples and the standards used for the HPLC-CAD analysis. Additionally, two bile acids with a molecular weight of 465.6 g/mol and 449.6 g/mol were present in the TIM-1 samples. Due to the same molecular weight, it is likely that the bile acids are glycohyocholic acid (GHC) and glycohyodeoxycholic acid (GHDC), which have previously been reported as major components of porcine bile.[73]

In the duodenum and jejunum compartment, the ratio of bile salts in CD conditions to healthy conditions is stable after 1.5 h with CD conditions showing approximately 50% of the bile salt intensity of healthy conditions. During the first hour of the experiment, the concentration of bile salts in CD conditions is closer to the bile salt concentration in healthy conditions, most likely due to the starting residues or preconditioning of the filters. In the ileum compartment, the bile salt concentration in CD conditions compared to healthy conditions was initially lower compared to the duodenum and ileum. However, the overall bile salt concentration in CD conditions in the ileum was also approximately half of the concentration in healthy conditions. The lower concentration of porcine bile in the CD conditions (43% of healthy conditions) was therefore, approximately reflected in the bile salt concentrations in all TIM-1 compartments. The presented semi-quantitative UPLC/MS analysis of luminal bile salt concentrations can thus, be used to monitor the difference between two different experimental setups in a time-efficient way.

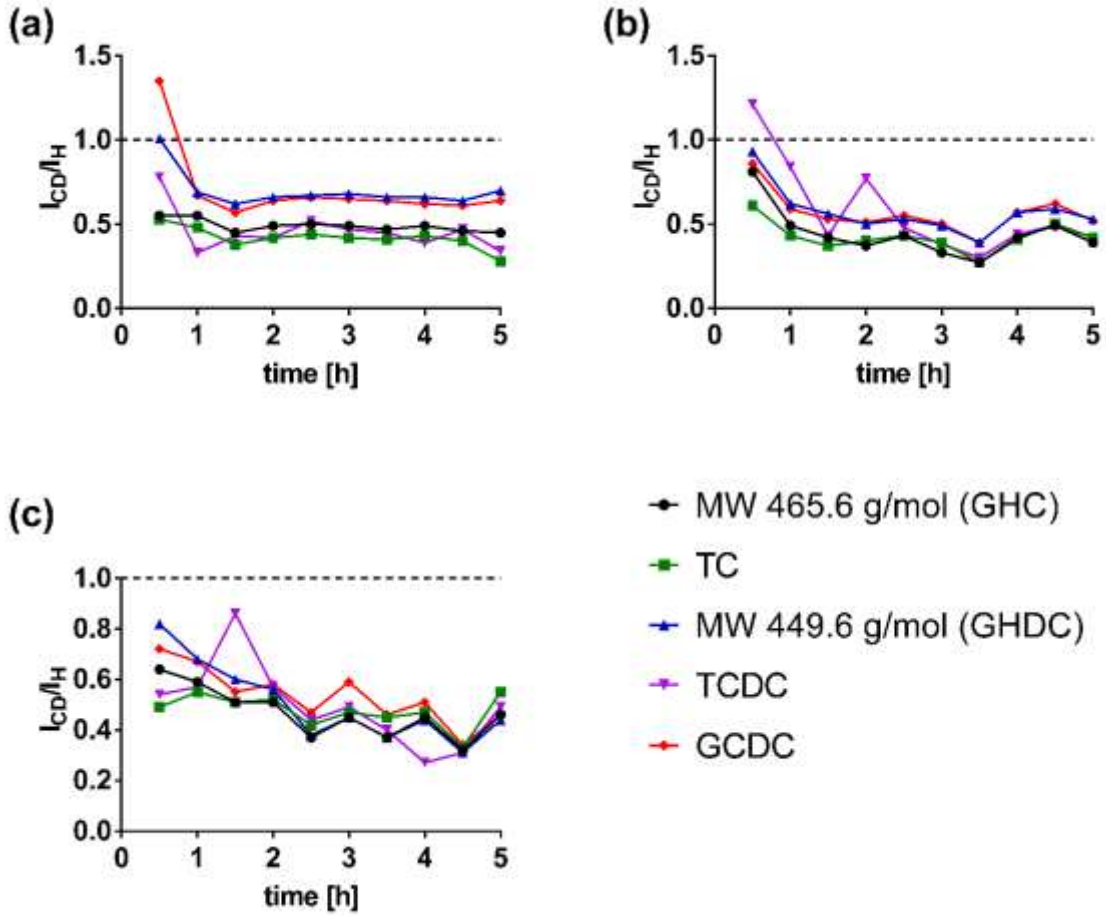


Figure 8: UPLC/MS intensity of specific bile salts in TIM-1 illustrated as ratio of the intensity in CD conditions to the intensity in healthy conditions (GHC: Glycohyocholic acid, TC: Taurocholic acid, TCDC: Taurochenodeoxycholic acid, GCDC: Glycochenodeoxycholic acid, GHDC: Glycohyodeoxycholic acid) in the duodenum compartment (a), in the jejunum compartment (b) and in the ileum compartment (c).

