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Determination of fetal bile acids in biological fluids from neonates by gas chromatography–negative ion chemical ionization mass spectrometry

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Abstract

A method has been developed for microanalysis of fetal bile acids in biological fluids from neonates by capillary gas chromatography–mass spectrometry using negative-ion chemical ionization of pentafluorobenzyl ester–dimethylethylsilyl ether derivatives of bile acids. Calibration curves for the bile acid derivatives are useful over the range 0.1–100 pg and the detection limit for bile acids was 1 fg ($S/N=5$) using isobutane as a reagent gas. Recoveries of the bile acids and their glycine and taurine conjugates from bile acid-free serum and dried blood discs ranged from 92 to 101% and from 93 to 108%, respectively, of the added amounts of their standard samples. The analysis of bile acids on a dried blood disc, meconium and urine from infants, exhibited significant hydroxylation at the 1β-, 2β-, 4β- and 6α-positions of the usual bile acids, cholic and chenodeoxycholic acids, for the urinary or fecal excretion of bile acids in the fetal and neonatal periods. The present method was applied clinically to analyze bile acids on a dried blood disc from neonatal patients with congenital biliary atresia and hyper-bile-acidemia.

Keywords: Bile acids

1. Introduction

Since unusual bile acids having a hydroxyl group at the 1β- or 6α-position have been found in the urine of patients with cholestatic liver disease [1], women in late pregnancy [2] and newborn infants [3], and in human meconium [4], considerable attention has been focused on the biological synthesis and metabolism of these bile acids in connection with fetal development and hepatobiliary diseases of infants [5–9].

In our previous papers, the quantitative determination of 1β- and 6α-hydroxylated bile acids in biological fluids during fetal and neonatal periods has been carried out by chemical synthesis of the standard bile acids and gas chromatography–mass spectrometry (GC–MS) in the electron impact ionization (EI) mode, and a number of fetal bile acids have been found progressively in neonatal urine [10–16]. However, the sensitivity of the determination of these bile acids in neonatal serum is still

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unsatisfactory, because no sufficient amounts of blood samples for analysis are available from immature neonates. Recently, capillary gas chromatography–negative-ion chemical ionization mass spectrometry (GC–NICI-MS) has been applied to the analysis of bile acids providing much higher sensitivity than EI-MS [17], and we reported its use in the analysis of fetal bile acids [18]. The present paper describes a highly sensitive method for the determination of the fetal bile acids, including common bile acids, on a dried blood disc (3 mm I.D.) and biological fluids obtained from neonates by capillary GC–NICI-MS using selected ion monitoring (SIM) for the characteristic fragments of these bile acids. We further describe the application of this method for clinical study of fetal bile acids in neonates with congenital biliary atresia (CBA) and hyper-bile-acidemia (HB) as reported briefly in a preliminary letter [19].

2. Experimental

2.1. Materials and reagents

The following abbreviations of compounds are used: CA=cholic acid (**1**), CDCA=chenodeoxycholic acid (**2**), DCA=deoxycholic acid (**3**), LCA=lithocholic acid (**4**), UDCA=ursodeoxycholic acid (**5**), CA-1β-ol=1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid (**6**), CDCA-1β-ol=1β,3α,7α-trihydroxy-5β-cholanoic acid (**7**), DCA-1β-ol=1β,3α,12α-trihydroxy-5β-cholanoic acid (**8**), CA-2β-ol=2β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid (**9**), CDCA-2β-ol=2β,3α,7α-trihydroxy-5β-cholanoic acid (**10**), CA-4β-ol=3α,4β,7α,12α-tetrahydroxy-5β-cholanoic acid (**11**), CDCA-4β-ol=3α,4β,7α-trihydroxy-5β-cholanoic acid (**12**), CA-6α-ol=3α,6α,7α,12α-tetrahydroxy-5β-cholanoic acid (**13**), CDCA-6α-ol(HCA)=3α,6α,7α-trihydroxy-5β-cholanoic acid (**14**), Δ^5-3β-ol=3β-hydroxy-5-cholenoic acid (**15**), Δ^5-3β,12α-diol=3β,12α-dihydroxy-5-cholenoic acid (**16**), 3β,4β,7α,12α-tetrol=3β,4β,7α,12α-tetrahydroxy-5β-cholanoic acid (**17**).

