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Quantification of the 15 major human bile acids and their precursor 7α-hydroxy-4-cholesten-3-one in serum by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Bile acids are increasingly gaining attention since they were discovered to be activators of the transcription factor farnesoid X receptor (FXR) in addition to their well-established role in dietary lipid emulsification. Moreover, the differential activation potency of bile acids on FXR, which is due to structural variation of the ligands, generates the need for new analytical tools that are sensitive and specific enough to quantify the individual species of this complex class of compounds. Because bile acids undergo enterohepatic circulation, the additional assessment of a bile acid precursor as a marker for bile acid biosynthesis is used to differentiate between newly synthesised bile acids and bile acids reabsorbed from the intestine. This paper describes two new methods using liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the quantification of the major unconjugated bile acids in human serum (cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid and ursodeoxycholic acid) with their glycine- and taurine-conjugates as well as their precursor 7α-hydroxy-4-cholesten-3-one (C4). Intra- and inter-day variation was less than 12% and accuracy was between 84% and 102% for all analytes. Extraction recovery was between 78% and 100% for the bile acids whereas it was 62% for C4 and limit of quantification values ranged from 2 nmol/l to 50 nmol/l for all compounds. These two methods have the practical advantage of requiring low sample volume (100 μl serum for each method) and identical eluents, stationary phase as well as ionisation technique, so that they can be used in a combined way. Moreover, they provide information on the composition of the bile acid pool on one hand and on the relative amount of newly synthesised bile acids on the other, which taken together, gives new insights in the investigation of bile acid metabolism.

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1. Introduction

Bile acids play an important physiological role in the elimination of cholesterol from the human body since they represent the main degradation pathway for this poorly soluble lipid. Moreover, their emulsifying properties are essential for digestion of dietary fat and lipophilic vitamins. In the last decade, bile acids were additionally discovered to be natural ligands of the farnesoid X receptor (FXR) [1–3], a nuclear transcription factor through which they regulate their own biosynthesis via a negative feedback. Moreover, it is now well established that FXR activation by bile acids results in the regulation of various genes involved not solely in bile acid homeostasis but also in cholesterol, triglyceride and glucose metabolism [4–9]. Therefore, bile acids have gained much attention since they might be of interest as biomarkers or as pharmacological targets in several intestinal and metabolic conditions ranging from Crohn's disease to metabolic syndrome [5,10–12].

Biosynthesis of bile acids mainly takes place in the liver. Oxidation of the cholesterol steroid core by the microsomal cholesterol 7α-hydroxylase (CYP7A1) in the classical pathway represents the rate-limiting step in bile acid biosynthesis and leads to the formation of the precursor 7α-hydroxycholesterol. The final products of the enzymatic cascade are the two primary bile acids cholic acid and chenodeoxycholic acid, which are conjugated to an amino acid,

Abbreviations: C4, 7α-hydroxy-4-cholesten-3-one; CA, cholic acid; CDCA, chenodeoxycholic acid; CYP7A1, cholesterol 7α-hydroxylase; DCA, deoxycholic acid; ESI, electrospray ionisation; FDA, Food and Drug Administration; FXR, farnesoid X receptor; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GLCA, glycolithocholic acid; GUDCA, glycoursodeoxycholic acid; LCA, lithocholic acid; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LOQ, limit of quantification; QC, quality control; SPE, solid-phase extraction; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

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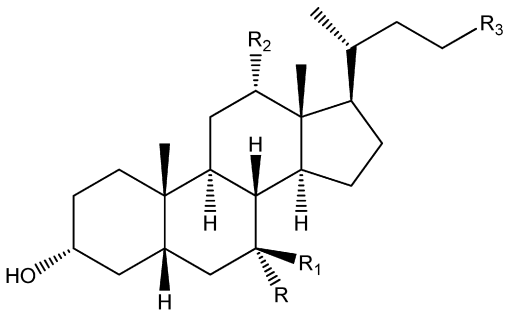
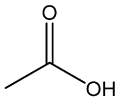
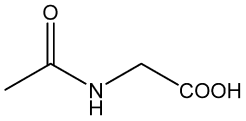
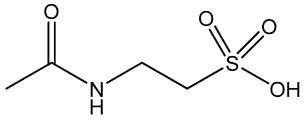
(a)



Bile acid	R	R_1	R_2
CA	-OH	-H	-OH
UDCA	-H	-OH	-H
CDCA	-OH	-H	-H
DCA	-H	-H	-OH
LCA	-H	-H	-H
	unconjugated		
R_3			
	glycine-conjugated		
R_3			
	taurine conjugated		
R_3			

(b)

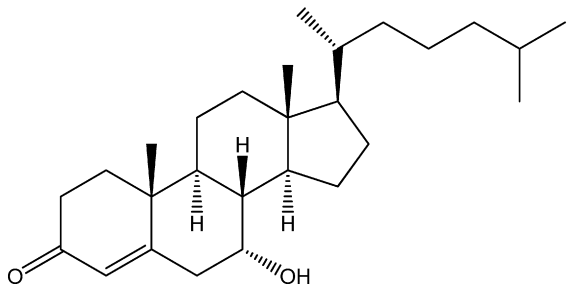


Fig. 1. Structures of (a) the major unconjugated, glycine- and taurine-conjugated bile acids and (b) the bile acid precursor C4.

usually glycine or taurine, prior to excretion. Once they are in the intestine, a fraction of conjugated cholic acid and chenodeoxycholic acid are deconjugated and/or converted to secondary bile acids by intestinal bacteria. The mixture of primary and secondary bile acids formed in this way is reabsorbed from the small intestine and returns to the liver via the portal system in order to be recycled and secreted again into the bile, thus completing the enterohepatic circulation [5]. The bile acid pool of an organism subsequently contains twenty or more different species varying in their abilities to activate FXR, in their susceptibilities to be metabolised by the intestinal bacteria flora and in their half-lives in the enterohepatic circulation. In order to have a better overview on the amount of newly synthesised bile acids out of cholesterol (in contrast to bile acids found in the circulation after reabsorption from the intestine), quantification of a bile acid precursor may be used. In this regard, it was shown that plasma levels of 7α-hydroxycholesterol, the precursor for the classical pathway, reflect bile acid synthesis in vivo [13]. However, this steroid may be formed from cholesterol as an artefact due to lipid peroxidation and therefore a sample might contain undefined amounts of 7α-hydroxycholesterol of non-enzymatic origin. 7α-hydroxy-4-cholesten-3-one (C4), the immediate enzymatic product of 7α-hydroxycholesterol is not formed by lipid peroxidation and was shown to reflect the activity of CYP7A1 in humans as well. Therefore, it can also be used as a marker for bile acid biosynthesis [14–15].

Because of the complexity of this class of compounds and their low concentration in the peripheral circulation as a result of efficient first-pass extraction by the liver, quantification of bile acids requires analytical techniques which offer high sensitivity and specificity. Moreover, due to the broad spectrum of activation potencies of the various bile acids on FXR, differentiated quantification of these metabolites is needed in order to understand the molecular mechanisms in which bile acids are involved. This was achieved as chromatographic techniques were coupled to mass spectrometry and has led to the expansion of this field of research. In contrast to gas chromatography, which requires laborious sample preparation including hydrolysis and derivatisation, liquid chromatography coupled with mass spectrometry (LC–MS(/MS)) nowadays represents the most convenient alternative for this type of analysis, as demonstrated by the number of new quantification methods developed in the past years for bile acids [16–24] and C4 [12,14,25–26].

