Concurrent occurrence of 3β,12α-dihydroxy-5-cholenoic acid associated with 3β-hydroxy-5-cholenoic acid and their preferential urinary excretion in liver diseases

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Abstract 3β-Hydroxy-(Δ^5-3β-ol), 3β,12α-dihydroxy-(Δ^5-3β,12α-ol), 3β,7α-dihydroxy-(Δ^5-3β,7α-ol) and 3β,7β-dihydroxy-(Δ^5-3β,7β-ol) 5-cholenoic acids were identified in patients with liver diseases by gas–liquid chromatography–mass spectrometry (GLC–MS). Of these unusual 3β-hydroxy-5-en-metabolites, Δ^5-3β-ol and Δ^5-3β,12α-ol were found as major components in the urine of patients with liver diseases (cholestasis, liver cirrhosis, chronic hepatitis, acute hepatitis). Other 3β-dihydroxy-5-en-metabolites, Δ^5-3β,7α-ol and Δ^5-3β,7β-ol, were found as minor components in the urine. The levels of Δ^5-3β-ol and Δ^5-3β,12α-ol in urine were correlated with their levels in serum, with total bile acids in the urine, and with liver function, implying that the degree of their increment correlated well with the severity of liver diseases. The most abundant amounts of Δ^5-3β-ol and Δ^5-3β,12α-ol were found in the urine as sulfate conjugates in comparison with bile, portal and hepatic venous sera, and liver tissue of the patients. The biliary excretion and hepatic extraction of these 3β-hydroxy-5-en-unsaturated bile acids were more impaired and inefficient than those of cholic and chenodeoxycholic acids.—**Shoda, J., T. Osuga, K. Matsuura, R. Mahara, M. Tohma, N. Tanaka, Y. Matsuzaki, and H. Miyazaki.** Concurrent occurrence of 3β, 12α-dihydroxy-5-cholenoic acid associated with 3β-hydroxy-5-cholenoic acid and their preferential urinary excretion in liver diseases. *J. Lipid Res.* 1989. **30:** 1233–1242.

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A number of unusual bile acids have been found in the urine of patients with liver disease (1–3). 3β-Hydroxy-5-cholenoic acid (Δ^5-3β-ol) (4, 5), which may be an intermediate in an altered pathway of bile acid synthesis via 26-hydroxycholesterol (6–8), was first identified in the urine of infants with congenital biliary atresia (9). Since then, this bile acid has also been detected in the biological fluids of patients with cholestasis (1, 2) or liver cirrhosis (10, 11). In particular, biological samples from term newborns, such as human meconium (12, 13), umbilical cord blood (14), and amniotic fluid (14, 15) have shown abundant amounts of $\Delta^5$3β-ol and 3β,12α-dihydroxy-5-cholenoic acid (Δ^5-3β,12α-ol), indicating that the altered metabolism was active during the fetal period, the so-called underdevelopmental state. 3β,7α-Dihydroxy-5-cholenoic acid (Δ^5-3β,7α-ol), which was identified in human bile (16, 17), has been suggested as another altered pathway via 26-hydroxylation of 7α-hydroxycholesterol (18). Recently Clayton et al. (19, 20) found a large amount of Δ^5-3β,7α-ol in a single infant with severe cholestasis due to a defect of microsomal 3β-hydroxy-5-en-steroid dehydrogenase/isomerase. Appearance of 3β-hydroxy-5-en-metabolites from biological fluids in the diseased states suggests a decrease of enzyme activity relative to normal bile acid synthesis and the activation of an altered metabolism.

This report deals with the analysis of 3β-hydroxy-5-en-metabolites in biological samples from patients with liver

Abbreviations: LCA, lithocholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; DCA, deoxycholic acid; HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography; Δ^5-3β-ol, 3β-hydroxy-5-cholenoic acid; Δ^5-3β,12α-ol, 3β,12α-dihydroxy-5-cholenoic acid; DMES, dimethylethylsilyl; SIM, selected ion monitoring; MS, mass spectrometry; UDCA, ursodeoxycholic acid; TLC, thin-layer chromatography.

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disease using gas-liquid chromatography-mass spectrometry (GLC-MS), and with metabolic and clinical aspects of the liver diseases.

METHODS

Patients

The present experiments were carried out in 111 male and female subjects (17 patients without liver disease and 94 patients with liver disease) ranging in age from 20 to 69 yr. The patients with liver diseases included 20 intra- and extrahepatic cholestasis, 21 liver cirrhosis in compensated state, 19 liver cirrhosis in decompensated state, 15 chronic hepatitis, and 19 acute hepatitis. The severity of the liver cirrhosis was judged using criteria described previously (21); according to these criteria, the liver cirrhosis was divided into two groups such as compensated and decompensated states. **Table 1** summarizes pertinent clinical and laboratory data of the 111 patients.

Sample collections

Urine and peripheral venous blood. Urine was collected over a 24-h period and aliquots were kept refrigerated at −20°C until analyzed. Blood was taken from an antecubital vein from fasting subjects, and the serum was obtained by centrifugation and frozen at −20°C until analyzed.

