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LC/ESI-tandem mass spectrometric determination of bile acid 3-sulfates in human urine 3β-Sulfooxy-12α-hydroxy-5β-cholanoic acid is an abundant nonamidated sulfate

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Abstract

We developed a highly sensitive and quantitative method to detect bile acid 3-sulfates in human urine employing liquid chromatography/electrospray ionization-tandem mass spectrometry. This method allows simultaneous analysis of bile acid 3-sulfates, including nonamidated, glycine-, and taurine-conjugated bile acids, cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), and lithocholic acid (LCA), using selected reaction monitoring (SRM) analysis. The method was applied to analyze bile acid 3-sulfates in human urine from healthy volunteers. The results indicated an unknown compound with the nonamidated common bile acid 3-sulfates on the chromatogram obtained by the selected reaction monitoring analysis. By comparison of the retention behavior and MS/MS spectrum of the unknown peak with the authentic specimen, the unknown compound was identified as 3β,12α-dihydroxy-5β-cholanoic acid 3-sulfate.

Keywords: Bile acid; Sulfate; Liquid chromatography/tandem mass spectrometry; Electrospray ionization; Human; Urine

1. Introduction

Bile acids, major cholesterol metabolites in the liver, are excreted into the small intestine via the bile duct. In the intestinal lumen, bile acids assist in lipolysis and the absorption of fats, and are reabsorbed through the intestinal wall to migrate over to the liver. Because of their efficient hepatic uptake, bile acids are present only at low concentrations in the peripheral blood. In humans, the two primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), are biosynthesized from cholesterol in the liver under negative feedback regulation of the farnesoid X receptor [1,2]. Secondary bile acids, deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA), are produced by the action of enzymes existing in the microbial flora of the colonic environment. In hepatocytes, both primary and secondary bile acids undergo amino acid conjugation at the C-24 carboxylic acid on the side chain, and almost all bile acids in the bile duct therefore exist as glycine- or taurine-conjugated forms. Glucuronides of bile acids have been found in the urine of jaundiced patients [3,4], and exist as not only ether-type 3-glucuronides [5] but also ester-type 24-glucuronides [6,7] in the urine of healthy subjects. In addition, amino acid conjugated bile acids strongly suppress the acyl glucuronidation of drugs possessing carboxyl groups [8], and, hence, inhibit the formation of protein-drug adducts, which may cause hypersensitive reactions [9–12]. The major phase II metabolism of bile acids is sulfation [13,14], mainly catalyzed by the hydroxysteroid sulfotransferase SULT2A1, which is expressed in hepatocytes [15–17]. The concentration of bile acid sulfates in urine is significantly increased in hepatobiliary diseases with

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breakdown of enterohepatic circulation [13,18–20]. Therefore, the separate determination of 3-sulfates with high sensitivity and selectivity is required for the diagnosis of hepatobiliary diseases.

Kato et al. [21] have reported an enzymatic method to determine the total concentration of serum bile acid 3-sulfates. This method consists of the deconjugation of a 3α-sulfooxy group by bile acid sulfate sulfatase and the subsequent oxidation of the liberated 3β-hydroxy bile acids by 3β-hydroxysteroid dehydrogenase. Finally, the diformazan formed by enzymatic reaction with diaphorase is monitored. This method can be used to determine the total concentration of 3α-sulfates. Ikegawa et al. [22] have reported a differentiated determination method for bile acid 3-sulfates by liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS). They have also described the liquid chromatographic behavior of the 3-sulfates of common human bile acids and the features of electrospray ionization of nonamidated, glycine-, and taurine-conjugated bile acid 3-sulfates. The results indicate that electrospray ionization with the negative ion detection is suitable for high sensitive detection of bile acid 3-sulfates. Moreover, Murray et al. [23] have also reported the fragmentation patterns of the low-energy collision-induced dissociation (CID) of some bile acid sulfates.

In this study, we report a sensitive and reliable method for simultaneous analysis of bile acid 3-sulfates in human urine employing LC/ESI-MS/MS. In addition, an uncommon bile acid 3β-sulfate was found as an abundant nonamidated sulfate in human urine from healthy subjects.

2. Experimental

2.1. Materials

CA, CDCA, DCA, LCA, and UDCA were purchased from Nacalai Tesque (Kyoto, Japan). 3β,12α-Dihydroxy-5β-cholanoic acid was synthesized as previously reported [24]. Glycine- and taurine-conjugated bile acids were prepared by the carbodiimide method [25], and all bile acid 3-sulfates were synthesized in our laboratory [26]. The stable isotope-labeled bile acids, 3,7,12-[^{18}O,^{2}H]$_3$-CA [27], nonamidated, glycine-, and taurine-conjugated 12-[^{18}O,^{2}H]-DCA 3-sulfates [22] were prepared in our laboratory. An OASIS HLB cartridge (30 mg) (Waters, Milford, MA, USA) was washed successively with ethanol (1 mL) and water (2 mL) prior to use. Urine creatinine contents were measured by the Jaffé method using a Creatinine Test Kit (Wako Pure Chemical Industries, Osaka, Japan). Glassware was silanized, and all other chemicals and solvents were analytical reagent grade.

