Occurrence of bile alcohol glucuronides in bile of patients with cerebrotendinous xanthomatosis[1]

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Abstract Using thin-layer chromatography, bile alcohol glucuronides were found with taurine- and glycine-conjugated bile acids in the bile of four patients with cerebrotendinous xanthomatosis. The concentration of the bile alcohol glucuronides was 1.7–5.2 times higher than that of the conjugated bile acids. Detectable amounts of unconjugated bile alcohols were not found in the bile of these patients. The bile alcohol glucuronides were isolated from the bile of one of the patients by means of preparative thin-layer chromatography. Treatment with β-glucuronidase of the bile alcohol glucuronides liberated glucuronic acid and a mixture of bile alcohols. More than 90% of the liberated bile alcohols was 5β-cholestane-3α,7α,12α,25-tetrol, and lesser amounts of 5β-cholestane-3α,7α,12α,23-tetrol, 5β-cholestane-3α,7α,12α,24-tetrol, 5β-cholestane-3α,7α,12α,23,25-pentol, and 5β-cholestane-3α,-7α,12α,24α,25-pentol were also obtained. The bile alcohol glucuronides were not oxidized by the treatment with 3α-hydroxysteroid dehydrogenase, indicating that the glucuronide moiety was at 3α-hydroxyl position of the bile alcohols. Comparison of the mass spectra of the acetylated and methylated derivatives of the natural glucuronides and the synthetic 7α,12α,25-triacetoxy-5β-cholestan-3α-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate) also indicated that the bile alcohol glucuronides consisted of mainly 5β - cholestane - 3α,7α,12α,25 - tetrol - glucuro - nide.—**Hoshita, T., M. Yasuhara, M. Une, A. Kibe, E. Itoga, S. Kito, and T. Kuramoto.** Occurrence of bile alcohol glucuronides in bile of patients with cerebrotendinous xanthomatosis. *J. Lipid Res.* 1980. **21:** 1015–1021.

Supplementary key words bile acids · gas–liquid chromatography–mass spectrometry

The presence of bile alcohols in patients with cerebrotendinous xanthomatosis (CTX) is a topic of current interest (1). Up to now the following bile alcohols have been isolated from the bile and feces of the CTX patients: 5β-cholestane-3α,7α,12α,23ξ-tetrol[2] (2); two 24-epimeric 5β-cholestane-3α,7α,12α,24-tetrols (2); 5β-cholestane-3α,7α,12α,25-tetrol (3); 5β-cholestane-3α,7α,12α,23ξ,25-pentol[2] (4–6); and 5β-cholestane-3α,7α,12α,24α,25-pentol (3, 4). These bile alcohols have been shown to be excreted into the bile and feces in the unconjugated forms (2, 3). However, since all bile alcohols that occurred in the bile of lower vertebrates were found as the sulfate esters (7), it seems to be of interest to ascertain whether conjugated bile alcohols are present in the bile of the CTX patients. In this report we describe the occurrence of bile alcohol glucuronides as major constituents in the bile of four patients with CTX.

MATERIALS AND METHODS

General

Melting points were determined with a Kofler-hot stage apparatus, and are uncorrected.

IR spectra were taken on a JASCO IRA-1 spectrometer using KBr discs. Absorption frequencies are quoted in reciprocal centimeters.

NMR spectra were obtained in pyridine-d_5 solution at 100 MHz on a JEOL JNM-PS-100 spectrometer. Chemical shifts are given in the δ ppm scale with tetramethylsilane as internal standard.

TLC was carred out on 0.25 mm thick silica gel G plates (Merck). Bile salt spots were made visible with a spray reagent which consisted of 10% phosphomolybdic acid in ethanol. Glucuronides were detected by spraying with 0.2% naphthoresorcinol in ethanol–phosphoric acid 10:1, followed by heating at 110°C for 5 min (8). Glucuronides give blue spots.

Abbreviations: CTX, cerebrotendinous xanthomatosis; TLC, thin-layer chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; TMS, trimethylsilyl; IR, infrared; NMR, nuclear magnetic resonance; RRT, relative retention time.

[1] This study is Part XVI of a series entitled "Comparative biochemical studies of bile acids and bile alcohols". (Part XV. Kibe, A., S. Nakai, T. Kuramoto, and T. Hoshita. *J. Lipid Res.* 1980. **21:** 594–599.)

[2] The β configuration for the 23-hydroxyl group of these tetrols and pentols was tentatively assigned on the basis of optical rotation differences (2, 5). However, it has recently been reported by Dayal et al. (6) that this assignment should be reversed. We retain the original nomenclature (3, 4) until the configuration is established by X-ray crystallography.

Preparative TLC was carried out on 1 mm thick silica gel H plates (Merck). Samples were applied to the plates as bands using a semiautomatic sample streaker (Applied Science Laboratories Inc.). After development in chloroform–methanol–acetic acid–water 13:4:2:1 (by vol), bile salt bands were detected by exposure of the dried plates to iodine vapor. The pertinent bands were scraped off the plates and the bile salts were eluted from the silica gel with methanol–acetic acid 97:3.

