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ALTERED METABOLISM OF BILE ACIDS IN CHOLESTASIS: DETERMINATION OF 1β- AND 6α-HYDROXYLATED METABOLITES

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SUMMARY

Trihydroxy and tetrahydroxy bile acid metabolites substituted at the C-1 or C-6 position were studied using the urine, serum and liver tissue from sixteen patients with cholestatic liver diseases. Following extraction, isolation and hydrolysis, bile acids were converted into the dimethylethylsilyl derivatives and assayed by capillary gas chromatography–mass spectrometry. Five 1β-hydroxylated bile acids, viz. 1β,3α,12α-trihydroxy-, 1β,3α,7α-trihydroxy-, 1β,3α,7β-trihydroxy-, 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acids and an epimer of the first compound, and two 6α-hydroxylated bile acids, viz. 3α,6α,7α-trihydroxy-, 3α,6α,7α,12α-tetrahydroxy-5β-cholanoic acids, were completely or partially identified. Large amounts of 1β-hydroxylated and 6α-hydroxylated bile acids were found in the urine, whereas only trace amounts were detected in the serum and liver tissue. These findings indicate that altered metabolism, such as 1β- or 6α-hydroxylation of bile acids, is enhanced in cholestasis, and that the resulting hydroxylated metabolites are eliminated in the urine.

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INTRODUCTION

Unusual bile acids have been found in human body fluids [1–5] especially in the urine of patients with cholestatic liver diseases [2,4]. Palmer [6] suggested that altered metabolism might be of significance in the cholestatic state. A number of trihydroxy and tetrahydroxy bile acids substituted at the C-1 or C-6 position have been found in urine [1–5]. The previous report [7] described possible hepatic hydroxylation of cholic, chenodeoxycholic and deoxycholic acids in patients with intrahepatic cholestasis. Thus, our interest has been focused on the significance of C-1 or C-6 hydroxylation of bile acids as a possible reflection of altered metabolism and subcellular function in the damaged liver. Little is yet known about the C-1 and C-6 hydroxylated bile acids. The former has been tentatively identified, but their stereochemistry is unknown as yet. In addition, little is known about the rate of synthesis of these bile acids and their enterohepatic circulation. Recently, Tohma and co-workers synthesized standards of 1β-hydroxylated bile acids [8,9] and found them to be present in human meconium [8–10], umbilical cord blood [11] and amniotic fluid [11]. Urinary bile acid excretion increases with increasing cholestasis and, in patients with biliary obstruction, urinary excretion becomes the major route for elimination of bile acids [12,13]. Therefore, the study of bile acids in urine as well as in serum and liver tissue may provide useful information about C-1 and C-6 hydroxylated bile acids.

This paper describes a sensitive and specific method for quantitation of C-1 and C-6 hydroxylated bile acids by gas chromatography (GC) with selected-ion monitoring (SIM), and the application of the method to a study of the metabolism and clinical significance of these bile acids.

EXPERIMENTAL

Patients

Sixteen patients with hepatobiliary diseases associated with cholestasis (five with intrahepatic and eleven with extrahepatic cholestasis) and fourteen control subjects were selected for this study. The diagnoses were based on biochemical data, image findings obtained from diagnostic procedures such as echography, endoscopy, computed-tomographic scan and angiography, and histological findings obtained from operation or autopsy. All patients including control subjects were hospitalized and on a regular diet with constant amounts of fat, protein and carbohydrate. For at least 3 weeks prior to the study, the patients and control subjects did not receive any drug that might interfere with bile acid metabolism.

Sample collection

Blood samples were taken from fasting patients and the sera were stored at $-20°C$ until analysis. Spontaneously voided 24-h urine was collected, and after measurement of total volume, aliquots were taken and stored at $-20°C$. Liver samples were obtained at autopsy from seven patients with extrahepatic cholestasis. Immediately after the resection, a part of the tissue was removed, rinsed

with chilled saline, sliced into small blocks corresponding to ca. 10 mg of liver tissue, rinsed again, briefly dried on a filter paper, weighed and stored at −80°C.

Reference compounds

Authentic bile acids were obtained from Steraloids (Wilton, NH, U.S.A.). Standards of 1β-hydroxylated bile acids were synthesized by Tohma and co-workers [8,9]. Deuterated bile acids, [6,6,7,7-$^2H_4$]lithocholic acid (LCA), [11,11,12,12-$^2H_4$]chenodeoxycholic acid (CDCA) and [11,11,12β-$^2H_3$]cholic acid (CA), were prepared in the Research Laboratory of Nippon Kayaku [12,12,13-$^2H_3$]Deoxycholic acid (DCA) and [11,11,12,12-$^2H_4$]ursodeoxycholic acid (UDCA) were kindly supplied by Dr. T. Beppu (Faculty of Medicine, Juntendo University, Tokyo, Japan) and by Tokyo Tanabe (Tokyo, Japan), respectively. The purity of these samples was checked by thin-layer chromatography (TLC), and all of them gave only a single spot on the chromatograms.

