Occurrence of bile alcohols in the bile of a patient with cholestasis[1]

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Abstract Considerable amounts (6.0 μmol/ml of bile) of bile alcohols were found in the bile of a patient with cholestasis due to gallstones in a common bile duct. Acid hydrolysis of the bile salts followed by chromatographic separation yielded three bile alcohols, which were identified as 26,27-dinor-5β-cholestane-3α,7α,12α,24,25-pentol, 24-methyl-26,-27-dinor-5β-cholestane-3α,7α,12α,24-tetrol, and 3α,7α,12α-trihydroxy-26,27-dinor-5β-cholestan-24-one by comparison with synthetic reference standards.—**Kibe, A., S. Nakai, T. Kuramoto, and T. Hoshita.** Occurrence of bile alcohols in the bile of a patient with cholestasis. *J. Lipid Res.* 1980. **21:** 594–599.

Supplementary key words bile acids

Bile alcohols are not only the likely evolutionary precursors of bile acids but are also intermediates in bile acid biosynthesis. Although earlier studies (1) indicated that the distribution of bile alcohols in nature was confined to lower vertebrates such as fish and amphibians, it has recently been found that these polyhydroxylated steroids can also be detected in mammals, including humans. In patients with the rare inherited disease, cerebrotendinous xanthomatosis (CTX), relatively large amounts of bile alcohols are excreted in the bile and feces (2–5). Further, bile alcohols resembling those excreted by CTX patients are found in the bile of normal rabbits, although in much less quantity (6). It seems, therefore, that bile alcohols can be produced both in healthy and in diseased mammals. Such normally minor bile alcohols are thought to be side products arising by a deviation of the normal pathway for the bile acid biosynthesis, and could become prominent under certain pathological conditions.

In connection with studies of bile alcohols in CTX patients, we have examined the bile of patients with various hepatobiliary diseases to find out whether bile alcohols could accumulate in patients with impaired hepatic functions other than CTX. The present study describes the occurrence of bile alcohols in the gallbladder of a patient with cholestasis.

MATERIALS AND METHODS

Case history and patient material

A 65-year-old Japanese man was examined. He was admitted to the hospital because of jaundice and right hypochondralgia. He had none of the symptoms seen in CTX patients, such as cerebellar ataxia, corticospinal tract dysfunction, dementia, cataracts, and xanthomatosis. Quantitatively, no abnormalities were noted in serum lipids, such as total cholesterol, free fatty acids, and total lipids. The values of serum total bilirubin and conjugated bilirubin were 12.8 mg/dl and 11.4 mg/dl, respectively. Alkaline phosphatase was elevated but not aspartate aminotransferase and alanine aminotransferase. Percutaneous-transhepatic cholangiography showed remarkably dilated bile ducts and gallstones incarcerated in a common bile duct. Judging from his case history, the patient was cholestatic for about a month. Gallbaldder bile was obtained by needle aspiration during an abdominal operation. The bile sample was stored at −20°C until analysis.

Thin-layer chromatography (TLC)

TLC was performed on silica gel G (in a layer of 0.25 mm) using a 10% solution of phosphomolybdic acid in ethanol (spraying followed by heating) as detection reagent.

Acid hydrolysis of bile alcohol conjugates (7)

The bile sample (about 3 ml) was extracted with 10 volumes of ethanol at room temperature. The ethanol extract was evaporated to leave crude bile salts as a solid (120 mg).

The crude bile salts (50 mg) were dissolved in 3 ml of ethanol. Ethyl acetate (27 ml) equilibrated with 2M H_2SO_4 was added to the ethanol solution. The mixture was incubated at 37°C for 24 hr. After dilution with 30 ml of water, the incubation mixture was extracted with two 30-ml portions of n-butanol–ethyl acetate 1:1. The extracts were combined, and each was washed two times with water, 3% Na_2CO_3 solution, and water, and then dried over anhydrous Na_2SO_4, and evaporated to dryness, leaving a neutral residue (9 mg) containing "hydrolyzed" bile alcohols.

