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# Preparation of Specific Antiserum to Estradiol 17-Sulfate (Clinical Analysis on Steroids. XXVIII[1])

Itsuo Yoshizawa,* Masayuki Kameyama and Kazuhiro Watanabe

*Hokkaido Institute of Pharmaceutical Sciences, 7–1, Katsuraoka-cho, Otaru, Hokkaido 047–02, Japan*

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The preparation and antigenic properties of estradiol 17-sulfate-bovine serum albumin conjugate in which the hapten is linked to the carrier protein through the C-6 position on the steroid nucleus are described. Antibody raised against antigen in the rabbit possessed extremely high specificity to estradiol 17-sulfate, exhibiting no significant cross-reactions with other estrogen sulfates except estriol 17-sulfate (0.2%), and no cross-reactions with free estrogens, their glucuronides, or other related steroids (<0.001%).

**Keywords**——radioimmunoassay; steroidal sulfate; estradiol 17-sulfate; cross-reaction; ion chromatography

In a recent investigation, we demonstrated the sulfate-specific 2-hydroxylation of estradiol 17-sulfate (E-17-S) by male rat liver microsomes.[2] The formation of the catechol, 2-hydroxyestradiol 17-sulfate (2-OH-E-17-S), was found to be induced by treatment of animals with phenobarbital.[3]

Previously, we have shown the directive effect of the 17-sulfate group on the *O*-methylation of 2-OH-E-17-S by the enzyme catechol *O*-methyltransferase (COMT), in that *O*-methylation of the C-2 phenolic hydroxyl group exceeded that of the C-3 group.[4] This result is reminiscent of the endogenous metabolism of 2-hydroxyestradiol, where 2-methoxyestradiol is predominantly formed accompanied with a minor amount of the 3-methoxy isomer.[5] From these results, we consider that the following pathways may operate in estradiol metabolism in the rat and/or human: estradiol → E-17-S → 2-OH-E-17-S → 2-methoxyestradiol 17-sulfate.

Although the liver has the capacity to convert estradiol into E-17-S,[6] there have been no reports on the presence of this sulfate in urine or plasma. This may be owing to the very low concentration of this conjugate in biological fluids.

In the course of our research on the metabolism of E-17-S and also on the changes of this conjugate during the menstrual cycle, it became necessary to determine the plasma and urinary E-17-S, preferably by a simple method. We have, therefore, developed a radioimmunoassay for E-17-S, and this paper presents the details of the methodology.

## Materials and Methods

### Chemicals and Reagents

(6,7-$^3$H)-Estradiol (54 Ci/mmol) was obtained from New England Nuclear (Boston, Mass, U.S.A.), and was used without further purification. Steroidal standards used for cross-reaction studies were prepared by the known methods as cited in Table I. Other steroids were obtained from Steraloids Inc. (Wilton, NH, U.S.A.). Bovine serum albumin (BSA, Fraction V) was purchased from Armour Pharm. Co. (Kankakee, Ill., U.S.A.), bovine serum gamma-globulin from ICN Pharm. Inc. (Cleaveland, U.S.A.), Freund's complete adjuvant from Difco Lab. (Detroit, U.S.A.), Sep-pak $C_{18}$ cartridges from Waters Ltd. (Milford, Mass., U.S.A.), Amberlite XAD-2 resin from Rohm and Haas Co.