Chemicals

All solvents were of analytical grade. Bond Elut C_{18} (octadecylsilane-bonded silica) cartridges were obtained from Analytichem International (Harbor City, CA, U.S.A.), cholylglycine hydrolase from Sigma (St. Louis, MO, U.S.A.), Sephadex LH-20 from Pharmacia (Uppsala, Sweden) and dimethylethylsilyl imidazole (DMESI) [14] from Tokyo Kasei Kogyo (Tokyo, Japan). Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) [15] was kindly supplied by Professor T. Nambara and Dr. J. Goto.

Sample preparation

The analysis of bile acids in biological samples was performed by the method of Yanagisawa and co-workers [16,17] with minor modifications as follows: serum (0.5–1.0 ml) and urine (5 ml) were diluted with 0.5 *M* potassium phosphate buffer (pH 7.0) (5–10 ml), respectively, and internal standards [$^2H_4$]LCA, [$^2H_3$]DCA, [$^2H_4$]CDCA, [$^2H_4$]UDCA and [$^2H_3$]CA (each 0.1–1.0 μg) were added to the solution. After ultrasonication, bile acids were extracted from the serum and urine using a Bond Elut C_{18} cartridge (6 ml). The cartridge was washed with water (5 ml), and the bile acids were eluted with 90% aqueous ethanol (5 ml). After evaporation of solvent under reduced pressure, the residue was subjected to solvolysis according to the method described by Kornel [18]. After solvolysis, the residue was subjected to enzymic hydrolysis according to the method described by Karlaganis et al. [19]. After the reaction mixture had been diluted with 0.5 *M* potassium phosphate buffer (10 ml), bile acids were extracted with a Bond Elut C_{18} cartridge. After evaporation, the hydrolysed sample was dissolved in 4 ml of 90% aqueous ethanol. This solution was applied to a PHP-LH-20 column (20 mm × 6 mm I.D.) prepared in 90% aqueous ethanol. The column was washed with 2 ml of 90% aqueous ethanol to remove neutral compounds, then bile acids were eluted with 4 ml of 0.1 *M* acetic acid in 90% aqueous ethanol. The combined solution was evaporated to dryness under reduced pressure.

The residue was dissolved in 0.5 ml of 5% (w/v) hydrogen chloride in ethanol and was allowed to stand for 60 min at room temperature. After evaporation

under reduced pressure, the residue of bile acid ethyl ester derivatives was treated with 100 μl of distilled pyridine and 25 μl of DMESI [14,20] and allowed to stand for 15 min. Excess silylating reagent was removed using a Sephadex LH-20 column (60 mm×6 mm I.D.) equilibrated with *n*-hexane–chloroform–ethanol (10:10:1, v/v/v). The DMES ether derivatives of bile acid ethyl esters were recovered in the first 2.5 ml of effluent. After evaporation under reduced pressure, the residue was dissolved in pyridine–*n*-hexane (5:95, v/v) solution.

Liver tissue equivalent to ca. 300 mg of wet liver tissue was homogenized in 2 ml of 95% aqueous ethanol containing 0.1% ammonium hydroxide, using a PTFE pestle homogenizer driven by a motor at ca. 700 rpm for 5 min on ice. The homogenate was transferred to a centrifuge tube and washed with three 3-ml portions of 95% aqueous ethanol–0.1% ammonium hydroxide with the aid of ultrasonication. The combined washings were heated in a water-bath at 80°C for 10 min under continuous stirring, and then centrifuged in the cold for 10 min at 5000 *g*. The supernatant was taken and stored at −20°C. To an aliquot of the pooled supernatants, internal standards were added as a mixture of [$^2H_4$]LCA, [$^2H_3$]DCA, [$^2H_4$]CDCA, [$^2H_4$]UDCA and [$^2H_3$]CA (each 0.1–1.0 μg). These samples were then subjected to enzymic hydrolysis [19], solvolysis [18], and derivatization [14,20] to the ethyl ester DMES ethers.

Thin-layer chromatography

TLC was carried out using precoated plates with Kieselgel 60 (Merck). Solvent systems used were isopropyl ether–isooctane–acetic acid (10:5:5, v/v/v) [21] for unconjugated bile acids, and *n*-butanol–acetic acid–water (10:1:1, v/v/v) [22] for conjugated bile acids.

Gas chromatography–mass spectrometry (GC–MS)

GC–MS was performed on a JMA-DX303 instrument equipped with a data processing system JMA DA-5000 (JEOL, Tokyo, Japan). The GC column (25 m×0.32 mm I.D.) was a fused-silica capillary coated with methylsilicone, coupled to the mass spectrometer. The flow-rate of the carrier gas (helium) was 2.0 ml/min. The operating conditions were: injection port temperature, 270°C; column oven programmed from 150 to 280°C at 30°C/min after a 1-min delay from the start time; separator and ion source temperature, 280°C; ionization energy, 70 eV; trap current, 300 μA. Mass spectra were taken by repetitive scanning of mass range *m/z* 100–800 (10 scans/s). The MS resolution was ca. 1000 at *m/z* 693. Selected-ion monitoring (SIM) was carried out under the control of the data system.