Bile and liver tissue. Paired bile samples and liver tissue were obtained simultaneously during laparotomy or autopsy from 8 of the control subjects and 14 of the cirrhotic subjects described above. Liver tissue was also obtained from 7 cholestatic patients. Bile samples were taken by puncture aspiration of gallbladder contents, and kept refrigerated at −20°C until analyzed. The resected liver samples were immediately rinsed with chilled saline, sliced into small blocks, briefly dried on filter paper, weighed, and kept refrigerated at −80°C until analyzed.

Portal venous blood, hepatic venous blood, and bile. Portal and hepatic venous blood were obtained during the examination of percutaneous portography for the evaluation of esophageal varices in 8 cirrhotic patients. Simultaneously, peripheral venous blood was taken from an antecubital vein. Bile was obtained as bile-rich duodenal fluid by aspirating duodenal juice after contracting the gallbladder using magnesium sulfate.

Reference compounds

Authentic bile acids were obtained from Steraloids, Inc. (Wilton, NH). Standards of 3β-dihydroxy-5-en-metabolites, such as 3β,12α-dihydroxy-, 3β,7α-dihydroxy-, and 3β,7β-dihydroxy-5-cholenoic acids were synthesized by Tohma et al. (22). Deuterated bile acids such as [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, Co. [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 Co. (Tokyo, Japan), respectively. Deuterated 3β-hydroxy-5-en-metabolites, such as [2,2,4,23,23-$^2H_5$]3β-hydroxy-5-cholenoic acid (Δ^5-3β-ol) and [2,2,4,4,6,23-$^2H_6$]3β,12α-dihydroxy-5-cholenoic acid (Δ^5-3β,12α-ol) were synthesized by Tohma et al. (23). Purity of bile acids was evaluated using thin-

TABLE 1. Biochemical data

	n	Alb	T-Bil	GOT	GPT	ALP
		g/dl	*mg/dl*	*U/l*	*U/l*	*K-AU*
Controls	17	4.3 ± 0.4[a] 3.7 - 5.3[b]	0.5 ± 0.2 0.2 - 1.1	14.1 ± 9.4 5 - 35	10.1 ± 8.4 4 - 35	7.2 ± 6.4 3.7 - 32.4
CHOL	20	3.6 ± 0.7 2.4 - 4.8	17.0 ± 8.4 5.0 - 34.6	137.2 ± 105.1 19 - 338	117.5 ± 113.6 14 - 273	42.9 ± 25.6 11.7 - 89.7
LC (com)[c]	21	3.6 ± 0.4 2.5 - 4.4	0.8 ± 0.3 0.5 - 1.8	87.6 ± 67.7 25 - 263	64.8 ± 59.4 10 - 275	11.7 ± 5.3 6.0 - 27.7
LC (dec)[c]	19	3.0 ± 0.6 1.7 - 3.8	5.3 ± 4.0 0.5 -14.5	129.9 ± 100.7 31 - 348	68.4 ± 67.5 8 - 224	21.2 ± 13.0 6.6 - 55.1
CH	15	4.3 ± 0.3 3.7 - 5.1	0.9 ± 0.5 0.3 - 1.8	76.0 ± 56.8 20- 213	126.4 ± 109.5 28 - 394	7.9 ± 1.5 5.3 - 9.8
AH	19	4.4 ± 0.4 3.6 - 5.1	8.4 ± 7.2 1.9 - 37.1	523.7 ± 478.9 82 - 1566	859.0 ± 636.2 186 - 2490	16.2 ± 7.2 6.8 - 24.1

Abbreviations: CHOL, cholestasis; LC (com), compensated liver cirrhosis; LC (dec), decompensated liver cirrhosis; CH, chronic hepatitis; AH, acute hepatitis; Alb, albumin; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; ALP, alkaline phosphatase.

[a]Values represent mean ± SD.

[b]Range of values.

[c]Patients with liver cirrhosis were divided into two groups of LC (com) (total clinical score 2.7 ± 0.8 points (mean ± SD) (1-4 points)) and LC (dec) (14.1 ± 2.7 points (10-17 points)) according to an index of the severity of liver disease described by McCormick et al. (21).

layer chromatography and high-performance liquid chromatography; all chromatograms showed only a single spot and a single peak, respectively.

Chemicals

All solvents were of analytical grade. Bond Elut C_{18} cartridges (octadecylsilane-bonded silica) were obtained from Analytichem International Inc. (Harbor City, CA). Sulfatase (aryl-sulfate sulfohydrolase from *Helix promatia*) (EC 3.1.6.1), cholylglycine hydrolase (3α,7α.12α-trihydroxy-5β-cholan-24-oylglycine amidohydrolase from *Clostridium perfingens*) (EC 3.5.1.24), and β-glucuronidase (β-D-glucuronide glucuronosohydrolase from *Helix promatia*) (EC 3.2.1.31) were from Sigma Chemical Co. (St. Louis, MO). Sephadex LH-20 was from Pharmacia Fine Chemicals (Uppsala, Sweden), and dimethylethylsilylimidazole (DMESI) and *p*-toluenesulfonyl-N-methyl-N-nitrosoamide were from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Piperidinohydroxypropyl Sephadex LH-20 was kindly supplied by Prof. T. Nambara and Dr. J. Goto (Pharmaceutical Institute, Tohoku University, Miyagi, Japan).

