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A Direct Radioimmunoassay of Estradiol 3-Glucuronide Using Specific Antiserum[1)]

TOSHIO NAMBARA,* TADASHI OHKUBO, TERUKO YAMAKI, and KAZUTAKE SHIMADA

Pharmaceutical Institute, Tohoku University, Aobayama, Sendai 980, Japan

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The preparation and antigenic properties of estradiol 3-glucuronide–bovine serum albumin conjugate in which the hapten is linked to the carrier protein through an (*O*-carboxymethyl)oxime bridge at the C-6 position on the steroid nucleus, are described. Antiserum elicited in the rabbit by the immunogen possessed high affinity and specificity to estradiol 3-glucuronide, exhibiting hardly any cross-reactivities with other estrogen conjugates except for estrone 3-glucuronide (18.66%) and no cross-reactions with free estrogens and other related steroids. This antiserum was purified by affinity chromatography on estrone 3-glucuronide–aminoethyl cellulose conjugate. The purified antiserum exhibited no cross-reaction with estrone 3-glucuronide. A direct radioimmunoassay method for urinary estradiol 3-glucuronide was developed by using the purified antiserum. The values obtained with the purified antiserum were somewhat lower than those with the native one. The within- and between-assay variations were 3.32 and 4.35%, respectively. The values obtained by the present method correlated closely with those obtained by the established indirect radioimmunoassay (RIA) method.

Keywords——radioimmunoassay; estradiol 3-glucuronide; 6-(*O*-carboxymethyl)oxime; hapten–BSA conjugate; affinity chromatography; cross-reaction; urine level

In recent years considerable attention has been focused on the physiological significance of steroid hormone conjugates. A simple method for the determination of steroid conjugates in biological fluids is needed in the biomedical field. Several attempts have previously been made to develop a radioimmunoassay (RIA) of intact steroid conjugates without prior hydrolysis.[2,3)] In these studies the preparation of antisera was carried out by use of an immunogen in which the steroid conjugate was coupled to a carrier protein *via* the carboxyl group in the glucuronyl moiety. However, antisera elicited with these immunogens were unsatisfactory with respect to the specificity. For instance, anti-estradiol 3-glucuronide antisera showed significant cross-reactivities with estrogen ring A glucuronides and free estrogens.[4)] Therefore, a separation step was essential for RIA using these antisera.

Recently, we obtained antisera highly specific to estrone sulfate,[5)] estradiol 17-glucuronide, and estriol 16-glucuronide[6)] by utilizing the C-6 position on the steroid nucleus for the formation of hapten–bovine serum albumin (BSA) conjugates. Also, highly specific antiserum to estradiol 3-glucuronide was prepared by the use of hapten-[C-6]–BSA conjugate.[7)] This method, however, requires many steps and tedious procedures. An urgent need for specific anti-estradiol 3-glucuronide antiserum prompted us to develop a more convenient method for the preparation of immunogen. The present paper deals with the preparation of antiserum with a new type of hapten-[C-6]–BSA conjugate, its purification by means of affinity chromatography and the direct RIA of estradiol 3-glucuronide in urine.

Materials and Methods

Chemicals——[6,7-^{3}H]-Estradiol (54 Ci/mmol) was supplied by New England Nuclear (Boston, MA), and the

radiochemical purity was checked by thin-layer chromatography (TLC) prior to use. The conjugated steroids were prepared in these laboratories by the known methods. All free steroids were kindly donated by Teikoku Hormone Mfg. Co. (Tokyo). BSA Fraction V and bovine serum gamma-globulin were purchased from Sigma Chemical Co. (St. Louis, MO). Aminoethyl (AE) cellulose was from Nakarai Chemicals Ltd. (Kyoto) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide·HCl was purchased from Kokusan Chemical Works (Tokyo). Other general reagents were obtained from Nakarai Chemicals (Kyoto).

