CHROMBIO. 3802

DETERMINATION OF 3β,12α-DIHYDROXY-5-CHOLEN-24-OIC ACID AND RELATED BILE ACIDS IN HUMAN SERUM BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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(First received March 16th, 1987; revised manuscript received May 25th, 1987)

SUMMARY

The glycine, taurine and sulphate conjugates of 3β,12α-dihydroxy-5-cholen-24-oic acid were synthesized as authentic samples for the analysis of this unusual bile acid. A highly sensitive and specific quantitative assay of the bile acid and related compounds has been developed by selected-ion monitoring in gas chromatography–mass spectrometry of their methyl ester-trimethylsilyl ether derivatives, using the deuterium labelled internal standards: [$^2H_6$]3β,12α-dihydroxy-5-cholen-24-oic acid, [$^2H_5$]3β-hydroxy-5-cholen-24-oic acid, [$^2H_7$]cholic acid and their sulphates. Calibration curves for these bile acids were linear over the range 0.01–100 μg/ml in human serum. Recoveries of the bile acids and their conjugates ranged from 95 to 103% of the added amounts of their standard samples. The unsaturated bile acid was identified in a significant amount of 25.2 μg/ml in serum of an infant with liver disease, and its sulphate comprised 55.1% of the amount of the bile acid.

INTRODUCTION

Sjövall and co-workers [1–4] identified an unusual bile acid, 3β,12α-dihydroxy-5-cholen-24-oic acid, together with 3β-hydroxy-5-cholen-24-oic acid [5], in urine from patients with cholestatic liver diseases by means of gas chromatography–mass spectrometry (GC–MS). It is of interest to study the excretion of these unsaturated bile acids in biological fluids of patients with hepatobiliary diseases, because these bile acids may reflect a specific function of the liver.

It is necessary to develop a specific microassay of the 3β,12α-dihydroxy-Δ^5-

unsaturated bile acid to facilitate stoichiometric studies on the relationship of liver function and the abnormal metabolism of bile acids. Quantitative analysis of bile acids in human biological fluids is usually carried out after considerable pretreatment, involving extraction, solvolysis and alkaline hydrolysis of bile acid conjugates [6]. We reported previously that the recovery of the sulphate of 3β-hydroxy-5-cholen-24-oic acid was only 40% from extraction using an Amberlite XAD-2 column, and this difficulty was overcome by use of XAD-7 and addition of the corresponding deuterium-labelled bile acid as an internal standard prior to pretreatment for GC–MS analysis [7].

In a continuation of our investigation into the quantitative analysis of unusual bile acids in liver diseases [7–11], we now report on the syntheses of the glycine, taurine and sulphate conjugates of $3\beta,12\alpha$-dihydroxy-5-cholen-24-oic acid and their deuterated derivatives, and on a new method for the determination of these compounds in human serum by selected-ion monitoring in GC–MS using three deuterium-labelled compounds as internal standards.

EXPERIMENTAL

Reagents and instruments

Reagents were obtained from Wako (Osaka, Japan) unless otherwise noted. Deuterium-labelled compounds were purchased from Daiichi (Tokyo, Japan).

Melting points were determined with a micro hot-stage apparatus and are uncorrected. Infrared (IR) spectra were measured with a JASCO IR A-102 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded with a Hitachi R-40 spectrometer at 90 MHz using tetramethylsilane as an internal standard.

Glyco-3β,12α-dihydroxy-5-cholen-24-oic acid (I)

Diethylphosphoryl cyanide [12] (500 mg), methyl glycinate hydrochloride (500 mg) and triethylamine (2 ml) were added to a solution of $3\beta,12\alpha$-dihydroxy-5-cholen-24-oic acid [10] (500 mg) in dimethylformamide (5 ml). After stirring at room temperature for 30 min, the reaction mixture was diluted with ice-water and extracted with ethyl acetate. The organic layer was washed with 2 M hydrochloric acid, 5% sodium hydrogencarbonate and saturated sodium chloride, dried over anhydrous sodium sulphate and evaporated to dryness. The residue was chromatographed on silica gel with n-hexane–ethyl acetate (2:3), and the eluate gave methyl ester of the conjugate (460 mg) as colourless plates by recrystallization from methanol, m.p. 116–119°C. IR (Nujol): 3350 (OH), 1710, 1650 (CONH) cm^{-1}. NMR (C^{2}HCl$_3$) δ: 0.72 (3H, s, 18-CH$_3$), 0.98 (3H, s, 19-CH$_3$), 3.47 (1H, m, 3α-H), 3.70 (3H, s, COOCH$_3$), 4.00 (2H, d, $J=5$ Hz, NCH$_2$ and 1H, m, 12β-H), 5.32 (1H, m, 6-H), 6.27 (1H, t, $J=5$ Hz, NH).

