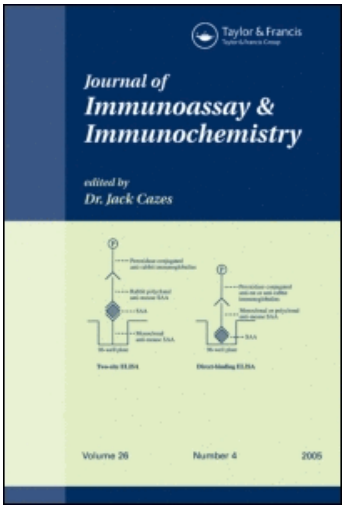

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Online publication date: 30 April 2001

To cite this Article Yoshimura, Teruki , Taniguchi, Tioshiyasu , Kobayashi, Daisuke , Komiya, Tomohiro , Murai, Tsuyoshi , Kimura, Akihiko , Kurosawa, Takao and Tohma, Masahiko(2001) 'ENZYME IMMUNOASSAY FOR CONJUGATED 7α-HYDROXY-3-OXO-4-CHOLENOIC ACID IN HUMAN URINE', Journal of Immunoassay and Immunochemistry, 22: 1, 1 — 13
To link to this Article: DOI: 10.1081/IAS-100102894
URL: http://dx.doi.org/10.1081/IAS-100102894

J. IMMUNOASSAY & IMMUNOCHEMISTRY, 22(1), 1–13 (2001)

ENZYME IMMUNOASSAY FOR CONJUGATED 7α-HYDROXY-3-OXO-4-CHOLENOIC ACID IN HUMAN URINE

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ABSTRACT

A microplate enzyme immunoassay (EIA) was developed for the measurement of glycine- and taurine-conjugated 7α-hydroxy-3-oxo-4-cholenoic acids (CDCA-Δ^4-3-one) in human urine. The antiserum was prepared by immunizing rabbits with N-(7α-hydroxy-3-oxo-4-cholen-24-oyl)-3-amino-propionic acid - bovine serum albumin conjugate. A colorimetric EIA was established using horseradish peroxidase-labeled antigen having a shorter bridge length than that of the immunogen, and 3, 3', 5, 5'-tetramethylbenzidine /hydrogen peroxide for the measurement of the enzyme activity.

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The reactivities of the antiserum for glycine and taurine conjugates of CDCA-Δ^4-3-one was almost the same. The specificity of the antiserum was investigated by determining the cross-reactivities of various bile acids and related compounds.

An appropriate dose-response curve for conjugated CDCA-Δ^4-3-one was obtained in the range of 0.05-10 ng/well. This method was used for direct analysis of conjugated CDCA-Δ^4-3-one in urine of healthy infants and patients with liver diseases.

INTRODUCTION

Clayton et al. reported the presence of 3-oxo-Δ^4-bile acids, 7αa-hydroxy-3-oxo-4-cholenoic (CDCA-Δ^4-3-one) and 7a,12a-dihydroxy-3-oxo-4-cholenoic (CA-Δ^4-3-one), in the urine of some infants with severe liver disease.(1) Also, a novel disorder in bile acid biosynthesis, named '3-oxo-Δ^4-steroid 50-reductase deficiency,' which is characterized by markedly elevated urinary levels of the amidates of CDCA-Δ^4-3-one and CA-Δ^4-3-one, was described by Setchell et al.(2) Their measurements in urine are important from a clinical point of view, since the elevated amounts of these bile acids in urine may reflect liver diseases, including the above disorder. On the other hand, we also have identified these 3-oxo-Δ^4-bile acids in the urine of some patients with severe cholestasis, and we have shown that the presence of large amounts of CDCA-Δ^4-3-one was positively correlated with a poor prognosis.(3, 4) These observations led to the speculation that urinary excretion of CDCA-Δ^4-3-one may be useful as an important indicator of poor prognosis in patients with liver diseases. Therefore, measurements of the urinary concentrations of 3 CDCA-Δ^4-bile acids, especially CDCA-Δ^4-3-one, are of particular interest.

