Similarity of unusual bile acids in human umbilical cord blood and amniotic fluid from newborns and in sera and urine from adult patients with cholestatic liver diseases

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Abstract Unusual bile acids in umbilical cord blood and amniotic fluid of term newborns and in sera and urine from adult patients with cholestatic liver diseases were analyzed by use of gas–liquid chromatography–mass spectrometry. These bile acids were compared in order to elucidate possible similarities of bile acid metabolism between fetal and cholestatic liver. In both umbilical cord blood and amniotic fluid, 14 unusual bile acids were found in addition to normal bile acids (cholic, chenodeoxycholic, deoxycholic, and lithocholic acids), and 15, excluding ursodeoxycholic acid, were found in sera and urine from patients with cholestatic liver diseases. Of the unusual bile acids detected, 12 were common to both samples. Six unusual bile acids, 3β-hydroxy- and 3β,12α-dihydroxy-5-cholenoic acids, 3α,6α,7α-trihydroxy-5β-cholanoic acid, 1β,3α,12α-trihydroxy-, 1β,3α,7α-trihydroxy-, and 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acids were more abundant than others. They could be classified into three groups, i.e., unsaturated, 6-hydroxylated, and 1β-hydroxylated bile acids. 1β-Hydroxylated bile acids, which were not found in serum specimens, were detected in sera from umbilical cord blood and from patients with cholestatic liver diseases. The presence of these unusual bile acids suggested similarities between the altered metabolic states of the two groups examined. — **Shoda, J., R. Mahara, T. Osuga, M. Tohma, S. Ohnishi, H. Miyazaki, N. Tanaka, and Y. Matsuzaki.** Similarity of unusual bile acids in human umbilical cord blood and amniotic fluid from newborns and in sera and urine from adult patients with cholestatic liver diseases. *J. Lipid Res.* 1988. **29:** 847–858.

Supplementary key words gas–liquid chromatography–mass spectrometry • fetus • 1β-hydroxylated bile acids

Cholestasis is occasionally associated with disturbances in bile acid metabolism. Production of unusual bile acids has been observed in patients with cholestatic liver diseases (1–3). The presence of identical unusual bile acids in human amniotic fluid (4) or meconium (5) has also been detected, e.g., 3β-hydroxy-5-cholenoic acid (4), hyocholic acid (5), and 1,3,7,12-tetrahydroxycholanoic acid (5). Therefore, it is speculated that there are common altered metabolic pathways of bile acids in term newborns and in patients with cholestatic liver disease. These unusual bile acids have been detected in a variety of specimens, but information about them is fragmentary. Because of a lack of analytical methods, analysis of biological fluids, such as umbilical cord blood, has not been performed. In order to investigate the hypothesis of common altered pathways, our study was designed to analyze bile acids in fetal body fluids (umbilical cord blood and amniotic fluid) and in body fluids from adults with cholestatic liver diseases (serum and urine) by systematic use of gas–liquid chromatography–mass spectrometry (6) and to compare the findings in both conditions. We found 14 unusual bile acids in umbilical cord blood, 11 in amniotic fluid, 12 in cholestatic disease sera, and 15 in cholestatic disease

Abbreviations and trivial names: TMS, trimethylsilyl; DMES, dimethylethylsilyl; GLC–MS, gas–liquid chromatography–mass spectrometry; lithocholic (LCA), 3α-hydroxy-5β-cholanoic; deoxycholic (DCA), 3α,12α-dihydroxy-5β-cholanoic; chenodeoxycholic (CDCA), 3α,7α-dihydroxy-5β-cholanoic; ursodeoxycholic (UDCA), 3α,7β-dihydroxy-5β-cholanoic; cholic (CA), 3α,7α,12α-trihydroxy-5β-cholanoic; 3β-Δ^5, 3β-hydroxy-5-cholenoic; 3β,12α-Δ^5, 3β,12α-dihydroxy-5-cholenoic; hyocholic (HyoCA), 3α,6α,7α-trihydroxy-5β-cholanoic; 1β,3α,12α, 1β,3α,12α-trihydroxy-5β-cholanoic; 1β,3α,7α, 1β,3α,7α-trihydroxy-5β-cholanoic; 1β,3α,7α,12α, 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic; 3β,7α, 3β,7α-dihydroxy-5β-cholanoic; 3β,7α,12α, 3β,7α,12α-trihydroxy-5β-cholanoic; nordeoxycholic, 24-nor-3α,12α-dihydroxy-5β-cholan-23-oic; and norcholic (NorCA), 24-nor-3α,7α,12α-trihydroxy-5β-cholan-23-oic acids. DMESOH represents dimethylethylsilanol. The term allo is used for substituted 5α-cholan 24-oic acids.

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urine. Of these unusual bile acids, 12, excluding ursodeoxycholic acid, were common and 6 were more abundant. These bile acids could be classified into three groups, i.e., unsaturated, 6-hydroxylated, and 1β-hydroxylated bile acids. The findings of similar unusual bile acids in fetal and cholestatic liver imply the existence of similarly altered metabolic pathways in both groups.

METHODS

Sample collections

Blood samples were taken from umbilical cord veins of 20 term newborns after delivery and from 20 patients with cholestatic liver diseases (two cases of common bile duct stone, four cases of bile duct carcinoma, two cases of gall bladder carcinoma, one case of pancreatic cyst, four cases of pancreatic carcinoma, three cases of intrahepatic cholestasis, and four cases with various other diagnoses).

Umbilical cord arterial blood was also collected from five of the newborns, and maternal blood from nine subjects. Amniotic fluid from the latter subjects was collected by needle centesis of the amniotic membrane. All specimens were stored at −20°C until analyzed. Urine samples were collected in polyethylene bottles which were kept refrigerated during the 24-hr collection period and aliquots were then stored at −20°C until analyzed. Seventeen samples of sera and urine were collected from healthy subjects as controls.

Reference compounds

Authentic bile acids were obtained from Steraloids, Inc. (Wilton, NH). [Carboxyl-^{14}C]-labeled cholic acid, [1-^{14}C]glycine-labeled glycocholic acid, [24-^{14}C]-labeled taurocholic acid, and [11,12-$^{3}H_2$]chenodeoxycholic acid were obtained from New England Nuclear Co. (Boston, MA). [Carboxyl-^{14}C]-labeled lithocholic acid was obtained from Amersham International Ltd. (Buckinghamshire, UK). 3β,12α-Dihydroxy-5-cholenoic acid (7) and 1β-hydroxylated bile acids (8, 9) were synthesized by Tohma et al. (7–9), and 24-nor-3α,7α,12α-trihydroxy-5β-cholan-23-oic acid was kindly supplied by Prof. T. Hoshita. [$^{3}H_2$]glycine-conjugated chenodeoxycholic acid-3-sulfate and [^{14}C]glycine-conjugated lithocholic acid-3-sulfate were prepared in our laboratory. Synthesis of these labeled bile acids was carried out using [$^{3}H_2$]chenodeoxycholic acid (sp act, 38.5 Ci/mmol) and [^{14}C]lithocholic acid (sp act, 57 mCi/mmol). Glycine-conjugated bile acids were synthesized using coupling reagent (10) from the corresponding unconjugated bile acids. Labeled glycochenodeoxycholic acid-3-sulfate and glycolithocholic acid-3-sulfate were synthesized from the corresponding glycine-conjugated bile acids according to the methods described by Parmentier and Eyssen (11) and Tserng and Klein (12), respectively. The purity of the synthesized bile acids was checked by thin-layer chromatography; all compounds showed only a single spot on the chromatograms.

