Developmental pattern of 3-oxo- Δ^4 bile acids in neonatal bile acid metabolism

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Abstract

Aims—To investigate whether a fetal pathway of bile acid synthesis persists in neonates and infants.

Methods—3-oxo- Δ^4 bile acids were determined qualitatively and quantitatively in the urine, meconium, and faeces of healthy neonates and infants, using gas chromatography-mass spectrometry.

Results—The mean percentage of 3-oxo- Δ^4 bile acids in total bile acids in urine at birth was significantly higher than that at 3 or 7 days, and at 1 or 3 months of age. The concentration of this component in meconium was significantly higher than that in faeces at 7 days and at 1 or 3 months of age.

Conclusions—The presence of large amounts of urinary 3-oxo- Δ^4 bile acids may indicate immaturity in the activity of hepatic 3-oxo- Δ^4 -steroid 5 β -reductase in the first week of postnatal life. Large amounts of this component in meconium may be due to the ingestion of amniotic fluid by the fetus during pregnancy.

(*Arch Dis Child* 1997;77:F52-F56)

Keywords: ketonic bile acid; 3-oxo- Δ^4 bile acid; 3-oxo- Δ^4 -steroid 5 β -reductase; meconium; gas chromatography-mass spectrometry

Infants with severe liver disease have a high urinary excretion of such ketonic bile acids as 3-oxo- Δ^4 bile acids (7 α , 12 α -dihydroxy-3-oxocholesterol-4-en-24-oic acid; Δ^4 -3-one, 12 α -hydroxy-3-oxocholesterol-4,6-dien-24-oic acid; $\Delta^{4,6}$ -3-one).¹ A newly identified inborn error in bile acid synthesis, 3-oxo- Δ^4 -steroid 5 β -reductase (5 β -reductase) deficiency, is associated with idiopathic neonatal hepatitis syndrome.² Because the amount of ketonic bile acids was suspected to be related to the severity of hepatic damage, the concentrations of bile acids were examined in various biological fluids obtained from patients with liver disease and from normal controls.¹⁻⁵ Ketonic bile acids were detected in amniotic fluid collected from healthy pregnant women for studies of bile acid metabolism.³ However, little is known about the amounts of ketonic bile acids present in the biological fluids of neonates and young infants.

Our objective was to evaluate qualitatively and quantitatively 3-oxo- Δ^4 bile acids in urine, meconium, and faeces from normal neonates and young infants using gas chromatography-mass spectrometry (GC-MS) with selected ion monitoring (SIM).

Methods

These healthy neonates (mean gestational age: 40 weeks, range 39–41 weeks) (mean birth-weight: 3050 g, range 2580–3655 g) showed normal development. Spot urine samples were collected from 24 infants (8 boys and 16 girls), six in each of six age groups: 0, 3, and 7 days and 1, 2, and 3 months. The concentration of individual bile acids in urine was corrected for creatinine concentration ($\mu\text{mol}/\text{mmol}$ of creatinine) in each case. Meconium (five specimens) was obtained from healthy neonates (three boys and two girls). Spot faecal samples were obtained from 13 healthy infants (seven boys and six girls) at the following ages: 7 days ($n=6$), 1 month ($n=2$), and 3 months ($n=5$). None of the subjects had a history of, or showed signs of, hepatobiliary or gastrointestinal disease. Infants were breastfed until 2 months of age. Thereafter, the diet consisted of breast milk supplemented with formula or baby food.

We synthesised the following agents, as described before: 1 β , 3 α , 7 α , 12 α -tetrahydroxy-5 β -cholestan-24-oic acid (CA-1 β -ol), 1 β , 3 α , 7 α -trihydroxy-5 β -cholestan-24-oic acid (CDCA-1 β -ol), 3 β -hydroxy-cholesterol-5-en-24-oic acid (Δ^5 -3 β -ol), 7 α , 12 α -dihydroxy-3-oxo-5 β -cholestan-24-oic acid (Δ^4 -3-one), Δ^4 -3-one, $\Delta^{4,6}$ -3-one, and 3 α , 7 α -dihydroxy-24-nor 5 β -cholestan-23-oic acid (Nor-CDCA) (6-8). 3 α , 7 α , 12 α -trihydroxy-5 β -cholestan-24-oic acid (cholic acid; CA), 3 α , 7 α -dihydroxy-5 β -cholestan-24-oic acid (chenodeoxycholic acid; CDCA), 3 α , 6 α , 7 α -trihydroxy-5 β -cholestan-24-oic acid (hyocholic acid; HCA), and 3 α , 7 β -dihydroxy-5 β -cholestan-24-oic acid (ursodeoxycholic acid; UDCA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). 3 α , 12 α -dihydroxy-5 β -cholestan-24-oic acid (deoxycholic acid; DCA), and 3 α -hydroxy-5 β -cholestan-24-oic acid (lithocholic acid; LCA) were obtained from Wako Junyaku (Osaka, Japan) and from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA), respectively.

