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Development and Application of a Radioimmunoassay for 2-Hydroxyestriol[1)]

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A radioimmunoassay method for the quantitative determination of plasma 2-hydroxyestriol has been developed. For the purpose of obtaining antiserum for this compound, 6-oxo-2-hydroxyestriol was converted to its 6-(*O*-carboxymethyl)oxime derivative and then coupled with bovine serum albumin by means of the mixed anhydride technique. The immunization of rabbits with this conjugate produced anti-2-hydroxyestriol antiserum which showed high affinity and specificity with low cross-reactivities to structurally related estrogens. After extraction of pregnancy plasma with ethyl acetate and purification on a Sephadex LH-20 column, 2-hydroxyestriol was measured by radioimmunoassay. The following mean concentrations were found in pregnancy plasma: 1st trimester (28.4 pg/ml), 2nd trimester (37.7 pg/ml), and 3rd trimester (104.1 pg/ml).

Keywords——2-hydroxyestriol; radioimmunoassay; catechol estrogen; pregnancy plasma; 2-hydroxyestriol–BSA conjugate; anti-2-hydroxyestriol antiserum; 2-hydroxyestriol 6-(*O*-carboxymethyl)oxime; cross-reaction

Ring A hydroxylation at either C-2 or C-4 is a major pathway of primary estrogen metabolism, leading to catechol estrogen.[2)] Considerable attention has been focused on the physiological significance of these catechol estrogens, especially in regulating the secretion of gonadotropic hormones.[3)] In recent years, 2-hydroxyestriol (2-OHE_3) has been quantitatively determined in human pregnancy urine by gas-liquid chromatography.[4)] However, this method did not have sufficient sensitivity, because of the very low concentration of plasma 2-OHE_3. This problem may be overcome by the development of a highly sensitive and specific radioimmunoassay (RIA). Although many studies on RIA for 2-hydroxyestrone and 2-hydroxyestradiol have been presented since the first report of Yoshizawa and Fishman,[5)] there have been no reports on RIA of 2-OHE_3. It is desirable to investigate the concentration of 2-OHE_3 in human pregnancy plasma in connection with estriol, which is one of the most reliable indices of fetal well-being in late pregnancy. The present paper therefore deals with the preparation of antiserum by immunization with hapten–bovine serum albumin (BSA) conjugate, and with the measurement of 2-OHE_3 in human pregnancy plasma by using the RIA method consequently developed.

Experimental

Materials——[6,7-^{3}H]Estrone (60.0 Ci/mmol), [6,7-^{3}H]estradiol (53.0 Ci/mmol), and [6,7-^{3}H]estriol (58.0 Ci/mmol) were supplied by New England Nuclear (Boston, Mass., U.S.A.). Mushroom tyrosinase and BSA (fraction V) were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Estriol and silica gel pre-coated on aluminum sheets for thin-layer chromatography (TLC) were purchased from E. Merck (Darmstadt, Germany),

Freund's complete adjuvant from Difco Lab. (Detroit, Mich., U.S.A.), bovine gamma-globulin from Miles Lab. (Elkhart, Ind., U.S.A.), Sephadex LH-20 from Pharmacia Fine Chemicals (Uppsala, Sweden), and other general reagents from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2-OHE_3 was synthesized by the method of Stubenrauch and Knuppen.[6] The catechol estrogens and guaiacol estrogens used for cross-reaction studies were prepared in these laboratories by the known methods.[6,7]

[6,7-^{3}H]2-Hydroxyestrone, [6,7-^{3}H]2-hydroxyestradiol, and [6,7-^{3}H]2-OHE_3 were prepared enzymatically from labeled estrone, estradiol, and estriol, respectively, by treatment with mushroom tyrosinase.[8] The resulting catechol estrogens were purified by Sephadex LH-20 column chromatography using a benzene–MeOH solvent system saturated with ascorbic acid,[5f] and then stored in MeOH containing 2% ascorbic acid and 1% AcOH at −18 °C. The radiochemical purity was checked by TLC prior to use.