2.2. Apparatus

The LC/MS/MS analysis was performed using a Quattro II triple stage quadrupole tandem mass spectrometer (Micromass, Manchester, UK) equipped with an ESI probe. The capillary voltage and source temperature were set at −3600 V and 160 °C, respectively. The cone voltage and collision energy were set at −50, −50, −30 V and 50, 30, 20 eV for nonamidated, glycine-, and taurine-conjugated bile acid 3-sulfates, respectively. The collision gas (Ar) pressure was set at 2.0×10^{-3} mbar. In the selected reaction monitoring (SRM) analysis of nonamidated bile acid 3-sulfates, the monitoring ions were *m/z* 455.3 as a precursor ion to *m/z* 97.0 as a product ion, *m/z* 471.3 to *m/z* 97.0, and *m/z* 487.3 to *m/z* 97.0, for mono-, di-, and trihydroxy bile acids, respectively. In the case of glycine-conjugated bile acid 3-sulfates, *m/z* 512.3 to *m/z* 432.3, *m/z* 528.3 to *m/z* 448.3, and *m/z* 544.3 to *m/z* 464.3 were used as SRM monitoring ions for mono-, di-, and trihydroxy derivatives, respectively. In the SRM analysis of taurine-conjugated bile acid 3-sulfates, the doubly charged ions at *m/z* 280.7, *m/z* 288.7, and *m/z* 296.7 were selected as precursor ions, and the $[M - H - H_2SO_4]^-$ at *m/z* 464.3, *m/z* 480.3, and *m/z* 496.3 were selected as monitoring product ions for mono-, di-, and trihydroxy derivatives, respectively. The reversed-phase column used in this study was a YMC Pack Pro C18 column (3 μm, 100 mm × 2.0 mm i.d., YMC, Kyoto, Japan). The mobile phase was 20 mM ammonium acetate (pH 7.0 adjusted by adding aqueous ammonia solution), acetonitrile, and ethanol (60:23:17, v/v/v), used at a flow rate of 0.16 mL/min and delivered by a Nanospace SI-1 pump (Shiseido, Tokyo, Japan).

LC/MS analysis to identify the unknown sulfated bile acid was performed using a JMS-LCmate (JEOL, Tokyo, Japan) double focusing mass spectrometer equipped with an LC-10AD solvent delivery system (Shimadzu, Kyoto, Japan). The resolution of the mass spectrometer was set at 750, and the voltages for electrospray, orifice, and ring lens were −2500, −10, and −100 V, respectively. The temperatures of the orifice and desolvating plate were 150 and 250 °C, respectively.

2.3. Analysis of bile acid 3-sulfates in human urine

Urine samples were collected from healthy volunteers aged 22–33 years and frozen until use. One hundred micro liters of human urine and 900 μL of 100 mM potassium phosphate buffer (pH 7.5) were added to 100 μL of the internal standard (IS) solution, thoroughly mixed, and passed through an OASIS HLB cartridge. After washing with 2 mL of water, bile acid 3-sulfates were eluted with 2 mL of a 1:9 mixture of water and ethanol. After the solvents were evaporated *in vacuo* at 35 °C, the residue was dissolved in 100 μL of water/ethanol (1:1, v/v), and 5 μL of the solution was subjected to LC/ESI-MS/MS analysis. The urinary concentrations of bile acid 3-sulfates were corrected by the creatinine concentrations.

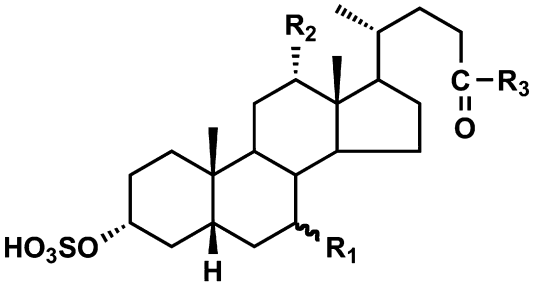
2.4. Method validation

For preparation of standard stock solutions, 3-sulfates of nonamidated, glycine-, and taurine-conjugated CA, CDCA, DCA, UDCA, and LCA were dissolved in water/ethanol (1:1, v/v) at a concentration of 0.1 mg/mL. Samples were diluted to concentrations of 20, 60, 200, 600, and 2000 ng/mL using water/ethanol (1:1, v/v). IS stock solutions containing 1 μg/mL of nonamidated, glycine-, and taurine-conjugated 12-[^{18}O,^{2}H]-DCA 3-sulfates were also prepared in water/ethanol (1:1, v/v).

In the calibration study, a 100-μL aliquot of each standard solution was mixed with 100 μL of the IS solution and evaporated under a nitrogen gas stream at room temperature. The residue was dissolved in 100 μL of water/ethanol (1:1, v/v) and 5 μL of this solution was injected into the LC/ESI-MS/MS system. The calibration curves were constructed by $1/y^2$ weighted least-squares linear regression of the peak area ratio of the analyte to the IS, against the concentration of each bile acid 3-sulfate. For the accuracy and precision studies, we prepared human blank urine by treating urine with activated charcoal. One hundred micro liters aliquot of blank human urine was added to 900 μL of 100 mM potassium phosphate buffer (pH 7.5), and 100-μL aliquots of 20, 200, or 2000 ng/mL stock solution were separately spiked into the mixture. The relative error (R.E.%) was calculated as [(observed concentration − theoretical concentration)/spiked concentration] × 100 (%), and the precision was obtained in terms of the coefficient of variation (R.S.D.%).

