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SHORT COMMUNICATION

SPECIFIC ANTISERA FOR THE RADIOIMMUNOASSAY OF ESTRADIOL-3-SULFATE

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Summary—Antisera were prepared against two types of estradiol-3-sulfate-bovine serum albumin (BSA) conjugates. The haptens were coupled to BSA through the C-6 position in the steroid molecule by the glutaraldehyde (A) or the carbodiimide method (B). In comparison the antiserum produced by method A had a high affinity for estradiol-3-sulfate ($K_a = 5.64 \times 10^8\ M^{-1}$); that produced by method B had an even higher affinity ($K_a = 2.62 \times 10^9\ M^{-1}$). Furthermore the latter had no significant cross-reaction with other estrogen sulfates (<3.83%), and no cross-reaction with other steroids (<0.03%). The former revealed a little cross-reactivity with some of related steroids.

INTRODUCTION

A major part of the estrogens in pregnancy plasma is present in the conjugate form. Measurement of estrogen conjugates in pregnancy plasma is particularly useful for monitoring fetal well-being. Most assays for estrogen conjugates need a hydrolytic step achieved by enzymolysis, solvolysis, or oxidation to release the hydrophobic steroids moiety [1, 2]. This step lacks the reliability required for analytical methods because hydrolysis may not proceed quantitatively and may provide by-products. Accordingly it is desirable that estrogen conjugates are determined by direct radioimmunoassay without hydrolysis. In a recent study estradiol sulfotransferase levels in receptor-positive breast carcinomas were found to be significantly higher than those in receptor-negative tissues [3, 4]. Martin *et al.*, have reported estrone sulfate concentrations in plasmas of women with breast cancer [5]. It is therefore important to measure the estradiol-3-sulfate level in various stages of the disease. Recently, Nambara *et al.* reported the preparation of a highly specific antiserum to estrone sulfate by use of a hapten–BSA conjugate having the bridge at the C-6 position [6]. For the purpose of obtaining specific antisera for the direct radioimmunoassay of estradiol-3-sulfate, we have prepared anti-estradiol-3-sulfate antisera by using two types of antigens in which the haptens are linked to BSA through the C-6 position of the steroid molecule.

EXPERIMENTAL

Reagents

[6,7-^{3}H]Estrone sulfate (59 Ci/mmol) was purchased from New England Nuclear (Boston, MA), the labeled compound was purified by thin layer chromatography (TLC) in suitable systems prior to use. Estradiol-3-sulfate and other conjugated steroids were prepared in our laboratory by known methods. All the free steroids, bovine gamma-globulin and BSA Fraction V were supplied by Sigma Chemical Co. (St Lous, MO) and Sephadex LH-20 by Pharmacia Fine Chemicals (Uppsala, Sweden). Complete Freund's adjuvant and other general reagents were bought from Nakarai Chemicals Ltd (Kyoto, Japan) and Amberlite XAD-2 resin from Rohm and Haas Co. (Philadelphia, PA).

6-Oxoestradiol-17-acetate (II)

To a solution of 6-oxoestradiol-3,17-diacetate (I) [500 mg] in methanol (50 ml) was added 5% K_2CO_3 (10 ml) under ice-cooling, and the solution was allowed to stand at 0°C for 2 h. After neutralization with 0.1 M HCl, the reaction mixture was concentrated, and then extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The residue was recrystallized from methanol to give II (320 mg) as colorless prisms. m.p. 278–280°C. NMR (4% solution in $CDCl_3$)δ ppm 0.84 (3 H, s, 18—CH_3), 2.06 (3 H, s, —$OCOCH_3$), 4.68 (1 H, t, J=8 Hz, 17α-H), 7.20 (1 H, q, J=2, 8 Hz, 2—H), 7.44 (1 H, d, J=8 Hz, 1—H), 7.70 (1 H, d, J=4 Hz). i.r. $\nu_{max}^{KBr}\ cm^{-1}$: 3650 (HO—Ar), 1735 (—$OCOCH_3$), 1685 (>C=O). Treatment of II with trimethylsilylchloride and hexamethyldisilazane in pyridine gave the trimethylsilyl (TMS) derivative. MS (*m/z*) : 400 (M^+).

