Journal of Chromatography B, 878 (2010) 51–60

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

High performance liquid chromatography–tandem mass spectrometry for the determination of bile acid concentrations in human plasma

Xiaoqiang Xiang, Yi Han, Mikko Neuvonen, Jouko Laitila, Pertti J. Neuvonen, Mikko Niemi*

Department of Clinical Pharmacology, University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland

ARTICLE INFO

Article history:
Received 3 May 2009
Accepted 11 November 2009
Available online 27 November 2009

Keywords:
Bile acids
Liquid chromatography–mass spectrometry
Healthy subjects
Bile acid-free plasma
Bile acid stability

ABSTRACT

We report a sensitive and robust method to determine cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), and their taurine- and glycine-conjugate concentrations in human plasma using liquid chromatography–tandem mass spectrometry. Activated charcoal was utilized to prepare bile acid-free plasma, which served as the biological matrix for the preparation of standard and quality control samples. Plasma sample preparation involved solid-phase extraction. A total of 16 bile acids and 5 internal standards were separated on a reverse column by gradient elution and detected by tandem mass spectrometry in negative ion mode. The calibration curve was linear for all the bile acids over a range of 0.005–5 μmol/L. The extraction recoveries for all the analytes fell in the range of 88–101%. Intra-day and inter-day coefficients of variation were all below 10%. A stability test showed that all the bile acids were stable in plasma for at least 6 h at room temperature, at least three freeze–thaw cycles, in the −70 °C or −20 °C freezer for 2 months, and also in the reconstitution solution at 8 °C for 48 h. Comparison of the matrix effect of bile acid-free plasma with that of real plasma indicated that the charcoal purification procedure did not affect the properties of charcoal-purified plasma as calibration matrix. This method has been used to determine the bile acid concentrations in more than 300 plasma samples from healthy individuals. In conclusion, this method is suitable for the simultaneous quantification of individual bile acids in human plasma.

1. Introduction

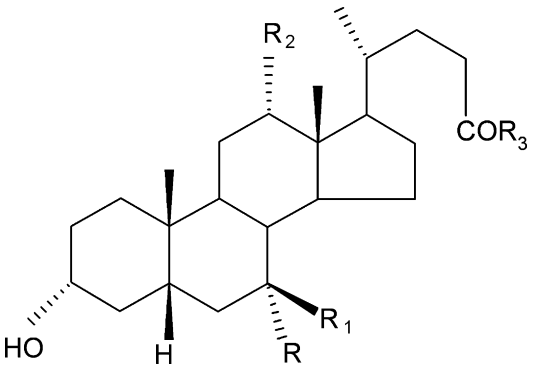
Bile acids are a class of structurally similar compounds that play essential roles in cholesterol homeostasis, lipid absorption, and intestinal signaling [1,2]. The primary bile acids, synthesized in the liver, are cholic acid (CA) and chenodeoxycholic acid (CDCA) [3]. A portion of these primary bile acids is subsequently converted by microbial flora into secondary bile acids such as deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) during the enterohepatic cycling [4]. These unconjugated bile acids form taurine- and glycine-conjugates via amidation on carbon 24, which leads to enhanced solubility, yet the potential physiological roles of these conjugates remain unknown [5] (Fig. 1).

Liver and gastrointestinal diseases can affect bile acid synthesis and disposition. Elevated bile acid concentrations in plasma occur in cholestasis and other types of liver injury [6,7]. For hepatic and intestinal diseases, serum or plasma bile acid concentrations have therefore long been utilized as prognostic and diagnostic markers. In line with the need for taking bile acid concentrations as biomarkers, numerous analytical methods have emerged to determine bile acid concentrations in human plasma or serum. GC–MS has been used as a sensitive method to determine the bile acid concentrations in biological samples from human or other mammals [8,9]. However, the use of GC–MS is limited by tedious work required by the GC–MS sample preparation. In the recent years, the high sensitivity and simple sample preparation have made HPLC–MS/MS an ideal option for the analysis of bile acids [10–14]. For the most developed HPLC–MS/MS methods, SPE has proven to be an efficient and relatively simple way of extracting of bile acids from serum or plasma [11,12]. More recently, analytical reports of bile acids have addressed the use of novel technologies in sample extraction

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; CV, coefficient of variation; DCA, deoxycholic acid; GC–MS, gas chromatography-mass spectrometry; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glycoursodeoxycholic acid; HDCA, hyodeoxycholic acid; HPLC, high performance liquid chromatography; HPLC–MS/MS, high performance liquid chromatography–tandem mass spectrometry; LCA, lithocholic acid; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; LTQ-FTMS, linear ion trap Fourier transform mass spectrometer; MRM, multiple reaction monitoring; QC, quality control; RAM, restricted access material; SPE, solid-phase extraction; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

* Corresponding author at: Department of Clinical Pharmacology, University of Helsinki, Tukholmankatu 8 C, FI-00014 Helsinki, Finland. Tel.: +358 9 471 73592; fax: +358 9 471 74039.
E-mail address: mikko.niemi@helsinki.fi (M. Niemi).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.11.019



Bile acid	R	R_1	R_2
UDCA	-H	-OH	-H
CA	-OH	-H	-OH
CDCA	-OH	-H	-H
DCA	-H	-H	-OH
LCA	-H	-H	-H

R_3, -OH, unconjugated;
-$NHCH_2COOH$, glycine conjugates;
-$H(CH_2)_2SO_3H$, taurine conjugates

Fig. 1. Structures of unconjugated, glycine- and taurine-conjugated bile acids.

and quantification, such as an on-line method combining restricted access material (RAM) or the linear ion trap Fourier transform mass spectrometer (LTQ-FTMS) [13,14]. Despite these advantages, the validation of most of the reported HPLC–MS/MS methods still suffer from the interference of endogenous plasma or serum bile acids. In some methods, a known amount of bile acid standards has been added to normal plasma, which served as a "blank" biological matrix [10–12]. The presence of endogenous bile acids in the "blank" plasma makes it difficult to determine the limit of quantification. A number of other methods chose an aqueous phase to prepare calibration standards [13,14]. Unfortunately, in such cases, the matrix effect of plasma cannot be taken into account. Most of these currently available methods therefore still require improvement for the preparation of the calibration standard. In this regard, a bile acid-free plasma is desirable to make the method reproducible. Finally, although the detection and quantification of bile acid concentrations have become a routine analysis in many biomedical laboratories, few reports have addressed the stability of the major bile acids. We therefore also aim to investigate the stability of bile acids under certain storage and experimental conditions.