2.5. Identification of unknown peak A

A human urine sample was injected into an HPLC, and the fraction corresponding to unknown peak A was collected. The solution was evaporated *in vacuo* below 35 °C, and chemical solvolysis of the sulfated bile acid was performed as described [28]. After addition of 3,7,12-[^{18}O,^{2}H]$_3$-CA as the IS, the sample was injected into the LC/ESI-MS system to compare the chromatographic behavior of the liberated bile acid with that of an authentic specimen. The fraction before solvolysis was also subjected to LC/ESI-MS/MS analysis to compare retention time and fragment pattern on low-energy CID with the authentic specimen.



	R_1	R_2
Cholic acid 3-sulfate	α-OH	OH
Chenodeoxycholic acid 3-sulfate	α-OH	H
Deoxycholic acid 3-sulfate	H	OH
Lithocholic acid 3-sulfate	H	H
Ursodeoxycholic acid 3-sulfate	β-OH	H

	R_3
Nonamidated form	OH
Glycine-conjugated form	$NHCH_2COOH$
Taurine-conjugated form	$NHCH_2CH_2SO_3H$

Fig. 1. Structures of bile acid 3-sulfates.

3. Results

The retention behavior of bile acid 3-sulfates in the reversed-phase HPLC is greatly influenced by the pH of the mobile phase, because the side chain acidic groups have different properties (Fig. 1), as described for the non-sulfated bile acids [29]. Using a mobile phase with a pH lower than 5, the simultaneous determination of 3-sulfates was very difficult, due to long retention times of nonamidated, glycine-, and taurine-conjugated LCA 3-sulfates, which exist in human urine [30,31]. Gradient elution, which is an effective mode for the reduction of analyzing time, failed to reproduce the ionization efficiency of 3-sulfates in the

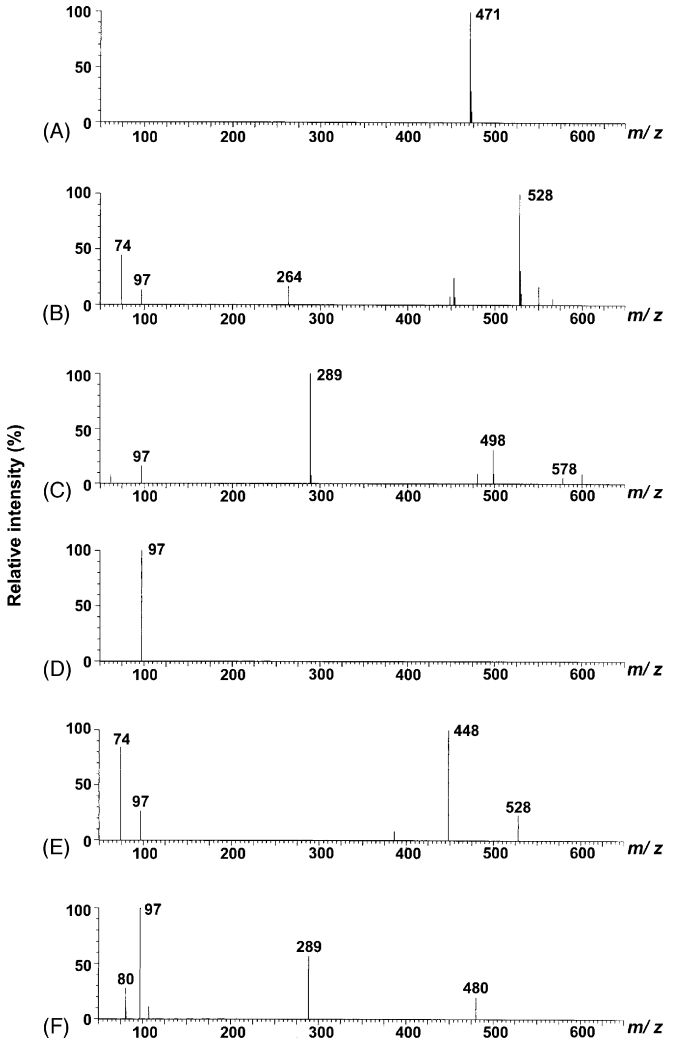


Fig. 2. Typical electrospray ionization mass spectra (A–C) and product ion mass spectra (D–F) of nonamidated (A, D), glycine-conjugated (B, E), and taurine-conjugated (C, F) chenodeoxycholic acid 3-sulfates. The singly charged deprotonated molecules at *m/z* 471 and 528 were selected as precursor ions for the nonamidated and glycine-conjugated forms, and the doubly charged deprotonated molecule at *m/z* 289 was used for the taurine-conjugated form. Injection amounts were 5 ng for nonamidated and glycine-conjugated forms and 10 ng for taurine-conjugated form. Other analytical conditions are described in the experimental section.

ESI mode. Therefore, we selected a neutral mobile phase using an ODS column, the YMC Pack Pro C18. Organic modifiers influenced the peak shape of the 3-sulfates. The use of acetonitrile as an organic modifier in a buffer solution resulted in the broadening of the 3-sulfate peaks, especially of LCA 3-sulfates. Bile acid 3-sulfates have both a lipophilic steroid nucleus and hydrophilic groups, such as hydroxyl, sulfate, and side chain acidic groups. Therefore, these sulfates are soluble in methanol and ethanol. In addition, these alcohols may inhibit hydrogen bonding between residual silanol groups on the surface of the packing material and polar functional groups of the bile acid 3-sulfates, which may be a major cause of peak tailing. Accordingly, a solution of 20 mM ammonium acetate (pH 7.0 adjusted by adding aqueous ammonia solution), acetonitrile, and ethanol was used for the simultaneous separation of bile acid 3-sulfates.