Identification and quantitation of individual bile acids

The identification of individual bile acid derivatives was based on the comparison of the methylene unit (MU) values [23] of the peaks on reconstructed ion profiles and their mass spectra with those of reference standards. Quantitation of individual bile acids was carried out by GC–SIM, using the base peak or prominent ions of the ethyl ester DMES ethers. All the monitored ions except those of CDCA/[$^2H_4$]CDCA and 1β- and 6α-hydroxylated bile acids were the charac-

teristic ions of $[M-C_2H_5]^+$ at m/z 461/465 for LCA/$[^2H_4]$LCA, m/z 563/566 for DCA/$[^2H_3]$DCA, m/z 563/567 for UDCA/$[^2H_4]$UDCA, and m/z 665/668 for CA/$[^2H_3]$CA, whereas the following ions were used for the monitored ions of CDCA/$[^2H_4]$CDCA and 1β- and 6α-hydroxylated bile acids: [M− dimethylethylsilanol (DMESOH)$-C_2H_5]^+$ at m/z 459/463 for CDCA/$[^2H_4]$CDCA, the inherent ion at m/z 245 (the well-known ion of m/z 143 with an additional DMES group) for 1β-hydroxylated bile acids, and $[M-3(4)DMESOH+H]^+$ at m/z 383 (381) for 6α-hydroxylated bile acids. The 1β- and 6α-hydroxylated bile acids were quantitated using $[^2H_3]$CA as a convenient internal standard. Good relative recoveries of individual bile acids (83–91%) were obtained by the developed procedure using deuterated bile acids as internal standards. Details of the recovery experiments were described in our recent reports [10,11].

RESULTS

Qualitative composition of bile acids

Table I summarizes the bile acids identified in the biological samples (urine, serum, liver tissue) of control subjects and those of patients with cholestasis. Fig. 1 shows representative selected-ion recordings of urinary bile acids of a patient with extrahepatic cholestasis. The ions of the base peak or a prominent peak in the mass spectra were used to quantitate individual bile acids by GC–SIM-MS. The derivatives of these bile acids (compounds α, γ, ω, ψ and ϵ) gave a base peak at m/z 245, corresponding to the well-known ion of m/z 143 with the additional DMES group. The appearance of this ion in a spectrum of a bile acid derivative strongly suggested a 1,3-bis-dimethylethylsiloxy structure. When MU values and mass spectra were compared with those of the authentic standards of 1β-hydroxylated bile acids, compounds α, ω and ϵ were identified as 1β,3α,12α-trihydroxy-(DCA-1β-ol), 1β,3α,7α-trihydroxy-(CDCA-1β-ol) and 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acids (CA-1β-ol), respectively. Compounds γ and ψ appeared to be an epimer of DCA-1β-ol (B-1β,3,12-ol) and 1β,3α,7β-trihydroxy-5β-cholanoic acid (UDCA-1β-ol), respectively. Although DCA-1β-ol was found in the urine of control subjects, none or trace amounts of the other 1β-hydroxylated bile acids were detected in either their urine or serum.

By comparing its MU value and mass spectrum with those of the authentic standard, compound β was identified as 3α,6α,7α-trihydroxy-5β-cholanoic acid (CDCA-6α-ol) (hyocholic acid; HyoCA). The derivative of compound δ gave a base peak at m/z 381 $[M-4DMESOH+H]^+$ and other prominent peaks at $[M-DMESOH]^+$, m/z 588 $[M-2DMESOH]^+$, m/z 251 [M−4DMESOH−side chain]$^+$ and m/z 129, suggesting that there are four hydroxyl groups in the ring system. The base peak at m/z 381, the intense peak at m/z 559, which corresponds to m/z 561 in the spectrum of the HyoCA derivative, and the peak at m/z 209, which is typical of the 6,7-bis-dimethylethylsiloxy structure, were very similar to that of the derivative of HyoCA. These data strongly suggest that this bile acid is 3α,6α,7α,12α-tetrahydroxy-5β-cholanoic acid (CA-6α-ol), which has been found previously in the urine of cholestatic patients [1]. In the control subjects, no CA-6α-ol was detected, although HyoCA was found.

TABLE I

COMPLETELY AND PARTIALLY IDENTIFIED BILE ACIDS IN THE BIOLOGICAL MATERIALS OF PATIENTS WITH CHOLESTASIS

MU = Methylene unit value of ethyl ester DMES derivative; M_r = molecular mass Fragment ions. chemical structures after M indicate mass of fragments lost; DMESOH = dimethylethylsilanol; italicized fragment ions were used to quantitate the individual bile acids.