Quantification of bile acid has been carried out mainly by gas chromatography-mass spectrometry (GC-MS).(1-6). This method, however, is tedious and time-consuming. A more simple and rapid method has been needed for periodic screening of a large number of liver disease subjects. Thus, an immunoassay that does not require prior deanup and enables direct analysis of a large number of samples appears to be suitable for routine analysis.

This paper describes an EIA for determination of conjugated CDCA-Δ^4-3-one and its application to the determination of bile acid concentration in urine samples from both healthy infants and patients with liver diseases.

EXPERIMENTAL

Chemicals and Reagents

Horseradish peroxidase (EC 1, 11, 1, 7, HRP) (grade I-C, 200 units/mg) and bovine serum albumin (BSA) were purchased from 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. Polystyrene 96-well microtiter plates were purchased from Sumitomo Bakelite Co. (Tokyo, Japan).

CDCA-Δ^4-3-one was chemically synthesized as reported previously.(7) The reference bile acids and steroids were either synthesized in our laboratory or were commercially obtained. 3-Oxo-Δ^4-steroid 5β-reductase was purified from rat livers according to the procedure described by Okuda et al.(8) All other reagents used were of analytical grade.

Apparatus

Melting points (mp) were determined with a Mitamura micro hot-stage apparatus and are uncorrected. 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; bs, broad singlet). Infrared (IR) spectra were obtained using a JASCO FT/IR 300 spectrometer and are expressed in cm^{-1}. The microplate reader was a Bio-Rad Model 2550 EIA Reader (Richmond, CA, USA).

Urine Samples

Urine samples were collected without preservatives from four patients (1-2 months old) with liver disease and from ten healthy infants (1-2 months old) as controls. All specimens were stored at –25°C until analysis.

Preparation of the Haptenic Derivatives of CDCA-Δ^4-3-one

Trichloroethyl N-(7α-hydroxy-3-oxo-4-cholen-24-oyl)-aminopropionate and trichloroethyl N-(7α-hydroxy-3-oxo-4-cholen-24-oyl)-2-aminoacetate [2 and 5 in Figure l]: 2, 2, 2-trichloroethyl 3- aminopropionate

p-toluenesulfonate (2.5 g) or 2, 2, 2-trichloroethyl 2-aminoacetate hydrochloride (1.6 g), diethyl cyanophosphonate (0.9 mL) and triethylamine (1 mL) were added to a solution of CDCA-Δ^4-3-one (**1**, 1 g) in dimethyl formamide (5 mL) under ice-cooling. The mixture was stirred for 60 min under ice-cooling and then diluted with ethyl acetate. The organic layer was washed with cold hydrochloric acid adjusted to pH 4-5 and water, and dried over anhydrous sodium sulfate. After evaporation of the solvent at room temperature, the crude products were purified by column chromatography on silica gel using chloroform/methanol (50/1, v/v) as an eluent to give **2** (900 mg) and **5** (1.3 g) as colorless oily products.

Compound **2** : IR (neat): 3330 (OH, NH), 1752 (C=O, ester), 1659 (C=O, amide, ketone). ^{1}H-NMR (CDCl,): 0.71(3H,s,18-CH_3), 0.92 (3H, d, J=6.4 Hz, 2 1-CH_3) 1.19 (3H, s, 19-CH_3). 2.72 (2H, t, J=5.9 Hz, -NHCH$_2$CH$_2$CO$_2$-), 3.58 (2H, t, J=5.9 Hz, -NHCH$_2$CH$_2$CO$_2$-), 3.97 (1H, bs, 7b-OH), 4.78 (2H, S, _CO$_2$CH$_2$CCl$_3$), 5.80 (1H, s, 4H), 6.00 (1H, bs, -CONHCH$_2$-)-

Compound **5**: IR (neat): 3330 (OH, NH), 1750 (C=O, ester), 1655 (C=O, amide, ketone). ^{1}H-NMR ($CDCl_3$): 0.72 (3H, S,18-CH_3), 0.95 (3H, d, J=6.4 Hz, 21-CH_3), 1.19 (3H, s, 19-CH_3), 3.97 (1H,bs, 7b-OH), 4.21 (2H, d, J=5.4 Hz, -NHCH$_2$CO$_2$-), 4.80 (2H, s, -C0$_2$CH$_2$CCl$_3$), 5.80 (1H, s, 4-H), 6.00 (1H,bs, -CONHCH$_2$-)-N-(7α-hydroxy-3-oxo-4-cholen-24-oyl)-3-aminopropiomc acid and N-(7α-hydroxy-3-oxo-4-cholen-24-oyl)-2-aminoacetic acid (**3** and **6**): 1 M potassium hydrogen phosphate (1 mL) was added to a solution of trichloroethyl ester (**2**: 450 mg, **5**: 500 mg) and zinc powder (1 g) in tetrahydrofuran (5 mL).

