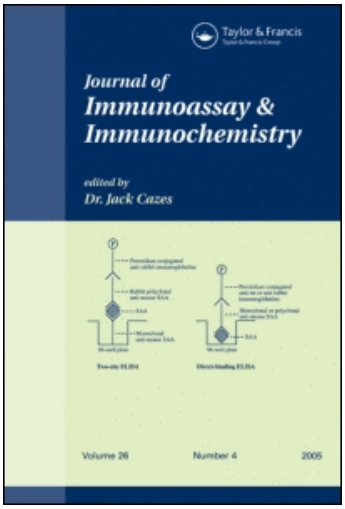

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A RADIOIMMUNOASSAY OF BILE ACIDS

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ABSTRACT

A radioimmunoassay for β-muricholic acid was developed using an antiserum which was prepared by injecting β-muricholic acid conjugated with bovine serum albumin into rabbits. The antiserum reacted with glyco-β-muricholic, tauro-β-muricholic and β-muricholic acids, but not with other bile acids. The radioimmunoassay showed good reproducibility with inter- and intra-assay coefficients of variations of 6 % to 15 %. When the validity of the method was examined by comparing it with a gas-liquid chromatography method, a linear correlation was obtained.
(KEY WORDS: radioimmunoassay, β-muricholic acid, tauro-β-muricholic acid, cholic acid, taurocholic acid).

INTRODUCTION

β-Muricholic acid (3α,6β,7β-trihydroxy-5β-cholan-24-oic acid), specific to rats and mice, is a major component of bile acids and, together with cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) which is a common bile acid in a large number of species, accounts for 60-80% of the total bile aids in rat and mouse bile and small intestines. Although the physiological role of

β-muricholic acid is not clear, the mechanism of bile acid metabolism can not be studied in detail unless β-muricholic acid is taken into account. β-Muricholic acid is synthesized from chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid) by the liver and secreted into bile. It circulates between the liver and small intestines via portal blood (enterohepatic circulation) in the same way as the other bile acids.

For the identification and quantitation of bile acids in tissues, urine and feces, gas-liquid chromatographic (GLC) methods are useful but necessitate a series of manipulations (extraction, hydrolysis, and derivative formation). Also large amounts of sera are needed since a lowest limit of detection is more than 0.1 μg. Recently, many research groups have developed radioimmunoassays (RIA) for cholic acid, chenodeoxycholic acid and ursodeoxycholic acid (1-6) and quantitated serum levels of bile acids. Therefore, to examine bile acid metabolism in serum, we prepared an antiserum of β-muricholic acid for RIA.

In this paper, we describe the cross-reactivity of β-muricholic acid antiserum and that of glycocholic acid antiserum which was prepared by another group in our laboratories. To measure validity of our method, we tested its correlation with the usual GLC method.

MATERIALS AND METHODS

Animals and Reagents

Animals: Female Japanese white rabbits were obtained from Kitayam rabes (Kyoto, Japan) and fed at Shionogi Aburahi Laboratories in Shiga Prefecture. Male Wistar rats were obtained from Japan Clea Lab (Tokyo, Japan) and maintained in an air-conditioned room (25°C and 50% humidity) lighted 12 h/day (8:00 to 20:00).

Reagents: Bovine serum albumin (BSA) was purchased from Sigma (St. Louis MO, USA) and treated with Norit A to remove bile acids. Bovine serum γ-globulin was purchased from Sigma. Freund's complete adjuvant was obtained from Difco Lab (Detroit, MI, USA). Polyethyleneglycol(M.W. 6000) was purchased from Yoneyama Chemical Ind (Osaka, Japan). PHP-LH-20 (piperidinohydroxypropyl Sephadex LH-20) was obtained from Shimadzu (Kyoto, Japan). Tri-n-butylamine and isobutylchlorocarbonate were obtained from Wako Chemical (Tokyo Japan). SEP PAK C_{18} cartridges were purchased from Waters Associates (Milford, MA, US).

