Enzyme-linked immunoassay of ursodeoxycholic acid in serum

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Abstract A sensitive and specific enzyme-linked immunoassay for the measurement of ursodeoxycholic acid in human serum was developed. Ursodeoxycholic acid conjugated to alkaline phosphatase (from calf intestine) was used as a tracer. An antiserum to ursodeoxycholic acid serum was raised in rabbits using ursodeoxycholic acid–bovine serum albumin conjugate as an antigen. The binding required 1 hr at 42°C; separation of the bound tracer was achieved by addition of a second antibody, and alkaline phosphatase activity of this bound complex was measured colorimetrically. The ratio of bound to total enzyme activity decreased linearly with a logarithmic increase in ursodeoxycholic acid concentration from 20 to 900 pmol. The specificity and sensitivity of this enzyme-linked immunoassay were similar to those of a radioimmunoassay reported previously. The serum ursodeoxycholic acid levels measured by this method correlated well with those determined by gas–liquid chromatography and radioimmunoassay. Based on these findings, this enzyme-linked immunoassay of bile acid might be useful as a tool for the routine clinical analysis of serum bile acids.—**Ozaki, S., A. Tashiro, I. Makino, S. Nakagawa, and I. Yoshizawa.** Enzyme-linked immunoassay of ursodeoxycholic acid in serum. *J. Lipid Res.* 1979. **20:** 240–245.

Supplementary key words serum bile acid · gas–liquid chromatography · radioimmunoassay · hepatic disease

In a recent study dealing with bile acid metabolism, serum bile acids were routinely analyzed by gas–liquid chromatography (1). However, this method requires many steps and relatively large samples of serum. In 1973, Simmonds et al. (2) developed a sensitive and specific radioimmunoassay (RIA) for estimating serum conjugated cholic acid, and it became possible to measure serum bile acids in a very small sample. Thereafter, RIA methods for various bile acids were reported by several investigators (3–5), and we established a RIA for ursodeoxycholic acid (UDCA) (6).

In 1971, Engvall and Perlmann (7) developed an enzyme-linked immunoassay (EIA) for the quantitation of immunoglobulin G, which used the same principle as RIA but employed an enzyme–antigen conjugate instead of a radioactive isotope tracer. The EIA procedure has been applied to the measurement of peptide hormone (8–10), steroid hormone (11–13), and morphine (14), but there is no information concerning EIA of bile acids.

This report describes an application of the EIA method for the determination of serum UDCA.

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade. Nonradioactive UDCA was synthesized by Tokyo Tanabe Company (Tokyo, Japan), and had a greater than 99% purity as judged by gas–liquid and thin-layer chromatography. [11,12-$^3H_2$]UDCA (sp act 6.02 mCi/mg, supplied by Tokyo Tanabe Company) showed greater than 99% purity by thin-layer chromatography and zonal scanning. Bovine serum albumin (BSA) was obtained from Armour Laboratories (Kankakee, IL). 1-Ethyl-3(3-dimethyl-aminopropyl)carbodiimide and alkaline phosphatase (AP) from calf intestine were purchased from Sigma Chemical Co., (St. Louis, MO). Goat anti-rabbit γ-globulin, which was used as a second antibody, was obtained from Eiken Chemical (Tokyo).

Antigen and antiserum

UDCA–BSA conjugate was prepared by the method of Erlanger et al. (15). Antiserum to UDCA was prepared in rabbits by the method of Simmonds et al. (2). Both procedures were reported in our previous paper (6).

Preparation of UDCA–AP conjugate

The UDCA–AP conjugate was prepared as follows. UDCA (0.1 mg) containing [11,12-$^3H_2$]UDCA (1.67×10^6 dpm) was dissolved in a small volume of ethanol,

Abbreviations: RIA, radioimmunoassay; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; EIA, enzyme-linked immunoassay; BSA, bovine serum albumin; AP, alkaline phosphatase.