In this paper, we present two new LC–MS/MS methods for the combined quantification in serum of the 15 major human bile acids on one hand and their precursor for the classical pathway, namely C4, on the other (Fig. 1). The main assets of these methods are that they require low sample volume (100 μl for each method) and that they enable a detailed description of bile acid metabolism since they provide information not only on the composition of the circulating bile acid pool but also on the amount of newly synthesised bile acids via C4 quantification. Moreover, these methods can be combined since they require identical eluents for the mobile phase, an identical stationary phase and the same ionisation technique. This simplifies the procedure and spares hardware modifications because neither the column nor the source needs to be changed between different types of samples. Together with another method previously developed in our group for the quantification of the bile acid precursor for the alternative biosynthesis pathway, namely 27-hydroxycholesterol [27], these tools allow a detailed description of bile acid metabolism in humans.

2. Experimental

2.1. Chemicals

Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), taurochenodeoxycholic acid (TCDCA) and taurodeoxycholic acid (TDCA) were purchased from Sigma-Aldrich (Buchs, Switzerland), whereas the glycine- and taurine-conjugates of CA

(GCA and TCA), LCA (GLCA and TLCA) and UDCA (GUDCA and TUDCA) were obtained from Calbiochem (Darmstadt, Germany). The deuterated bile acid internal standards cholic acid-2,2,4,4-d_4 (CA-d_4) and chenodeoxycholic acid-2,2,4,4-d_4 (CDCA-d_4) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA), whereas deoxycholic acid-2,2,4,4-d_4 (DCA-d_4), lithocholic acid-2,2,4,4-d_4 (LCA-d_4), ursodeoxycholic acid-2,2,4,4-d_4 (UDCA-d_4), glycocholic acid-2,2,4,4-d_4 (GCA-d_4) and glycochenodeoxycholic acid-2,2,4,4-d_4 (GCDCA-d_4) were purchased from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada).

The bile acid precursor C4 was purchased from Toronto Research Chemicals, Inc. (North York, Ontario, Canada) and its deuterated internal standard 7α-hydroxy-4-cholesten-3-one-25,26,26,26,27,27,27-d_7 (C4-d_7) was obtained from Medical Isotopes, Inc. (Pelham, NH, USA).

The salts for buffers and methanol were of HPLC grade. Activated charcoal and ammonium acetate were purchased from Sigma-Aldrich (Buchs, Switzerland), ammonium carbonate was obtained from Scharlau Chemie (Barcelona, Spain), methanol was obtained from Honeywell Burdick & Jackson (Seelze, Germany) and hydrochloric acid 32% (GR for analysis) was obtained from Merck (Darmstadt, Germany). Human serum from healthy volunteers was obtained from PAA Laboratories (Pasching, Austria) and purified water was obtained using a central water purification installation from Burkhalter (Worblaufen, Switzerland).

2.2. Preparation of a bile acid/C4-free serum pool

In order to construct calibration curves in a matrix free from endogenous bile acids and C4 but similar to the real sample matrix, serum was mixed overnight with 100 mg/ml activated charcoal, known for its high adsorption capacity. The mixture was then centrifuged at 13,000 × *g* and 10 °C for 20 min, after which the supernatant was collected and centrifuged again. After three centrifugation cycles, the supernatant was filtered with a 0.45 μm filter and stored at −20 °C until analysis. Prior to use, newly prepared charcoal treated serum batches were tested for the absence of any remaining endogenous bile acids or C4 (data not shown).

2.3. Preparation of standard stock solutions

Bile acid standard stock solutions were prepared by dissolving the respective 15 bile acids in the appropriate amount of methanol in order to obtain individual stock solutions of 5 μmol/ml. The 15 individual stock solutions were then pooled together to obtain a 100 μmol/l solution in methanol, which was further diluted with purified water to obtain standard stock solutions containing 25, 10, 5, 0.5 and 0.05 μmol/l of each bile acid, respectively.

A 1 μmol/ml C4 standard stock solution was prepared by dissolving C4 in the appropriate amount of acetonitrile. This solution was further diluted with methanol to obtain 0.5, 0.1, 0.05 and 0.01 μmol/l standard stock solutions.

2.4. Preparation of deuterated internal standard stock solutions

The 7 deuterated bile acids were dissolved in the appropriate amount of methanol in order to obtain individual 3 μmol/ml solutions. These solutions were then pooled together to obtain a 75 μmol/l solution in methanol, which was further diluted with water to obtain an internal standard solution containing 15 μmol/l of each deuterated bile acid.

A 1 μmol/ml C4-d_7 solution was prepared in acetonitrile and further diluted with methanol to obtain a 0.1 μmol/l C4-d_7 internal standard solution.

2.5. Sample preparation

Calibrators and quality control samples (QCs) were prepared by adding the appropriate amount of the different standard stock solutions to 100 μl of charcoal treated serum and extracting these using the corresponding sample preparation procedure described below.

2.5.1. Sample preparation for the 15 bile acids

100 μl serum sample (respectively 100 μl charcoal treated serum with defined amounts of the appropriate standard stock solution) were mixed with 100 μl ammonium carbonate buffer 100 mM, pH 9.3. The volume was completed to 700 μl with purified water and 30 μl of the 15 μmol/l deuterated bile acids internal standard solution were added, after which the samples were vortexed.

Sample preparation was performed by solid-phase extraction (SPE) with 200 mg Bond Elut C18 cartridges from Varian, Inc. (Palo Alto, CA, USA) using a procedure previously described by our group [24], after minor adaptations (mainly reduction of sample volume). Pre-conditioning of the cartridges was achieved by activation with 2 ml methanol followed by 2 ml purified water and finally 2 ml ammonium carbonate buffer 100 mM, pH 9.3, upon which the sample was loaded onto the cartridge and allowed to pass through it by gravity. Thereafter, the cartridge was washed with 2 ml purified water and dried under vacuum. The retained compounds were eluted with 3 ml of methanol using gravity and the samples were evaporated at 30 °C under vacuum using a rotavapor from Büchi (Flawil, Switzerland). The residue was dissolved in 60 μl methanol and mixed with 30 μl ammonium acetate buffer 10 mM, pH 6.5, upon which the sample was centrifuged at 13,400 × *g* and 10 °C for 10 min. The supernatant was collected and 30 μl were injected into the LC–MS system for analysis.

2.5.2. Sample preparation for C4

100 μl serum sample (respectively 100 μl charcoal treated serum with defined amounts of the appropriate standard stock solution) were mixed with 500 μl purified water and 50 μl of the 0.1 μmol/l C4-d_7 internal standard solution. The methanol volume (including 50 μl internal standard solution and various amounts of standard stock solutions) was completed to 150 μl in all samples in order to avoid variations due to different methanol contents while loading the samples on the SPE cartridges. Finally, 60 μl of 1 M hydrochloric acid were added to all samples and these were vortexed immediately afterwards in order to loosen the C4 fraction presumably bound to plasma proteins.

Sample preparation was performed using the same solid-phase extraction cartridges as for the bile acids sample preparation. Preconditioning of the cartridges was achieved by activation with 2 ml methanol followed by 2 ml purified water, upon which the sample was loaded onto the cartridge and allowed to pass through it by gravity. The cartridge was washed and samples were eluted and evaporated as described in the bile acid sample preparation procedure (Section 2.5.1). The residue was dissolved in 80 μl methanol and mixed with 10 μl ammonium acetate buffer 10 mM, pH 6.5, upon which the sample was centrifuged at 13,400 × *g* and 10 °C for 10 min. The supernatant was collected and 30 μl were injected into the LC–MS system for analysis.