In the present study, we have developed a simple, sensitive, and robust HPLC–MS/MS method to determine specific bile acid concentrations in human plasma. It was applied to determine the quantitative profile of bile acids in the plasma of healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

A total of 16 bile acids and five isotopic internal standards were included in this study: CA, DCA, CDCA, LCA, UDCA, their taurine- and glycine-conjugates taurocholic acid (TCA), glycocholic acid (GCA), taurodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCA), taurochenodeoxycholic acid (TCDCA), glycochenodeoxycholic acid (GCDCA), taurolithocholic acid (TLCA), tauroursodeoxycholic acid (TUDCA), and hyodeoxycholic acid (HDCA), and the internal standards chenodeoxycholic acid-2,2,4,4-d_4 (CDCA-d_4) and glycocholic acid-(glycine-1-^{13}C) (GCA-^{13}C) were from Sigma Chemical (St. Louis, MO, USA). Glycoursodeoxycholic acid (GUDCA), taurocholic acid-d_4 (TCA-d_4), taurochenodeoxycholic acid (TCDCA-d_4), and tauroursodeoxycholic-2,2,3,4,4-d_5 acid (TUDCA-d_5) were from Toronto Research Chemicals Inc. (Toronto, Canada). Glycolithocholic acid (GLCA) was purchased from Steraloids UK Ltd. (London, England).

Deionized water ($R \geq 18\,M\Omega$) used for HPLC mobile phase and sample preparation was prepared on an Elga Prima/Maxima reversed osmosis system (Elga Labwater, Lane End, Buckinghamshire, UK). Methanol and acetonitrile were of HPLC grade and obtained from VWR International Ltd. (Poole, England); formic acid and ammonium acetate were from Sigma Chemical (St. Louis, MO, USA); activated charcoal was from Merck (Darmstadt, Germany).

Pooled drug-free human plasma was supplied from the blood bank of the Helsinki University Central Hospital (Helsinki, Finland). Plasma was stored at −70 °C until use and analysis.

2.2. Bile acid-free plasma preparation

Pooled drug-free human plasma was purified using activated charcoal to remove endogenous bile acids. Such bile acid-free plasma was used as biological matrix in this study. To be specific, 400 mL of the plasma was mixed with 20 g of activated charcoal and the mixture was shaken moderately on an orbital shaker overnight (about 17 h) at room temperature. After centrifugation at 19,500 rpm for 1 h, the supernatant of purified plasma was transferred to clean tubes and kept at −70 °C until use.

2.3. Calibration and quality control standard preparation

Bile acid stock solutions were prepared separately in methanol to achieve the concentration of 10 mmol/L. Calibration standards were prepared by adding appropriate amounts of bile acid stock solutions into the bile acid-free plasma to give the following concentrations: 0, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5 μmol/L. QC standards of 0.012, 0.12, and 1.2 μmol/L were similarly prepared. The calibration and QC samples were kept at −70 °C until analyzed.

2.4. Sample preparation

Plasma samples (500 μL) were mixed with 1.3 mL of 0.05% formic acid. After being mixed thoroughly by vortexing, the mixture was loaded to the extraction cartridges (C18 Bond Elut, Varian, Palo Alto, CA, USA), which had been pre-conditioned with 1 mL of methanol and 1 mL of 0.05% formic acid. The cartridge was subsequently washed with 1 mL of water and 1 mL of 5% methanol. The bile acids were eluted out with 1 mL of methanol and 2 mL of acetonitrile. The eluent was dried at 60 °C under a nitrogen stream. The residue was dissolved in 100 μL of 60% methanol by vigorous vortexing, followed by 1 min of ultrasonication. Fifteen microliters of the sample was injected to the HPLC–MS/MS system.

2.5. HPLC–MS/MS analysis

The chromatographic system consisted of an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany). The chromatographic separation was carried out on an Atlantis®T3 column (2.1 mm × 100 mm, 3 μm) (Waters Corp., Milford, MA, USA), protected by an Atlantis®T3 (2.1 mm × 10 mm, 3 μm) guard cartridge (Waters Corp.). The mobile phase consisted of (A) 10 mmol/L ammonium acetate and 0.005% formic acid in water and (B) 10 mmol/L ammonium acetate and 0.005% formic acid in methanol. The total running time was 29.3 min, with the mobile phase gradient of 2 min at 40% A, 13 min to 5% A, 4.3 min at 5% A, 0.1 min to

40% A, and 9.9 min at 40% A. The injection volume was 15 μL and the mobile phase flow rate was 200 μL/min. The internal standards used were GCA-^{13}C, CDCA-d_4, TCA-d_4, TCDCA-d_4, and TUDCA-d_5.

Mass spectra were obtained using an Applied Biosystems SCIEX API2000 mass spectrometer (Sciex Division of MDS, Toronto, Ontario, Canada) equipped with a TurboIonSpray interface operated in the negative ion mode. MRM mode was chosen to acquire the quantitative data. The ion source temperature was set at 300 °C. Nitrogen was used as curtain gas and collision gas and set at 25 and 9 psi, respectively. The ion spray voltage was set at −4000 V. The interface heater was kept on. The whole chromatographic run was divided into four periods. The ion transition and dwell time for all the compounds as well as the corresponding internal standard for each bile acid are listed in Table 1. Data were acquired with Analyst software, Version 1.4.

2.6. Stability

The stability test was performed using the low- and high-concentration QC samples (0.012 and 1.2 μmol/L, respectively) according to the FDA guidance for bioanalytical method validation [15]. To be specific, the following stability tests were conducted.

Short-term stability: Low and high concentrations of QC samples were thawed at room temperature and kept at this temperature for 6 h and analyzed together with freshly processed QC samples.
Freeze and thaw stability: The freeze–thaw stability evaluation was conducted by comparing the back-calculated concentrations of the stability samples after three–freeze and thaw cycles with the plasma samples thawed only once. For each freeze and thaw cycle, high and low QC samples were stored at −20 °C for 24 h and thawed unassisted at room temperature.
Long-term stability: The stability of spiked human plasma samples after 60 days of storage at −20 and −70 °C was compared with freshly spiked QC samples. The analysis was performed on the same day.
Post-preparative stability: The post-preparative stability of the extracted bile acids was evaluated by comparing the extracted plasma samples that were injected immediately (time 0) with the samples that were re-injected 48 h after sitting in the autosampler at 8 °C. Evaluation was based on back-calculated concentrations. All the above stability tests were performed with five repeats and the results are expressed as percentages of freshly processed QC samples.

2.7. Accuracy, precision, linearity, and extraction recovery

LODs of all bile acids were defined as the lowest concentrations which could provide a signal-to-noise (S/N) ratio of 3:1. LOQs were defined as the lowest concentration of the calibration curve which yielded a response at least five times that of blank plasma control and also an acceptable accuracy (between 80 and 120%) and precision (≤15%). The intra-day accuracy was assessed by extraction and analysis of six replicates of high, middle and low QC samples in 1 day. The inter-day accuracy and precision were evaluated by extraction and analysis of the QC samples on 6 different days. The accuracy is expressed as the percentage of determined concentration to the spiked concentrations. Precision, expressed as CV, was calculated as the relative standard deviation of the determined concentration. The linearity of each of the 16 bile acids was determined by analyzing plasma standards prepared to contain 0.005–5 μmol/L of bile acids, and the mean correlation coefficient for each regression equation was generated on 6 different days. The SPE extraction recovery was evaluated by comparing the analyte peaks of extracted QC samples to those of post extracted plasma blanks spiked at equal concentrations.