A mixture of the methyl ester (214 mg) in ethanol (5 ml) and 0.2 M sodium hydroxide (1 ml) was stirred at room temperature for 1 h. The reaction mixture was acidified with 1 M sulphuric acid, concentrated under reduced pressure and diluted with water. A precipitate was separated by filtration and recrystallized from methanol–diethyl ether to give the conjugate I (172 mg) as colourless needles,

m.p. 184–185°C. IR (KBr): 1634, 1574, 1214 (CONH) cm^{-1}. NMR (C^{2}H$_3$O^{2}H) δ: 0.71 (3H, s, 18-CH$_3$), 0.96 (3H, s, 19-CH$_3$), 3.45 (1H, m, 3α-H), 3.84 (2H, s, NCH$_2$), 3.96 (1H, m, 12β-H), 5.29 (1H, m, 6-H).

Tauro-3β,12α-dihydroxy-5-cholen-24-oic acid (II)

Diethylphosphoryl cyanide (100 mg) and triethylamine were added to a solution of 3β,12α-dihydroxy-5-cholen-24-oic acid [10] (12 mg) and taurine (10 mg) in dimethylformamide (3 ml) under ice-cooling. After stirring for 1 h, the reaction mixture was adjusted to pH 12 with 1 M sodium hydroxide and placed on an Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, U.S.A.) column, which was washed with water. The eluate with methanol (50 ml) was evaporated and the residue was dissolved in methanol and reprecipitated with diethyl ether to give the taurine conjugate II (7 mg), m.p. 220–224°C. IR (KBr): 1646, 1548 (CONH), 1210, 1044 (SO$_3$H) cm^{-1}.

Glyco-3β,12α-dihydroxy-5-cholen-24-oic acid 3-sulphate (III)

Chlorosulphonic acid (0.1 ml)–pyridine (0.5 ml) complex in dimethylformamide (1 ml) was added dropwise to a solution of the glycine conjugate I (50 mg) in pyridine (1 ml) and dimethylformamide (1 ml) under ice-cooling as described previously [7]. After standing for 3 h, the resulting solution was poured into ice-water, adjusted to pH 10 with 1 M sodium hydroxide and placed on an Amberlite XAD-2 column, which was neutralized by washing with water. The eluate with methanol (100 ml) was evaporated and the residue was dissolved in chloroform–methanol (1:1) and chromatographed on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column. The eluate with chloroform–methanol (1:2) gave the 3-sulphate III (32 mg), m.p. 215–218°C. IR (KBr): 1634, 1598, 1224 (CONH), 1400, 1255, 1066 (OSO$_3$H) cm^{-1}.

[2,2,4,4,6,23-^{2}H$_6$] 3β,12α-Dihydroxy-5-cholen-24-oic acid (IV)

The labelled compound (IV) was synthesized from methyl 12α-acetoxy-3-oxo-4-cholen-24-oate [10] by deuterium exchange using potassium *tert.*-butoxide in [1-^{2}H]*tert.*-butanol, deconjugation with [1-^{2}H]acetic acid and reduction with sodium borohydride by the method described previously [11]. MS (Fig. 1b) showed the following isotopic composition: [^{2}H$_0$] and [^{2}H$_1$], 0.0%; [^{2}H$_2$], 1.6%; [^{2}H$_3$], 2.9%; [^{2}H$_4$], 10.8%; [^{2}H$_5$], 34.6%; [^{2}H$_6$], 42.4%; [^{2}H$_7$], 7.7%.