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and the solution was adjusted to pH 4 with 0.001 N HCl. Then, 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide (10.0 mg) and 0.5 mg of AP were added with stirring. After 2 hr, an additional 15.0 mg of 1-ethyl-3(3-dimethyl-aminopropyl)carbodiimide was added and the solution was stirred for 24 hr. The reaction mixture was dialyzed in a cellulose tube (Visking Company, Chicago, IL) against cold water for 72 hr. All procedures were carried out at 4°C, and the UDCA–AP conjugate solution was stored at 4°C. The number of steroid molecules linked to an AP molecule was calculated from a small amount of radioactive UDCA that was added with cold UDCA to AP; the results showed that this conjugate contained 7.6 ± 0.9 (mean ± SD) UDCA molecules per AP (steroid number, 7.6). As indicated in **Table 1,** the steroid number was enhanced after addition of cold UDCA: e.g., 21.4 for 0.5 mg of UDCA, and 42.0 for 2.0 mg of UDCA.

Radioimmunoassay

The procedure of UDCA–RIA was reported in a previous paper (6).

Enzyme-linked immunoassay

The assay system consisted of 0.1 ml of bile acid-free serum containing standard UDCA or an unknown serum sample from which AP activity was removed by heating at 60°C, for 1 hr, 0.1 ml of UDCA–AP conjugate, 0.1 ml of antiserum (dilution 1:20), and 0.2 ml of buffer. The components of the incubation mixture were diluted with 0.01 M potassium phosphate buffer, pH 7.4. The tubes were incubated at 42°C for 1 hr. The second antibody was then added, and a second incubation was carried out at 4°C overnight. This incubation mixture was centrifuged at 900 *g* for 15 min at 4°C; the supernatant was then aspirated and discarded. The precipitate was washed with 1 ml of cold buffer and recentrifuged under the same conditions.

The AP activity of the bound complexes was determined as follows. Two ml of 0.05 M sodium carbonate buffer (pH 10.0) containing 0.95 mg/ml disodium phenylphosphate and 1.1 mg/ml 4-aminoantipyrin was added to the bound complex. After the reaction mixture was allowed to stand at 37°C for 60 min, the test tube was immediately placed into ice-cold water, and 2 ml of color reagent containing 12 mg/ml $K_3Fe(CN)_6$ was added. The absorbance at 570 nm was measured by spectrophotometry.

TABLE 1. Enzyme activity and steroid number of UDCA–AP conjugates

	UDCA-AP Conjugate Preparation Number		
	1	2	3
Amount of UDCA used (mg)	0.1	0.5	2.0
UDCA incorporated into enzyme (steroid number)	7.6	21.4	42.0
Enzyme activity[a] (percent of control)	37.3	25.9	1.3

[a] A control enzyme was treated with carbodiimide in the absence of UDCA, and AP activity of a control enzyme was 15.8% of untreated enzyme activity.

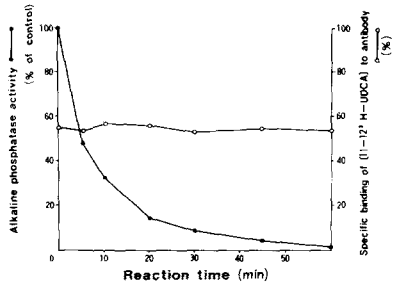

Fig. 1. Effect of reaction time on AP activity. A serum with a high AP activity (46 King-Armstrong units) was reacted at 60°C with [11,12-$^3H_2$]UDCA.

Subjects

Nine healthy persons, eight patients with gallstones undergoing UDCA treatment (oral, 450 mg/day) and six patients with hepatic diseases were used in this study. The normal subjects were persons without hepatobiliary diseases selected from the medical staff of our clinic. Of the group of hepatic patients, one had chronic hepatitis, three had acute hepatitis, and two had compensated liver cirrhosis. Blood samples were obtained in the morning before breakfast after an overnight fast; separated sera were kept frozen at −20°C.

