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Differentiated quantification of human bile acids in serum by high-performance liquid chromatography–tandem mass spectrometry

Ines Burkard, Arnold von Eckardstein, Katharina M. Rentsch *

Institute of Clinical Chemistry, University Hospital Zurich, Rämistrasse 100, CH-8091 Zurich, Switzerland

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Abstract

Determination of quantitative changes in the pattern of serum bile acids is important for the monitoring of diseases affecting bile acid metabolism. A sensitive and specific high-performance liquid chromatography (HPLC)–MS/MS method was developed for the differentiated quantification of unconjugated as well as glycine- and taurine-conjugated cholic, chenodeoxycholic (CDCA), deoxycholic (DCA), ursodeoxycholic (UDCA) and lithocholic acid (LCA) in serum samples. After solid-phase extraction and reversed-phase HPLC separation, detection of the conjugated bile acids was performed using electrospray ionization (ESI)-MS/MS and selected reaction monitoring mode, whereas unconjugated bile acids were determined by ESI-MS and selected ion monitoring mode. The within-day and between-day coefficients of variation were below 7% for all bile acids and the recovery rates of the extraction procedure were between 84.9 and 105%. The developed method was applied to a group of 21 healthy volunteers and preliminary reference intervals in serum were established. In patients with drug-induced cholestasis, an elevation of primary bile acids has been shown.

Keywords: Bile acids; High-performance liquid chromatography–tandem mass spectrometry; Electrospray ionization; Serum

1. Introduction

Bile acids, the major metabolites of cholesterol, play an important physiological role in the elimination of cholesterol from the body. In addition to being catabolic products of cholesterol, bile acids facilitate the absorption of dietary lipids and fat-soluble vitamins by formation of micelles. Most recently, bile acids have been identified as endogenous activators of a nuclear transcription factor, namely farnesoid-X-receptor, which plays a pivotal role in the homeostatic regulation of cholesterol and bile acid metabolism.

The major bile acids are cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA) and lithocholic acid (LCA). Most of these bile acids in peripheral blood are conjugated with the amino acids glycine and taurine on carbon 24 (Table 1), even though unconjugated forms can also be detected [1].

Under physiological conditions, serum bile acid concentrations are determined by hepatic extraction and intestinal absorption [2]. As a result of efficient first-pass extraction, bile acids are normally present at micromolar concentrations in the peripheral circulation. However, in hepatobiliary and intestinal diseases, the hepatic synthesis and clearance of bile acids and their intestinal absorption are disturbed, giving rise to both quantitative and qualitative changes in the pattern of serum bile acids. Therefore, the differentiated quantification of bile acids may be an important tool for the diagnosis, follow-up and prognostics of liver and intestinal disorders and other diseases affecting bile acid metabolism. In addition, bile acids have therapeutic applications, since CDCA and UDCA have been widely used for cholesterol gallstone dissolution [3] and, more recently, UDCA has been introduced for the therapy of cholestatic liver disease [4]. Thus, analysis of these bile acids may be useful for monitoring bile acid therapy in such diseases.

The separation and selective quantification of bile acids are challenging since they are present at micromolar concentrations in biological fluids, and have structural similarities as exemplified by the three isomeric forms CDCA, DCA and UDCA.

* Corresponding author. Tel.: +41 1 255 22 90; fax: +41 1 255 45 90.
E-mail address: rentsch@ikc.unizh.ch (K.M. Rentsch).

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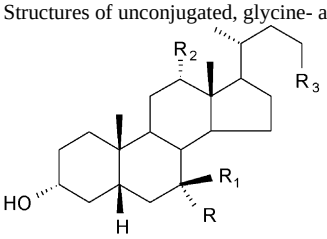
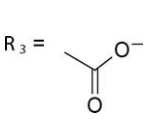
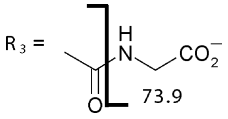
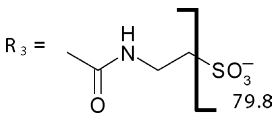
Table 1
Structures of unconjugated, glycine- and taurine-conjugated bile acids and their ions used in the HPLC–MS and HPLC–MS/MS analysis









	R	R_1	R_2	Unconjugated bile acids	Glycine-conjugated bile acids	Taurine-conjugated bile acids
				$[M-H]^-$ (m/z)	Precursor ion $[M-H]^-$ → product ion (m/z)	Precursor ion $[M-H]^-$ → product ion (m/z)
Cholic acid (CA)	−OH	−H	−OH	407.3	464.4 → 73.9	514.4 → 79.8
Chenodeoxycholic acid (CDCA)	−OH	−H	−H	391.3	448.4 → 73.9	498.4 → 79.8
Deoxycholic acid (DCA)	−H	−H	−OH	391.3	448.4 → 73.9	498.4 → 79.8
Ursodeoxycholic acid (UDCA)	−H	−OH	−H	391.3	448.4 → 73.9	498.4 → 79.8
Lithocholic acid (LCA)	−H	−H	−H	375.3	432.4 → 73.9	482.4 → 79.8

A number of analytical methods has been described for the quantitative determination of bile acids in various matrices [5–7]. High-performance liquid chromatography (HPLC) has become a popular technique for the analysis of individual bile acids in biological fluids. However, in combination with UV detection, it suffers from limited sensitivity and specificity due to the lack of a strong chromophore in the bile acid molecule. Various derivatization procedures have been described for modifying the bile acid structure to overcome the poor UV absorption [8]. However, such methods are time-consuming. In contrast, mass spectrometry (MS) represents a sensitive and selective detection technique that is effective for the analysis of biological samples. Gas chromatography (GC) [9] – most often combined with MS [10,11] – has been extensively used for bile acid quantification. However, GC requires a series of laborious pre-analytical procedures, such as preliminary group fractionation of bile acids based on their mode of conjugation (unconjugated, glycine- and taurine-conjugated), hydrolysis of conjugates, as well as preparation of volatile and thermostable derivatives. This complex sample pre-treatment represents the limiting step in GC/MS analysis. In contrast, coupling of HPLC with MS offers the advantage that bile acids can be analyzed directly in simple extracts as intact conjugates.

Several ionization techniques have been utilized for the HPLC–MS/MS analysis of bile acids. Early methods based on fast atom bombardment [12] and thermospray ionization [12–14] have largely been replaced by softer techniques, such as electrospray ionization (ESI). HPLC in combination with ESI-MS/MS has been applied to the determination of glycine- and taurine-conjugated bile acids in plasma [15,16] as well as in other biological fluids, such as human gallbladder bile [17]. Unconjugated bile acids, in contrast, are not yet directly detectable by conventional MS/MS, due to the lack of any specific fragment ions. However, an alternative MS/MS detection mode, which monitors the precursor ion itself as survivor of the fragmentation process, has recently been described [16].