6-Oxoestradiol-O-(p-*nitrobenzyl*)*oxime-17-acetate* (III)

To a solution of II (300 mg) in methanol (20 ml) was added O-(*p*-nitrobenzyl)hydroxylamine·HCl (300 mg) and neutralized with 1 M NaOH, allowed to stand at room temperature overnight, the resulting solution was concentrated, and extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated. A residue obtained was chromatographed on silica gel (25 g). Elution with n-hexane–ethyl acetate (2:1) and recrystallization of the eluate from methanol gave III (145 mg) as yellow prisms. m.p. 207–214°C. NMR (4% solution in $CDCl_3$) δ ppm 0.80 (1 H, s, 18—CH_3), 2.06 (3 H, s, —$COCH_3$), 4.66 (1 H, t, J=6 Hz, 17α—H), 6.76 (1 H, q, J=3, 12 Hz, 2—H), 7.10 (1 H, d, J=8 Hz, 1—H), 7.31 (1 H,

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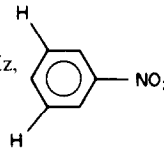
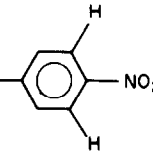
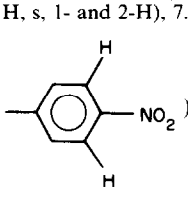
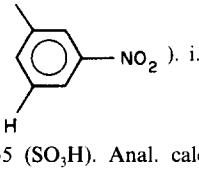
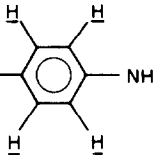
The following trivial names are used in this paper: estrone = 3-hydroxy-1,3,5(10)-estratrien-17-one; estradiol = 1,3,5(10)-estratriene-3,17β-diol; 6-oxoestradiol = 3,17β-dihydroxy-1,3,5(10)-estratrien-6-one

d, J=4 Hz, 4—H), 7.48 (2 H, d, J=9 Hz, ), 8.16 (2 H, d, J=8 Hz, ). i.r. ν_{max}^{KBr} cm^{-1}: 3690 (HO—Ar), 1735 (C=O), 1520, 1320 (NO_2). Anal. calcd for $C_{27}H_{29}O_6N \cdot H_2O$: C, 65.44, H, 6.01, N, 5.65, Found: C, 64.80, H, 6.02, N, 5.18. Trimethylsilylation of **III** led to the TMS derivative. MS (*m/z*); 550 (M^+).

6-Oxoestradiol-3-sulfate-O-(p-*nitrobenzyl*)*oxime-17-acetate* (**IVa, IVb**)

To a solution of **III** (120 mg) in pyridine (2 ml) was added chlorosulfonic acid (0.5 ml) in pyridine (7 ml) under ice-cooling. After stirring at 37°C for 2.5 h, the reaction mixture containing the 3-sulfate (**IVa**) was poured into ice-cooled 0.05 M NaOH solution (200 ml), and then allowed to stand at room temperature overnight. The resulting solution was diluted with water (300 ml), passed through Amberlite XAD-2 (2 × 40 cm), and washed with water (100 ml). The adsorbed compound was eluted with methanol (200 ml). The desired fraction was evaporated to dryness under reduced pressure at 25°C to give the crude sulfate as colorless oily product. The residue was subjected to preparative TLC using chloroform–methanol–NH_4OH (15:5:1, by vol as a solvent. The adsorbent corresponding to the band (R_f 0.32) was eluted with chloroform–methanol–NH_4OH (15:7:1, by vol) to produce **IVb** (110 mg) as a yellow amorphous substance. m.p. 225–227°C. NMR (4% solution in CD_3OD) δ ppm 0.75 (3 H, s, 18—CH_3), 3.64 (1 H, t, J=4 Hz, 17α—H), 7.25 (2 H, s, 1- and 2-H), 7.66 (1 H, s, 4—H), 7.60 (2H, d, J=8 Hz ), 8.16 (2 H, s, J=8 Hz, Hz, ). i.r. ν_{max}^{KBr} cm^{-1}: 1520, 1350 (NO_2), 1055 (SO_3H). Anal. calcd for $C_{25}H_{28}O_8N_2S \cdot NH_3 \cdot H_2O$: C, 54.46, H, 6.03, N, 7.62. Found: C, 54.18, H, 5.64, N, 7.40.