The ascorbic acid buffer (pH 7.4) containing bovine gamma-globulin and ethylenediaminetetraacetic acid disodium salt (EDTA) was prepared according to Ball *et al.*[5b]

Synthesis of Hapten–BSA Conjugate——Instruments: All melting points were taken on a Yanagimoto micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 digital polarimeter. Ultraviolet (UV) spectra were obtained on a Hitachi 323 recording spectrophotometer. Electron impact mass spectral (MS) measurements were run on a JEOL JMS-D 100 instrument. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded using tetramethylsilane as an internal standard on a JEOL FX-100 spectrometer at 100 MHz. Abbreviations used: s = singlet, d = doublet, and m = multiplet. Silica gel (70—230 mesh; E. Merck) was used for column chromatography.

6-Oxo-2-hydroxyestriol Tetraacetate (II): A solution of CrO_3 (600 mg) in H_2O (0.45 ml)–AcOH (3.4 ml) was added dropwise to a solution of 2-hydroxyestriol tetraacetate (I) (670 mg) in AcOH (2 ml), and the mixture was stirred at room temperature for 3 h. The excess reagent was then reduced with EtOH (1.5 ml). After dilution with H_2O, the reaction mixture was extracted with AcOEt and the extract was washed with 1% $NaHCO_3$, then dried over anhydrous Na_2SO_4. Removal of AcOEt gave an oily residue, which was chromatographed on silica gel (60 g) with hexane–AcOEt (2 : 1). The eluate was recrystallized from AcOEt–MeOH to give II (220 mg) as colorless needles. mp 188—189 °C. $[\alpha]_D^{26}$ −61.8° (c = 0.08 in $CHCl_3$). *Anal.* Calcd for $C_{26}H_{30}O_9$: C, 64.18; H, 6.22. Found: C, 64.19; H, 6.28. MS m/z: 486 (M^+), 444, 402. UV λ_{max}^{EtOH} nm: 254, 295. ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, s, 18-CH_3), 2.06 (3H, s, 16α- or 17β-$OCOCH_3$), 2.10 (3H, s, 17β- or 16α-$OCOCH_3$), 2.31 (6H, s, 2- and 3-$OCOCH_3$), 4.99 (1H, d, J = 5.9 Hz, 17α-H), 5.21 (1H, m, 16β-H), 7.24 (1H, s, 1-H), 7.87 (1H, s, 4-H).

6-Oxo-2-hydroxyestriol (III): A solution of II (180 mg) in MeOH (45 ml) was treated with 2.4 M H_2SO_4 (15 ml); the mixture was allowed to stand at room temperature for 42 h, then diluted with H_2O saturated with NaCl. After evaporation of the MeOH, the remaining solution was extracted three times with AcOEt. The organic phase was washed twice with 0.17 M AcOH and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the crude product obtained was chromatographed on silica gel (40 g) impregnated with ascorbic acid[9] using $CHCl_3$–MeOH–AcOH (90 : 10 : 0.8). The eluted product was recrystallized from AcOEt–hexane to give III (85 mg) as colorless prisms. mp 177—179 °C (dec.). $[\alpha]_D^{17}$ −19.5° (c = 0.08 in MeOH). *Anal.* Calcd for $C_{18}H_{22}O_5$: C, 67.91; H, 6.97. Found: C, 68.20; H, 6.71. MS m/z: 318 (M^+). UV λ_{max}^{MeOH} nm: 238, 283, 325. ^{1}H-NMR (CD_3OD) δ: 0.75 (3H, s, 18-CH_3), 3.49 (1H, d, J = 5.4 Hz, 17α-H), 4.06 (1H, m, 16β-H), 6.79 (1H, s, 1-H), 7.38 (1H, s, 4-H).

6-Oxo-2-hydroxyestriol 6-(*O*-Carboxymethyl)oxime (IV): (*O*-Carboxymethyl)hydroxylamine · 1/2HCl (48 mg) was added to a solution of III (40 mg) in EtOH (3.2 ml) containing ascorbic acid buffer (pH 10.5)[10] (0.14 ml), and the mixture was refluxed for 80 min under nitrogen. The reaction mixture was acidified by adding a few drops of AcOH and H_2O (2 ml), then EtOH was removed under reduced pressure. The oily residue obtained was extracted with AcOEt, and the extract was washed with H_2O and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the crude product was recrystallized from MeOH–AcOEt to give IV (30 mg) as colorless prisms. mp 225—227 °C (dec.). $[\alpha]_D^{16}$ −31.0° (c = 0.10 in MeOH). *Anal.* Calcd for $C_{20}H_{25}NO_7 \cdot H_2O$: C, 58.67; H, 6.65; N, 3.42. Found: C, 58.40; H, 6.31; N, 2.92. UV λ_{max}^{MeOH} nm: 272, 315. ^{1}H-NMR (CD_3OD) δ: 0.76 (3H, s, 18-CH_3), 3.47 (1H, d, J = 5.9 Hz, 17α-H), 4.05 (1H, m, 16β-H), 4.62 (2H, s, $-OCH_2CO-$), 6.74 (1H, s, 1-H), 7.33 (1H, s, 4-H).

