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Separatory Determination of Bile Acid 3-Sulfates by Liquid Chromatography/Electrospray Ionization Mass Spectrometry

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A method for the separatory determination of bile acid 3-sulfates by liquid chromatography (LC)/electrospray ionization (ESI) mass spectrometry (MS) was developed. The sulfates were characterized by an abundant pseudo-molecular ion $[M-H]^-$ with a doubly charged ion $[M-2H]^{2-}$, and the ratio of these negative ions was markedly influenced by an acidic component of salt added to the mobile phase according to the pK_a value of the conjugated form at C-24. The resolution into unconjugated and glycine-and taurine-conjugated cholic acid, chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid and lithocholic acid 3-sulfates was achieved on a Capcell Pak C_{18} column with 20 mM ammonium acetate–methanol (10:12, v/v) by selected ion monitoring, monitored with their corresponding pseudo-molecular ions. The combined use of stable isotope-labeled compounds as internal standards in LC/MS was applied to the determination of bile acid 3-sulfates in human urine. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Recently, considerable attention has been focused on the metabolic significance of bile acids in hepatobiliary diseases. Since bile acids are commonly conjugated with sulfuric acid at the 3α-hydroxy group on the steroid nucleus and then excreted into urine, a reliable method for the determination of these 3-sulfates in biological fluids is required. Bile acids have usually been determined by high-performance liquid chromatography (HPLC) coupled with an immobilized enzyme reactor of 3α-hydroxysteroid dehydrogenase or derivatization through the 3α-hydroxy group.[1] As bile acid 3-sulfates are very polar and lacking in volatility and thermal stability, HPLC on a reversed-phase column also appears to be more suitable for the separation and determination of these sulfates in biological fluids. However, bile acid 3-sulfates have no signal groups that are highly responsive toward common detectors and no inherent functional groups making possible detection-oriented derivatization to enhance sensitivity and specificity.

In recent years, mass spectrometry (MS) combined with liquid chromatography (LC) has been developed as a new approach for the separation and determination of trace compounds in biological fluids. Several interfaces such as moving belt,[2] continuous-flow fast atom bombardment,[3] thermospray,[4–6] ionspray[7] and electrospray ionization[8] have been applied to the determination of unconjugated and glycine-and taurine-conjugated bile acids. However, no attempts have been made at the simultaneous and quantitative determination of bile acid 3-sulfates in biological fluids. Among various interfaces, electrospray ionization (ESI) is considered to be a powerful tool for the determination of biologically important substances having an ionic function such as amino and sulfate groups. This paper deals with the development of a sensitive and reliable method for the separatory determination of bile acid 3-sulfates in human urine by LC/ESI-MS.

EXPERIMENTAL

LC/ESI-MS

LC/MS was carried out using a Hitachi M-1000H quadrupole mass spectrometer equipped with an ESI system in the negative ion mode. The MS parameters were set at drift voltage −50 V and nebulizer temperature 250 °C. The LC separation was performed on a Capcell Pak C_{18} column (5 μm, 150 mm × 1 mm i.d.) (Shiseido, Tokyo, Japan) with a flow rate of 50 μl min^{-1}.

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Materials

Bile acid 3-sulfates (Fig. 1) were synthesized by the method reported previously.[9,10] Sephadex LH-20 was purchased from Pharmacia (Uppsala, Sweden) and piperidinohydroxypropyl-Sephadex LH-20 (PHP-LH-20; acetate form, 0.6 mequiv. g^{-1})[11] and carboxymethyl-Sephadex LH-20 (CM-LH-20; K^+ form, 1 mequiv. g^{-1})[12] were prepared according to the cited methods. All chemicals were of analytical-reagent grade. A Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) was washed successively with chloroform (5 ml), ethanol (5 ml), water (10 ml), 5% bovine serum albumin (5 ml) and then water (5 ml) prior to use. All glasswares was silanized with trimethylchlorosilane.