Separation and identification of bile alcohols

Separation of bile alcohols was carried out by preparative TLC using 1 mm layers of silica gel H. Samples were applied to the plates as a band using a semiautomatic sample streaker (Applied Science Laboratories Inc.). Bile alcohol bands were detected by exposure of the developed plates to iodine vapor. The bands corresponding to the R_f of the known standards, 26,27-dinor-5β-cholestane-3α,7α,12α,24,25-pentol, 24-methyl-26,27-dinor-5β-cholestane-3α,7α, 12α,24-tetrol and 3α,7α,12α-trihydroxy-26,27-dinor-5β-cholestan-24-one, were scraped off the plates and bile alcohols were eluted from the silica gel with methanol. The solvent system for preparative TLC was ethyl acetate–acetone 7:3 (v/v).

The bile alcohols thus isolated were converted to the trimethylsilyl (TMS) ethers, prepared with hexamethyldisilazane and trimethylchlorosilane in pyridine, and then analyzed by gas–liquid chromatography (GLC) and mass spectrometry. GLC was carried out on a Shimadzu GC-6A gas chromatograph using a glass column (2 m × 3 mm) packed with 2% OV-1, 3% OV-17, 2% Poly I-110, or 3% QF-1 on gas Chrom Q (80–100 mesh). Mass spectra were obtained by a JEOL D-300 combined gas chromatography–mass spectrometer with a glass column (1 m × 3 mm) packed with 2% OV-17. The following operating conditions were employed: ionization voltage, 70 eV; ionization current, 300 μA; accelerating voltage, 3000 V; column temperature, 280°C; ion source temperature, 270°C. The samples were analyzed as their TMS derivatives.

Detection of unconjugated bile alcohols

The crude bile salts (10 mg) were dissolved in 50 ml of water containing 3 ml of conc. NH_4OH, and 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 resulting residue, containing neutral lipids, was examined by TLC and GLC for the detection of unconjugated bile alcohols.

β-Glucuronidase treatment (8)

The crude bile salts (10 mg) were dissolved in 10 ml of 0.5 M acetate buffer (pH 5.0) containing 7500 units of β-glucuronidase (Sigma) and a drop of $CHCl_3$. The incubation period was 24 hr at 37°C. The incubation mixture was adjusted to pH 10 with 0.1 N KOH and extracted with four 30-ml portions of ethyl acetate. The extracts were combined, washed with water, and evaporated to dryness. The resulting residue was examined by TLC and GLC for the detection of "β-glucuronidase-liberated" bile alcohols.

Quantitative analysis of bile lipids

An aliquot of bile sample was directly applied to a silica gel G plate which was developed in chloroform–methanol–acetic acid–water 9:3:2:1 (v/v) or ethyl acetate–n-heptane 11:9 (v/v). Spots due to bile salts, lecithin, and cholesterol were visualized with the phosphomolybdic acid reagent, and their color intensities were estimated by direct densitometry using a Shimadzu CS-910 dual-wavelength chromatoscanner. Detailed technical descriptions have been presented elsewhere (9).

Hydrolysis of bile acid conjugates

The solution of the crude bile salts (20 mg) in 20 ml of 2 N NaOH was heated in a sealed tube at 110°C for 6 hr. The hydrolysate was acidified with 2 N HCl and extracted with four 20-ml portions of ether. The extracts were combined, washed with water, dried, and evaporated to dryness, leaving a residue (9.6 mg) consisting mainly of "deconjugated" bile acids.

Analysis of bile acids

The bile acid mixture was treated with ethereal diazomethane solution and the resulting methyl esters were silylated and analyzed by GLC using 2% OV-1, 3% OV-17, 2% Poly I-110, and 3% QF-1.

