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Radioimmunoassay of Estradiolusing Estradiol-6-oxim-BSA Anti Serum

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A radioimmunoassay (RIA) of estradiol was performed with the use of antiserum produced by estradiol-6-oxim-BSA. The precision of the assay was such as to result in a within-assay variance of 10.1% and a between-assay variance of 31.8%.

The concentrations of estradiol thus determined in the serum of women with normal menstrual cycles were 69.0±40.6 pg/ml inthe menstrual phase, 127.8±70.3 pg/ml in the follicular phase, 281.9±113.5 pg/ml in the ovulatory phase, and 165.4±79.5 pg/ml in the luteal phase.

The blood estradiol level durinh pregnancy ranged from 0.28 to 13.17 ng/ml in the first trimester, 2.6 to 48.1 ng/ml in the second trimester, and 13.5 to 130.0 nh/ml in the third trimester.

INTRODUCTION

There have been a number of reports[2)~7)] on the radioimmunassay of serum estradiol since 1969 when this assay method was first revealed by ABRAHAM[1)]. The study reported on here involved the RIA of estradiol using antiserum produced by estradiol-6-oxim BSA.

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METHODS

1) Reagents and Appliances

Radioactive estradiol : Estradiol-17β-6.7-^{3}H (New England Nuclear Corporation, Boston, U. S. A., specific activity 46.6 Ci/mM, E_2-^{3}H) was stored at 4°C after purification in a Sephadex LH-20 microcolumn and dilution to a concentration of 1×10^5 dpm/ml with methanol.

Non-radioactive estradiol : A pure substance (Teikokuzoki Co., Tokyo) was used, and a series of dilutions ranging in concentration from 5 ng/ml to 125 pg/ml were prepared and stored at 4°C. Bovine plasma γ-globulin (ICN Pharmaceuticals Inc., Ohio, U.S.A.) and bovine serum albumin (BSA) were used as serums to be added to the buffer.

Dextran charcoal solution : One gram of purified charcoal and 0.1 g of Dextran T-70 (Pharmacia Fine Chemicals Co., Sweden) were dissolved in 100 ml of 0.05M borate buffer and then stored at 4°C.

Scintillation solution : A dioxane scintillation solution was prepared by dissolving 0.5 g of 1.4-Bis (5-Phenyl-oxazolyl)-benzene (POPOP), 20 g of 2.5-D phenyloxazole (PPO), and 200 g of naphthalene in 2,000 ml of dioxane.

Sephadex LH-20 column chromatography: The chromatographic procedure was in accordance with the method of WU et al[5]. Sephadex LH-20 was suspended in a 85 : 15 mixture of benzene and methanol and packed to a height of 60 mm in a 7 × 100 mm microcolumn.

Liquid scintillation counter : The ALOKA LSC-651 (Tokyo) liquid scintillation counter was utilized. Each sample was subjected to a scintillation count for five minutes. Counting vials were of the low background type.

Table 1. Cross reaction with various steroidsof estradiol-6-oxim BSA antibody (From Yoshida et al[5].)

Steroids	Cross reaction (%)	Steroids	Cross reaction (%)
estradiol-17β	100	androst-4-ene, 3-17-dione	0.44
16 oxoestradiol	0.80	dehpdroepiandrosterone	<0.08
estradion-17β	0.80	pregnenolone	<0.08
estrone	3.20	progesterone	<0.08
2-methoxy-estrone	<0.08	17β-hydroxyprogesterone	<0.08
estrone-3-sulfate	8.00	cortisol	<0.08
estriol	1.77	corticosterone	<0.08
16-epiestriol	5.33	cortisone	<0.08
estriol-16-glucuronide	<0.08	cholesterol	<0.08
testosterone	0.29		

Estradiol antiserum : Estradiol was combined with BSA by introducing at OXO group at C-6, a position that has no bearing on the biologic activity of the substance, while the active OH groups at C-3 and C-17 were kept intact. The resulting estradiol-6-oxim-BSA was injected into rabbits to obtain an estradiol antiserum. The specificity of the antiserum is shown in Table 1.