Synthesis of stable isotope-labeled sulfates

The unconjugated and glycine-and taurine-conjugated 12-oxolithocholic acids were subjected to sulfation with chlorosulfonic acid according to the method reported previously.[9,10] The 12-oxolithocholic acid 3-sulfates obtained were then converted into corresponding potassium salt using CM-LH-20 (K^+ form), and each 10 mg portion of the salt was dissolved in $H_2^{18}O$ (isotopic purity 98 at.%, 180 μl) and heated at 90 °C for 50 h.[13] After cooling, the solution was stirred with NaB^2H_4 (isotopic purity 99 at.%, 1.5 mg) for 30 min with ice cooling, and then acidified with 5% hydrochloric acid to decompose the excess reagent. The reaction mixture was neutralized and then passed through a column (30 mm × 35 mm i.d.) of Pre Pak-500/C_{18} (Waters) (2 g). After washing with water (20 ml), unconjugated or glycine-or taurine-conjugated [12α-^{18}O,12β-^{2}H]deoxycholic acid 3-sulfate was eluted with methanol (20 ml) and then purified by HPLC.[1] The structures of stable isotope-labeled 3-sulfates were confirmed by comparing the HPLC behavior and mass spectrometric properties in LC/ESI-MS with those for authentic specimens.

Extraction of bile acid 3-sulfates in human urine

To a urine specimen (2 ml) from a healthy volunteer or a patient with obstructive jaundice were added unconjugated and glycine-and taurine-conjugated [12α-^{18}O, 12β-^{2}H]deoxycholic acid 3-sulfates as internal standards (ISs, each 5 μg), and the whole was passed through a Sep-Pak C_{18} cartridge. After washing with water (10 ml), bile acids were eluted with 90% ethanol (4 ml), and the eluate was applied to a column (18 mm × 6 mm i.d.) of PHP-LH-20 (0.6 mequiv. g^{-1}, 100 mg). Elution was carried out at a flow rate of 0.2 ml min^{-1}. After removal of co-existing neutral and weakly acidic compounds by washing with 90% ethanol (4 ml) and 0.3 M acetic acid–potassium acetate in 90% ethanol (pH 6.3) (4 ml), the desired bile acid 3-sulfates were eluted with 1% ammonium carbonate in 70% ethanol (pH 8.5) (6 ml), followed by evaporation in vacuo below 40 °C. A 10 μl aliquot of the redissolving solution in water–acetonitrile (1:1, v/v) (100 μl) was subjected to the LC/ESI-MS analysis.

RESULTS AND DISCUSSION

In the ESI mode, a buffer consisting of a non-volatile salt, such as sodium phosphate, which has commonly been used for the separation of bile acid 3-sulfates on a reversed-phase column,[1] is not advantageous and volatile electrolytes such as ammonium acetate and ammonium formate are recommended for promoting the proton transfer reaction in the LC separation. Accordingly, an initial effort was directed to elucidating the effect of these organic salts in the mobile phase on formation of a characteristic negative ion. The ESI mass spectra of 15 kinds of unconjugated and glycine-and taurine-conjugated bile acid 3-sulfates were obtained in a solution of 50 mM ammonium acetate (pH 7.0)–acetonitrile (1:1, v/v) in the flow injection mode, and the typical spectra of cholic acid 3-sulfates are illustrated in Fig. 2. Unconjugated trihydroxy bile acid 3-sulfate exhibited a singly charged ion $[M - H]^-$ at m/z 487.2 as a base peak, whereas those of glycine and taurine conjugates showed doubly charged ions $[M - 2H]^{2-}$ at m/z 271.6 and 296.6 as an intense peak with a low abundance of singly charged ions at m/z 544.3 and 594.2, respectively. Furthermore, formation of a doubly charged ion increased with decreasing pK_a value of an acidic moiety at C-24: unconjugated, 6.5;

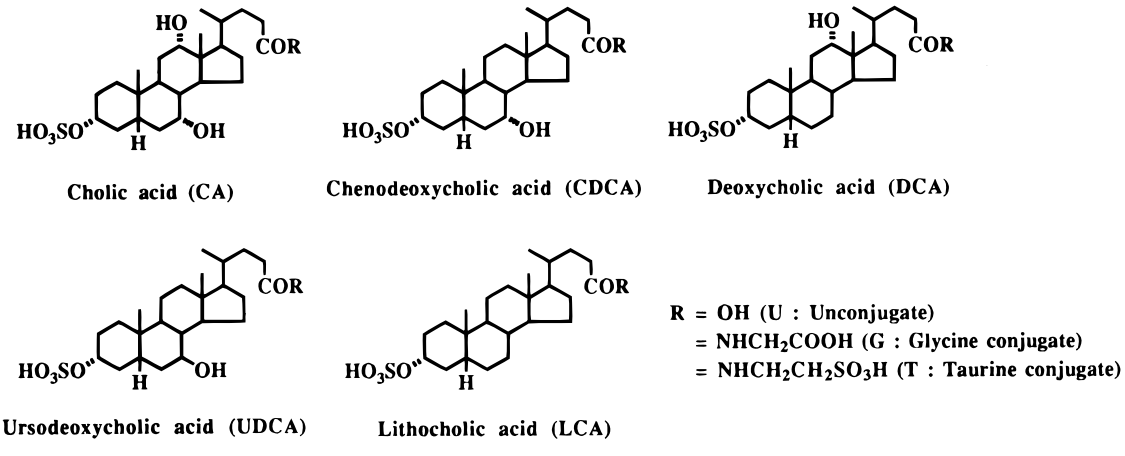

Figure 1. Structures of bile acid 3-sulfates.