Glycocholic acid was obtained from Nacalai tesque Inc (Kyoto, Japan). β-Muricholic acid was synthesized by the method of Hsia et al.(7). Tauro-β-muricholic acid was synthesized by the method of Norman (8). The other taurine-conjugated, glycine-conjugated and unconjugated bile acids were purchased from PL Biochemicals (Milwaukee, WI, USA). Other reagents were of guaranteed grade.

Radioactive compounds: [2,4-^{3}H]Cholic acid (925 GBq/mmol) and [2-^{3}H]glycine (2.06 TBq/mmol) were obtained from New England Nuclear (Boston, MA, USA). β-Muricholic acid was conjugated with the radioactive glycine by the mixed carboxylic anhydride method of Norman (8) without diluting their activities.

[2,4-^{3}H]Cholic acid was conjugated with cold glycine to yield glycocholic acid by the method used to prepare radioactive glyco-β-muricholic acid.

Buffer and solution: Phosphate buffer (0.1 M) containing 0.1% sodium azide and 0.2% bovine serum γ-globulin was prepared and adjusted to pH 7.4 (BSG buffer) at room temperature. Bovine serum albumin was dissolved in saline to make a 4% solution (BSA solution).

Preparation of β-Muricholic Acid Antigen

β-Muricholic acid (50.6 mg) was dissolved in tetrahydrofuran (2 ml). While cooling this in an ice-water bath with stirring, tri-n-butylamine (30 μl) was added to the solution and then isobutylchlorocarbonate (17 μl) was added. After stirring at 5°C for 30 min, the reaction mixture was added dropwise to a solution of BSA (174 mg, crystals) dissolved in water (2 ml) and tetrahydrofuran (2 ml). The mixture was adjusted to pH 8.0 with 0.1 N NaOH and stirring was continued at 4°C for 4 h. The reaction mixture was dialyzed using a Visking tube (20/32 inch)

clamped at both ends and then lyophilized. The binding number of β-muricholic acid/mole of BSA was 32.1 when determined by trinitrobenzenesulfonic acid procedure (9).

Immunization with β-Muricholic Acid

The antigen prepared as described above (3 mg) was suspended in a mixture of Freund's complete adjuvant (7.5 ml) and saline (7.5 ml). This solution (1 ml) was intradermally injected to a rabbit (2.9-3.20 kg) on its sheared back. The injection was repeated six times every 3 weeks. Blood was collected from the rabbits via their carotid artery. Antisera were obtained by centrifugation of the blood for 15 min at 3000 rpm.

Antiserum of glycocholic acid, a generous gift of Dr. Kohno in these laboratories, had been prepared in a similar manner.

Treatment of Rat Serum for Radioimmunoassay

The fresh rat serum (100 μl) was diluted with 4 ml of 0.5 M phosphate buffer (pH 7) and passed through a SEP PAK C_{18} cartridge which had been washed with methanol and subsequently with water. The bile acids were eluted with 90% ethanol (8 ml). The eluent was applied to a PHP-LH-20 gel (200 mg) packed in a disposable 5 ml-graduated pippet (Corning Glass Works, NY, USA) to separate taurine-conjugated and unconjugated bile acids by the method of

Goto et al.(10). The taurine-conjugate fraction was further passed through a SEP PAK C_{18} cartridge to remove the potassium acetate which had been used to elute the taurine-conjugate fraction.