Thin-layer chromatography (TLC)

Thin-layer chromatography was carried out according to the method described by Subbian and Kuksis (24).

High performance liquid chromatography (HPLC)

High-performance liquid chromatography was carried out according to the method described by Hirano et al. (25) with minor modifications: a Kaseisorb LC ODS-120-5 column, 4.6 × 250 mm (Tokyo Kasei Kogyo, Tokyo, Japan) was used for the separation of the bile acids. The flow rate was 1 ml/min.

Gas-liquid chromatography (GLC)

Gas-liquid chromatography was carried out as previously described (14).

Gas-liquid chromatography-mass spectrometry (GLC-MS)

A JMS-DX 303 gas chromatograph-mass spectrometer equipped with a data processing system JMA DA 5000 (JEOL Ltd., Tokyo., Japan) was used. The temperature of the injection port was 270°C; the column oven was programmed from 150° to 280°C at a rate of 30°C. Gas-liquid chromatography-selected ion monitoring (GLC-SIM) was carried out under computerized operation.

Clean-up procedure

Urine, serum, bile, and liver samples were purified according to the method described by Yanagisawa et al. (26, 27) with minor modifications. A detailed description of the clean-up procedure is described in our previous report (14).

Urine, serum, and bile. To 1-2 ml of serum, 5-10 ml of urine, and 5-10 μl of bile or bile-rich duodenal juice, adequate amounts of internal standards were added as a mixture of [$^2H_4$]LCA, [$^2H_3$]DCA, [$^2H_4$]CDCA, [$^2H_4$]UDCA, [$^2H_3$]CA, [$^2H_5$]Δ^5-3β-ol, and [$^2H_6$]Δ^5-3β,12α-ol in 0.5 M potassium phosphate buffer (pH 7.0). Each of the resulting mixtures was applied to Bond Elut C_{18}. The resulting eluted bile acids were subjected to enzymatic solvolysis using sulfatase (28); the bile acids were incubated with 180 U of sulfatase in 2 ml of 0.1 M acetic buffer (pH 5.1) and 0.2 ml ethanol at 37°C for 16 h. The reaction mixtures were applied to Bond Elut C_{18}. The solvolyzed bile acids were subjected to enzymatic hydrolysis using cholylglycine hydrolase (29), followed by methylation with diazomethane (freshly distilled diazomethane in diethyl ether-methanol 9:1 (v/v) and derivatization to the DMES ethers (30). After removal of excess silylating reagent by a Sephadex LH-20 column, the resulting bile acid methyl ester DMES ethers were analyzed by GLC-MS.

Conjugation forms of 3β-hydroxy-5-en-metabolites. In the case of samples from some cirrhotic patients, after enzymatic cleavage of amino acid conjugates, the resulting deconjugated bile acids were separated into three fractions using a column of piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) (31). Nonglucuronidated-nonsulfated bile acids, deglucuronidated bile acids after glucuronic acid cleavage by treatment with β-glucruronidase (32), and desulfated bile acids after solvolysis (33) were converted into the methyl ester DMES ether derivatives.

Liver. Liver tissue, corresponding to about 300 mg wet weight, was homogenized in 2 ml of 95% aqueous ethanol containing 0.1% ammonium hydroxide, using a Teflon pestle homogenizer driven by a motor at about 700 rpm, for 5 min on ice. The homogenate was transferred into 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 continuous stirring, and then centrifuged in the cold (at 0°C) for 10 min at 5000 *g*. The supernatant was stored at −20°C. To an aliquot of the pooled supernatants, adequate amounts of internal standards were added as a mixture and then purified.

Identification and quantitation of individual bile acids. The identification of individual bile acid derivatives was based on the comparison of the methylene unit values (MUv) (34) of the peaks on reconstructed ion profiles, and their mass spectra, with those of authentic standards. Quantitation of individual bile acids was carried out by GLC-SIM, using the ions of the methyl ester DMES ethers at *m/z* 447/451 for LCA/[$^2H_4$]LCA, 549/552 for DCA/[$^2H_3$]DCA, 371/375 for CDCA/[$^2H_4$]CDCA, 549/553 for UDCA/[$^2H_4$]UDCA, 369/372 for CA/[$^2H_3$]CA, 445/449 for Δ^5-3β-ol/[$^2H_5$]Δ^5-3β-ol, and 547/552 for Δ^5-3β,12α-ol/[$^2H_6$]Δ^5-3β,12α-ol, respectively. All the monitoring ions except those of CDCA/[$^2H_4$]CDCA, CA/[$^2H_3$]CA, Δ^5-3β,

