Conjugated 1β-Hydroxycholic Acid in the Urine of Newborns and Pregnant Women Measured by Radioimmunoassay Using Antisera Raised against *N*-(1β-Hydroxycholyl)-2-aminopropionic Acid–Bovine Serum Albumin Conjugate

Shigeo IKEGAWA, Tsuyoshi MURAI, Teruki YOSHIMURA and Masahiko TOHMA*

Faculty of Pharmaceutical Sciences, Higashi-Nippon-Gakuen University, Ishikari-Tobetsu, Hokkaido 061-02, Japan. *Received November 25, 1991*

Anti-tauro 1β-hydroxycholic acid antisera were prepared by immunizing rabbits with *N*-(1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oyl)-2-aminopropionic acid–bovine serum albumin (BSA) conjugate. The antisera raised had high affinity ($1.25—1.46 \times 10^9$ M^{-1}) and specificity for conjugated 1β-hydroxycholic acid; cross-reactivity for glyco 1β-hydroxycholic acid was 100% and that for the glycine and taurine conjugates of other 1β-hydroxylated bile acids ranged from 11.30 to 0.23%. Urinary concentrations of conjugated 1β-hydroxycholic acid were determined by radioimmunoassay in newborns, 0—20 d after birth, in amounts ranging from 0.29 to 18.51 μg/ml and in women in late pregnancy (< 1.13 μg/ml), as well as in normal women (< 0.12 μg/ml).

Keywords radioimmunoassay; tauro 1β-hydroxycholic acid; conjugated 1β-hydroxycholic acid; bile acid; human urine; *N*-(1β-hydroxycholyl)-2-aminopropionic acid–bovine serum albumin

1β-Hydroxycholic acid (CA-1β-ol), which may be a metabolite of cholic acid, has been found in the urine of healthy adults[1] and infants,[2] in meconium,[3-8] in amniotic fluid[9,10] and in fetal gallbladder bile.[11] Recently, much interest has been focused on the formation of this bile acid in connection with fetal development and biliary disease.[12,13] In order to establish an assay to study the physiology and pathophysiology of this bile acid and assess its diagnostic utility, high-performance liquid chromatographic and gas chromatography-mass spectrometric methods have been widely used.[5,8,14] However, because of its low sample throughput, a requirement for sample extraction, and its susceptibility to analytical interference from unknown substances, it is inacceptable for routine use. A simple method for the determination of this bile acid is needed for biomedical use. In our preceding paper,[15] we reported a method for its determination by radioimmunoassay (RIA) using antiserum raised against glyco CA-1β-ol–bovine serum albumin (BSA) conjugate. This method, is still unsatisfactory due to its lack of specificity, resulting in minor cross reactivity with other 1β-hydroxylated bile acids. This is likely due to the lower binding affinity of the tracer for the antibody, since the hapten side chain differs from that of the [^{3}H]tauro CA-1β-ol used as the labeled antigen. In order to elicit an antibody with enhanced specificity, we have prepared a new hapten whose structure closely resembles the side chain of tauro CA-1β-ol. The present paper deals with the preparation of antisera using the 2-aminopropionic acid derivative of CA-1β-ol as the hapten and its use for direct RIA of conjugated CA-1β-ol in urine.

Materials and Methods

Chemicals and Reagents [2-^{3}H]Tauro 1β-hydroxycholic acid (743.7 GBq/mmol) was prepared as previously described.[15] Prior to use, the radiochemical purity was checked by thin layer chromtography using *n*-BuOH–AcOH–H_2O (25 : 4 : 10, v/v). The reference bile acids were either synthesized in our laboratory or were obtained commercially and purified to homogenity by chromatography and/or recrystallization. BSA (fraction V) and complete Freund's adjuvant were obtained from Sigma Chemical Co. (U.S.A.) and Iatron Laboratories (Tokyo), respectively. All organic solvents and chemicals were of analytical reagent grade.

