

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

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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 (BAs). A solid-phase extraction (SPE) method using C18 and silica columns for the separation of BAs, phospholipids (PLs), and neutral lipids (NLs), including free fatty acids, 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 before SPE to maximize lipid recoveries. BAs, PLs, and NLs were isolated using lipophilic and polar solvents to promote elution from the SPE columns. The different lipid classes were subsequently analyzed 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 baseline separation of nearly all lipid classes investigated. In conclusion, these methods provide a means of lipid class analysis of NLs, PLs, and BAs in human fed-state small intestinal fluid, with potential use in other fluids from the intestinal tract and animals.—Persson, E., L. Löfgren, G. Hansson, B. Abrahamsson, H. Lennernäs, and R. Nilsson. Simultaneous assessment of lipid classes and bile acids in human intestinal fluid by solid-phase extraction and HPLC methods. *J. Lipid Res.* 2007. 48: 242–251.

Supplementary key words solid-phase extraction • high-performance liquid chromatography • evaporative light scattering • neutral lipids • phospholipids

Poor correspondence between solubility data for simulated and real intestinal fed-state fluid has indicated a need for investigations of 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, after liquid-liquid extraction.

The liquid extraction methods optimized for lipid classes are commonly based on chloroform-methanol (MeOH) (3) or isopropanol-hexane (4) but result in poor recoveries of conjugated bile acids (BAs) 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 SPE methods for lipids in plasma and tissue extracts have been published (6–14). However, none of these methods include BAs in their separation, nor have they been validated for the separation of lipids in intestinal fluids. BAs represent a broad range in polarity, with the taurine-conjugated trihydroxy BAs being the most polar and the unconjugated monohydroxy BAs being the least polar. Techniques for the extraction and separation of BAs (5, 15) and phospholipids (PLs) (16) in biological samples have been thoroughly reviewed. C18 SPE has been shown to efficiently isolate BAs and their conjugates from biological fluids such as bile, serum, urine, stool, etc. (17). We present here a new C18 SPE protocol based on the simultaneous adsorption of BAs and total lipids in human small intestinal fluid and subsequent elution for further analysis by silica (Si) SPE and HPLC.

HPLC with evaporative light-scattering (ELS) detection provides a simple and affordable detection system for high-concentration samples such as intestinal fluids, bile,

Abbreviations: BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DAG, diacylglycerol; DCA, deoxycholic acid; ELS, evaporative light scattering; FeSSIF, fed simulated small intestinal fluid; GCA, glycocholic acid; HAC, acetic acid; HIF, human intestinal fluid; LOD, limit of detection; LOQ, limit of quantification; LPC, lysophosphatidylcholine; MAG, monoacylglycerol; MeOH, methanol; MTBE, methyl *tert*-butyl ether; NL, neutral lipid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; Si, silica; SPE, solid-phase extraction; TAG, triacylglycerol.

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or feces. In addition, upper small intestinal fluid in normal subjects contains almost exclusively conjugated BAs, which can be analyzed directly with HPLC without prior removal of the conjugating moiety, in contrast to gas chromatographic methods, which require ion-exchange separation into taurine, glycine, and free BA classes before deconjugation, derivatization, and analysis. Numerous applications of HPLC for the separation of BAs (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 (NLs) using a 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 PLs, BAs, and NLs, including FFAs, in human intestinal fluid (HIF). The application of this new procedure to the assay of BAs, PLs, and NLs in HIF has been demonstrated, and the results obtained were compared with the lipid content in simulated intestinal fluids used in drug development.

MATERIALS AND METHODS

Chemicals

Orlistat was purchased from Apin Chemicals. The following BA standards were supplied by Sigma-Aldrich: cholic acid (CA), glycocholic acid (GCA), taurocholic acid, deoxycholic acid (DCA), glycodeoxycholic acid, taurodeoxycholic acid, chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid, taurochenodeoxycholic acid, and lithocholic acid. PL standards were ordered from Avanti Polar Lipids, as follows: phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidylglycerol, phosphatidylserine, diphosphatidylglycerol, phosphatidic acid (PA), and sphingomyelin. Phosphatidylinositol was ordered from ICN Biomedicals. The NL standards, cholesterol, cholesteryl oleate, dipalmitin, tripalmitin, and palmitic acid, were purchased from Sigma-Aldrich, and monoolein was from Larodan AB. Radioactive lipid standards were obtained as follows and used without further purification: [1-¹⁴C]palmitic acid, [7(n)-³H]cholesterol, phosphatidylcholine-1-palmitoyl-2-[¹⁴C]linoleoyl, lyso-phosphatidylcholine-1-[¹⁴C]palmitoyl, [1-¹⁴C]GCA sodium salt, tauro[carbonyl-¹⁴C]cholic acid sodium salt was obtained from Amersham and tripalmitin[palmitic-1-¹⁴C] was from American Radiolabeled Chemicals. 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. All solvents were of analytical grade. Triethylamine was purchased from Sigma Chemical Co. Acetic acid (HAc) and chloroform were purchased from Merck; all other solvents came from Rathburn Chemicals, Ltd.