6-Oxoestradiol-3-sulfate-O-(p-*aminobenzyl*)*oxime* (**V**)

To a solution of **IVb** (80 mg) in acetone–1 M NaOH (5:1) [30 ml] was added $Na_2S_2O_4$ (610 mg) and the mixture was stirred at room temperature for 1 h. The resulting solution was passed through a column of Amberlite XAD-2 resin. After washing with distilled water the desired fraction was eluted with methanol. The eluate was purified by preparative TLC using $CHCl_3$–methanol–NH_4OH (15:7:1, by vol) as a solvent. The adsorbent corresponding to the band (R_f 0.1) was eluted with chloroform–methanol (2:1, v/v). The eluate was extracted with methanol containing a drop of NH_4OH to give **V** (35 mg) as a colorless amorphous substance. m.p. < 300°C NMR (4% solution in CD_3OD) δ ppm 0.72 (3 H, s, 18—CH_3), 3.64 (1 H, t, J=8 Hz, 17α—H), 6.72–7.64 (4 H, m, ), 7.24 (2 H, s, 1— and 2—H), 7.78 (1 H, s, 4—H). i.r. ν_{max}^{KBr} cm^{-1}: 3200–3500 (OH, NH_2), 1060 (SO_3H).

6-Oxoestradiol-O-carboxymethyloxime-17-acetate (**VII**)

To a solution of **II** (97 mg) in methanol (50 ml) was added O-carboxymethylhydroxylamine·HCl (70 mg) and neutralized with 1 M NaOH; the mixture was allowed to stand at room temperature overnight. The resulting solution was concentrated, and extracted with ethyl acetate. The organic layer was purified by silica gel column chromatography in the manner described above to give **VII** (85 mg) was colorless prisms. m.p. 178–180°C. NMR (4% solution in $CDCl_3$) δ ppm 0.78 (3 H, s, 18—CH_3), 2.05 (3 H, s, —$COCH_3$), 4.56 (2 H, m, =$NOCH_2$), 6.68–7.04 (3 H, m, Ar—H). i.r. ν_{max}^{KBr} cm^{-1}: 3400 (Ar—OH), 1610–1550 (>C=N—, —COOH). Anal. calcd for $C_{22}H_{27}O_6N \cdot H_2O$: C, 63.15, H, 6.96, N, 3.34, Found: C, 62.89, H, 6.89, N, 3.35.

6-Oxoestradiol-3-sulfate-O-carboxymethyloxime (**VIIIa, VIIIb**)

To a solution of **VII** (100 mg) in pyridine (10 ml) was added chlorosulfonic acid (0.5 ml) in pyridine (10 ml) cooled at −10°C and stirred at 37°C for 2.5 h. The resulting solution of **VIIIa** obtained was poured into 0.05 M NaOH solution and then treated as described above. The reaction mixture was purified by preparative TLC using chloroform–methanol–NH_4OH (15:5:1, by vol) to yield a colorless amorphous substance. m.p. < 300°C. i.r. ν_{max}^{KBr} cm^{-1}: 1055 (SO_3H). NMR (4% solution in CD_3OD) δ ppm 0.76 (1 H, s, 18—CH_3), 4.52 (2 H, s, =$NOCH_2$), 7.24, (2 H, s, 1— and 2—H), 7.76 (1 H, s, 4—H).