Each bile acid or mixture of bile acids was converted to the methyl ester by incubation with diazomethane ether (1 ml) at room temperature for 10 minutes. After evaporation, the trimethylsilyl ethers were prepared by heating the residue with *N*-trimethylsilylimidazole (50 μl) (Tokyo Kasei, Tokyo, Japan) in acetonitrile at 38°C for 60 minutes. Excess reagents were evaporated in an N_2 stream, and the residue was dissolved in acetone before GC-MS analysis with SIM.

An internal standard (Nor-CDCA) was added to the samples of urine (1 ml) and lyophilised meconium (1 mg) or faeces (1

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Accepted 4 February 1997

mg). The latter were dissolved in NaOH, 0.1 mol/l (2 ml). All samples were applied to a Bond-Elut C18 cartridge (6 ml) (Analytichem, Harbor City, CA, USA). The cartridge was washed with 5 ml of water and the bile acid conjugates were eluted with 5 ml 90% ethanol. The solvent was evaporated and the residue was treated with 0.05 M sodium acetate buffer (pH 5.6). Each sample was added to 0.75% (v/v) 2-mercaptoethanol (200 μ l) (Sigma), 0.05M EDTA (200 μ l) (Sigma), H₂O (100 μ l), choloylglycine hydrolase (3 units) (Sigma) and sulphatase (50 units) (Sigma) and incubated at 38°C for 16 hours. The sample was then applied to a Bond-Elut C18 cartridge. The cartridge was washed with 5 ml of water, and the bile acid conjugates were eluted with 5 ml 90% ethanol. Free bile acids were extracted with piperidinoxypropyl dextran gel (Shimadzu Corp, Kyoto, Japan), eluted with 5 ml of acetic acid in 90% ethanol (0.1 mol/l), and converted to the methyl ester-trimethylsilyl ethers (50 μ l) for GC-MS analysis. After conversion of the methyl ester-trimethylsilyl ethers (50 μ l) the sample was added to 50 μ l of acetone. Next, 1 μ l of the sample was injected into a splitless injection port of the GC-MS system. The mean recovery of unconjugated bile acids was 97.3% (range 78.6–119.8%); the lowest recovery rate was for 3-oxo- Δ^4 bile acids (78.6%).

After extracting bile acids from urine (using the Bond-Elut C18 cartridge), each sample was applied directly to a column of piperidinoxypropyl dextran gel. Steps in the step-wise elution of unconjugated bile acids and taurine-, glycine-, and sulphate-conjugated bile acids were separated by washing with the buffers, eluted with 5 ml of AcOH-AcOK (pH 6.5) in 90% ethanol (0.3 mol/l), of HCOOH in 90% ethanol (0.2 mol/l) and 1% (NH₄)₂ CO₃ in 70% ethanol, respectively. After adding the internal standard (Nor-CDCA), hydrolysis of the glycine- and taurine-conjugated fractions was carried out using choloylglycine hydrolase. Sulphate conjugated fractions were solvolysed and hydrolysed using sulphatase and choloylglycine hydrolase. The respective fractions were converted first to unconjugated bile acids and then to methyl ester-trimethylsilyl ether derivatives, then analysed by GC-MS with SIM.

GC-MS was performed using a Hitachi-M-80B instrument equipped with a data processing system (Hitachi M-0101; Hitachi Ltd, Tokyo, Japan). A Megabore DB-1 GC capillary column (25 m by 0.53 mm, internal diameter, glass coil; J and W Scientific, Folsom, CA, USA) was used. The temperature of the column oven was programmed to rise from 200 to 290°C at 18°C/minute; the temperature of both the injection port and the detector was 260°C. The flow rate of helium gas was 25 ml/minute. Ionisation energy was set at 70 eV, multiplier voltage at 1400 V, acceleration voltage at 3000 V, source slit at 400 μ m, and collector slit at 300 μ m.

