Measurement of Conjugated 1β-Hydroxycholic Acid in Urine of Newborns by Specific Radioimmunoassay

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Anti-tauro 1β-hydroxycholic acid antisera were prepared by immunizing rabbits with *N*-(1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oyl)glycine bovine serum albumin conjugate. The immunoglobulin G fraction was obtained by ammonium sulfate precipitation, followed by diethylaminoethyl cellulose column chromatography. The antibody was characterized using [2-^{3}H]tauro 1β-hydroxycholic acid which has a high affinity ($K_a = 1.09 \times 10^9\ M^{-1}$) and reasonable specificity. Cross-reactivity for glyco 1β-hydroxycholic acid was 100% and those for other 1β-hydroxylated bile acids ranged from 4.32 to 29.6%. Concentrations of conjugated 1β-hydroxycholic acid in urine of newborns at 0—20 d after birth were determined by radioimmunoassay to be significant (0.2—11.1 μg/ml), exhibiting a tendency to increase during the 20 d after birth.

Keywords radioimmunoassay; tauro 1β-hydroxycholic acid; glyco 1β-hydroxycholic acid; bile acid; [2-^{3}H]tauro 1β-hydroxycholic acid

Physiologic cholestasis has been defined during the first months of life,[1-3] but the exact mechanisms responsible are not completely understood. As a result of physiologic cholestasis, the newborn infant has a tendency to develop a true neonatal cholestasis when subjected to such stresses as sepsis, hypoxia and total parenteral nutrition.[3] Abnormalities in bile acid metabolism have been suggested to be a factor in the development of certain forms of cholestasis in newborns.[4]

1β-Hydroxycholic acid (CA-1β-ol), which may be a metabolite of cholic acid, has been found in the urine of healthy adults[5] and infants,[6] in meconium,[7-12] in amniotic fluid[13,14] and in fetal gallbladder bile.[15] Of particular interest is the urinary excretion of this bile acid, which is preferentially conjugated with taurine and is the principal ingredient of urinary bile acid in newborn babies.[16,17] A recent study has also shown that the proportion of this bile acid is greater in urine than in the serum and liver tissue in patients with cholestasis.[18] The 1β-hydroxylation has been demonstrated to occur *in vivo* by identification of radioactive 1β-hydroxylated bile acid in the urine of patients administered ^{14}C-labeled cholic acid,[19] and was confirmed to be caused *in vitro* by human fetal liver microsomes.[20] These observations have led to the speculation that the formation of tauro 1β-hydroxycholic acid (tauro-CA-1β-ol) is most likely an important excretion mechanism of cholic acid in the fetus. Therefore, cholestatic disease forces cholic acid to a more polar area and promotes clearance from the body.[21]

In order to clearly understand this bile acid synthesis and metabolism in early life and in patients with specific disease, it is essential to provide a background for determination of the physiological and pathophysiological role of this bile acid during development. Accordingly, measurement of the urinary concentration of tauro-CA-1β-ol is of considerable interest.

Recently, gas chromatography-mass spectrometry and high-performance liquid chromatography have been widely used for analyzing bile acids in biological fluids. These methods, however, are tedious and time-consuming, and require instruments. Radioimmunoassay (RIA) is useful method for routine analysis, and this paper deals with the development of an RIA of tauro-CA-1β-ol and its application to the measurement of this substance in the urine of newborns.

Materials and Methods

Chemicals and Reagents [2-^{3}H(N)]Taurine (743.7 GBq/mmol) was purchased from New England Nuclear Co. (U.S.A.). Bovine serum albumin (BSA, fraction V) and complete Freund's adjuvant were obtained from Sigma Chemical Co. (U.S.A.) and Iatron Laboratories (Tokyo), respectively. Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was synthesized according to the method previously reported.[22] The reference bile acids were either synthesized in our laboratory or were obtained commercially and purified to homogeneity by chromatography and/or recrystallization. All organic solvents and chemicals were of an analytical reagent grade.

