Perinatal bile acid metabolism: analysis of urinary bile acids in pregnant women and newborns

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Abstract The metabolism of bile acids in 30 pregnant women was evaluated by analyzing the urinary composition of bile acids during late gestation (weeks 30–41) and again in these women and their newborn infants during the first week after delivery. The levels of individual bile acids were determined by gas chromatography–mass spectrometry after solvolysis and hydrolysis of bile acid conjugates. The mean total bile acid/ creatinine ratio in pregnant women decreased from 1.22 μmol/mmol creatinine at 30–32 weeks of gestation to 0.15 μmol/mmol creatinine at 6–7 days after delivery. The mean percentage of 1β-hydroxylated bile acids peaked at 27% at 3–4 days after delivery. In newborn infants, the mean total bile acid/creatinine ratio rapidly increased from 3.39 μmol/mmol creatinine at birth to 54.33 μmol/mmol creatinine at 7 days. During this period, large amounts (40–50%) of unsaturated ketonic bile acids, especially 7α,12α-dihydroxy-3-oxo-5β-chol-1-en-24-oic acid and 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acid, were observed in the infants' urine. These data suggest that, during the perinatal period, the formation of polyhydroxylated and unsaturated ketonic bile acids probably represents a mechanism for the excretion of bile salts, and that the metabolism of bile acids in both the mother and the infant changes significantly after birth.—**Kimura, A., M. Suzuki, T. Murai, T. Inoue, H. Kato, D. Hori, Y. Nomura, T. Yoshimura, T. Kurosawa, and M. Tohma.** Perinatal bile acid metabolism: analysis of urinary bile acids in pregnant women and newborns. *J. Lipid Res.* 1997. **38:** 1954–1962.

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A variety of unusual bile acids have been identified in human urine (1–4), amniotic fluid (5, 6), meconium (4, 7, 8), and in the bile from the fetal gallbladder (9, 10). These findings suggest the existence of some altered pathways of bile acid metabolism in the fetal liver and in the liver of patients with cholestasis.

Large amounts of unusual bile acids are synthesized by the fetal liver late in gestation. These compounds are mostly transferred from the fetus to the mother (11), with some being excreted into the amniotic fluid (5, 6). Polyhydroxylated bile acids are more abundant in the body fluids of the fetus as compared with adult (5). Analysis of umbilical cord blood and amniotic fluid indicates that the bile acid levels are generally elevated in the fetus (5, 11). Serum bile acid concentrations may also be increased in pregnant women, with higher levels being observed late in gestation (12).

Our objective was to examine in detail the qualitative and quantitative bile acid composition of urine from healthy pregnant women obtained at different times during late gestation and within the first week after delivery. Such data will provide baseline information for future comparisons with the urinary bile acid composition of healthy nonpregnant women. We also analyzed the urinary bile acids of newborn infants during the first week of life to elucidate some aspects of fetal bile acid metabolism during the perinatal period.

METHODS

Sample collection

Spot urine samples were collected from 25 healthy pregnant women (mean age: 31 years, range 20–38 years): who were divided into five groups with 5 subjects in each group: 30–32 weeks of gestation, 35–36 weeks of gestation, delivery, 3–4 days after delivery, and 6–7 days after delivery. Urine samples were obtained from

Abbreviations: Cr, creatinine; GC–MS, gas chromatography–mass spectrometry; Me-DMES, methyl ester-dimethylethylsilyl ether.

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a sixth group of 5 healthy nonpregnant women (mean age: 27 years, range 25–33 years). Spot urine samples also were collected from 15 healthy newborn infants (9 males and 6 females) (mean gestational age: 39 weeks, range 38–41 weeks; mean birth weight: 3205.6 g, range 2860–3480 g) in three age groups, with 5 subjects in each group: 0, 3, and 7 days. All infants exhibited normal development. Urine samples were stored at −25°C before analysis. The concentration of individual bile acids in the urine was corrected for creatinine (Cr) concentration in each subject and expressed as μmol/mmol of Cr. None of the subjects had a history of, or showed signs of, hepatobiliary or gastrointestinal disease. Infants were fed breast milk during this study. Informed consent was obtained from the pregnant women and from the parents of the newborn infants.

Material and reagents

The following bile acids were synthesized as described previously (2,13–17): 1β,3α,7α-trihydroxy-5β-cholan-24-oic acid; 1β,3α,12α-trihydroxy-5β-cholan-24-oic acid; 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid; 2β,3α,7α-trihydroxy-5β-cholan-24-oic acid; 2β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid; 3β,4β,7α,12α-tetrahydroxy-5β-cholan-24-oic acid; 3β,7α,12α-trihydroxy-5β-cholan-24-oic acid; 3α,4β,7α-trihydroxy-5β-cholan-24-oic acid; 3α,4β,7α,12α-tetrahydroxy-5β-cholan-24-oic acid; 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid; 3α,6α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid; 3α,6α,12α-trihydroxy-5β-cholan-24-oic acid; 3β-hydroxy-5-cholen-24-oic acid; 3β,12α-dihydroxy-5-cholen-24-oic acid; and 3α,7α,12α,19-tetrahydroxy-5β-cholan-24-oic acid. Cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, hyocholic acid, and ursodeoxycholic acid were obtained from Sigma Chemical (St. Louis, MO). We synthesized 7α,12α-dihydroxy-3-oxo-5β-chol-1-en-24-oic acid; 7α-hydroxy-3-oxo-4-cholen-24-oic acid; 3-oxo-4,6-choladien-24-oic acid; 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acid; 12α-hydroxy-3-oxo-4,6-choladien-24-oic acid; allochenodeoxycholic acid; and allocholic acid as described previously (18, 19).