RESULTS

UDCA–AP conjugate

As shown in Table 1, three different UDCA–AP conjugates with the steroid numbers 7.6, 21.4, and 42.0, respectively, were prepared by increasing the amount of cold UDCA. Although all conjugates still possessed AP activity, the values decreased with increasing steroid number. All experiments described in the present study were carried out with the preparation having the steroid number 7.6.

Measurement of AP activity

Effect of heating time on AP activity. [11,12-$^3H_2$]UDCA was added to 0.1 ml of an abnormal serum (AP, 46 King-Armstrong units) and allowed to stand at 60°C for 5–60 min. As shown in **Fig. 1**, a decrease of

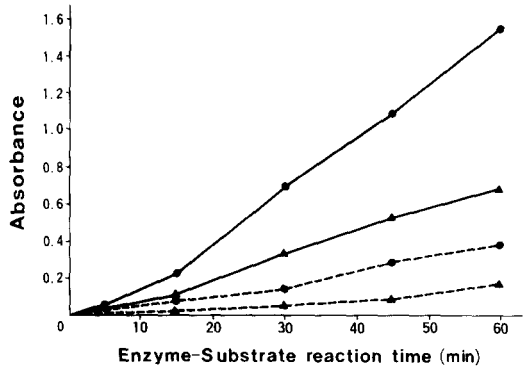

Fig. 2. Effect of substrate–enzyme reaction time on AP activity of the bound complex. Different concentrations of UDCA–AP conjugate (high, circle; low, triangle) were incubated at 37°C with (– – –) or without (——) an excess of nonradioactive UDCA (5.1 nmol/tube).

AP activity was observed with time. The enzyme activity fell to 50% of control values after 5 min and to 2% after 60 min. However, specific binding of radioactive UDCA to antibody was not affected under these experimental conditions.

Effect of substrate–enzyme reaction time on AP activity of the bound complex. Two different concentrations of UDCA–AP conjugate solution were assayed by the standard method with or without an excess of nonradioactive UDCA (5.1 nmol), and the bound complex was allowed to react with the substrate at 37°C over time periods from 5 to 60 min. As shown in **Fig. 2,** the enzyme activity of the bound complex increased linearly with time in each case, even in the presence of UDCA. This result showed that there was a sufficient amount of substrate in the reaction mixture and that neither substrate nor enzyme were destroyed during 60 min. Based on these data, a reaction temperature of 37°C and a time period of 60 min were chosen for further experiments.

Effect of reaction temperature on activity of the bound complex. The bound complex was reacted at 0°C, 24°C, and 37°C for periods of 5–60 min, respectively (**Fig. 3**). At 24°C and 37°C, the enzyme activity increased linearly with time. At 0°C, the reaction rate remained very low over the 60-min period; therefore, the tube was immediately placed in ice-cold water after 60 min in order to stop the reaction.

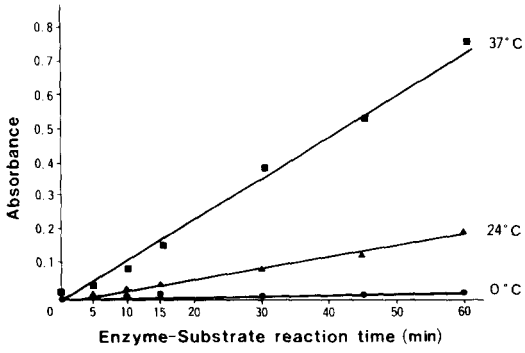

Fig. 3. Effect of temperature on AP activity of the bound complex. The complex was reacted at 0°C, 24°C, and 37°C for different reaction time periods.

