Journal of Chromatography B, 923–924 (2013) 120–127

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Determination of 3β-hydroxy-Δ^5-bile acids and related compounds in biological fluids of patients with cholestasis by liquid chromatography–tandem mass spectrometry

Tsuyoshi Murai[a,*], Kana Oda[a], Terutake Toyo[a], Hiroshi Nittono[b], Hajime Takei[b], Akina Muto[b], Akihiko Kimura[c], Takao Kurosawa[a]

[a] School of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Kanazawa, Ishikari-Tobetsu, Hokkaido 061-0293, Japan
[b] Junshin Clinic Bile Acid Institute, Haramachi, Meguro-ku, Tokyo 152-0011, Japan
[c] Department of Pediatrics and Child Health, Kurume University School of Medicine, Asahi-machi, Kurume-shi, Fukuoka 830-0011, Japan

A R T I C L E I N F O

Article history:
Received 9 November 2012
Accepted 3 February 2013
Available online 19 February 2013

Keywords:
3β-Hydroxy-Δ^5-bile acids
3β-Hydroxy-Δ^5-C_{27}-steriod dehydrogenase/isomerase deficiency
High-performance liquid chromatography–electrospray ionization-tandem mass spectrometry

A B S T R A C T

A method for the determination of conjugated and unconjugated 3β-hydroxy-Δ^5-bile acids and related bile acids in human urine and serum has been developed using high-performance liquid chromatography–electrospray ionization-tandem mass spectrometry. Calibration curves for the bile acids were linear over the range of 10–2000 pmol/mL, and the detection limit was less than 4 pmol/mL for all bile acids using selected reaction monitoring analysis. The bile acids in urine and serum samples from two patients with severe cholestatic liver disease were measured by this analytical method. Glycine-conjugated 3β-hydroxy-Δ^5-bile acid 3-sulfates were determined to be the major bile acids in the urine and serum from patients with a 3β-hydroxy-Δ^5-C_{27}-steriod dehydrogenase/isomerase deficiency or dysfunction.

1. Introduction

In 1987 Clayton et al. discovered a disorder in bile acid biosynthesis, named 3β-hydroxy-Δ^5-C_{27}-steriod dehydrogenase/isomerase (HSD3B7) deficiency [1]. It is characterized by markedly elevated urine levels of glycine and taurine amidates or 3-sufates of 3β-hydroxy-Δ^5-bile acids, such as 3β,7α12α-trihydroxy-5-cholenoic acid (Δ^5-3β,7α,12α-ol, Fig. 1) and 3β,7α-dihydroxy-5-cholenoic acid (Δ^5-3β,7α-ol, Fig. 1), and these bile acids have been identified in more than thirty children with liver diseases [1–9]. Deficiency of HSD3B7 is thought to cause chronic liver injury in childhood. We also recently identified and quantified these unusual bile acids in urine of a 22-year-old patient with liver disease [6]. Since the presence of these 3β-hydroxy-Δ^5-bile acids in biological fluids reflects this disorder, their measurement in biological fluids is important from a clinical point of view. There have been several reports on the separation and quantification of 3β-hydroxy-Δ^5-bile acids by gas chromatography–mass spectrometry (GC–MS) [1,3,4,6,7]. However, these procedures have some disadvantages, such as tedious sample clean-up and insufficient information concerning the conjugation mode of the bile acids. Moreover, under the alkaline or acidic conditions usually used for deconjugation in the GC–MS method, 3β-hydroxy-Δ^5-bile acids are expected to be converted into their dehydrated products or unknown degradation products.

In recent years, high-performance liquid chromatography-electrospray ionization coupled to tandem mass spectrometry (LC/ESI-MS/MS) has been developed for the determination of common bile acids in human biological fluids [9–16]. The LC/ESI-MS/MS method, which makes prior deconjugation unnecessary, appears to be suitable for the determination of 3β-hydroxy-Δ^5-bile acid conjugates in human biological fluids. The present paper deals with a highly sensitive method for the determination of conjugated and unconjugated 3β-hydroxy-Δ^5-bile acids, including common bile acids, with an LC/ESI-MS/MS method. It is also reported for the application of this method to determine these bile acids in biological fluids obtained from patients with liver diseases and healthy volunteers.

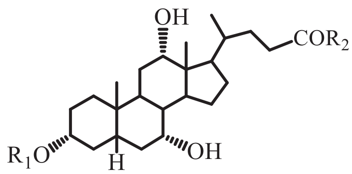
2. Experimental

2.1. Reagents and chemicals

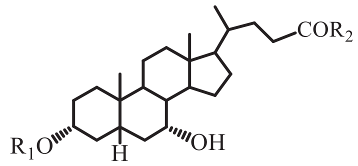
Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA) and urusodeoxycholic acid

* Corresponding author at: School of Pharmaceutical Sciences, Health Sciences University of Hokkaido, 1757 Kanazawa, Ishikari-Tobestu, Hokkaido 061-0293, Japan. Tel.: +81 0133 23 1211; fax: +81 0133 23 1266.
E-mail address: murai@hoku-iryo-u.ac.jp (T. Murai).

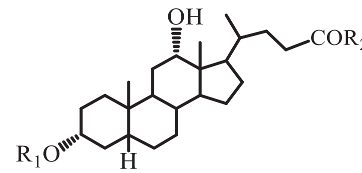
1570-0232/$ – see front matter
http://dx.doi.org/10.1016/j.jchromb.2013.02.003



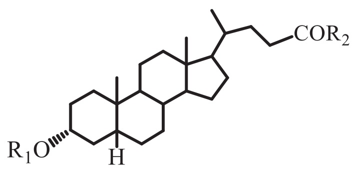
	R_1	R_2
CA (**1**)	H	OH
G-CA (**2**)	H	$NHCH_2COOH$
T-CA (**3**)	H	$NH(CH_2)_2SO_3H$
CA-S (**4**)	SO_3H	OH
G-CA-S (**5**)	SO_3H	$NHCH_2COOH$
T-CA-S (**6**)	SO_3H	$NH(CH_2)_2SO_3H$



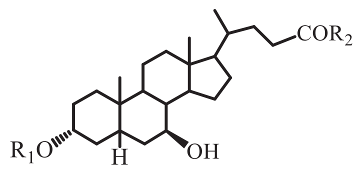
	R_1	R_2
CDCA (**7**)	H	OH
G-CDCA (**8**)	H	$NHCH_2COOH$
T-CDCA (**9**)	H	$NH(CH_2)_2SO_3H$
CDCA-S (**10**)	SO_3H	OH
G-CDCA-S (**11**)	SO_3H	$NHCH_2COOH$
T-CDCA-S (**12**)	SO_3H	$NH(CH_2)_2SO_3H$



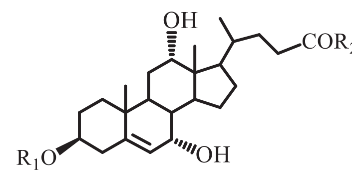
	R_1	R_2
DCA (**13**)	H	OH
G-DCA (**14**)	H	$NHCH_2COOH$
T-DCA (**15**)	H	$NH(CH_2)_2SO_3H$
DCA-S (**16**)	SO_3H	OH
G-DCA-S (**17**)	SO_3H	$NHCH_2COOH$
T-DCA-S (**18**)	SO_3H	$NH(CH_2)_2SO_3H$