Chemicals

All solvents were of analytical grade; pyridine was redistilled before use over potassium oxide. Bond Elut C_{18} (octadecylsilane-bonded silica) cartridges were obtained from Analytichem International, Inc. (Harbor City, CA), cholylglycine hydrolase was from Sigma Chemical Co. (St. Louis, MO), Sephadex LH-20 was from Pharmacia Fine Chemicals (Uppsala, Sweden), and dimethylethylsilylimidazole (DMESI) was from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Piperidinohydroxypropyl Sephadex LH-20 was kindly supplied by Prof. T. Nambara and Dr. J. Goto.

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

The Shimadzu GC-7A gas chromatograph was equipped with a flame ionization detector and a Van den Berg solventless injector. The Shimadzu-LKB 9000B and Shimadzu 9020 DF were equipped with a data processing system (PAC-90 and SCAP 11/23, PAC-1100, respectively) and the Van den Berg solventless injector. The capillary column (25 m × 0.31 mm i.d.) was coated with methylsilicone (Hewlett Packard). The temperature of the column oven was programmed from 220 to 300°C at 2°C/min after a 4-min delay from the starting time; the injection port and detector temperatures were 300°C. The flow rate of the carrier gas (helium) was 1.5 ml/min. GLC-MS was carried out as follows. The ionization energy was set at 22.5 eV, acceleration voltage 3.5 kV, and trap current 100 μA. The temperature of separator and ion source was maintained at 270°C and 290°C, respectively. Mass spectra were taken by repetitive scanning of mass range m/z 100–800 (20 scans/min).

Liquid scintillation counting (LSC) and thin-layer chromatogram scanner

In recovery experiments using ^{14}C-labeled bile acids, the radioactivity of the sample before and after the procedure was measured by liquid scintillation counting (Beckman), and the completeness of solvolysis or hydrolysis was checked by direct scanning of the plate using an Aloka Thin-Layer Chromatogram Scanner (Aloka, Tokyo, Japan).

Clean-up procedure

Sera, urine, and amniotic fluid were stored at −20°C until analyzed and were treated according to the method described by Yanagisawa et al. (13) with a few modifications. One to two ml of sera or urine and 7–8 ml of amniotic fluid, with adequate amounts of 24-nor-3α,12α-dihydroxy-5β-cholan-23-oic acid (nordeoxycholic acid) as an internal standard, were diluted with 0.5 M potassium phosphate buffer (pH 7.0). The sample was applied to a

Bond Elut C_{18} cartridge (prewashed with 10 ml of ethanol and distilled water) and washed with 4 ml of distilled water; bile acids were eluted with 90% aqueous ethanol. After evaporation of solvent under reduced pressure, the residue was subjected to solvolysis according to the method described by Kornell (14). After solvolysis, the residue was subjected to enzymatic hydrolysis according to the modified method described by Karlaganis, Schwarzenbach, and Paumgartner (6). The sample was dissolved in 0.5 ml of 1.86% EDTA solution, 0.2 M β-mercaptoethanol, and 5 ml of 0.025 M sodium acetic buffer (pH 5.6). After addition of 5 U of cholylglycine hydrolase, the mixture was incubated at 37°C for 16 hr. After dilution with 0.5 M potassium phosphate buffer, bile acids were extracted with Bond Elut C_{18}, and the evaporated, hydrolyzed sample was dissolved in 4 ml of 90% aqueous ethanol. This solution was applied to a piperidinohydroxypropyl Sephadex LH-20 column (15) (2 mm × 20 mm) prepared in 90% aqueous ethanol. After washing with 2 ml of 90% aqueous ethanol to remove neutral compounds, 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) HCl 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 (16, 17) and allowed to stand for 15 min. Excess silylating reagent was removed on a Sephadex LH-20 column (6 × 60 mm) 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 redissolved in 5% (v/v) pyridine–*n*-hexane solution.

The efficiency of the extraction procedure was examined by addition of unconjugated, glycine-, and taurine-conjugated [^{14}C]cholic acids to the serum sample before clean-up. The recoveries of added [^{14}C]cholic acid and its conjugated form at each step through the clean-up procedure were as follows. Bond Elut C_{18} yielded a recovery of 95.6 ± 0.5% for unconjugated (n = 4, mean ± SD), 88.8 ± 0.3% for glycine-conjugated, and 93.8 ± 0.7% for taurine-conjugated cholic acid. The completeness of solvolysis was examined for glycine-conjugated lithocholic acid-3-sulfate and glycine-conjugated chenodeoxycholic acid-3-sulfate; it was 98.1 ± 0.7% (n = 5) and 98.4 ± 1.0% (n = 5), respectively (using glycine-conjugated [^{14}C]lithocholic acid-3-sulfate and glycine-conjugated [^{14}C]chenodeoxycholic acid-3-sulfate, measured by a radiochromatoscanner).

Unconjugated bile acids (chenodeoxycholic, cholic, deoxycholic, lithocholic, ursodeoxycholic, 3β-hydroxy-5-cholenoic, hyocholic, and 1β,3α,12α-trihydroxy-5β-cholanoic acids) added to the serum sample in amounts half of that of the endogenous bile acids were recovered in the range of 83 to 91%. Recovery was independent of concentration and there was no statistically significant difference for individual bile acids.

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) (18) of peaks on reconstructed ion profiles and their mass spectra to those of authentic standards.

Quantitation of individual bile acids was based on peak areas appearing on the ion current chromatograms. The base peak or prominent ion in mass spectra was selected for the monitoring ion (**Table 1**). Nordeoxycholic acid was used as an internal standard.

RESULTS

Umbilical cord blood, amniotic fluid, and maternal blood. Qualitative composition of bile acids

A representative, reconstructed ion profile obtained from analysis of bile acids in umbilical cord blood and amniotic fluid is shown in **Fig. 1.** Table 1 shows a list of completely and partially identified bile acids found in this study. The results revealed that 14 unusual bile acids were detected in both umbilical cord blood and amniotic fluid, and 4, excluding ursodeoxycholic acid, were detected in maternal blood, in addition to normal bile acids.

Monohydroxy bile acids. Two monohydroxycholanoic acids were found in all the samples. Compound a was identified as lithocholic acid (LCA). Compound b was found to be an unsaturated bile acid as judged from the peaks at m/z 345 $[M-143]^+$ and 143, indicative of 3-dimethylethylsiloxy-Δ^5 structure; it was identified as 3β-hydroxy-5-cholenoic acid (3β-Δ^5). Trace amounts of this compound were also detected in maternal blood in this study. The compound 3β-Δ^5 has been identified in meconium (5) and amniotic fluid (4).