2) Plasma Extraction, Separation and Purification

Blood samples were immediately centrifuged at 2,500-3,000 rpm for 10 minutes and then the serum was stored at -25°C. A1$\times 10^3$ dpm volume of ^{3}H-estradiol was added to the serum to produce an internal standard for recovery estimations. The substance under investigation was then extracted with 4ml of ethyl ether, following which the extract was

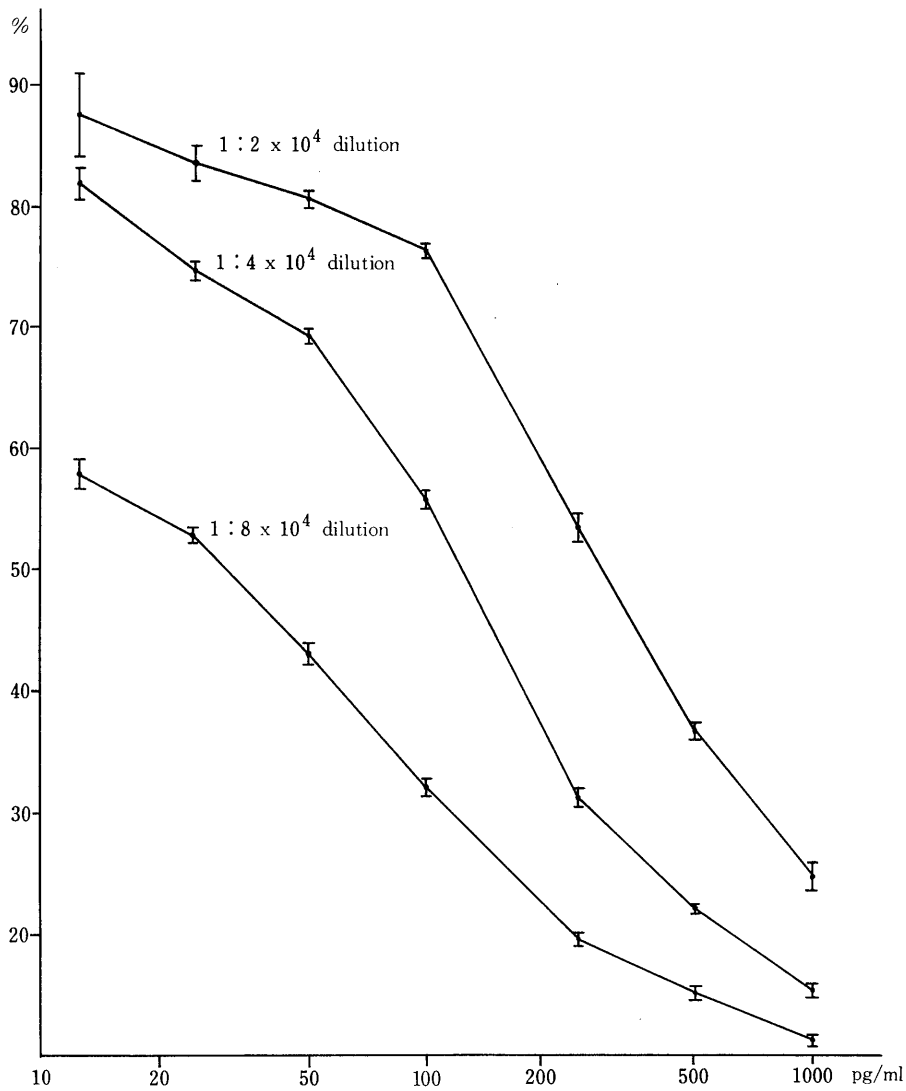


Fig. 1 Standard curves with different antserum dilutions

dried by spraying of N_2 gas in a thermostat at 37–40°C. The dried residue was dissolved in 0.2ml of an 85 : 15 mixture of benzene and methanol.

