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Detection of Δ^4-3-oxo-steroid 5β-reductase deficiency by LC–ESI-MS/MS measurement of urinary bile acids

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ABSTRACT

The synthesis of bile salts from cholesterol is a complex biochemical pathway involving at least 16 enzymes. Most inborn errors of bile acid biosynthesis result in excessive formation of intermediates and/or their metabolites that accumulate in blood and are excreted in part in urine. Early detection is important as oral therapy with bile acids results in improvement. In the past, these intermediates in bile acid biosynthesis have been detected in neonatal blood or urine by screening with FAB-MS followed by detailed characterization using GC–MS. Both methods have proved difficult to automate, and currently most laboratories screen candidate samples using LC–MS/MS. Here, we describe a new, simple and sensitive analytical method for the identification and characterization of 39 conjugated and unconjugated bile acids, including Δ^4-3-oxo- and $\Delta^{4,6}$-3-oxo-bile acids (markers for Δ^4-3-oxo-steroid 5β-reductase deficiency), using liquid chromatography-electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS). In this procedure a concentrated, desalted urinary sample (diluted with ethanol) is injected directly into the LC–ESI-MS/MS, operated with ESI and in the negative ion mode; quantification is obtained by selected reaction monitoring (SRM). To evaluate the performance of our new method, we compared it to a validated method using GC–MS, in the analysis of urine from two patients with genetically confirmed Δ^4-3-oxo-steroid 5β-reductase deficiency as well as a third patient with an elevated concentration of abnormal conjugated and unconjugated Δ^4-3-oxo-bile acids. The Δ^4-3-oxo-bile acids concentration recovered in three patients with 5β-reductase deficiency were 48.8, 58.9, and 49.4 μmol/mmol creatinine, respectively by LC–ESI-MS/MS.

1. Introduction

Bile acids are biosynthesized from cholesterol in a process that utilizes multiple partially overlapping enzymatic pathways to make substantial changes to the steroid ring nucleus and side-chain, a process that utilizes a minimum of 16 enzymes [1]. Some of the first inborn errors in these pathways were detected nearly 30 years ago [2,3] by the detection of intermediates in the bile acid biosynthetic pathway. Since that time, inborn errors of bile acid formation involving isolated defects in most of the enzymes in the biosynthetic pathway have been reported [4]. However, such enzymatic defects are extremely rare. The most common genetic defect in bile acid biosynthesis appears to be Δ^4-3-oxo-steroid 5β-reductase deficiency, first described by Setchell et al. [5]. In children with defects in the gene or promoter region of Δ^4-3-oxo-steroid 5β-reductase (gene *AKR1D1*), the urinary bile acid profile contains 7α-hydroxy-3-oxo-4-cholenoic acid (as its glycine *m/z* 444 and taurine *m/z* 494 conjugates), as well as its 12α-dihydroxy-metabolite (again as glycine *m/z* 460 and taurine *m/z* 510 conjugates), as shown in Fig. 1. Chenodeoxycholic acid and cholic acid, the normal primary bile acids in man are nearly undetectable. However,

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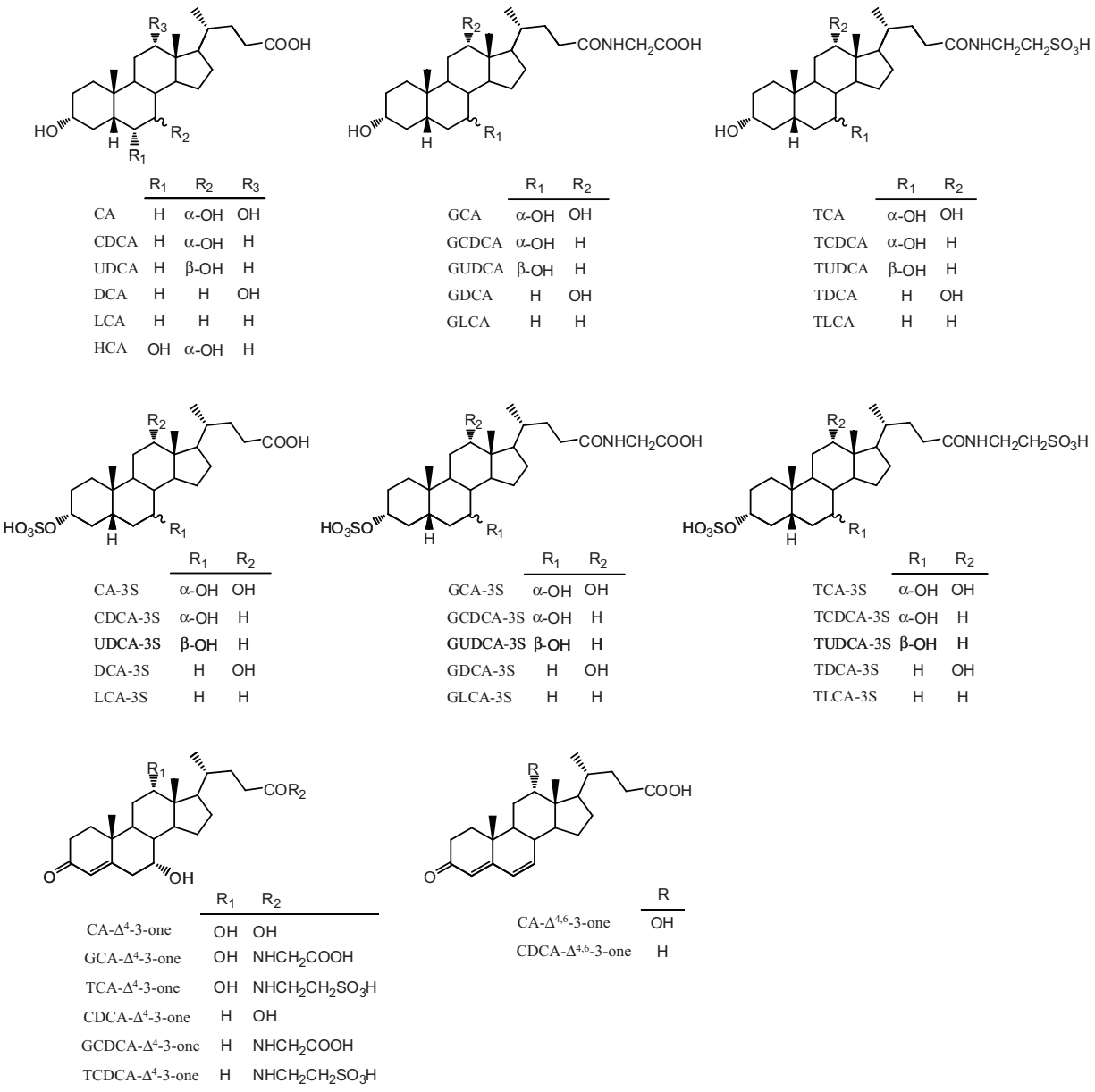