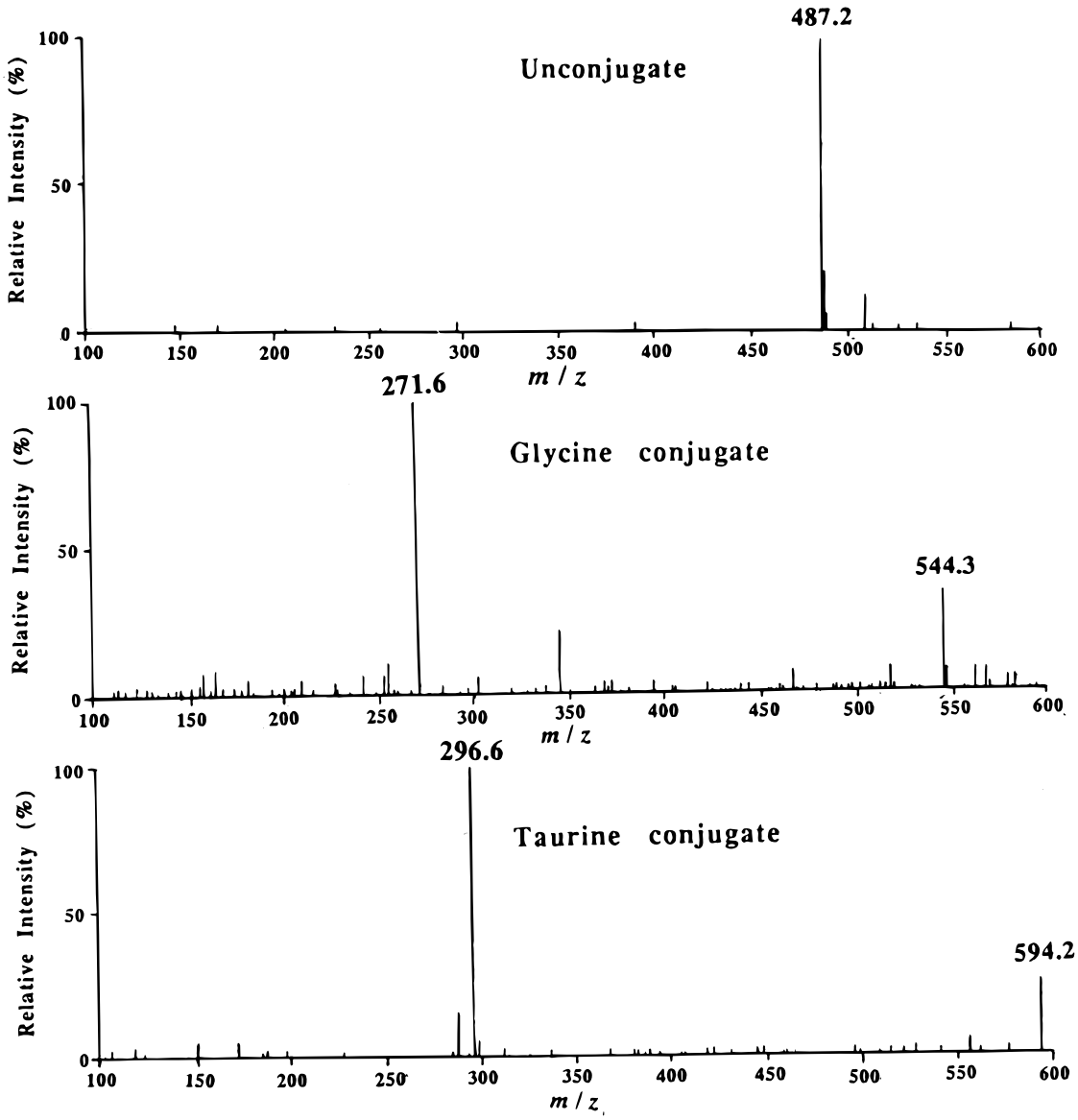

Figure 2. Electrospray negative ion mass spectra of cholic acid 3-sulfates obtained with the flow injection mode using 50 mM ammonium acetate buffer (pH 7.0)–acetonitrile (1 : 1, v/v) as the mobile phase at a flow rate of 50 μl min^{-1}.