Apparatus

BA separation was accomplished by reversed-phase HPLC. The equipment was fitted with a binary pump (Gynkotek P580M HPG; Dionex), autosampler (Gynkotek Gina 50; Dionex), and degasser (Scantec Lab AB). The chromatographic column, a Zorbax C18 Extend Column (150 mm × 4.6 mm, 3.5 μm; Agilent

Technologies), was held at 40°C by a column heater (Croco-cil; Scantec Lab AB). A light-scattering detector was used for the detection (PL-ELS 1000; Polymer Laboratories). The nebulizer and evaporation chamber temperatures were 80°C and 85°C, respectively. The detector gas flow was 1.2 ml/min.

PLs and NLs, including free fatty acids, were separated by straight-phase HPLC using a binary pump (Dionex P580A LPG; Dionex), autosampler (Gynkotek Gina 160; Dionex), and degasser (Scantec Lab AB). PLs were separated on a YMC-Pack Diol column (250 mm × 2.1 mm, 5 μm; YMC, Inc.) held at 55°C, and NLs, including free fatty acids, were separated on an Apex II Diol column (150 mm × 4.6 mm, 5 μm; Jones Chromatography) held at 15°C. A Sedex 75 light-scattering detector (Sedere) was used for the detection of PLs. 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 NLs, including free fatty acids, a PL-ELS 1000 (Polymer Laboratories) was used. The nebulizer and evaporation chamber temperatures were 65°C and 80°C, respectively. The detector gas flow was 1.2 ml/min.

Sample collection

The sampling of 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 HIF was collected from the jejunum of six healthy volunteers using the Loc-I-Gut method (44, 45), after informed consent was given. 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 min intervals over a period of 90 min. The fractions obtained from 20 to 60 min, 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 before the analysis.

SPE

The lipids in the intestinal fluid were separated into PLs, NLs, and BAs using prepacked C18 and Si columns.

BA extraction on C18 SPE columns. The extraction of BAs in the intestinal fluid was performed using a C18 column preactivated and conditioned with MeOH and water. An intestinal fluid sample of 1 ml was loaded on the column. The pH in the intestinal fluid was measured before conducting SPE. The lipids were eluted in two different fractions, BAs and PLs/NLs. The procedure was optimized according to **Table 1**. Fraction 2 from the C18 SPE was dried under N₂ and redissolved in 700 μl of MeOH for HPLC analysis of individual BAs. The solvent fraction containing PLs and NLs was evaporated under N₂ at 40°C and redissolved in 1 ml of chloroform before loading on Si columns.

Separation of NLs and PLs on Si SPE columns. Separation of NLs and free fatty acids from polar lipids was accomplished using prepacked Si columns as described previously (46), with some modifications made for the SPE. The procedure is shown in **Fig. 1**. The CHCl₃ eluate (sample load + fraction 5) was dried under N₂ and redissolved in 700 μl of mobile phase A, for subsequent analysis of NLs. The PL fraction (fraction 6) from the Si SPE was dried under N₂ and redissolved in 700 μl of CHCl₃, for subsequent determination of PL content by HPLC.

TABLE 1. Solvents used in the C18 solid-phase extraction of lipid classes in human intestinal fluid

Variable	Solvent	Modification	Eluted Lipids
Activation	MeOH	—	—
Conditioning	H ₂ O	—	—
Sample load	—	Addition of MeOH (0–40%) and HAc (0–10%, pH 6–3.5)	—
Fraction 1	MeOH/H ₂ O (15:85)	—	—
Fraction 2	MeOH/H ₂ O (75:25)	—	Bile acids
Fraction 3	MTBE/MeOH	Adjusting the amount of MTBE (25–100%)	Neutral lipids, phospholipids
Fraction 4	MeOH/HAc (98:2)	—	Neutral lipids, phospholipids

HAc, acetic acid; MeOH, methanol; MTBE, methyl *tert*-butyl ether.