Preparation of Hapten–BSA Conjugate——All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 automatic polarimeter. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL FX-100 spectrometer at 100 MHz (^{1}H) using tetramethylsilane as an internal standard. Abbreviations used: s = singlet, d = doublet, dd = doublet of doublets, and m = multiplet. Silica gel HF_{254} and silica gel (70—230 mesh) (E. Merck AG, Darmstadt) were used for preparative TLC and column chromatography, respectively.

17-*O*-Acetyl-6-oxoestradiol 3-Glucuronide Acetate-Methyl Ester 6-(*O*-Carboxymethyl)oxime (2)——(*O*-Carboxymethyl)hydroxylamine·1/2HCl (153 mg) and 10% sodium acetate in water (1.5 ml) were added to a solution of 17-*O*-acetyl-6-oxoestradiol 3-glucuronide acetate-methyl ester (**1**)[7] (62 mg) in MeOH (10 ml)–ethyl acetate (4 ml), and the whole was stirred at 35 °C for 12 h. The resulting solution was diluted with water and extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated down under reduced pressure. The residue was recrystallized from ether to give **2** (51 mg) as a colorless amorphous material. mp 185—188 °C. $[\alpha]_D^{20}$ +64.3° (c = 0.11 in MeOH). *Anal.* Calcd for $C_{35}H_{43}NO_{15}\cdot H_2O$: C, 57.14; H, 6.16; N, 1.90. Found: C, 56.91; H, 5.97; N, 1.81. NMR ($CDCl_3/CD_3OD$) δ: 0.84 (3H, s, 18-CH_3), 2.06 and 2.08 (each 6H, each s, 17-, 2′-, 3′-, 4′-$OCOCH_3$), 3.74 (3H, s, $COOCH_3$), 4.69 (2H, s, $NOCH_2$), 6.98 (1H, dd, J = 9, 2 Hz, 2-H), 7.16 (1H, d, J = 9 Hz, 1-H), 7.54 (1H, d, J = 2 Hz, 4-H).

6-Oxoestradiol 3-Glucuronide 6-(*O*-Carboxymethyl)oxime–BSA Conjugate (4)——Tri-*n*-butylamine (50 μl) and isobutyl chloroformate (13 μl) were added to a solution of **2** (48 mg) in dry dimethylformamide (DMF) (1.5 ml), and the whole was stirred for 30 min under ice-cooling. BSA (123 mg) in DMF (3.0 ml)–water (2.2 ml)–1 N NaOH (0.05 ml) was added to the above solution and stirred at 4 °C for 12 h. The resulting solution was dialyzed against cold running water for 24 h. Lyophilization of the solution gave 17-*O*-acetyl-6-oxoestradiol 3-glucuronide acetate-methyl ester 6-(*O*-carboxymethyl)oxime–BSA conjugate (**3**) (166 mg). A solution of **3** (165 mg) in water (50 ml) was adjusted to pH 12 with 5 N NaOH and stirred at room temperature for 48 h. The resulting solution was dialyzed against cold running water for 24 h. Lyophilization of the solution gave **4** (131 mg) as a fluffy powder. The molar steroid : protein ratio of the conjugate was determined to be 13 by ultraviolet (UV) spectrometric analysis.

Preparation of AE Cellulose Estrone 3-Glucuronide Conjugate——A suspension of AE cellulose (100 mg) in water was added to a solution of estrone 3-glucuronide (15 mg) in DMF (2 ml), and the whole was adjusted to pH 5.3 with 0.1 N HCl. After addition of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide·HCl (60 mg), the suspension was stirred at room temperature for 72 h. The precipitate was collected by filtration, washed alternately with water and methanol several times, and dried in air. Determination of the unconjugated steroid in the filtrate revealed that 1 mg of the conjugate contained 73 μg of estrone 3-glucuronide.

