Hydroxylation of cholic, chenodeoxycholic, and deoxycholic acids in patients with intrahepatic cholestasis

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Abstract The metabolism of ^{14}C-labeled chenodeoxycholic, cholic, and deoxycholic acids was studied in patients with intrahepatic cholestasis. Radioactively labeled metabolites were isolated from urine and were identified by gas–liquid chromatography–mass spectrometry. About 5% of the radioactivity was recovered in urine after administration of labeled *chenodeoxycholic* acid to a patient with mild intrahepatic cholestasis. In urine collected 0–24 hr after the injection, 20% of the radioactivity appeared in the combined glycine and taurine conjugate fractions, and the predominant metabolite in these fractions was identified as hyocholic acid. Eighty percent of the activity was eluted in the sulfate fraction presumably representing mainly sulfated chenodeoxycholic acid conjugates. Twenty percent of the radioactivity was recovered in urine following administration of labeled *cholic* acid to a patient with biliary cirrhosis and severe cholestasis. In urine collected on the fifth day, half of this radioactivity appeared in the glycine and taurine conjugate fractions, and 10% of this activity was present as tetrahydroxycholanoates. The major metabolites in this fraction were 3α,6α,7α,12α-tetrahydroxy-5β- and 1ξ,3α,7α,12α-tetrahydroxy-5β-cholanoic acids. The former compound constituted about 50% of the tetrahydroxycholanoates. Three additional minor tetrahydroxy bile acids were present, one of which was tentatively identified as 6β-hydroxycholic acid. About 5% of the radioactivity appeared in urine after oral administration of labeled *deoxycholic* acid to a patient with mild intrahepatic cholestasis. Twenty-two percent of the activity appeared in the glycine and taurine conjugate fractions isolated from urine collected on the second day after the administration. About 75% of this activity was associated with trihydroxycholanoates. The main metabolite was 1β-hydroxydeoxycholic acid with small amounts of, tentatively, 6α-hydroxydeoxycholic acid.—**Bremmelgaard, A., and J. Sjövall.** Hydroxylation of cholic, chenodeoxycholic, and deoxycholic acids in patients with intrahepatic cholestasis. *J. Lipid Res.* 1980. **21:** 1072–1081.

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

Several investigators have suggested that impaired or altered bile acid metabolism might be of etiological importance in the cholestatic syndrome in man (1–5). Decreased bile acid transport in the hepatocyte due to diminished activity of the enzymes involved in the transport mechanisms has been considered a cause of recurrent benign intrahepatic cholestasis (6). In patients with cholestasis, the urinary excretion of bile acids is increased (7–19). Tetrahydroxycholanoates carrying hydroxyl groups at positions C-1 or C-6 appear in significant amounts (15–19), and their occurrence might discriminate between the normal condition and liver disease. In studies of bile acid profiles in urine of patients with liver disease, a positive correlation was found between the excretion rates of 1- and 6-hydroxylated bile acids and the common major bile acids, i.e., cholic, chenodeoxycholic, and deoxycholic acids (18, 19). This indicated a metabolic relationship between cholic and tetrahydroxycholanoic acids, between chenodeoxycholic and hyocholic acids, and between deoxycholic and 1,3,12-trihydroxycholanoic acids. The present study was undertaken to investigate the role of cholic, chenodeoxycholic, and deoxycholic acids as precursors of tri- and tetrahydroxycholanoates carrying hydroxyl groups at C-1 and C-6.

MATERIALS AND METHODS

Reference bile acids

Authentic bile acids were those used in previous studies (17). Dr. K. Takeda, Shionogi Research Laboratory, Osaka, kindly supplied 3α,6α-dihydroxy-12α-acetoxy-7-oxo-5β-cholanoic acid from which

Abbreviations: Cholic, 3α,7α,12α-trihydroxy-5β-cholanoic; chenodeoxycholic, 3α,7α-dihydroxy-5β-cholanoic; deoxycholic, 3α,12α-dihydroxy-5β-cholanoic; lithocholic, 3α-hydroxy-5β-cholanoic; hyocholic, 3α,6α,7α-trihydroxy-5β-cholanoic and hyodeoxycholic, 3α,6α-dihydroxy-5β-cholanoic acids. TMS, trimethylsilyl; Me, methyl ester; t_R, retention time relative to that of the TMS ether of methyl cholate; GLC/MS, gas–liquid chromatography–mass spectrometry.

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methyl 3α,6α,7α,12α-tetrahydroxy-5β-cholanoate was prepared. About 0.7 mg of the oxo acid was dissolved in 0.5 ml of ethanol and treated with 1 mg of sodium borohydride for 1 hr at room temperature. The reaction was terminated with 10 μl of acetone, the solution was concentrated, 1 ml of water was added, and the products were extracted with ethyl acetate from the acidified solution. After treatment with diazomethane, a small sample was converted to the TMS ether derivative and analyzed by gas–liquid chromatography–mass spectrometry (GLC/MS). Two products were detected having t_R values on SE-30 of 1.23 and 1.39 as compared to 1.53 for the derivatized starting material. The compound with shorter retention time gave a mass spectrum with peaks at *m/z* 711 (M-15), 636 (M-90), 546 (M-2 × 90, base peak), 457 (M-2 × 90-89), 367 (M-3 × 90-89) and 251 (ABCD-rings) and 195 (A-ring + C-6 and one TMS group) indicating that reduction at C-7 and C-12 (or hydrolysis) had occurred to produce a TMS ether of a tetrahydroxycholanoate. The other product gave the expected mass spectrum of a TMS ether of the trihydroxymonoacetoxycholanoate (*m/z* 636 (M-60), 606 (M-90), 546 (M-90-60), 516 (M-2 × 90), 456 (M-2 × 90-60), 367 (M-2 × 90-60-89 base peak), 341 (ABCD-ring with retention of one TMS group), 285 (A-ring + C-6 and two TMS groups), 251 (ABCD-rings) and 195 (285-90).

