Simultaneous assessment of lipid classes and bile acids in human intestinal fluid by solid phase extraction and HPLC methods

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Running footline: SPE and HPLC methods for characterization of lipids in intestinal fluids

ABBREVIATIONS
BA, bile acids; CA, cholic acid; CDCA, chenodeoxycholic acid; CE, cholesterol ester; CV, coefficient of variation; DAG, diacylglycerols; DCA, deoxycholic acid; DPG, diphosphatidylglycerol; ELS, evaporative light scattering; FeSSIF, fed simulated small intestinal fluid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; HAc, acetic acid; HIF, human intestinal fluid; LCA, lithocholic acid; LOD, limit of detection; LOQ, limit of quantification; LPC, lyso-phosphatidylcholine; MAG, monoacylglycerols; MeOH, methanol; mM, millimolar (moles/L); MTBE, methyl tert-butyl ether; NH₂, aminopropyl; NL, neutral lipids; PA, phosphatidic acid; PC, phosphatidylcholine, PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipids; PS, phosphatidylserine; Si, silica; SM, sphingomyelin; SPE, solid phase extraction; TAG, triacylglycerols; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; UC, unesterified cholesterol.
ABSTRACT

The purpose of the study reported here was to develop a method for the determination of lipid classes in intestinal fluids, including bile acids. A solid phase extraction (SPE) method using C18 and silica columns for the separation of bile acids (BA), phospholipids (PL) and neutral lipids including free fatty acids (NL) has been developed and validated. Fed state small intestinal fluid collected from humans was treated with orlistat to inhibit lipolysis and mixed with acetic acid and methanol prior to the SPE to maximize lipid recoveries. BA, PL and NL were isolated using lipophilic and polar solvents to promote elution from the SPE columns. The different lipid classes were subsequently analysed using three separately optimized HPLC methods with evaporative light scattering detectors. High recoveries (> 90%) of all lipids evaluated were observed, with low coefficients of variation (< 5%). The HPLC methods developed were highly reproducible and allowed base-line separation of nearly all lipid classes investigated. In conclusion, these methods provide a means of lipid class analysis of neutral lipids, phospholipids, as well as, bile acids, in human fed state small intestinal fluid, with potential use in other fluids from the intestinal tract and animals.

KEY WORDS: solid phase extraction, HPLC, ELS, neutral lipids, phospholipids, bile acids, intestinal fluid.
INTRODUCTION

Poor correspondence between solubility data for simulated and real intestinal fed state fluid has indicated that there is a need for investigations on the composition of components in gastrointestinal fluids collected from humans (1,2). Conventionally, TLC has been used to provide efficient separation of lipids in this matrix, following liquid-liquid extraction. The liquid extraction methods optimized for lipid classes are commonly based on chloroform:methanol (3) or isopropanol:hexane (4) but result in poor recoveries of conjugated bile acids (BA) in the organic phase (5). Thus, simultaneous liquid-liquid extraction and TLC separation of BA and other lipids is very difficult. In addition, TLC often results in low lipid recoveries.

Solid phase extraction (SPE) represents an alternative to TLC, which makes it possible to combine the extraction of large samples with lipid class separation. Several solid phase extraction methods for lipids in plasma and tissue extracts have been published (6-14). However, none of these methods include bile acids in their separation, nor have they been validated for the separation of lipids in intestinal fluids. Bile acids (BA) represent a broad range in polarity, with the taurine conjugated trihydroxy bile acids being the most polar and the unconjugated mono-hydroxy bile acid being the least polar. Techniques for extraction and separation of bile acids (5, 15) and phospholipids (16) in biological samples have been thoroughly reviewed. C18 SPE have been shown to efficiently isolate bile acids and their conjugates from biological fluids such as bile, serum, urine, stools etc (17). We present here a new C18 SPE protocol based on simultaneous adsorption of bile acids and total lipids in human small intestinal fluid and subsequent elution for further analysis by silica SPE and HPLC.
HPLC with evaporative light scattering (ELS) detection provides a simple and affordable detection system for high-concentration samples like intestinal fluids, bile or feces. In addition, upper small intestinal fluid in normal subjects contains almost exclusively conjugated bile acids, which can be analysed directly with HPLC without prior removal of the conjugating moiety, in contrast to gas chromatographic methods requiring ion-exchange separation into taurine-, glycine- and free bile acid classes prior to deconjugation, derivatisation and analysis. Numerous applications of HPLC for the separation of bile acids (18-26) and lipid classes have been reported (27-41). Silversand and Haux (42) have published a method for the analysis of neutral and polar lipids in fish eggs that uses two different straight phase systems. For the separation of polar lipids, they used a diol column in combination with a binary gradient. Another method for the separation of both polar and neutral lipids using a silica (Si) column has been published by Homan and Andersson (43).

