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Preparation of Estradiol - HRP Conjugate and its Use in Different ELISA

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***Abstract*: In the present study estradiol was conjugated with HRP and polyclonal antibodies against estradiol, produced in rabbits. For the characterization of these antibodies and Estradiol-HRP conjugate, different immunoassays were performed mainly Competitive ELISA and Antibody Capture assay. Purification of Anti-Estradiol was done by ammonium salt precipitation and ion exchange chromatography. Preparation of enzyme conjugate was done by conjugation of Estradiol 6-CMO with HRP. Various diagnostic assays were performed like checker board assay, Assay to determine enzyme-conjugate titer, Standard competitive ELISA assay, Antibody capture assay.**

***Keywords*: Antibody capture assay, Competitive ELISA, Estradiol, HRP.**

1. Introduction

Estradiol (17β-estradiol, E2, 1,3,5 (10)-estratriene-3, 17β-diol), a steroid hormone, is produced primarily by the ovarian follicles from testosterone. [Abraham, 1975; Faiman et al., 1976] Estradiol is the most active naturally secreted estrogen. [Abraham, 1975] In men, estradiol originates in the testes and from extraglandular conversion of androgens. [Abraham, 1975] Circulating estradiol levels are relatively high at birth in both males and females, but decrease postnatally. [Faiman et al., 1976] In prepubertal children and men, levels are non-cyclic and low. During puberty, there are gradual increases in estradiol levels in both males and females. Interactions between luteinizing hormone (LH) and follicle-stimulating hormone (FSH) cause the release of estradiol from the ovaries in premenopausal women. Estradiol secretion is low in postmenopausal women. Research concerning estradiol has focused predominantly on reproductive issues such as conception, ovulation, infertility, and menopause. [Lipson et al., 1996; Choe et al., 1983] Yet, estradiol affects a diversity of biological processes involved with reproductive capacity, [Scheffer et al., 2003] establishment and maintenance of preg¬nancy, [Bazer et al., 2009] parenting, [Maestripieri, 1999] coronary artery disease, [Perez Lopez et al., 2010] immunocompetence, [Gameiro et al., 2010] cancer susceptibility, [Tsuchiya et al., 2005] and neuro protection. [McEven, 1999] Estradiol is also believed to affect individual differences in cognitive and socio¬ emotional processes as well as psychopathology. [Uvnas Moberg et al., 1990; Seeman 1997] Estrogens have been measured by many immunoassay methods. Studies suggest that estradiol can be accurately measured in saliva. [Lipson et al., 1996; Choe et al., 1983; Shirtcliff et al., 2001; Shirtcliff et al., 2000]

2. Materials

Anti-Estradiol, Estradiol HRP conjugates, 0.09% saline-NaCI, DEAE Sephadex, Estradiol 6-CMO (Carboxy Methyl Oxime), Dioxane, DMF (Dimethyl Formamide), NHS (N-Hydroxy succinimide), EDAC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide), Stabilizers- Ethylene glycol, 3% BSA (Bovine Serum Albumin), Sucrose, ARGG (Anti rabbit gamma globulin), Estradiol-6 CMO- BSA, ARGG-HRP, Saturated ammonium sulphate solution (pH-7), Ammonium Carbonate Buffer (pH- 7 to 7.4), Phosphate buffer (l0 mM).

A. Purification of Anti-Estradiol

1) Ammonium Salt Precipitation

5ml of serum was taken and mixed with 10 ml of Protein. Saline was added to the same. The beaker was kept on a stirrer and 15ml of ammonium sulphate solution was added drop wise. It was then stirred for 2 hours at room temperature. It was centrifuged at 6000 rpm for 20 min at 4C. The supernatant was discarded and 10ml l5mm phosphate buffer (pH-6.3). The above solution was dialyzed against 15mm phosphate buffer giving 2 to 3 changes. Stored at -20° C.

2) Ion Exchange Chromatography

Ammonium sulphate dialysate was taken, centrifuged and supernatant was taken. DEAE sephadex was taken; the buffer was removed by centrifugation and adds precipitated ammonium sulphate supernatant. Kept in a beaker and stirred for 30 minutes at room temperature. Centrifuged at 5000 rpm and kept the supernatant dialyzed the supernatant against NH_4C0_3 (0.09%) pH- 7.0 to 7.4. DEAE sephadex was washed with 1N NaCl. Supernatant was collected and a solution was prepared by diluting it 20 times. Absorbance was measured at UV 260 and 280 nm.