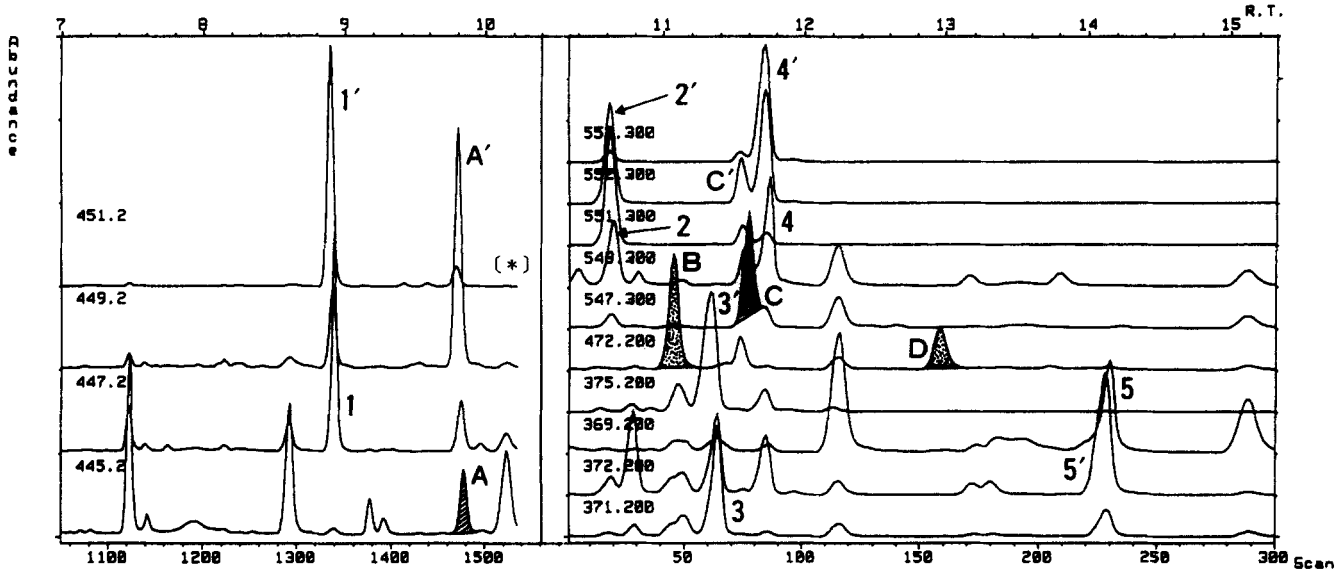

Fig. 1. A representative selected ion recording of urinary bile acids of a patient with extrahepatic cholestasis; 1, LCA; 1', [$^2H_4$]LCA; 2, DCA; 2', [$^2H_3$]DCA; 3, CDCA; 3', [$^2H_4$]CDCA; 4, UCDA; 4', [$^2H_4$]UCDA; 5, CA, 5', [$^2H_3$]CA; A, Δ^5-3β-ol; A', [$^2H_5$]Δ^5-3β-ol; B, Δ^5-3β,7α-ol; C, Δ^5-3β,12α-ol; C', [$^2H_6$]Δ^5-3β,12α-ol; D, Δ^5-3β,7β-ol. Monitoring ions were selected as follows: *m/z* 447/451 for LCA/[$^2H_4$]LCA, 549/552 for DCA, 371/375 for CDCA, 549/553 for UCDA, 369/372 for CA/[$^2H_3$]CA, 445/449 for Δ^5-3β-ol/[$^2H_5$]Δ^5-3β-ol, 547/552 for Δ^5-3β,12α-ol/[$^2H_6$]Δ^5-3β,12α-ol, and 472 for Δ^5-3β,7α-ol. Monitoring ions were changed at the position indicated by the asterisk.

7α-ol, and Δ^5-3β,7β-ol were the characteristic ions of $[M-C_2H_5]^+$, whereas the following ions were used as the monitoring ions: $[M-2 \times DMESOH + H]^+$ for CDCA/[$^2H_4$]CDCA, $[M-3 \times DMESOH + H]^+$ for CA/[$^2H_3$]CA, and $[M-DMESOH]^+$ for Δ^5-3β,7α-ol and Δ^5-3β,7β-ol.

Good relative recoveries of individual bile acids (84–91%) were obtained by the present procedure using deuterated bile acids as internal standards. Details of the recovery experiments were described in our previous reports (14, 23).

RESULTS

Identification of urinary 3β-hydroxy-5-en bile acids in liver diseases

Fig. 1 shows a representative selected ion recording obtained by the analysis of urinary bile acids of a patient with extrahepatic cholestasis. Four compounds of 3β-hydroxy-5-en bile acids, 3β-hydroxy-, 3β,12α-dihydroxy-, 3β,7α-dihydroxy-, and 3β,7β-dihydroxy-5-cholenoic acids,

TABLE 2. 3β-Hydroxy-5-en bile acid in urine and serum of patients with liver disease

		Δ^5-3β-ol		Δ^5-3β,12α-ol		Δ^5-3β-ol		Δ^5-3β,12α-ol	
	n	Excretion	Proportion[a]	Excretion	Proportion	Concentration	Proportion	Concentration	Proportion
		mg/day	%	*mg/day*	%	*μg/ml*	%	*μg/ml*	%
Control	17	0.6 ± 0.2	6.5 ± 1.2	Tr (< 0.1)	0.2 ± 0.09	0.8 ± 0.02	2.3 ± 0.8	Tr (< 0.05)	-
CHOL	20	27.8 ± 5.4****	29.8 ± 3.5****	2.1 ± 0.4****	2.5 ± 0.5****	1.69 ± 0.38****	2.9 ± 0.3	0.38 ± 0.23	0.4 ± 0.1**
LC (com)	21	2.1 ± 0.5***	11.1 ± 2.0*	0.2 ± 0.1	0.9 ± 0.4	0.57 ± 0.16**	3.9 ± 1.0	0.06 ± 0.04	0.1 ± 0.04
LC (dec)	19	6.3 ± 1.3****	21.2 ± 3.9****	3.2 ± 2.0	5.1 ± 2.6	1.95 ± 0.75**	6.1 ± 1.9*	0.53 ± 0.49	0.2 ± 0.04*
CH	15	1.3 ± 0.4	13.3 ± 2.7**	Tr	0.3 ± 0.05	0.17 ± 0.13	0.9 ± 0.4	Tr	-
AH	19	83.3 ± 32.3***	32.0 ± 5.8****	2.0 ± 0.5****	1.2 ± 0.2****	1.18 ± 0.38***	1.9 ± 0.4	0.11 ± 0.01***	0.3 ± 0.06***