Fig. 1. Chemical structures of unconjugated and conjugated bile acids used in this study.

because gene alterations cannot be found in the majority of patients excreting increased amounts of these urinary Δ^4-3-oxo-bile acids, it was eventually recognized that such abnormal bile acids could also accumulate and be excreted in urine in children with severely damaged liver functions [4]. This turned out to be the case for the first Japanese patient with possible Δ^4-3-oxo-steroid 5β-reductase deficiency [6].

The effects of an accumulation of potentially toxic bile acid intermediates, and the absence of completed bile acid structures, often results in life threatening cholestatic liver disease, and as a result, great emphasis has been placed on early diagnosis and treatment with replacement bile acid therapy. The most non-invasive means of obtaining a preliminary diagnosis is through the analysis of urine for altered bile acids. Most laboratories currently screen candidate samples using LC–ESI-MS/MS [7,8]. Urine samples are examined in the negative mode and searched for the dominant ions present between *m*/*z* 350 and *m*/*z* 700 using both the total ion current but also with selected precursor ion scans (parents of 74 for glycine conjugates; parents of 85 for glucuronide conjugates; parents of 97 for sulfate conjugates, and parents of 124 for taurine conjugates). A large number of methods based on LC–MS, with or without CID or multiple reaction monitoring (MRM), have been published, for a summary see Griffiths and Sjövall [9]. Many of these methods produce results that do not always agree with the more comprehensive ion exchange separation followed by GC–MS methods [10]. Recently, Griffiths & Sjövall [11] published a powerful LC–MS/MS method for the complete analysis of oxysterol metabolomes, however, this method is too sensitive and labor intensive for the routine analysis of neonatal urine samples.

Here we describe a new LC–MS/MS method for the analysis of human urinary bile acids. Our aims were to find a method that matched the results obtained by GC–MS methodology, to enable direct analysis of intact bile acid conjugates, to simplify sample preparation, and thus to develop an accurate analytical method that requires a minimum of time and labor. To evaluate the performance of our new method, we compared it to a validated GC–MS method, and then used both methods to analyze the urine from two patients with genetically confirmed Δ^4-3-oxo-steroid 5β-reductase

Table 1
LC–ESI-MS/MS date for unconjugated and conjugated bile acids examinated.

