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Bile acid profiling in human biological samples: Comparison of extraction procedures and application to normal and cholestatic patients

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ABSTRACT

The role of bile acids in cell metabolism, membrane biology and cell signaling is increasingly recognized, thus making necessary a robust and versatile technique to extract, separate and quantify a large concentration range of these numerous molecular species. HPLC–MS/MS analysis provides the highest sensitivity to detect and identify bile acids. However, due to their large chemical diversity, extraction methods are critical and quite difficult to optimize, as shown by a survey of the literature. This paper compares the performances of four bile acid extraction protocols applied to either liquid (serum, urine, bile) or solid (stool) samples. Acetonitrile was found to be the best solvent for deproteinizing liquid samples and NaOH the best one for stool extraction. These optimized extraction procedures allowed us to quantitate as much as 27 distinct bile acids including sulfated species in a unique 30 min HPLC run, including both hydrophilic and hydrophobic species with a high efficiency. Tandem MS provided a non ambiguous identification of each metabolite with a good sensitivity (LOQ below 20 nmol/l except for THDCA and TLCA). After validation, these methods, successfully applied to a group of 39 control patients, detected 14 different species in serum in the range of 30–800 nmol/l, 11 species in urine in the range of 20–200 nmol/l and 25 species in stool in the range of 0.4–2000 nmol/g. The clinical interest of this method has been then validated on cholestatic patients. The proposed protocols seem suitable for profiling bile acids in routine analysis.

1. Introduction

Bile acids are a group of end products of cholesterol catabolism bearing a pentanoic acid side chain and one to three hydroxyl groups at position α3, α7, and α12 of the cholane cycle. Two primary bile acids, cholic (CA) and chenodeoxycholic acid (CDCA) are primarily synthesized in hepatocytes from cholesterol and conjugated with glycine or taurine (shown in Fig. 1) [1]. Bile acids play a critical role in the digestion and absorption of dietary lipids within the intestinal lumen before their deconjugation and dehydroxylation by different bacterial phyla into secondary bile acids, deoxycholic (DCA) and lithocholic acids (LCA). In addition, CDCA is partially epimerized into α3 β7-OH ursodeoxycholic (UDCA) acid, the major tertiary bile acid. Dihydroxylated bile acids are then extensively reabsorbed through the ileal intestinal wall into the portal circulation. Due to their efficient uptake by the liver, bile acids remain at a low concentration in the peripheral blood circulation [2]. Besides amidation by glycine or taurine, bile acids can be also conjugated as 3α sulfated metabolites [3] or with sugars such as N-acetyl glucosamine, glucose or glucuronate. The sulfated species are water-soluble structures abundant in normal urine and consistently increased in cholestasis or intestinal dysfunctions.

Besides their role as natural detergents, some bile acids have been recently identified as signalling molecules interacting with two types of specific receptors: the G-protein-coupled transmembrane receptor TGR5, and the nuclear transcription factor Farnesoid X receptor (FXR). Consequently, bile acids appear as metabolic integrators involved in the regulation of cholesterol homeostasis [4]

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; CV, coefficient of variation; DCA, deoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GC–MS, gas chromatography coupled to mass spectrometry; GDCA, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glyco ursodeoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; HPLC–MS/MS, high pressure liquid chromatography coupled to tandem mass spectrometry; LCA, lithocholic acid; LOQ, limit of quantification; SPE, solid phase extraction; TCA, taurocholic acid; TCDCA, tauro chenodeoxycholic acid; TDCA, Tauro deoxycholic acid; THDCA, tauro hyodeoxycholic acid; TLCA, Tauro lithocholic acid; UDCA, ursodeoxycholic acid; -3S, 3 sulfate; RE, relative error.

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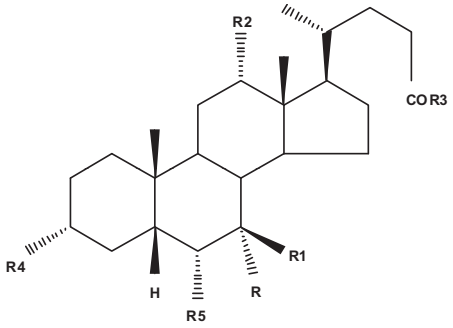

	R	R1	R2	R3	R4	R5
CA	-OH	-H	-OH	-OH	-OH	-H
CDCA	-OH	-H	-H	-OH	-OH	-H
DCA	-H	-H	-OH	-OH	-OH	-H
LCA	-H	-H	-H	-OH	-OH	-H
UDCA	-H	-OH	-H	-OH	-OH	-H
HCA	-OH	-H	-H	-OH	-OH	-OH
HDCA	-H	-H	-H	-OH	-OH	-OH
Glycine Conjugated				$-NHCH_2CO_2^-$		
Taurine Conjugated				$-NH(CH_2)_2SO_3^-$		
Sulfated					$-SO_3O^-$	

Fig. 1. Chemical structure of bile acids and conjugates.

and of energy metabolism [5,6]. Because of the involvement of specific bile acids species in these various regulatory processes, an increasing attention has been given to their detailed profiling in various biomedical samples (serum, urine, bile and stool).

Bile acid analysis in serum and urine has been performed since many years to screen and follow up hepatobiliary and intestinal disorders [7], mainly to characterize cholestasis and to follow UDCA treatment of cholestatic liver diseases.

Various methods are currently used to identify and quantify bile acids in human serum (for review, see [8]). Gas chromatography coupled to mass spectrometry (GC–MS) is sensitive and specific but is time-consuming because of the multiple steps required for the processing of samples including the cleavage of amido- and sulfoconjugates and the methylation of carboxylic and hydroxyl groups [9]. However, GC–MS remains the reference method to ascertain bile acid structure and assign the position and stereochemistry of the hydroxyl groups on the cholane cycle. This method is still the reference to elucidate inborn errors in bile acid metabolism.

On the contrary, high pressure liquid chromatography coupled to tandem mass spectrometry (HPLC–MS/MS) allows in a single step the measurement of both non-conjugated (free) and conjugated bile acids as native metabolites. For routine quantification of clinical samples, HPLC–MS/MS thus appears as the most suitable method to screen the bile acid profiles without tedious prior fractionation of conjugates (for review, see [8]).

Recent papers have described bile acid profiles in biological samples using various extraction methods followed by HPLC–MS/MS. However, bile acids, being amphipathic molecules dispersed in an aqueous medium are not evenly extracted by the methods described. Surprisingly, this central question has not been addressed until now and there is no available data that compare the critical efficiency for nanomolar and micromolar concentration of metabolites for the first step of the different techniques used.

In the present study, we compare four bile acids extraction techniques on liquid samples including, or not, a solid phase extraction (SPE) step and followed by HPLC–MS/MS. The aim of this comparative study is to select a simple and robust technique prior analysis with HPLC–MS/MS to profile bile acids in liquid samples such as human plasma, urine, and bile. We have also compared four extraction methods to extract bile acids from solid samples such as human stool. Finally we have set two techniques able to quantify 27 distinct human bile acids including sulfated species in the same run. These techniques were found the most efficients to measure simultaneously both hydrophobic and hydrophilic bile acid species in liquid or solid samples. The technique for liquid samples was applied to a group of healthy subjects and to another group of cholestatic patients. This new protocol can now be used to monitor bile acid markers of hepatobiliary and intestinal diseases which could be investigated in routine.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and solvents were of the highest purity commercially available. Bile acid standards CA, DCA, CDCA, UDCA, LCA, HCA, HDCA, and their corresponding glycine and taurine derivatives, as well as TLCA3S and GLCA3S were obtained from Sigma–Aldrich (Saint Quentin Fallavier, 38297, France). LCA3S was synthetized from LCA in the laboratory. GUDCA-3S and TUDCA-3S were a generous gift from Dr. J. Goto. The three internal standards were respectively 23-nor-5β-cholanoic acid-3α,12α diol from Steraloids Inc. (Newport, USA), ursodeoxycholic-2,2,4,4-d4 acid and lithocholic-2,2,4,4-d4 acid from CDN isotopes (Pointe-Claire, Quebec, Canada). Acetic acid, ammonium carbonate, ammonium acetate, trichloroacetic acid, acetonitrile and 2-propanol for HPLC were from Sigma–Aldrich (Saint Quentin Fallavier, 38297, France). Methanol (Chromanorm grade) was from VWR (Fontenay sous Bois, 94126, France). NaOH was from Merck (Darmstadt 64271, Germany).

