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RADIOIMMUNOLOGICAL DETERMINATION OF PLASMA ESTRADIOL-17β USING PRECIPITATION OF THE PLASMA GLOBULIN FRACTION

SIRKKA ONIKKI and HERMAN ADLERCREUTZ
Department of Clinical Chemistry, University of Helsinki, Meilahti Hospital, SF-00290 Helsinki 29, Finland

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SUMMARY

A practical and specific radioimmunological method for the determination of human plasma estradiol is described. The procedure involves the partial purification of the steroid with ammonium sulfate precipitation of sex hormone binding globulin (SHBG). A specific antiserum against 11α-hydroxyestradiol-11-succinyl-bovine serum albumin is used in the final assay. The method has been shown to fulfill the reliability criteria.

INTRODUCTION

IN ORDER to avoid chromatographic steps in the clinical determination of estradiol in human plasma and still retain a reasonable degree of specificity of the assay, we used the same principle in the purification and extraction as Ismail *et al.* [1] utilized in their plasma testosterone assay. In this method a complex containing sex hormone binding globulin (SHBG) is precipitated and the steroids bound to it are extracted. The estradiol assay is performed radioimmunologically using dextran-coated charcoal to separate antiserum-bound and free steroid fractions.

Although SHBG binds other steroids in addition to estradiol, the use of a highly specific antiserum against 11α-hydroxyestradiol-11-succinyl-bovine serum albumin has made it possible to obtain a specific quantification of estradiol. If a less specific antiserum is used, the method measures the so-called "immunoreactive estrogens" in plasma [2].

EXPERIMENTAL

Materials

Tracers: [6,7-^{3}H]-estrone (S. A. 40 Ci/mmol), [6,7-^{3}H]-estradiol (S. A. 40 Ci/mmol) and 6,7-^{3}H-estriol (S. A. 50 Ci/mmol) were purchased from the New England Nuclear Company (Boston, Mass., U.S.A.).

Reference standards. Estrone, estradiol-17β (estradiol), estriol, 16α-hydroxyestrone, 2-methoxyestrone, 16-oxoestradiol and 16-epiestriol were obtained from Ikapharm (Ramat-Gan, Israel), and estradiol-17α and testosterone from Steraloids Inc. (Pawling, N. Y., U.S.A.). The following reference steroids were obtained as gifts: 15α-hydroxyestrone from Dr. Klaus Kieslich (Schering AG, Berlin, Germany), 2-hydroxyestradiol from Dr. M. M. Coombs and 2-methoxyestradiol from Steroid Reference Collection (London, England).

Solvents and reagents. Benzene (Merck AG, Darmstadt, Germany), petroleum ether (Mallincrodt, St. Louis, Mo., U.S.A.) and methanol (Fluka AG, Bucks, Switzerland) were analytical grade and were used without further purification. Other reagents used were: boric acid, sodium hydroxide, hydrochloric acid,

ammonium sulfate, toluene (Merck), Tween 20 (Atlas Chemie GmbH, Essen, Germany), gelatine (Difco Laboratories, Detroit, Michigan, U.S.A.), 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene (dimethyl-POPOP, Packard Instrument Company Ltd, Downers Grove, Ill., U.S.A.), 2,5-diphenyloxazole (Koch Light Laboratories, Colnbrook, Bucks., England), Triton 100 X (BDH Chemicals Ltd, Poole, England), Dextran T 70 (Pharmacia, Uppsala, Sweden), Norit A (Amend Inc., New York, N.Y. 10010, U.S.A.) and Insta-gel (Packard Instrument Company, Inc., Downers Grove, U.S.A.).

Saturated ammonium sulphate solution. The saturated ammonium sulphate solution (at +4°C) is prepared by heating and mixing for about 20 min. The solution is allowed to equilibrate at +4°C at least over night.

Assay buffer. Borate buffer, pH 8·0 was prepared as follows: 8·25 g boric acid and 2·7 g NaOH were dissolved in 500 ml of quartz-distilled water and 3 ml concentrated HCl were added. The volume was then brought to 1000 ml with dist. water and the pH adjusted to pH 8·0 with 5N HCl. Tween 20 was added to a final concentration of 0·00001% and gelatine to a final concentration of 0·02%.

Scintillation fluids. The scintillation fluids used were the commercially available Insta-gel and the toluene/Triton 100 X liquid scintillant. The latter was prepared by mixing two parts of a solution containing 100 mg dimethyl-POPOP and 4 g PPO in 100 ml toluene and one part Triton 100 X. When 0·5 ml of an aqueous sample is added to 10 ml of this scintillation fluid, 0·5 ml of 0·2 N HCl is required to dissolve the resultant precipitate.