We here describe a method that employs a simple and efficient clean-up procedure using reversed-phase C_{18} cartridges, utilization of serum calibrators, as well as the addition of seven isotopically labeled internal standards that were added before sample processing. These deuterated bile acid analogs behave almost identically to their analytes and differ in their mass-to-charge ratio (m/z).

The goal of this method is the differentiated quantification of the five most common bile acids as well as their glycine- and taurine-conjugated derivatives in serum samples. Previously, several specific transporters for bile acid uptake across the basolateral membrane into the hepatocyte as well as for the subsequent export via the canalicular membrane have been described. Since these transporters have distinct specificities for individual bile acid species [18,19], changes in the abundance of these transporters will probably lead to specific changes in the serum bile acid pattern.

Whereas secondary bile acids (DCA, LCA) are extremely toxic, an elevation of the primary bile acids (CA, CDCA) is considered as less dangerous. It has been published that in chronic cholestasis, the concentrations of primary bile acids are markedly increased, whereas the concentrations of secondary bile acids decrease [20].

2. Experimental

2.1. Chemicals and reagents

All chemicals and solvents were of the highest purity available. CA, CDCA, UDCA, LCA, glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDCA) were purchased from Fluka (Buchs, Switzerland). DCA, glycodeoxycholic acid (GDCA) and taurodeoxycholic acid (TDCA) were obtained from Sigma–Aldrich (Buchs, Switzerland). Glycine- and taurine-conjugated forms of CA, UDCA and LCA were purchased from Calbiochem (JURO Supply, Lucerne, Switzer-

land). The internal standards cholic-2,2,4,4-d_4 acid (CA-d_4) and chenodeoxycholic-2,2,4,4-d_4 acid (CDCA-d_4) were obtained from Isotec Inc. (Miamisburg, USA) and deoxycholic-2,2,4,4-d_4 acid (DCA-d_4), ursodeoxycholic-2,2,4,4-d_4 acid (UDCA-d_4), lithocholic-2,2,4,4-d_4 acid (LCA-d_4), glycocholic-2,2,4,4-d_4 acid (GCA-d_4) and glycochenodeoxycholic-2,2,4,4-d_4 acid (GCDCA-d_4) were obtained from CDN Isotopes (Quebec, Canada).

Ammonium carbonate, ammonium acetate and methanol were of HPLC grade and purchased from Scharlau (Barcelona, Spain). Acetic acid was obtained from Fluka (Buchs, Switzerland) and formic acid was obtained from Merck (Darmstadt,

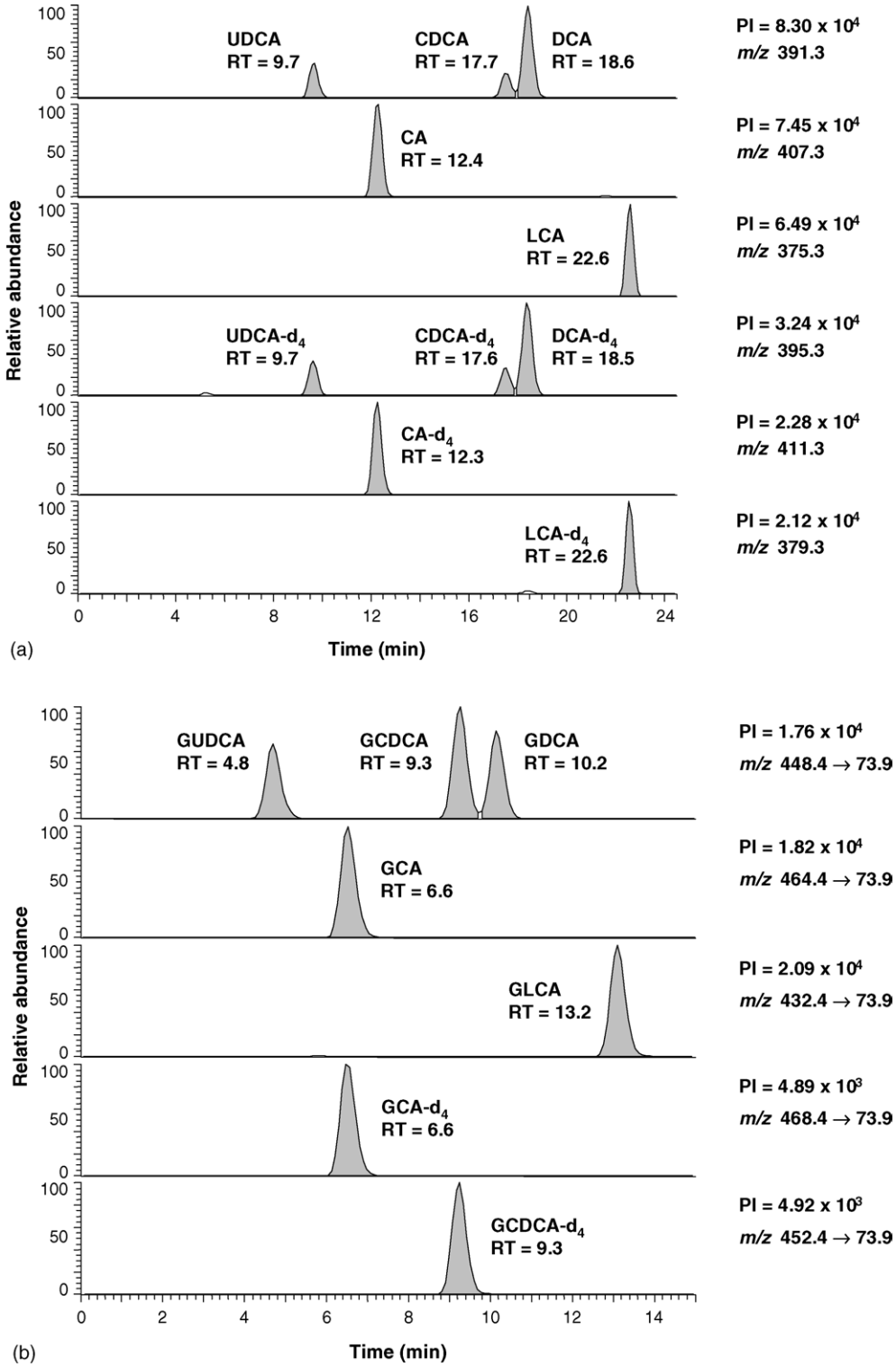


Fig. 1. Mass chromatograms of an extracted serum standard spiked with 6 μmol/l of each unconjugated (a); glycine-conjugated (b) and taurine-conjugated (c) bile acid. RT: retention time (measured in minutes), PI: peak intensity. Analyte abbreviations, see text.

