Identification of bile alcohols in human bile

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Abstract Human gallbladder bile was examined for bile alcohols. Following isolation and hydrolysis, the bile alcohols were analyzed by capillary gas–liquid chromatography–mass spectrometry. The following bile alcohols were identified with certainty by direct comparison with reference standards: 5β-cholane-3α,7α,23,24-tetrol; 5β-cholane-3α,7α,12α,24-tetrol; 24-nor-5β-cholestane-3α,7α,12α,25-tetrol; 27-nor-5β-cholest-25-ene-3α,7α,12α,24-tetrol; 3α,7α,12α-trihydroxy-27-nor-5β-cholestan-24-one; 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol; 27-nor-5β-cholestane-3α,7α,12α,24,25,26-hexol; 5β-cholestane-3α,7α,24-triol; 5β-cholestane-3α,7α,25-triol; 5β-cholestane-3α,7α,26-triol; 5α-cholestane-3α,7α,12α,24-tetrol; 5β-cholestane-3α,7α,12α,24-tetrol; 5β-cholestane-3α,7α,12α,25-tetrol; 5β-cholestane-3α,7α,12α,26-tetrol; (24R)- and (24S)-5β-cholestane-3α,7α,12α,24,25-pentols; 5β-cholestane-3α,7α,12α,24,26-pentol; 5β-cholestane-3α,7α,12α,-25,26-pentol; 5β-cholestane-3α,7α,12α,26,27-pentol; 26-methoxy-5β-cholestane-3α,7α,12α,25-tetrol. There also existed two norcholestanetetrols and three cholestanetetrols with two hydroxyl substituents on the nucleus and two in the side chain. The human biliary bile alcohols occurred mainly as sulfate esters and in lesser amounts as glucuronoconjugated and unconjugated forms. The amount of total bile alcohols was about 0.9 mg (0.7–1.2 mg) in 1 g of bile solid, or 0.16 μmol (0.07–0.24 μmol) in 1 ml of gallbladder bile. —**Kuroki, S., K. Shimazu, M. Kuwabara, M. Une, K. Kihira, T. Kuramoto, and T. Hoshita.** Identification of bile alcohols in human bile. *J. Lipid Res.* 1985. **26:** 230–240.

Supplementary key words gas–liquid chromatography–mass spectrometry • ion-exchange chromatography • bile alcohol sulfate • bile alcohol glucuronide • cholelithiasis

Bile alcohols are not only major biliary constituents of evolutionarily primitive vertebrates such as fishes and amphibians but are also considered to be intermediates in the pathway for the formation of bile acids from cholesterol in mammals (1). Until recently, it had been thought that the distribution of bile alcohols is confined to the primitive vertebrates. In 1974, however, Setoguchi et al. (2) reported the accumulation of bile alcohols in the bile and feces of patients with a rare inherited disease, cerebrotendinous xanthomatosis (CTX). Since then, many investigations have been performed to indicate occurrence of bile alcohols in mammals (3–9). Nowadays, bile alcohols are known to be present in urine (6–9) not only of patients with liver diseases, but also of healthy individuals. However, in bile, the major route of bile acid excretion, little is known about the occurrence of bile alcohols. The aim of the present investigation is to identify bile alcohols in the bile of patients whose liver functions are not severely affected.

MATERIALS AND METHODS

General

Melting points, IR spectra, and PMR spectra were obtained as described previously (9).

Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was prepared according to the procedure described by Goto et al. (10). Bile alcohols were fractionated into three groups, based upon their mode of conjugation by ion-exchange chromatography on PHP-LH-20, which was achieved essentially as described by Goto et al. (10). Unconjugated bile alcohols, glucuronoconjugated bile alcohols, and sulfated bile alcohols were eluted stepwisely with 90% ethanol, 0.2 M formic acid in 90% ethanol, and 1% ammonium carbonate in 70% ethanol, respectively (9).

'The usual work up' refers to dilution with a large amount of water, extraction with organic solvent, washing to neutrality with water, drying over anhydrous Na_2SO_4, filtration, and evaporation of the solvent under a reduced pressure.

Reference bile alcohols and bile acids

5β-Petromyzonol (5β-cholane-3α,7α,12α,24-tetrol) (11), 24-nor-5β-cholestane-3α,7α,23,25-tetrol (12), 24-nor-5β-cholestane-3α,7α,12α,25-tetrol (13), 3α,7α,12α-trihydroxy-

Abbreviations: CTX, cerebrotendinous xanthomatosis; IR, infrared; PMR, proton nuclear magnetic resonance; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; TMS, trimethylsilyl; PHP-LH-20, piperidinohydroxypropyl Sephadex LH-20; RRT, relative retention time; TLC, thin-layer chromatography.

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27-nor-5β-cholestan-24-one (14), 5β-deoxyranol (27-nor-5β-cholestane-3α,7α,12α,24-tetrol) (14), 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol (9), 5β-cholestane-3α,7α,24-triol (15), 5β-cholestane-3α,7α,25-triol (16), 5β-cholestane-3α,-7α,26-triol (15), 5β-cholestane-3α,7α,12α,24-tetrol (17), 5β-cholestane-3α,7α,12α,25-tetrol (18), 5β-cholestane-3α,7α,-12α,26-tetrol (19), and (24R)- and (24S)-5β-cholestane-3α,7α,12α,24,25-pentols (20, 21) were synthesized as described previously. 5α-Cholestane-3α,7α,12α,24-tetrol, 5β-chimaerol (5β-cholestane-3α,7α,12α,24,26-pentol), 5β-bufol (5β-cholestane-3α,7α,12α,25,26-pentol), and 5β-cyprinol (5β-cholestane-3α,7α,12α,26,27-pentol) were isolated from the solvolyzed biles of the rabbit (4); the sting-ray, *Dasyatis akajei* (22); the toad, *Bufo vulgaris formosus* (11); and the frog, *Rana nigromaculata* (23), respectively. 5β-Bufol sulfate (5β-cholestane-3α,7α,12α,25,26-pentol 26-sulfate) was obtained from the bile of the toad, *Bufo vulgaris formosus* (11). Lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid), deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid), and cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) were commercial products (Sigma Chemical Co., St. Louis, MO). Chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid) and ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid) were supplied by Tokyo Tanabe Pharmaceutical Co. (Tokyo, Japan). 7α,12α-Dihydroxy-5β-cholan-24-oic acid was prepared as described previously (24).

