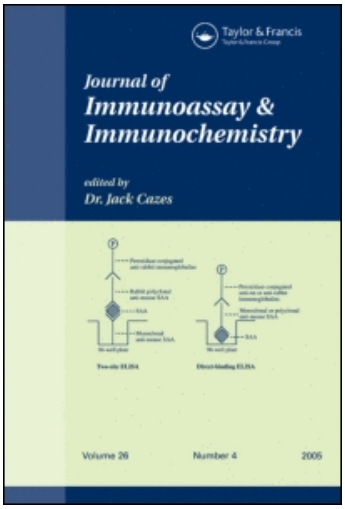

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ENZYME IMMUNOASSAY FOR CONJUGATED CHOLIC AND 1β-HYDROXYCHOLIC ACIDS IN URINE OF EARLY INFANCY

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ABSTRACT

A direct competitive heterologous enzyme immunoassay (EIA) for conjugated cholic acid (CA) was developed using horseradish peroxidase labeled antigen having a shorter bridge length than that of the immunogen. An appropriate dose-response curve for conjugated CA was obtained in the range of 0.05-50 ng/well. Specificity of the EIA proved satisfactory in terms of cross-reactivities to 23 kinds of related bile acids. The proposed method was evaluated to be useful for the determination of conjugated CA in urine with acceptable accuracy and inter- and intra-assay precision. The results of analysis showed a reverse relationship between age and urinary excretion ratio of conjugated 1β-hydroxy-CA to conjugated CA in the first 9 months after birth. (KEY WORDS: enzyme immunoassay, horseradish peroxidase, conjugated bile acid, cholic acid, 1β-hydroxycholic acid, infant urine).

INTRODUCTION

Recently, attention has been focused on bile acid metabolism in connection with fetal-neonatal development and hepatobiliary diseases

(1). Of paticular interest is the formation of 1β-hydroxycholic acid (CA-1β-ol) in the fetus and neonate, since it has been found in the urine of infants (2-4), in meconium (5,6), in amniotic fluid (7,8) and in fetal gallblader bile (9). Our previous study has shown that the proportion of this bile acid is greater in urine than in serum and liver tissue (10), and bile acid is preferentially conjugated with taurine and is the principal ingredient of urinary bile acids in newborn babies (11,12). These observations have led to speculation that the formation of CA-1β-ol may be an important excretion mechanism of cholic acid (CA) in the fetus and newborn (13-15). In order to understand the metabolic role of CA-1β-ol in early life, determination of age-related changes in the urinary excretion ratio of conjugated CA-1β-ol to conjugated CA is required.

It is generally accepted that since immunoassay is favorable for the sensitive and specific determination of bile acids and steroid hormones in biological fluids without tedious and time consuming pretreatment, the method is useful for routine assay. In the preceding paper (16), we developed an enzyme immunoassay (EIA) for the sensitive determination of conjugated CA-1β-ol. The present paper deals with the development of an EIA for conjugated CA and its application to the determination of the urinary excretion ratio of conjugated CA-1β-ol to conjugated CA in early life.

MATERIALS AND METHODS

Chemicals and Reagents

Horseradish peroxidase (EC1,11,1,7, HRP) (Grade I-C, Reinheits > 3.0, 200 units/mg) and bovine serum albumin (BSA) were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Complete Freund's adjuvant was obtained from Iatron Laboratories (Tokyo, Japan). Goat anti-rabbit

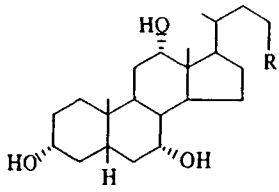
immunoglobulin G (H+L) and 3,3',5,5'-tetramethylbenzidine were obtained from Wako Pure Chemicals (Osaka, Japan) and Dojindo Co. (Kumamoto, Japan), respectively. The reference bile acids were collected by these laboratories. The anti-tauro CA-1β-ol antiserum and glyco CA-1β-ol-HRP conjugate used were those reported in the previous paper (16). Flat bottomed polystyrene 96-well microtiter plates were purchased from Sumitomo Bakelite Co. (Tokyo, Japan).