6-Oxo-2-hydroxyestriol 6-(*O*-Carboxymethyl)oxime–BSA Conjugate (V): Tri-*n*-butylamine (24 μl) and isobutyl chlorocarbonate (9 μl) were added to a solution of IV (20 mg) in dry dimethylformamide (DMF) (0.8 ml) under ice-cooling, and the mixture was stirred for 15 min, then added to a solution of BSA (45 mg) in H_2O (2 ml)–DMF (1.5 ml) containing 1 N NaOH (0.03 ml) under ice-cooling. After being stirred for 3 h while the pH was maintained at *ca.* 7.5, the resulting solution was dialyzed overnight at 4 °C against a constant flow of cold 0.001% ascorbic acid solution (20 l), and the turbid protein solution was brought to pH 4.5 with 0.1 N HCl. After standing at 4 °C overnight, the precipitate was collected by centrifugation at 3000 rpm for 10 min, then dissolved in 0.01% ascorbic acid (5 ml)–1% $NaHCO_3$ (5 ml), and dialyzed in the same manner as above. Lyophilization of the solution afforded V (42 mg) as a fluffy powder. The molar steroid–protein ratio of the conjugate, as determined spectrophotometrically[11] at 272 nm, was 16.

Immunization Procedure——The steroid–protein conjugate (V) (3 mg) was dissolved in sterile isotonic saline (1.2 ml) and emulsified with complete Freund's adjuvant (1.8 ml). Immunization was carried out by injecting this emulsion into multiple subcutaneous sites along both sides of the back of three domestic strain male albino rabbits.

The rabbits were injected at two-week intervals for three months and then were boosted twice a month. After confirmation of the increase of the antibody titer, the blood was collected by bleeding from the marginal ear veins. The sera were promptly separated by centrifugation and stored at −18 °C in small aliquots. The antisera were thawed and diluted with ascorbic acid buffer (pH 7.4) and used in the assay at a final dilution of 1 : 11000.

Radioimmunoassay Procedure——All dilutions of the standard or sample, tracer, and antiserum were performed in ascorbic acid buffer (pH 7.4) containing gamma-globulin and EDTA. Dextran-coated charcoal suspension was prepared by continuously stirring Norit A (250 mg) and dextran T-70 (25 mg) in cold ascorbic acid buffer (40 ml) for 15 min prior to use.

To test tubes containing standard or unknown amounts of 2-OHE_3 dissolved in assay buffer (0.1 ml), [6,7-^{3}H]-2-OHE_3 (*ca.* 8000 cpm) in assay buffer (0.1 ml) and diluted antiserum (0.5 ml) were added. All tubes were shaken in a vortex mixer and incubated at 4 °C overnight. Dextran-coated charcoal suspension (0.3 ml) was pipetted into each tube, vortexed, incubated for 10 min in an ice water bath, and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant (0.5 ml) was withdrawn into counting vials containing a scintillation cocktail (8 ml) and the radioactivity of tritium was measured in a Beckman LS-9000 liquid scintillation spectrometer.

The radioactivity bound to the antibody was calculated after correction for the blank value of the assay buffer. The standard curve was prepared in duplicate with doses ranging from 2 to 1000 pg.

Specificity of Antiserum——The specificity of antibody was tested by calculating the percentage of cross-reaction with other steroids and catecholamines. Cross-reactivity was determined under the above assay procedure, by comparing the concentrations of non-labeled 2-OHE_3 and test compounds necessary for 50% displacement of the antibody-bound labeled 2-OHE_3.