The usual bile acids (CA, CDCA, DCA and LCA, **1–4** in Fig. 1), UDCA and HCA (**5**, **14**) were purchased from Sigma Chemical (St. Louis, MO, USA). The 1β-, 4β- and 6α-hydroxylated and unsaturated bile acids (**6–8**, **11–14**) and their glycine and taurine conjugates and [2-^{3}H]tauro-CA (663.0 GBq/mmol) were synthesized by methods developed in our laboratory [10,11,20–22].

The 2β-hydroxylated bile acids and the

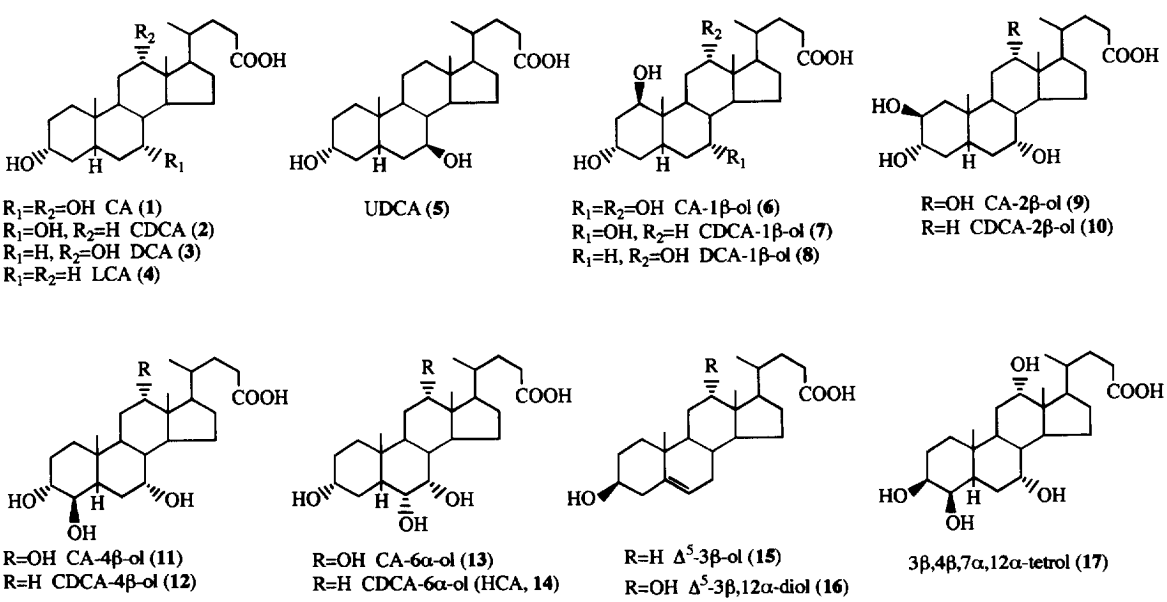

Fig. 1. Structures of bile acids **1–17**.

3β,4β,7α,12α-tetrol (**9**, **10**, **17**) were prepared according to the methods of Haslewood and Tokes [23] and Iida et al. [24]. Dimethylethylsilyl (DMES) imidazole and pentafluorobenzyl (PFB) bromide were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). The Bond Elut C_{18} cartridge was obtained from Varian (Harbor City, CA, USA).

Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was synthesized by the method described by Goto et al. [25].

2.2. Collection of biological samples

Dried blood discs were obtained from normal neonates ($n=17$) and congenital biliary atresia patients ($n=43$) at 5 days after birth by Guthrie's method [26]. These samples were kindly supplied by Dr. Masaru Fukushi (Sapporo Institute of Public Health) and Dr. Akira Matsui (Department of Pediatrics, Jitiika University School of Medicine).

Urine was obtained from normal neonates ($n=14$) at 0–24 days after birth. Meconium was obtained from normal neonates ($n=5$). These samples were kindly supplied by Dr. Hiroshi Nittono (Juntendo University School of Medicine). The samples were stored at −20°C before analysis. Bile acids in urine or meconium were determined as in previous studies [13].

2.3. Preparation of bile acid-free serum and blood

The bile acid-free serum and blood were prepared by the partially modified procedure of the National Committee for Clinical Laboratory Standards, removing bile acids from pooled human serum by charcoal treatment. Erythrocytes from apparently normal heparinized blood were washed with isotonic saline, and then recombined with bile acid-free serum to adjust the hematocrit to 55% reflecting the higher hematocrit in newborns.