Bile acid	RT (min)	Precursor ion (m/z)	Product ion (m/z)	CE (eV)	LOD (pmol/mL)	Correlation coefficient (r^2)
Saturated bile acids						
CA	31.1	407.2	343.2	33	0.50	0.9931
CDCA	34.6	391.3	373.2	32	8.79	0.9903
UDCA	29.5	391.3	373.2	32	14.31	0.9994
DCA	35.2	391.3	345.5	35	1.45	0.9929
LCA	37.9	375.2	357.2	33	21.54	0.9876
HCA	29.2	407.3	389.4	34	1.91	0.9954
GCA	29.5	464.3	74.0	39	0.08	0.9990
GCDCA	32.7	448.3	74.1	42	0.14	0.9995
GUDCA	27.6	448.3	74.1	42	0.19	0.9987
GDCA	33.6	448.3	74.1	42	0.12	0.9999
GLCA	36.0	432.3	74.1	39	0.03	0.9998
TCA	29.4	514.3	124.0	51	1.17	0.9944
TCDCA	32.5	498.3	124.0	51	0.34	0.9985
TUDCA	27.5	498.3	124.0	51	0.14	0.9922
TDCA	33.3	498.3	124.0	51	0.14	0.9933
TLCA	35.7	482.3	124.1	49	0.05	0.9973
CA-3S	24.5	487.3	97.0	46	0.03	0.9960
CDCA-3S	28.4	471.3	97.0	55	1.61	0.9974
UDCA-3S	23.2	471.3	97.0	55	0.30	0.9970
DCA-3S	28.8	471.3	97.0	55	0.64	0.9991
LCA-3S	31.7	455.3	97.0	44	0.01	0.9989
GCA-3S	22.1	271.7	97.0	41	0.05	0.9972
GCDCA-3S	25.5	263.7	97.0	40	0.03	0.9982
GUDCA-3S	20.3	263.7	97.0	40	0.03	0.9987
GDCA-3S	26.1	263.7	97.0	40	0.03	0.9990
GLCA-3S	28.5	255.7	97.0	40	0.37	0.9995
TCA-3S	22.2	296.7	97.0	38	0.04	0.9977
TCDCA-3S	25.4	288.7	97.0	39	0.03	0.9983
TUDCA-3S	20.4	288.7	97.0	39	0.03	0.9967
TDCA-3S	26.1	288.7	97.0	39	0.02	0.9975
TLCA-3S	28.4	280.7	97.0	37	1.13	0.9953
Unsaturated bile acids						
CA-Δ^4-3-one	23.8	403.3	123.1	39	0.09	0.9900
GCA-Δ^4-3-one	22.3	460.3	74.0	37	0.16	0.9992
TCA-Δ^4-3-one	22.4	510.3	124.1	50	0.33	0.9965
CDCA-Δ^4-3-one	29.0	387.3	369.4	27	0.83	0.9880
GCDCA-Δ^4-3-one	27.1	444.3	74.1	35	0.08	0.9980
TCDCA-Δ^4-3-one	27.0	494.3	124.0	44	0.76	0.9946
CA-$\Delta^{4,6}$-3-one	27.6	385.3	341.5	27	0.99	0.9822
CDCA-$\Delta^{4,6}$-3-one	33.2	369.3	325.5	28	0.39	0.9859

RT, retention time; CE, collision energy; LOD, limit of detection (S/N = 5).

deficiency as well as from a third patient with an elevated concentration of abnormal conjugated and unconjugated Δ^4-3-oxo-bile acids.

2. Experimental procedure

2.1. Materials and reagents

Authentic reference bile acids (see Appendix A) used in this study were as follows: cholic acid (CA), glycocholic acid (GCA), taurocholic acid (TCA), chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), ursodeoxycholic acid (UDCA), glycoursodeoxycholic acid (GUDCA), tauroursodeoxycholic acid (TUDCA), lithocholic acid (LCA), glycolithocholic acid (GLCA), taurolithocholic acid (TLCA), deoxycholic acid (DCA), taurodeoxycholic acid (TDCA) and hyocholic acid (HCA) were purchased from Sigma Chemicals (St. Louis, MO, USA). [2,2,4,4-d_4]-CA (d_4-CA, internal standard (IS) for unconjugated bile acids), [2,2,4,4-d_4]-GCA (d_4-GCA, IS for glycine conjugated bile acids), and [2,2,4,4-d_4]-TCA (d_4-TCA, IS for taurine conjugated and double conjugated bile acids) were obtained from CDD Isotopes Inc. (Quebec, Canada). The 3-sulfates for the following bile acids: CA, CDCA, UDCA, DCA, LCA, GCA, GCDCA, GUDCA, GDCA, GLCA, TCA, TCDCA, TUDCA, TDCA, and TLCA were synthesized by a previously reported method [12,13]. Unsaturated bile acids with the Δ^4-3-one configuration in the steroid nucleus for CA, GCA, TCA, CDCA, GCDCA, TCDCA, and for the $\Delta^{4,6}$-3-one for CA, and CDCA were synthesized by a previously reported method [14]. In this paper, we have used semi-trivial nomenclature for the Δ^4-3-one and $\Delta^{4,6}$-3-one derivatives of the common bile acids by using the abbreviation for the saturated compound. Ethanol, methanol, and water were of HPLC grade, ammonium acetate was analytical grade, and all were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

2.2. Preparation of standards

Individual stock solutions of bile acids were prepared separately at 10 μmol/mL in ethanol and the stock solutions were stored at −20 °C. These solutions were mixed in equal amounts for the analysis of unknown samples, and five point calibration standard solutions (30, 100, 300, 1000, 3000 pmol/mL) were prepared in 50% ethanol. The calibration standard solutions were stable in analytical glass vials for 4 weeks at 4 °C.

2.3. Urine specimens

Urine samples used for the present analysis were as follows: urines from two patients with 5β-reductase deficiency (as determined by genetic diagnosis, case 2 patient was receiving UDCA); urine from a patient with 5β-reductase deficiency (as determined by urinary bile acids analysis and clinical diagnosis); urine from 9 healthy children (ages 2–3 years); and urine from 5 healthy

Table 2
Recovery test of unconjugated and conjugated bile acids examined by LC–ESI-MS/MS.