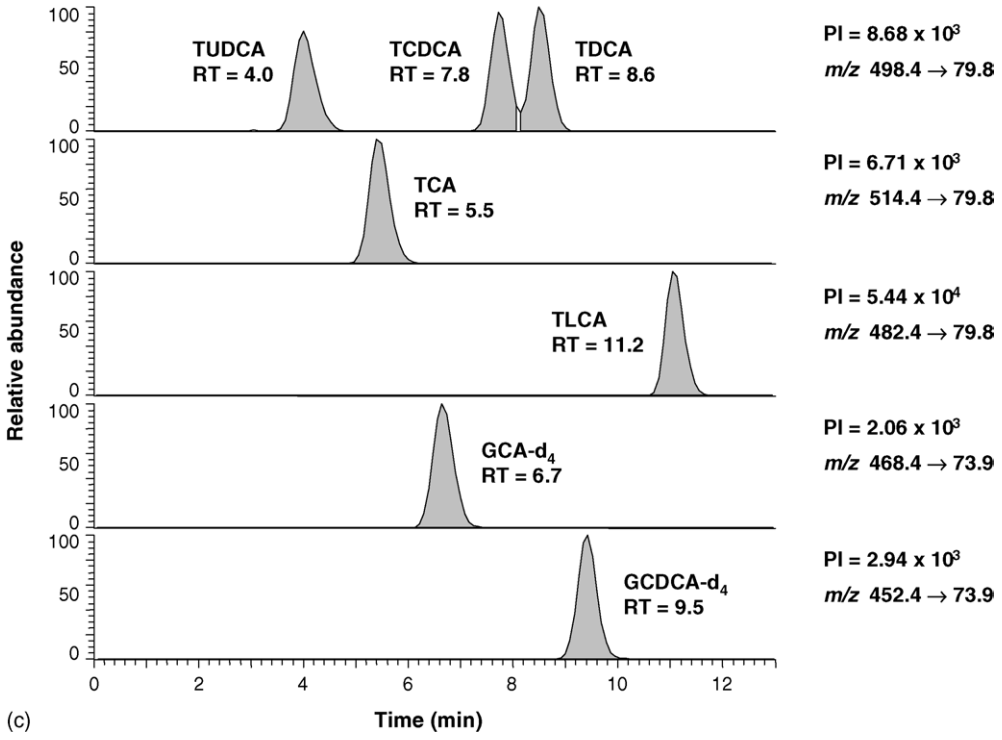


Fig. 1. (*Continued*).

Germany). Water (18 MΩ/cm) was purified in-house using a Maxima mk II water purification system (USF Elga, Labtec Services AG, Wohlen, Switzerland).

2.2. Preparation of standard solutions

Standard stock solutions were prepared by dissolving the respective compounds in methanol or water to obtain concentrations ranging from 20 to 100 mmol/l. These stock solutions were further diluted with methanol to obtain mixed working solutions containing 2, 0.2 mmol/l, 20 and 2 μmol/l of each bile acid, respectively.

2.3. Preparation of calibration standards in serum

For purposes of quantification, calibration samples were prepared by adding the appropriate amount of the corresponding bile acid working solution to serum of healthy volunteers (obtained from the Swiss Red Cross, Zurich, Switzerland).

2.4. Sample preparation

0.75 ml serum were mixed with 50 μl internal standard solution (containing 0.65 μg of CA-d_4, CDCA-d_4, DCA-d_4, UDCA-d_4, LCA-d_4, GCA-d_4 and GCDCA-d_4, respectively), followed by an addition of 0.75 ml 100 mM ammonium carbonate buffer pH 9.3. The clean-up procedure was performed by solid-phase extraction. The Varian Bond Elut C_{18} cartridges (200 mg) were pre-conditioned with 2 ml methanol, 2 ml water and 2 ml 100 mM ammonium carbonate buffer pH 9.3. The serum sample was then applied onto the cartridge and allowed to pass through the cartridge using gravity. Afterwards, the cartridge was washed with 2 ml water only using gravity and briefly dried under vacuum. The bile acids were desorbed with 3 ml methanol with gravity elution. The eluted substances were dried at 30 °C by evaporation (Rotavapor, Büchi, Flawil, Switzerland), and the residue dissolved in 100 μl methanol and mixed with 100 μl water. Thirty microlitres of the extracts were injected separately three times into the HPLC–MS system since unconjugated, glycine- and taurine-conjugated bile acids were quantified by three separate runs. Serum samples with concentrations higher than the standard curve were reinjected after dilution with methanol/water (1:1, v/v). Calculation of serum samples with concentrations below the first calibration standard (0.12 μmol/l) was performed by proportional conversion using the respective peak area ratios of the first calibration standard. Due to the large calibration range (0.12–60 μmol/l), the intercept contributes substantially to the peak area ratios of samples with low concentrations. Therefore, only the first five calibrators (0.12, 0.6, 1.2, 3.2 and 6 μmol/l) were used for the calculation of serum samples below 6 μmol/l. Samples between 6 and 60 μmol/l were calculated including additional 3 calibrators (12, 32 and 60 μmol/l).

2.5. HPLC–MS/MS

The HPLC system consisted of a Rheos 2000 pump (Flux Instruments, Basel, Switzerland) and a HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland). Separation was achieved using an Uptisphere C_{18} ODB, 5 μm particle size column (125 mm × 2 mm, Interchim, Montluçon, France),

protected with a guard column (10 mm × 2 mm, Interchim). The mobile phase consisted of 10 mM ammonium acetate buffer pH 4.5 containing 0.012% formic acid (eluent A) and methanol containing 0.012% formic acid (eluent B). The eluents were linearly changed from 30% A and 70% B to 20% A and 80% B within 9 min (glycine- and taurine-conjugated bile acids) and within 12 min (unconjugated bile acids), respectively. This final ratio was maintained for 2 min (glycine- and taurine-conjugated bile acids) and for 5 min (unconjugated bile acids), respectively. Afterwards, the eluents were changed to 5% A and 95% B within