The GC-MS data for bile acid derivatives and related compounds are summarised in table 1. Figure 1 shows a chromatogram obtained using SIM of the characteristic

fragments of the methyl ester-trimethylsilyl ether derivatives of a mixture of reference bile acids.

We obtained calibration curves for the determination of bile acids by plotting the peak area ratios corresponding to the monitored ions for each bile acid and the corresponding internal standard versus the amount of each bile acids. A linear relation ($r > 0.976$ to 0.997) was obtained over a range of 1.5 to 10 ng for each bile acid. Because Δ^4 -3-one and $\Delta^{4,6}$ -3-one had the same retention time (22.2 minutes) and the same fragment ions (267 382 mass:charge ratio; m/z), we could not tell one from the other. We therefore mixed them in equal amounts and expressed the result of the analysis as 3-oxo- Δ^4 bile acids. These chromatographic responses are appropriate for the assay of bile acids in urine, meconium, and faeces with the addition of adequate amounts of internal standard.

Data are reported as mean (SD). One-way ANOVA was used to determine the significance of differences between groups. Comparisons of groups of categorical data were made using Student's t test. A P value of less than 0.05 was accepted as significant.

Table 1 GC-MS data for methyl ester-trimethylsilyl ether derivatives of bile acids

Bile acid	Base peak (m/z)	Fragment ion (m/z)
CA	253*	343 368
CDCA	370*	255 355
DCA	255*	370 460
LCA	215	257 372*
UDCA	460	255 370*
CA-1 β -ol	217*	251 366
CDCA-1 β -ol	217*	368 458
HCA	458	147 369*
Δ^4 -3 β -ol	129	249* 370
Δ^4 -3-one	267*	382 472
Δ^4 -3-one	382	267* 472
$\Delta^{4,6}$ -3-one	267*	382

* Fragment ions used for selected ion monitoring.

Figure 1 Selected ion GC-MS chromatogram of the methyl ester-trimethylsilyl ether derivatives of a mixture of reference bile acids: 1 Nor-CDCA; 2 LCA; 3 DCA; 4 Δ^4 -3 β -ol; 5 CDCA; 6 UDCA; 7 HCA; 8 CA; 9 Δ^4 -3-one; 10 3-oxo- Δ^4 bile acids; 11 CA-1 β -ol; 12 CDCA-1 β -ol.

Results

URINE (TABLE 2)

The highest mean total bile acids (TBA):creatinine ratio (19.5 (24.8) $\mu\text{mol}/\text{mmol}$ of creatinine) was observed in the urine of 7 day old infants. The mean value decreased gradually thereafter. The TBA:creatinine ratio at 3 and 7 days of age significantly exceeded that at 3 months of age ($P < 0.05$) and the TBA:creatinine ratio at 3 and 7 days of age significantly exceeded that on the first day of life ($P < 0.05$).

Urinary 3-oxo- Δ^4 bile acids (Δ^4 -3-one, $\Delta^{4,6}$ -3-one) were detected in the urine at each test period. The percentage of this component in TBA in neonatal urine at birth was significantly higher than that at 3 and 7 days and at 1 and 3 months of age ($P < 0.01$, $P < 0.05$, $P < 0.05$, and $P < 0.05$, respectively). The mean percentage of 3-oxo- Δ^4 bile acids in TBA did not differ significantly after 3 days of age. The concentration of this component in urine did not differ in each period. The mean Δ^4 -3-one:creatinine increased between days 1 and 3 of postnatal life. The mean Δ^4 -3-one:creatinine at 3 or 7 days of age significantly exceeded that at 2 or 3 months of age (all $P < 0.05$). The mean percentage of Δ^4 -3-one to TBA excretion on the first day of life and at 3 days of age significantly exceed that at 1, 2, or 3 months of age ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively). The mean percentage of Δ^4 -3-one to TBA excretion at 7 days of age significantly exceeded that at 1, 2, or 3 months of age ($P < 0.05$, $P < 0.05$, and $P < 0.05$, respectively).