Antibodies. Two antisera were used. The antibody to estradiol-17β-monohemisuccinate-human serum albumin (batch no. S-52 # 5; = antiserum I) was kindly provided by Dr. G. E. Abraham[3]. The antibody against 11α-hydroxy-estradiol-11-succinyl-bovine serum albumin (= antiserum II) was raised in a rabbit. If not otherwise stated, antiserum II was used.

Methods

Purification of estradiol-17β. 1·5 ml of plasma or serum from normal cycling women was required in the assay. For the measurement of estradiol in males or postmenopausal females, 2–4 ml plasma should be used. For the estimation of procedural losses, 12 pg [6,7-^{3}H]-estradiol in 20 μl methanol were pipetted into each tube before the addition of the plasma. After mixing the tracer and the plasma sample with a Vortex mixer, the tubes were gently shaken while being incubated for 1 h at +40°C, followed by 1 h at +4°C. The precipitation was performed at +4°C first with nine volumes of an ammonium sulfate solution (saturated $(NH_4)_3SO_4$ at +4°C: quartzdistilled water, 10:9, v/v). After standing at +4°C for about ten min the tubes were centrifuged (at +4°C) at 2000 *g* for 30 min. 3 ml quartz-distilled water was used to dissolve this precipitate. The second precipitation was performed by adding 3 ml of saturated (+4°C) ammonium sulfate. The contents were mixed and centrifuged as described above.

The resulting precipitate was dissolved in one ml of quartz-distilled water (+4°C) and extracted once with 8 ml and once with 3 ml of benzene-petroleum ether mixture (2:3, v/v). The extract was dried under nitrogen and then redissolved in 1·1 ml of the assay buffer. For recovery estimation a 0·5 ml aliquot of the dissolved sample was taken.

Radioimmunoassay. The radioimmunoassay was performed in disposable Eppendorf microtubes (no. 3810) and the samples and reagents dissolved in buffer

were added with Oxford Samplers (Oxford Laboratories, San Mateo, California, U.S.A.). 0·5 ml of the dissolved sample was used for radioimmunoassay. 100 μl of the antiserum solution were added and the tubes mixed on an Eppendorf rotamixer 3300. The antiserum was diluted to the point that, in the absence of unlabeled hormone, 40–60% binding is obtained (in our system usually: antiserum I, final dilution 1:248000 and antiserum II, final dilution 1:17500). Before adding the tracer solution ([6,7-^{3}H]-estradiol diluted in methanol) the tubes were incubated at room temperature for 30 min. 50 μl of the tracer solution containing about 53 pg = 7·8 nCi [6,7-^{3}H]-estradiol were then added and the contents were mixed and incubated at + 4°C overnight. The separation of the free fraction from the one bound to the antibody was performed at + 4°C by adding 0·2 ml of dextran-coated charcoal solution (625 mg Norit A and 625 mg Dextran T 70 in 100 ml assay buffer). After mixing the tubes were incubated for 15 min (+ 4°C). After centrifugation for 2·5 min at 8000 *g* (Eppendorf centrifuge 3200), 0·5 ml of the supernatant were used for counting the radioactivity in the bound fraction.

A series of standards were prepared in duplicates for each assay and the results were read on estradiol standard curves. Per cent radioactivity bound was plotted against the mass of unlabeled estrogen (log scale), ranging from 10 to 560 pg, each point representing the mean of the duplicates. The linearisation of the standard curve by logit transformation was also used for the calculation of the results [4].

Liquid scintillation counting. Radioactivity was measured in a Wallac 81000 automatic liquid scintillation counter (Wallac Oy, Turku, Finland). The volume of water phase in each counting vial was equal and no correction for quenching effect was needed. The counting efficiences for tritium as determined by the internal standard method were ~ 29% for the toluene/Triton 100 X liquid scintillant and ~ 41% for Insta-gel in our assay system.

Mass fragmentographic determination of estradiol. This was carried out using the same extract as used for the radioimmunoassay in order to obtain positive proof of the specificity of the method. The extract was silylated with 20 μl hexamethyldisilazane and 2 μl trimethylchlorosilane in 200 μl of pyridine overnight. The solvents were evaporated and the silylated steroid fraction extracted with n-hexane. A known amount of standard estradiol was silylated in the same way with hexamethyl-d_{18}-disilazane and trimethyl-d_9-chlorosilane (Supelco, Inc.) and the n-hexane extract of the silylated product combined with the n-hexane extract of the sample in a graduated microtube.