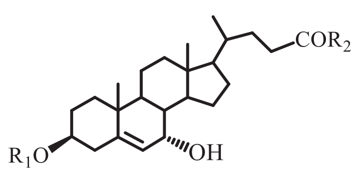
	R_1	R_2
LCA (**19**)	H	OH
G-LCA (**20**)	H	$NHCH_2COOH$
T-LCA (**21**)	H	$NH(CH_2)_2SO_3H$
LCA-S (**22**)	SO_3H	OH
G-LCA-S (**23**)	SO_3H	$NHCH_2COOH$
T-LCA-S (**24**)	SO_3H	$NH(CH_2)_2SO_3H$



	R_1	R_2
UDCA (**25**)	H	OH
G-UDCA (**26**)	H	$NHCH_2COOH$
T-UDCA (**27**)	H	$NH(CH_2)_2SO_3H$
UDCA-S (**28**)	SO_3H	OH
G-UDCA-S (**29**)	SO_3H	$NHCH_2COOH$
T-UDCA-S (**30**)	SO_3H	$NH(CH_2)_2SO_3H$



	R_1	R_2
Δ^5-3β,7α,12α-ol (**31**)	H	OH
G-Δ^5-3β,7α,12α-ol (**32**)	H	$NHCH_2COOH$
T-Δ^5-3β,7α,12α-ol (**33**)	H	$NH(CH_2)_2SO_3H$
Δ^5-3β,7α,12α-ol-S (**34**)	SO_3H	OH
G-Δ^5-3β,7α,12α-ol-S (**35**)	SO_3H	$NHCH_2COOH$
T-Δ^5-3β,7α,12α-ol-S (**36**)	SO_3H	$NH(CH_2)_2SO_3H$



	R_1	R_2
Δ^5-3β,7α-ol (**37**)	H	OH
G-Δ^5-3β,7α-ol (**38**)	H	$NHCH_2COOH$
T-Δ^5-3β,7α-ol (**39**)	H	$NH(CH_2)_2SO_3H$
Δ^5-3β,7α-ol-S (**40**)	SO_3H	OH
G-Δ^5-3β,7α-ol-S (**41**)	SO_3H	$NHCH_2COOH$
T-Δ^5-3β,7α-ol-S (**42**)	SO_3H	$NH(CH_2)_2SO_3H$

Fig. 1. Chemical structures of the unconjugated and conjugated bile acids used in this study.

(UDCA) were purchased from Sigma Chemical Co (St. Louis, MO, USA) and used after chromatographic purification. 3β,7α-Dihydroxy-5β-cholenoic acid (Δ^5-3β,7α-ol) and 3β,7α,12α-trihydroxy-5β-cholenoic acidΔ^5-3β,7α,12α-ol) were synthesized as previously reported [17]. Glycine- and taurine-conjugated bile acids and bile acid 3-sulfates were all stock samples synthesized in our laboratory. Chemical structures of all bile acids and their abbreviations are shown in Fig. 1. Stable isotope-labeled bile acids, [2,2,3,4,4-$^2H_5$]-CA (CA-d_5), [2,2,3,4,4-$^2H_5$]-CDCA (CDCA-d_5), [2,2,3,4,4-$^2H_5$]-LCA (LCA-d_5), and [2,2,3,4,4-$^2H_5$]-UDCA (UDCA-d_5), and their glycine- and taurine-conjugates and 3-sulfates (isotopic purity > 98.5%) were all stock samples that were synthesized in our laboratory. These stable isotope-labeled bile acids were used as internal standards (ISs) for the quantitative determination of bile acids by LC/ESI-MS/MS. An Oasis HLB 96-well plate cartridge (Waters Co., Milford, MA, USA) was washed successively with ethanol (0.5 mL) and water (1 mL) prior to use. All other reagents were of analytical grade.

2.2. Collection of biological samples

Urine and serum samples used for the present study were obtained from two patients (1 year and 23 years old) with HSD3B7 deficiency. As a control, urine samples were collected from eight healthy volunteers (13–22 years old). Informed consent was obtained from all subjects. The samples were stored at −25 °C before analysis.

2.3. LC/ESI-MS/MS

LC/ESI-MS/MS analysis was performed using a API 4000 Q-Trap hybrid triple quadrupole linear ion-trap mass spectrometer (AB SCIEX, Foster City, CA, USA) equipped with an ESI probe. High-purity nitrogen was produced by a nitrogen generator, 12E-SDA (System Instruments, Tokyo, Japan). The ion source was operated in the negative ion-mode using the following settings: ion spray voltage, −4000 V; ion source heater temperature, 550 °C; source gas 1, 55 psi; source gas 2, 85 psi; and curtain gas setting, 30 psi. Analytes were monitored by selected reaction monitoring (SRM). Mass transitions and MS parameters are shown in Table 1. The chromatographic system consisted of an Agilent 1200 series HPLC system (Agilent Technologies, Palo Alto, CA, USA). Gradient chromatographic separation of bile acids was performed on a Kinetex XB-C18 column (50 mm × 2.1 mm i.d., 2.6-μm particles, Phenomenex, Torrance, CA, USA) at ambient temperature. Mobile phase A was 15% acetonitrile (MeCN)/10 mM ammonium acetate (adjusted to pH 7.0 with aqueous ammonia solution), and mobile phase B was 90% MeCN/10 mM ammonium acetate (adjusted to pH 7.0 with aqueous ammonia solution). The mobile phase was delivered at a flow rate of 0.35 mL/min. The gradient program was as follows: mobile phase B was increased from 0 to 48%

Table 1
LC/ESI-MS/MS parameters of reference bile acids.