2.8. Sample preparation for comparing the matrix effect of real plasma with that of charcoal-purified plasma

Three sets of plasma samples were prepared to evaluate the differences of matrix effect between charcoal-purified plasma and three different lots of real plasma. The first set was prepared in extracts of blank charcoal-purified plasma and, after extraction, spiked with 100 μL internal standard working solution, and 120 μL 0.5 μmol/L bile acid working solution. The second set was prepared in real plasma extracts originating from three different subjects and, after extraction, spiked equally with internal standard and

Table 1
The optimum HPLC–MS/MS parameters for the analytes, and the internal standard for each bile acid.

Bile acid	Q1 mass (amu)	Q3 mass (amu)	Dwell (ms)	Retention time (min)	Internal standard
Period 1					
TUDCA	498.20	79.90	200.00	10.7	TUDCA-d_5
GUDCA	448.14	448.14	200.00	11.0	GCA-^{13}C
TUDCA-d_5	503.36	79.90	200.00	10.7	
Period 2					
UDCA	391.10	391.10	80.00	13.9	GCA-^{13}C
HDCA	391.10	391.10	80.00	14.8	GCA-^{13}C
TCDCA	498.20	79.90	80.00	15.4	TCDCA-d_4
TDCA	498.20	79.90	80.00	15.8	TCDCA-d_4
GCDCA	448.14	448.14	80.00	15.6	CDCA-d_4
GDCA	448.14	448.14	80.00	16.1	CDCA-d_4
GCA	464.20	464.20	80.00	13.5	CDCA-d_4
GCA-^{13}C	465.20	74.90	80.00	13.5	
CA	407.10	407.10	80.00	15.4	CDCA-d_4
TCA	514.20	514.20	80.00	13.2	TCA-d_4
TCDCA-d_4	502.13	79.94	80.00	15.4	
TCA-d_4	518.15	518.15	80.00	13.1	
Period 3					
CDCA	391.10	391.10	120.00	17.4	CDCA-d_4
DCA	391.10	391.10	120.00	17.7	CDCA-d_4
TLCA	482.20	79.90	120.00	17.3	TCDCA-d_4
GLCA	432.10	73.79	120.00	17.6	GCA-^{13}C
CDCA-d_4	395.20	395.20	120.00	17.4	
Period 4					
LCA	375.00	375.00	200.00	19.1	GCA-^{13}C

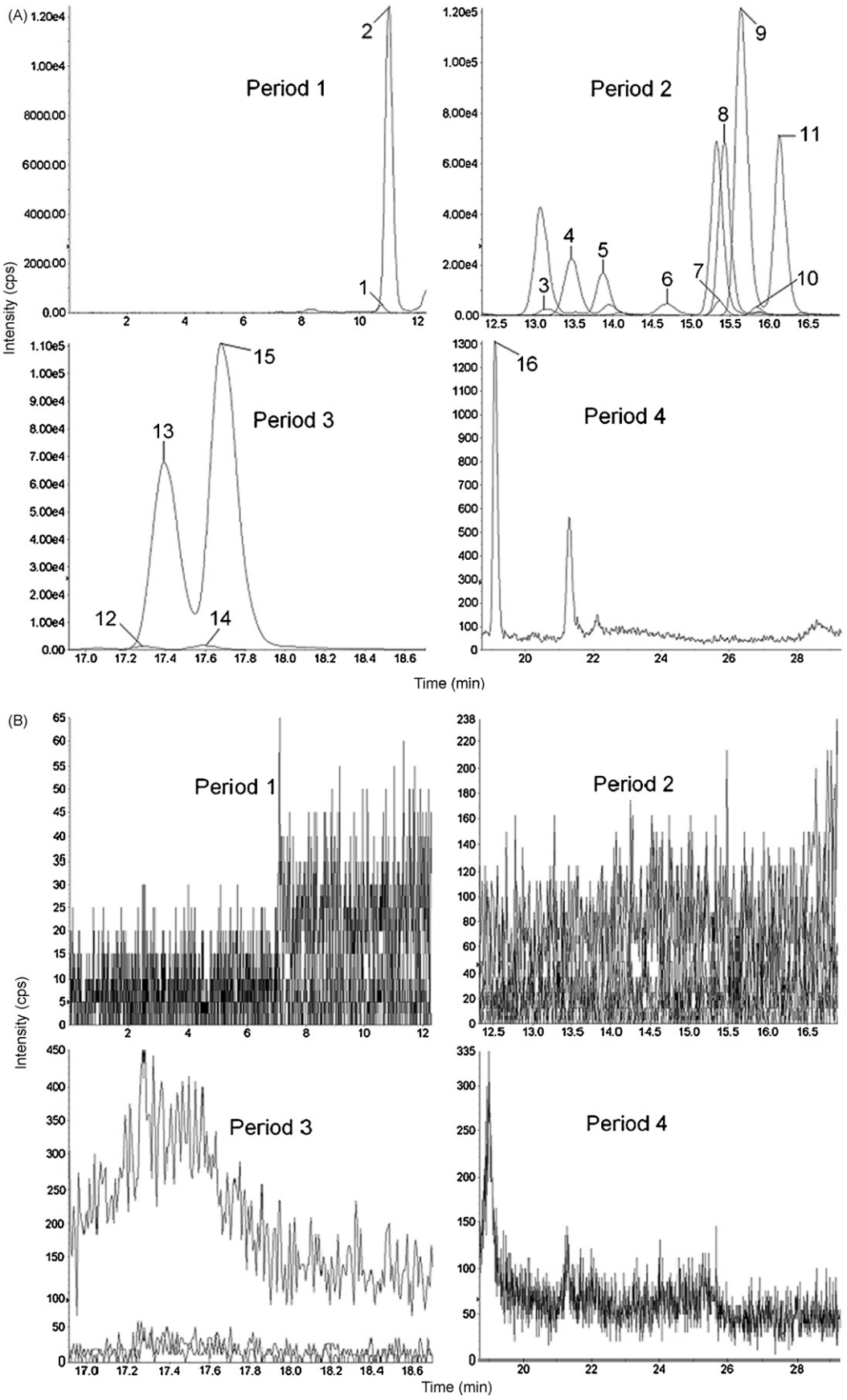


Fig. 2. Extracted ion chromatograms of pooled human plasma samples prior to (A) and after (B) the removal of endogenous bile acids using activated charcoal. (1) TUDCA, (2) GUDCA, (3) TCA, (4) GCA, (5) UDCA, (6) HDCA, (7) TCDCA, (8) CA, (9) GCDCA, (10) TDCA, (11) GDCA, (12) TLCA, (13) CDCA, (14) GLCA, (15) DCA, and (16) LCA.