Bile acid identified[a]		MU	Fragment ions (m/z) (relative intensities)				
			M_r	$[M]^+$	$[M-C_2H_5]^+$	$[M-DMESOH]^+$	$[M-C_2H_5-DMESOH]^+$ or $[M-C_2H_5-DMESOH+H]^+$
†A	5β-B-3α-ol	33 00	490	490 (1.6)	*461* (100.0)	386 (61.5)	358 (1 3)
†B	5β-B-3α,12α-ol	34.48	592	592 (–)	563 (100.0)	488 (0.6)	459 (2.3)
†C	5β-B-3α,7α-ol	34 82	592	592 (–)	563 (2.4)	488 (0.8)	*459* (61 3)
†D	5β-B-3α,7β-ol	35.24	592	592 (–)	*563* (100.0)	488 (22.5)	459 (11.8)
†E	5β-B-3α,7α,12α-ol	36 03	694	694 (–)	*665* (100 0)	590 (0.6)	561 (6 9)
†α^T	5β-B-1β,3α,12α-ol	36.55	694	694 (–)	665 (33 3)	590 (14.2)	561 (8.4)
†β^T	5β-B-3α,6α,7α-ol	36 62	694	694 (–)	665 (3.7)	590 (1 3)	561 (9 2)
γ	B-1β,3,12-ol[b]	36 99	694	694 (–)	665 (28.8)	590 (21 3)	561 (8 8)
ω^T	5β-B-1β,3α,7α-ol	37.40	694	694 (–)	665 (3 5)	590 (2 2)	561 (1 7)
ψ	5β-B-1β,3α,7β-ol	37.68	694	694 (–)	665 (15.1)	590 (16.9)	561 (4.8)
δ	5β-B-3α,6α,7α,12α-ol	37.76	796	796 (–)	767 (8.6)	692 (–)	663 (3 8)
ϵ	5β-B-1β,3α,7α,12α-ol	37.97	796	796 (–)	767 (32.0)	692 (4.4)	663 (4 8)

[a]B = cholanoic acid. Configurations at C-5 and of hydroxyl groups are indicated by Greek letters Capitals and Greek letters correspond to the peaks in Fig 1; daggers mark those bile acids that were also found in control subjects; superscript T represents polyhydroxylated bile acids that were also found in the liver tissue of patients with cholestasis.

[b]Positions of hydroxyl groups and stereochemistry are tentative.

Quantitative composition of bile acids

The levels and composition of urinary bile acids found in control subjects and in the patients are shown in Table II. The data for the serum bile acids are listed in Table III. The patients with cholestasis excreted considerable amounts, 85.2 mg/day, as total bile acids into urine. In proportion to the increase of daily excretion, 1β- and 6α-hydroxylated bile acids also increased in comparison with control subjects; CDCA-1β-ol, 0.7 mg/day; CA-1β-ol, 2.9 mg/day ($p<0.05$, vs. control subjects); HyoCA, 7.7 mg/day ($p<0.01$); and CA-6α-ol, 5.5 mg/day ($p<0.01$). However, the daily excretion of DCA-1β-ol decreased. Other hydroxylated bile acids, UDCA-1β-ol and B-1β,3,12-ol, were excreted in trace amounts (less than 0.1 mg/day). Similarly, the proportions of these hydroxylated bile acids showed a significant increase in comparison with those of control subjects: CA-1β-ol, 3.2% ($p<0.01$); HyoCA 9.0% ($p<0.05$); CA-6α-ol, 5.3% ($p<0.01$). However, the proportion of DCA-1β-ol (0.2%, $p<0.05$) decreased significantly.

Two compounds of tetrahydroxylated bile acids, which were not detectable in control subjects, were abundant in the urine of the patients with cholestasis, and the ratio of tri plus tetra to dihydroxy bile bile acids of cholestatic patients as an index of urinary bile acid profiles (1.49) was significantly higher than that of control subjects (0.76) ($p<0.01$).

[M−2DMESOH]$^+$ or [M−2DMESOH+H]$^+$	[M−3DMESOH]$^+$ or [M−3DMESOH+H]$^+$	[M−4DMESOH]$^+$ or [M−4DMESOH+H]$^+$	Other ions
–	–	–	323 (24.7), 257 (35.2), 215 (50.9)
384 (6.8)	–	–	359 (4.5), 255 (74.6)
385 (100.0)	–	–	369 (10.5), 339 (18.1), 255 (30.7)
383 (21.2)	–	–	339 (10.0), 255 (12.1)
486 (6.0)	383 (30.3)	–	357 (7.3), 337 (4.6), 353 (24.7)
486 (5.3)	382 (4.0)	–	330 (8.4), 253 (9.8), *245* (100.0)
487 (2.2)	*383* (100.0)	–	337 (9.6), 319 (1.9), 275 (3.0), 161 (24.6), 159 (23.8)
486 (10.6)	382 (12.0)	–	330 (4.8), 253 (12.8) *245* (100.0)
486 (4.4)	382 (5.3)	–	330 (3.5), 253 (–) *245* (100.0)
486 (27.6)	382 (4.4)	–	330 (4.9), 253 (4.0) *245* (100.0)
588 (3.6)	485 (81.2)	*381* (100.0)	559 (30.4), 251 (5.2), 209 (10.6), 161 (13.4), 129 (12.7)
588 (4.4)	485 (15.6)	380 (17.8)	355 (5.7), 251 (11.1), 245 (100.0), 209 (17.7), 196 (14.2)