Dihydroxy bile acids. Four 3,7- and 3,12-dihydroxycholanoic acids were found; these compounds were chenodeoxycholic (CDCA) (compound d), ursodeoxycholic (UDCA) (compound g), deoxycholic (DCA) (compound c), and unsaturated dihydroxycholenoic (compound f) acids. The peaks at m/z 343 $[M-143-DMESOH]^+$ and 143 in the mass spectrum of compound f, which correspond to m/z 329 and 129 in the methyl ester TMS ether derivatives (19), are both indicative of a 3-dimethylethylsiloxy-Δ^5 structure and the base peak at m/z 561 $[M-29]^+$ is indicative of the 3,12-bis-dimethylethylsiloxy structure in the ethyl ester DMES ether derivatives. The mass spec-

TABLE 1. Completely and partially identified bile acids in the umbilical

		Bile Acids[a] Identified	MUv[b]	Mr	Fragment Ions[c] (m/z) [M]‡	[M-C_2H_5]⁺	[M-DMESOH]‡	[M-C_2H_5-DMESOH]‡ or [M-C_2H_5-DMESOH + H]⁺
*a[d]	A[e]	5βB-3α-ol	33.00	490	490 (1.6)[g]	461 (100.0)	386 (61.5)	358 (1.3)
*b	B	B[5]-3β-ol	33.80	488	488 (6.4)	459 (69.8)	384 (20.5)	355 (−)
	C	5βB-3β,7α-ol	34.46	592	592 (−)	563 (4.8)	488 (20.0)	459 (14.5)
*c	D	5βB-3α,12α-ol	34.48	592	592 (−)	563 (100.0)	488 (0.6)	459 (2.3)
*d	E	5βB-3α,7α-ol	34.82	592	592 (−)	563 (2.4)	488 (0.8)	459 (61.3)
e	F	24-nor-5βB-3α,7α,12α-ol	34.97	680	680 (−)	651 (100.0)	576 (−)	547 (−)
f	G	B[5]-3β,12α-ol	35.16	590	590 (−)	561 (100.0)	486 (5.3)	457 (8.2)
*g	H	5βB-3α,7β-ol	35.24	592	592 (−)	563 (100.0)	488 (22.5)	459 (11.8)
h	I	5βB-3β,7α,12α-ol	35.61	694	694 (−)	665 (100.0)	590 (2.0)	561 (−)
*i	J	5βB-3α,7α,12α-ol	36.03	694	694 (−)	665 (100.0)	590 (0.6)	561 (6.9)
	K	5βB-3α,12α-ol-7-oxo	36.41	606	606 (−)	577 (100.0)	502 (3.9)	473 (2.7)
*j	L	5βB-1β,3α,12α-ol	36.52	694	694 (−)	665 (55.0)	590 (19.0)	561 (7.8)
*k	M	5βB-3α,6α,7α-ol	36.60	694	694 (−)	665 (3.7)	590 (1.3)	561 (9.2)
*l	N	B_1-3,6,7-ol[f]	36.79	694	694 (−)	665 (2.2)	590 (−)	561 (6.0)
*m	O	B_2-3,6,7-ol[f]	37.24	694	694 (−)	665 (7.7)	590 (34.2)	561 (29.5)
n	P	5βB-1β,3α,7α-ol	37.54	694	694 (−)	665 (2.0)	590 (9.6)	561 (1.8)
o	Q	B_1-3,6,7,12-ol[f]	37.76	796	796 (−)	767 (8.6)	692 (−)	663 (3.8)
*p	R	5βB-1β,3α,7α,12α-ol	37.92	796	796 (−)	767 (39.0)	692 (5.4)	663 (1.7)
q	S	B_2-3,6,7,12-ol[f]	38.45	796	796 (−)	767 (18.5)	692 (45.9)	663 (9.7)
r	T	B_3-3,6,7,12-ol[f]	38.94	796	796 (−)	767 (7.8)	692 (0.9)	663 (1.6)

[a] B, cholanoic acid. Configuration at C-5 and of hydroxyl groups are indicated by Greek letters. Superscript denotes position of double bond.
[b] Methylene unit values of ethyl ester DMES ester derivatives.
[c] Chemical formulas after M indicate mass of fragments lost. DMESOH represents dimethylethylsilanol.
[d,e] Small and large characters of alphabet correspond to the peaks of mass chromatograms in Fig. 1 (umbilical cord blood and amniotic fluid) and Fig. 2 (serum and urine of cholestatic patient), respectively. Asterisks indicate the bile acids found in the maternal blood.
[f] Positions of hydroxyl groups and stereochemistry are tentative.
[g] Fragment ions underlined represent those for quantitation of the individual bile acids.

trum of compound f was identical to that of the authentic standard of 3β,12α-dihydroxy-5-cholenoic acid (3β,12α-Δ^5) reported by Tohma et al. (20). Thus, compound f was identified as 3β,12α-Δ^5. This compound was also identified in meconium by Back and Walter (5).

Trihydroxy bile acids. Several trihydroxycholanoic acids were found. Compounds h and i were estimated to be substituted at the C-3, C-7, and C-12 positions on steroidal rings. Compound i was identified as cholic acid (CA) and compound h as the 3β-epimer of CA. The peak corresponding to compound h showed the nonspecific 3,7,12-dimethylethylsiloxy bile acid pattern with the base peak at m/z 665 [M−29]⁺. The mass spectrum of this compound showed that it was 3α,7α,12α-trihydroxy-5α- (allocholic acid) or 3β,7α,12α-trihydroxy-5β-cholanoic acid (3β,7α, 12α). The MUv (35.61) of this compound was identical to that of the authentic standard of 3β,7α,12α (35.65) and thus compound h was newly identified as 3β,7α,12α. Compound h was not detected in either amniotic fluid or maternal blood in this study. In addition, this bile acid has not been found in either meconium or amniotic fluid.

Three trihydroxycholanoic acids, which were estimated to be substituted at C-3, C-6, and C-7, were found. Compound k was hyocholic acid (HyoCA), which was one of the predominant compounds and was found in all the samples. HyoCA has been identified in meconium (5). Compounds l and m yielded mass spectra analogous to those of HyoCA, derived from fragmentation of the ethyl ester DMES ether of trihydroxycholanoic acid substituted at C-3, C-6, and C-7. These two bile acids have not been identified yet.

The derivatives of two trihydroxy bile acids (compounds j and n) gave a base peak at m/z 245 in their mass spectra. This ion corresponds to the well-known ion of m/z 143 with an additional DMES group. Consequently, the appearance of this ion in a spectrum of bile acid derivatives strongly suggested a 1,3-bis-dimethylethylsiloxy structure. Compared to authentic standards (9), compounds j and n were identified as 1β,3α,12α-trihydroxy-(1β,3α,12α) and 1β,3α,7α-trihydroxy-5β-cholanoic acids (1β,3α,7α), respectively. Although 1β,3α,7α was not detected, 1β,3α,12α was detected in trace amounts in maternal blood. These 1β-hydroxylated trihydroxycholanoic acids were identified in meconium (8, 9).