Radioimmunoassay of β-Muricholic Acid and Its Taurine-conjugate

The reaction was carried out in duplicate in 12 x 75 mm disposable culture tubes (Corning Glass Works). To obtain the standard curve of β-muricholic acid or its taurine-conjugate, standards of the bile acid were dissolved in BSA solution from 0.05 to 1,300 pmol/50 µl. Antiserum and radiolabeled glyco-β-muricholic acid were diluted with BSG buffer, and 0.2 ml of each aliquot was added to the tube. An aliquot (50 µl) of standard β-muricholic acid or of rat serum samples which were dissolved in BSA solution was added to the tube. The tubes were warmed at 39°C for 2 h and then kept at 4°C overnight. After these equilibration procedures, 0.2 ml of 40% polyethyleneglycol solution in BSG buffer was added, and after mixing for 10 seconds, the reaction mixture was stored at 4°C for 15 min. The tubes were then centrifuged at 3000 rpm for 30 min. An aliquot (0.25 ml) of the supernatant was added to Scintisol EX-H (5 ml; Dojin Chemical Research Lab., Osaka, Japan), and its radioactivity was counted in a Packard liquid scintillation counter Model 2000CA.

Radioimmunoassay of Cholic Acid and Its Taurine-conjugate

Radioimmunassay of cholic acid and taurocholic acid was carried out essentially in the same way as that of β-muricholic acid and its conjugate. But the standard bile acid was taurocholic acid or cholic acid, and the antiserum was glycocholic acid antiserum.

Recovery of Bile Acids in the Extraction Procedure

To examine the recovery of bile acids in the separation procedure, three to five different concentrations (3 samples for each concentration) of β-muricholic acid cholic acid and their taurine-conjugates were dissolved in rat serum and treated by the same procedure as the fractionation of conjugated and unconjugated bile acids described above. The concentration was determined by RIA. The slope of the regression line of the recovered values against the added amounts gave the recovery % which is shown in Table III.

Gas-Liquid Chromatography

To examine the validity of the values obtained by RIA, their correlation with those from the usual GLC assay method was examined for the plasma samples from ten rats. The gas chromatograph was a Model GC-7A (Shimadzu), and the analytical methods were those described previously (11).

RESULTS

Specificity

Approximately 55% of a tracer dose of [2-^{3}H]glyco-β-muricholic acid and glyco-[2,4-^{3}H]cholic acid were bound by the corresponding antiserum diluted 1:8000 and 1:200, respectively. The cross-reactivity of various bile acids are shown in Table I. The antiserum of β-muricholic acid bound to β-muricholic acid and its glycine- and taurine-conjugate but did not react with the other bile acids examined thus far. The antiserum of glycocholic acid showed fair cross-reactivity with taurocholic acid, less with cholic acid and glycodeoxycholic acid and even lesser extent with glycochenodeoxycholic acid, taurochenodeoxycholic acid, taurodeoxycholic acid and 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid (Table I).

Calibration Curve and Detection Limit

The standard curves of bile acids were plotted in bound % (ordinate) vs the concentration of bile acid (abscissa)(Fig.1). The sensitivity of the assay for the bile acids examined as the confidence limits to the zero standard estimate (12) was 16.0 pmol/ml for β-muricholic acid (n = 4), 12.0 pmol/ml for tauro-β-muricholic acid (n = 6), less than 40 pmol/ml for cholic acid (n = 3) and 120 pmol/ml for taurocholic acid (n = 8).

TABLE I

Cross reactivities of antisera for β-muricholic acid and for glycocholic acid with various bile acids

Compound	Antiserum for GCA*	Antiserum for β-MCA**
Glycine conjugate		
Cholic acid	100	<1
Chenodeoxycholic acid	6.5	<1
Deoxycholic acid	38.5	<1
Lithocholic acid	<1	<1
Ursodeoxycholic acid	<1	<1
β-Muricholic acid	<1	69.0
Taurine conjugate		
Cholic acid	66.0	<1
Chenodeoxycholic acid	5.8	<1
Deoxycholic acid	10.8	<1
Lithocholic acid	<1	<1
Ursodeoxychlic acid	<1	<1
β-Muricholic acid	<1	90.0
Unconjugate		
Cholic acid	41.0	<1
Chenodeoxycholic acid	1.6	<1
Deoxycholic acid	<1	<1
Lithocholic acid	<1	<1
Ursodeoxychlic acid	<1	1.0
β-Muricholic acid	<1	100
α-Muricholic acid	<1	1.0
ω-Muricholic acid	<1	1.0
Hyocholic acid	<1	<1
Hyodeoxycholic acid	<1	<1
Ursocholic acid	<1	<1
3α,12α-Dihydroxy-7-oxo-5β-cholan-24-oic acid	10.0	<1