Mass spectra were obtained by direct sample introduction technique with a JEOL JMS-01SG-2 mass spectrometer under conditions of ion source temperature, 200°C and ionization voltage, 75 eV. Samples were analyzed as the methyl ester-polyacetates which were prepared by methylation with diazomethane followed by acetylation with acetic anhydride–pyridine 2:1 at 90°C for 6 hr.

GLC-MS of bile acids, bile alcohols, and glucuronic acid was carried out on a JEOL D-300 gas chromatograph–mass spectrometer. The bile acids to be analyzed were methylated with diazomethane and trimethylsilylated with hexamethyldisilazane–trimethylchlorosilane–pyridine 10:1:25 at room temperature for 2 hr, and the resulting methyl ester-TMS derivatives were injected into the gas chromatograph. The bile alcohols and glucuronic acid were analyzed as their TMS derivatives, which were prepared by the same procedure as the trimethylsilylation of the methylated bile acids. The following operating conditions were employed: column (2 m × 3 mm), 2% Poly I-110 on 80/100 mesh Gas Chrom Q for bile acids, 3% OV-17 on 80/100 mesh Gas Chrom Q for bile alcohols, or 3% QF-1 on 80/100 mesh Gas Chrom Q for glucuronic acid; column temperature, 230°C, 280°C, and 165°C for bile acids, bile alcohols, and glucuronic acid, respectively; emission current and electron energy, 300 μA and 70 eV for bile acids and bile alcohols, or 60 μA and 20 eV for glucuronic acid.

Patients

Patient 1 was a 33 year-old Japanese man who manifested bilateral Achilles tendon xanthomata, cerebellar ataxia, mental retardation, hyperactive deep reflexes, bilateral Babinski's sign, muscular atrophy, and peripheral neuropathy (9). Analysis of the fecal bile alcohols of this patient has been published (2). Detailed analysis of biliary bile salts was carried out in this patient.

Patients 2, 3, and 4, whose biles were analyzed only by TLC, were, respectively, 22, 27, and 30 year-old Japanese women. Detailed clinical descriptions of these patients will be presented elsewhere.

Each patient was fasted for 12 hr, and bile was collected by duodenal intubation after stimulation of the gallbladder with cholecystokinin. The bile samples were stored at −20°C until analyzed.

Determination of bile salt composition

For determination of biliary bile salt composition, TLC-direct densitometry was carried out according to the procedures described previously (10). Aliquots of the bile samples were directly applied as spots 2–5 mm in diameter on a silica gel G plate (0.5 mm thick). The plates were developed in chloroform–methanol–acetic acid–water 13:4:2:1 (by vol), and bile salts were visualized with the phosphomolybdate reagent. Color intensities were estimated by direct densitometry using a Shimadzu CS-910 dual-wavelength chromatoscanner.

Detection of unconjugated bile alcohols

Extraction of neutral lipids was carried out according to the procedures described by Setoguchi et al (3). A portion of the bile sample from patient 1 was diluted with 50 ml of water containing 1 ml of concentrated NH_4OH, and was extracted with four 50-ml portions of ethyl acetate. The extracts were combined, washed with water to neutrality, and the solvent was evaporated to dryness. The residue was examined by TLC using solvent systems ethyl acetate–acetone 7:3 (by vol) and benzene–isopropanol–acetic acid 30:10:1 (by vol) and by GLC-MS using 3% OV-17 column for the detection of unconjugated bile alcohols.

β-Glucuronidase treatment

Hydrolysis of bile alcohol glucuronides by β-glucuronidase was carried out according to the procedures described previously for the hydrolysis of bile acid glucuronides (11). The sample to be hydrolyzed was dissolved in 10 ml of 0.5 M acetate buffer (pH 5.0) containing 7500 units of β-glucuronidase (EC 3.2.1.31) (Sigma Chemical Co.) and a drop of chloroform. The incubation period was 24 hr at 37°C. The incubation mixture was then adjusted to pH 10 with 0.1 N KOH and extracted with two 30-ml portions of ethyl acetate. The ethyl acetate extracts were combined, washed with water to neutrality, dried over anhydrous Na_2SO_4, and the ethyl acetate was removed in a rotary evaporator. The resulting residue was examined by GLC–MS for the determination of "β-glucuronidase liberated" bile alcohols. The water phase left from the ethyl acetate extraction was adjusted to pH 7 with 0.1 N HCl and percolated through 30 g of Amberlite XAD-2 resin in a column (1.3 × 36 cm) at a rate of about one drop every 2 sec. The

column was washed with 100 ml of water, and then with 100 ml of methanol. The methanol eluate was evaporated to dryness and the resulting residue was examined by GLC–MS for the presence of glucuronic acid.

