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Determination of conjugated bile acids in human urine by high-performance liquid chromatography with chemiluminescence detection

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ABSTRACT

A qualitative and quantitative analysis of the conjugated 1β- and 6α-hydroxy bile acids, including common bile acids, in human urine using high-performance liquid chromatography with chemiluminescence detection is described. After extraction of urine with C_{18} silica cartridges, the bile acids were separated into non-conjugated, glycine, taurine and sulphate fractions by ion-exchange chromatography on a lipophilic gel. Solvolysis of the sulphate was carried out by treatment with trifluoroethanol in acetone containing hydrochloric acid, and the liberated amino acid conjugates were fractionated again. The individual bile acids were separated on a reversed-phase C_{18} column (Bile Pak II), with detection by an immobilized 3α-hydroxysteroid dehydrogenase enzyme reactor and chemiluminescence reaction of the generated NADH using 1-methoxy-5-methylphenazinium methylsulphate–isoluminol–microperoxidase system. The assay method showed the detection limits ranging from 8 to 250 pmol for the bile acids tested. Analysis of urine samples obtained from newborns, non-pregnant women and women in late pregnancy showed a large difference in bile acid composition and conjugation mode, suggesting that bile acid metabolism is different during fetal and neonatal periods.

INTRODUCTION

Recently, interest has been focused on bile acid synthesis and metabolism in early life. With newly developed gas chromatographic–mass spectrometric methods a number of trihydroxy and tetrahydroxy bile acids substituted at the 1β or 6α positions have been found in human biological fluids [1–7], especially in the urine of newborns [8], pregnant women [9], and patients with cholestatic liver disease [10–14]. The occurrences of these unusual bile acids have been suggested to be a factor in certain forms of cholestasis in infants and in patients with specific liver disease [15]. A clearer understanding of the conjugation mode of these bile acids is essential to provide a background for understanding the physiological and pathophysiological roles in fetus and neonate.

We have previously shown that the urinary excretion of 1β-hydroxycholic acid (CA-1β-ol) increases in late pregnancy and accounts for more than 50% of total bile acids in newborns; it then decreases with the maturation [16,17]. Accordingly, a qualitative and quantitative analysis of these bile acid conjugates in urine of newborns and normal and pregnant women will serve to

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highlight the marked differences that occur in the synthesis and metabolism of bile acids in the fetal and neonatal periods.

In recent years, high-performance liquid chromatographic (HPLC) methods using 3α-hydroxysteroid dehydrogenase (3α-HSD) with fluorimetric [18,19], electrochemical [20] and chemiluminescent [21] detection have been developed for the direct analysis of conjugated bile acids in human biological fluids. The HPLC method with a chemiluminescence detector is extremely sensitive and allows intact bile acid conjugates to be analysed without prior hydrolysis. In this study, an HPLC method using a 3α-HSD enzyme reactor and isoluminol–microperoxidase chemiluminescent detection was applied to analyse non-conjugated and conjugated bile acids excreted in the urine of newborns, normal women and women in late pregnancy.

EXPERIMENTAL

Materials

Cholic (CA), chenodeoxycholic (CDCA), deoxycholic (DCA), hyocholic (HCA) and lithocholic (LCA) acids were purchased from Sigma (St. Louis, MO, USA), and their glycine and taurine conjugates were prepared by the conventional method. 1β-Hydroxycholic (CA-1β-ol), 1β-hydroxychenodeoxycholic (CDCA-1β-ol), 1β-hydroxydeoxycholic (DCA-1β-ol) and 6α-hydroxycholic (CA-6α-ol) acids, and their glycine and taurine conjugates were synthesized by the cited methods as developed in our laboratory [1,6]. NAD^+ and NADH were obtained from Oriental Yeast (Tokyo, Japan), 1-methoxy-5-methylphenazinium methylsulphate (1-MPMS) from Dojin Kagaku (Kumamoto, Japan), isoluminol (IL) from Tokyo Kasei Kogyo (Tokyo, Japan), and 3α-HSD (grade II) and microperoxidase (m-POD) from Sigma. Amino glass beads, used as the solid phase of the immobilized enzyme, were Amino Propyl-CPG (151 Å) from Electro Nucleonics (Milford, MA, USA). Bond Elut C_{18} cartridges were from Analytichem International (Harbor City, CA, USA). Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was synthesized by the method described previously [22].