Analyses Melting point was determined with a Mitamura micro hot-stage apparatus, and was uncorrected. Optical rotation was measured with a Union Giken-201 polarimeter. Proton nuclear magnetic resonance (^{1}H-NMR) was recorded with a Hitachi model R-40 spectrometer (90 MHz). Chemical shifts are given as the δ value with tetramethylsilane as the internal standard (s, singlet; d, doublet; m, multiplet).

Preparation of Immunogen A solution of glyco 1β-hydroxycholic acid (glyco-CA-1β-ol, 0.03 mmol) in dry dimethylformamide (DMF, 0.3 ml) was treated with tri-*n*-butylamine (0.013 ml) and isobutyl chlorocarbonate (0.006 ml) at 10 °C, and the entire mixture was stirred for 30 min. Then, BSA (40 mg) in H_2O (3.1 ml)–DMF (0.6 ml)–1 M NaOH (0.034 ml) was added under ice cooling and the mixture was stirred for 3 h. The resulting solution was dialyzed against cold running water for 2 d, and the turbid protein solution was brought to pH 4.5 with 0.1 M HCl. After standing at 4 °C overnight, the suspension was centrifuged at 3000 rpm for 15 min. The precipitate was dissolved in 5% $NaHCO_3$ and dialyzed in the manner described above. Lyophilization of the solution afforded the glyco-CA-1β-ol–BSA conjugate (37 mg) as a fluffy powder.

Immunization and Blood Collection Two female domestic albino rabbits (2—2.5 kg) were used for immunization with the conjugate. The immunogen (1 mg) was dissolved shortly before injection in 0.5 ml of sterile isotonic saline and emulsified with 0.5 ml of complete Freund's adjuvant. This emulsion was injected subcutaneously at multiple sites over the entire back. This procedure was repeated at one week intervals for a one month period and then once a month. Blood was obtained from the ear vein one week after the booster injection. The sera were separated by centrifugation at 3000 rpm and stored at −20 °C.

Preparation of Immunoglobulin G (IgG) Antiserum (10 ml) was diluted with an equal volume of 1/15 M phosphate buffer (pH 7.0) containing 0.9% NaCl and fractionated with a 40 percent saturation of $(NH_4)_2SO_4$. The precipitates were collected by centrifugation, dissolved in 10 mM phosphate buffer (pH 7.6) and dialyzed against three changes of the same buffer for 2 d at 4 °C. The dialyzed sample was chromatographed on a diethylaminoethyl (DEAE)-cellulose column (2 × 17 cm) which had been equilibrated with the above buffer. The IgG that showed absorbance at 280 nm was combined and stored at −20 °C. For use in RIA, the IgG was thawed and diluted with the assay buffer (PBS): 10 mM phosphate buffer

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(pH 7.4) containing 0.1% gelatin, 0.01% NaN_3 and 0.9% NaCl.

CA-1β-ol *p*-Nitrophenyl Ester A solution of CA-1β-ol (10 mg), *p*-nitrophenol (15 ml) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (15 mg) in 95% dioxane (0.6 ml) was stirred at room temperature for 4 h. The resulting solution was diluted with AcOEt, washed with H_2O, dried, and then evaporated. The residue was chromatographed on silica gel (5 g). Elution with $CHCl_3$–MeOH (10:1) and recrystallization of the eluate from MeOH gave the ester (8 mg) as colorless plates. mp 228—232 °C, $[\alpha]_D^{15}$ +17.5° (c=0.06, $CHCl_3$). ^{1}H-NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 1.04 (3H, d, J=6 Hz, 21-CH_3), 3.8—4.2 (4H, m, 1α-, 3β-, 7β- and 12β-H), 7.25, 8.28 (each 2H, d, J=9 Hz, aromatic H). *Anal.* Calcd for $C_{30}H_{43}NO_8$: C, 66.03; H, 7.94; N, 2.57. Found: C, 66.01; H, 8.02; N, 2.65.

