

Identification of short side chain bile acids in urine of patients with cerebrotendinous xanthomatosis

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Abstract Urine from patients with cerebrotendinous xanthomatosis (CTX) was found to contain a number of minor bile acids along with three major bile acids, 7-epicholic acid, norcholic acid, and cholic acid. The following minor bile acids were identified by combined gas-liquid chromatography-mass spectrometry: 7-ketobisnordeoxycholic acid; 12-ketobisnorchenodeoxycholic acid; 7-ketonordeoxycholic acid; 12-ketochenodeoxycholic acid; 7-ketodeoxycholic acid; 12-ketochenodeoxycholic acid; bisnorcholic acid; allonorcholic acid; allocholic acid; 1 β -hydroxybisnorcholic acid; 1 β -hydroxynorcholic acid; 1 β -hydroxycholic acid; 2 β -hydroxybisnorcholic acid; 2 β -hydroxynorcholic acid; 2 β -hydroxycholic acid. The presence of C₂₂ and C₂₃ bile acids in urine of the CTX patients suggests that bile alcohols having a hydroxyl group at C₂₂ or C₂₃ in the side chain may be further degraded to these bile acids. — Kuramoto, T., Y. Furukawa, T. Nishina, T. Sugimoto, R. Mahara, M. Tohma, K. Kihira, and T. Hoshita. Identification of short side chain bile acids in urine of patients with cerebrotendinous xanthomatosis. *J. Lipid Res.* 1990. 31: 1895–1902.

Supplementary key words cerebrotendinous xanthomatosis • short side chain bile acids • urine.

Cerebrotendinous xanthomatosis (CTX) is a rare familial sterol storage disease caused by an inborn error of cholesterol catabolism, in which there is a deficiency of enzymes that catalyze 26-hydroxylation of 5 β -cholestane-3 α ,7 α ,12 α -triol and 24-S hydroxylation of 5 β -cholestane-3 α ,7 α ,12 α ,25-tetrol, intermediate in the pathway leading to cholic acid from cholesterol (1). The enzymatic block in the bile acid synthesis results in defective bile acid production and accumulation of abnormal metabolites, neutral bile alcohols, and unusual bile acids in the bile and urine (2, 3).

Wolthers et al. (4) found that three unusual bile acids, 23-hydroxycholic acid and two isomeric norcholic acids, were excreted in predominantly unconjugated form in urine of CTX patients. Kihira et al. (5) have examined urinary bile acids of a CTX patient and determined with certainty that the two isomeric C₂₃ bile acids are allonorcholic and norcholic acids.

In order to obtain information about the biogenesis of bile acids, especially of the short side chain bile acids, in CTX, a more complete knowledge of the urinary bile acids of CTX patients must be secured. We have now examined the minor bile acids in the urine of CTX patients by means of a combination of gas-liquid chromatography and mass spectrometry.

MATERIALS AND METHODS

General

Melting points and PMR spectra were obtained as described previously (6). Piperidinoxypropyl Sephadex LH-20 (PHP-LH-20) was prepared according to the

Abbreviations and trivial names: cholic acid, 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid; allocholic acid, 3 α ,7 α ,12 α -trihydroxy-5 α -cholan-24-oic acid; 7-ketodeoxycholic acid, 3 α ,12 α -dihydroxy-7-oxo-5 β -cholan-24-oic acid; 12-ketochenodeoxycholic acid, 3 α ,7 α -dihydroxy-12-oxo-5 β -cholan-24-oic acid; 7-epicholic acid, 3 α ,7 β ,12 α -trihydroxy-5 β -cholan-24-oic acid; 1 β -hydroxycholic acid, 1 β ,3 α ,7 α ,12 α -tetrahydroxy-5 β -cholan-24-oic acid; 2 β -hydroxycholic acid, 2 β ,3 α ,7 α ,12 α -tetrahydroxy-5 β -cholan-24-oic acid; norcholic acid, 3 α ,7 α ,12 α -trihydroxy-24-nor-5 β -cholan-23-oic acid; allonorcholic acid, 3 α ,7 α ,12 α -trihydroxy-24-nor-5 α -cholan-23-oic acid; 7-ketonordeoxycholic acid, 3 α ,12 α -dihydroxy-7-oxo-24-nor-5 β -cholan-23-oic acid; 12-ketonorchenodeoxycholic acid, 3 α ,7 α -dihydroxy-12-oxo-24-nor-5 β -cholan-23-oic acid; 1 β -hydroxynorcholic acid, 1 β ,3 α ,7 α ,12 α -tetrahydroxy-24-nor-5 β -cholan-23-oic acid; 2 β -hydroxynorcholic acid, 2 β ,3 α ,7 α ,12 α -tetrahydroxy-24-nor-5 β -cholan-23-oic acid; bisnorcholic acid, 3 α ,7 α ,12 α -trihydroxy-23,24-dinor-5 β -cholan-22-oic acid; 7-ketobisnordeoxycholic acid, 3 α ,12 α -dihydroxy-7-oxo-23,24-dinor-5 β -cholan-22-oic acid; 12-ketobisnorchenodeoxycholic acid, 3 α ,7 α -dihydroxy-12-oxo-23,24-dinor-5 β -cholan-22-oic acid; 1 β -hydroxybisnorcholic acid, 1 β ,3 α ,7 α ,12 α -tetrahydroxy-23,24-dinor-5 β -cholan-22-oic acid; 2 β -hydroxybisnorcholic acid, 2 β ,3 α ,7 α ,12 α -tetrahydroxy-23,24-dinor-5 β -cholan-22-oic acid; CTX, cerebrotendinous xanthomatosis; PMR, proton nuclear magnetic resonance; PHP-LH-20, piperidinoxypropyl Sephadex LH-20; GLC, gas-liquid chromatography; TMS, trimethylsilyl; RRT, relative retention time; GLC-MS, gas-liquid chromatography-mass spectrometry.