3α-Hydroxysteroid dehydrogenase treatment

The procedures for this treatment were those of Mashige, Imai, and Osuga (12). The sample to be tested was treated with the Sterognost-3α Kit (Nyegard Co., Oslo) which contained 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50), NAD, and hydrazine hydrate. The intensity of fluorescence of the NADH produced was measured at 450 nm with excitation at 350 nm.

Hydrolysis of conjugated bile acids

The procedure for hydrolysis of conjugated bile acids was basically that of Nair and Garcia (13). The sample to be hydrolyzed was dissolved in 2.0 ml of 1 M acetate buffer (pH 5.6), and the solution was incubated at 37°C for 25 min in the presence of 50 units of choloylglycine hydrolase (EC 3.5.1.24) (Sigma Chemical Co.), 0.5 ml of 0.2 M disodium salt of ethylenediaminetetraacetic acid, and 0.5 ml of 0.2 M β-mercaptoethanol. The incubation mixture was cooled to room temperature, acidified to pH 1 with 2 N HCl, and extracted with three 20-ml portions of ethyl acetate. The ethyl acetate extracts were combined, washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue was examined by GLC–MS for the determination of bile acid composition.

Reference compounds

Unconjugated bile acids, conjugated bile acids, and glucuronic acid were commercial products. 5α-Cyprinol (5α-cholestane-3α,7α,12α,26,27-pentol) sulfate was obtained from carp bile by means of preparative TLC (7). 5β-Cholestane-3α,7α,12α,23ξ-tetrol (14), two 24-epimeric 5β-cholestane-3α,7α12α,24-tetrols (15), 5β-cholestane-3α,7α,12α,25-tetrol (16, 17), 5β-cholestane-3α,7α,12α,23ξ,25-pentol (5), and 5β-cholestane-3α,7α,12α,24α,25-pentol (17, 18) were prepared according to the methods described previously.

Bile Alcohol glucuronide synthesis (Fig. 1)

5β-Cholestane-3α,7α,12α,25-tetrol tetraacetate (II). 5β-Cholestane-3α,7α,12α,25-tetrol (I) (2 g) was acetylated with acetic anhydride (30 ml) and anhydrous sodium acetate (2 g) by heating on a water bath for 18 hr. After the usual workup, the product was recrystallized from ethyl acetate to give crystals

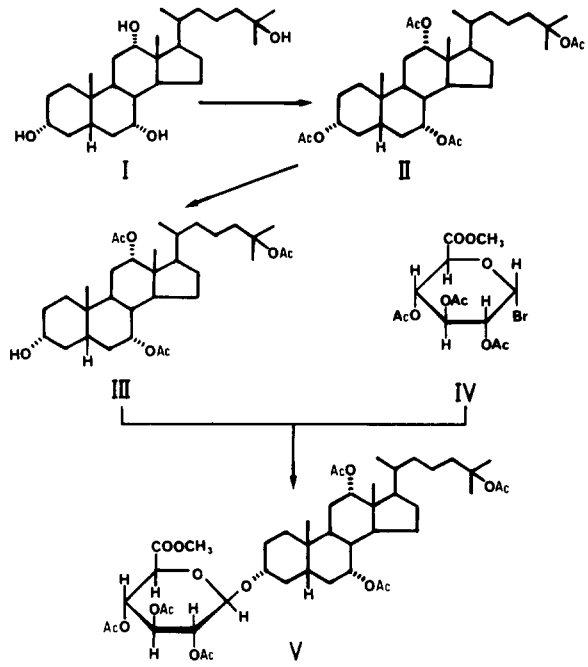

Fig. 1. Synthesis of the methyl ester-acetate of 5β-cholestane-3α,7α,12α,25-tetrol-3-glucuronide. I, 5β-Cholestane-3α,7α,12α,25-tetrol; II, 5β-cholestane-3α,7α,12α,25-tetrol tetraacetate; III, 7α,12α,25-triacetoxy-5β-cholestane-3α-ol; IV, methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranouronate; V, 7α,12α,25-triacetoxy-5β-cholestan-3α-*O*-(methyl 2,3,4-tri-*O*-*acetyl*-β-D-glucopyranosyluronate).

(1.3 g) of II, mp 140°C (reported (16) mp 142.5°C).

7α,12α,25-Triacetoxy-5β-cholestan-3α-ol (III). To a solution of the tetraacetate (II) (1.1 g) in 10 ml of methanol, a solution of potassium carbonate (0.25 g) in aqueous methanol was added at room temperature. After being allowed to stand at room temperature for 2 hr, the mixture was diluted with 10 volumes of water and extracted with ether (50 ml × 3). The ether extracts were combined, washed with water, and evaporated to dryness. The residue was chromatographed on a column of silica gel (Merck) (100 g). Elution with benzene–acetone 85:15 and crystallization from ether and methanol gave crystals (681 mg) of III, mp 64–5°C. IR $\nu_{max}^{KBr}(cm^{-1})$:3340(hydroxyl), 1735, 1250, and 1050 (acetate). NMR (δ ppm): 0.71 (s, 3H, 18—CH_3), 0.87 (s, 3H, 19—CH_3), 0.95 (d, J=6 Hz, 3H, 21—CH_3), 1.42 (s, 6H, 26—CH_3 and 27—CH_3), 1.91 (s, 3H, —$OCOCH_3$), 1.97 (s, 3H, —$OCOCH_3$), 2.01 (s, 3H, —$OCOCH_3$), 3.70 (m, 1H, 3β—H), 5.16 (m, 1H, 7β—H), and 5.38 (m, 1H, 12β—H).

Methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranosyluronate (IV). This compound was prepared according to the procedures of Bollenback et al (19). Glucuronolactone was dissolved in 200 ml of methanol

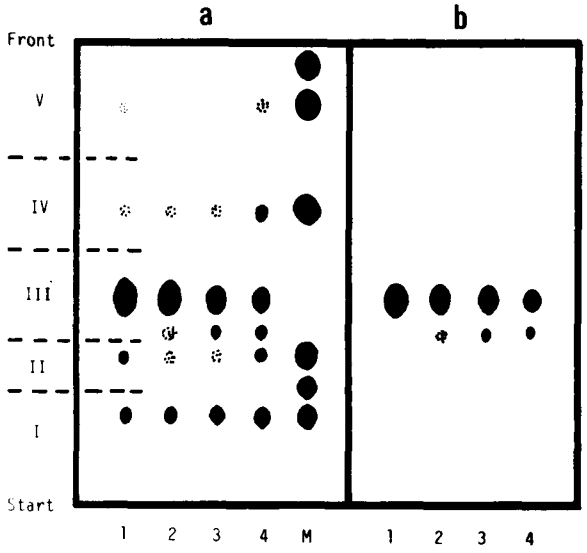

Fig. 2. Representative thin-layer chromatogram of the bile samples from patients with CTX and standards. Developing solvent: chloroform–methanol–acetic acid–water 13:4:2:1 (by volume). Silica gel G. Spray reagent: a, phosphomolybdate reagent; b, naphthoresorcinol reagent. Lanes 1–4 represent patients 1–4, respectively. M, mixture of the following standards in ascending order: taurocholate, 5α-cyprinol sulfate, taurodeoxycholate/taurochenodeoxycholate, glycocholate, glycodeoxycholate/glycochenodexycholate, and 5β-cholestane-3α,7α,12α,25-tetrol.

which contained 70 mg of NaOH. The mixture was stirred at room temperature for 2 hr, and the solvent was removed under reduced pressure. The resulting syrup was dissolved in 100 ml of absolute ethanol and allowed to stand at 0°C for 48 hr. Unchanged glucuronolactone was removed by filtration. The ethanolic filtrate was concentrated to dryness in vacuo. The residue, methyl glucuronate, was dissolved in 50 ml of pyridine and 75 ml of acetic anhydride and the solution was stored at 0°C. Crystalline material separated overnight. It was isolated by filtration. Recrystallization of the crystals from acetone gave 9.9 g of methyl tetra-*O*-acetyl-β-D-glucopyranuronate, mp 176–8°C (reported (19) mp 176.5–8°C). Five grams of methyl tetra-*O*-acetyl-β-D-glucopyranuronate were dissolved in 30 ml of 25% hydrobromic acid in acetic acid and the mixture was allowed to stand at 0°C overnight. The solution was evaporated to dryness under reduced pressure and the residue was dissolved in 50 ml of chloroform. The chloroform solution was washed with cold saturated aqueous sodium bicarbonate and then with water, dried over anhydrous Na_2SO_4, and the solvent was evaporated. The residue was recrystallized from isopropyl ether to give crystals (3.4 g) of IV, mp 105–7°C (reported (19) mp 106–7°C).

7α,12α,25-Triacetoxy-5β-cholestan-3α-O-(methyl 2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate) (V). To a solution of the triacetoxycholestanol (III) (100 mg) and methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranouronate (IV) (200 mg) in 10 ml of chloroform, 200 mg of silver carbonate were added under stirring at room temperature. The mixture was stirred for 3 days at room temperature. The precipitate was removed by filtration and washed with chloroform. The filtrate and the washings were combined and the solvent was evaporated. The residue was chromatographed on a column of silica gel (Merck) (10 g). Elution with benzene–ethyl acetate 4:1 and recrystallization from ether gave crystals (23.3 mg) of V, mp 184–5°C. IR ν_{max}^{KBr}(cm^{-1}): 1750, 1725, 1240, and 1030 (methyl ester and acetate). NMR (δ ppm): 0.76 (s, 3H, 18-CH_3), 0.86 (s, 3H, 19-CH_3), 0.98 (d, J=6 Hz, 3H, 21—CH_3), 1.30 (s, 6H, 26—CH_3 and 27—CH_3), 1.98 (s, 3H, —$OCOCH_3$), 2.02 (s, 6H, —$OCOCH_3$ × 2), 2.05 (s, 3H, —$OCOCH_3$), 2.12 (s, 3H, —$OCOCH_3$), 2.16 (s, 3H, —$OCOCH_3$), 3.86 (s, 3H, —$COOCH_3$), and 4.76–5.96 (m, 7H, 3β-H, CH—$OCOCH_3$ × 5, and anomeric H).