Preparation of antigens

(*i*) *Glutaraldehyde method.* To a solution of **V** (40 mg) and BSA (68 mg) in 0.1 M phosphate buffer (pH 8.0) [15 ml] was added 1% (w/v) aq. glutaraldehyde solution (1 ml) dropwise and the reaction mixture was allowed to stand at 4°C overnight. The resulting solution was dialyzed purified by gel filtration and lyophilized to give a hapten–BSA conjugate (**VI**) [60 mg]. Approximately 10 molecules of steroid were bound to a molecule of BSA when measured by using the color reaction with conc. sulfuric acid.

(ii) *Carbodiimide method.* To a solution of BSA (60 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (100 mg) in 0.1 M phosphate buffer (pH 7.1) [1.5 ml] was added **VIIIb** (50 mg) in pyridine (1.5 ml) and the mixture was stirred at 4°C overnight. The resulting solution was dialyzed and then lyophilized as usual to give a hapten–BSA conjugate (**IX**) [48 mg]. The molar hapten:BSA ratio of conjugate was evaluated to be 41.

Immunization

Three male guinea pigs were used for immunization. The conjugate (0.5 mg) was dissolved in sterile isotonic saline (0.25 ml) and emulsified with Freund's complete adjuvant (0.25 ml). This emulsion was injected subcutaneously into each thigh and below each shoulder blade. Subcutaneous injection was repeated 14 and 28 days after the initial injection, and every 30 days thereafter. The animals were bled 10 days after the booster injection. The sera were separated by centrifugation at 3500 rpm for 20 min and stored at −20°C.

Preparation of [6,7-^{3}*H*]*estradiol-3-sulfate*

To a solution of [6,7-^{3}H]estrone sulfate (200 μCi) [1.28 μg] was added $NaBH_4$ (10 μg) in methanol (200 μl)

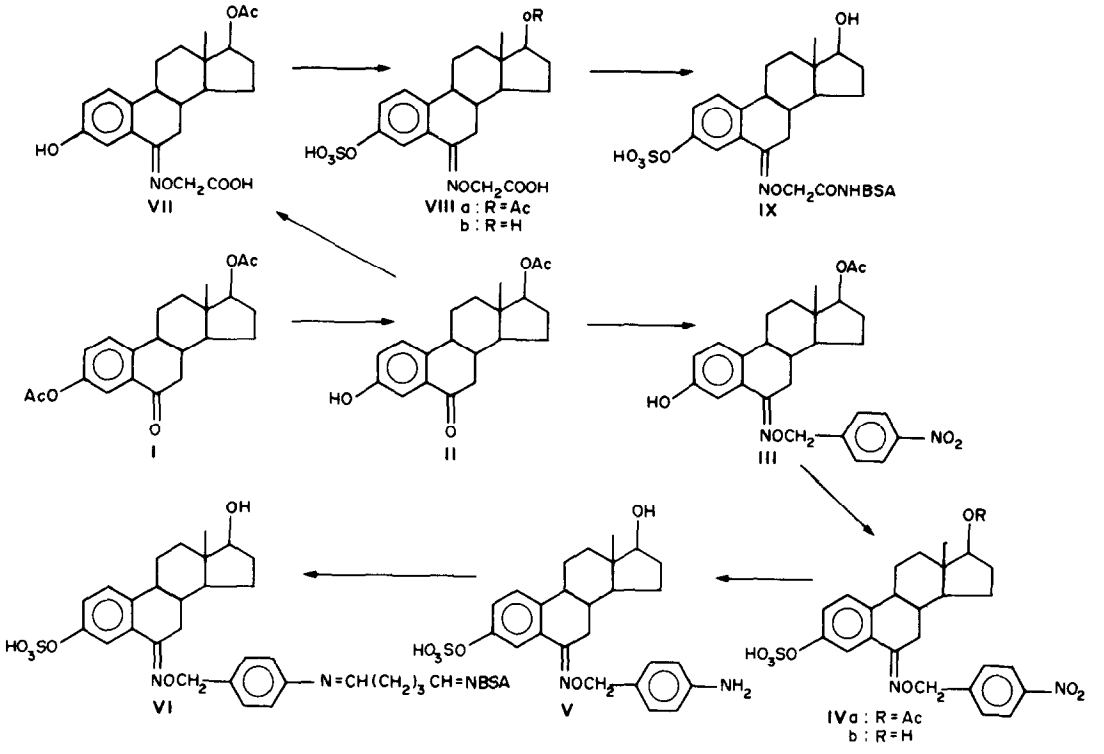


Fig. 1. Preparation of estradiol-3-sulfate–BSA conjugate.