In addition to the common trihydroxy C_{24} bile acids, norcholic acid (NorCA) (compound e) was found in minor amounts.

Tetrahydroxy bile acids. One of the tetrahydroxycholanoic acids (compound p) was identified. The ethyl ester DMES ether of compound p gave a base peak at m/z 245 indica-

(Relative Intensities)

[M-2 × DMESOH]⁺ or [M-2 × DMESOH + H]⁺	[M-3 × DMESOH]⁺ or [M-3 × DMESOH + H]⁺	[M-4 × DMESOH]⁺ or [M-4 × DMESOH + H]⁺	Other Ions
			323 (24.7) 256 (35.2) 215 (50.9)
			369 (13.0) 345 (45.3) 255 (12.1) 143 (100.0)
384 (100.0)			369 (20.8) 399 (5.4) 255 (19.8)
384 (6.8)			359 (4.5) 255 (74.6)
385 (100.0)			369 (10.5) 339 (18.1) 255 (30.7)
472 (15.7)	369 (25.6)		357 (4.2) 323 (5.9) 253 (12.8)
382 (9.2)			367 (6.0) 343 (14.6) 253 (28.7) 143 (19.8)
383 (21.2)			339 (10.0) 255 (12.1)
486 (25.6)	383 (14.9)		357 (27.2) 330 (5.5) 275 (19.3) 253 (40.3)
486 (6.0)	383 (30.3)		357 (7.3) 337 (4.6) 253 (24.7)
398 (1.9)			381 (13.7) 355 (16.5) 269 (16.6) 251 (28.2)
486 (5.8)	382 (22.4)		330 (4.1) 253 (7.2) 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 (2.1)	383 (100.0)		337 (6.0) 319 (2.2) 253 (6.4) 161 (23.6) 159 (12.3)
486 (38.7)	383 (100.0)		337 (2.3) 319 (5.5) 275 (9.8) 161 (32.1) 159 (24.0)
486 (19.0)	382 (38.6)		330 (4.9) 253 (5.0) 245 (100.0) 209 (17.7) 196 (13.9)
588 (3.6)	485 (81.2)	381 (100.0)	335 (19.4) 251 (5.2) 161 (13.4) 159 (6.9)
588 (5.0)	484 (14.5)	380 (21.3)	355 (9.8) 251 (12.4) 245 (100.0) 209 (27.8) 196 (24.3)
588 (31.6)	485 (21.2)	381 (100.0)	335 (12.5) 251 (22.4) 161 (53.2) 159 (41.9)
588 (3.0)	485 (3.7)	381 (100.0)	335 (15.5) 251 (11.7) 161 (36.3) 159 (15.7)

tive of a 1,3-bis-dimethylethylsiloxy structure. The mass spectrometric pattern was identical to that of the authentic standard of 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid reported by Tohma et al. (8, 9). Therefore, compound p was identified as 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid (1β,3α,7α,12α). Although 1β-hydroxylated bile acids were found only in urine of adults (19) and in meconium (5, 8, 9) and urine of healthy newborns, the data in this study newly reveal the existence of 1β-hydroxylated bile acids in umbilical cord blood and amniotic fluid. Furthermore, the reconstructed ion profile, as shown in Fig. 1, exhibited three other ion peaks derived from fragmentation of tetrahydroxycholanoic acids (compounds o, q, and r). These spectrometric patterns are analogous in many respects to that of the derivative of HyoCA. These three compounds were therefore tentatively identified as 3,6,7,12-tetrahydroxycholanoic acids (B_1-3,6,7,12, B_2-3,6,7,12, and B_3-3,6,7,12), previously detected in urine from patients with cholestatic disease by Almé et al. (19).

Quantitative composition of bile acids

The data in **Table 2** show quantitative analysis of bile acids in the arterial and venous blood of umbilical cord, amniotic fluid, and maternal blood.

In the umbilical cord blood, the mean concentrations of total bile acids were found to be 3.5 μg/ml (2.1 μg/ml in arterial blood and 3.8 μg/ml in venous blood), values higher than those found in maternal venous blood (mean 3.1 μg/ml). The quantitative composition of bile acids showed little difference between arterial and venous blood. CDCA and CA were found as the predominant bile acids detected, accounting for 10.3 to 65.6% and 11.2 to 42.8% of total bile acids, respectively. The ratio of cholic/chenodeoxycholic acid ranged from 0.2 to 4.1 (mean 1.0). Secondary bile acids, LCA and DCA, were detected in small amounts, accounting for trace to 6.0% and trace to 12.7% of total bile acids, respectively.

In amniotic fluid, the mean concentration of total bile acids was 1.4 μg/ml, also lower than that of maternal blood. CA (compound i) and 3β-Δ^5 (compound b) were the predominant bile acids, accounting for 9.7 to 21.2% and 1.9 to 26.7%, respectively.

Fourteen unusual bile acids were detected in umbilical cord blood and amniotic fluid. Among them, 3β-Δ^5 (0.1–26.7% of total bile acids), 3β,12α-Δ^5 (0.2–13.1%), and HyoCA (compound k) (0.7–31.3%) were found as the more abundant components.

Bile acids with a hydroxyl group at C-1, which were in umbilical cord and amniotic fluid but not detected in serum samples, were: 1β,3α,12α (1.1–12.3% of total bile acids), 1β,3α,7α (0.5–13.2%), and 1β,3α,7α,12α (0.2–35.7%).

The unusual bile acids in the umbilical cord blood and amniotic fluid can be mainly classified into three groups: unsaturated, 6-hydroxylated, and 1β-hydroxylated bile acids. The relative amounts of these bile acid groups were present up to 35.8%, 27.7%, and 55.6% of total bile acids, respectively.

In quantitative analysis of bile acid content in umbilical cord blood and amniotic fluid, the ratio of tri- plus tetra-