Gas chromatography–mass spectrometry (GC–MS)

GC–MS was performed on a JEOL JMS-AM 150 instrument (JEOL Co., Tokyo, Japan) using a gas chromatographic column DB-1 (30 m × 0.2 mm I.D., fused silica capillary column bonded with methylsilicon; J & W Scientific, Folsom, CA) with the column temperature, programmed from 170 to 230°C at 10°C/min and 230 to 310°C at 3°C/min. Helium was used as the carrier gas with a flow rate of 45 cm/s. The mass spectra were recorded at an ionization energy of 70 eV with an ion source temperature of 290°C. **Figure 1** shows a chromatogram obtained by selective ion monitoring of the characteristic fragments of the methyl ester-dimethylethylsilyl ether (Me-DMES)-methoxime derivatives of the bile acids in the standard sample.

Derivatization of bile acids for GC-MS analysis

Each bile acid (2 μg) or a mixture of bile acids was dissolved in distilled water (1.0 ml) An equal volume of pyridinium acetate buffer (1.5 M, pH 5) containing 1.0 M of O-methylhydroxylamine hydrochloride was added to the solution and the mixture was kept at 37°C for 2 h. The bile acids were extracted from the solution using a Bond Elut C18 cartridge (3 ml, Varian, Harbor City, CA). The cartridge was washed with water (5 ml) and the bile acids were eluted with ethanol (5 ml). After evaporation of the solvents, the residue was dissolved in 1 ml of 90% aqueous ethanol. The solution was applied to a piperidinohydroxypropyl dextran gel (Shimadzu Co., Kyoto, Japan) column (30 × 6 mm I.D.) that was equilibrated with 90% aqueous ethanol. The column was washed with 90% ethanol (4 ml) to remove neutral compounds and the bile acids were eluted with 0.1 M acetic acid in 90% ethanol (5 ml). After evaporation, the purified bile acid methoximes were derivatized to the methyl ester with diazomethane at room temperature for 10 min. After the removal of excess reagent, the dimethylethylsilyl ether was obtained by heating the residue with 30 μl of dimethylethylsilylimidazole (Tokyo Kasei, Tokyo, Japan) at 60°C for 40 min. The resulting preparation was applied to a silica gel column (30 × 6 mm I.D.) and eluted with n-hexane/ethyl acetate (3:1 by volume). The derivatized bile acids were recovered in the first 5 ml of effluent and the solvent was evaporated to dryness under reduced pressure. The residue was dissolved in n-hexane (50 μl) and an aliquot (1 μl) was injected into the CG–MS system.

Analysis of bile acids in urine

In the standard procedure, samples of human biological fluids were routinely prepared for CG–MS analysis as described in a previous paper (20) as follows. An internal standard (3α,7α-dihydroxy-24-nor-5β-cholan-23-oic acid, 2 μg) was added to 1 ml of a urine sample. The 3-oxo bile acids were derivatized to methoximes, and the conjugated bile acids were extracted from the solution using a Bond Elut C18 cartridge as described above. After the solvents were evaporated, the residue was subjected to enzymatic hydrolysis and solvolysis using 30 U choloylglycine hydrolase (Sigma Chemical, St. Louis, MO) and 150 U sulfatase Type H-1: from *Helix pomatia* (Sigma Chemical, St. Louis, MO) in 200 μl 0.05 M sodium acetate buffer (pH 5.6), 200 μl 0.6 mM dithiothreitol, 200 μl 0.05 M EDTA, and 100 μl distilled wa-

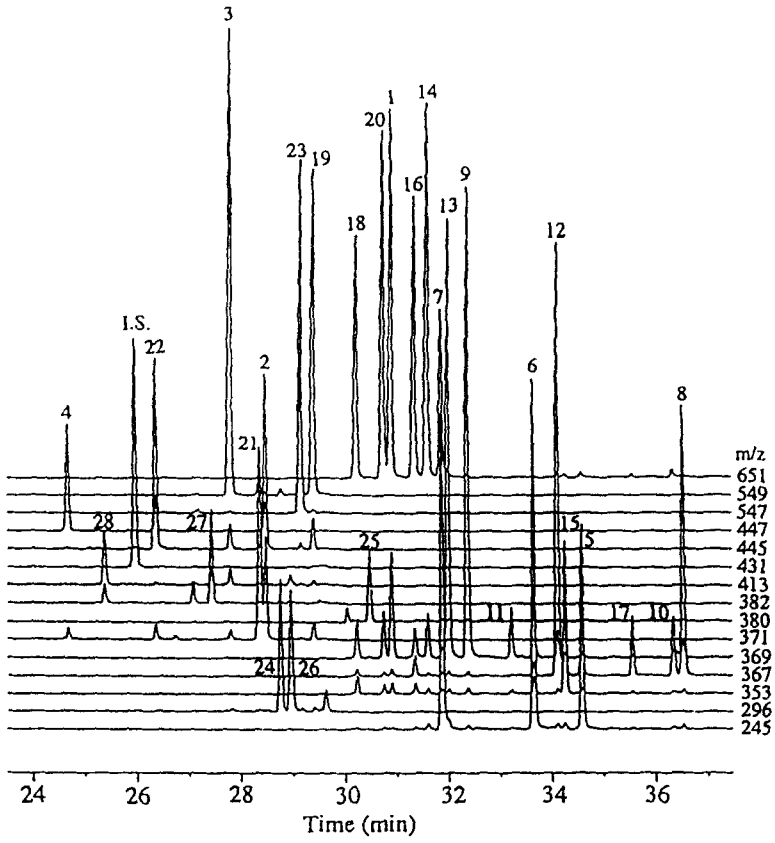

Fig. 1. Selected ion GC–MS chromatogram of the Me-DMES-methoxime derivatives of bile acids. Peak numbers and compounds are the same as in Table 1.