[2,2,4,4,6,23-^{2}H$_6$] Glyco-3β,12α-dihydroxy-5-cholen-24-oic acid (V)

Treatment of the deuterated IV (200 mg) as described for the unlabelled compound (I) afforded the glycine conjugate V (146 mg) as colourless needles, m.p. 184–185°C.

[2,2,4,4,6,23-^{2}H$_6$] Glyco-3β,12α-dihydroxy-5-cholen-24-oic acid 3-sulphate (VI)

Treatment of the conjugate V (50 mg) as described for the unlabelled compound (III) afforded the sulphate VI (28 mg) as colourless crystals, m.p. 217–221°C.
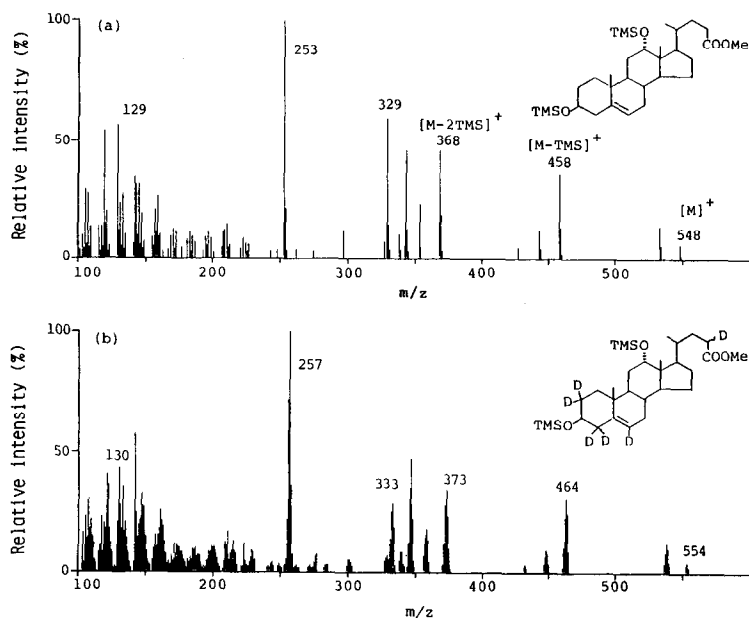
Fig. 1. Mass spectra of the methyl ester-trimethylsilyl ethers of 3β,12α-dihydroxy-5-cholen-24-oic acid (a) and its deuterated derivative (b).

[2,2,3,4,4,23,23-$^2H_7$] 3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid (VII)

A mixture of methyl 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oate [13] (165 mg) and sodium methoxide (40 mg) in [1-^2H]methanol (4 ml) was refluxed for 12 h. After distillation of the solvent, the residue was redissolved in [1-^2H]methanol (4 ml) and again refluxed for 3 h. The solution was treated with [$^2H_4$]sodium borohydride (40 mg) under stirring at room temperature for 15 min. The reaction mixture was evaporated, acidified with 2 M hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with water and saturated sodium chloride, dried over anhydrous sodium sulphate and evaporated. After methylation of the oily product with diazomethane, the mixture was chromatographed to give the 3α-hydroxy ester. Finally, the ester was hydrolysed with 2 M lithium-hydroxide (2 ml) in methanol (10 ml) under reflux for 1 h. After concentration, the solution was diluted with water and acidified with 2 M hydrochloric acid. The precipitate was recrystallized from ethyl acetate to give [$^2H_7$]cholic acid (VII, 125 mg), m.p. 199–200°C. The isotopic composition was calculated from MS: [$^2H_0$]–[$^2H_3$], 0.0%; [$^2H_4$], 2.9%; [$^2H_5$], 12.9%; [$^2H_6$], 27.0%; [$^2H_7$], 57.2%.