2.2. Preparation of calibration standards

Stock solutions of the bile acids and the 3 internal standards were prepared in methanol (1 mg/ml) and stored in sealed vials at −20 °C. The 27 standard stock solutions were then pooled together to obtain a 30 μg/ml solution, further diluted in methanol to obtain an 6 levels in the calibration curve ranging from 0.006 to 30 μg/ml (corresponding to 0.01–80 μmol/l).

2.3. Extraction from liquid samples (serum, urine, bile)

Serum (500 μl), urine (2 ml of a 24 h urine pool) or bile (1 ml of a1/1000 dilution) were stored at −20 °C until measurement.

Four different methods adapted from previously published works [10–14] were compared. A comparative scheme of these methods is shown in supplementary data 1A. Five microliter of the stock solution of the three internal standards were added at the beginning of the extraction procedure to calculate the extraction yield.

2.3.1. Deproteinization with methanol (L1 protocol) or with trichloroacetic acid followed by methanol extraction (L2 protocol)

Proteins were precipitated by addition of methanol (80% final concentration (v/v)) or trichloroacetic acid (1% final concentration (v/v)). After mechanical stirring (1 min vortex) samples were incubated at room temperature for 20 min and clarified by centrifugation (4000 × *g*, 15 min). The supernatant was recovered and dried under a nitrogen stream at 50 °C. The residue was then dissolved in 150 μl methanol and 5 μl were injected into the HPLC–MS/MS system.

2.3.2. Deproteinization with acetonitrile (L3 protocol) or ammonium carbonate (L4 protocol) followed by SPE

Biological samples were deproteinized by addition of ammonium carbonate 0.4 M (final concentration 80 mmol/l) or acetonitrile (final concentration 80%, v/v). After stirring (1 min vortex mixing) acetonitrile-treated samples were incubated at room temperature for 20 min and centrifuged. The supernatant was evaporated under a nitrogen stream at 50 °C and the residue resuspended in 4 ml ammonium acetate 15 mmol/l, pH 5.3. Ammonium carbonate-treated samples were incubated at 60 °C for 30 min and centrifuged at 4000 × *g* for 20 min. The supernatants were then loaded onto a reverse-phase 30 mg SPE cartridge (Chromabond® C_{18} cartridges, Macherey-Nagel, Düren, Germany, 30 μm bed size) for a 3-step "clean up". After pre-conditioning with 5 ml methanol and 5 ml water, the cartridge was loaded and rinsed successively with 20 ml water to discard salts and hydrophilic metabolites, 10 ml hexane to discard neutral lipids and again with 20 ml water. The bile acids were then eluted with 5 ml methanol. The methanol fraction was collected and dried under a nitrogen stream at 50 °C. The residue was dissolved in 150 μl methanol of which 5 μl were injected into the HPLC–MS/MS.

2.4. Extraction from stool

Stool samples were thoroughly homogenized after reception and then stored at −80 °C. Before use, the samples were lyophilized, and 100 mg of dried stool were then treated according to 4 extraction protocols (S1–S4) [15–18], summarized in supplementary data 1B. Five microliter of the stock solution of the three internal standards were added to samples before extraction.

2.4.1. Extraction with NaOH (S1 protocol)

Two ml of NaOH (0.1 mol/l) were added to 100 mg dried feces and incubated for 1 h at 60 °C before addition of 4 ml water. The sample was thoroughly homogenized using a Polytron® homogenizer (KINEMATICA GMBH, Lucern, Switzerland) (30 s, maximal speed) and clarified at 20 000 × *g* for 20 min. The supernatant was collected and extracted using a 60 mg SPE cartridge as described above for L3–L4 protocols.

2.4.2. Extraction with acetonitrile (S2 protocol) or isopropanol (S3 protocol)

Two milliliter of water were added to 100 mg dried feces. The sample was homogenized with Polytron® homogenizer for 30 s and mixed with acetonitrile or isopropanol (final concentration 80%, v/v). After incubation at room temperature for 20 min, the extract was clarified by centrifugation at 20 000 × *g* for 20 min. The supernatant was collected and extracted using a SPE cartridge as described for L3–L4 protocols.

2.4.3. Extraction with water (S4 protocol)

Ten milliliter of water were added to 100 mg dried feces. The sample was homogenized using a Polytron® homogenizer for 30 s and clarified by centrifugation at 20 000 × *g* for 20 min. The supernatant was collected and extracted using a SPE cartridge as described above for L3–L4 protocols.

2.5. High pressure liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS)

The chromatographic separation of bile acids was carried out on a Zorbax eclipse XDB-C18 (Agilent Technology, Garches, 92380, France) fitted on an Agilent 1100 HPLC system (91745 Massy, France). The column was thermostated at 35 °C. The mobile phases consisted of (A) (ammonium acetate 15 mmol/l, pH 5.3) and (B) (methanol) at 65:35 (v/v). Bile acids were eluted by increasing B in A from 65 to 95 (v/v) for 30 min. Separation was achieved at a flow rate varying between 0.3 and 0.5 ml/min for 30 min.Mass spectra were obtained using an API® 2000 Q-Trap (AB-Sciex, Concord, Ontario, Canada) equipped with a TurboIon electrospray (ESI) interface set in the negative mode (needle voltage – 4500 V) with nitrogen as the nebulizer set at 40 (arbitrary pressure unit given by the equipment provider). Curtain and heater pressures were set at 20 and 40, respectively (arbitrary unit). The ion source temperature was set at 400 °C. Declustering and entrance potentials were set at −60 V and −10 V, respectively. The MS/MS detection was operated at unit/unit resolution. The acquisition dwell time for each transition monitored was 70 ms. Data were acquired by the Analyst® software (version 1.4.2, AB-Sciex) in the Multiple Reaction Monitoring (MRM) mode.

2.6. Validation

2.6.1. Recovery

In order to assess the efficiency of extraction, six different serum samples (3 normal and 3 cholestatic ones) were spiked with a pool of standards (0.3 μg/ml corresponding to 0.5–0.8 μmol/l), supplemented with internal standards and extracted following L3 protocol. In parallel, the same amount of standards without serum, and the same six different unspiked serum samples were extracted as blanks. In order to evaluate matrix effect on ion suppression, the same six serum samples were extracted as blanks before spiking with the same amount of standards and compared to the values of standards in the absence of matrix.

The same experiments were performed on six urine samples and six stool samples.

For each bile acid, the matrix effect was evaluated by calculating the ratio of the peak area in the presence of matrix to the peak area in absence of matrix, normalized with the internal standard. The requirements for the matrix effects and the ion suppression calculated from 6 batches of matrix were a coefficient of variation (CV) < 20%.

A fortification assay was performed by adding 3 increasing concentrations of 3 standard bile acids with short, medium and long retention times: TUDCA (11 min), TCDCA (18 min) and CDCA (24 min) to serum, urine and stool samples. The respective concentrations were chosen in ranges varying from 0.4 to 4 μmol/l for serum, from 0.1 to 100 nmol/l for urine and from 8 to 800 nmol/g for stool. Samples were extracted following L3 protocol (described in Section 2.3.2) for serum and urine or S1 protocol (described in Section 2.4.1) for stool and analyzed as described above. The extracts were analyzed in triplicates.