Our main objective was to develop a method for the separation and subsequent analysis of phospholipids (PL), bile acids and neutral lipids (NL), including free fatty acids (FFA), in human intestinal fluid. The application of this new procedure to the assay of bile acids, phospholipids and neutral lipids in human intestinal fluid has been demonstrated and the results obtained compared to the lipid content in simulated intestinal fluids used in drug development.

MATERIALS AND METHODS

Chemicals

Orlistat was purchased from Apin Chemicals, UK. The following bile acid standards, were supplied by Sigma-Aldrich, Germany: cholic acid (CA), glycocholic acid (GCA), taurocholic acid (TCA), deoxycholic acid (DCA), glycodeloxycholic acid
(GDCA), taurodeoxycholic acid (TDCA), chenodeoxycholic acid (CDCA),
glycochenodeoxycholic acid (GCDDCA), taurochenodeoxycholic acid (TCDDCA) and
lithocholeic acid (LCA). Phospholipid standards were ordered from Avanti Polar
Lipids, USA, including/as follows: phosphatidylcholine (PC), lyso-
phosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidylglycerol
(PG), phosphatidylserine (PS), diphosphatidylglycerol (DPG), phosphatidic acid (PA)
and sphingomyelin (SM)). Phosphatidylinositol (PI) was ordered from
ICN Biomedicals, Germany. The neutral lipid standards, cholesterol,
cholesteryloleate, dipalmitin, tripalmitin and palmitic acid, were purchased from
Sigma-Aldrich, Germany and monoolein from Larodan AB, Sweden. Radioactive
lipid standards were obtained from Larodan AB, Sweden as follows and used without
further purification: [1-14C]palmitic acid, [7(n)-3H]cholesterol, phosphatidylcholine-1-
palmitoyl-2-[14C]linoleoyl, lyso-phosphatidylcholine-1[1-14C]palmitoyl, [1-
14C]glycocholic acid, sodium salt, tauro[carbonyl-14C]cholic acid, sodium salt was
obtained from Amersham, USA and tripalmitin[palmitic-1-14C] from American
Radiolabeled Chemicals, USA. The radioactive standards were diluted in ethanol to
obtain a concentration of 0.3 MBq/ml. C18 (500 mg, 3 ml) and Si (100 mg, 3 ml)
columns were obtained from Isolute, International Sorbent Technology, UK. All
solvents were of analytical grade. Triethylamine was purchased from Sigma Chemical
Company, USA. Acetic acid and chloroform were purchased from Merck, Germany;
all other solvents came from Rathburn Chemicals Ltd, Scotland.

**Apparatus**

Bile acid separation was accomplished by reversed-phase HPLC. The equipment was
fitted with a binary pump (Gynkotek P580M HPG, Dionex Germany), autosampler
Gynkotek Gina 50, Dionex Germany) and degasser (Scantec Lab AB, Sweden). The chromatographic column, a Zorbax C18 Extend Column (150 mm*4.6 mm, 3.5 µm), Agilent Technologies, USA), was held at 40 °C by a column heater (Croco-cil, Scantec Lab AB, Sweden). A light scattering detector was used for the detection (PL-ELS 1000, Polymer Laboratories, UK). The nebuliser and evaporation chamber temperature were at 80 and 85 °C, respectively. The detector gas flow was 1.2 ml/min.