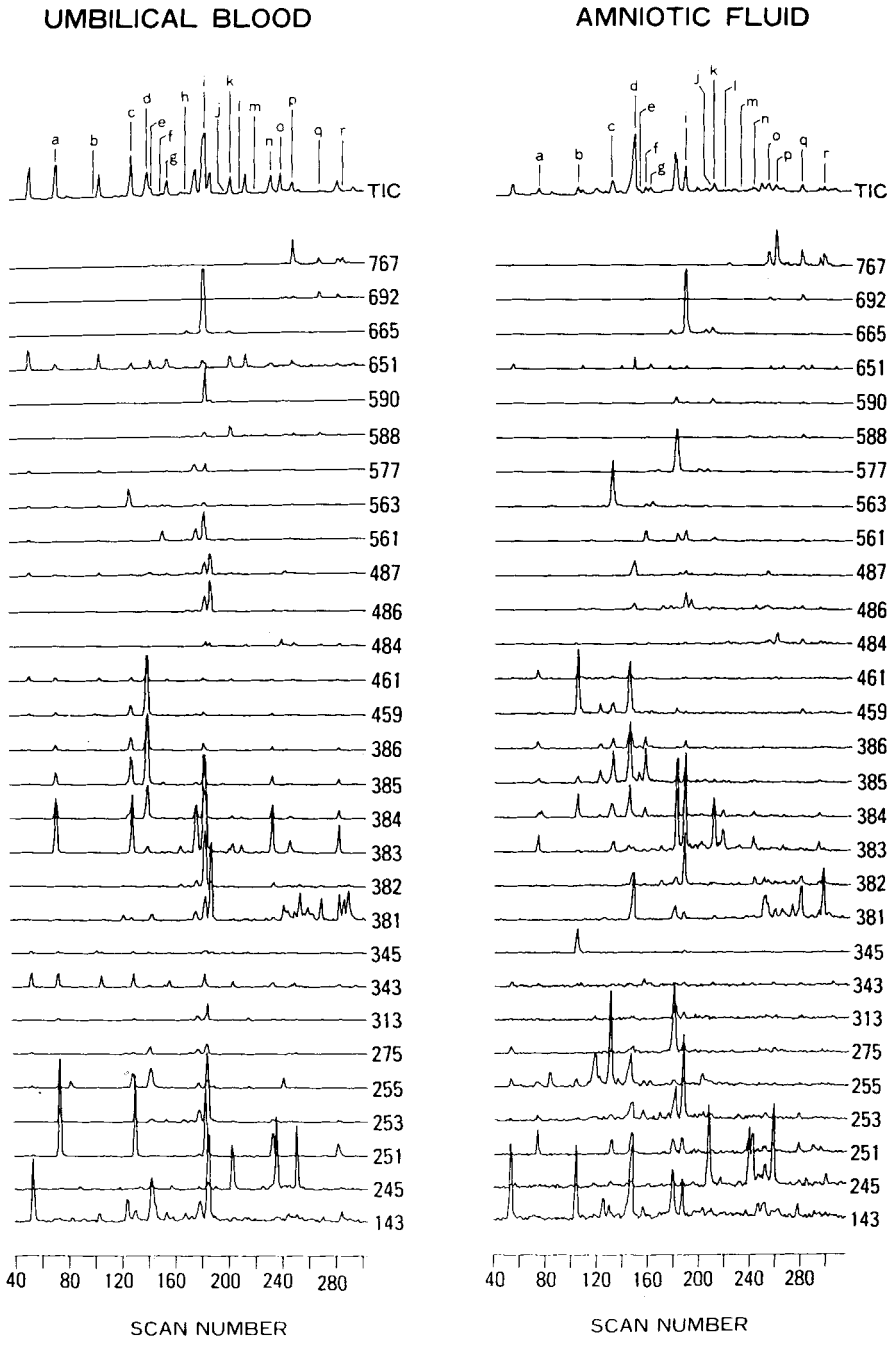

Fig. 1. Total and fragment ion chromatograms obtained in the analysis of umbilical cord blood and amniotic fluid. Peaks a–r represent bile acids completely or partially identified in the present study.

hydroxy to dihydroxy bile acids[2] was examined, and their values were found to be greater than 1 in most cases in both (umbilical cord blood 0.3–5.7, mean 1.5; amniotic fluid 0.7–5.1, mean 3.2).

Cholestatic liver diseases. Qualitative composition of bile acids

Fig. 2 shows a representative reconstructed ion profile obtained from analysis of bile acids in sera and urine from

[2]Ratios of tri- plus tetrahydroxy to dihydroxy bile acids in the text represent values of (sum of cholic acid, three components of 1β-hydroxylated bile acids, six components of 6α-hydroxylated bile acids, and 3β,7α,12α-trihydroxy-5β-cholanoic acid)/(sum of deoxycholic, chenodeoxycholic, ursodeoxycholic, and 3β,12α-dihydroxy-5-cholenoic acids). The ratios were estimated to be 1.5 ± 0.2 (mean ± SEM) in umbilical cord blood, 3.2 ± 0.5 in amniotic fluid, 0.7 ± 0.1 in maternal blood, 1.1 ± 0.1 in cholestatic serum, 1.3 ± 0.2 in cholestatic urine, 0.5 ± 0.1 in serum of healthy subjects, and 0.7 ± 0.2 in urine of healthy subjects.

TABLE 2. Bile acid levels and composition in umbilical cord blood, amniotic fluid, and maternal blood

Compound	Position of Substituents[a]	Umbilical Cord Blood: Artery (A) (n = 5)	Umbilical Cord Blood: Vein (V) (n = 20)	Umbilical Cord Blood: A + V (n = 25)	Amniotic Fluid (n = 9)	Maternal Blood (n = 9)
		Total (μg/ml)				
		2.1 ± 0.2	3.8 ± 0.4	3.5 ± 0.5	1.4 ± 0.4	3.1 ± 0.5
		Percent of Total				
a[b]	3α	0.9 ± 0.2	1.3 ± 0.2	1.2 ± 0.2	1.9 ± 0.4	1.9 ± 0.5
c	3α,12α	5.9 ± 2.1	4.1 ± 0.8	4.3 ± 0.8	10.5 ± 1.8	28.8 ± 2.4
d	3α,7α	32.2 ± 5.1	32.8 ± 2.8	32.6 ± 2.3	7.4 ± 1.4	26.8 ± 3.8
i	3α,7α,12α	25.9 ± 4.8	24.7 ± 2.3	24.8 ± 2.0	16.3 ± 1.7	30.3 ± 4.6
g	3α,7β	0.8 ± 0.4	0.6 ± 0.1	0.6 ± 0.1	1.2 ± 0.1	2.4 ± 0.5
h	3β,7α,12α	2.8 ± 1.6	2.7 ± 0.5	2.7 ± 0.5	N.D.	N.D.
b	3β-Δ[5]	4.3 ± 0.9	5.2 ± 0.8	5.4 ± 0.8	11.6 ± 3.1	1.7 ± 0.8
f	3β,12α-Δ[5]	2.9 ± 1.1	4.4 ± 0.9	4.2 ± 0.8	3.5 ± 1.5	0.7 ± 0.5
j	1β,3α,12α	3.5 ± 0.6	3.6 ± 0.4	3.5 ± 0.3	4.7 ± 1.0	1.5 ± 0.3
n	1β,3α,7α	5.7 ± 1.4	4.0 ± 0.8	4.3 ± 0.6	2.1 ± 0.7	N.D.
p	1β,3α,7α,12α	3.6 ± 1.6	3.1 ± 0.7	3.3 ± 0.6	15.5 ± 3.3	0.3 ± 0.3
k	3α,6α,7α	8.2 ± 2.1	7.5 ± 1.3	7.6 ± 1.1	9.0 ± 1.0	2.1 ± 0.3
l	B_1-3,6,7[c]	1.6 ± 0.8	2.7 ± 0.2	2.5 ± 0.2	1.5 ± 0.3	N.D.
m	B_2-3,6,7[c]	1.5 ± 0.7	1.3 ± 0.3	1.3 ± 0.3	3.9 ± 1.6	N.D.
o	B_1-3,6,7,12[c]	0.8 ± 0.3	0.9 ± 0.4	0.9 ± 0.3	3.5 ± 0.6	N.D.
q	B_2-3,6,7,12[c]	0.3 ± 0.1	1.9 ± 0.2	1.6 ± 0.4	3.5 ± 0.6	N.D.
r	B_3-3,6,7,12[c]	0.4 ± 0.2	1.7 ± 0.4	1.4 ± 0.5	4.9 ± 1.3	N.D.