Bile acids	100 pmol/mL Recovery (n = 5, %)			1000 pmol/mL Recovery (n = 5, %)		
	Average	(Range)	R.S.D. (%)	Average	(Range)	R.S.D. (%)
Saturated bile acids						
CA	90.6	(87.0–94.8)	3.2	105.6	(86.5–114.7)	10.6
CDCA	91.6	(88.9–97.3)	3.8	107.8	(93.5–120.0)	11.9
UDCA	77.2	(67.0–95.6)	14.1	106.9	(82.8–120.0)	13.8
DCA	91.1	(85.9–95.7)	4.1	106.8	(85.5–117.9)	12.0
LCA	76.5	(70.0–85.4)	8.8	104.7	(96.1–112.5)	5.9
HCA	87.0	(83.2–93.1)	4.2	106.1	(88.0–118.0)	11.1
GCA	104.3	(98.3–106.4)	3.3	91.9	(82.9–108.4)	10.6
GCDCA	101.3	(98.8–103.7)	2.2	93.0	(86.4–109.6)	10.3
GUDCA	100.5	(96.4–104.1)	3.2	89.8	(82.3–109.0)	12.1
GDCA	98.4	(95.0–103.8)	8.2	89.7	(83.3–109.0)	12.0
GLCA	100.0	(95.5–106.2)	4.7	86.7	(83.5–94.0)	5.1
TCA	102.4	(97.4–104.2)	8.9	94.2	(88.9–99.5)	4.6
TCDCA	94.1	(92.2–99.0)	3.0	103.4	(94.8–111.0)	6.0
TUDCA	91.5	(84.0–99.8)	7.2	97.1	(86.4–107.6)	9.5
TDCA	100.9	(89.8–111.4)	7.6	95.2	(89.7–101.6)	5.0
TLCA	97.3	(90.6–100.7)	4.3	99.6	(95.4–105.3)	3.8
CA-3S	101.2	(98.2–003.8)	2.2	97.6	(89.1–104.6)	6.8
CDCA-3S	98.1	(95.5–102.1)	2.7	100.2	(92.7–109.7)	7.2
UDCA-3S	98.3	(94.0–103.2)	4.3	96.2	(89.1–105.8)	6.8
DCA-3S	101.6	(96.9–104.6)	3.2	118.8	(112.8–123.9)	3.5
LCA-3S	98.9	(95.0–102.8)	3.3	98.7	(91.6–103.7)	5.6
GCA-3S	99.3	(96.0–104.3)	3.4	101.2	(94.0–109.8)	6.3
GCDCA-3S	103.2	(98.4–106.7)	3.5	95.3	(88.1–106.2)	7.8
GUDCA-3S	101.1	(97.5–103.5)	2.9	100.8	(88.7–115.0)	9.3
GDCA-3S	103.2	(101.2–104.6)	1.3	106.2	(99.8–108.5)	3.4
GLCA-3S	99.8	(93.6–104.9)	5.6	109.1	(101.0–114.6)	5.2
TCA-3S	100.8	(98.8–103.8)	2.3	102.9	(95.7–112.2)	6.6
TCDCA-3S	98.2	(95.0–99.8)	1.9	103.0	(94.9–110.1)	6.6
TUDCA-3S	99.4	(96.8–102.6)	2.9	94.6	(92.1–98.7)	2.9
TDCA-3S	99.5	(95.4–104.3)	4.1	101.6	(92.3–110.5)	7.6
TLCA-3S	99.5	(96.3–103.0)	2.9	107.4	(99.1–117.9)	6.6
Unsaturated bile acids						
CA-Δ^4-3-one	85.0	(81.8–93.1)	4.4	105.9	(84.2–116.4)	11.9
GCA-Δ^4-3-one	100.1	(96.1–105.6)	3.6	88.2	(79.1–110.1)	14.2
TCA-Δ^4-3-one	94.7	(84.8–103.9)	8.7	94.1	(79.1–105.1)	11.9
CDCA-Δ^4-3-one	92.2	(89.9–94.7)	2.5	104.2	(88.1–112.7)	9.3
GCDCA-Δ^4-3-one	99.3	(96.9–100.9)	1.6	87.7	(81.5–105.4)	11.4
TCDCA-Δ^4-3-one	99.3	(95.1–104.5)	4.0	94.5	(86.5–98.4)	5.1
CA-$\Delta^{4,6}$-3-one	91.3	(87.0–96.0)	4.5	118.0	(96.3–128.3)	11.0
CDCA-$\Delta^{4,6}$-3-one	94.3	(89.4–99.5)	4.1	113.1	(88.7–121.9)	12.4

children (ages 6–8 months). All urine samples were stored at −25 °C until the pretreatment for analysis.

2.4. Sample preparation

For the LC–ESI-MS/MS analysis, 0.05 mL of the urine samples was used for analysis. 0.45 mL of 50% ethanol and IS, 0.5 mL, containing (d_4-CA, d_4-GCA and d_4-TCA 200 pmol/mL in 50% ethanol), was added to the urine. Precipitated solids were moved by filtration through a 0.45 μm millipore filter (Millex®-LG, Billerica, MA, USA). A 10 μL aliquot of the above filtrate was injected directly into the LC–ESI-MS/MS instrument.

2.5. LC–ESI-MS/MS conditions

The LC–ESI-MS/MS system consisted of a TSQ Quantum Discovery Max mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an ESI probe and Surveyor HPLC system (Thermo Fisher Scientific). A trapping column, Hypersil GOLD column (50 mm × 2.1 mm I.D., 5 μm of particle size; Thermo Fisher Scientific) and a chromatographic separation column, Inertsil Sustain C18 column (150 mm × 2.1 mm I.D., 3 μm particle size; G&L Science, Tokyo, Japan) were employed at 40 °C. A trapping column *via* a column-switching valve was used for the on-line desalting and concentration of urine specimens [15]. After injection of the sample solution, the trapping column was washed with 5 mM ammonium acetate (AA) for 5.5 min at flow rate of 0.1 mL/min, eluted with ethanol, and then transferred into the separation column. A mixture of 5 mM ammonium acetate, ethanol and methanol was used as the eluent, and the separation carried out by linear gradient elution at a flow rate of 0.2 mL/min. The mobile phase composition of ethanol and methanol was gradually changed as follows: ammonium acetate for 3.5 min, ammonium acetate–ethanol (9:1, v/v) for 3.5–4 min, ammonium acetate–ethanol (7:3, v/v) for 4–10 min, ammonium acetate–ethanol–methanol (57:10:33, v/v/v) for 10–16 min, ammonium acetate–ethanol–methanol (2:3:95, v/v/v) for 16–43 min, and then ammonium acetate–ethanol–methanol (2:3:95, v/v/v) for 43–47 min; the column was re-equilibrated for 5 min. Altogether, the total run time was 52 min.