The mixture was stirred for 60 min at room temperature. Then the reaction mixture was diluted with ethyl acetate and filtered by suction, and the filtrate was combined and dried over anhydrous sodium sulfate. The solvent was evaporated in vacuo. The crude products were recrystallized from methanol/ether to give **3** (230 mg) as colorless prisms and **6** (150 mg) as colorless crystals.

Compound **3** : mp : 186-188°C. IR (nujol): 3330 (OH, NH), 1654 (C=O, carboxyl, amide, ketone). ^{1}H-NMR (C_5D_5N): 0.68 (3H, s, 18-CH_3), 0.93 (3H, d, J=5.9 Hz, 21-CH_3), 1. 10 (3H, s, 19-CH_3), 2.92 (2H, t, J=6.4 Hz, -NHCH$_2$CH$_2$CO$_2$-), 3.96 (2H, t, J=6.4 Hz, -NHCH$_2$CH$_2$CO$_2$-), 4.06 (1H, bs, 7b-OH), 6.02 (1H, s, 4-H), 8.37 (1H, bs, -CONHCH$_2$-). Anal. Calcd. for $C_{27}H_{41}NO_5.H_2O$: C, 67.89; H, 9.07; N, 2.93, Found: C 68.12; H, 8.85; N, 2.61.

Compound **6**: mp: 192-194 T. IR (nujol) 3340 (OH, NH), 1653 (C=O, carboxyl, amide, ketone). ^{1}H-NMR (C_5D_5N): 0.67 (311, s, 18-CH_3), 0.92 (3H, d, J=4.4 Hz, 21-CH_3), 1.10 (3H, s, 19-CH_3), 4.06 (1H, bs, 7b-OH), 4.48 (2H, d, J=3.9 Hz, -NHCH$_2$CO$_2$-), 6.00 (1H, s5 4-H), 8.39 (1H, bs,

-CONHCH$_2$-). Anal. Calcd. for $C_{26}H_{39}NO_5 \cdot H_2O$: C, 67.36; H, 8.91; N, 3.02, Found: C, 67.55; H, 8.68; N, 2.69.

p-Nitrophenyl N-(7α-hydroxy-3-oxo-4-cholen-24-oyl)-aminoacetate: (7) p-Nitrophenol (54 mg) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (104 mg) were added to a solution of compound **6** (140 mg) in dioxane (3 mL) containing dimethylformamide (10 μL). The mixture was stirred overnight at room temperature and was then diluted with ethyl acetate. The organic layer was washed with water and dried over anhydrous sodium sulfate. After evaporation of the solvent, the crude product was purified by column chromatography on silica gel using chloroform / acetone (5/1, v/v) as an eluent to give 7 (50 mg) as a pale yellow amorphous powder. IR (nujol): 3330 (OH, NH), 1770 (C=O, ester), 1660 (C=O, amide, ketone). ^{1}H-NMR ($CDCl_3$): 0.71 (311, s, 18-CH_3), 0.95 (3H, d, J=6.4 Hz, 21-CH_3), 1.19 (3H, s, 19-CH_3), 3.97 (1H, bs, 7b-OH), 4.33 (2H, d, J=5.9 Hz, -NHCH$_2$CO$_2$–), 5.81 (1H, s, 4-H), 6.04 (1H, bs, -CONHCH$_2$), 7.32, 8.29 (each 2H, d, j=8.8 Hz, aromatic H).