Reference compounds

26,27-Dinor-5β-cholestane-3α,7α,12α,24,25-pentol (mp, 188–189°C) (10), 24-methyl-26,27-dinor-5β-cholestane-3α,7α,12α,24-tetrol (mp, 129–131°C) (11), and 3α,7α,12α-trihydroxy-26,27-dinor-5β-cholestan-24-one (mp, 166–168°C) (12) were synthesized in this laboratory according to the methods previously described.

RESULTS

Two-dimensional development of TLC of the crude bile salts from a patient with cholestasis is shown **Fig. 1.** There were three unusual spots, A, B, and C, along
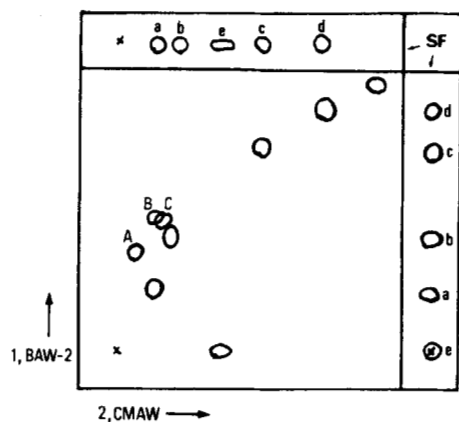
Fig. 1. Two-dimensional TLC of crude bile salts from a patient with cholestasis. Three unusual spots, A, B, and C, were found along with spots due to taurine-conjugated bile acids. Layer thickness 0.25 mm, silica gel G, solvent system BAW-2, n-butanol–acetic acid–water 85:10:5 (v/v); CMAW, chloroform–methanol–acetic acid–water 65:20:10:5 (v/v). X, origin; a, taurocholate; b, taurochenodeoxycholate; c, glycocholate; d, glycochenodeoxycholate; e, lecithin; SF, solvent front.

with spots due to conjugated bile acids, lecithin, and cholesterol. The mobilities of the unusual spots were close to those of known bile alcohol sulfates.

Acid hydrolysis of the crude bile salts gave a mixture of neutral steroids; GLC (**Fig. 2**) indicated the presence of three different bile alcohols, X_1 (59% of the total bile alcohol), X_2 (30%), and X_3 (11%). The mixture was subjected to preparative TLC (see Methods), yielding bands of R_f 0.09 (band X_1), R_f 0.23 (band X_2), and R_f 0.34 (band X_3). Bile alcohols X_1, X_2, and X_3 were obtained in chromatographically pure state from the
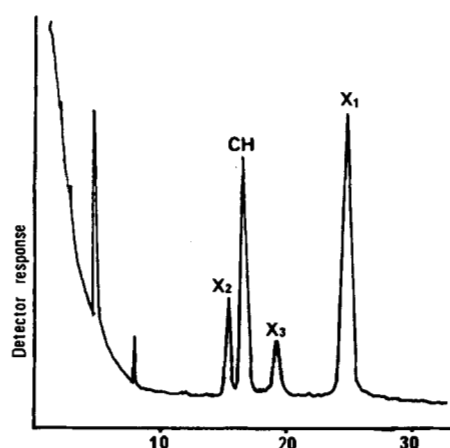

Fig. 2. GLC of the TMS ethers of neutral steroids obtained from crude bile salts after acid hydrolysis. Column, 3% OV-17; column temperature, 280°C. CH, cholesterol. X_1, X_2, and X_3 were identified as 26,27-dinor-5β-cholestane-3α,7α,12α,24,25-pentol, 24-methyl-26,27-dinor-5β-cholestane-3α,7α,12α,24-tetrol, and 3α,7α,12α-trihydroxy-26,27-dinor-5β-cholestan-24-one, respectively.