Synthesis of 5β-cholane-3α,7α,23,24-tetrol

Phocaecholic acid (3α,7α,23-trihydroxy-5β-cholanoic acid) was isolated from the bile of the seal, *Phoca vitulina*. A solution of 100 mg of methyl phocaecholate in 10 ml of tetrahydrofuran was added dropwise to a suspension of $LiAlH_4$ (200 mg) in 10 ml of tetrahydrofuran and the reaction mixture was stirred for 16 hr at room temperature. After dilution with 0.1 N H_2SO_4, the usual work up (ethyl acetate) gave a residue, which was recrystallized from ethyl acetate–acetone to give crystals of 5β-cholane-3α,7α,23,24-tetrol, mp 224.5–226.5°C. IR, ν_{max}^{KBr} (cm^{-1}): 3350 (OH); PMR (δppm): 0.74 (3H, s, 18-H_3), 0.96 (3H, s, 19-H_3), 1.11 (3H, d, J = 6Hz, 21-H_3), 3.4–4.5 (3H, m, 3β-H and 7β-H and 23-H), 3.90 (2H, d, J = 6Hz, 24-H_2).

Synthesis of 27-nor-5β-cholest-25-ene-3α,7α,12α,24-tetrol

A solution of 3α,7α,12α-triacetoxy-5β-cholan-24-al (9) (2.5 g) dissolved in 30 ml of anhydrous ether was added to an anhydrous ethereal solution containing Grignard reagent prepared from vinyl bromide (10 ml) and magnesium (2 g) and a piece of iodine. The reaction mixture was refluxed for 3 hr. After addition of 0.1 N H_2SO_4, the usual work up (ether) gave an oily residue, which was then hydrolyzed with 100 ml of 5% methanolic KOH at 60°C for 2 hr. After the usual work up (ethyl acetate), the product was chromatographed on a column of 100 g of silica gel 60 (Merck). Elution with ethyl acetate and recrystallization from ethyl acetate gave crystals of 27-nor-5β-cholest-25-ene-3α,7α,12α,24-tetrol, mp 195–197°C. IR, ν_{max}^{KBr} (cm^{-1}): 3450 (OH), 1636 and 883 (CH_2 = CH–); PMR (δppm): 0.83 (s, 3H, 18-H_3), 1.00 (s, 3H, 19-H_3), 1.24 (d, J = 6Hz, 3H, 21-H_3), 3.78 (m, 1H, 3β-H), 4.09 (m, 1H, 7β-H), 4.26 (m, 1H, 12β-H), 4.35 (m, 1H, 24–H), 5.35 and 5.55 (2H, m, 26-H_2), 6.15 (1H, m, 25–H).

Synthesis of 26-methoxy-5β-cholestane-3α,7α,12α,25-tetrol

5β-Bufol sulfate (67 mg) was dissolved in 2.5 N potassium hydroxide in 50% aqueous methanol and heated in a sealed metal container at 160°C for 20 hr. The hydrolyzate was diluted with water and acidified with dilute HCl. The usual work up [n-butanol–ethyl acetate 1:1 (v/v)] gave a residue, which was analyzed by silica gel TLC [developing solvent, ethyl acetate–acetone 7:3 (v/v)] showing a main spot less polar than 5β-bufol and slightly more polar than 5β-cholestane-3α,7α,12α,25-tetrol. The residue was applied on a reversed phase partition column (LiChroprep RP-8, Merck) and eluted with 80% aqueous methanol; the eluate was monitored by silica gel TLC. Fractions containing the major product were combined and evaporated in vacuo to give 26-methoxy-5β-cholestane-3α,7α,12α,25-tetrol as amorphous powder. IR, ν_{max}^{KBr} (cm^{-1}): 3350 (OH); PMR (δppm): 0.84 (s, 3H, 18-H_3), 1.02 (s, 3H, 19-H_3), 1.27 (d, J = 6Hz, 3H, 21-H_3), 1.43 (s, 3H, 27-H_3), 3.36 (s, 3H, -OCH_3), 3.45 (s, 2H, 26-H_2), 3.74 (m, 1H, 3β-H), 4.12 (m, 1H, 7β-H), 4.28 (m, 1H, 12β-H).

Synthesis of 27-nor-5β-cholestane-3α,7α,12α,24,25,26-hexol

A solution of 27-nor-5β-cholest-25-ene-3α,7α,12α,24-tetrol (50 mg) dissolved in 10 ml of anhydrous pyridine and 10 ml of acetic anhydride was heated on a water bath for 3 hr. The usual work up (ether) gave an oily residue, which was dissolved in 10 ml of anhydrous ether and 0.1 ml of anhydrous pyridine and treated with 40 mg of osmium tetraoxide in a closed flask at room temperature for 18 hr. The solvent was evaporated under reduced pressure and the residue was refluxed with 20 ml of 50% aqueous ethanol containing 1 g of sodium sulfite for 3 hr. The reaction mixture was diluted with 200 ml of ethanol and filtered. The filtrate was evaporated under reduced pressure and the residue was hydrolyzed with 5% methanolic KOH (20 ml) for 2 hr. After the usual work up [n-butanol–ethyl acetate 1:1 (v/v)], recrystallization from methanol–ethyl acetate gave crystals of 27-nor-5β-cholestane-3α,7α,12α,24,25,26-hexol, mp 174–175°C. IR, ν_{max}^{KBr} (cm^{-1}): 3350 (OH); PMR (δppm): 0.80 (s, 3H, 18-H_3),

0.99 (s, 3H, 19-H_3), 1.32 (d, J = 6Hz, 3H, 21-H_3), 3.50–4.40 (m, 7H, 3β-H, 7β-H, 12β-H, 24-H, 25-H, and 26-H_2).

Patients

Six patients were included in the studies, four females (one with carcinoma of the pancreas head and three with cholelithiasis) and two males (one with carcinoma of the choleduchus and another with cholelithiasis). Diagnoses were based on clinical findings, laparotomy, and pathological examinations of resected specimens. The gallbladder bile was obtained by needle aspiration from the gallbladder during the laparotomy and stored at −20°C to avoid the bacterial transformation of the bile contents until analysis.

Analysis of bile alcohols in bile

Extraction of bile alcohols from the gallbladder bile. Each bile sample (about 10 ml) was extracted with 10 volumes of ethanol at room temperature. The ethanolic extract was concentrated to dryness under reduced pressure. The resulting bile solid (about 1 g) was dissolved in 10 ml of 90% ethanol and applied to PHP-LH-20 column (15 cm × 3 cm i.d.) and eluted successively with 300 ml of 90% ethanol, 800 ml of 0.2 M formic acid in 90% ethanol, and 500 ml of 1% ammonium carbonate in 70% ethanol, to give unconjugated bile alcohols along with cholesterol, glucuronoconjugated bile alcohols along with glycine conjugated bile acids and glucuronoconjugated bile acids, and sulfated bile alcohols along with taurine conjugated bile acids and sulfated bile acids, respectively.

β-Glucuronidase treatment. The glucuronoconjugated bile alcohol fraction eluted from the PHP-LH-20 column was evaporated in vacuo. The residue was dissolved in 40 ml of 0.1 M phosphate buffer, pH 6.8, containing 10,000 units of β-glucuronidase (EC 3.2.1.31) (Sigma Chemical Co., Type IX) and two drops of $CHCl_3$, and the mixture was incubated at 37°C for 48 hr (25). The incubation mixture was lyophilized and extracted with 100 ml of methanol. The methanolic extract was evaporated to dryness and the residue was dissolved in 5 ml of 90% ethanol and the solution was applied on a column of PHP-LH-20 (10 cm × 1.6 cm i.d.). The column was eluted with 100 ml of 90% ethanol to give deconjugated bile alcohols.