RESULTS

On TLC (**Fig. 2a**) using the phosphomolybdate spray reagent, the bile samples from patients 1–4 gave unusual spots with mobilities different from any hitherto known bile salt, along with lesser spots with R_f values that corresponded to known taurine- and glycine-conjugated bile acids. There were no spots corresponding to unconjugated bile alcohols and sulfated bile alcohols. When sprayed with the naphthorescorcinol reagent, the unusual spots were positive (blue spots) for the glucuronide moiety, and other spots could not be visualized (Fig. 2b).

Bile salt composition was determined by TLC-direct densitometry (10). The results are shown in **Table 1.**

TABLE 1. Biliary bile salt composition of patients with CTX[a]

Bile Salts	Composition			
	Patient 1	Patient 2	Patient 3	Patient 4
	(% of total)			
Taurocholate	13.2	14.2	19.9	20.7
Taurodeoxycholate/ taurochenodeoxycholate[b]	3.9	0.8	1.5	5.1
More polar glucuronides[c]	tr[e]	2.3	7.4	7.7
Less polar glucuronides[d]	80.6	81.6	68.4	55.4
Glycocholate	1.7	1.1	2.8	10.1
Glycodeoxycholate/ glycochenodeoxycholate[b]	0.6	tr	tr	1.0

[a] The direct densitometric measurements were performed as described previously (10), using TLC and a dual-wavelength chromatoscanner.
[b] These compounds could not be resolved on TLC.
[c] R_f 0.36.
[d] R_f 0.44.
[e] Tr, trace: less than 0.5%.

The concentration of the unusual bile salts was 1.7–5.2 times higher than that of the bile acid conjugates.

A portion of the bile sample from patient 1 was examined for the confirmation of the absence of unconjugated bile alcohols as described in the section of Materials and Methods. No detectable amounts of unconjugated bile alcohols were found.

The bile sample from patient 1 was subjected to preparative TLC, yielding five bile salt bands (I–V in Fig. 2). Band III was removed from the plate and eluted with methanol and acetic acid. The extract was divided into three aliquots.

One aliquot was used for mass spectrometry after evaporation of the solvents, followed by methylation and acetylation. The mass spectrum of the methyl ester–polyacetates is shown in **Fig. 3a.** The presence of the acetylated methyl glucuronide residue was suggested by the presence of the fragment ions at m/e 317, 257, 197, and 155. The fragment ion at m/e 253 is recognized as an ion consisting of the steroid nucleus including three double bonds. Although the molecular ion was not observed, there were two series of peaks, one at m/e 818, 758, 698, 638, 578, 518, and a second at m/e 545, 485, 425, 365. The former series results from the consecutive loss of one to six molecules of acetic acid from the molecular ion. The 545 fragment results from the loss of the acetylated methyl glucuronide residue, and the other fragments in the latter series are due to the loss of one, two, and three molecules of acetic acid from the 545 fragment.

The second aliquot was evaporated and the residue was subjected to enzymatic hydrolysis using β-glucuronidase according to the procedures described previously for the hydrolysis of bile acid glucuronides (11). Extraction with ethyl acetate of the incubation mixture gave "β-glucuronidase liberated" bile alcohols, which were examined by GLC–MS as the TMS derivatives (**Table 2**). Five major peaks appeared at the retention times corresponding to those of the TMS ethers of authentic 5β-cholestane-3α,7α,12α,23ξ-tetrol, 5β-cholestane-3α,7α,12α,24α-tetrol/5β-cholestane-3α,7α,12α,24β-tetrol, 5β-cholestane-3α,7α,12α,25-tetrol, 5β-cholestane-3α7α,12α,23ξ,25-pentol, and 5β-cholestane-3α,7α,12α,24α,25-pentol. The identity of these peaks was further confirmed by mass spectrometry. Detailed descriptions of the mass spectra of these bile alcohols have been published (2–5). The presence of glucuronic acid in the aqueous layer left from the ethyl acetate extraction was confirmed not only by GLC but also by mass spectrometry. Silylated derivative of the material present in the aqueous layer gave a GLC peak which had the same retention time (12.3 min on QF-1 column) as that of a known sample of glucuronic acid treated in the same