Values given as mean ± SEM. Abbreviations: Δ^5-3β-ol, 3β-hydroxy-5-cholenoic acid; Δ^5-3β,12α-ol, 3β,12α-dihydroxy-5-cholenoic acid; CHOL, cholestasis; LC (com), compensated liver cirrhosis; LC (dec), decompensated liver cirrhosis; CH, chronic hepatitis, AH, acute hepatitis; Tr, trace amounts.
[a]Proportion of each bile acid to total bile acids (%).
*, $P < 0.05$; **, $P < 0.025$; ***, $P < 0.01$; ****, $P < 0.005$ compared with controls.

TABLE 3. Nonsulfate-nonglucuronide, sulfate, and glucuronide fractions of bile acids in the urine of patients with liver disease (n = 7)

Compound	Nonsulfate-Nonglucuronide		Sulfate		Glucuronide	
	Percentage	Mean ± SEM	Percentage	Mean ± SEM	Percentage	Mean ± SEM
LCA	0.3 - 13.9	5.6 ± 2.0	71.7 - 96.4	89.4 ± 3.3	0 - 14.3	4.7 ± 1.9
DCA	0 - 25.8	17.2 ± 3.8	47.6 - 88.4	69.2 ± 5.0	7.1 - 20.8	13.6 ± 1.9
CDCA	7.3 - 32.1	17.6 ± 3.4	67.9 - 87.3	76.6 ± 2.5	0 - 8.5	3.4 ± 0.9
UDCA	6.0 - 58.9	22.5 ± 7.2	51.6 - 93.9	70.0 ± 8.1	0 - 22.8	7.4 ± 3.0
CA	36.4 - 66.2	49.5 ± 3.9	25.3 - 42.6	35.9 ± 2.4	4.8 - 25.4	14.4 ± 2.4
Δ^5-3β-ol	3.7 - 38.8	14.9 ± 4.8	54.9 - 92.7	80.8 ± 5.2	2.3 - 6.2	4.1 ± 0.4
Δ^5-3β,12α-ol	7.6 - 25.3	16.3 ± 2.1	68.9 - 86.8	78.3 ± 2.1	3.1 - 6.5	4.8 ± 0.4

were completely identified by comparison with authentic standards (22).

Quantitative determinations of 3β-hydroxy-5-en bile acids in the liver diseases

Urine. The levels of four 3β-hydroxy-5-en-metabolites in the urine of patients with various liver diseases were determined by the present method and compared with those of control subjects. **Table 2** summarizes these results. The increased excretion of Δ^5-3β-ol and Δ^5-3β,12α-ol and their proportions in total bile acids were observed in the liver diseases, especially cholestasis and acute hepatitis; the increases were significant in comparison with the other groups.

The relationship between Δ^5-3β-ol and Δ^5-3β,12α-ol was examined, and there were positive correlations between the amounts excreted in urine ($r = 0.28$, $P < 0.01$) and between the relative amount ($r = 0.52$, $P < 0.01$) in urine. For 7-hydroxy-5-en bile acids, Δ^5-3β,7α-ol and Δ^5-3β,7β-ol, however, excretion increased slightly and their proportions showed no significant difference in comparison with those of control subjects.

Table 3 shows the conjugation forms of Δ^5-3β-ol and Δ^5-3β,12α-ol in the urine. It is worthy of note that the major portions of these two bile acids in the urine were as sulfate conjugates.