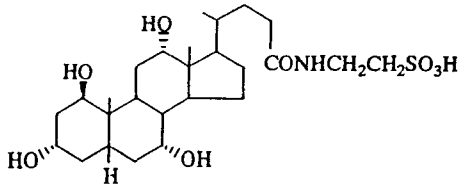
Apparatus

Melting points (mp) were determined with a Mitamura micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-360 digital polarimeter. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNM-EX 400 spectrometer at 400 MHz with tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet, q, quartet, m, multiplet). Infrared (IR) spectra were obtained with a JASCO IRA-102 spectrometer in Nujol and are expressed in cm^{-1}. The microplate reader was BIO-RAD Model 2550 EIA Reader (Richmond, CA, USA).

Preparation of the Haptenic Derivative of Cholic Acid

Trichloroethyl N-(3α, 7α, 12α-Trihydroxy-5β-cholan-24-oyl)-3-aminopropionate [2 in Figure 1]: Diethyl cyanophosphonate (0.9 ml), 2,2,2-trichloroethyl 3-aminopropionate p-toluenesulfonate (2.88 g) and triethylamine (1 ml) were added to a solution of cholic acid [1] (1.0 g) in dimethylformamide (1 ml), and the mixture was stirred at room temperature for 15 min. The reaction mixture was diluted with ethyl acetate, and the resulting solution was washed with 5% HCl, 5% $NaHCO_3$, and sat. NaCl, and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the crude product was purified by column





1 : R = COOH
2 : R = $CONHCH_2CH_2COOCH_2CCl_3$
3 : R = $CONHCH_2CH_2COOH$
4 : R = $CONHCH_2CH_2CONH$ - BSA
5 : R = $CONHCH_2CH_2SO_3H$
6 : R = $CONHCH_2COOH$
7 : R = $CONHCH_2CONH$ - HRP

Tauro 1β-Hydroxycholic Acid

FIGURE 1. Structures of cholic and 1β-hydroxycholic acids and their conjugates.

chromatography on silica gel using acetone-hexane (1:1, v/v) as an eluant to give 2 (1.83 g) as colorless amorphous. $[\alpha]_D$ +144° (C= 0.100, methanol). IR : 3300 (OH), 1745 (C=O, ester), 1640 (C=O, amide). ^{1}H-NMR (C_5D_5N) : 0.79 (3H, s, 18-CH_3), 0.99 (3H, s, 19-CH_3), 1.17 (3H, d, J=6.4 Hz, 21-CH_3), 2.91 (2H, t, J=6.4 Hz, -$NHCH_2\underline{CH_2}COO$-), 3.73 (1H, m, 3β-H), 3.80 (2H, q, J=6.4 Hz, -$NH\underline{CH_2}CH_2COO$-), 4.04 (1H, m, 7β-H), 4.21 (1H, m, 12β-H), 4.97 (2H, s, -$COOCH_2$-).

N-(3α, 7α, 12α-Trihydroxy-5β-cholan-24-oyl)-3-aminopropionic Acid [3]: Acetic acid (24 ml) and zinc powder (4 g) were added to a solution of 2 (500 mg) in tetrahydrofuran (24 ml), and the mixture was stirred at room temperature for 30 min. After filtration of zinc on a bed of Celite, the solvent was evaporated *in vacuo*. The crude product was recrystallized from methanol-ether to give 3 (440 mg) as colorless prisms. mp 206.5-207.5 °C. $[\alpha]_D$ +170° (C= 0.100, methanol). IR : 3340 (OH), 1700 (carboxyl), 1640 (amide). ^{1}H-NMR (C_5D_5N) : 0.78 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 1.17 (3H, d, J=5.9 Hz, 21-CH_3), 2.89 (2H, t, J=6.4 Hz,

-$NHCH_2\underline{CH_2}COO$-), 3.72 (1H, m, 3β-H), 3.89 (2H, q, J=6.4 Hz, -$NH\underline{CH_2}CH_2COO$-), 4.05 (1H, m, 7β-H), 4.19 (1H, m, 12β-H). Anal. Calcd. for $C_{27}H_{45}NO_6$: C, 67.45; H, 9.61; N, 2.84. Found : C, 67.61; H, 9.46; N, 2.24.