Values are given as mean ± SEM. Numbers in parentheses represent number of cases studied. N.D., not detectable.
[a] In 5β-cholanoic acid, unless otherwise noted. Greek letter denotes configuration of hydroxyl groups; Δ[5] denotes 5,6 double bond.
[b] Small characters of the alphabet correspond to peaks of reconstructed ion profile in Fig. 1 (umbilical cord blood and amniotic fluid).
[c] Tentative; see text.

patients with intrahepatic cholestasis. Table 1 shows a list of completely or partially identified bile acids in sera and urine from patients with cholestatic liver diseases. The results of GLC-MS analyses revealed that 15 unusual bile acids were found in addition to 5 bile acids normally found in humans, including UDCA.

Serum and urine samples from 17 healthy subjects were analyzed as control samples. In serum, unusual bile acids, such as 3β-Δ[5] and 3β,7α, were found as minor components. In urine, minor amounts of 1β,3α,12α and HyoCA were detected, in addition to 3β-Δ[5] and 3β,7α, but no tetrahydroxy bile acids were detected.

Quantitative composition of bile acids

The data in **Table 3** show analysis of bile acids in serum and urine from patients with cholestatic liver diseases. The serum concentration of total bile acids ranged from 9.1 to 135.4 μg/ml, and the urinary concentration from 11.7 to 206.4 μg/ml.

In the sera, monohydroxylated bile acids were present in all patients, ranging from 0.3 to 11.6% of total bile acids. 3β-Δ[5] was found as the major monohydroxylated bile acid, accounting for a trace to 10.7% of total bile acids. Another unsaturated bile acid, 3β,12α-Δ[5] was found, accounting for a trace to 3.6% of total bile acids. HyoCA was the major unusual bile acid in addition to 3β-Δ[5], accounting for a trace to 15.9% of total bile acids. Bile acids with a hydroxyl group at C-1 were found in small amounts. Only trace amounts of tetrahydroxy bile acid substituted at C-3, C-6, C-7, and C-12 were detected. Unsaturated, 6-hydroxylated, and 1β-hydroxylated groups were present at levels up to 10.7%, 15.9%, and 15.4%, respectively. The ratio of tri- plus tetrahydroxy to dihydroxy bile acid was greater than 1 in most cases (0.19–3.1, mean 1.1).

In urine, unusual bile acids 3β-Δ[5] and HyoCA (compound M) were abundantly present along with CDCA and CA, accounting for 3.1 to 70.8% and for 0.6 to 28.6% of total bile acids, respectively. The unsaturated bile acid, 3β,12α-Δ[5], also increased with the elevation of 3β-Δ[5], accounting for 0.2 to 10.6% of total bile acids. Tetrahydroxy bile acids with a hydroxyl group at C-1 or C-6 were more abundant in urine in contrast to the sera; 1β,3α,7α,12α and B_1-3,6,7,12 were present up to 13.6% and 7.2% of total bile acids, respectively. Unsaturated, 6-hydroxylated, and 1β-hydroxylated groups were present up to 72.1%, 34.9%, and 19.6% of total bile acids, respectively. The ratio of tri- plus tetrahydroxy to dihydroxy bile

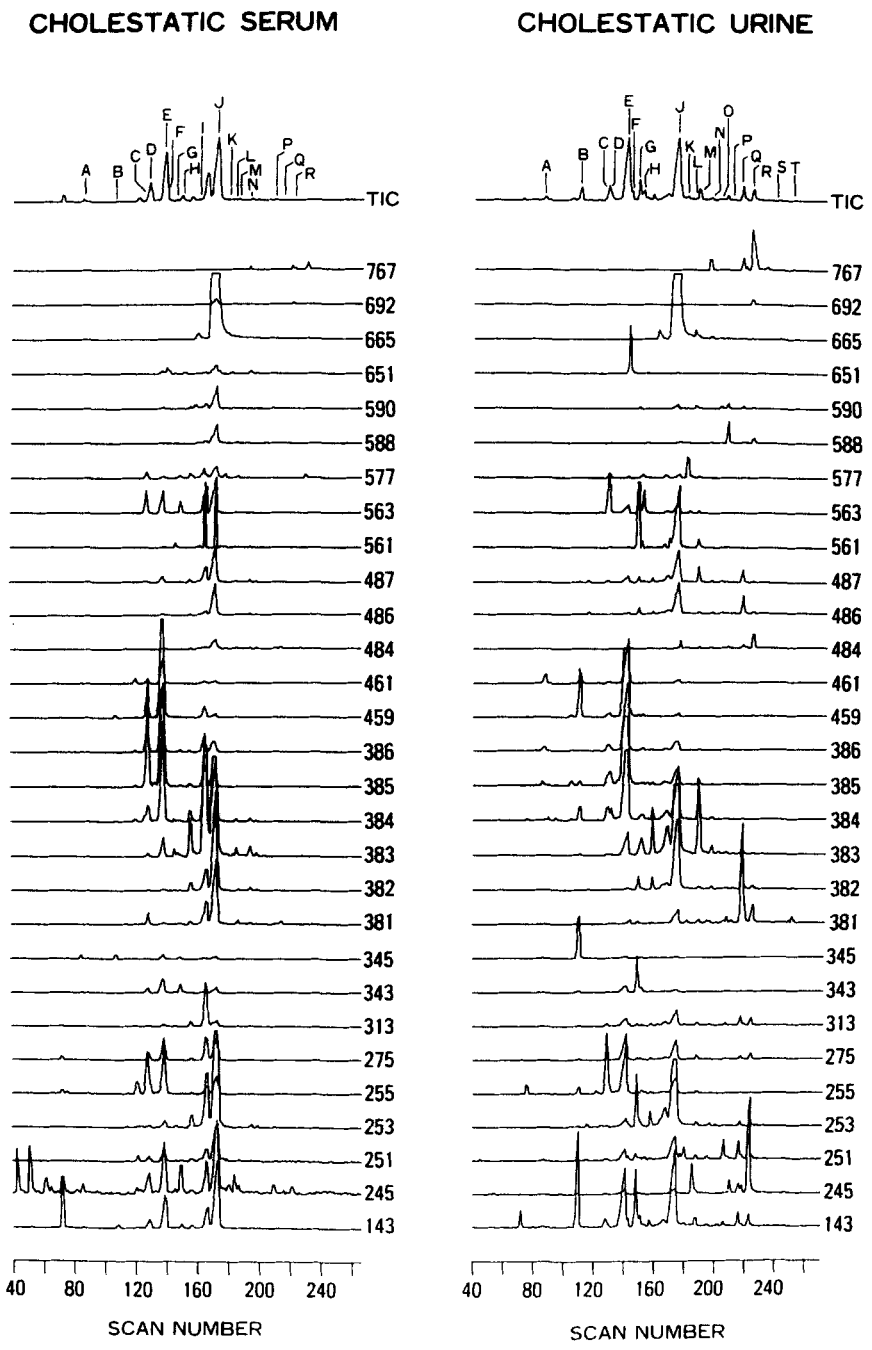

Fig. 2. Total and fragment ion chromatograms obtained from the serum and urine from a patient with intrahepatic cholestasis. Peaks A-T represent bile acids completely or partially identified in the present study.

acids[2] was greater than 1, except in one case (0.2–3.2, mean 1.3).