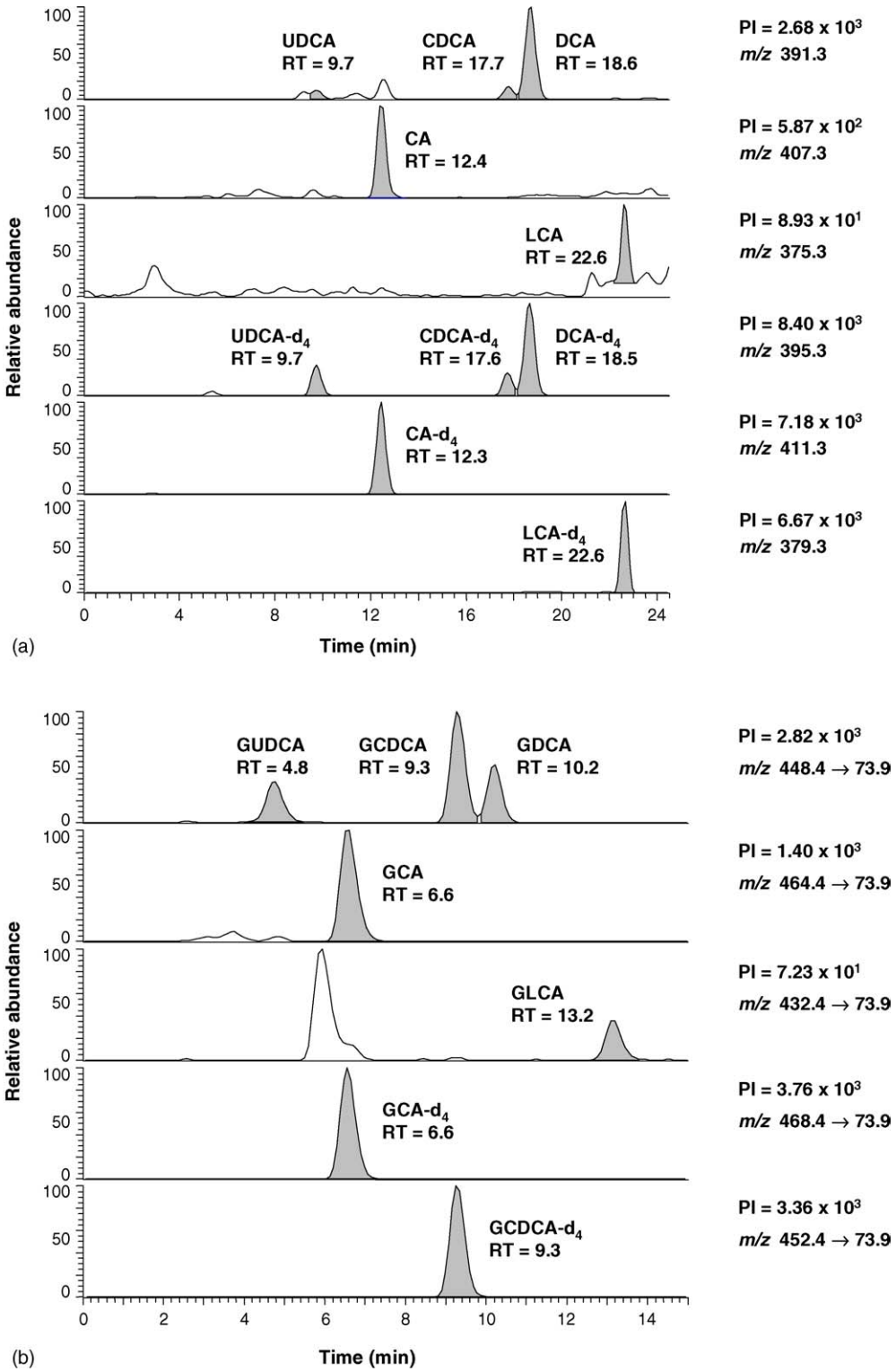


Fig. 2. Mass chromatograms of an extracted serum sample of a healthy volunteer. Unconjugated (a); glycine-conjugated (b) and taurine-conjugated (c) bile acids. RT: retention time (measured in minutes), PI: peak intensity. Analyte abbreviations, see text.

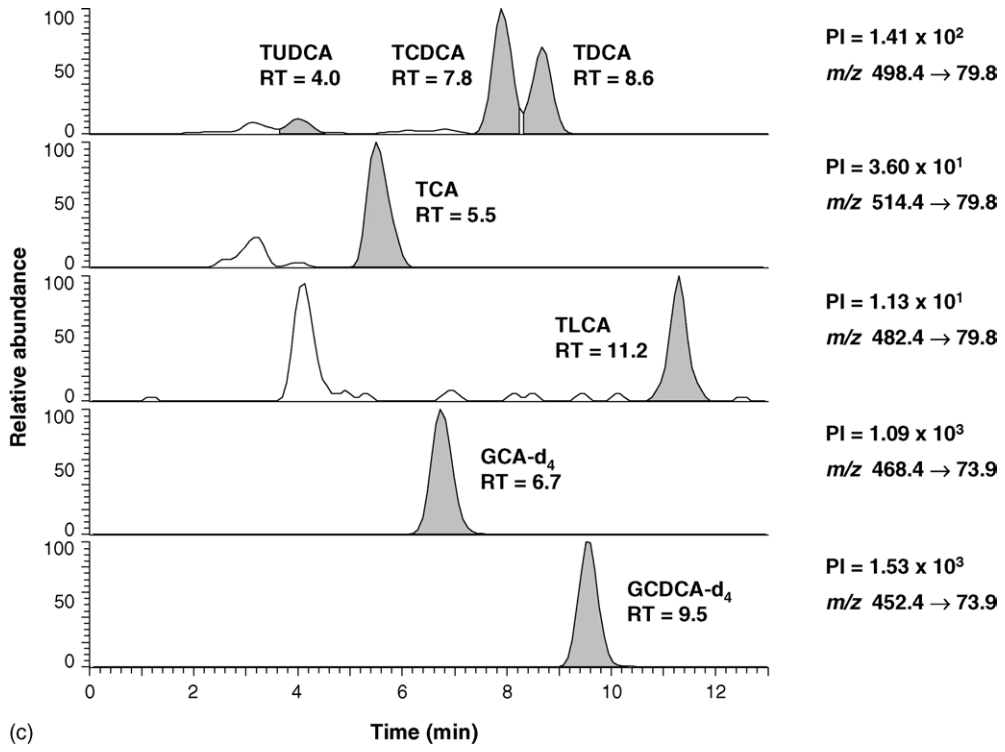


Fig. 2. (*Continued*).

1 min and finally adjusted to the original ratio of 30% A and 70% B within 8 min in order to enable equilibration of the column. Including the re-equilibration time, the resulting total runtime was 20 min for the glycine- and taurine-conjugated bile acids and 26 min for the unconjugated bile acids, respectively. The column flow-rate was 200 μl/min throughout the entire analysis.

The flow from the analytical column was split 1:2.5, with 80 μl/min directed to a Thermo-Quest Finnigan TSQ 7000 triple quadrupole mass spectrometer (San Jose, CA, USA), operating with an ESI ionization source in the negative mode. The capillary temperature was kept at 280 °C and the spray voltage was set at 4.5 kV. The sheath gas pressure was held at 50 psi. Instrument optimization was performed by direct infusion and manual tuning. Due to the lack of any specific and stable fragment ions, detection of the unconjugated bile acids was performed using single ion monitoring of the deprotonated molecules at *m/z* 407.3 (CA), *m/z* 391.3 (CDCA, DCA and UDCA) and *m/z* 375.3 (LCA) (Table 1). The respective deuterated internal standards were recorded at *m/z* 411.3 (CA-d_4), *m/z* 395.3 (CDCA-d_4, DCA-d_4 and UDCA-d_4) and *m/z* 379.3 (LCA-d_4). Detection of the glycine- and taurine-conjugated bile acids was performed using the selected reaction-monitoring mode. The transitions of the deprotonated molecules to their corresponding product ions were recorded at *m/z* 464.4 → 73.9 (GCA), *m/z* 448.4 → 73.9 (GCDCA, GDCA, GUDCA) and *m/z* 432.4 → 73.9 (GLCA) for the glycine-conjugated bile acids and at *m/z* 514.4 → 79.8 (TCA), *m/z* 498.4 → 79.8 (TCDCA, TDCA, TUDCA) and *m/z* 482.4 → 79.8 (TLCA) for the taurine-conjugated bile acids (Table 1). The respective deuterated internal standards were monitored at the transitions *m/z* 468.4 → 73.9 (GCA-d_4) and *m/z* 452.4 → 73.9 (GCDCA-d_4). The collision energy used was 40 eV for the glycine derivatives and 75 eV for the taurine derivatives.