[2-^{3}H]Tauro-CA-1β-ol A solution of the *p*-nitrophenyl ester (500 μg) in pyridine (0.2 ml) was added to a solution of [2-^{3}H(N)]taurine (7.25 MBq) in 0.1 M HCl (1 ml), and the mixture was stirred at room temperature overnight. After removal of pyridine by blowing N_2 gas, the residue was acidified with 0.1 M HCl and was loaded on a Bond Elut C18 cartridge. The cartridge was washed with H_2O, and the bile acid was eluted with MeOH, and then evaporated by blowing N_2 gas. The residue was applied to a PHP-LH-20 column (13 × 7.5 mm i.d.) and bile acids were stepwise eluted with 90% EtOH (5 ml), 0.1 M AcOH in 90% EtOH (5 ml) for free, and 0.3 M AcOH–AcOK (pH 6.3) (5 ml) in 90% EtOH for taurine conjugate. The crude taurine conjugate fraction containing 3.8 MBq was diluted to 10 ml with 0.01 M phosphate buffer (pH 7.4) and applied to a Sep-pak C_{18} column for removal of salts. After washing with H_2O, the bile acid was eluted with MeOH and submitted to preparative thin layer chromatography (TLC) using *n*-BuOH–AcOH–H_2O (25:4:10) as a developing solvent. The radioactive spot was scrapped off and eluted with MeOH to give 2.6 MBq of the labeled compound. After removal of silica gel particles by filtration, the filtrate was kept at 4 °C. An aliquot was scanned following the above TLC and a single radioactive peak coincident with the unlabeled tauro-CA-1β-ol was found.

Characterization of Antibody A standard curve was obtained by setting up duplicate centrifuge tubes (7 ml) containing 50—1500 pg of non-labeled tauro-CA-1β-ol and [^{3}H]tauro-CA-1β-ol (15000 dpm), which were dissolved in PBS (0.1 ml). The appropriately diluted antibody (0.1 ml) was added to all tubes and incubated at 4 °C overnight. After addition of 0.5 ml of dextran (0.05% w/v)–charcoal (1.5% w/v) suspension in PBS, the resulting mixture was vortexed and allowed to stand at 4 °C for 10 min. After centrifugation (3000 rpm, 10 min), the supernatants were transferred by decantation into vials, and the radioactivities were counted.

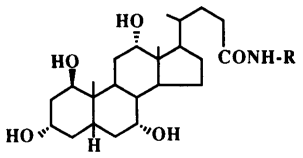
Specificities were investigated according to Abraham,[23] where the degree of cross reaction is expressed numerically on the basis of the mass of each bile acid required to produce 50% displacement of labeled tauro-CA-1β-ol. Association constant was obtained from the Scatchard plot.[24]

Measurement of Urinary Tauro-CA-1β-ol Content A diluted urine (1 ml) in buffer was loaded on a Bond Elut C18 cartridge. The cartridge was washed with H_2O (5 ml) and the bile acids were eluted with $CHCl_3$–MeOH (1:9). After removal of the solvent by a centrifugal evaporator at 60 °C, the remainder was dissolved in PBS (1 ml). An aliquot of 0.1 ml was submitted to RIA as described above. Background was determined by processing samples of pooled urine exactly as described above. Values of tauro-CA-1β-ol content were read off a standard curve which was concurrently carried out.

Results and Discussion

Conjugation at the side chain which leaves a functional group in tauro-CA-1β-ol was desirable in order to elicit an antibody with high specificity to the ring structure. Accordingly, glyco-CA-1β-ol was chosen as a bile acid hapten which could be linked to the carrier protein at the C-24 position, and was coupled to BSA using the mixed anhydride method. The number of bile acid residues incorporated per molecule of BSA was determined by gas chromatography-mass spectrometry after alkaline hydrolysis of the conjugate to be 10.