To make bile acid stock solutions, each bile acid was dissolved in methanol and made up to 10 μ*M* with methanol.

A 0.3 m*M* NAD^+ solution was prepared by dissolving NAD^+ in 10 m*M* KH_2PO_4 containing 1 m*M* sodium EDTA and adjusted to pH 8.5 with 3 *M* KOH.

The 1-MPMS solution was prepared by dissolving 1-MPMS in water (4 mg/ml).

The IL–m-POD solution contained $2.4 \cdot 10^{-4}$ *M* IL and 10^{-6} *M* m-POD in 0.25 *M* carbonate buffer (pH 10.5).

To make the immobilized 3α-HSD column, 3α-HSD was coupled to amino glass beads (120–200 mesh) by the glutaraldehyde method through Schiff base formation [23] and packed in a stainless-steel column (35 mm × 4.6 mm I.D.), which was placed in an HPLC system.

Apparatus and chromatographic conditions

The apparatus consisted of a Tri-Rotar-VI system (JASCO, Tokyo, Japan), which was equipped with a Soma S-3400 chemiluminescence detector (Soma Spectroscopic, Tokyo, Japan). The separation of individual bile acids was carried out on a Bile Pak II column (5 μm, 125 mm × 4.6 mm I.D.) at 35°C. The mobile phases were acetonitrile–methanol–10 m*M* phosphate buffer (pH 7.3) [(A) 80:16:16 and (B) 40:40:20, v/v]. Isocratic elution was performed with mobile phase A for 10 min, and then with 85% A for 20 min. A linear gradient to 100% B over 10 min was then applied. The flow-rate was 1.0 ml/min. The eluent from the column was mixed with the NAD^+ solution on the immobilized column with the use of a JASCO BIP-1 pump at a flow-rate of 0.6 ml/min, and the generated NADH was mixed with the 1-MPMS solution and IL–m-POD with the use of two pumps (BIP-1 and 880-PU, JASCO) at flow-rates of 0.6 and 1.5 ml/min, respectively. The chemiluminescence was monitored with a flow-cell (cell volume, *ca.* 10 μl).

Determination of Michaelis constant (K_M) and maximum velocity (V_{max})

A solution (1.8 ml) of bile acids (0.8–512 μ*M*)

in 0.01 *M* Tris buffer (pH 9.5) and 0.1 ml of 0.02 *M* Tris–10 m*M* EDTA buffer (pH 7.5) was injected into the quartz cell (1 cm path length) and pre-incubated at 37°C for 3 min. The reaction was initiated by injecting 0.1 ml of 55 m*M* NAD^+ in water. The NADH fluorescence signals were monitored on a Hitachi 650-60 fluorimeter, using an emission wavelength of 568 nm and an excitation wavelength of 336 nm, for *ca.* 2 min. The initial reaction rate was calculated from the slope of the initial linear part of the reaction curve ($\Delta F/\Delta t$). Absolute reaction rates, $\Delta[NADH]/\Delta t(1\ mol^{-1}s^{-1})$, were estimated using an NADH standard. The K_M values were estimated by Lineweaver–Burk plot method.

Procedure for determination of bile acids in human urine

Extraction of bile acids from urine. A urine sample (3 or 5 ml) was adjusted to pH 1 with 2 *M* hydrochloric acid and loaded on a Bond Elut C_{18} cartridge. The cartridge was washed with water (5 ml), and bile acids were eluted with 10% (v/v) chloroform in methanol (4 ml) and evaporated under reduced pressure at 40°C.