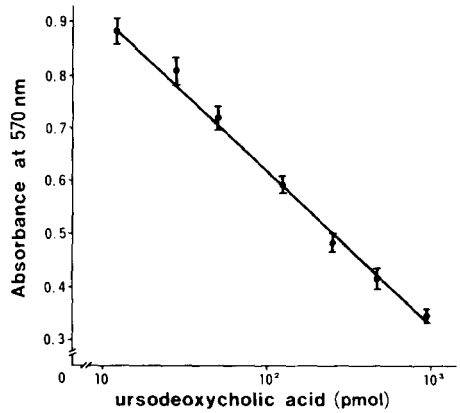

Fig. 4. Standard curve for measurement of UDCA by EIA.

Effect of serum concentration on AP activity of the bound complex. Bile acid-free serum without AP was prepared by heating the serum at 60°C for 60 min and by charcoal extraction (2); a standard assay was carried out using different amounts of this serum. However, the enzyme activity of the bound complex was not affected by the serum protein concentration (data are not shown here).

Immunoassay

Standard curve. The percentage of the bound AP activity decreased linearly with a logarithmic increase in UDCA from 20 to 900 pmol (**Fig. 4**).

Specificity. The percent cross reactivity of antiserum with other bile acids was calculated at 50% displacement of the bound AP activity of UDCA–AP conjugate. As shown in **Table 2**, this antiserum possessed an extremely high specificity for UDCA, glyco-UDCA,

TABLE 2. Cross reactivity of bile acids with the antiserum

Bile Acid	Cross Reactivity (%)
Ursodeoxycholic	100
Glycoursodeoxycholic	180
Tauroursodeoxycholic	180
Chenodeoxycholic	<0.1
Glycochenodeoxycholic	<0.1
Taurochenodeoxycholic	<0.1
Cholic	none
Glycocholic	none
Taurocholic	none
Lithocholic	<0.1
Glycolithocholic	<0.1
Deoxycholic	none

TABLE 3. Recovery of UDCA added to serum (n = 3)

	UDCA Added (pmol)			
	59	120	235	477
	(pmol)			
UDCA measured[a] (mean ± SD)	55.4 ± 0.9	131.4 ± 2.7	226.4 ± 4.9	436.4 ± 9.5
	(%)			
Recovery (mean ± SD)	93.9 ± 1.5	109.5 ± 2.3	96.3 ± 2.1	91.5 ± 2.0

[a] The values mean an increase over amount prior to addition.

Increasing amounts of UDCA were added to aliquots of three different sera, assays were carried out and recovery of added UDCA was calculated.

and tauro-UDCA. However, cross reactivity of conjugated UDCA was almost twice as much as that of free UDCA. This finding was similar to that from RIA reported previously.

Recovery of added UDCA. Recovery of UDCA was determined by the addition of different amounts of UDCA (59–477 pmol/0.1 ml serum) to three serum samples. The recoveries were 91.5–109.5% (**Table 3**).

Dilution test. Three serum samples (940, 500, and 240 pmol of UDCA/0.1 ml) were diluted 1:2, 1:4, and 1:8 with bile acid-free serum without AP. The results are indicated in **Fig. 5**. Dilutions up to 1:8 did not significantly alter the measured amount.

Reproducibility of assay results. To assess within-assay variability, the coefficient of variation was calculated for eight sets of triplicate measurements randomly chosen from five assays; the mean ± SEM of the coefficient of variation was 3.22 ± 1.28%. A high degree of between-assay reproducibility is illustrated in **Table 4** by the replicate values of 10 serum assays on two occasions at 1-week intervals.