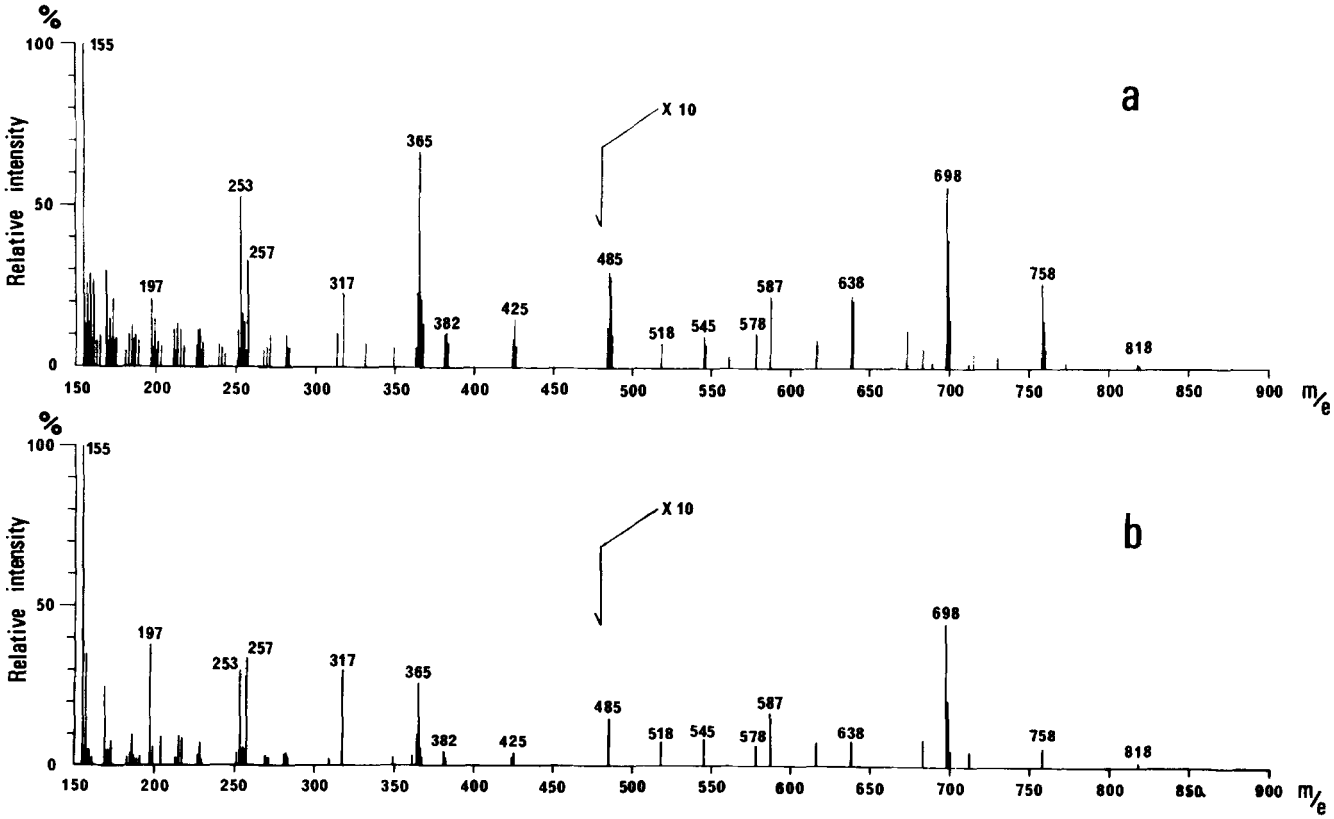

Fig. 3. Mass spectra of a) the methyl ester-polyacetates of the biosynthetic bile alcohol glucuronides and b) 7α,12α,25-triacetoxy-5β-cholestan-3α-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate).

TABLE 2. Bile alcohols in the bile of patient 1

Bile Alcohols	RRT[a] on GLC[b]	Composition
		(% of total)
5β-Cholestane-3α,7α,12α,23ξ-tetrol	0.83	5.3
5β-Cholestane-3α,7α,12α,24α-tetrol and 5β-cholestane-3α,7α,12α,24β-tetrol[c]	0.99	0.9
5β-Cholestane-3α,7α,12α,25-tetrol	1.10	90.9
5β-Cholestane-3α,7α,12α,23ξ,25-pentol	1.46	1.2
5β-Cholestane-3α,7α,12α,24α,25-pentol	1.52	1.7

[a] Relative to TMS ether of methyl cholate (RRT = 1.00).
[b] Bile alcohols were chromatographed on 3% OV-17 column as their TMS ethers.
[c] Two epimers could not be resolved on GLC.

manner. In the mass spectrum of the peak from the biological sample, the molecular ion (M) was not detected but the ion that corresponded to the molecular ion minus a methyl group was observed at m/e 539. Also present were the characteristic ions at m/e (relative intensity) 465(3) (M-89), 450(2) [M-(89 + 15)], 360(4) [M-(89 + 90 + 15)], 333(5) [TMS—OOC—C(O—TMS)=CH—CH=O$^+$—TMS], 217(76) (TMS—O—CH=CH=O$^+$—TMS), 204(8) (TMS—O—CH=CH—O—TMS), 189(8) (204-15), 147(32) [$(TMS)_2O$-15], and 73(100) (TMS). This fragmentation pattern was identical with that obtained with the silylated glucuronic acid.

