Characterization of serum and urinary bile acids in patients with primary biliary cirrhosis by gas-liquid chromatography-mass spectrometry: effect of ursodeoxycholic acid treatment

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Abstract We have studied the effect of ursodeoxycholic acid on the serum and urinary bile acids in seven patients with moderate to severe primary biliary cirrhosis. Bile acids were characterized by gas-liquid chromatography-mass spectrometry and quantified by capillary gas-liquid chromatography. Serum bile acids were elevated 26-fold over control values, with 2.2 times more cholic acid than chenodeoxycholic acid. Urinary bile acid output was elevated 22-fold over control values with a cholic acid:chenodeoxycholic acid ratio of 1.6. In addition, lithocholic acid, deoxycholic acid, ursodeoxycholic acid, 1β-hydroxycholic acid, 1β-hydroxydeoxycholic acid, and hyocholic acid were identified in both serum and urine; the proportions of the 1- and 6-hydroxylated bile acids were much higher in urine than in serum of the patients (32.1% versus 4.2%). Three months of placebo administration did not change the serum and urinary bile acid composition. In contrast, ursodeoxycholic acid feeding (12-15 mg/kg body weight per day) for 6 months resulted in a 25% decline in the total serum bile acid concentration from the pretreatment values. The proportion of ursodeoxycholic acid increased from 2.1 to 41.2% of total bile acids, so that total fasting serum endogenous bile acid levels decreased 62.4%. Ursodeoxycholic acid feeding substantially increased urinary bile acid output, with ursodeoxycholic acid comprising 58.1%. The proportion of 1- and 6- hydroxylated endogenous bile acids was reduced by 45.5% from pretreatment levels and approximately 4.5% of the urinary bile acids were ω-muricholic acid, 1β-hydroxyursodeoxycholic acid, and 21-hydroxyursodeoxycholic acid. These results demonstrate significant changes in the serum and urinary bile acid pattern in primary biliary cirrhosis during ursodeoxycholic acid treatment. The beneficial effect of ursodeoxycholic acid may be due to reduction of the hydroxylated derivatives of endogenous bile acids together with the appearance of hydroxylated derivatives of ursodeoxycholic acid or it may be due to displacement of the more hydrophobic endogenous bile acids by the hydrophilic ursodeoxycholic acid. —**Batta, A. K., R. Arora, G. Salen, G. S. Tint, D. Eskreis, and S. Katz.** Characterization of serum and urinary bile acids in patients with primary biliary cirrhosis by gas-liquid chromatography-mass spectrometry: effect of ursodeoxycholic acid treatment. *J. Lipid Res.* 1989. **30:** 1953-1962.

Supplementary key words 1β-hydroxy bile acids • ω-muricholic acid

Primary biliary cirrhosis is a chronic cholestatic liver disease characterized by destruction of interlobular bile ducts, which leads to cirrhosis and often death from liver failure. The exact mechanism underlying the development of this disease is not clear; however, it is considered that alterations in the immune functions lead to the initiation of bile duct damage (1). Damaged bile ducts result in impaired hepatic clearance of bile acids which is associated with increased concentration of bile acids in the blood and liver and an increased urinary excretion (2-5). Several abnormalities in bile acid metabolism have been noted in this disease (6-15), the most remarkable being a decreased cholic acid synthesis with progression of the disease (6).

It was shown that, in the rat, ursodeoxycholic acid administration may prevent or reduce hepatic damage and cholestasis induced by high concentrations of other bile acids (16-18). In a recent study, Poupon et al. (19) have examined the effect of replacing the endogenous bile acids by ursodeoxycholic acid in patients with primary biliary

Abbreviations and trivial names: UDCA, ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholan-24-oic acid; iso-ursodeoxycholic acid, 3β,7β-dihydroxy-5β-cholan-24-oic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; nor-cholic acid, 24-nor-3α,7α,12α-trihydroxy-5β-cholan-23-oic acid, lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholan-24-oic acid; hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholan-24-oic acid; ursocholic acid, 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid; hyocholic acid, 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid; ω-muricholic acid, 3α,6α,7β-trihydroxy-5β-cholan-24-oic acid; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; MS, mass spectrometry; TMSi, trimethylsilyl.

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cirrhosis and found improvement in liver function tests after 2 years of treatment. However, the metabolism of this bile acid and its effect on the endogenous bile acids in this disease are not known. In the present study, we have examined the effect of ursodeoxycholic acid on the urinary and serum bile acids in patients with primary biliary cirrhosis and we have characterized several unusual metabolites of ursodeoxycholic acid in the serum and urine of these patients with the help of gas-liquid chromatography-mass spectrometry.

MATERIALS AND METHODS

Materials

Sep-pak C_{18} cartridges were purchased from Waters Associates (Milford, MA). The acetone powder of cholylglycine hydrolase [from *C. perfringens (welchii)*] and β-glucuronidase (type H-1, with approximately 10% sulfatase activity; from *Helix pomatia*) were from Sigma Chemical Co. (St. Louis, MO). Ursodeoxycholic acid was a gift from Ciba-Geigy (Summit, NJ). The bile acids used as standards for GLC and thin-layer chromatography were purchased from Steraloids, Inc. (Wilton, NH). Iso-ursodeoxycholic acid (3β,7β-dihydroxy-5β-cholan-24-oic acid) and ω-muricholic acid were synthesized as described in the literature (20, 21).

Thin-layer chromatography

The TLC of the serum and urinary bile acids was performed on silica gel O plates (Analabs, New Haven, CT) in a solvent system of chloroform-isopropanol-acetic acid- water 30:30:4:1 (solvent system A) (22) or in chloroform-methanol-acetic acid 40:2:1 (solvent system B). Spots were visualized by spraying the appropriate portions of the plate with 10% H_2SO_4 followed by a solution of 3.5% phosphomolybdic acid and then heating at 110°C for 2 min.