2.6. Quantification

The calibration domain for the 15 bile acids ranged from 0.02 μmol/l to 50 μmol/l and could be extended to 100 μmol/l without affecting linearity by adding two additional calibrators if required (for highly concentrated samples). Due to the large calibration range, the intercept contributed significantly to the peak area ratios of low concentrated samples. Therefore sam-

Table 1
Parent and product ions of the analytes with individually optimised detection parameters (tube lens and collision energy) as well as corresponding internal standard used for quantification. If more than one fragment was reported, the sum of the signals of the different product ions was used for quantification.

Bile acids	Parent ion $[M-H]^-$ (m/z)	Product ion $[M-H]^-$ (m/z)	Tube lens (V)	Collision energy (eV)	Internal standard
CA	407.2	407.2	−135	15	CA-d_4
CDCA	391.2	391.2	−135	15	CDCA-d_4
DCA	391.2	391.2	−135	15	DCA-d_4
LCA	375.2	375.2	−125	20	LCA-d_4
UDCA	391.2	391.2	−135	15	UDCA-d_4
GCA	464.2	464.2	−120	15	GCA-d_4
	464.2	74.4	−120	38	
GCDCA	448.2	448.2	−110	15	GCDCA-d_4
	448.2	404.1	−110	32	
	448.2	386.1	−110	35	
	448.2	74.4	−110	35	
GDCA	448.2	448.2	−110	15	DCA-d_4
	448.2	404.1	−110	32	
	448.2	386.1	−110	35	
	448.2	74.4	−110	35	
GLCA	432.2	432.2	−105	10	LCA-d_4
	432.2	388.1	−105	34	
	432.2	74.4	−105	38	
GUDCA	448.2	448.2	−110	15	UDCA-d_4
	448.2	404.1	−110	32	
	448.2	386.1	−110	35	
	448.2	74.4	−110	35	
TCA	514.2	514.2	−145	15	CA-d_4
	514.2	124.2	−145	46	
	514.2	107.2	−145	50	
TCDCA	498.2	498.2	−135	20	CDCA-d_4
	498.2	124.2	−135	47	
	498.2	107.2	−135	50	
TDCA	498.2	498.2	−135	20	DCA-d_4
	498.2	124.2	−135	47	
	498.2	107.2	−135	50	
TLCA	482.2	482.2	−135	15	LCA-d_4
	482.2	124.2	−135	45	
	482.2	107.2	−135	50	
TUDCA	498.2	498.2	−135	20	UDCA-d_4
	498.2	124.2	−135	47	
	498.2	107.2	−135	50	
CA-d_4	411.2	411.2	−135	15	–
CDCA-d_4	395.2	395.2	−135	15	–
DCA-d_4	395.2	395.2	−135	15	–
LCA-d_4	379.2	379.2	−125	15	–
UDCA-d_4	395.2	395.2	−135	15	–
GCA-d_4	468.2	468.2	−120	15	–
	468.2	74.3	−120	38	
GCDCA-d_4	452.2	452.2	−110	10	–
	452.2	74.3	−110	38	
C4	**Parent ion $[M+H]^+$ (m/z)**	**Product ion $[M+H]^+$ (m/z)**	**Tube lens (V)**	**Collision energy (eV)**	**Internal standard**
C4	401.3	383.3	108	16	C4-d_7
	401.3	177.0	108	23	
C4-d_7	408.3	390.3	109	16	–
	408.3	177.0	109	24	

ples with concentrations above 5 μmol/l were calculated using all six (respectively eight) calibrators 0.02, 0.5, 5, 10, 30 and 50 μmol/l (respectively 75 and 100 μmol/l), whereas samples with concentrations below 5 μmol/l were calculated using only the first three calibrators. Samples with concentrations below 0.02 μmol/l as well as samples for which the peak area ratio was below the absolute value of the intercept of its respective three points calibration curve were calculated by proportional conversion using the respective peak area ratio of the first calibration standard. Since deuterated internal standards with identical structure were not commercially available for each of the 15 bile acids at the time the method was developed, those lacking an identical deuterated homologue were attributed the corresponding unconjugated deuterated bile acid (Table 1). Although the deuterated bile acids used as internal standards were of high purity, they contained a fraction of corresponding undeuterated bile acid which contributed to the peak area of the analytes in the samples as well as in the calibrators and the QCs. The percentage of undeuterated bile acids contained in the various internal standards varied from 0.1% to 1.2% and this effect was corrected by

subtracting a defined percentage of the internal standard peak from the corresponding undeuterated analyte peak in every analysis.

The calibration domain for C4 ranged from 0.01 μmol/l to 0.4 μmol/l and sample concentration was calculated similarly as described above. Samples with concentrations above 0.05 μmol/l were calculated using all six calibrators 0.01, 0.02, 0.05, 0.1, 0.2 and 0.4 μmol/l, whereas samples with concentrations below 0.05 μmol/l were calculated using only the three first calibrators. Samples with concentrations below 0.01 μmol/l as well as samples for which the peak area ratio was below the absolute value of the intercept of its respective three points calibration curve were calculated by proportional conversion using the respective peak area ratio of the first calibration standard. Since only insignificant amounts of undeuterated C4 impurity could be detected in the C4-d_7 internal standard solution, the peak area of C4 did not need to be corrected in contrast to that of bile acids.

2.7. Instrumentation

The LC–MS system consisted of an HTC PAL autosampler from CTC Analytics (Zwingen, Switzerland) coupled with a Rheos 2200 HPLC pump from Flux Instruments (Reinach, Switzerland) and a TSQ Quantum Access mass spectrometer from Thermo Scientific (Waltham, MA, USA). Separation was achieved using a 125 mm x 2.0 mm Uptisphere C_{18} 5 μm particle size ODB column from Interchim (Montluçon, France), which was protected by a 10 mm x 2.0 mm modulo-cart QS guard column also from Interchim. Data acquisition was performed using Xcalibur software (version 2.0.6) from Thermo Scientific.

2.8. Separation, ionisation and detection conditions

Mobile phase for both methods consisted of methanol (eluent A) and 10 mM ammonium acetate buffer at pH 6.5 (eluent B).

For separation of the bile acids, the eluents were set to 65% A and 35% B during the first 10 min, after which the proportions were changed within 2 min to 70% A and 30% B and maintained for 4 min. After 16 min of analysis, the proportions were changed again within 1 min to 80% A and 20% B and maintained for 8 min. At 25 min, the proportions were changed to 100% A and maintained for 5 min in order to flush the column, upon which the conditions were set to the beginning proportions of 65% A and 35% B for 20 min to equilibrate the chromatographic column. The flow rate was set to 300 μl/min during the whole analysis.

The separation of the more hydrophobic C4 was achieved with a mobile phase containing 93% A and 7% B at a flow rate of 250 μl/min. After 10 min the column was flushed for 5 min with 400 μl/min of eluent A and finally the column was equilibrated for 10 min at a flow rate of 300 μl/min with the beginning proportions of 93% A and 7% B.

In both methods, ionisation was performed using an electrospray ionisation (ESI) source, which was operating in the negative mode for bile acids and in the positive mode for C4. Ionisation parameters were tuned for the different compounds and optimal conditions are shown in Table 2. The tube lens and collision energy values were individually optimised for each analyte and are reported in Table 1. Collision pressure was set to the default value of 1.5 mTorr. Scan time was set to 0.05 s for the bile acids and to 0.5 s for C4, while scan width was set to 0.002 *m*/*z* for both methods. Due to the lack of stable fragments for unconjugated bile acids, their detection was performed in the pseudo-selected reaction monitoring mode. This detection mode uses low collision energy, which does not fragment the parent ion, but decreases the background noise and therefore leads to a better signal-to-noise ratio. In contrast, all of the conjugated bile acids as well as C4 showed reproducible fragments which are reported in Table 1.