Validation of the SPE method. For quantitative recovery experiments, 50 μ l of a radioactive polar lipid (phosphatidylcholine-1-palmitoyl-2-[¹⁴C]linoleyl or lysophosphatidylcholine-1[¹⁴C]palmitoyl), free fatty acid ([¹⁴C]palmitic acid), NL ([¹⁴C]tripalmitin or [³H]cholesterol), or BA ([¹⁴C]GCA or [¹⁴C]taurocholic acid) was added to 1 ml of the intestinal fluid before performing the SPE. Of this sample, 100 μ l 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

BAs. A binary gradient was used for the separation of BAs. The mobile phases were as follows: (A) MeOH/buffer (15 mM ammonium acetate, 0.2% triethylamine, and 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 min, and thereafter increased to 50% B over 10 min and then to 100% B over 5 min. Thereafter, the gradient was decreased to 10% B over 0.1 min and held there throughout the run. The gradient took 30 min in total. The flow rate was set to 1 ml/min, and the sample volume was 25 μ l. Standards in the concentration range

0.04–1.00 mM were prepared in MeOH. Nor-deoxycholic acid was included as an internal standard in the analysis.

PLs and NLs, including free fatty acids. The methods adopted for the HPLC analysis of PLs and NLs, including free fatty acids, were modifications of that described by Silversand and Haux (42). For PL 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). The gradient and the column temperature were varied in the NL 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 PL and NL 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 analyzed 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) was determined at a signal-to-noise ratio of 10:1.

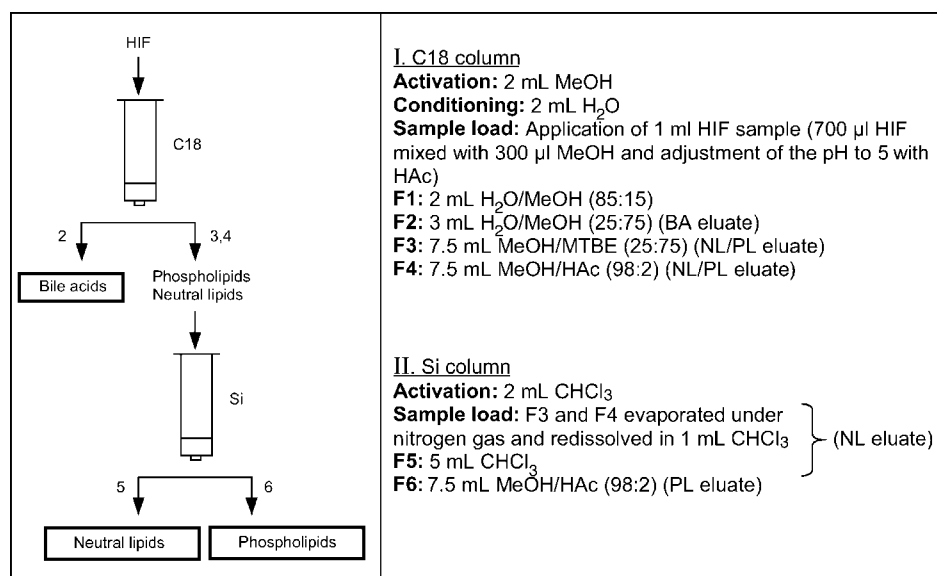


Fig. 1. Schematic illustration of the final solid-phase extraction (SPE) method for human intestinal fluids (HIFs). Bile acids (BAs) were separated from phospholipids (PLs) and neutral lipids (NLs) by use of a C18 column. A silica (Si) column was subsequently used for the separation of PLs and NLs. Each fraction was analyzed for their species with HPLC and evaporative light-scattering (ELS) detection. HAc, acetic acid; MeOH, methanol; MTBE, methyl *tert*-butyl ether.

RESULTS

SPE

The final SPE method is shown in Fig. 1. Two different columns were used for the separation of all 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 Fig. 2.