glycine conjugate, 4.5; and taurine conjugate, 1.5. These results indicate that formation of a prominent ion is dependent on the acidity of the conjugated form at C-24. It is well known that the chromatographic behavior of acidic compounds is distinctly influenced by the pH of a mobile phase, owing to the dissociation equation of the analytes.[1,14] Accordingly, the ESI spectra of these bile acids in the pH range 4.0–8.0 were then measured. However, no substantial changes in the relative abundance of $[M - 2H]^{2-}$ with respect to $[M - H]^-$ were observed.

It is generally accepted that the ES dispersion of a sample solution would result in the production of ultimate droplets containing only one charge and one solute molecule.[15] As the solvent evaporated from a droplet, its charge would be retained by the solute molecule. It seems, therefore, that ionization of bile acid 3-sulfates is influenced by dissociation of the acidic component of the salt. Hence, negative ion mass spectra of 3-sulfates in a buffer solution (pH 7.0) consisting of the 20 mM ammonium salt of formic (pK_a 3.77) or trifluoroacetic acid (pK_a 0.23), instead of acetic acid (pK_a 4.76), were then measured. As shown by the unconjugated and glycine-and taurine-conjugated ursodeoxycholic acid 3-sulfates in Fig. 3, the relative abundance of $[M - 2H]^{2-}$ with respect to $[M - H]^-$ decreased along with a decrease in the pK_a of the acidic component in the buffer. The abundance of the doubly charged ion of the glycine conjugate in ammonium acetate was three times stronger than that of the unconjugated form and significantly decreased in ammonium formate. Moreover, the taurine conjugate indicated a singly charged ion as an intense peak in ammonium trifluoroacetate. An increase in the concentration of salt to 100 mM resulted in an enhancement of the relative abundance of $[M - H]^-$ with respect to $[M - 2H]^{2-}$ but in a decrease in the absolute abundance of an individual ion. This effect of an electrolyte added to a mobile phase for production of a doubly charged ion of the analyte is consistent with previous results obtained by Ikonomou *et al.*[16] and Wang and Cole.[17] These observations along with the previous findings for non-

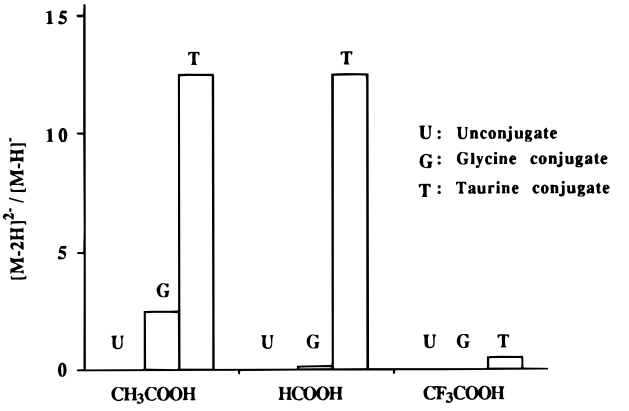

Figure 3. Effect of the ammonium salt of a volatile organic acid on the ionization of ursodeoxycholic acid 3-sulfates. 50 mM ammonium salt of acetic acid, formic acid or trifluoroacetic acid (pH 7.0)–acetonitrile (1 : 1, v/v) was used at a flow rate of 50 μl min^{-1}. The relative abundance of $[M-2H]^{2-}$ with respect to $[M-H]^{-}$ was calculated from the peak heights.

sulfated bile acids,[8] in which the formation of a negative ion of unconjugated bile acids was not reported, indicate that the use of a salt consisting of an organic acid having a pK_a value relatively smaller than that of an acidic moiety at C-24 results in the depression of charge transfer from ultimate droplets to an amino acid residue of bile acids. Typical spectra of taurine-conjugated ursodeoxycholic acid 3-sulfate obtained in ammonium acetate and trifluoroacetate (pH 7.0) are illustrated in Fig. 4. For the quantitative determination of bile acid 3-sulfates, the use of ammonium acetate with an organic solvent as a mobile phase is much more preferable, because of the effective formation of a characteristic negative ion of 3-sulfates. Even though singly charged ions are predominantly produced, the use of ammonium trifluoroacetate elicits a decrease in sensitivity as a result of the depression of the formation of negative ions.