Analyses Melting points were determined with a Mitamura micro hot-stage apparatus, and were uncorrected. Optical rotations were measured with a Union Giken-201 polarimeter. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded at 400 MHz with a JEOL JNM-EX 400 spectrometer. Chemical shifts are given as the δ vlaue with tetramethylsilane as the internal standard (s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet).

Methyl *N*-(1β,3α,7α,12α-Tetrahydroxy-5β-cholan-24-oyl)-2-aminopropionate (2) Diethyl cyanophosphonate (0.2 ml) and 2-aminopropionic acid methyl ester hydrochloride (200 mg) were added to a solution of 1β-hydroxycholic acid (**1**) (50 mg) in dimethylformamide (DMF) (0.35 ml) containing triethylamine (0.1 ml), and the mixture was stirred at room temperature overnight. After addition of H_2O, the resulting solution was mixed with Amberlite XAD-2 resin (65 ml). The resin was washed with water in a sintered glass funnel, then the bile acid was eluted with MeOH. After evaporation of the solvent *in vacuo*, the crude product was chromatographed on silica gel using $CHCl_3$–MeOH (8 : 1) as an eluant to give **2** (35 mg). mp 208—209 °C (colorless prisms, acetone). $[\alpha]_D^{20}$ −112.1° (c = 0.12, MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 0.97 (3H, d, J = 5.9 Hz, 21-CH_3), 1.01 (3H, s, 19-CH_3), 2.54 (2H, dd, J = 5.9, 6.4 Hz, –NHCH_2CH_2COO–), 3.47 (2H, dd, J = 5.9, 6.4 Hz, –NHCH_2CH_2COO–), 3.70 (3H, s, $COOCH_3$), 3.83—3.92 (4H, m, 1α, 3β, 7β, 12β-H), 6.64 (1H, t, J = 5.9 Hz, –NH–). *Anal.* Calcd for $C_{28}H_{49}NO_7$: C, 65.72; H, 9.65; N, 2.74. Found: C, 65.65; H, 9.43; N, 2.75.

***N*-(1β,3α,7α,12α-Tetrahydroxy-5β-cholan-24-oyl)-2-aminopropionic Acid (3)** 2 M LiOH (1 ml) was added to a solution of **2** (140 mg) in MeOH (6 ml), and the mixture was stirred at room temperature for 4 h. The MeOH was evaporated *in vacuo*, and the residue was loaded on Amberlite XAD-2 resin (200 ml) and was allowed to stand at room temperature overnight. After washing with H_2O (1 l), the bile acid was eluted with MeOH (300 ml). After evaporation of the solvent *in vacuo*, the crude product was chroamtographed on silica gel using $CHCl_3$–MeOH (1 : 1) as an eluant to give **3** (128 mg). mp 251.8—252.7 °C (colorless granulous, MeOH). $[\alpha]_D^{20}$ +51.4° (c = 0.06, MeOH). NMR (CD_3OD–$CDCl_3$ = 3 : 1) δ: 0.71 (3H, s, 18-CH_3), 1.00 (3H, d, J = 6 Hz, 21-CH_3), 1.02 (3H, s, 19-CH_3), 3.4—4.0 (4H, m, 1α, 3β, 7β, 12β-H). *Anal.* Calcd for $C_{27}H_{45}NO_7 \cdot H_2O$: C, 63.13; H, 9.22, N, 2.73. Found: C, 63.17; H, 9.24; N, 2.85.

Preparation of Antigen A solution of **3** (75 μmol) in dry DMF (0.7 ml) was treated with tri-*n*-butylamine (25 μl) and isobutyl chlorocarbonate (50 μl) at 10 °C, and the whole mixture was stirred for 30 min. Then BSA (90 mg) in H_2O (2.2 ml)–DMF (1.4 ml)–1 M NaOH (0.08 ml) was added with cooling under ice and the mixture was stirred for 3 h. The resulting solution was dialyzed and then lyophylized as previously reported[15] to give the BSA conjugate (96.9 mg) as a fluffy powder.