2.6. Method validation

2.6.1. Linearity

Defined amounts of bile acid working solutions were added to human serum for preparation of eight standards ranging from 0.12 to 60 μmol/l for all bile acids. These standard samples were extracted as described above. The standard curves were plotted as the peak area ratio of the respective compound to the internal standard versus the concentration and then corrected for endogenous bile acids in the human serum by subtracting blank peak area ratios. To assess linearity, the line of best fit was determined by least square regression for the lower (0.12–6 μmol/l) and the higher calibration range (0.12–60 μmol/l), respectively.

2.6.2. Precision and accuracy

For the determination of the between-day and within-day precision, serum samples were prepared by addition of defined amounts of each bile acid. Calibration curves for all bile acids as well as two samples of different bile acid concentrations were analyzed eight times on the same day (within-day precision) and once on eight different days (between-day precision), respectively. After correction for endogenous bile acids in the human serum, the accuracy of the method was assessed by expressing the mean of the assayed concentration as percent of the weight-in concentration.

2.6.3. Recovery

To determine the efficiency of the extraction procedure, all bile acids were analyzed without addition of the internal standards. Defined amounts of all analytes were added to 0.75 ml of serum ("sample") or into a clean vial ("standard"), respectively. The samples were extracted six times as described above, and the standards simply evaporated. Afterwards both the samples and standards were dissolved in methanol and water. The average peak area of all bile acids of the sample was compared to the corresponding peak area of the standard.

2.6.4. Ion suppression

To evaluate the effect of the serum matrix on the signal intensities of the analytes, a solution containing either unconjugated, glycine-or taurine-conjugated bile acids (10 μmol/l) was infused into the column effluent via a T-valve at a flow-rate of 80 μl/min. While the solution was being infused, an extracted serum sample was injected.

2.6.5. Limit of quantification, limit of detection

The limit of quantification (LOQ) of the different analytes was calculated using a signal-to-noise ratio of 5 from data obtained for spiked serum samples. In addition, the coefficients of variation at this concentration were calculated for each bile acid.

The limit of detection (LOD) of the different analytes was calculated using a signal-to-noise ratio of 3 from data obtained for spiked serum samples.

2.7. Determination of preliminary reference intervals

Twenty-one healthy volunteers (10 males and 11 females) from the staff of our institute, between 19 and 57 years of age (mean age, 34 years) and with serum cholesterol concentration of 4.5 ± 1.0 mmol/l of (mean ± S.D.) were studied for the determination of reference intervals. The blood samples were taken in the morning after an overnight fast. Informed consent was obtained from all volunteers.

Table 2
Least square regression data of the different bile acids ($n = 8$)

Bile acid	Calibration range (μmol/l)	Linear regression parameters		
		Slope (mean ± S.D.)	Intercept (mean ± S.D.)	Correlation coefficient (mean ± S.D.)
CA	0.12–6	0.5476 ± 0.0113	0.0036 ± 0.0115	0.9973 ± 0.0010
	0.12–60	0.4881 ± 0.0125	0.1423 ± 0.0934	0.9966 ± 0.0022
CDCA	0.12–6	0.3790 ± 0.0077	0.0005 ± 0.0078	0.9976 ± 0.0011
	0.12–60	0.3401 ± 0.0198	0.1094 ± 0.0788	0.9975 ± 0.0016
DCA	0.12–6	0.4098 ± 0.0086	−0.0031 ± 0.0054	0.9969 ± 0.0011
	0.12–60	0.3616 ± 0.0116	0.0957 ± 0.0723	0.9972 ± 0.0019
UDCA	0.12–6	0.4247 ± 0.0109	0.0039 ± 0.0076	0.9974 ± 0.0011
	0.12–60	0.3765 ± 0.0136	0.1260 ± 0.0778	0.9972 ± 0.0016
LCA	0.12–6	0.5226 ± 0.0153	−0.0043 ± 0.0096	0.9967 ± 0.0012
	0.12–60	0.4680 ± 0.0167	0.0896 ± 0.1152	0.9970 ± 0.0020
GCA	0.12–6	0.6070 ± 0.0157	0.0056 ± 0.0119	0.9976 ± 0.0013
	0.12–60	0.5450 ± 0.0238	0.1901 ± 0.1260	0.9965 ± 0.0033
GCDCA	0.12–6	0.5203 ± 0.0090	0.0047 ± 0.0030	0.9974 ± 0.0013
	0.12–60	0.4607 ± 0.0254	0.1794 ± 0.1321	0.9959 ± 0.0039
GDCA	0.12–6	0.4452 ± 0.0118	−0.0015 ± 0.0053	0.9973 ± 0.0013
	0.12–60	0.3984 ± 0.0266	0.1464 ± 0.1305	0.9959 ± 0.0040
GUDCA	0.12–6	0.3910 ± 0.0150	−0.0032 ± 0.0062	0.9972 ± 0.0011
	0.12–60	0.3602 ± 0.0214	0.0971 ± 0.1012	0.9958 ± 0.0041
GLCA	0.12–6	0.7479 ± 0.0424	0.0210 ± 0.0278	0.9975 ± 0.0010
	0.12–60	0.6668 ± 0.0325	0.2884 ± 0.2147	0.9957 ± 0.0039
TCA	0.12–6	0.5768 ± 0.0486	0.0168 ± 0.0152	0.9981 ± 0.0012
	0.12–60	0.4957 ± 0.0484	0.2339 ± 0.1463	0.9930 ± 0.0094
TCDCA	0.12–6	0.4589 ± 0.0324	0.0222 ± 0.0119	0.9981 ± 0.0011
	0.12–60	0.3919 ± 0.0352	0.2742 ± 0.0985	0.9956 ± 0.0042
TDCA	0.12–6	0.5098 ± 0.0541	0.0171 ± 0.0184	0.9978 ± 0.0015
	0.12–60	0.4412 ± 0.0493	0.2434 ± 0.1356	0.9959 ± 0.0049
TUDCA	0.12–6	0.4529 ± 0.0373	0.0205 ± 0.0135	0.9982 ± 0.0011
	0.12–60	0.3932 ± 0.0346	0.2207 ± 0.0957	0.9959 ± 0.0041
TLCA	0.12–6	3.1350 ± 0.2289	0.0670 ± 0.0845	0.9975 ± 0.0015
	0.12–60	2.6423 ± 0.1900	1.6448 ± 0.8075	0.9964 ± 0.0040