and allowed to stand at 0°C for 1 h. The reaction mixture was diluted with water and passed through Amberlite XAD-2 column. The sulfate fraction was eluted with methanol and concentrated. The eluate was purified by preparative TLC using chloroform–methanol–NH_4OH (15:5:1, by vol) as a solvent. The band corresponding to an authentic sample was eluted with aq. methanol containing NH_4OH, the eluate was further purified by column chromatography on Sephadex LH-20, and the desired [^{3}H]estradiol-3-sulfate (120 μCi) was obtained. Radiochemical purity was greater than 98% by TLC on silica gel G.

Assay procedure

The labeled compound (59 Ci/mmol) [8×10^4 dpm/ml] was stored in methanol containing a drop of NH_4OH at 4°C for routine use. Standards were prepared in duplicate by adding a solution of estradiol-3-sulfate (0, 50, 100, 200, 500, 1000, 2000 pg) in methanol (0.1 ml) to [^{3}H]estradiol-3-sulfate (10 pg) in methanol (0.1 ml), and then organic solvent was evaporated under a stream of nitrogen gas. The diluted antiserum (0.25 ml) was added to all tubes, and the mixture was incubated at room temperature for 1 h. After addition of 50% saturated $(NH_4)_2SO_4$ solution (0.25 ml) the resulting suspension was allowed to stand at room temperature for 15 min and then centrifuged at 2500 rpm for 10 min. A $\frac{2}{10}$ ml aliquot of each supernatant was transferred into a counting vial containing scintillator solution (10 ml) and radioactivity was counted.

Cross-reaction

Cross-reaction of other steroids against the antiserum is defined as 100 X/Y, where X is the mass of nonlabeled estradiol-3-sulfate, and Y is the mass of steroid required to produce 50% binding inhibition of the labeled estradiol-3-sulfate to the antiserum.

RESULTS AND DISCUSSION

The preparation of two types of antigens for production of anti-estradiol-3-sulfate antisera was carried out (Fig. 1). First, 6-oxoestradiol diacetate (**I**) was synthesized according to the method of Dean *et al.* [7]. Upon treatment with K_2CO_3 partial hydrolysis of the acetoxyl groups was affected to furnish 6-oxoestradiol-17-acetate (**II**), which transformed into the trimethylsilyl ether was unequivocally characterized by mass spectrometry. Condensation of **II** with O-(*p*-nitrobenzyl)hydroxylamine in the usual way afforded the desired nitrobenzyloxime derivative (**III**). Treatment of **III** with chlorosulfonic acid gave the 3-sulfate (**IVa**). The aminobenzyloxime derivate (**V**) obtained from **IVa** in two steps was coupled to BSA using glutaraldehyde as a cross-linking agent to yield a hapten–BSA conjugate (**VI**). The second antigen was synthesized in the following manner. Condensation of **II** with *O*-carboxymethylhydroxylamine and subsequent reaction of **VII** with chlorosulfonic acid led to the sulfate (**VIIIa**). After hydrolysis of **VIIIa**, the desired compound (**VIIIb**) was provided in the reasonable yield. In the consecutive step, coupling of estradiol-3-sulfate to BSA was undertaken by employment of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide under the mild condition. Subsequently we succeeded in the preparation of hapten–BSA conjugate (**IX**) with intact functional groups using the carbodiimide method instead of the usual mixed anhydride method and the activated ester method. The spectra of hapten–BSA conjugates applying the color reaction demonstrated that more than 10 molecules of steroid needed for recognizing hapten structures were attached to each BSA in these two antigens.