2.4. Gas chromatography–mass spectrometry

Capillary GC–MS was carried out using a JMS-AM150 instrument (JEOL, Tokyo, Japan). Isobutane was used as the reagent gas. The ionization energy was 100 eV for the chemical ionization mode. The ion source temperature was 295°C and the transfer line was 300°C. A fused-silica capillary column (30 m×0.25 mm I.D.) bonded with methylsilicon DB-1HT (J&W Scientific, Folsom, CA, USA), was coupled to the mass spectrometer. The carrier gas was helium at a linear velocity of 45 cm/s. Test samples were introduced by splitless injection at 310°C. The column oven temperature was set at 200°C for the initial 2 min and was raised by programming to 280°C at 20°C/min and kept at that temperature for 3 min. At a later stage, the temperature was raised to 320°C at 3°C/min and kept there for 10 min.

2.5. Derivatization of bile acids for GC–MS analysis

Each bile acid or mixture of bile acids (ca. 1 ng) was derivatized to PFB ester with PFB bromide–acetonitrile (1:1, 30 μl) and dimethylethylamine–acetonitrile (1:2, 30 μl) by warming at 40°C for 30 min. After removal of excess reagents under a stream of nitrogen, the DMES ether of the residue was prepared by heating with DMES-imidazole (40 μl) at 60°C for 45 min. Excess reagents were removed on a silica-gel column (20×6 mm I.D.) equilibrated with *n*-hexane–ethyl acetate (3:1). The derivatized bile acids were recovered from the first 4 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 GC–MS system.

2.6. Purification procedure for biological fluids

In the standard procedure, the sample preparation for GC–MS analysis was performed from human biological fluids as follows. A mixture of serum (1–10 μl) and the internal standard (3α,6β,7β,12α-tetrahydroxy-5β-cholanoic acid, 1 ng) was evaporated to dryness on a centrifugal evaporator. The dried blood disc (3 mm I.D.) obtained was placed in methanol (1 ml) and sonicated for 1 h. After the addition of the internal standard (3α,6β,7β,12α-tetrahydroxy-5β-cholanoic acid, 1 ng), the solution was evaporated to dryness on a centrifugal evaporator. The prepared samples were solvolyzed at pH 1 with 2 *M* HCl in ethanol–acetone (1:9, 2 ml) at 40°C for 1 h, then neutralized with 1 *M* NaOH and

evaporated to dryness. The residue was hydrolyzed with 4 *M* NaOH–methanol (1:1, 2 ml) at 80°C for 16 h, and acidified to pH 1 with 6 *M* HCl (0.8 ml) under ice cooling. The free bile acids were extracted with a Bond Elut C_{18} cartridge (3 ml). The cartridge was washed with water (3 ml), and the bile acids were eluted with 90% aqueous ethanol (4 ml). After evaporation of the solvents, the residue was dissolved in 90% aqueous ethanol (2 ml). The solution was applied to a column (20×6 mm I.D.) of PHP-LH-20. After washing with 90% aqueous ethanol (4 ml) to remove neutral compounds, bile acids were eluted with 0.1 *M* acetic acid in 90% aqueous ethanol (5 ml). After evaporation to dryness under reduced pressure, unconjugated bile acids were derivatized to PFB ester–DMES ethers for GC–MS analysis.

2.7. Recovery

A mixture of the synthetic bile acids (1.00 ng each) and the internal standard (1.00 ng) were added to 1 ml of bile acid-free serum. A 10-μl aliquot of the serum sample was assayed by the proposed method. Relative recoveries were calculated for the peak area ratio of the standard mixture of bile acids and the internal standard was used throughout the procedure. Known amounts of bile acids and the internal standard were added to 1 ml of bile acid-free blood. A 20-μl aliquot of the blood was spotted onto a filter paper card. One disc punched from the dried blood sample was used for recovery experiments. Relative recoveries of the bile acids from a dried blood disc were also calculated for the peak-area ratio of the bile acids and the internal standard, as described above. In the experiments with a dried blood disc using [2-^{3}H]tauro-CA (6.5×10^5 dpm), the radioactivities of the sample before and after the extraction procedure were measured by liquid scintillation counting.