TABLE 1. Chromatographic data of the bile alcohols isolated from bile of a patient with cholestasis

Bile Alcohols[a]	Relative RT on GLC[b]				R_f Value on TLC	
	OV-17	OV-1	Poly I-110	QF-1	EA-2[c]	CE-2[d]
X_1	1.52	2.23	0.64	2.02	0.09	0.16
X_2	0.94	1.37	0.45	1.31	0.23	0.46
X_3	1.16	1.14	0.67	2.85	0.34	0.68

[a] Bile alcohols X_1, X_2, and X_3 were identified as 26,27-dinor-5β-cholestane-3α,7α,12α,24,25-pentol, 24-methyl-26,27-dinor-5β-cholestane-3α,7α,12α,24-tetrol, and 3α,7α,12α-trihydroxy-26,27-dinor-5β-cholestan-24-one, respectively.
[b] Bile alcohols were chromatographed as their TMS ethers.
[c] EA-2, ethyl acetate–acetone 7:3(v/v).
[d] CE-2, chloroform–ethanol 4:1(v/v).

bands X_1, X_2, and X_3, respectively. Chromatographic data of these bile alcohols are listed in **Table 1** and mass spectra are shown in **Fig. 3**.

The mobilities on TLC (two different solvent systems) and the relative retention times (RTs) on GLC (four different columns) of bile alcohol X_1 were identical with those of known 26,27-dinor-5β-cholestane-3α,7α,12α,24,25-pentol. Further, the mass spectrum of the TMS ether of bile alcohol X_1 (Fig. 3) was identical with that of the corresponding derivative of 26,27-dinor-5β-cholestane-3α,7α,12α,24α,25-pentol. Although the molecular ion was not seen, there was a series of fragments at m/e 694, 604, 514, 424, and 335, which arose from the consecutive loss of one to five of the TMS groups. Another series of fragments at m/e 681, 591, 501, 411, and 321 were produced as follows: the fragment at m/e 681 arose from splitting between the C-24 and C-25 carbon atoms with loss of m/e 103; the other fragments were due to the loss of one, two, three, and four molecules of trimethylsilanol from the 681 fragment. The ions at m/e 253 and 343 represent loss of the side chain plus three and two nuclear TMS groups, respectively. The base peak at m/e 129 is the side chain fragment resulting from the scission of the bond between the C-23 and C-24 carbon atoms followed by the loss of a TMS group.