The main part of the reduction products was hydrolyzed in 1 ml of 1.5 M NaOH in 50% aqueous methanol at 60°C for 3 hr. After dilution with water and acidification, the bile acids were extracted with ethyl acetate and methylated. GLC/MS analysis of the TMS ether derivative revealed the presence of one predominant compound (90–95% of the total area of peaks). The relative retention time and mass spectrum were identical with those of the tetrahydroxycholanoate mentioned above (t_R 1.23).

Part of the tetrahydroxycholanoate was treated with dimethoxypropane/HCl as described below. The TMS ether derivative of the product had a t_R of 1.45. The mass spectrum showed that an acetonide had been formed (see Results).

Labeled bile acids

[24-^{14}C]Cholic acid, sp act 51 mCi/mmol, and [24-^{14}C]deoxycholic acid, sp act 52 mCi/mmol, were from the Radiochemical Center, Amersham; [24-^{14}C]chenodeoxycholic acid, sp act 54 mCi/mmol, was from NEN Chemicals GmbH, Frankfurt/Main, West Germany. Each patient was given about 10 μCi of one of the labeled bile acids intravenously or orally. After the administration, urine was collected in 24-hr portions and stored at −20°C until analyzed.

Patients studied

Two patients with intermittent intrahepatic cholestasis of unknown etiology in resolution phase and one with stage II primary biliary cirrhosis were studied. Clinical and routine laboratory data are given in **Table 1.** The patient with primary biliary cirrhosis (EH, given labeled cholic acid intravenously) had severe itching and was treated with cholestyramine; she did not receive immunosuppressive treatment. The other two patients had moderate (JML, given labeled deoxycholic acid orally) and no itching (JPD, given labeled chenodeoxycholic acid intravenously), and were not treated with bile acid-binding resin. The quantitative liver function was estimated by galactose elimination capacity and antipyrin clearance. The values were normal for all patients in the time before performance of the bile acid studies. None of the patients had kidney disease.

Informed consent was given by the patients. The studies that were carried out conformed to the Helsinki Declaration II.

Bile acid analyses

Solvents and reagents were of analytical grade and were distilled before use. Bile salts were extracted and purified as described by Almé et al. (17). The pH of 300–400 ml of urine was adjusted to

TABLE 1. Routine laboratory and clinical data of three patients with intrahepatic cholestasis

Patient	Sex	Disease	Stage of Disease	Plasma[c] Bilirubin (2–17)	Plasma[c] Alkaline Phosphatases (70–275)	Plasma[c] Alanine Amino-transferases (10–40)	Galactose[c] Elimination Capacity (1.38–3.50)
				μmol/l	*U/l*	*U/l*	*mmol/min*
EH	F	PBC[a]	II	113	760	138	1.75
JML	M	IICUE[b]	resolution	14	1680	97	
JPD	M	IICUE[b]	resolution	140	183	200	1.91

[a] Primary biliary cirrhosis.
[b] Intermittent intrahepatic cholestasis of unknown etiology.
[c] Normal range of values is given in parentheses.

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3–4 and the bile acids were extracted on a column of 25 g Amberlite XAD-2. Bile salts were eluted with ethanol containing 0.25% aqueous ammonium hydroxide. The eluate was evaporated, dissolved in 72% ethanol, and passed through a column of 16 g Amberlyst A-15 in the H^+ form. Bile acids in the eluate were then fractionated according to mode of conjugation on a column of 1.5 g diethylaminohydroxypropyl Sephadex LH-20 (DEAP-LH-20). The fractions containing glycine- and taurine-conjugated bile acids were collected, evaporated, and dissolved in 18 ml 15% NaOH in 50% ethanol. After hydrolysis at 115°C for 10 hr, the mixture was acidified, diluted with water, and the bile acids were extracted with ethyl acetate. They were then rechromatographed on a column of 0.6 g DEAP-LH-20 in 72% ethanol. The purified unconjugated bile acids were methylated using freshly prepared diazomethane. The methyl esters were dissolved in chloroform–hexane 1:4 (v/v), and separated according to number of hydroxyl groups on a column of 4 g Lipidex 5000® (Packard Instrument Co., La Grange, IL) packed in the same solvent (**Fig. 1**). Methyl monohydroxycholanoates were eluted with 40 ml of chloroform–hexane 1:4. Methyl dihydroxycholanoates were eluted with 70 ml of chloroform–hexane 3:7, methyl trihydroxycholanoates with 100 ml of chloroform–hexane 1:1, and methyl tetrahydroxycholanoates with 50 ml of methanol. The effluent was collected in 5-ml fractions. Aliquots were taken for liquid scintillation counting using 15 ml of Instagel® as scintillating fluid. Fractions containing labeled bile acids were analyzed by GLC/MS and the specific activity of the quantitated bile acids was calculated.