In serum, these hydroxylated metabolites were found in small amounts. However, their concentrations increased in proportion to increase of the serum concentrations of total bile acids: CDCA-1β-ol, 0.09 μg/ml; CA-1β-ol, 0.04 μg/ml; HyoCA, 2.3 μg/ml ($p<0.05$, vs. control subjects); CA-6α-ol, 0.05 μg/ml ($p<0.05$). Two hydroxylated metabolites, DCA-1β-ol and HyoCA, were detected in small amounts in the serum of control subjects. As with the proportions of total bile acids, these hydroxylated metabolites increased slightly in comparison with control subjects: CDCA-1β-ol, 0.7%; CA-1β-ol, 0.2%; HyoCA, 3.7%; CA-6α-ol, 0.1% ($p<0.05$, vs. control subjects). However, DCA-1β-ol decreased significantly. In the serum bile acid profile, the ratio of tri plus tetra to dihydroxy bile acids of cholestatic patients (1.31) was larger than that in control subjects (0.54, $p<0.01$). There was no significant difference between intrahepatic and extrahepatic cholestasis on qualitative and quantitative analyses of bile acids. The amounts of DCA-1β-ol decreased with a decrease of DCA in the patients with cholestasis, but the amounts of HyoCA, 1β- and 6α-tetrahydroxylated bile acids increased in proportion to the increase of primary bile acids.

Table IV shows the levels and composition of bile acids in the liver tissue of the patients with extrahepatic cholestasis. As shown in this table, DCA-1β-ol, CDCA-1β-ol and HyoCA were found as minor components. No tetrahydroxy bile acids were found in the liver tissue analysed.

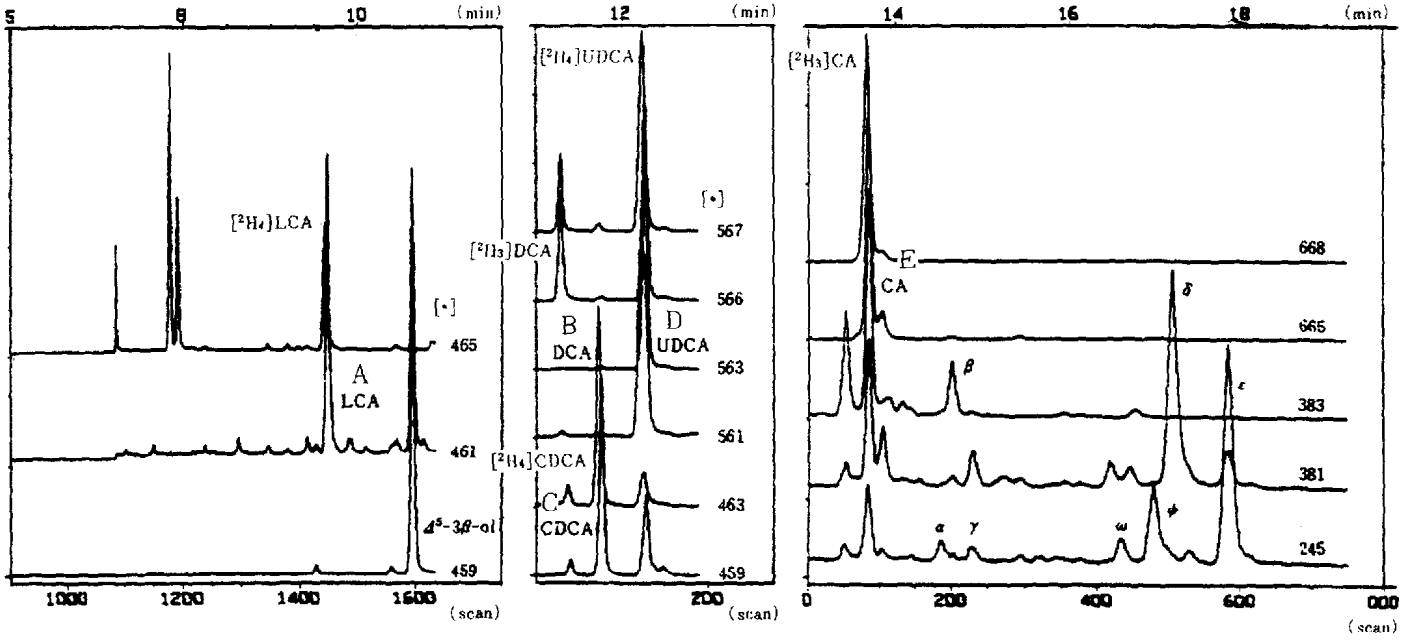

Fig. 1. Selected-ion recordings of urinary bile acids of a patient with extrahepatic cholestasis. Peaks α, β, γ, ω, ψ, δ and ϵ represent 1β- and 6α-hydroxylated bile acids completely or partially identified in the present study (Table I). Monitored ions were selected as follows: m/z 461/465 for LCA/[$^2H_4$]LCA; 563/566 for DCA/[$^2H_3$]DCA; 459/463 for CDCA/[$^2H_4$]CDCA; 563/567 for UDCA/[$^2H_4$]UDCA; 665/668 for CA/[$^2H_3$]CA; 459 for 3β-hydroxy-5-cholenoic acid, 245 for 1β-hydroxylated bile acids, and 383 (or 381) for 6α-hydroxylated bile acids. Monitored ions were changed at the positions indicated by the asterisks.