The third aliquot was evaporated to dryness and the resulting residue was treated with 3α-hydroxysteroid dehydrogenase in the presence of NAD. No significant amounts of NADH were formed. The fact that the natural bile alcohol glucuronides have no oxidizable 3α-hydroxyl group provides satisfactory proof that the glucuronide moiety is at 3α-hydroxyl position.

Since the above-mentioned results indicated that the material in band III consisted of mainly (more than 90%) 5β-cholestane-3α,7α,12α,25-tetrol-3α-glucuronide, chemical synthesis of the methyl ester-polyacetate of this compound was undertaken (Fig. 1). The mass spectrum (Fig. 3b) of 7α,12α,25-triacetoxy-5β cholestan-3α-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate) (V) was identical in all important respects to that (Fig. 3a) of the methylated and acetylated derivative of the material in band III.

TABLE 3. Bile acids in the bile of patient 1

Bile Acids	RRT[a] on GLC[b]	Composition
		(% of total)
Cholic acid	1.00	75.8
Deoxycholic acid	1.26	7.3
Chenodeoxycholic acid	1.49	16.9

[a] Relative to TMS ether of methyl cholate (RRT = 1.00).
[b] Bile acids were chromatographed on 2% Poly I-110 column as their methyl ester-TMS ethers.

Bands I, II, IV, and V in Fig. 2 contained taurine- and glycine-conjugated bile acids. The materials in these bands were combined and treated with choloylglycine hydrolase according to the procedures described previously (13), and the resulting deconjugated bile acids were methylated with diazomethane, converted to the TMS ethers, and then examined by GLC–MS (**Table 3**). There were three major peaks with the retention times corresponding to the TMS ethers of methyl cholate, methyl deoxycholate, and methyl chenodeoxycholate. The identity of these bile acids was established through comparison of mass spectra with authentic samples.

DISCUSSION

The present studies revealed that the major bile salts in the bile of four patients with CTX were bile alcohol glucuronides. This identification was based on a) the naphthoresorcinol test, b) enzymatic (β-glucuronidase) liberation of glucuronic acid and a mixture of bile alcohols, which consisted mainly of 5β-cholestane-3α,7α,12α,25-tetrol, and c) mass spectral data of the methyl ester polyacetates, and their comparison with synthetic 7α,12α,25-triacetoxy-5β-cholestan-3α-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyl uronate). Since the natural glucuronides were not dehydrogenated by 3α-hydroxysteroid dehydrogenase in the presence of NAD, it can be assumed that the glucuronide moiety is at the 3α-hydroxyl position. Although the existence of bile acid glucuronides in urine and blood plasma of patients with chronic intrahepatic cholestasis has been reported (11, 20), the present finding is the first evidence for the occurrence of bile alcohol glucurono-conjugates in biological sources.

In contrast to the previous observation (3, 21) that in CTX patients biliary bile alcohols as well as fecal bile alcohols occur as the unconjugated forms, the CTX patients examined in the present studies had no unconjugated bile alcohols. This discrepancy cannot yet

be fully explained. Possibly the formation of bile alcohol glucuronides is related to the amount of accumulated bile alcohols. In the present cases, the percentage composition of biliary bile alcohols exceeds that of biliary bile acids, which is also contrary to the previously reported cases (3). Bile alcohol glucuronides are much more polar compounds and much more soluble in aqueous medium than are unconjugated bile alcohols. Thus, bile alcohol glucuronides may be readily excreted into the intestine with the bile. It is tempting to speculate that glucuronidation of bile alcohols is a more suitable step for hepatic elimination of larger amounts of bile alcohols.

Bile alcohol glucuronides were not present in the feces of patients 2–4[3] nor in those of patient 1 (2). This may be because bile alcohol glucuronides, like bilirubin glucuronides, were hydrolyzed to form unconjugated bile alcohols by the action of intestinal microorganisms during their passage through the intestinal tract.

The formation and occurrence of bile alcohol glucuronides in the CTX patients provide a number of interesting questions. For example, a) can the glucuronide be excreted in urine? b) can the glucuronides be reabsorbed? and c) do the glucuronides affect the metabolism of cholesterol and bile salts? Studies to answer these questions are now in progress.