We analysed such polyhydroxylated bile acids as 1 β -hydroxylated bile acids (CA-1 β -ol, CDCA-1 β -ol) and 6 α -hydroxylated bile acid (HCA), and unsaturated bile acid (Δ^5 -3 β -ol). The non-ketonic fetal bile acids (CA-1 β -ol, CDCA-1 β -ol, HCA, Δ^5 -3 β -ol):creatinine ratio in urine remained increased from 3 days to 1 month, then decreased gradually thereafter ($P < 0.05$ vs 3 months). The ratio of non-ketonic fetal bile acids:creatinine in urine on the first day of life was significantly lower than that at 3 days, 7 days, and 1 month of age ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively). The mean percentage of non-ketonic fetal bile acids in TBA was lowest on the first day of life (18.6 (5.9)%) and increased gradually thereafter. These non-ketonic fetal bile acids in urine exhibited a similar percentage at 7 days, 1 month, 2 month, and 3 months of age (7 days,

41.4(16.3)%; 1 month, 44.7(11.4)%; 2 months, 46.0(10.0)%; 3 months, 43.9 (5.4)%).

The urinary concentration of the usual bile acids (CA, CDCA, DCA, LCA) was lowest on the first day of life and increased gradually thereafter ($P < 0.05$ vs 3 days, $P < 0.01$ vs 1 month). The mean percentage of the usual bile acids in TBA on the first day of life and at 3 days of age differed from that at 1, 2, or 3 months of age ($P < 0.001$, $P < 0.05$, and $P < 0.01$, respectively). The mean percentage of the usual bile acids in TBA at 7 days of age differed from that at 1 or 3 months of age ($P < 0.01$ and $P < 0.05$, respectively). The mean UDCA:creatinine ratio in urine on the first day of life and at 3 and 7 days of age was significantly lower than that at 1 month of age ($P < 0.01$). The mean UDCA:creatinine ratio on the first day of life and at 3 days of age was significantly lower than that at 3 months of age ($P < 0.01$). The mean UDCA:creatinine ratio on the first day of life was significantly lower than that at 2 months of age ($P < 0.01$). The mean percentage of UDCA in TBA increased gradually after birth. The mean percentage of UDCA in TBA at 3 days of age was significantly lower than that at 1, 2, and 3 months of age ($P < 0.01$, $P < 0.01$, and $P < 0.001$, respectively). UDCA increased gradually after birth.

The percentage of conjugated bile acids in the urine was analysed in the first week after birth. Of the 3-oxo- Δ^4 bile acids, DCA, LCA, and UDCA, glycine-conjugated bile acids (73, 27.6, 55.8, and 27.1%, respectively) predominated over the other conjugated bile acids. Of the 1 β - (CA-1 β -ol, CDCA-1 β -ol), 6 α -hydroxylated (HCA) bile acid, Δ^4 -3-one and CA, taurine-conjugated bile acids (49.0, 34.4, 49.1, 45.6, and 41.5%, respectively) predominated over the other conjugated bile acids. The sulphate conjugated bile acids predominated over the other conjugated bile acids in Δ^5 -3 β -ol and CDCA (42.0 and 47.5 %, respectively) (fig 2).

MECONIUM AND FAECES (TABLE 3)

The highest value for TBA (mean (SD), 14.2 (6.9) $\mu\text{mol}/\text{g}$) in meconium was observed on the first day of life and the mean value decreased gradually thereafter. The TBA in meconium on the first day of life significantly exceeded that in faeces at 7 days and at 1 or 3 months of age ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively).