We have previously investigated the ionization of bile acid 3-sulfates using the ESI process, and have reported that organic anion used as a mobile phase modifier greatly influences ionization efficiency [22]. Although the obtained mass spectra of nonamidated, glycine-, and taurine-conjugated bile acid 3-sulfates were similar to those in our previous report, the relative intensities of doubly charged ions of glycine- and taurine-conjugated bile acid 3-sulfates to the corresponding singly charged ions were slightly different, due to the instrumental differences (Fig. 2A–C). The doubly charged deprotonated molecule had one-fifth the intensity of the singly charged ion for glycine-conjugated bile acid 3-sulfates. Moreover, for taurine-conjugated bile acid 3-sulfates, the doubly charged deprotonated molecule was more intense than $[M - HSO_3]^-$ and $[M - H]^-$.

The steroid nucleus is very stable under low-energy CID [32]; however, the glycine- or taurine-residues on the side chain and sulfate group at the 3α position are easily fragmented. The product ion mass spectra of nonamidated, glycine-, and taurine-conjugated CDCA 3-sulfates are illustrated in Fig. 2D–F. The sole product ion of nonamidated sulfates was HSO_4^- at *m/z* 97. Murray et al. [23] have reported that glycine- and taurine-conjugated sulfates produce acidic group product ions at *m/z* 74, 80, 107, and 124, as well as HSO_4^-. In addition, we also found a steroid nucleus-containing product ion $[M - HSO_3]^-$ under mild CID conditions, especially for glycine conjugates. In the product ion mass spectra of taurine-conjugated 3-sulfates, we found another steroid nucleus-containing product ion $[M - H - H_2SO_4]^-$, whereas HSO_4^- at *m/z* 97 was the most intense peak, with a low noise level; this may lead to higher sensitivity under SRM analysis. Therefore, we selected HSO_4^- formed from $[M - H]^-$ for nonamidates, $[M - HSO_3]^-$ formed from $[M - H]^-$ for glycine-conjugates, and $[M - H - H_2SO_4]^-$ formed from $[M - 2H]^{2-}$ for taurine-conjugates as monitoring ions under SRM analysis.

Typical SRM chromatograms for authentic specimens of fifteen bile acid 3-sulfates are shown in Fig. 3, indicating the simultaneous separation and determination of all targeted bile acid 3-sulfates within 25 min. This method has sufficient linearity for all bile acid 3-sulfates tested in this study, and analysis

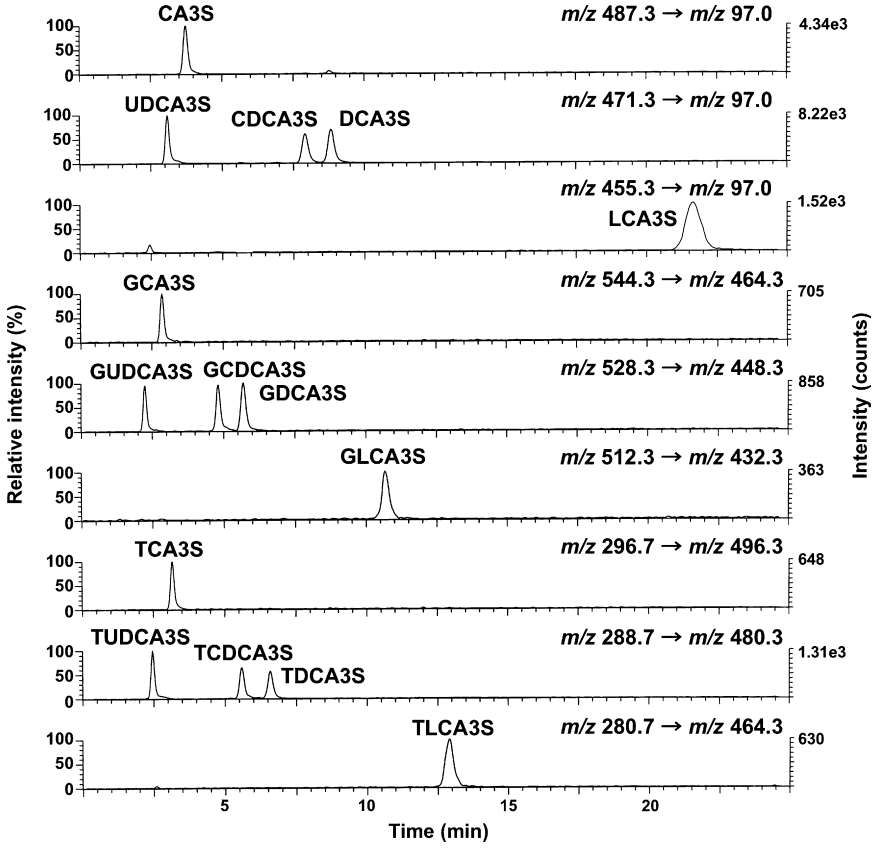


Fig. 3. Typical MRM chromatograms of the authentic standards of 15 bile acid 3-sulfates. Chromatographic and mass spectrometric conditions are detailed in the experimental section.