ter, at 37°C for 12 h. The resulting unconjugated bile acids were extracted again with a Bond Elut C18 cartridge. The cartridge was washed with 5 ml of distilled water, and eluted with 5 ml of 90% ethanol. The unconjugated bile acids were extracted with piperidinohydroxypropyl dextran gel, eluted with 5 ml of 0.1 M acetic acid in 90% ethanol, and converted to the Me-DMES derivatives for GC–MS analysis.

With this method, the recovery of bile acids and their conjugates from urine, relative to the internal standard, ranged from 94.2 to 105.9% of the amount added to the samples.

Identification and quantitation of individual bile acids

The GC–MS data of individual bile acids are summarized in **Table 1,** including the relative retention times with respect to the internal standard, their characteristic fragment ions, and their relative abundance.

Statistical analysis

All data are reported as the mean ± SD in the tables. One-way ANOVA was used to determine the significance of the differences between groups. Groups were compared using Student's *t*-test. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Total bile acids

The mean total bile acid/Cr ratio in the urine of pregnant women was highest at 30–32 weeks of gestation (1.22 μmol/mmol Cr), and it gradually decreased thereafter (**Table 2**). At 6–7 days after delivery, the ratio was comparable to that in healthy nonpregnant women (0.15 μmol/mmol Cr). In general, the mean total bile acid/Cr ratios were significantly higher before delivery than afterwards.

The mean total bile acid/Cr ratio in the urine of the newborn infants rapidly increased between birth (3.39 μmol/mmol Cr) and 7 days postpartum (54.53 μmol/mmol Cr) (**Table 3**). The differences in mean total bile acid/Cr ratio between 7-day-old infants versus the newborn or 3-day-old infants were statistically significant ($P < 0.01$ and $P < 0.05$, respectively).

TABLE 1. Gas chromatography–mass spectrometric data of the methyl ester-dimethylethylsilyl ether and methoxime derivatives of bile acids

No.	Bile Acid	Relative Retention Time	Base Peak *m/z*	Fragment Ions, *m/z* (Relative Intensity, %)
Common bile acids				
1.	Cholic acid	1.19	651[a]	369(51), 253(42)
2.	Chenodeoxycholic acid	1.10	445[a]	371(95), 355(10)
3.	Deoxycholic acid	1.07	549[a]	255(87), 371(9)
4.	Lithocholic acid	0.95	447[a]	323(50), 372(45)
1β-, 2β-, 4β-, 6α- and 19-Hydroxylated bile acids				
5.	1β,3α,7α,12α-Tetrahydroxy-5β-cholan-24-oic acid	1.33	245[a]	367(23), 471(10)
6.	1β,3α,7α-Trihydroxy-5β-cholan-24-oic acid	1.29	245[a]	369(18), 472(7)
7.	1β,3α,12α-Trihydroxy-5β-cholan-24-oic acid	1.23	245[a]	651(21), 547(7)
8.	2β,3α,7α,12α-Tetrahydroxy-5β-cholan-24-oic acid	1.44	367[a]	545(10), 357(5)
9.	2β,3α,7α-Trihydroxy-5β-cholan-24-oic acid	1.25	369[a]	337(13), 457(5)
10.	3α,4β,7α,12α-Tetrahydroxy-5β-cholan-24-oic acid	1.40	367[a]	357(32), 471(15)
11.	3α,4β,7α-Trihydroxy-5β-cholan-24-oic acid	1.28	369[a]	329(45), 457(20)
12.	3α,6α,7α,12α-Tetrahydroxy-5β-cholan-24-oic acid	1.31	367[a]	471(20), 545(10)
13.	Hyocholic acid	1.24	369[a]	337(20), 547(7)
14.	3α,6α,12α-Trihydroxy-5β-cholan-24-oic acid	1.22	651[a]	253(51), 369(24)
15.	3α,7α,12α,19-Tetrahydroxy-5β-cholan-24-oic acid	1.32	353[a]	457(52), 367(49)
Isomerized 3β- and 7β-hydroxy and 5α- (allo) bile acids				
16.	3β,7α,12α-Trihydroxy-5β-cholan-24-oic acid	1.16	651[a]	357(44), 369(30)
17.	3β,4β,7α,12α-Tetrahydroxy-5β-cholan-24-oic acid	1.37	367[a]	327(44), 575(20)
18.	3α,7β,12α-Trihydroxy-5β-cholan-24-oic acid	1.21	651[a]	253(97), 357(34)
19.	Ursodeoxycholic acid	1.13	549[a]	369(50), 459(30)
20.	Allocholic acid	1.18	651[a]	357(31), 369(27)
21.	Allochenodeoxycholic acid	1.09	371[a]	549(12), 355(10)
3β-Hydroxy-Δ^5-bile acids				
22.	3β-Hydroxy-5-cholen-24-oic acid	1.03	445[a]	331(88), 370(34)
23.	3β,12α-Dihydroxy-5-cholen-24-oic acid	1.12	547[a]	253(69), 329(31)
Unsaturated ketonic bile acids				
24.	7α,12α-Dihydroxy-3-oxo-5β-chol-1-en-24-oic acid	1.11	296[a]	380(40), 591(19)
25.	7α,12α-Dihydroxy-3-oxo-4-cholen-24-oic acid	1.17	296	380[a](89), 590(68)
26.	12α-Hydroxy-3-oxo-4,6-choladien-24-oic acid	1.12	296[a]	411(59), 515(5)
27.	7α-Hydroxy-3-oxo-4-cholen-24-oic acid	1.06	382[a]	488(47), 517(30)
28.	3-Oxo-4,6-choladien-24-oic acid	0.98	413[a]	249(88), 382(35)
I.S.	3α,7α-Dihydroxy-24-nor-5β-cholan-23-oic acid	1.00[b]	431[a]	