Table 2 Mean (SD) bile acids in urine of healthy infants

Bile acid ($\mu\text{mol}/\text{mmol}$ Cr)	0 days (n=6)	3 days (n=6)	7 days (n=6)	1 month (n=6)	2 months (n=6)	3 months (n=6)
Usual bile acids	0.40 (0.19) ^{a,b}	2.69 (2.67)	1.84 (1.24)	3.47 (1.83)	1.96 (0.74) ^c	1.12 (0.37)
% in total bile acids	(14.19 (5.74)) ^{c,d,e}	(13.60 (7.82)) ^{c,d,f}	(17.36 (8.43)) ^{b,g}	(29.10 (5.45))	(23.19 (2.75))	(25.61 (3.86))
Fetal bile acids	0.54 (0.19) ^{b,h,i}	6.26 (3.81) ^g	5.59 (3.55)	6.29 (6.21) ^g	3.87 (1.64)	1.93 (0.70)
% in total bile acids	(18.63 (5.86)) ^{a,b,c,i,k}	(35.95 (19.02))	(41.40 (16.34))	(44.66 (11.42))	(45.96 (10.01))	(43.88 (5.40))
Ursodeoxycholic acid	0.04 (0.02) ^{b,c,f}	0.08 (0.04) ^{b,c,g}	0.14 (0.07) ^b	0.35 (0.18)	0.27 (0.10)	0.29 (0.24)
% in total bile acids	(1.48 (0.92)) ^{c,l,m}	(0.52 (0.33)) ^{b,f,m}	(1.99 (1.77)) ^m	(3.25 (1.47)) ^c	(3.45 (1.36)) ^c	(5.90 (1.98))
3-oxo- Δ^4 bile acid	1.09 (0.88) ^g	6.94 (4.83) ^{c,g,i}	5.63 (8.89) ^{c,g}	0.79 (0.37)	0.54 (0.21)	0.35 (0.12)
% in total bile acids	(31.44 (17.24)) ^{d,k,m}	(34.57 (18.00)) ^{d,k,m}	(22.93 (9.55)) ^{c,g}	(6.81 (1.76))	(6.60 (2.00))	(8.17 (1.68))
3-oxo- Δ^4 bile acids	1.15 (1.05)	3.27 (3.15)	6.26 (13.75)	2.00 (1.38)	1.81 (1.24)	0.81 (0.62)
% in total bile acids	(34.27 (16.22)) ^{g,h,i,l}	(15.36 (9.30))	(16.32 (17.19))	(16.18 (5.33))	(20.80 (11.76))	(16.44 (4.48))
Total bile acids	3.22 (1.86) ^{a,i}	19.24 (8.93) ^g	19.46 (24.81) ^g	12.91 (9.46)	8.45 (2.88)	4.50 (1.93)

Usual bile acids: CA, CDCA, DCA, LCA. Fetal bile acids: CA-1 β -ol, CDCA-1 β -ol, HCA, Δ^5 -3 β -ol. 3-oxo- Δ^4 bile acid: Δ^4 -3-one. 3-oxo- Δ^4 bile acids: Δ^4 -3-one, $\Delta^{4,6}$ -3-one.

^a $P < 0.05$ vs 3 days; ^b $P < 0.01$ vs 1 month; ^c $P < 0.01$ vs 3 months; ^d $P < 0.001$ vs 1 month; ^e $P < 0.05$ vs 2 months; ^f $P < 0.01$ vs 2 months; ^g $P < 0.05$ vs 3 months;

^h $P < 0.01$ vs 3 days; ⁱ $P < 0.05$ vs 7 days; ^j $P < 0.01$ vs 7 days; ^k $P < 0.001$ vs 2 months; ^l $P < 0.05$ vs 1 month; ^m $P < 0.001$ vs 3 months.

Figure 2 Percentage of unconjugated bile acids and of taurine, glycine, and sulphate conjugated bile acids in the urine of neonates in the first week after birth.

The concentration of 3-oxo- Δ^4 bile acids in meconium on the first day of life significantly exceeded that in faeces at 7 days and at 1 or 3 months of age ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively). The mean concentration of this component in faeces did not differ significantly after 7 days of age. The concentration of Δ^1 -3-one did not differ in each period. The mean percentage of Δ^1 -3-one to TBA excretion at birth was significantly lower than those at 7 days and at 1 or 3 months of age ($P < 0.05$, $P < 0.01$, and $P < 0.05$, respectively).

Non-ketonic fetal bile acids were increased in meconium on the first day of life, but decreased gradually thereafter ($P < 0.01$ vs 7 days). The mean percentage of non-ketonic fetal bile acids in TBA did not differ significantly after 7 days of age. These non-ketonic fetal bile acids remained at about the same level at 7 days, 1 and 3 months of age (7 days, 31.7 (11.6)%; 1 month, 39.7 (9.3)%; 3 months, 38.3 (11.1)%).

The concentration of the usual bile acids in meconium was higher on the first day of life ($P < 0.05$ vs 7 days) and remained at about the same level in faeces at 7 days and at 1 and 3 months of age. The mean percentage of the usual bile acids in TBA did not differ in each period.