3) Antiserum Titer

Binding rate of 1×10^4 dpm of ^{3}H-estradiol to the antiserum was 86.3±3.8% at an antiserum dilution of 2×10^4, 83.1±1.0% at an antiserum dilution of 4×10^4, and 71.6±1.8% at an antiserum dilution of 8×10^4 (Fig. 1).

The binding rate of standard estradiol in a range of 0–1,000 pg varied in an almost linear fashion when the antiserum dilution rate was 4×10^4. Within this standard range, the binding values displayed a standard deviation of ±0.2–±1.8, with a coefficient of 0.5–6.0%. These findings led us to utilize an antiserum dilution of 4×10^4 in the present study.

4) Incubation Time and Temprature

A study, using a 4×10^4-fold dilution of antiserum, was made as to the relationship between incubation time and incubation temperature. It was found that the incubation time required for ^{3}H-estradiol to be bound to antiserum at a rate exceeding 80% was 90–120 minutes at 4°C and 20–30 minutes at room temperature (15–28°C).

5) Chromatography

A chromatographic study was made with a Sephadex LH-20 microcolum in accordance with the method of Wu[5] et al. The elution patterns thus obtained are shown in Fig. 2.

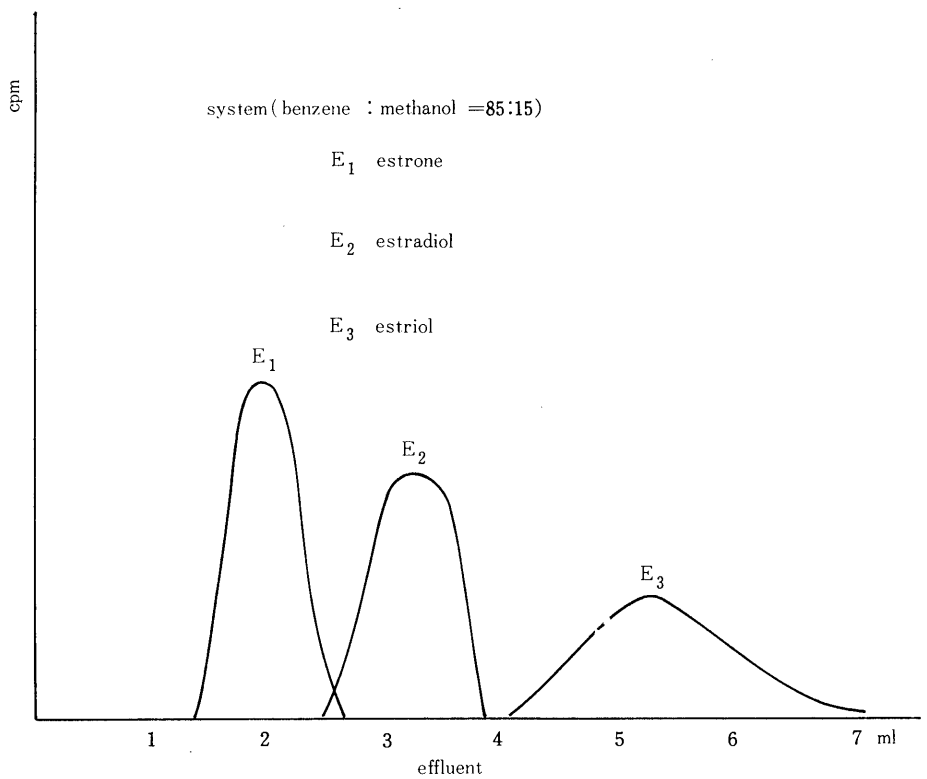


Fig. 2 Fractionation of estrogen in Sephadex LH-20 column chromatography

6) Precision

A coefficient of variation of 2.5–17.0% was obtained with estradiol added, in amounts of 0–500 pg, to 1ml of distilled water. The precision was such as to give (1) within-assay variance of 10.1% (n=10) and a between-assay variance of 31.8% (n=9).

7) RIA

After considering all of the afore-mentioned results and the conditions involved in the present study, we decided on the estradiol radioimmunoassay procedure as outlined in Fig. 3.