Mass fragmentography was carried out using a 1% SE-30 column (250°C). A VARIAN MAT CH7 mass spectrometer with a multiple ion detector was used. The ions m/e 416 and 434 (deuterized internal standard derivative) were monitored using a twin pen recorder with the possibility of separate amplification of the two signals.

RESULTS

Recovery

The recovery of added [6,7-^{3}H]-estradiol depends on the concentration of SHBG in the plasma sample. In sera from women the recovery was 51 ± 3·0% (n = 147) and from men 32 ± 6·5% (n = 35).

Sensitivity

At the 95% confidence limit, 10 pg was significantly different from zero pg on

the standard curve for both antisera. The coefficient of variation was 6·4% at this level (32 duplicate determinations). At each point on the standard curves between 10 and 560 pg the coefficient of variations was less than 7%. Taking into account the recovery of the hormone after purification and the fraction of the purified estradiol used in the radioimmunoassay, the practical sensitivity of the method is about 30 pg/ml of plasma for cycling women. When more than 1·5 ml of plasma is used for the assay, the practical sensitivity is greater.

Precision

The method's precision was evaluated with both antisera by duplicate determinations of estradiol in male and female plasma using all the samples which fell between 25 and 85% on the inhibition curve. The coefficient of variation was 11·4% in the concentration range of 35–475 pg/ml (n = 69).

Replicate determinations performed the same day on plasma pools in the concentration range of 66–352 pg/ml, yielded coefficients of variation, varying from 3·7 to 14·0% (mean 8·9%, 7 pools). The day to day variation at three concentration levels, 114, 215 and 370 pg/ml yielded the coefficients of variation of 10·4, 7·1 and 4·7%, respectively.

Both serum and plasma values of estradiol were determined with antiserum II in 14 samples. The mean values for serum and plasma were 166 pg/ml (range 31–475 pg/ml) and 171 pg/ml (range 33–472 pg/ml), respectively, and the coefficient of variation was 7·5% in these duplicate determinations. The results indicate that plasma and serum can be used for the assay.

Accuracy

Pure estradiol in amounts of 70–560 pg was added to 1·5 ml aliquots of a plasma pool containing endogenous estradiol and the recovery determined. The recoveries obtained are presented in Table 1. The mean overall recovery of estradiol-17β added to other plasma pools and processed on consecutive days was 96·9 ± 14·1% (mean ± S.D., n = 33, pure estradiol added in amounts of 35–560 pg).

Effect of plasma volume on the estradiol value

Estradiol was quantitated in different volumes (1–4 ml) from two plasma pools, and it could be shown that the amount of plasma used did not influence the values obtained (pool 1: range 350–373 pg/ml; pool 2: range 200–216 pg/ml).

Table 1. Recovery of estradiol added to 1·5 ml of plasma through the assay procedure

pg estradiol added	values obtained pg estradiol	pg estradiol recovered	recovery of estradiol %
0	174		
0	167		
0	183		
–	—		
mean	175		
70 (6 assays) mean ± S.D.	259 ± 8·0	84	120·0
140 (3 assays) mean	306	131	93·6
280 (5 assays) mean ± S.D.	466 ± 31·8	291	103·9
560 (2 assays) mean	665	490	87·5

Specificity

The ability of estrone to displace ^{3}H-estradiol bound to the estradiol antiserum I was 46·5% and to antiserum II it was only 18·2%, as determined according to Abraham [5]. The recovery of [6,7-^{3}H]-estrone in the precipitation and extraction system is 35 ± 6·4% (mean ± S.D.) in female plasma (n = 38), as compared to the recovery of 51 + 3·0% (mean ± S.D., n = 147) of [6,7-^{3}H]-estradiol. The overall effect of estrone in our estradiol assay is therefore small when the highly specific antiserum is used.

Parallel determinations in pregnancy plasma by radioimmunoassay using antiserum II and mass fragmentography in extracts of 10 plasma samples with a concentration range from 8 to 78 ng/ml gave results which did not differ from each other by more than what would be expected from the methodological errors inherent in both methods. The calculated correlation coefficient was found to be 0·97 (Fig. 1). These results indicate that the method is specific for estradiol-17β because all other estrogens are excluded from the measurement when this specific mass fragmentographic method is used. The small amount of estrone present which should interfere, does not do so to a significant extent.

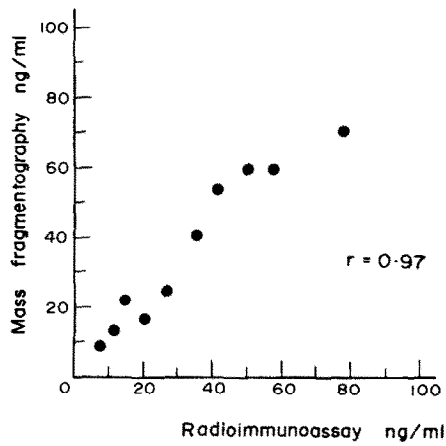

Fig. 1. Correlation between values for pregnancy plasma estradiol-17β obtained by radioimmunoassay (antibody to 11α-hydroxyestradiol-11-succinyl-bovine serum albumin; final dilution 1 : 17 500) and by mass fragmentography.