TABLE II

BILE ACID LEVELS AND COMPOSITIONS IN URINE OF CONTROL SUBJECTS AND PATIENTS WITH CHOLESTASIS

Values are mean ± S.D.; N.D. = not detectable.

Compound[a]	Position of substituents[b]	Controls ($n=14$)		Cholestasis					
				Intrahepatic ($n=5$)		Extrahepatic ($n=11$)		Total ($n=16$)	
		mg/day	%	mg/day	%	mg/day	%	mg/day	%
	Total	23.4 ± 43.6		87.3 ± 72.8		84.1 ± 72.6[c]		85.2 ± 70.2[d]	
A	3α	7.0 ± 13.6	24.0 ± 16.6	2.1 ± 2.6	2.7 ± 3.6[f]	1.0 ± 0.6	1.7 ± 1.3[f]	1.4 ± 1.5	2.1 ± 2.2[f]
B	3α,12α	3.2 ± 4.4	18.0 ± 7.4	1.2 ± 1.1[e]	1.3 ± 1.4[f]	1.2 ± 1.1	1.8 ± 2.4[f]	1.1 ± 1.1	1.6 ± 2.1[f]
C	3α,7α	6.5 ± 14.8	14.7 ± 9.6	23.5 ± 11.6	33.0 ± 16.9[f]	18.9 ± 15.0	25.0 ± 13.9	20.3 ± 13.8[c]	27.5 ± 14.8[d]
D	3α,7β	3.1 ± 5.5	10.7 ± 6.6	1.4 ± 1.1	1.6 ± 1.1[f]	1.4 ± 1.8	1.8 ± 1.6[f]	1.4 ± 1.5	1.7 ± 1.5[f]
E	3α,7α,12α	2.2 ± 2.5	17.6 ± 22.2	22.4 ± 31.3	18.7 ± 12.1	10.3 ± 4.5[d]	16.2 ± 9.7	14.1 ± 17.6[d]	17.0 ± 10.1
α	1β,3α,12α	0.1 ± 0.1	4.1 ± 5.1	0.07 ± 0.04	0.11 ± 0.06[e]	0.11 ± 0.07	0.19 ± 0.15[e]	0.10 ± 0.06	0.16 ± 0.13[e]
ω	1β,3α,7α	N.D.	–	0.12 ± 0.06[e]	0.2 ± 0.1[e]	1.0 ± 2.1	1.0 ± 1.7	0.7 ± 1.8	0.7 ± 1.4
ϵ	1β,3α,7α,12α	N.D.	–	2.9 ± 3.8	3.8 ± 5.3	2.9 ± 5.0	2.9 ± 3.8[c]	2.9 ± 4.5[c]	3.2 ± 4.1[d]
β	3α,6α,7α	0.4 ± 1.1	3.2 ± 5.5	6.8 ± 8.0	6.6 ± 5.0	8.2 ± 7.0[d]	10.2 ± 8.9[c]	7.7 ± 7.1[d]	9.0 ± 7.9[c]
δ	3α,6α,7α,12α[g]	N.D.	–	6.6 ± 11.1	4.6 ± 4.9	5.0 ± 5.6[c]	5.6 ± 5.7[d]	5.5 ± 7.4[d]	5.3 ± 5.3[d]
	$\Delta^5-3\beta$	0.9 ± 0.9	7.5 ± 5.1	19.1 ± 12.5	27.3 ± 10.1	33.5 ± 16.6	33.6 ± 12.6	29.0 ± 16.5	31.6 ± 11.9

[a] Capitals and Greek letters correspond to the peaks in Fig 1.

[b] In 5β-cholanoic acid, unless otherwise noted. Greek letter donates configuration of hydroxyl groups. Superscript denotes position of double bond.

[c] $p < 0.05$, significantly increased in comparison with controls.

[d] $p < 0.01$, significantly increased in comparison with controls.

[e] $p < 0.05$, significantly decreased in comparison with controls

[f] $p < 0.01$, significantly decreased in comparison with controls.

[g] Tentative; see text.

TABLE III

BILE ACID LEVELS AND COMPOSITIONS IN SERUM OF CONTROL SUBJECTS AND PATIENTS WITH CHOLESTASIS

Values are mean ± S.D.; Tr. = trace amounts (< 0.01 μg/ml); N.D. = not detectable.