Preparation of Antiserum for Conjugated Cholic Acid

A solution of the hapten [3] (40 mg) in dry dioxane (2 ml)-dimethylformamide (0.5 ml) was treated with tri-n-butylamine (26 μl) and isobutyl chloroformate (12 μl) at 10 °C, and the whole mixture was stirred for 90 min. Then BSA (90 mg) in water (2.2 ml)-dioxane (1.4 ml)-1M NaOH (1.4 ml) was added with ice cooling and the mixture was stirred for 3 hr. The resulting solution was dialyzed and then lyophilized as previously reported to afford the BSA conjugate [4] (139 mg) as a fluffy powder. The number of hapten molecules incorporated into a BSA molecule was determined spectrophotometrically (390 nm) on the basis of coloration with 83% H_2SO_4 to be 18.

The antigen (3 mg) was dissolved in saline (1.5 ml) and emulsified with complete Freund's adjuvant (1.5 ml). The emulsion was injected into domestic female rabbits subcutaneously at multiple sites along the back. This procedure was repeated once a week for a first month and then once a month. Blood withdrawn was centrifuged at 3000 rpm for 20 min. The antiserum collected was stored at 4 °C in the presence of 0.1% (w/v) of sodium azide and stored at -20 °C.

Preparation of Glyco-Cholic Acid -HRP Conjugate

The HRP conjugate [7] was prepared from glyco CA [6] by the mixed anhydride method as previously reported (16). The conjugate mixture was purified on a column (1 cm: i.d. x 1 m) of Bio-Gel P-60 (BIO-RAD) with 0.01 M phosphate buffer (pH 7.0) containing 0.9 % NaCl. Fractions were

collected and their absorbances were measured at 403 nm. HRP activities of the fractions were determined by colorimetry and the immune activities were examined by the EIA described below. Fractions of the purified conjugate were collected and stored at 4 °C. This solution was stable for at least 6 months with only a slight loss of activity.

Preparation of Second Antibody Immobilized Plates

Flat-bottomed polystyrene 96-well microtiter plates were coated with 100 μl of goat anti-rabbit IgG (H+L) (10 μg/ml) in 0.05 M phosphate buffer (pH 7.4) containing 0.1 M NaCl. The plates were sealed and incubated overnight at room temperature, emptied by inversion, and washed three times with 0.01 M phosphate buffer (pH 7.4). The plates were covered with 0.05 M phosphate buffer containing 0.1 M NaCl and 0.1% BSA and stored at 4 °C until use.

EIA Procedure

All solutions were diluted with 0.05 M phosphate buffer (pH 7.0) containing 0.1 M NaCl and 0.1% BSA, and all incubations were performed at room temperature in a total volume of 150 μl/well. An appropriately diluted urine sample or standard solution (50 μl), the HRP-labeled antigen (50 μl), and the first antibody (1 : 20000, 50 μl) were added to the microtiter plates coated with the second antibody. The plates were lightly patted and shaken, then incubated overnight at 4 °C. After washing 5 fill/aspirate cycles with 0.4 ml of 0.1% Tween 20 in saline and tapping on a paper towel, a 150 μl solution (9:1, v/v) of 0.023% hydrogen peroxide and 0.005% 3,3',5,5'-tetramethylbenzidine in 0.05 M acetic acid-citric acid buffer (pH 5.5) containing 0.6 ml of dimethylsulfoxide was added and incubated at 25 °C for 1 hr. The enzyme reaction was terminated by the addition of 0.5 M H_2SO_4 (150 μl), and absorbances were measured at 450 nm.