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

HAc was added to the intestinal fluid to alter the pH in the samples. The recovery of PLs and FFAs was increased from 75% to 100% and from 88% to 98%, respectively, when decreasing the pH in the intestinal fluid. At pH 5, the recovery of PLs was 95%. At pH values for the intestinal fluid <5, the BAs were retained longer on the column and led to a coelution with the other lipids. This was more pronounced for the glycoconjugated BAs. These 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, NLs, and PLs is shown in Fig. 3. The recovery of FFAs 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 to >50% did not increase the recovery further. The recovery of the PLs was unchanged up to 75% MTBE in the mobile phase. When 100% MTBE was present, the recovery of PLs was reduced by 50%. The best recovery for all of the lipids was obtained with 75% MTBE in the solvent.

Chromatographic procedures

BAs. A typical chromatogram of standard BAs is shown in Fig. 4A. All BAs were baseline separated. The LOQ was 0.04 mM, and the LOD was 0.012 mM. The standard curves followed a second degree polynomial fitting ($r = 0.99$) in

TABLE 2. Recoveries of lipid classes isolated by solid-phase extraction

Lipid	Percentage Recovery
Taurocholate	94.3 ± 2.0
Glycocholate	96.4 ± 2.0
Triacylglycerol	95.5 ± 2.9
Free fatty acid	94.5 ± 2.8
Cholesterol	95.9 ± 3.5
Phosphatidylcholine	90.2 ± 4.6
Lysophosphatidylcholine	97.1 ± 2.5

The results are presented as means ± SD (n = 9).

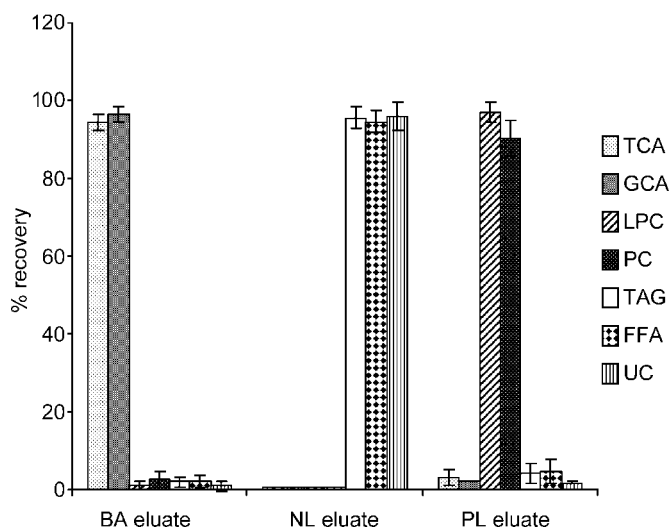


Fig. 2. Recoveries of the different lipid species in the BA, NL, and PL fractions presented as means ± SD (n = 9). TCA, taurocholic acid; GCA, glycocholic acid; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; TAG, triacylglycerol; UC, unesterified cholesterol.

the range 0.04–1.00 mM. The coefficient of variation was <20% for low, medium, and high concentrations (n = 6 for each concentration), and the accuracy varied between 7% and 20%. The interday and intraday variations were <9% and 7%, respectively (n = 6).

PLs. The compositions of the mobile phases resulting in best resolution of the PLs were as follows: *a*) hexane/2-propanol/HAc/triethylamine, 82:18:0.5:0.014 (v/v/v/v), and *b*) 2-propanol/water/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 PLs is shown in Fig. 4B. All lipids eluted within the first 32 min, and the rest of the program was needed for equilibration of the column. All polar lipid classes were baseline separated, with the exception of PA and PE, and nearly all eluted as single peaks, the exceptions being phosphatidylglycerol and LPC. The contents of the latter two lipids were calculated from the sum of the two unresolved peaks. The LOQ was 0.1 mM and the LOD was 0.03 mM for all PLs, apart from PC, for which the LOQ was 0.04 mM and the LOD was 0.012 mM. The standard curves followed a second degree polynomial fitting ($r = 1$) in the range 0.04–1 mM for PC and 0.1–1 mM for the other PLs. The coefficient of variation was <20% for low, medium, and high concentrations (n = 6 for each concentration). The interday and intraday variations were <15% and 8%, respectively (n = 6).