Group separation by ion-exchange chromatography on PHP-LH-20. The bile acids extracted from urine were dissolved in 1 ml of 90% ethanol and applied to a PHP-LH-20 column in acetate form (20 mm × 4.6 mm I.D.). After washing with 4 ml of 90% ethanol, the free acids, the glycine, taurine conjugates, and the sulphates were fractionated by stepwise elution with 4-ml volumes of 0.1 *M* acetic acid, 0.2 *M* formic acid, 0.3 *M* acetic acid–potassium acetate (pH 6.5) in 90% ethanol, and 1% ammonium carbonate in 70% ethanol. The sulphate fraction was evaporated to dryness under reduced pressure, and the residue was redissolved in water (2 ml) and applied to a Bond Elut C_{18} cartridge to remove inorganic salts. After washing with water (5 ml), the bile acids were eluted with methanol (4 ml) and then reevaporated under reduced pressure. The residue was submitted to solvolysis in 4 ml of acetone–trifluoroethanol (9:1, v/v) containing 80 μl of 2 *M* hydrochloric acid at 37°C for 1 h. The liberated bile acid conjugates were fractionated again into their mode of conjugation by the method described above. To all six fractions, 5β-pregnane-3α,11α,20α-triol (650 pmol) was added as an internal standard (I.S.). The resulting mixture was evaporated under reduced pressure, and the residue was dissolved in methanol (100 μl) and then applied to the HPLC analysis.

Optimization of solvolysis using standard bile acid sulphates

A methanolic solution of the I.S. (1 mg/ml, 0.5 ml) was added to an aqueous solution (0.5 ml) of standard 3-sulphates of CA, CDCA, DCA and LCA (400–500 μ*M*). After removal of the solvent, the residue was incubated at 37°C in the following reagents: (A) 4 ml of a mixture of acetone–ethanol (9:1, v/v) containing 80 μl of 2 *M* hydrochloric acid; (B) 10 ml of a mixture of acetone–50% trifluoroacetic acid (9:1, v/v); (C) 4 ml of a mixture of acetone–trifluoroethanol (9:1, v/v) containing 80 μl of 2 *M* hydrochloric acid. An aliquot from each incubation mixture was taken, and the solvent was evaporated to dryness under nitrogen. The liberated bile acid was dissolved in methanol (0.5 ml), and an aliquot of 10 μl was used in the HPLC analysis.

Recovery test

A synthetic mixture of each bile acid (1–2 nmol) was added to 5 ml of pooled urine and then assayed by the proposed method. Recoveries were calculated against a standard mixture of bile acids carried through the procedure.

RESULTS AND DISCUSSION

Determination of kinetic parameters for 3α-HSD

In the present study, the assay method utilizes an immobilized 3α-HSD enzyme column reactor in a flow mode that converts the bile acids into the corresponding 3-oxo bile acids, in the presence of NAD^+, after chromatographic separation on an ODS column. In the enzyme column reactor NADH is generated and monitored by the chemiluminescence reaction with 1-MPMS–IL–m-POD. Therefore, the affinity of the substrate for the enzyme affects the detectability of

individual bile acids. Accordingly, the kinetics of each bile acid for 3α-HSD were systematically studied. We determined the kinetic parameters by fluorometrically monitoring the NADH generated by enzymic reaction according to the similar method reported previously [24]. As can be seen in Table I, it is apparent that the positions and the number of hydroxyl groups differentiate the K_M values and have an important effect on the rate of the enzymic reaction. The bile acids with a hydroxyl group at the 1β position of CA, CDCA and DCA showed the higher K_M values than the corresponding common bile acids. This may be due to the lower affinity for the enzyme of the 1β-hydroxyl group, which is close to the 3α-position. In addition, the K_M values increased as the number of hydroxyl groups increased. Similar observations concerning both 3α-HSD and 7α-HSD have been reported previously [25–27]. The lowest affinity as substrate for 3α-HSD is shown by CA-1β-ol, which is the most hydrophilic of all, with four hydroxyl gorups. No significant differences of K_M and V_{max} were observed between free, glyco and tauro conjugates of the individual bile acids, as they have same numbers of hydroxyl groups, in the same positions. These findings suggest that the 1β-hydroxylated bile acids are somewhat less readily detected by the proposed HPLC method than the common bile acids.

Chromatographic separation

Various solvent systems and columns for the separation of bile acids and their conjugates have been reported. The separation of bile acids should be carried out under nearly neutral conditions when enzymic reactions are used in the detection system. The chromatographic conditions were examined in order to obtain complete separation of non-conjugated acids and their glycine and taurine conjugates. From the results, the gradient elution mode and the mobile phase acetonitrile–methanol–10 m*M* phosphate buffer (pH 7.3) were selected.

A typical chromatogram of tauro conjugates is shown in Fig. 1. All the bile acids were completely separated within 75 min. Previous studies have given similar results at neutral pH. Close similarities in chromatographic behaviour were observed between free acids and glyco and tauro conjugates (Table II).