Table 2
Optimised ionisation and detection parameters and polarity for the 15 bile acids and C4.

Optimised parameter	Unit	bile acids	C4
Ionisation polarity	–	Negative	Positive
Spray voltage	kV	4.0	5.0
Sheath gas	Arbitrary	35	40
Auxiliary gas	Arbitrary	10	35
Ion sweep gas	Arbitrary	10	0
Ion transfer capillary temperature	°C	350	310
Skimmer offset	V	10	−5

2.9. Method validation

A detailed validation procedure was performed according to the Food and Drug Administration (FDA) guidelines [28].

2.9.1. Linearity

Defined amounts of standard stock solutions were added to charcoal treated serum for preparation of 6 calibration points ranging from 0.02 μmol/l to 50 μmol/l for bile acids and from 0.01 μmol/l to 0.4 μmol/l for C4. The samples were extracted as described in Sections 2.5.1 and 2.5.2 respectively. Calibration curves were plotted as the peak area ratio of the respective bile acid over its internal standard against the nominal concentration of the calibrator. The line of best fit was determined by least square linear regression for the lower calibration domain (three lowest calibrators) as well as for the entire calibration domain for both methods and for six freshly prepared calibration curves. Calculation of the mean correlation coefficient was used to assess linearity.

2.9.2. Precision and accuracy

In order to assess intra-day precision and accuracy, six QCs of a low and of a high concentration (0.5 μmol/l and 30 μmol/l for bile acids versus 0.02 μmol/l and 0.2 μmol/l for C4) were extracted and analysed using a freshly prepared calibration curve on a given day. Additional QCs for each the low and the high concentrations were also analysed on five different days, each time with a freshly prepared calibration curve, in order to assess inter-day precision and accuracy. Precision of the method was represented by the coefficient of variation while accuracy was obtained by expressing the mean of the measured concentrations as a percentage of the nominal concentration.

2.9.3. Recovery

Since matrix constituents are known to influence the extraction process, the recovery experiment was performed using untreated serum instead of a charcoal treated serum in order to have extraction conditions as close as possible to those of real samples.

In order to assess efficiency of the extraction procedure, twelve serum samples were extracted for each a low and a high concentration (0.5 μmol/l and 30 μmol/l for bile acids versus 0.02 μmol/l and 0.2 μmol/l for C4). From these twelve samples, six were spiked with the appropriate amount of standard stock solutions and internal standards before extraction. The six remaining samples were extracted as blanks and spiked with the same amount of standard stock solutions and internal standards after the extraction. Six additional blank samples containing only endogenous bile acids or C4 were extracted without addition of any standard stock solutions or internal standards. Thereafter all samples were evaporated and dissolved in mobile phase according to Sections 2.5.1 and 2.5.2 respectively. After correction of the spiked serum samples by subtraction of endogenous amounts of the respective analyte, recovery was obtained by expressing the mean peak area of samples spiked

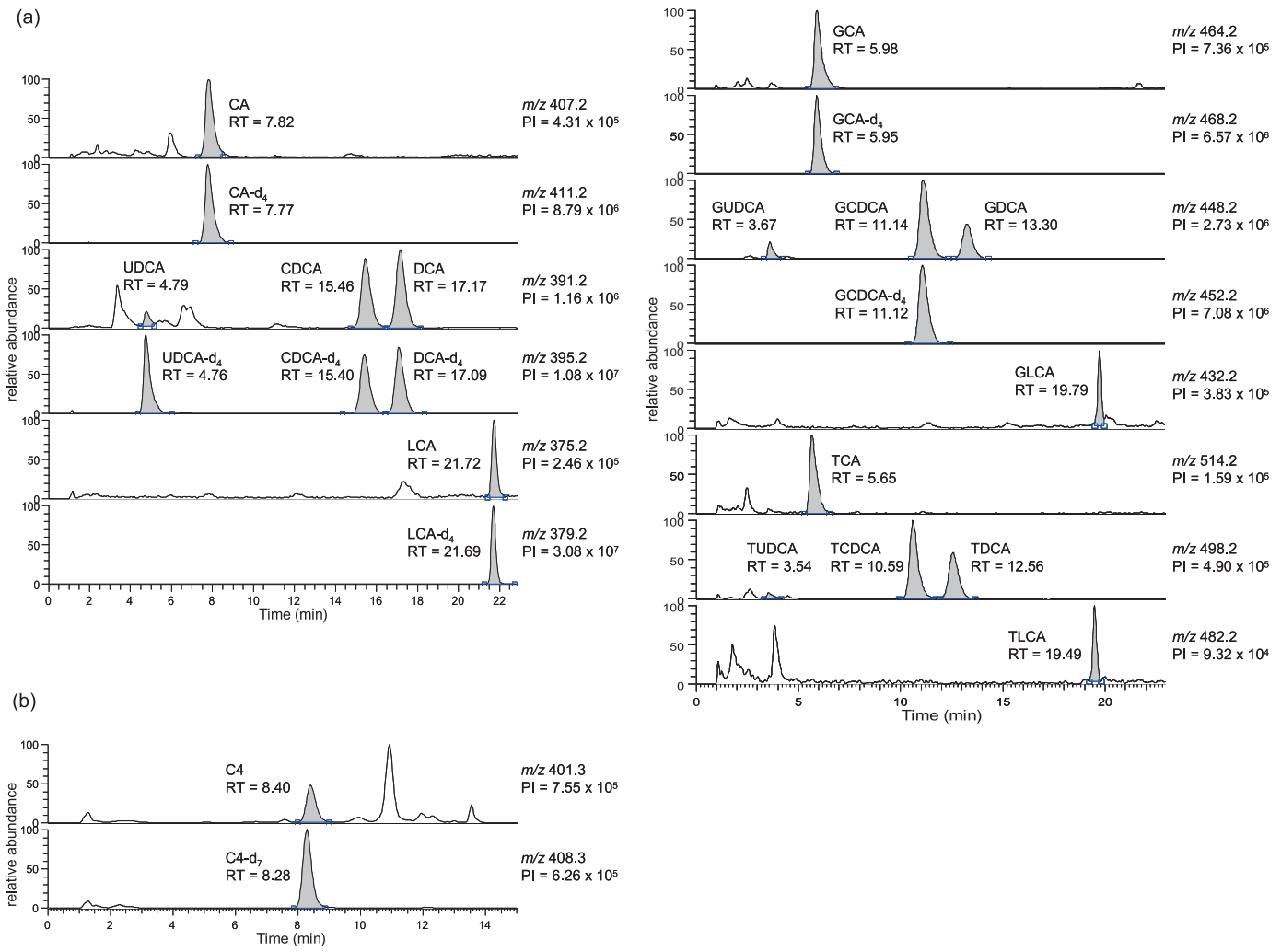


Fig. 2. Representative chromatograms of an extracted serum (100 μl) from a healthy volunteer, spiked with (a) seven deuterated bile acids (30 μl, 15 μmol/l) and showing endogenous amounts of the 15 major bile acids; (b) C4-d_7 (50 μl, 0.1 μmol/l) and showing endogenous amounts of C4. The total bile acid concentration represented 5.0 μmol/l, whereas the C4 concentration was 24.5 nmol/l. RT: retention time (min), PI: peak intensity.

before extraction as a percentage of that of samples spiked after extraction.