Gas chromatography–mass spectrometry

GC–MS was performed on a Shimadzu-LKB 9000 system equipped with a multiple-ion detector and a data-processing system (Shimadzu GC-MS PAC 300M). A gas chromatographic column (2 m × 2.5 mm I.D., glass coil) packed with 1.5% Poly I-110 or 1.5% SE-30 on Gas Chrom Q (100–120 mesh) was used at 250°C. The flow-rate of helium carrier gas was 30 ml/min. The temperatures of the flash

Serum (0.5 ml)

internal standards (0.1—1.0 µg) added

Extraction by Bond Elut C$_{18}$

eluted with methanol—chloroform (9:1)

Sephadex LH-20 chromatography

0.02 M sodium chloride in methanol

0.02 M sodium chloride in methanol—chloroform

Total bile acid   Sulphate   Non-sulphate

Solvolysis at pH 1 in ethanol—acetone (1:9)
38°C for 1 h

Hydrolysis in 4 M sodium hydroxide—methanol (1:1) at 80°C for 16 h

washed with n-hexane

Extraction by Bond Elut C$_{18}$

Derivatization (methyl ester-trimethylsilyl ether)
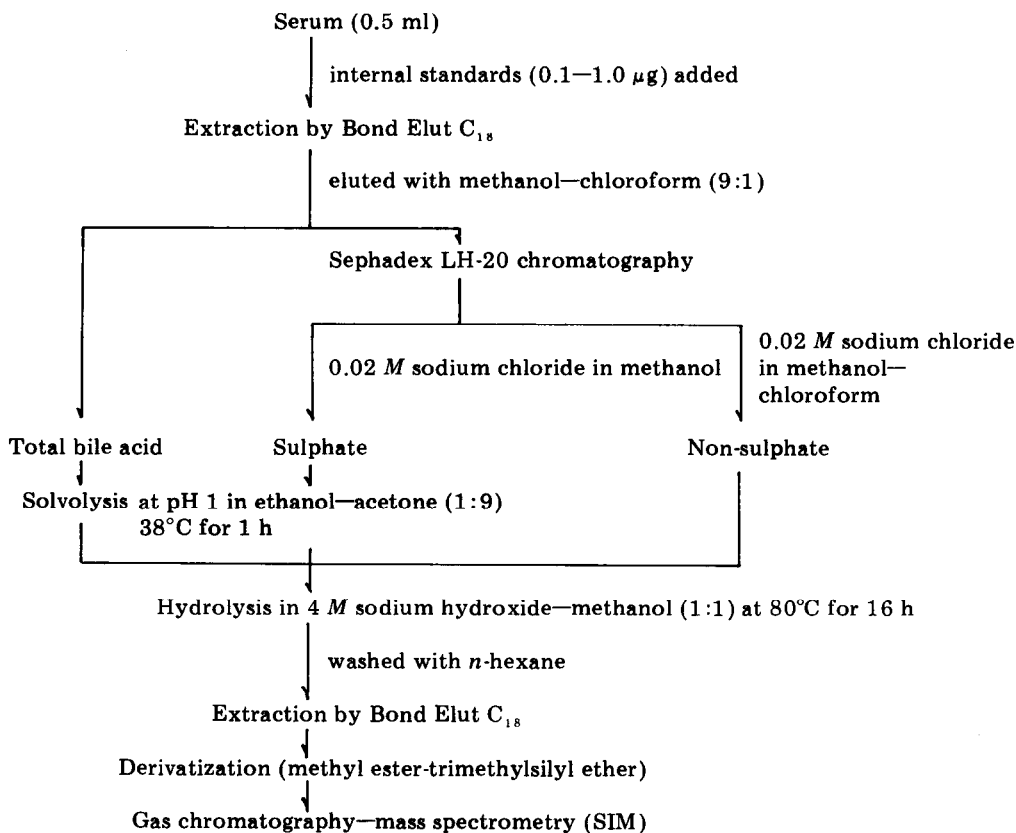
Gas chromatography—mass spectrometry (SIM)

Fig. 2. Procedure for the determination of bile acids in human serum.

heater and separator were 280 and 300°C, respectively. The mass spectra were recorded at 70 eV with an ion source temperature of 290°C.

Derivatization of bile acids for GC–MS measurement

Each bile acid or a mixture of bile acids was derivatized to the methyl ester with diazomethane–diethyl ether at room temperature. After removal of excess reagents, the trimethylsilyl ethers were prepared by heating the residue with N-trimethylsilylimidazole in acetonitrile at 60°C for 30 min. Excess reagents were evaporated off in a stream of nitrogen, and the residue was redissolved in n-hexane prior to GC–MS analysis.