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REFERENCES

1. Salen, G., and E. H. Mosbach. 1976. The metabolism of sterols and bile acids in cerebrotendinous xanthomatosis. *In* The Bile Acids. Vol. 3. P. P. Nair and D. Kritchevsky, editors. Plenum Press, New York and London. 115–153.
2. Yasuhara, M., T. Kuramoto, T. Hoshita, E. Itoga, and S. Kito. 1978. Identification of 5β-cholestane-3α,7α,12α,23β-tetrol, 5β-cholestane-3α,7α,12α,24α-tetrol, and 5β-cholestane-3α,7α,12α,24β-tetrol in cerebrotendinous xanthomatosis. *Steroids.* **31:** 333–345.
3. Setoguchi, T., G. Salen, G. S. Tint, and E. H. Mosbach. 1974. A biochemical abnormality in cerebrotendinous xanthomatosis. Impairment of bile acid biosynthesis associated with incomplete degradation of the cholesterol side chain. *J. Clin. Invest.* **53:** 1393–1401.
4. Shefer, S., B. Dayal, G. S. Tint, G. Salen, and E. H. Mosbach. 1975. Identification of pentahydroxy bile alcohols in cerebrotendinous xanthomatosis: characterization of 5β-cholestane-3α,7α,12α,24ξ,25-pentol and 5β-cholestane-3α.7α,12α,23ξ,25-pentol. *J. Lipid Res.* **16:** 280–286.

[3] Hoshita, T., M. Yasuhara, M. Une, A. Kibe, E. Itoga, S. Kito, and T. Kuramoto. Unpublished observation.

5. Hoshita, T., M. Yasuhara, K. Kihira, and T. Kuramoto. 1976. Identification of (23*S*)-5β-cholestane-3α,7α,12α,23,25-pentol in cerebrotedinous xanthomatosis. *Steroids.* **27:** 657–664.
6. Dayal, B., G. S. Tint, S. Shefer, and G. Salen. 1979. Configurational assignment of 5β-cholestane-3α,7α,12α,23,25-pentol excreted by patients with cerebrotendinous xanthomatosis (a circular dichroism study). *Steroids.* **33:** 327–338.
7. Hoshita, T., and T. Kazuno. 1968. Chemistry and metabolism of bile alcohols and higher bile acids. *Adv. Lipid Res.* **6:** 207–254.
8. *In* Handbook of Chromatography. Vol. II. 1972. G. Zweig and J. Sherma, editors. CRC Press, Cleveland, OH. 127.
9. Kito, S., E. Itoga, Y. Yamamura, and T. Hoshita. 1980. Cerebrotendinous xanthomatosis with emphasis on bile acid metabolism. *In* Spinocerebellar Degeneration. Edited by Japan Medical Research Foundation. University of Tokyo Press, Tokyo. 355–364.
10. Kibe, A., T. Kuramoto, and T. Hoshita. 1979. A rapid and sensitive method for the determination of the lithogenic index of bile by thin-layer chromatography and densitometry using a dual-wavelength chromatoscanner. *Anal. Biochem.* **100:** 146–151.
11. Back, P., K. Spaczynski, and W. Gerok. 1974. Bile-salt glucuronides in urine. *Z. Physiol. Chem.* **355:** 749–752.
12. Mashige, F., K. Imai, and T. Osuga. 1976. A simple and sensitive assay of total serum bile acids. *Clin. Chim. Acta.* **70:** 79–86.
13. Nair, P. P., and C. Garcia. 1969. A modified gas–liquid chromatographic procedure for the rapid determination of bile acids in biological fluids. *Anal. Biochem.* **29:** 164–171.
14. Kuramoto, T., N. Matsumoto, and T. Hoshita. 1978. Syntheses of 22- and 23-hydroxylated bile alcohols. *Chem. Pharm. Bull. (Tokyo).* **26:** 2788–2792.
15. Masui, T., and E. Staple. 1967. The separation of the stereo-isomers of bile steroids, 5β-cholestane-3α,-7α,12α,24α-tetrol and 5β-cholestane-3α,7α,12α,24β-tetrol by thin-layer chromatography. *Steroids.* **9:** 443–450.
16. Pearlman, W. H. 1947. The preparation of C-27 steroids from the bile acids. I. Coprostanetetrol-3(α),7(α),12(α),25. *J. Am. Chem. Soc.* **69:** 1475–1476.
17. Dayal, B., S. Shefer, G. S. Tint, G. Salen, and E. H. Mosbach. 1976. Synthesis of 5β-cholestane-3α,7α,12α,25-tetrol and 5β-cholestane-3α,7α,12α,24ξ,25-pentol. *J. Lipid Res.* **17:** 74–77.
18. Hoshita, T. 1962. Syntheses of 3α,7α,12α,25,26- and 3α,7α,12α,24,25-pentahydroxycoprostanes. *J. Biochem. (Tokyo).* **52:** 176–179.
19. Bollenback, G. N., J. W. Long, D. G. Benjamin, and J. A. Lindquist. 1955. The synthesis of aryl-D-glucopyranosiduronic acids. *J. Am. Chem. Soc.* **77:** 3310–3315.
20. Back, P. 1976. Bile acid glucuronides, II. Isolation and identification of a chenodeoxycholic acid glucuronide from human plasma in intrahepatic cholestasis. *Z. Physiol. Chem.* **357:** 213–217.
21. Salen, G., S. Shefer, F. W. Cheng, B. Dayal, K. Batta, and G. S. Tint. 1979. Cholic acid biosynthesis. The enzymatic defect in cerebrotendinous xanthomatosis. *J. Clin. Invest.* **63:** 38–44.