To operate the LC–ESI-MS/MS, the spray voltage and vaporizer temperature were set at 3500 V and 330 °C, respectively. The sheath and auxiliary gas (nitrogen) pressure were set at 50 and 10 arbitrary units, respectively, and the ion transfer capillary temperature was carried out at 330 °C. The collision gas (argon) pressure and the collision energy were kept at 1.3 mm Torr and 27–55 eV, respectively, all in the negative ion mode.

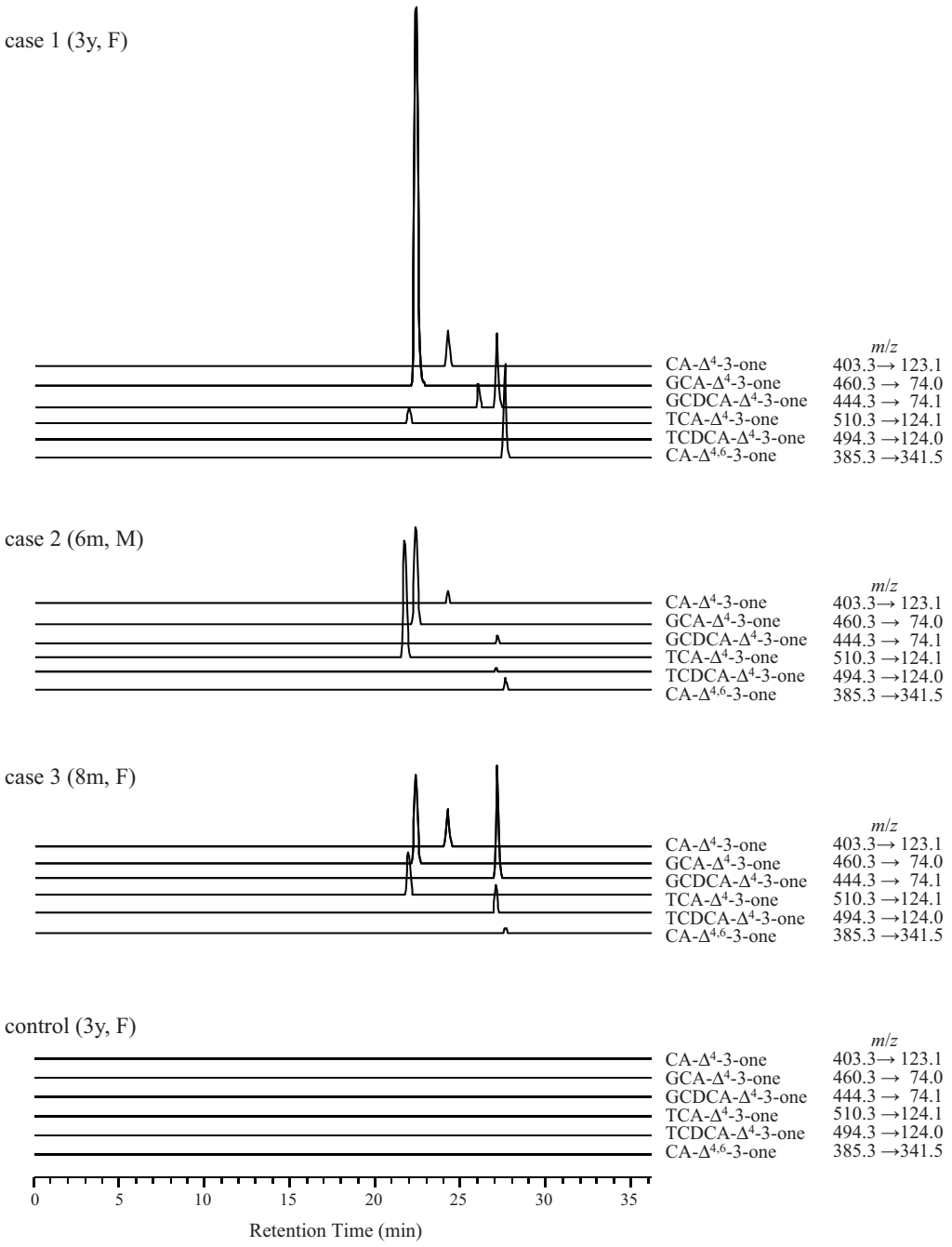

Fig. 2. Selected reaction monitoring chromatograms of urinary bile acids in three patients with 5β-reductase deficiency, as well as healthy control, by LC–ESI-MS/MS.