Phospholipids and neutral lipids, including free fatty acids were separated by straight-phase HPLC using a binary pump (Dionex P580A LPG, Dionex Germany), autosampler (Gynkotek Gina 160 autosampler, Dionex Germany) and degasser (Scantec Lab AB, Sweden). Phospholipids were separated on an YMC-Pack Diol column (250 mm*2.1 mm, 5 µm) (YMC Inc, USA), held at 55 °C and neutral lipids, including free fatty acids, were separated on an Apex II Diol column (150mm*4.6mm, 5µm) (Jones chromatography, USA), held at 15 °C. A Sedex 75 light scattering detector (Sedere, France) was used for the detection of phospholipids. The detector temperature was 52 °C and the air pressure was 2.5 bar. The gain of the photomultiplier was set to 5. For the detection of neutral lipids, including free fatty acids, a PL-ELS 1000 (Polymer Laboratories, UK) was used. The nebuliser and evaporation chamber temperature were at 65 and 80 °C, respectively. The detector gas flow was 1.2 ml/min.

**Sample collection**

The sampling of human intestinal fluid (HIF) was performed at the Clinical Research Department of the University Hospital in Uppsala, Sweden and was approved by the Ethics Committee of the Medical Faculty at Uppsala University.
Pooled fed human intestinal fluid (HIF) was collected from the jejunum of six healthy volunteers using the Loc-I-Gut method (44, 45), after given informed consent. In brief, the tube was positioned in the jejunum under the guidance of fluoroscopy. The subjects were given a well-defined nutritional drink intended for parenteral administration to patients with mild to moderate catabolism (NuTRIflex®, Braun) (nitrogen 0.8 g, amino acids 5.8 g, glucose 11.5 g, lipids 7.2 g, energy 576 kJ) to simulate fed conditions. The intestinal fluid was collected in 10 minute intervals over a period of 90 minutes. The fractions obtained from 30 to 60 minutes, inclusive, were pooled and used for analysis. Orlistat was added to the intestinal fluid to reduce lipolysis. The intestinal fluid was kept at -70 °C prior to the analysis.

**Solid phase extraction**

The lipids in the intestinal fluid were separated into phospholipids, neutral lipids and bile acids using pre-packed C18 and Si columns.

*Bile acid extraction on C18 SPE columns*

The extraction of bile acids in the intestinal fluid was performed using a C18 column pre-activated and conditioned with MeOH and H₂O. An intestinal fluid sample of 1 ml was loaded on the column. The pH in the intestinal fluid was measured prior to conducting the SPE. The lipids were eluted in two different fractions, bile acids and phospholipids/neutral lipids. The procedure was optimised according to Table 1. Fraction 2 from the C18 SPE was dried under N₂ and redissolved in 700 µl MeOH for HPLC analysis of individual bile acids. The solvent fraction containing phospholipids and neutral lipids was evaporated under N₂ at 40 °C and redissolved in 1 mL chloroform prior to loading on Si columns.
Separation of NL and PL on Si SPE columns

Separation of neutral lipids and free fatty acids from polar lipids was accomplished using pre-packed Si columns as described previously (46), with some modifications made for the SPE. The procedure is shown in Figure 1. The CHCl₃ eluate (sample load+F5) was dried under N₂ and redissolved in 700 µl of mobile phase A, for subsequent analysis of neutral lipids. The PL fraction (fraction 6) from the Si SPE was dried under N₂ and redissolved in 700 µl of CHCl₃, for subsequent determination of phospholipid content by HPLC.

Validation of the SPE method

For quantitative recovery experiments, 50 µl of a radioactive polar lipid (Phosphatidylcholine-1-palmitoyl-2-[1⁴C]linoleyl or lyso-phosphatidyl-choline-1[1-⁴C]palmitoyl), free fatty acid (⁴C-palmitic acid), neutral lipid (⁴C-tripalmitin or ³H-cholesterol) or bile acid (⁴C-glycocholic acid or ⁴C-taurocholic acid) was added to 1 mL of the intestinal fluid prior to performing the SPE. Of this sample, 100 µl was transferred to an empty glass vial as the control. The radioactivity in the different fractions was analyzed using a Wallac Win-Spectral 1414 liquid scintillation counter (Wallac, Turku, Finland). The elution of the different lipid species was also confirmed by HPLC with ELS detection.

HPLC procedures

Bile acids

A binary gradient was used for separation of the bile acids. The mobile phases were as follows: (A) MeOH:buffer (ammonium acetate 15 mM, 0.2% triethylamine,
0.5% formic acid, pH 3.15) 60:40 (v/v), and (B) MeOH:buffer 95:5 (v/v). The
gradient started at 10% B, at which it was held for 10 minutes, and thereafter increased
to 50% B over 10 minutes and then to 100% B over 5 minutes. The gradient was,
thereafter, decreased to 10% B over 0.1 minutes and held there throughout the run.
The gradient took 30 minutes in total. The flow rate was set to 1 ml/min and the
sample volume was 25 µl. Standards in the range of concentration 0.04-1.00 mM were
prepared in MeOH. Nor-deoxycholic acid was included as internal standard in the
analysis.