Gas-liquid chromatography

The various bile acids in the serum and urine were methylated with methanolic HCl and the methyl esters were silylated with 100 μl of Sil-Prep (Alltech Associates, Inc., Deerfield, IL) for 30 min at 55°C. After evaporation of solvents under N_2, the silyl ethers were dissolved in 100 μl of hexane and 1-5 μl was injected into a Hewlett-Packard 5880A gas chromatograph equipped with a split/splitless device for capillary columns. A fused silica CP-Sil-5 CB or CP-Sil-19 CB capillary column (25 m), i.d. 0.20-0.22 mm, was used and helium was used as the carrier gas. The GLC operating conditions for both columns were as follows. Injector and detector temperatures were 260°C and 290°C, respectively. After injection, the oven temperature was kept at 100°C for 2 min, then programmed at a rate of 35°C/min to a final temperature of 265°C. The retention times of the bile acids (**Table 1**) were reproducible and for amounts of bile acids ranging from 4 ng to 120 ng injected onto the column, the detector response, as shown by the integrator, was linear. The response factors of the various standard bile acids were found to be similar and the correction factor corresponding to the peak area of 5α-cholestane was of the order of 1.3. This correction factor was also used for the quantitation of bile acids in the serum or urine for which standards were not available.

Gas-liquid chromatography-mass spectrometry

The mass spectra of the various bile acids in the biological fluids were obtained on a Hewlett-Packard model 5988 capillary gas-liquid chromatograph-mass spectrometer (Paramus, NJ) operating in the electron impact mode with an energy of 70 electron volts. The GLC operating conditions were identical to those described above.

Clinical

Studies were conducted in seven women with primary biliary cirrhosis. Their clinical, biochemical, and histological data were described earlier (23). Patients were on no medication at least 1 month prior to ursodeoxycholic acid administration. For the first 3 months, each patient received three placebo capsules daily followed by ursodeoxycholic acid at a dose of 12-15 mg/kg body weight per day (300 mg capsules, three capsules per day). Each patient was evaluated before entering the trial and every month thereafter. Serum samples were analyzed for liver function tests by automated techniques.

TABLE 1. GLC retention times of the methyl ester-trimethylsilyl ether derivatives of some bile acids

Bile Acid	GLC (RRT)[a]
Nor-cholic acid	1.31
Lithocholic acid	1.34
Deoxycholic acid	1.46
Chenodeoxycholic acid	1.50
Cholic acid	1.53
Hyodeoxycholic acid	1.54
Ursodeoxycholic acid[b]	1.59
Iso-ursodeoxycholic acid[b]	1.59
Ursocholic acid	1.65
Hyocholic acid	1.72
ω-Muricholic acid	2.08

A fused silica CP-Sil-5 CB capillary column (25 m × 0.22 mm) was used for GLC. Chromatographic conditions: injector temperature, 260°C; detector temperature, 290°C; column temperature, 100°C for 2 min, then programmed at a rate of 35°C/min to a final temperature of 265°C. Helium was used as the carrier gas at a flow rate of 1.5 ml/min.

[a]Retention time of 5α-cholestane, 11.65 min.

[b]Relative retention times for ursodeoxycholic acid and iso-ursodeoxycholic acid were 1.80 and 1.82, respectively, when injected into a fused silica CP-Sil-19 CB capillary column (25 m × 0.22 mm) under identical chromatographic conditions as above. When this column was used, the retention time of 5α-cholestane was 11.56 min.

Sample collection

Fasting serum samples were collected before and during treatment with ursodeoxycholic acid and refrigerated until used for analysis. Urine (50–100 ml) was collected and refrigerated until used for analysis.

Analysis of serum and urinary bile acids

Serum (0.5–1 ml) or urine (1–2 ml) was passed through a pre-washed C_{18} Sep-pak cartridge and the bile acids were eluted with methanol. After evaporation of the solvent, the residue was subjected to hydrolysis with cholylglycine hydrolase and β-glucuronidase in acetate buffer at pH 5.0 for 36 h at 37°C as previously described (24). The liberated bile acids were isolated by passing the incubation mixture through a second Sep-pak and eluting with methanol. Bile acids were methylated with methanolic HCl and subjected to GLC (or GLC–MS) as the trimethylsilyl ether derivatives.

For recovery purposes, ^{14}C-labeled taurocholate was added to selected serum and urine samples and the entire procedure, excepting derivatization and GLC, was repeated. Radioactivity was determined in the recovered bile acids. It was found that 80–85% radioactivity was recovered after the hydrolysis procedure.

Urinary sulfated and nonsulfated bile acids

In order to determine the sulfated and nonsulfated bile acids in the urine, 10 ml of pretreatment urine or urine during ursodeoxycholic acid therapy was subjected to Sep-pak treatment. The concentrated bile acids were applied as a band on a TLC plate. Free cholic acid, glycolithocholate, glycocholate, taurolithocholate, and taurocholate were applied on the side of the plate and the plate was developed in solvent system A. Four bands were scraped from the plate corresponding roughly to the free bile acids [band from the R_f value of cholic acid up to the solvent front (fraction 1)], glycine-conjugated bile acids [band from the R_f value of glycocholate up to the R_f value of glycolithocholate (fraction 2)], taurine-conjugated bile acids [band from the R_f value of taurocholate up to the R_f value of taurolithocholate (fraction 3)], and the sulfated-conjugated bile acids [band from the origin up to just below the R_f value of taurocholate (fraction 4)]. The bile acids in each fraction were eluted with methanol–ethyl acetate 1:1 and the solvents were evaporated. Since the fractions eluted from the plate could contain sulfated or glucuronidated bile acids also, the residue obtained from each fraction was divided in half, one portion was subjected to treatment with β-glucuronidase and the other portion was subjected to β-glucuronidase plus cholylglycine hydrolase treatment (24). The liberated bile acids were isolated from the incubation mixture by passing through Sep-pak followed by methanol elution as described above. The bile acids were then methylated with methanolic HCl and subjected to GLC as the trimethylsilyl ether derivatives.