*Glycocholic acid, **β-muricholic acid

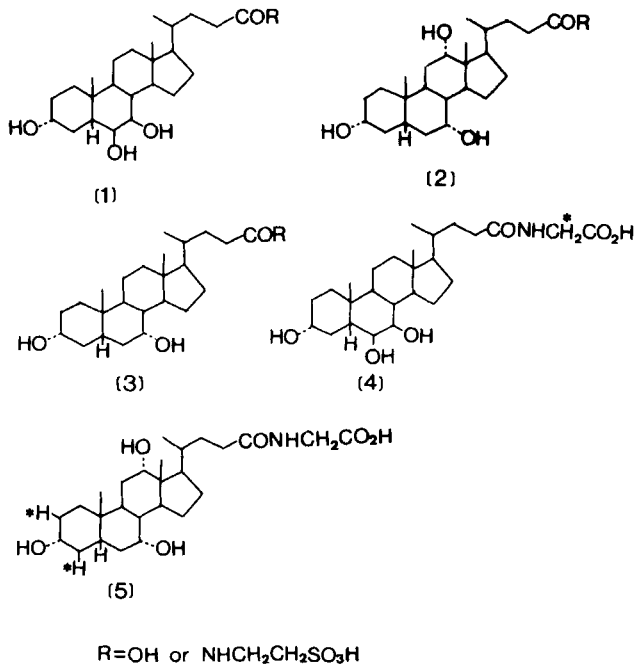

FIGURE 1. Structures of β-muricholic acid(1:R=OH), tauro-β-muricholic acid (1:R=$NH(CH_2)_2SO_3H$), cholic acid (2:R=OH), taurocholic acid (2:R=$NH(CH_2)_2SO_3H$), chenodeoxycholic acid (3: R=OH) and taurochenodeoxycholic acid (3:R=$NH(CH_2)_2SO_3H$). Compounds (4) and (5) are radiolabelled glyco-β-muricholic acid and glycocholic acid respectively, and a labelled position is shown by an asterisk.

Precision of Assay

The intra- and inter-assay reproducibility was examined using serum treated with charcoal to remove bile acids. The bile acids were added to the serum to an appropriate level (15 to 2500 pmol/ml). Coefficients of variation ranged from 6.3 to 14.8% (Table II).