bile acid. The third set was also prepared in three lots of real plasma but, after extraction, spiked with only internal standard. From peak areas of post-extraction spiked charcoal-purified plasma samples (A_{ps}), peak areas of post-extraction spiked real plasma samples (A_{rs}), and peak areas of endogenous bile acids in real plasma samples (A_{re}) as well as peak areas of internal standards (A_{IS}), one can calculate the relative matrix effect (RME) of real plasma compared with charcoal-purified plasma from:

$$\mathrm{RME} = \frac{A_{rs}/A_{IS} - A_{re}/A_{IS}}{A_{ps}/A_{IS}}$$

3. Results and discussion

3.1. Bile acid-free plasma preparation

Endogenous bile acids in human plasma or serum have been a problem for the validation of bile acid analysis methods. Several methods have used pooled human serum or plasma as a blank matrix to prepare calibration standard and quality control samples [10,12]. In these methods, LOD and LOQ were calculated based on the following formula: LOD = $3.3\sigma/S$ and LOQ = $10\sigma/S$, where σ is the standard deviation of peak area for each individual bile acid endogenous in pooled serum or plasma; S represents the slope of calibration curve. The σ values may vary considerably when different batches of pooled serum/plasma are used as a blank matrix, which contains varying concentrations of endogenous bile acids. Therefore LOD and LOQ obtained in such a way are not reproducible and consistent across laboratories or for a long period. To overcome this shortcoming, some other methods adopted the calibration standards in aqueous matrix to determine the LOD and LOQ, as well as the calibration curve [13,14]. Such methods fail to take into account the matrix effect of biological fluids, which is an important factor not only for the chromatographic separation and mass spectrometric signal, but also for the sample extraction process. In our study, activated charcoal was used to remove the endogenous bile acids from the pooled plasma. In the clinic, a charcoal column coupled with porcine hepatocytes has been utilized as a bioartificial liver to decrease the abnormally high levels of serum bile acids in patients with liver failure [16]. In the field of immunoassays, use of dextran-coated activated charcoal has been proven to be a simple way to remove solubilising detergents such as sodium dodecyl sulfate from the protein extract [17]. All these indicate that activated charcoal is an efficient adsorbent of amphiphilic compounds such as bile acids from the protein matrix. Our results demonstrate that activated charcoal efficiently removes (more than 99.99%; calculated from total peak areas) of the endogenous bile acids from human plasma, as shown in Fig. 2. This kind of bile acid-free plasma is a suitable blank matrix for the preparation of calibration standard and quality control samples for bile acid analysis.

3.2. Mass spectrometry optimization

The mass spectrometry parameters for each compound were automatically optimized using "quantitative optimization" functionality provided by Analyst 1.4. We found that at MRM mode, the highest sensitivity was achieved for all the bile acids when the product ion was set the same as the precursor ion. Such "unchanged" ion transition, however, could impair the specificity of some conjugated bile acids, such as TUDCA, TCDCA, TDCA, TLCA, and GLCA. For

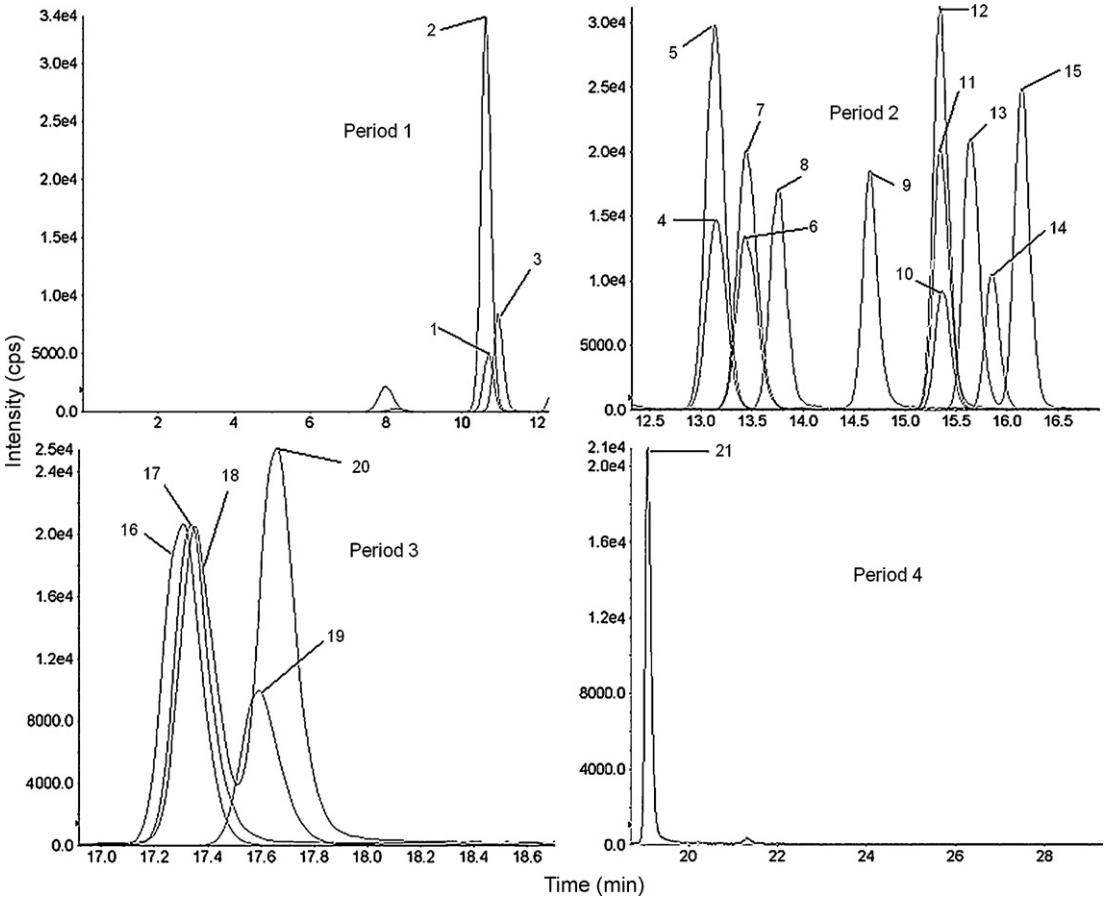


Fig. 3. Representative chromatograms of an extracted middle QC (0.12 μmol/L) sample. (1) TUDCA, (2) TUDCA-d_5, (3) GUDCA, (4) TCA, (5) TCA-d_4, (6) GCA, (7) GCA-^{13}C, (8) UDCA, (9) HDCA, (10) TCDCA, (11) CA, (12) TCDCA-d_4, (13) GCDCA, (14) TDCA, (15) GDCA, (16) TLCA, (17) CDCA-d_4, (18) CDCA, (19) GLCA, (20) DCA, and (21) LCA.

Table 2
Linear regression parameters generated for 6 different days (mean ± SD, $n = 6$).