3. Results and discussion

3.1. Quantitative analysis of fetal bile acids by GC–NICI-MS

The mass spectra of the PFB ester–DMES ether derivatives of the reference 1β-hydroxylated bile acids, CA-1β-ol and CDCA-1β-ol, are shown in Fig. 2. The characteristic fragment ions at *m/z* 665 and *m/z* 767 indicated loss of a PFB group from the original molecules. The GC–MS data from bile acid derivatives are summarized in Table 1, showing retention times and characteristic fragment ions accompanied by their relative abundance. These data suggest that the selected ion monitoring of the prominent ion at $[M-PFB]^-$ of the bile acid derivatives would permit sensitive determination. Fig. 3 shows a mass chromatogram of PFB ester–DMES ether derivatives of the reference bile acids using SIM of the characteristic fragments in GC–NICI-MS. It can be seen that the simultaneous determination of 17 kinds of bile acids could be achieved in 15–23 min using the SIM in GC–NICI-MS.

Calibration curves for the determination of bile acids were obtained by plotting the peak-area ratios between the monitoring ions for the derivative of each bile acid and the internal standard versus the amounts of the bile acid. Good linearity was found over the range 0.1–100 pg for each bile acid (correlation coefficient, $r>0.999$). Approximately 1 fg of tetrahydroxylated bile acids (CA-1β-ol, CA-2β-ol, CA-4β-ol, CA-6α-ol and 3β,4β,7α,12α-tetrol) could be detected at a signal-to-noise ratio of 5 in the NICI mode using isobutane as a reagent gas (Fig. 4). The developed method offered about 10 000 times the sensitivity (1–10 ng) of the EI method [12].

Bile acids and their conjugates in biological fluids were treated with the following procedure prior to GC–MS for the determination of the total amounts of individual bile acids including their conjugates. Relative recoveries of the bile acids and their conjugates from the bile acid-free serum ranged from 92 to 101% of the added amounts of their standard samples (Table 2). Extraction of the bile acids from a dried blood disc was done by ultrasonic treatment in methanol. The efficiency of the extraction procedure was determined by the addition of [2-^{3}H]tauro-CA to the blood. The recovery of added [2-^{3}H]tauro-CA at this step was 97.9±1.2% ($n=5$, mean±S.D.). Moreover, the whole blood volume in a dried blood disc (3 mm I.D.) calculated from the radioactivity of [2-^{3}H]tauro-CA was 2.4 μl. Relative recoveries of the bile acids and their conjugates from dried blood discs were obtained from 93 to 108% by the above procedure, as shown in Table 2.

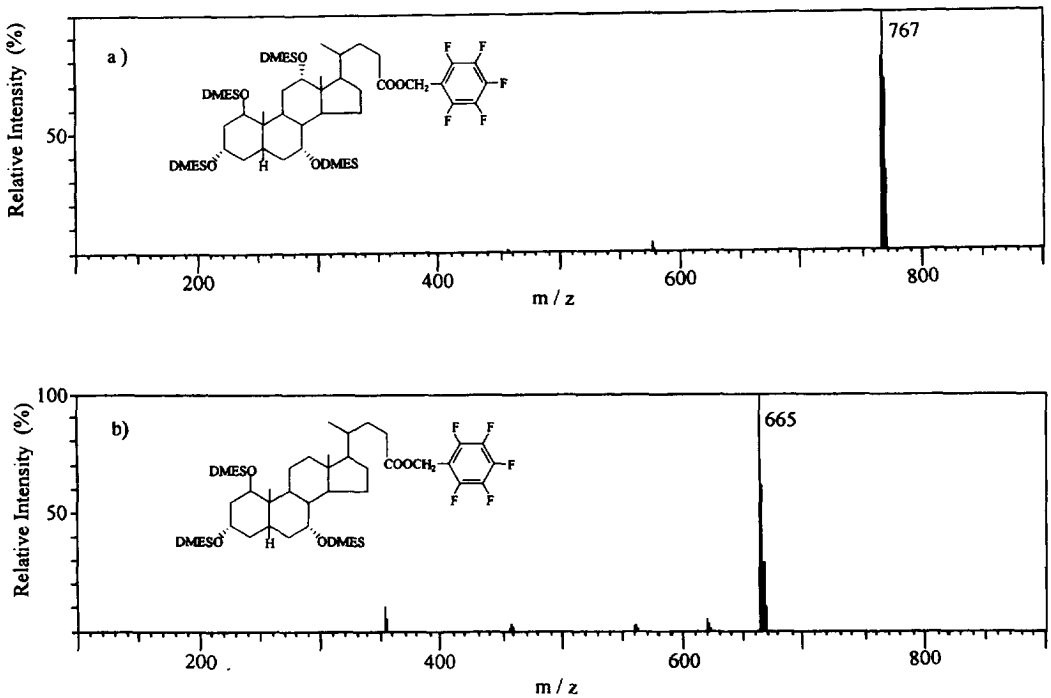

Fig. 2. Mass spectra of the PFB ester–DMES ether derivatives of (a) 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid, and (b) 1β,3α,7α-trihydroxy-5β-cholanoic acid.