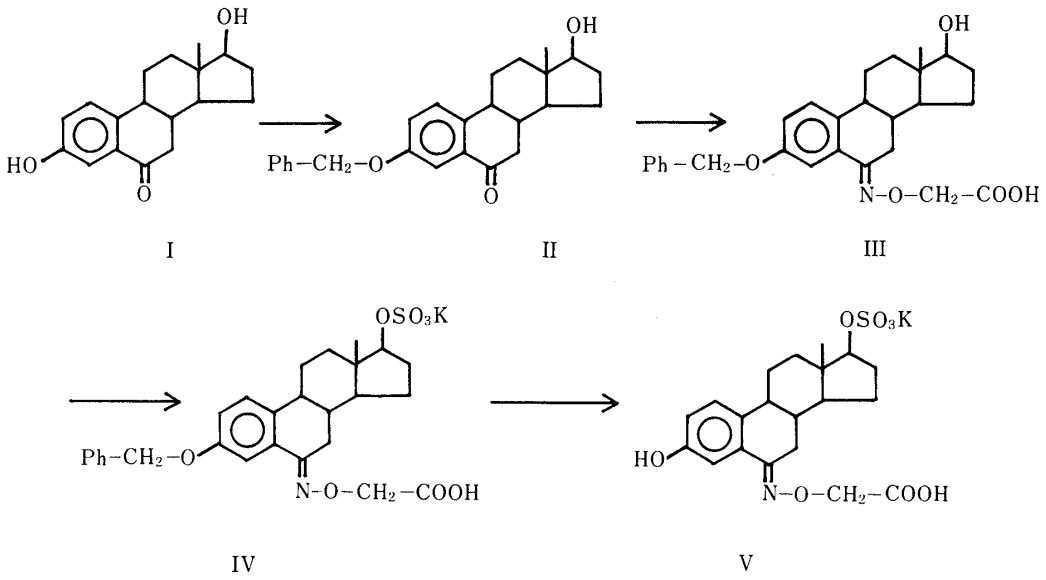


Chart 1

(Philadelphia, U.S.A.), and Mylase P and other general reagents from Wako Pure Chemicals Ltd. (Tokyo, Japan).

**Synthesis of Hapten–BSA Conjugate**

**Instruments**——Melting points were determined on a Kofler-type micro-hot stage (Mitamura, Tokyo, Japan) and are uncorrected. Thin-layer chromatography (TLC) was performed with Merck precoated Silica gel 60 $F_{254}$ plates. Nuclear magnetic resonance (NMR) spectra were measured on a JNM FX-100 spectrometer (JEOL, Tokyo) at 100 MHz and chemical shifts are expressed as $\delta$ values relative to 1% tetramethylsilane as an internal standard. Abbreviations used s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet. Infrared spectra (IR, $\nu_{max}$) in KBr disks were recorded on a JASCO IR-2 machine (Nihon Bunko, Tokyo) and are given in $cm^{-1}$. Mass spectra (MS) were taken by the direct insertion method with a 9000 B spectrometer (Shimadzu, Kyoto, Japan). Ultraviolet (UV) spectra were measured in MeOH with a model 200-20 spectrometer equipped with an X-Y recorder (Hitachi, Tokyo) and are expressed as $\lambda_{max}$ nm ($\varepsilon$) values.

**3-Benzyloxy-17$\beta$-hydroxyestra-1,3,5(10)-trien-6-one (II)**——An ethanolic solution of 6-oxoestradiol[7] (I, 3.04 g in 250 ml) containing benzyl chloride (6.0 g) and anhydrous $K_2CO_3$ (2.0 g) was refluxed for 4 h. After being cooled to room temperature, the mixture was concentrated under reduced pressure to give a residue, which was dissolved in 300 ml of EtOAc. The solution was washed with water, dried over anhydrous $Na_2SO_4$, and concentrated. The oily product obtained was chromatographed on an $Al_2O_3$ column (20 cm × 1.0 cm, I.D.). The fraction obtained by elution with $CHCl_3$ was recrystallized from EtOH to afford 3.0 g of fine needles, mp 177—178 °C. *Anal.* Calcd for $C_{25}H_{28}O_3$ (376.47): C, 79.75; H, 7.50. Found: C, 79.64; H, 7.38. UV: 256 (9100), 322 (3400). IR: 3490 (OH), 3000—2880 (CH), 1660 (C=O), 1600 (aromatic C=C). NMR (DMSO-$d_6$): 7.5—7.3 (8H, m, aromatic H), 5.12 (2H, s, Ph–$\underline{CH_2}$–O), 3.77 (1H, t, $J$ = 8.0 Hz, 17$\alpha$-H), 0.79 (3H, s, 18-H). MS, *m/e*: 376 ($M^+$), 91 (tropylium ion).