Incidentally, the concentration in 1ml of serum of estradiol of the unconjugated type was calculated from the following formula :

$$\text{Estradiol level (Pg/ml)} = \left[\frac{E}{R} \cdot \%\text{Pg} \cdot \frac{100}{Re} - (M+B)\right] \times \frac{1}{\text{Volume of serum under examination (ml)}}$$

Serum, 0.1–2.0ml

Addition of estradiol-^{3}H (1000 dpm) for correction of recovery rate

Extraction, with 4ml of ethyl ether (×2 mal)

Drying with N_2 gas spray in thermostat at 37–40°C

Sephadex LH-20 column chromatography

0.5ml for correction of recovery rate
1.0ml submitted to assay

Radioimmunoassay

1×10^4dpm of estradiol-^{3}H added
Dried by N_2 gas spray
Incubated for 30 minuttes at room temperature with
0.3ml of antiserum (1: 4×10^4 dilution) added.

Separation of free from bound from

0.3ml of dextran-coated charcoal addedin ice water bath. Allowed to stand for minutes.
Centrifuged at 2,600–3,000 rpm for 10 minutes.

Determination

Concentrationcalculated from the standard curve.

Fig. 3 Sequential steps in estradiol RIA

where E denotes the total amount of eluate in chromatography ; Re, recovery rate (%); R, the amount of eluate used in RIA ; M, the concentration of ^{3}H-estradiol for correction of the recovery rate (Pg), % Pg, the concentration of estradiol as obtained from the standard curve (Pg) ; and B, the concentration of estradiol for the blank (Pg).

RESULTS

In women with a normal menstrual cycle, the serum level of estradiol was 69.0± 40.6 Pg/ml (n=57) for the menstrual phsae, 281.9±113.5 Pg/ml (n=15) for the ovulatory phase, and 165.4±79.5 Pg/ml (n=63) for the luteal phase. Determinations of serum estradiol levels were made daily throughout each cycle of four women experiencing normal menstrual cycles. The results are shewn in Fig. 4.

The serum estradiol level during pregnancy ranged from 0.28 to 13.17 ng/ml (n= 50) for the first trimester (7th-16th weeks), from 2.6 to 48.1 ng/ml (n=17) for the second trimester (17th-28th weeks), and from 13.5 to 130.0 ng/ml (n=111) for the third trimester (29th-43rd weets). The measurements made every week after the 36th week in 18 cases of pregnancy are plotted in Fig. 5.