Immunization and Blood Collection Three female domestic albino rabbits (2—2.5 kg) were used for immunization with the conjugate. The antigen (1 mg) was dissolved shortly before injection in 0.5 ml of sterile isotonic saline and was emulsified with 0.5 ml of complete Freund's adjuvant. This emulsion was injected subcutaneously in multiple sites over the back. This procedure was repeated at intervals of one week for an additional month 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 was stored at −20 °C. For RIA, the antisera were thawed and were diluted with 10 mM phosphate buffer (pH 7.4) containing 0.1% gelatin, 0.01% NaN_3 and 0.9% NaCl. Rabbit

serum with the highest titer was selected for characterization.

Radioimmunoassay Procedure All dilutions of the standard, tracer and antisera were made in 10 mM phosphate buffer (pH 7.4) containing 0.1% gelatin, 0.01% NaN_3 and 0.9% NaCl. Diluted antiserum (0.1 ml) and [^{3}H]tauro CA-1β-ol (15000 dpm) were added to a series of standard solutions of tauro CA-1β-ol (50—2000 pg) or to the diluted urine in buffer, and the mixture was incubated overnight at 4 °C. The incubation mixture was then treated with 0.5 ml of dextran (0.05% (w/v))–charcoal (1.5% (w/v)) suspension in buffer, vortexed, allowed to stand 10 min at 4 °C and then centrifuged at 4 °C at 3000 rpm for 10 min. The supernatants were decanted into vials, containing 10 ml EX-H scientisol for counting.

Control and back ground were determined by processing samples of buffer and charcoal treated bile acid free urine exactly as above. Tauro CA-1β-ol content was determined by comparison with standards which were run concurrently.

Characterization of Antisera Antiserum spceificity was investigated according to Abraham.[16] The relative amounts required to reduce the initial binding of [^{3}H]tauro CA-1β-ol by 50% compared with nonlabeled tauro CA-1β-ol were calculated from the standard curve. Association constants were obtained from Scatchard plots.[17]

Results and Discussion

The bile acid hapten used in this study was *N*-(1β,3α,-7α,12α-tetrahydroxy-5β-cholan-24-oyl)-2-aminopropionic acid (**3**), in which the methylene of the amino acid residue on the side chain was one unit longer than that of the glycine conjugate used for the previous study. The haptenic derivative was covalently coupled to BSA to provide CA-1β-ol–BSA conjugate by the mixed anhydride method. The number of bile acid residues incorporated per molecule of BSA was determined spectrophotometrically (390 nm) on the basis of coloration with 83% sulfuric acid to be 25.

The hapten–BSA conjugate thus prepared was used for immunization of three rabbits. The sera obtained from rabbits immunized with the conjugate for six months exhibited satisfactory binding abilities. Final dilutions of 1 : 3000—1 : 7000 giving 50% binding of [^{3}H]tauro CA-1β-ol were used for the study. Standard curves were obtained with these antisera and are presented in Fig. 1. Scatchard analysis of the antisera disclosed high affinity constants as listed in Table I.

The specificity of the antisera was investigated by determining the cross-reactivities of various bile acids. As can be seen in Table II, the greatest reactivity was found with glyco CA-1β-ol to be 100—110%. A low reactivity was observed with taurine and glycine conjugates of 1β-hydroxylated bile acids (0.23—11.3%). All other bile acids revealed negligible competition with the antibody ranging from <0.01 to 0.94%.

The cross-reactivities of the above antisera with selected bile acids were compared with that of antiserum elicited by

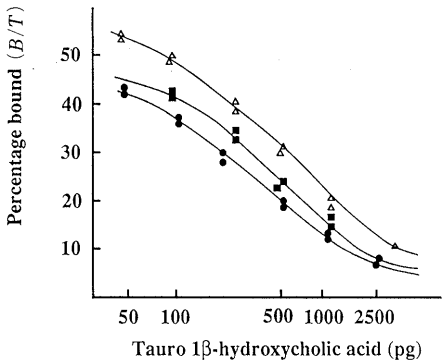
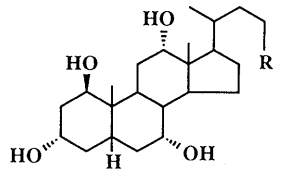


Fig. 1. Dose–Response Curves for Tauro 1β-Hydroxycholic Acid Using Antisera Obtained from Three Rabbits

●, R-1; △, R-2; ■, R-3.