Iso-ursodeoxycholic acid in the serum and urine

Serum (2 ml) or urine (10 ml) from two patients who were fed ursodeoxycholic acid for 1 month was passed through Sep-pak and the concentrated bile acids were applied as a band on a TLC plate. The plate was developed in solvent system A as described above and the four bands were scraped from the plate and products were eluted and hydrolyzed exactly as described above. The residue from each hydrolysate was rechromatographed using solvent system B. Standards of ursodeoxycholic acid and iso-ursodeoxycholic acid were applied on the side of the plate. Ursodeoxycholic acid and iso-ursodeoxycholic acid did not show good separation in the solvent system (R_f value of ursodeoxycholic acid, 0.70 and that of iso-ursodeoxycholic acid, 0.72), and the band corresponding to the mixture of the two bile acids was scraped from the TLC plate and the compounds were eluted with methanol. After evaporation of methanol, ursodeoxycholic acid and iso-ursodeoxycholic acid in each fraction were characterized as the methyl ester trimethylsilyl ether derivatives by GLC on a fused silica CP-Sil-19 CB capillary column (25).

RESULTS

Clinical

All patients had severe pruritus, which persisted during the placebo period but was abolished in four patients and was significantly suppressed in the other three patients when ursodeoxycholic acid was administered for 6 months. All had elevated serum alkaline phosphatase, serum glutamic-oxalo transaminase, and serum glutamic-pyruvic transaminase levels (4.3, 5, and 3 times, respectively, above control values). The liver enzyme levels also persisted during the 3 months of placebo period but were significantly lowered (by 34%, 40%, and 30%, respectively) after 6 months of ursodeoxycholic acid. The details of the clinical and biochemical data before and during ursodeoxycholic acid treatment have been described elsewhere (23).

Characterization of serum and urinary bile acids

Fig. 1a and **Fig. 2a** show the gas–liquid chromatograms of the methyl ester-trimethylsilyl ether derivatives of the serum and urinary bile acids in a patient with primary biliary cirrhosis (patient F. I.). Several peaks were found in both spectra, but the GLC pattern of the urinary bile acids was more complex (Fig. 2a). Most of the major bile acids could be characterized from their mass spectra. Thus, the mass spectra of GLC peaks 1–7 were completely compatible with the methyl ester-trimethylsilyl ether

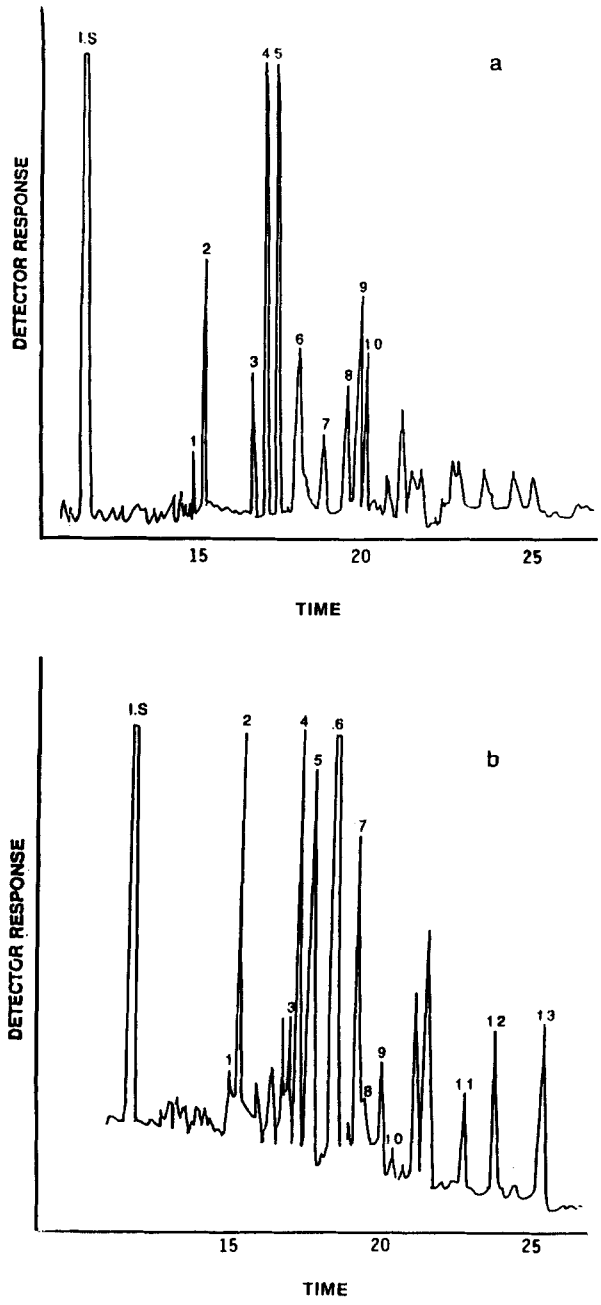

Fig. 1. Capillary GLC profile of the urinary bile acids of a patient with primary biliary cirrhosis. Fig. 1a, before treatment; Fig. 1b, after ursodeoxycholic acid treatment. The methyl ester-trimethylsilyl ether derivatives of the bile acids were separated on fused silica capillary CP-Sil 5 CB column, using the conditions described in Methods. The peak numbers denote the following compounds as identified by GLC-MS: peak 1, nor-cholic acid; 2, lithocholic acid; 3, deoxycholic acid; 4, chenodeoxycholic acid; 5 cholic acid; 6, ursodeoxycholic acid; 7, ursocholic acid; 8, 1β,3α,12α-trihydroxy-5β-cholan-24-oic acid; 9, hyocholic acid; 10, 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid; 11, 1β,3α,7β-trihydroxy-5β-cholan-24-oic acid; 12,ω-muricholic acid; 13,21- hydroxyursodeoxycholic acid.