The next effort was directed to the separation of 3-sulfates. The chromatographic behavior obtained on a Capcell Pak C_{18} column with 0.3% ammonium acetate–acetonitrile as the mobile phase is depicted in Fig. 5. The k′ values of 3-sulfates of unconjugated, glycine and taurine conjugated chenodeoxycholic, deoxycholic, ursodeoxycholic and cholic acids relative to tauroursodeoxycholic acid 3-sulfate were plotted against pH. The k′ values were influenced by the pH of a mobile phase. In the neutral condition, unconjugated and glycine-and taurine-conjugated bile acid 3-sulfates exhibited similar k′ values. On the other hand, the k′ values of unconjugated and glycine-conjugated 3-sulfates increased with decreasing pH from 6.5 and 4.5, respectively, according to their pK_a values. A similar phenomenon has been reported previously, and such

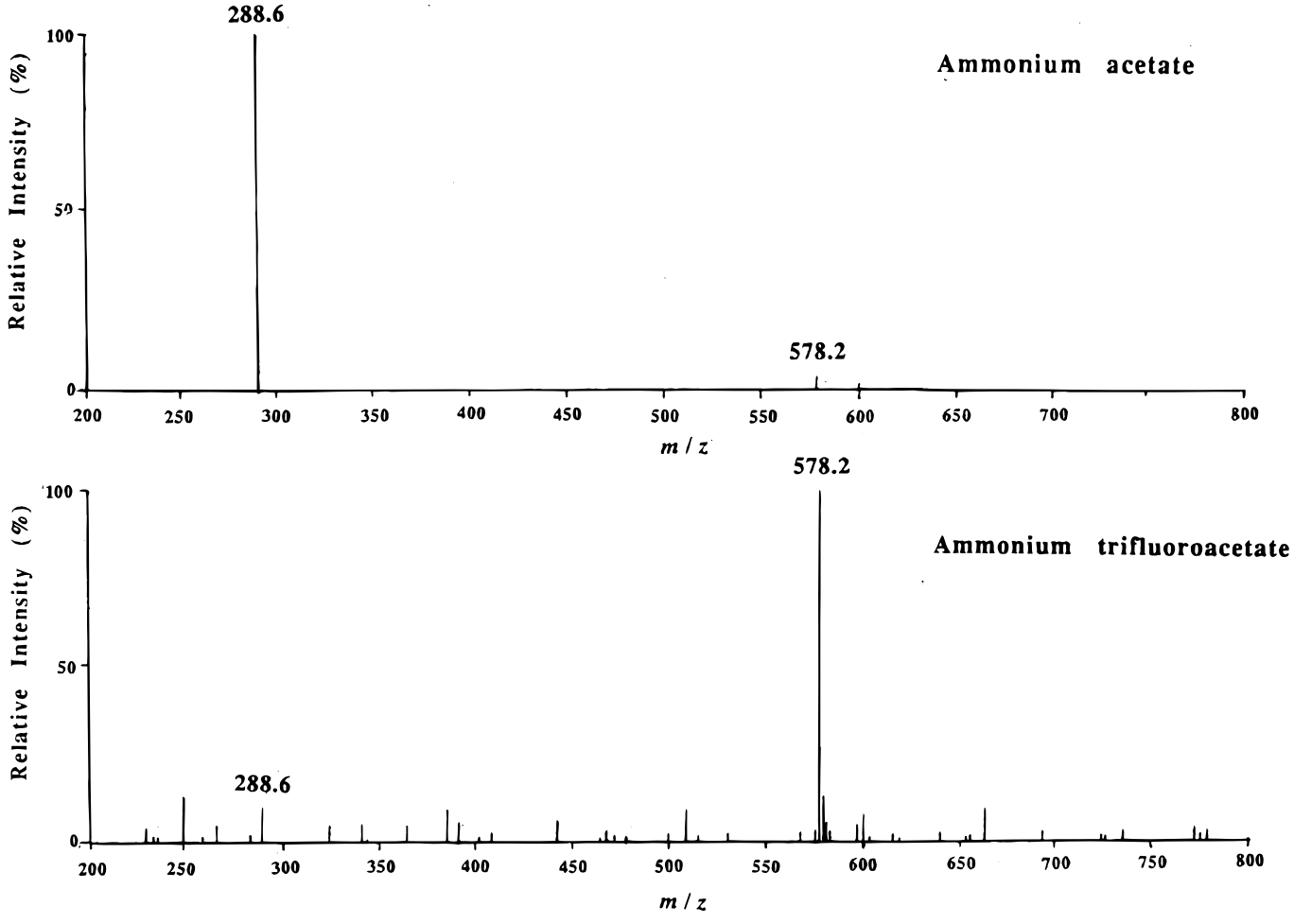

Figure 4. Electrospray negative ion mass spectra of tauroursodeoxycholic acid 3-sulfate using the flow injection mode. 50 mM ammonium salt of acetic acid or trifluoroacetic acid (pH 7.0)–acetonitrile (1 : 1, v/v) was used at a flow rate of 50 μl min^{-1}.

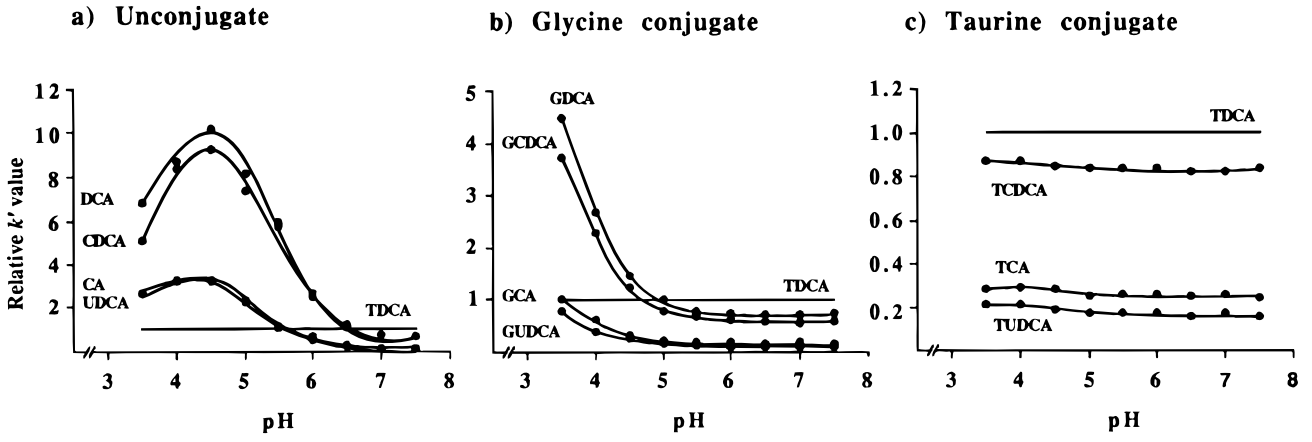

Figure 5. Effect of pH of mobile phase on k' values of bile acid 3-sulfates relative to tauroursodeoxycholic acid 3-sulfates. Column, Capcell Pak C_{18}; mobile phase, 50 mM ammonium acetate buffer–acetonitrile (1 : 1, v/v). For abbreviations, see Fig. 1.

behavior can be explained in terms of the dissociation of the amino acid residue at C-24.[1] Although no substantial differences in the chromatographic separation of 3-sulfates were found between acetonitrile and methanol as the organic solvent in the mobile phase, the use of the latter is preferred for the effective formation of the inherent negative ion.