[a]Fragment ions used for selected ion monitoring.
[b]Retention time is 25.56 min, I.S.: internal standard.

Common bile acids

The mean ratio of common bile acids found in healthy adults (cholic, chenodeoxycholic, deoxycholic, and lithocholic acids) to total bile acids in the urine did not differ significantly according to stage of gestation in either the pregnant women or newborn infants (Tables 2 and 3). However, the mean percentage of common bile acids differed between the pregnant women (25–50%) and the newborn infants (6–13%) (Tables 2 and 3). The mean ratio of primary bile acids (cholic and chenodeoxycholic acids) in the total common bile acids in urine of pregnant women ranged from 36% to 51% among the stages of gestation analyzed (Table 2). However, large amounts of primary bile acids were detected in the urine of newborn infants, especially at 3 and 7 days after birth (99.2 and 99.8%, respectively) (Table 3).

1β-, 2β-, 4β-, 6α-, and 19-Hydroxylated bile acids

The mean percentage of 1β-hydroxylated bile acids (1β,3α,7α,12α-tetrahydroxy-,1β,3α,7α-trihydroxy-, and 1β,3α,12α-trihydroxy-5β-cholan-24-oic acids) in the total bile acids in the urine of pregnant women increased throughout late gestation, reaching 19% at delivery ($P < 0.05$ vs. 6–7 days after delivery) (Table 2). The percentage of 1β-hydroxylated bile acids showed a further increase in the first 3–4 days after delivery (27%) ($P < 0.05$ vs. 30–32 and 35–36 weeks of gestation, $P < 0.01$ vs. 6–7 days after delivery). However, at 6–7 days after delivery, the percentage of 1β-hydroxylated bile acids declined sharply to values approaching those seen in healthy nonpregnant women. The 1β-hydroxylated bile acids in the pregnant and the nonpregnant women consisted mainly of 1β,3α, 12α-trihydroxy-5β-cholan-24-oic acid. The percentage of 1β-hydroxylated bile acids in the newborn infants, which consisted mainly of 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid, gradually increased from 7% to 15% during the first week of life (Table 3).

The percentage of 2β-hydroxylated bile acids (2β,3α,7α,12α-tetrahydroxy- and 2β,3α,7α-trihydroxy-5β-cholan-24-oic acids) did not change significantly in

TABLE 2. Bile acid composition of urine in women before and after delivery

Bile acid (Compound number in Table 1)	Gestational Age 30–32 Weeks (n=5)	Gestational Age 35–36 Weeks (n=5)	At Delivery (n=5)	3–4 Days after Delivery (n=5)	6–7 Days after Delivery (n=5)	Healthy Nonpregnant Women (n=5)
Common bile acids (1–4)	24.93 ± 9.26 (51.2 ± 40.2)	45.08 ± 20.90 (36.4 ± 19.5)	31.76 ± 18.08 (38.3 ± 25.0)	39.38 ± 20.86 (44.6 ± 26.1)	50.73 ± 17.06[a] (44.2 ± 22.4)	34.17 ± 21.16 (45.3 ± 27.0)
1β-Hydroxylated bile acids (5–7)	8.67 ± 7.74	11.14 ± 5.36	19.73 ± 10.64[b]	27.08 ± 21.82[c,d]	2.13 ± 1.87	5.99 ± 5.14
2β-Hydroxylated bile acids (8,9)	0.30 ± 0.43	0.22 ± 0.21	0.40 ± 0.46	0.45 ± 0.70	0.76 ± 0.82	0.09 ± 0.17
4β-Hydroxylated bile acids (10,11)	0.67 ± 0.30	0.32 ± 0.30	0.48 ± 0.49	0.32 ± 0.71	3.09 ± 2.35[e,f]	0.57 ± 0.70
6α-Hydroxylated bile acids (12–14)	26.37 ± 14.72	17.62 ± 11.90	26.42 ± 11.09	11.55 ± 7.02	12.78 ± 7.62	12.41 ± 20.19
19-Hydroxylated bile acid (15)	trace	0.05 ± 0.11	0.03 ± 0.07	0.30 ± 0.68	0.14 ± 0.32	trace
3β-Hydroxylated bile acids (16,17)	7.86 ± 10.77	3.76 ± 6.54	1.93 ± 4.00	1.32 ± 1.62	4.46 ± 6.95	6.53 ± 9.91
Urso bile acids (18,19)	20.38 ± 12.95	11.28 ± 6.87	15.57 ± 14.56	14.10 ± 12.30	11.86 ± 6.49	32.97 ± 22.2[g]
Allo bile acids (20,21)	0.74 ± 1.29	1.30 ± 1.58	0.34 ± 0.36	2.86 ± 5.60	1.78 ± 2.10	1.89 ± 0.60
3β-Hydroxy-Δ^5-bile acids (22,23)	0.69 ± 0.44	1.30 ± 0.85	1.40 ± 1.87	2.19 ± 1.36	11.12 ± 5.17[h,i]	4.02 ± 1.80
3-Oxo-Δ^1-bile acid (24)	0.12 ± 0.22	0.06 ± 0.08	0.25 ± 0.41	not detectable	not detectable	0.14 ± 0.27
3-Oxo-Δ^4-bile acids (25–28)	9.26 ± 13.91	6.74 ± 8.88	3.31 ± 4.06	0.52 ± 1.17	1.95 ± 3.03	1.33 ± 2.67
Total bile acids [μmol/mmol Cr]	1.22 ± 0.82[j,k]	1.05 ± 0.23[l,m]	0.47 ± 0.38	0.32 ± 0.20	0.15 ± 0.10	0.14 ± 0.08

