# Development of a solid-phase enzyme immunoassay for ursodeoxycholic acid: application to plasma disappearance of injected ursodeoxycholic acid in the rabbit

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**Abstract** A bile acid disappearance test using an enzyme immunoassay for ursodeoxycholic acid (UDCA) is presented. The immunoassay employs an antiserum produced in rabbits with UDCA coupled by amide linkage to egg albumin. An antigen (UDCA)–enzyme (β-D-galactosidase) complex was prepared by adding the *N*-hydroxysuccinimide ester of UDCA to β-D-galactosidase in a molar ratio of 5000:1. The anti-UDCA serum was coupled to glass beads and a competitive reaction between bile acids and UDCA coupled to the enzyme on the glass beads was measured by determining enzyme activity. One bead was used for each test tube. Thus it was convenient to wash and transfer the bead to a fresh test tube after incubation. The procedure requires 2.5 hr at 30°C for the competitive reaction and enzyme assay. Using a 1:100 dilution of antiserum, the intensity of fluorescence of 4-methylumbelliferone produced from 4-methylumbelliferyl-β-D-galactoside by the enzyme decreased linearly with a logarithmic increase of UDCA concentration over a range of from 0.1 to 10 pmol per assay tube. The antiserum exhibited high specificity for UDCA and its glycine and taurine conjugates, and good recovery data were obtained. The development of the enzyme immunoassay using glass beads shortens analysis time; furthermore, the method makes it possible to detect obstructive jaundice in rabbits before the serum bilirubin level is elevated. —**Maeda, Y., T. Setoguchi, T. Katsuki, and E. Ishikawa.** Development of a solid-phase enzyme immunoassay for ursodeoxycholic acid: application to plasma disappearance of injected ursodeoxycholic acid in the rabbit. *J. Lipid Res.* 1979. **20:** 960–965.

**Supplementary key words** plasma clearance test · biliary obstruction · β-D-galactosidase

The determination of serum bile acid levels is an important factor in the estimation of liver pathophysiology (1–3). Gas–liquid chromatography (GLC) has often been the technique of choice for the quantitative determination of this parameter (4–6), but this method has limited sensitivity. Therefore, radioimmune procedures were developed (7–12) that are more sensitive and more specific and require only small amounts of sample. Several bile acid tolerance tests have been reported in the literature (13–19) and recent studies using an enzyme-linked immunoassay system (20–25) have demonstrated that such methods could overcome some of the drawbacks inherent in radioimmunoassay (RIA). The study presented here was designed to develop a bile acid disappearance test using an enzyme immunoassay with highly specific rabbit anti-UDCA serum. This sensitive test should have potential value in discovering acute obstructive jaundice at a stage where serum bilirubin remains normal.

## MATERIALS

Chenodeoxycholic acid, more than 99% pure by GLC, was obtained from Eisai Co., Ltd., Tokyo, Japan. Deoxycholic acid and cholic acid were purchased from Sigma Chemical Co., Saint Louis, MO. Ursodeoxycholic acid and sodium ursodeoxycholate (50 mg/5 ml water) of more than 99% purity by GLC were obtained from Tokyo Tanabe Co., Ltd., Tokyo, Japan.

Abbreviations: CDCA, chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; UDCA, ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanoic acid; DCA, deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; CA, cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; GLC, gas–liquid chromatography; TLC, thin-layer chromatography. The prefixes glyco (G) and tauro (T) are used for bile acids having glycine or taurine in amide linkage at C-24.

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Glycine and taurine conjugates of CDCA, CA, and DCA were purchased from Calbiochem, La Jolla, CA, and were used without further drying. Glycine and taurine conjugates of UDCA were prepared by the mixed anhydride method (26) as described in the experimental section. Water-soluble carbodiimide [1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide hydrochloride] was purchased from Nakarai Chemical Co., Kyoto, Japan. *N*-Hydroxysuccinimide and *N,N′*-dicyclohexylcarbodiimide were obtained from Tokyo Kasei Kogyo Co., Ltd.

## EXPERIMENTAL

### Preparation of glycine and taurine conjugates of UDCA

The reaction products of the mixed anhydride procedure were isolated by preparative TLC on 20 × 20 cm glass plates coated with 1.0-mm thick layers of silica gel using the solvent system chloroform–methanol–acetic acid–water 65:20:10:5 (v/v). The appropriate band was scraped from the plate and the conjugated bile acid was eluted with ethanol. After evaporation of the ethanol the residue was dissolved in *n*-butanol; the butanol solution was washed with water and the solvent was evaporated in vacuo. The dried bile acid conjugates, which appeared as foamy solid flakes, were ground up and dried in vacuo at 80°C for 16 hr; further drying did not produce any weight change.