2.9.4. Limit of quantification

QCs were spiked with concentrations ranging from 0.002 μmol/l to 0.05 μmol/l for the bile acids and 0.001 μmol/l to 0.01 μmol/l for C4. Five replicates were extracted and analysed for each concentration and limit of quantification (LOQ) was determined as being the lowest concentration at which the analyte could be quantified with sufficient precision and accuracy.

2.9.5. Matrix effects

Matrix effects were assessed by two different methods, in both a quantitative and a qualitative manner. Because of the major role played by matrix components, these experiments were performed using untreated serum instead of charcoal treated serum.

In order to assess matrix effects in a quantitative manner, six blank serum samples were extracted using the procedures described for bile acids and C4 respectively. After extraction, three samples were spiked with defined amounts of standard stock solutions (10 μmol/l for bile acids versus 0.1 μmol/l for C4) and internal standards, while the remaining three samples were kept as blank samples. Simultaneously, identical amounts of standard stock solutions and internal standards were pipetted into three clean vials. All samples were then evaporated and dissolved in mobile phase according to Sections 2.5.1 and 2.5.2 respectively. After correction of the spiked serum samples by subtraction of endogenous amounts of the respective analyte, quantitative matrix effects were assessed by expressing the peak area of spiked serum samples (with matrix) as a percentage of the peak area of samples containing only the pure standard stock solutions (without matrix).

The qualitative evaluation of potential ion suppression or enhancement effects due to matrix components was preformed by post-column infusion of a solution containing the analyte of interest while an extracted blank serum sample was simultaneously injected by the autosampler. Matrix components interfering with the ionisation process show an increase (ion enhancement) or more often a decrease (ion suppression) in the signal.

3. Results and discussion

3.1. Preparation of standard stock solutions

During method development, a rapid decrease in peak intensity was observed in aqueous solutions of C4 (data not shown). This decrease was probably due to poor aqueous solubility of C4 because it was not observed when C4 was dissolved in methanol. Therefore all C4 standard stock solutions were prepared in methanol,

Table 3
Precision, accuracy and recovery data for the 15 bile acids and C4 ($n = 6$).

Analyte	Nominal concentration (μmol/l)	Intra-day ($n = 6$)			Inter-day ($n = 6$)			Extraction recovery (%)
		Mean (μmol/l)	CV (%)	Accuracy (%)	Mean (μmol/l)	CV (%)	Accuracy (%)	
CA	0.5	0.483	2.2	96.6	0.496	2.0	99.2	91.2
	30.0	30.0	0.6	100	30.0	0.7	99.9	96.4
CDCA	0.5	0.474	3.6	94.7	0.470	2.6	94.0	82.5
	30.0	29.9	0.9	99.7	29.9	1.4	99.6	96.7
DCA	0.5	0.479	3.6	95.9	0.470	2.6	93.9	88.4
	30.0	30.0	0.5	100	29.8	1.9	99.2	98.4
LCA	0.5	0.438	4.2	87.6	0.423	7.4	84.7	78.3
	30.0	29.2	2.9	97.2	29.2	6.1	97.2	99.0
UDCA	0.5	0.493	1.2	98.6	0.490	2.0	98.1	92.1
	30.0	29.9	0.8	99.7	29.9	1.0	99.6	97.3
GCA	0.5	0.485	1.7	97.0	0.496	1.4	99.2	92.1
	30.0	29.8	0.3	99.4	30.1	0.8	100	99.1
GCDCA	0.5	0.485	2.0	97.0	0.494	1.8	98.9	84.4
	30.0	29.8	0.5	99.2	30.2	1.5	101	97.9
GDCA	0.5	0.492	1.9	98.3	0.492	2.0	98.4	83.7
	30.0	28.8	0.9	96.1	29.5	2.1	98.3	97.4
GLCA	0.5	0.461	4.4	92.2	0.468	10.1	93.7	80.1
	30.0	29.7	3.1	98.9	30.1	3.7	100	97.4
GUDCA	0.5	0.483	2.2	96.6	0.497	1.0	99.4	92.4
	30.0	30.7	0.8	102	30.6	1.4	102	96.7
TCA	0.5	0.456	2.5	91.2	0.464	1.7	92.8	92.1
	30.0	29.7	0.8	99.0	29.9	1.4	99.8	99.3
TCDCA	0.5	0.471	2.2	94.3	0.481	2.9	96.1	85.5
	30.0	29.6	0.6	98.7	30.0	1.7	100	98.9
TDCA	0.5	0.475	2.4	95.0	0.486	2.5	97.3	85.8
	30.0	29.7	0.9	98.9	29.9	1.8	99.6	98.1
TLCA	0.5	0.479	4.8	95.7	0.489	11.9	97.8	82.7
	30.0	28.7	3.5	95.5	29.2	5.0	97.2	96.5
TUDCA	0.5	0.461	1.1	92.2	0.469	1.6	93.8	92.6
	30.0	29.3	0.9	97.7	29.4	1.2	98.0	96.4
C4	0.02	0.018	5.2	89.1	0.020	3.6	97.7	62.5
	0.2	0.197	2.1	98.4	0.197	1.6	98.3	62.1

CV: coefficient of variation.

Table 4
Comparison of extraction recoveries, limit of quantification and sample volume between the new methods and methods previously described. LOQ values given in ng/ml were converted to nmol/l based on an average molecular mass of 400. LOQ values were extrapolated for a corresponding sample volume of 100 μl in order to allow comparison between the methods.

Source	Extraction recoveries (%)	Limit of quantification (LOQ)		Sample volume (μl)	LOQ extrapolated to 100 μl sample volume (nmol/l)
		nmol/l	ng/ml		
Bile acids					
New method	78–99	2–50	n.r.	100	2–50
[16] Xiang et al.	88–103	5	n.r.	500	25
[17] Scherer et al.	> 80	n.r.	n.r.	100	n.r.
[18] Anouti et al.	83–98	~25–100	10–40	100	~25–100
[19] Bentayeb et al.	n.r.	~1–400	~0.4–160	100	~1–400
[20] Ye et al.	86–110	3–18	n.r.	300	9–54
[21] Ando et al.	n.r.	n.r.	n.r.	100	n.r.
[24] Burkard et al.	85–105	1–103	n.r.	750	7.5–770
[22] Tagliacozzi et al.	93–105	n.r.	n.r.	250	n.r.
[23] Tessier et al.	80–87	~25	10	300	~75
C4					
New method	62	5	n.r.	100	5
[25] DeBarber et al.	79–105	125	50	5	6.25
[12] Camilleri et al.	93–107	n.r.	n.r.	200	n.r.
[26] Honda et al.	92–94	0.25[a]	0.1[a]	50	0.125[a]
[14] Lövgren-Sandblom et al.	105	n.r.	n.r.	500–1000	n.r.

n.r.: not reported.
[a] LOQ was measured using a standard stock solution (in contrast to spiked sample matrix).