The elution order of bile acids with different numbers of hydroxyl groups on the steroid nucleus was found to be identical with that previously reported [21]. Of particular interest is the finding that 1β-hydroxy derivatives of CA and CDCA were eluted faster than their corresponding 6α-hydroxy derivatives. This may be due the higher polarity of 1β-hydroxylated bile acids compared with that of 6α-hydroxylated bile

TABLE I

KINETIC PARAMETERS OF 3α-HSD FOR BILE ACIDS

Values for K_M (μ*M*) and V_{max} (μmol/min/mg) were determined for the negative intercept and slope from the least-squares regression line at each substrate concentration in the Lineweaver–Burk plots.

Bile acid	Free		Glycine		Taurine	
	K_M	V_{max}	K_M	V_{max}	K_M	V_{max}
CA-1β-ol	151.5	2.5	33.3	0.4	133.3	8.3
CDCA-1β-ol	109.9	3.0	80.0	0.7	14.3	5.6
DCA-1β-ol	16.0	2.0	41.7	7.4	6.8	3.3
CA-6α-ol	20.0	3.8				
HCA	9.8	2.0	4.6	5.9	20.0	8.3
CA	13.2	9.1	5.9	7.7	18.9	5.5
CDCA	8.3	15.4	1.9	6.3	3.2	5.9
DCA	2.5	3.5	1.8	2.7	7.8	2.6
LCA	1.4	2.5	2.9	3.6	12.1	1.4

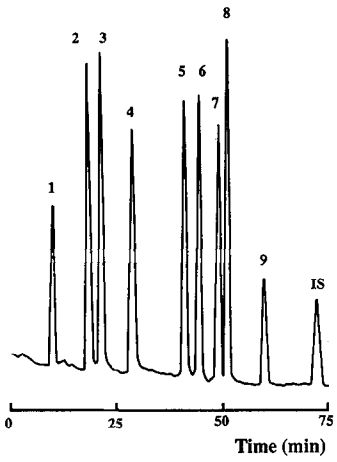

Fig. 1. Typical chromatogram of standard taurine-conjugated bile acids. Peaks: 1 = CA-1β-ol; 2 = CDCA-1β-ol; 3 = CA-6α-ol; 4 = DCA-1β-ol; 5 = HCA; 6 = CA; 7 = CDCA; 8 = DCA; 9 = LCA; IS = internal standard.

acids, where the two hydroxyl groups at the 6α and 7α positions are located so that the protons can form hydrogen bonds.

Calibration graphs were obtained by injecting a standard mixture of each bile acid. As shown in Table III, the detection limits of common bile acids were *ca.* 10 pmol (signal-to-noise ratio = 5), which were comparable with those reported previously [21]. In contrast, the 1β- and 6α-hydroxylated bile acids, especially with CA-1β-ol, showed lower detection limits because of their lower affinity for the enzyme, as suggested by the kinetic study.

TABLE II

RELATIVE CAPACITY FACTORS OF BILE ACIDS

The figures are k' values relative to 5β-pregnane-3α,11α,20α-triol (k' = 34.2).

Bile acid	Free acid	Glycine	Taurine
CA-1β-ol	0.075	0.098	0.122
CDCA-1β-ol	0.161	0.212	0.243
DCA-1β-ol	0.223	0.253	0.284
CA-6α-ol	0.283	0.347	0.399
HCA	0.533	0.560	0.567
CA	0.612	0.617	0.614
CDCA	0.677	0.679	0.678
DCA	0.698	0.700	0.699
LCA	0.817	0.815	0.817

TABLE III

DETECTION LIMITS OF BILE ACIDS

Values are pmol (signal-to-noise ratio = 5).

Bile acid	Free acid	Glycine	Taurine
CA-1β-ol	250	230	200
CDCA-1β-ol	75	69	70
DCA-1β-ol	18	22	24
CA-6α-ol	13	18	23
HCA	9	9	10
CA	15	9	11
CDCA	12	10	11
DCA	10	8	11
LCA	10	9	11

Extraction and group separation

The liquid–solid extraction procedure, which has been used successfully for many years for the isolation and concentration of bile acids from human biological fluids, was also used in this study. For the simultaneous determination of bile acids in urine, fractionation into the free acids and the glyco, tauro and sulpho conjugates was required prior to HPLC. This was done by ion-exchange chromatography on PHP-LH-20, according to the method of Goto *et al.* [22].