derivatives of nor-cholic acid, lithocholic acid, deoxycholic acid, chenodeoxycholic acid, cholic acid, ursodeoxycholic acid, and ursocholic acid, respectively. The GLC peak 9 was confirmed as hyocholic acid. The mass spectra of GLC peaks 8 and 10 (**Fig. 3**) showed the base ion fragment at *m/z* 217 characteristic of 1,3-dihydroxy bile acid (26, 27). Fragment ions at *m/z* 548 (M^+-TMSi), *m/z* 458 (M^+-2 × TMSi), *m/z* 368 (M^+-3 × TMSi), and *m/z* 253 suggested the structure of peak 8 as 1β-hydroxydeoxycholic acid (28). The mass spectra of the methyl ester-trimethylsilyl ethers

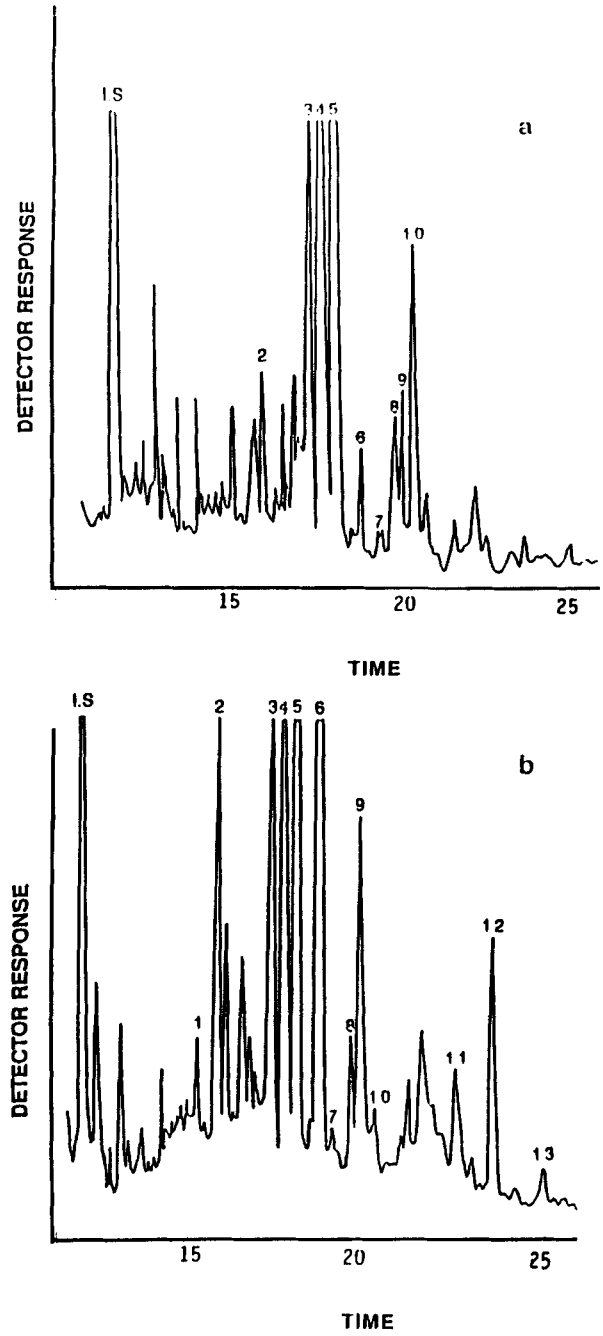

Fig. 2. Capillary GLC profile of the serum bile acids of a patient with primary biliary cirrhosis. Fig. 2a, before treatment; Fig. 2b, after ursodeoxycholic acid treatment. For GLC conditions and peak identification, see legend to Fig. 1.