**3-Benzyloxy-6-carboxymethoxyimino-17$\beta$-hydroxyestra-1,3,5(10)-trien (III)**——A pyridine solution (200 ml) containing II (1.50 g) and carboxymethoxylamine hydrochloride (1.70 g) was heated for 6 h at 80 °C. The mixture was cooled to room temperature, and pyridine was removed under reduced pressure to give a residue, which was dissolved in 300 ml of EtOAc. The solution was washed with 0.1 N HCl (50 ml × 3) and water (50 ml × 4), then dried, and concentrated. The product (1.75 g) was recrystallized from EtOAc to give fine needles, mp 180—183 °C. *Anal.* Calcd for $C_{27}H_{31}NO_5$ (499.53): C, 72.14; H, 6.95; N, 3.12. Found: C, 72.24; H, 7.04; N, 3.02. UV: 263 (11750), 308 (4400). IR: 3420 (OH), 3100—2950 (CH), 2800—2400 (COOH), 1730 (C=O), 1620 (aromatic C=C), 1590 (C=N). NMR (DMSO-$d_6$): 14.20 (1H, s, COOH), 7.3—6.9 (8H, m, aromatic H), 5.08 (2H, s, Ph–$\underline{CH_2}$–O), 4.66 (2H, s, O–$\underline{CH_2}$–COOH), 0.64 (3H, s, 18-H). MS, *m/e*: 499 ($M^+$), 91 (tropylium ion).

**Potassium 3-Benzyloxy-6-carboxymethoxyiminoestra-1,3,5(10)-trien-17$\beta$-yl Sulfate (IV)**——Freshly prepared sulfur trioxide–pyridine complex (1.0 g) was added to a pyridine solution (260 ml) of III (1.70 g), and the mixture was heated at 50 °C overnight. Pyridine was removed under reduced pressure to give an oily product, which was dissolved in 50 ml of water. The solution was neutralized by addition of 0.1 N KOH, and passed through a column packed with Amberlite XAD-2 resin (30 cm × 5 cm, I.D.). After thorough washing of the column with water, the desired product was eluted with MeOH. The eluate was concentrated under reduced pressure to give a residue, which was applied to a column (10 cm × 1.0 cm, I.D.) packed with Dowex 50W (× 8, 200—400 mesh, $K^+$ form) and eluted with water. The eluate was concentrated under reduced pressure below 50 °C to give a crude product (1.60 g), which was recrystallized from MeOH to afford fine needles, mp 191.5—195 °C. *Anal.* Calcd for $C_{27}H_{30}KNO_8S$ (567.69): C, 57.12; H, 5.32; N,

2.46; S, 5.65. Found: C, 57.00; H, 5.55; N, 2.18; S, 5.49. UV: 261 (10950), 308 (3850). NMR (MeOH-$d_4$): 7.5—6.9 (8H, m, aromatic H), 5.06 (2H, s, Ph–$\underline{CH_2}$–O), 4.70 (2H, s, O–$\underline{CH_2}$–COOH), 4.32 (1H, t, $J$=8.8 Hz, 17α-H), 0.82 (3H, s, 18-H). IR: 3600—3400 ($H_2O$), 3100—2800 (CH), 2800—2400 (COOH), 1730 (C=O), 1620 (aromatic C=C), 1590 (C=N), 1220 ($OSO_3K$).

**Potassium 6-Carboxymethoxyimino-3-hydroxyestra-1,3,5(10)-trien-17β-yl Sulfate (V)**——A methanolic solution (80 ml) containing IV (1.30 g) and 10% Pd/C (1.30 g) was stirred under an $H_2$ stream at atmospheric pressure for 40 h at room temperature. After removal of the catalyst, the solution was concentrated under reduced pressure to give a white powder (670 mg), which was recrystallized from MeOH to afford fine needles, mp 232—237 °C. *Anal.* Calcd for $C_{20}H_{24}KNO_8S$ (477.57): C, 50.30; H, 5.07; N, 2.93; S, 6.72. Found: C, 50.04; H, 5.22, N, 2.76; S, 6.56. UV: 261 (11400), 311 (4200). IR: 3500—3200 (OH), 3000—2950 (CH), 2800—2400 (COOH), 1710 (C=O), 1580 (C=N), 1220 ($OSO_3K$). NMR (MeOH-$d_4$): 7.33 (1H, d, $J$=2.7 Hz, 4-H), 7.17 (1H, d, $J$=8.4 Hz, 1-H), 6.75 (1H, q, $J_1$=8.4 Hz, $J_2$=2.7 Hz, 2-H), 4.61 (2H, s, O–$\underline{CH_2}$–COOH), 4.33 (1H, t, $J$=8.8 Hz, 17α-H), 0.83 (3H, s, 18-H).