Bile acid	$[M-H]^-$ (m/z)	SRM transition (m/z)	C.E. (V)	R.T. (min)	LOD (pmol/mL)	I.S.
Common bile acids						
CA	407.3	407.3 → 407.3	5	6.9	2.0	CA-d_5
G-CA	464.3	464.3 → 74.0	78	6.6	0.5	G-CA-d_5
T-CA	514.3	514.3 → 80.0	124	6.8	0.5	T-CA-d_5
CA-S	487.2	487.2 → 97.0	90	5.0	0.5	CA-S-d_5
G-CA-S	544.3	544.3 → 464.3	30	4.7	2.0	G-CA-S-d_5
T-CA-S	594.2	296.6 → 496.3	44	4.9	2.0	T-CA-S-d_5
CDCA	391.3	391.3 → 391.3	5	8.9	2.0	CDCA-d_5
G-CDCA	448.3	448.3 → 74.0	70	7.9	1.0	G-CDCA-d_5
T-CDCA	498.3	498.3 → 80.0	122	8.1	1.0	T-CDCA-d_5
CDCA-S	471.2	471.2 → 97.0	88	6.4	1.0	CDCA-S-d_5
G-CDCA-S	528.3	528.3 → 448.3	54	5.7	1.0	G-CDCA-S-d_5
T-CDCA-S	578.2	288.6 → 480.3	100	5.9	1.4	T-CDCA-S-d_5
DCA	391.3	391.3 → 391.3	5	9.1	2.0	DCA-d_5
G-DCA	448.3	448.3 → 74.0	70	8.2	0.5	G-DCA-d_5
T-DCA	498.3	498.3 → 80.0	122	8.5	1.0	T-DCA-d_5
DCA-S	471.2	471.2 → 97.0	88	6.5	1.0	DCA-S-d_5
G-DCA-S	528.3	528.3 → 448.3	54	5.9	1.0	G-DCA-S-d_5
T-DCA-S	578.2	288.6 → 480.3	100	6.1	2.0	T-DCA-S-d_5
LCA	375.3	375.3 → 375.3	5	10.3	2.0	LCA-d_5
G-LCA	432.3	432.3 → 74.0	62	9.5	0.5	G-LCA-d_5
T-LCA	482.3	482.3 → 80.0	110	9.6	0.3	T-LCA-d_5
LCA-S	455.2	455.2 → 97.0	86	8.0	0.5	LCA-S-d_5
G-LCA-S	512.3	512.3 → 432.3	30	6.8	1.0	G-LCA-S-d_5
T-LCA-S	562.3	280.6 → 464.3	50	7.0	4.0	T-LCA-S-d_5
UDCA	391.3	391.3 → 391.3	5	6.7	2.0	UDCA-d_5
G-UDCA	448.3	448.3 → 74.0	70	6.1	0.5	G-UDCA-d_5
T-UDCA	498.3	498.3 → 80.0	122	6.4	0.5	T-UDCA-d_5
UDCA-S	471.2	471.2 → 97.0	88	4.7	0.5	UDCA-S-d_5
G-UDCA-S	528.3	528.3 → 448.3	54	4.2	0.5	G-UDCA-S-d_5
T-UDCA-S	578.2	288.6 → 480.3	100	4.5	1.0	T-UDCA-S-d_5
3β-Hydroxy-Δ⁵- bile acids						
Δ^5-3β,7α-ol	389.3	389.3 → 389.3	5	6.6	2.0	UDCA-d_5
G-Δ^5-3β,7α-ol	446.3	446.3 → 74.0	62	5.9	0.5	G-UDCA-d_5
T-Δ^5-3β,7α-ol	496.3	496.3 → 80.0	124	6.1	0.5	G-UDCA-d_5
Δ^5-3β,7α-ol-S	469.2	469.2 → 97.0	130	5.6	1.0	G-UDCA-d_5
G-Δ^5-3β,7α-ol-S	526.2	526.2 → 446.3	42	5.0	1.0	G-UDCA-d_5
T-Δ^5-3β,7α-ol-S	576.2	287.6 → 478.3	32	5.2	0.5	G-UDCA-d_5
Δ^5-3β,7α,12α-ol	405.3	405.3 → 405.3	5	4.8	2.0	T-UDCA-S-d_5
G-Δ^5-3β,7α,12α-ol	462.3	462.3 → 74.0	70	4.7	0.5	G-UDCA-S-d_5
T-Δ^5-3β,7α,12α-ol	512.3	512.3 → 80.0	124	4.9	0.3	G-UDCA-S-d_5
Δ^5-3β,7α,12α-ol-S	485.2	485.2 → 97.0	84	4.2	1.2	G-UDCA-S-d_5
G-Δ^5-3β,7α,12α-ol-S	542.2	542.2 → 462.3	46	4.0	1.2	T-UDCA-S-d_5
T-Δ^5-3β,7α,12α-ol-S	592.2	295.6→494.3	34	4.3	1.2	T-UDCA-S-d_5

C.E.: collision energy; R.T.: retention time; LOD: limit of detection (S/N = 5); I.S.: internal standard.

Table 2
Matrix effects for 3β-hydroxy-Δ^5-bile acids and their internal standards.

Bile acid	Urine samples		Seume samples	
	Relative peak area (%, mean, n = 5)	C.V. (%)	Relative peak area (%, mean, n = 5)	C.V. (%)
Δ^5-3β,7α-ol	98.5	4.9	98.3	3.5
G-Δ^5-3β,7α-ol	100.2	2.0	97.9	4.5
T-Δ^5-3β,7α-ol	99.4	3.2	100.4	5.2
Δ^5-3β,7α-ol-S	98.7	4.6	98.4	4.6
G-Δ^5-3β,7α-ol-S	99.3	3.3	99.7	4.9
T-Δ^5-3β,7α-ol-S	97.6	3.9	98.6	4.3
Δ^5-3β,7α,12α-ol	98.4	4.8	99.2	3.3
G-Δ^5-3β,7α,12α-ol	98.8	4.6	97.9	3.7
T-Δ^5-3β,7α,12α-ol	99.6	2.9	99.2	2.9
Δ^5-3β,7α,12α-ol-S	100.1	2.4	99.3	3.9
G-Δ^5-3β,7α,12α-ol-S	102.6	3.8	100.5	4.1
T-Δ^5-3β,7α,12α-ol-S	99.8	2.9	100.3	2.8
UDCA-d_5	97.8	5.3	99.7	4.5
G-UDCA-d_5	98.9	3.6	97.8	4.3
G-UDCA-S-d_5	97.4	2.7	98.7	5.2
T-UDCA-S-d_5	101.2	5.1	99.2	3.1

C.V.: coefficient value.