The mass spectrum of the TMS ether of bile alcohol X_2 (Fig. 3) was identical with that of the TMS ether of known 24 - methyl - 26,27 - dinor - 5β - cholestane - 3α,7α,12α,24-tetrol. The base peak at m/e 131 is the side chain fragment resulting from the scission of the bond between the C-23 and C-24 carbon atoms. A series of peaks at m/e 620, 530, 440, and 350 results from the successive loss of the TMS groups from the molecular ion which could not be seen. The ions m/e 384 and 294 are attributed to the rupture of the bond between carbons 22 and 23 (loss of 145 amu) together with the transfer of a hy-
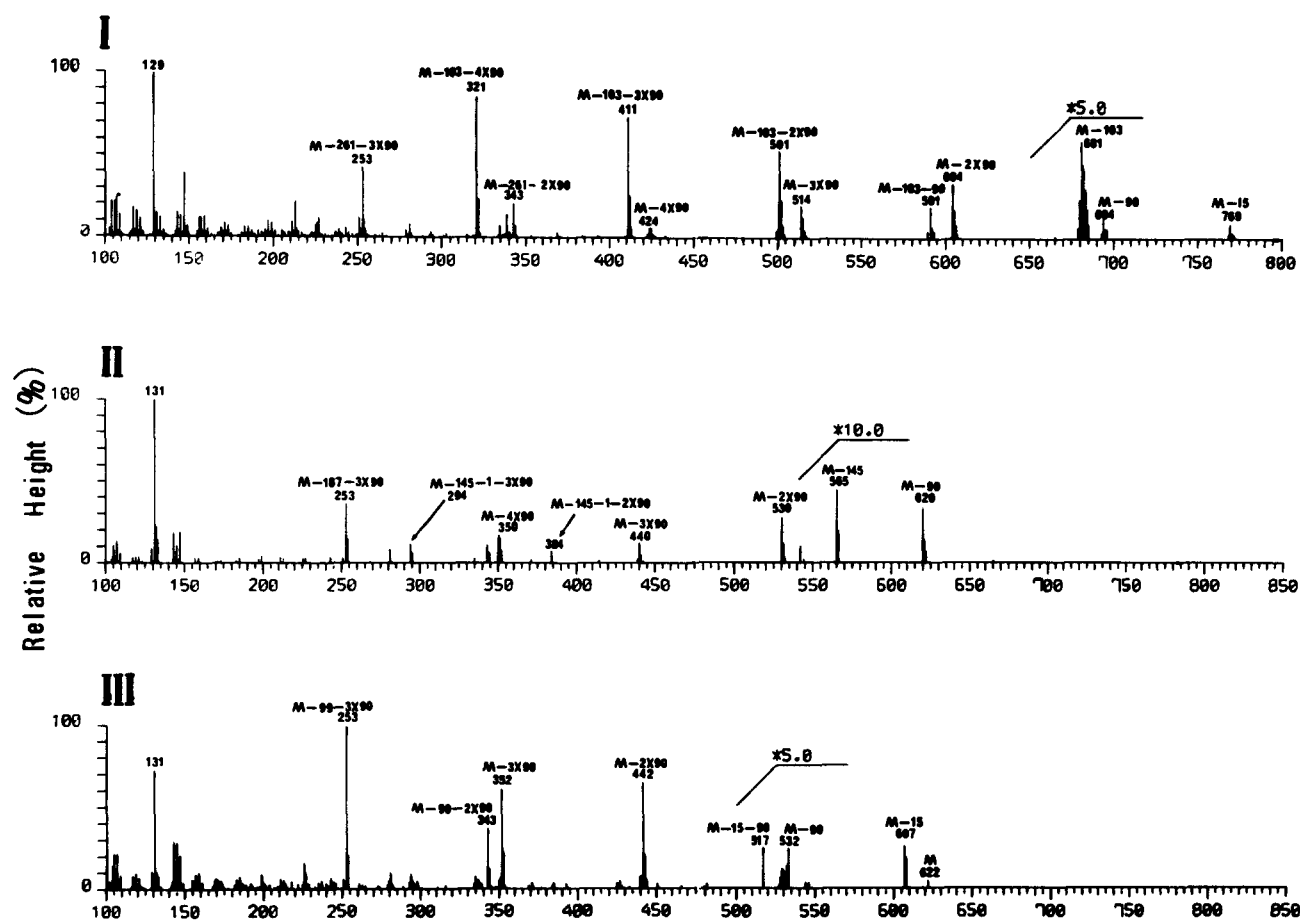
Fig. 3. Mass spectra of three bile alcohols (TMS ethers) isolated from the bile of a patient with cholestasis. Mass spectra I, II, and III were identical with those of 26,27-dinor-5β-cholestane-3α,7α,12α,24,25-pentol, 24-methyl-26,27-dinor-5β-cholestane-3α,7α,12α,24-tetrol, and 3α,7α,12α-trihydroxy-26,27-dinor-5β-cholestan-24-one, respectively. For detailed description see Results section.

drogen atom (1 amu) from the charged fragment and the loss of three and two nuclear TMS groups. The identity of bile alcohol X_2 and 24-methyl-26,27-dinor-5β-cholestane-3α,7α,12α,24-tetrol was further established by direct comparison of their chromatographic properties.