NLs, including free fatty acids. Optimum resolution of NLs, 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

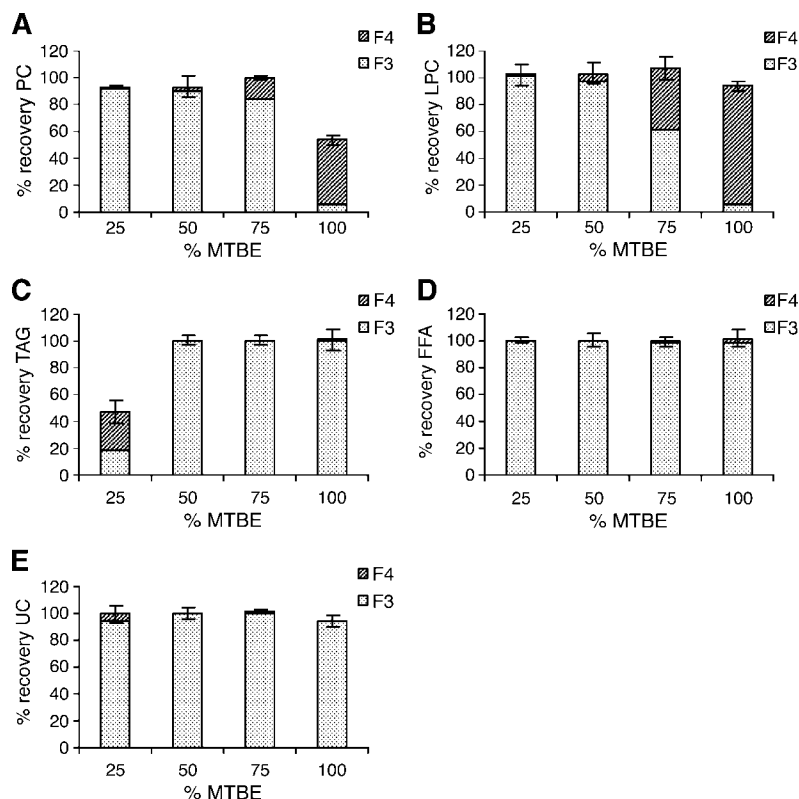


Fig. 3. Effect of increasing the amount of MTBE from 25% to 100% in fraction 3 (F3) on the total recovery (recovery in F3 and F4, NL and PL eluates) of PC (A), LPC (B), TAG (C), FFA (D), and UC (E) from the SPE C18 column ($n = 3$). Values are presented as means \pm SD.

gradient was changed to 5% B over 16 min and then to 100% B over 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 min in total to run, and all lipids eluted within the first 32 min, 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 NLs is shown in Fig. 4C. All polar lipid classes were baseline separated, and nearly all eluted as single peaks, exceptions being diacylglycerols (DAGs). 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 cholesteryl esters, FFAs, and triacylglycerols (TAGs). A steeper gradient led to a poor resolution of unesterified cholesterol and DAG. The LOQ was 0.1 mM for FFAs and 0.04 mM for the NLs, and the LOD was 0.03 mM for FFAs and 0.012 mM for the NLs. The standard curves were fitted with a second degree polynomial ($r = 1$) in the range 0.1–1 mM for FFAs and 0.04–1 mM for the NLs. The coefficient of variation was <20% for low, medium, and high concentrations ($n = 6$ for each concentration). The interday and intraday variations were <16% and 17%, respectively ($n = 6$).

HIF

The chromatograms from the HIF analyzed are shown in Fig. 5. All lipid classes present in intestinal fluid were

well separated with minor molecular species separation. Chromatography of BAs showed that intestinal fluid contained the glycoconjugates and tauroconjugates of CA, CDCA, and DCA and that no unconjugated BAs were found. GCA was the major BA found in the HIF (Fig. 5A). Figure 5B shows the elution profiles of PLs. Chromatography of the intestinal fluid revealed that PC and its degradation product, LPC, were the only PLs present. The concentration of LPC was higher than the concentration of PC. Figure 5C shows the chromatography of NLs in the intestinal fluid. All NLs included in the analysis were present in the intestinal fluid. Most of the TAGs from the nutritional drink were degraded in the intestinal fluid. FFA was the major NL found in the analysis of the HIF. The concentrations of monoacylglycerols (MAGs) and DAG were higher than the concentration of TAG. The concentration of unesterified cholesterol was higher than the concentration of cholesteryl ester. The peaks for both FFA and MAG were split in two. This is probably attributable 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 BAs, NLs, and PLs 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 BAs in the separation of lipids. The method was found to produce highly reproducible results, and Table 2