For the determination of sulphated bile acids, a solvolytic step is essential. Current chemical solvolytic methods require a relatively long time for complete solvolysis, and occasionally lead to the formation of the ester derivative of carboxylic side-chain. In recent years, a new solvolysis procedure has been developed by Hirano *et al.* [28]. It is fast and simple, but we have also attempted to developed a new procedure by using trifluoroethanol as a nucleophilic agent in acetone. A comparative study was therefore undertaken to estimate the optimal condition by the proposed and two established methods [29,30] used for GC analysis. Fig. 2 shows that, in comparison with the methods using hydrochloric acid and trifluoroacetic acid, our method using trifluoroethanol shortened the solvolysis time and did not lead to

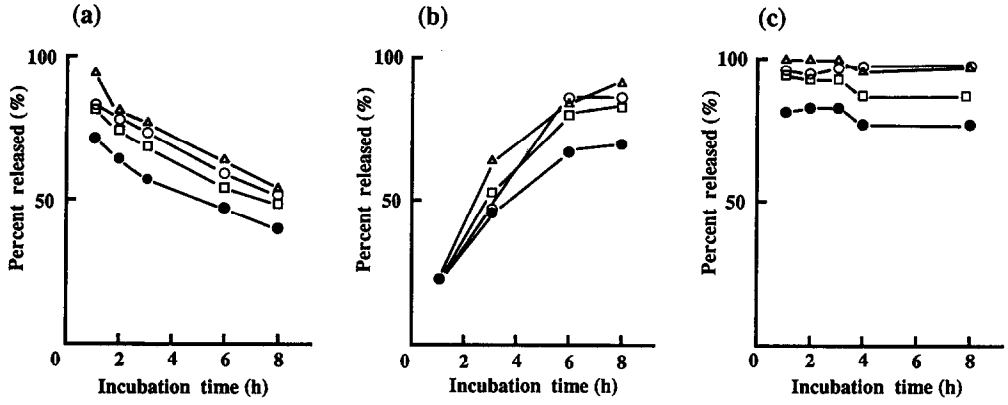

Fig. 2. Effects of reaction conditions on the solvolysis of sulphated bile acids: (a) acetone–ethanol (9:1, v/v) + 2 *M* HCl; (b) acetone–50% CF_3COOH (9:1, v/v); (c) acetone–CF_3CH_2OH (9:1, v/v) + 2 *M* HCl. Symbols: (○) CA; (△) CDCA; (□) DCA; (●) LCA.

formation of the undesired ester derivative. In the present study, we did not test the solvolysis procedure with sulphated 1β- and 6α-hydroxy bile acids because of the unavailability of authentic samples. In our preliminary experiment, satisfactory recoveries of tauro CA-6α-ol and tauro HCA were obtained after solvolysis and extraction, which suggests that the acetonides were not formed from the 6α,7α-structure. Accordingly, solvolysis was carried out by treatment with trifluoroethanol and hydrochloric acid in acetone, and the liberated bile acids were fractionated again into the free acids and the glyco and tauro-conjugates. Known amounts of representative non-conjugated and conjugated bile acids were added to the pooled urine, and their recovery rates were estimated after extraction and fractionation by the analysis with HPLC system as described above. As listed in Table IV, each bile acid was recovered at a rate of more than 82%.

Application to human urine samples

Application of the HPLC method to the analysis of non-conjugated and conjugated bile acids in urine was carried out for newborns at three days after birth, and for healthy non-pregnant and pregnant women. The results are given in Tables V–VII, and a typical chromatogram of taurine conjugates in newborn urine is shown in Fig. 3. A conspicuous difference was found between infant and adult samples. In the newborns, the predominant bile acids identified were 1β-

TABLE IV

RECOVERIES OF CONJUGATED BILE ACIDS ADDED TO POOLED URINE

Values are mean ± S.D. (%), obtained from three experiments. N.D. = not determined.