Table 1
Accuracy and precision of the analysis of bile acid 3-sulfates in human urine ($n = 5$)

Bile acid 3-sulfate	20 ng/mL			200 ng/mL			2000 ng/mL		
	Observed conc: (ng/mL)	R.S.D.%	R.E.%	Observed conc: (ng/mL)	R.S.D.%	R.E.%	Observed conc: (ng/mL)	R.S.D.%	R.E.%
(A) Intra-day validation									
Unconjugate									
CA 3S	21.1	2.6	5.4	211	3.2	5.3	2051	2.1	2.5
CDCA 3S	20.9	1.6	4.4	203	2.9	1.4	2004	1.9	0.2
DCA 3S	20.5	3.2	2.4	200	4.0	0.0	1973	1.9	−1.3
LCA 3S	20.5	5.3	2.3	187	3.3	−6.3	1894	2.9	−5.3
UDCA 3S	21.4	1.8	7.2	201	1.5	0.4	2057	2.2	2.8
Glycine conjugate									
CA 3S	20.5	6.3	2.6	207	4.3	3.3	2156	2.7	7.8
CDCA 3S	21.2	4.2	5.9	201	4.2	0.3	2028	2.1	1.4
DCA 3S	20.9	5.6	4.4	203	3.9	1.4	2090	1.6	4.5
LCA 3S	21.4	2.7	7.2	185	6.0	−7.5	1892	2.1	−5.4
UDCA 3S	18.8	4.5	−5.9	196	3.9	−2.0	2155	2.8	7.8
Taurine conjugate									
CA 3S	20.3	6.6	1.3	217	1.3	8.3	2091	2.8	4.6
CDCA 3S	21.3	6.4	6.7	216	1.7	8.2	2138	1.6	6.9
DCA 3S	20.4	1.2	2.2	215	2.5	7.5	2149	1.9	7.5
LCA 3S	21.2	8.3	6.2	217	2.6	8.7	2125	2.0	6.3
UDCA 3S	20.4	7.5	1.9	216	2.3	7.9	2151	2.4	7.6
(B) Inter-day validation									
Unconjugate									
CA 3S	20.5	7.3	2.6	202	4.7	0.8	2047	5.4	2.3
CDCA 3S	21.1	2.1	5.3	203	6.3	1.7	2068	5.0	3.4
DCA 3S	20.8	6.2	3.9	199	4.1	−0.4	2036	3.3	1.8
LCA 3S	20.7	3.1	3.5	202	8.5	0.9	2023	7.1	1.2
UDCA 3S	22.3	7.8	9.6	212	8.9	5.9	2158	6.0	7.9
Glycine conjugate									
CA 3S	20.1	6.2	0.3	203	7.9	1.4	2040	6.4	2.0
CDCA 3S	21.3	4.8	6.5	199	1.4	−0.3	2020	4.2	1.0
DCA 3S	21.3	4.7	6.7	200	6.0	0.1	2052	3.2	2.6
LCA 3S	20.5	8.6	2.6	199	4.1	−0.6	1949	7.8	−2.6
UDCA 3S	19.1	6.2	−4.5	210	6.4	4.9	2141	4.7	7.0
Taurine conjugate									
CA 3S	20.9	5.6	4.7	218	4.0	8.8	2106	3.0	5.3
CDCA 3S	21.7	4.2	8.6	209	7.4	4.6	2044	7.1	2.2
DCA 3S	19.8	3.5	−1.1	217	0.5	8.5	2106	2.0	5.3
LCA 3S	20.9	2.6	4.7	204	5.6	1.9	1980	8.7	−1.0
UDCA 3S	20.0	6.1	−0.2	216	0.9	8.2	2143	2.2	7.1

can be performed with a 100-fold dynamic range and excellent correlations (greater than 0.999). The deviations of calibration standards from nominal concentrations were less than 10% for the all points in the calibration range. The detection limits (S/N = 5) were 0.7–1.5, 1.6–2.5, and 1.5–3.6 ng/mL for nonamidated, glycine-, and taurine-conjugated bile acid 3-sufates in urine, respectively. The assay validation in Table 1 demonstrates that the method simultaneously determines 3-sulfates of common human bile acids in urine in a range of 20–2000 ng/mL.

The method was applied to the determination of bile acid 3-sulfates in human urine from six male and six female healthy volunteers. The typical SRM chromatograms of sample No. 8, obtained from a male volunteer, are shown in Fig. 4. All bile acid 3-sulfates detected in this study were well separated without endogenous contaminants. The assay results of all healthy volunteers are summarized in Table 2. In the glycine- and taurine-conjugated fraction, the secondary bile acid 3-sulfates, DCA and LCA 3-sulfate, were determined at relatively high concentrations. In particular, GDCA 3-sulfate was the most abundant sulfate in 12 samples, and glycine-conjugated sulfates accounted for approximately 85% of the total sulfates of bile acids. In all urine samples, only a small quantity of nonamidated sulfates was found with a slightly higher concentration in the six female samples than the six male samples.

