DETERMINATION OF 3β-HYDROXY-5-CHOLEN-24-OIC ACID AND ITS SULFATE IN HUMAN SERUM BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

The glycine conjugate of 3β-hydroxy-5-cholen-24-oic acid and its sulfate labeled with deuterium at the C-2, -4, and -23 positions were synthesized. A highly sensitive and specific quantitative assay of the bile acid has been developed by selected ion monitoring in gas chromatography-mass spectrometry of the methyl ester trimethylsilyl ether derivatives using the deuterium labeled conjugates as internal standards. Calibration curves for the bile acid and its sulfate exhibited a linear relationship over the range of 0.01-100 μg/ml in human serum.

INTRODUCTION

An unusual bile acid, 3β-hydroxy-5-cholen-24-oic acid(I), was found in the urine of infants with biliary atresia by Makino, *et al* (1), and it has been detected also in patients with cholestasis (2), human meconium (3) and amniotic fluid (4). This bile acid is an intermediate in the alternative biosynthesis of bile acids proposed by Mitropoulos *et al* (5) which begins with oxidation of the cholesterol side chain and differs from the major pathway initiated by the 7α-hydroxylation of cholesterol. Therefore, it may be desirable to develop a specific microassay of this unusual bile acid to facilitate further studies on the relationship of the liver function and the abnormal metabolism of bile acids. The

present paper describes syntheses of the deuterium labeled compounds of the bile acid(I), its glycine conjugate and sulfate, and a new method for determination of the bile aci (I) and its sulfate in human serum by gas chromatography-mass spectrometry(GC-MS) using the deuterated conjugates as internal standards.

EXPERIMENTAL

Glyco-3β-hydroxy-5-cholen-24-oic Acid (II) To a solutio of the bile acid(I, 83 mg) and ethyl glycinate hydrochlorid (42 mg) in dimethylformamide was added dropwise diethylphosphoryl cyanide(70 mg) and triethylamine(58 mg) in dimethylformamide under ice-cooling. After stirring for 30 min, the reaction mixture was stirred at room temperature for 4 hr, then diluted with benzene-AcOEt(1:4 v/v, 40 ml), washed with 5% HCl, H_2O, 5% $NaHCO_3$, sat NaCl, dried over anhydrous Na_2SO and evaporated to dryness. The residue was dissolved in 0.2 NaOH(5 ml) and EtOH(5 ml). After stirring at room tempera ture for 4 hr, the solution was condensed *in vacuo*, adjusted to pH 2 with 1N HCl, and extracted with AcOEt. The organic layer was washed with sat NaCl, dried over anhydrous Na_2SO_4 and evaporated. The residue was recrystallized from MeOH-ether to give the glycine conjugate(II, 46 mg) as colorless plates, mp 218-221°C, IR(nujol) ν : 3340, 1660, 1550, 1260 (CONH), 1720(COOH) cm^{-1}.

Glyco-3β-hydroxy-5-cholen-24-oic Acid 3-Sulfate (III) To a solution of the conjugate(II, 200 mg) in pyridine(2 ml) was added $ClSO_3H$(0.3 ml) in pyridine(2 ml) under ice-cooling and the solution was heated at 50°C for 1 hr. The reaction mixture was poured into ice-water, and submitted to an Amberlite XAD-2(50 ml) column. After washing with H_2O, the eluate with MeOH was evaporated. The residue was dissolved in H_2O, acidified with conc HCl, the precipitate was centrifuged and washed with H_2O, then recrystallized from MeOH-eth to give the sulfate(III, 116 mg) as colorless needles, mp 16 161°C, IR(KBr) ν: 3425, 1640, 1545, 1220(CONH), 1730(COOH), 1380, 1060(OSO_3H) cm^{-1}.