**Estradiol 17-Sulfate-(C-6)-BSA Conjugate**——Isobutyl chloroformate (6 mg) was added to a solution (2.5 ml) of V (19 mg) and tri-*n*-butylamine (10 mg) in dioxane and dimethylformamide (1 : 2). The mixture was stirred under cooling for 1 h, then BSA (83 mg) dissolved in 60% aq. dioxane (6 ml) was added. The whole was stirred under cooling for 5 h followed by additional stirring for 6 h at room temperature; it was kept neutral by addition of a few drops of 1 N KOH. The resulting solution was dialyzed against distilled water for 70 h at room temperature. The steroid–protein conjugate was obtained by lyophilization as a white powder (83 mg). The steroid: protein molar ratio of the conjugate was spectrophotometrically (at 270 nm in MeOH) determined to be 28.5.

**Determination of Sulfate Group by the Ion Chromatographic Method**

**Equipment**——The ion chromatograph used was a type 2000i (Dionex, Midland, MI, U.S.A.) with a stainless steel column (5 cm × 4.6 mm, I.D.) packed with TSK GEL IC-620 SA (Toyo Soda, Tokyo) and equipped with an electric conductivity detector. The column was maintained at 30 °C. A solution (pH 8.5) containing 1.3 mM sodium borate and 1.3 mM gluconic acid was used as the mobile phase at a flow rate of 1.2 ml/min, and a pressure of 8 kg/cm$^2$.

**Hydrolysis of Antigen**——An exact amount (*ca.* 3.0 mg) of hapten–BSA conjugate was dissolved in 4 N HCl (3 ml), and the solution was refluxed for 6 h. The solution was cooled and a known amount of $Na_2HPO_4$ was added as an internal standard. The whole was diluted with water to about 10 ml. The whole was centrifuged at 3000 rpm for 10 min to remove the precipitate, followed by extraction with EtOAc (2 ml × 3). The aq. layer was centrifuged again at 3000 rpm, and injected into a chromatograph with a microliter syringe. Relative retention times of $Cl^-$ and $SO_4^{2-}$ were 0.32 and 1.11 ($HPO_4^{2-}$ = 1.00, 9.01 min), respectively.

**Standard Curve**——To obtain the standard curve, 1.0, 2.0, 3.0, 5.0, 10.0, 20.0, 30.0, or 50.0 ppm of $SO_4^{2-}$ ($Na_2SO_4$) was added to a solution of $HPO_4^{2-}$ (10.0 ppm). Each mixture was injected into the chromatograph. The calibration curve was constructed by plotting the peak height of $SO_4^{2-}$ relative to that of the internal standard ($HPO_4^{2-}$) against the amount of the former.

**Preparation of (6,7-$^3$H)-Estradiol 17-Sulfate**

**Radioactivity Counting**——Radioactivity was counted with a Packard Tri-Carb 2650 liquid scintillation spectrophotometer. Toluene containing 6 g/l of 2,5-diphenyloxazole and 300 mg/l of 1,4-bis(5-phenyloxazol-2-yl)-benzene was used as a scintillant. Aq. samples were counted in Bray's system. The radio thin-layer scannograph used was an Aloka JTC-203 machine (Nihon Musen, Tokyo).

**Preparation Method**——Chlorosulfonic acid (0.2 ml) was added to a pyridine solution (5.0 ml) containing (6,7-$^3$H)-estradiol (0.5 mCi), and the mixture was heated at 50 °C for 4 h. Complete sulfation of the labeled steroid was confirmed as follows. A part of the reaction mixture was taken into 0.5 ml of water, and the solution was passed through a Sep-pak $C_{18}$ cartridge. After thorough washing of the cartridge with water (1.0 ml), the conjugate was obtained by elution with MeOH, and was subjected to TLC with EtOAc–MeOH–$H_2O$ (8 : 2 : 1) as the solvent system. After development, the plate was scanned with a radio thin-layer scannograph. The sole radioactive peak coincided in *Rf*-value with authentic estradiol 3,17-disulfate developed concurrently.