B. Preparation of Enzyme conjugates

1) Conjugation of Estradiol 6-CMO with HRP

3mg of Estradiol was dissolved in 100 µl DMF and 100 µl Dioxine. This solution was then added to 6 mg NHS and 12 mg EDAC solution which was prepared with 100 µl distilled water. The solution obtained was kept at room temperature for 3 to 4 hours. After 4 hours, 2mg HRP was dissolved in 200 µl of 15 mM phosphate buffer (pH 6.3). Now activated steroid was added to HRP and kept it overnight at 4°C. Estradiol HRP was subjected to Size exclusion Chromatography on G-25 column using 10mM PBS. All the colored fractions were collected and pooled together. It was then stored at -20°C. Equal amount of 50% Ethylene Glycol, 2 % BSA with a pinch of sucrose and pinch of ammonium sulphate were added to it.

C. Checker Board Assay to Determine Enzyme Conjugate Titer

A single strip was taken and coated with 100µl ARGG (15µl/200µl/well, ARGG in 10 mM phosphate buffer). It was then incubated overnight at room temperature. The strip was decanted and washed with tap water four to five times. 100µl of Anti-E2 0.5 µg/well was coated on the strip and again incubate overnight. The strip was again washed with tap water. Blocking buffer was added to it and incubated for 2 hours. It was then decanted, dried and packed with silica pouches. 100µl Estradiol conjugated with HRP was then added to it and incubated for 2 hours. Washed 4-5 times and substrate was added to it. Kept for 15 to 20 mints for color reaction. 100µl of stop solution was added to it. Absorbance was taken at 260 and 280 nm.

D. Standard Competitive ELISA Assay

In this assay, the standards of Estradiol were added and a competitive ELISA was carried out in which the standards of Estradiol competes with HRP conjugated Estradiol. Two strips coated with ARGG were taken. 200 µl of 0.5 µg of Anti-E_2, was added to all the wells. Now the standards of E_2 (2ng/mI, l0ng/ml, 50ng/mI, 100ng/ml, 200ng/ml) were coated in a regular fashion. It was then incubated for 2 hours. It was decanted, washed and dried with silica packets. Now E_2-HRPwas added to the wells with a dilution of 1:200 obtained from enzyme conjugate titer assay. Incubated for 2 hours. Decanted, washed and dried. 100 µl of substrate was added to it and kept for 15 to 20 mins. Stop solution was added. O.D. was taken at 450nm. Information about the assay and depicts how the 2 Antigens have competed for the same antibody.

E. Antibody Capture Assay

3 dilutions of Estradiol 6CMO-BSA were prepared of concentrations (1µg/100µl, 2µg/100µl, 5µg/100µl). Six strips were taken and 2 were coated with 100µl each concentration. Incubated at 37°C for 1 and half an hour. Decanted, washed and blotted on the paper. Anti-estradiol of various concentrations was added in a known manner. The concentrations taken were 0.1µg /ml, 0.2 µg/ml, 0.5µg/ml and 1µg/ml. Again incubated for 1and half an hrs at 37°C. Decanted, washed 4 to 5 times and dried with silica pouches. 100 µl of ARGG-HRP was added to each well. Incubated for 2 hours. Decanted and dried. Substrate was added and kept for 15 to 20 mints. Finally stop solution was added and reading was taken at 450nm.

3. Results and Discussion

A. Purification of Anti Estradio

1) Estimation of protein content obtained after ammonium salt precipitation

Absorbance at 260nM (UV range) = 0.321
Absorbance at 280nM (UV range) = 0.509
Protein concentration = 1.55 x A_{28o}- O.75 x A_{26o}
= 1.55x 0.509-0.75x0.321
=0.5482mg

The initial solution was prepared by twenty times dilution, so protein concentration =0.5482x20 =10.964mg

This protein content was only for 1ml but the volume of serum was 5ml, so final concentration of protein in serum =10.964x5 =54.82mg.