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procedure described by Goto et al. (7) and bile acids were fractionated based upon their mode of conjugation on PHP-LH-20. Unconjugated, glycine- and glucurono-conjugated, taurine-conjugated, and sulfated bile acids were eluted stepwise with 0.1 M acetic acid in 90% ethanol, 0.2 M formic acid in 90% ethanol, 0.3 M potassium acetate in 90% ethanol (pH 6.3), and 2% ammonium carbonate in 70% ethanol, respectively. Gas-liquid chromatography (GLC) was carried out on a Shimadzu GC-6A gas chromatograph using a glass column (2 m × 3 mm) packed with 3% Poly I-110. Bile acids to be analyzed were converted to the methyl ester-trimethylsilyl (TMS) ether derivatives. Gas-liquid chromatography-mass spectrometry (GLC-MS) was carried out on a Shimadzu QP-1000 gas chromatograph-mass spectrometer. The following operating conditions were used: column SE-30 capillary column (0.25 mm × 25 m); column temperature, programmed operation from 220°C to 270°C with increments of 2°C/min; ionization current 300 μA; ionization voltage 70 eV.

Reference bile acids

Cholic acid was a commercial product. Allocholic (8), 7-ketodeoxycholic (9), 12-ketochenodeoxycholic (10), 7-epicholic (11), 1β-hydroxycholic (12), 2β-hydroxycholic (13), norcholic (14), allonorcholic (15), 7-ketonorchoxycholic (16), 12-ketonorchenodeoxycholic (16), and bisnorcholic (17) acids were prepared in these laboratories according to previously described methods.

Synthesis of 7-ketobisnordeoxycholic acid

To a solution of bisnorcholic acid (250 mg) dissolved in 70 ml of acetone was added a solution of N-bromosuccinimide (370 mg) in 30 ml of water at room temperature for 2 h. The reaction mixture was diluted with 500 ml of water and extracted with ethyl acetate. Evaporation of the solvent followed by recrystallization from ethyl acetate gave 113 mg of colorless needles of 7-ketobisnordeoxycholic acid: mp 199.0–200.0°C; PMR(δ, C₅D₅N) 0.78 (3H, s, 18-CH₃), 1.06 (3H, s, 19-CH₃), 1.15 (3H, d, J = 6 Hz, 21-CH₃), 3.69 (1H, m, 3β-H), 4.13 (1H, m, 12β-H).

Synthesis of 12-ketobisnorchenodeoxycholic acid

Six hundred milligrams of bisnorcholic acid was methylated by the usual manner with diazomethane. A solution of the methyl ester in a mixture of acetic anhydride (3.0 ml), pyridine (2.0 ml), and benzene (3.0 ml) was allowed to stand at room temperature for 5 h. The reaction mixture was then poured into water and extracted with ether. The extract was washed with 0.2 N HCl, water, 5% NaHCO₃, and water and evaporation of the solvent followed by recrystallization from ether gave 376 mg of colorless needles of methyl 3α,7α-diacetoxy-12α-hydroxy-23,24-dinor-5β-cholan-22-oate, mp 180–

181°C. The diacetoxy compound (300 mg) was then oxidized with CrO₃ to give the 3α,7α-diacetoxy-12-oxo derivative which was then hydrolyzed with 5% methanolic KOH. The hydrolyzed product was recrystallized from methanol and yielded 145 mg of the colorless crystals of 12-ketobisnorchenodeoxycholic acid, mp 262°C; PMR, (δ, C₅D₅N) 1.04 (3H, s, 18-CH₃), 1.09 (3H, s, 19-CH₃), 1.13 (3H, d, J = 6 Hz, 21-CH₃), 3.88 (1H, m, 3β-H), 4.01 (1H, m, 7β-H).