Acetonides were prepared for isolation and identification of bile acids with a 6α,7α-dihydroxy structure. Bile acid methyl esters were dissolved in 1 ml of dimethoxypropane and 50 μl of 0.1 N HCl was added. The sample was incubated at 37°C for 30 min. Incubation at a lower temperature or with a higher concentration of HCl resulted in incomplete reaction. To remove the acid, the reaction mixture was passed through a 0.2 g column of DEAE Sephadex A 25 in OH^- form prepared by washing with 10 ml of 0.2 N NaOH, 10 ml of H_2O, and 10 ml of 72% methanol prior to application of the sample. To reduce the time of elution, a slight pressure was applied to the column during the chromatography. The column was rinsed with 6 ml of methanol. The combined eluate was evaporated under reduced pressure at 20°C. The resulting acetonides were dissolved in chloroform. After addition of hexane to the appropriate ratio, the acetonides were isolated and purified on a column of 4 g of Lipidex as described above. The eluate was collected in 5-ml fractions and aliquots from these fractions were taken for counting and GLC/MS analysis. Prior to the latter, the compounds were converted into TMS ether derivatives using pyridine–hexamethyldisilazane–trimethylchlorosilane 3:2:1 (by vol) as the reagent. The reaction was performed at 60°C for 30 min. GLC columns were 1.5% Hi Eff 8 BP for analysis of compounds without an acetonide group and 1% SE-30 for determination of compounds containing this substituent.

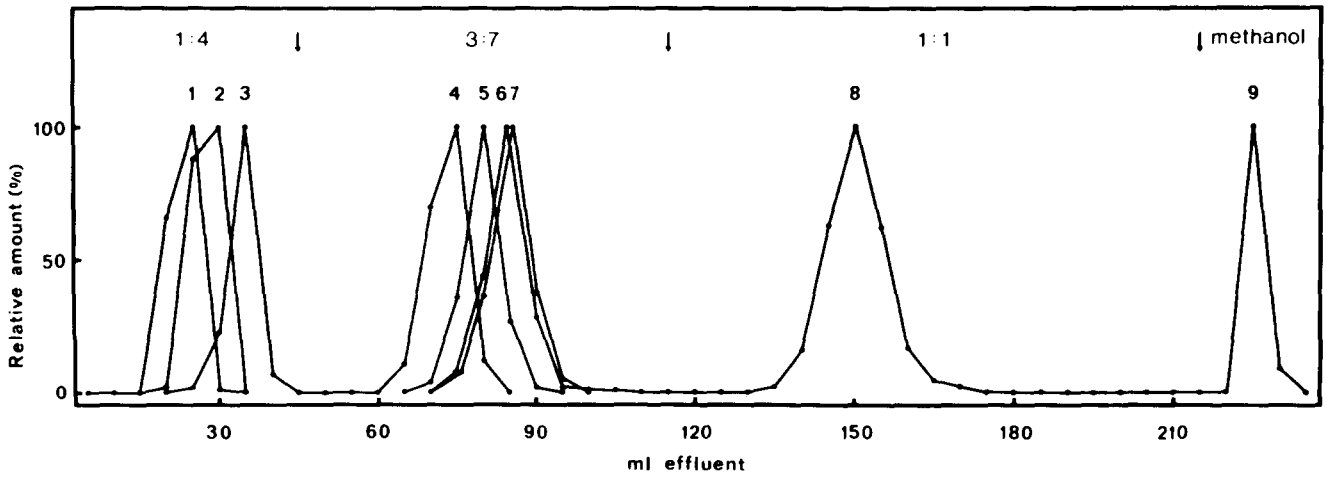

Fig. 1. Chromatography on a column of Lipidex 5000 (4 g, 20 × 1 cm) of a mixture of bile acid methyl esters. Solvent: chloroform–hexane 1:4 (40 ml), 3:7 (70 ml), 1:1 (100 ml), and methanol (25 ml). The peaks represent coprostanol (1), and methyl esters of lithocholic (2), 3β-hydroxy-5-cholenoic (3), chenodeoxycholic (4), apocholic (5), deoxycholic (6), ursodeoxycholic (7), cholic (8) and tetrahydroxycholanoic acids (9).

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TABLE 2. Radioactivity recovered in the extraction and group separation of urinary metabolites of chenodeoxycholic (CDC), cholic (C), and deoxycholic (DC) acids

	CDC[a]	C[b]	DC[c]
		cpm × 10^{-3}	
Urine	67.1	128.6	1166.8
Amberlite XAD-2	68.9	116.5	1111.7
Amberlyst A-15	70.7	117.3	1108.4
DEAP-LH-20 (total)	64.1	121.1	944.9
Neutral fraction	1.1	0	14.7
Unconjugated fraction	0	0	54.9
Glycine + taurine conjugated fraction	12.5	68.5	252.6
Monosulfated fraction	50.5	52.7	580.4
Di- and trisulfated fraction	0	0	42.3

[a] Urine collected 0–24 hr after i.v. injection of [24-^{14}C]chenodeoxycholic acid.

[b] Urine collected on the 5th day after i.v. injection of [24-^{14}C]-cholic acid (containing 14% of the radioactivity excreted during the first 5 days).

[c] Urine collected on the second day after oral administration of [24-^{14}C]deoxycholic acid (containing 40% of the radioactivity excreted during the first 2 days).