1 : R= COOH
2 : R= $CONH(CH_2)_2COOC_2H_5$
3 : R= $CONH(CH_2)_2COOH$
4 : R= $CONH(CH_2)_2CONH$-BSA

Chart 1

TABLE I. Affinity Constants and Titers of Antisera

Antiserum	Final dilution	K_a ($M^{-1} \times 10^9$)
R-1	1 : 3000	1.25
R-2	1 : 7000	1.46
R-3	1 : 6000	1.32

TABLE II. Per Cent Cross-Reactivity of Antisera

Bile acid	Antiserum (Final dilution)					
	R-1 (1 : 3000)		R-2 (1 : 7000)		R-3 (1 : 6000)	
Tauro 1β-hydroxycholic acid	100	(100)	100	(104)	100	(110)
Tauro 1β-hydroxychenodeoxycholic acid	3.67	(3.67)	4.00	(6.76)	4.20	(5.60)
Tauro 1β-hydroxydeoxycholic acid	5.18	(5.50)	8.57	(4.31)	11.30	(11.1)
Tauro 1β-hydroxylithocholic acid	0.23	(0.36)	0.32	(0.39)	0.42	(0.83)
Tauro 2β-hydroxycholic acid	<0.01	(<0.01)	<0.01	(<0.01)	<0.01	(<0.01)
Tauro 4β-hydroxycholic acid	<0.01	(<0.01)	<0.01	(<0.01)	<0.01	(<0.01)
Tauro 6α-hydroxycholic acid	<0.01	(0.09)	<0.01	(<0.01)	0.10	(0.07)
Taurocholic acid	0.12	(0.94)	0.13	(0.09)	0.05	(0.08)
Taurochenodeoxycholic acid	<0.01	(<0.01)	0.02	(<0.01)	<0.01	(<0.01)
Taurodeoxycholic acid	<0.01	(<0.01)	0.02	(0.03)	0.02	(0.02)
Taurolithocholic acid	<0.01	(<0.01)	<0.01	(<0.01)	<0.01	(<0.01)
Taurohyocholic acid	<0.01	(0.10)	<0.01	(<0.01)	<0.01	(<0.01)
Taurohyodeoxycholic acid	<0.01	(<0.01)	<0.01	(<0.01)	<0.01	(<0.01)
Tauroursodeoxycholic acid	<0.01	(<0.01)	<0.01	(<0.01)	<0.01	(<0.01)

Values in parenthesis represent the cross-reactivity of glycine conjugates.

TABLE III. Comparison of Per Cent Cross-Reactivity of Antisera with Selected Bile Acids

Bile acid	Cross-reactivities (%)			
	R-1		Glyco-CA-1β-ol–BSA[a]	
Tauro 1β-hydroxycholic acid	100	(100)	100	(100)
Tauro 1β-hydroxychenodeoxycholic acid	3.67	(3.67)	24.5	(29.6)
Tauro 1β-hydroxydeoxycholic acid	5.18	(5.50)	12.9	(22.7)
Tauro 1β-hydroxylithocholic acid	0.23	(0.36)	4.32	(5.89)

Values in parenthesis represent the cross-reactivity of glycine conjugates.
a) Reference 15.