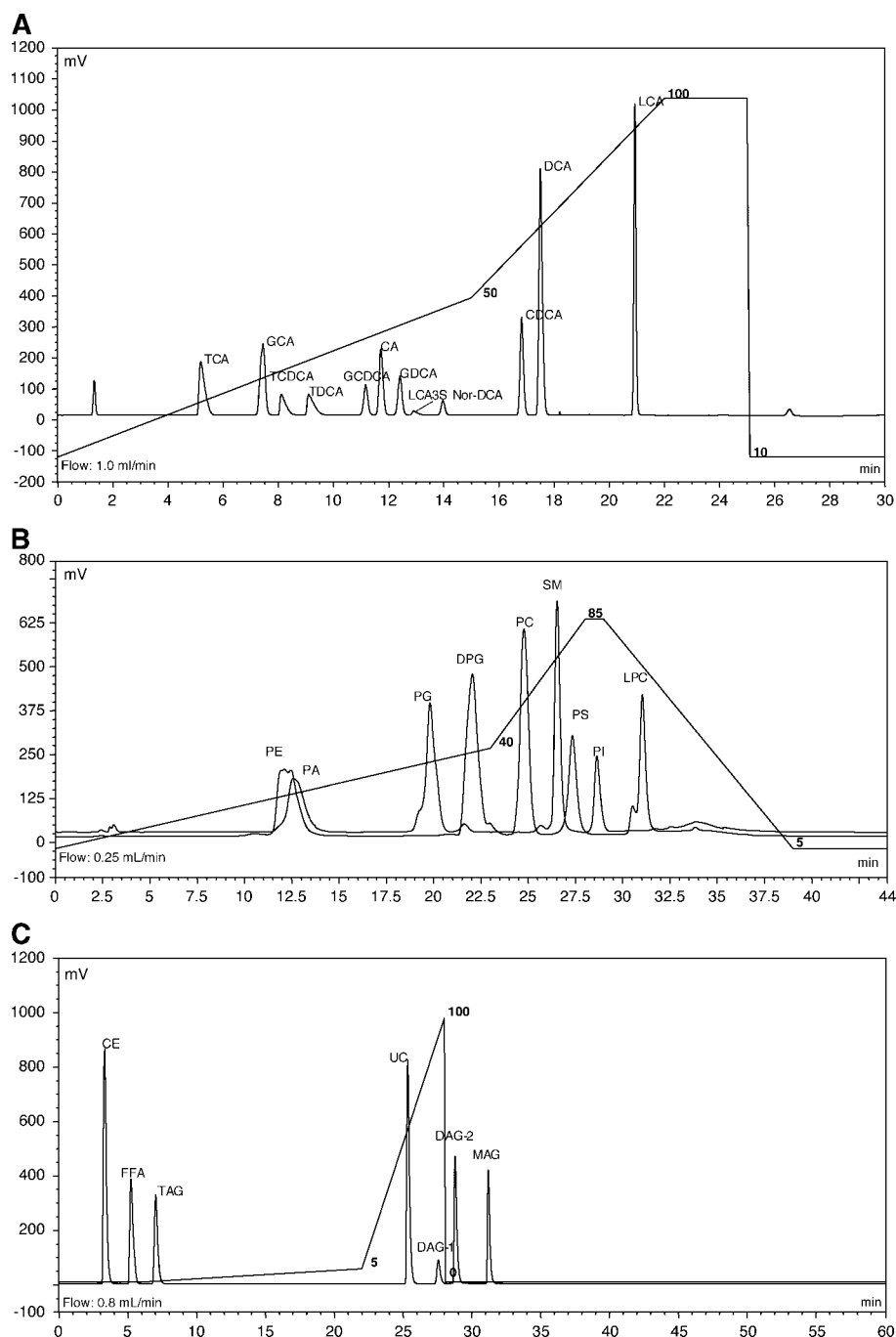


Fig. 4. Chromatograms of standard BAs (A), PLs (B), and NLs (C) using HPLC with ELS detection. The concentration of the components in the samples analyzed was 0.5 mg/ml. Details concerning the HPLC conditions for each of these measurements are discussed in Materials and Methods. TCA, taurocholic acid; GCA, glycocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; CA, cholic acid; LCA-3S, lithocholic acid 3-sulfate; nor-DCA, nor-deoxycholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; PE, phosphatidylethanolamine; PA, phosphatidic acid; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; CE, cholesteryl ester; FFA, free fatty acid; UC, unesterified cholesterol; DAG, diacylglycerol; MAG, monoacylglycerol.