Pyridine was removed from the reaction mixture under reduced pressure at 50 °C to give a residue, which was neutralized by addition of 0.1 N $NaHCO_3$. The solution was passed through a Sep-pak $C_{18}$ cartridge. After washing of the cartridge with water (3 ml), the desired labeled steroid was obtained by elution with MeOH (the recovery of radioactivity was 87%). The radioactive product was then dissolved in 5 ml of acetate buffer (10 mM, pH 6.0) containing 500 mg of Mylase P. The whole was incubated at 37 °C for 3 d. After filtration, the mixture was passed through a Sep-pak $C_{18}$ cartridge. The labeled conjugate fraction obtained by elution with MeOH was subjected to preparative TLC using the same solvent as described above. The band corresponding to the spot of authentic E-17-S was scraped off, and eluted with MeOH. The desired tracer was kept in a refrigerator. The radiochemical purity was found to be over 98% by the reverse isotope dilution method.

**Immunization of Rabbits**

Three male albino rabbits were used for immunization. The antigen (2 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with Freund's complete adjuvant (0.5 ml). This emulsion was injected into rabbits subcutaneously at multiple sites of the back and foot pads. This procedure was repeated at intervals of two weeks for a further two months and then once a month. The rabbits were bled 10 d after the final booster injection. The sera

were separated by centrifugation at 3000 rpm for 10 min, and stored at −20 °C. The antisera were thawed and diluted with 0.05 M borate buffer (pH 8.0) containing 0.06% BSA and 0.05% bovine serum gamma-globulin.

**Assay Procedure**

A standard curve was constructed by setting up duplicate centrifuge tubes (10 ml) containing 0, 5.0, 10, 20, 30, 50, 100, 200, 300, 500, 700, and 1000 pg of non-labeled E-17-S and (6,7-$^3$H)-E-17-S (38 pg, 10000 dpm). A 1 : 7500 dilution of antiserum (0.25 ml) was added to each mixture and the tubes were incubated for 60 min at room temperature. After addition of 50% aq. $(NH_4)_2SO_4$ solution (0.25 ml), the whole was allowed to stand for 15 min at room temperature, then centrifuged at 3000 rpm for 10 min. A one-fifth ml aliquot of each supernatant was transferred into a counting vial containing a scintillation cocktail (5 ml), and the radioactivities were counted.

**Cross-Reaction Study**

The specificity of antiserum raised against the estradiol 17-sulfate-BSA conjugate was tested by cross-reaction studies with thirty kinds of purified steroids (Table I). The relative amounts required to reduce the initial binding of labeled steroid by half, where the mass of non-labeled E-17-S was arbitrarily taken as 100%, were calculated from the standard curve.

## Results and Discussion

A haptenyl compound V was prepared by the method summarized in Chart 1, and was covalently linked to BSA by the mixed anhydride method. The amount of $SO_4^{2-}$ liberated by the hydrolysis of antigen was determined by the ion chromatographic method to be 29.0 per mol of the antigen. The calibration curve for $SO_4^{2-}$ obtained by the internal standard method (internal standard: $HPO_4^{2-}$) showed satisfactory linearity in the range of 0.5—30 ppm. The value obtained by the UV method (28.5) was essentially identical with the above result. Thus, it was confirmed that the ethereal sulfate group at the C-17 position of the hapten was not hydrolyzed during the coupling reaction with BSA. It may be concluded, therefore, that a satisfactory amount of steroid sulfate was coupled with BSA without any structural change of the hapten moiety.

The present method using an ion chromatograph is convenient for the determination of ethereal sulfate of the hapten moiety in the antigen molecule. This procedure may also be useful in radioimmunoassay for sulfoconjugated steroids or drugs.

The serum sample from the immunized rabbits showed increased binding activity to estradiol 17-sulfate, though there was considerable individual variation. At three or four months after the first injection, several samples showed a significant increase in binding activity. The anti-estradiol 17-sulfate antiserum obtained was used for the assay, in which free and bound antigens were readily separated by addition of ammonium sulfate followed by centrifugation. The standard curve obtained with 1 : 7500 dilution of the antiserum is shown in Fig. 1. The percentage of free radioactivity increased linearly with logarithmic increase in non-labeled E-17-S concentration from 20 to 600 pg.