2.6.2. Linearity

Linearity was measured using a freshly prepared calibration pool of the 27 bile acid standards that was diluted to 1/10, 1/50, 1/100, 1/250, 1/500, and 1/1000 to obtain 6 final standard concentrations ranging from 0.05 to 8 μmol/l. The same volume of calibration pool was added to either 500 μl of normal serum, 0.5 ml of a bile dilution at 1/1000, 1 ml of urine, 10 mg of stool samples, or to 500 μl of methanol as blank. The samples were then extracted following L3 procedure (described in Section 2.3.2) for serum, bile and urine, and S1 (described in Section 2.4.1) for stool. Measurements were repeated in triplicates. The peaks were quantified and calibration curves were established by plotting the peak area of each bile acid as a function of concentration after correction for endogenous bile acids in the biological samples. Linear regression analysis of the calibration curves was performed. Linearity was followed by the correlation coefficient (r^2) value.

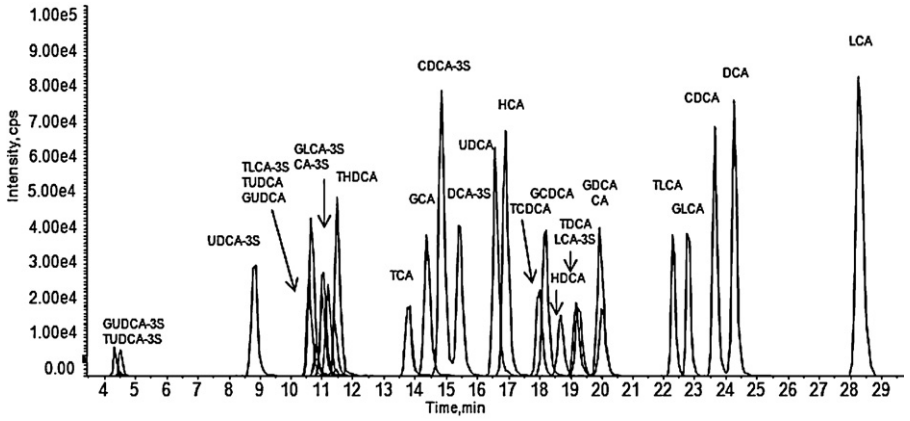

Fig. 2. Chromatogram of 27 bile acids. A reverse phase of C_{18}-silica (Pinnacle® II analytical column (250 mm × 3.2 mm, 5 μmol/l) was eluted by increasing the amount of methanol in aqueous ammonium acetate 15 mmol/l, pH 5.3). The HPLC was coupled with ESI-tandem mass spectrometry for the detection by Multiple Reaction Monitoring mode.

The limit of quantification (LOQ) was calculated as the bile acid concentration at the lowest measured concentration for which the variation coefficient (CV) was lower than 15% [19].

2.6.3. Precision

Precision was tested using calibration curves containing 6 concentrations (0.01–80 μmol/l) of the 27 bile acid standards. Measurements were repeated in triplicate on the same day (within-run) and also on three different days in triplicate (between-run). For precision of biological samples, serum with low and high concentrations of bile acids, urine, bile and stool samples were extracted and measured 6 times on the same day (within-run) and on 3 different days (between-run) in triplicate. The CV was ≤15% for any sample.

2.6.4. Accuracy

Accuracy was performed by spiking samples (4 sera, 4 urines and 4 stools) with 3 known concentrations (a low, medium and high quality control) of 3 bile acids (TUDCA (11 min), TCDCA (18 min) and CDCA (24 min)). After analysis, accuracy was calculated for each sample and each class of bile acid as the relative error (RE) from the nominal expected concentration as follows:

$$\text{Relative error \%} = \frac{\text{recovered BA concentration} - \text{expected BA concentration}}{\text{expected BA concentration}}$$

The recovery rates must be within 100 ± 15%.

2.6.5. Sample stability of liquid samples

Sample stability of liquid samples was evaluated under 3 different conditions of storage: −20 °C, 4 °C and room temperature. The same serum, bile or urine sample was divided in 7 aliquots: the first aliquot was analyzed immediately, three others after seven days and three others after 30 days.

Stool was immediately frozen at −80 °C upon reception. The samples were then dry-lyophilized after a few hours, or a week, or a month. The lyophilized samples were then analyzed by LC–MS/MS.

3. Results and discussion

Thanks to the C18-reverse phase chromatography, the sulfo-conjugates, tauro-conjugates, glyco-conjugates and non-conjugated bile acids were eluted sequentially as a function of their hydrophilicity/hydrophobicity balance. Specific detection of precursor/product ion pairs in the Multiple Reaction Monitoring mode allowed the measurement of overlapping elution peaks. Separation of the 27 bile acid standards was achieved in a single run of 30 min (Fig. 2). Some recent papers develop techniques with short runs that separate only 13–18 species but do not include sulfated species [20,22,23]. The long run time used here allowed to separate in the same run the main endogenous species present in various human samples including urine, that contains high amounts of doubly conjugated species, and stool that is rich in hydrophobic bile acids. This contrasted with the separation of bile acids by gas chromatography where bile acids were deconjugated and for which separation was only dependent on hydroxyl substituents (for review, see [8]). Table 1 summarizes the main parameters for MS/MS detection of the 27 bile acids usually found in human samples. Three internal standards with different retention times were used in a typical routine assay, in order to ensure that bile acids with short or long retention times were equally quantified.

3.1. Sample extraction

In order to develop a single method for different types of liquid biological samples, we have compared four different protocols adapted from literature [10–14] using precipitation of plasma proteins and, for two of them, SPE extraction (supplementary data 1). They were first compared on a serum sample from a cholestatic patient treated with UDCA.

The acetonitrile protocol (L3 protocol, supplementary data 1A), adapted from Tagliacozzi et al. [12] appeared as the most efficient one. Therefore, it was set at 100% to be compared to the 3 other protocols. As shown in Fig. 3A, trichloroacetic extraction (L2 protocol, filled bars) adapted from Bentayeb et al. [11] gave lower concentrations for 11 out of the 17 bile acids detected with acetonitrile. Moreover, the most hydrophobic bile acids eluted with a long retention time, namely, TDCA, LCA-3S, GDCA, and non conjugated CA, GLCA, and CDCA were not detected. Ammonium carbonate extraction (L4 protocol, empty bars) adapted from Buckard et al. [13] detected 15 out of the 17 bile acids already detected with acetonitrile. Non-conjugated CA and sulfated species gave much lower results (<20%) than acetonitrile. Surprisingly, methanol extraction without SPE (L1 protocol, hashed bars), adapted from Bobeldijk et al. [10] gave results close to the acetonitrile extraction, as 17 bile acids were detected although at a lower level than acetonitrile protocol. Consequently the acetonitrile protocol followed by C_{18}-SPE (L3 protocol) was found the most suitable technique for serum extraction of conjugated and non conjugated bile acids. It was therefore selected for further experiments.

Table 1
Analytical settings for HPLC–MS/MS analysis.