Extraction and fractionation of bile acid mixture from the urine

Sep-Pak C₁₈ cartridge extraction was carried out by the method previously described (18). Urine, which was collected during 24 h, was passed, in 100-ml portions, through Sep-Pak C₁₈ cartridges (Waters Associates, Milford, MA). After a wash with 5 ml of water, the adsorbed steroids were eluted with 5 ml of methanol. The cartridges were washed with 50 ml of water and used again for extraction of a second portion of urine. The combined methanol eluates were taken to dryness. The residue (1.1 g) was dissolved in 20 ml of 90% ethanol and passed through a column of PHP-LH-20 (20 cm × 3 cm) in acetate form, followed by 300 ml of 90% ethanol to remove neutral compounds. The column was eluted successively with 1000 ml of 0.1 M acetate in 90% ethanol and 2% ammonium carbonate in 70% ethanol to give unconjugated bile acids and conjugated bile acids, respectively.

Hydrolysis with cholyglycine hydrolase

The conjugated bile acid fraction eluted from the PHP-LH-20 column was evaporated to dryness. The residue was dissolved in 30 ml of 0.1 M acetate buffer (pH 5.6) containing 160 μmol each of mercaptoethanol and EDTA. After addition of 50 units of cholyglycine hydrolase (EC 3.5.1.24 Sigma Chemical Co., St. Louis, MO), the solution was incubated at 37°C for 16 h. The incubation mixture was passed through Sep-Pak C₁₈ cartridges followed by washing with 5 ml of water. The adsorbed steroids were eluted with 5 ml of methanol and the combined methanol eluate was evaporated to dryness. The residue was dissolved in 10 ml of 90% ethanol, and the solution was applied on a column of PHP-LH-20 (15 × 3 cm). After washing with 270 ml of 90% ethanol, the column was eluted successively with 900 ml of 0.1 M acetate in 90% ethanol, 0.2 M formic acid in 90% ethanol, 2% ammonium carbonate in 70% ethanol, to give deamidated bile acids, glucurono-conjugated bile acids, and sulfated bile acids, respectively.

β-Glucuronidase treatment

β-Glucuronidase treatment was carried out using the method previously described (19). The glucurono-conjugated bile acid fraction eluted from the second PHP-

LH-20 column was evaporated to dryness. The residue was dissolved in 20 ml of 0.1 M phosphate buffer (pH 6.8), containing 10,000 units of β -glucuronidase (EC3.2.1.31, Sigma Chemical Co., Type IX) and the mixture was incubated at 37°C for 24 h. The incubation mixture was applied to Sep-Pak C₁₈ cartridges. The methanol extracts were evaporated to dryness and the residue was dissolved in 5 ml of 90% ethanol and applied on a column of PHP-LH-20 (15 × 2 cm). After washing with 150 ml of 90% ethanol, the column was eluted with 500 ml of 0.1 M acetate in 90% ethanol to give deconjugated bile acids. Then, the column was eluted with 0.2 M formic acid in 90% ethanol; however, no bile acids were found in this fraction.

Solvolysis

Solvolysis was carried out by the method described previously (20). The sulfated bile acid fraction eluted from the second PHP-LH-20 column was evaporated to dryness. The residue was dissolved in water and applied on Sep-Pak C₁₈ cartridges and the methanol extract was evaporated in vacuo. To a solution of the residue dissolved in 5 ml of ethanol, 50 ml of ethyl acetate equilibrated with 2 M sulfuric acid was added and the solution was incubated at 37°C for 72 h. The solution was then neutralized with 10% sodium hydroxide and taken to dryness. The residue was hydrolyzed with 5% potassium hydroxide and purification on a PHP-LH-20 column performed as described above yielded a solvolized bile acid fraction. After eluting the solvolized bile acid, the column was eluted with 0.3 M potassium acetate in 90% ethanol (pH 6.3); however, no bile acid was found in this fraction.

Silica gel column chromatography

The unconjugated, deamidated, deconjugated, and solvolized bile acid fractions were methylated by the usual manner with diazomethane and then chromatographed on a silica gel column (10 g) eluted with benzene-ethyl acetate-acetone mixtures. The column effluents were monitored by thin-layer chromatography (silica gel G plate, Merck; solvent system, ethyl acetate).