Bile acid	Calibration range (μmol/L)	Linear regression parameters		
		Slope	Intercept	Correlation coefficient
UDCA	0.005–5	5.77 ± 0.170	0.00214 ± 0.00168	0.9982 ± 0.0005
GUDCA	0.005–5	4.56 ± 0.112	0.000436 ± 0.000697	0.9992 ± 0.0003
TUDCA	0.005–5	1.19 ± 0.034	0.000797 ± 0.000224	0.9996 ± 0.0004
CA	0.005–5	8.97 ± 0.189	0.0323 ± 0.00233	0.9974 ± 0.0008
GCA	0.005–5	8.47 ± 0.184	0.0153 ± 0.00278	0.9995 ± 0.0003
TCA	0.005–5	3.92 ± 0.219	0.0122 ± 0.00209	0.9981 ± 0.0010
CDCA	0.005–5	8.09 ± 0.182	0.0100 ± 0.00495	0.9993 ± 0.0002
GCDCA	0.005–5	10.62 ± 0.214	−0.000296 ± 0.00427	0.9989 ± 0.0008
TCDCA	0.005–5	2.38 ± 0.058	0.0132 ± 0.0037	0.9989 ± 0.0012
DCA	0.005–5	10.33 ± 0.354	0.00591 ± 0.00629	0.9993 ± 0.0002
GDCA	0.005–5	11.57 ± 0.225	0.00126 ± 0.00237	0.9989 ± 0.0010
TDCA	0.005–5	2.60 ± 0.048	0.00317 ± 0.00289	0.9990 ± 0.0003
LCA	0.005–5	4.62 ± 0.300	0.0121 ± 0.00389	0.9971 ± 0.0013
GLCA	0.005–5	2.82 ± 0.076	0.00226 ± 0.00096	0.9975 ± 0.0012
TLCA	0.005–5	4.74 ± 0.091	0.00424 ± 0.00090	0.9976 ± 0.0009
HDCA	0.005–5	5.75 ± 0.108	−0.000284 ± 0.00243	0.9988 ± 0.0006

these bile acids, a characteristic fragment generated from taurine or glycine moiety was chosen as the product ion to increase the specificity, e.g., 79.9 m/z for taurine-conjugated bile acids and 73.9 m/z for glycine-conjugated bile acids.

3.3. Selection of the analytical column

The use of Waters Atlantis®T3 column, which is designed to increase the retention of polar compounds, offered alternative selectivity compared to conventional C18 columns. The T3 column provided better separation and sharper peaks as well as less retention time shift as compared with a Waters Symmetry®C8 column (2.1 mm × 150 mm, 3.5 μm). The T3 column is more suitable for routine analysis of bile acids for a long period. The concentration of buffer salt is a crucial factor for the separation performance of T3 column. Higher concentrations of ammonium acetate could improve the resolution, but also result in a lower sensitivity. Our results demonstrated that 10 mmol/L of ammonium acetate represented a good balance between the resolution and sensitivity. Typical chromatograms of a middle QC sample are shown in Fig. 3.

3.4. Sample preparation

The direct SPE method was used to extract bile acids from plasma samples without any pre-precipitation of plasma protein using an organic solvent. This has been demonstrated to be an effective way for the extraction of endogenous bile acids [11,12]. We found components of washing solution to play essential roles in bile acid recovery and eluting efficiency. Using 0.05% formic acid solution as the first washing solution, as suggested previously [12], the relative SPE extraction recovery for taurine-conjugated bile acids ranged between 28.7% and 77.4%, compared to using pure water in the first washing step. In addition, the concentration of methanol in the second washing solution has been suggested to be critical for the extraction efficiency of bile acids [12]. Therefore, we also investigated the role of different methanol concentrations in the second wash step. However, our results were on the contrary to the previously described method [12] in that the SPE extraction recovery was only 2.3% for TCA, 2.6% for TUDCA, 11.2% for GUDCA, 27.9% for TCDCA, 42.8% for TDCA, and 63.2% for GCA when using 40% methanol in the second washing step. The very low recoveries suggested that a large amount of these bile acids might have been washed out by such a high concentration of methanol. This was supported by subsequent analysis of the second washing solution in which high concentrations of the corresponding bile acids were observed. Several factors may contribute to the discrepancy between our method and the previously described method [12]. One possible explanation is that the C18 SPE cartridges from different manufacturers may have differing extraction performances for bile

Table 3
Comparison of LOD and LOQ of the new HPLC–MS/MS method for bile acids with those of reported methods.

Bile acid	Ref. [10]	Ref. [11]	Ref. [12]		Ref. [19]	New method	
	LOD (μmol/L)	LOQ (μmol/L)	LOD (μmol/L)	LOQ (μmol/L)	LOQ (μmol/L)	LOD (μmol/L)	LOQ (μmol/L)
UDCA	0.004	0.010	0.003	0.009	0.051	0.00070	0.005
GUDCA	–	0.009	–	–	0.022	0.00094	0.005
TUDCA	0.004	0.005	0.003	0.009	0.080	0.00080	0.005
CA	0.002	0.006	0.001	0.003	0.049	0.00059	0.005
GCA	0.008	0.032	0.005	0.015	0.021	0.00087	0.005
TCA	0.006	0.021	0.006	0.018	0.078	0.00038	0.005
CDCA	0.003	0.027	0.002	0.006	0.051	0.00084	0.005
GCDCA	0.006	0.103	0.004	0.012	0.022	0.00067	0.005
TCDCA	0.006	0.011	0.005	0.015	0.080	0.00048	0.005
DCA	0.002	0.007	0.002	0.006	0.051	0.00085	0.005
GDCA	0.006	0.013	0.004	0.012	0.022	0.00050	0.005
TDCA	0.005	0.008	0.006	0.018	0.080	0.00067	0.005
LCA	0.001	0.007	0.002	0.006	0.053	0.00075	0.005
GLCA	0.005	0.021	0.005	0.015	0.023	0.00050	0.005
TLCA	0.004	0.001	0.003	0.009	0.083	0.00035	0.005
HDCA	–	–	0.003	0.009	–	0.00071	0.005
Sample volume (μL)	250	750	300		100	500	
Sample type	Human plasma	Human serum	Human serum		Rat serum	Human plasma	

acids; we used Bond Elut C18 cartridges from Varian whereas the previous method employed Chromabond C18 cartridges from Macherey-Nagel GmbH & Co. We have therefore, adapted the method by using pure water and 5% methanol for washing purposes, which resulted in a high recovery for all bile acids investigated.

3.5. Selection of internal standards

Deuterium-labeled internal standards are useful to improve the quantification of bile acids [11]. In our study, besides deuterium-labeled bile acids, a carbon 13 labeled bile acid, GCA-^{13}C, was also used as an internal standard. Either CDCA-d_4 or GCA-^{13}C performed well as an internal standard for both unconjugated bile acids and glycine-conjugated bile acids. For these two categories of bile acids, the final selection of CDCA-d_4 or GCA-^{13}C for each analyte therefore depended on the best accuracy and precision results obtained when the method was validated. However, taurine-conjugated bile acids are not compatible with CDCA-d_4 and GCA-^{13}C, especially TCA whose accuracy and precision were acceptable only when TCA-d_4 was used as the respective internal standard. In our study, three deuterium-labeled taurine-conjugated bile acids proved suitable as the internal standards for taurine-conjugated bile acids based on the validation results.

Table 4
Accuracy, precision, and recovery data of bile acid plasma quality control samples.