Regression analysis showed that the amount of Δ-3β-ol

TABLE 4. Bile acids in bile and supernatant of liver tissue

Compound	Controls		Liver Cirrhosis		Cholestasis
	Bile (n = 8) (mg/ml)	Liver (n = 8) (μg/g)	Bile (n = 14) (mg/ml)	Liver (n = 14) (μg/g)	Liver (n = 7) (μg/g)
Total bile acids	10.1 ± 3.4	36.6 ± 8.6	6.7 ± 1.6	65.9 ± 9.4	112.5 ± 33.8
			percentage		
LCA	1.9 ± 0.4	3.0 ± 1.0	2.5 ± 1.1	1.2 ± 0.2	2.2 ± 1.0
DCA	11.6 ± 2.5	28.2 ± 5.0	6.8 ± 1.0	4.5 ± 1.1	1.9 ± 0.4
CDCA	51.7 ± 3.3	37.1 ± 5.1	55.5 ± 5.9	62.2 ± 3.9	61.1 ± 6.2
UDCA	5.2 ± 1.3	3.4 ± 1.5	9.5 ± 1.7	0.9 ± 0.1	1.2 ± 0.4
CA	28.1 ± 2.2	23.4 ± 3.4	28.6 ± 4.5	25.2 ± 3.2	22.6 ± 5.1
Δ^5-3β-ol	2.1 ± 0.7	2.6 ± 0.9	1.2 ± 0.3	3.2 ± 1.0	9.8 ± 7.1
Δ^5-3β,12α-ol	ND	ND	Tr	Tr	Tr
CA/CDCA (Bile/liver)	0.54 ± 0.06	0.87 ± 0.27 (0.82 ± 0.09)	0.85 ± 0.26	0.38 ± 0.08 (1.71 ± 0.16)**	
CA/Δ^5-3β-ol (Bile/liver)	24.1 ± 14.4	17.1 ± 6.7 (1.08 ± 0.15)	36.5 ± 7.4	15.2 ± 2.4 (2.27 ± 0.20)**	
CDCA/Δ^5-3β-ol (Bile/liver)	39.9 ± 18.5	52.7 ± 29.4 (1.10 ± 0.15)	94.8 ± 28.5	60.0 ± 13.2 (1.62 ± 0.17)*	

Values are mean ± SEM; ND, nondetectable; Tr, trace (< 0.1%).
*, $P < 0.05$; **, $P < 0.01$.

TABLE 5. Total bile acids, cholic, chenodeoxycholic, 3β-hydroxy-5-cholenoic, and 3β,12α-dihydroxy-5-cholenoic acids in portal (P), hepatic (H), and peripheral (V) venous sera of eight cirrhotic patients

Patient No.	Total (μg/ml)					CA (%)					CDCA (%)					Δ^5-3β-ol (%)					Δ^5-3β,12α-ol (%)				
	P	H	V	P/V	P/H	P	H	V	P/V	P/H	P	H	V	P/V	P/H	P	H	V	P/V	P/H	P	H	V	P/V	P/H
1	6.06	4.53	2.58	2.35	1.33	37.1	34.7	16.4	2.26	1.06	27.7	33.3	47.6	0.58	0.83	3.8	3.8	3.5	1.08	1.00	0.5	0.6	0.6	0.83	0.83
2	16.38	11.36	13.07	1.25	1.44	22.6	25.5	26.7	0.85	0.87	70.2	54.5	55.7	1.26	1.28	0.1	0.5	0.4	0.33	0.20	Tr	Tr	0.1	0.50	0.34
3	15.99	12.45	13.17	1.21	1.28	33.8	30.5	29.9	1.13	1.11	60.8	65.8	65.9	0.91	0.92	0.3	0.4	0.3	1.00	0.75	0.1	0.2	0.1	1.00	0.50
4	22.39	19.15	14.74	1.52	1.16	23.4	22.2	23.8	0.98	1.05	70.5	71.5	65.1	1.08	0.98	0.1	0.3	0.1	1.00	0.33	0.1	0.1	0.2	0.50	1.00
5	41.19	-	39.59	1.04	-	10.1	-	9.4	1.07	-	87.1	-	86.8	0.98	-	0.4	-	0.6	0.66	-	Tr	-	0.1	0.50	-
6	4.51	-	3.18	1.41	-	33.5	-	15.3	2.19	-	54.9	-	70.9	0.75	-	0.3	-	3.2	0.09	-	0.5	-	0.5	1.00	-
7	25.70	-	19.07	1.34	-	27.5	-	17.7	1.55	-	66.1	-	68.1	0.97	-	0.2	-	5.1	0.03	-	0.1	-	0.5	1.00	-
8	11.13	-	7.90	1.43	-	20.9	-	23.1	0.90	-	40.9	-	56.9	0.71	-	0.1	-	0.9	0.11	-	0.2	-	0.2	1.00	-
Mean ± SEM	17.91 ± 4.21	11.87 ± 2.97	14.16 ± 4.13	1.44 ± 0.13	1.30 ± 0.05	26.1 ± 3.0	28.2 ± 2.7	20.3 ± 2.3	1.37 ± 0.17	1.02 ± 0.05	59.7 ± 6.5	56.2 ± 8.4	64.6 ± 4.1	0.90* ± 0.07	1.00 ± 0.09	0.7 ± 0.4	1.2 ± 0.8	1.7 ± 0.6	0.53* ± 0.15	0.65 ± 0.16	0.2 ± 0.07	0.2 ± 0.1	0.3 ± 0.07	0.69* ± 0.10	0.66 ± 0.15

Tr, trace; -, no measurement.
*, Significant decrease ($P < 0.05$) in comparison with CA.
**, Significant decrease ($P < 0.01$) in comparison with CA.

and Δ^5-3β,12α-ol excreted and their proportions in total bile acids correlated well with the serum level of total bile acid concentrations; the coefficients (r) of Δ^5-3β-ol and Δ^5-3β,12α-ol were 0.35 ($P < 0.01$) and 0.42 ($P < 0.01$) for the amounts excreted, and 0.28 ($P < 0.05$) and 0.24 ($P < 0.05$) for the proportions in total bile acids, respectively. The proportions were also well correlated with the excretion level of total bile acids; the coefficients (r) of Δ^5-3β-ol and Δ^5-3β,12α-ol were 0.25 ($P < 0.05$) and 0.25 ($P < 0.05$), respectively.