### Preparation of rabbit anti-UDCA serum

UDCA was purified by preparative thin-layer chromatography. The resultant UDCA was more than 99.95% pure by GLC. It was coupled to egg albumin (2× crystallized, Seikagaku, Kogyo Co., Ltd., Tokyo) by the carbodiimide method, molar ratio 14:1 (9). Two mg of purified UDCA and 1 mg of the water-soluble carbodiimide were added to 1 ml of ethanol in a vial and kept in the refrigerator for 30 min. The mixture was added to 7 ml of distilled water containing 16 mg of egg albumin, stirred in the cold for 2 hr, and emulsified with an equal volume of Freund's adjuvant (Difco Laboratories, Detroit, MI). One ml of this mixture was injected subcutaneously into the legs and foot pads of four New Zealand white rabbits. The antigen was administered at 1-wk intervals for 4 wk and after that at longer intervals according to the antibody titer.

Male New Zealand white rabbits, 2.5–3.5 kg, were used in all studies. The animals were housed in individual metabolic cages and maintained on a diet of Nippon CLEA CR-2 Rabbit Chow pellets. Food and water were supplied ad libitum. All animals were acclimated to this food for at least 3 wk before the experiment was begun. Ligation of the choledochus was performed under sodium nembutal anesthesia (30–50 mg/kg). The sham operation (laparotomy only) was performed under the same conditions. The sham-operated and the obstructed group were compared to normal rabbits by measuring the remaining amounts of the injected UDCA in their serum.

### Preparation of the enzyme–antigen complex

*N,N′*-Dicyclohexylcarbodiimide (1.3 g, 6.3 mmol) was added to a solution of UDCA (2.47 g, 6.3 mmol) and *N*-hydroxysuccinimide (0.72 g, 6.3 mmol) in 100 ml of cold (10°C) dioxane and allowed to stand overnight in the refrigerator. The dicyclohexyl urea was filtered off and washed with dioxane. The filtrate was evaporated to dryness and the solvent was removed in vacuo. A yield of 2.9 g of *N*-hydroxysuccinimide ester was obtained.

The β-D-galactosidase–UDCA complex was prepared by adding the *N*-hydroxysuccinimide ester (27) of UDCA (0.5 mg in 10 μl of acetone) to 0.1 mg of β-D-galactosidase (Boehringer-Mannheim, Mannheim, Germany) (dissolved in 1 ml of 0.1 M phosphate buffer, pH 6.3) in a molar ratio of 5000:1. The complex was purified on a Sepharose-6B column (1 × 30 cm), which had been equilibrated with 0.05 M sodium phosphate buffer, pH 8.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% $NaN_3$, and 0.1% bovine serum albumin (Armour Pharmaceutical Co., Chicago, IL) (buffer A). The complex solution was stable for more than 2 yr when stored at 4°C.

### Coupling of rabbit anti-UDCA serum to glass beads

The rabbit antiserum-coupled glass beads were prepared by the method of Hamaguchi et al. (25). The surfaces of the glass beads (4 mm diameter) were roughened by shaking the beads in a vessel containing carborundum suspended in water. The rough beads were washed with water, then with acetone, dried, and immersed in a 2% solution of 3-aminopropyl-triethoxysilane in acetone for 24 hr at 45°C. After washing with acetone and drying, the beads were immersed in a 1% aqueous solution of glutaraldehyde for 1 hr and washed with 0.25 M sodium phosphate buffer, pH 7.5. They were then placed in a rabbit antiserum which had been diluted with 0.25 M sodium phosphate buffer, pH 7.5 (1:100 dilution) overnight at 4°C. The beads, thus coupled with anti-UDCA serum, were washed with 0.25 M sodium phosphate buffer, pH 7.5, then with buffer A, and were stored in this buffer at 4°C until used.

