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Specific Antiserum to 2-Hydroxyestradiol 17-Sulfate (Clinical Analysis on Steroids. XXX[1])

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A bovine serum albumin conjugate of 2-hydroxyestradiol 17-sulfate was used for antibody preparation in rabbits. The hapten was linked to the carrier protein through the C-6 position on the steroid nucleus. The antibody obtained possessed extremely high specificity to 2-hydroxyestradiol 17-sulfate, exhibiting essentially no cross-reactions with other related steroids and their conjugates.

Keywords——radioimmunoassay; steroid sulfate; 2-hydroxyestradiol 17-sulfate; cross-reaction; catechol estrogen

Recently, steroids have been analyzed directly as conjugate forms by employing such methods as radioimmunoassay (RIA), high-performance liquid chromatography (HPLC), or gas chromatography-mass spectrometry (GC-MS). This is largely because it is now recognized that conjugated steroids play important physiological roles, and that the position of the conjugate group can provide valuable information on the biotransformation of steroids in the body.

Because of its simplicity, convenience, and ability to deal with many samples, RIA has been used for direct measurement of such esterogen conjugates as estrone sulfate,[2] estradiol 17-glucuronide,[3] or estriol 16-glucuronide.[4] Estrone sulfate is endocrinologically the most important plasma circulating estrogen, and the RIA of this conjugate has been investigated by numerous laboratories.[2] Estradiol 3-sulfate, which is the reduction product of estrone sulfate at the C-17 position, can now be measured by RIA.[5]

Recently, we prepared specific antiserum to estradiol 17-sulfate by utilizing the C-6 position on the steroidal nucleus for the formation of hapten–bovine serum albumin (BSA) conjugate.[6] This RIA study was started for the measurement of estradiol 17-sulfate in biological fluids. Application of this highly specific antiserum to human (female) urine revealed that a considerable amount of this sulfate is excreted in urine.[7]

Previously, we have observed the 2-hydroxylation of estradiol 17-sulfate by rat liver microsomes with a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system,[8] and such catechol formation was found to occur even for estradiol 17-glucuronide, resulting in the formation of 2-hydroxyestradiol 17-glucuronide.[9]

Thus, a part of circulating estradiol 17-sulfate may undergo 2-hydroxylation to 2-hydroxyestradiol 17-sulfate, which should consequently be found in the biological fluids. To examine this possibility, it was necessary to measure plasma or urinary 2-hydroxyestradiol 17-sulfate. We have, therefore, developed an RIA method for this sulfate without prior deconjugation, and this paper describes the details of the methodology.

Materials and Methods

Chemicals and Reagents——[6,7-^{3}H]-Estradiol 17-sulfate (54 Ci/mmol) was obtained by the method described previously.[6] The starting material, 2,3,17β-trihydroxyestra-1,3,5(10)-trien-6-one (I)[10] and other steroidal standards[6]

used for cross-reaction studies were prepared in this laboratory by the methods cited. 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. (Detroit, U.S.A.), Sep-Pak C_{18} cartridges from Waters Ltd. (Milford, Mass., 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 on 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 in δ values relative to 1% tetramethylsilane as an internal standard. Abbreviations used: s = singlet and t = triplet. Infrared (IR) spectra (ν_{max}) in KBr disks were recorded on a JASCO IR-2 machine (Nihon Bunko, Tokyo) and are expressed in cm^{-1}. Mass spectra (MS) were taken by the direct insertion method with a 9000B instrument (Shimadzu, Kyoto, Japan). Ultraviolet (UV) spectra were measured in methanol with a model 200-20 spectrometer equipped with an X-Y recorder (Hitachi, Tokyo) and are expressed as λ_{max} nm (ε). HPLC was carried out on a model 803 chromatograph (Toyo Soda, Tokyo) with absorbance monitoring at 270 nm. A stainless steel column (30 cm × 4 mm, i.d.) packed with TSK Gel ODS-120A (5 μm, Toyo Soda) was used. The flow rate of the mobile phase was 1.0 ml/min and the column pressure was 130 kg/cm^2. The temperature of the column was maintained at 40 °C in a circulating water bath. Preparative HPLC was carried out on the same machine using a 30 cm × 7.5 mm (i.d.) column packed with the same stationary phase under similar conditions. The following solvent was used for HPLC as the mobile phase, 0.5% $NH_4H_2PO_4$ (pH 3.0)–methanol (60 : 40, v/v). 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) 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.