Preparation of Immunogen and Antiserum

Tri-n-butylamine (28.3 μL) and isobutyl chloroformate (13 μL) were added to a solution of the hapten (3, 41.3 mg) in dimethylformamide (0.5 ml) and dioxane (2 ml), and the whole mixture was stirred for 60 min at 10°C. Then a solution of BSA (100 mg) in dioxane (0.4 mL) and water adjusted to pH 9 with sodium hydroxide (2.2 mL) was added, and the mixture was further stirred for 3 h at 4°C. The reaction mixture was dialyzed against cold water for 2 d and was then filtered through a cellulose acetate membrane filter. The filtrate was lyophilized to afford the bile acid-BSA conjugate (93 mg) as a fluffy powder. The molar ratio of the bile acid to BSA was determined to be 27 by measuring the UV absorption of the conjugate in 0.1 M sodium bicarbonate using molar absorptivity 1.5 x 10^4 at 240 nm.

The immunogen (1.5 mg) was dissolved in saline (1.5 mL) and emulsified with complete Freund's adjuvant (1.5 mL). The emulsion was subcutaneously injected into domestic female rabbits (2-2.5 kg) at multiple sites along the back. The injection was repeated once a week for a first month and then once a month thereafter. Antiserum was obtained by centrifugation of blood at 2800 rpm for 20 min, and it was stored at -25°C in the presence of 0.1% sodium azide.

Preparation of HRP-Labeled Antigen

p-Nitropheny ester 7 (596 mg) in dioxane (120 μL) was added to a solution of HRP (1 mg) in 50 mM borate buffer (pH 8.5) (200 μL), and the mixture was stirred at 4°C overnight. The resulting solution was dialyzed against 10 mM phosphate buffer (pH 7.4) overnight, and it was then purified by gel chromatography using Bio Gel P-60 (Bio Rad) equilibrated with 10 mM phosphate buffer (pH 7.4) containing 0.9% sodium chloride. Fractions containing enzyme activity were pooled, diluted 10-fold with 50 mM phosphate buffer (pH 7.4) containing 0.1 M sodium chloride and 0.1% BSA (assay buffer), and stored at 4°C in the presence of 1% sucrose until use. The HRP-labeled antigen solution was diluted to 154 ng/mL with the assay buffer just before its use.

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 50 mM phosphate buffer (pH 7.4) containing 0.1 M sodium chloride. The plates were allowed to stand overnight at 4°C. The wells were washed three times with 10 mM phosphate buffer (pH 7.4), post-coated by the addition of 250 ml of the assay buffer, and stored at 4°C until use.

EIA Procedure

All solutions were diluted with the assay buffer. An appropriately diluted urine sample or standard solution (50 μL), the HRP-labeled antigen (50 mL), and the first antibody (1:100000, 50 μL) were added to the microtiter plates that had been coated with the second antibody. The plates were 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 solution (9:1, v/v, 150 μL) of 0.023% hydrogen peroxide and 0.21 mM 3,3',5,5'-tetramethybenzidine in 0.05 M acetic acid-citric acid buffer (pH 5.5) containing 3% of dimethylsulfoxide was added, and the solution was incubated at room temperature for 1 h. The reaction was terminated by adding 150 μL of 0.5 M sulfuric acid, and the absorbances were measured at 450 nm using a microplate leader.

In some experiments, urine samples were treated with purified 3-oxosteroid 5β-reductase and then analyzed by the EIA method to confirm whether the antibody reacted with compounds present in urine other than

3-oxo-Δ^4-ssteroids. Urine (10 μL) was incubated with the purified enzyme (10 μg) in a total volume of 0.2 mL of 0.1 M Tris-HCl buffer (pH 7.4) containing 0.1 mM NADPH at 37°C for 20 min. The mixture was appropriately diluted with the assay buffer and analyzed by the EIA.

Cross-Reaction Study

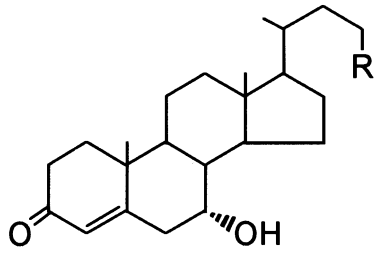
Specificity of the assay system was assessed by cross-reactivity with structurally related bile acids and steroids. The cross-reactivity was calculated from the ratio of the concentration of taurine-conjugated CDCA-Δ^4-3-one to that of the tested steroids at 50% inhibition of binding of the enzyme-labeled antigen.