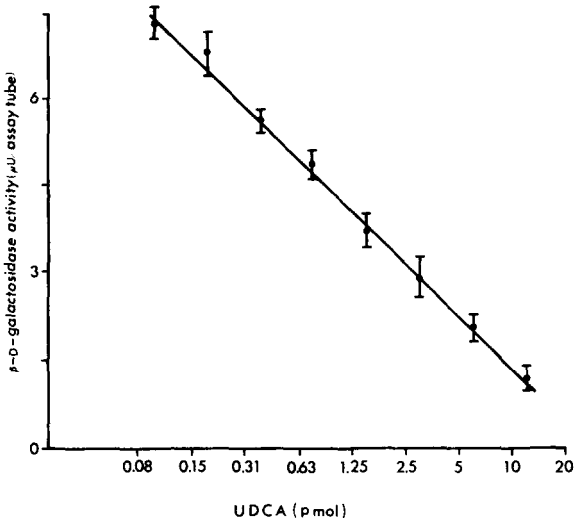


**Fig. 1.** Standard curve (antiserum dilution 1:100) showing intensity of fluorescence of 4-methylumbelliferone produced by antibody-bound UDCA–β-galactosidase complex in relation to amounts (log scale) of UDCA present in assay. Vertical lines show standard deviations.

### Enzyme immunoassay using antibody-coupled glass beads as solid phase

The assay was performed using the competitive binding method. A single glass bead coupled with antibody was incubated for 2 hr at 30°C in a tube containing 0.2 ml of 0.01 M sodium phosphate buffer, pH 8.0 (4-fold dilution) to which various amounts of bile acids and $10^{-2}$ units of the antigen–enzyme complex were added. The beads were then washed in the test tube with 1 ml of buffer A followed by a wash with 1 ml of 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% $NaN_3$, and 0.1% bovine serum albumin (buffer B). The washed beads were then transferred to fresh test tubes to eliminate nonspecific binding.

The β-D-galactosidase activity bound to the glass beads was assayed using 4-methylumbelliferyl-β-D-galactoside as reported by Hamaguchi (25). The beads were immersed in 0.2 ml of buffer B and preincubated at 30°C for 5 min, then the enzyme reaction was started by adding 50 μl of $3 \times 10^{-4}$ M 4-methylumbelliferyl-β-D-galactoside. The reaction was performed in a shaking incubator at 30°C for 30 min and was stopped by the addition of 2.5 ml of 0.1 M glycine–NaOH buffer, pH 10.3. The amount of 4-methylumbelliferone formed was measured by fluorometry as described by Woolen (28). One unit of enzyme activity was defined as that which hydrolyzed 1 μmol of the substrate per min under the assay conditions.

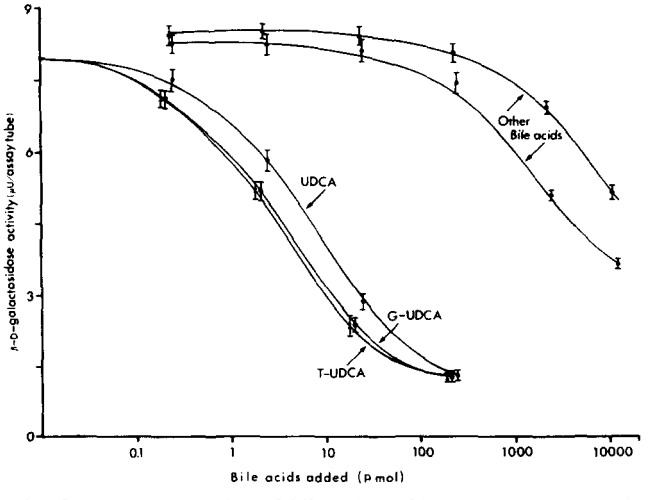


**Fig. 2.** Cross reactivity of bile acids with antiserum. G-UDCA and T-UDCA were most sensitive. Other bile acids (BA) tested without significant cross-reactivity (less than 0.1%) are specified in the text.

## RESULTS

### Sensitivity, specificity, and recovery

Using a 1:100 dilution of the antiserum, a standard curve was obtained (**Fig. 1**). The intensity of fluorescence decreased linearly with a logarithmic increase in UDCA concentration over a range of 0.1–10 pmol per assay tube. These concentrations are adequate to cover the UDCA disappearance test. Since we

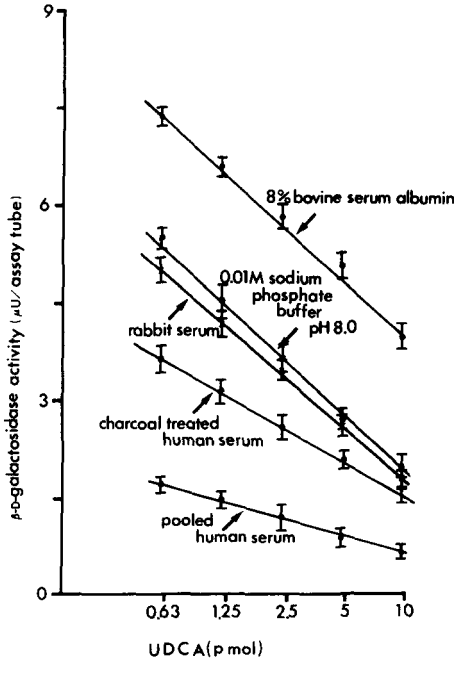


**Fig. 3.** Effect of serum and its components on the reaction. The standard curve prepared with 0.01 M sodium phosphate buffer, pH 8.0, was changed by additions of sera or serum components.