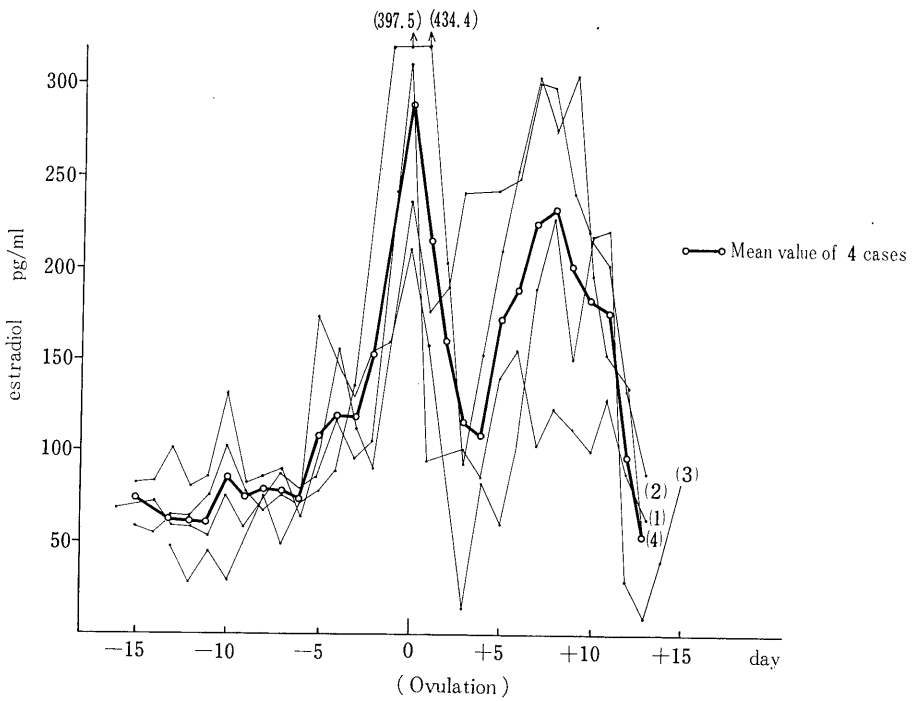
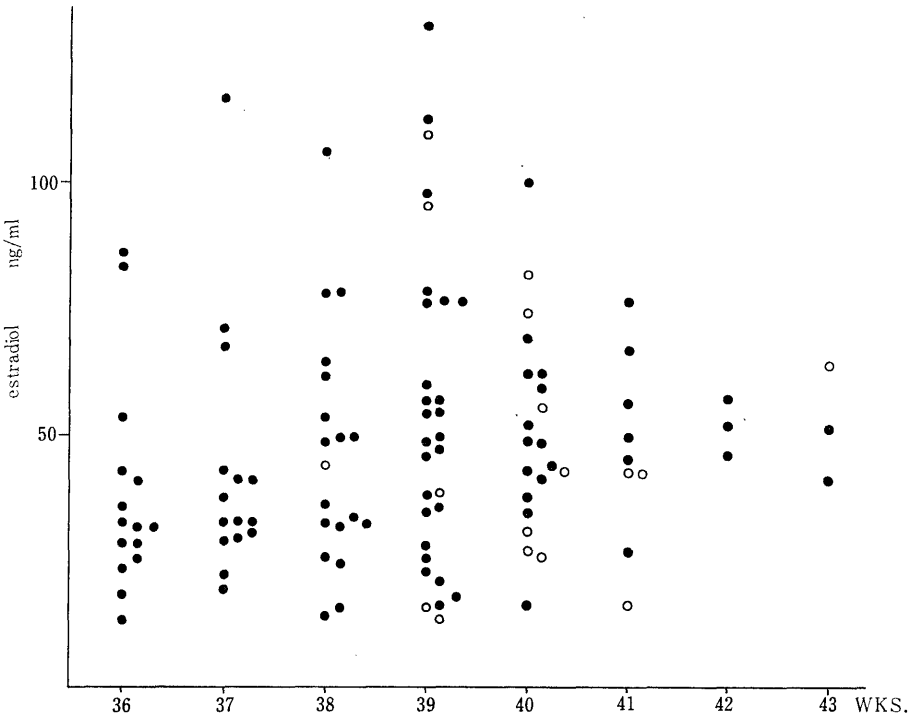


Fig. 4 Variatlon in estradiol level in 4 women with normal menstrual cycle (The thick line indicates mean values for 4 cases.)



Hollow circles indicate values at onset of paids.

Fig. 5 Variation in estradiol level at the 36th and later weeks of pregnancy

DISCUSSION

Antiserum produced by estradiol-6-oxim BSA is more specific for estradiol and also of better quality respect to sensitivity and precision that conventional antiserum produced by estradiol-17β-succinyl BSA. On the other hand, it must be mentioned that estradiol is contained in the lipid extracted from serum, giving rise to a variation in the results of RIA, as pointed out by MAKINO[4] and HASHIMOTO[3]. In the present study, a microcolumn was used with a view to removing lipid and purifying estradiol. As a result, a mean recovery rate of $68.7 \pm 10.2\%$ was attained for the entire process of extraction, the rate being as high as $94.3 \pm 8.0\%$ in as much as the microcolumn alone was concerned. The use of a Sephadex LH-20 column as a means of removing impurities in the blood involves a somewhat complicated procedure, whereas the microcolumn is capable of dealing with a number of specimens in a short period of time. The latter also is economical, permitting repeated use.