6-*O*-Carboxymethoxyimino-2,3,17β-trihydroxyestra-1,3,5(10)-triene (II)——A pyridine solution (50 ml) containing I (300 mg) and *O*-carboxymethylhydroxylamine hydrochloride (600 mg) was heated under a nitrogen stream for 6 h at 40 °C. The mixture was cooled to room temperature, and pyridine was removed under reduced pressure to give a residue, which was dissolved in 200 ml of ethyl acetate. The solution was washed with 0.1 N HCl (30 ml × 3), and water (30 ml × 4), then dried over anhydrous Na_2SO_4, and concentrated. The product (400 mg) was recrystallized from ethyl acetate to give fine needles, mp 216—218 °C. *Anal.* Calcd for $C_{20}H_{25}NO_6$ (375.41): C, 63.98; H, 6.71; N, 3.73. Found: C, 63.84; H, 6.79; N, 3.68. UV: 272 (11720), 312 (7850). IR: 3400 (OH), 2850 (CH), 1730 (C=O), 1590 (C=N). NMR (methanol-d_4): 7.32 (1H, s, 4-H), 6.74 (1H, s, 1-H), 4.62 (2H, s, $\underline{CH_2}$–COOH), 4.10 (1H, t, J = 6.0 Hz, 17α-H), 0.75 (3H, s, 18-H). MS: 375 (M^+), 301 [$M^+ - 74$(O–CH_2–COO^-)].

6-*O*-Carboxymethoxyimino-2,3,17β-trihydroxyestra-1,3,5(10)-triene Trisulfate (III)——Freshly prepared sulfur trioxide–pyridine complex (1.0 g) was added to a pyridine solution (100 ml) of II (500 mg), and the mixture was heated at 55 °C overnight. Pyridine was removed under reduced pressure to give an oily product, which was dissolved in 10 ml of water. After being neutralized by addition of 1 N Na_2CO_3, the aq. solution was diluted with 200 ml of *n*-butanol. The butanolic solution was washed with KCl-saturated water (30 ml × 3), and once with water (20 ml), then evaporated under reduced pressure to give a residue (560 mg). Complete sulfation of II was checked in the following way.

Compound III (20 mg) was dissolved in a mixture (3.0 ml) of pyridine and acetic anhydride (2 : 1). The resultant solution was allowed to stand for 20 h at room temperature, then concentrated under reduced pressure. The oily product obtained was dissolved in 1.0 ml of water, and the solution was passed through a Sep-Pak C_{18} cartridge. The cartridge was washed with water (2.0 ml), then eluted with methanol (3.0 ml). The product obtained from the methanolic eluate was identical with III in terms of chromatographic behavior on HPLC.

Dipotassium 6-*O*-Carboxymethoxyimino-2,3-dihydroxyestra-1,3,5(10)-trien-17β-yl Sulfate (IV)——The trisulfate (III, 500 mg) was dissolved in 100 ml of acetate buffer solution (10 mM, pH 6.0) containing Mylase P (25 g, enzyme activity: unknown) and ascorbic acid (100 mg), and the mixture was allowed to stand for a week at 37 °C. After filtration, the mixture was extracted with *n*-butanol. The extract was concentrated under reduced pressure to give the residue, which was applied to a column (20 cm × 1 cm, i.d.) packed with Dowex 50W ion-exchange resin (× 8, 200—400 mesh, K^+ form), and eluted with water. The eluate was concentrated under reduced pressure below 50 °C to give a white powder (420 mg), which was recrystallized from methanol to afford fine needles, mp 258—262 °C. *Anal.* Calcd for $C_{20}H_{23}K_2NO_9S$ (531.59): C, 45.19; H, 4.36; N, 2.64; S, 6.02. Found: C, 44.70; H, 4.69; N, 2.18; S, 5.68. UV: 271 (9270), 310 (5800). NMR (methanol-d_4): 7.20 (1H, s, 4-H), 6.59 (1H, s, 1-H), 4.67 (2H, s, $\underline{CH_2}$–COOK), 4.36 (1H, t, J = 6.0 Hz, 17α-H), 0.68 (3H, s, 18-H).

2-Hydroxyestradiol 17-Sulfate-(C-6)–BSA Conjugate——Isobutyl chloroformate (0.6 mg) was added to a mixture (1.5 ml) of dioxane and dimethylformamide (1 : 2, v/v) containing IV (10 mg) and tri-*n*-butylamine (0.3 mg). The mixture was stirred under cooling for 1 h, then BSA (30 mg) dissolved in 60% aq. dioxane (3 ml) was added. The whole was stirred under cooling for 5 h followed by additional stirring for 6 h at room temperature. The solution was kept neutral by addition of 0.1 N Na_2CO_3. The resulting solution was dialyzed against distilled water for 72 h at room temperature. The steroid–BSA conjugate was obtained by lyophilization as a white powder (40 mg). The steroid:

protein molar ratio of the conjugate was spectrophotometrically (at 270 nm in methanol) determined to be 29.0.