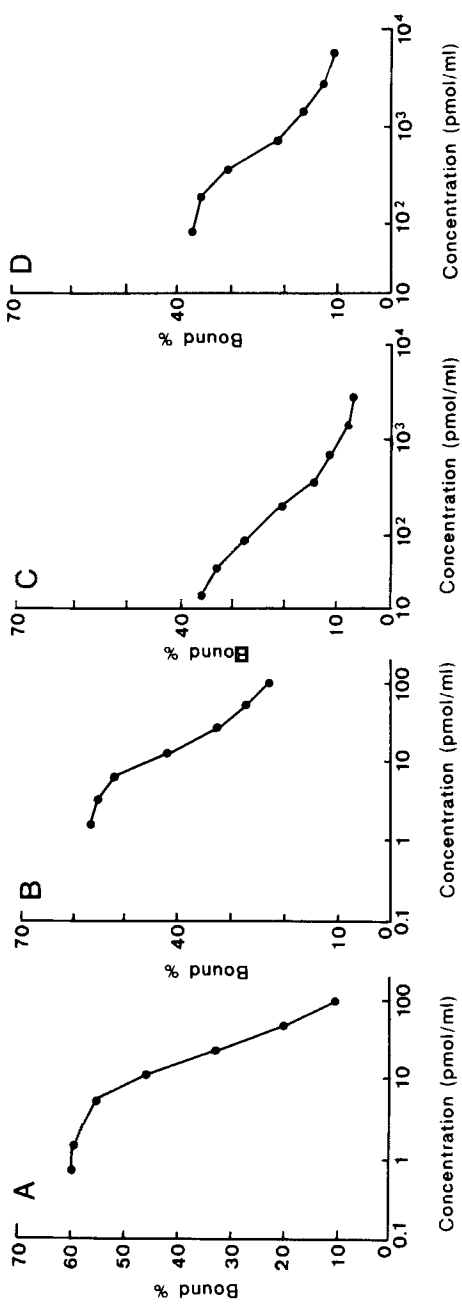

FIGURE 2. Standard curves of the antiserum of β-muricholic acid and glycocholic acid using tauro-β-muricholic acid (A), β-muricholic acid (B), taurocholic acid (C) and cholic acid (D) as ligands. The ordinate indicates % binding and the abscissa shows the concentration of standards (pmol/ml). Antiserum for β-muricholic acid was used in the dilution of 1:8000 and that for glycocholic acid in the dilution of 1:200.

TABLE II

Reproducibility of the assay

Bile acid and number of determination	Concentration (pmol/ml) M ± SD	Coefficient variation (%)
Intraassay		
Tauro-β-muricholic acid		
5	81.0 ± 5.07	6.3
5	37.9 ± 4.24	11.2
5	18.6 ± 2.75	14.8
Taurocholic acid		
5	2,389 ± 58.6	2.5
5	491 ± 11.5	2.3
4	127 ± 5.5	4.4
Interassay		
Tauro-β-muricholic acid		
4	48.6 ± 6.87	14.1
4	23.5 ± 2.33	9.9
Taurocholic acid		
4	1,916 ± 81.3	4.2
4	240 ± 34.4	14.3

Intra- and interassay variations were determined with a charcoal-treated rat serum containing an elevated level of tauro-β-muricholic acid and taurolcholic acid by using β-muricholic acid antiserum and glycocholic acid antiserum, respectively.

Validation

Rat plasma concentrations of the taurine conjugates of cholic acid and β-muricholic acid measured by RIA showed a linear correlation with the values from the GLC method. Regression lines were $Y = 0.81X+0.006$ ($r = 0.98$, $n = 10$) for tauro-β-muricholic acid and $Y = 0.93X+0.15$ ($r = 0.92$, $n = 10$) for taurocholic acid. The values in