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.

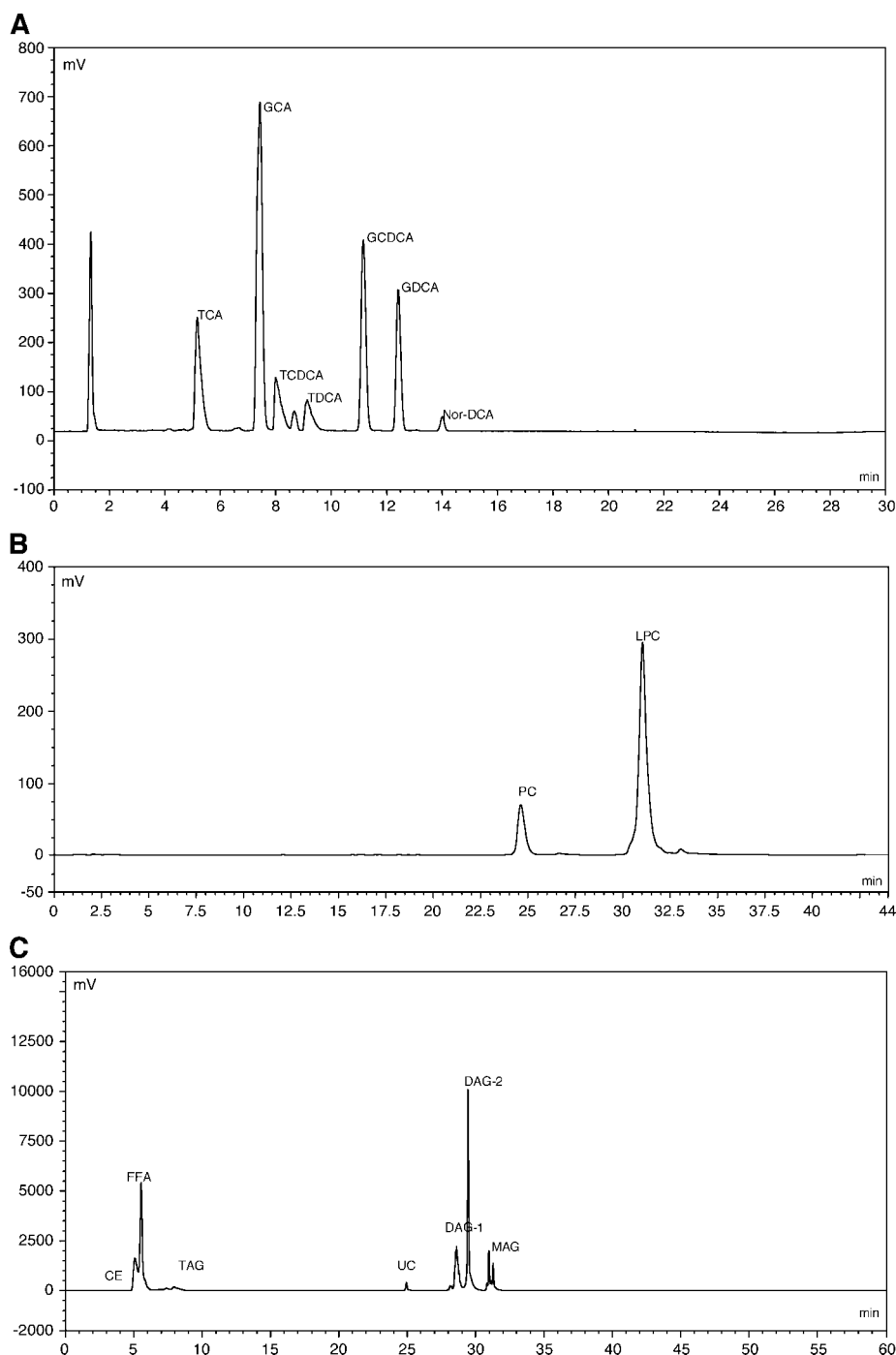


Fig. 5. HPLC of BAs (A), PLs (B), and NLs (C) found in fed HIF after SPE. TCA, taurocholic acid; GCA, glycocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; CA, cholic acid; nor-DCA, nor-deoxycholic acid; CE, cholesterol ester; FFA, free fatty acid; UC, unesterified cholesterol; DAG, diacylglycerol; MAG, monoacylglycerol.