2.6. Validation of LC–ESI-MS/MS methodology

To characterize the LC–ESI-MS/MS method, we examined specific product ions generated by selecting parent ions and altering the ESI collision energy. Initially, a simultaneous analysis of unconjugated and conjugated bile acids found that a portion of the bile acids were quite sensitive to detection in the positive mode; however, overall, the negative mode was found to be much more appropriate for the measurement of all bile acids as has been reported previously [8]. For the detection of parent ions by ESI, it was possible to select two types of negative ion charges-$[M-H]^-$ for unconjugated bile acids, *N*-acylamidated bile acids and nonamidated bile acid 3-sulfates and $[M-2H]^{2-}$ for the *N*-acylamidated bile acid 3-sulfates [13]. Optimal conditions to conduct the selected reaction monitoring (SRM) were established by the collision-induced dissociation (CID) experiments carried out for each bile acid, and the most suitable collision energy determined by observing the characteristic product ions. The product ions of *N*-acylamidated conjugates were best detected at *m*/*z* 74.0 (glycine conjugates) and 124.0 (taurine conjugates); sulfated bile acids were best detected at *m*/*z* 97.0.

2.7. GC–MS analysis

For the GC–MS analysis, an aliquot of 0.5 mL of urine samples before dried and derivatized as a methyl ester-dimethylethyl silyl ether product, as previously described [16]. Ten unconjugated bile acids (CA, CDCA, UDCA, DCA, LCA, HCA, CA-Δ^4-3-one, CDCA-Δ^4-3-one, CA-$\Delta^{4,6}$-3-one and CDCA-$\Delta^{4,6}$-3-one) were used for the GC–MS analysis, which was performed on a Hewlett

Table 3
Urinary bile acid profile in three patients with 5β-reductase deficiency and healthy controls by LC–ESI-MS/MS.

	Healthy control (2–3y, $n=9$)	Healthy control (6–8m, $n=5$)	Case 1 (3y)	Case 2[a] (6m)	Case 3 (8m)
Saturated bile acids					
CA	–	–	–	–	–
CDCA	–	–	–	–	–
UDCA	–	–	–	–	–
DCA	–	–	–	–	–
LCA	–	–	–	–	–
HCA	–	–	–	–	–
GCA	–	–	–	–	–
GCDCA	–	–	–	–	–
GUDCA	–	–	–	0.99	–
GDCA	–	–	–	–	–
GLCA	–	–	–	–	–
TCA	–	–	–	–	
TCDCA	–	–	–	–	
TUDCA	–	–	–	–	–
TDCA	–	–	–	–	–
TLCA	–	–	–	–	–
CA-3S	–	–	–	–	–
CDCA-3S	–	–	–	–	–
UDCA-3S	–	–	–	–	–
DCA-3S	–	–	–	–	–
LCA-3S	–	–	–	–	–
GCA-3S	–	–	–	1.51	0.10
GCDCA-3S	0.19 ± 0.14	0.31 ± 0.13	–	–	–
GUDCA-3S	–	–	–	1.64	–
GDCA-3S	0.11 ± 0.16	–	–	–	–
GLCA-3S	0.02 ± 0.04	–	–	–	–
TCA-3S	–	–	–	–	–
TCDCA-3S	0.02 ± 0.03	0.11 ± 0.05	–	–	0.16
TUDCA-3S		–	–	1.10	
TDCA-3S		–	–	–	
TLCA-3S	0.00 ± 0.01	–	–	–	–
Unsaturated bile acids					
CA-Δ^4-3-one	–	–	1.59	0.82	2.58
GCA-Δ^4-3-one	–	–	40.12	22.64	18.23
TCA-Δ^4-3-one	–	–	2.91	33.26	11.85
CDCA-Δ^4-3-one	–	–	–	–	–
GCDCA-Δ^4-3-one	–	–	3.97	1.18	10.38
TCDCA-Δ^4-3-one	–	–	0.17	1.02	6.39
CA-$\Delta^{4,6}$-3-one	–	–	1.10	0.54	0.38
CDCA-$\Delta^{4,6}$-3-one	–	–	–	–	–

Unit: μmol/mmol Cr; Cr, creatinine.
[a] Case 2 patient was receiving UDCA.

Packard 5890 gas chromatograph (Agilent, Santa Clara CA, USA) and Hewlett Packard 5973 mass selective detector instrument (Agilent, Santa Clara CA, USA). A fused-silica capillary column bonded with methylsilicon, DB5MS (30 m × 0.25 mm I.D., 0.25 μm film thickness; Agilent) was used to separate the derivatized bile acids. A carrier gas (helium) of flow rate was 1.4 mL/min. was used, and the column temperature was held at 170 °C for 2 min and then ramped at 10 °C/min until 230 °C ramped again at 3 °C/min. up to 310 °C. Mass spectra were recorded at 70 eV for the ionization energy and at 250 °C for the ion source temperature.

2.8. Method validation

2.8.1. Recoveries of bile acids and ISs during pretreatment

The recoveries of bile acids were calculated from the peak area ratios of unconjugated bile acids/d_4-CA, glycine conjugated bile acids/d_4-GCA, and taurine conjugated and double conjugated bile acids/d_4-TCA, respectively, in sample A and B as described below. The recoveries of ISs were calculated from the peak area ratios of d_4-CA/CA, d_4-GCA/GCA, and d_4-TCA/TCA in sample A and B as described below.

Sample A: the blank urine (0.05 mL) spiked with 39 reference bile acids (100 pmol) was pretreated. After addition of ISs (100 pmol each) to this pretreated urine, the resulting sample was subjected to LC–ESI-MS/MS.