Quantitative analysis of bile acids in human serum

Sample preparation from human serum for GC–MS was performed in two ways, as shown in Fig. 2.

Human serum (0.5 ml) was diluted with 0.1 M sodium hydroxide (2 ml), and internal standards, [2,2,4,4,6,23-^{2}H$_6$]3β,12α-dihydroxy-, [2,2,4,23,23-^{2}H$_5$]3β-hydroxy-5-cholen-24-oic acids [7] and [2,2,3,4,4,23,23-^{2}H$_7$]cholic acid and/or their sulphates (each 0.1–1.0 µg) were added to the solution. The bile acid con-

jugates were extracted from the serum using Bond Elut C_{18} cartridge (6 ml, Analytichem International, Harbor City, CA, U.S.A.). The cartridge was washed with water (5 ml), and the bile acid conjugates were eluted with methanol–chloroform (9:1, 5 ml). The eluate was divided into two portions and evaporated for determination of the total amounts of individual bile acids or separation of their sulphates. One portion of the eluate was solvolysed at pH 1 with 2 M hydrochloric acid in ethanol–acetone (1:9, 4 ml) at 38°C for 1 h, neutralized with 1 M sodium hydroxide and evaporated to dryness. The residue was hydrolysed with 4 M sodium hydroxide–methanol (1:1, 4 ml) at 80°C for 16 h, then washed with two 1-ml portions of n-hexane to remove cholesterol and acidified to pH 1 with 6 M hydrochloric acid (1.4 ml) under ice-cooling. The free bile acids were extracted again with Bond Elut C_{18}, as described above, and derivatized to the methyl ester-trimethylsilyl ethers for GC–MS analyses. Another portion was subjected to the separation of sulphate and non-sulphate fractions by means of Sephadex LH-20 chromatography [14], then solvolysis of the sulphates, hydrolysis of the amino acid conjugates and GC–MS analysis were carried out according to the above procedure.

RESULTS AND DISCUSSION

Synthesis of the unsaturated bile acid conjugates and their deuterium-labelled compounds

In a previous paper [10], we reported a convenient synthesis of the unsaturated bile acids, $3\beta,12\alpha$-dihydroxy-5-cholen-24-oic acid, together with the $3\beta,7\alpha$- and $3\beta,7\beta$-isomers. The latter 7-hydroxylated bile acids had been identified in an alternative pathway of bile acid formation proposed by Yamasaki et al. [15] and Harano et al. [16].

The glycine, taurine and sulphuric acid conjugates of the $3\beta,12\alpha$-dihydroxy bile acid were synthesized as standard samples for the quantitative analysis of these conjugates. The glycine and taurine conjugates were prepared by mixing the bile acid and methyl glycinate or taurine with diethylphosphoryl cyanide in the presence of triethylamine at room temperature [12]. The sulphate was synthesized from the glycine conjugate with a chlorosulphonic acid–pyridine complex in dimethylformamide [7].

The deuterium-labelled compounds of their conjugates were also prepared as internal standards for GC–MS analysis from $[2,2,4,4,6,23-^2H_6]3\beta,12\alpha$-dihydroxy-5-cholen-24-oic acid by the above procedure.

GC–MS of $3\beta,12\alpha$-dihydroxy-5-cholen-24-oic acid and related compounds

Mass spectra of the methyl ester-trimethylsilyl ether derivatives of the unsaturated bile acid and its deuterium-labelled compound are shown in Fig. 1. The molecular ion peaks of those compounds are presented at m/z 548 and m/z 554, respectively. The characteristic fragment ions at m/z 458 and 464 indicate facile loss of the 12α-trimethylsiloxy group from the original molecules. Further loss of trimethylsilanol from the C-3 position gives rise to fragment ions at m/z 368, $[M-90-90]^+$, and m/z 373, $[M-90-91]^+$, respectively. The peaks at m/z 129

TABLE I

GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC DATA OF THE METHYL ESTER-TRIMETHYLSILYL ETHER DERIVATIVES OF BILE ACIDS