Solvolysis (26). The sulfated bile alcohol fraction eluted from the PHP-LH-20 column was evaporated to dryness. The residue was dissolved in 10 ml of water and the solution was adjusted to pH 1 by adding dilute HCl; then 8 g of NaCl and 60 ml of ethyl acetate were added. The mixture was incubated at 37°C for 48 hr. After dilution with 100 ml of 5% KOH solution, the incubation mixture was extracted with n-butanol–ethyl acetate 1:1 (v/v). The organic layer was washed with water and evaporated to dryness. The residue was then hydrolyzed with 5% methanolic KOH at 60°C for 30 min. After the usual work up [n-butanol–ethyl acetate 1:1 (v/v)], the residue was dissolved in 2 ml of 90% ethanol and applied on a column of PHP-LH-20 (5 cm × 1 cm i.d.). Elution with 20 ml of 90% ethanol gave solvolyzed bile alcohols.

Silica gel chromatography. The unconjugated, deglucuronidated, and solvolyzed bile alcohol fractions were evaporated and each residue was transferred onto a column of silica gel 60 (10 g, Merck). The column was successively eluted with each: 20 ml of benzene, benzene–ethyl acetate 1:1 (v/v), ethyl acetate, ethyl acetate–acetone 1:1 (v/v), acetone, acetone–ethanol 1:1 (v/v), and ethanol. Each fraction was monitored by silica gel TLC (Merck) using the solvent system of ethyl acetate–acetone 3:2 (v/v). Fractions containing bile alcohols (the last three fractions, i.e., acetone, acetone–ethanol, and ethanol) were collected and analyzed for bile alcohols.

Gas–liquid chromatography and mass spectrometry. Each bile alcohol fraction eluted from the silica gel column was evaporated under reduced pressure. To the residue, 5α-cholestane (10.0 μg) was added as an internal standard for both quantitative and relative retention time measurements. The residue was treated with pyridine–hexamethyldisilazene–trimethylchlorosilane 5:2:1 (v/v/v) for 2 hr at room temperature (9). The resulting TMS ether derivatives were analyzed by GLC and GLC–MS.

Gas–liquid chromatography (GLC) was run on a Shimadzu model GC-6A gas chromatograph equipped with a flame ionization detector and Van den Berg's solventless injector. The columns used were a WCOT glass capillary column (25 m × 0.35 mm i.d.) coated with SE-30 (LKB-Produkter, Stockholm, Sweden) and a glass column (2 m × 3 mm i.d.) packed with 3% OV-17 on 80–100 mesh Gas Chrom Q. All the retention times listed in the text are given relative to 5α-cholestane (RRT = 1.00). Measurements of peak areas were accomplished with a Shimadzu E1A automatic integrator.

Gas–liquid chromatography–mass spectrometry (GLC–MS) was carried out on a Shimadzu model GCMS-9000 gas chromatograph–mass spectrometer equipped with a data processing system (Shimadzu GCMSPAC-90) and Van den Berg's solventless injector. The following operating conditions were employed: column, SE-30 (25 m × 0.35 mm i.d.); injection port temperature, 285°C; column oven temperature, 270°C; separator temperature, 270°C; ionization source temperature, 290°C; flow rate of helium carrier gas, 1.5 ml/min; ionization energy, 22.5 eV; acceleration voltage, 3.5 kV; trap current, 100 μA.

Analysis of biliary bile acid

To each bile sample (0.1 ml) were added 100 μg of 7α,12α-dihydroxy-5β-cholan-24-oic acid as an internal standard and 2 ml of ethanol. The mixture was filtered and

evaporated to dryness. The residue was dissolved in 2 ml of 2 N KOH solution and hydrolyzed at 130°C for 3 hr. After acidification with dilute HCl and the usual work up (ethyl acetate), the residue was converted to methyl ester-dimethylethylsilyl ether derivative and analyzed by GLC (3% QF-1) as described previously (27).

RESULTS

Gallbladder bile samples obtained from the six patients with cholelithiasis or malignant diseases were examined for bile alcohols. The bile solid extracted with ethanol from the gallbladder bile was submitted to ion-exchange chromatography on PHP-LH-20 to give unconjugated, glucuronoconjugated, and sulfated bile alcohol fractions. β-Glucuronidase treatment of the glucuronoconjugated bile alcohol fraction and solvolysis of the sulfated bile alcohol fraction gave deconjugated and solvolyzed bile alcohols, respectively. These fractions, as well as unconjugated bile alcohol fraction, were then preliminarily cleaned up by silica gel column chromatography, because TLC analysis of these fractions revealed a complex mixture of neutral compounds. Fractions containing bile alcohols were derivatized and analyzed by GLC and combined GLC-MS. A representative gas chromatogram is shown in **Fig. 1**. GLC and combined GLC-MS analyses revealed that these fractions contained at least 27 bile alcohols, which were tentatively named 1-27 in order of increasing retention times (**Table 1**).

The following bile alcohols were identified by direct comparison of the RRTs on both SE-30 and OV-17 columns and mass spectra with those of the authentic compounds: bile alcohol 1, 5β-cholane-3α,7α,12α,24-tetrol; bile alcohol 3, 24-nor-5β-cholestane-3α,7α,12α,25-tetrol; bile alcohol 4, 3α,7α,12α-trihydroxy-27-nor-5β-cholestan-24-one; bile alcohol 5, 5α-cholestane-3α,7α,12α,24-tetrol; bile alcohol 6, 5β-cholestane-3α,7α,12α,24-tetrol; bile alcohol 7, 5β-cholestane-3α,7α,24-triol; bile alcohol 9, 5β-cholestane-3α,7α,25-triol; bile alcohol 10, 5β-cholestane-3α,7α,12α,-25-tetrol; bile alcohol 11, 5β-cholestane-3α,7α,26-triol; bile alcohol 12, 5β-cholestane-3α,7α,12α,26-tetrol; bile alcohol 16, 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol; bile alcohols 19 and 20, (24R)- and (24S)-5β-cholestane-3α,7α,-12α,24,25-pentols; bile alcohol 21, 5β-chimaerol (5β-cholestane-3α,7α,12α,24,26-pentol); bile alcohol 24, 5β-bufol (5β-