Determination of Sulfate Group by the Ion Chromatographic Method[6]——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 and centrifuged at 3000 rpm for 10 min to remove the precipitate, followed by extraction with ethyl acetate (2 ml × 3). The aq. layer was centrifuged again at 3000 rpm, and an aliquot was injected into the ion chromatograph with a microliter syringe. Relative retention times of Cl^- and SO_4^{2-} were 0.33 and 1.12 (HPO_4^{2-} = 1.00, 9.04 min), respectively.

Preparation of [6,7-^{3}H]-2-Hydroxyestradiol 17-Sulfate

Radioactivity Counting——Radioactivities were 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-phenyloxazole-2-yl)benzene was used as a scintillant. Aqueous samples were counted in Bray's system. The radioscannograph used was an Aloka JTC-203 machine (Nihon Musen, Tokyo).

Preparation Method——[6,7-^{3}H]-Estradiol 17-sulfate (6.25 μCi) was added to 4.0 ml of Tris-HCl buffer solution (0.1 M, pH 7.4) containing mushroom tyrosinase (120 μg) and reduced nicotinamide adenine dinucleotide (NADH) (2.4 mg). The mixture was incubated for 20 min at 37 °C. Ascorbic acid (10 mg) was added, and the mixture was heated at 90 °C for 1 min. The resulting solution was passed through Sep-Pak C_{18} cartridge, followed by elution with water (2.0 ml) and then with methanol (3.0 ml). The methanolic eluate was evaporated under a nitrogen stream, and the residue obtained was subjected to preparative HPLC. Fractions corresponding to 2-hydroxyestradiol 17-sulfate[1] were collected to give the desired tracer (14.2 μCi). Radiochemical purity was checked with the radioscannograph, and a mixture of ethyl acetate : methanol : water (8 : 2 : 1, by volume) containing 1% ascorbic acid was used as the mobile phase for TLC.

The radiochemical purity was determined by the reverse isotope dilution method as follows. A solution of ethereal diazomethane was added to a methanolic solution (20 ml) containing 2-hydroxyestradiol (*ca.* 30 mg) and a known amount of [6,7-^{3}H]-2-hydroxyestradiol 17-sulfate. The solution was allowed to stand for 16 h in a refrigerator, then evaporated. The crystalline product was then dissolved in 10 ml of 10% H_2SO_4. The solution was heated at 60 °C for 2 h, then extracted with ether for 72 h. The ether layer was washed with water, dried, and evaporated. The product was acetylated in the usual way to give 2,3-dimethoxyestra-1,3,5(10)-trien-17β-yl acetate, which was repeatedly recrystallized until constant specific radioactivity was obtained.

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 ten days 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 50 mM Tris-HCl buffer (pH 7.0) 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, 10, 20, 30, 50, 100, 200, 300, 500, 700, and 1000 pg of non-labeled 2-hydroxyestradiol 17-sulfate in 0.1 ml Tris-HCl buffer solution (50 mM, pH 7.0) and labeled tracer (10000 dpm, 40 pg) in the same buffer solution. A 1 : 3000 dilution of antiserum (0.8 ml) was added to each mixture and the tubes were incubated for 60 min at room temperature. After addition of 50% aq. ammonium sulfate (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-filfth ml aliquot of each supernatant was transferred into a counting vial containing a scintillation cocktail (5 ml), and the radioactivity was counted.

Investigation of the Incubation Conditions——Incubation conditions were examined in the same way as described in the assay procedure using non-labeled steroid (40 pg) and labeled steroid (10000 dpm, 40 pg).

Cross-Reaction Study——The specificity of antiserum raised against the 2-hydroxyestradiol 17-sulfate–BSA conjugate was tested by cross-reaction studies with several 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 2-hydroxyestradiol 17-sulfate was arbitrarily taken as 100%, were calculated from the standard curve.