Values in parentheses represent percentage of primary bile acids (cholic and chenodeoxycholic acids) to common bile acids. All data are means ± SD (% of total bile acids).

[a] $P < 0.05$ vs. 30–32 weeks of gestation; [b] $P < 0.05$ vs. 6–7 days after delivery; [c] $P < 0.05$ vs. 30–32 and 35–36 weeks of gestation; [d] $P < 0.01$ vs. 6–7 days after delivery and healthy nonpregnant women; [e] $P < 0.01$ vs. 30–32 weeks of gestation, at delivery, and healthy nonpregnant women; [f] $P < 0.001$ vs. 35–36 weeks of gestation and 3–4 days after delivery; [g] $P < 0.05$ vs. 35–36 weeks of gestation and 3–4 and 6–7 days after delivery; [h] $P < 0.0001$ vs. 30–32 and 35–36 weeks of gestation, at delivery, and 3–4 days after delivery; [i] $P < 0.001$ vs. healthy nonpregnant women; [j] $P < 0.01$ vs. at delivery and 3–4 days after delivery; [k] $P < 0.001$ vs. 6–7 days after delivery and healthy nonpregnant women; [l] $P < 0.05$ vs. at delivery; [m] $P < 0.01$ vs. 3–4 and 6–7 days after delivery and healthy nonpregnant women.

the pregnant women before or after delivery. In the newborn infants, however, the percentage of urinary 2β-hydroxylated bile acids was significantly elevated at 3 days (5%) and 7 days postpartum (6%) as compared with the percentage at birth (2%) ($P < 0.05$).

The proportion of the 4β-hydroxylated bile acids (3α,4β,7α,12α-tetrahydroxy- and 3α,4β,7α-trihydroxy-5β-cholan-24-oic acids) in the urine of pregnant women did not change significantly during the late stages of gestation of early after delivery (0.3–0.7%). At 6–7 days postpartum, however, their levels were significantly elevated to 3% ($P < 0.01$ vs. 30–32 weeks of gestation, at delivery, and healthy nonpregnant women; $P < 0.001$ vs. 35–36 weeks of gestation and 3–4 days after delivery). The levels of 4β-hydroxylated bile acids in newborn infants increased significantly to 7% ($P < 0.05$) at 3 days postpartum and 9% ($P < 0.01$) at 7 days postpartum (Table 3).

The mean ratio of 6α-hydroxylated bile acids (3α,6α,7α,12α-tetrahydroxy- and 3α,6α,12α-trihydroxy-5β-cholan-24-oic acids and hyocholic acid) to total bile acids in pregnant women was the highest be-

TABLE 3. Bile acid composition of urine in newborn infants

Bile Acid (Compound number in Table 1)	0 Days (n = 5)	3 Days (n = 5)	7 Days (n = 5)
Common bile acids (1–4)	13.23 ± 20.63 (77.7 ± 20.1)	5.75 ± 5.98 (99.2 ± 0.4)[a]	6.18 ± 5.64 (99.8 ± 0.2)
1β-Hydroxylated bile acids (5–7)	7.03 ± 5.59	16.61 ± 8.32	15.02 ± 12.23
2β-Hydroxylated bile acids (8,9)	1.95 ± 1.08	5.12 ± 2.41[b]	5.82 ± 2.49[a]
4β-Hydroxylated bile acids (10,11)	2.01 ± 1.54	6.68 ± 2.27[c]	8.96 ± 4.52[d]
6α-Hydroxylated bile acids (12–14)	7.35 ± 5.61	13.01 ± 9.94	10.81 ± 11.87
19-Hydroxylated bile acid (15)	0.82 ± 0.69	0.48 ± 0.12	0.43 ± 0.23
3β-Hydroxylated bile acids (16,17)	5.61 ± 4.59	9.22 ± 3.90	4.72 ± 1.81
Urso bile acids (18,19)	0.85 ± 0.97	0.24 ± 0.29	0.26 ± 0.43
Allo bile acids (20,21)	0.29 ± 0.23	0.23 ± 0.31	0.09 ± 0.03
3β-Hydroxy-Δ^5-bile acids (22,23)	2.59 ± 3.17	0.68 ± 0.38	0.86 ± 0.87
3-Oxo-Δ^1-bile acid (24)	25.40 ± 16.14	31.96 ± 16.04	32.11 ± 16.01
3-Oxo-Δ^4-bile acids (25–28)	33.04 ± 21.52[e]	8.96 ± 5.56	14.72 ± 13.25
Total bile acids [μmol/mmol Cr]	3.39 ± 4.34	10.16 ± 7.64	54.53 ± 39.19[f,g]

Values in parentheses represent percentage of primary bile acids (cholic and chenodeoxycholic acids) to common bile acids. All data are means ± SD (% of total bile acids).