Table 3
Precision and accuracy data of the different bile acids

Bile acid	Concentration (μmol/l)	*n*	Mean (μmol/l)	S.D. (μmol/l)	CV (%)	Accuracy (%)
CA	Within-day					
	0.6	8	0.589	0.029	5.0	98.2
	32	8	31.2	1.2	3.9	97.4
	Between-day					
	0.6	8	0.609	0.018	3.0	102
	32	8	30.5	0.8	2.7	95.3
CDCA	Within-day					
	0.6	8	0.579	0.027	4.7	96.4
	32	8	32.3	1.5	4.7	101
	Between-day					
	0.6	8	0.601	0.025	4.2	100
	32	8	30.7	1.5	5.0	96.0
DCA	Within-day					
	0.6	8	0.590	0.021	3.6	98.3
	32	8	31.3	1.2	3.7	97.9
	Between-day					
	0.6	8	0.602	0.015	2.4	100
	32	8	30.3	0.9	3.0	94.6
UDCA	Within-day					
	0.6	8	0.614	0.028	4.6	102
	32	8	32.5	2.1	6.4	102
	Between-day					
	0.6	8	0.609	0.019	3.2	102
	32	8	31.3	1.9	5.9	97.8
LCA	Within-day					
	0.6	8	0.603	0.025	4.1	101
	32	8	32.1	1.6	4.9	100
	Between-day					
	0.6	8	0.596	0.023	3.8	99.3
	32	8	30.4	1.0	3.2	95.1
GCA	Within-day					
	0.6	8	0.602	0.024	3.9	100
	32	8	30.3	1.6	5.1	94.8
	Between-day					
	0.6	8	0.604	0.023	3.8	101
	32	8	30.3	0.8	2.5	94.7
GCDCA	Within-day					
	0.6	8	0.582	0.031	5.3	97.0
	32	8	30.8	1.5	5.0	96.2
	Between-day					
	0.6	8	0.616	0.020	3.2	103
	32	8	30.5	1.0	3.1	95.2
GDCA	Within-day					
	0.6	8	0.599	0.025	4.1	99.8
	32	8	32.6	1.6	5.0	102
	Between-day					
	0.6	8	0.610	0.011	1.8	102
	32	8	30.6	0.9	3.0	95.5
GUDCA	Within-day					
	0.6	8	0.579	0.027	4.7	96.4
	32	8	30.9	1.4	4.6	96.5
	Between-day					
	0.6	8	0.604	0.017	2.9	101
	32	8	30.4	0.8	2.6	94.9

Table 3 (*Continued*)

Bile acid	Concentration (μmol/l)	*n*	Mean (μmol/l)	S.D. (μmol/l)	CV (%)	Accuracy (%)
GLCA	Within-day					
	0.6	8	0.604	0.021	3.5	101
	32	8	31.2	1.4	4.4	97.3
	Between-day					
	0.6	8	0.591	0.022	3.8	98.6
	32	8	30.6	1.1	3.5	95.6
TCA	Within-day					
	0.6	8	0.605	0.025	4.1	101
	32	8	31.0	1.6	5.0	96.9
	Between-day					
	0.6	8	0.610	0.014	2.3	102
	32	8	30.0	0.9	2.9	96.8
TCDCA	Within-day					
	0.6	8	0.608	0.043	7.0	101
	32	8	32.7	0.9	2.7	102
	Between-day					
	0.6	8	0.611	0.012	2.0	102
	32	8	31.1	0.7	2.3	97.1
TDCA	Within-day					
	0.6	8	0.602	0.033	5.5	100
	32	8	32.3	0.8	2.4	101
	Between-day					
	0.6	8	0.609	0.015	2.4	102
	32	8	31.0	1.2	3.9	96.9
TUDCA	Within-day					
	0.6	8	0.601	0.028	4.6	100
	32	8	31.9	1.1	3.3	99.8
	Between-day					
	0.6	8	0.614	0.016	2.7	102
	32	8	30.5	1.0	3.2	95.2
TLCA	Within-day					
	0.6	8	0.600	0.026	4.3	99.9
	32	8	32.7	0.8	2.5	102
	Between-day					
	0.6	8	0.614	0.013	2.1	102
	32	8	31.6	1.1	3.6	98.8

3. Results and discussion

3.1. HPLC–MS/MS

A crucial parameter in the method development was the chromatographic separation of unconjugated, glycine- and taurine-conjugated UDCA, CDCA and DCA. Since these three analytes – either unconjugated, glycine- or taurine-conjugated – have the same molecular mass and also behave identically through the ionization process, a sufficient peak resolution was a prerequisite for further analyses.

Although both the elution gradient and the detection mode were identical, glycine- and taurine-conjugated bile acids were quantified by two separate runs. Thereby, the run is shared by a smaller number of analytes with consequential fewer mass transitions as well as a longer dwell time. This finally results in a better signal-to-noise ratio for each analyte.

To account for any analyte losses throughout sample preparation and injection, quantification of bile acids was accomplished by the use of deuterium-labeled internal standards. This is an improvement as compared to a publication which also described quantification of glycine- and taurine-conjugated bile acids in plasma by HPLC–MS/MS, however, without the use of any internal standards [16]. Since taurine-conjugated deuterated analogs are not commercially available at present, GCA-d_4 and GCDCA-d_4 were used as internal standards for determination of taurine-conjugated bile acids. This did obviously not affect the quantification of taurine-conjugated compounds, as demonstrated by excellent precision and accuracy data in the course of the validation procedure with different serum matrices.

In contrast to conjugated bile acids, product ion scans of the unconjugated bile acids did not give any specific and stable product ions that could be used for selected reaction-monitoring mode. Therefore, quantification of the unconjugated compounds

was performed using single ion monitoring mode of the deprotonated molecules. The major common product ion resulting from fragmentation of the deprotonated glycine-conjugated bile acid molecules is observed at *m/z* 73.9 and arises from the loss of the deprotonated glycine molecule. The product ion suitable for reliable quantification of the taurine-conjugated bile acids is observed at *m/z* 79.8 and represents a fragment of the taurine moiety.

Representative mass chromatograms of a serum standard for the unconjugated, glycine- and taurine-conjugated bile acids are shown in Fig. 1. All peaks were symmetric and sufficiently resolved. The retention times are indicated on the respective chromatograms. Representative mass chromatograms of a serum sample of a healthy volunteer are shown in Fig. 2. As has been shown previously, 3-sulfated [21] and 3-glucuronidated [22] derivatives do not give the same precursor ions as described in Fig. 1, and therefore, do not interfere with our assay.