Bile acid	Acronym	*m/z*	MRM	CE (V)	Retention time (min)
Tauro ursodeoxycholic 3 sulfate	TUDCA-3S	288.9*	288.9/97.0	−70	4.33
Glyco ursodeoxycholic 3 sulfate	GUDCA-3S	528.4	528.4/97.0	−70	4.54
Ursodeoxycholic 3 sulfate	UDCA-3S	471.4	471.4/97.0	−70	8.87
Tauro ursodeoxycholic	TUDCA	498.4	498.4/80.0	−110	10.66
Taurolithocholic 3 sulfate	TLCA-3S	280.8*	280.8/97.0	−70	10.80
Glyco ursodeoxycholic	GUDCA	448.4	448.4/74.0	−70	11.07
Cholic 3 sulfate	CA-3S	487.4	487.4/97.0	−70	11.21
Glycolithocholic 3 sulfate	GLCA-3S	512.4	512.4/74.0	−70	11.40
Taurohyodeoxycholic	THDCA	498.4	498.4/80.0	−110	11.49
Taurocholic	TCA	514.4	514.4/80.0	−110	13.76
Glycocholic	GCA	464.4	464.4/74.0	−70	14.37
Chenodeoxycholic 3 sulfate	CDCA-3S	471.4	471.4/97.0	−70	14.86
Deoxycholic 3 sulfate	DCA-3S	471.4	471.4/97.0	−70	15.41
Ursodeoxycholic	UDCA	391.4	391.4/391.4	−10	16.57
Hyocholic	HCA	407.4	407.4/407.4	−10	16.92
Taurochenodeoxycholic	TCDCA	498.4	498.4/80.0	−110	18.02
Glycochenodeoxycholic	GCDCA	448.4	448.4/74.0	−70	18.23
Hyodeoxycholic	HDCA	391.4	391.4/391.4	−10	18.77
Taurodeoxycholic	TDCA	498.4	498.4/80.0	−110	19.19
Lithocholic 3 sulfate	LCA-3S	455.4	455.4/97.0	−70	19.26
Glycodeoxycholic	GDCA	448.4	448.4/74.0	−70	19.94
Cholic	CA	407.4	407.4/407.4	−10	20.01
Taurolithocholic	TLCA	482.4	482.4/80.0	−110	22.28
Glycolithocholic	GLCA	432.4	432.4/74.0	−70	22.76
Chenodeoxycholic	CDCA	391.4	391.4/391.4	−10	23.66
Deoxycholic	DCA	391.4	391.4/391.4	−10	24.28
Lithocholic	LCA	375.4	375.4/375.4	−10	28.27
Internal standards ursodeoxycholic-2,2,4,4-d4	UDCA-D4	395.6	395.6/395.6	−10	16.46
23-Nor-5β-cholanoic	Nor	377.4	377.4/377.4	−10	21.31
Lithocholic 2,2,4,4-d4	LCA-D4	379.6	379.6/379.6	−10	28.08

CE, collision energy; MRM, Multiple Reaction Monitoring; *m/z* corresponds to $[M-H]^{1-}$, for ion* *m/z* correspond to $[M-2H]^{2-}$ (ESI conditions has been optimized and the major ion peak selected); CE was obtained by direct infusion. Retention times are derived from Fig. 2.

One urine sample collected from a cholestatic patient was then submitted to the same 4 extraction techniques. Eighteen different bile acids species were detected using acetonitrile extraction (given as 100% in Fig. 3B). Methanol (hashed bars) was close to acetonitrile except for TLCA-3S showing a higher yield of extraction. DCA-3S, LCA-3S and GDCA show similar yields of extraction by ammonium carbonate (white bars). However, other sulfoconjugates and hydrophilic bile acids were much lower. Interestingly, the urinary metabolite CA-3S was detected in this sample after extraction with acetonitrile or methanol but it was not detected either with trichloroacetic acid (filled bars) or with ammonium carbonate extraction (empty bars).

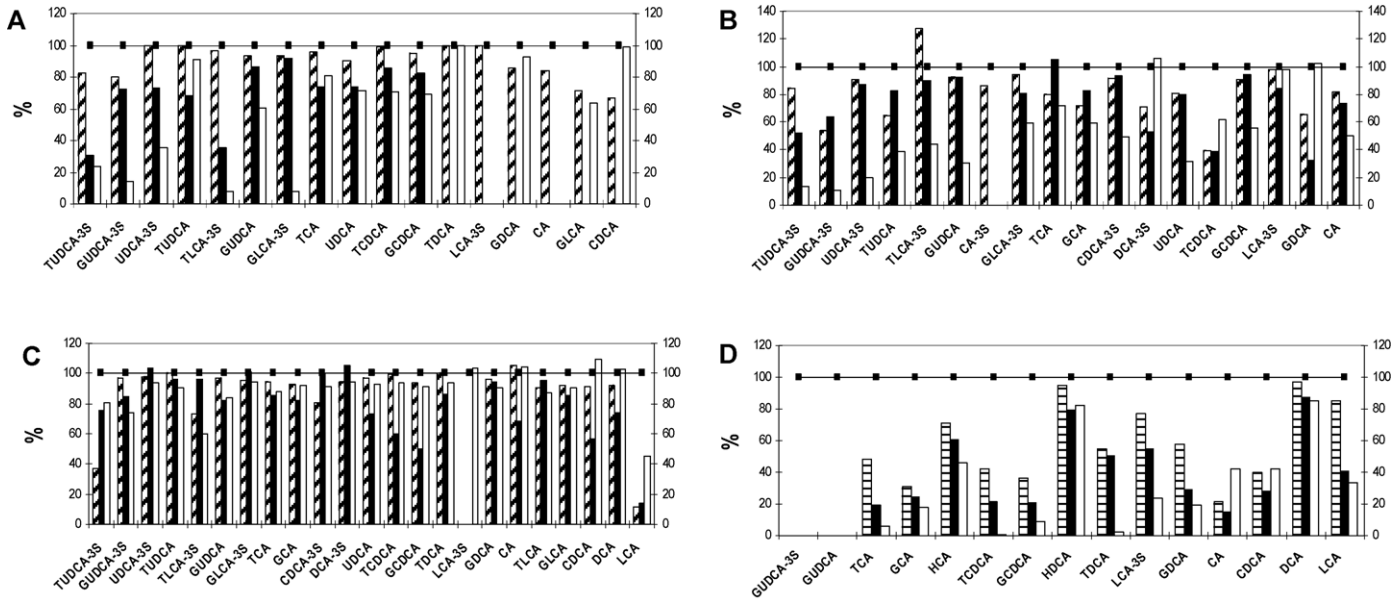

Fig. 3. Comparison of bile acids profiles following extraction by four different protocols: (A) serum, (B) urine, (C) bile, extracted with methanol (hashed bars), trichloroacetic acid (filled bars) or ammonium carbonate (empty bars) as compared to acetonitrile extraction taken as a reference (line with squares). The protocols are carried out on the same sample and results are expressed as % of the maximum value obtained with acetonitrile (given as 100%). (D) stool, extracted with acetonitrile (filled bar), isopropanol (hashed bar) and water (empty bar) as compared to NaOH extraction (line with squares). Results are expressed as % of the maximum value obtained with NaOH (given as 100%).

Table 2
Matrix effect of serum, urine and stool on extraction recovery expressed as percentage of the standard extracted in the absence of matrix and relative analytical response as a percentage of the analytical response in the absence of matrix. Each experiment is performed on six samples in triplicate.