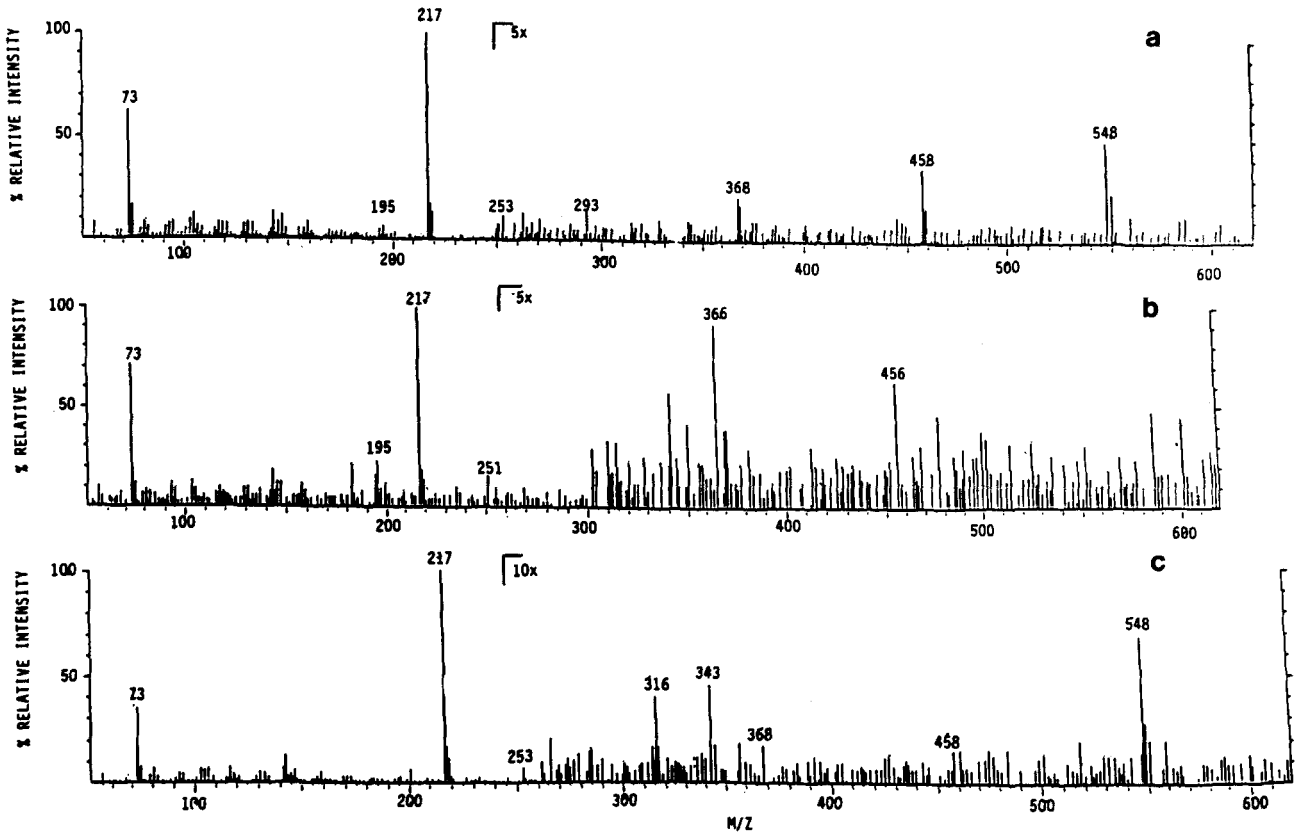

Fig. 3. Mass spectra of GLC peaks 8, 10, and 11. Peak 8 was identified as 1β,3α,12α-trihydroxy-5β-cholan-24-oic acid, peak 10 was identified as 1β,3α, 7α,12α-tetrahydroxy-5β-cholan-24-oic acid, and peak 11 was identified as 1β,3α,7β-trihydroxy-5β-cholan-24-oic acid.

of 1β-hydroxychenodeoxycholic acid and 1β-hydroxydeoxycholic acid have been reported in literature (28). Both compounds have very similar mass spectra, only the fragment ion at *m/z* 548 (M^+–TMSi) is more prominent in the mass spectrum of the 1β-hydroxydeoxycholic acid derivative, while the fragment ion at *m/z* 368 (M^+–3 × TMSi) is more prominent in the mass spectrum of 1β-hydroxychenodeoxycholic acid derivative. Since the fragment ion at *m/z* 548 is more prominent in the mass spectrum of GLC peak 8, we have assigned the structure of 1β-hydroxydeoxycholic acid to this compound. The fragment ions at *m/z* 456 (M^+–3 × TMSi), *m/z* 366 (M^+–4 × TMSi), and *m/z* 251 confirmed the structure of GLC peak 10 as 1β-hydroxycholic acid (28). **Table 2** shows the amounts of the completely and tentatively identified bile acids in the urine of patients with primary biliary cirrhosis. Thus, cholic acid was excreted in larger amounts as compared with chenodeoxycholic acid and 1β-hydroxycholic acid was found in larger amounts as compared with 1β-hydroxydeoxycholic acid. Similar to the previous reports in patients with cholestatic liver disease (9, 29), small amounts of deoxycholic acid, ursodeoxycholic acid, hyocholic acid, ursocholic acid, nor-cholic acid, and lithocholic acid were also excreted in the urine of patients with primary biliary cirrhosis. The serum bile acid pattern in these patients is shown in **Table 3**. Again, more cholic acid was found in the serum than chenodeoxycholic acid and the ratio between cholic acid and chenodeoxycholic acid in the serum was higher than in the urine (2.2 versus 1.6). In contrast, a significantly larger proportion of the polyhydroxylated bile acids was found in the urine than in the serum (29; Tables 2 and 3).

Urinary and serum bile acids after feeding ursodeoxycholic acid

The urinary as well as serum bile acid concentrations remained virtually unchanged during the 3-month placebo period; this suggested the constancy of the abnormal bile acid pattern in this disease (Tables 2 and 3). However, during ursodeoxycholic acid treatment, the urinary excretion pattern as well as the serum bile acid pattern changed significantly (Tables 2 and 3). The total excretion of bile acids in the urine increased greatly after ursodeoxycholic acid feeding; however, ursodeoxycholic acid constituted 58.1% of the total urinary bile acids. The amounts of cholic acid and chenodeoxycholic acid actually decreased from their pretreatment values.

TABLE 2. Urinary bile acids in patients with primary biliary cirrhosis before and after ursodeoxycholic acid treatment[a]