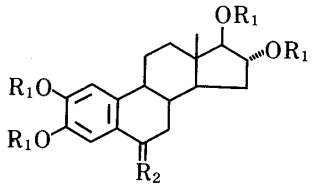
Measurement of Plasma 2-OHE_3 Content——Blood was collected into vacutainer tubes (5 ml) containing EDTA (10 mg) and ascorbic acid (15 mg), and then centrifuged at 4 °C. The plasma sample was promptly removed from red blood cells and frozen at −18 °C until assayed.

Plasma (2 ml) was pipetted into a centrifuge tube containing [6,7-^{3}H]2-OHE_3 (*ca.* 600 cpm) to estimate the recovery during the procedure. After addition of AcOEt (15 ml), the mixture was shaken on a vortex mixer for 1 min and centrifuged in order to break the emulsion. The organic phase was evaporated at 40 °C under a gentle stream of nitrogen. The residue was applied to Sephadex LH-20 column (13 × 0.6 cm, i.d.) using benzene–MeOH (89 : 11) saturated with ascorbic acid. Eluates corresponding to 2-OHE_3 fraction (28—42 ml, Fig. 3) were combined and dried under reduced pressure. The dried eluate was dissolved in EtOH (0.5 ml), and an aliquot (0.1 ml) of this solution was measured for radioactivity to determine the recovery. Another aliquot (0.3 ml) was evaporated to dryness under a nitrogen gas stream and the residue was subjected to RIA as described above.

Recovery Test——Aliquots (2 ml) from a pool of normal male plasma were pipetted into each of the centrifuge tubes containing 21, 53, 106, and 212 pg/ml of non-labeled 2-OHE_3 and [6,7-^{3}H]2-OHE_3 (*ca.* 600 cpm). The sample preparation and assay were then carried out in the manner described above.

Results and Discussion

Our initial effort was directed to the synthesis of 6-oxo-2-OHE_3 6-(*O*-carboxymethyl)oxime–BSA conjugate (V) as an antigen. For this purpose, 2-OHE_3 tetraacetate (I) was prepared from estriol in several steps, employing the methods developed by Stubenrauch and Knuppen[6] and Rao and Axelrod.[12] Oxidation of I with chromium trioxide in acetic acid[13] provided the 6-oxo derivative (II). Simultaneous removal of the acetyl groups at C-2, C-3, C-16, and C-17 by exposure to sulfuric acid in methanol under mild conditions furnished the desired 6-oxo-2-OHE_3 (III). Accordingly, condensation of III with (*O*-carboxymethyl)hydroxylamine under ascorbic acid protection to avoid oxidative decomposition of the labile catechol estrogen proceeded readily to afford IV in a fairly good yield. The structure of IV was confirmed by inspection of the ^{1}H-NMR spectrum, which showed the methylene protons of the *O*-carboxymethyl group at 4.62 ppm and the aromatic ring protons as two singlets at 6.74 and 7.33 ppm. The results of other instrumental analyses of this



I: $R_1 = Ac$, $R_2 = H_2$
II: $R_1 = Ac$, $R_2 = O$
III: $R_1 = H$, $R_2 = O$
IV: $R_1 = H$, $R_2 = NOCH_2COOH$
V: $R_1 = H$, $R_2 = NOCH_2CONH$–BSA

Chart 1

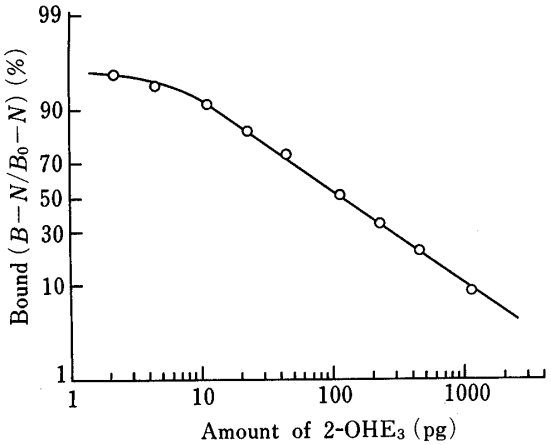


Fig. 1. Standard Curve for 2-OHE_3 with Anti-2-OHE_3 Antiserum (1 : 11000 Dilution)