Guinea pigs were immunized against two different types of antigens. Binding activity of estradiol-3-sulfate was present in all antisera at 3 months after the primary immunization. The titre was determined by measuring 50% binding ability of labeled steroid to diluted antiserum. The titre (1:100 dilution) of the antiserum raised against **VI** was lower than that (1:5000 dilution) raised against **IX**. The length of the bridge linkage to the carrier protein has been suggested to be important in the titre of antiserum, as it is shown that the antiserum to estrogen glucuronide conjugate attained by Mannich method had the highest titre in three

Table 1. Per cent cross-reaction of various steroids with anti-estradiol-3-sulfate antisera

Steroid	Antisera A	Antisera B
Estradiol-3-sulfate	100	100
Estrone-sulfate	4.54	3.83
Estriol-3-sulfate	7.01	0.28
Dehydroepiandrosterone sulfate	0.19	0.008
Cholesterol sulfate	0.56	<0.001
Testosterone sulfate	<0.001	<0.001
Estradiol-3-glucuronide	0.01	0.002
Estradiol-17β-glucuronide	<0.001	<0.001
Estriol-16α-glucuronide	<0.001	<0.001
Estradiol-3-methyl ether	<0.001	<0.001
2-Hydroxyestrone	<0.001	<0.001
Estrone	<0.001	<0.001
Estradiol	0.056	0.034
Estriol	<0.001	<0.001
Progesterone	<0.001	0.006
Corticosterone	0.02	<0.001
Cortisol	0.05	<0.001

(A) Glutaraldehyde method; (B) Carbodiimide method.

types of antigens [8, 9]. Accordingly the immunization of the hapten–protein conjugate which possesses a comparatively short bridge can be expected to produce an antiserum of the high titre.

In binding affinity, a constant volume of diluted antiserum was incubated with various amounts of the labeled antigen. By varying the labeled antigen a relationship between the concentration of antigen and the ratio of bound to free was evaluated by a Scatchard plot [10]. The linearity of plots indicated that antiserum with estradiol-3-sulfate was homogenous, having an association constant (K_a) of $5.64 \times 10^8\ M^{-1}$ (**VI**) and K_a of $2.62 \times 10^9\ M^{-1}$ (**IX**) each. Both antisera raised against the two types of antigens exhibited high affinity for estradiol-3-sulfate. A standard curve was constructed with 1:100 or 1:2000 dilution of antiserum. The accuracy of the method was examined with various known amounts of authentic estradiol-3-sulfate ranging from 20–2000 pg.

The specificity of antisera was established by testing the ability of the analogous steroid to compete for binding site on the antibody. The per cent cross-reaction of antiserum was calculated by the method of Abraham [11]. The cross-reactivity of anti-estradiol-3-sulfate antisera with various steroids is listed in Table 1. These antisera are highly specific to estradiol-3-sulfate. The antiserum raised against **IX** exhibited somewhat low cross-reactivity compared to **VI** with estrogen sulfates. It is reasonable that cross-reaction of 0.28, 7.01% with estriol-3-sulfate and of 3.83, 4.54% with estrone-sulfate is dependent on the close similarity of the structure of these compounds to estradiol-3-sulfate. There was no significant cross-reactivity with free estrogens, estrogen glucuronides and other steroids. The antisera raised against estrogens indicated a high degree of cross-reactivity with related steroids where the inherent groups of the steroid nucleus, namely C-3, C-17, have been used for coupling with a carrier protein [12, 13], while those against estradiol-3-glucuronide-6-BSA, and estrone-sulfate-6-BSA were highly specific [6, 14].

In order to obtain specific antiserum for assay of estradiol-3-sulfate, the steroid hapten should be bound to the carrier protein through the C-6 position, remote from particular functional groups in the steroid molecule. In this study, it has been reported that a specific anti-estradiol-3-sulfate antisera for radioimmunoassay can be obtained by the preparation of a hapten–BSA conjugate having the intact sulfate moiety. To make specific antiserum of other steroid conjugate for immunoassay this method would be applicable.

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