RESULTS AND DISCUSSION

In this study, N-(7α-hydroxy-3-oxo-4-cholen-24-oyl)-3-amino-propionic acid and N-(7α-hydroxy-3-oxo-4-cholen-24-oyl)-3-aminoacetic acid were used as the haptenic derivatives for preparation of the immunogen and the enzyme-labeled antigen, respectively, on the basis of our previous data.(9) CDCA-Δ^4-3-one is thermally unstable and is easily converted into its dehydrated products, 3-oxochola-4,6-dien-24-oic acid, or into unknown degradation products, under alkaline or acidic conditions. Therefore, a series of preparations of haptenic derivatives was carried out under mild conditions. The BSA conjugate, as an immunogen, was prepared by the mixed anhydride method. Preparation of HRP-labeled antigen was performed by the activated ester method.

The immunogen emulsified with complete Freund's adjuvant was subcutaneously administered to three rabbits. The appropriate antiserum was obtained from rabbits at 4 months after the first administration of the immunogen. No significant differences in titer or specificity were observed between the antisera elicited in two rabbits; however, the antiserum elicited in the other rabbit was more specific for the dehydrated product, 3-oxochola-4,6-dien-24-oic acid, than for the conjugated CDCA-Δ^4-3-one. This is attributed to the conversion of a part of CDCA-Δ^4-3-one residue of the immunogen into 3-oxochola-4,6-dien-24-oic acid in the rabbits.

The appropriate dilution of the antiserum and the appropriate amount of HRP-labeled antigen were first examined to establish an EIA based on the principle of competitive inhibition. From the results obtained by incubation of various dilutions of the antiserum (1:5000-500000 dilution) with 3.8-15.2 ng of HRP-labeled antigen, we chose to use 1: 100000 dilution of



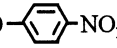
1 : R = COOH
2 : R = $CONHCH_2CH_2COOCH_2CCl_3$
3 : R = $CONHCH_2CH_2COOH$
4 : R = $CONHCH_2CH_2CONH$ - BSA
5 : R = $CONHCH_2COOCH_2CCl_3$
6 : R = $CONHCH_2COOH$
7 : R = $CONHCH_2COO$-  -NO_2
8 : R = $CONHCH_2CONH$ - HRP

Figure 1. Structures of CDCA-Δ^4-3-one and its conjugates. BSA; bovine serum albumin, HRP: horseradish peroxidase.

the antiserum and 7.7 ng of HRP-labeled antigen in all subsequent assays. A typical dose-response curve for taurine-conjugated CDCA-Δ^4-3-one is shown in Figure 2. The dose-response curve gave a useful assay range of 0.05-10 ng per well.

Specificity of the method was investigated by examining the cross-reactivities of various bile acids and related steroids (Tables 1 and 2). As expected from our previous data,(9) the greatest reactivity was observed with glycine-conjugated CDCA-Δ^4-3-one to be 98.0%. This was advantageous to our purpose because CDCA-Δ^4-3-one in urine is present as glycine or taurine conjugate. Conjugated CA-Δ^4-3-one, one of major 3-oxo-Δ^4-bile acids in urine, showed negligible competition with the antibody ranging from 0.59 to 1.44% relative to taurine-conjugated CDCA-Δ^4-3-one. On the other hand, significant cross-reactivities were observed with the dehydrated product, 3-oxochola-4,6-dien-24-oic acid (8.01-15.6%), and 7α-hydroxy-3-oxo-4-cholestenoic acid (20.6%). The proportion of the former to CDCA-Δ^4-3-one in urine of the patients with liver diseases has been shown to be less than 20% by GC-MS(3) and HPLC analysis,(10) and the