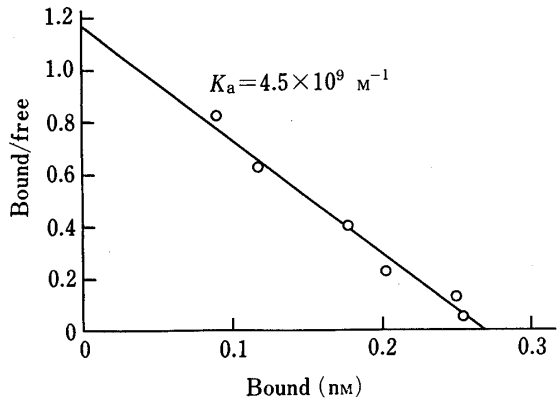


Fig. 2. Scatchard Plot for Anti-2-OHE_3 Antiserum

TABLE I. Cross-Reactions of Anti-2-OHE_3 Antiserum with Selected Steroids and Catecholamines

Compound	% cross-reactivity (50%)	Compound	% cross-reactivity (50%)
2-OHE_3	100	Estriol	4.3
2-Hydroxyestrone	0.098	Testosterone	0.009
2-Hydroxyestradiol	2.4	Androsterone	<0.005
2-Hydroxyestrone 3-methyl ether	0.067	Dehydroepiandrosterone	<0.005
2-Hydroxyestradiol 3-methyl ether	0.80	Etiocholanolone	<0.005
2-Hydroxyestriol 3-methyl ether	0.93	Corticosterone	<0.005
2-Methoxyestrone	0.078	Cortisone	<0.005
2-Methoxyestradiol	0.15	Hydrocortisone	<0.005
2-Methoxyestriol	4.4	Pregnanediol	<0.005
4-Hydroxyestrone	0.064	Progesterone	<0.005
4-Hydroxyestradiol	0.13	Cholesterol	<0.005
4-Hydroxyestriol	3.1	Dopamine hydrochloride	<0.005
Estrone	0.059	Noradrenaline hydrochloride	<0.005
Estradiol	0.12		

material also supported this structure. The oxime derivative was then coupled with BSA by the mixed anhydride method using tri-*n*-butylamine and isobutyl chlorocarbonate, followed by dialysis of the reaction mixture against 0.001% ascorbic acid solution to give the conjugate (V). As judged from the UV absorption due to the aromatic ring structure, satisfactory numbers of hapten molecules were joined to each BSA molecule.

The immunogen thus prepared was administered to three rabbits in order to produce antibody. At four months after the initial injection, the immunized rabbits produced antibody showing binding activity towards 2-OHE_3. The highest titer antiserum among the antisera elicited in the three rabbits was adopted for detailed characterization. The anti-2-OHE_3 antiserum was diluted with the ascorbic acid buffer (pH 7.4) and [6,7-^{3}H]2-OHE_3 was used as a labeled antigen. The separation of bound and free fractions was carried out by using a dextran-coated charcoal suspension. The antiserum bound approximately 50% of [6,7-^{3}H]2-OHE_3 (*ca.* 8000 cpm) at a final dilution of 1 : 11000. The standard curve in Fig. 1 was constructed by plotting the logit percent bound radioactivity *vs.* the logarithm of the amount of non-labeled 2-OHE_3. The range of linearity of this log-logit plot extends from 10 to 1000 pg. The association constant (K_a) of anti-2-OHE_3 antiserum was determined to be

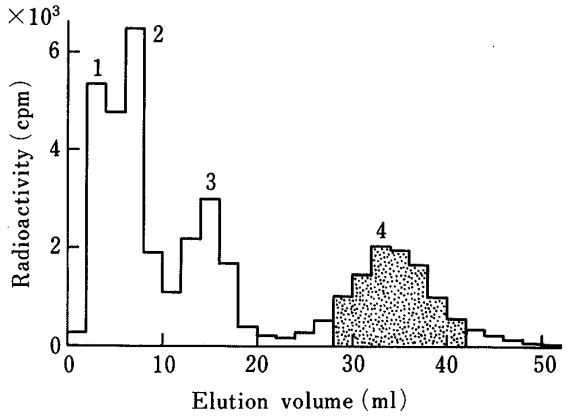


Fig. 3. Elution Pattern of Some Estrogens from a Sephadex LH-20 Column (13 × 0.6 cm, i.d.) Using Benzene–MeOH (89 : 11) Saturated with Ascorbic Acid

1, [6,7-^{3}H]estrone; 2, [6,7-^{3}H]estradiol and [6,7-^{3}H]2-hydroxyestrone; 3, [6,7-^{3}H]estriol and [6,7-^{3}H]2-hydroxyestradiol; 4, [6,7-^{3}H]2-OHE$_3$.