Compound[a]	Position of substituents[b]	Controls ($n=14$)		Cholestasis					
				Intrahepatic ($n=5$)		Extrahepatic ($n=11$)		Total ($n=16$)	
		μg/ml	%	μg/ml	%	μg/ml	%	μg/ml	%
	Total	2.4 ±0.7		69.2 ±46.5[c]		63.3 ±39.6[d]		65.1 ±40.3[d]	
A	3α	0.11 ± 0.03	2.6 ± 1.8	0.09 ± 0.03	0.4 ± 0.5[f]	0.15 ± 0.08	0.4 ± 0.6[f]	0.13 ± 0.08	0.4 ± 0.5[f]
B	3α,12α	0.5 ±0.3	23.8 ± 10.3	0.5 ± 0.3	2.9 ± 6.5[f]	3.8 ± 10.8	3.5 ± 7.8[f]	2.7 ± 8.9	2.9 ± 6.5[f]
C	3α,7α	0 8 ±0.7	34.6 ± 16.6	30.9 ± 24.9[d]	46.2 ± 15.6	26.5 ± 18.2[d]	45.2 ± 13.1	27.9 ± 19.7[d]	46.2 ± 15.6
D	3α,7β	0.12 ± 0.07	6.3 ± 4.8	0.6 ± 0.3[e]	1.5 ± 2.9[e]	0.3 ± 0.4	0.8 ± 0.7[f]	0.4 ± 0.4[e]	1.5 ± 2.9[f]
E	3α,7α,12α	0.5 ±0.2	21.1 ± 7 4	33.7 ±32.8	37.1 ± 16.8	27.2 ± 17.3[d]	35.6 ± 14.7[d]	29.3 ± 22.2[d]	37.1 ± 16.8[d]
α	1β,3α,12α	Tr.	1.0 ± 0.7	0.02 ± 0.03	0.2 ± 0.3[f]	0.06 ± 0.04[d]	0.2 ± 0.3[f]	0.05 ± 0.04[d]	0.2 ± 0.3[f]
ω	1β,3α,7α	N.D.	–	0.02 ± 0.03	0.7 ± 2.7	0.1 ± 0.2	1.1 ± 3.3	0.09 ± 0.24	0.7 ± 2.7
ε	1β,3α,7α,12α	N.D.	–	Tr.	0.3 ± 0.8	0.05 ± 0.08	0.3 ± 1 0	0.04 ± 0.07	0.2 ± 0.8
β	3α,6α,7α	0.1 ±0.2	6.1 ± 8.1	1.4 ± 1.2	3.7 ± 4 8	2.7 ± 4.4	4.3 ± 5.8	2.3 ± 3.7[c]	3.7 ± 4.8[c]
δ	3α,6α,7α,12α[g]	N.D.	–	0.03 ± 0 03	0.08 ± 0.11	0.06 ± 0.07[c]	0.07 ± 0.09[c]	0.05 ± 0.07[c]	0.08 ± 0.11
	$\Delta^5-3\beta$	0.2 ±0 1	4.1 ± 3.9	1.9 ± 0.4	6.9 ± 3.0	2.4 ± 2.1	8.7 ± 3.5	2.2 ± 1.8	8.1 ± 3.3

[a] Capitals and Greek letters correspond to the peaks in Fig. 1.

[b] In 5β-cholanoic acid, unless otherwise noted. Greek letter donates configuration of hydroxyl groups. Superscript denotes position of double bond.

[c] $p < 0.05$, significantly increased in comparison with controls.

[d] $p < 0.01$, significantly increased in comparison with controls.

[e] $p < 0.05$, significantly decreased in comparison with controls

[f] $p < 0.01$, significantly decreased in comparison with controls.

[g] Tentative; see text

TABLE IV

BILE ACID LEVELS AND COMPOSITION IN LIVER TISSUE OF PATIENTS WITH EXTRAHEPATIC CHOLESTASIS

Values in parentheses are expressed as a percentage of the total bile acids in liver; N.D. = not detectable.

Patient No.	Total (μg/g of liver)	Individual bile acids (μg/g of liver)									
		3α[a]	3α,12α	3α,7α	3α,7β	3α,7α,12α	1β,3α,12α	1β,3α,7α	1β,3α,7α,12α	3α,6α,7α	3α,6α,7α,12α
1	251.9	0.59 (0.02)	1.72 (0.6)	163.98 (65.1)	6.80 (2.7)	74.31 (29.5)	0.25 (0.1)	N.D. (–)	N.D. (–)	N.D. (–)	N.D. (–)
2	87.0	0.78 (0.9)	2.34 (2.7)	38.36 (44.1)	2.95 (3.4)	38.01 (43.7)	0.44 (0.5)	N.D. (–)	N.D. (–)	1.91 (2.2)	N.D. (–)
3	61.1	0.36 (0.6)	1.83 (3.0)	41.73 (68.3)	0.12 (0.2)	15.15 (24.8)	N.D. (–)	N.D. (–)	N.D. (–)	N.D. (–)	N.D. (–)
4	49.3	0.24 (0.5)	1.13 (2.3)	30.76 (62.4)	0.19 (0.4)	15.03 (30.5)	0.10 (0.2)	N.D. (–)	N.D. (–)	N.D. (–)	N.D. (–)
5	33.6	2.04 (6.1)	1 10 (3.3)	22.10 (65.8)	0.33 (1.0)	5.34 (15.9)	0.13 (0.4)	N.D. (–)	N.D. (–)	0.53 (1.6)	N.D. (–)
6	74.9	0.37 (0.5)	1.12 (1.5)	64.48 (86.1)	0.44 (0 6)	7.26 (9.7)	0.15 (0.2)	0.07 (0.1)	N.D. (–)	N.D. (–)	N.D. (–)
7	229.7	15.16 (6.6)	0.68 (0.3)	81.77 (35.6)	0.91 (0.4)	9.87 (4.3)	0.23 (0.1)	0.01 (t)[b]	N.D. (–)	0.46 (0.2)	N.D. (–)

[a]In 5β-cholanoic acid, unless otherwise noted. Greek letter donates configuration of hydroxyl groups.
[b]Trace ($<0.1\%$).