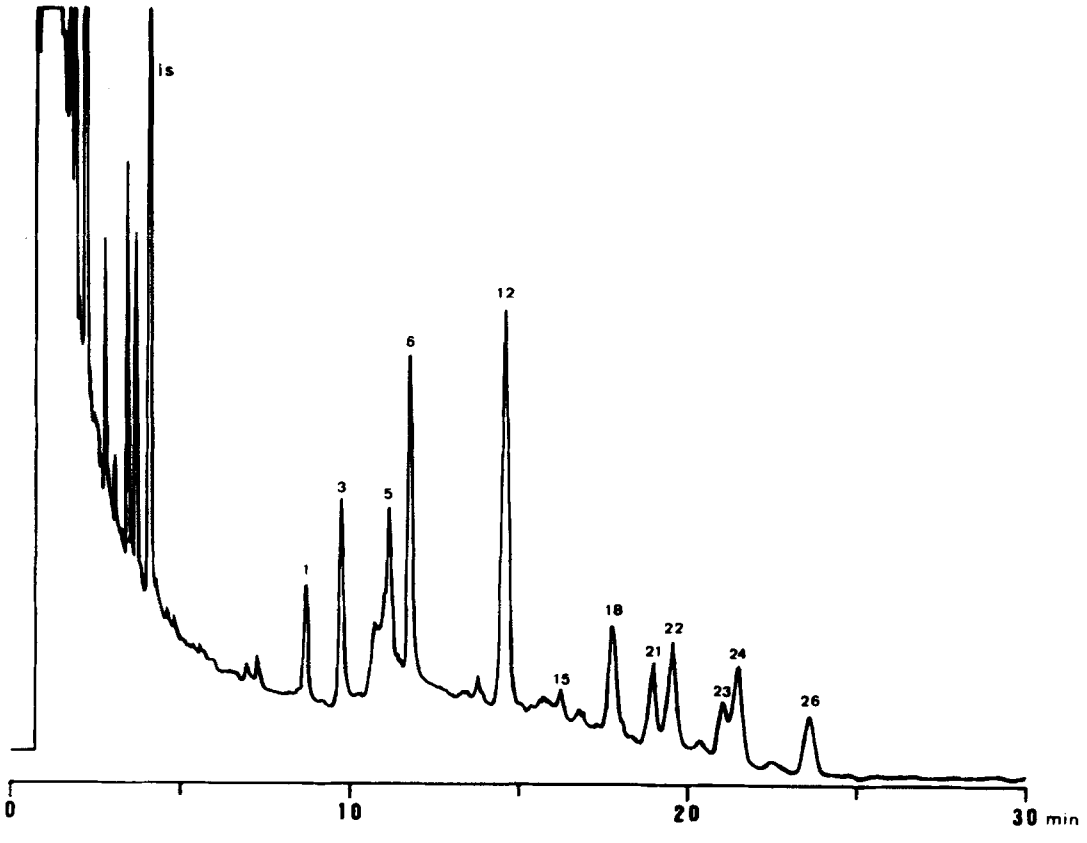

Fig. 1 Gas chromatogram of TMS ether derivatives of bile alcohols from the sulfate fraction of bile obtained from a patient. Column, capillary SE-30 (25 m × 0.35 mm i.d.); column temperature, 270°C; is, internal standard (5α-cholestane). Each number of the peak corresponds to that of a bile alcohol shown in Table 1.

TABLE 1. Relative retention times of bile alcohols detected in human gallbladder bile

No.	Compounds	RRT
	5α-Cholestane (internal standard)	1.00
1	5β-Cholane-3α,7α,12α,24-tetrol (5β-petromyzonol)	2.13
2	27-Nor-5β-cholest-25-ene-3α,7α,12α,24-tetrol	2.37
3	24-Nor-5β-cholestane-3α,7α,12α,25-tetrol	2.39
4	3α,7α,12α-Trihydroxy-27-nor-5β-cholestan-24-one	2.51
5	5α-Cholestane-3α,7α,12α,24-tetrol	2.73
6	5β-Cholestane-3α,7α,12α,24-tetrol	2.88
7	5β-Cholestane-3α,7α,24-triol	2.93
8	5β-Cholane-3α,7α,23,24-tetrol	3.03
9	5β-Cholestane-3α,7α,25-triol	3.15
10	5β-Cholestane-3α,7α,12α,25-tetrol	3.22
11	5β-Cholestane-3α,7α,26-triol	3.56
12	5β-Cholestane-3α,7α,12α,26-tetrol	3.57
13	27-Nor-5β-cholestane-3α,7α,12α,24,25-pentol[a]	3.78
14	Norcholestanetetrol[b]	3.93
15	27-Nor-5β-cholestane-3α,7α,12α,24,25-pentol[a]	3.94
16	27-Nor-5β-cholestane-3α,7α,12α,24,25-pentol	4.00
17	Norcholestanetetrol[b]	4.04
18	26-Methoxy-5β-cholestane-3α,7α,12α,25-tetrol	4.36
19	(24R)-5β-Cholestane-3α,7α,12α,24,25-pentol	4.49
20	(24S)-5β-Cholestane-3α,7α,12α,24,25-pentol	4.67
21	5β-Cholestane-3α,7α,12α,24,26-pentol (5β-chimaerol)	4.68
22	Cholestanetetrol[b]	4.82
23	Cholestanetetrol[b]	5.18
24	5β-Cholestane-3α,7α,12α,25,26-pentol (5β-bufol)	5.29
25	27-Nor-5β-cholestane-3α,7α,12α,24,25,26-hexol	5.81
26	5β-Cholestane-3α,7α,12α,26,27-pentol (5β-cyprinol)	5.82
27	Cholestanetetrol[b]	5.85

RRT, relative retention time relative to 5α-cholestane (capillary column coated with SE-30).

[a]Diastereoisomers of bile alcohol 16.

[b]Positions of hydroxyl substituents and stereochemistry are unknown.

cholestane-3α,7α,12α,25,26-pentol); bile alcohol 26, 5β-cyprinol (5β-cholestane-3α,7α,12α,26,27-pentol).

The mass spectrum of the TMS ether of bile alcohol 2 (**Fig. 2A**) was very similar to that of TMS ether of 27-nor-5β-cholestane-3α,7α,12α,24-tetrol (5β-deoxyranol), except for a downfield shift of two mass units for the high field fragments containing the entire side chain (m/z 708, 618, 528, 438, and 349) and the side chain fragment with m/z 129. The ion at m/z 708 was the molecular ion for a C_{26} tetrol with a double bond. The series of the fragment ions at m/z 618, 528, 438, and 349 is formed from the successive loss of one to four TMS groups (90 or 89 mass units) from the molecular ion. The ion at m/z 129 corresponds to the ion at m/z 131 in the spectrum of 5β-deoxyranol TMS ether. These side chain fragments composed of carbons 24 to 26 result from the cleavage of the bond between carbons 23 and 24. These results suggested that bile alcohol 2 is a derivative of 5β-deoxyranol with unsaturation at the end of the side chain. The structural assignment was confirmed by direct comparison with the synthetic sample of 27-nor-5β-cholest-25-ene-3α,7α,12α,-24-tetrol. The RRTs on GLC and mass spectrum of the synthetic bile alcohol were completely identical with those of the natural bile alcohol 2.