Cross-Reaction Study

Specificity of the assay system was assessed by cross-reactivity to structurally related bile acids. The cross-reactivity was calculated from the ratio of the concentrations of tauro CA [5] to those of the tested bile acids at 50% inhibition of binding of the enzyme labeled antigen.

RESULTS AND DISCUSSION

There have been some reports on the EIA for conjugated CA in human biological fluids (17,18). But these methods lack the sensitivity to determine the trace amounts of this bile acid in urine samples obtainable from newborn babies. It has been reported that a bridge heterologous combination of enzyme labeled antigen having a shorter bridge structure than that of the immunogen is more favorable for developing a sensitive assay system in EIA for steroid hormones (19,20). CA possess a characteristic structure on the steroid nucleus and is predominantly conjugated at C-24 with taurine in newborn infants. Therefore, N-(3α,7α,12α-trihydroxy-5β-cholan-24-oyl)-3-aminopropionic acid [3], which had the same number of methylene on the side chain as that of tauro CA [5], and glyco CA [6] were chosen as the haptenic derivatives for preparation of the immunogen and the enzyme-labeled antigen, respectively. The immunogen thus obtained was administered to rabbits with complete Freund's adjuvant subcutaneously. The appropriate antiserum was obtained from rabbits 5 months after the first administration of the immunogen.

An evaluation of the titer was carried out by incubating various dilutions of antiserum with a 10 ng of glyco CA-HRP conjugate [7]. The optimal dilution of the antiserum was found to be 1: 20000, where 50% of the enzyme activity was precipitated. With this dilution of antiserum,

the dose-response curve for tauro CA was sufficiently sensitive in the range of 0.05 -50 ng per well as shown in Figure 2. The dynamic range was judged to be useful for metabolic study. The amount of tauro CA needed to reduce the enzyme activity in the immune precipitate by half was determined to be 0.4 ng per well.

Specificity of the EIA was assessed by ascertaining the ability of various related bile acids to compete with the enzyme-labelled antigens for binding to the antibody, and was expressed as per cent cross-reactivity (Table 1). The anti-CA antibody discriminated tauro CA from the other bile acids presented in the urine of infants with their cross-reactivities of a few percent or less. The greatest reactivity was observed with glyco CA to be 96 %. It is noteworthy that the reactivity with conjugated CA-1β-ol reveals negligible competition with the antibody. It was confirmed that the developed EIA is sufficiently specific and applicable to determine the conjugated CA.

EIA for conjugated CA-1β-ol was performed by using 3,3'5,5'-tetramethylbenzidine for the measurement of the enzyme activity in place of o-phenylenediamine which was used in the previous study (16). The valid range of the assay was 0.01 ng to 3 ng (Figure 2). The ratio of bound enzyme activity to that at 0 dose (B/B_0) was approximately 68 % at 0.01 ng per well, and 40% at 0.05 ng per well.

The assessment of the assay reliability in these EIAs were carried out by measuring tauro CA (0.25-1.0 ng) or tauro CA-1β-ol (0.054-0.269 ng) added to bile acid free urine prepared as described by Simmonds (21). The acceptable recovery rates (87-106 and 90-109%) of these bile acids added to urine at three levels justified the reliability of the proposed EIAs (Table 2, 3). The reproducibilities of the intra- and inter-assay coefficients of variation (CV) were 7-21 % for the former and 4-12 % for the latter, respectively. The results show that these assay systems can be applied to urine samples.

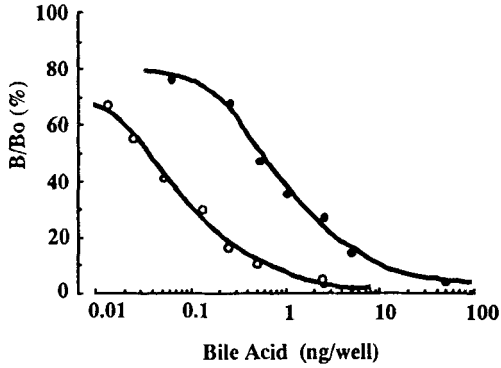


FIGURE 2. Typical standard curves for tauro cholic acid (●) and tauro 1β-hydroxycholic acid (○).