Comparisons

The present study revealed that 14 unusual bile acids were present in umbilical cord blood and amniotic fluid and 15 in the sera and urine from patients with cholestatic liver disease. Of these unusual bile acids, 12 were common to both groups. These were mainly classified into three groups: unsaturated, 6-hydroxylated, and 1β-hydroxylated bile acids.

In order to discuss the important differences in the bile acid compositions of the various groups, quantitative data for individual bile acids were statistically analyzed according to the F-test method. **Table 4** shows the results. As for the proportions of individual bile acids, 1β-

TABLE 3. Bile acid levels and composition in sera and urine of cholestatic patients

Compound	Positions of Substituents[a]	Cholestasis	
		Serum (n = 20)	Urine (n = 20)
		Total (μg/ml)	
		64.2 ± 14.3	59.9 ± 13.4
		Percent of Total	
A[b]	3α	0.5 ± 0.1	2.0 ± 0.4
D	3α,12α	3.3 ± 0.7	2.5 ± 0.5
E	3α,7α	53.9 ± 12.1	21.1 ± 4.7
J	3α,7α,12α	40.3 ± 9.0	17.4 ± 3.8
H	3α,7β	3.2 ± 0.7	1.4 ± 0.3
I	3β,7α,12α	0.2 ± 0.1	0.2 ± 0.1
B	3β-Δ^5	4.6 ± 1.0	26.0 ± 5.8
G	3β,12α-Δ^5	0.5 ± 0.1	1.8 ± 0.4
L	1β,3α,12α	0.3 ± 0.1	0.2 ± 0.1
P	1β,3α,7α	0.6 ± 0.1	0.5 ± 0.1
R	1β,3α,7α,12α	0.2 ± 0.1	2.1 ± 0.4
M	3α,6α,7α	3.1 ± 0.6	8.7 ± 1.9
N	B_1-3,6,7[c]	0.6 ± 0.1	0.2 ± 0.1
O	B_2-3,6,7[c]	Tr	0.1 ± 0.1
Q	B_1-3,6,7,12[c]	Tr	1.1 ± 0.2
S	B_2-3,6,7,12[c]	N.D.	Tr
T	B_3-3,6,7,12[c]	Tr	3.7 ± 0.8

Values are given as mean ± SEM. Numbers in parentheses represent number of cases studied. Tr, represents trace (= < 0.1%); N.D., not detectable.

[a] In 5β-cholanoic acid, unless otherwise noted. Greek letter denotes configuration of hydroxy groups; Δ^5 = 5,6 double bond.

[b] Large characters of the alphabet correspond to peaks of reconstructed ion profile in Fig. 2 (serum and urine of cholestatic patients).

[c] Tentative; see text.

hydroxylated bile acids were significantly higher in umbilical cord blood and amniotic fluid. Unsaturated and 6-hydroxylated bile acids were also significantly higher in umbilical cord blood, amniotic fluid, and cholestatic urine. Furthermore, these three groups were present in much higher proportion in amniotic fluid and cholestatic urine than in umbilical cord blood and cholestatic serum, respectively.

DISCUSSION

Background

Various unusual bile acids have been identified, mainly in urine from patients with cholestatic liver diseases (1, 3, 21). Some of them were identical to bile acids previously identified in human meconium (5, 8, 9) and amniotic fluid (4), and also to those in umbilical cord blood and amniotic fluid in this study, as summarized in **Table 5.**

These reports and findings suggested the existence of some altered pathways of bile acid metabolism in fetal liver and cholestatic liver.

Individual unusual bile acids found in fetal body fluids

Fourteen unusual bile acids were completely or partially identified by the present technique. Since very few of these unusual bile acids were found in the maternal blood, and since the proportion of unusual bile acids to total bile acids was much higher in umbilical cord blood and amniotic fluid than in maternal blood, materno-fetal transfer of bile acids might be unlikely. Colombo et al. (22) have also reported that the extent of placental transfer of bile acids from fetus to mother was very limited. Therefore, unusual bile acids found in umbilical cord blood and amniotic fluid were thought to be most likely derived from the fetus.

Two bile acids possessing a 3β-hydroxy-Δ^5 structure were found. The major one, 3β-Δ^5, may be an intermediate in an altered pathway for bile acid biosynthesis starting with degradation of the sterol chain (23). The origin of 3β,12α-Δ^5 is not yet known. Bile acids with a planar A/B ring junction may be 12α-hydroxylated in rat liver (24), and if this is also the case in human liver, 3β-Δ^5 might be the precursor.

Several bile acids were present in the form of hydroxylated products of primary and secondary bile acids. Two positions of steroidal rings were hydroxylated at positions C-1 and C-6. Our study revealed that HyoCA was found as a major unusual component and this finding agreed with that in the case of human meconium reported by Back et al. (5).

Three bile acids with a hydroxyl group at C-1 (1β,3α,12α, 1β,3α,7α, and 1β,3α,7α,12α) were found. Although the excretion of urinary 1β-hydroxylated bile acids has been known (1, 3, 16, 25), the presence of these bile acids in sera from fetus and adult has not been previously reported. In this study, the proportions of 1β-hydroxylated bile acids in umbilical cord blood and amniotic fluid were significantly higher than those in the sera and urine from cholestatic patients. This higher proportion of urinary 1β-hydroxylated bile acids suggested that 1β-hydroxylation might be concerned with elimination of bile acids. Takikawa et al. (26) reported that in the neonatal stage the capacity of the liver for glucuronidation of bile acid seemed to be low, and Balistreri et al. (27) reported that the fetal level of hepatic sulfotransferase was very low in rats with a progressive increase in its activity during the first 3 weeks of life. 1β-Hydroxylation might be substituted for sulfation and glucuronization of bile acids, and might function in addition to other polyhydroxylating reactions for elimination of bile acids during the fetal stage. This proportional difference might be partly attributable to the low capacity of fetal liver for sulfation and glucuronidation of bile acids or the different enzyme activity, as suggested by Back et al. (5). The presence of 1β-hydroxylated bile acids in umbilical cord blood and

TABLE 4. Statistical analysis of individual bile acids in umbilical cord blood, amniotic fluid, maternal blood, cholestatic serum and urine, and serum and urine of normal controls