[a] $P < 0.05$ vs. 3 and 7 days; [b] $P < 0.05$ vs. 0 days; [c] $P < 0.05$ vs. 0 days; [d] $P < 0.01$ vs. 0 days; [e] $P < 0.05$ vs. 3 days; [f] $P < 0.01$ vs. 0 days; [g] $P < 0.05$ vs. 3 days.

fore delivery (17–26%), whereas in newborn infants it was the highest between 3 and 7 days postpartum (11–13%) (Tables 2 and 3).

The mean proportion of 19-hydroxylated bile acid (3α,7α,12α,19-tetrahydroxy-5β-cholan-24-oic acid) in the total urinary bile acids did not change significantly before or after delivery in the pregnant women (0.03–0.3%) or in the newborn infants (0.4–0.8%).

The mean ratio of fetal bile acids (1β, 2β, 4β, 6α-, and 19-hydroxylated bile acids) among the total bile acids in urine of pregnant women increased during late gastation, reaching 47% at delivery; this percentage decreased sharply thereafter (19% at 6–7 days postpartum). In newborn infants, the mean percentage of fetal bile acids in the total bile acids increased from 19% at delivery to 40% at 7 days postpartum.

Isomerized bile acids

The mean ratio of 3β-hydroxylated bile acids (3β,7α,12α-trihydroxy- and 3β,4β,7α,12α-tetrahydroxy-5β-cholan-24-oic acids) among the total bile acids gradually decreased from 8 to 2% in pregnant women during the late stages of gestation, but increased from 1 to 4% after delivery (Table 2).

The percentage of the urinary urso bile acids (3α,7β,12α-trihydroxy-5β-cholan-24-oic acid and ursodeoxycholic acid) remained at a high relatively constant level in pregnant women before and after delivery (11–20%). However, the levels at 35–36 weeks of gestation and those at 3–4 days and 6–7 days postpartum were significantly lower than those observed in healthy nonpregnant women (33%) ($P < 0.05$) (Table 2). The levels of urso bile acids were low in newborn infants (0.2–0.9%). Low levels of allo (5α) bile acids (allocholic and allochenodeoxycholic acids) were detected in the pregnant women both before and after delivery (0.3–2.9%) as well as in the newborn infants (0.1–0.3%).

3β-Hydroxy-Δ^5-bile acids

The mean ratio of 3β-hydroxy-Δ^5-bile acids (3β,12α-dihydroxy- and 3β-hydroxy-5-cholen-24-oic acids) among the total urinary bile acids in pregnant women remained relatively constant during late gestation and early after delivery (0.7–2.2%). At 6–7 days after delivery, however, the levels (11%) were significantly higher than at all other times ($P < 0.0001$ vs. values at 30–32 and 35–36 weeks of gestation, at delivery and 3–4 days after delivery, $P < 0.001$ vs. values in healthy nonpregnant women) (Table 2). The newborn infants exhibited no change in the mean percentage of 3β-hydroxy-Δ^5-bile acids during the first week of life (0.7–2.6%).

Unsaturated ketonic bile acids

In the pregnant women, the 3-oxo-Δ^1-bile acid (7α,12α-dihydroxy-3-oxo-5β-chol-1-en-24-oic acid) was barely detectable in the urine (0.1–0.3%). In contrast, in the newborn infants, 3-oxo-Δ^1-bile acid consistently represented a large proportion of the total urinary bile acids (25–32%)

The mean ratio of the 3-oxo-Δ^4-bile acids (7α,12α-dihydroxy-3-oxo-4-cholen-24-oic acid, 7α-hydroxy-3-oxo-4-cholen-24-oic acid, 12α-hydroxy-3-oxo-4,6-choladien-24-oic acid and 3-oxo-4,6-choladien-24-oic-acid) to the total bile acids in the urine of pregnant women gradually decreased between 30–32 weeks of gestation and 3–4 days after delivery (9 to 0.5%) (Table 2). In newborn infants, however, the levels of urinary 3-oxo-Δ^4-bile acids remained high throughout the first week of life, although their proportion declined significantly from 33% at birth to 9% at 3 days after birth ($P < 0.05$) (Table 3).

Thus, the bile acids that predominated in the urine of pregnant women in the late stages of gestation were the common bile acids observed in the urine of healthy adults, such as cholic, chenodeoxycholic, and deoxycholic acids. In newborn infants, the unsaturated ketonic bile acids predominated, and included 7α, 12α-dihydroxy-3-oxo-5β-chol-1-en-24-oic, and 7α,12α-dihydroxy-3-oxo-4-cholen-24-oic and 12α-hydroxy-3-oxo-4,6-choladien-24-oic acids.