A review of actual measurements disclosed that the serum concentration of estradiol in women with a normal menstrual cycle roughly corresponded with the figures reported by other investigators (Table 2). Daily determination of blood estradiol level revealed that the level was at its lowest during the menstrual phase, then rose gradually during the

follicular phase, and sharply for4 to 5 days before the LH peak until a peak was reached during the ovulatory phase. It then showed a rapid decline, followed around 7 to 8 days after ovulation by a rise up to a peak for the luteal phase, and then finally declined.

The serum level of estradiol at different weeks of pregnancy varied markedly in different individuals. There was a prominent individual difference observed in the maximal value-ranging from 24.5 to 130 ng/ml-obtained in 18 cases from the 36th week until the onset of labor. The estradiol level at onset of pains was lower than that at 1-2 weeks before delivery in 14, but higher in 3, of 18 cases. No difference was noted in the one remaining case. Blood estradiol level rises sharply around 40 days after fertilization[6], and the luteoplacental shift occurs around 50-60 days after fertilization[2] with the subsequent

Table 2. Blood estradiol levels in women with normal menstrual cycles

Method of determination	Time of determinatino	Value obtained (pg/ml) (number of cases)	Person in change of determination
DIDD	Follicular phase Ovulatory phase Luteal phase	29～85 (9) 318±98 (13) 191±49 (6)	Baird (1969)
CPBA	Follicular phase Ovulatory phase Luteal phase	65.6±14.3 (15) 124.5±49.6 (12) 137.2±35.6 (6)	Korenman (1969,1970)
CPB	Follicular phase Ovulatory phase Luteal phase	10～100 (12) 140～770 (12) (350±145) 190～340 (12)	Dufan (1970)
RIA	Follicular phase (pooling)	108±13	Abraham (1969)
RIA	Women (pooling) Ovulatory phase	145±24 (6) 513±42 (8)	Mikhail (1970)
RIA	3rd day of menstrual cycle 27th day of menstrual cycle	21.4±2.62 (4) 68.5±4.70 (4)	Wu (1971)
RIA	Follicular phase Ovulatory phase Luteal pphase	87± 34.5 (12) 352±128.8 (12) 110± 89.2 (16)	Yamaji (1972)
RIA	Follicular phase Ovulatory phase Luteal phase	102.5±28.2 (10) 284.4±99.2 (5) 150.4±60.2 (17)	Hashimoto(1974)
RIA	Follicular phase Intermediate phase Luteal phase	62.8±22.9 204.9±84.7 119.4±46.7	Kawagoe (1974)
RIA	Menstrual phase Follicular phase Ovulatory phase Luteal phase Peak of ovulation	69.0± 40.6 (29) 127.8± 70.3 (57) 281.9±113.5 (15) 165.4— 79.5 (63) 384.7±127.7 (5)	Miura (1976)

biosynthesis of estrogens in large quantities in the chorionic villi. It is evident that DHAS from the fetal adrenal is the precursor of estrogens, particularly estriol synthesized in the placenta. Hence, the urine or blood level of estriol is a reflection of the functional status of the fetus or placenta. At the same time, the variation in the level of estriol, whose activity is much less potent than that of estrogens, is also of importance in analyzing the physiological phenomena that occur during pregnancy. Further investigation is needed in this respect.

REFERENCES

1) ABRAHAN, G. E. : *J. Clin. Endocr.*, 29: 866 (1969).
2) CSAPO, A. I., et al. : *Am. J. Obst. & Gynecol.*, 112: 1061 (1972).
3) HASHIMOTO, K. : *Medical Postgraduates*, 12 : 83 (1974) (Japanese).
4) MAKINO, T. : *Folia Endocrinologica Japonica*, 49: 629 (1972) (Japanese).
5) WU, C. H. & LUDY, L. E. : *Steroids*, 18: 19 (1971).
6) YAMACHI, M. : *Nippon Sanka-Fujinka Gakkai Zasshi*, 24: 1045 (1972) (Japanese).
7) YOSHIDA, T. & MAKino, T. : *Clinical Endocrinology*, 21: 651 (1973) (Japanese).