RESULTS

Urine samples obtained from the three CTX patients were examined for bile acids (Fig. 1). The methanol extracts from the urine were submitted to ion-exchange column chromatography on PHP-LH-20 to give unconjugated and conjugated bile acid fractions. Treatment of the conjugated bile acid fraction with cholyglycine hydrolase followed by separation with PHP-LH-20 gave deamidated, glucurono-conjugated, and sulfated bile acid fractions. β -Glucuronidase treatment of the glucurono-conjugated bile acid fraction and solvolysis of the sulfated bile

acid fraction gave deconjugated and solvolized bile acid fractions, respectively.

Since preliminary GLC-MS analysis of the unconjugated bile acid fraction revealed a complex mixture of bile acids, this fraction was methylated and further chromatographed on a silica gel column to give five subfractions as shown in Table 1. GLC analysis of five subfractions revealed the presence of at least eighteen bile acids, tentatively named as bile acids 1-18 (Table 2). Bile acids 7, 9, 10, 12, and 18 were identified as 7-epicholic acid, allonorcholic acid, norcholic acid, cholic acid, and 2 β -hydroxycholic acid, respectively, by direct comparison of the mass spectra and GLC data with those of the authentic compounds. The occurrence of these bile acids in urine of CTX patients had been reported previously (5).

Bile acid 8 was identified as bisnorcholic acid by direct comparison of the RRT and mass spectrum of the authentic compound. In the mass spectrum, the molecular ion was absent, but there were two series of peaks, one at m/z 595 (M-15), 505 (M-15-90), 415 (M-15-2 × 90), and 325 (M-15-3 × 90), and a second at 520 (M-90), 430 (M-2 × 90, base peak), and 340 (M-3 × 90). The peaks at m/z 253 and 343 represent loss of the side chain plus three and two TMS groups, respectively.

Bile acid 11 was identified as allocholic acid by comparison of the RRT and mass spectrometry data with the authentic compound. In the mass spectrum of the methyl ester-TMS derivative of bile acid 11, the molecular ion was absent, but the peak due to the loss of a methyl group from the molecular ion was seen at m/z 623. There were

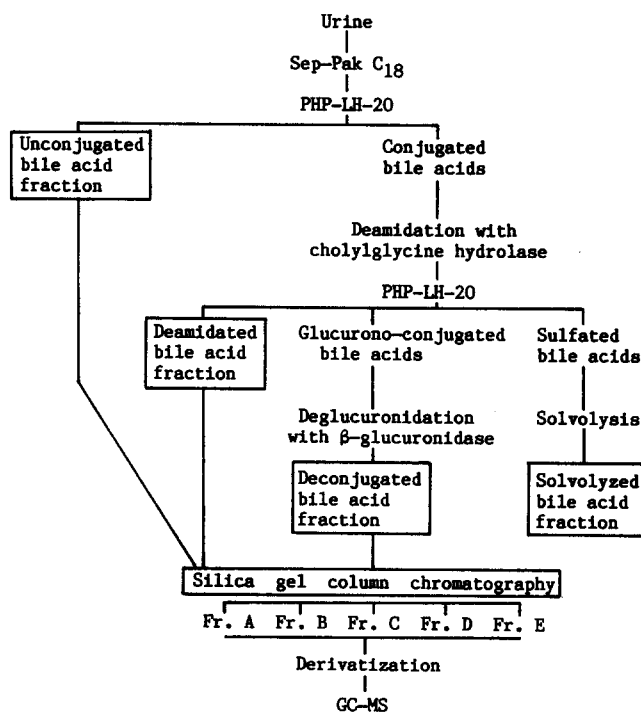


Fig. 1. Procedure for separation of urinary bile acids.

TABLE 1. Silica gel column chromatography of bile acids from the urine of CTX patients as methyl esters

Subfraction	Eluant	Eluate	Bile Acid
A	50% Benzene in ethyl acetate	Dihydroxymonooxo bile acids	Bile acids 1, 2, 3, 4, 5, and 6,
B	30% Benzene in ethyl acetate	Trihydroxy bile acid	Bile acid 7
C	Ethyl acetate	Trihydroxy bile acids	Bile acids 8, 9, 10, 11, and 12,
D	10% Acetone in ethyl acetate	Tetrahydroxy bile acids	Bile acids 13, 14, and 15
E	20% Acetone in ethyl acetate	Tetrahydroxy bile acids	Bile acids 16, 17, and 18

two series of fragments, one at m/z 548, 458 (base peak), and 368 which are formed by the successive loss of the TMS groups from the molecular ion, and the other at m/z 623, 533, 443, and 353, which are formed by the successive loss of the TMS groups and a methyl group. The peaks at m/z 253, 343, and 433 represent loss of the side chain plus three, two, and one TMS groups, respectively.