3.2. Method validation

3.2.1. Linearity

Since the intercept increased as more standard samples were included in the high calibration range, separate standard curves were determined for the lower (0.12–6 μmol/l) and the higher (0.12–60 μmol/l) calibration range. The standard curves were linear in both calibration ranges for all bile acids. Least square regression data of the calibration curves are summarized in Table 2.

3.2.2. Precision and accuracy

The results of precision and accuracy experiments are summarized in Table 3. For within-day analysis, the coefficients of variation (CV) were between 2.4 and 7.0%, whereas the values for between-day coefficients of variation were between 1.8 and 5.9%. The lower CV sometimes found from day to day does not significantly differ from the intra day CV (Student's *t*-test, data not shown). The precision data were in the same range as those reported by authors who likewise used HPLC–MS/MS for bile acid quantification in human blood [16]. Accuracies varied between 94.8 and 102% for within-day analysis, and between 94.6 and 103% for between-day analysis, respectively.

3.2.3. Recovery

The recoveries of the different bile acids are shown in Table 4. The mean recovery rates of the extraction procedures were between 84.9 and 105%. Unconjugated, glycine- as well as taurine-conjugated LCA showed markedly lower recoveries than the other analytes, presumably because of their more lipophilic nature.

Using our quantification method, we found recovery rates which were lower than those reported by authors who just performed protein precipitation before analysis of plasma bile acids with HPLC–MS/MS [16]. However, when comparing our results with a publication in which glycine- and taurine-conjugated bile acids were determined in human bile after sample clean-up by solid-phase extraction as well [17], we found significantly higher recovery rates.

Table 4
Recoveries of the different bile acids ($n = 6$)

Bile acid	Concentration (μmol/l)	Recovery (%)
CA	0.6	98.3
	32	103
CDCA	0.6	91.8
	32	96.0
DCA	0.6	94.8
	32	104
UDCA	0.6	99.6
	32	102
LCA	0.6	86.5
	32	89.6
GCA	0.6	94.3
	32	98.1
GCDCA	0.6	88.9
	32	95.7
GDCA	0.6	93.3
	32	95.0
GUDCA	0.6	96.3
	32	98.2
GLCA	0.6	84.9
	32	90.9
TCA	0.6	91.1
	32	101
TCDCA	0.6	87.3
	32	105
TDCA	0.6	96.8
	32	95.5
TUDCA	0.6	95.0
	32	101
TLCA	0.6	87.1
	32	94.6

3.2.4. Ion suppression

The effect of the serum matrix on the signal intensities of bile acids is shown in Fig. 3. In the timeframes relevant for the detection of the unconjugated, glycine- and taurine-conjugated bile acids, no significant matrix effects were observed.

During the last years, ion suppression as become generally known as one important factor that can affect the quantitative performance of a mass spectrometer, especially when operating with an ESI interface. However, to our knowledge, this has not been evaluated in published HPLC–ESI-MS/MS methods for determination of unconjugated, glycine- and taurine-conjugated bile acids in biological fluids so far.

3.2.5. Limit of quantification, limit of detection

The LOQ (signal-to-noise ratio, 5) for serum samples of 0.75 ml were between 0.001 and 0.103 μmol/l (Table 5). The coefficients of variation were below 20% for all compounds at the respective LOQ (data not shown).

The LOD (signal-to-noise ratio, 3) for serum samples of 0.75 ml were between 0.001 and 0.062 μmol/l (data not shown).

With the exception of CDCA and GCDCA, which showed higher values for the LOD, the levels for the LOD determined with the present method were comparable to literature data [16]. As far as CDCA and GCDCA are concerned, the limit of quantification is still well below the preliminary reference intervals of the respective bile acids.

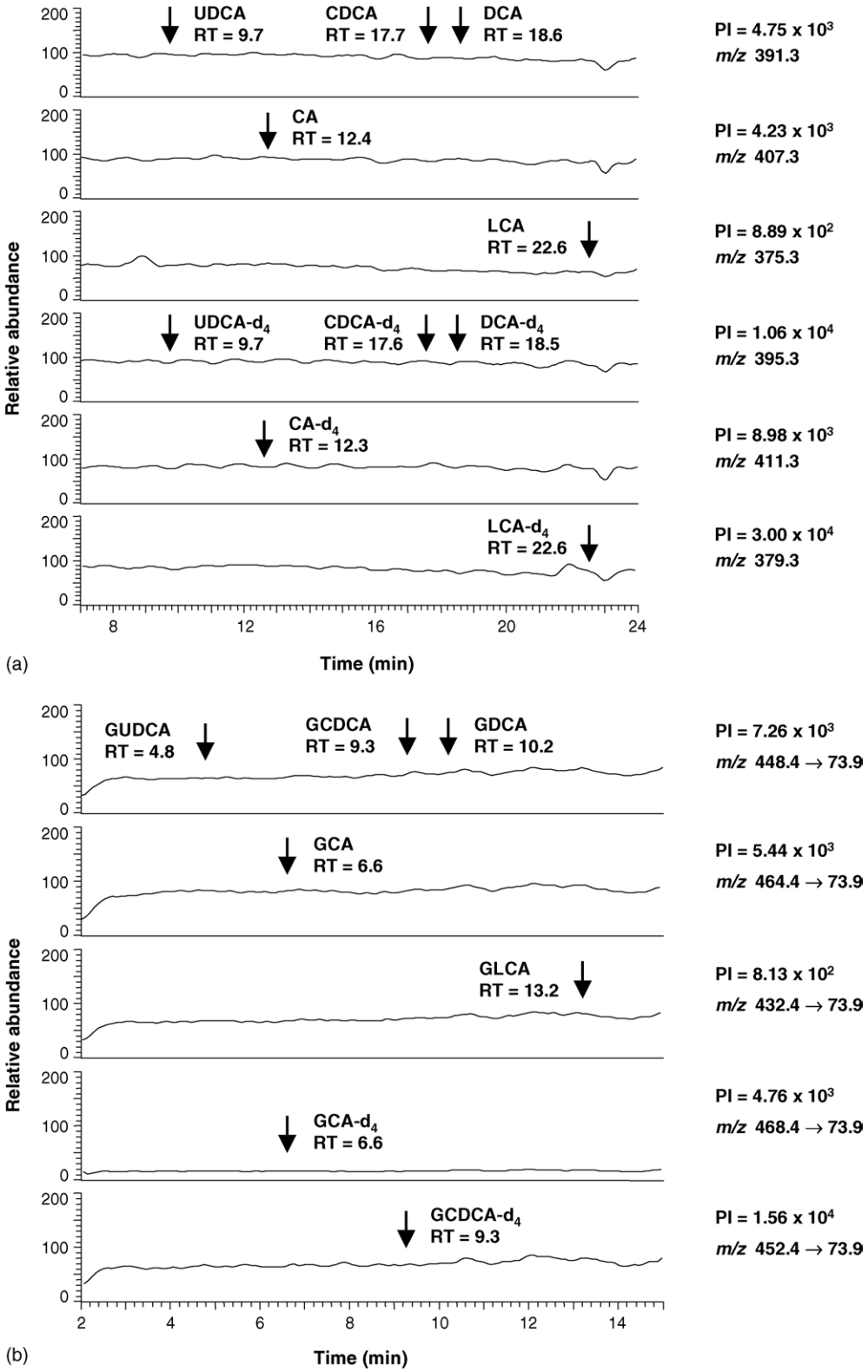


Fig. 3. Effect of the serum matrix on the signal intensities of unconjugated (a); glycine-conjugated (b) and taurine-conjugated (c) bile acids. RT: retention time (measured in minutes) is indicated by an arrow for each bile acid, PI: peak intensity. Analyte abbreviations, see text.