The mean percentage of UDCA in TBA increased gradually after birth. The mean percentage of UDCA in TBA in meconium on the first day of life was significantly lower than that

in faeces at 7 days and 3 months ($P < 0.05$ and $P < 0.05$, respectively).

Discussion

The serum concentration of TBA in healthy neonates significantly exceeds that in children over 1 year of age, a condition called physiological cholestasis.⁹ The urinary TBA:creatinine ratio was raised in the first week after birth, then decreased gradually. The high concentration of TBA in urine may be attributable to either an enhanced stimulation of the enterohepatic circulation of bile acids or an impaired hepatic clearance or excretion.¹⁰ The highest value for TBA in meconium was in neonates. This value is greatly influenced by events or conditions during pregnancy, such as the presence of biliary bile in the fetal duodenum or the ingestion of amniotic fluid by the fetus.^{10, 11}

Ketonic bile acids are usually considered to result from the bacterial oxidation of primary bile acids.¹² In this study we detected ketonic bile acids early in life. The intestine may be colonised by bacterial flora during the first week.¹³ A high concentration of 3-oxo Δ^4 bile acids in serum or urine has been associated with a deficiency in, or a reduction of, 5 β -reductase activity, the enzyme which catalyses the conversion of 3-oxo- Δ^4 C27 sterol intermediates to 3-oxo-5 β products in the normal pathway for primary bile acid synthesis.² In this study the urinary TBA:creatinine ratio was increased in the first week after birth. The mean percentage of urinary 3-oxo- Δ^4 bile acids in TBA was significantly higher in the first day after birth than at any other age. This condition reflects the normal development of bile acid metabolism, including the initial immaturity of hepatic enzymes, such as 5 β -reductase. After 3 months of age, the urinary TBA:creatinine ratio stabilises as a result of the maturation of liver function and of the enterohepatic circulation.¹⁰ At 3 months of age, we detected a small amount of 3-oxo- Δ^4 bile acids in urine, probably associated with intestinal bacterial flora.¹³ Healthy infants show small amounts of 3-oxo- Δ^4 bile acids in urine, because 3-oxo- Δ^4 bile acids absorbed from the intestine are metabolised by hepatic 5 β -reductase. Two possible sources of 3-oxo- Δ^4 bile acids in urine are bacterial metabolism of cholic acid and side chain oxidation of intermediates in bile acid synthesis. The immaturity of hepatic enzymes

Table 3 Mean (SD) bile acids in faeces of healthy infants

Bile acid ($\mu\text{mol/g}$)	0 days (n=5)	7 days (n=6)	1 month (n=2)	3 months (n=5)
Usual bile acids	4.31 (3.55) ^a	1.35 (0.29)	1.70 (0.17)	1.94 (0.61)
% in total bile acids	(27.64 (9.81))	(21.52 (6.47))	(29.36 (7.75))	(31.36 (13.88))
Fetal bile acids	4.41 (1.94) ^b	1.99 (0.60)	2.39 (0.95)	2.69 (1.29)
% in total bile acids	(32.65 (11.06))	(31.68 (11.35))	(39.68 (9.32))	(38.33 (11.05))
Ursodeoxycholic acid	0.31 (0.09)	0.36 (0.18)	0.23 (0.01)	0.32 (0.09)
% in total bile acids	(2.41 (0.92) ^{a,c})	(5.23 (1.44))	(3.86 (0.54))	(5.42 (3.32))
3-oxo- Δ^4 bile acid	0.93 (0.54)	0.70 (0.32)	0.82 (0.02)	0.66 (0.25)
% in total bile acids	(6.44 (1.47) ^{a,c,d})	(10.59 (3.10))	(14.01 (2.77) ^c)	(9.72 (1.67))
3-oxo- Δ^4 bile acids	4.27 (2.10) ^{a,e,f}	2.29 (1.63)	0.79 (0.23)	1.07 (0.51)
% in total bile acids	(30.86 (16.28))	(30.98 (14.97))	(13.09 (1.73))	(15.17 (5.01))
Total bile acids	14.22 (6.86) ^{b,e,f}	6.69 (2.07)	5.92 (1.00)	6.67 (1.67)

Usual bile acids: CA, CDCA, DCA, LCA. Fetal bile acids: CA-1 β -ol, CDCA-1 β -ol, HCA, Δ^5 -3 β -ol. 3-oxo- Δ^4 bile acid: Δ^1 -3-one. 3-oxo- Δ^4 bile acids: Δ^1 -3-one, Δ^4 -6-3-one.