Bile acids 5 and 6 were identified as 7-ketodeoxycholic and 12-ketochenodeoxycholic acids by direct comparison of the RRT and mass spectrum with authentic compounds, respectively. The mass spectra of the methyl ester-TMS derivative of these dihydroxymonooxocholanoic acids were similar to each other except for the difference of the intensities of the fragments. There were three series of fragments, one at m/z 564 (M), 474 (M-90), 384 (M-2 × 90), a second at 549 (M-15), 459 (M-15-90), and 369 (M-15-2 × 90), and a third at 449 (M-side chain), 359 (M-side chain-90), and 269 (M-side chain-2 × 90), and peaks at m/z 456 (M-18-90), 366 (M-18-2 × 90), 341 (M-side chain-18-90), 269 (M-side chain-2 × 90), and 251 (M-side chain-18-2 × 90).

The mass spectra of the methyl ester-TMS derivatives of bile acids 1 and 3 were closely similar to that of the corresponding derivative of bile acid 5, 7-ketodeoxycholic acid, except for downfield shifts of 28 and 14 mass units for the high field fragments containing the entire side chains, respectively. The methyl ester-TMS derivatives of bile acids 1 and 3 showed GLC peaks that had RRTs of 0.47 and 0.72 relative to that of the corresponding derivative of 7-ketodeoxycholic acid. These ratios were in good agreement with constant separating factors of bisnorcholic acid (0.48), norcholic acid (0.73), and cholic acid (1.00). These results indicate that bile acids 1 and 3 are the C₂₂ and C₂₃ homologs of 7-ketodeoxycholic acid, respectively. To confirm these structural assignments, 7-ketobisnordeoxycholic acid and 7-ketonordeoxycholic acid were prepared as described in the section of Materials and Methods. The RRTs on GLC and the mass spectra of the synthetic C₂₂ and C₂₃ bile acids were identical with those of the natural bile acids 1 and 3, respectively.

Bile acids 2 and 4 were identified as 12-ketobisnorchenodeoxycholic acid and 12-ketonorchenodeoxycholic acid, respectively. The mass fragmentation patterns of the methyl ester TMS-derivatives of bile acids 2 and 4 were very similar to that of the corresponding derivative of bile acid 6, 12-ketochenodeoxycholic acid, except for shifts of 28 and 14 mass units for the fragment ions containing the entire side chains, respectively. The methyl ester-TMS derivatives of bile acids 2 and 4 showed GLC peaks that had RRTs of 0.49 and 0.73 relative to the corresponding derivative of 12-ketochenodeoxycholic acid. The GLC data also indicated that bile acids 2 and 4 are the C₂₂ and C₂₃ homologs of 12-ketochenodeoxycholic acid. Thus, 12-ketobisnorchenodeoxycholic acid and 12-ketonorchenodeoxycholic acid were prepared as described in the section of Materials and Methods. The chemically synthesized C₂₂ and C₂₃ bile acids had the same RRT on GLC and mass spectra data as the naturally occurring bile acids 2 and 4, respectively.

TABLE 2. Relative retention times of bile acids detected in the urine of CTX patients

Bile Acid Number	Trivial Name	RRT
1	7-Ketobisnordeoxycholic acid	1.25
2	12-Ketobisnorchenodeoxycholic acid	1.44
3	7-Ketonordeoxycholic acid	1.93
4	12-Ketonorchenodeoxycholic acid	2.13
5	7-Ketodeoxycholic acid	2.68
6	12-Ketochenodeoxycholic acid	2.92
7	7-Epicholic acid	1.18
8	Bisnorcholic acid	0.48
9	Allonorcholic acid	0.65
10	Norcholic acid	0.73
11	Allocholic acid	0.89
12	Cholic acid	1.00
13	1β-Hydroxybisnorcholic acid	0.41
14	1β-Hydroxynorcholic acid	0.61
15	1β-Hydroxycholic acid	0.84
16	2β-Hydroxybisnorcholic acid	0.66
17	2β-Hydroxynorcholic acid	0.98
18	2β-Hydroxycholic acid	1.36

RRT, relative retention time relative to methyl cholate-TMS derivative (1.00) for 17.98 min on 3% Poly I-110.