Two rabbits were immunized periodically with the conjugate emulsified with complete Freund's adjuvant several months. Initial attempts to characterize the antibody titer in the serum were examined using ^{125}I-labeled histamine conjugate of glyco-CA-1β-ol. However, the method resulted in only a slight binding ability of the ligand (data not shown). This might be ascribable to a structural difference at the C-24 linkages between the radioligand and the immunogen used for production of the antibody. Tritium labeled tauro-CA-1β-ol with high specific activity was thefefore prepared for the RIA procedure. Use of the commercially available [^{3}H]tauro cholic acid as a starting material was impractical in view of the number of chemical steps required to achieve the transformation to tauro-CA-1β-ol. We selected a procedure in which the *p*-nitrophenyl ester of CA-1β-ol reacted with [2-^{3}H]taurine under mild conditions. The product was separated from the unreacted starting material and taurine by PHP-LH-20 column chromatography and was further purified by preparative TLC on silica gel. TLC analysis coincided with the carrier tauro-CA-1β-ol which showed the product to be 100% radiohomogeneous, and with a specific activity of 743.7 GBq/mmol. Subsequently, the titers of the antisera were determined from the ability of the antibody at several dilutions after each bleeding to bind to a certain amount of this labeled compound. The antisera exhibited significant binding abilities ranging from 2 to 30% at the dilution of 1:1000 (Fig. 1). No substantial differences in the titer were observed between the antisera elicited in the two rabbits.



tauro-CA-1β-ol : R=$CH_2CH_2SO_3H$
immunogen : R=CH_2CONH-BSA
[2-^{3}H]tauro-CA-1β-ol : R=C[$^3H_2$]CH_2SO_3H

Chart 1

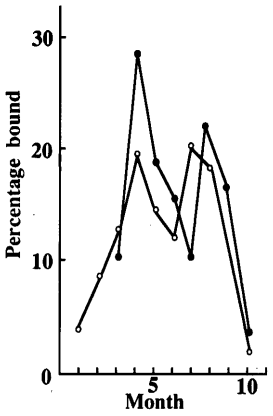


Fig. 1. Binding Abilities at the 1:1000 Dilution of Antisera Obtained from Two Rabbits

○, R-1; ●, R-2.

To avoid the nonspecific binding by other serum proteins, a dose–response curve was constructed with IgG, which was prepared from R-1 antiserum by precipitation with ammonium sulfate and following DEAE-cellulose column chromatography (Fig. 2). Scatchard analysis of the antibody disclosed a K_a value of 1.09×10^9 M^{-1}.

The specificity of the antibody was characterized by determining the cross-reactivities of 27 kinds of related bile

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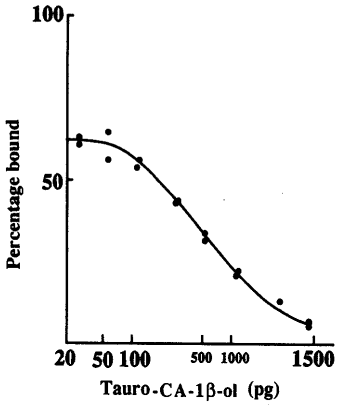

Fig. 2. Dose–Response Curve for RIA of Tauro-CA-1β-ol

TABLE I. Cross-Reactivities with Selected Bile Acids

Bile acid	Cross-reactivity (%)	
	Taurine conjugate	Glycine conjugate
CA-1β-ol	100	100
1β-Hydroxychenodeoxycholic acid	24.5	29.6
1β-Hydroxydeoxycholic acid	12.9	22.7
1β-Hydroxylithocholic acid	4.32	5.89
2β-Hydroxycholic acid	<0.01	<0.01
4β-Hydroxycholic acid	<0.01	<0.01
6α-Hydroxycholic acid	<0.01	0.07
Cholic acid	0.02	0.01
Chenodeoxycholic acid	<0.01	0.03
Deoxycholic acid	0.01	<0.01
Lithocholic acid	<0.01	<0.01
Hyocholic acid	<0.01	0.03
Hyodeoxycholic acid	<0.01	<0.01
Ursodeoxycholic acid	<0.01	<0.01

TABLE II. Recovery of Tauro-CA-1β-ol Added to Pooled Urine

Added (pg)	Found (pg)	Expected (pg)	Recovery (%)
0	32.5	—	—
265.5	338	298	113
573.5	605	606	100
1062	1162	1095	106
		Mean	106±5

TABLE III. Precision of the RIA

Urine sample	Tauro-CA-1β-ol (pg)	
	Intra-assay (n=5) Mean±S.D.	Inter-assay (n=3) Mean±S.D.
A	305±13 (4.1)	279± 6 (2.0)
B	573±19 (3.4)	567±14 (2.5)
C	1129±40 (3.5)	1137±62 (5.5)

The figures in parentheses represent C.V. (%).