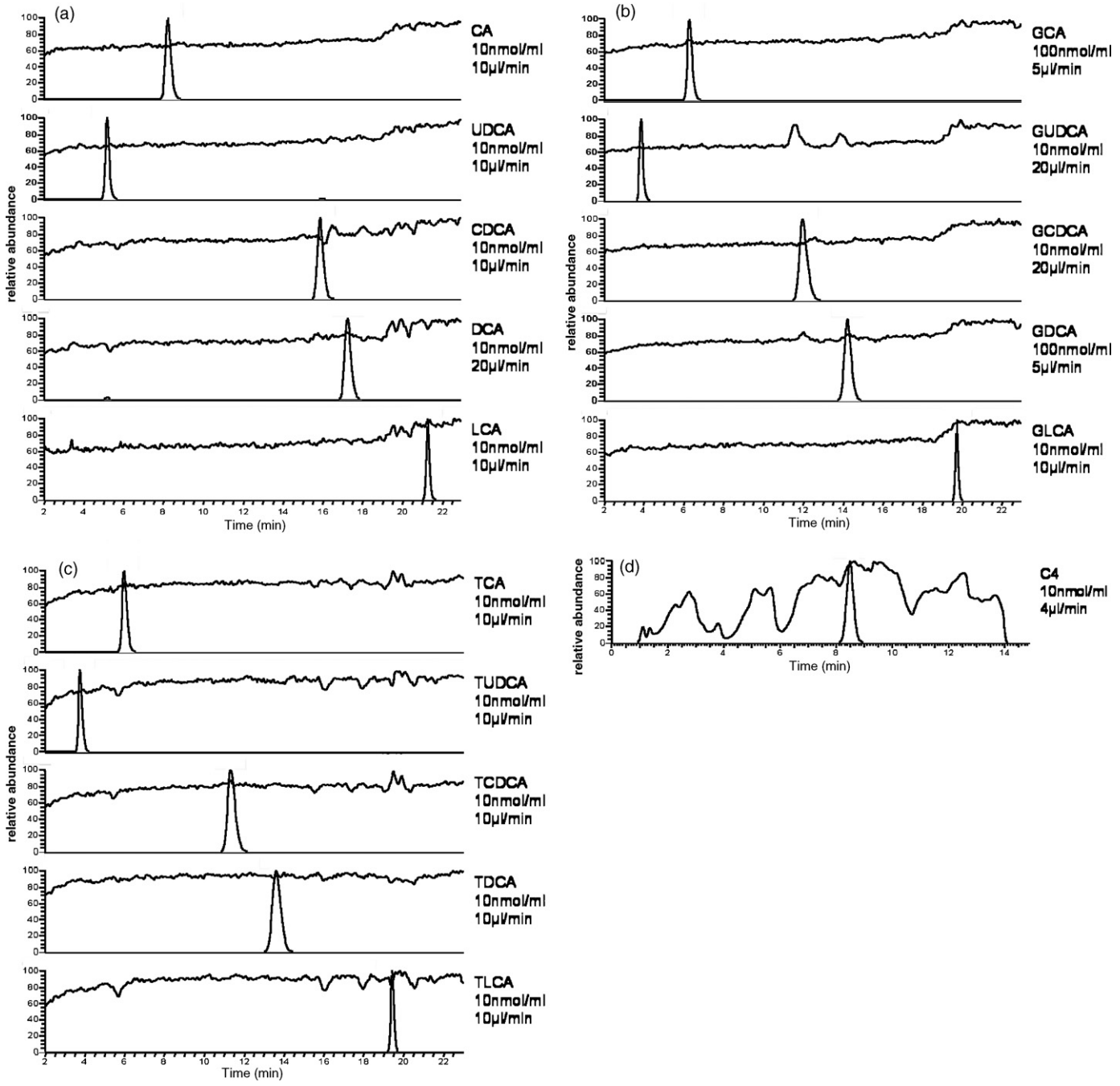


Fig. 3. Qualitative assessment of matrix effects for the 15 bile acids and C4. The figure shows the effect of an extracted serum matrix on the signal of directly infused analytes for (a) unconjugated bile acids; (b) glycine-conjugated bile acids; (c) taurine-conjugated bile acids and (d) C4. Chromatograms of standard stock solutions (bile acids: 10 μl, 10 μmol/l; C4: 10 μl, 1 μmol/l) were superimposed to facilitate visualisation of relevant detection timeframes. Concentrations and flow rates of the infused solutions were chosen as the minimum still covering the signal of endogenous peaks and are reported for every compound.

although this should be avoided as much as possible when using solid-phase extraction. Nevertheless, in order to have identical extraction conditions, the methanol volume was completed to 150 μl in all C4 samples. In contrast to C4, bile acids were dissolved in water and showed no decrease in peak intensity.

3.2. Sample preparation

Our new methods each required only 100 μl of serum sample, which was less than for many of the existing methods [12,14,16,20,22–24] although methods for quantification of C4 in even smaller sample volumes exist [25–26]. A low sample volume represents an important advantage if the method is to be adapted for analysis of mouse samples (muricholic acids), since mice have a very small blood volume. In addition, our C4 method offers the advantage of requiring a rather short sample preparation in comparison to other existing methods [14,25–26] and no derivatisation [25–26]. However, in order for these methods to be improved, efforts should be made to find a combined extraction procedure for bile acids and C4, which would reduce sample volume to only 100 μl instead of twice 100 μl and would lead to a shorter total analysis time. This was not achieved yet since C4 seems to be bound to plasma proteins and required the addition of hydrochloric acid in order to obtain sufficient recovery, which