*Phospholipids and Neutral lipids including free fatty acids*

The methods adopted for the HPLC analysis of phospholipids and neutral
lipids including free fatty acids were modifications of that described by Silversand
and Haux (42). For phospholipid analysis, the compositions of the mobile phases and
the column temperature, for optimum resolution of critical pairs, were determined by
a central experimental composite design using MODDE 6.0 (Modeling and design,
Umetrics, Sweden). The gradient and the column temperature were varied in the
neutral lipid analysis to obtain the best resolution. Standards in the concentration
range 0.04-1.00 mM were prepared in CHCl₃ and mobile phase A for phospholipid
and neutral lipid analysis, respectively.

*Validation of the final chromatographic method*

The chromatograms of pure lipid standards were obtained individually to
confirm retention times and purity. Furthermore, three control samples with known
(weighed in) amounts, 0.9, 0.15 and 0.05 mM, were analysed to determine the
accuracy and precision of within and between-day runs (n = 12). The limit of
detection (LOD) was determined at a signal-to-noise ratio of 3/1 and the limit of quantification (LOQ) at a signal-to-noise ratio of 10/1.

RESULTS

Solid phase extraction

The final SPE method is shown in Figure 1. Two different columns were used for the separation of all the lipid classes in intestinal fluid. The recovery of the radioactive lipid standards used is shown in Table 2. The eluted lipid species in the BA, NL and PL fractions are shown in Figure 2.

The effect of adding MeOH to the intestinal fluid sample, prior to the C18 SPE separation, on the adsorption of NL and BA to the column during sample load was studied. The adsorption of NL to the column improved as the amount of MeOH added increased. The amount retained of NL increased from 50 to 98 % when the amount of MeOH added increased from 0 to 30 %. The BA adsorbed well to the column when up to 30 % MeOH was added to the intestinal fluid, however amounts above 30% resulted in a decrease of up to 65% in the recovery of BA. When 30% MeOH was added to the intestinal fluid, the best adsorption of both bile acids and NL to the column material, was obtained.

HAc was added to the intestinal fluid to alter the pH in the samples. The recovery of PL and FFA was increased from 75 to 100 % and 88 to 98 %, respectively, when lowering the pH in the intestinal fluid. At pH 5, the recovery of PL was 95 %. At pH values for the intestinal fluid of below 5, the BA were retained longer on the column and led to a co-elution with the other lipids. This was more pronounced for the glycoconjugated bile acids. The findings were confirmed by HPLC analysis of the different fractions.
The effect of altering the ratio of methyl tert-butyl ether (MTBE) and MeOH on the recovery of free fatty acids, NL and PL is shown in Figure 3. The recovery of FFA increased from 50 to 100 % when the amount of MTBE in the mobile phase increased from 25 to 50 %. Increasing the MTBE in the solvent above 50 % did not increase the recovery further. The recovery of the PL was unchanged up to 75 % MTBE in the mobile phase. When 100% MTBE was present, the recovery of PL was reduced by 50 %. The best recovery for all the lipids was obtained with 75 % MTBE in the solvent.

**Chromatographic procedures**

*Bile acids*

A typical chromatogram of standard bile acids is shown in Figure 4a. All bile acids were base-line separated. The limit of quantification (LOQ) was 0.04 mM and the limit of detection (LOD) was 0.012 mM. The standard curves followed a 2° polynomial fitting ($r = 0.99$) in the range 0.04-1.00 mM. The coefficient of variance (CV) was below 20 % for low, medium and high concentrations ($n = 6$ for each concentration) and the accuracy varied between 7 and 20 %. The inter- and intraday variations were below 9 % and 7 %, respectively ($n=6$).