Immunization of Rabbits——Three male albino rabbits were used for immunization. The immunogen (2 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with complete Freund's adjuvant (0.5 ml). This emulsion was injected into rabbits subcutaneously at multiple sites over the scapulae and in the thigh. This procedure was repeated at intervals of two weeks for a further two months and then once a month. The rabbits were bled ten days after the booster injection. The sera were separated by centrifugation at 3000 rev/min for 10 min and stored at −20 °C.

Elimination of Cross-Reactive Antibodies by Affinity Chromatography——The 1 : 100 diluted antiserum (8 ml) was shaken with estrone 3-glucuronide AE cellulose immunoadsorbent (50 mg) at 4 °C for 1 h. The purified antiserum was separated by centrifugation at 3000 rev/min for 10 min and used for RIA at the optimum dilution. After being washed with 1 N HCl and 0.5 M NaCl, the immunoadsorbent was usable repeatedly. No contamination of unbound estrone 3-glucuronide in the AE cellulose immunoadsorbent was found.

Assay Procedure——A diluted urine specimen or standard solution (0.05 ml) was mixed with [^{3}H]-estradiol 3-glucuronide (75 pg, 20000 dpm) in 0.05 M phosphate buffer (pH 7.4) (0.05 ml), and the whole was allowed to stand at room temperature for 1 h. The diluted antiserum (0.1 ml) was added and the mixture was incubated at 4 °C for 12 h. On treatment with dextran-coated charcoal (0.5% Norit and 0.1% dextran in assay buffer) (0.2 ml) for 10 min at 0°C followed by centrifugation at 3000 rev/min, the bound and free fractions were separated. A two-tenths ml aliquot of each supernatant was transferred into a counting vial containing a scintillation cocktail tT21 (4 ml),[8] and the radioactivity was counted on a Beckman LS 7000 liquid scintillation spectrometer.

Characterization of Antiserum——The specificity of antiserum was tested by cross-reaction studies with 18 kinds of steroids related to estradiol 3-glucuronide. The relative amounts required to reduce the initial binding of [^{3}H]-estradiol 3-glucuronide by half, where the mass of non-labeled estradiol 3-glucuronide was arbitrarily defined as 100%, were calculated by use of the standard curve.[9]

Recovery Test——Hexaplicate 0.1 ml aliquots of diluted normal male urine were added to each of the assay tubes containing 50, 100, 200, and 500 pg of non-labeled estradiol 3-glucuronide and [^{3}H]-estradiol 3-glucuronide in assay

buffer. The assay was then carried out in the manner described above.

Results and Discussion

Our initial effort was directed to the preparation of estradiol 3-glucuronide–BSA conjugate having a linkage at the C-6 position on the steroid nucleus. For this purpose 17-*O*-acetyl-6-oxoestradiol 3-glucuronide acetate-methyl ester (**1**) was adopted as a pertinent starting material. Treatment of **1** with (*O*-carboxymethyl)hydroxylamine gave the 6-(*O*-carboxymethyl)oxime (**2**) in a reasonable yield. The oxime derivative was covalently linked to BSA by the mixed anhydride technique.[10] Finally, elimination of the protecting groups in the hapten with alkali under mild conditions[11] afforded the desired 6-oxoestradiol 3-glucuronide 6-(*O*-carboxymethyl)oxime–BSA conjugate.

The hapten–BSA conjugate thus prepared was used for immunization of rabbits. Several months after an initial injection of the conjugate, all the rabbits yielded antibody exhibiting a remarkably increased binding activity to estradiol 3-glucuronide. No substantial difference in the affinity and specificity was observed among the antisera elicited in the three rabbits.