On the basis of these data, 20 mM ammonium acetate (pH 7.0–methanol (10 : 12, v/v) was chosen as a suitable mobile phase. The simultaneous separation of 3-sulfates of unconjugated and glycine-and taurine-conjugated bile acids can be readily attained by selected ion monitoring (SIM), monitored with their characteristic negative ions $[M - H]^-$ (Fig. 6) and the limit of detection was 200 fmol, 1000 times lower than that obtained with UV detection.

In the mass spectrometric determination of trace compounds in biological specimens, the use of a stable isotope-labeled compound as the IS is recommended for obtaining reliable results. In a previous study, we developed a method for the preparation of bile acids labeled with an ^{18}O atom at the hydroxyl group on a steroid nucleus by the exchange reaction of a carbonyl oxygen atom.[13] Since the formation of singly and doubly charged ions is dependent on the pK_a value of the acidic moiety at C-24, unconjugated and glycine-and taurine-conjugated [12α-^{18}O,12β-^{2}H]deoxycholic acid 3-sulfates were synthesized in the same manner. The 3-sulfates of 12-oxolithocholic acid and its conjugates with amino acids were converted into the potassium salt by passage through a lipophilic cation-exchange gel, CM-LH-20 (K^+ form). The salts were then dissolved in $H_2{}^{18}O$, allowed to stand at 90 °C and then the ^{18}O-labeled oxo bile acids formed were subjected to reduction with sodium borodeuteride (NaB^2H_4). The ratio of the unlabeled component to the fully labeled