Bile acid	Relative retention time[*]		Base peak, m/z	Fragment ions, m/z (%)
	Poly I-110	SE-30		
5β-Cholanoic acid	1.00	1.00	217[**]	374 (15), 359 (17), 257 (24)
3β-Hydroxy-5-cholenoic acid	1.88	2.14	129	460 (13), 370[**] (35), 255 (11)
3β,7α-Dihydroxy-5-cholenoic acid	1.11	1.88	458[**]	548 (4), 368 (1), 253 (2)
3β,7β-Dihydroxy-5-cholenoic acid	1.74	2.80	458[**]	548 (6), 368 (2), 253 (1)
3β,12α-Dihydroxy-5-cholenoic acid	1.28	2.63	253[**]	548 (3), 458 (18), 368 (23)
Lithocholic acid	1.48	1.90	215	462 (1), 372[**] (80), 257 (27)
Deoxycholic acid	1.06	2.36	255[**]	535 (6), 460 (2), 370 (16)
Chenodeoxycholic acid	1.15	2.41	370[**]	460 (3), 355 (23), 255 (25)
Cholic acid	0.78	2.48	253[**]	458 (26), 368[**] (51), 343 (28)
Cholesterol	1.18	2.07	129	458 (28), 368[**] (62), 329 (91)

[*]Relative retention times were calculated for 7.9 min (Poly I-110) and 7.4 min (SE-30) of the retention times of methyl 5β-cholanoate at 250°C.
[**]Fragment ions used for selected-ion monitoring.

and m/z 329, $[M-90-129]^+$ in the spectrum of the unlabelled bile acid (Fig. 1a) are indicative of a 3-trimethylsiloxy-Δ^5 structure [17,18], and the base peak at m/z 253 is the ABCD-ring fragment ion. These fragmentations are identical with those in the spectrum of the bile acid derivative obtained from the urine of a patient with intrahepatic cholestasis by Almé et al. [1]. The deuterium-labelled compound shows the corresponding ions at m/z 130, 333 and 257 in Fig. 1b, and the preferential deuterium incorporation in the bile acid was assigned at the C-2,2,4,4,6 and C-23 positions from inspection of the mass spectrum.

The GC–MS data of the bile acid derivative and related compounds are summarized in Table I, involving retention times and characteristic fragment ions accompanied by their relative abundances. On the basis of these data, it seems likely that selected-ion monitoring of the prominent ion at m/z 253 of the 3β,12α-dihydroxy unsaturated bile acid derivative would permit sensitive determination of the bile acid using the corresponding ion at m/z 257 of the deuterated compound as an internal standard without interferance by other bile acids in the subsequent GC–MS analysis using Poly I-110 or SE-30 column.

Fig. 3 shows a chromatogram (Poly I-110) by selected-ion monitoring of the characteristic fragments of the methyl ester-trimethylsilyl ether derivatives of the standard unsaturated and usual bile acids. It can been seen that simultaneous determination of these bile acids could be achieved by selected-ion monitoring in GC–MS even if chromatographic separation is not complete, such as 3β,12α-dihydroxy-5-cholenoic and chenodeoxycholic acids and cholesterol derivatives.

Bile acids and their conjugates in human serum were treated in two different ways prior to GC–MS measurements (Fig. 2). For the determination of the total amounts of individual bile acids, a portion of the serum extract was solvolysed at
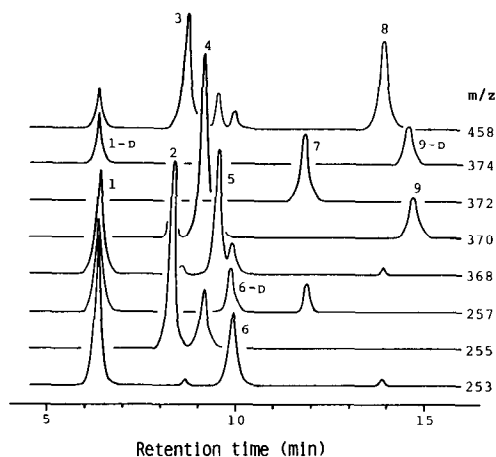
Fig. 3. Selected-ion monitoring of the methyl ester–trimethylsilyl ether of a mixture of bile acids by GC–MS using a Poly I-110 column. Peaks: 1 = cholic acid; 2 = deoxycholic acid; 3 = 3β,7α-dihydroxy-5-cholenoic acid; 4 = chenodeoxycholic acid; 5 = cholesterol; 6 = 3β,12α-dihydroxy-5-cholenoic acid; 7 = lithocholic acid; 8 = 3β,7β-dihydroxy-5-cholenoic acid; 9 = 3β-hydroxy-5-cholenoic acid.