routinely diluted the sample 10-fold and then used 10 μl for the assay, the working limit of sensitivity was 0.1 nmol per ml. **Fig. 2** shows cross-reactivities for various free and conjugated bile salts. The assay is more sensitive for T-UDCA and G-UDCA. Other bile salts showed activities less than 0.1%. Bile acids examined were CA, CDCA, and DCA and their glycine and taurine conjugates.

**Fig. 3** presents the effect of different sera and of serum components on the reaction. The control solution consisted of 0.01 M sodium phosphate buffer, pH 8.0, to which increasing amounts of UDCA had been added. In order to measure quantitatively the amount of UDCA retained in the blood when performing the disappearance test, a standard curve was prepared using pre-injection serum i.e., serum obtained just before loading with UDCA. Recovery of added UDCA in rabbit serum is shown in **Fig. 4;** 85–130% of the injected UDCA was recovered.

Three serum samples (controls 1–3) were tested for precision and accuracy of the assay (**Table 1**). The coefficient of variation was 11–15% within assay and 16–18% between assays.

TABLE 1. Precision of rabbit serum UDCA assay

| | $n^a$ | M[b] | SD[c] | CV[d] |
|---|---|---|---|---|
| | | *ng/ml* | | % |
| Within-assay | | | | |
| Control 1 | 10 | 2525 | 379 | 15 |
| Control 2 | 10 | 811 | 120 | 15 |
| Control 3 | 10 | 380 | 42 | 11 |
| Between-assay | | | | |
| Control 1 | 4 | 2725 | 439 | 16 |
| Control 2 | 4 | 919 | 148 | 16 |
| Control 3 | 4 | 389 | 69 | 18 |

[a] Number of replicate samples within an assay, or consecutive numbers of assays.
[b] Mean value.
[c] Standard deviation from mean value.
[d] Coefficient of variation.

### Plasma tolerance test in rabbits

Sodium-UDCA was injected intravenously into a rabbit ear vein at a dose of 0.25 μmol/kg. Blood samples were collected every min from the other ear vein or artery and the plasma UDCA concentration was determined using the proposed immunoassay. Delayed disappearance of the injected sodium-UDCA was observed in all rabbits with biliary obstruction as early as 4 hr postoperatively (**Fig. 5**) at which time bilirubin levels were not yet elevated. The values obtained 7 min after the injection were about 10 times as high as the values obtained from normal rabbits. The values obtained in sham-operated animals were almost the same as those of normal rabbits. Antisera from three rabbits showed a very

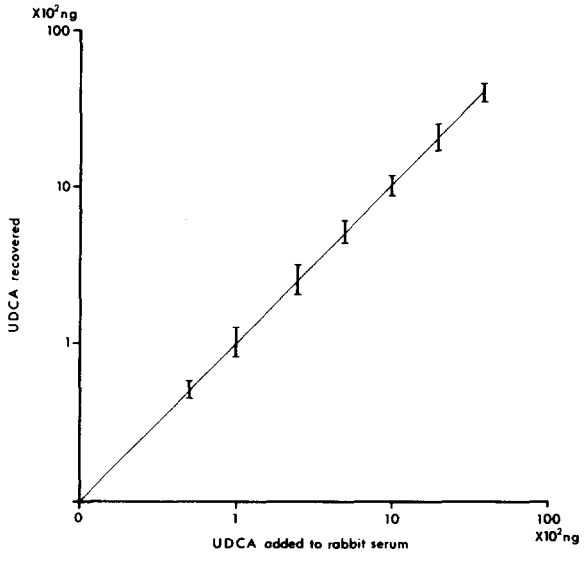


**Fig. 4.** Recovery of ursodeoxycholic acid added to rabbit serum. This study was carried out using free UDCA for preparation of the standard curve. Recovery of UDCA was about 85–130%.