Bile acid	Nominal concentration (μmol/L)	Intra-day (n = 6)		Inter-day (n = 6)		SPE recovery (%)
		Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	
UDCA	0.012	94.0	4.9	98.1	2.6	97.3
	0.12	98.1	2.2	101.9	3.3	92.2
	1.2	98.8	2.8	101.4	2.0	92.7
GUDCA	0.012	94.6	4.5	99.7	2.9	96.8
	0.12	96.3	2.0	100.2	1.4	92.4
	1.2	101.0	1.6	101.8	2.1	90.4
TUDCA	0.012	96.3	3.3	98.5	2.0	91.4
	0.12	101.9	1.7	101.4	2.5	93.0
	1.2	99.9	2.0	102.1	4.1	91.1
CA	0.012	100.8	6.2	102.0	3.0	91.0
	0.12	103.5	4.2	104.3	1.7	92.0
	1.2	99.1	1.9	101.9	3.9	91.6
GCA	0.012	103.0	4.3	102.6	2.9	91.8
	0.12	100.5	2.6	100.3	1.6	90.6
	1.2	102.4	1.9	102.2	6.2	90.7
TCA	0.012	96.3	6.3	98.7	4.3	89.2
	0.12	105.2	2.0	104.1	3.1	94.2
	1.2	97.9	2.1	102.4	5.5	92.4
CDCA	0.012	103.0	6.0	103.2	5.7	94.7
	0.12	100.4	1.7	102.2	1.9	94.3
	1.2	102.4	2.2	101.5	5.7	92.1
GCDCA	0.012	94.9	2.4	101.0	4.9	94.6
	0.12	95.3	2.2	98.4	1.2	94.7
	1.2	101.6	2.7	100.6	2.4	92.3
TCDCA	0.012	102.0	3.9	99.5	5.0	90.5
	0.12	103.5	3.7	103.5	2.4	92.9
	1.2	101.9	3.1	103.8	5.7	91.2
DCA	0.012	106.7	3.5	102.4	7.3	92.2
	0.12	103.7	2.7	102.4	3.0	98.2
	1.2	108.7	2.4	106.9	6.2	95.8
GDCA	0.012	94.4	3.0	100.5	3.1	91.5
	0.12	96.5	2.4	99.4	1.0	92.2
	1.2	105.7	2.1	102.4	6.5	92.2
TDCA	0.012	101.1	8.2	104.0	3.9	95.7
	0.12	109.8	4.1	105.2	1.5	93.7
	1.2	105.2	3.1	101.5	2.8	90.7
LCA	0.012	114.8	9.8	103.0	2.0	97.6
	0.12	99.3	6.9	113.5	9.8	101.2
	1.2	108.2	5.3	113.1	7.8	100.1
GLCA	0.012	104.0	4.3	101.3	3.3	101.8
	0.12	103.1	3.7	106.3	3.1	99.1
	1.2	100.5	2.7	100.1	2.2	95.4
TLCA	0.012	109.6	3.3	103.9	5.5	102.6
	0.12	110.6	4.9	108.3	4.0	92.6
	1.2	98. 8	4.0	99.4	2.8	87.6
HDCA	0.012	97.1	6.4	101.9	4.1	89.3
	0.12	98.3	4.2	101.2	1.5	91.7
	1.2	102.8	7.3	96.9	5.9	91.0

Table 5
Stability results, expressed as percentage of control samples (mean ± SD, $n = 5$).

Bile acid	Short term		Freeze–thaw		Long term −20 °C		Long term −70 °C		Post-preparative	
	0.012 μmol/L	1.2 μmol/L	0.012 μmol/L	1.2 μmol/L	0.012 μmol/L	1.2 μmol/L	0.012 μmol/L	1.2 μmol/L	0.012 μmol/L	1.2 μmol/L
UDCA	94.2 ± 5.2	96.2 ± 0.8	96.9 ± 3.9	96.9 ± 1.5	99.5 ± 3.9	100.9 ± 4.0	92.9 ± 2.3	100.0 ± 2.2	102.3 ± 8.3	100.1 ± 4.9
GUDCA	95.4 ± 1.4	98.9 ± 1.3	95.2 ± 1.3	98.5 ± 1.6	100.2 ± 1.9	99.5 ± 2.2	99.0 ± 0.7	99.2 ± 2.2	99.7 ± 4.7	98.6 ± 1.3
TUDCA	99.7 ± 3.7	102.2 ± 0.5	100.1 ± 3.8	99.7 ± 0.8	97.0 ± 3.1	99.5 ± 1.5	95.6 ± 2.1	99.2 ± 1.3	97.7 ± 2.9	98.3 ± 2.1
CA	103.1 ± 3.1	103.1 ± 1.6	107.1 ± 1.7	101.4 ± 2.5	104.5 ± 2.3	100.3 ± 3.9	102.6 ± 4.2	101.2 ± 1.8	107.4 ± 7.0	100.7 ± 1.9
GCA	105.6 ± 2.4	104.5 ± 1.7	106.0 ± 4.4	103.4 ± 2.4	106.7 ± 2.3	99.5 ± 2.7	103.3 ± 1.2	98.8 ± 1.6	102.7 ± 0.6	101.6 ± 3.2
TCA	99.9 ± 3.6	101.5 ± 2.2	99.6 ± 4.1	101.9 ± 2.4	99.6 ± 4.8	100.7 ± 2.0	99.3 ± 2.3	100.8 ± 4.3	100.7 ± 4.0	102.1 ± 4.1
CDCA	99.8 ± 1.5	100.7 ± 2.7	104.1 ± 6.9	101.0 ± 1.9	98.7 ± 1.7	100.0 ± 3.9	92.2 ± 2.6	98.7 ± 2.0	100.9 ± 3.5	99.5 ± 3.1
GCDCA	106.1 ± 1.2	105.1 ± 2.7	104 ± 3.1	103.0 ± 2.4	99.0 ± 2.3	99.9 ± 2.7	100.3 ± 2.4	99.7 ± 1.3	108.8 ± 2.9	104.8 ± 4.3
TCDCA	95.0 ± 2.1	102.9 ± 3.3	98.2 ± 4.3	97.4 ± 3.4	98.0 ± 4.4	99.9 ± 2.3	96.1 ± 3.4	100.1 ± 3.3	98.4 ± 4.2	100.6 ± 4.5
DCA	104.5 ± 2.3	99.5 ± 2.3	100.8 ± 3.7	101.3 ± 3.1	99.7 ± 4.8	98.2 ± 2.8	92.8 ± 1.2	96.8 ± 1.9	100.0 ± 4.9	103.7 ± 2.4
GDCA	107.0 ± 2.2	104.2 ± 2.0	104.5 ± 2.9	104.3 ± 2.7	99.3 ± 2.7	100.6 ± 4.3	99.0 ± 1.2	96.8 ± 1.3	107.2 ± 4.9	101.5 ± 4.8
TDCA	102.5 ± 4.0	105.2 ± 2.8	103.3 ± 6.9	102.3 ± 2.1	100.1 ± 8.6	100.2 ± 2.1	98.4 ± 2.7	99.7 ± 2.6	104.6 ± 4.5	101.0 ± 6.2
LCA	105.7 ± 5.1	100.0 ± 1.3	109.3 ± 7.3	100.3 ± 2.1	98.5 ± 2.2	99.4 ± 0.9	100.9 ± 6.6	99.8 ± 1.7	101.3 ± 6.4	99.8 ± 2.7
GLCA	98.1 ± 6.0	98.7 ± 2.9	93.7 ± 3.7	101.7 ± 6.4	100.7 ± 7.1	98.8 ± 2.8	97.3 ± 3.3	97.7 ± 1.4	95.8 ± 8.4	104.1 ± 4.9
TLCA	101.5 ± 3.5	97.4 ± 2.0	101.2 ± 3.2	99.3 ± 5.1	108.5 ± 4.6	96.8 ± 1.9	102.6 ± 5.7	99.0 ± 3.6	96.1 ± 6.0	104.5 ± 10.3
HDCA	101.7 ± 4.9	105.1 ± 1.9	96.1 ± 4.2	102.0 ± 3.5	100.0 ± 3.3	101.1 ± 2.2	94.2 ± 7.2	97.1 ± 2.5	101.3 ± 7.2	101.5 ± 2.4