Results and Discussion

A hapten compound (IV) was prepared by the method summarized in Chart 1. The 6-oxo-catechol (I) was treated with *O*-carboxymethylhydroxylamine hydrochloride to give the C-6 oxime derivative (II) quantitatively, and this was further converted to the trisulfate (III). Because this trisulfate (III) was not obtained in a crystalline state, it was necessary to check whether the C-17 hydroxyl group was sulfated or not. This was confirmed by the finding that the material had no hydroxyl group reacting under ordinary acetylation conditions. The

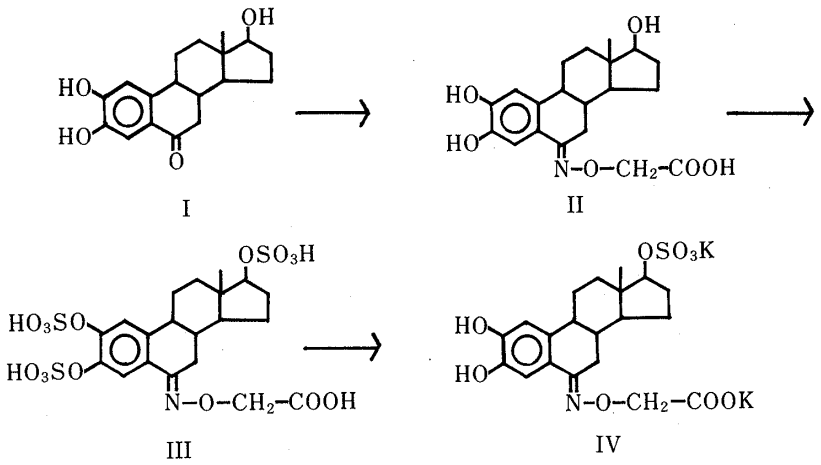

Chart 1

trisulfate thus confirmed was treated with Mylase P to give the desired product (IV).

The hapten (IV) was then covalently linked to BSA by the mixed anhydride method. The lyophilized hapten–BSA conjugate was found to have a hapten number of 29.0 by means of the UV method. The amount of SO_4^{2-} liberated by the hydrolysis of the antigen was determined by ion chromatographic method[6] to be 30.4 per mol of the antigen, which is essentially identical with the above result obtained by the UV method. 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.

Radioactive antigen for the RIA study was prepared by the method of Jellinck *et al.*[11] from [6,7-^{3}H]-estradiol 17-sulfate by using tyrosinase. As prolonged incubation gave the 4-hydroxy isomer as a by-product, the incubation mixture was subjected to preparative HPLC, by which pure radioactive 2-hydroxyestradiol 17-sulfate was easily separated. The carrier-free tracer thus prepared showed over 96% radiochemical purity (reverse isotope dilution method), and gave on a radioscannograph only one peak which was coincident with that of an authentic sample developed concurrently.

The sera from immunized rabbits showed increased binding activity to 2-hydroxyestradiol 17-sulfate, though there was considerable individual variation. At four or five months after the first injection, samples from several rabbits showed a significant increase in binding activity. The anti-2-hydroxyestradiol 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.

Because catechol estrogens are extremely labile, it was necessary to select suitable incubation conditions for the antigen–antibody reaction. Figure 1 shows the effect of incubation time and hydrogen ion concentration (pH) using Tris-HCl buffer solution upon the antigen–antibody reaction. The binding ratio has a maximum between pH 6.8 and 7.0 as shown in (a). In the alkaline pH region, the binding gradually decreased, probably due to decomposition of the catechol estrogen. The effect of incubation time upon the binding was found to be linear up to about 60 min, and no further incubation is needed, as shown in (b). The optimum incubation temperature was found to be 37 °C. From these results, the incubation for the RIA for 2-hydroxyestradiol 17-sulfate was done at 37 °C at pH 7.0 in Tris-HCl buffer solution for 60 min.

The standard curve obtained with 1 : 3000 dilution of the antiserum is shown in Fig. 2. The percentage of free radioactivity increased linearly with logarithmic increase in non-

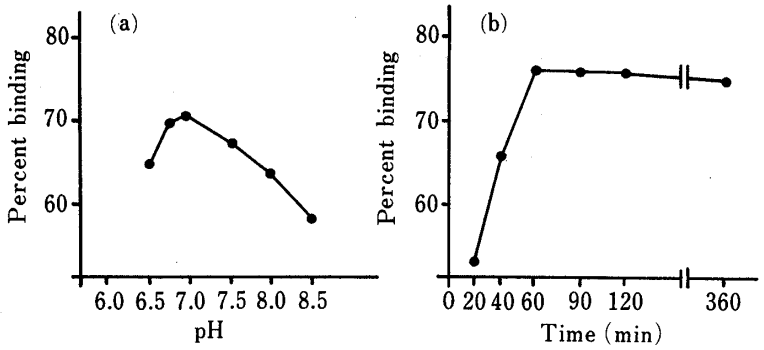

Fig. 1. Effects of Hydrogen Ion Concentration (a) and Incubation Time (b) upon the Antigen–Antibody Reaction

Each point represents the mean value of triplicate experiments.