The mass spectrum of the TMS ether of bile alcohol X_3 (Fig. 3) was identical with that of the TMS ether of known 3α,7α,12α-trihydroxy-26,27-dinor-5β-cholestan-24-one. The molecular ion was seen at m/e 622. There were two series of fragments, one at m/e 532, 442, 352, and a second at m/e 607, 517, 427, and 337. The former series are formed by the successive loss of the TMS groups from the molecular ion. The latter series resulted from the loss of the C-25 methyl group and the consecutive loss of one, two, and three TMS groups. Bile alcohol X_3 had the same R_f values and relative RTs when subjected to TLC and GLC as the synthetic keto bile alcohol. All of the evidence established that the bile alcohol X_3 is 3α,7α,12α-trihydroxy-26,27-dinor-5β-cholestan-24-one.

The crude bile salt was examined for unconjugated bile alcohols as described above. No significant quantities were found.

In order to check the presence of bile alcohol glucuronides, the crude bile salt was treated with β-glucuronidase according to the procedure previously described (8). No significant quantities of "β-glucuronidase-liberated" bile alcohols were formed by this treatment.

Quantitative analysis of the bile salts, lecithin, and cholesterol, was carried out by TLC and direct densitometry (9). When developed on a silica gel plate with chloroform–methanol–acetic acid–water, bile alcohol conjugate A, bile alcohol conjugates B/C, taurocholate, taurodeoxycholate/taurochenodeoxycholate, lecithin, glycocholate and glycodeoxycholate/glycochenodeoxycholate were separated with R_f values of 0.07, 0.15, 0.26, 0.35, 0.61, 0.76, and 0.89, respectively. Cholesterol migrated with an R_f of 0.42 on another plate developed in ethyl acetate–n-heptane. The results are shown in **Table 2.** The molar percent ratio of bile

TABLE 2. Quantitative determination of bile lipid composition by densitometry[a]

Bile Components	μmol/ml Bile
Bile acids	
Taurocholate	4.68
Taurochenodeoxycholate/Taurodeoxycholate	5.14
Glycocholate	6.41
Glycochenodeoxycholate/Glycodeoxycholate	5.47
Bile alcohols	
A	3.54
B/C	2.49
Lecithin	4.63
Cholesterol	3.39

[a] The direct densitometric measurements were performed as described previously (9), using TLC and a dual-wavelength chromatoscanner.

acids, bile alcohols, lecithin, and cholesterol was 60.7%, 16.9%, 13.0%, and 9.5%, respectively.

The identification and quantitation of bile acids obtained after hydrolysis of the bile salts were carried out by GLC. The results are shown in **Table 3.** Both cholic acid and chenodeoxycholic acid were major bile acids in this patient.

DISCUSSION

In the bile of a patient with cholestasis caused by gallstones incarcerated in a common bile duct, we have found considerable amounts (6.0 μmol/ml of bile) of the following bile alcohols: 26,27-dinor-5β-cholestane-3α,7α,12α,24,25-pentol; 24-methyl-26,27-dinor-5β-cholestane-3α,7α,12α,24-tetrol; 3α,7α,12α-trihydroxy-26,27-dinor-5β-cholestan-24-one. These three bile alcohols accounted for, respectively, 59%, 30%, and 11% of total bile alcohols. Although there have been several reports of the appearance of unusual bile acids, i.e., 3β-hydroxy-5-cholenoic and 3α-hydroxy-5α-cholanoic acids (13) and 3α,7α,12α-trihydroxy-5β-cholestanoic acid (14), in patients with cholestasis due to biliary atresia, this is the first case for the accumulation of the bile alcohols in patients with cholestasis due to gallstones in a common bile duct.