DISCUSSION

The production of urine by the human fetus increases after 30 weeks of gestation (21, 22). At that time, fetal urine and amniotic fluid volumes are elevated as demonstrated by real-time ultrasonography (22). This suggests that the total urinary levels of bile acid in the mother would decrease after 30 weeks of gestation when the fetal bile acids are excreted into the amniotic fluid. Our present results confirm this hypothesis. Moreover, the mean total urinary bile acid/Cr ratio at 25 weeks of gestation (0.93 μmol/mmol Cr) was lower than that at 30 weeks of gestation (data not shown). Thus, the total bile acid/Cr ratio in the urine of the mother appeared to peak at about 30 weeks of pregnancy. Similarly, we observed that the levels of 2β- and 4β-hydroxylated bile acids and 3-oxo-Δ^4-bile acids, which are elevated in the amniotic fluid at fullterm (6), were decreased in the urine of pregnant women late in gestation (weeks 30–41).

The proportion of primary bile acids in the urine of newborn infants significantly exceeded that of the pregnant women. This proportion may differ according to the type of bacterial colonization in the mother's intestine as well as that of the neonate due to the ingestion of food supplements (23, 24). We detected small

amounts of secondary bile acids (deoxycholic and lithocholic acids) in neonatal urine on the first day of life. Such bile acids appeared to be mainly of maternal origin with transfer to the fetus occurring via the placenta (11). The profile of urinary bile acids in the neonate on the first day of life resembles that of the meconium (24).

The proportion of 1β-hydroxylated bile acids, especially 1β,3α,12α-trihydroxy-5β-cholan-24-oic acid, was increased in the urine of pregnant women between delivery and 3–4 days postpartum, but decreased again thereafter. These increased levels reflect the presence of subclinical intrahepatic cholestasis due to pregnancy (12). Because hydroxylation increases the solubility of bile acids, the formation of 1β-hydroxylated bile acids probably represents an additional mechanism of bile salt excretion during cholestasis (4, 5). According to a previous report (25), 1β,3α,12α-trihydroxy-5β-cholan-24-oic acid is conjugated with glycine, whereas 1β,3α,7α,12α-tetrahydroxy- and 1β,3α,7α-trihydroxy-5β-cholan-24-oic acids are conjugated only with taurine. The 1β,3α,7α,12α-tetrahydroxy- and 1β,3α,7α-trihydroxy-5β-cholan-24-oic acids excreted in the urine of pregnant women may have been secreted by the fetus. Accordingly, at 6–7 days after delivery, the mean percentage of 1β-hydroxylated bile acids in the total bile acids in the mother's urine returned to the levels found in healthy nonpregnant women.

A high proportion of 3β-hydroxy-Δ^5-bile acids was detected at 6–7 days after delivery. This also reflects the presence of subclinical intrahepatic cholestasis related to pregnancy (12). However, the relationship between the high proportion of 3β-hydroxy-Δ^5-bile acids and the fetoplacental metabolism of bile acids remains to be elucidated (5).

In pregnant women, and especially, in healthy nonpregnant women, we detected large amounts of isomerized 7β-hydroxylated bile acids. The urinary levels of these bile acids were low in newborn infants. In fact, urso bile acids, especially 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid, may be the important bile acids in the urine of adults. It has been suggested that their high levels are related to the epimerization of 7α-hydroxy group of cholic acid by intestinal microorganisms (26) and to the ingestion of food supplements (23, 24).

The urinary total bile acids/Cr ratio in newborn infants increased between birth and the seventh postpartum day. This increase, which reflects an increase in serum total bile acids, may be attributable to oral intake, the enhanced stimulation of enterohepatic circulation of bile acids, or their impaired hepatic clearance or excretion into the urine (27–29). Bile acids are excreted in urine to protect against hypercholemia. Mainly excreted in the urine of newborn infants were the polyhydroxylated bile acids, such as 1β-, 2β-, 4β-, 6α-, and 19-tetrahydroxylated bile acids (41%), and unsaturated ketonic bile acids (48%), including 3-oxo bile acids. The formation of bile acids, especially of polyhydroxylated bile acids, is probably a mechanism for the excretion of bile salts in the newborn with physiologic cholestasis. Thus, the pathway for the synthesis of polyhydroxylated bile acids may differ in the liver of the fetus and newborn infant as compared with the adult (2, 4–10).

During the first week of life, when the infant's intestine may become colonized by bacterial flora (30), we observed high levels of unsaturated ketonic bile acids, specifically of 3-oxo-Δ^1- and 3-oxo-Δ^4-bile acids. High levels of 3-oxo-Δ^4-bile acids in the urine have been associated with a deficiency (31, 32), or reduced activity (33), of 3-oxo-Δ^4-steroid 5β-reductase, the enzyme that catalyzes the conversion of 3-oxo-Δ^4-C_{27} sterol intermediates to 3-oxo-5β-configurational products in the normal pathway of primary bile acid synthesis (34). In this study, the mean ratio of urinary 3-oxo-Δ^4-bile acids (especially 7α,12α-dihydroxy-3-oxo-4-cholen-, 12α-hydroxy-3-oxo-4,6-choladine-, and 7α-hydroxy-3-oxo-4-cholen-24-oic acids) to total bile acids was significantly higher at birth than at 3 days after birth. This decline in 3-oxo-Δ^4-bile acid levels reflects the normal development of bile acid metabolism, including the initial immaturity of hepatic 3-oxo-Δ^4-steroid 5β-reductase.