Serum					Urine				Stool			
Analyte	Extraction recovery (%)	CV (%)	Relative analytical response (%)	CV (%)	Extraction recovery (%)	CV (%)	Relative analytical response (%)	CV (%)	Extraction recovery (%)	CV (%)	Relative analytical response (%)	CV (%)
TUDCA-3S	97.7	6.5	93.3	7.7	93.3	7.7	97.1	4.5	95.3	5.0	97.9	6.9
GUDCA-3S	99.9	7.2	89.9	9.4	89.7	9.4	98.5	7.5	95.8	3.6	99.9	4.1
UDCA 3S	97.5	5.8	95.4	4.0	95.3	4.0	99.0	3.3	96.1	3.4	94.3	3.1
TUDCA	96.8	8.1	94.6	5.5	94.6	5.5	99.5	3.0	94.6	4.0	98.6	3.0
TLCA-3S	102.2	7.6	96.6	3.1	96.6	3.1	102.2	4.9	97.8	3.0	100.6	4.8
GUDCA	100.1	5.9	94.2	5.8	94.2	5.8	100.5	5.5	95.4	3.5	96.2	4.0
CA-3S	99.3	9.7	96.7	6.8	96.7	6.8	100.9	4.6	96.2	2.9	97.3	2.5
GLCA 3S	99.0	4.7	97.6	4.9	97.6	4.9	99.1	3.6	96.5	2.4	99.3	2.5
THDCA	96.4	6.0	91.8	10.6	91.8	10.6	96.7	2.2	96.2	3.5	97.1	4.9
TCA	96.8	8.5	93.7	7.5	93.7	7.5	99.9	2.6	97.1	3.1	99.1	1.9
GCA	96.8	9.3	96.1	4.3	96.1	4.3	98.8	7.1	95.9	3.2	98.7	3.0
CDCA-3S	95.2	9.1	97.6	4.2	97.6	4.2	98.2	4.6	96.5	4.2	96.6	3.5
DCA-3S	93.3	9.7	97.9	6.5	97.9	6.5	99.8	5.8	95.0	3.5	96.9	3.0
UDCA	98.9	9.1	95.6	4.1	95.6	4.1	98.4	2.9	96.5	2.6	97.3	1.5
HCA	93.7	8.0	95.5	4.8	95.5	4.8	97.3	3.1	97.4	2.3	99.5	3.9
TCDCA	93.9	9.6	94.7	5.8	94.7	5.8	99.2	6.3	97.2	3.0	98.8	2.2
GCDCA	97.5	6.7	99.8	5.5	99.8	5.5	103.0	3.5	96.4	3.1	98.1	2.3
HDCA	96.3	7.7	96.3	3.5	96.3	3.5	98.6	1.9	96.6	3.2	97.4	3.5
TDCA	95.4	9.6	96.6	6.3	96.6	6.3	99.8	2.2	96.6	3.0	98.4	2.6
LCA-3S	93.6	9.2	96.1	4.1	96.1	4.1	99.5	4.1	97.3	3.5	98.4	2.1
GDCA	96.5	9.7	97.5	3.8	97.5	3.8	100.0	3.4	97.9	2.8	96.9	3.0
CA	99.3	4.8	98.8	5.1	98.8	5.1	102.9	4.7	97.8	9.3	93.0	7.7
TLCA	95.7	10.0	95.3	8.0	95.3	8.0	97.2	4.6	95.7	3.7	97.1	2.9
GLCA	98.5	9.6	94.7	6.8	94.7	6.8	98.7	6.0	94.9	3.9	97.9	2.7
CDCA	99.8	5.4	98.0	4.9	98.0	4.9	98.1	5.7	95.2	4.1	97.3	3.0
DCA	101.3	7.3	97.2	6.4	97.3	6.4	98.4	5.1	96.1	3.8	96.7	3.2
LCA	99.0	5.5	98.1	2.5	98.1	2.5	102.6	3.5	95.7	3.8	97.0	2.8

The four extraction protocols were also compared for extraction of a bile sample collected from gallbladder during a cholecystectomy. The results are shown in Fig. 3C. Due to their high concentration in bile, up to 23 different molecular species were detected using the L3 acetonitrile/SPE extraction. This protocol gave higher yields than methanol, trichloroacetic acid and ammonium carbonate techniques. Noticeably, LCA-3S was not detected in methanol or trichloroacetic extracts.

Altogether, our results indicate that although it takes a longer time than protein precipitation without SPE, the acetonitrile deproteinization protocol followed by SPE extraction seems to give the best yield for the three biological fluids tested. This protocol was therefore tested in validation assays in order to be used in routine analysis.

Four different extractions were also compared for stool samples. These techniques were necessarily different from those used for fluid samples because solid samples like stool require preincubation and homogenization before extraction. The four protocols tested used SPE extraction (for protocol comparison, see supplementary data 1B). NaOH extraction (S1 protocol) adapted from Perwaiz et al. [15], and Batta et al. [16] detected 15 molecular species and was used as the 100% reference method in Fig. 3D. Acetonitrile (S2 protocol, filled bars) adapted from liquid sample, and isopropanol (S3 protocol, hashed bars) adapted from Baiocchi et al. [17] detected 13 molecular species at a lower level than NaOH extraction for 13 out of 15 bile acids. Finally water extraction previously reported as the reference method [18] (S4 protocol, empty bars) gave a low yield for most species. Noticeably, minor hydrophilic bile acids such as GUDCA and GUDCA-3S were only detected by NaOH technique. Inferred from these results, the S3 NaOH protocol although longer than the others because of 1 h incubation before SPE, was chosen in further experiments as the routine extraction protocol for stool.

3.2. Validation of the method

3.2.1. Recovery and matrix effect

The extraction recoveries for the different bile acids in six different samples of each matrix are shown in Table 2. They ranged from 93% to 102% for acetonitrile extraction of serum and urine, and from 95 to 98% for NaOH extraction of stool. This result confirms that the two methods chosen were highly efficient to extract all the bile acid species tested, as expected from the results obtained in the acetonitrile [12] and NaOH [15] methods. The recovery was higher than in many results from literature (compared in [20]) in which overall recovery was ≥80%. Recovery of 3 bile acids at 3 concentrations was also measured in a fortification assay performed in serum, urine and stool, which showed the same extraction recovery (supplementary data 2).

3.2.2. Matrix effects on ion suppression

In addition to extraction recovery, standards were added to extracted serum, urine or stool and compared to the values obtained with standards in the absence of matrix (Table 2). The signal in presence of matrix did not show a decrease of the relative analytical response below 89% for serum, 96% for urine, and 93% for stool, showing a very limited quantitative matrix effect (<11%).

3.2.3. Linearity

Linearity was tested by analyzing bile acid standards at 6 concentrations ranging from 0.01 to 80 μmol/l [12]. The range investigated was much larger than in other studies, due to the wide range observed in the various matrices tested. Calibration plots were linear for the 27 bile acids as correlation coefficients for most bile acids were higher than 0.99 (Table 3) except for 4 species for which the correlation coefficient was between 0.98 and 0.99. Linearity was also checked in serum, urine and stool samples spiked with standards. Calibration curves were linear with correlation coefficients >0.98, except for some hydrophobic species in urine and stool which were >0.96 (supplementary data 3). The limit of quantification LOQ was estimated as the lowest value for which the coefficient of variation CV was lower than 15% [19]. It was estimated at 20 nmol/l except for TLCA and THDCA (Table 2). Taking into account the low volume of serum actually tested in the assay (about 20 μl out of the 500 μl extracted), LOQ values were in agreement with those described recently using HPLC–MS/MS [compared in 20].

3.2.4. Precision and accuracy

Within run and between run precision of the methods were estimated <10% (Table 3). Within run precision of unspiked serum, urine, bile, and stool was lower than 15% (3–13% for serum, 3–13% for urines, 4–15% for bile and 3–13% for stool) (supplementary data 4) whereas between run precision ranged from 1% to 14% (1–13% for serums, 4–18% in urines, 1–14% for bile and 1–15% for stool) (supplementary data 5). Within run precision was also determined in a fortification assay and ranged from 2% to 11%. The relative errors (RE) were found between 1 and 10% (supplementary data 2).

The accuracy was determined by 3 levels of quality control (low, medium and high). The relative errors (RE) were found <10% (supplementary data 2).

3.2.5. Stability

Stability was measured on serum, bile, urine and stool with high and low levels of total bile acids. Table 4a shows that recovery was above 94% for normal and high serum samples stored at −20 °C either for 1 week or for 1 month. The same recovery was found in urine (Table 4b) and bile (supplementary data 6) conserved in the same conditions. Stability at 4 °C was lower, but still acceptable with a recovery above 80% for normal serum, urine or bile. In contrast, the recovery was lower for high urine and serum samples with low recoveries for sulfated species (below 60%).