Although nonamidated bile acid 3-sulfates existed at trace levels, we found an abundant unknown peak A, with a retention time of 5.2 min on the SRM chromatogram (*m/z* 471 to *m/z* 97), corresponding to nonamidated dihydroxylated bile acid mono-

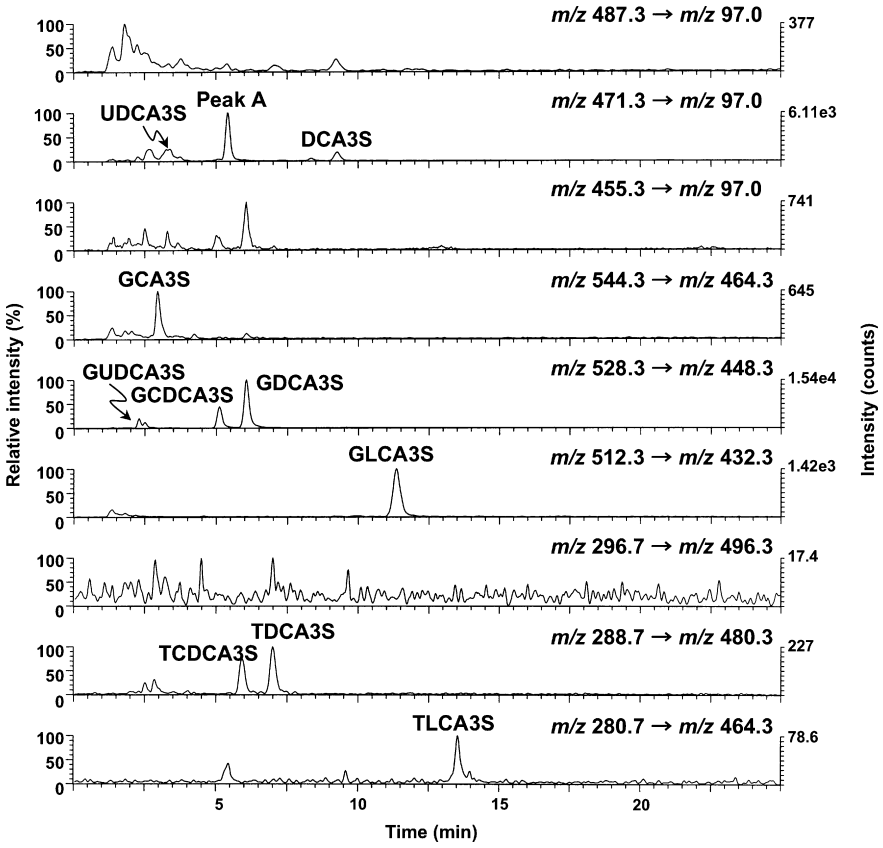


Fig. 4. Typical MRM chromatograms of bile acid 3-sulfates in urine from healthy volunteers. Chromatographic and mass spectrometric conditions are detailed in the experimental section.

Table 2
Bile acid 3-sulfates in urine from healthy volunteers

Observed (ng/mg creatinine)													
	1 f	2 f	3 f	4 f	5 f	6 f	7 m	8 m	9 m	10 m	11 m	12 m	Average ± S.D.
Unconjugate													
CA 3S	–	–	–	–	–	–	–	–	–	–	–	–	–
CDCA 3S	NQ	11.5	–	–	–	–	NQ	NQ	–	–	NQ	–	0.96 ± 3.3
DCA 3S	NQ	NQ	NQ	59.6	NQ	NQ	25.8	12.2	NQ	NQ	NQ	NQ	8.13 ± 18
LCA 3S	–	–	–	NQ	–	–	NQ	–	–	–	–	NQ	–
UDCA 3S	20.3	NQ	NQ	100	NQ	50.6	40.5	23.8	15.2	27.7	37.0	8.60	27.0 ± 28
Glycine conjugate													
CA 3S	NQ	11.8	40.0	NQ	NQ	NQ	NQ	39.6	NQ	–	37.0	14.3	11.9 ± 17
CDCA 3S	338	285	348	422	325	316	152	300	111	95.4	578	216	291 ± 136
DCA 3S	20.8	53.6	337	1411	1386	1019	690	592	363	318	335	261	566 ± 474
LCA 3S	NQ	39.5	28.9	971	389	810	555	112	282	377	127	331	336 ± 312
UDCA 3S	249	110	341	NQ	69.1	81.9	29.8	85.4	15.6	NQ	603	24.5	134 ± 181
Taurine conjugate													
CA 3S	–	–	–	NQ	NQ	–	–	–	–	–	–	–	–
CDCA 3S	54.3	42.4	71.1	74.4	72.6	54.1	21.8	22.5	NQ	34.9	52.4	29.7	44.2 ± 23
DCA 3S	NQ	NQ	59.6	235	241	146	46.9	30.2	28.5	54.4	32.5	35.2	75.9 ± 85
LCA 3S	NQ	NQ	NQ	280	187	174	74.4	10.8	63.5	123	30.9	77.6	85.2 ± 90
UDCA 3S	24.6	NQ	38.8	NQ	NQ	–	–	NQ	–	–	29.6	NQ	7.75 ± 14
Total	709	555	1265	3555	2672	2654	1637	1230	881	1031	1864	999	1588 ± 929

f, female; m, male; NQ, less than the limit of quantitation.