During the first 7 days after birth, 7α,12α-dihydroxy-3-oxo-5β-chol-1-en-24-oic acid predominated in the infants' urine (25–32% of total urinary bile acids). Thereafter, however, the levels of this unsaturated ketonic bile acid decreased, whereas the levels of 1β,3α,12α-tetrahydroxy-5β-cholan-24-oic acid increased (data not shown). These findings suggest that 7α,12α-dihydroxy-3-oxo-5β-chol-1-en-24-oic acid may be formed by dehydration of a precursor for the synthesis of 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid. The former unsaturated ketonic bile acid, however, may be not synthesized in the human body. Alternatively, 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid may be synthesized from 1β,7α,12α-trihydroxy-3-oxo-5β-cholan-24-oic acid by reduction of the 3-oxo group, after 7α,12α-dihydroxy-3-oxo-5β-cholan-24-oic acid has been converted into its corresponding 1β-hydroxylated bile acid (**Fig. 2**). We found that more than 95% of the 1β,7α,12α-trihydroxy-3-oxo-5β-cholan-24-oic acid in the urine was converted into 7α,12α-dihydroxy-3-oxo-5β-chol-1-en-24-oic acid. Moreover, Wahlén et al. (3) detected the presence of 1β,7α,12α-trihydroxy-3-oxo-5β-cholan-24-oic acid in the urine of newborn infants early in life. These findings strongly suggest that

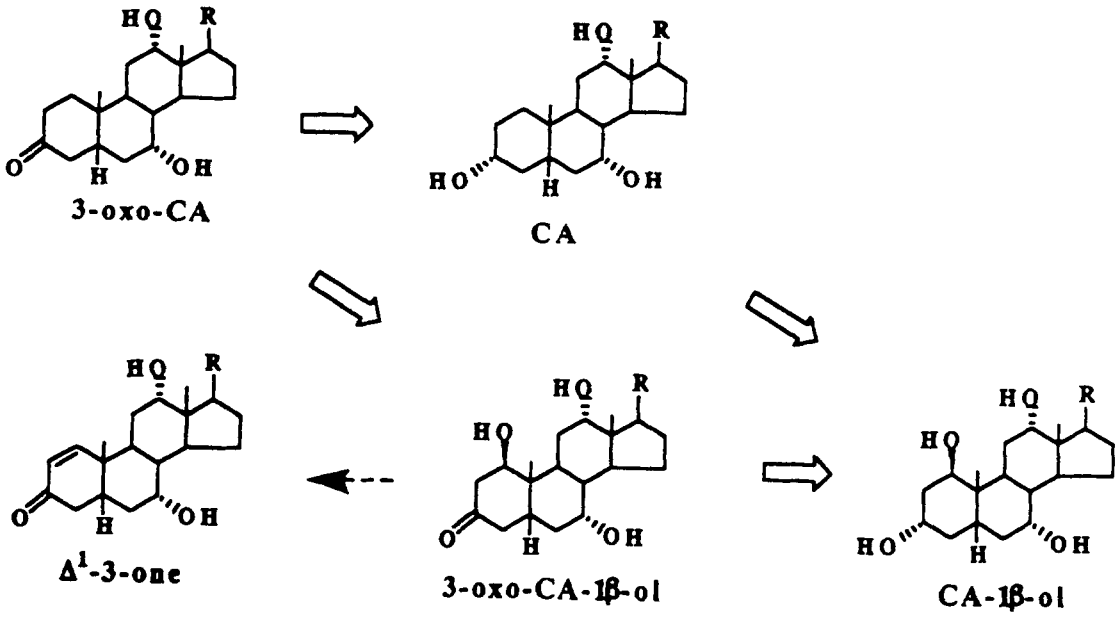

Fig. 2. Sythetic pathway for 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic and 7α,12α-dihydroxy-3-oxo-5β-chol-1-en-24-oic acids. CA: cholic acid; CA-1β-ol: 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid; 3-oxo-CA: 7α,12α,dihydroxy-3-oxo-5β-cholan-24-oic acid; 3-oxo-CA-1β-ol: 1β,7α,12α-trihydroxy-3-oxo-5β-cholan-24-oic acid; Δ^1-3-one: 7α,12α-dihydroxy-3-oxo-5β-chol-1-en-24-oic acid.

1β,7α,12α-trihydroxy-3-oxo-5β-cholan-24-oic acid is converted into 7α,12α-dihydroxy-3-oxo-5β-chol-1-en-24-oic acid by dehydration of the β-ketol. In addition, we suggest that 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid is produced from cholic acid by hydroxylation at C-1 and/or from 1β,7α,12α-trihydroxy-3-oxo-5β-cholan-24-oic acid by a redox reaction at C-3 (Fig. 2).

The physiologic relevance of polyhydroxylated and unsaturated ketonic bile acids is currently unclear. Unless they are metabolized into more polar compounds, bile acids have a pronounced hepatotoxic effect on the fetus and the newborn infants. Therefore, it is likely that the formation of polyhydroxylated bile acids, especially 1β-tetrahydroxylated bile acid, and of unsaturated ketonic bile acids, especially 3-oxo-Δ^1 bile acid, together with the conjugation with taurine (25) play a role in the excretion of bile salts in the perinatal period. Our data and those of previous studies (2–10, 35–37) support the notion that the metabolism of bile acids changes significantly in the mother and infant after birth.

Further studies are under way to determine the profile of biosynthetic intermediates for unsaturated ketonic bile acids, 3-oxo-C_{27}-bile acids and minor allylic bile acids.

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