TABLE IV. Reliability for the Measurement of Tauro 1β-Hydroxycholic Acid

Added (pg)	n	Recovery (%) Mean±S.D.	CV (%)
Inter assay			
265	5	95.8±3.3	3.4
531	5	99.1±4.3	4.4
1062	5	97.0±9.3	9.5
Intra assay			
265	8	102±7.3	7.2
531	8	105±7.0	6.7
1062	8	101±8.2	8.2

CV: coefficients of variation.

antigen having a shorter bridge length (Table III). It is evident that the specificities of the antisera prepared in this study are higher than that of the antibody elicited by the glyco CA-1β-ol–BSA conjugate.[15] This can be attributed to a difference in binding of the tracer to the antibody. Since the antisera are elicited by the CA-1β-ol-2- amino-propionic acid–BSA conjugate, which closely resembles part of the side chain of the ^{3}H-labeled tracer, binding with these antisera is tighter than that with antibody elicited by glyco CA-1β-ol–BSA conjugate.

Development of an assay system with the highest specificity was then undertaken to measure conjugated CA-1β-ol in human urine. From the cross-reaction data, contamination with other 1β-hydroxylated bile acids is not likely to introduce significant errors.

Recoveries of cold tauro CA-1β-ol added to bile acid-free urine as measured by RIA are shown in Table IV. These data indicate excellent concordance between added and measured material. Inter- and intra-assay coefficients of variation were 3.4—9.5% and 7.0—8.2%, respectively. Thus the present method is both precise and accurate. The blank value obtained with charcoal-treated urine was not significantly different from zero. The minimum detectable amounts averaged 50 pg.

Urinary conjugated CA-1β-ol levels were then measured. In the RIA, dilutions ranged from 1 : 100 to 1 : 1000 were used for urine samples obtained from normal women, women in late pregnancy and newborns. All samples were run at least as duplicates and on occasion as triplicates and quadruplicates. As shown in Fig. 2, conspicuous differences were found among these samples. The urinary conjugated CA-1β-ol content of the newborn at 0—20 d after birth

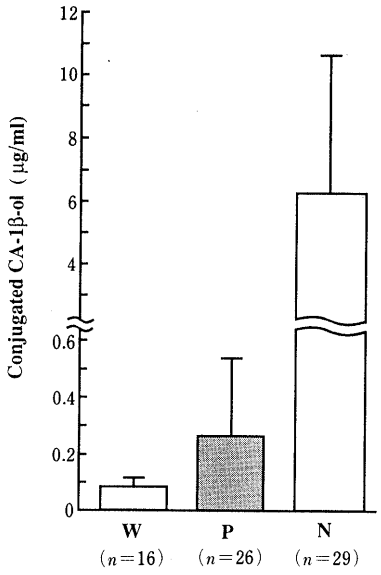


Fig. 2. Urinary Concentrations of Conjugated 1β-Hydroxycholic Acid in Normal Women (W, $n=16$), Women in Late Pregnancy (P, $n=26$) and Infants (N, $n=29$)

was much higher than that of normal and pregnant women. Excreted levels (0.29—18.51 μg/ml) were similar to those reported previously.[15] In normal women, urinary excretion of conjugated CA-1β-ol was extremely low, ranging from 0.03 to 0.18 μg/ml (0.09±0.04 μg/ml, mean±S.D.). However levels in pregnant women were elevated significantly, ranging from 0.04 to 1.13 μg/ml (0.28±0.28 μg/ml, mean±S.D.).

A recent study has shown that the proportion of CA-1β-ol is greater in urine than in serum and liver of patients with cholestasis and is relatively low in plasma of premature infants.[18,19] Abnormalities in bile acid metabolism have been related to the development of certain forms of cholestasis in newborns.[20] The relatively high concentration of this cholic acid metabolite in urine of newborns compared with that in plasma, suggest that this metabolite has a rapid metabolic clearance. As suggested from early studies,[8,21] the increased conjugated CA-1β-ol excretion in newborns and women in late pregnancy may reflect differences in bile acid metabolism during fetal and neonatal periods. Further studies are underway to determine whether the levels of this metabolite are elevated in diseases associated with increased CA-1β-ol formation such as congenital biliary atresia and cholestasis in adult life.

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