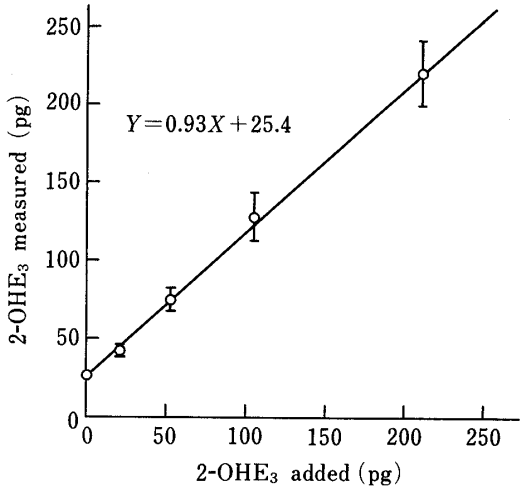


Fig. 4. Recovery Test for 2-OHE$_3$ Added to Pooled Male Plasma

Each point and bar indicate the mean value and standard deviation, respectively, for four determinations.

TABLE II. Plasma Concentrations of 2-OHE$_3$ in Normal Pregnant Women

Case	Number	Mean ± S.D. (pg/ml)	Range (pg/ml)
1st trimester	3	28.4 ± 4.2	24.0— 32.3
2nd trimester	6	37.7 ± 6.6	28.6— 48.6
3rd trimester	8	104.1 ± 29.6	66.1—157.4

$4.5 \times 10^9\ M^{-1}$ from a Scatchard plot as shown in Fig. 2.

The specificity of the antiserum was investigated by cross-reaction tests with various steroids and catecholamines competing for the binding site on the antibody. The percentage cross-reactivities were calculated at 50% displacement of the antibody-bound tritiated 2-OHE$_3$ and the results are presented in Table I. The antiserum exhibited considerable cross-reactions with 2-methoxyestriol (4.4%), estriol (4.3%), 4-hydroxyestriol (3.1%), and 2-hydroxyestradiol (2.4%). 2-Hydroxyestradiol 3-methyl ether and 2-hydroxyestriol 3-methyl ether also showed somewhat high values of 0.80% and 0.93%, respectively. However, there was a cross-reaction of less than 0.2% with all other compounds tested. It is evident from the data that the antibody prepared is more specific for estrogen derivatives modified in the D-ring than in the A-ring.

Development of an assay system for plasma 2-OHE$_3$ was then undertaken. From the cross-reaction data, estriol exerts a significant influence on the determination of 2-OHE$_3$ in the pregnancy plasma because of its large amount. Therefore, it is necessary to introduce a purification step by column chromatography before equilibration with the antiserum, in order to separate 2-OHE$_3$ from interfering substances. The elution pattern of a mixture of tritiated classical monophenolic estrogens and 2-hydroxyestrogens on a Sephadex LH-20 column using benzene–methanol (89 : 11) saturated with ascorbic acid is depicted in Fig. 3. The blood was collected into vacutainer tubes containing ascorbic acid to avoid decomposition of catechol estrogens. The plasma sample (2 ml) was extracted with ethyl acetate and purified on

Sephadex LH-20. The eluate containing 2-OHE_3 was dried and then used for RIA. The observed values were corrected on the basis of the recovery rate of [6,7-^{3}H]2-OHE_3 (*ca.* 600 cpm) added to each plasma sample.

The accuracy of the assay method was examined by analysis of pooled normal male plasma to which known amounts of 2-OHE_3 (21—212 pg/ml) had been added. The regression line was $Y=0.93X+25.4$, as shown in Fig. 4. The inter-assay coefficient of variation was 10.2%, and the recovery rates of various amounts of 2-OHE_3 added were satisfactory.