*Phospholipids*

The composition of the mobile phases resulting in best resolution of the phospholipids were the following: (A) hexane:2-propanol:HAc:triethylamine 82:18:0.5:0.014 (v/v/v/v), and (B) 2-propanol:H$_2$O:HAc:triethylamine 85:15:0.5:0.014 (v/v/v/v). The temperature of the column was held at 55 °C. A typical chromatogram of standard phospholipids is shown in Figure 4b. All lipids eluted
within the first 32 minutes, the rest of the program was needed for equilibration of the column. All polar lipid classes were base-line separated, with the exception of PA and PE, and nearly all eluted as single peaks, the exceptions being PG and LPC. The contents of the latter two were calculated from the sum of the two unresolved peaks. The LOQ was 0.1 mM and the LOD 0.03 mM for all phospholipids, apart from PC, for which LOQ was 0.04 mM and LOD 0.012 mM. The standard curves followed a second degree polynomial fitting ($r = 1$) in the range 0.04 to 1 mM for PC and 0.1 to 1 mM for the other phospholipids. The CV was below 20% for low, medium and high concentrations ($n = 6$ for each concentration). The inter and intraday variations were below 15 and 8%, respectively ($n = 6$).

Neutral lipids including free fatty acids

Optimum resolution of neutral lipids, including free fatty acids, was obtained using the following settings. The two mobile phases were as follows: (A) hexane:HAc 99:1 (v/v), and (B) isohexane:2-propanol:HAc 84:15:1 (v/v/v). The samples were injected at time 0, and the gradient profile was started at 0% for solvent mixture B where it was kept for 6 min. The gradient was changed to 5% B over a period of 16 min and then to 100% B over a period of 6 min. Thereafter, the gradient was decreased to 0% B over 0.1 min and kept at 0% B for 32 min. The solvent program took 60.1 minutes in total to run and all lipids eluted within the first 32 minutes, with the rest of the program being needed for equilibration of the column. The temperature of the column was held at 15 °C. A typical chromatogram of standard neutral lipids is shown in Figure 4c. All polar lipid classes were base-line separated and nearly all eluted as single peaks, exceptions being diacylglycerols (DAG). The DAG content was calculated from the sum of the two peaks. It was found that the equilibration time,
the steepness of the gradient and the column temperature were all important in
determining the retention time of the lipid species. Decreasing the column
temperature and increasing the equilibration time at the end of the program led to a
better resolution of cholesterol ester (CE), FFA and triacylglycerols (TAG). A steeper
gradient led to a poor resolution of unesterified cholesterol (UC) and DAG. The LOQ
was 0.1 mM for FFA and 0.04 mM for the neutral lipids and the LOD was 0.03 mM
for FFA and 0.012 mM for the neutral lipids. The standard curves were fitted with a
second degree polynomial (r=1) in the range 0.1 to 1 mM for FFA and 0.04 to 1 mM
for the neutral lipids. The CV was below 20 % for low, medium and high
concentrations (n = 6 for each concentration). The inter and intraday variations were
below 16 and 17 %, respectively (n = 6).

Human intestinal fluid

The chromatograms from the human intestinal fluid analyzed are shown in
Figure 5. All lipid classes present in intestinal fluid were well separated with minor
molecular species separation. Chromatography of BA showed that intestinal fluid
contained the glyco- and tauroconjugates of CA, CDCA and DCA and that no
unconjugated bile acids were found. GCA was the major bile acid found in the human
intestinal fluid (Figure 5a). Figure 5b shows the elution profile of PL.
Chromatography of the intestinal fluid revealed that PC and its degradation product,
LPC, were the only phospholipids present. The concentration of LPC was higher than
the concentration of PC. Figure 5c shows the chromatography of NL in the intestinal
fluid. All neutral lipids included in the analysis were present in the intestinal fluid.
Most of the triacylglycerols from the nutritional drink were degraded in the intestinal
fluid. FFA was the major neutral lipid found in the analysis of the human intestinal
fluid. The concentration of monoacylglycerols (MAG) and DAG were higher than the concentration of TAG. The concentration of UC was higher than the concentration of CE. The peaks for both FFA and MAG were split in two. This is probably due to the presence of different fatty acids in the intestinal fluid, with different retention coefficients.