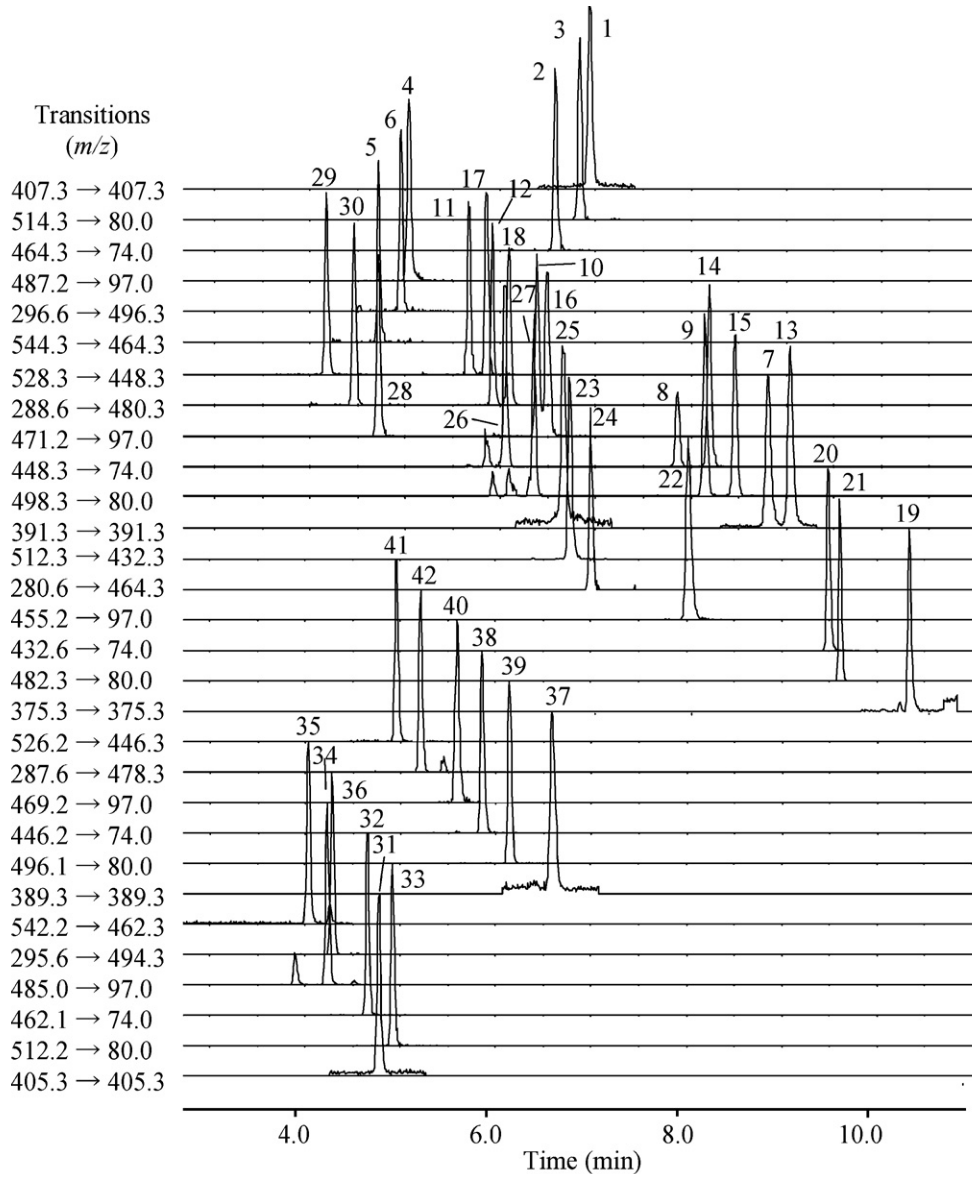


Fig. 2. Typical SRM chromatograms of authentic standards of bile acids. Peak number and compounds are the same as those in Fig. 1.

over 8 min and then from 48% to 100% over 3 min. The column was washed at 100% B for 1 min and re-equilibrated at 0% B for 5 min.

2.4. Quantitative analysis of bile acids in human urine and serum

Sample preparation of human urine and serum for LC/ESI-MS/MS analysis using a solid phase extraction (SPE) was performed by the method previously reported [18,19]. SPE was performed using Oasis HLB 96-well plate cartridges, a vacuum manifold and a vacuum source. After addition of internal standards (each 20 pmol) to urine or serum (5–100 μL), the mixture was diluted with 0.1 M phosphate buffer (pH 7.4, 0.2 mL), and the solution was loaded onto an Oasis HLB 96-well plate cartridge at a flow rate of approximately 4 mL/min. The cartridge was sequentially washed with water (0.4 mL). After the cartridge was dried under vacuum for 2 min, the bile acids were eluted with ethanol (0.4 mL). The eluate was evaporated to dryness under reduced pressure. The residue was dissolved with 50 μL of mobile phase A, and an aliquot (5 μL) was injected into the LC/ESI-MS/MS system.

2.5. Method validation

For preparing standard stock solutions, glycine- and taurine-conjugated bile acids and sulfated bile acids were dissolved in methanol at a concentration of 200 μM. Samples were diluted to concentrations of 10, 20, 50, 100, 200, 500, 1000, and 2000 pmol/mL using methanol. An IS stock solution containing 400 pmol/mL of stable isotope-labeled bile acids was also prepared in methanol. A 50-μL aliquot of each standard solution was mixed with 50 μL of IS solution and evaporated under nitrogen gas at room temperature. The residue was dissolved in 50 μL of mobile phase A, and 5 μL of this solution was injected into the LC/ESI-MS/MS system. Calibration curves were constructed by plotting the peak-area ratio of each bile acid to those of ISs *versus* the weights of the bile acid. For the accuracy studies, blank samples (bile acid-free samples) were prepared from urine or serum of healthy volunteers by treating with activated charcoal. In order to assess matrix effects, blank urine and serum samples were extracted using the assay procedure described above. After extraction, these samples were spiked with defined amounts of standard stock solution (10 pmol/mL) and IS stock solution (400 pmol/mL). Matrix effects were assessed by comparing the peak area of spiked urine and serum samples (A) to those of samples containing only standard stock solutions (B). The peak area ratio (A/B × 100) % was used to evaluate the matrix effects. The recovery rates through the assay procedure were tested by adding known concentrations of bile acids (10, 100, and 1000 pmol/mL) to blank urine and serum.

3. Results and discussion

3.1. LC/ESI-MS/MS analysis of 3β-hydroxy-Δ^5-bile acids and related compounds

It has been reported that glycine- and taurine-conjugated bile acids and nonamidated bile acid 3-sulfates are easily fragmented under the condition of low-energy collision-induced dissociation (CID), and those product ions formed from the deprotonated molecule $[M-H]^-$ were observed at *m/z* 74 for glycine-conjugated bile acids, at *m/z* 80 for taurine-conjugate bile acids, and at *m/z* 97 for nonamidated bile acid 3-sulfates [10–14]. It has also been reported that glycine- and taurine-conjugated bile acid 3-sulfates give doubly charged $[M-2H]^{2-}$ ions in an ammonium acetate-MeCN mobile phase [10,12,14]. Goto et al. [12] reported that taurine-conjugated bile acid 3-sulfates give a steroid nucleus-containing product ion $[M-H-H_2SO_4]^-$, formed from the doubly charged ion $[M-2H]^{2-}$, with a low noise level and also that glycine-conjugated bile acid 3-sulfates give the steroid nucleus-containing product ion $[M-HSO_3]^-$, formed from the deprotonated molecule $[M-H]^-$, under mild CID conditions. In contrast to conjugated bile acids, it has been reported that unconjugated bile acids show no prominent product ion [13,14]. First, we investigated the fragmentation of deprotonated bile acids in the negative ion mode using 50% MeCN/10 mM ammonium acetate (adjusted to pH 7.0 by adding an aqueous ammonia solution) as the mobile phase. The glycine- and taurine-conjugated 3β-hydroxy-Δ^5-bile acids (G-Δ^5-3β,7α-ol, G-Δ^5-3β,7α,12α-ol and T-Δ^5-3β,7α-ol, T-Δ^5-3β,7α,12α-ol) and the nonamidated 3β-hydroxy-Δ^5-bile acid 3-sulfates (Δ^5-3β,7α-ol-S and Δ^5-3β,7α,12α-ol-S) gave product ions at *m/z* 74, *m/z* 80 and *m/z* 97, respectively. T-Δ^5-3β,7α,12α-ol-S gave a doubly charged ion $[M-2H]^{2-}$ at *m/z* 295.6 and a product ion $[M-H-H_2SO_4]^-$ at *m/z* 494.3. G-Δ^5-3β,7α,12α-ol-S gave the deprotonated ion

Table 3
Relative recoveries of bile acids from urine.

