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Specific and Sensitive Estradiol-17 β Radioimmunoassay

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Abstract

A specific and highly sensitive radioimmunoassay of estradiol-17 β (E_2-17 β) was developed. Ether was used to extract E_2-17 β from 0.5 ml plasma samples and the extract was then purified by Sephadex LH-20 column chromatography and measured using an anti-serum to E_2-6-BSA and I^{125}-labelled E_2-17 β. Sensitivity of the method was 2.5 pg/ml. The coefficient of variation in accuracy and precision of the method was under 15%. The unconjugated E_2-17 β concentration in sera of 21 postmenopausal women ranged from 2.5 to 14.1 pg/ml. In serum samples over 10 pg/ml investigated by the direct method, E_2-17 β values determined by the present method correlated well with the direct method ($r=0.91$, $p<0.001$, $N=87$). In samples under 10 pg/ml determined by the direct method, E_2-17 β value ranged from 2.5 to 23.5 pg/ml by the present method.

The present method might be useful for more accurate evaluations of ovarian functions.

Introduction

Radioimmunoassay (RIA) of serum estrogen, and sometimes estradiol-17 β (E_2-17 β) is generally performed as an ovarian function test. There are three RIA methods : 1) RIA in combination with extraction of hormone from serum with an organic solvent [1,2]. 2) RIA in combination with extraction and purification [3-8], and 3) direct RIA of serum samples without extraction or purification, which is the most recently used method [9]. However, none of these methods is sufficiently enough, because the minimal limit of measurement is 10~20 pg/ml. Therefore, accurate evaluation of the pathophysiology of ovarian function during climacterium or puberty is difficult.

With that problem in mind, we combined the advantageous features of the conventional

methods to develop a more specific and sensitive RIA method. E_2-17β was extracted with ether and purified by column chromatography, and E_2-I^{125} was measured as the radiolabelled antigen.

Materials and Methods

A. Collection of samples

Blood samples were collected from the cubital vein of healthy adult women between the age of 22 and 69. The samples were immediately centrifuged at 4℃ at 3000 rpm for 10 min. The sera were then frozen at-60℃ until the assay.

B. Preparation of Sephadex LH-20 column

Sephadex LH-20 (Pharmacia Chemicals Corp.) was washed with methanol and, to expand it, was immersed overnight in an eluting solution (benzene : methanol=85 : 15). Tuberuculin syringes were plugged in the bottom with glass wool, stuffed with 2 ml of the washed Sephadex LH-20, and then rinsed 5 times with 2 ml of the eluting solvent.

C. Extraction and purification of E_2-17β

Each 0.5 ml of serum and distilled water were pipetted into a glass tube with cap. Samples were extracted with 5.5 ml of diethyl ether by shaking for 3 min. The water layer was discarded. The extracts were evaporated in a 50℃ water bath under a nitrogen stream. The residues were then dissolved in the eluting solvent, applied to the pretreated LH-20 column and eluted. Eluates corresponding to the E_2-17β fraction (2.8~4.05 ml) were evaporated in the hot bath under a nitrogen stream.

D. Assay procedure

The E_2-17β fractions obtained by chromatography and the standard E_2-17β solutions (0, 2.5, 5.0, 12.5, 50.0, 100.0 pg in 0.1 ml of methanol) were evaporated in the hot bath (50℃) under a nitrogen stream, and the residue was dissolved in 0.05 ml diluent of a phosphate buffer, pH 7.4, containing 0.06% r-globulin and 0.05% bovine serum albumin.

Finally, 0.01 μCi of E_2-I^{125} (Sp. act. 3540 μCi/㎎, CIS Corp.) in 0.1 ml diluent and 0.1ml antiserum optimally diluted with the diluent (antibody to E_2-6-oxime-BSA, CIS Corp.) were added to tubes containing the purified samples or E_2-17β standard solutions. After mixing, the tubes were incubated at room temperature. Then 1.0ml of an optimally diluted 2nd antibody containing polyethylene glycol was added to each tube. The tubes were then mixed, incubated at room temperature for 15 min, and centrifuged at 3000 rpm at 4℃ for 15 min. The supernatant in the tube was discarded, and the residue was counted with a r-counter (Aloka LSC 903).

Results

On a log-logit scale, the standerd curve for E_2-17β was linear (Fig. 1) The minimal detectable dose averaged 1.25 pg. The mean recovery through the extraction and purification steps was 65.9±2.0 (SD)%, (N=3).