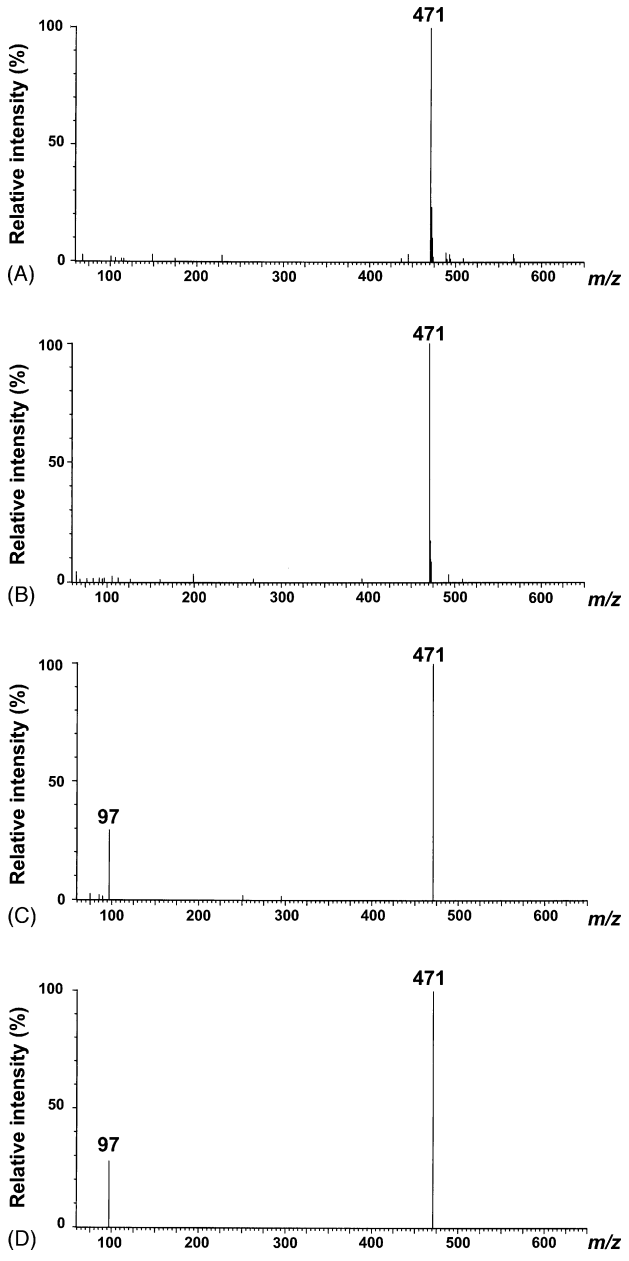


Fig. 5. Electrospray ionization mass spectra (A, B) and product ion mass spectra (C, D) of unknown peak A (A, C) and 3β,12α-dihydroxy-5β-cholanoic acid 3-sulfate (B, D). The singly charged deprotonated molecules at *m/z* 471 were selected as precursor ions. Collision energy was set at 30 eV. Other analytical conditions are described in the experimental section.

sulfate, in all urine specimens measured. As shown in Fig. 5A, we found a deprotonated molecule at *m/z* 471 as a base peak corresponding to a nonamidated dihydroxylated bile acid mono sulfate. The product ion mass spectrum of this deprotonated molecule is illustrated in Fig. 5C. The characteristic fragment ion at *m/z* 97 corresponding to the HSO_4^- was found with the neutral loss of 374. To identify this compound, we collected the fraction containing peak A and subjected it to chemical solvolysis followed by LC/ESI-MS analysis. One intense peak was observed on the chromatogram at *m/z* 391, corresponding to a deprotonated molecule of dihydroxylated bile acid (data not shown). The retention time was almost identical to that of 3α,7α,12α-trihydroxy-5β-cholanoic acid. Nambara and Goto [29] have provided the retention factors of nonamidated 5β-cholanoic acids, and 3β-hydroxylated bile acids are rapidly eluted with relative retention factors that are approximately one-half of those of the 3α-epimers. The results for the bile acid produced by solvolysis of unknown peak A suggested that it was 3β-hydroxylated bile acid 3-sulfate. Therefore the retention behaviors of the liberated unknown bile acid were compared to those of the authentic 3β,12α-dihydroxy-5β-cholanoic acid, with the use of stable isotope-labeled CA as an IS. The retention behaviors at different pH of the mobile phase agreed completely (Table 3). Next, we prepared 3β,12α-dihydroxy-5β-cholanoic acid 3-sulfate and compared the retention time and the mass and MS/MS spectra with those of unknown peak A. The retention time of unknown peak A was identical to that of the authentic specimen, 3β,12α-dihydroxy-5β-cholanoic acid 3-sulfate (Fig. 6), and an ESI mass spectrum and a product ion spectrum of unknown peak A were identical to those of the authentic specimen, respectively (Fig. 5). In consequence, unknown peak A was identified as 3β,12α-dihydroxy-5β-cholanoic acid 3-sulfate.