Bile acid	Relative recovery (%, mean ± S.D., *n* = 5)		
	Concentration added 10 pmol/mL	Concentration added 100 pmol/mL	Concentration added 1000 pmol/mL
CA	105.1 ± 2.2 (2.1)	101.3 ± 1.6 (1.6)	100.1 ± 0.9 (0.9)
G-CA	100.9 ± 1.8 (1.8)	100.7 ± 2.2 (2.2)	102.1 ± 1.1 (1.1)
T-CA	99.2 ± 2.4 (2.4)	100.4 ± 1.2 (1.2)	100.8 ± 1.9 (1.9)
CA-S	95.9 ± 1.3 (1.4)	99.8 ± 3.0 (3.0)	101.1 ± 1.7 (1.7)
G-CA-S	95.5 ± 3.7(3.9)	98.3 ± 1.9(1.9)	99.4 ± 2.1(2.1)
T-CA-S	95.4 ± 3.8(4.0)	100.7 ± 2.8(2.8)	100.2 ± 1.8(1.8)
CDCA	99.6 ± 1.5(1.5)	101.5 ± 1.2(1.2)	100.5 ± 0.8(0.8)
G-CDCA	101.9 ± 4.6(4.5)	101.3 ± 1.3(1.3)	101.1 ± 1.3(1.3)
T-CDCA	100.0 ± 0.9(0.9)	99.0 ± 2.4(2.4)	99.2 ± 0.8(0.8)
CDCA-S	99.9 ± 3.9(3.9)	100.6 ± 1.8(1.8)	99.5 ± 1.1(1.1)
G-CDCA-S	100.8 ± 2.8(2.8)	97.6 ± 1.6(1.6)	100.6 ± 1.3(1.3)
T-CDCA-S	101.4 ± 3.4(3.4)	101.6 ± 4.1(4.0)	100.0 ± 2.2(2.2)
DCA	102.6 ± 3.5(3.4)	104.1 ± 1.7(1.6)	100.4 ± 1.1(1.1)
G-DCA	102.2 ± 3.8(3.7)	101.4 ± 2.1(2.1)	101.7 ± 1.7(1.7)
T-DCA	99.0 ± 1.2(1.2)	98.9 ± 2.3(2.3)	102.5 ± 1.3(1.3)
DCA-S	99.9 ± 3.8(3.8)	100.6 ± 2.1(2.1)	99.5 ± 1.7(1.7)
G-DCA-S	100.8 ± 2.9(2.9)	99.8 ± 2.9(2.9)	99.5 ± 2.8(2.8)
T-DCA-S	99.7 ± 6.3(6.3)	101.3 ± 1.1(1.1)	101.8 ± 4.6(4.5)
LCA	105.8 ± 1.6(1.5)	102.3 ± 1.2(1.2)	99.5 ± 1.0(1.0)
G-LCA	104.8 ± 2.0(1.9)	99.7 ± 2.6(2.6)	101.1 ± 0.7(0.7)
T-LCA	98.4 ± 1.1(1.1)	98.9 ± 1.8(1.8)	99.4 ± 1.4(1.4)
LCA-S	98.5 ± 2.0(2.0)	102.7 ± 1.2(1.2)	99.3 ± 1.8(1.8)
G-LCA-S	99.5 ± 4.1(4.1)	96.1 ± 2.6(2.7)	97.2 ± 1.5(1.5)
T-LCA-S	99.4 ± 4.2(4.2)	98.6 ± 3.3(3.3)	98.1 ± 1.3(1.3)
UDCA	100.1 ± 3.5(3.5)	101.1 ± 1.7(1.7)	100.0 ± 0.9(0.9)
G-UDCA	98.9 ± 1.7(1.7)	99.9 ± 2.0(2.0)	101.5 ± 2.3(2.3)
T-UDCA	97.5 ± 2.3(2.4)	101.7 ± 2.6(2.6)	100.8 ± 1.2(1.2)
UDCA-S	98.0 ± 4.4(4.5)	100.9 ± 1.5(1.5)	99.5 ± 1.3(1.3)
G-UDCA-S	96.0 ± 4.6(4.8)	98.2 ± 0.7(0.7)	100.6 ± 1.5(1.5)
T-UDCA-S	99.3 ± 3.0(3.0)	101.7 ± 3.8(3.7)	100.9 ± 1.7(1.7)
Δ^5-3β,7α-ol	100.2 ± 4.2(4.2)	103.1 ± 1.2(1.2)	98.5 ± 1.2(1.2)
G-Δ^5-3β,7α-ol	97.1 ± 1.8(1.9)	95.4 ± 4.2(4.4)	97.5 ± 2.9(3.0)
T-Δ^5-3β,7α-ol	97.2 ± 4.6(4.7)	104.2 ± 4.1(3.9)	97.2 ± 4.3(4.4)
Δ^5-3β,7α-ol-S	101.6 ± 3.4(3.3)	101.9 ± 2.2(2.2)	106.6 ± 1.2(1.1)
G-Δ^5-3β,7α-ol-S	99.0 ± 4.7(4.7)	95.2 ± 3.4(3.5)	99.9 ± 3.3(3.3)
T-Δ^5-3β,7α-ol-S	102.4 ± 3.7(3.6)	100.8 ± 4.9(4.9)	98.1 ± 3.4(3.5)
Δ^5-3β,7α,12α-ol	101.3 ± 1.7(1.7)	104.2 ± 4.0(4.7)	99.3 ± 1.0(1.0)
G-Δ^5-3β,7α,12α-ol	102.0 ± 2.6(2.5)	99.3 ± 2.5(2.5)	100.2 ± 2.2(2.2)
T-Δ^5-3β,7α,12α-ol	100.5 ± 3.8(3.8)	99.5 ± 4.9(4.9)	104.9 ± 1.2(1.1)
Δ^5-3β,7α,12α-ol-S	100.1 ± 2.7(2.6)	95.3 ± 2.7(2.9)	97.8 ± 2.1(2.1)
G-Δ^5-3β,7α,12α-ol-S	97.0 ± 2.3(2.4)	99.7 ± 4.9(4.9)	99.5 ± 1.9(1.9)
T-Δ^5-3β,7α,12α-ol-S	98.0 ± 3.5(3.6)	98.2 ± 3.0(3.1)	102.3 ± 3.3(3.2)

S.D.: standard deviation; values in parentheses represent coefficient values.

$[M\text{-}H]^-$ at *m/z* 542.2 and a product ion $[M\text{–}HSO_3]^-$ at *m/z* 462.3. Unconjugated 3β-hydroxy-Δ^5-bile acids did not yield any major fragments except deprotonated ion $[M-H]^-$. These results are consistent with previously reported findings. Therefore, we selected these product ions as monitoring ions for SRM analysis, and we optimized the collision energy to obtain the highest signal-to-noise ratio. For unconjugated bile acids, the same mass was monitored for both precursor and product ions (Table 1). Typical SRM chromatograms for authentic samples of 3β-hydroxy-Δ^5-bile acids and related compounds are shown in Fig. 2, indicating the simultaneous separation and determination of all bile acids within 11 min. Calibration curves for all bile acids were linear over the range of 10–2000 pmol/mL, with linear correlation coefficients of more than 0.999. The deviations of calibration standards were less than 10% ($n = 5$) for all points in the calibration range. The detection limit was less than 4 pmol/mL (S/N = 5) for all bile acids in blank urine. The LC/ESI-MS/MS parameters of the reference bile acids are summarized in Table 1. The results of the matrix effects test are shown in Table 2. The mean matrix effect values of 3β-hydroxy-Δ^5-bile acids and their ISs were 97.4–102.6% of the peak area of samples containing only standard stock solutions with coefficients of variation that were less than 5.3%. The results showed that no significant ion suppression or enhancement effects were observed. In addition, significant matrix effects for other bile acids were not observed (data not shown).