pH 1 in ethanol–acetone (1:9) following by the hydrolysis in 4 M sodium hydroxide–methanol (1:1) and derivatization to the methyl ester–trimethylsilyl ethers for GC–MS analysis. On the other hand, for the determination of the sulphates, the separation of the sulphate and non-sulphate fractions from the serum extract using Sephadex LH-20 chromatography before solvolysis were first performed. Extraction of bile acids and their conjugates from human serum was carried out on reversed-phase octadecylsilane bonded silica (Bond Elut C_{18} cartridge) instead of on Amberlite XAD-7 as described previously [7] because the resin gave low recoveries for less polar bile acids such as lithocholic acid (65% yield). The suitability of the cartridge was confirmed by the following recovery tests.

Calibration curves for the determination of the unsaturated bile acid and related compounds were obtained by plotting the peak-area ratios between the monitoring ions for each bile acid and the corresponding internal standard versus amounts of the bile acid. A good linear relationship (correlation coefficient, $r > 0.9988$) was found over the range 0.01–100 μg/ml of each bile acid in serum. Excellent relative recoveries of these bile acids and their conjugates from human serum were obtained (Table II) by the developed procedure using three deuterium-labelled compounds as internal standards, [$^2H_6$] 3β,12α-dihydroxy-5-cholenoic acid for its natural bile acid and lithocholic acid, [$^2H_5$] 3β-hydroxy-5-cholenoic acid for its natural bile acid and [$^2H_7$] cholic acid for the other usual bile acids.

Decomposition of 3β,7α- and 3β,7β-dihydroxy-5-cholenoic acids during solvolysis

The 7-hydroxy-Δ^5-unsaturated bile acids could not be determined by the present analytical procedure using selected-ion monitoring of a characteristic ion at m/z 458 because these allyl alcohols decomposed to form 3β-hydroxy-5,7-choladienoic acid (UV: λ_{max} 232, 239 and 248 nm) by the acid incubation for solvolysis. It is similar to the decomposition of the 7α-hydroxy bile acid in a methanolic

TABLE II

RECOVERIES OF BILE ACIDS FROM HUMAN SERUM

Bile acid	Internal standard[*]	Added amount (μg)	Relative recovery (mean ± S.D., $n=4$) (%)
3β-Hydroxy-5-cholenoic acid	a	1.09	99 ± 3
3β,12α-Dihydroxy-5-cholenoic acid	b	1.06	101 ± 1
Glyco-3β-hydroxy-5-cholenoic acid	a	1.03	97 ± 1
Glyco-3β,12α-dihydroxy-5-cholenoic acid	b	1.00	100 ± 1
Tauro-3β,12α-dihydroxy-5-cholenoic acid	b	1.02	101 ± 2
3β-Hydroxy-5-cholenoic acid 3-sulphate	a	1.02	95 ± 6
Glyco-3β,12α-dihydroxy-5-cholenoic acid 3-sulphate	b	0.90	98 ± 3
Cholic acid	c	1.05	101 ± 3
Chenodeoxycholic acid	c	1.08	95 ± 2
Deoxycholic acid	c	1.08	99 ± 2
Lithocholic acid	b	1.12	103 ± 3

[*]a = [^{2}H$_5$]3β-hydroxy-5-cholenoic acid (1.06 μg); b = [^{2}H$_6$]3β,12α-dihydroxy-5-cholenoic acid (1.02 μg); c = [^{2}H$_7$]cholic acid (1.04 μg).

hydrogen chloride treatment described by Harano et al. [19]. Consequently, determination of the sulphates of bile acids with an allyl alcohol group would require hydrolysis under rather mild conditions, such as enzymatic hydrolysis.