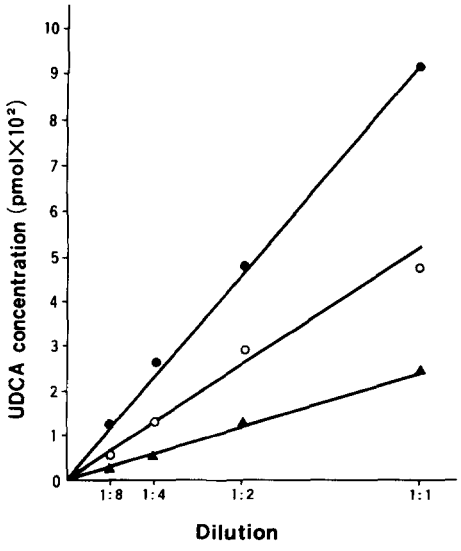

Fig. 5. Dilution test. Three serum samples (940, 500, and 240 pmol of UDCA/0.1 ml) were diluted 1:2, 1:4, and 1:8 with bile acid free serum and the assay was carried out.

Assay of human serum. Serum UDCA concentrations in nine normal subjects, in eight patients with gallstones during UDCA therapy, and in six patients with hepatic diseases were determined by gas–liquid chromatography, RIA, and EIA. However, serum UDCA values obtained by the EIA method were divided by 1.8 because serum bile acid is mainly composed of conjugate and the cross reactivity of this antiserum with conjugated UDCA was 1.8-fold as much as that of free UDCA. A high correlation was found between the results obtained by the three methods: EIA vs. gas–liquid chromatography r = 0.92 (n = 9), and EIA vs. RIA r = 0.94 (n = 24) (**Fig. 6**). In normal subjects a small amount of UDCA was detected in fasting serum: 0.27 ± 0.12 nmol/ml (mean ± SD, n = 9).

Stability of the UDCA–AP conjugate. When the UDCA–AP conjugate solution diluted with buffer was stored, the enzyme activity of the conjugate decreased rapidly; the AP activity was reduced by almost 50% after 1 day (**Fig. 7**). In order to maintain the level of enzyme activity, 0.2 M $MgCl_2$ as stabilizer was added

TABLE 4. Between-assay reproducibility

No. of Sample	Serum UDCA Concentration	
	Assay 1	Assay 2
	pmol/ml	
1.	87.5	95.0
2.	112.5	130.0
3.	185.0	171.0
4.	235.0	205.0
5.	1500.0	1650.0
6.	950.0	1100.0
7.	550.0	540.0
8.	2000.0	1650.0
9.	4200.0	4500.0
10.	3800.0	4000.0

The replicates of 10 serum assays were carried out on two occasions at 1-week intervals.

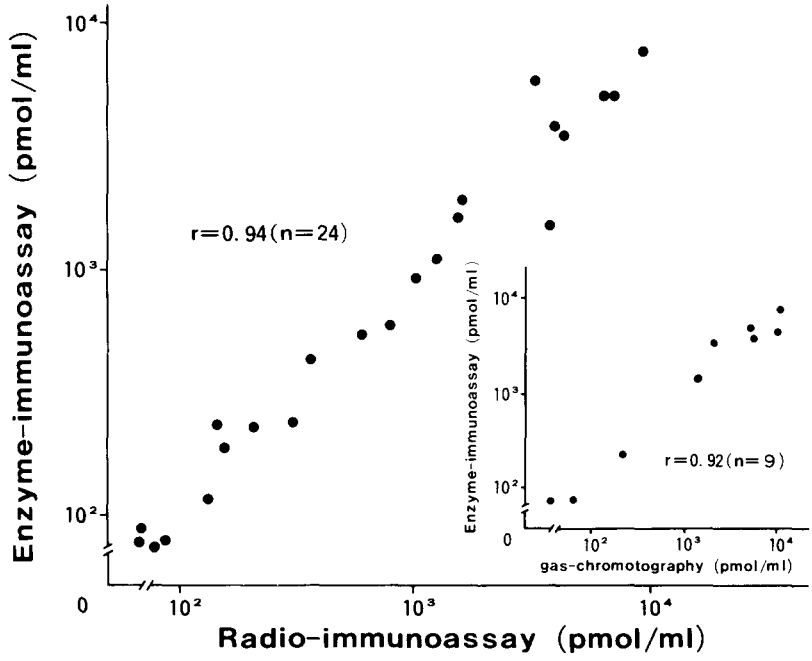

Fig. 6. Correlation among serum UDCA values by EIA, RIA, and GLC.