3.2. Determination of 3β-hydroxy-Δ^5-bile acids in human urine and serum

The LC/ESI-MS/MS method was applied to determination of 3β-hydroxy-Δ^5-bile acids in human urine and serum from patients with liver disease, in which an abnormality in HSD3B7 was suggested. Urine and serum samples were submitted to a clean-up procedure using conventional reversed-phase extraction prior to LC/ESI-MS/MS analysis. The recovery rates through the assay procedure described in the Section 2 were tested by adding known concentrations of 3β-hydroxy-Δ^5-bile acids and related compounds to blank urine and serum. The relative recoveries of bile acids were 95.2–106.6% of the added amounts of their standard samples with coefficients of variation that were less than 6.3%, as shown in Tables 3 and 4. Decomposition or dehydration of the 3β-hydroxy-Δ^5-bile acids was not observed in the clean-up procedure. Typical SRM chromatograms of urine samples obtained from a healthy volunteer and from a patient with liver disease

Table 4
Relative recoveries of bile acids from serum.

Bile acid	Relative recovery (%, mean ± S.D., $n = 5$)		
	Concentration added 10 pmol/mL 100 pmol/mL	Concentration added 1000 pmol/mL	Concentration added 1000 pmol/mL
CA	105.9 ± 2.3(2.2)	102.0 ± 1.1(1.1)	101.0 ± 1.2(1.2)
G-CA	101.8 ± 2.5(2.5)	99.5 ± 2.3(2.3)	101.1 ± 2.7(2.7)
T-CA	99.6 ± 3.8(3.8)	101.7 ± 3.5(3.4)	102.1 ± 0.8(0.8)
CA-S	99.9 ± 2.6(2.6)	102.9 ± 2.1(2.0)	100.0 ± 2.3(2.3)
G-CA-S	99.0 ± 3.8(3.8)	102.3 ± 3.3(3.2)	97.6 ± 1.4(1.4)
T-CA-S	97.3 ± 4.9(5.0)	100.8 ± 2.4(2.4)	101.1 ± 2.8(2.8)
CDCA	102.3 ± 2.8(2.7)	104.0 ± 1.1(1.1)	99.7 ± 1.1(1.1)
G-CDCA	100.8 ± 1.3(1.3)	100.8 ± 2.5(2.5)	99.9 ± 1.6(1.6)
T-CDCA	99.6 ± 3.0(3.0)	102.8 ± 1.4(1.4)	99.1 ± 1.2(1.2)
CDCA-S	103.1 ± 3.5(3.4)	102.0 ± 1.6(1.6)	99.5 ± 1.3(1.3)
G-CDCA-S	100.8 ± 1.9(1.9)	102.8 ± 0.4(0.4)	100.0 ± 0.9(0.9)
T-CDCA-S	101.5 ± 3.7(3.6)	101.4 ± 2.7(2.7)	100.8 ± 2.8(2.8)
DCA	106.2 ± 3.3(3.1)	103.6 ± 2.0(1.9)	100.9 ± 2.0(2.0)
G-DCA	99.7 ± 1.7(1.7)	104.2 ± 1.8(1.7)	97.4 ± 1.0(1.0)
T-DCA	105.7 ± 4.5(4.3)	103.4 ± 2.3(2.2)	105.0 ± 1.9(1.8)
DCA-S	99.9 ± 1.7(1.7)	102.9 ± 1.8(1.7)	100.0 ± 1.0(1.0)
G-DCA-S	101.6 ± 3.2(3.1)	103.7 ± 1.3(1.3)	101.6 ± 3.4(3.3)
T-DCA-S	102.6 ± 4.1(4.0)	98.6 ± 2.0(2.0)	97.1 ± 2.1(2.2)
LCA	98.4 ± 2.6(2.6)	100.9 ± 0.4(0.4)	97.2 ± 1.1(1.1)
G-LCA	100.2 ± 3.4(3.4)	102.2 ± 1.0(1.0)	100.8 ± 1.8(1.8)
T-LCA	99.7 ± 1.0(1.0)	98.7 ± 2.0(2.0)	100.2 ± 3.4(3.4)
LCA-S	100.8 ± 1.8(1.8)	100.6 ± 3.6(3.6)	99.8 ± 2.1(2.1)
G-LCA-S	98.7 ± 3.4(3.4)	99.7 ± 1.0(1.0)	97.2 ± 1.5(1.5)
T-LCA-S	98.4 ± 2.6(2.6)	100.9 ± 0.4(0.4)	98.1 ± 1.3(1.3)
UDCA	99.6 ± 2.5(2.5)	98.6 ± 3.3(3.3)	97.7 ± 3.1(3.2)
G-UDCA	101.5 ± 4.8(4.7)	99.8 ± 2.1(2.1)	101.5 ± 1.8(1.8)
T-UDCA	104.2 ± 4.5(4.3)	101.8 ± 1.3(1.3)	98.1 ± 2.7(2.8)
UDCA-S	96.3 ± 4.6(4.5)	102.4 ± 1.9(1.9)	100.2 ± 2.2(2.2)
G-UDCA-S	101.5 ± 1.3(1.3)	103.9 ± 1.8(1.7)	99.1 ± 1.2(1.2)
T-UDCA-S	100.2 ± 2.8(2.8)	102.6 ± 2.7(2.6)	96.1 ± 4.5(4.7)
Δ^5-3β,7α-ol	98.8 ± 3.3(3.3)	101.4 ± 1.0(1.0)	98.6 ± 1.2(1.2)
G-Δ^5-3β,7α-ol	100.7 ± 2.0(2.0)	100.9 ± 3.1(3.0)	99.2 ± 3.0(3.1)
T-Δ^5-3β,7α-ol	99.5 ± 4.9(4.9)	99.5 ± 4.9(4.9)	98.4 ± 4.3(4.4)
Δ^5-3β,7α-ol-S	100.7 ± 1.2(1.2)	101.9 ± 1.0(1.0)	100.4 ± 2.4(2.4)
G-Δ^5-3β,7α-ol-S	104.3 ± 3.4(3.3)	101.9 ± 0.9(0.9)	100.0 ± 2.5(2.5)
T-Δ^5-3β,7α-ol-S	96.8 ± 2.7(2.8)	99.7 ± 1.3(1.3)	99.6 ± 2.1(2.1)
$\Delta\Delta^5$-3β,7α,12α-ol	99.8 ± 4.3(4.3)	104.1 ± 1.3(1.3)	98.5 ± 1.1(1.1)
G-Δ^5-3β,7α,12α-ol	100.9 ± 4.6(4.6)	99.8 ± 1.8(1.8)	100.3 ± 3.3(3.3)
T-$\Delta\Delta^5$-3β,7α,12α-ol	99.7 ± 2.9(2.9)	102.7 ± 3.2(3.2)	101.9 ± 2.8(2.8)
Δ^5-3β,7α,12α-ol-S	100.8 ± 2.4(2.4)	101.6 ± 1.8(1.8)	101.7 ± 1.2(1.2)
G-Δ^5-3β,7α,12α-ol-S	102.6 ± 1.6(1.5)	101.9 ± 0.9(0.9)	102.0 ± 3.6(3.5)
T-Δ^5-3β,7α,12α-ol-S	101.0 ± 1.7(1.7)	103.9 ± 1.8(1.8)	101.2 ± 4.7(4.6)

S.D.: standard deviation; values in parentheses represent coefficient values.