Evaluation of the titer was performed by incubating various dilutions of antiserum with a constant amount of [^{3}H]-estradiol 3-glucuronide.[7] The dilution of antiserum which was capable of binding 50% of the labeled antigen was defined as a titer. The dose–response curve was constructed with 1 : 3000 diluted anti-estradiol 3-glucuronide antiserum. The percent bound radioactivity *vs.* the logarithm of the amount of estradiol 3-glucuronide showed a linear relationship in the range of 20 to 1000 pg. The antiserum exhibited high affinity to estradiol 3-glucuronide with the association constant (K_a) of $3.50 \times 10^9\,\mathrm{M}^{-1}$.

The specificity of antiserum was assessed by testing the ability of closely related compounds to compete for binding sites on the antibody. The percent cross-reactions determined by the method of Abraham[9] with native antiserum are collected in Table I. There was some cross-reaction with estrone 3-glucuronide (18.66%) and estradiol 3-methyl ether (6.00%). Other selected steroids exhibited no significant cross-reactivities.

This antiserum was purified by immunoadsorption on affinity chromatography media to eliminate cross-reactive antibodies. The 1 : 1000 diluted antiserum showed a linear relationship in the range of 20 to 1000 pg. It is evident from the data in Table I that the specificity of the antiserum was remarkably improved by this purification. There were no

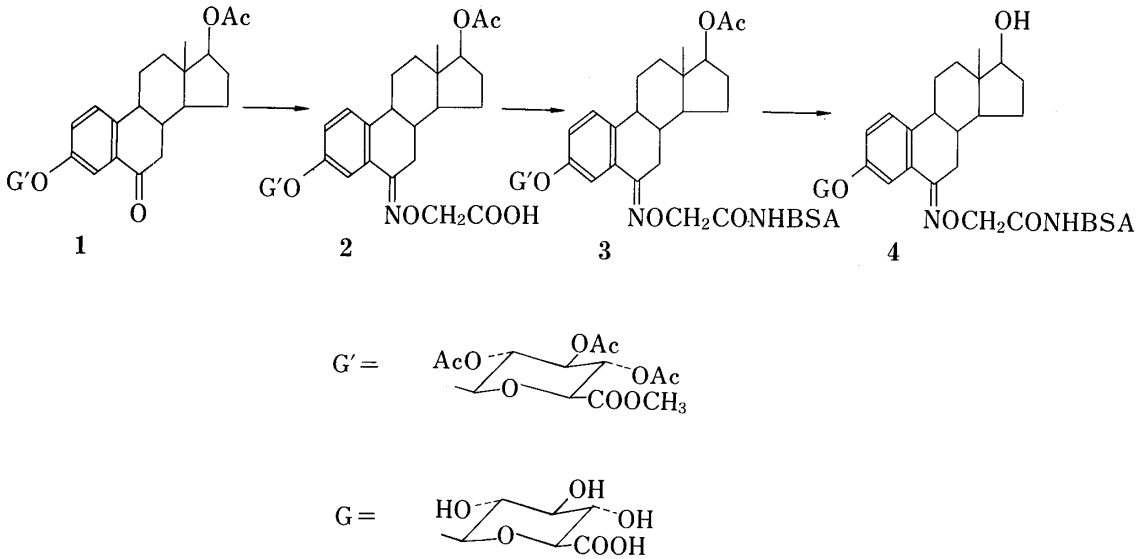

Fig. 1. Preparation of the Estradiol 3-Glucuronide-[C-6]–BSA Conjugate

Table I. Cross-Reactivities of Purified and Native Anti-Estradiol 3-Glucuronide Antisera with Selected Steroids

Steroid	% Cross-reaction (50%)	
	Purified antiserum	Native antiserum
Estradiol 3-glucuronide	100	100
Estrone glucuronide	0.04	18.66
Estriol 3-glucuronide	0.19	0.47
Estradiol 17-glucuronide	<0.001	<0.001
Estriol 16-glucuronide	<0.001	<0.001
2-Hydroxyestradiol 2-glucuronide	<0.001	<0.001
Estrone sulfate	<0.001	0.93
Estradiol 3-sulfate	<0.001	2.80
Estriol 3-sulfate	<0.001	<0.001
Estradiol	<0.001	2.10
Estradiol 3-methyl ether	0.01	6.00
Estrone 3-methyl ether	<0.001	<0.001
Estriol 3-methyl ether	<0.001	<0.001
2-Hydroxyestrone	<0.001	<0.001
2-Methoxyestrone	<0.001	<0.001
2-Hydroxyestrone 3-methyl ether	<0.001	<0.001
2-Hydroxyestradiol	<0.001	0.20
2-Methoxyestradiol	<0.001	0.07