[2,2,4,23,23-$^2H_5$] 3-Acetoxy-3,5-choladien-24-oic Acid Methyl Ester (V) To a solution of KH(706 mg) in tert-BuOD(12 ml was added dropwise methyl 3-oxo-4-cholenoate(IV, 400 mg)(6) in tetrahydrofuran(2 ml) in an Ar atmosphere. After stirrin at room temperature for 1 hr, acetic anhydride(2.2 g) in tetrahydrofuran(5 ml) was added dropwise and stirring was continued for 1 hr. The reaction mixture was poured into ice-water and extracted with ether. The organic layer was washed with H_2O, 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. Recrystallization of the

residue from EtOH gave the deuterate(V, 389 mg) as colorless needles, mp 147-148°C, UV(EtOH) λ: 235 nm(ε=17500). IR(nujol) ν: 1755(AcO), 1740(COOMe), 1665, 1615(C=C) cm^{-1}.

[2,2,4,23,23-$^2H_5$] 3β-Hydroxy-5-cholen-24-oic Acid (I-d_5) To a solution of the dienol acetate(V, 379 mg) in EtOH(36 ml) was added dropwise $NaBH_4$(270 mg) in EtOH(25 ml) under stirring at 0°C for 4 hr. Refluxing of the mixture for 1 hr was followed by addition of conc HCl(11 ml) and continued for another hr. After cooling, the reaction product was extracted with AcOEt, and worked up in the usual manner. The methyl ester of the product was submitted to SiO_2 chromatography, and the eluate with 10% AcOEt-hexane gave the ester (72 mg) as colorless crystals by recrystallization from MeOH, mp 109-110°C, IR(nujol) ν: 3425, 3300(OH), 1735, 1270(COOMe) cm^{-1}. Hydrolysis of the ester(48 mg) in 0.5N KOH-MeOH gave the deuterate(I-d_5, 40 mg) crystallized from MeOH, mp 228-229°C, IR(KBr) ν: 3300(OH), 1675(COOH) cm^{-1}, NMR(pyridine-d_5) δ: 0.66(3H, s, 18-Me), 1.04(3H, s, 19-Me), 3.80(1H, d, 3α-H), 5.40(1H, m, 5-H). MS(Fig 1-b) shows the following isotopic composition: 0.7% d_0(m/z 370), 1.8% d_1(m/z 371), 9.7% d_2(m/z 372), 22.0% d_3(m/z 373), 36.4% d_4(m/z 374), 29.4% d_5(m/z 375).

[2,2,4,23,23-$^2H_5$] Glyco-3β-hydroxy-5-cholen-24-oic Acid (II-d_5) Treatment of the deuterate (I-d_5, 43 mg) as described for the unlabeled compound(II) and recrystallization from MeOH-AcOEt afforded the conjugate(II-d_5, 31 mg) as colorless plates, mp 215-218°C, IR(KBr) ν:3320, 1615, 1570, 1220(CONH), 1740(COOH) cm^{-1}.

[2,2,4,23,23-$^2H_5$] Glyco-3β-hydroxy-5-cholen-24-oic Acid 3-Sulfate (III-d_5) Treatment of the conjugate(II-d_5, 20 mg) as described for the unlabeled compound(III) afforded the sulfate(III-d_5, 8 mg) as colorless crystals, mp 160-161°C, IR(KBr) ν: 3425, 1635, 1545, 1215(CONH), 1735(COOH), 1380, 1060(OSO_3H) cm^{-1}.

Gas Chromatography-Mass Spectrometry (GC-MS) GC-MS was performed on a Shimadzu-LKB 9000 system equipped a multiple ion detector and a data-processing system (Shimadzu GC-MS PAC 300M). A gas chromatographic column(2 m x 2.5 mm I.D. glass coil) packed with 1.5% Poly I-110 on Gas Chrom Q(80-100 mesh) was used at 255°C (7). The flow rate of He carrier gas was 30 ml/min. The temperatures of flash heater and separator were 280°C and 300°C, respectively. The mass spectra were recorded at 70 eV, with an ion source temperature of 290°C.