Determination of the unsaturated bile acids in human serum with liver disease

The developed procedure was applied to determination of the bile acids in serum of an infant with severe intrahepatic cholestasis. 3β,12α-Dihydroxy-5-cholen-24-oic acid was identified in a significant amount, 25.2 μg/ml (18.0% of total bile acids). 3β-Hydroxy-5-cholen-24-oic acid was not detected (Fig. 4). Further, the mass spectrum of the peak in GC–MS shows an identical fragment pattern to that of the bile acid in Fig. 1a. This unsaturated bile acid was also identified in umbilical blood (0.11 ± 0.05 μg/ml, 6.7%) accompanied by the 3β-monohydroxy bile acid (0.38 ± 0.14 μg/ml, 23.3%) from ten normal subjects. Although the 3β,12α-dihydroxy bile acid was not detected in any serum of thirteen healthy adults, it was found in the serum of four patients with intrahepatic cholestasis (0.60 ± 0.43 μg/ml, 1.2%) together with the 3β-monohydroxy bile acid (1.11 ± 0.99 μg/ml, 2.2%). These facts imply that the similarity of the bile acid metabolism between foetus and cholestatic patients and the determination of the unusual bile acid in serum provides useful diagnostic information in the patient with liver disease, and further details will be presented in another paper.

Determination of the sulphates

Analyses of sulphate and non-sulphate fractions of the unsaturated and related bile acids in serum of patients with liver diseases were carried out by the method described above and the results are summarized in Table III. The conjugates of the less polar bile acids, such as 3β-hydroxy-5-cholenoic acid and lithocholic acid, are composed of their sulphates in high levels, while that of the polar cholic acid
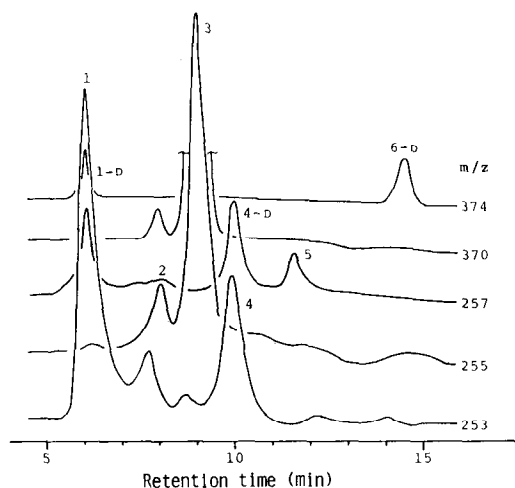
Fig. 4. Selected-ion monitoring of the methyl ester-trimethylsilyl ether derivatives of the bile acids in serum of an infant with intrahepatic cholestasis. Peaks: 1 = cholic acid; 2 = deoxycholic acid; 3 = chenodeoxycholic acid; 4 = $3\beta,12\alpha$-dihydroxy-5-cholenoic acid; 5 = lithocholic acid; 6 = 3β-hydroxy-5-cholenoic acid.

TABLE III

ANALYSIS OF SULPHATE AND NON-SULPHATE FRACTIONS OF BILE ACIDS IN SERUM OF PATIENTS WITH LIVER DISEASES ($n = 4$)

Bile acid	Sulphate		Non-sulphate	
	μg/ml	Mean (%)	μg/ml	Mean (%)
3β-Hydroxy-5-cholenoic acid	0.16–1.55	80.4	0.05– 0.21	19.6
Lithocholic acid	0.13–0.43	75.6	0.01– 0.16	24.4
$3\beta,12\alpha$-Dihydroxy-5-cholenoic acid	0.03–0.22	55.1	0.02– 0.64	44.9
Deoxycholic acid	0.06–0.73	32.8	0.06– 1.57	67.2
Chenodeoxycholic acid	0.20–2.21	11.5	2.86–14.40	88.5
Cholic acid	0.10–0.42	2.6	2.75–11.88	97.4

is present mainly in the non-sulphate fraction. $3\beta,12\alpha$-Dihydroxy-5-cholenoic acid was almost equally divided between the sulphate and the non-sulphate fractions (55.1:44.9). These results suggested that conjugation of bile acids with sulphuric acid occurred in order to increase renal excretion of the less polar bile acids.

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