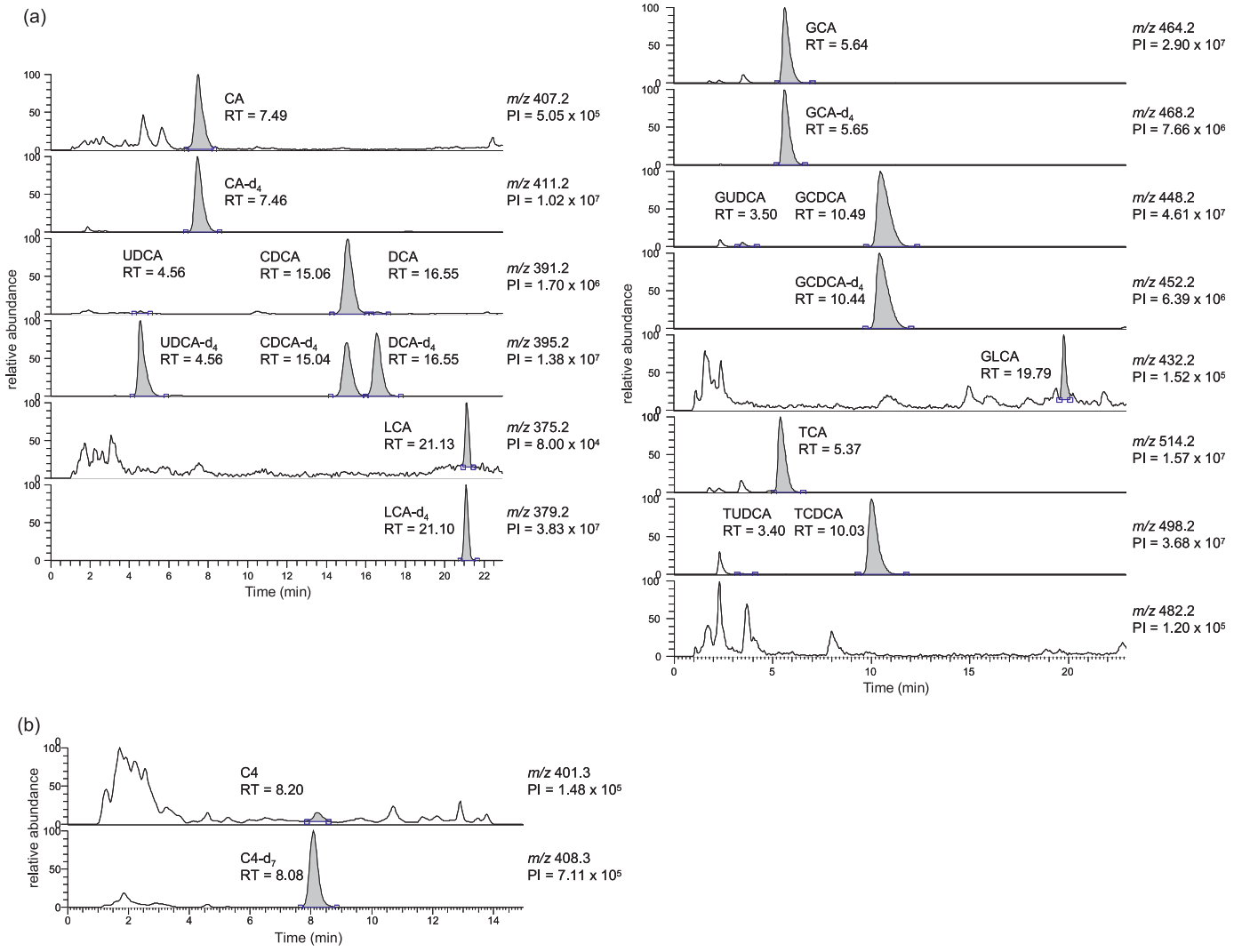


Fig. 4. Chromatograms of an extracted serum (100 μl) from a patient suffering from liver cirrhosis and cholecystolithiasis spiked with (a) seven deuterated bile acids (30 μl, 15 μmol/l); (b) C4-d_7 (50 μl, 0.1 μmol/l). The total bile acid concentration represented 56.5 μmol/l, whereas the C4 concentration was 1.2 nmol/l (<LOQ). RT: retention time (min), PI: peak intensity.

in turn would probably hydrolyse the gylcine and taurine moieties of conjugated species if the same procedure was applied to bile acids.

3.3. Separation, ionisation and detection conditions

The baseline separation of the two isomers CDCA and DCA as well as their glycine- and taurine-conjugates represented the main limitation for the chromatographic separation of the 15 bile acids since these isomers behave identically with respect to fragmentation and therefore they cannot be distinguished from each other by mass spectrometry only. Separation of the 15 major bile acids was consequently achieved in 20 min. In the case of C4, the peak retention time was delayed to approximately 8 min because it needed to be separated from a 100 times more intense co-eluting peak of unknown origin in order to minimise ion suppression effects (data not shown).

Selectivity is undeniably lower for unconjugated bile acids due to the absence of reproducible fragments, which was also the case in previously developed LC–MS methods for bile acids. However, the relevance of this analytical limitation depends on the context of the intended application.

Examples of mass chromatograms of bile acids and C4 in serum from a healthy volunteer is shown in Fig. 2. The total bile acid concentration in this serum reached 5.0 μmol/l with GCDCA being the most concentrated species (1.4 μmol/l), while the concentration obtained for C4 was 24.5 nmol/l.

Table 5
Limit of quantification values for the 15 bile acids and C4.

Analyte	Limit of quantification (nmol/l)
CA	20
CDCA	20
DCA	20
LCA	50
UDCA	20
GCA	20
GCDCA	20
GDCA	20
GLCA	50
GUDCA	5
TCA	5
TCDCA	5
TDCA	2
TLCA	5
TUDCA	2
C4	5

ESI was chosen for the ionisation of bile acids since these molecules possess a carboxyl group (unconjugated and glycine-conjugated bile acids) or a sulfonyl group (taurine-conjugated bile acids) which easily generates negatively charged ions in solution. Ionisation of C4 was also performed using ESI although atmospheric pressure chemical ionisation (APCI) would probably have been a better suited ionisation technique regarding the polarity of the analyte. Nevertheless, ESI was preferred because it offered the possibility of combined application of both methods in addition to a sufficient sensitivity for the analysis of C4. The combined application of both methods represents an advantage, particularly for the analysis of large batches of samples. Indeed, the fact that both methods require the same eluents, stationary phase and ionisation source allows to run sequences containing both types of samples without any modifications of hardware (column, source), thereby sparing time and reducing the risk of handling errors.

3.4. Method validation

3.4.1. Linearity

The calibration curves were linear in both calibration ranges for the 15 bile acids and C4. For the bile acids, the mean correlation coefficients r ($n = 6$) ranged from 0.9983 to 1.0000 for the lower calibration domain (0.02–5 μmol/l) and from 0.9994 to 1.0000 for the entire calibration domain (0.02–50 μmol/l). Linearity was also observed if the calibration domain was extended to 100 μmol/l for high concentrated samples (data not shown). For C4, the mean correlation coefficient r ($n = 6$) was 0.9993 for both the lower (0.01–0.05 μmol/l) and the entire (0.01–0.4 μmol/l) calibration domain.

3.4.2. Precision and accuracy

Intra- and inter-day precision and accuracy data are summarized in Table 3. For bile acids, intra-day (inter-day) variation ($n = 6$) ranged from 0.3% to 4.8% (0.7% to 11.9%). Intra-day (inter-day) accuracy ($n = 6$) ranged from 87.6% to 102% (84.7% to 102%). For C4, intra-day (inter-day) variation ($n = 6$) was below or equal to 5.2% (3.6%). Intra-day (inter-day) accuracy ($n = 6$) was 89.1% and 98.4% (97.7% and 98.3%) for the low and the high QCs respectively. The precision and accuracy data were within the interval set by the FDA concerning validation of bioanalytical methods [28].

3.4.3. Recovery

The extraction recoveries ($n = 6$) of the different bile acids and C4 are shown in Table 3. This experiment only took in account the efficiency of the extraction procedure *per se* and not the matrix effects which are discussed below. In this case, matrix effects were identical for all samples since serum samples spiked before extraction were compared to serum samples spiked after extraction.

The extraction recoveries for bile acids ranged from 78.3% to 99.3% and were slightly higher for 30 μmol/l than for 0.5 μmol/l samples, which might be due to imprecision in low concentrated samples when correcting for endogenous amounts, since error is proportionally higher on low concentrations. However, this did not affect linearity as demonstrated in Section 3.4.1.

Table 4 summarizes and compares extraction recoveries of the here and previously reported methods for the measurement of bile acids and C4. Extraction recoveries for bile acids were quite similar to those reported for other existing methods [16–18,20,22–24]. The extraction recovery for C4 was found to be approximately 62%, which was markedly lower than for bile acids and for other C4 methods [12,14,25–26]. This is presumably explained by its lipophilic properties and by the fact that the others methods used different sample preparation techniques (protein precipitation, liquid-liquid extraction followed by SPE, derivatisation and salting-out respectively).

Table 6
Quantitative assessment of matrix effects for the 15 bile acids and C4 as well as for their internal standards ($n = 3$). The analytical response in presence of matrix was expressed as a percentage of the analytical response in the absence of matrix.

Analyte	Relative analytical response (%)	CV (%)
CA	92.6	4.0
CDCA	93.8	1.9
DCA	92.0	2.8
LCA	96.9	6.2
UDCA	76.3	5.6
GCA	93.2	4.4
GCDCA	101	1.6
GDCA	100	3.0
GLCA	96.4	3.0
GUDCA	87.5	6.0
TCA	82.1	4.0
TCDCA	94.6	2.6
TDCA	97.3	3.4
TLCA	103	2.3
TUDCA	73.7	6.6
C4	96.2	8.5
CA-d_4	91.7	2.9
CDCA-d_4	94.0	1.4
DCA-d_4	92.7	1.9
LCA-d_4	102	7.7
UDCA-d_4	78.2	6.0
GCA-d_4	92.0	3.8
GCDCA-d_4	101	1.9
C4-d_7	87.9	8.4

CV: coefficient of variation.