TABLE 3. Radioactivity recovered in the separation of glycine and taurine conjugated urinary metabolites of chenodeoxycholic (CDC), cholic (C), and deoxycholic (D) acids

	CDC	C	DC
		cpm × 10^{-3}	
Hydrolyzed conjugates	11.7	55.3	187.7
DEAP-LH-20 (Total)	13.5	57.6	177.8
Neutral fraction	6.5	0	26.0
Unconjugated fraction	7.0	57.6	151.8
Lipidex (Total)	5.9	50.5	137.5
Monohydroxy fraction	0	2.0	11.9
Dihydroxy fraction	1.4	2.7	25.8
Trihydroxy fraction	3.4	40.4	99.9
Tetrahydroxy fraction	1.1	5.5	0
Lipidex (Total)[a]	2.1	4.3	89.7
Monohydroxy fraction	2.1		2.8
Dihydroxy fraction	0	2.1	1.3
Trihydroxy fraction	0	0	85.6
Tetrahydroxy fraction	0	2.2	0

[a] After reaction of the trihydroxy (CDC and D) and tetrahydroxy (C) fractions, from the first Lipidex column, with dimethoxypropane/HCl.

RESULTS

Tables 2 and **3** show the recovery of labeled compounds during the isolation of urinary metabolites in each experiment. Since the majority of bile acids containing hydroxyl groups at C-1 and C-6 are found as glycine and taurine conjugates (17–19), these fractions were combined for further analysis.

Chenodeoxycholic acid

About 5% of the administered radioactivity was excreted in urine in 15 days (patient JPD). The sample collected during the first day was studied in detail. The combined glycine and taurine conjugate fractions contained 18.6% of the total radioactivity in this sample. The major part (71%) was recovered in the trihydroxy fraction after separation on Lipidex. Analysis by GLC/MS showed that the radioactivity was correlated to the appearance of methyl hyocholate. After reaction with dimethoxypropane and rechromatography on Lipidex, all the radioactivity was displaced into the monohydroxy fraction, where it coincided with the elution of a single compound. The spectrum of the TMS ether of this compound (**Fig. 2**) was identical with the spectrum of the TMS ether of the acetonide of authentic methyl hyocholate. The specific activity of the acetonide was calculated to about 30 cpm/μg. The specific activity of chenodeoxycholic acid in the glycine and taurine conjugate fraction was 36 cpm/μg.

Cholic acid

Twenty percent of the administered radioactivity was excreted in urine in 20 days (patient EH). Urine

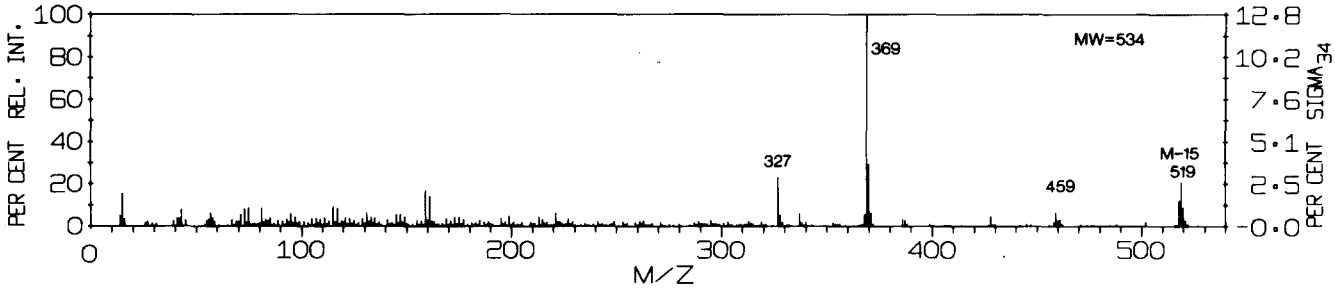

Fig. 2. Mass spectrum of the TMS ether derivative of the acetonide of methyl hyocholate obtained in the analysis of metabolites of [24-^{14}C]chenodeoxycholic acid in urine from a patient with intrahepatic cholestasis. Numbers to the left of the spectrum indicate intensities relative to the base peak, numbers to the right indicate intensities relative to the sum of intensities of peaks from *m/z* 34 to the molecular weight (*m/z* 534). Diagnostically important peaks arise by loss of the elements of the acetonide group (*m/z* 459, M-75) together with trimethylsilanol (*m/z* 369, M-75-90).

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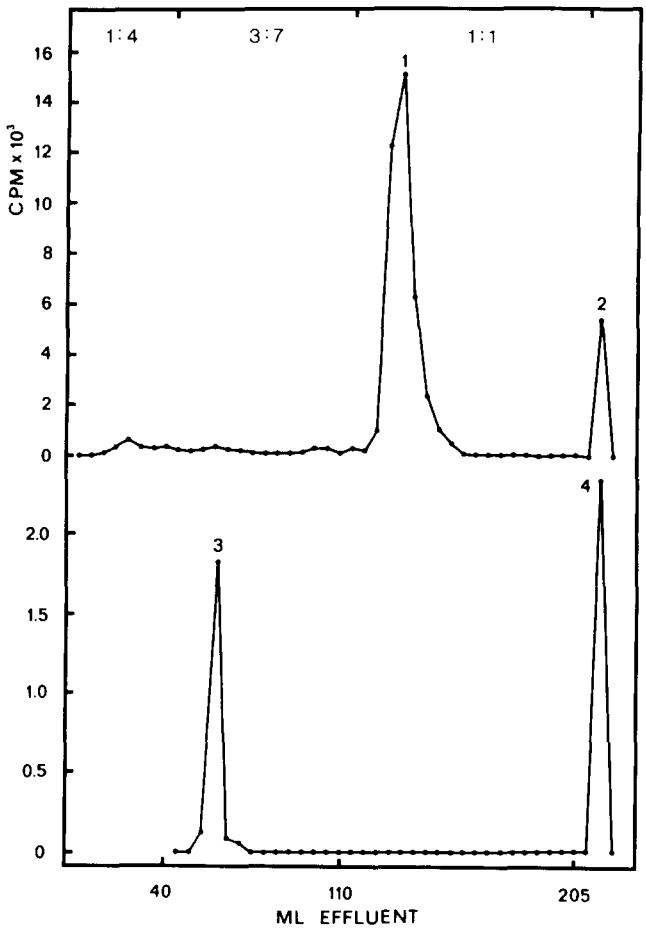