2) The protein content serum after DEAE (ion exchange) chromatography

Absorbance at 260nM (UV range) = 0.221
Absorbance at 280nM (UVrange) = 0.503
Protein concentration = 1.55xA_{28o}- 0.75xA_{260}
=1.55x0.221-0.75x0.503

The initial solution was prepared by twenty times dilution, so protein concentration

= 0.5482x20
= 10.9640mg

This protein content was only for 1 ml but the volume of serum was 5 ml, so final concentration of protein in serum =10.9640x5 =54.8200mg.

Checker Board Assay: A checker board was prepared in which a range of concentrations of Anti Estradiol were made to interact with a range of dilutions of estradiol conjugated with HRP. The manner in which they were interacted is depicted in the Table 1.

Table 1, explains the interaction between Antibodies and Estradiol conjugated with HRP in a range of concentrations. The same is depicted in the form of absorbance. It gives us the most appropriate concentration of both the reagents for a sensitive assay.

From the above assay the estimation of the concentration of Anti-estradiol was obtained. The most appropriate concentration is that concentration in which a most sensitive assay can be obtained at the minimum antigen concentration. The desirable concentration is 0.5µg/100µl.

Enzyme conjugate titer assay:

This test was carried out to estimate the dilution of Estradiol conjugated with HRP appropriate for the assay. The pattern is depicted in table 2.

Table 1
Interaction between Antibodies and Estradiol conjugated with HRP in a range of concentrations

S.No.	Anti E_2 (100µl)	E_2-HRP(100µl)	Absorbance at 450nM
1.	0.5 µg	1:200	1.341
2.	0.5 µg	1:200	1.543
3.	0.5 µg	1:400	0.690
4.	0.5 µg	1:400	0.765
5.	0.5 µg	1:800	0.531
6	0.5 µg	l:800	0.518
7.	0.5 µg	1 :1600	0.291
8.	0.5 µg	1: 1600	0.407
9.	1 µg	1:200	1.695
10.	1 µg	1:200	0.902
11.	1 µg	1:400	0.775
12.	1 µg	1:400	0.634
13.	1 µg	1:800	0.462
14.	1 µg	1:800	0.478
15.	1 µg	1: 1600	0.370
16.	1 µg	1: 1600	0.428
17.	2 µg	1:200	1.355
18.	2 µg	1 :200	1.162
19.	2 µg	1:400	0.900
20.	2 µg	1 :400	0.939
2l.	2 µg	1:800	0.834
22.	2 µg	1:800	0.902
23.	2 µg	1:1600	0.414
24.	2 µg	1: 1600	0.586
25.	($P0_4$ buffer)	1:200	0.743
26.	($P0_4$ buffer)	1:200	1.225
27.	($P0_4$ buffer)	1:200	0.547
28.	($P0_4$ buffer)	1:400	0.217
29.	($P0_4$ buffer)	1 :800	0.364
30.	($P0_4$ buffer)	1 :800	0.349
3l.	($P0_4$ buffer)	1: 1600	0.161
32.	($P0_4$ buffer)	1:1600	0.114

Table 2
Interaction between Antibodies and Estradiol conjugated with HRP in a range of concentrations

Well num.	Dilutions of E2- HRP	Absorbance at 450nm
9.	1 :100	2.884
10.	1 :100	2.786
11.	1:200	2.010
12.	1:200	2.289
13.	1:400	1.885
14.	1:400	2.640
15.	1:800	1.082
16.	1:800	1.057

Table 2, explains the interaction between Antibodies and Estradiol conjugated with HRP in a range of concentrations and only E2-HRPis in different dilutions. The same is depicted in the form of absorbance. It gives us the most appropriate estimation of the conjugate titer for a sensitive assay.

Since the absorbance was maximum in the range of 1:200 dilutions is 2.289, hence this was selected appropriate for a sensitive assay.

Standard competitive ELISA assay:

In this assay, the Anti-Estradiol and E2- HRPs elected above were used and a competitive assay designed with standards of different but known concentrations the pattern is as shown in table 3.