The specificity of the antiserum was assessed by testing the ability of related steroids to

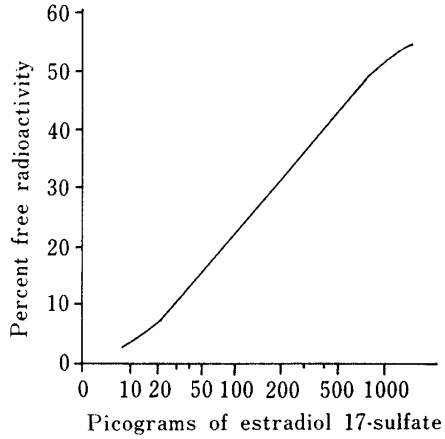


Fig. 1. Standard Curve with Anti-estradiol 17-Sulfate Antiserum, 1 : 7500 Dilution

TABLE I. Cross-Reactions of Anti-estradiol 17-Sulfate Antiserum with Selected Steroids

| Steroid | % Cross-reactivity (50%) |
|---|---|
| Estradiol 17-sulfate[a] | 100 |
| Estriol 17-sulfate[b] | 0.20 |
| 2-Hydroxyestradiol 17-sulfate[c] | 0.03 |
| 2-Methoxyestradiol 17-sulfate[d] | 0.02 |
| Estriol 16-sulfate[b] | 0.003 |
| Estradiol 3-sulfate[a] | <0.001 |
| Estriol 3-sulfate[b] | <0.001 |
| Estradiol 3,17-disulfate[a] | <0.001 |
| Estrone sulfate[e] | <0.001 |
| 4-Hydroxyestradiol 17-sulfate[f] | <0.001 |
| Dehydroepiandrosterone sulfate[g] | <0.001 |
| Pregnanediol 3-sulfate[h] | <0.001 |
| Pregnanediol 20-sulfate[h] | <0.001 |
| Pregnanediol 3,20-disulfate[h] | <0.001 |
| Cholesterol sulfate[i] | <0.001 |
| Testosterone sulfate[j] | <0.001 |
| Estradiol 3-glucuronide[k] | <0.001 |
| Estradiol 17-glucuronide[k] | <0.001 |
| Estrone glucuronide[k] | <0.001 |
| 2-Hydroxyestradiol 17-glucuronide[c] | <0.001 |
| 2-Methoxyestradiol 17-glucuronide[d] | <0.001 |
| 2-Hydroxyestradiol 3-methyl ether 17-glucuronide[d] | <0.001 |
| Estriol 16-glucuronide[l] | <0.001 |
| Pregnanediol 3-glucuronide[m] | <0.001 |
| Estradiol | <0.001 |
| Estrone | <0.001 |
| Estriol | <0.001 |
| 2-Hydroxyestradiol[n] | <0.001 |
| 4-Hydroxyestradiol[o] | <0.001 |
| Progesterone | <0.001 |
| Pregnanediol | <0.001 |

a) Ref. 13. b) Ref. 14. c) Ref. 15. d) Ref. 16. e) Ref. 17.
f) Ref. 18. g) Ref. 19. h) Ref. 20. i) Ref. 21. j) Ref. 22.
k) Ref. 23. l) Ref. 24. m) Ref. 25. n) Ref. 26. o) Ref. 27.

compete for binding sites on the antibody. The percent cross-reaction of the antiserum was determined by the method of Abraham[8] from the displacement of labeled steroid added. The results on cross-reactions of anti-E-17-S antiserum with various closely related steroids are listed in Table I. It is evident that the antiserum is highly specific to E-17-S. There were no significant cross-reactivities with other estrogen sulfates and no cross-reactivities with free estrogens, their glucuronides or other related steroids. Even the most cross-reactive steroid, estriol 17-sulfate, showed less than 0.2% cross-reactivity. Estradiol 17-glucuronide, which is known to be a major urinary estradiol in humans,[9] cross-reacted to the extent of less than 0.001%, *i.e.*, it showed essentially no cross-reaction.

It is considered that the structure remote from the attachment position of the carrier protein is readily recognized with a hapten–protein conjugate.[10] Nambara *et al.*[11] showed that highly specific antisera discriminating the structure around the conjugated position of estrogen D-ring glucuronides can be raised against hapten–BSA conjugate having a linkage through a position remote from the glucuronyl moiety. A similar result was obtained in the case of sulfate: antiserum specific to estrone sulfate was elicited with hapten–BSA conjugate

having a bridge at C-6 on the steroid nucleus.[12] In the present work, it has also been demonstrated that the use of an immunologic complex which has an intact ethereal sulfate group and a linkage to the carrier through the C-6 position can provide antibody highly specific to E-17-S.

Development of a simple and reliable radioimmunoassay system for E-17-S in biological fluids using this antiserum will be the subject of a future communication.

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