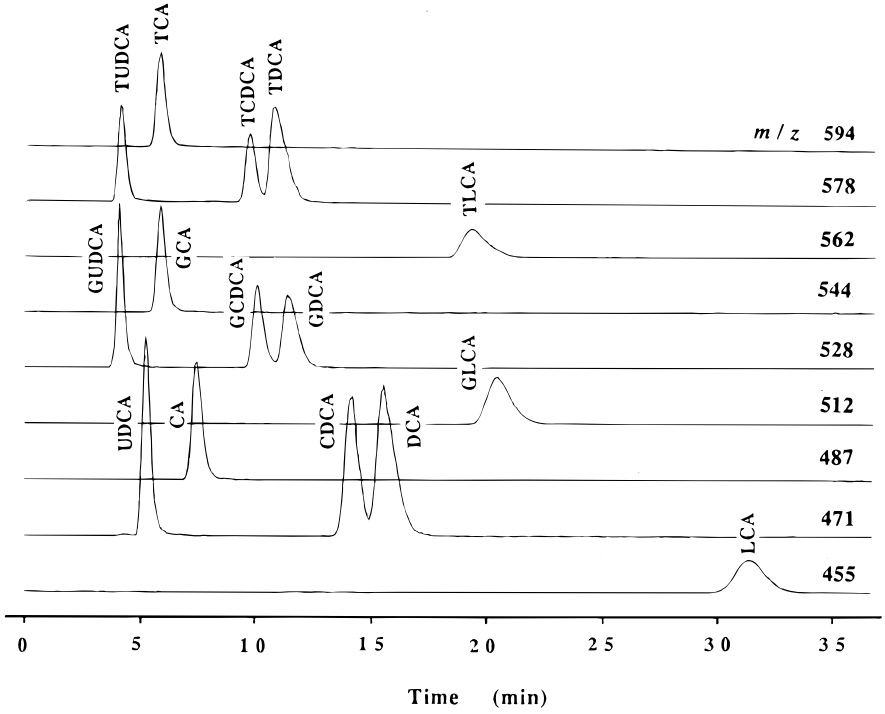

Figure 6. SIM recording for 15 bile acid 3-sulfates. Column, Capcell Pak C_{18}; mobile phase, 20 mM ammonium acetate buffer (pH 7.0)–methanol (10 : 12, v/v); flow rate, 50 µl min^{-1}. For abbreviations, see Fig. 1.

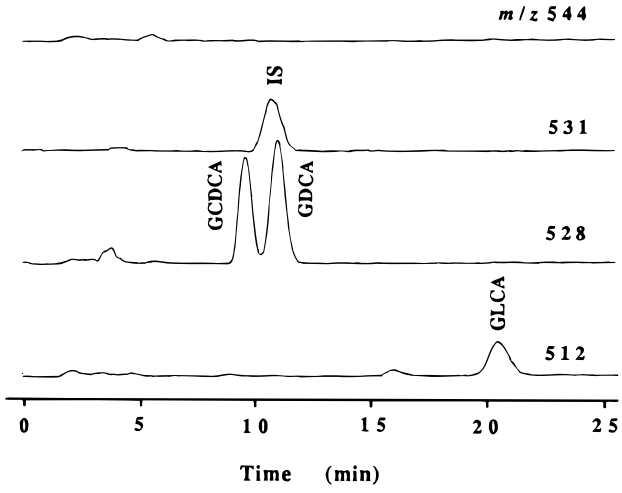

Figure 7. SIM recording for glycine-conjugated bile acid 3-sulfates in urine from a healthy volunteer. LC/MS conditions as in Fig. 6. For abbreviations, see Fig. 1.

component was determined by ESI-MS to be less than 1/900 for all the labeled 3-sulfates. A calibration graph was constructed by plotting the ratio of the peak area of each bile acid 3-sulfate to that of corresponding unconjugated and glycine-and taurine-conjugated [12α-^{18}O,12β-^{2}H]deoxycholic acid 3-sulfates against the amount of bile acid. The linearity was good from 50 ng ml^{-1} for all bile acid 3-sulfates using SIM with the individual ions.