^a $P < 0.05$ vs 7 days; ^b $P < 0.01$ vs 7 days; ^c $P < 0.05$ vs 3 months; ^d $P < 0.01$ vs 1 month; ^e $P < 0.05$ vs 1 month; ^f $P < 0.01$ vs 3 months.

such as 5 β -reductase is particularly important in early life. Thus our findings of large amounts of unsaturated ketonic bile acids in urine support the results of previous studies.^{4,5,12}

The concentration of 3-oxo- Δ^4 bile acids in faeces on the first of life was significantly higher than at any other age. A high mean percentage of 3-oxo- Δ^4 bile acids in TBA was also present in the meconium of the neonates and in faeces at 7 days of age. The 3-oxo- Δ^4 bile acids found in meconium may originate from amniotic fluid ingested by the fetus during pregnancy.³ Large amounts of 3-oxo- Δ^4 -steroid intermediate may be produced in the intestine by conversion of primary bile acids into secondary bile acids during the neonatal period.¹⁴ 3-oxo- Δ^4 bile acids may be absorbed from the intestine less easily than other bile acids. Analysis of meconium and faeces is difficult because of disintegration of bile acids, such as 3-oxo- Δ^4 bile acids, and/or a low recovery rate, when lyophilised meconium or faeces was dissolved in 0.1 mol/l NaOH in this study. Despite this, we detected large amounts of 3-oxo- Δ^4 bile acids in the meconium and faeces.

Using our method, $\Delta^{4,6}$ -3-one is produced from Δ^4 -3-one during the preparation of the sample. We therefore suggest that 3-oxo-bile acids are derived to methoximes after the addition of an internal standard to sample.

Large amounts of 3-oxo- Δ^1 bile acid were excreted in the urine on the first day of life, and again at 3 and 7 days of age. Elimination of water from the 1 β -hydroxy structure may be the mechanism for formation of Δ^1 bile acids.¹² It seems likely that 3-oxo- Δ^1 bile acids are more readily excreted in the form of hydroxylates. In urine, the percentage of 1 β -hydroxylated bile acids in TBA gradually increased after a decrease in the amount of Δ^1 -3-one. The latter may be converted to CA-1 β -ol.

The usual bile acids and fetal bile acids showed the same pattern of excretion as we had found before.¹⁰ We detected small amounts of DCA and LCA in meconium and urine on the first day of life. These bile acids seemed to be mostly of maternal origin, entering the fetus via placental transfer.¹⁵ Interestingly, we detected UDCA in both urine and faeces. This finding may be related to the use of food supplements.⁴ Their formation is probably linked to mechanisms for bile salt excretion in infants with physiological cholestasis. Bile acids have a pronounced hepatotoxic effect in the fetus unless they are metabolised into more polar compounds. CA and CDCA are transformed by polyhydroxylation into 1 β -hydroxylated bile acids.¹⁶ The process of 6 α -hydroxylation is probably also important in detoxification.¹⁷

The urinary data in the present study showed that most of the conjugated bile acids were glycine conjugates (40% of TBA, 73% of 3-oxo- Δ^4 bile acids), whereas the fetal bile acids were predominantly taurine and sulphate conjugates. Taurine conjugates constituted 17.4 to 49.1% of each fetal bile acid, and sulphate conjugates constituted 31.1 to 42% of each

fetal bile acid. Conjugation increases the polarity of the molecule, thereby facilitating its renal excretion and minimising the membrane damaging potential of the more hydrophobic unconjugated species.¹⁸

In conclusion, this study has shown that healthy newborn infants excrete large amounts of 3-oxo- Δ^4 bile acids in urine and faeces. The presence of large amounts of this urinary component may indicate an immaturity of hepatic 5 β -reductase activity in the first week after birth. After 1 month of age, this urinary component is thought to be derived from 3-oxo- Δ^4 -steroid intermediates in the intestine. The presence of large amounts of this component in meconium may be due to the ingestion of amniotic fluid by the fetus during pregnancy.