Fig. 3. Separation of the methyl esters of urinary metabolites of [24-^{14}C]cholic acid before (upper curve) and after (lower curve) reaction of the tetrahydroxycholanoates with dimethoxypropane/HCl. Column: Lipidex 5000, 4 g; solvents: see Fig. 1. The peaks are 1: methyl cholate; 2: mixture of methyl tetrahydroxycholanoates; 3: acetonide of methyl 3α,6α,7α,12α-tetrahydroxy-5β-cholanoate, 4: mixture of tetrahydroxycholanoates.

from the fifth day was analyzed. The combined glycine and taurine conjugate fractions contained about 55% of the total radioactivity in the urine sample. After separation on Lipidex, 80% of this radioactivity appeared in the trihydroxy fraction associated with the elution of methyl cholate, the specific activity of which was 8.8 cpm/μg. The tetrahydroxy fraction contained 9.2% of the radioactivity. GLC/MS analysis showed that several tetrahydroxycholanoates were present. The TMS ether derivative of one of these (compound I), had retention times and mass spectrum corresponding to those of the derivative of the 3,6,7,12-tetrahydroxycholanoate, tentatively identified in a previous study (17). The retention times were also identical with those of authentic methyl 3α,6α,7α,12α-tetrahydroxy-5β-cholanoate TMS ether. All the diagnostically important peaks in the mass spectrum of the latter compound were present in the same abundances in the spectrum of the mixture of urinary tetrahydroxycholanoates appearing at the retention time of the reference compound.

After reaction with dimethoxypropane and rechromatography on Lipidex, half of the radioactivity due to tetrahydroxycholanoates moved to the dihydroxy fraction (**Fig. 3**). GLC/MS analysis of this fraction (peak 3, Fig. 3) showed that the radioactivity coincided with the elution of a single compound. The mass spectra of the TMS ethers of this compound and the acetonide of authentic methyl 3α,6α,7α,12α-tetrahydroxy-5β-cholanoate are shown in **Fig. 4.** The specific activity of the metabolite was 11 cpm/μg. Based on these results compound I is identified as 3α,6α,7α,12α-tetrahydroxy-5β-cholanoic acid.

Half of the radioactivity from the second Lipidex chromatography remained in the tetrahydroxy fraction. Since a mixture of several bile acids was expected, the fraction was chromatographed on a column of 4 g Sephadex LH-20 using cyclohexane–ethanol 4:1 as eluting solvent (16). The eluate was collected in 2.5-ml fractions. The methyl tetrahydroxycholanoates were eluted between 50–100 ml, and 95% of the radioactivity was recovered in this volume. At least five methyl tetrahydroxycholanoates were present. The bile acids are listed in **Table 4.** Compound I, found in small amounts in fractions 23–33, was found to be methyl 3α,6α,7α,12α-tetrahydroxy-5β-cholanoate, which had not reacted with dimethoxypropane.

Fractions 23–29 contained a hitherto unknown bile acid (compound II). The relative retention time on Hi Eff 8 BP (t_R = 1.41) was longer than for any previously identified tetrahydroxy bile acid. The mass spectrum of the TMS ether is shown in **Fig. 5.** Peaks at *m/z* 431, 341, and 251 indicate that the four hydroxyl groups are in the ring skeleton. The intense peak at *m/z* 243 is an unusual feature in a spectrum of a bile acid derivative. A small peak at this mass is usually seen in bile acids with a 3,7-bis-trimethylsiloxy structure and corresponds to the C-3 to C-7 part of the molecule (20). However, a more intense peak at this mass is seen in spectra of the TMS ethers of methyl 3,12,15-trihydroxy-5β-cholanoate.[1] Whether or not the new tetrahydroxy bile acid carries a hydroxyl group at C-15 remains to be established.

The predominant tetrahydroxycholanoate, compound III, was eluted in fractions 25–38. Its TMS ether derivative had the retention time and mass spectrum of 1,3,7,12-tetrahydroxycholanoate, tentatively identified in a previous study (17). The specific

[1] Carlström, K., D. Kirk, and J. Sjövall. Unpublished observations.