Bile Acid	Pretreatment[a]	UDCA Treatment[b]
	μmol/g creatinine	
Nor-cholic acid	0.2 (tr-0.5)[c]	0.1 (tr-0.3)
Lithocholic acid	2.9 (0.9-4.6)	10.1 (3.7-21.6)
Deoxycholic acid	4.5 (1.0-11.7)	2.9 (0.6-7.7)
Chenodeoxycholic acid	12.5 (1.3-26.3)	9.9 (0.4-29.8)
Cholic acid	20.3 (1.2-58.6)	17.3 (1.0-64.5)
Hyodeoxycholic acid	tr	tr
Ursodeoxycholic acid	2.6 (0.5-8.7)	111.1 (6.3-477.2)
Iso-ursodeoxycholic acid	0.4 (tr-1.5)	10.1 (0.8-40.2)
Ursocholic acid	0.8 (tr-3.0)	9.7 (1.2-24.7)
1β,3α,12α-Trihydroxy-5β-cholanoic acid	4.3 (1.0-9.0)	3.9 (1.6-8.2)
Hyocholic acid	10.2 (1.8-20.5)	5.2 (1.4-12.1)
1β,3α,7α,12α-Tetrahydroxy-5β-cholanoic acid	6.4 (1.3-9.6)	2.3 (tr-5.5)
1β,3α,7β-Trihydroxy-5β-cholanoic acid	N.D.	1.9 (0.2-4.8)
ω-Muricholic acid	N.D.	5.6 (0.7-14.9)
3α,7β,21-Trihydroxy-5β-cholanoic acid	N.D.	1.2 (0.1-3.1)
Total bile acids	65.1	191.3
Control[d]	3.0	49.0

The urinary bile acids were quantitated by gas-liquid chromatography. For details of the analysis, see Methods; tr, trace, less than 0.1 μmol/g creatinine; N.D., not detected.

[a]Early morning urine was obtained during the pretreatment period and every month during the placebo period. The relative proportions of the urinary bile acids were the same during the pretreatment and placebo periods, and an average of the pretreatment and placebo urinary bile acids is shown in the Pretreatment column.

[b]Early morning urine was obtained every month during administration of ursodeoxycholic acid and an average of the bile acids during the last 3 months is shown.

[c]The urinary bile acids reported represent a mean for seven patients. The range of the amounts of the various bile acids obtained in these patients is shown in parentheses.

[d]Reference 23.

Fig. 1b shows the GLC pattern of the urinary bile acid methyl ester-trimethylsilyl ether derivatives in the patient F. I. after 900 mg/day of ursodeoxycholic acid treatment for 6 months and the values in all patients are given in Table 2. Thus, cholic acid and chenodeoxycholic acid and the 1-hydroxylated bile acids were excreted in larger amounts in urine before treatment than after treatment with ursodeoxycholic acid. In addition to lithocholic acid, nor-cholic acid, hyocholic acid, and ursocholic acid, hyodeoxycholic acid was identified in most of the urine samples analyzed. Several metabolites of ursodeoxycholic acid were also identified in the urine after treatment and small amounts of iso-ursodeoxycholic acid were always detected. Iso-ursodeoxycholic acid could not be detected by GLC using a CP-Sil-5 capillary column, since the methyl ester-trimethylsilyl ether derivative of iso-ursodeoxycholic acid showed the same retention time as that of ursodeoxycholic acid. However, the two compounds were partially resolved on a CP-Sil-19 CB capillary column (25).

Tentative identification of hydroxylated derivatives of ursodeoxycholic acid

In addition to iso-ursodeoxycholic acid, at least three other metabolites of ursodeoxycholic acid were identified; all had higher GLC retention times and were not detected during analysis of pretreatment urine samples. The metabolite with GLC relative retention time of 1.91 (GC peak 11) showed a base ion fragment at *m/z* 217 (Fig. 3) suggesting a 1,3-dihydroxy bile acid. The fragments at *m/z* 548, 458, 368, 343, 316, and 253 strongly indicated the compound to be 1ξ-hydroxyursodeoxycholic acid. The mass spectral fragmentation pattern of the compound was found to be identical with that reported for 1β-hydroxyursodeoxycholic acid (25). The metabolite with relative retention time of 2.08 on GLC (GC peak 12) showed mass fragments at *m/z* 548, 458, 369, 235, and 195 (**Fig. 4**). Both the GLC retention time and the mass spectral fragmentation pattern were consistent with ω-muricholic acid. The structure was confirmed by a direct comparison with an authentic reference sample (21). ω-Muricholic acid has been detected in the urine of patients with cerebrotendinous xanthomatosis during ursodeoxycholic acid feeding (25) and, recently, in the urine of patients with cholestasis (29). The metabolite with relative retention time of 2.15 (peak 13) was tentatively assigned the structure of 21-hydroxyursodeoxycholic acid by comparison of its GLC retention time and mass spectral fragments [at *m/z* 548 (M^+-90), 535 (M^+-103), 458 (M^+-2 × 90), 369 (M^+-3 × 90), and 329 (M^+-129-2 × 90) (Fig. 4)] with

TABLE 3. Serum bile acids in patients with primary biliary cirrhosis before and after ursodeoxycholic acid treatment[a]

Bile Acid	Pretreatment[a]	UDCA Treatment[b]
	μM	
Nor-cholic acid	tr	tr
Lithocholic acid	1.0 (tr-4.8)[c]	1.5 (0.5-3.0)[c]
Deoxycholic acid	2.5 (1.2-5.8)	1.5 (0.2-8.0)
Chenodeoxycholic acid	22.0 (3.3-46.3)	11.0 (0.8-28.0)
Cholic acid	48.5 (2.8-124.5)	13.8 (1.2-36.5)
Hyodeoxycholic acid	tr	tr
Ursodeoxycholic acid	1.7 (0.7-3.0)	24.5 (2.3-51.3)
Iso-ursodeoxycholic acid	0.2 (tr-0.7)	1.3 (0.3-5.0)
Ursocholic acid	0.2 (tr-1.7)	2.2 (tr-11.7)
1β,3α,12α-Trihydroxy-5β-cholanoic acid	0.8 (tr-2.0)	0.2 (tr-0.7)
Hyocholic acid	1.0 (0.3-3.0)	1.5 (tr-5.0)
1β,3α,7α,12α-Tetrahydroxy-5β-cholanoic acid	1.5 (tr-3.5)	0.2 (tr-1.0)
1β,3α,7β-Trihydroxy-5β-cholanoic acid	N.D.	0.2 (tr-1.5)
ω-Muricholic acid	N.D.	1.3 (0.2-4.0)
3α,7β,21-Trihydroxy-5β-cholanoic acid	N.D.	0.7 (tr-3.3)
Total bile acids	79.4	59.5
Control[d]	3.0	8.0

Fasting serum bile acids were quantitated by gas-liquid chromatography. For details of the analysis, see Methods; tr, trace, less than 0.2 μM; N.D., not detected.