This study was supported in part by Grant-in Aid 07670924 for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

- 1 Clayton PT, Patel E, Lawson AM, Carruthers RA, Tanner MS, Strandvik B, *et al.* 3-Oxo- Δ^4 bile acids in liver disease. *Lancet* 1988;i;1283-4.
- 2 Setchell KDR, Suchy FJ, Welsh MB, Zimmer-Nechemias L, Heubi J, Balistreri WF. Δ^4 -3-Oxosteroid 5 β -reductase deficiency described in identical twins with neonatal hepatitis: a new inborn error in bile acid synthesis. *J Clin Invest* 1988;82:2148-57.
- 3 Nakagawa M, Setchell KDR. Bile acid metabolism in early life: studies of amniotic fluid. *J Lipid Res* 1990;31:1089-98.
- 4 Wählén E, Strandvik B. Effects of different formula feeds on the developmental pattern of urinary bile acid excretion in infants. *J Pediatr Gastroenterol Nutr* 1993;18:9-19.
- 5 Strandvik B, Wählén E, Wikström S. The urinary bile acid excretion in healthy premature and full-term infants during the neonatal period. *Scand J Clin Lab Invest* 1994;54:1-10.
- 6 Leppik RA. Improved synthesis of 3-keto, 4-ene-3-keto, and 4,6-diene-3-keto bile acids. *Steroids* 1983;41:475-84.
- 7 Tohma M, Mahara R, Takeshita H, Kurosawa T, Ikegawa S. Synthesis of the 1 β -hydroxylated bile acids, unusual bile acids in human biological fluids. *Chem Pharmacol Bull (Tokyo)* 1986;34:2890-9.
- 8 Tohma M, Takeshita H, Mahara R, Kurosawa T. Determination of 3 β , 12 α -dihydroxy-5-cholesten-24-oic acid and related bile acids in human serum by gas chromatography-mass spectrometry. *J Chromatogr* 1987;421:9-19.
- 9 Suchy FJ, Balistreri WF, Heubi JE, Searcy JE, Levin RS. Physiologic cholestasis: elevation of the primary serum bile acid concentration in normal infants. *Gastroenterology* 1981;80:1037-41.
- 10 Kimura A, Yamakawa R, Ushijima K, Fujisawa T, Kuriya N, Kato H, *et al.* Fetal bile acid metabolism during infancy: analysis of 1 β -hydroxylated bile acids in urine, meconium and feces. *Hepatology* 1994;20:819-24.
- 11 Yamakawa R, Ushijima K, Kimura A, Inokuchi T, Mahara R, Tohma M, *et al.* Origin of bile acids in meconium: analysis of bile acids in biliary bile, amniotic fluid and meconium [In Japanese]. *J Clin Pediatr* 1992;40:59-62.
- 12 Wählén E, Egestad B, Strandvik B, Sjövall J. Ketonic bile acids in urine of infants during the neonatal period. *J Lipid Res* 1989;30:1847-57.
- 13 Rotimi VD, Duerden BI. The development of the bacterial flora in normal neonates. *J Med Microbiol* 1981;14:51-62.
- 14 Coleman JP, White WB, Egestad B, Sjövall J, Hylemon PB. Biosynthesis of a novel bile acid nucleotide and mechanism of 7 α -dehydroxylation by an intestinal Eubacterium species. *J Biol Chem* 1987;262:4701-7.
- 15 Colombo C, Roda A, Roda E, Buscaglia M, Alberto Dell'Agnola C, Filippetti P, *et al.* Correlation between fetal and maternal serum bile acid concentration. *Pediatr Res* 1985;19:227-31.
- 16 Kimura A, Mahara R, Tohma M, Ushijima K, Yuge K, Ono E, *et al.* Unusual 1 β -hydroxylated bilir acids in children with a paucity in interlobular bile ducts. *Clin Chim Acta* 1989;185:215-18.
- 17 Shoda J, Tanaka N, Osuga T, Matsuura K, Miyazaki H. Altered bile acid metabolism in liver disease: concurrent occurrence of C-1 and C-6 hydroxylated bile acid metabolites and their preferential excretion into urine. *J Lipid Res* 1990;31:249-59.
- 18 Scholmerich J, Becher MS, Schmidt K, Schubert R, Kremer B, Feldhaus S, *et al.* Influence of hydroxylation and conjugation of bile salts on their membrane-damaging properties: studies on isolated hepatocytes and lipid membrane vesicles. *Hepatology* 1984;4:661-6.