The mass spectrum of the TMS ether of bile alcohol 8 (Fig. 2B) resembled that of 24-nor-5β-cholestane-3α,7α,-23,25-tetrol in the presence of a series of fragment ions at m/z 579, 489, 399, and 309 and also of ions at m/z 283 and 255. The series results from the scission of the bond between carbons 23 and 24 (or 25) and successive loss of one to three TMS-OH groups. The ions at m/z 283 (cleavage between C-20 and C-22 plus two TMS-OH groups) and 255 (cleavage between C-17 and C-20 plus two TMS-OH groups) are characteristic of a bile alcohol with two hydroxyl substituents on the nucleus. A striking difference between the two spectra was seen in the presence or absence of a side chain fragment. In the spectrum of the norcholestanetetrol, the base peak at m/z 131 arises from the scission of the bond between C-23 and C-25. However, such a fragment was not seen in the spectrum of bile alcohol 8, indicating that this compound has no tertiary carbon atom with a hydroxyl substituent in the side chain. These results suggested that bile alcohol 8 is a homolog of the norcholestanetetrol having a shortened side chain. Thus, 5β-cholane-3α,7α,23,24-tetrol was prepared from methyl phocaecholate by reduction with lithium aluminum hydride. The RRT on GLC and the mass spectrum of the synthetic bile alcohol were completely identical with those of the natural bile alcohol 8.

The mass spectra of the TMS ethers of bile alcohols 13 and 15 were the same as those of the corresponding derivative of bile alcohol 16 or synthetic 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol, although their RRTs were different from one another. 27-Nor-5β-cholestane-3α,7α,12α,24,25-pentol has two asymmetric carbons at positions 24 and 25; thus there are four diastereoisomers on variation at these positions. The synthetic C_{26} pentol (bile alcohol 16) should be one of the four isomers, and bile alcohols 13 and 15 might be diastereoisomers of bile alcohol 16.

The mass spectrum of the TMS ether of bile alcohol 18 (Fig. 2C) resembled that of the TMS ether of 5β-bufol in the presence of a series of fragment ions at m/z 709, 619, 529, 439, and 349 and of ions at 253 and 343. The series results from the scission of the bond between C-25 and C-26 and successive loss of one to four TMS-OH groups. Although the molecular ion was not seen in the spectrum of bile alcohol 18 TMS ether, there were two other series of fragment ions: one at m/z 739, 649, 559, 469, and 379, and a second at m/z 664, 574, 484, and 394. The former series results from the loss of a methyl group and the consecutive loss of one to four TMS-OH groups from the molecular ion, and the latter series is formed by the successive loss of one to four TMS-OH groups from the molecular ion. The base peak was seen at m/z 161. This ion may be $[CH_3C^+(OTMS)CH_2OCH_3]$, which corresponds to the side chain fragment ion at m/z 219, $[CH_3C^+(OTMS)CH_2OTMS]$, in the spectrum of 5β-bufol TMS ether. These ions composed of carbons 25, 26,

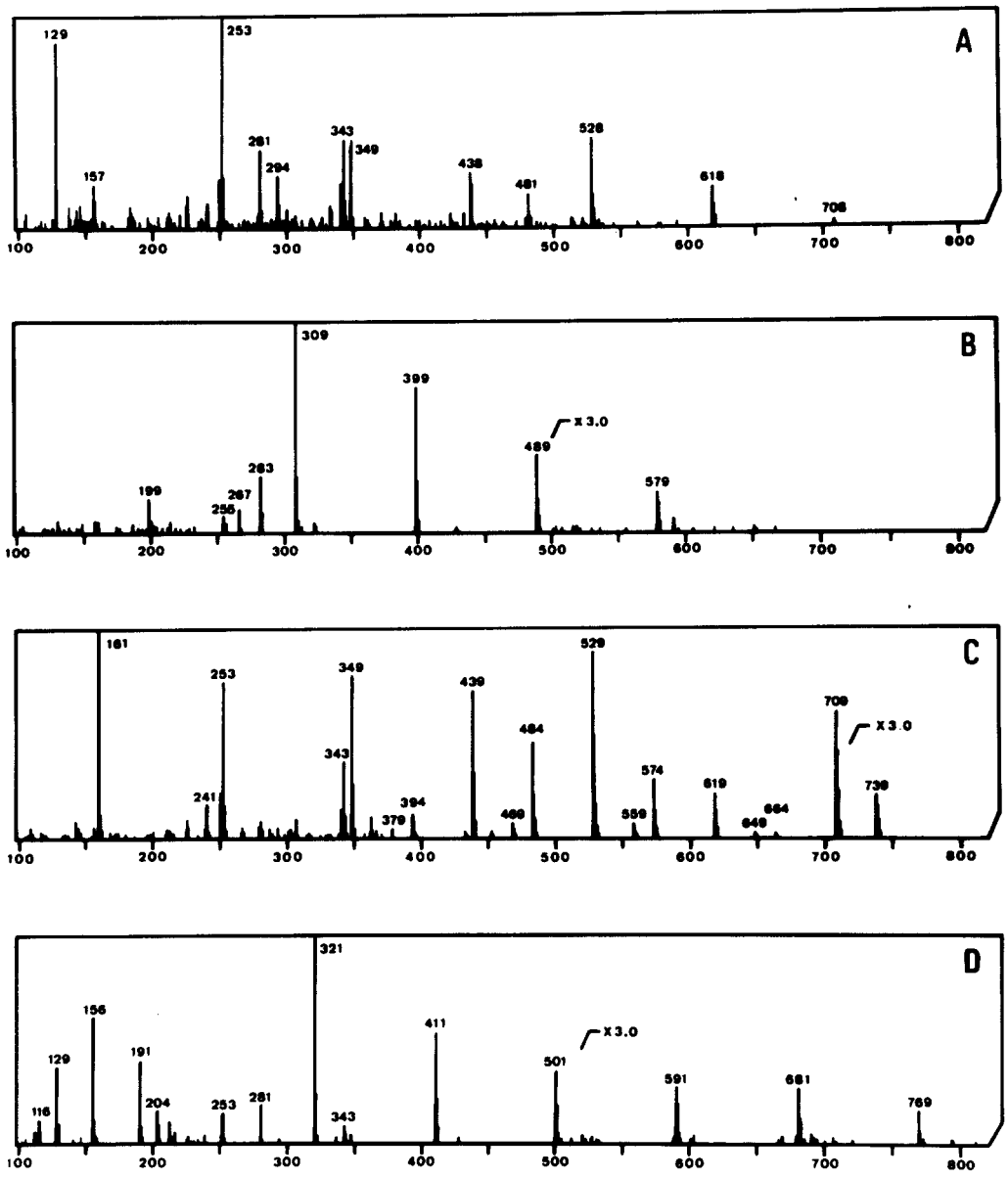

Fig. 2 Mass spectra of TMS ethers of four natural bile alcohols found in human bile. Mass spectra A (bile alcohol 2), B (bile alcohol 8), C (bile alcohol 18), and D (bile alcohol 25) were identical with those of TMS ethers of 27-nor-5β-cholest-25-ene-3α,7α,12α,24-tetrol, 5β-cholane-3α,7α,23,24-tetrol, 26-methoxy-5β-cholestane-3α,7α,-12α,25-tetrol, and 27-nor-5β-cholestane-3α,7α,12α,24,25,26-hexol, respectively. For detailed description, see the Results section.