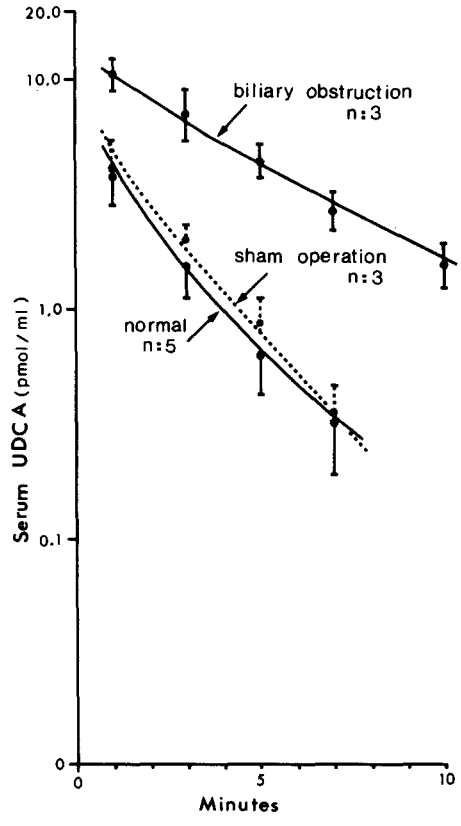


**Fig. 5.** Plasma disappearance curve of ursodeoxycholic acid (0.25 μmol/kg body weight) intravenously injected in the rabbit. Concentration of UDCA in peripheral venous or arterial blood, as determined by enzyme immunoassay, is shown on a logarithmic scale.

similar degree of specificity, although their titers varied.

## DISCUSSION

A radioimmunoassay for the determination of bile acids has been reported by Simmonds et al. (7) using an anti-glycocholic acid serum. This radioimmunoassay satisfies the criteria of specificity, sensitivity, reproducibility, and technical ease (7–12); however, its applicability is limited to a few laboratories because of the use of radioisotopes. Therefore the enzymeimmunoassay was developed to overcome this disadvantage (20–25) and to enhance its clinical usefulness. We propose the enzymeimmunoassay using a UDCA-β-D-galactosidase as a label for several reasons: *a*) the β-D-galactosidase is not present in human or animal biological fluids, *b*) the enzyme can be obtained from *Escherichia coli* in large amounts, *c*) it has a high turnover number, and *d*) it can be obtained in a highly purified and stable form. Furthermore, the hapten–β-D-galactosidase complex is very stable when stored at 4°C; we have been using the same preparation for more than 2 yr.

The quantitative aspect of the preparation of the UDCA–enzyme complex has not been examined in detail; the complex was prepared by adding the *N*-hydroxysuccinimide ester of UDCA to 0.1 mg of β-D-galactosidase in a molar ratio of 5000:1.

The sensitivity and specificity of the proposed method were the same as those observed with the radioimmunoassay (7–12). However, recovery from human serum proved difficult. The amounts recovered were always higher than the theoretical ones when determined from the standard curve which was prepared without adding human serum to the reaction. Even when the standard curve was prepared with charcoal-treated serum as reported by Simmonds et al. (7), the recovery was too high. Simmonds et al. (7) have reported that human bile acids can be determined using charcoal-treated serum as a blank. Therefore the interference noted in our experiments is probably not due to bile acids. Rabbit serum does not show this interference, but bovine serum albumin did interfere (Fig. 3). Thus, in our enzyme immunoassay of UDCA, the determination of UDCA in rabbit serum was performed using a standard curve without rabbit serum, while the determination of UDCA in human serum must be performed with a standard curve using pre-injection serum, that is, serum obtained just before loading with UDCA. In this fashion the plasma disappearance test could be made applicable to clinical studies.

The rabbit anti-UDCA serum we used in the present experiments was highly specific for UDCA. The cross-reactivities with CDCA, DCA, or CA as well as with their glycine or taurine conjugates were less than 0.1%. It has been reported by Schalm et al. (11) that when using rabbit anti-glycochenodeoxycholic acid serum, glycoursodeoxycholic acid cross-reacted with glycochenodeoxycholic acid up to 10%. This may be due to certain cross reactions caused by the glycine-peptide moiety. It has also been reported that, when using anti-cholic acid serum, cholic acid showed high cross-reactivities with CDCA or DCA (7–10). Using anti-UDCA serum, Makino et al. (12) have reported cross-reactivities of UDCA with other bile acids at a level of less than 0.1%, the same value as found in our experiments. It can be concluded that anti-UDCA serum can be prepared that has very little cross-reactivity with common bile acids containing a 7α-OH group as well as with their glycine and taurine conjugates. Furthermore, the antigenic hapten, sodium ursodeoxycholate, is moderately soluble in aqueous systems and UDCA is not a major bile acid in normal human serum or bile. Therefore, the anti-UDCA serum should be a very good anti-bile acid serum to be used for a human plasma disappearance test.

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