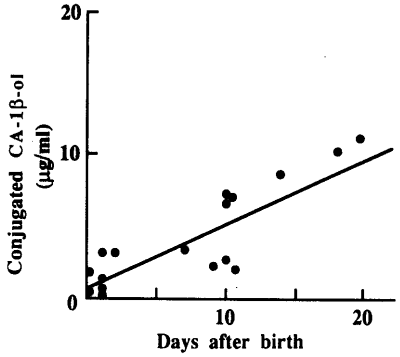

Fig. 3. Effect of Age on the Concentration of Conjugated CA-1β-ol in Urine of Newborns as Measured by RIA

$y=0.48x+0.67$. $\gamma=0.886$.

acids. As can be seen in Table I, the greatest reactivity with glyco-CA-1β-ol was found to be 100%. This may be due to the structural features of the immunogen, in which the bile acid is coupled to the carrier protein through the C-24 carboxyl group. Contrary to expectation, significant cross-reactivities were observed with the taurine and glycine conjugates of 1β-hydroxylithocholic (4.32 and 5.89%), 1β-hydroxydeoxycholic (12.9 and 22.7%) and 1β-hydroxychenodeoxycholic (24.5 and 29.6%) acids. All the other bile acids exhibited negligible competition for the antibody ranging from <0.01 to 0.07% relative to tauro-CA-1β-ol. Among these 1β-hydroxylated bile acids tested, only 1β-hydroxychenodeoxycholic acid has been detected in the urine of newborn babies, in which the proportion of this bile acid to tauro-CA-1β-ol represents less than 30%. To the best of our knowledge, 1β-hydroxylithocholic acid has not yet been found in any human biological fluids, though the existence of 1β-hydroxydeoxycholic acid has been reported in the meconium, amniotic fluid and in the urine of pregnant women and patients with liver disease. It has shown that the conjugation of bile acids by human fetus is preferential amidation with taurine.[25] As the infant matures (and as the diet is altered), glycine conjugate appears by the time of adulthood and is predominant. In fact, we observed that the urinary CA-1β-ol in newborns was mainly conjugated with taurine.[17] Our findings suggest that the participation of other bile acids in the RIA for conjugated CA-1β-ol in the urine of newborns is not likely to introduce significant errors.

Assessment of the assay was carried out by checking the recovery rate and the intra-assay and inter-assay coefficients of variation at several dilutions of pooled urine. A known amount of tauro-CA-1β-ol added to pooled urine was recovered at the rate of 106±5% (S.D.) (Table II). Intra-assay and inter-assay coefficients of variation were 3.4—4.1% and 2.0—5.5%, respectively (Table III). The blank values obtained by the assay procedure using 1 ml of pooled urine ranged from 12 to 33 pg.

The RIA was thus applied to the measurement of conjugated CA-1β-ol in urine samples obtained from newborns at 0—20 d after birth. All the samples were run at least as duplicates and on occasion as triplicates and quadruplicates. The urinary levels of conjugated CA-1β-ol found after correction for blank values ranged from 0.2 to 11.1 μg/ml, which were similar to those hitherto reported in infants.[6] The values obtained in this study showed a tendency to increase with the passage of time after birth (Fig. 3). This excretion pattern probably reflects the maturation of these infants, since the synthesis of bile acid in infants progressively rises during the first week of life. The pattern corresponds to a period of increased oral intake and

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enhanced enterohepatic circulation.[2,26] Our data suggest that the maturation in early life may effect a change of this bile acid concentration in urine. Further studies are needed to evaluate the metabolic significance of this bile acid during early life. Studies are underway on whether this substance plays an important role under normal conditions and in diseases which have been associated with increased bile acid formation such as congenital biliary atresia and cholestasis.