Table 5
Concentration of bile acids in urine and serum from patients with liver disease and from healthy controls.

Bile acid	Urine (nmol/mL)			Serum (nmol/mL)	
	Patient 1	Patient 2	Healthy control (mean ± S.D., $n=8$)	Patient 1	Patient 2
Common bile acids					
CA	0.36	0.37	0.41 ± 0.50	0.04	0.05
G-CA	1.70	1.48	0.44 ± 0.80	0.53	0.21
T-CA	0.89	0.84	0.03 ± 0.03	0.16	0.09
CA-S	0.24	0.24	0.01 ± 0.02	0.02	0.03
G-CA-S	3.02	1.03	0.20 ± 0.35	0.19	0.05
T-CA-S	0.69	0.30	0.01 ± 0.04	0.06	0.03
CDCA	n.d.	n.d.	0.03 ± 0.07	n.d.	n.d.
G-CDCA	9.40	3.17	0.12 ± 0.13	0.35	0.52
T-CDCA	1.45	1.13	n.d.	n.d.	n.d.
CDCA-S	n.d.	n.d.	0.07 ± 0.10	n.d.	n.d.
G-CDCA-S	5.80	7.20	2.31 ± 3.66	0.66	0.49
T-CDCA-S	2.53	1.55	0.22 ± 0.37	0.52	0.18
DCA	n.d.	n.d.	0.15 ± 0.23	n.d.	n.d.
G-DCA	n.d.	n.d.	0.04 ± 0.02	n.d.	n.d.
T-DCA	n.d.	n.d.	n.d.	n.d.	n.d.
DCA-S	n.d.	n.d.	n.d.	n.d.	n.d.
G-DCA-S	n.d.	n.d.	1.80 ± 1.71	n.d.	n.d.
T-DCA-S	n.d.	n.d.	0.15 ± 0.06	n.d.	n.d.
LCA	n.d.	n.d.	0.01 ± 0.02	n.d.	n.d.
G-LCA	n.d.	n.d.	n.d.	n.d.	n.d.
T-LCA	n.d.	n.d.	n.d.	n.d.	n.d.
LCA-S	n.d.	n.d.	0.02 ± 0.06	n.d.	n.d.
G-LCA-S	n.d.	n.d.	0.87 ± 0.56	n.d.	n.d.
T-LCA-S	n.d.	n.d.	0.52 ± 0.32	n.d.	n.d.
UDCA	n.d.	n.d.	0.04 ± 0.07	n.d.	n.d.
G-UDCA	n.d.	n.d.	0.12 ± 0.13	n.d.	n.d.
T-UDCA	n.d.	n.d.	n.d.	n.d.	n.d.
UDCA-S	n.d.	n.d.	0.09 ± 0.13	n.d.	n.d.
G-UDCA-S	1.13	2.03	0.56 ± 0.74	n.d.	n.d.
T-UDCA-S	n.d.	n.d.	0.02 ± 0.03	n.d.	n.d.
3β-Hydroxy-Δ^5-bile acids					
Δ^5-3β,7α-ol	n.d.	1.18	n.d.	6.40	16.9
G-Δ^5-3β,7α-ol	12.4	97.5	0.09 ± 0.06	0.71	2.89
T-Δ^5-3β,7α-ol	n.d.	1.56	n.d.	n.d.	0.13
Δ^5-3β,7α-ol-S	261	545	0.01 ± 0.02	38.7	28.7
G-Δ^5-3β,7α-ol-S	1165	1360	0.14 ± 0.11	118	60.5
T-Δ^5-3β,7α-ol-S	121	56.5	n.d.	18.0	5.70
Δ^5-3β,7α,12α-ol	23.0	107	n.d.	0.99	2.85
G-Δ^5-3β,7α,12α-ol	142	1095	0.06 ± 0.03	1.50	5.15
T-Δ^5-3β,7α,12α-ol	10.2	39.7	n.d.	0.28	0.78
Δ^5-3β,7α,12α-ol-S	291	368	0.01 ± 0.01	18.2	14.2
G-Δ^5-3β,7α,12α-ol-S	2040	1400	0.03 ± 0.03	74.5	24.7
T-Δ^5-3β,7α,12α-ol-S	150	43.9	0.01 ± 0.01	6.70	1.52
Total	4242	5134	8.59 ± 8.40	288	166

S.D.: standard deviation; n.d., not detectable.

are shown in Fig. 3. 3β-Hydroxy-Δ^5-bile acids were identified in the urine of patients and were hardly detected in normal urine. The assay results obtained from healthy volunteers and patients with liver disease are summarized in Table 5. The levels of 3β-hydroxy-Δ^5-bile acids in urine of liver disease patients were markedly increased compared with those in urine of normal subjects. The proportion of 3β-hydroxy-Δ^5-bile acids and their conjugates in urine of liver disease patients was greater than that in urine of normal volunteers (*ca.* 99% of total bile acids). Although common bile acids, such as CA, CDCA and UDCA, were also detected in urine of liver disease patients, the proportion of these common bile acids was less than 1.0% of total bile acids. G-Δ^5-3β,7α,12α-ol-S and G-Δ^5-3β,7α-ol-S were predominant components in liver disease patients, accounting for more than 50% of the total amount of bile acids. Clayton et al. [2,9] investigated the bile acid profiles in urine of patients with HSD3B7 deficiency using FAB-MS, ESI-MS, and GC-MS methods and reported that sulfated 3β-hydroxy-Δ^5-bile acids and their glycine conjugates are the major bile acids excreted in urine of the patients. Our results are in good accordance with previous reports.

Subsequently, the profiles of serum bile acids from patients with liver disease were also determined using the LC/ESI-MS/MS method. Although the total amounts of 3β-hydroxy-Δ^5-bile acids in serum of liver disease patients were much lower than those in urine of the patients, the proportion of total 3β-hydroxy-Δ^5-bile acids and their conjugates was more than 98% of the total bile acids (Table 5). G-Δ^5-3β,7α,12α-ol-S and G-Δ^5-3β,7α-ol-S were the predominant bile acids in serum of liver disease patients as well as in urine of the patients. These results suggest that 3β-hydroxy-Δ^5-bile acids are mainly converted into sulfated glycine conjugates and excreted predominantly in urine.

In conclusion, this LC/ESI-MS/MS method is suitable for simultaneous determination of 3β-hydroxy-Δ^5-bile acids in routine clinical analysis. It is suggested that these 3β-hydroxy-Δ^5-bile acids are the major components in urine and/or serum of patients with liver disease by the malfunction of HSD3B7. Analysis of the profile of these bile acids in biological fluids may provide useful information on the function of HSD3B7. Further studies on the clinical application of this LC/ESI-MS/MS method are now in progress and the details will be reported in the near future.