Sample B: the blank urine (0.05 mL) spiked with 39 reference bile acids (1000 pmol) was pretreated. After addition of ISs (100 pmol each) to this pretreated urine, the resulting sample was subjected to LC–ESI-MS/MS.

2.8.2. Reproducibility

The reproducibility was assessed by determining two urine samples at different concentration levels ($n=5$ for each sample) and determined as the relative standard deviation (R.S.D.%).

2.8.3. Assay accuracy (analytical recovery)

50% ethanol (0.45 mL) was added to the urine (the spiked concentrations of bile acids were 100 and 1000 pmol, respectively). After the addition of ISs (100 pmol each), each of the resulting samples were pretreated and analyzed by LC–MS/MS. The assay accuracy (analytical recovery) of bile acids was defined as $F/(F_0+A)\times 100\%$, where F is the concentration of bile acids in the spiked sample, F_0 is the concentration of bile acids in the unspiked sample and A is the spiked concentration.

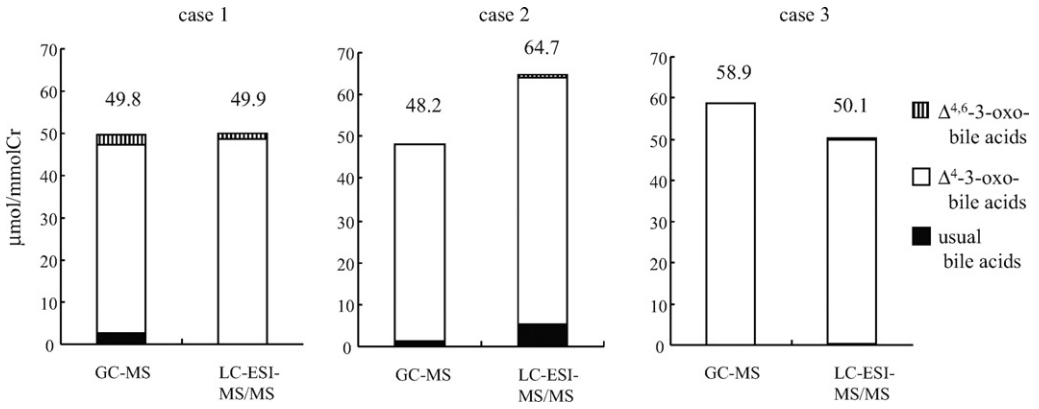

Fig. 3. Comparison of urinary bile acids levels by GC/MS and LC–ESI-MS/MS. Levels shown by GC/MS are the concentration of total bile acids following deconjugation by enzymatic hydrolysis. The results using LC–ESI-MS/MS are the concentration of total unconjugated bile acids combined with their glycine, taurine, and unconjugated bile acid sulfates as well as the *N*-acylamidates of the bile acid sulfates.

3. Results

Fig. 1 shows the chemical structures of the 39 variants of unconjugated and conjugated C_{24} bile acids examined in this study, which include the unconjugated bile acids, *N*-acylamidate conjugates with glycine or taurine at C-24 in the side chain, as well as the C-3 sulfated bile acids in unconjugated and *N*-acylamidated forms. For the initial LC–ESI-MS/MS analysis, we examined the optimum conditions for generating specific product ions arising from the respective parent ions (protonated molecule $[M+H]^+$ or deprotonated molecule $[M-H]^-$) in both the positive and negative ion charge modes. Most of the bile acids showed a high sensitivity and selectivity in the negative mode. Tube lens offset voltage and collision energy of each bile acid, and their conjugates under negative-ion ESI-MS/MS were optimized by directly injecting the standard solution. The most abundant transitions that could be used for monitoring ion are listed in Table 1. The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials. When using an Inertsil Sustain C18 column and a linear gradient elution of ammonium acetate for 3.5 min, ammonium acetate–ethanol (9:1, v/v) for 3.5–4 min, ammonium acetate–ethanol (7:3, v/v) for 4–10 min, ammonium acetate–ethanol–methanol (57:10:33, v/v/v) for 10–16 min, ammonium acetate–ethanol–methanol (2:3:95, v/v/v) for 16–43 min, and then ammonium acetate–ethanol–methanol (2:3:95, v/v/v) for 43–47 min at a flow rate of 0.2 mL/min, satisfactory separation of each bile acid was achieved, and the chromatographic run time was 52 min; retention times and transitions used in SRM for bile acids are given in Table 1. Calibration graphs were then constructed by plotting the peak-area ratio of each bile acid to those of [2,2,4,4-d_4]-CA (IS for unconjugated bile acids), [2,2,4,4-d_4]-GCA (IS for glycine conjugated bile acids), and [2,2,4,4-d_4]-TCA (IS for taurine conjugated and double conjugated bile acids) *versus* the weights of the bile acid. The response was linear with correlation coefficient (r^2) of 0.9822–0.9999 within the range of 30–3000 pmol/mL. The assay reproducibility was examined by 5 repetitive measurement of healthy volunteer's, which contained different concentrations of bile acid. The assay of R.S.D for all the bile acids was less than 14.2%. The assay accuracy was evaluated as the analytical recovery. As shown in Table 2, satisfactory recovery rates ranging from 76.5 to 118.8% were obtained. These data indicate that the present method is highly reproducible and accurate.