DISCUSSION

In this study we present a new SPE method for the separation of bile acids, neutral lipids and phospholipids in fed state human small intestinal fluid. To the best of our knowledge, this is the first method developed for small intestinal fluid that includes bile acids in the separation of lipids. The method was found to produce highly reproducible results and Table III displays the excellent quantitative data (recovery > 90%) obtained when appropriate internal standards were added in the intestinal fluid. In addition, three methods for quantitative determination of the lipids by HPLC with ELS detection are presented. These methods have successfully been applied in several studies for the determination of lipid content in fasted and fed small intestinal fluid in human and dog (47, 48). The data obtained are crucial for the improved development of in vivo relevant dissolution media for pharmaceutical purposes.

Several silica-based SPE materials were tested, but only by combining the use of C18 and Si columns we were able to separate NL, PL and BA from each other. The most lipophilic unconjugated bile acid LCA, however, eluted together with the PL and NL in the present study. Whether this is true for di- and tri-hydroxylated bile acids is not known. It has been shown previously that the retention of unconjugated (pKa ca. 6) and glycine conjugated (pKa ca. 4.5) bile acids on C18 columns increased when the
pH was decreased from 7.5 to 2.5, owing to ionic suppression, which enhances the lipophilic character of the molecules (21, 22). This has to be taken into consideration when analysing samples that might contain unconjugated bile acids, like plasma and feces. However, simply increasing the amount of MeOH in the eluent could overcome this difficulty, but some neutral lipids might be eluted in the BA fraction as well as a result.

The retention of lipids on the small disposable C18 columns was a function of lipid polarity and of solvent strength and polarity. It has previously been noted that tracer amounts of cholesterol or other nonpolar compounds in water are not efficiently sorbed by C18 cartridges (49-51). This might be due to the physical state in which these compounds occur in water, i.e., not in monomolecular solution, or to the immiscibility of water and octadecyl groups at the interface. Addition of at least 50-60% methanol results in dispersion of lipid aggregates and improved sorption to the stationary phase (52, 53). In the method developed in the present study, addition of 30% methanol and lowering of the pH to 5 in the intestinal fluid prior to the SPE was sufficient for improved sorption of TAG, FFA and PL. This is also in accordance with previous studies (54, 55, 56-58). More than 30% MeOH in the intestinal fluid resulted in breakthrough of conjugated bile acids in the present method. The amount of methanol needed for sorption to the stationary phase is likely depending on the concentration and composition of lipids in the fluid as well as properties of the specific SPE material. The present method has been applied to samples with concentrations prevailing in the fasted state intestinal fluid to fed state concentrations (47, 48). Thus, the method presented in the present study should be applicable on samples with wide variations in bile acid concentration as well as phospholipids and neutral lipids concentrations. Lowering the pH in the intestinal fluid probably
increases the fraction of uncharged FFA, which would be expected to improve the interaction with the stationary phase. In addition, unspecific binding of HAc (H⁺) to charged silanol groups (SiO⁻²) and ion pair effects caused by Ac⁻, may increase the interaction of phospholipids with the C18 chains. Addition of MeOH to the water-wash further increased the recovery of TAG and FFA, by increased wettability of the analytes. The amount of MTBE in F4 was optimised to increase the recovery of NL and PL (Figure 3). We were not able to completely separate NL and PL by changing the amount of MTBE and still have a good recovery. Thus, we chose to use 75 % MTBE in the mobile phase. If 50 % MTBE had been selected, the total extraction time would have been a bit shorter, since F4 would have been unnecessary, but this would also render a somewhat lower recovery of LPC, in particular.

The composition of the intestinal fluid will vary along the gastrointestinal tract and differ with meal composition, disease state and fasted/fed state of the intestine and in different animal species. The SPE method developed in the present study was optimised for the quantification of lipids in fed state small intestinal fluid. It has also been successfully applied to the analysis of lipids in fasted and fed state human and dog intestinal fluid (47, 48). Thus, the method has shown potential to be used not only for the specified samples in this study. However, the lipid composition also differ between different body fluids and the present method may therefore be used with caution when analysing fluids from other parts of the body than the small intestine, as discussed previously in this paper.