DISCUSSION

Recent studies have revealed the occurrence of unusual trihydroxy and tetrahydroxy bile acids, such as C-1 or C-6 hydroxylated metabolites of DCA, CDCA and CA, in the urine of patients with cholestasis [2,4]. It is thought that these bile acids might reflect altered metabolism of bile acids in the cholestatic state. Bremmelgaard and Sjövall [7] reported that hydroxylation of CA, CDCA and DCA might occur preferentially at the C-1 or C-6 position in cholestatic patients. However, there is little information about the profiles of C-1 and C-6 hydroxylated bile acids in biological samples other than urine.

In this study, GC–SIM was used to determine trace amounts of C-1 or C-6 hydroxylated bile acids in urine, serum and liver tissue. The SIM of the 1β-hydroxylated bile acid derivatives using the base peak at m/z 245 made it possible to perform microdetermination of these bile acids without interference from other bile acids. Excellent recoveries were obtained using [$^2H_3$]CA as an internal standard. The resulting data about the distribution of 1β- and 6α-hydroxylated bile acids in various biological samples revealed the metabolic significance of hydroxylation of bile acids in the cholestatic state.

Five 1β-hydroxylated bile acids, DCA-1β-ol, CDCA-1β-ol, UDCA-1β-ol, CA-1β-ol and an epimer of DCA-1β-ol, were found in the urine of patients with cholestasis. Tohma and co-woerkers [8,9] identified the 1β-hydroxylated bile acids in human meconium by comparison with synthesized standards [8,9]. Further 1β-hydroxylated bile acids were found in umbilical cord blood [11] and amniotic fluid [11]. In comparison with authentic standards, these hydroxylated metabolites were proved to have a 1β-hydroxyl as well as those in meconium [8,9], umbilical cord blood [11] and amniotic fluid [11]. Previous studies have shown that DCA is hydroxylated to very small extent in human males [24–28]. It has been reported that DCA exists as sulphate in urine [1–3]. This study suggested that 1β-hydroxylation is another metabolic transformation of DCA in cholestasis, and that 1β- or 6α-hydroxylation is also a metabolic transformation of CA in this state. Other 1β-hydroxylated bile acids can also be postulated to be metabolites from 1β-hydroxylation of CDCA and UDCA in cholestasis.

All 1β- and 6α-hydroxylated bile acids found in the cases of intrahepatic cholestasis were also present in the samples from patients with extrahepatic cholestasis. Indeed, there was no qualitative difference between intra- and extrahepatic cholestasis. Tetrahydroxy bile acids were found only in cholestasis, whereas trihydroxy bile acids, such as DCA-1β-ol and HyoCA, were detected in the urine of control subjects. This suggests that, in the normal state, DCA and CDCA are transformed into 1β- and 6α-hydroxylated compounds, respectively.

Quantitative analysis of 1β-hydroxylated bile acids in the urine demonstrated that, in cholestasis, CDCA-1β-ol and CA-1β-ol increased in the daily excretion and in proportion to the total bile acids, but DCA-1β-ol decreased. This was considered to reflect the bile acid profile in serum and liver tissue in cholestasis. Cholestasis seems to accelerate 1β-hydroxylation of excessive bile acids. Similarly in the case of 6α-hydroxylated bile acids, the increase of HyoCA and CA-6α-ol

in the daily excretion might reflect accelerated 6α-hydroxylation of excessive CA and CDCA. The ratio of tri plus tetra to dihydroxy bile acids in the bile acid profile of urine and serum was much higher in cholestasis patients than in control subjects. This is due to the increase of 1β- and 6α-hydroxylated bile acids in the urine.

Little is known about the organs in which 1β- or 6α-hydroxylation of bile acids occurs. Foetal liver microsomes have been shown to be capable of 1β-hydroxylation of steroids [29,30] and bile acid [31]. Also, Trülsch et al. [32] reported the existence of microsomal 6α-hydroxylase activity toward taurolithocholic acid in liver biopsies. The bile acid composition in liver tissue likely reflects the biosynthesis [33]. The 1β- and 6α-hydroxylated bile acids appearing in liver tissue suggest possible hepatic 1β- or 6α-hydroxylation of bile acids.

The proportions of 1β- and 6α-hydroxylated bile acids were greater in urine than in serum and liver tissue. This situation seems to be analogous to those of sulphate [34,35] and glucuronide bile acids [36,37]. For example, sulphated bile acids are predominant in urine but are only minor components in bile. Serum bile acids show intermediate sulphation between urine and bile [38–40]. Thus, the enrichment of these hydroxylated metabolites in urine suggests that they are efficiently eliminated into urine owing to an increase in their polarity. In patients with cholestasis, sulphation [34,35] and glucuronidation [36,37] of bile acids are important for bile acid metabolism. High renal clearance rates contribute to efficient elimination of sulphated bile acids from the body [34]. In this situation, cholestatic liver may accelerate hydroxylation of excessive bile acids at the 1β- or 6α-position for their efficient elimination.

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