and 27 are formed from the cleavage of the bond between C-24 and C-25. These data suggested that bile alcohol 18 is the 26-methoxy derivative of 5β-bufol. To confirm this structural assignment, 26-methoxy-5β-cholestane-3α,7α,-12α,25-tetrol was prepared from the natural 5β-bufol 26-sulfate by alkaline treatment in methanol. The RRT on GLC and the mass spectrum of the synthetic 26-methoxy-5β-bufol were entirely identical with those of the natural bile alcohol 18.

The mass spectrum of the TMS ether of bile alcohol 25 (Fig. 2D) closely resembled that of 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol TMS ether. Both spectra showed a series of fragment ions at m/z 681, 591, 501, 411, and 321, resulting from the scission of the bond between C-24 and C-25 and consecutive loss of one to four TMS-OH groups. The RRT (5.81 on SE-30 column) of the TMS ether of bile alcohol 25 was longer than those (3.78–4.00) of the TMS ethers of four epimeric 27-nor-5β-cholestane-3α,7α,-12α,24,25-pentols, suggesting that bile alcohol 25 is a hexahydroxy bile alcohol. In order to confirm the structure of bile alcohol 25, 27-nor-5β-cholestane-3α,7α,12α,-24,25,26-hexol was prepared from 27-nor-5β-cholest-25-

TABLE 2. Mass spectral data of TMS ethers of partially identified bile alcohols found in human bile

No.	Postulated Structure	Important Fragment Ions
		m/z
14	Norcholestanetetrol	117, 129, 156, 255, *323*, 413, 503, 593
17	Norcholestanetetrol	117, *159*, 213, 255, 413, 503, 710
22	Cholestanetetrol	103, 131, 159, 213, 233, 255, 296, *413*, 503, 544
23	Cholestanetetrol	219, 255, 351, *441*, 531, 621, 709
27	Cholestanetetrol	147, 213, 228, 255, 364, 454, 529, *544*, 634, 724

Italicized figure means the base peak. Number of each bile alcohol is the same as shown in Table 1.

ene-3α,7α,12α,24-tetrol by osmium tetraoxide oxidation. The RRT of GLC and mass spectrum of the synthetic bile alcohol TMS ether were identical with those of the natural bile alcohol 25.

The mass spectral data of the TMS ethers of bile alcohols whose structures were partially identified are shown in **Table 2**. It was suggested that bile alcohols 14 and 17 are norcholestanetetrols with two hydroxyl substituents on the nucleus and two in the side chain, and that bile alcohols 22, 23, and 27 are cholestanetetrols with two hydroxyl substituents on the nucleus and two in the side chain. Their structures are now being studied.

Results of quantitative analysis of biliary bile alcohol are shown in **Table 3**. The mean excretion of total bile alcohol was 2.06 μmol/g of bile solid (range 1.59–2.74) or 0.159 μmol/ml of gallbladder bile (range 0.071–0.240). The bile alcohols were excreted into bile mainly as sulfated forms and in lesser amounts as glucuronoconjugated and unconjugated forms.

The identification and quantitation of the bile acids in the gallbladder bile from patients with cholelithiasis or malignant diseases were carried out by GLC. Major biliary bile acids were cholic acid and chenodeoxycholic acid. Deoxycholic acid was found in five of six cases. Two patients with cholelithiasis had received the oral administration of ursodeoxycholic acid (300 mg/day) for about a

TABLE 3. Bile alcohol excretion in human gallbladder bile

Bile Alcohol	Unconjugated	Glucuronoconjugated	Sulfated	Total
1	15.1 (7.6-27.2:6)	2.0 (0-11.2:2)	68.5 (0-355:5)	85.6 (10.2-373:6)
2	2.7 (0-8.7:2)	0.6 (0-3.5:1)	nd	3.2 (0-10.8:2)
3	4.8 (0-17.7:3)	1.1 (0-3.8:2)	9.6 (0-57.6:1)	15.4 (0-63.2:5)
4	12.5 (0-22.7:4)	5.6 (0-13.7:4)	nd	18.0 (3.1-29.9:6)
5	nd	nd	99.0 (0-169:5)	99.0 (0-169:5)
6	0.5 (0-2.2:2)	0.5 (0-3.1:1)	212 (89.8-278:6)	213 (92.9-453:6)
7	nd	7.1 (0-25.8:3)	nd	7.1 (0-25.8:3)
8	0.8 (0-4.7:1)	1.7 (0-5.1:2)	nd	2.5 (0-9.8:2)
9	nd	33.1 (0-105:4)	nd	33.1 (0-105:4)
10	nd	17.2 (0-34.8:5)	nd	17.2 (0-34.8:5)
11	nd	11.6 (0-33.9:4)	nd	11.6 (0-33.9:4)
12	nd	18.5 (2.8-41.5:6)	562 (179-1049:6)	580 (186-1055:6)
13	5.6 (0-16.1:3)	11.8 (0-26.5:3)	0.9 (0-5.2:1)	18.3 (0-42.6:4)
14	nd	3.8 (0-22.7:1)	nd	3.8 (0-22.7:1)
15	4.3 (0-26.0:1)	nd	1.4 (0-8.6:1)	5.8 (0-26.0:2)
16	39.7 (0-118:5)	59.7 (0-171:3)	1.4 (0-8.4:1)	101 (0-210:5)
17	nd	10.3 (0-51.0:2)	nd	10.3 (0-51.0:2)
18	nd	nd	50.5 (0-136:3)	50.5 (0-136:3)
19	nd	0.9 (0-5.4:1)	nd	0.9 (0-5.4:1)
20	nd	11.3 (0-25.5:4)	nd	11.3 (0-25.5:4)
21	18.2 (5.3-37.2:6)	4.6 (0-24.6:2)	84.1 (43.8-168:6)	107 (57.8-149:6)
22	15.9 (0-77.9:4)	118 (0-287:5)	97.6 (64.3-142:6)	232 (97.5-506:6)
23	6.8 (0-38.1:2)	85.3 (0-215:5)	42.9 (0-79.4:5)	135 (21.1-333:6)
24	18.7 (9.7-35.3:6)	27.4 (2.1-51.5:6)	105 (36.9-279:6)	152 (70.4-301:6)
25	25.7 (9.6-79.6:6)	nd	nd	25.7 (9.6-79.6:6)
26	0.2 (0-0.8:2)	0.4 (0-2.6:1)	60.8 (28.8-117:6)	61.5 (29.6-120:6)
27	nd	35.3 (4.1-77.3:6)	23.5 (5.2-31.6:6)	58.8 (25.7-105:6)
Total	171 (71.7-388)	468 (46.9-913)	1420 (865-2290)	2060 (1590-2740)

The values are represented as mean of the six patients (nmol of bile alcohol excreted per g of bile solid); nd: not detected. Figures in parentheses indicate range and incidence of each bile alcohol in each fraction of the six patients. Systematic and trivial names of bile alcohols 1-27 are shown in Table 1.

month before the operations and ursodeoxycholic acid occupied about 36% of total bile acid in these patients. The mean amount of total bile acid was 590 mg/g of bile solid (range 320–790). The amount and the composition of the bile acid in the gallbladder bile of the patients were almost normal. The mean ratio of total bile alcohol to total bile acid was 1/625 (range 1/833–1/455).