Table 1
GC–NICI-MS data of the PFB–DMES derivatives of bile acids

Bile acid	Peak No.	Relative retention time	Base peak $[M-PFB]^-$, m/z	Fragment ions, m/z (Relative intensity, %)
CA	1	1.00[a]	665*	353 (11.4), 457 (2.7)
CDCA	2	0.92	563*	355 (14.6)
DCA	3	0.89	563*	355 (10.1), 459 (2.6)
LCA	4	0.80	461*	357 (21.7)
CA-1β-ol	6	1.12	767*	577 (4.0)
CA-2β-ol	9	1.19	767*	577 (6.4)
CA-4β-ol	11	1.18	767*	577 (10.3)
CA-6α-ol	13	1.10	767*	577 (6.5)
CDCA-1β-ol	7	1.09	665*	353 (7.9), 457 (2.5)
CCDA-2β-ol	10	1.06	665*	353 (8.4)
CDCA-4β-ol	12	1.08	665*	353 (4.5), 370 (2.9)
CDCA-6α-ol (HCA)	14	1.04	665*	353 (3.8), 370 (4.3)
DCA-1β-ol	8	1.03	665	353 (7.6), 475* (10.6)
UDCA	5	0.94	665*	353 (11.5), 459 (2.8)
3β,4β,7α,12α-tetrol	17	1.15	767*	577 (11.6)
Δ^5-3β-ol	15	0.87	459*	355 (11.1)
Δ^5-3β,12α-diol	16	0.95	561*	353 (6.6), 457 (4.8)

* Fragment ions used for selected ion monitoring.
[a] Retention time is 18.57 min.

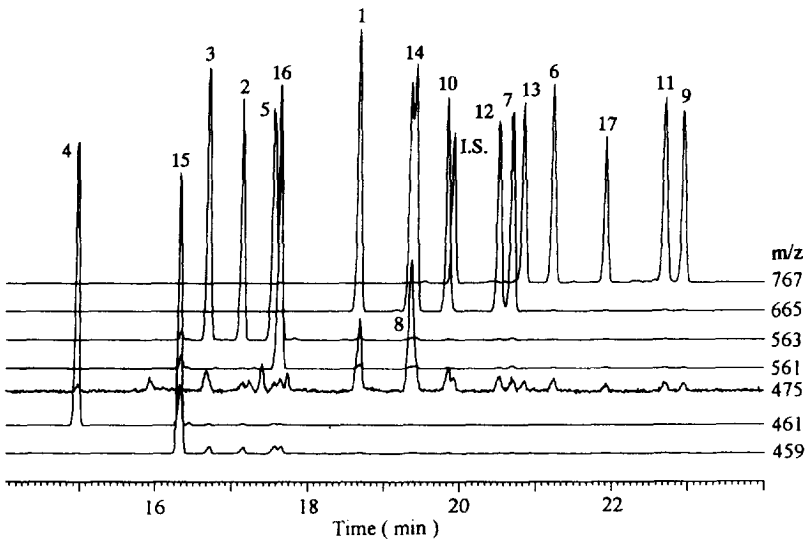

Fig. 3. Mass chromatogram of the reference bile acids as the PFB ester–DMES ether derivatives. 1=CA, 2=CDCA, 3=DCA, 4=LCA, 5=UDCA, 6=CA-1β-ol, 7=CDCA-1β-ol, 8=DCA-1β-ol, 9=CA-2β-ol, 10=CDCA-2β-ol, 11=CA-4β-ol, 12=CDCA-4β-ol, 13=CA-6α-ol, 14=HCA, 15=Δ^5-3β-ol, 16=Δ^5-3β,12α-diol, 17=3β,4β,7α,12α-tetrol, I.S.=internal standard (3α,6β,7β,12α-tetrahydroxy-5β-cholanoic acid).