Several Si-based SPE materials were tested, but only by combining the use of C18 and Si columns were we able to separate NLs, PLs, and BAs from each other. The most lipophilic unconjugated BA, lithocholic acid, however, eluted together with the PLs and NLs in this study. Whether this is true for dihydroxylated and trihydroxylated BAs is not known. It has been shown previously that

the retention of unconjugated ($Pk_a \sim 6$) and glycine-conjugated ($Pk_a \sim 4.5$) BAs 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 analyzing samples that might contain unconjugated BAs, such as plasma and feces. However,

simply increasing the amount of MeOH in the eluent could overcome this difficulty, although some NLs 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 been noted previously that tracer amounts of cholesterol or other nonpolar compounds in water are not efficiently sorbed by C18 cartridges (49–51). This might be attributable 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% MeOH results in the dispersion of lipid aggregates and improved sorption to the stationary phase (52, 53). In the method developed in this study, addition of 30% MeOH and decreasing the pH to 5 in the intestinal fluid before SPE was sufficient to improve the sorption of TAGs, FFAs, and PLs. This is also in accordance with previous studies (54–58). More than 30% MeOH in the intestinal fluid resulted in a breakthrough of conjugated BAs in this study. The amount of MeOH needed for sorption to the stationary phase likely depends on the concentration and composition of lipids in the fluid as well as on the properties of the specific SPE material. This 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 here should be applicable on samples with wide variations in BA concentration as well as PL and NL concentrations. Decreasing the pH in the intestinal fluid probably increases the fraction of uncharged FFAs, 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 PLs with the C18 chains. Addition of MeOH to the water wash further increased the recovery of TAGs and FFAs, by increased wettability of the analytes. The amount of MTBE in fraction 4 was optimized to increase the recovery of NLs and PLs (Fig. 3). We were not able to completely separate NLs and PLs by changing the amount of MTBE and still have 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, because fraction 4 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 the fasted/fed state of the intestine and in different animal species. The SPE method developed in this study was optimized for the quantification of lipids in fed-state small intestinal fluid. It has also been applied successfully 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 differs between different body fluids; therefore, this method should be used with caution when

analyzing fluids from other parts of the body than the small intestine, as discussed above.

The HPLC analysis methods developed were highly reproducible and resulted in a good separation of all lipids present in 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 setup. 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°C to 55°C and changing the proportion of HAc and triethylamine in the mobile phases from 1% to 0.5% and from 0.08% to 0.014%, respectively, in the PL system, in accordance with the results from the chemometric optimization, we were able to separate all PLs, except PE and PA (Fig. 4B). The NL system was sensitive to changes in column temperature, gradient steepness, and equilibration time at the end of the gradient. By optimizing these parameters, we were able to separate all of the NLs (Fig. 4C). In our method used for BA separation, both conjugated and unconjugated BAs were separated within 30 min, or 15 min if the conjugated BAs are taken into account, using a gradient of MeOH and an ammonium acetate buffer, pH 3.15 (Fig. 4A). This is in accordance with earlier investigations (18–26). However, those methods used ultraviolet detection at 200–210 nm and, therefore, are limited by poor absorptivity of BA and high absorptivity of impurities. In addition, with ultraviolet detection, the baseline shifts with the gradient, in contrast to ELS detection, as used in this study. This method was suitable for the analysis of BAs in HIF.

The analysis of HIF performed in this study showed that, compared with the fed simulated small intestinal fluid (FeSSIF) (59) used in drug development today, the concentration of BAs in HIF was only half that in FeSSIF. The concentration of PLs was similar to the concentration in FeSSIF, and the ratio of BAs to PLs varied between 1:1 and 3:1. The major BAs and PLs were GCA and LPC, respectively, in HIF, whereas taurocholic acid and PC were added in FeSSIF. The nutritional lipids found in the highest concentration (12 mM) were the FFAs. The FFA/MAG/DAG/TAG ratio in HIF was 6:1:1:1.

Conclusions

We have developed a highly reproducible method to separate the major NL classes and polar lipids, including BAs. 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 reevaluation and the development of new physiologically based *in vitro* drug dissolution test media. 

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