[a]Fasting serum was obtained during the pretreatment period and every month during the placebo period. The relative proportions of the various bile acids in the serum were the same during the pretreatment and placebo periods, and an average of the pretreatment and placebo serum bile acids is shown under the Pretreatment column.

[b]Fasting was obtained every month during administration of 900 mg/day of ursodeoxycholic acid and an average of the bile acids during the last 3 months is shown.

[c]The serum bile acids reported represent a mean for seven patients. The range of concentration of the various bile acids obtained in these patients is shown in parentheses.

[d]Reference 23.

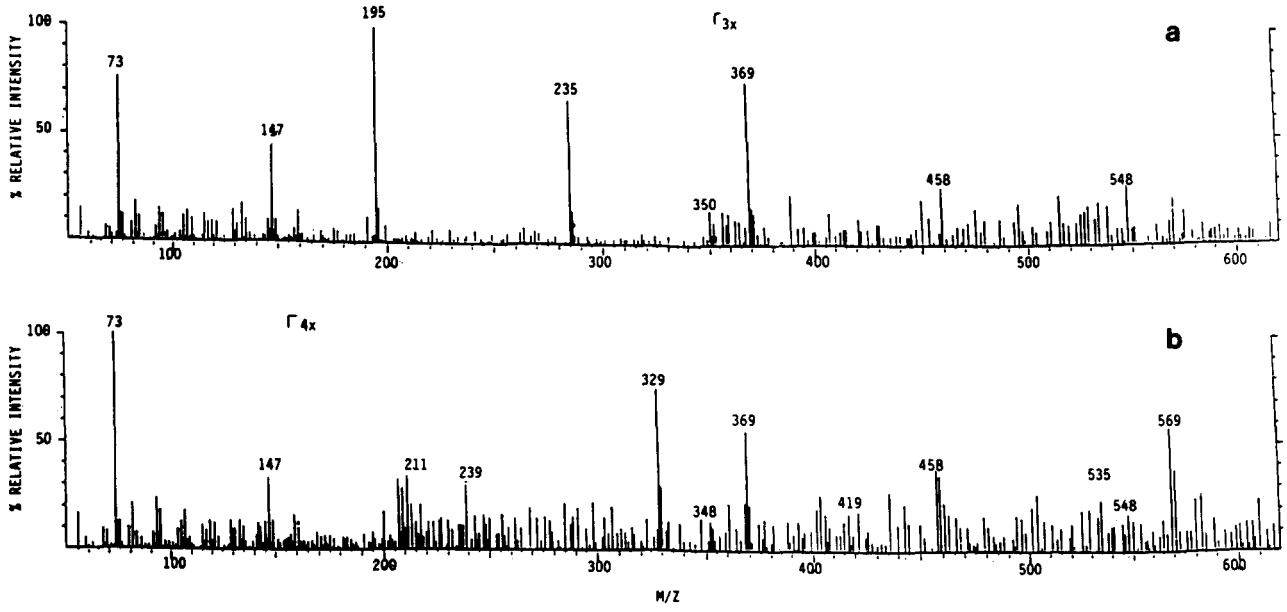

Fig. 4. Mass spectra of GLC peaks 12 and 13. Peak 12 was identified as ω-muricholic acid and peak 13 was tentatively identified as 21-hydroxyursodeoxycholic acid.

those reported by Koopman et al. (25) for a side chain hydroxylated bile acid excreted in the urine of patients with cerebrotendinous xanthomatosis during oral administration of ursodeoxycholic acid.

Capillary GLC analysis of the serum bile acids during treatment showed that ursodeoxycholic acid became the major bile acid. Total bile acids in the serum decreased slightly. However, the amounts of both cholic acid and chenodeoxycholic acid declined markedly compared to pretreatment serum. Unlike results in healthy humans, deoxycholic acid was found only in relatively small proportions (26). Several other bile acids were also characterized. Small amounts of lithocholic acid and ursocholic acid and variable amounts of hyocholic acid were seen in the serum of all patients before as well as after ursodeoxycholic acid feeding. Although iso-ursodeoxycholic acid could not always be detected in the serum of these patients before ursodeoxycholic acid was given, it was invariably present in the sera of all patients after treatment.

Urinary sulfated and nonsulfated bile acids

The urinary bile acids were separated by TLC in solvent system A and the free bile acids and their glycine and taurine conjugates were obtained by methanol–ethyl acetate elution. Since these fractions could also contain sulfated or glucuronidated bile acids, an aliquot from each fraction was subjected to hydrolysis with β-glucuronidase in order to estimate the glucuronidated/sulfated bile acids. Complete hydrolysis of a second aliquot with β-glucuronidase plus cholylglycine hydrolase gave the total amount of each bile acid in the fractions and by subtraction, the nonglucuronidated/sulfated bile acids were estimated. The glucuronides/sulfates of the conjugated bile acids were estimated in the conjugated bile acid fraction, thereby underestimating somewhat the proportion of the sulfated bile acids. **Table 4** shows the percentages of the various bile acids in the free, conjugated, and sulfated/glucuronidated forms in the urines of these patients before and after treatment with ursodeoxycholic acid. We noted that 1β-hydroxydeoxycholic acid, 1β-hydroxycholic acid, and hyocholic acid were present conjugated with glycine/taurine as also observed by Bremmelgaard and Sjövall (9). Ursodeoxycholic acid was isolated mainly as the sulfate/glucuronide before treatment, whereas it was present in significant amounts in the free as well as conjugated forms after feeding, although it was still excreted predominantly as the sulfate/glucuronide. Iso-ursodeoxycholic acid was present only as the sulfate/glucuronide. All hydroxylated derivatives of ursodeoxycholic acid (1β-hydroxyursodeoxycholic acid, ω-muricholic acid, and 21-hydroxyursodeoxycholic acid) were present predominantly in the conjugated form.