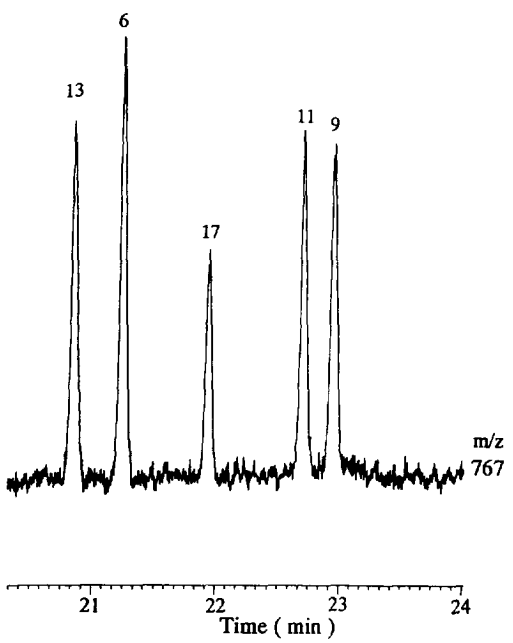

Fig. 4. Detection limit of bile acids (1 fg each) as PFB ester–DMES derivatives. 6=CA-1β-ol, 9=CA-2β-ol, 11=CA-4β-ol, 13=CA-6α-ol, 17=3β,4β,7α,12α-tetrol.

The developed GC–NICI-MS method has enabled determination of fetal bile acids in 1–10 μl of serum and one dried blood disc involving approximately 2.4 μl of blood.

3.2. Determination of the fetal bile acids in blood and other biological fluids from normal neonates

The procedure developed was employed to determine bile acids in a dried blood disc obtained from normal neonates at 5 days after birth, and the SIM chromatogram obtained is shown in Fig. 5. Table 3 summarizes the results and also provides composition of bile acids in urine from neonates and human meconium determined by previous studies [13]. The mean concentration of total bile acids in blood was found to be 7.40 μg/ml, and CA and CDCA were found to be the predominant bile acids, accounting for 38.1% and 18.3% of total bile acids, respectively. Fetal bile acids CA-1β-ol and HCA were found to be minor components accounting for 4.2% and 0.8%, respectively, in neonatal blood, although they had been found to be the predominant bile acids in neonatal urine and meconium, as shown in Table 3. Primary bile acids, CA and CDCA, are synthesized from cholesterol in fetal liver and consti-

Table 2
Recoveries of the bile acids and their conjugates from serum and dried blood disc

Bile acid	Serum		Dried blood disc	
	Added amount (ng)	Relative recovery (%)	Added amount (ng)	Relative recovery (%)
Tauro CA	1.00	95.2±2.9*	1.00	93.2±1.4*
Glyco CDCA	1.00	92.0±6.7	1.00	98.3±8.1
DCA	1.00	95.8±7.4	1.00	98.9±2.3
LCA-3-sulfate	1.00	98.4±1.9	1.00	94.6±4.4
Tauro CA-1β-ol	1.00	94.5±3.2	1.00	95.9±2.5
CDCA-1β-ol	1.00	94.7±7.6	1.00	107.6±7.6
Tauro CA-6α-ol	1.00	95.2±4.7	1.00	95.9±3.2
Glyco HCA	1.00	100.6±6.2	1.00	98.2±7.9

3α,6β,7β,12α-Tetrahydroxy-5β-cholanoic acid (1.00 ng) was added as an internal standard for GC–MS analysis to serum and dried blood disc.
*Mean±S.D. (n=5).

tute the major bile acids in human blood, but they are not necessary in the fetal period for lipolysis and the intestinal absorption of fats. Consequently, it seems reasonable to assume that hydroxylation at the 1β-, 2β-, 4β- and 6α-positions of the primary bile acids in liver might be carried out to facilitate the elimination of excess bile acids in the fetal and neonatal periods.

This consideration is also supported by comparison of the ratios of bile acids in urine to that in blood (U/B) or meconium (M/B). These urinary and fecal excretion ratios (U/B and M/B) were calculated, as was the percentage in urine and meconium for each bile acid in the blood (Table 3). M/B and U/B ratios for the usual bile acids are less than 1, but are higher than 1 in the case of the hydroxylated fetal bile acids. In particular, CA-1β-ol and CA-6α-ol hydroxylated at the 1β- and 6α-positions of CA indicated higher values of U/B (CA-1β-ol: 7.29, CA-6α-ol: 3.50) than the corresponding values of M/B (1.48 and 0.60). Conversely, M/B values for CDCA-1β-ol (3.47) and HCA (36.3) are higher than those of U/B (1.47 and 8.50). These results suggest that the elevated CA was hydroxylated mainly at the