Storage at room temperature gave unequal results between high and normal serum (Table 4a). Results for urine at room temperature are not shown, because a heavy flocculation appeared after a few days, preventing a reliable analysis. This limitation has to be taken into account for routine assays where urines should be aliquoted prior freezing.

These results confirm that although bile acids are stable molecules, liquid biological samples must be analyzed rapidly, or stored at −20 °C to obtain reliable results [21].

Stool samples were found stable after storage for a month at −80 °C (supplementary data 6). Due to the high level of bacteria present in these samples, they were not tested after storage at 4 °C nor at room temperature.

3.3. Application to human samples

The acetonitrile extraction protocol was applied to a series of 39 sera from healthy donors. The profile is presented in Fig. 4A as percentages of the total concentration of bile acids. The respective concentrations of each of the fourteen different bile acids detected above their LOQ values are presented in Table 5. As expected from previous studies, large inter individual differences in the concentration of bile acids are observed for each type of biological samples, shown as large SD in Table 5 [21]. The serum profile is characterized by the presence of three major bile acids, CDCA (44% of total bile acids), DCA (25%), and CA (15%) in their free and tauro or glyco conjugates with a concentration above 0.1 μmol/l each. The tauro- and glyco-conjugates percentages (10 and 53%, respectively) are in agreement with previously published data using either GC–MS or LC–MS/MS [12,13,20–24]. Low amounts of non conjugated UDCA

Table 3
Linearity, limit of quantification (LOQ), within run and between run precisions for the 27 bile acids analyzed.

	LOQ (nM)	Slope, mean ± SD ($\times 10^6$)	Correlation coefficient (r^2)	Within run precision (CV%)	Between run precision (CV%)
TUDCA-3S	12.6	0.604 ± 0.009	0.9983	6.45	6.35
GUDCA-3S	13.8	1.131 ± 0.050	0.9942	7.95	8.84
UDCA-3S	15.5	8.365 ± 0.035	0.9977	7.23	4.26
TUDCA	14.6	16.450 ± 0.257	0.9991	6.27	2.84
TLCA-3S	13.0	0.463 ± 0.012	0.9894	6.73	6.13
GUDCA	16.3	5.777 ± 0.227	0.9988	7.27	3.00
CA-3S	15.0	5.130 ± 0.9954	0.9954	4.49	6.35
GLCA-3S	14.2	6.927 ± 0.102	0.9955	9.28	7.92
THDCA	73.2	7.318 ± 0.602	0.9968	3.25	9.27
TCA	14.2	5.648 ± 0.179	0.9923	4.22	6.66
GCA	15.7	7.220 ± 0.337	0.9985	9.79	7.34
CDCA-3S	15.5	6.130 ± 0.094	0.9964	5.80	2.64
DCA-3S	15.5	11.680 ± 0.375	0.9966	9.58	1.80
UDCA	18.6	11.730 ± 0.349	0.9905	7.97	9.82
HCA	17.9	7.047 ± 0.048	0.9968	6.81	7.51
TCDCA	14.6	3.139 ± 0.276	0.9842	1.48	8.81
GCDCA	16.3	3.304 ± 0.014	0.9964	5.45	8.21
HDCA	18.6	6.945 ± 0.122	0.9808	7.33	8.35
TDCA	14.6	2.715 ± 0.104	0.9837	5.40	9.07
LCA-3S	16.0	5.856 ± 0.459	0.9951	3.85	5.79
GDCA	16.3	3.024 ± 0.024	0.9988	6.65	4.73
CA	17.9	7.979 ± 0.031	0.9898	9.71	9.46
TLCA	75.6	5.694 ± 0.045	0.9979	4.14	5.70
GLCA	16.9	13.180 ± 0.173	0.9908	5.12	5.74
CDCA	18.6	15.430 ± 0.276	0.9988	9.46	1.69
DCA	18.6	17.620 ± 0.213	0.9982	6.98	2.10
LCA	19.4	22.110 ± 0.428	0.9992	8.69	7.43

and glyco-UDCA were also found. HCA was under the LOQ value (<15 nmol/l) and HDCA was not detected. In accordance with literature, glyco-conjugates were found at higher concentration than non conjugated bile acids (X2 folds) and tauro-conjugated (X3 folds) in these healthy subjects. Interestingly, sulfated conjugates (8% of total bile acids) were detected as two major species represented by almost all of LCA and 1/3 of UDCA. The presence of sulfated bile acids in the serum of healthy donors is in the range of

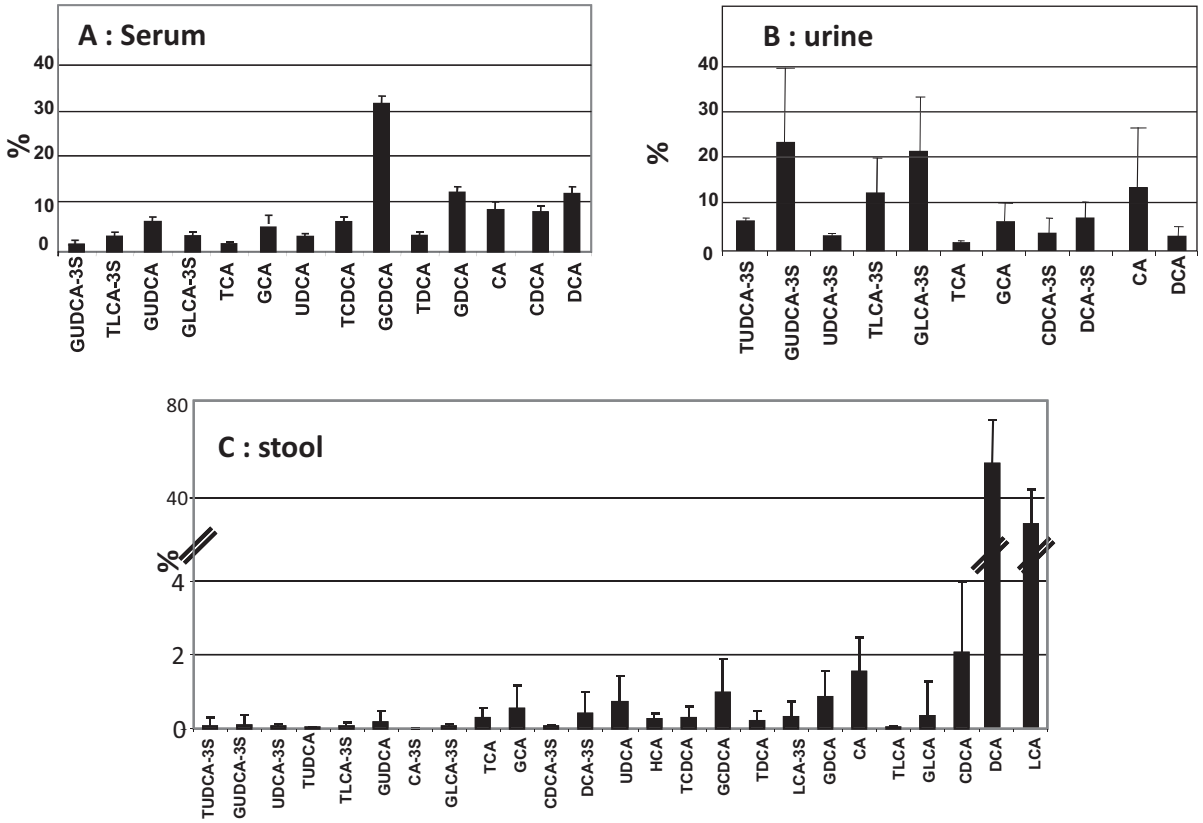

Fig. 4. Bile acid profiles in serum (A), urine (B) and stool (C) from a group of healthy patients. Extraction from serum and urine was performed using acetonitrile method. Results from 18 serum and 24 urine samples from normal patients are shown. Extraction from stool was performed using NaOH method. Results from 17 normal patients are shown in % of total bile acids.