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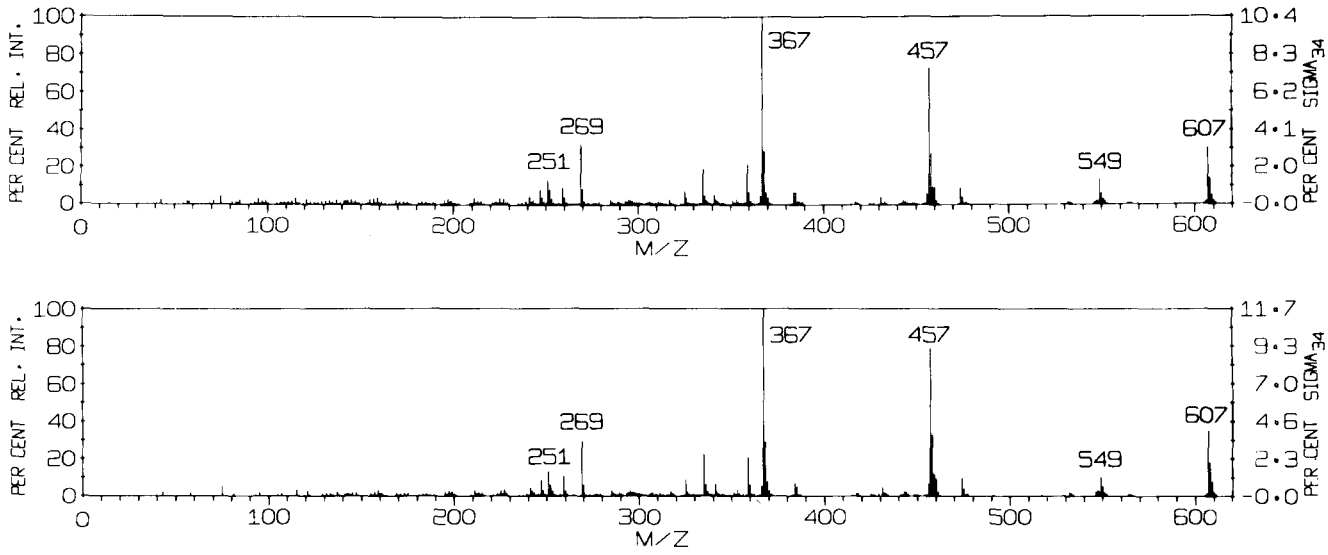

Fig. 4. Mass spectra of the TMS ether derivatives of the acetonides of methyl 3α,6α,7α,12α-tetrahydroxy-5β-cholanoate from urine (upper spectrum) and authentic 3α,6α,7α,12α-tetrahydroxy-5β-cholanoate (lower spectrum). The peak at *m/z* 547 is due to loss of the acetonide group (622-75), and the peaks at *m/z* 457 and 367 result from further loss of one and two TMS groups.

activity of this compound, calculated from its peak fraction (no. 31), was about 12 cpm/μg, i.e., close to that of cholic acid. Based on the mass spectrum as discussed previously (17) and on the information that it is a metabolite of cholic acid, this tetrahydroxy bile acid is assigned the structure 1ξ,3α,7α,12α-tetrahydroxy-5β-cholanoic acid.

Fractions 29–38 contained two tetrahydroxycholanoates (IV and V) whose TMS ether derivatives had closely similar retention times (Table 4). Compound V was quantitatively the least important tetrahydroxycholanoate. The mass spectra (**Fig. 6**) showed that they were tetrahydroxycholanoates. Decreasing specific activity during elution of these compounds may indicate that only one of these compounds is a metabolite of cholic acid. The presence of peaks at *m/z* 285 and 195 are indicative of a 3,6,7-tris-trimethylsiloxy structure (20), and a peak at *m/z* 401 given by the derivatized compound V may arise by loss of the A-ring (546–145) in analogy with the fragmentation of TMS ether derivatives of methyl 3α,6β-dihydroxycholanoates (21). The retention times of both compounds are compatible with a 3α,6β,7α,-12α-tetrahydroxy-5β-cholanoate structure (**Table 5**). The short retention times on Hi Eff 8 BP are also compatible with a 5α-H configuration.

Deoxycholic acid

About 5% of the administered radioactivity was excreted in urine in 10 days (patient JML). The sample collected on the second day was analyzed. About 22% of the radioactivity was recovered in the glycine and taurine conjugate fractions. After hydrolysis, extraction, and separation of bile acids on Lipidex, the major part of the radioactivity was recovered in the trihydroxy fraction. This fraction was treated with dimethoxypropane–HCl and rechromatographed on Lipidex. Practically all of the radioactivity remained in the trihydroxy fraction, while unlabeled hyocholate was removed into the monohydroxy fraction. The radioactivity was correlated with the elution of two compounds, the major one being the 1,3,12-trihydroxycholanoate tentatively identified in a previous study (17). The retention times on SE-30 and Hi Eff 8 BP and the mass spectrum of the TMS ether of this compound were identical with those of the TMS ether of methyl 1β,3α,12α-trihydroxy-5β-cholanoate prepared by microbial hydroxylation of deoxycholic acid.[1] The major hydroxylation product of deoxycholic acid in man is therefore identified as 1β,3α,12α-trihydroxy-5β-

TABLE 4. Retention times[a] of TMS ether derivatives of methyl tetrahydroxycholanoates partially separated on Sephadex LH-20

Tetrahydroxy-cholanoate	Elution Volume	Retention Time		Tentative Structure[b]
		Hi Eff 8 BP	SE-30	
	ml			
I	57–82	0.80	1.25	5βB-3α,6α,7α,12α-ol
II	57–72	1.41	1.01	B-tetrol
III	62–95	0.80	1.25	5βB-1β,3α,7α,12α-ol
IV	72–95	0.53	1.01	B-3,6,7,12-ol
V	72–95	0.56	1.01	B-3,6,7,12-ol

[a] Relative to that of the TMS ether of methyl cholate.
[b] B = cholanoic acid. Configuration at C-5 and of hydroxyl groups is indicated by Greek letters.