3.3. Light microscopy

The contents of the gastric and duodenal compartment were examined with light microscopy as shown in Figure 9. In the stomach compartment, the emulsion droplets showed a polydisperse particle size distribution with similar droplet sizes for the different time points. In the duodenum compartment, the emulsion droplets were bigger during the first hour and their diameter decreased subsequently. Differences between healthy and CD conditions were not observed.

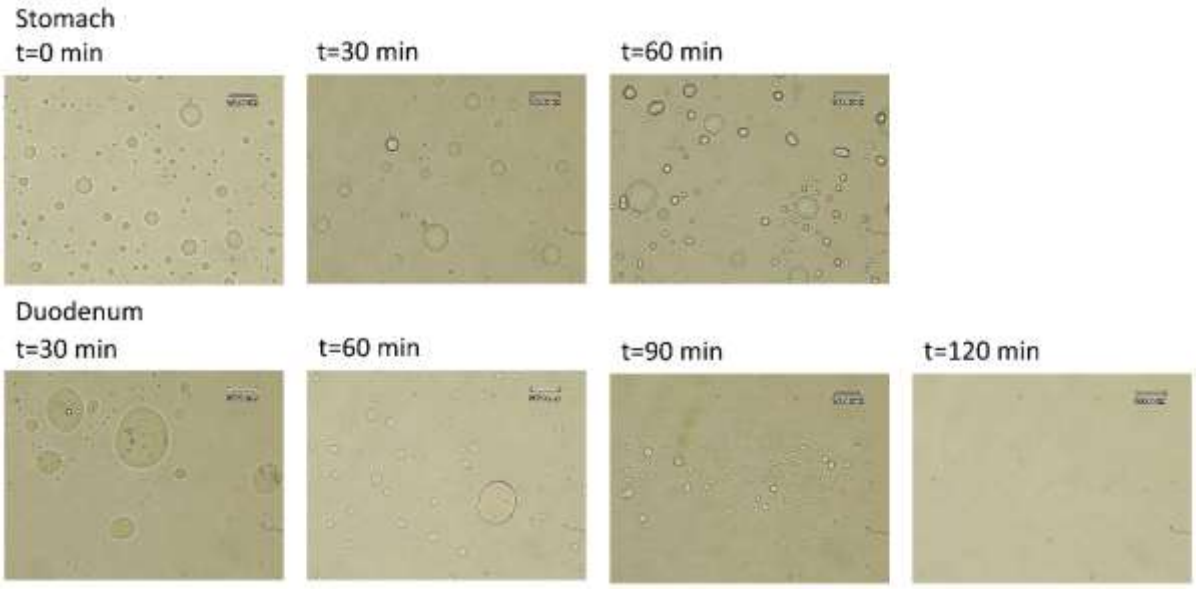


Figure 9: Light microscopy pictures of the contents of the gastric and duodenal compartment after administration of Ciproxin® oral suspension in healthy conditions (scale bar is 30 µm).

4. Conclusion

The performance of Ciproxin® oral suspension was not impacted by CD conditions, most likely due to the low lipophilicity of ciprofloxacin. The digestion of excipients of a LBF can be followed in the TIM-1 system. By comparing the lipolysis of the medium chain TGs in healthy and CD conditions, reduced FA and MG concentration in CD conditions during the first hours and higher concentrations at the end of the experiment were observed. This indicates a delayed and reduced digestion process in CD. Consequently, the GI luminal environment is expected to be different in CD patients compared to healthy subjects, suggesting a possible impact on the performance of LBFs in CD.

For more lipophilic compounds, differences in drug product performance of LBFs are expected due to the differences observed in the luminal environment and suggest an increased risk of altered drug product performance in patients with CD.

In terms of the biorelevance of the TIM-1 conditions, bile acid concentrations were higher in healthy TIM-1 fasted state conditions compared to reported concentrations in human intestinal fluids. Interestingly, the conditions defined for CD patients showed similar bile salt

650 concentrations compared to human intestinal fluids. Cholesterol concentrations in healthy
651 conditions were in the range of the levels observed in human intestinal fluids.

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