Serum. The serum concentrations of Δ^5-3β-ol and Δ^5-3β,12α-ol concurrently increased in the liver diseases. The proportions of Δ^5-3β,12α-ol to total bile acids significantly increased in cholestasis, decompensated liver cirrhosis, and acute hepatitis in comparison with the control subjects.

The urine/serum (U/S) ratios of the proportions of Δ^5-3β-ol and Δ^5-3β,12α-ol to total bile acids were found to be 16.8 ± 8.8 (mean ± SD) and 16.4 ± 7.3, respectively, indicating that these unusual bile acids were easily excreted into urine.

Bile and liver tissue. **Table 4** shows levels and composition of individual bile acids in the paired bile and liver samples. The amounts of Δ^5-3β-ol in the bile decreased in the liver cirrhosis, and there were only trace levels of Δ^5-3β,12α-ol in all bile samples, including those of control subjects.

The paired bile and liver samples from the cirrhotic patients were analyzed according to the report by Akashi et al. (35). The results indicated that the respective ratios—1.71 for [CA/CDCA] in bile to [CA/CDCA] in liver tissue and 2.27 for [CA/Δ^5-3β-ol] in bile to [CA/Δ^5- 3β-ol] in liver tissue—of the cirrhotic patients were significantly higher than 0.82 for [CA/CDCA] and 1.08 for [CA/Δ^5-3β-ol] of control subjects. The ratio of [CDCA/Δ^5-3β-ol] in bile to that in liver tissue was also significantly higher in liver cirrhosis (1.62) than in control subjects (1.10). This can be explained by postulating the impaired biliary excretion of CDCA and Δ^5-3β-ol in comparison with that of CA in diseased states: biliary excretion may be inefficient in the order Δ^5-3β-ol > CDCA > CA.

Enterohepatic circulation of 3β-hydroxy-5-en bile acids in liver diseases

Bile acids were analyzed simultaneously in the urine, bile and blood samples taken from portal, hepatic, and peripheral veins of eight cirrhotic patients during the examination of percutaneous portography, in order to elucidate the possibly different enterohepatic circulation of 3β-hydroxy-5-en-metabolites compared to that of other normal bile acids. **Table 5** shows the results. The mean ratio between portal and peripheral venous serum concentrations (P/V ratio) of total bile acids was calculated to be 1.44 in this study, which seemed to be lower than the P/V

ratio of healthy subjects previously reported (36, 37). The mean ratio between portal and hepatic venous serum concentrations (P/H ratio) also showed a lower value of 1.30 in comparison with the P/V ratio of healthy subjects (36, 37).

The P/V ratio for CA (1.37) was significantly higher than that for CDCA (0.90) ($P < 0.05$), Δ^5-3β-ol (0.53) ($P < 0.01$), and Δ^5-3β,12α-ol (0.09) ($P < 0.05$). The mean P/H ratio for CA (1.02) was also significantly higher than that for CDCA (1.00), Δ^5-3β-ol (0.65), and Δ^5-3β,12α-ol (0.66). These data suggest that CA is more efficiently extracted by liver from portal venous flow than CDCA and the 3β-hydroxy-5-en-metabolites in the cirrhotic patients. The mean ratio of [CA/CDCA] in portal venous serum to [CA/CDCA] in hepatic venous serum was estimated to be 1.06, and those of the corresponding [CA/ Δ^5-3β-ol] and [CA/Δ^5-3β,12α-ol] were estimated to be 2.54 and 1.79, respectively. This is explained by postulating a more inefficient extraction of Δ^5-3β-ol and Δ^5-3β,12α-ol than CDCA in the cirrhotic patients.

Correlation between 3β-hydroxy-5-en-metabolites and liver function

Table 6 shows the results of a multiple regression analysis for the proportions of 3β-hydroxy-5-en-metabolites in total bile acids in urine and liver function tests (Table 1) according to the statistical method of the Regression Service Program (38). Multiple correlation coefficients were 0.73 ($P < 0.001$) for Δ^5-3β-ol and 0.78 ($P < 0.001$) for Δ^5-3β,12α-ol, which indicates the close relationship between these 3β-hydroxy-5-en-metabolites and liver functions.

DISCUSSION

3β-Hydroxy-, 3β,12α-dihydroxy-, 3β,7α-dihydroxy-, and 3β,7β-dihydroxy-5-cholenoic acids were identified in the urine of patients with liver disease by the present method, but Δ^5-3β,7α-ol and Δ^5-3β,7β-ol were found to be minor components. On the other hand, the occurrence of Δ^5-3β-ol and Δ^5-3β,12α-ol seemed to be related to cholestasis or liver dysfunction and the degree of their increment correlated well with the severity of the liver disease. Parallel increment of Δ^5-3β-ol and Δ^5-3β,12α-ol suggested that 12α-hydroxylation of Δ^5-3β-ol might take precedence over 7α-hydroxylation. It has been reported that 12α-hydroxylation proceeds preferentially in bile acids (39–41) and sterols with a planar A/B junction. These facts suggest that Δ^5-3β-ol may be a precursor for biosynthesis of Δ^5-3β,12α-ol, especially in the diseased liver.