Table 3
Retention factors of the authentic specimens and the liberated products relative to ^{18}O-labeled cholic acid

pH	Retention factors relative to ^{18}O-labeled cholic acid	
	The liberated product after chemical solvolysis	3β,12α-dihydroxy-5β-cholanoic acid
7.0	1.08	1.08
6.5	1.11	1.11
6.0	1.18	1.18
5.0	1.30	1.30
4.0	1.29	1.29

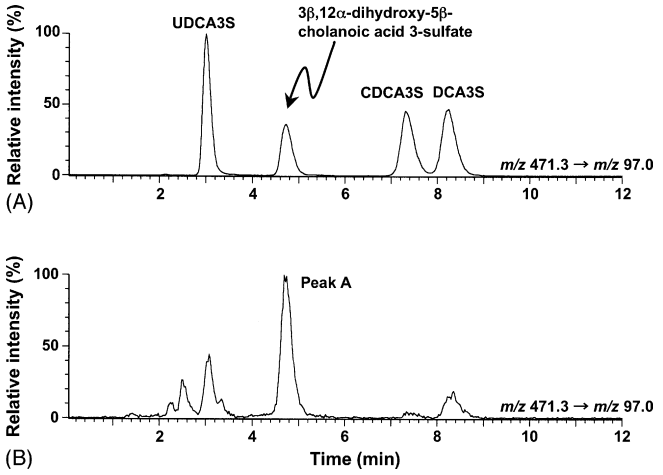


Fig. 6. MRM chromatograms of the authentic standards of dihydroxylated bile acid 3-sulfates (A) and human urine extract (B). Chromatographic and mass spectrometric conditions are detailed in the experimental section.

4. Discussion

In hepatobiliary diseases, the excretion of bile acid 3-sulfates into the urine commonly increases [13,18–20]; therefore, the

analysis of the sulfates is useful in diagnosing liver diseases. In this paper, we report a highly sensitive and selective LC/ESI-MS/MS method for simultaneous analysis of bile acid 3-sulfates in human urine. The separate determination of 3-sulfates and comparison with normal state are required for the diagnosis of hepatobiliary diseases. This method improves on a previously reported method [21] that can be used to determine the total bile acid 3α-sulfate concentration in human serum, but has a disadvantage in the simultaneous analysis of individual bile acid sulfates. We have also previously reported a separatory determination method using LC/ESI-MS, where the sulfates are monitored by only deprotonated molecules [22]. However, it is difficult in the respect of sensitivity to apply this method to urine from healthy subjects. The present method using an LC/ESI-MS/MS is enough sensitive for analyzing bile acid 3-sulfates in human urine not only from patients with liver diseases but also from healthy subjects.

Using the new method, we found that urine contains a tiny quantity of nonamidated bile acid 3-sulfates, which can be determined with a good signal to noise ratio. Almost all bile acids are converted into glycine or taurine conjugates in hepatocytes, where hydroxysteroid sulfotransferase converts bile acids into their sulfates [15–17]. Interestingly, UDCA 3α-sulfate is relatively abundant among nonamidated bile acid 3-sulfates, except 3β,12α-dihydroxy-5β-cholanoic acid 3-sulfate. On the other hand, the concentration of glycine-conjugated UDCA 3-sulfate is lower than other 3-sulfates. Nakamura et al. [33] have reported that UDCA 3-sulfate levels increase in the serum and urine of patients with chronic liver disease after UDCA therapy. This result indicates that nonamidated UDCA is easily converted into the corresponding 3α-sulfate by the action of sulfotransferase in the liver. In the case of glycine conjugates, the average concentration of urinary DCA 3-sulfate for all specimens is the highest. It has been reported that the relatively lipophilic secondary bile acids, glycine-conjugated DCA and LCA, tend to undergo sulfation [17]. Sulfation is a phase II metabolism and plays a key role in the detoxification of endogenous and exogenous lipophilic compounds. Although human serum commonly contains higher concentrations of the nonsulfated form of CDCA than DCA, sulfation may be more favorable for DCA than CDCA. The carboxyl and sulfonyl groups on the side chain of DCA form intramolecular hydrogen bonds with the 12α-hydroxyl group [29] at neutral pH, resulting in increased hydophobicity, which may be favorable for sulfation.

We have identified the uncommon sulfated bile acid, 3β,12α-dihydroxy-5β-cholanoic acid, in urine from healthy subjects. The corresponding glycine- and taurine-conjugated sulfates were not observed in the same urine specimens. Previously, the existence of this 3β-hydroxy bile acid sulfate has been reported in urine from pregnant women [34], and increased excretion of the 3β,12α-dihydroxy derivative into feces has been observed after administration of deoxycholic acid [18]. In 1990, Yoneda et al. [35] reported that intravenous administration of the dehydrocholic acid, 3,7,12-trioxo-5β-cholanoic acid, to two cholangiocarcinoma patients who underwent percutaneous transhepatic cholangial drainage led to elevated blood levels of 3α-hydroxy-7,12-dioxo-cholanoic acid. In addition, Goto et al. [36,37] demonstrated that the incubation of 3-oxo-bile acid with human blood produced 3β-hydroxylated bile acid as a major metabolite with minor levels of 3α-hydroxylated bile acid. These observations suggest that deoxycholic acid is the precursor for the formation of 3β,12α-dihydroxy-5β-cholanoic acid. The 3-sulfate of 3β,12α-dihydroxy-5-cholenoic acid has been observed at high concentrations in the urine of patients with biliary atresia [38]. This unsaturated bile acid has a 3β-hydroxy-Δ^5 skeleton that is similar to dehydroepiandrosterone, which is the most favorable endogenous substrate for the hydroxysteroid sulfotransferase, SULT2A1 [16]. Thus, SULT2A1 may be involved with the sulfation of the 3β,12α-dihydroxy-5β-cholanoic acid.

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