to the concentrated UDCA–AP conjugate solution (approximately 6.25×10^{-9}mol/dl 0.01 M potassium phosphate buffer, pH 7.4), and this solution was stored at 4°C in a dark room. The working conjugate solution was diluted with buffer containing 0.2 M $MgCl_2$ just before performing the assay. Fig. 7 shows that $MgCl_2$ was useful for stabilization of AP enzyme activity, and that UDCA–AP conjugate should be kept in the state of the concentrated solution containing 0.2 M $MgCl_2$.

DISCUSSION

In the present study we developed an EIA for serum UDCA using an assay system similar to that for UDCA–RIA, with an UDCA–AP conjugate as a tracer. The specificity, accuracy, and reproducibility of this UDCA–EIA were similar to those of UDCA–RIA we reported previously (6).

For the EIA, it would be advantageous to select an enzyme that is not present in human serum. We chose the AP from calf intestine because its activity could be easily measured. The UDCA–AP conjugate was synthesized by using 1-ethyl-3(3-dimethyl-aminopropyl)carbodiimide. This compound still possessed AP activity, but the value decreased with the increased number of UDCA molecules coupled to the enzyme. This change in enzyme activity could be due to the following: the AP molecule is a dimer (mol wt 80,000; subunit 40,000), and this configuration is necessary to maintain enzyme activity. The dimer might be destroyed by an increase in UDCA molecules coupled to the AP. In our experiments, the conjugate preparation of steroid number 7.6 was used, but we have not as yet determined the optimal steroid number to be used.

In the present experiment, a double antibody method was used for the separation of bound and free UDCA–AP conjugate. Because of the large size of the UDCA–AP conjugate molecule, the polyethylene glycol method for RIA could not be used for EIA. However, the double antibody procedure is time-consuming and is perhaps not suitable for the

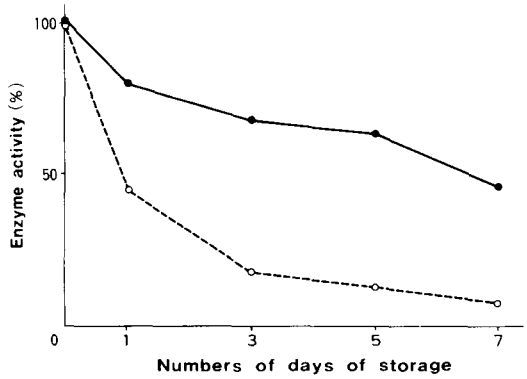

Fig. 7. Effect of $MgCl_2$ on AP activity of the diluted UDCA–AP conjugate solution. ● —— ●. The working solution diluted with buffer containing 0.2 M $MgCl_2$. ○ —— ○. The conjugate solution diluted with only buffer not containing $MgCl_2$. The diluted conjugate solution was stored at 4°C in a dark room, and the AP activity was measured the following day.

routine determination of a large number of serum samples in clinical studies.

According to our method, AP present in human serum did not interfere with the EIA, but it is preferable to inactivate the serum AP at 60°C for 60 min when a high level of AP is found in such samples.

It is well known that UDCA is the main bile acid in bear bile; there is only a small amount found in human serum and bile. In our present study, the normal level of UDCA in serum was 0.27 ± 0.12 nmol/ml (n = 9), this value was very similar to that which we reported previously (6).

In the near future much information concerning UDCA metabolism during chenodeoxycholic acid (CDCA) and UDCA therapy in patients with gallstones could be obtained by using this convenient EIA for the determination of serum UDCA.

It should be feasible to develop other bile acid EIA methods according to this procedure, but further investigation of possible conjugates and the automation of analytical procedures are needed to apply EIA to the measurement of various serum bile acids in clinical studies.

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