Accuracy of the method was determined by the addition of 2.5, 5.0, 12.5, and 50.0 pg E_2-17β to the serum blank of a postmenopausal woman (E_2-17β concentration : 3.3 pg/ml). Recovery in the accuracy of method was 98.0~128.0% (Table 1). Intra-and inter-assay precision of the method was estimated using sera of healthy women in the periods of maturity and postmenopause. The intra-assay precision was satisfactory, since the

coefficient of variation (CV) was 3.6~14.8% (Table 2). The inter-assay precision was estimated in 2 or 4 different assays The coefficient of variation in inter-assay precision was 6.4% in 16 serum samples under 10pg/ml, (Table 3). and 7.2% in 35 serum samples over 10pg/ml.

In 21 women who were from 3 to 14 years postmenopause, the un conjugated E_2-17β concentration in the serum was below 2.5 to 14.1 pg/ml, with concentrations below 2.5 pg/ml in 8 cases, 2.5~5.0 pg/ml in 9 cases, 5.0~10.0pg/ml in 3 cases, and over 10pg/ml in one case. Fig. 2 shows the correlation between the E_2-17β values determined by the present method and the direct method (sensitivity : 10 pg/ml). In serum samples over 10 pg/ml obtained by the direct method, E_2-17β values determined by the two methods correlated significantly (Fig. 2-A). In serum samples under 10 pg/ml by the dierct method, the E_2-17β value ranged from 2.5 to 23.5 pg/ml by the present method (Fig. 2-B).

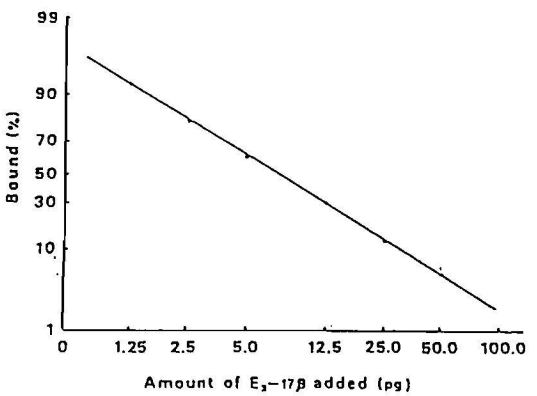

Fig. 1. Standard curve for E_2-17β. The mean values of duplicate determinations for each point are represented on a log-logit scale.

Discussion

We developed an improved method for the measurement of unconjugated E_2-17β with very high sensitivity (2.5 pg per 1 ml serum) and accuracy.

There are three RIA methods of E_2-17β in serum : RIA in conjunction with extraction, RIA in conjunction with extraction and purification, and direct RIA. Reported E_2-17β concentrations in sera of postmenopausal women measured by these methods are not consistent : 15 pg/ml or less[10),11)] 20~30 pg/ml[5),12)], and 30~40 pg/ml in 1 to 4 years postmenopause and 7~10 pg/ml after

Table 1. Accuracy of the method.

No. of determination	Standard E_2-17β added (pg/ml)			
	2.5	5.0	12.5	50.0
1	3.7	5.2	12.1	45.8
2	3.1	5.6	13.5	54.9
3	2.8	5.0	10.7	51.4
4	2.9	4.2	13.2	61.9
5	3.4	4.6	19.1	31.7
Mean	3.2	4.9	13.7	49.1
SD	0.3	0.6	3.2	11.4
CV (%)	9.4	12.2	23.4	23.2
Recovery (%)	128.0	98.0	109.6	98.2

5 years postmenopause[2]. The unconjugated E_2-17β concentrations in sera of 16 postmenopausal women measured by our method varied from less than 2.5 pg/ml to 14.1 pg/ml with the exception of one 10pg/ml or less. These values were considerably lower than those previously reported. This may be due to the following reasons. In our method, the use of antibody at high titers and E_2-I^{125} with a higher measurement of sensitivity (1.25

Table 2. Intra-assay precision of the method.

No. of determination	Sample (pg/ml)						
	A*	B*	C*	D	E	F	G
1	3.3	5.8	5.8	37.7	96.5	140.8	267.4
2	3.1	6.0	7.3	36.7	95.4	139.1	217.8
3	3.7	6.0	6.8	35.4	91.5	132.0	230.3
4	3.7	6.5	4.6	34.2	93.8	143.0	198.6
5	3.8	6.4	6.2	37.6	84.8	150.5	213.9
Mean	3.5	6.1	6.1	36.3	92.4	141.1	225.6
SD	0.3	0.3	0.9	1.3	4.2	6.0	23.2
CV (%)	8.6	4.9	14.8	3.6	4.5	4.3	10.3

Footnote ; Asterisks show the values obtained from postmenopausal women.

Table 3. Inter-assay precision of the method.