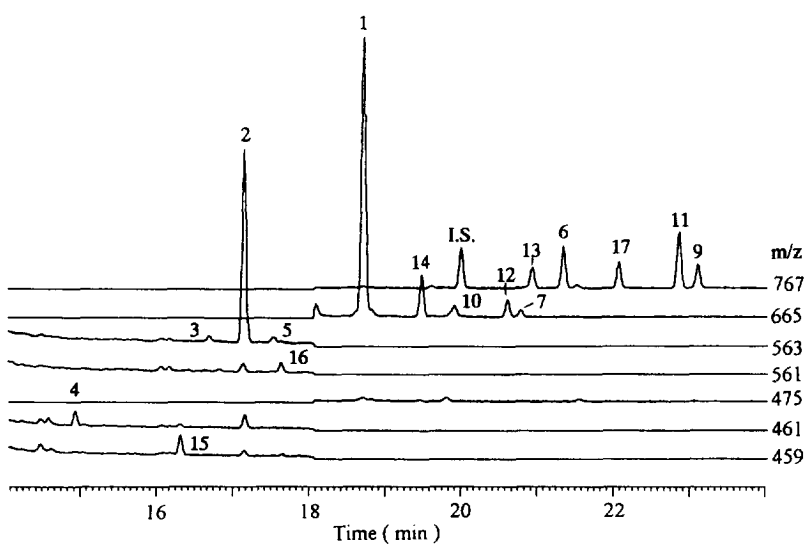

Fig. 5. Selected ion chromatogram of bile acids on a dried blood disc obtained from a normal neonate as PFB ester–DMES ether derivatives. Peak identity is the same as in Fig. 3.

Table 3
Differential compositions of the bile acids in biological fluids from normal neonates

Bile acid	Blood[a] ($n=17$)		Urine ($n=14$)			Meconium ($n=5$)		
	(μg/ml)	(%)	(μg/ml)	(%)	U/B[b]	(μg/mg)	(%)	M/B[c]
CA	2.82±1.98*	38.1	1.22±1.02*	10.8	0.28	0.70±0.50*	27.0	0.71
CDCA	1.36±0.65	18.3	0.15±0.13	1.3	0.07	0.42±0.28	16.2	0.89
DCA	0.10±0.04	1.4	n.d.			0.06±0.02	2.3	1.64
LCA	0.30±0.32	4.1	n.d.			0.16±0.03	6.2	1.51
CA-1β-ol	0.31±0.26	4.2	3.45±2.45	30.6	7.29	0.16±0.09	6.2	1.48
CA-2β-ol	0.30±0.21	4.1	2.03±2.39	18.0	4.39	–		
CA-4β-ol	0.72±0.98	9.7	0.79±0.69	7.0	0.72	–		
CA-6α-ol	0.15±0.15	2.0	0.79±0.67	7.0	3.50	0.03±0.03	1.2	0.60
CDCA-1β-ol	0.14±0.12	1.9	0.32±0.60	2.8	1.47	0.17±0.10	6.6	3.47
CDCA-2β-ol	0.08±0.07	1.1	0.09±0.09	0.8	0.73	–		
CDCA-4β-ol	0.23±0.17	3.1	0.15±0.20	1.3	0.42	–		
CDCA-6α-ol (HCA)	0.06±0.06	0.8	0.77±1.09	6.8	8.50	0.75±0.62	29.0	36.3
DCA-1β-ol	0.11±0.13	1.5	0.01±0.01	0.1	0.07	0.06±0.04	2.3	1.53
UDCA	0.05±0.08	0.7	n.d.			–		
3β,4β,7α,12α-tetorol	0.30±0.29	4.1	1.44±1.55	12.8	3.12	–		
Δ^5-3β-ol	0.31±0.41	4.2	0.01±0.02	0.1	0.02	0.06±0.04	2.3	0.53
Δ^5-3β,12α-diol	0.06±0.05	0.8	0.05±0.04	0.4	0.50	0.02±0.01	0.8	1.00
Total[d] (±S.D.)	7.40±3.12		11.27±7.48			2.59±1.66		

[a] Blood was extracted from dried blood disc.
[b] Ratio of bile acids (%) in urine to blood.
[c] Ratio of bile acids (%) in meconium to blood.
[d] Mean concentration of total bile acids.
n.d.: not detected.
* Mean±S.D.

1β-position in the liver and excreted predominantly in urine, and CDCA was hydroxylated mainly at the 6α-position and excreted in feces and meconium.