TABLE 3. Urinary bile acid profile of CTX patients

Compound Number ^a	Bile Acids ^b	Patient P			Patient Q			Patient R		
		U	A	G	U	A	G	U	A	G
<i>µg/l urine</i>										
	C₂₂									
1	5 β ,3 α ,12 α ,7-oxo	19			30			12		
2	5 β ,3 α ,7 α ,12-oxo	4			6			ND		
8	5 β ,3 α ,7 α ,12 α	116		27	88		22	30		16
13	5 β ,1 β ,3 α ,7 α ,12 α	19			16			ND		
16	5 β ,2 β ,3 α ,7 α ,12 α	4			8			9		
	C₂₃									
3	5 β ,3 α ,12 α ,7-oxo	48			26			9		
4	5 β ,3 α ,7 α ,12-oxo	ND			10			6		
9	5 α ,3 α ,7 α ,12 α	209			63			48		
10	5 β ,3 α ,7 α ,12 α	1216		10	554		20	336		6
14	5 β ,1 β ,3 α ,7 α ,12 α	19			28			6		
17	5 β ,2 β ,3 α ,7 α ,12 α	28			28			24		
	C₂₄									
5	5 β ,3 α ,12 α ,7-oxo	166			188			126		
6	5 β ,3 α ,7 α ,12-oxo	125			69			57		
7	5 β ,3 α ,7 β ,12 α	336	185		753			264	111	
11	5 α ,3 α ,7 α ,12 α	9	325		5	347		24	213	
12	5 β ,3 α ,7 α ,12 α	109			643	99		606	150	
15	5 β ,1 β ,3 α ,7 α ,12 α	ND			5			9		
18	5 β ,2 β ,3 α ,7 α ,12 α	6			13			33		
	Total	2433	510	223	2533	446	104	1599	474	22

Abbreviations: U, unconjugated bile acids; A, amidated bile acids; G, glucurono-conjugated bile acids; ND, not detected.

^aThese numbers correspond to those in Table 2.

^bConfigurations at C-5 and of hydroxyl groups are indicated by Greek letters; C₂₂, 23,24-dinorcholan-22-oic acid; C₂₃, 24-norcholan-23-oic acid; C₂₄, cholan-24-oic acid.

Bile acid 15 was identified as authentic 1 β -hydroxycholeic acid by comparison of the RRT and mass spectrum. In the mass spectrum, a series of fragment ions at *m/z* 636, 546, 456, and 366 resulted from the successive loss of the TMS groups from the molecular ion. The peaks at *m/z* 251 and 341 represent loss of side chain plus four and three nuclear TMS groups, respectively. The base peak at *m/z* 217 is derived by cleavage of the C-1,C-10 and C-3,C-4 bonds.

The mass spectra of the methyl ester-TMS derivative of bile acids 13 and 14 were closely similar to that of the corresponding derivative of bile acid 15, 1 β -hydroxycholeic acid, with respect to peak intensities and fragmentation patterns. The only difference was that the peaks in the spectra of bile acids 13 and 14 were shifted 28 and 14 mass units downfield, respectively. A series of fragment ions at *m/z* 622, 532, 442, and 352 in bile acid 14 and at *m/z* 608, 518, 428, and 338 in 13 resulted from the successive loss of the TMS groups from the molecular ion. The base peaks of both bile acids 13 and 14 were seen at *m/z* 217 as that of 1 β -hydroxycholeic acid. Fragments at *m/z* 251 and 341 observed for both bile acids 13 and 14 were due to the loss of side chain plus four and three TMS groups, respectively. The ratios of the RRTs of bile acids 13, 14, and 1 β -

hydroxycholeic acid were in good agreement with the constant separating factor of a series of C₂₂, C₂₃, and C₂₄ bile acid homologs. These results strongly indicate that bile acids 13 and 14 are 1 β -hydroxybisnorcholeic and 1 β -hydroxynorcholeic acids, respectively.

Bile acids 16 and 17 were identified as 2 β -hydroxybisnorcholeic and 2 β -hydroxynorcholeic acids, respectively. The mass fragmentation patterns of the methyl ester-TMS derivative of bile acids 16 and 17 were similar to that of bile acid 18, 2 β -hydroxycholeic acid. The only difference was that the peaks in the spectra of bile acids 16 and 17 were shifted 28 and 14 mass units downfield, respectively, suggesting the shortened methylene groups in the side chain. A series of fragments at *m/z* 622, 532, 442, and 353 in bile acid 17 and *m/z* 608, 518, 428, and 339 in bile acid 16 correspond to the successive loss of one, two, three, and four TMS groups from the steroid nucleus, respectively. The base peaks of both bile acids 16 and 17 were seen at *m/z* 147 as that of 2 β -hydroxycholeic acid. Fragments at *m/z* 251 and 341 were observed for both bile acids 16 and 17 as the loss of side chain plus four and three TMS groups, respectively. The ratios of the RRTs of bile acids 16 and 17 to that of 2 β -hydroxycholeic acid were 0.49 and 0.72, respectively. The GLC data also supported the

identification of bile acids 16 and 17 as the C₂₂ and C₂₃ homologs of 2β-hydroxycholeic acid.