Sample	Inter-assay (pg/ml)			
	1st assay	2nd assay	Mean ± SD	CV(%)
1	5.6	5.9	5.8±0.2	3.4
2	4.2	4.3	4.3±0.1	2.3
3	7.9	8.3	8.1±0.2	2.5
4	3.5	4.5	4.0±0.5	13.0
5	8.6	8.3	8.5±0.2	2.4
6	7.6	6.1	6.9±0.8	11.6
7	5.8	5.7	5.8±0.1	1.7
8	5.4	6.5	6.0±0.6	10.0
9	5.5	5.4	5.5±0.1	1.8
10	4.2	3.6	3.9±0.3	7.7
11	3.9	4.3	4.1±0.2	4.9
12	8.0	9.4	8.7±0.7	8.0
13	7.0	7.1	7.1±0.1	1.4
14	9.2	6.9	8.1±1.2	14.8
15	5.1	7.1	6.1±1.0	16.4
16	3.8	3.8	3.8±0.0	0.0
Mean				6.4

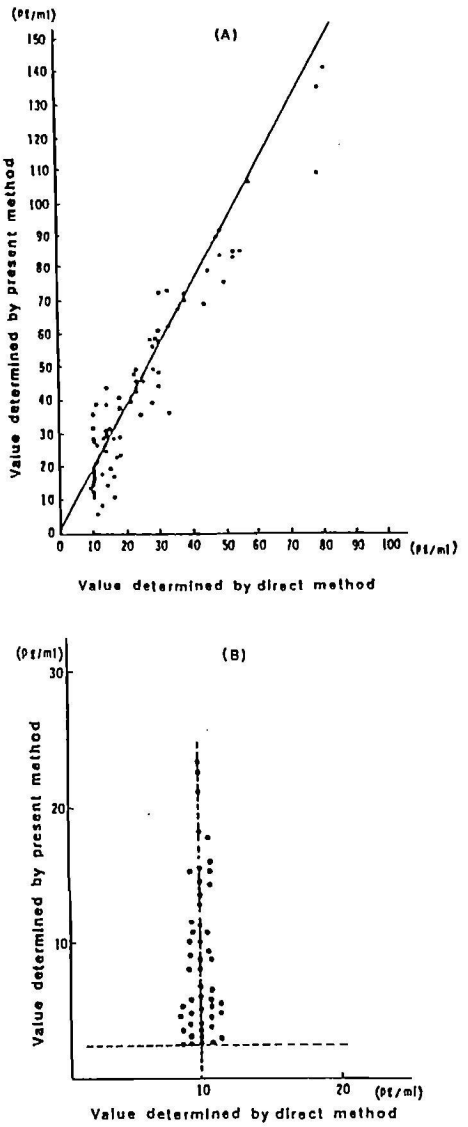

Fig. 2. Correlation between E_2-17β values determined by the present method and the direct method is a slope given by 1.67X 1.65 ($r=0.91$, $p<0.001$, $N=87$) in serum samples over 10 pg/ml by the direct method (A), while in samples under 10pg/ml (B), E_2-17β values ranged from 2.5 to 14.1 pg/ml by the revised method. Non-detectable E_2-17β values were assigned the limit of assay sensitivity.

pg/tube), and purification of 0.5 ml of serum samples by Sephadax LH-20 column chromatography with no column blank ; these facilitated measurement of E_2-17β at low concentrations (2.5 pg/1ml serum).

E_2-17β is generally measured in Japan by direct RIA using 50μl of serum samples without extraction or purification. Values obtained by this conventional method correlated well with those by our method in serum samples containing 10 pg/ml or more E_2-17β. However, the values obtained by our method were considerably higher than those obtained by the conventional method. These differences in values between the two methods may be due to the fact that only the unconjugated non-protein-bound form of E_2-17β is measured in the direct method.

There are some proteins in serum that bind to E_2-17β. Among them, testosterone-estradiol binding globulin (TeBG) with high binding affinity (Ka for E_2-17β : $0.43\sim6.4\times10^8$ 1/mol [13-16] which competes with E_2-17β antibody for the labelled antibody, seems to be the cause of lower measurement values. Since TeBG changes markedly with physiological changes such as aging [17,18] and pregnancy [19] and pathological changes such as hyperthyroridism [5], obesity [18,20], and liver diseases [21], caution is needed in interpreting values obtained by the direct method.

Our improved method for the measurement of E_2-17β allows measurement of unconjugated E_2-17β in serum at very low levels. This method is clinically very useful not only for evaluating ovarian function, but also for studying accurately the dynamics of administered E_2-17β.

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