TABLE 1

Cross-Reactivities of Antiserum with Related Bile Acids

Bile acid	Cross-reactivity, %	
	Taurine conjugate	Glycine conjugate
Cholic acid	100	96.0
Chenodeoxycholic acid	7.44	8.54
Deoxycholic acid	1.82	2.07
Lithocholic acid	1.06	0.04
Hyocholic acid	0.17	<0.01
Hyodeoxycholic acid	0.03	0.17
Ursodeoxycholic acid	0.13	<0.01
1β-Hydroxycholic acid	0.31	0.86
1β-Hydroxychoenodeoxycholic acid	0.17	0.09
1β-Hydroxydeoxycholic acid	0.03	0.23
2β-Hydroxycholic acid	1.28	0.06
4β-Hydroxycholic acid	0.72	1.05
6α-Hydroxycholic acid	0.17	----

TABLE 2
Reliability of Tauro Cholic Acid Measurement

Added (ng)	n	Recovery (%) Mean ± SD	CV (%)
Inter assay			
0.25	5	106 ± 21	20
0.50	5	95 ± 12	13
1.00	5	94 ± 19	21
Intra assay			
0.25	10	87 ± 3	8
0.50	10	98 ± 8	8
1.00	10	90 ± 13	7

TABLE 3
Reliability of Tauro 1β-Hydroxycholic Acid Measurement

Added (ng)	n	Recovery (%) Mean ± SD	CV (%)
Inter assay			
0.054	5	96 ± 9	10
0.108	5	102 ± 4	4
0.269	5	96 ± 7	7
Intra assay			
0.054	10	109 ± 13	12
0.108	10	102 ± 6	6
0.269	10	90 ± 6	7

These assay methods were then applied to the measurement of conjugated CA and CA-1β-ol in urine samples obtained from infants aged 1 month to 3 years. Wide variabilities in conjugated CA-1β-ol concentrations were observed in 21 newborns from the first day to 30 days after birth, although no clear age-related changes were observed. The mean value, 3.6 ± 3.9 μg/ml (mean ± SD), was similar to those reported

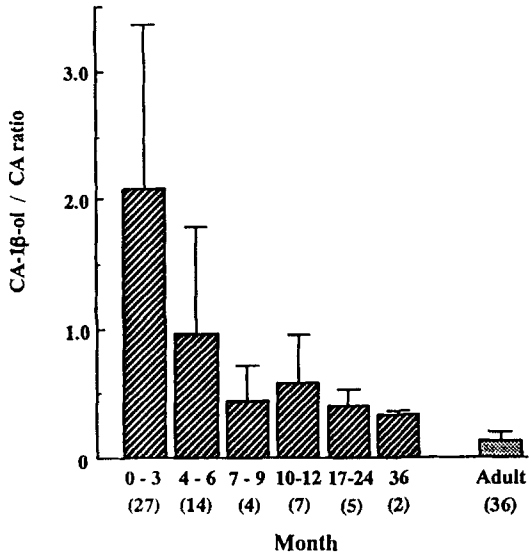


FIGURE 3. Change of urinary excretion ratio of conjugated 1β-hydroxycholic acid to conjugated cholic acid in infants. (Number of samples in parenthesis)

previously (22,23). In contrast, a reverse relationship was observed between the ratio of conjugated CA-1β-ol to conjugated CA and age as shown in Figure 3. The ratio in the 3 month old infants, 2.08 ± 1.30 (mean ± SD, n=27), progressively decreased with age and reached the level of adults (0.13 ± 0.07, mean ± SD, n=36). These observations strongly suggest that 1β-hydroxylation of CA in early infancy declines in inverse proportion to aging.

It is hoped that the availability of this analytical method may provide more precise knowledge on the physiological and pathophysiological roles of these bile acids in relation to hepatobiliary diseases.

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