Table 1. Ratios of $[M - 2H]^{2-}$ to $[M - M]^{-}$ of bile acid 3-sulfates[a]

Bile acid 3-sulfate	pH 5.5	pH 7.0
Glycochenodeoxycholic acid 3-sulfate	0.47	0.43
Urine	0.45	0.42
Glycodeoxycholic acid 3-sulfate	0.30	0.28
Urine	0.30	0.30
Glycolithocholic acid 3-sulfate	0.78	0.76
Urine	0.78	0.78

[a] Mobile phase, 20 mM ammonium acetate buffer–methanol (10:12, v/v); flow rate, 50 μl min^{-1}.

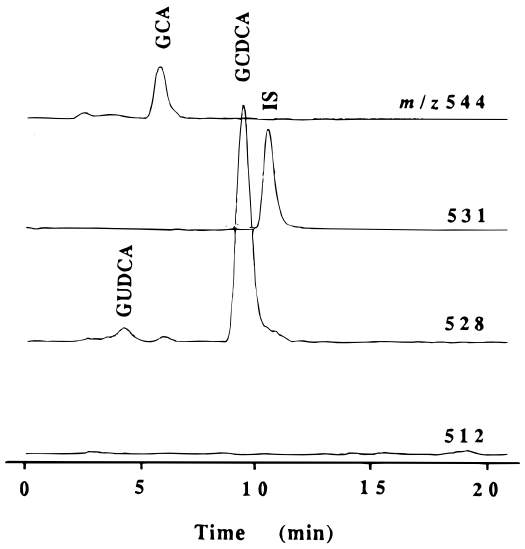

Figure 8. SIM recording for glycine conjugated bile acid 3-sulfates in urine from a patient with obstructive jaundice. LC/MS conditions as in Fig. 6. For abbreviations, see Fig. 1.

The method was then applied to the simultaneous determination of bile acid 3-sulfates in human urine. The elimination of coexisting substances such as protein and inorganic salts in biological fluids is usually a prerequisite for the separatory determination of trace compounds. The urine specimen was subjected to liquid–solid extraction with a Sep-Pak C_{18} cartridge and then ion-exchange chromatography on a lipophilic gel, PHP-LH-20, as described under Experimental. The monosulfate fraction obtained was submitted to the LC/ESI-MS analysis in the SIM mode, monitored at *m/z* 455 (monohydroxy), 471 (dihydroxy), 474 (IS) and 487 (trihydroxy) for unconjugates, 512 (monohydroxy), 528 (dihydroxy), 531 (IS) and 544 (trihydroxy) for glycine conjugates and 562 (monohydroxy), 578 (dihydroxy), 581 (IS) and 594 (trihydroxy) for taurine conjugates. The 3-sulfates of unconjugated and glycine- and taurine-conjugated cholic, chenodeoxycholic, deoxycholic, ursodeoxycholic and lithocholic acids added to human urine from a healthy volunteer (each 100 ng ml^{-1}) were recovered at a rate of more than 90%. As shown in Fig. 7, the result obtained for a healthy volunteer allowed the detection of chenodeoxycholic, deoxycholic and lithocholic acid 3-sulfates as the glycine conjugates (594, 996 and 310 ng ml^{-1}, respectively) with very small amounts of unconjugated and taurine-conjugated bile acid 3-sulfates.

Although the SIM technique has high sensitivity and selectivity, this method is not always satisfactory for the identification of peaks on the chromatogram. We have demonstrated that the k′ values of bile acid 3-sulfates were influenced by the pH of the mobile phase with a characteristic ratio of the corresponding singly charged ion to the doubly charged ion. The identity of 3-sulfates was therefore further confirmed with two different mobile phases. As shown in Table 1, the ratios of these ions formed from 3-sulfates on the chromatogram were identical with those of the corresponding authentic samples. LC determination combined with ESI-MS was also applied to the determination of 3-sulfates in urine specimens from patients with obstructive jaundice and a representative chromatogram for glycine conjugates is illustrated in Fig. 8. The result allows the detection of glycine conjugates of chenodeoxycholic acid and cholic acid 3-sulfates (30 and 4.8 μg ml^{-1}, respectively) with a small amount of unconjugated and taurine-conjugated chenodeoxycholic acid 3-sulfates (2.6 and 1.2 μg ml^{-1}, respectively).

It is hoped that the availability of this novel method for the simultaneous determination of bile acid 3-sulfates in urine with sufficient reliability and sensitivity may provide a much more precise knowledge of the metabolic profile of bile acids and may serve as a diagnosis for hepatobiliary diseases.

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