Quantitative Analysis of Bile Acids in Human Serum Human serum(1 ml) was diluted with 0.1N NaOH-saline(9 ml) and internal standards(II-d_5 and III-d_5, each 1-5 μg) added. The bile conjugates were extracted from the serum with an Amberlite XAD-7(5 g) column, and sulfate and non-sulfate fractions were separated using a Sephadex LH-20 column (8). Solvolysis of the sulfates was carried out at pH 1 in EtOH-acetone(1:9) at 37°C for 1 hr, and the amino acid conjugates were hydrolyzed with 4N NaOH-MeOH(1:1, 10 ml) at 80°C for 16 hr. The bile acids were extracted with AcOEt at pH 1, the organic

layer was dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue was derivatized to the methyl ester with diazomethane-ether, and to the trimethylsilyl ethers of hydroxyl groups with N-trimethylsilylimidazole in acetonitrile at 60°C for 30 min. Excess reagents were removed under a stream of N_2, and the residue was dissolved in n-hexane prior to GC-MS.

RESULTS AND DISCUSSION

Syntheses of 3β-Hydroxy-5-cholen-24-oic Acid Conjugates and Their Deuterium Labeled Compounds

The glycine and sulfuric acid conjugates of 3β-hydroxy-5-cholen-24-oic acid were synthesized in order to develop the quantitative analysis of the bile acid and its conjugates. The glycine conjugate(II) was prepared by mixing of the bile acid(I) and ethyl glycinate with diethylphosphoryl cyanide in the presence of triethylamine at room temperature followed by alkaline hydrolysis. The bile acid sulfate(III) was synthesized from the glycine conjugate(II) with chlorosulfonic acid in pyridine at 50°C for 1 hr.

The deuterium labeled compounds of their conjugates were prepared as internal standards for GC-MS analysis as shown in

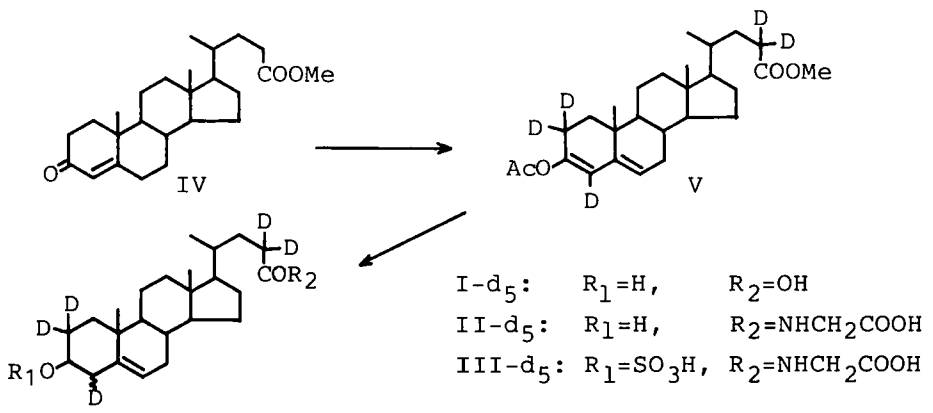

Chart 1.

chart 1. The labeling of methyl 3-oxo-4-cholen-24-oate(IV) (7) was achieved by deuterium exchange via enolization with potassium hydride and tert-butanol-d_1 in tetrahydrofuran, and the enolacetate(V) was isolated after acetylation of the reaction product with acetic anhydride, and then reduced with sodium borohydride in ethanol and hydrolyzed to give [2,2,4, 23,23-$^2H_5$] 3β-hydroxy-5-cholen-24-oic acid(I-d_5). The deuterate(I-d_5) was converted to the glycine conjugate(II-d_5) and the glyco-sulfate(III-d_5) by the procedure as mentioned above.