There were some differences between bile salts of the present patient and those of the CTX patients. First, the side chain structure of the bile alcohols found in the present patient differed from those excreted by the CTX patients, though the nuclear structure of bile alcohols in both patients was identical to each other and to that of cholic acid. The biliary and fecal bile alcohols of the CTX patients were 5β-cholestane-polyols (2–5), which are believed to be formed from cholesterol without loss of carbon atoms (2). Some of them could be metabolized into cholic acid with

cleavage of the terminal three carbon atoms (15). On the other hand, the biliary bile alcohols of the present patient were C_{25} and C_{26} steroids containing the unusual side chain. The two C_{25} bile alcohols have not yet been identified in any other natural source, although the C_{26} bile alcohol has been identified only as a minor constituent of normal rabbit bile (6). It is not certain from the data currently available whether the C_{25} and C_{26} bile alcohols were formed from cholesterol or whether these bile alcohols can be degraded to cholic acid. According to present knowledge (16), the side chain oxidation of cholesterol in the biosynthetic pathway for cholic acid is thought to proceed via 5β-cholestane-3α,7α,12α,26-tetrol and 3α,7α,12α-trihydroxy-5β-cholestanoic acid. Further metabolism of the C_{27} bile acid then proceeds via β-oxidation resulting in the formation of cholic acid (C_{24}) plus the C_3 fragment, propionic acid. It is hardly conceivable that the C_{25} and C_{26} bile alcohols are the intermediates in the normal pathway for the cholic acid synthesis, because the biosynthetic pathway of these bile alcohols from cholesterol must include reactions involving the loss of one or two carbon atoms.

The second difference between the bile alcohols of the present patient and the CTX patients was the presence of conjugation. In contrast to the CTX patients, in whom the bile alcohols occur as the glucuronides,[5] no bile alcohol glucuronides were detected in the bile of the present patient. In the present study, "deconjugated" bile alcohols were obtained from the bile salts by the treatment with sulfuric acid but not with β-glucuronidase. Based on the chromatographic properties, the bile alcohols of the present patient seemed to be excreted as sulfate esters, though direct evidence is lacking.

Third, bile acid production in CTX was subnormal

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[5] Hoshita, T., M. Yasuhara, M. Une, A. Kibe, E. Itoga, S. Kito, and T. Kuramoto. Occurrence of bile alcohol glucuronides in bile of a patient with cerebrotendinous xanthomatosis. Submitted to *J. Lipid Res.*

TABLE 3. Bile acid composition in bile[a]

Bile Acids	Composition (% total)
Cholic acid	47.2
Chenodeoxycholic acid	46.8
Hyodeoxycholic acid	2.1
Ursodeoxycholic acid	2.0
Deoxycholic acid	1.2
Allocholic acid	0.7
Lithocholic acid	<0.1

[a] Identification was carried out by direct comparison with authentic samples by GLC, using 2% OV-1, 3% OV-17, 2% Poly I-110, and 3% QF-1. Quantification was performed by 2% Poly I-110.

and biliary bile acid composition was also abnormal with cholic acid accounting for over 80% of the total bile acid (17). In the present patient, the concentration of bile acids in the bile was approximately 18 mg/ml, and cholic acid and chenodeoxycholic acid accounted for 47.2% and 46.8% of the total bile acid, respectively. These figures are comparable with those of normal subjects. It might be postulated that in the present patient the normal pathway for bile acid production is still operative.

Fourth, bile alcohols have been found in all of the CTX patients examined, but no bile alcohol has been found in other cases with cholestasis.[6] It seems unlikely, therefore, that cholestasis by itself was the primary cause of the accumulation of bile alcohols. It also remains to be established whether the occurrence of bile alcohols could have contributed to the cholesterol gallstone formation. The molar percent ratio of the composition of bile acids/bile alcohols, lecithin, and cholesterol in the present patient was 77.6%, 13.0%, and 9.5%, respectively, and the lithogenic index calculated according to the data relating total lipid concentration to lithogenicity (18) was 2.7, indicating that the bile of this patient was hypersaturated with cholesterol and thus lithogenic.

The present study demonstrated that the accumulation of bile alcohols can be a specific feature of some cases with cholestasis or hepatic dysfunction. Of course, more cases will have to be studied to elucidate the mechanism of the formation of these bile alcohols and the possible role of these compounds.▮▮

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