This assay procedure was applied to the measurement of 2-OHE_3 in plasma samples obtained from normal pregnant women. The plasma concentration levels throughout pregnancy are summarized in Table II. The 2-OHE_3 levels were low in the first trimester and in the second trimester. However, during the third trimester of pregnancy, the concentration increased to a mean value of 104.1 ± 29.6 pg/ml (mean $\pm$ S.D.), ranging from 66.1 to 157.4 pg/ml. From these results, it seems that the plasma 2-OHE_3 concentration tends to increase in late pregnancy, as is the case with plasma estriol.

The RIA method described in the present study allows sensitive and reliable quantitation of 2-OHE_3 in plasma samples. It would be of interest to investigate the difference in plasma 2-OHE_3 levels between normal and toxemic pregnancies using this assay system. The development of an RIA system for conjugated 2-OHE_3 (such as sulfate and glucuronide) as well as free 2-OHE_3 will be the subject of a future communication.

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References and Notes

1) A part of this work has been presented as a preliminary communication; Y. Fujii, M. Teranishi, M. Mizukami, H. Ukari, M. Yamazaki, Y. Horikawa, S. Kishida, and S. Miyabo, *Horm. Metab. Res.*, **16**, 268 (1984). The following trivial name is used in this paper: 2-hydroxyestriol = 1,3,5(10)-estratriene-2,3,16α,17β-tetrol.
2) P. Ball and R. Knuppen, *Acta Endocrinol. Suppl.*, **232**, 1 (1980).
3) J. Fishman, *J. Endocrinol.*, **89**, 59P (1981); N. J. MacLusky, F. Naftolin, L. C. Krey, and S. Franks, *J. Steroid Biochem.*, **15**, 111 (1981).
4) H. P. Gelbke and R. Knuppen, *J. Steroid Biochem.*, **5**, 1 (1974); H. P. Gelbke, H. Hoogen, and R. Knuppen, *ibid.*, **6**, 1187 (1975); S. L. Cohen, P. Ho, Y. Suzuki, and F. E. Alspector, *Steroids*, **32**, 279 (1978); T. Fotsis, P. Järvenpää, and H. Adlercreutz, *J. Steroid Biochem.*, **12**, 503 (1980).
5) *a*) I. Yoshizawa and J. Fishman, *J. Clin. Endocrinol. Metab.*, **32**, 3 (1971); *b*) P. Ball, G. Emons, O. Haupt, H.-O. Hoppen, and R. Knuppen, *Steroids*, **31**, 249 (1978); *c*) S. C. Chattoraj, A. S. Fanous, D. Cecchini, and E. W. Lowe, *ibid.*, **31**, 375 (1978); *d*) P. Ball, G. Reu, J. Schwab, and R. Knuppen, *ibid.*, **33**, 563 (1979); *e*) S. Kono, D. Brandon, G. R. Merriam, D. L. Loriaux, and M. B. Lipsett, *ibid.*, **36**, 463 (1980); *f*) D. Berg, F. Thaler, and E. Kuss, *Acta Endocrinol.*, **100**, 154 (1982); *g*) S. Kono, G. R. Merriam, D. D. Brandon, D. L. Loriaux, and M. B. Lipsett, *J. Clin. Endocrinol. Metab.*, **54**, 150 (1982).
6) G. Stubenrauch and R. Knuppen, *Steroids*, **28**, 733 (1976).
7) M. Teranishi, Y. Fujii, M. Yamazaki, and S. Miyabo, *Chem. Pharm. Bull.*, **31**, 3309 (1983).
8) P. H. Jellinck and B. J. Brown, *Steroids*, **17**, 133 (1971).
9) H. P. Gelbke and R. Knuppen, *J. Chromatogr.*, **71**, 465 (1972).
10) Y. Fujii, M. Teranishi, H. Ukari, M. Yamazaki, and S. Miyabo, *Chem. Pharm. Bull.*, **31**, 1082 (1983).
11) B. F. Erlanger, F. Borek, S. M. Beiser, and S. Lieberman, *J. Biol. Chem.*, **234**, 1090 (1959).
12) P. N. Rao and L. R. Axelrod, *J. Chem. Soc.*, **1961**, 4769.
13) T. Nambara, M. Numazawa, Y. Tsuchida, and T. Tanaka, *Chem. Pharm. Bull.*, **24**, 1510 (1976).