Bile acid	Glycine	Taurine	Sulphate
CA-1β-ol	97.8 ± 3.5	96.8 ± 1.4	N.D.
CDCA-1β-ol	89.2 ± 0.5	100.3 ± 12.5	N.D.
DCA-1β-ol	91.1 ± 3.6	92.8 ± 15.3	N.D.
CA-6α-ol	80.5 ± 3.0	90.8 ± 6.8	N.D.
HCA	91.0 ± 9.6	108.3 ± 12.7	N.D.
CA	98.7 ± 8.1	91.7 ± 8.5	101.5 ± 5.7
CDCA	89.7 ± 6.0	97.3 ± 1.1	105.0 ± 4.2
DCA	95.5 ± 8.5	96.3 ± 12.7	94.8 ± 4.5
LCA	84.7 ± 7.5	99.7 ± 5.5	82.3 ± 7.1

TABLE V

URINARY LEVELS OF BILE ACIDS IN NEONATES

Values are ng/ml (%) (n = 3). N.D. = not detected.

Bile acid	Free acid	Glycine	Taurine	Total
CA-1β-ol	N.D.	N.D.	1430 (100)[a]	1430 (33)[b]
CDCA-1β-ol	N.D.	N.D.	664 (100)	664 (15)
DCA-1β-ol	N.D.	N.D.	N.D.	
CA-6α-ol	N.D.	98 (17)	478 (83)	576 (13)
HCA	N.D.	N.D.	760 (100)	760 (17)
CA	22 (2)	74 (8)	811 (90)	907 (21)
CDCA	37 (100)	N.D.	N.D.	37 (1)
DCA	N.D.	N.D.	N.D.	
LCA	N.D.	N.D.	N.D.	

[a] Values in parentheses are expressed as a percentage of the conjugates in individual bile acids.
[b] Values in parentheses are expressed as a percentage of the individual bile acids in the total bile acids.

and 6α-hydroxylated bile acids of CA and CDCA, accounting for *ca.* 80% of the total urinary bile acids. These bile acids have been also detected in the meconium of newborns [31]. The principal secondary bile acids of adult humans, DCA and LCA, were not present, and the primary bile acids, CA and CDCA, accounted for 21 and 1%, respectively. The absence of secondary bile acids may be explained by rapid hydroxylation to other metabolites, the novel 4β- and 6α-hydroxylated bile acids, previously found in fetal gall bladder bile [5]. Particularly noteworthy was the fact that all these bile acids were preferentially conjugated with taurine. Sulphated bile acids were not found, or when detected were in low and variable amounts. These findings are in agreement with a previous report describing the total bile acids in the urine of newborns [8] and in fetal gall bladder bile [4,5].

In contrast, the excretion patterns of non-preg-

TABLE VI

URINARY LEVELS OF BILE ACIDS IN NON-PREGNANT WOMEN

Values are ng/ml (%) (n = 3). N.D. = not detected.

Bile acid	Free acid	Glycine	Taurine	Sulphates			Total
				Free acid	Glycine	Taurine	
CA-1β-ol	N.D.	N.D.	N.D.	N.D.	Trace	N.D.	
CDCA-1β-ol	N.D.	41 (100)[a]	N.D.	N.D.	N.D.	N.D.	41 (2)[b]
DCA-1β-ol	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	
CA-6α-ol	115 (100)	N.D.	N.D.	N.D.	N.D.	N.D.	115 (4)
HCA	N.D.	27 (13)	100 (51)	N.D.	76 (36)	N.D.	203 (7)
CA	212 (12)	1395 (76)	72 (4)	N.D.	113 (6)	32 (2)	1824 (65)
CDCA	N.D.	22 (13)	10 (6)	N.D.	116 (69)	19 (12)	167 (6)
DCA	8 (3)	33 (10)	12 (4)	26 (8)	187 (58)	53 (17)	320 (11)
LCA	N.D.	N.D.	N.D.	N.D.	79 (57)	60 (43)	139 (5)
Total							2809 (100)

[a,b] See Table V.

TABLE VII

URINARY LEVELS OF BILE ACIDS IN PREGNANT WOMEN

Values are ng/ml (%) (n = 3). N.D. = not detected.