Table 4
Stability results (a) for normal and high serum, and (b) for normal and high urine, expressed as % of the values in fresh samples (mean ± SEM). Total concentrations were 1.38 and 96.80 μmol/l for normal and high serum, and 2.5 and 76.7 μmol/l for normal and high urine.

a	Normal serum						High serum					
	Room T°		+4 °C		−20 °C		Room T°		+4 °C		−20 °C	
	One week	One month	One week	One month	One week	One month	One week	One month	One week	One month	One week	One month
TUDCA-3S							78.7 ± 4.1	93.3 ± 1.1	67.9 ± 1.6	86.4 ± 5.6	104.0 ± 0.6	101.9 ± 0.2
GUDCA-3S							78.0 ± 3.0	84.6 ± 1.1	65.8 ± 1.9	77.9 ± 1.0	98.0 ± 0.8	95.0 ± 1.2
UDCA-3S							95.3 ± 2.6	121.5 ± 0.4	74.3 ± 2.9	82.6 ± 4.3	96.6 ± 4.8	95.1 ± 0.7
TUDCA							99.8 ± 6.3	104.2 ± 0.5	104.7 ± 2.5	86.4 ± 5.6	95.8 ± 4.6	97.0 ± 0.2
TLCA-3S							92.9 ± 1.3	56.2 ± 2.0	72.2 ± 6.0	64.1 ± 4.6	103.1 ± 1.4	100.0 ± 0.5
GUDCA							103.9 ± 2.2	96.8 ± 2.8	102.1 ± 3.0	87.1 ± 0.4	102.9 ± 2.2	103.7 ± 1.1
GLCA-3S							82.9 ± 4.8	80.4 ± 25.5	106.9 ± 1.2	71.5 ± 4.3	104.9 ± 4.9	104.5 ± 3.3
TCA	102.4 ± 5.3	110.0 ± 1.4	92.9 ± 3.8	85.7 ± 7.3	100.0 ± 6.3	100.0 ± 5.5	104.7 ± 7.0	98.1 ± 1.3	101.1 ± 0.5	99.4 ± 2.2	96.4 ± 0.5	101.1 ± 1.3
GCA	101.5 ± 4.5	88.1 ± 5.8	92.9 ± 2.7	85.7 ± 3.6	95.7 ± 4.8	95.7 ± 1.3	97.4 ± 6.1	93.8 ± 1.1	100.3 ± 2.4	92.8 ± 2.7	98.1 ± 2.5	98.0 ± 0.9
UDCA							109.7 ± 5.7	99.6 ± 0.4	95.5 ± 2.5	85.7 ± 1.6	105.0 ± 5.0	103.5 ± 4.1
TCDCA	103.7 ± 2.5	102.2 ± 2.7	88.2 ± 2.6	89.1 ± 4.2	98.6 ± 4.8	102.5 ± 5.3	104.6 ± 6.3	96.7 ± 2.4	94.5 ± 0.7	84.8 ± 1.5	97.9 ± 1.8	104.1 ± 2.1
GCDCA	102.5 ± 4.2	91.1 ± 2.6	86.5 ± 0.8	89.6 ± 1.5	103.8 ± 2.5	96.3 ± 2.2	101.2 ± 0.8	95.0 ± 1.3	98.8 ± 2.2	77.5 ± 1.5	99.1 ± 4.1	98.1 ± 0.4
TDCA	105.4 ± 23.2	104.1 ± 5.9	90.0 ± 4.9	92.2 ± 8.6	100.0 ± 6.3	100.0 ± 3.5	98.8 ± 1.5	85.0 ± 0.7	97.2 ± 0.2	83.1 ± 17.9	101.1 ± 1.2	96.3 ± 0.5
GDCA	114.6 ± 3.6	123.3 ± 4.2	96.7 ± 10.4	85.5 ± 3.7	100.0 ± 2.7	97.5 ± 5.3	106.3 ± 1.1	97.8 ± 2.2	99.1 ± 1.1	80.5 ± 1.6	103.1 ± 2.4	100.2 ± 1.2
CA	105.4 ± 2.3	80.2 ± 4.7	90.0 ± 3.5	93.0 ± 2.9	97.4 ± 20.7	95.1 ± 1.8	104.3 ± 0.8	107.9 ± 2.0	99.1 ± 1.0	88.7 ± 20.2	98.4 ± 2.3	101.4 ± 2.4
DCA	110.1 ± 5.8	132.7 ± 3.5	98.9 ± 10.0	98.5 ± 4.6	100.0 ± 32.5	103.6 ± 4.3	109.6 ± 3.1	120.7 ± 5.3	97.7 ± 3.0	70.0 ± 4.8	94.5 ± 0.6	99.8 ± 8.0

b	Normal urine				High urine			
	+4 °C		−20 °C		+4 °C		−20 °C	
	One week	One month	One week	One month	One week	One month	One week	One month
TUDCA-3S	111.1 ± 1.8	73.0 ± 0.8	103.1 ± 0.4	103.1 ± 2.3	97.2 ± 2.7	98.1 ± 3.3	99.2 ± 2.0	104.4 ± 0.8
GUDCA-3S	122.4 ± 1.1	88.8 ± 6.6	103.4 ± 0.6	103.3 ± 1.2	89.6 ± 0.2	65.2 ± 0.7	95.8 ± 2.3	99.4 ± 0.4
UDCA-3S					84.2 ± 3.5	88.3 ± 2.5	101.2 ± 6.8	97.0 ± 2.9
TUDCA								
TLCA-3S	107.7 ± 0.1	120.8 ± 10.4	100.0 ± 1.4	101.4 ± 1.5	117.3 ± 3.0	146.3 ± 2.9	103.9 ± 2.3	100.1 ± 1.4
GUDCA					97.3 ± 0.9	93.0 ± 0.7	99.0 ± 1.8	102.0 ± 1.0
GLCA-3S	97.9 ± 1.2	80.8 ± 1.9	95.5 ± 4.9	100.3 ± 3.3	104.2 ± 4.9	83.9 ± 0.7	100.2 ± 3.5	103.8 ± 1.9
TCA	97.4 ± 5.5	95.8 ± 3.3	100.0 ± 0.5	97.5 ± 3.2				
GCA	106.6 ± 2.1	93.2 ± 2.7	104.2 ± 2.5	103.3 ± 1.5	97.1 ± 2.2	95.3 ± 0.5	102.4 ± 3.6	105.7 ± 2.8
CDCA-3S	98.8 ± 2.0	116.4 ± 3.5	100.6 ± 11.8	98.1 ± 10.8	92.7 ± 0.8	101.2 ± 4.1	98.1 ± 5.9	96.4 ± 2.3
DCA-3S	100.0 ± 0.8	106.3 ± 1.3	99.4 ± 13.3	96.8 ± 6.1	93.2 ± 7.6	116.3 ± 4.0	104.3 ± 3.7	103.5 ± 3.9
UDCA					126.5 ± 2.2	103.6 ± 2.5	104.2 ± 5.0	95.8 ± 4.5
LCA-3S					92.1 ± 2.5	96.2 ± 1.9	104.7 ± 6.1	96.4 ± 3.6
GDCA	95.6 ± 2.3	87.7 ± 5.1	100.3 ± 2.4	103.8 ± 1.8				
CA	102.9 ± 1.4	90.7 ± 1.8	100.0 ± 2.3	96.2 ± 2.4	90.7 ± 0.7	92.7 ± 0.3	102.3 ± 1.7	105.2 ± 3.6

Table 5
Bile acid profiles in serum ($n = 39$), urine ($n = 39$) and stool ($n = 19$) from healthy patients.