DISCUSSION

The present study has shown that bile alcohols were excreted in the bile of six patients with cholelithiasis or malignant diseases, although the concentration was much less than that of bile alcohols accumulated in the bile of patients with cerebrotendinous xanthomatosis (CTX) (2, 28, 29). The biliary bile alcohols in each sample from our patients resembled one another with respect to both total amount and composition. Furthermore, analysis of bile acids in these samples indicated that bile acid production in these patients, in contrast to the CTX patients (2), is normal. These results strongly suggested that the bile alcohols found in trace amounts in the bile of our patients, like bile alcohols excreted in urine from healthy man (8, 9), are normal components but not abnormal metabolites in pathological conditions.

As described in the preceding sections, the biliary bile alcohols were separated into unconjugated, glucuronoconjugated, and sulfated bile alcohol fractions by means of ion-exchange chromatography. Since intact conjugated bile alcohols have not been isolated in this study, the types of conjugation must be regarded as tentative. However, analysis of bile alcohols in each fraction has revealed that bile alcohol profiles in three fractions were quite different from one another. This clearly indicated the occurrence of at least three different groups of bile alcohols in conjugation and also selective mechanisms for the conjugations and biliary elimination of bile alcohols.

In these patients approximately 69% of total biliary bile alcohols was found in the sulfate fraction, and almost all of the major bile alcohols found in this fraction have previously been found in the bile of lower vertebrates such as fishes and amphibians (1), in which these bile alcohols also occur as the conjugates with sulfuric acid at a hydroxyl group in the side chain. It seems likely, therefore, that sulfation produces a more excretable form of bile alcohols into the bile, and it may be speculated that the site of the sulfation of bile alcohols in humans is the same as that in the lower vertebrates. In three samples from six patients, a C_{28} bile alcohol was detected only in the desulfated bile alcohol mixture, which was obtained from the sulfate fraction by solvolysis followed by treatment with alkali in methanol. The bile alcohol was identified as 26-methoxy-5β-cholestane-3α,7α,12α,25-tetrol, the 26-methoxy derivative of 5β-bufol. In this connection, it should be noted that it has been reported (30) that alkaline treatment in ethanol of the toad bile whose principal bile salt is 5β-bufol 26-sulfate afforded the 26-ethoxy derivative of 5β-bufol as a major product. If the presently identified C_{28} bile alchol is an artifact formed from 5β-bufol 26-sulfate during the procedures for desulfation, it should be evidence that the sulfate group is located at the end of the side chain, C-26.

Approximately 23% of total biliary bile alcohols was found in the glucuronoconjugated bile alcohol fraction. Three cholestanetriols carrying the chenodeoxycholic acid type nucleus were found only in this fraction. The structures of the major constituents, bile alcohols 22, 23, and 27, of this fraction, could not be elucidated conclusively, but the mass spectral data suggested that these bile alcohols are cholestanetetrols carrying two hydroxyl groups on the nucleus and two in the side chain. Thus, more than 65% of total glucuronoconjugated bile alcohols possessed dihydroxylated nuclear structures. This may be a reflection of the selective glucuronidation of bile alcohols carrying two nuclear hydroxyl groups.

The unconjugated bile alcohol fraction contained 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol and 27-nor-5β-cholestane-3α,7α,12α,24,25,26-hexol as its major bile alcohols. The former was also found in the glucuronoconjugated bile alcohol fraction. The excretion of the C_{26} pentol as both unconjugated and glucuronoconjugated forms in human urine has recently been reported (9). The latter C_{26} hexol was found only in the unconjugated bile alcohol fraction. This highly hydroxylated bile alcohol might be able to be excreted into the bile without any conjugation.

Disregarding the nature of conjugation, the biliary bile alcohols may be divided into several groups according to type of their biosynthesis. The possible pathways for the biosynthesis of some bile alcohols in man are shown in **Fig. 3**.

One group may be classified as 5β-cholestane-3α,7α,-12α,26-tetrol (III) and its hydroxylation products. The most abundant bile alcohol found in the present patients was 5β-cholestane-3α,7α,12α,26-tetrol (III), which has been known to be an intermediate in the pathway for the biosynthesis of cholic acid (VI) in man (31). Since the cholestanetetrol (III) in human bile occurred mainly as the sulfate ester, it is conceivable that in human liver a greater amount of the cholestanetetrol (III) is converted into 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (IV) by an oxidation of 26-hydroxyl group and then into cholic acid (VI) by a β-oxidation (31), while a lesser fraction of the cholestanetetrol (III) is sulfated presumably at 26-hydroxyl group, and hence the resulting sulfated cholestanetetrol could not be oxidized to the C_{27} bile acid (IV) and then to cholic acid (VI), but would be excreted into the bile because of its increased solubility in water.

The present study has shown the presence in human bile of 5β-chimaerol (VII), 5β-bufol (VIII), and 5β-cyprinol