Table II. Recovery Test for Estradiol 3-Glucuronide Added to Normal Male Urine

Added	Purified antiserum		Native antiserum	
	Expected	Found ± S.D. (pg/tube)	Expected	Found ± S.D. (pg/tube)
0	—	68 ± 3.3 (4.9)[a]	—	84 ± 4.9 (4.7)
50	118	108 ± 3.0 (2.8)	134	128 ± 7.5 (4.8)
100	168	165 ± 4.3 (2.6)	184	186 ± 10.0 (5.4)
200	268	258 ± 5.2 (2.2)	284	276 ± 12.5 (4.5)
500	568	535 ± 31.0 (5.8)	584	530 ± 60 (11.3)

a) The figures in parentheses represent C.V. (%). $n=8$.

significant cross-reactions with estrone 3-glucuronide (0.04%), estradiol 3-methyl ether (0.01%), or other conjugated and free steroids.

A direct radioimmunoassay method for urinary estradiol 3-glucuronide was developed by using purified and native antisera, respectively. The recovery rates of various amounts of estradiol 3-glucuronide added to normal male urine samples are listed in Table II. A good correlation was observed between the expected and found values of estradiol 3-glucuronide with both antisera. The values obtained with native antiserum were slightly higher than those with purified antiserum. In addition, the results on urine specimens obtained with purified and native antisera showed a good correlation ($r=0.97$; $n=22$), the regression equation being $Y=0.96X+142.2$ (Fig. 2). These data indicate that the use of native antiserum overestimates the amount of estradiol 3-glucuronide in the urine sample.

The within- and between-assay variations were examined with three urine specimens taken from two males and one female. The results are collected in Table III. As shown in Fig.

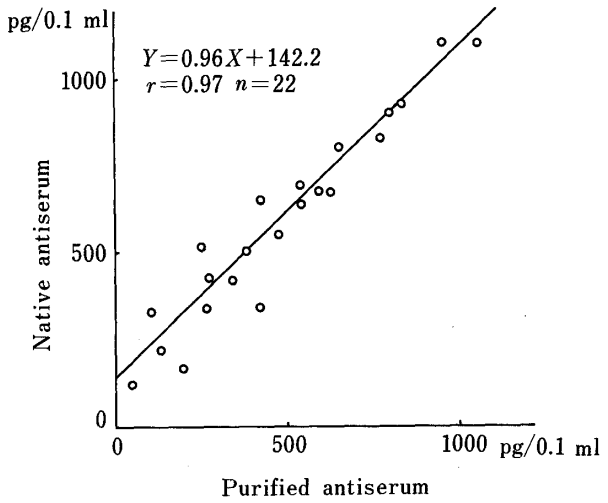

Fig. 2. Correlation of Estradiol 3-Glucuronide Levels in Urine Determined by Direct RIA with Purified and Native Antisera