Bile acids in the other fractions were analyzed by the same procedure used for the analysis of the unconjugated bile acids.

The results are shown in Table 3. No detectable amounts of bile acids were found in the solvolyzed bile acid fraction.

DISCUSSION

The bile acid profile in the urine of patients with CTX is characteristic of the presence of relatively large amounts of bile acids having a shortened side chain.

In the present study, five C₂₂ bile acids and six C₂₃ bile acids were identified in the urine of three CTX patients, amounting to 24–60% of the total urinary bile acids (Fig. 2). These C₂₂ and C₂₃ bile acids were present mainly as the unconjugated form and lesser amounts as the glucurono-conjugates, but not as the glycine- and taurine-conjugated forms. These findings were in agreement with previous studies (16, 21) that have shown that short side chain bile acids are poor substrates for the amino acid conjugating enzyme systems, and that non-bile acids are conjugated with glucuronic acid more rapidly than their corresponding C₂₄ bile acids in human liver microsomes.

Norcholeic acid (XIII) has been found in the urine of healthy persons, although in much less amounts. In normal humans, the C₂₃ bile acid (XIII) may be formed from

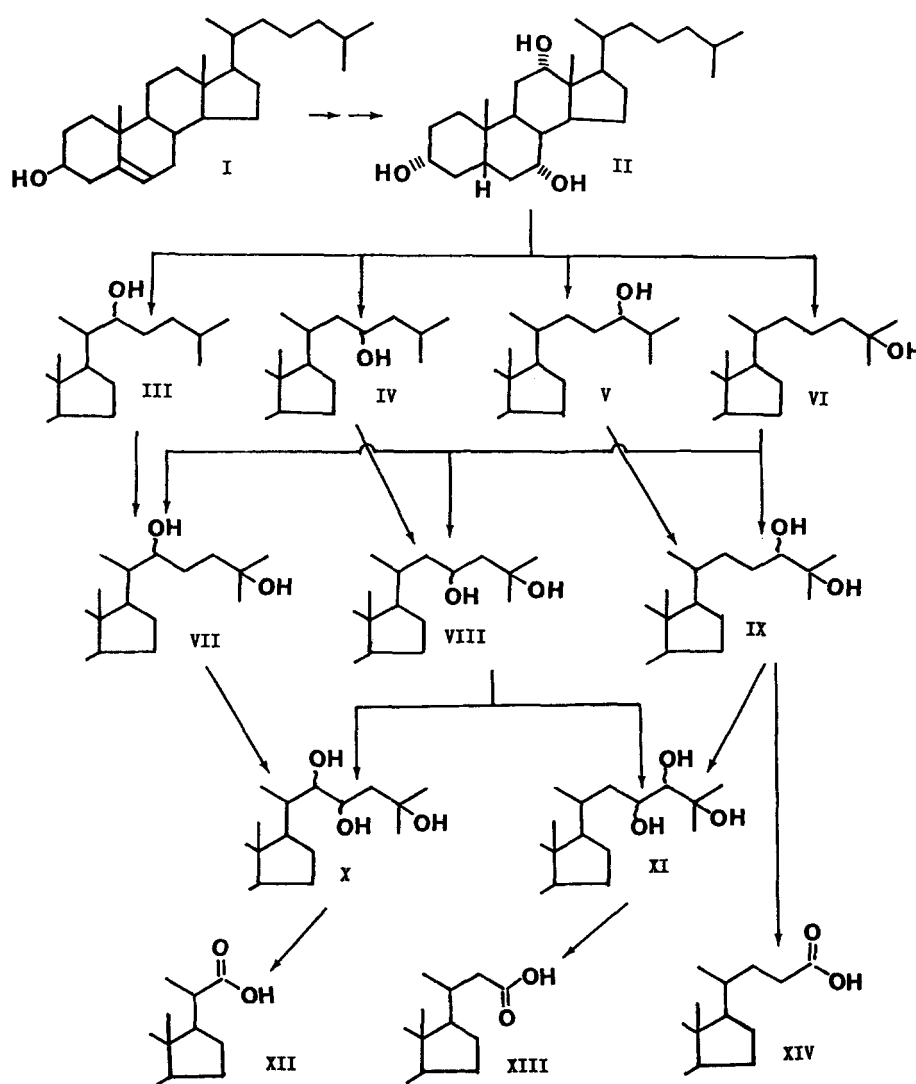


Fig. 2. Possible pathway for the formation of bile acids in CTX patients. I, cholesterol; II, 5β-cholestane-3α,7α,12α-triol; III, 5β-cholestane-3α,7α,12α,22-tetrol; IV, 5β-cholestane-3α,7α,12α,23-tetrol; V, 5β-cholestane-3α,7α,12α,24-tetrol; VI, 5β-cholestane-3α,7α,12α,25-tetrol; VII, 5β-cholestane-3α,7α,12α,22,25-pentol; VIII, 5β-cholestane-3α,7α,12α,23,25-pentol; IX, 5β-cholestane-3α,7α,12α,24,25-pentol; X, 5β-cholestane-3α,7α,12α,22,23,25-hexol; XI, 5β-cholestane-3α,7α,12α,23,24,25-hexol; XII, bisnorcholeic acid; XIII, norcholeic acid; XIV, cholic acid.

the corresponding C₂₄ bile acid, cholic acid (XIV) by the shortening of the side chain by one carbon atom (α -oxidation). It seems, however, unlikely that the major part of the norcholic acid excreted in considerable amounts in the urine of CTX patients is formed from cholic acid by α -oxidation, since the production of C₂₄ bile acids is below normal in this disease. We postulated, therefore, that the increased formation of norcholic acid (XIII) in CTX is ascribable to the degradation of some bile alcohols that accumulate in patients with this disease. Patients with CTX accumulate 5 β -cholestanepolyols (III–XI) having the cholic acid type of nuclear structure and the cholesterol type side chain carrying one, two, or three hydroxyl groups at C-22, C-23, C-24, and/or C-25, but not at C-26. It is well known that one of the accumulated bile alcohols, 5 β -cholestane-3 α ,7 α ,12 α ,24,25-pentol (X), is the biosynthetic precursor of cholic acid (XIV) in CTX. By analogy to the degradation of the 24,25-pentol (IX) to form