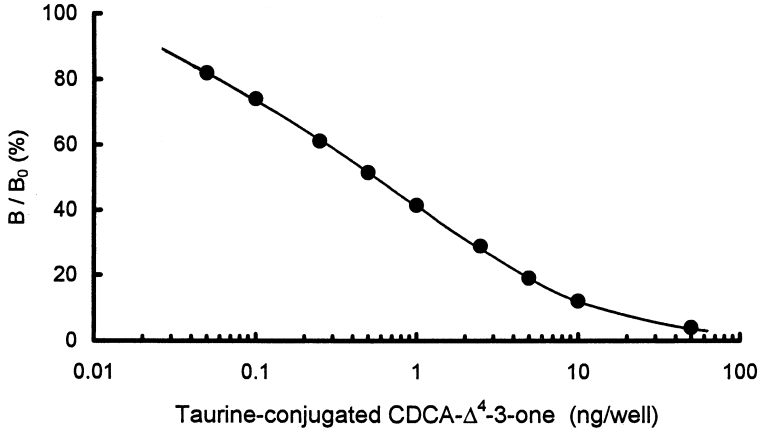


Figure 2. Dose-response curve for taurine-conjugated CDCA-Δ^4-3-one.

Table 1. Cross-Reactivities of Antiserum with Related Bile Acids

Bile Acid	Cross-Reactivity, %		
	Unconjugated	Glycine Conjugated	Taurine Conjugated
7α-Hydroxy3-oxo-4-cholenoic acid (CDCA-Δ^4-3-one)	51.0	98.0	100
7α, 12α-Dihydroxy-3-oxo-4-cholenoic acid (CA-Δ^4-3-one)	0.59	0.73	1.44
3-oxochola-4,6-dienoic acid	8.01	14.4	15.6
12α-Hydroxy-3-oxochola-4,6-dienoic acid	1.06	0.98	1.36
Cholic acid	0.03	0.03	< 0.01
Chenodeoxycholic acid	0.06	0.28	0.03
Deoxycholic acid	0.07	0.06	0.03
Lithocholic acid	< 0.01	0.01	0.01
1β-Hydroxycholic acid	0.03	0.02	< 0.01
2β-Hydroxycholic acid	< 0.01	< 0.01	< 0.01
4β-Hydroxycholic acid	< 0.01	< 0.01	< 0.01
6α-Hydroxycholic acid	< 0.01	< 0.01	< 0.01
1β-Hydroxychenodeoxycholic acid	0.03	0.02	< 0.01
2β-Hydroxychenodeoxycholic acid	0.08	< 0.01	0.03
4β-Hydroxychenodeoxycholic acid	0.12	< 0.01	< 0.01
6α-Hydroxychenodeoxycholic acid	< 0.01	8.92	0.15
1β-Hydroxydeoxycholic acid	0.05	< 0.01	0.05
4β-Hydroxydeoxycholic acid	< 0.01	< 0.01	< 0.01

Table 2. Cross-Reactivities of Antiserum with Related Bile Acids and Steroids

Bile Acid	Cross-Reactivity, %
7α-Hydroxy3-oxo-4-cholestenoic acid	20.6
7α, 12α-Dihydroxy-3-oxo-4-cholestenoic acid	0.34
7α-Hydroxy-4-cholesten-3-one	0.07
7α, 12α-Dihydroxy-4-cholesten-3-one	0.68
3β-Hydroxy-5-cholenoic acid	0.58
3β, 12α-Dihydroxy-5-cholenoic acid	0.09
7α, 12α-Dihydroxy-3-oxo-5β-chol-1-enoic acid	<0.01
7α-Hydroxy-3-oxo-5β-cholanoic acid	4.35
7α, 12α-Dihydroxy-3-oxo-5β-cholanoic acid	<0.01
Cortisol	0.08
Dexamethasone	<0.01
Androstenedione	1.64
Testosterone	2.81
Progesterone	7.25

Table 3. Reliability for Taurine Conjugated CDCA-Δ^4-3-one Measurement

		Recovery (%)	
Added (ng)	n	Mean $\pm$ SD	CV (%)
Intra-assay			
0.25	10	114 $\pm$ 15	14
0.5	10	96 $\pm$ 9	9
1.0	10	111 $\pm$ 3	3
Inter-assay			
0.25	5	110 $\pm$ 8	7
0.5	5	111 $\pm$ 16	14
1.0	5	96 $\pm$ 10	10

latter has not been detected in urine from infants.(11) Thus, the cross-reactivities with the above bile acids would not lead to serious errors in the assay. All the other bile acids and steroids, including 1β-, 2β-, 4β-, and 6α-hydroxylated bile acids, which are major components of urinary bile acids in the fetal and neonatal periods, showed practically negligible cross-reactivities.