Gas chromatography-Mass Spectrometry of 3β-Hydroxy-5-cholen-24-oic Acid and Related Bile Acids

Mass spectra of the methyl ester trimethylsilyl ether derivatives of the bile acid(I) and its deuterate(I-d_5) are shown in Fig. 1. Preferential deuterium incorporation in the bile acid at the C-2, -2, -4, -23 and -23 positions was determined by analyses of these spectra. The isotopic

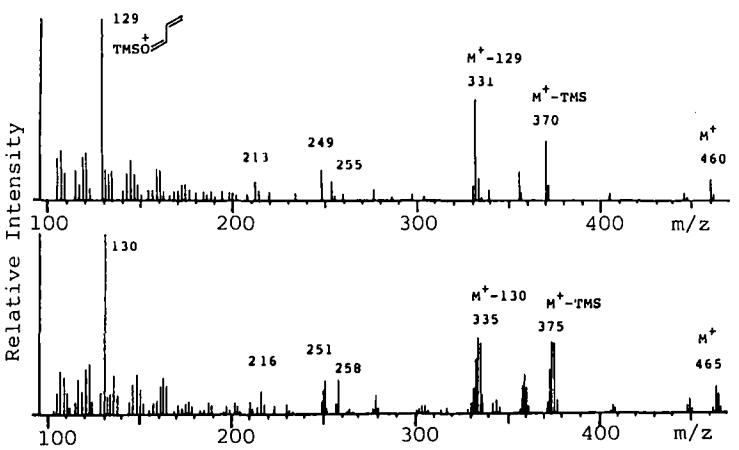

Fig.1. Mass Spectra of the Methyl-Trimethylsilyl Derivatives of 3β-Hydroxy-5-cholen-24-oic Acid(a) and Its Deuterate(b

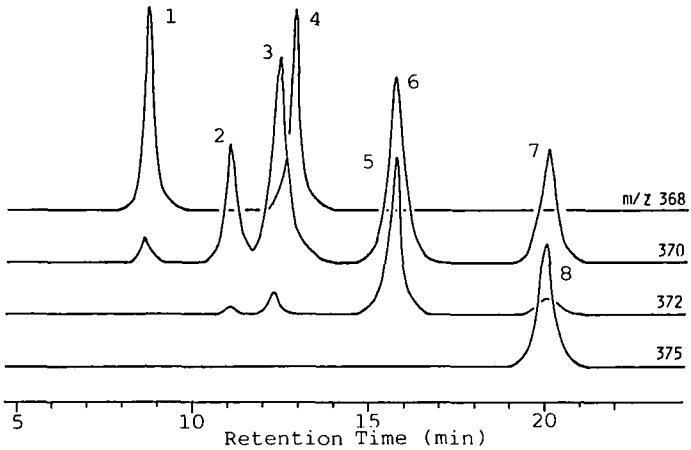

Fig.2. Selected Ion Monitoring of the Methyl-Trimethylsilyl Derivatives of Bile Acids by GC-MS
1. Cholic Acid, 2. Deoxycholic Acid, 3. Chenodeoxycholic Acid
4. Cholesterol, 5. Lithocholic Acid, 6. Ursodeoxycholic Acid
7. 3β-Hydroxy-5-cholen-24-oic Acid, 8. 3β-Hydroxy-5-cholen-24-oic Acid-d_5

composition of the deuterate(I-d_5) was 0.7% d_0, 1.8% d_1, 9.7 d_2, 22.0% d_3, 36.4% d_4 and 29.4% d_5 from the peak area ratio in the selected ion monitoring of the corresponding ions nea m/z 370, and it was not influenced by alkaline hydrolysis. It seems likely that the prominent ions at m/z 370 and m/z 375 would permit sensitive determinations of the bile acid and its deuterate without interference by each other, and the deuterated compounds(II-d_5 and III-d_5) were used as internal standards in the following GC-MS.

Fig. 2 shows a chromatogram by selected ion monitoring of the characteristic fragments near to m/z 370 (M^+-nTMSOH) of the methyl-trimethylsilyl derivatives of the standard bil acid(I) and related compounds. It can be seen that the simultaneous determination of these bile acids could be

achieved by selected ion monitoring in GC-MS even if chromatographic separation was not complete.