Consequently, the concentration of the usual bile acids, CA and CDCA, in blood was maintained at a lower level and homeostasis in neonates was analogous to that in adult humans. It appears that the enzymes concerned with these hydroxylations have different specificities for their substrates, and the reabsorption of these hydroxylated bile acids at the intestine or kidney differs according to the structure of the individual bile acids in the entero-hepatic circulation.

3.3. Determination of the fetal bile acids in dried blood discs obtained from neonatal patients with cholestasis

Congenital biliary atresia (CBA) is a disease in which the bile duct is naturally blocked. The incidence of CBA is said to be about 1:10 000 for live births in Japan [27]. Measurement of the total bile acids or glycocholic acid in dried blood discs from neonates was done earlier for the diagnosis of the disease [28,29]. However, the efficiency of this screening method for CBA is deficient. Recent studies have shown that bile acid metabolism during fetal and neonatal periods is different than for adults [3,4,8], and that the predominant bile acids in the urine of neonates have a hydroxyl group at the 1β or 6α-position of the usual bile acids [10–15]. The method developed to analyze fetal bile acids on a dried blood disc was applied clinically in neonatal patients with CBA and hyper-bile-acidemia (HB), which is a disease caused by cholestasis of unknown origin. Results are given in Table 4. Although the mean concentrations of total bile acids and primary bile acids (CA, CDCA) in CBA and HB were much higher than those in normal subjects, no significant difference between CBA and HB was observed.

There were obvious differences in concentrations of fetal bile acids hydroxylated at the 1β- and 6α-

Table 4
The concentration of bile acids in dried blood discs from neonatal patients with cholestasis

Bile acid	Congenital biliary atresia (n=43)		Hyper-bile-acidemia (n=4)	
	(μg/ml)	(%)	(μg/ml)	(%)
CA	10.98±9.14*	60.2	10.68±4.45*	43.5
CDCA	2.72±1.71	14.9	6.49±0.84	26.4
DCA	0.11±0.06	0.6	0.11±0.03	0.4
LCA	0.16±0.23	0.9	0.45±0.45	1.8
CA-1β-ol	0.54±0.51	3.0	0.53±0.40	2.2
CA-2β-ol	0.22±0.15	1.2	0.03±0.04	0.1
CA-4β-ol	0.40±0.30	2.2	0.13±0.13	0.5
CA-6α-ol	0.24±0.16	1.3	0.26±0.17	1.1
CDCA-1β-ol	0.19±0.14	1.0	2.61±0.68	10.6
CDCA-2β-ol	0.30±0.22	1.6	0.39±0.22	1.6
CDCA-4β-ol	0.55±0.31	3.0	0.78±0.51	3.2
CDCA-6α-ol (HCA)	0.58±0.13	3.2	0.18±0.12	0.7
DCA-1β-ol	0.17±0.20	0.9	1.53±0.96	6.2
UDCA	0.06±0.05	0.3	0.02±0.03	0.1
3β,4β,7α,12α-tetrol	0.39±0.48	2.1	tr.	
Δ^5-3β-ol	0.47±0.26	2.6	0.28±0.13	1.1
Δ^5-3β,12α-diol	0.16±0.13	0.9	0.08±0.03	0.3
Total[a]±S.D.	18.24±11.51		24.57±7.35	

tr.=trace.
* Mean±S.D.
[a]Mean concentration of total bile acids.

positions among these subjects. The mean levels of CDCA-1β-ol in HB (2.61±0.68 μg/ml) were significantly higher than those in CBA (0.19±0.14 μg/ml) and normal subjects (0.14±0.12 μg/ml). The concentrations of HCA in CBA (0.58±0.13 μg/ml) were higher than those in HB (0.18±0.12 μg/ml) and normal subjects (0.06±0.06 μg/ml). Moreover, the standard deviations (S.D.) of the mean concentrations of CDCA-1β-ol and HCA in CBA and HB were smaller than for the other fetal bile acids. These results suggested that the activities of the enzyme concerned with 1β- or 6α-hydroxylation of CDCA differ from the case of these diseases in the neonatal period, and profile analysis of the fetal bile acids in dried blood discs by the present method is useful for the diagnosis of these diseases. Further studies are under way to clarify the physiological and pathophysiological roles of these bile acids in relation to hepatobiliary diseases, and a mild method for analysis of very labile 7α-hydroxy-3-oxo-Δ^4-bile acids under the conditions of hydrolysis and silyl derivatization from urine of patients with 3-oxo-Δ^4-steroid 5β-reductase deficiency [30,31] will be reported in the near future.

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