The reliability of the EIA was assessed by measuring taurine-conjugated CDCA-Δ^4-3-one (0.25-1.0 ng) added to bile acid-free urine prepared by charcoal extraction.(12) As shown in Table 3, the recovery of taurine-

conjugated CDCA-Δ^4-3-one ranged from 96 to 114% for samples containing 0.25-1.0 ng of taurine-conjugated CDCA-Δ^4-3-one. The intra-assay and inter-assay coefficients of variation (CV) were 3-14% at all levels. The results show that the present assay system can be applied to urine samples.

The assay method was then applied to the measurement of conjugated CDCA-Δ^4-3-one in urine samples obtained from healthy infants and from patients with liver disease.

Urinary excretion levels of conjugated CDCA-Δ^4-3-one in four liver disease patients and ten normal infants are shown in Figure 3. The mean of conjugated CDCA-Δ^4-3-one in the urine of the healthy 1-2-month-old

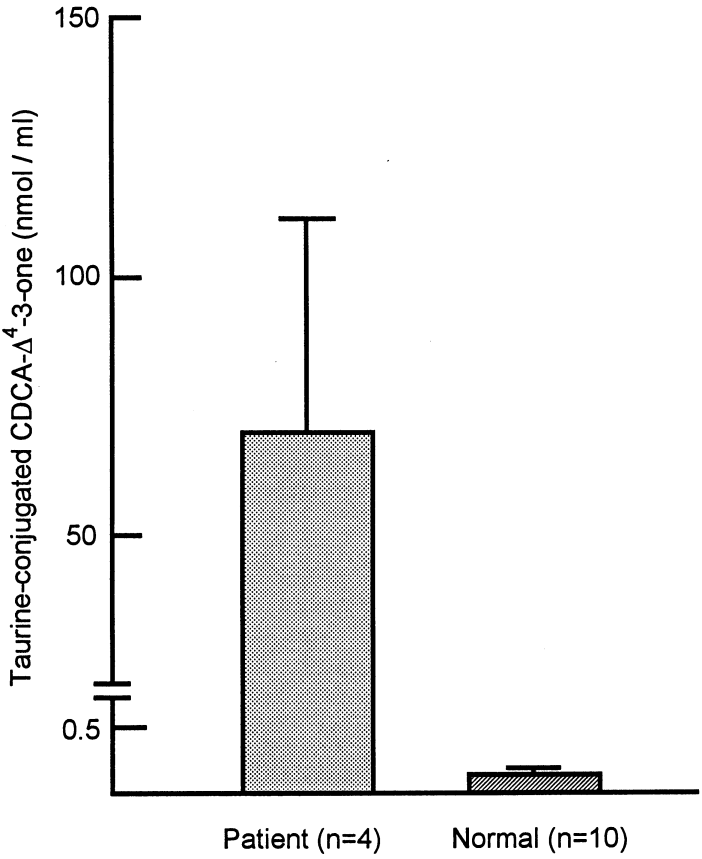


Figure 3. Concentration of conjugated CDCA-Δ^4-3-one in urine from the patients with liver diseases and healthy infants.

infants was estimated to be 0.18 nmol/mL. On the other hand, the bile acid level in urine from the liver disease patients, 71.5 ± 41 nmol/mL (mean $\pm$ SD, range; 41-131 nmol/mL), was significantly higher than that in healthy subjects. Treatment of the urine with purified 3-oxo-Δ^4-steroid 5β-reductase, which showed the activity for many of the 3-oxo-Δ^4- steroids,(8) showed no significant inhibition of bound enzyme activity, suggesting that the antibody did not react with components present in the urine other than 3-oxo-Δ^4- steroids.

The proposed method is suitable for the determination of conjugated CDCA-Δ^4-3-one in routine clinical analysis. Further studies on the urinary excretion of the bile acid in patients with hepatobiliary disease using this method are in progress.

ACKNOWLEDGMENTS

This work was supported, in part, by grants from Japan Society for the Promotion of Science and the Foundation for Scientific Research in High-Tech Research Center from the Ministry of Education, Science, Sports, and Culture of Japan; correspondence should be sent to Dr. Kurosawa.

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Received August 12, 2000 Manuscript 3006
Accepted October 1, 2000