Quantitative Analysis of 3β-Hydroxy-5-cholen-24-oic Acid in Human Serum

Determination of bile acids in human serum was usualy carried out after several steps of pretreatments involving extraction, solvolysis and alkaline hydrolysis of the bile acid conjugates. As pointed out by some workers (9), recoveries of the conjugates in bile acid analysis were reduced by Amberlite XAD-2 extraction. The sulfate of glyco-3β-hydroxy-5-cholen-24-oic acid gave a low recovery of only 40% from a XAD-2 column, and an insufficient increase to 67% from a XAD-7 column. This difficulty was overcome by adding of the conjugates of deuterated bile acid(II-d_5 and III-d_5) as internal standards to the serum prior to pretreatments, and the results are presented in Table 1. Calibration curves for the determination of the bile acid(I) and related

Table 1. Recovery of 3β-Hydroxy-5-cholen-24-oic Acid in Serum

Bile acid	Amount added (μg/ml)	recovered (μg/ml)	Recovery (% ± S.D.)
3β-Hydroxy-5-cholen-24-oic acid	10.4	9.9	95.2 ± 3.7
Glyco-3β-hydroxy-5-cholen-24-oic acid	81.3	78.8	96.9 ± 2.1
Glyco-3β-hydroxy-5-cholen-24-oic acid 3-sulfate	22.1	21.4	96.9 ± 2.1

Glyco-3β-hydroxy-5-cholen-24-oic acid-d_5 (15.1 μg) and its sulfate-d_5 (3.7 μg) were added as internal standards.

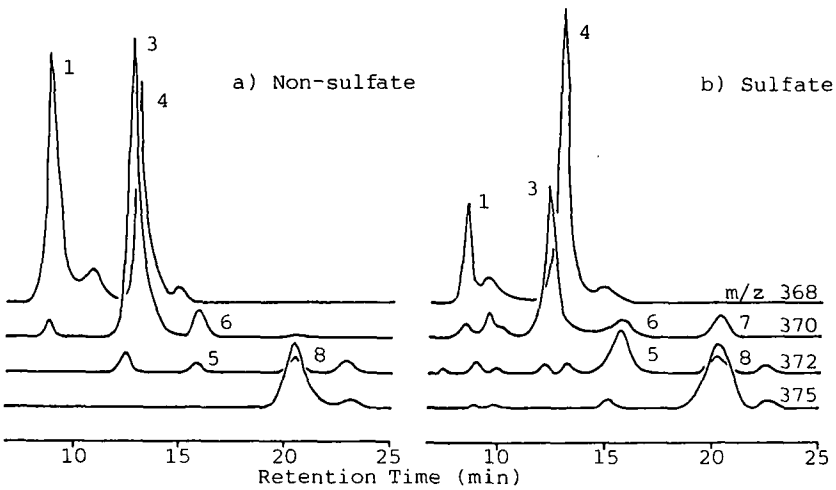

Fig.3. Selected Ion Monitoring of the Methyl-Trimethylsilyl Derivatives of Bile Acids in the Serum of a Patient with Cholestasis. a) Non-sulfate fraction, b) Sulfate fraction
Numbering of peaks indicates the same bile acids as Fig.2.

bile acids were obtained by plotting the peak area ratios of the monitoring ions for the bile acids and the internal standard *vs* amounts of the bile acids. A good linear relationship (correlation coefficient>0.998) was found over the range of 0.01-100 μg/ml for each bile acid in serum.

The established procedure was applied to determination of the bile acids in serum of a patient with cholestasis, an 3β-hydroxy-5-cholen-24-oic acid was identified in significan amounts of 0.482 μg/ml from only the sulfate fraction as shown in Fig. 3. On the basis of these data, this method is currently being applied in a study of the unusual bile acid in normal men and patients with liver disease.

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