Short term, 6 h at room temperature; freeze–thaw, 3 cycles; long term, 2 months at −20 °C and −70 °C. Post-preparative, prepared samples in the autosampler (8 °C) for 48 h.

Table 6
Relative matrix effect (RME) of real plasma from three subjects compared with charcoal-purified plasma.

	Real plasma: lot 1 ($n = 6$)		Real plasma: lot 2 ($n = 6$)		Real plasma: lot 3 ($n = 6$)	
	Average (%)	CV (%)	Average (%)	CV (%)	Average (%)	CV (%)
UDCA	95.2	6.6	101.0	6.1	97.7	4.7
GUDCA	107.7	2.7	113.4	3.3	107.5	1.8
TUDCA	97.2	3.3	100.8	0.9	99.4	1.0
CA	88.7	5.4	107.9	5.1	107.0	3.4
GCA	95.3	4.2	104.0	1.2	95.0	4.0
TCA	103.5	3.4	103.5	1.6	105.0	2.6
CDCA	106.6	2.3	113.5	1.6	94.4	3.0
GCDCA	98.4	5.1	97.6	2.0	92.6	2.1
TCDCA	110.6	4.4	97.9	3.5	101.0	3.6
DCA	96.8	3.5	94.8	2.3	103.5	4.3
GDCA	88.7	4.3	96.3	3.1	104.0	4.0
TDCA	109.8	2.8	104.2	3.3	105.4	6.0
LCA	93.9	6.5	96.1	4.9	88.7	5.6
GLCA	91.9	2.4	96.1	3.3	92.0	2.3
TLCA	94.1	4.2	94.7	3.0	95.4	2.6
HDCA	103.1	2.7	96.7	6.2	97.5	3.5

3.6. Linearity, limit of quantification, accuracy, precision, and recovery

The lowest bias over the calibration curve range of 0.005–5 μmol/L was calculated by a regression analysis of the data to a linear fit with a weighting factor of $1/x^2$ for the ratio of the peak area of bile acids and the IS against the nominal concentration. The calibration curve was linear over the range tested with a mean correlation coefficient better than 0.995 for all the compounds. The results of the linear regression analysis for all

Table 7
Bile acid concentrations in normal human plasma.

Bile acid	Mean ± SD[a] (μmol/L)	Range (μmol/L)	Number of samples above LOQ
UDCA	0.141 ± 0.137	n.q.–0.795	323
GUDCA	0.265 ± 0.248	0.028–1.248	328
TUDCA	0.009 ± 0.012	n.q.–0.081	195
CA	0.411 ± 0.437	0.021–2.039	328
GCA	0.269 ± 0.197	0.021–0.928	328
TCA	0.060 ± 0.059	n.q.–0.377	317
CDCA	0.641 ± 0.626	0.015–2.885	328
GCDCA	0.805 ± 0.537	0.129–2.547	328
TCDCA	0.154 ± 0.115	0.024–0.537	328
DCA	0.475 ± 0.329	n.q.–1.633	317
GDCA	0.308 ± 0.241	n.q.–1.309	318
TDCA	0.066 ± 0.055	n.q.–0.263	313
LCA	0.008 ± 0.005	n.q.–0.025	210
GLCA	0.015 ± 0.014	n.q.–0.084	257
TLCA	n.q.	n.q.–0.075	92
HDCA	0.053 ± 0.032	0.008–0.162	328

n.q.: not quantifiable.
[a] Mean values of 328 fasting plasma samples from 65 healthy volunteers.

the 16 bile acids are summarized in Table 2. The LODs and LOQs of the present and the previously published methods [11,12,18,19] are summarized in Table 3.

Intra-day and inter-day accuracy and precision data are summarized in Table 4. Good intra- and inter-day precisions were obtained for all the bile acids studied. The intra-day and inter-day accuracy were in the ranges of 94.0–114.8% and 96.9–113.5%, respectively. CV values for intra-day analysis were between 1.6 and 9.8% and for inter-day analysis they were between 1.5 and 9.8%. The recovery of bile acids was calculated by comparing the bile acid standards, which were supplemented into the bile acid-free plasma before or after the SPE procedure. High recoveries were observed for all the bile acids as shown in Table 4, with the average value of 93.54%.

3.7. Bile acid stability

The freeze–thaw, short term, long term, and post-preparative stability of the bile acids were investigated and the results are summarized in Table 5. No significant degradation was found in these specific conditions. The data indicate that the bile acids are stable during at least three freeze and thaw cycles and the prepared samples for analysis are stable for at least 48 h at 8 °C. Moreover, no stability related problems are to be expected if bile acid plasma samples are stored at room temperature for up to 6 h or when they are stored in −20 °C or −70 °C for 2 months.

3.8. The difference of matrix effect between real plasma and charcoal-purified plasma

The difference of matrix effect between charcoal-purified plasma and real plasma was measured by the relative matrix effect of real plasma compared with charcoal-purified plasma, and RME obtained from three different lots of real plasma is shown in Table 6. The RME ranged between 88.7 and 113.5% for all 16 bile acids. There was no significant difference in matrix effect between charcoal-purified plasma and real plasma. Our results are consistent with a previous report, which observed no significant ion suppression of human serum on unconjugated, glycine- and taurine-conjugated bile acids [11]. In another study, the extraction recoveries of two stable-labeled isotope bile acids in charcoal-stripped serum were found to be similar to those in real rat serum [19]. These data indicate that the charcoal purification process did not change the properties of bile acid-free plasma as a biological matrix for the preparation of calibration standards and quality controls.

3.9. Application

This method was applied in determining the bile acid concentrations in 328 fasting plasma samples from 65 healthy volunteers. The study was approved by the Coordinating Ethics Committee of the Helsinki and Uusimaa Hospital District, and all volunteers gave a written informed consent. The overall range and mean values of each bile acid are shown in Table 7. Among the six unconjugated bile acids, CDCA, DCA, and CA were the most abundant ones in plasma. The plasma concentrations of their glycine-conjugates were about three to four times higher than those of their corresponding taurine-conjugates. These three major unconjugated bile acids, together with their glycine-conjugates, accounted for 79% of total plasma bile acids. These values are similar to those reported previously [10–11]. Representative chromatograms are shown in Fig. 4.