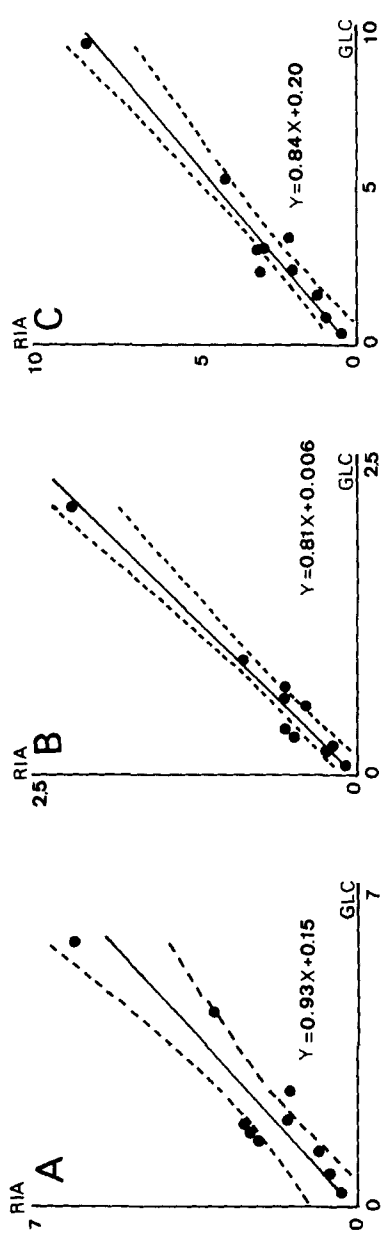

FIGURE 3. Correlation of values obtained by the RIA and GLC methods. The values of the ordinate line are expressed as nmol/ml obtained by the RIA method, and those of the abscissa for those obtained by the GLC method. Both values are corrected by the recovery % in the extraction procedure. Figure A is for tauro-β-muricholic acid, and B is for taurocholic acid. Figure C is for the sum of both bile acids including β-muricholic acid and cholic acid.

TABLE III

Recoveries of bile acids in extraction with SEP PAK C_{18} and separation with PHP-LH-20

Bile acid	Recovery %
Tauro-β-muricholic acid	106.0
β-Muricholic acid	84.4
Taurocholic acid	65.8
Cholic acid	75.6

Fig. 2 were corrected by the recovery %, and the dotted lines show the 95% confidence band. No correlation was observed for the unconjugated bile acids due to their low concentration in the plasma. However, the regression line for the sum of both taurine-conjugated and unconjugated bile acids was $Y = 0.84X+0.20$ ($r = 0.93$, $n = 10$). The values obtained by the GLC method were slightly higher than those found by the RIA method.

DISCUSSION

This paper reports the preparation of an antiserum for β-muricholic acid and its practical use in a RIA for the determination of taurine-conjugated and unconjugated β-muricholic acid. The numbers of hapten bound to BSA were calculated by the method of Habeeb (9) which is an indirect method. Other research groups (1,4) have directly calculated the numbers of hapten bound to a protein by using a radioactive bile acid. For example,

Beckett et al.(4) added a labelled bile acid prior to coupling with BSA and precipitated a portion of a hapten-bound BSA with trichloroacetic acid. They counted the radioactivity of the precipitates to obtain the numbers of hapten. However, the radioactive β-muricholic acid was not available to us when the antigen was being prepared.

Injection of the β-muricholic acid-BSA immunogen to rabbit produced an antiserum specific for β-muricholic acid and tauro-β-muricholic acid, but less specific for glyco-β-muricholic acid. This cross-reactivity was consistent with that of β-muricholic acid antiserum obtained by Botham et al.(6), who reported that their antiserum was most specific for tauro-β-muricholic acid.

In our rats, unconjugated and taurine-conjugated bile acids are the major components while glycine-conjugated bile acids only present in trace amounts. The cross-reactivity of the antiserum for β-muricholic acid with tauro-β-muricholic acid (Table I) necessitated the separation of the unconjugated bile acid from the taurine-conjugated one. The approach used to the separate them was similar to that of Goto et al.(10) who developed PHP-LH-20 to separate glycine- and taurine-conjugated and unconjugated bile acids from each other. In this procedure, both taurine-conjugated and unconjugated β-muricholic acids were recovered at more than 80%, but those of cholic acids were less than 80% (Table III).

Bile acids that return via the portal vein in the enterohepatic circulation are thought to be taken up by the liver, but some of the bile acids (especially when the bile acid concentration is high in the portal vein) flow into the systemic circulation. In this sense, the low level of unconjugated bile acids in the plasma (used to show a correlation between the RIA and GLC methods) may be due to poor absorption of the unconjugated bile acid from the intestines, or may indicate that the deconjugation reaction did not occur in the upper part of the intestines. The concentration of bile acids in the plasma varied from rat to rat, which suggested that there are individual differences in the capability of absorption from the intestines and of hepatic uptake.

RIA is a valuable method for estimating the concentration in a small volume of serum without the need of an extraction procedure. However, in the case of contamination with a closely related substance, procedures of extraction and separation are necessary, as shown in this study. To simplify the assay method, further study is needed to obtain antibodies specific for a single form of bile acid conjugation and sensitive enough to eliminate serum (or plasma) effects caused by a large dilution.

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