The HPLC analysis methods developed were highly reproducible and resulted in a good separation of all lipids present in the fed state small intestinal fluid. The method developed by Silversand and Haux (42) and that of Homan and Andersson (43) were tested in our HPLC set-up. However, neither gave complete separation of
the lipids, using available column material. The best separation was obtained using the method introduced by Silversand and Haux (42), so this method was modified for our purposes. By increasing the column temperature from 45 to 55 °C and changing the proportion of acetic acid and triethylamine in the mobile phases from 1 to 0.5 % and 0.08 to 0.014 %, respectively, in the phospholipid system, in accordance with the results from the chemometric optimisation, we were able to separate all phospholipids, except PE and PA (Figure 4b). The neutral lipid system was sensitive to changes in column temperature, gradient steepness and equilibration time at the end of the gradient. By optimising these parameters, we were able to separate all the neutral lipids (Figure 4c). In the present method used for bile acid separation, both conjugated and unconjugated bile acids were separated within 30 minutes, or 15 minutes if the conjugated bile acids are taken into account, using a gradient of MeOH and a ammonium acetate buffer pH 3.15 (Figure 4a). This is in accordance with earlier investigations (18-26). However, these methods utilise UV detection at 200-210 nm and are, therefore, limited by poor absorptivity of BA and high absorptivity of impurities. In addition, with UV detection, the base-line shifts with the gradient, in contrast to ELS detection, used in the present study. This method was suitable for the analysis of bile acids in human intestinal fluid.

The analysis of human intestinal fluid performed in this study showed that, compared to fed simulated small intestinal fluid (e.g. FeSSIF) (59) used in drug development today, the concentration of bile acids in HIF was only half that in FeSSIF. The concentration of PL was similar to the concentration in FeSSIF and the ratio of BA:PL varied between 1:1-3:1. The major BA and PL were GCA and LPC, respectively, in HIF, while TCA and PC were added in FeSSIF. The nutritional lipids
found in the highest concentration (12 mM) were the FFA. The ratio between FFA:MAG:DAG:TAG in HIF was 6:1:1:1.

**CONCLUSIONS**

We have developed a highly reproducible method to separate the major neutral lipid classes and polar lipids, including bile acids. These analytical methods offer the possibility to quantify both endogenous and nutritional lipids in human upper small intestinal fluid, with a high level of recovery. The in vivo findings obtained are relevant for re-evaluation and the development of new physiological based in vitro drug dissolution test media.
ACKNOWLEDGEMENTS

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REFERENCES


**Figure legends**

**Figure 1.** Schematic illustration of the final solid phase extraction method for human intestinal fluids. Bile acids were separated from phospholipids and neutral lipids by use of a C18 column. A Si column was subsequently used for separation of phospholipids and neutral lipids. Each fraction was analysed for their species with HPLC and ELS-detection.

**Figure 2.** The recoveries of the different lipid species in the BA, NL and PL fractions presented as the mean ± SD (n = 9).

**Figure 3.** The effect of increasing the amount of MTBE from 25 to 100 % in F3 on the total recovery (recovery in F3 and F4, NL and PL elute) of a) PC, b) LPC, c) TAG, d) FFA and e) C from the SPE C18 column (n = 3).

**Figure 4.** Chromatograms of standard a) bile acids, b) phospholipids and c) neutral lipids using HPLC with ELS detection. The concentration of the components in the samples analysed were 0.5 mg/ml. The details concerning the HPLC conditions for each of these measurements is discussed in the section on materials and methods.