DISCUSSION

The results of the present study show that short-term treatment with ursodeoxycholic acid causes a significant change in the serum and urinary bile acid pattern in primary biliary cirrhosis. Thus, during treatment, ursodeoxycholic acid became the major bile acid both in serum and urine with a corresponding decrease in the endoge-

TABLE 4. Urinary sulfated and nonsulfated bile acids in patients with primary biliary cirrhosis before and after ursodeoxycholic acid treatment

Bile Acid	Free	Conjugated[a]	Sulfated[b]
		%	
Nor-cholic acid	100 (100)[c]		
Lithocholic acid			100 (100)
Deoxycholic acid		15 (15)	85 (85)
Chenodeoxycholic acid		10 (10)	90 (90)
Cholic acid	10 (10)	30 (30)	60 (60)
Hyodeoxycholic acid			100 (100)
Ursodeoxycholic acid	(20)	10 (20)	90 (60)
Iso-ursodeoxycholic acid			100 (100)
Ursocholic acid	80 (80)	20 (20)	
1β,3α,12α-Trihydroxy-5β-cholanoic acid		100 (100)	
Hyocholic acid		100 (100)	
1β,3α,7α,12α-Tetrahydroxy-5β-cholanoic acid		100 (100)	
1β,3α,7β-Trihydroxy-5β-cholanoic acid		(100)	
ω-Muricholic acid		(100)	
3α,7β,21-Trihydroxy-5β-cholanoic acid		(95)	(5)

The urinary bile acids were fractionated as described under Methods and analyzed by gas–liquid chromatography.
[a]The conjugated fraction represents mixture of the glycine and taurine conjugates of free bile acids and their sulfates/glucuronides.
[b]The sulfated fraction represents mixture of the sulfated/glucuronidated free bile acids.
[c]The values shown in parentheses aare those obtained after ursodeoxycholic acid feeding.

nous bile acid concentrations (Tables 2 and 3). In addition, several metabolites of ursodeoxycholic acid (products of hydroxylation at C-1, C-6, and C-21) were observed; their proportions were greater in the urine than in the serum (Tables 2 and 3). The various bile acids were characterized by gas-liquid chromatography-mass spectrometry and comparison with standard samples was made whenever possible. It was not possible to determine whether ursodeoxycholic acid treatment caused increase in the bile flow, nor was it possible in this short-term study to know if liver histology of the patients changed. Both of these should be important mechanisms to follow during the long-term treatment with ursodeoxycholic acid.

There is, so far, no accepted effective treatment for primary biliary cirrhosis, histologic abnormalities persist, and often the patient suffers from side effects of the treatment medication (30–38). Ursodeoxycholic acid is now widely used for the medical dissolution of gallstones and up to the present, no toxicity or significant side effects of ursodeoxycholic acid have been reported, although occasional diarrhea (1–2%) has been noted (39). In our patients, the drug was well tolerated and resulted in significant improvement in alkaline phosphatase and aminotransferase levels. It is not clear whether these satiatory effects represent improved cellular function or possibly could result from greater clearance of bile acids from the liver. The most remarkable improvement of ursodeoxycholic acid in these patients was the quality of life as reflected by diminished pruritus. Often this devastating symptom drives the patient to depression.

It is noteworthy that substantial amounts of bile acids and their metabolites are excreted in the urine of patients with primary biliary cirrhosis. The bile duct damage in these patients may result in interruption of hepatic flux of bile acids and lead to their back-up and urinary excretion. The proportion of the hydroxylated metabolites of bile acids as compared to the normal bile acids is substantially greater in urine than in serum. It is possible that these compounds are formed in the liver by hydroxylation of endogenous bile acids and preferentially cleared by the kidney. Shoda et al. (29) have recently detected small amounts of 1β-hydroxydeoxycholic acid and hyocholic acid in the liver tissue of patients with extrahepatic cholestasis and they have suggested that these compounds may have a hepatic origin. These compounds may also be formed by the renal hydroxylation of endogenous bile acids. This may be specially possible for 1-hydroxylation which is well known for renal hydroxylation of 25-hydroxy vitamin D. Thus, the kidney may play a helpful role in the detoxification and excretion of potentially harmful bile acids. After treatment, ursodeoxycholic acid became the major serum and urinary bile acid in these patients and there was a reduction in endogenous bile acids in both serum and urine. Small amounts of ursodeoxycholic acid were hydroxylated at C-1, C-6, and C-21. The hydroxylated derivatives of the endogenous bile acids were still present both in the serum and urine of the patients, although their relative proportions were diminished substantially. It is possible that these hydroxylated derivatives of the endogenous bile acids may play a role in causing pruritus and deterioration of the liver in this disease and their reduction during ursodeoxycholic acid feeding may result in improvement seen in these patients. Otherwise, ursodeoxycholic acid may act by displacement of the retained, more hydrophobic, endogenous bile acids which may be hepatotoxic (40, 41) and in this regard, the appearance of the 1-, 6-, and 21-hydroxylated derivatives of ursodeoxycholic acid (which are probably more hydrophilic than ursodeoxycholic acid) may add to the beneficial effect of ursodeoxycholic acid.

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