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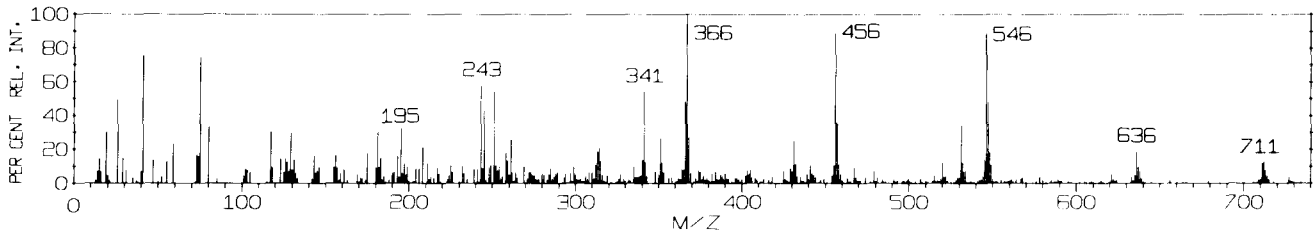

Fig. 5. Mass spectrum of the TMS ether of tetrahydroxycholanoate III formed from [24-^{14}C]cholic acid in a patient with primary biliary cirrhosis.

cholanoic acid. The specific activity of this compound was 184 cpm/μg, as compared to 125 cpm/μg for deoxycholic acid determined in the combined glycine and taurine conjugate fraction.

The minor trihydroxycholanoate (constituting about 5% of the trihydroxycholanoates formed from deoxycholic acid) appeared in the early part of the peak of the 1-hydroxylated metabolite eluted from the Lipidex column. Its TMS ether derivative had a relative retention time of about 1.30 on Hi Eff 8 BP. The retention time and mass spectrum were closely similar to those of the derivative of 3α,6α,12α-trihydroxy-5β-cholanoic acid which has recently been found in human urine (22). The amounts of material were too small for further confirmation of this structure.

DISCUSSION

Recent studies have shown that bile acids carrying hydroxyl groups at C-1 or C-6 are excreted in urine of most patients with intra- and extrahepatic cholestasis (16–19). In a group of 43 patients with various liver diseases, such bile acids constituted 5–15% of the total bile acids in urine (18). A striking finding in all patients with cholestasis is the appearance of 1,3,7,12- and 3,6,7,12-tetrahydroxycholanoates in urine (18, 19). These bile acids are usually not detected in the urine of healthy subjects although trace amounts have been found during pregnancy (19). The presence of a high percentage of tetrahydroxycholanoates in meconium has recently been reported (23). In contrast to the tetrahydroxycholanoates, trihydroxycholanoates carrying a hydroxyl group at C-1 or C-6 are found in most urine samples from healthy subjects (17–19). Two major bile acids in this group are hyocholic and 1β,3α,12α-trihydroxy-5β-cholanoic acids.

The analyses of urinary bile acid profiles in patients with cholestasis have given indirect evidence for 1- and 6-hydroxylation of the major common bile acids (18, 19). The present study has provided positive proof for hydroxylation of the common bile acids in man, C-1 and C-6 being the preferred sites of hydroxylation.

Hyocholic acid was the major hydroxylated metabolite of chenodeoxycholic acid. This is analogous to the formation of hyocholic acid in the pig (24), whereas 6β-hydroxylation is the major metabolic transformation of chenodeoxycholic acid in the rat (25). Conversion of chenodeoxycholic acid into compounds

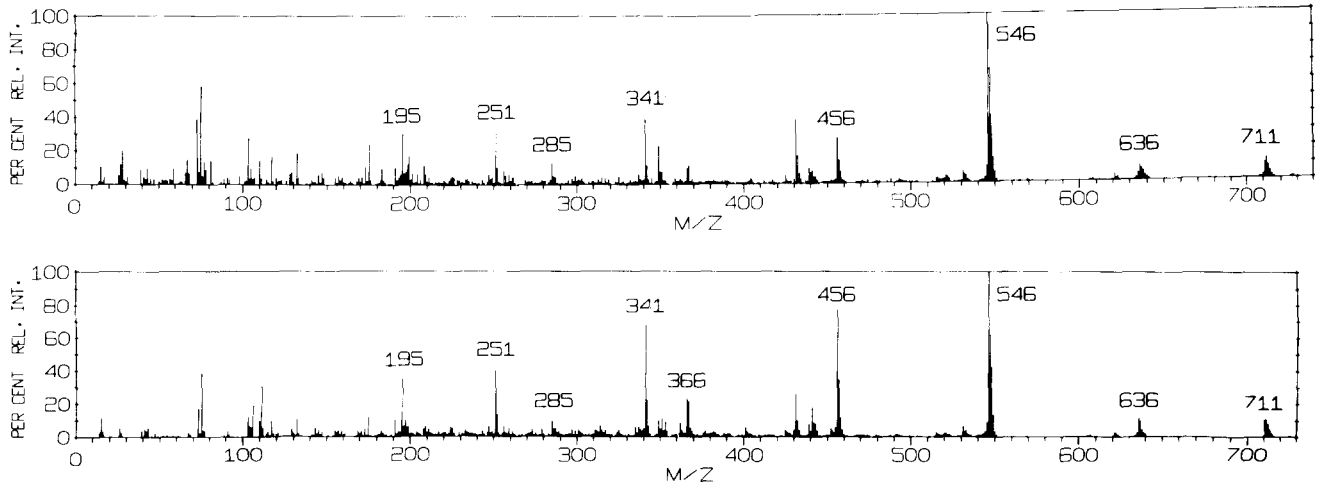

Fig. 6. Mass spectra of the TMS ether derivatives of tetrahydroxycholanoates IV (upper spectrum) and V (lower spectrum) having short relative retention times (0.53 and 0.56, respectively) on Hi Eff 8 BP.