3.4.4. Limit of quantification

LOQ was defined as the lowest concentration at which the analyte could be quantified with a coefficient of variation below 20% and accuracy between 80% and 120% ($n = 5$). For bile acids, LOQ ranged from 2 nmol/l to 50 nmol/l. After correction for sample volume, this was comparable or slightly better than what has been reported in recent publications providing LOQ values [16,18–20,23–24] (Table 4). For C4, LOQ was 5 nmol/l, which is similar to what was reported by DeBarber et al. [25]. The detailed LOQ values for the individual analytes are shown in Table 5.

3.4.5. Matrix effects

The results of the quantitative matrix effects test ($n = 3$) are shown in Table 6. In contrast to recovery, this experiment takes into account only the matrix effects (and not the loss that occurs during extraction) since the analytes were added to the matrix after extraction and compared to the same amount of analytes in pure solvents.

The signal in presence of matrix components was between 73% and 103% of the signal in pure standards for all analytes, which indicated some competition between the analytes and interfering matrix components during ionisation. However, assessment of matrix effects in a qualitative way with post-column infusion of the analytes showed no significant ion suppression or enhancement effects in relevant detection timeframes, as demonstrated in Fig. 3.

In this paper, recovery and matrix effects were evaluated in separate experiments (Sections 2.9.3 and 2.9.5) unlike in some publications where recovery is assessed by comparing an extracted spiked sample to a stock solution. In this latter case, it would not be clear whether a decrease in signal intensity is due to insufficient extraction recovery or to ion suppression.

3.5. Quantification of bile acids and C4 in a patient with cirrhosis and cholestasis

As an application example, bile acids and C4 were quantified in the serum of a 39-year old woman suffering from liver cirrhosis and cholecystolithiasis (gallstones) among other conditions (Fig. 4). Clinical findings showed increased levels of total bilirubin (32 μmol/l), gamma-glutamyl transferase (356 U/l) and alkaline phosphatase (189 U/l), which indicate the presence of cholestasis and liver damage. The total bile acid concentration in this patient was 10 times higher than in the serum from a healthy volunteer displayed in Fig. 2 (56.5 μmol/l versus 5.0 μmol/l), which is often observed in cholestatic conditions. While glycine and taurine-conjugates of the primary bile acids (GCA, GCDCA, TCA and TCDCA) showed up to an 80-fold increase in concentration compared to serum from a healthy volunteer, all secondary bile acids except GUDCA had concentrations below LOQ. Several of them (GDCA, TDCA and TLCA) were totally absent from the circulation as a consequence of breakdown of enterohepatic circulation. The concentration obtained for C4 was 1.2 nmol/l, (<LOQ) and indicates poor liver function due to cirrhosis, as C4 concentrations in healthy subjects have been reported to be between 12 nmol/l and 120 nmol/l (5–48 ng/ml) [14].

4. Conclusions

In addition to their traditionally recognized role in lipid emulsification, bile acids were found to be biologically important signaling molecules, which has resulted in an increased relevance of these metabolites in biology and medicine and in a greater demand for sensitive, precise and accurate methods for the quantification of bile acids in recent years.

In this paper, we described two new methods based on LC–MS/MS for the combined quantification of the 15 major human bile acids and their precursor for the classical biosynthesis pathway, namely C4, in serum. The advantage of these methods lies in their ability to describe both the composition of the circulating bile acid pool and the amount of newly synthesised bile acids, reflected by C4 concentrations. Combination of both methods is possible since the eluents for the mobile phase, the stationary phase as well as the ionisation source are identical. These new methods allow a detailed insight into bile acid metabolism in humans, which will provide additional valuable information while performing clinical studies to determine the prognostic and/or diagnostic value of cholesterol metabolites in various diseases.

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References

[1] M. Makishima, A.Y. Okamoto, J.J. Repa, H. Tu, R.M. Learned, A. Luk, M.V. Hull, K.D. Lustig, D.J. Mangelsdorf, B. Shan, Science 284 (1999) 1362.
[2] H. Wang, J. Chen, K. Hollister, L.C. Sowers, B.M. Forman, Mol. Cell 3 (1999) 543.
[3] D.J. Parks, S.G. Blanchard, R.K. Bledsoe, G. Chandra, T.G. Consler, S.A. Kliewer, J.B. Stimmel, T.M. Willson, A.M. Zavacki, D.D. Moore, J.M. Lehmann, Science 284 (1999) 1365.
[4] T. Claudel, B. Staels, F. Kuipers, Arterioscler Thromb. Vasc. Biol. 25 (2005) 2020.
[5] F. Kuipers, J.H. Stroeve, S. Caron, B. Staels, Curr. Opin. Lipidol. 18 (2007) 289.
[6] S. Caron, B. Cariou, B. Staels, Endocrinology 147 (2006) 4022.
[7] Y. Zhang, P.A. Edwards, FEBS Lett. 582 (2008) 10.
[8] D. Duran-Sandoval, B. Cariou, J.C. Fruchart, B. Staels, Biochimie 87 (2005) 93.
[9] P. Lefebvre, B. Cariou, F. Lien, F. Kuipers, B. Staels, Physiol. Rev. 89 (2009) 147.
[10] B. Cariou, B. Staels, Trends Pharmacol. Sci. 28 (2007) 236.
[11] B. Staels, F. Kuipers, Drugs 67 (2007) 1383.
[12] M. Camilleri, A. Nadeau, W.J. Tremaine, J. Lamsam, D. Burton, S. Odunsi, S. Sweetser, R. Singh, Neurogastroenterol. Motil. 21 (2009) 734.
[13] I. Bjorkhem, E. Reihner, B. Angelin, S. Ewerth, J.E. Akerlund, K. Einarsson, J. Lipid. Res. 28 (1987) 889.
[14] A. Lovgren-Sandblom, M. Heverin, H. Larsson, E. Lundstrom, J. Wahren, U. Diczfalusy, I. Bjorkhem, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 856 (2007) 15.
[15] G. Sauter, F. Berr, U. Beuers, S. Fischer, G. Paumgartner, Hepatology 24 (1996) 123.
[16] X. Xiang, Y. Han, M. Neuvonen, J. Laitila, P.J. Neuvonen, M. Niemi, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 878 (2010) 51.
[17] M. Scherer, C. Gnewuch, G. Schmitz, G. Liebisch, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 3920.
[18] Y. Alnouti, I.L. Csanaky, C.D. Klaassen, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 873 (2008) 209.
[19] K. Bentayeb, R. Batlle, C. Sanchez, C. Nerin, C. Domeno, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 869 (2008) 1.
[20] L. Ye, S. Liu, M. Wang, Y. Shao, M. Ding, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 860 (2007) 10.
[21] M. Ando, T. Kaneko, R. Watanabe, S. Kikuchi, T. Goto, T. Iida, T. Hishinuma, N. Mano, J. Goto, J. Pharm. Biomed. Anal. 40 (2006) 1179.
[22] 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.
[23] E. Tessier, L. Neirinck, Z. Zhu, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 798 (2003) 295.
[24] I. Burkard, A. von Eckardstein, K.M. Rentsch, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 826 (2005) 147.
[25] A.E. DeBarber, W.E. Connor, A.S. Pappu, L.S. Merkens, R.D. Steiner, Clin. Chim. Acta 411 (2010) 43.
[26] A. Honda, K. Yamashita, M. Numazawa, T. Ikegami, M. Doy, Y. Matsuzaki, H. Miyazaki, J. Lipid. Res. 48 (2007) 458.
[27] R. Karuna, A. von Eckardstein, K.M. Rentsch, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 261.
[28] FDA http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf (Accessed 2009).