cholic acid (XIV), the cleavage of the bond between C-23 and C-24 of 23,24-hydroxylated bile alcohols would directly lead to the formation of norcholic acid (XIII). The occurrence of 5 β -cholestane-3 α ,7 α ,12 α ,23,24,25-hexol (XI) in the urine and feces of a CTX patient has been reported previously from this laboratory (22). A lesser amount of bisnorcholic acid (XII) has been found in the bile of a CTX patient (23). The present study confirmed that CTX patients have the capacity to produce bisnorcholic acid and other C₂₂ bile acids. There are two possible pathways for the bisnorcholic acid production. First, the C₂₂ bile acid (XII) could be formed from the corresponding C₂₄ bile acid, cholic acid (XIV), by β -oxidation. Second, bisnorcholic acid (XII) could be formed by the cleavage of the bond between C-22 and C-23 of 5 β -cholestane-3 α ,7 α ,12 α ,22,23,25-hexol (X), which was recently identified in the feces of a CTX patient in this laboratory (Hatano, A., K. Kihira, and T. Hoshita, unpublished data). Although direct evidence for the relative importance of cholic acid (XIV) and the cholestanhexol (X) as the biosynthetic precursor of bisnorcholic acid (XII) is lacking, it seems probable that the pathway including the degradation of the 22,23-hydroxylated bile alcohol (X) is of greater quantitative importance, because of the decreased production of C₂₄ bile acids in CTX.

The present study demonstrates the presence of 1 β - and 2 β -hydroxylated derivatives of bisnorcholic, norcholic, and cholic acids in the urine of three CTX patients. Although the occurrence of 1 β - and 2 β -hydroxylated derivatives of common C₂₄ bile acids in biological fluids from healthy and diseased humans (24–27) has been known, this is the first demonstration of the natural occurrence of C₂₂ and C₂₃ bile acids having a 1 β - or 2 β -hydroxyl group. Hydroxylation at 1 β or 2 β of bile acids produces more polar metabolites with an increased ability for urinary excretion than the original bile acids. Thus,

the formation of these tetrahydroxy-C₂₂ and C₂₃ bile acids may reflect the operation of compensating mechanisms to the accumulation of unconjugated bisnorcholic and norcholic acids in CTX patients.

The present study is also the first demonstration of the natural occurrence of 7- and 12-oxo-derivatives of bisnorcholic and norcholic acids. These C₂₂ and C₂₃ keto bile acids are thought to be formed from bisnorcholic and norcholic acids by the action of either hepatic or bacterial enzymes that catalyze the dehydrogenation of 7 α - or 12 α -hydroxyl group to 7- or 12-oxo group. The occurrence of short side chain keto bile acids suggests that shortening the side chain of cholic acid does not affect the action of 7- and 12-hydroxysteroid dehydrogenases. ■

Manuscript received 16 April 1990 and in revised form 22 June 1990.

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