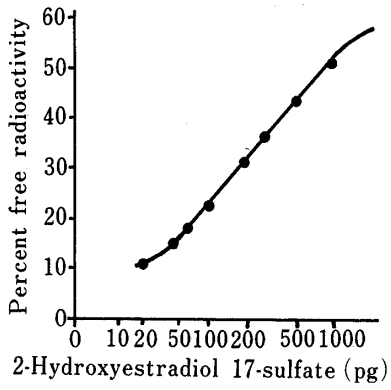

Fig. 2. Standard Curve with Anti-2-hydroxy-estradiol 17-Sulfate

Antiserum, 1: 3000 dilution.

TABLE I. Cross-Reactions of Anti-2-hydroxyestradiol 17-Sulfate Antiserum with Selected Steroids

Steroids	% cross-reactivity (50%)
2-Hydroxyestradiol 17-sulfate	100
Estradiol 17-sulfate	0.1
Estradiol 3,17-disulfate	0.05
2-Methoxyestradiol 17-sulfate	0.03
4-Hydroxyestradiol 17-sulfate	0.01
Estrone sulfate	<0.01
Estradiol 3-sulfate	<0.01
Estriol 16-sulfate	<0.01
Estriol 17-sulfate	<0.01
Estrone glucuronide	<0.01
Estradiol 3-glucuronide	<0.01
Estradiol 17-glucuronide	<0.01
2-Methoxyestradiol 17-glucuronide	<0.01
2-Hydroxyestradiol 17-glucuronide	<0.01
Estriol 16-glucuronide	<0.01
Estrone	<0.01
Estradiol	<0.01
2-Hydroxyestradiol	<0.01
4-Hydroxyestradiol	<0.01
Estriol	<0.01

labeled sulfate concentration from 50 to 1000 pg.

The specificity of the antiserum was assessed by testing the ability of related steroids to compete for binding sites on the antibody. The percent corss-reaction of the antiserum was determined by the method of Abraham[12] from the displacement of labeled steroid added. The results on cross-reaction of anti-2-hydroxyestradiol 17-sulfate antiserum with various closely related steroids are listed in Table I. It is evident that the antiserum is highly specific to 2-hydroxyestradiol 17-sulfate; 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, estradiol 17-sulfate, showed only 0.1% cross-reactivity.

In the previous paper dealing with the RIA of estradiol 17-sulfate,[6] it was shown that highly specific antiserum discriminating the structure around the conjugated position of the estrogen D-ring sulfate can be raised against hapten–BSA conjugate having a linkage through a position remote from the sulfooxy group moiety.[6] In the present work, a similar result was obtained for 2-hydroxyestradiol 17-sulfate.

The utilization of this highly specific antiserum for the measurement of 2-hydroxyestradiol 17-sulfate in biological fluids will be the subject of a future communication.

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References

1) Part XXIX: K. Watanabe, R. Kimura and I. Yoshizawa, *Chem. Pharm. Bull.*, **32**, 2745 (1984).
2) T. Nambara, T. Ohkubo and K. Shimada, *Clin. Chim. Acta*, **119**, 81 (1982), and references cited therein.
3) T. Nambara, K. Shimada, T. Ohkubo and T. Niwa, *J. Steroid Biochem.*, **16**, 533 (1982).
4) K. Wright, D. C. Collins, M. Virkler, P. I. Musey, E. Dale and J. P. K. Preedy, *Am. J. Obst. Gynecol.*, **131**, 255 (1978).
5) T. Tanaka, Y. Shiratori, N. Suguro and A. Kubodera, Abstract of Papers, 104th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, March 1984, p. 377.
6) I. Yoshizawa, M. Kameyama and K. Watanabe, *Chem. Pharm. Bull.*, **32**, 1885 (1984).
7) M. Kameyama and I. Yoshizawa, Abstract of Papers, 104th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, March 1984, p. 601.
8) K. Watanabe and I. Yoshizawa, *J. Pharmacobio-Dyn.*, **5**, 340 (1982).
9) K. Watanabe and I. Yoshizawa, *Steroids*, **42**, 163 (1983).
10) A. Nakagawa, R. Ohuchi and I. Yoshizawa, *Chem. Pharm. Bull.*, **26**, 3567 (1978).
12) P. H. Jellinck and B. J. Brown, *Steroids*, **17**, 133 (1971).
12) G. E. Abraham, *J. Clin. Endocr. Metab.*, **29**, 866 (1969).