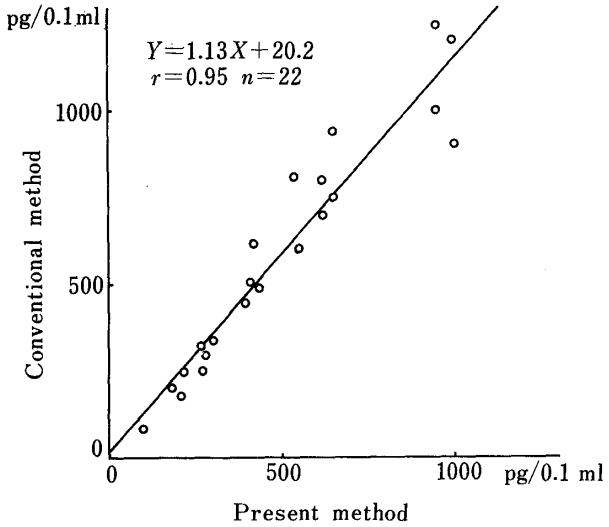

Fig. 3. Correlation of Estradiol 3-Glucuronide Levels in Urine Determined by the Present Direct RIA and Conventional RIA Methods

TABLE III. Assay Variations of the Present Method for Estradiol 3-Glucuronide in Urine

Subject No.	Found ± S.D. (pg/0.1 ml)	
	Within-assay	Between-assay
1 (Male)	204 ± 6.77 (3.32)[a]	220 ± 8.4 (3.82)
2 (Male)	393 ± 8.07 (2.05)	425 ± 15.3 (3.60)
3 (Female)	1310 ± 18.16 (1.38)	1380 ± 60.1 (4.35)

a) The figures in parentheses represent C.V. (%). $n=10$.

3, the values obtained by the proposed direct RIA method correlated closely with those obtained by the conventional RIA method[13,14] ($r=0.95$; $n=22$).

In recent years, several attempts have been made to develop direct RIA of steroid glucuronides without prior hydrolysis. Contrary to expectation, however, almost all the antisera were unsatisfactory with respect to specificity. The inadequate specificity appeared to be ascribable in part to the linkage of the immunogen, where the hapten was attached to a carrier protein *via* the carboxylic group in the glucuronyl moiety.[2,4] In order to improve the specificity, anti-estrogen glucuronide antisera were previously prepared by immunizing rabbits with three different types of antigens in which the steroid hapten was linked to BSA at the C-2 or C-4 position.[12] These antisera were actually specific to estrogen ring *D* glucuronides, but showed cross-reactivity with the ring *A* conjugates. In addition, we obtained antisera highly specific to estrone sulfate,[5] estradiol 17-glucuronide, and estriol 16-glucuronide[6] employing the [C-6]–BSA conjugates as immunogens. Similarly, specific anti-estradiol 3-glucuronide antiserum was prepared by immunizing animals with the [C-6]–BSA conjugate which was elaborately synthesized from 6-aminomethylestradiol 3-glucuronide.[7]

In the present study the preparation of 6-oxoestradiol 3-glucuronide 6-(*O*-carboxymethyl)oxime–BSA conjugate possessing a bridge at the C-6 position has been carried out. To achieve unequivocal attachment through a carboxymethyloxime bridge, the glucuronyl moiety was protected as the acetate-methyl ester until after the attachment to BSA and was then selectively hydrolyzed under mild conditions to expose the glucuronide.[11] Antibody

having a satisfactory titer was raised against the newly developed immunogen. The antiserum showed the expected specificity, discriminating free steroids and sulfates from glucuronides with the sole exception of estrone 3-glucuronide (18.66%). Therefore, cross-reacting antibodies were removed by immunoadsorption on estrone 3-glucuronide AE-cellulose conjugate. This procedure is much simpler than the usual one. The purified antiserum showed excellent specificity and was applicable to the direct RIA of estradiol 3-glucuronide in biological fluids without any pretreatment. The result obtained by the present direct RIA method was in good accord with that by the conventional RIA method. The use of specific antiserum in immunoassays means that no extraction or chromatographic separation is necessary, and such an antiserum is therefore favorable for routine assay. The newly developed methods for the preparation of the hapten–BSA conjugate and purification of elicited antibody are simple, convenient, and widely applicable. The clinical application of RIA of estradiol 3-glucuronide will be reported elsewhere in the near future.

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References and Notes

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