Having validated the method for LC–ESI-MS/MS, we then examined the urinary bile acid profiles in a control patient, and in three patients with 5β-reductase deficiency. The results are shown in Fig. 2, in which the abnormal Δ^4-3-oxo bile acids stand out prominently in the three 5β-reductase deficient patients (48.8 μmol/mmol creatinine (Cr), case 1; 58.9 μmol/mmol Cr, case 2; and 49.4 μmol/mmol Cr, case 3), and are completely absent in the normal control. The results of a quantitative determination of total urinary bile acids for two controls and the three 5β-reductase patients are shown in Table 3.

We then analyzed the urines from the three 5β-reductase patients using a validated GC–MS method, and compared the GC–MS results to the data obtained using our LC–ESI-MS/MS methodology. As shown in Fig. 3, both methods gave near identical results, as both the GC–MS and LC–ESI-MS/MS could detect Δ^4-3-oxo and $\Delta^{4,6}$-3-oxo bile acids in these urine samples in a similar proportion. In Fig. 3, the total bile acid concentration recovered in cases 1–3 was 49.8, 48.2, and 58.9 μmol/mmol Cr (respectively) by GC–MS, and 49.9, 64.7, and 50.1 μmol/mmol Cr (respectively) by LC–ESI-MS/MS. Values for urine samples from control subjects done under identical conditions yielded less than 1% Δ^4-3-oxo and $\Delta^{4,6}$-3-oxo bile acids (data not shown).

4. Discussion

The method reported describes a time and labor saving LC–ESI-MS/MS method for human urine that requires only a quick dilution step with alcohol, filtration through a standard 0.45 μm millipore filter, and direct injection into the instrument. In comparison with previously published methods for FAB-MS [17] and GC–MS [18,19], our method is able to separate and identify 39 conjugated and unconjugated bile acids found in urine. In addition, whereas other LC–ESI-MS/MS methods produce results that are often not in accordance with validated GC–MS methods, results obtained with our methodology agree quite well with those obtained by GC–MS. A clear advantage of our method over GC–MS is its ability to detect and quantify the bile acid conjugates and distinguish them from unconjugated bile acids. Thus, our method shows that the Δ^4-3-one bile acids were present as taurine and glycine conjugates. In contrast, the GC–MS method requires a prior enzymatic deconjugation step and thus does not provide such information.

The life-threatening severity of inborn errors of bile acids metabolism, and their need for early detection, has led to a proliferation of non-invasive screening methods. In patients either with confirmed 5β-reductase deficiency, or with severe liver disease, the output of normal bile acids is suppressed and bile acid precursors appear in the urine. Current treatment protocols call for the oral administration of primary bile acids in patients with inborn errors of bile acid biosynthesis, and following the response to treatment by monitoring tests of liver injury. Our quantitative method should

enable a real-time monitoring of the effects of the course of treatment, by following the gradual reduction and disappearance of bile acid precursors from the urine.

Appendix A.

Abbreviations and the corresponding trivial names of unconjugated, *N*-acylamidated (with glycine or taurine), and sulfated bile acids used in this study.

CA	Cholic acid
CDCA	Chenodeoxycholic acid
UDCA	Ursodeoxycholic acid
DCA	Deoxycholic acid
LCA	Lithocholic acid
HCA	Hyocholic acid
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GUDCA	Glycoursodeoxycholic acid
GDCA	Glycodeoxycholic acid
GLCA	Glycolithocholic acid
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TUDCA	Tauroursodeoxycholic acid
TDCA	Taurodeoxycholic acid
TLCA	Taurolithocholic acid
CA-3S	Cholic acid 3-sulfate
CDCA-3S	Chenodeoxycholic acid 3-sulfate
UDCA-3S	Ursodeoxycholic acid 3-sulfate
DCA-3S	Deoxycholic acid 3-sulfate
LCA-3S	Lithocholic acid 3-sulfate
GCA-3S	Glycocholic acid 3-sulfate
GCDCA-3S	Glycochenodeoxycholic acid 3-sulfate
GUDCA-3S	Glycoursodeoxycholic acid 3-sulfate
GDCA-3S	Glycodeoxycholic acid 3-sulfate
GLCA-3S	Glycolithocholic acid 3-sulfate
TCA-3S	Taurocholic acid 3-sulfate
TCDCA-3S	Taurochenodeoxycholic acid 3-sulfate
TUDCA-3S	Tauroursodeoxycholic acid 3-sulfate
TDCA-3S	Taurodeoxycholic acid 3-sulfate
TLCA-3S	Taurolithocholic acid 3 sulfate
CA-Δ^4-3-one	7α,12α-Dihydroxy-3-oxo-4-cholenoic acid
GCA-Δ^4-3-one	7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid *N*-(carboxymethyl)amide
TCA-Δ^4-3-one	7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid *N*-(2-sulfoethyl)amide
CDCA-Δ^4-3-one	7α-Hydroxy-3-oxo-4-cholen-24-oic acid
GCDCA-Δ^4-3-one	7α-Hydroxy-3-oxo-4-cholen-24-oic acid *N*-(carboxymethyl)amide
TCDCA-Δ^4-3-one	7α-Hydroxy-3-oxo-4-cholen-24-oic acid *N*-(2-sulfoethyl)amide
CA-$\Delta^{4,6}$-3-one	12α-Hydroxy-3-oxo-4,6-choladien-24-oic acid
CDCA-$\Delta^{4,6}$-3-one	3-Oxo-4,6-choladien-24-oic acid

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