It has been reported that the major fraction of Δ^5-3β-ol is excreted into urine as the sulfate (42, 43) or glucuronide (42, 43). We have obtained similar findings with regard to the conjugate forms of Δ^5-3β,12α-ol. It is worthy of note that the major fraction of Δ^5-3β,12α-ol was excreted in urine as the sulfate as with the less polar bile acids such as Δ^5-3β-ol and LCA. In addition, it is interesting that these bile acids existed only in trace amounts in the bile and portal venous blood but abundantly in urine, suggesting little excretion into bile in the diseased state.

Bile acid composition in liver tissue reflects both the state of the enterohepatic circulation of bile acids and their biosynthesis in liver (44). Simultaneous assessment of bile acid compositions in liver, bile and portal venous blood in the present study can shed light on the state of bile acid synthesis. The levels of Δ^5-3β-ol and Δ^5-3β,12α-ol and their proportions to total bile acids in the samples from patients with liver cirrhosis and cholestasis were high in comparison to those of control subjects. The low levels and proportions of these compounds in the portal venous blood are consistent with a hypothesis that they are formed in the liver rather than intestine, although this is inconsistent with a recent report that 3α-hydroxy-5β-6-cholen-24-oic acid has been shown to be formed from che-

TABLE 6. Multiple regression analysis between 3β-hydroxy- and 3β,12α-dihydroxy-5-cholenoic acids and the biochemical data of patients studied

	Correlation Coefficient	Alb	Bil	GOT	GPT	ALP
U-Δ^5-3β-ol[a]	Simple	0.05	0.31*	0.25*	0.28*	0.30*
	Partial	0.05	0.17	−0.27	0.76	0.41
U-Δ^5-3β,12α-ol[b]	Simple	0.06	0.56***	0.18	0.10	0.33***
	Partial	0.06	0.68	0.20	−0.19	0.41
U-Δ^5-3β-ol	Multiple	All biochemical data		0.73***		
U-Δ^5-3β,12α-ol	Multiple	All biochemical data		0.78***		

Alb, albumin; Bil, total bilirubin; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; ALP, alkaline phosphatase.
[a]Urinary (U) proportion of 3β-hydroxy-5-cholenoic acid to total bile acids.
[b]Urinary (U) proportion of 3β,12α-dihydroxy-5-cholenoic acid to total bile acids.
*F test 5% significant; ***, F test 0.1% significant.

nodeoxycholic acid by intestinal bacteria (45). The results suggest the increase of production of Δ^5-3β-ol and Δ^5-3β,12α-ol in the diseased liver. Analyses of paired liver and bile samples reveal that the biliary excretion of CDCA, which exists mainly as the sulfate in liver, was more impaired than that of CA, and that the biliary excretion of Δ^5-3β-ol, which exists mainly as the sulfate, was more impaired than that of CDCA.

A comparison of the bile acid levels and composition between portal and peripheral venous sera gives useful information on the dynamics of enterohepatic circulation of bile acids.

A significantly high portal/peripheral (P/V) ratio was observed between CA and other bile acids such as CDCA, Δ^5-3β-ol, and Δ^5-3β,12α-ol. A similar trend was observed for the portal/hepatic (P/H) ratios. This suggests a preferential hepatic extraction of CA in comparison with that of CDCA, Δ^5-3β-ol, and Δ^5-3β,12α-ol in cirrhotic patients, although it may be necessary to consider the existence of porto-systemic shunting. Further, the higher portal/hepatic venous ratios of [CA/Δ^5-3β-ol] and [CA/Δ^5-3β,12α-ol] in comparison with that of [CA/CDCA] suggested the inefficient hepatic extraction of Δ^5-3β-ol and Δ^5-3β,12α-ol than CDCA. Gärtner et al. (46) reported that the hepatic extraction of the sulfated or glucuronide lithocholic acid was much lower than that of glyco- and taurolithocholic acids. Since both Δ^5-3β-ol and Δ^5-3β, 12α-ol were mainly conjugated with sulfate, as was lithocholic acid as shown in the present study, all the above data might be attributable to the decreased extraction by the diseased liver of Δ^5-3β-opl and $\Delta^3$$\beta$-12$\alpha$-ol in comparison with CA and CDCA.

Interpretations are based on the assumption that the hepatic transport system of the sulfated bile acids is less efficient (47, 48) and impaired more easily by cholestasis than that of nonsulfated bile acids. Hepatic extraction (47), renal clearance (49), and intestinal absorption (50, 51) of the sulfated and nonsulfated bile acids are different from one another. The metabolic dynamics of the sulfated bile acids can be explained by the distribution of Δ^5-3β-ol and Δ^5-3β,12α-ol in the individual biological samples. Thus, the sulfate and glucuronide conjugates with high solubility in water are easily filtered in the glomeruli due to their lower affinities for albumin and lipoprotein (52, 53).

In conclusion, the major fractions of Δ^5-3β-ol and Δ^5-3β,12α-ol are efficiently excreted into the urine as sulfate rather than into bile in the diseased states due to impaired biliary excretion and hepatic extraction.

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