Table 3
Competition between standard Estradiol and Estradiol conjugated with standards in a range of concentrations

Well Number	Standards added in ng/ml(50µl)	E2-HRP (1:200) 100µl
1	2	1.993
2	2	1.799
3	10	1.510
4	10	1.963
5	50	2.703
6	50	2.617
7	100	1.979
8	100	2.352
9	200	1.915
10	200	2.342
11	2	2.758
12	2	1.942
13	10	2.586
14	50	2.684
15	100	2.151
16	200	1.946

Table 3, explains the competition between standard Estradiol and Estradiol conjugated with standards in a range of concentrations. The same is depicted in the form of absorbance. It gives us the actual information about the assay and depicts how the 2 Antigens have competed for the same antibody.

A regular result was not depicted in the above table. The explanation for the same is inefficiency of Estradiol -HRP. It was not able to compete with standards and hence, the results obtained were ambiguous. An alternative test was carried out using ARGG-HRP- as a secondary antibody and was named as Antibody Capture Assay was shown in table 4.

Table 4, explains the Antibody Capture Assay in which antibody (Anti-E2 is a primary antibody and ARGG-HRP is a secondary antibody) captures a target antigen which is E2-6CMO-BSA.

From this table we determine that antibody capture assay which is a type of Sandwich Elisa is a reliable technique than the rest of the other technique like standard competitive ELISA assay because it gives a linear curve.

Table 4
Antibody capture assay

S.No,	E2-6CMO-BSA	Ab (Anti-E2)	Absorbance at 450 nm
1	1µg/100µl	0.1	0.772
2	1µg/100µl	0.1	0.750
3	1µg/100µl	0.2	0.770
4	1µg/100µl	0.2	0.808
5	1µg/100µl	0.5	0.982
6	1µg/100µl	0.5	0.954
7	1µg/100µl	1.0	1.077
8	1µg/100µl	1.0	1.435
9	1µg/100µl	0.1	0.943
10	1µg/100µl	0.1	0.932
11	1µg/100µl	0.2	1.010
12	1µg/100µl	0.2	0.896
13	1µg/100µl	0.5	1.029
14	1µg/100µl	0.5	0.970
15	1µg/100µl	1.0	1.096
16	1µg/100µl	1.0	1.698
17	2µg/100µl	0.1	0.837

18	2µg/100µl	0.1	0.741
19	2µg/100µl	0.2	0.847
20	2µg/100µl	0.2	0.804
21	2µg/100µl	0.5	1.012
22	2µg/100µl	0.5	0.929
23	2µg/100µl	1.0	1.630
24	2µg/100µl	1.0	1.824
25	2µg/100µl	0.1	0.801
26	2µg/100µl	0.1	0.913
27	2µg/100µl	0.2	0.895
28	2µg/100µl	0.2	0.898
29	2µg/100µl	0.5	1.075
30	2µg/100µl	0.5	0.948
31	2µg/100µl	1.0	1.220
32	2µg/100µl	1.0	2.370
33	5µg/100µl	0.1	0.841
34	5µg/100µl	0.1	0.784
35	5µg/100µl	0.2	0.778
36	5µg/100µl	0.2	0.747
37	5µg/100µl	0.5	0.893
38	5µg/100µl	0.5	0.837
39	5µg/100µl	1.0	1.136
40	5µg/100µl	1.0	1.496
41	5µg/100µl	0.1	0.764
42	5µg/100µl	0.1	0.777
43	5µg/100µl	0.2	0.854
44	5µg/100µl	0.2	0.865
45	5µg/100µl	0.5	0.927
46	5µg/100µl	0.5	0.903
47	5µg/100µl	1.0	0.959
48	5µg/100µl	1.0	0.926

4. Conclusion

Final concentration of protein in serum after purification of Anti-Estradiol was 54.5mg. The protein content serum after DEAE (ion exchange) chromatography was 54.8200mg. In the checker board assay, a checker board was prepared in which a range of concentration of Anti Estradiol were made to interact with a range of dilution of Estradiol conjugated with HRP. From the above assay the estimation of the concentration of Anti-Estradiol was 0.5µg/100µl.Since the absorbance was maximum in the range of 1:200 dilution i.e. 2.289, hence this was selected for appropriate sensitive assay. In the Standard Competitive ELISA Assay, the Anti-Estradiol and E2-HRP selected above was used and a competitive assay was designed with standards of different but known concentrations. In the competitive assay results were not appropriate, Antibody Capture assay was performed and a linear curve was obtained which gives suitable results.

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