Bile acid	Free acid	Glycine	Taurine	Sulphates			Total
				Free acid	Glycine	Taurine	
CA-1β-ol	Trace	N.D.	105 (100)[a]	N.D.	N.D.	N.D.	105 (2)[b]
CDCA-1β-ol	N.D.	N.D.	118 (100)	N.D.	N.D.	N.D.	118 (2)
DCA-1β-ol	13 (3)	460 (97)	N.D.	N.D.	N.D.	N.D.	476 (10)
CA-6α-ol	N.D.	Trace	49 (100)	N.D.	N.D.	N.D.	49 (1)
HCA	N.D.	755 (78)	N.D.	N.D.	209 (22)	Trace	964 (20)
CA	123 (5)	1711 (71)	47 (2)	Trace	350 (14)	198 (8)	2429 (50)
CDCA	Trace	Trace	N.D.	Trace	152 (100)	Trace	152 (3)
DCA	Trace	Trace	14 (4)	30 (8)	283 (71)	69 (17)	396 (8)
LCA	Trace	Trace	N.D.	19 (12)	63 (39)	78 (49)	160 (3)
Total							4849 (100)

[a,b] See Table V.

nant women showed a predominance of primary and secondary bile acids, which were conjugated preferentially with glycine. Our data are in agreement with the previous finding that glycine-conjugated bile acids in the urine of healthy adults account for up to 50% of the total bile acids [32]. A relatively large proportion of sulpho conjugates of dihydroxy and monohydroxy bile acids were found, showing an increased water solubility by sulphation of less polar bile acids for elimination from the enterohepatic circulation. The percentage of sulphated bile acids was somewhat lower than those previously reported with normal subjects [32] and patients with liver disease [33–36], probably reflecting differences in methodology. The reported excretion of sulphated mono- and dihydroxy bile acids could be confirmed. The proportions of 1β- and 6α-hydroxylated bile acids were significantly decreased, representing only 13% of total bile acids. Whereas the excretion of HCA and DCA-1β-ol has been observed in healthy subjects [9,32], the presence of CDCA-1β-ol in normal women has not been reported.

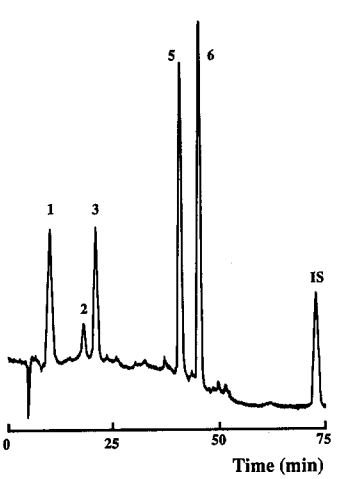

Fig. 3. Typical chromatogram of taurine-conjugated bile acids in the urine of newborn infants. For abbreviations, see Fig. 1.

On the other hand, women in their 35th week of pregnancy exhibited an increased excretion of 1β- and 6α-hydroxycholic acids (35% of total bile acids) compared with non-pregnant women though the common bile acids were the major components. The occurrence of these tetrahydroxy bile acids has been reported in the urine of normal pregnant women [9] and in pregnant women with intrahepatic cholestasis [9,32]. A conspicuous feature is the new finding that

CA-1β-ol, CDCA-1β-ol and CA-6α-ol were conjugated only with taurine, whereas DCA-1β-ol and HCA were conjugated with glycine. The glyco conjugate concentration of common bile acids exceeded that of the tauro conjugates both in non-sulphated and sulphated fractions, which resemble those of normal women.

As shown in an earlier study, the predominant mode of conjugation by the human fetus and neonate is amidation with taurine [37]. As the infant matures, glycine conjugates appear, and are the predominant mode by the period of adult life. Our data markedly contrast the conjugation pattern for newborn urine, where amidation with taurine is the predominant mode of conjugation. This conjugation pattern reflects the increased availability of taurine in the fetus and, in particular, the high concentration of taurine in the fetal liver [38]. CA-1β-ol, CDCA-1β-ol and CA-6α-ol excreted in the urine of pregnant women might be secreted by the fetus. This consideration is supported by the evidence that 1β-and 6α-hydroxylation occur *in vivo* and *in vitro* in the human fetal liver [11,39]. Although the physiological significance of 1β- and 6α-hydroxylated bile acids found in newborns is obscure, metabolic conversion of CA and CDCA into other bile acids via additional hydroxylation *e.g.* at the C-1 or C-6 position, may reflect the hepatic metabolism at a specific point during development. Further studies are under way to clarify the physiological and pathophysiological roles of these bile acids in relation to hepatobiliary disease.

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