	Serum (nmol/l)	Urine (nmol/l)	Stool (nmol/g)
TUDCA-3S	nd	53.90 ± 36.19	0.34 ± 1.21
GUDCA-3S	37.87 ± 73.21	201.25 ± 246.55	0.75 ± 1.58
UDCA-3S	nd	25.05 ± 38.87	1.43 ± 3.38
TUDCA	nd	nd	0.30 ± 0.37
TLCA-3S	53.27 ± 23.98	108.6 ± 95.39	0.77 ± 0.63
GUDCA	99.62 ± 204.16	nd	2.39 ± 2.1
CA-3S	nd	nd	0.20 ± 0.26
GLCA-3S	76.34 ± 53.96	185.73 ± 151.63	1.26 ± 1.24
THDCA	nd	nd	nd
TCA	41.15 ± 29.21	16.29 ± 11.82	5.78 ± 4.32
GCA	112.81 ± 115.51	47.69 ± 51.04	10.15 ± 7.51
CDCA-3S	nd	19.33 ± 40.55	1.06 ± 1.20
DCA-3S	nd	49.53 ± 85.42	9.36 ± 15.51
UDCA	57.91 ± 110.71	nd	27.05 ± 61.13
HCA	nd	nd	6.71 ± 4.46
TCDCA	83.43 ± 90.08	nd	6.03 ± 5.00
GCDCA	705.03 ± 680.16	nd	22.28 ± 15.65
HDCA	nd	nd	nd
TDCA	47.89 ± 65.63	nd	4.32 ± 5.81
LCA-3S	nd	nd	7.76 ± 9.24
GDCA	255.25 ± 293.31	nd	19.19 ± 13.69
CA	169.12 ± 336.08	124.84 ± 193.22	44.71 ± 47.79
TLCA	nd	nd	0.51 ± 0.40
GLCA	nd	nd	6.68 ± 18.49
CDCA	177.84 ± 295.01	nd	54.8 ± 72.07
DCA	242.90 ± 300.96	19.01 ± 15.32	1920.10 ± 1390.50
LCA	nd	nd	1016.60 ± 647.31
Total	2160 ± 1797	853 ± 532	3171 ± 2095

nd, not detectable. Results are means values ± SD.

Table 6
Bile acid profiles in 23 healthy and 9 cholestatic patients analyzed in the same series. Bile acid levels are expressed in nM or in relative values ± SD.

	Healthy nM	Cholestatic nM	Relevant changes
TUDCA-3S	nd	50 ± 80	↑**
GUDCA-3S	13 ± 30	110 ± 300	ns
UDCA-3S	nd	10 ± 10	ns
TUDCA	nd	30 ± 30	↑***
TLCA-3S	10 ± 22	180 ± 210	↑***
GUDCA	14 ± 34	40 ± 60	ns
GLCA-3S	17 ± 30	50 ± 70	ns
THDCA	nd	10 ± 20	ns
TCA	62 ± 70	53 920 ± 42 620	↑***
GCA	190 ± 230	25 030 ± 21 110	↑***
UDCA	20 ± 30	nd	↓*
TCDCA	95 ± 150	18 130 ± 10 430	↑***
GCDCA	720 ± 810	12 370 ± 8760	↑**
HDCA	35 ± 45	nd	↓*
TDCA	80 ± 140	1030 ± 2140	↑*
GDCA	390 ± 650	680 ± 1450	ns
CA	65 ± 100	40 ± 70	ns
CDCA	130 ± 380	nd	ns
DCA	240 ± 280	15 ± 25	↓*
LCA	30 ± 25	nd	↓**
Total	2135 ± 2120	111 700 ± 80 940	↑***
	% of total	% of total	Relevant changes
Primary BA	58 ± 14	97 ± 3	↑***
Secondary BA	40 ± 13	2 ± 3	↓***
CA/CDCA	0.5 ± 0.4	2.5 ± 1.1	↑***
Tauro/glyco	0.3 ± 0.3	3 ± 3.5	↑***

nd, not detectable; p values were determined using an F-test comparison; ns, not significant.
* $p \leq 0.05$.
** $p \leq 0.01$.
*** $p \leq 0.001$.

previous results obtained with an enzymatic assay (for review, see [5]) and also with LC–MS/MS [3,10,25].

Urines from healthy donors were also tested using the acetonitrile protocol. Eleven different bile acids were quantified (Table 5). As expected from previous results [25,26], the prominent metabolites in urine were sulfated, mainly UDCA and LCA which were also found as glyco- or tauro-conjugates (76% of total urinary bile acids) (Fig. 4B). In contrast, CA was found mainly non-conjugated (70% of total CA). As pointed out in a recent review [5], the proportions of individual sulfated bile acid decreased with the number of hydroxyl groups. This might reflect that active sulfation process is required for detoxification of the hydrophobic species. Strikingly, free CDCA was not eliminated in urine unlike CA and DCA and this lack of detoxification might take part in the high level of this bile acid in serum relative to its low synthesis rate in liver.

Profiling of stool samples from 19 control patients allowed the detection of 25 different bile acid species (Table 5). The few previous studies from literature used GC–MS based on the assumption that fecal bile acids are mainly non-conjugated due to their active deconjugation by the microflora within the intestinal lumen [14–16,18,27,28], in accordance with these previous results, we found 97% of free bile acids in healthy patients. The two major bile acids found in stool were DCA (55% of total bile acids) and LCA (29%) in accordance with previous results [27]. Despite the low amount of sample used (100 mg of dried stool) our protocol using NaOH extraction followed by LC–MS/MS detection allowed to detect many minor species such as CA, CDCA and UDCA and also numerous conjugated bile acids (3%) (Fig. 4C and Table 5). The glyco-conjugates (2%) were higher than tauro-conjugates. Noticeably, large inter individual differences in the concentration of bile acids were shown by the high values of SD in Table 5, possibly due to a poor homogeneity of fecal samples. Interestingly, SD is much lowered by expressing the results as % of total bile acids (Fig. 4C). The present LC–MS/MS analysis of bile acids in stool is thought to be useful for the follow up of digestive diseases associated with a change in proportions of non conjugated/conjugated metabolites. This type of variation has been described in stool from cholecystectomized patients [28]. Preliminary studies show that such variations might be associated with disturbance in intestinal bacterial activity (Henri Duboc, personal communication). In turn, the potentially toxic or protective effects of bile acids on intestinal wall can be investigated by the current protocol.

Finally, the acetonitrile and LC–MS/MS method was applied to sera from 9 patients with cholestasis resulting from various defaults of bile acid secretion [1]. The results for normal and cholestatic patients are summarized in Table 6. The expected increase in total bile acids (ranging from 28 to 235 μmol/l whereas control patients were from 2.5 to 9 μmol/l) was characterized by a very significant increase in 4 main species, TCA, GCA, TCDCA and GCDCA [12,29] and increased proportion in primary bile acids, CDCA balanced by a decrease in the secondary bile acids DCA and LCA. In accordance with previous results [12,22,29], the tauro-conjugates were higher than glycoconjugates in cholestatic sera, in contrast with healthy patients. We also found that the percentage of sulfo conjugates was increased in a lower proportion than unsulfated bile acids in cholestatic sera when compared to normal ones [3]. Noticeably, during cholestasis, the increase in CA is not balanced by an increase in CDCA which results in the increased ratio CA/CDCA [22,29].

4. Conclusion

Four extraction techniques followed by HPLC–MS/MS were compared to select a routine protocol for profiling bile acids in clinical samples. Acetonitrile extraction followed by a clean-up with a SPE C_{18} cartridge step gives the best results for serum, bile and urine samples analysis. NaOH extraction is shown to be the most efficient

extraction technique for stool. A major advantage of the method is the wide range of sensitivity which allows besides prominent bile acid species to assay the minor species potentially relevant as biomarkers or for a regulatory activity. A variety of intestinal conditions with consequences on the metabolism of bile acids will be now analyzed according to the protocol selected in this comparative study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.05.015.

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