- TCA, taurocholic acid, GCA, glycocholic acid, TCDCA, taurochenodeoxycholic acid, TDCA, taurodeoxycholic acid, GCDCA, glycochenodeoxycholic acid, GDCA, glycodeoxycholic acid, CA, cholic acid, LCA-3S, lithocholic acid 3-sulphate, nor-DCA, nor-deoxycholic acid, CDCA, chenodeoxycholic acid, DCA, deoxycholic acid, LCA, lithocholic acid, PE, phosphatidylethanolamine, PA, phosphatidic acid, PG, phosphatidylglycerol, DPG, diphosphatidylglycerol, PC, phosphatidylcholine, SM, sphingomyelin, PS, phosphatidylserine, PI, phosphatidylinositol, LPC, lysophosphatidylcholine, CE, cholesterol ester, FFA, free fatty acids, TAG, triacylglycerols, UC, cholesterol, DAG, diacylglycerol, MAG, monoacylglycerol.
Figure 5. HPLC of a) bile acids, b) phospholipids and c) neutral lipids, found in fed human intestinal fluid after solid phase extraction. TCA, taurocholic acid, GCA, glycocholic acid, TCDCA, taurochenodeoxycholic acid, TDCA, taurodeoxycholic acid, GCDCA, glycochenodeoxycholic acid, GDCA, glycodeoxycholic acid, CA, cholic acid, nor-DCA, nor-deoxycholic acid, PC, phosphatidylcholine, LPC, lyso-phosphatidylcholine, CE, cholesterol ester, FFA, free fatty acids, TAG, triacylglycerols, UC, cholesterol, DAG, diacylglycerol, MAG, monoacylglycerol.
TABLE 1. Solvents used in the C18 solid phase extraction of lipid classes in human intestinal fluid.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Modification</th>
<th>Eluted lipids</th>
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<tbody>
<tr>
<td>Activation</td>
<td>MeOH&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Conditioning</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>-</td>
</tr>
<tr>
<td>Sample load</td>
<td>-</td>
<td>Addition of MeOH (0-40%) and HAc&lt;sup&gt;2&lt;/sup&gt; (0-10%, pH 6-3.5)</td>
</tr>
<tr>
<td>Fraction 1 (F1)</td>
<td>MeOH/H&lt;sub&gt;2&lt;/sub&gt;O (15:85)</td>
<td>-</td>
</tr>
<tr>
<td>Fraction 2 (F2)</td>
<td>MeOH/H&lt;sub&gt;2&lt;/sub&gt;O (75:25)</td>
<td>-</td>
</tr>
<tr>
<td>Fraction 3 (F3)</td>
<td>MTBE&lt;sup&gt;3&lt;/sup&gt;/MeOH</td>
<td>Adjusting the amount of MTBE (25-100%)</td>
</tr>
<tr>
<td>Fraction 4 (F4)</td>
<td>MeOH/HAc (98:2)</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>1</sup>Methanol, <sup>2</sup>Acetic acid, <sup>3</sup>Methyl tert-butyl ether
TABLE 2. Recoveries of lipid classes isolated by solid phase extraction. The results are presented as the mean ± SD (n = 9).

<table>
<thead>
<tr>
<th>Lipid</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurocholate</td>
<td>94.3 ± 2.0</td>
</tr>
<tr>
<td>Glycocholate</td>
<td>96.4 ± 2.0</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>95.5 ± 2.9</td>
</tr>
<tr>
<td>Free fatty acid</td>
<td>94.5 ± 2.8</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>95.9 ± 3.5</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>90.2 ± 4.6</td>
</tr>
<tr>
<td>Lyso-phosphatidylcholine</td>
<td>97.1 ± 2.5</td>
</tr>
</tbody>
</table>
I. C18 column
Activation: 2 mL MeOH
Conditioning: 2 mL H₂O
Sample load: Application of 1 ml HIF sample (700 µl HIF mixed with 300 µl MeOH and adjustment of the pH to 5 with HAc)
F1: 2 mL H₂O/MeOH (85:15)
F2: 3 mL H₂O/MeOH (25:75) (BA eluate)
F3: 7.5 mL MeOH/MTBE (25:75) (NL/PL eluate)
F4: 7.5 mL MeOH/HAc (98:2) (NL/PL eluate)

II. Si column
Activation: 2 mL CHCl₃
Sample load: F3 and F4 evaporated under nitrogen gas and redissolved in 1 mL CHCl₃ (NL eluate)
F5: 5 mL CHCl₃
F6: 7.5 mL MeOH/HAc (98:2) (PL eluate)
Figure 2

% recovery

BA eluate  NL eluate  PL eluate

TCA  GCA  LPC  PC  TAG  FFA  UC
Figure 3

a) % recovery PC
0 20 40 60 80 100
25 50 75 100 % MTBE

b) % recovery LPC
0 20 40 60 80 100
25 50 75 100 % MTBE

c) % recovery TAG
0 20 40 60 80
25 50 75 100 % MTBE

d) % recovery FFA
0 20 40 60 80
25 50 75 100 % MTBE

e) % recovery UC
0 20 40 60 80
25 50 75 100 % MTBE
Figure 4

(a) Flow: 1.0 mL/min
(b) Flow: 0.25 mL/min
(c) Flow: 0.8 mL/min
Figure 5

(a) mV vs. time (min) showing peaks for various compounds.

(b) mV vs. time (min) focusing on LPC and PC.

(c) mV vs. time (min) with peaks for FFA, TAG, UC, DAG-1, and MAG.