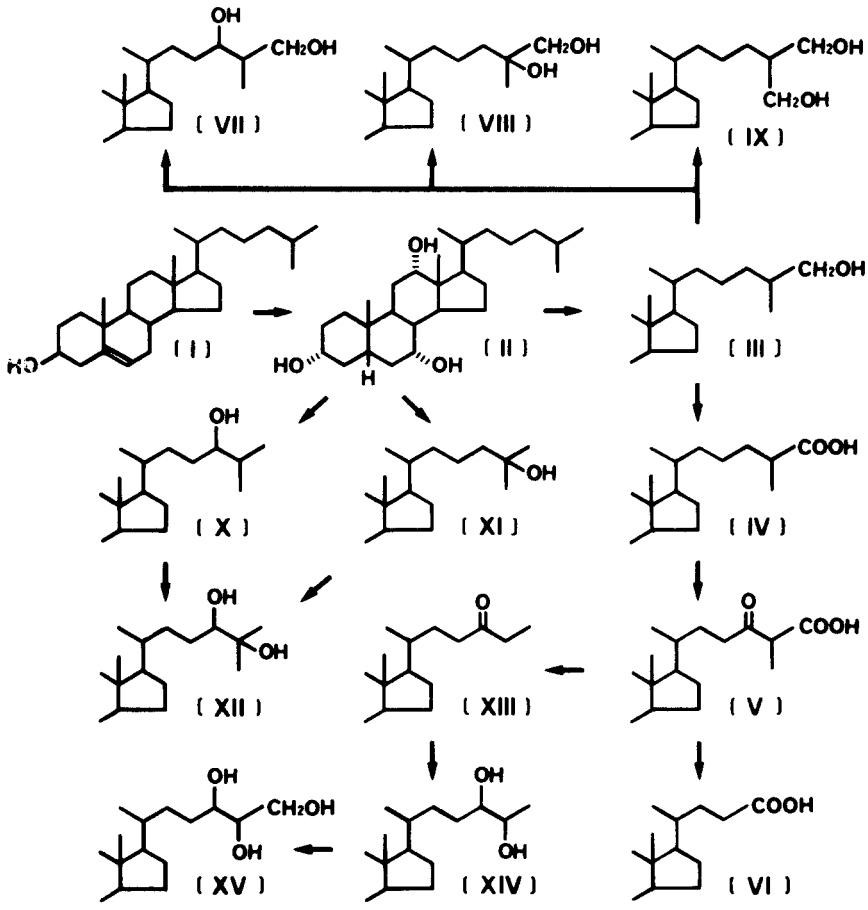

Fig. 3 Possible pathway for the formation of some bile alcohols found in human bile. I, cholesterol; II, 5β-cholestane-3α,7α,12α-triol; III, 5β-cholestane-3α,7α,12α,26-tetrol; IV, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; V, 3α,7α,12α-trihydroxy-24-oxo-5β-cholestan-26-oic acid; VI, cholic acid; VII, 5β-cholestane-3α,7α,12α,-24,26-pentol (5β-chimaerol); VIII, 5β-cholestane-3α,7α,12α,25,26-pentol (5β-bufol); IX, 5β-cholestane-3α,7α,12α,-26,27-pentol (5β-cyprinol); X, 5β-cholestane-3α,7α,12α,24-tetrol; XI, 5β-cholestane-3α,7α,12α,25-tetrol; XII, 5β-cholestane-3α,7α,12α,24,25-pentol; XIII, 3α,7α,12α-trihydroxy-27-nor-5β-cholestan-24-one; XIV, 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol; XV, 27-nor-5β-cholestane-3α,7α,12α,24,25,26-hexol.

(IX), which have previously been found in the bile of some lower vertebrates (1). In such animals, these cholestanepentols, (VII), (VIII), and (IX), are thought to be formed directly from 5β-cholestane-3α,7α,12α,26-tetrol (III) by 24-, 25-, and 27-hydroxylations, respectively (1). The present finding suggested that human liver has the capacity to synthesize such bile alcohols, though in much lower quantities, presumably by the same pathways as those in the primitive animals. Since these cholestanepentols, (VII), (VIII), and (IX), as well as the cholestanetetrol (III) in human bile occurred mainly as the sulfate esters, it is possible that the substrate for the 24-, 25-, and 27-hydroxylases in human liver is the sulfated cholestanetetrol rather than the cholestanetetrol itself.

Three of the presently identified bile alcohols, 5β-cholestane-3α,7α,12α,24-tetrol (X), 5β-cholestane-3α,7α,12α,-25-tetrol (XI), and 5β-cholestane-3α,7α,12α,24,25-pentol (XII), have previously been found in large amounts in the bile and feces of patients with CTX (2, 28, 32). The accumulation of these bile alcohols in CTX was ascribed to a relative deficiency of 26-hydroxylase (29, 33, 34), which catalyzes the formation of 5β-cholestane-3α,7α,-12α,26-tetrol (III) from 5β-cholestane-3α,7α,12α-triol (II). Thus, the cholestanetriol (II) would tend to accumulate in the liver cells of the CTX patients (29), exposing it to the action of 24- and 25-hydroxylases instead of 26-hydroxylase to result in the formation and accumulation of the bile alcohols hydroxylated at C-24 and/or C-25 but not at C-26. The present findings of the CTX bile alcohols in the bile of our patients indicated that 24- and 25-hydroxylations of 5β-cholestane-3α,7α,12α-triol (II) occur, although probably to a lesser extent than 26-hydroxylation, even when the normal pathway to yield cholic acid involving 26-oxygenated intermediates has not been blocked.

In the present study, a number of C_{26} bile alcohols were found. The most abundant biliary C_{26} bile alcohol was 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol (XIV), which has previously been found as the major bile alcohol in

human urine (6–9). Karlaganis et al. (35) have recently shown that cholesterol (I), but not sitosterol, is the biosynthetic precursor of the major urinary bile alcohol (XIV) in patients with primary biliary cirrhosis. Thus, the biosynthetic pathway of the C_{26} bile alcohol (XIV) from the C_{27} sterol (I) must include a reaction involving loss of only one carbon atom from the end of the cholesterol side chain. Recently, Kuwabara et al. (9) have proposed a possible pathway that involves a decarboxylation of 3α,7α,12α-trihydroxy-24-oxo-5β-cholestan-26-oic acid (V), which has been known as the direct biosynthetic precursor of cholic acid (VI) in man (1), to form 3α,7α,12α-trihydroxy-27-nor-5β-cholestan-24-one (XIII). This proposal might receive further support from the present finding of the presence of the postulated intermediate (XIII) in all samples from our patients. Recently Karlaganis et al. referred to the presence of 27-nor-5β-cholestane-3α,7α,12α,24,25,26-hexol (XV) in human urine (in reference 35, unpublished data). The present study has confirmed the occurrence of this bile alcohol in human bile by direct comparison with the chemically synthesized sample. The C_{26} hexol (XV) might be formed from 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol (XIV) by a simple hydroxylation at C-26.

The present results showed the occurrence of three cholestanetriols, 5β-cholestane-3α,7α,24-triol, 5β-cholestane-3α,7α,25-triol, and 5β-cholestane-3α,7α,26-triol. To the best of our knowledge, this is the first demonstration of the natural occurrence of bile alcohols carrying the chenodeoxycholic acid type nucleus. By analogy to the biosynthesis of chenodeoxycholic acid, which is probably formed by the same pathway as that for the biosynthesis of cholic acid except that no 12α-hydroxylation occurs (31), these cholestanetriols might be formed by similar pathways to those for the formation of the corresponding cholestanetetrols carrying the cholic acid type nucleus, in which the only difference is that the step for 12α-hydroxylation is omitted.

In summary, most of the bile alcohols found in the bile of the present patients appear to represent intermediates or simple modifications of intermediates in the biosynthetic courses of cholic acid and chenodeoxycholic acid, the primary bile acids in man.

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