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with chromatographic properties of trihydroxycholanoates has previously been demonstrated, but the products were not identified (26–28). The degree of conversion was small, less than 10% (27) or 5% (28) in healthy subjects. Björkhem, Einarsson, and Hellers (29) found no significant hydroxylation of taurochenodeoxycholic acid in microsomal fractions of human liver homogenates. On the other hand, 6α-hydroxylation of taurolithocholate to taurohyodeoxycholate has been demonstrated with human liver microsomes (30). Thus, hyodeoxycholic acid which is sometimes present in human urine (15–19), may be formed either by microbial 7-dehydroxylation during the enterohepatic circulation of hyocholic acid (as in the pig (24)) or by 6α-hydroxylation of lithocholic acid. In both cases, chenodeoxycholic acid is the primary precursor but further studies are required to determine the major pathway for formation of hyodeoxycholic acid.

Hydroxylation of cholic acid in man has not been described previously. In the experiment with [24-^{14}C]-cholate, 6.1% of the radioactivity in the urine sample was associated with tetrahydroxycholanoates. In a previous study, two major tetrahydroxy bile acids were noticed (17). The more detailed fractionation technique in the present study resulted in detection of at least five tetrahydroxycholanoates. The predominant ones were 3α,6α,7α,12α-tetrahydroxy-5β-cholanoic and 1ξ,3α,7α,12α-tetrahydroxy-5β-cholanoic acids. The former could be identified by comparison with authentic material, and one of the minor tetrahydroxycholanoates was tentatively identified as the 6β-epimer. The configuration at C-1 of the second major tetrahydroxycholanoate has not been established. However, the chromatographic behavior and analogies between the spectra of the methyl ester TMS ether derivatives of this compound and 1β,3α,12α-trihydroxy-5β-cholanoic acid make a 1β-configuration the most likely one. Furthermore, 1β-hydroxylation of cortisol metabolites having the same A/B-ring structure as cholic acid has been shown to occur in man (31).

The TMS ether of one of the minor tetrahydroxycholanoates gave a mass spectrum with a peak at *m/z* 243. A small peak at this mass is usually given by cholanoates with a 3,7-bis-trimethylsiloxy structure (20) but a more intense peak is formed when a 15-trimethylsiloxy group is present (representing the side chain and D-ring).[2] 15-Hydroxylation of C_{19} and C_{21} steroids has been demonstrated in pregnant women (32), and 15-hydroxylation is an important pathway for metabolism of C_{21} steroids in female rats (33). Further studies are needed to establish whether bile acids can be 15-hydroxylated.

Previous studies have shown that deoxycholic acid is hydroxylated to a very small extent, if at all in man (27, 28, 34–36). However, in these studies the excretion of metabolites in urine was not studied. In our investigation, about 20% of the labeled metabolites in urine was found in the trihydroxy fraction of glycine and taurine conjugated bile acids. The major metabolite in this group was identified as 1β,3α,12α-trihydroxy-5β-cholanoic acid, while a minor product was tentatively identified as 3α,6α,12α-trihydroxy-5β-cholanoic acid. Since most of the remaining radioactivity was recovered in the sulfate fraction, it may be concluded that sulfation and 1β-hydroxylation were the two major metabolic transformations of deoxycholic acid, in addition to the conjugation with glycine and taurine.

Both 1β-hydroxydeoxycholic acid and hyocholic acid are found in urine from healthy subjects. The former does not increase in patients with cholestasis when formation of deoxycholic acid is decreased (17–19). The excretion of hyocholic and tetrahydroxycholanoic acids on the other hand increases in these patients (14, 16–19). However, the quantitative importance of hydroxylation of primary bile acids in cholestasis cannot be assessed from the rate of excretion in urine. It is obvious from previous investigations that the profiles of bile acids in bile, blood, and urine are very different (e.g., 13–19). For example, sulfated bile acids are predominant in urine but minor components in bile. Serum bile acids show an intermediate degree of sulfation (13, 14, 37). An analogous situation seems to exist with respect to the hydroxylated metabolites described in this study. The ratio between chenodeoxycholic and hyocholic acids in three icteric patients with intermittent intrahepatic cholestasis was 1.5–12 in urine and 10–100 times higher in bile (18). The ratio between cholic acid and its 1- and 6-hydroxylated metabolites was 1–11 in urine while the tetrahydroxy acids were below the detection limit in bile (ratio above 100). Similar large differences between urine and bile were found for deoxycholic acid and its 1β-hydroxylated metabolite.

TABLE 5. Relative retention times (t_R) on gas-liquid chromatography of TMS ethers of methyl tri- and tetrahydroxycholanoates carrying a hydroxyl group at C-6

Compound[a]	SE-30	Hi Eff 8 BP
5βB-3α,6α,7α-ol	1.27	1.38
5βB-3α,6β,7α-ol	1.00	0.91
5βB-3α,6α,7α,12α-ol	1.31	0.82
5βB-3α,6β,7α,12α-ol	1.03[b]	0.54[b]

[a] For abbreviations see Table 3.
[b] Calculated from the ratio between the retention times of the trihydroxycholanoates.

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These results show that the hydroxylated metabolites are enriched in urine relative to the precursor bile acids.

In analogy with discussions concerning urinary bile acid sulfates (13, 14, 38, 39), the high proportion of 1- and 6-hydroxylated bile acid metabolites in urine could be due to a high renal clearance or to formation and excretion by the kidney. Since microsomes from human liver have been shown to 6α- and 1β-hydroxylate other substrates with a 3α-hydroxy-5β-H stereochemistry (30, 31), formation in the liver is still the most likely explanation for the appearance of hydroxylated bile acid metabolites. The concentration of primary bile acids in the liver increases markedly in cholestasis and this may result in increased formation of hyocholic and tetrahydroxycholanoic acids which is reflected in the metabolic profile of bile acids in urine.

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