Only small amounts (14%) of estriol bind to SHBG and estriol is soluble only to a limited extent in the solvent used for extraction. The recovery of [6,7-^{3}H]-estriol is, accordingly, less than 2% after precipitation and extraction. With both antibodies 100 and 200 pg of 2-hydroxyestradiol, 105 and 210 pg of 2-methoxy-estradiol, 95 and 190 pg of estradiol-17α, 98 and 196 pg of 16-oxoestradiol, 81 and 162 pg of 15α-hydroxyestrone, 288 and 575 pg of 16α-hydroxyestrone, 140 and 280 pg of 2-methoxyestrone, 125 and 250 pg of 16-epiestriol and 10 ng of testosterone added to 1·5 ml of female plasma pool did not have any noticeable effect on the quantification of estradiol by this method. It could be concluded that the use of a specific antiserum in combination with the precipitation and extraction techniques utilized adds a high degree of specificity to the method.

Blank

Blanks were measured according to Ismail *et al.* [1] and were always negligible.

Eight drops of saturated $(NH_4)_2SO_4$ and 1 ml of quartzdistilled water were extracted as plasma samples and assayed together with the estradiol-17β standards and samples. Correction for blank values was never needed.

Estradiol values in plasma

The estradiol concentration was determined in a female plasma pool, in individual females during the menstrual cycle and in male plasma samples. For cycling females, low values during the follicular phase, a noticeable rise in estradiol value at the midcycle and a second, smaller rise during the luteal phase were noted.

The following results were obtained: Female plasma pool: 115 ± 12 pg/ml (mean $\pm$ S.D.). Individual values for females: Follicular phase: $<$ 30–98 pg/ml, at midcycle: 130–475 pg/ml, and luteal phase peak: 33–260 pg/ml. Individual values for men: $<$ 20–68 pg/ml.

DISCUSSION

A number of methods with [6–10] and without [2,9,12] chromatographic steps for the determination of plasma estradiol have been published. The main advantages of the present method are its practicability and the comparatively high degree of specificity attainable when a highly specific antiserum is used. It is also important that no problems arise with the blanks. The main disadvantage of this method is the fact that 1·5 ml of plasma from cycling women and still more from men and postmenopausal women has to be used.

Because the precipitation method used does not completely exclude estrone, the use of a highly specific antiserum is necessary in order to obtain an exact determination of estradiol. From the results it can be calculated that, with the specific antiserum elicited against 11α-hydroxy-11-succinyl-BSA, the overall interference of estrone is about 12%, which means that usually only about 4–6% of the estradiol value obtained from a normal fertile female subject is due to estrone [6,11]. A great number of other estrogens were tested and there was no interference noticed in the method used. The mass fragmentographic results also indicated that the method is specific when pregnancy plasma is assayed.

When assays of plasma samples were carried out with both antisera we sometimes noticed differences in values obtained that cannot be caused exclusively by estrone. These experiments were carried out by reducing the estrone to estradiol with borohydride. This suggests that other compounds in addition to estrone interfere with the determination of estradiol, a possibility which obviously needs further studies. These compounds may be of a non-steroidal nature.

The direct methods are hampered by some disadvantages that are at least partly avoided in the present method. The nonspecificity of the direct methods is obvious [12]. By precipitating the fraction containing the sex hormone binding globulin all those steroids can be eliminated that remain free in the plasma or bound to other proteins not precipitated. Overestimations of the true values [8] and the effect of plasma volume on the results are frequent in the direct methods when non-pregnancy plasma is used [12]. We have noticed no influence of plasma volume [1–4 ml] on the quantitation of estradiol by the present method.

The radioimmunoassay of estrogens often suffers from interfering factors introduced by the purification and separation procedures. This causes variable blanks which necessitates extremely careful control of assay conditions and

reagents used. This is very inconvenient in routine clinical work. In our own experience the use of LH-20 Sephadex for chromatographic separation of estrogens almost invariably caused significant blanks. However, different antisera give different blank values and it is certainly possible to include a chromatograph step in the estrogen method without obtaining an increase in blank values. However, in the present method the radioimmunoassay of estradiol, using both antisera, has not proved very sensitive to any interference from the simple purification step utilized.

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