3β-Δ^5

Group	Mean	MB	CON-S	CHOL-S	UB	CON-U	AMN	CHOL-U
MB	1.7[a]							
CON-S	2.3							
CHOL-S	2.8							
UB	5.4							
CON-U	6.5							
AMN	11.5	**[b]	**	**	*			
CHOL-U	29.8	***	***	***	***	***	***	

3β 12α-Δ^5

Group	Mean	CON-S	CON-U	CHOL-S	MB	CHOL-U	AMN	UB
CON-S	—							
CON-U	0.2							
CHOL-S	0.4							
MB	0.7							
CHOL-U	2.5	**	**	*				
AMN	3.5	**	**	**	*			
UB	4.2	***	***	***	***	*		

HyoCA

Group	Mean	MB	CON-U	CHOL-S	CON-S	UB	CHOL-U	AMN
MB	2.0							
CON-U	2.6							
CHOL-S	3.2							
CON-S	5.4							
UB	7.5	*	*	*				
CHOL-U	8.8	**	**	**				
AMN	8.9	*	*	*				

1β 3α 12α

Group	Mean	CHOL-S	CHOL-U	CON-S	MB	UB	CON-U	AMN
CHOL-S	0.1							
CHOL-U	0.4							
CON-S	1.1							
MB	1.5							
UB	3.5	***	***	**	*			
CON-U	4.3	***	***	***	**			
AMN	4.7	***	***	***	**			

1β 3α 7α

Group	Mean	CON-S	MB	CON-U	CHOL-U	CHOL-S	AMN	UB
CON-S	—							
MB	—							
CON-U	0.1							
CHOL-U	0.5							
CHOL-S	0.5							
AMN	2.1	*	*	*				
UB	4.3	***	***	***	***	***	**	

1β 3α 7α 12α

Group	Mean	CON-S	CON-U	CHOL-S	MB	CHOL-U	UB	AMN
CON-S	—							
CON-U	—							
CHOL-S	0.2							
MB	0.3							
CHOL-U	2.4	*	*					
UB	3.2	**	**	**	*			
AMN	15.4	***	***	***	***	***	***	

Statistical analysis was performed according to F-test. Abbreviations used: 3β-Δ^5, 3β-hydroxy-5-cholenoic acid; $3\beta,12\alpha$-Δ^5, $3\beta,12\alpha$-dihydroxy-5-cholenoic acid; HyoCA, hyocholic acid; $1\beta,3\alpha,12\alpha$, $1\beta,3\alpha,12\alpha$-trihydroxy-5β-cholanoic acid; $1\beta,3\alpha,7\alpha$, $1\beta,3\alpha,7\alpha$-trihydroxy-5β-cholanoic acid; $1\beta,3\alpha,7\alpha,12\alpha$, $1\beta,3\alpha,7\alpha,12\alpha$-tetrahydroxy-5$\beta$-cholanoic acid; B-3,6,7, 3,6,7-trihydroxycholanoic acid; B-3,6,7,12, 3,6,7,12-tetrahydroxycholanoic acid. UB, umbilical blood; AMN, amniotic fluid; MB, maternal blood; CHOL-S, cholestatic serum; CHOL-U, cholestatic urine; CON-S, serum of normal controls; CON-U, urine of normal controls.

[a] Values represent the mean of relative amounts of bile acids (percentage).

[b] *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TABLE 5. Relationships of unusual bile acids identified between present study and previous studies

Bile Acids Identified in Present Study [a]	Present Study				Previous Studies				References
	UB [b]	AMN [c]	CH·S [d]	CH·U [e]	AMN	MEC [f]	CH·S	CH·U	
B^5-3β-ol	+	+	+	+	+	+	+	+	(1)(2)(3)(4)(5)(19)(20)(21)
$B^5$3β, 12α-ol	▨	+	+	+	−	+	+	+	(1)(2)(19)(20)(25)
5βB-3α, 6α, 7α-ol	▨	+	+	+	−	+	+	+	(1)(2)(5)(19)(20)(21)
5βB-1β, 3α, 12α-ol	▨	▨	▨	+	−	+	−	+	(1)(8)(9)(19)(20)(25)
5βB-1β, 3α, 7α-ol	▨	▨	▨	+	−	+	−	+	(1)(8)(9)
5βB-1β, 3α, 7α, 12α-ol	▨	▨	▨	+	−	+	−	+	(1)(8)(9)(19)(20)
5βB-3β, 7α-ol	−	−	+	+	−	−	+	+	(1)(2)(19)(20)
5βB-3β, 7α, 12α-ol	▨	−	−	▨	−	−	−	−	
5βB-3α, 12α-ol-7-oxo	−	−	+	+	−	−	−	+	(1)(2)(19)(25)
24-nor-5βB-3α, 7α, 12α-ol	▨	▨	+	+	−	+	+	+	(1)(2)(5)(19)(25)

Cross-hatched areas represent new findings in the present study.
[a] B, Cholanoic acid; configurations at C-5 and of hydroxyl groups are indicated by Greek letters; superscript denotes position of double bond. Abbreviations: [b], UB, umbilical cord blood; [c], AMN, amniotic fluid; [d], CH · S, cholestatic serum; [e], CH · U, cholestatic urine; [f], MEC, meconium.

amniotic fluid is strongly compatible with hepatic 1β-hydroxylation of bile acids during the fetal stage, since fetal liver microsomes have also been shown to be capable of 1β-hydroxylation of steroids (28, 29).

There are some possible explanations for the common occurrence of unusual bile acids in both conditions. Considering the serum concentration of total bile acids, the concentration in umbilical cord blood at birth of premature and term neonates has been shown to be slightly higher or almost equal to that in maternal blood (30, 31); and that of the fetus at an early gestational age has been shown to be somewhat higher than that in maternal blood (32). Our data on umbilical cord artery (2.1 μg/ml), vein (3.8 μg/ml), and maternal blood (3.1 μg/ml) did not contradict those of other reports mentioned above. Therefore, in relation to body surface area, the level of total bile acids was speculated to be markedly greater during the fetal stage. In the serum profile of bile acids, the cholic/chenodeoxycholic acid ratio (C/CDC) was 1.0 (0.2–4.1) in umbilical cord blood and 1.3 (0.2–3.2) in maternal blood. These ratios were much higher than those of healthy subjects and were very close to the C/CDC ratio (0.8, 0.2–0.9) observed in cholestasis as reported by Carey (33). Moreover, the ratios of tri- plus tetrahydroxy to dihydroxy bile acids[2] in umbilical cord blood and amniotic fluid were 1.5 and 3.2, respectively, and these values were much higher than those in healthy subjects, 0.5 in sera and 0.7 in urine.

More abundant polyhydroxylated bile acids were observed in fetal body fluids. Therefore, on the basis of the data concerning umbilical cord blood and amniotic fluid, it is speculated that the fetus is maintained under conditions of higher bile acid level and that the fetal liver attempts to excrete bile acids into urine (amniotic fluid) by increasing their polarity.

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