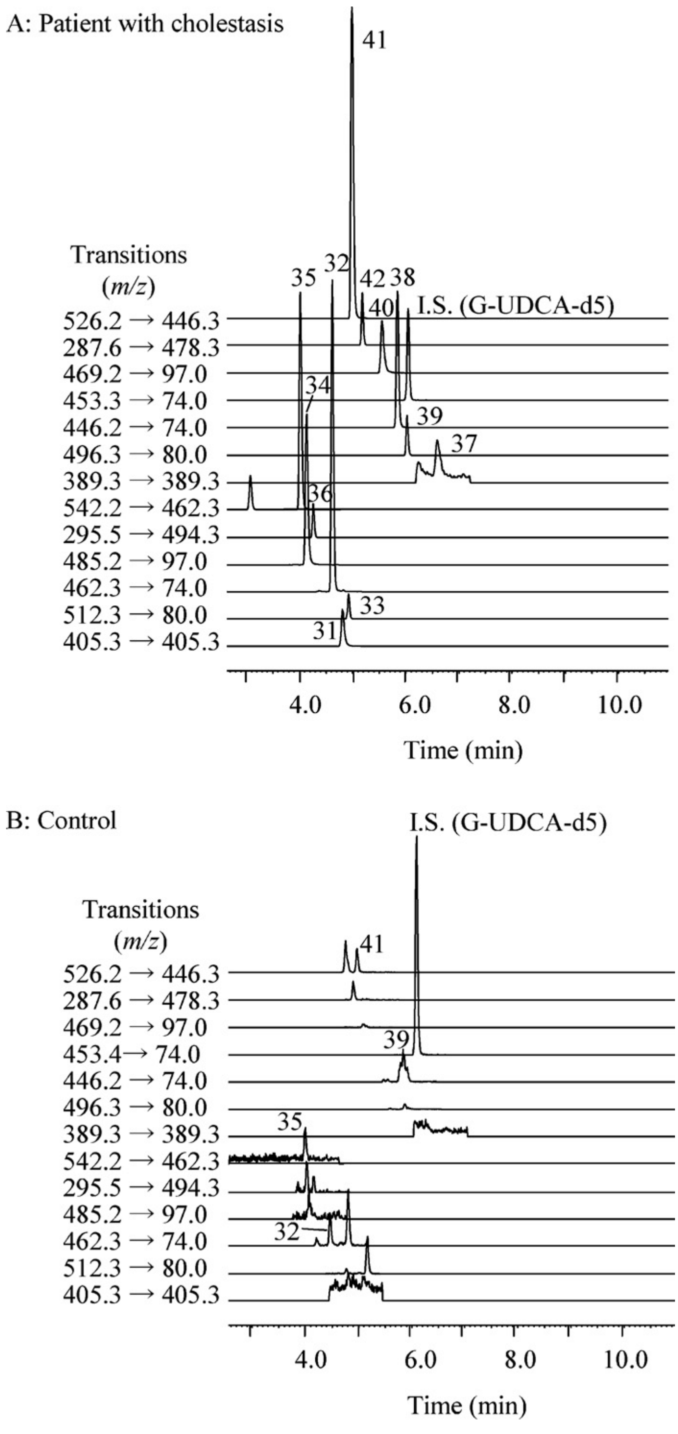


Fig. 3. Typical SRM chromatograms of bile acids in urine from a patient with cholestasis (A) and from a healthy control (B). Peak number and compounds are the same as those in Fig. 1.

References

[1] P.T. Clayton, J.V. Lenoard, A.M. Lawson, K.D.R. Setchell, S. Andersson, B. Egestad, J. Sjövall, J. Clin. Invest. 79 (1987) 1031.
[2] H. Ichimiya, H. Nazer, T. Gunasekaran, P. Clayton, J. Sjövall, Arch. Dis. Childhood 65 (1990) 1121.
[3] M.S. Buchmann, E.A. Kvittingen, H. Nazer, T. Gunasekaran, P.T. Clayton, J. Sjövall, I. Björkhem, J. Clin. Invest. 86 (1990) 2034.
[4] H. Ichimiya, B. Egestad, H. Nazer, E.S. Baginski, P.T. Clayton, J. Sjövall, J. Lipid Res. 32 (1991) 829.
[5] E. Jacquemin, K.D.R. Setchell, N.C. O'Connell, A. Estrada, G. Maggiore, J. Schmitz, M. Hadchouel, O. Bernard, J. Pediatr. 125 (1994) 379.
[6] M. Kobayashi, M. Koike, M. Sakiyama, S. Okuda, M. Okuda, T. Tanaka, A. Unno, H. Nittono, H. Takei, T. Murai, T. Yoshimura, T. Kurosawa, Pediatr. Int. 42 (2000) 685.
[7] P. Subramaniam, P.T. Clayton, B.C. Portmann, G.M. Vergani, N. Hadzïć, J. Pediartr. Gastroenterol. Nutr. 50 (2010) 61.
[8] D. Haas, H. Gan-Schreier, C.D. Langhans, T. Rohrer, G. Engelmann, M. Heverin, D.W. Russell, P.T. Clayton, G.F. Hoffmann, J.G. Okun, Word J. Gastroenterol. 14 (2012) 1067.
[9] P.T. Clayton, J. Inherit. Metab. Dis. 34 (2011) 593.
[10] S. Ikegawa, T. Yanagihara, N. Murao, H. Watanabe, J. Goto, T. Niwa, J. Mass Spectrom. 32 (1997) 401.
[11] I. Burkard, A. von Eckardstein, K.M. Rentsch, J. Chromatogr. B 826 (2005) 147.
[12] T. Goto, K.T. Myint, K. Sato, O. Wada, G. Kakiyama, T. Iida, T. Hishinuma, N. Mano, J. Goto, J. Chromatogr. B 846 (2007) 69.
[13] Y. Alnouti, I.L. Csanaky, C.D. Klaassen, J. Chromatogr. B 873 (2008) 209.
[14] M. Scherer, C. Gnewuch, G. Schmitz, G. Liebisch, J. Chromatogr. B 877 (2009) 3920.
[15] C. Steiner, A. von Eckardstein, K.M. Rentsch, J. Chromatogr. B 878 (2010) 2870.
[16] A. Muto, H. Takei, A. Unno, T. Murai, T. Kurosawa, S. Ogawa, T. Iida, S. Ikegawa, J. Mori, A. Ohtake, T. Hoshina, T. Mizuochi, A. Kimura, A.F. Hofmann, L.R. Hagey, H. Nittono, J. Chromatogr. B 900 (2012) 24.
[17] M. Tohma, R. Mahara, H. Takeshita, T. Kurosawa, Steroids 48 (1986) 331.
[18] K. Yamaguchi, T. Murai, H. Yabuuchi, T. Kurosawa, Anal. Sci. 25 (2009) 1155.
[19] K. Yamaguchi, T. Murai, H. Yabuuchi, T. Kurosawa, Anal. Sci. 26 (2010) 317.