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References

1) L. Barbara, R. Lassari, A. Roda, R. Aldini, D. Festi, C. Sama, A. M. Morselli, A. Collina, F. Bazzoli, G. Mazella and E. Roda, *Pediatr. Res.*, **14**, 1222 (1980).
2) F. J. Suchy, W. F. Balistreri, J. E. Heubi, J. E. Searcy and R. S. Levin, *Gastroenterology*, **80**, 1037 (1981).
3) W. F. Balistreri, J. E. Heubi and F. J. Suchy, *J. Pediatr. Gastroenterol. Nutr.*, **2**, 346 (1983).
4) D. K. Freese and R. F. Hason, *J. Pediatr. Gastroenterol. Nutr.*, **2**, 374 (1983).
5) B. Almé, A. Bremmelgaard, J. Sjövall and P. Thomassen, *J. Lipid Res.*, **18**, 339 (1977).
6) B. Strandvik and S-Å, Wikstrom, *Eur. J. Clin. Invest.*, **12**, 301 (1982).
7) P. Back and K. Walter, *Gastroenterology*, **78**, 671 (1980).
8) M. Tohma, R. Mahara, H. Takeshita, T. Kurosawa, S. Ikegawa and H. Nittono, *Chem. Pharm. Bull.*, **33**, 3071 (1985).
9) M. Tohma, R. Mahara, H. Takeshita, T. Kurosawa and S. Ikegawa, *Chem. Pharm. Bull.*, **34**, 2890 (1986).
10) K. D. R. Setchell and J. M. Street, *Semin. Liver Dis.*, **2**, 85 (1987).
11) K. D. R. Setchell and L. Zimmer-Nechemias, *Hepatology*, **7**, 1133 (1987).
12) R. Mahara, H. Takeshita, T. Kurosawa, S. Ikegawa and M. Tohma, *Anal. Sci.*, **3**, 449 (1987).
13) J. Shoda, R. Mahara, T. Osuga, M. Tohma, S. Ohnishi, H. Miyazaki, N. Tanaka and Y. Matsuzaki, *J. Lipid Res.*, **29**, 847 (1988).
14) M. Nakagawa and K. D. R. Setchell, *J. Lipid Res.*, **31**, 1089 (1990).
15) K. D. R. Setchell, R. Dumaswala, C. Colombo and M. Rouchi, *J. Biol. Chem.*, **263**, 16637 (1988).
16) A. Bremmelgaard and J. Sjövall, *Eur. J. Clin. Invest.*, **9**, 341 (1979).
17) S. Ikegawa, N. Hirabayashi, T. Yoshimura, M. Tohma, M. Maeda and A. Tsuji, *J. Pharmacobio-Dyn.*, **14**, s-92 (1991).
18) J. Shoda, T. Osuga, R. Mahara, M. Tohma, K. Matsuura and H. Miyazaki, *J. Chromatogr.*, **488**, 315 (1989).
19) A. Bremmelgaard and J. Sjövall, *J. Lipid Res.*, **21**, 1072 (1980).
20) J. Gustafsson, S. Andersson and J. Sjövall, *Biol. Neonat.*, **47**, 26 (1985).
21) R. Mahara, T. Kurosawa, M. Tohma, Y. Hata and S. Fujimoto, *J. Pharmacobio-Dyn.*, **12**, s-7 (1989).
22) J. Goto, H. Hasegawa, H. Kato and T. Nambara, *Clin. Chim. Acta*, **20**, 187 (1985).
23) G. E. Abraham, *J. Clin. Endocrinol. Metab.*, **29**, 866 (1969).
24) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660 (1949).
25) J. R. Poley, J. C. Dower, C. A. Owen and G. B. Sticker, *J. Lab. Clin. Med.*, **63**, 838 (1964).
26) S. Barnes, G. Berkowitz, B. I. Hirschowitz and D. Wirtschafter, *J. Clin. Invest.*, **68**, 775 (1981).

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