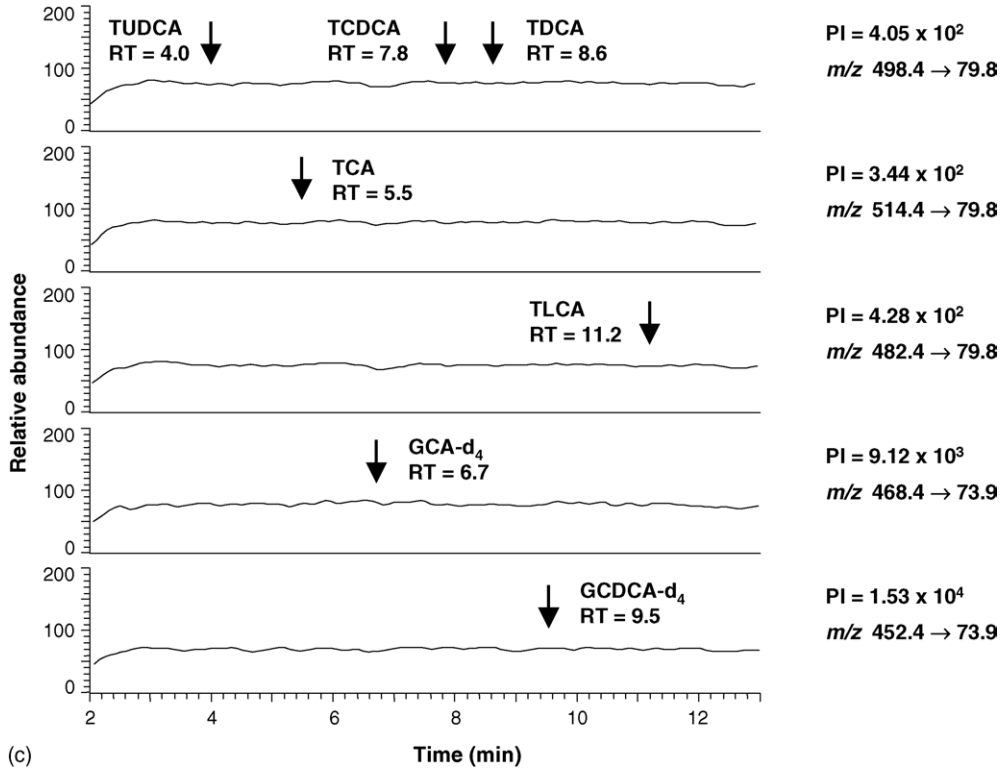


Fig. 3. (*Continued*).

3.3. Determination of preliminary reference intervals

Although several methods for bile acid determination have been published over the last two decades, only few publications specified individual bile acid concentrations in a reasonable number of healthy, adult subjects. Using our method, we determined bile acid concentrations (median and range) in serum samples from 21 healthy volunteers (Table 5). These preliminary reference intervals were in the same range as those recently reported using plasma samples from 10 healthy volunteers [16]. Two earlier publications [23,24], which only determined serum values for CA, CDCA and DCA, found levels that were higher than our reported median values. Yet, it is important to note that all studies, including our own, analyzed a very limited number of subjects, which decreases the reliability and significance of the reported median levels.

Table 5
Limit of quantification and preliminary reference intervals (median; range) of the different bile acids

Bile acid	Limit of quantification (μmol/l)	Preliminary reference interval ($n = 21$) (μmol/l)
CA	0.006	0.094; 0.009–0.497
CDCA	0.027	0.247; 0.028–1.258
DCA	0.007	0.356; 0.013–1.596
UDCA	0.010	0.031; n.d.[a]–0.371
LCA	0.007	0.017; n.d.[a]–0.034
GCA	0.032	0.143; 0.058–0.971
GCDCA	0.103	0.873; 0.142–3.421
GDCA	0.013	0.295; n.d.[a]–0.909
GUDCA	0.009	0.172; n.d.[a]–0.796
GLCA	0.021	n.d.[a]; n.d.[a]–0.060
TCA	0.021	0.029; n.d.[a]–0.433
TCDCA	0.011	0.074; 0.022–0.620
TDCA	0.008	0.062; n.d.[a]–0.177
TUDCA	0.005	n.d.[a]; n.d.[a]–0.023
TLCA	0.001	n.d.[a]; n.d.[a]–0.003

[a] n.d.: not detected.

3.4. Clinical applications

To yield some clinical experience, we also analyzed some samples from patients. In two patients treated with antibiotics, drug-induced cholestasis was diagnosed based on conventional clinical chemistry parameters (i.e. elevated plasma activities and concentrations of alkaline phosphatase, gamma glutamyltransferase, and bilirubine, respectively). Both patients had highly elevated concentrations of total bile acids in serum (289 and 149 μmol/l, reference range: <6 μmol/l). Differentiated bile acid analysis revealed tremendously elevated serum concentrations of glycine- and taurine-conjugated CA and CDCA (Table 6) as compared to our preliminary reference intervals (Table 5), whereas serum concentrations of other bile acids were either normal or less pronouncedly elevated. According to published data [20], these patients appear to be at lower risk for severe hepatic injury since only the concentrations of primary bile acids are increased.

Several carefully controlled studies will have to be performed in the next few years to elucidate the diagnostic and prognostic value of different free and conjugated bile acids in

Table 6
Concentrations of the different bile acids in serum samples from two patients with drug-induced cholestasis (under treatment with antibiotics)

Bile acid	Serum samples (μmol/l)	
CA	2.54	0.059
CDCA	2.31	0.061
DCA	1.88	n.d.[a]
UDCA	2.00	0.012
LCA	1.92	n.d.[a]
GCA	135	30.2
GCDCA	72.9	20.9
GDCA	1.57	0.049
GUDCA	0.939	0.255
GLCA	1.93	n.d.[a]
TCA	42.8	52.6
TCDCA	17.6	44.2
TDCA	1.87	0.072
TUDCA	2.64	0.711
TLCA	1.81	0.002

[a] n.d.: not detected.

various cholestatic liver diseases and intestinal malabsorption syndromes.

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