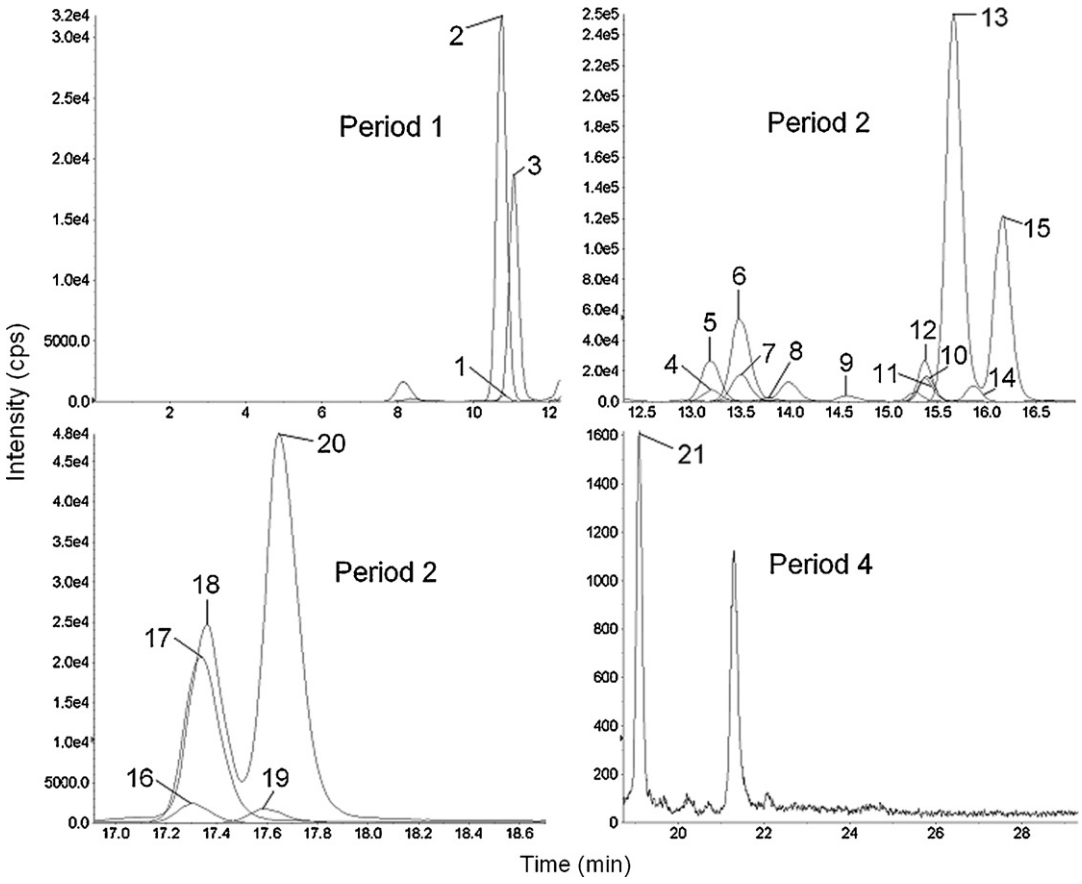


Fig. 4. Representative chromatograms of an extracted plasma sample of a healthy volunteer. (1) TUDCA, (2) TUDCA-d_5, (3) GUDCA, (4) TCA, (5) TCA-d_4, (6) GCA, (7) GCA-^{13}C, (8) UDCA, (9) HDCA, (10) TCDCA, (11) CA, (12) TCDCA-d_4, (13) GCDCA, (14) TDCA, (15) GDCA, (16) TLCA, (17) CDCA-d_4, (18) CDCA, (19) GLCA, (20) DCA, and (21) LCA.

4. Conclusion

We have developed a robust, sensitive, and simple method to determine the bile acid concentrations in human plasma. This method includes a direct SPE extraction procedure followed by HPLC–MS/MS detection. To our best knowledge, it is the first in the literature to describe the preparation of bile acid-free plasma, making the validation of bile acid analysis straightforward and reproducible.

Acknowledgements

This study was supported by grants from the Sigrid Jusélius Foundation (Helsinki, Finland) and the Helsinki University Central Hospital Research Fund (Helsinki, Finland).

References

[1] J.Y.L. Chiang, Am. J. Physiol. Gastrointest. Liver Physiol. 284 (2003) G349.
[2] A.F. Hofmann, Arch. Intern. Med. 159 (1999) 2647.
[3] A. Pellicoro, K.N. Faber, Aliment. Pharmacol. Ther. 26 (2007) 149.
[4] J.M. Ridlon, D.-J. Kang, P.B. Hylemon, J. Lipid Res. 47 (2006) 241.
[5] C.N. Falany, M.R. Johnson, S. Barnes, R.B. Diasio, J. Biol. Chem. 269 (1994) 19375.
[6] G. Zollner, M. Trauner, Clin. Liver Dis. 12 (2008) 1.
[7] C. Pauli-Magnus, P.J. Meier, Hepatology 44 (2006) 778.
[8] F. Courillon, M.F. Gerhardt, A. Myara, F. Rocchiccioli, F. Trivin, Eur. J. Clin. Chem. Clin. Biochem. 35 (1997) 919.
[9] A. Roda, F. Piazza, M. Baraldini, J. Chromatogr. B 717 (1998) 263.
[10] D. Tagliacozzi, A.F. Mozzi, B. Casetta, P. Bertucci, S. Bernardini, C. Di Ilio, A. Urbani, G. Federici, Clin. Chem. Lab. Med. 41 (2003) 1633.
[11] I. Burkard, A. von Eckardstein, K.M. Rentsch, J. Chromatogr. B 826 (2005) 147.
[12] L. Ye, S. Liu, M. Wang, Y. Shao, M. Ding, J. Chromatogr. B 860 (2007) 10.
[13] I. Bobeldijk, M. Hekman, J. de Vries-van der Weij, L. Coulier, R. Ramaker, R. Kleemann, T. Kooistra, C. Rubingh, A. Freidig, E. Verheij, J. Chromatogr. B 871 (2008) 306.
[14] K. Bentayeb, R. Batlle, C. Sanchez, C. Nerin, C. Domeno, J. Chromatogr. B 869 (2008) 1.
[15] FDA Guidelines for Industry: Bioanalytical Method Validation http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf, Accessed 9/9/2009.
[16] P. Pazzi, E. Morsiani, M.T. Vilei, A. Granato, J. Rozga, A.A. Demetriou, M. Muraca, Aliment. Pharmacol. Ther. 16 (2002) 1547.
[17] A.N. Malhas, R.A. Abuknesha, R.G. Price, J. Immunol. Methods 264 (2002) 37.
[18] E. Tessier, L. Neirinck, Z. Zhu, J. Chromatogr. B 798 (2003) 295.
[19] Y. Alnouti, I. Csanaky, C. Klaassen, J. Chromatogr. B 873 (2008) 209.