HOMOGENEOUS ENZYME IMMUNOASSAY OF ESTRADIOL USING ESTRADIOL-3-O-CARBOXYMETHYL ETHER AS HAPTEN

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ABSTRACT

A homogeneous enzyme immunoassay for estradiol estimation has been developed, which can be extended to other steriods. A new procedure for the preparation of estradiol -3-0- carboxymethyl ether by a simple one step reaction in high yield (90%) has been described. This hapten has been used for raising highly specific anti-estradiol antibody in rabbits and for preparation of enzyme conjugates. Two different enzymes, lysozyme and glucose -6- phosphate dehydrogenase have been studied for their suitability as enzyme labels. Our results indicate that lysozyme-conjugate meets the essential requirement for a practical enzyme immunoassay. The advantage of the present non-radioactive procedure is the overall simplicity, low cost and high stability of the reagents.

INTRODUCTION

The concentration of estradiol in peripheral blood of women has been proved to be a reasonable biochemical marker of follicular development. Despite the development of several heterogeneous enzyme immunoassay methods for estradiol (1-3), the related simpler homogeneous enzyme immunoassay has not found any application. The development of a useful enzyme immunoassay requires an antibody of high specificity and affinity for the compound to be measured and a stable efficient enzyme for which a simple sensitive assay is available.

Estradiol-6-0- carboxymethyl oxime conjugates has been so far used for raising anti-estradiol antibody, however, this hapten though commercially available is quite expensive. Exley(4) and Rao(5) reported

that a highly specific antiserum which discriminates well against D-ring metabolites such as estrone may be raised by coupling estradiol through the functional group at C-3 in the A-ring. However, their method of preparation of estradiol-3-hemisuccinate or estradiol-3-O-carboxymethyl ether was not satisfactory due to very low overall yield.

In this communication, we report a method for the preparation of estradiol -3-O-carboxymethyl ether in high yield, and the development for the first time a homogeneous enzyme immunoassay for estradiol. Since no single enzyme is suitable for EIA, we studied the utility of the enzymes lysozyme and glucose-6-phosphate dehydrogenase (G6PDH) as labels. In addition to the simplicity of the assay procedure and long shelf life of the enzyme conjugates, this technique has the advantage of utilizing a cheap hapten for raising highly specific antibody and making enzyme conjugates.

MATERIALS AND METHODS

Estradiol, bovine serum albumin (Cohn fraction V), hen egg white lysozyme (E.C.3.2.1.17), dried cell wall powder of Micrococcus lysodeikticus, glucose-6-phosphate dehydrogenase (G6PDH), nicotinamide adenine dinucleotide phosphate (NADP), N-hydroxy succinimide, dicyclohexyl carbodiimide (DCC) and trinitrobenzene sulphonic acid (TNBS) were obtained from Sigma Chemical Co., USA and Sephadex G-50 and Sepharose 4B from Pharmacia Fine Chemicals, Sweden. Estradiol-3-O-carboxymethyl ether was synthesized as described below. All other reagents used were of analytical grade.

Assay buffers - The enzyme assay was carried in potassium phosphate buffer 0.05M, pH 6.2 for lysozyme and Tris buffer (Tris-HCL 72 mM, magnesium chloride 4mM and BSA 1.0g/L, adjusted to pH 8.0 with HCl) for G6PDH.

Substrate solution - For lysozyme - Stock solution of the substrate was prepared by suspending dried cell wall powders of M. lysodeikticus in assay buffer (1 mg/mL).

For G6PDH - The optimized substrate solution contained glucose-6-phosphate (25 mM) and NADP (2mM) in assay buffer.

Synthesis of estradiol-3-O-carboxymethyl ether ($E_2$3CME) - Estradiol 3-O-carboxymethyl ether was prepared by alkylation[7] of estradiol with bromoacetic acid. To a solution of estradiol (300 mg, 1.10 mmole) in dry dimethylsulfoxide (6 mL) was added dry powdered KOH (1 g, 18 mmole). After stirring for 5 min, bromoacetic acid (300 mg, 2.15 mmole) was added. Stirring was continued for 2 h after which the reaction mixture was poured into ice cold water (50 mL) and extracted with ethyl acetate to recover unreacted estradiol. The aqueous solution was cooled and acidified with dilute HCL (2 N) and the separated solids were filtered. It was washed with water till neutral and dried under vacuum. The solid was crystallized from chloroform-methanol to give estra-1, 3, 5 (10)-triene-3, 17β-diol-3-O-carboxymethyl ether as colourless solid (335 mg, 90%, mp 188-90°C) [lit. mp 192-93°C(5)]. The product was fully characterized by the following spectral properties.

UV : $\lambda_{max}^{ethanol}$ 278 nm (ϵ 1500), 285 (ϵ 1330) nm, which remained unaffected on addition of alkali

IR : ν_{max}^{nujol} 3420(OH), 1720(COOH)cm^{-1}

PMR($CDCl_3$) : δ 0.68s (18-Me), 2.28 brs (H-6 and H-9), 3.74t (H-17, J=7Hz), 4.62s (O-CH_2-CO), 6.65 (H-4, J=1.5 Hz), 6.71dd (H-2, J=1.5, 8Hz), 7.22 d(H-1,J=8Hz).

MS : m/z 330 (M^+), 271

Preparation of antibodies - Estradiol-3-O-carboxymethyl ether was coupled to BSA by the activated ester method employing N-hydroxy-succinimide and dicyclohexylcarbodiimide as described by Mattox and Nelson (6). Analysis of the conjugate by TNBS method showed a coupling ratio of 25 estradiol residues per protein molecule. The steroid-protein conjugate was lyophilized and stored at -20°C.

Antibodies were raised in New Zealand white rabbits as described by Dawson and co-workers (7). Briefly, 1 mg of the steroid-protein conjugate in 0.5 mL of isotonic saline was emulsified with 0.5 mL of Freund's adjuvant (complete) and injected subcutaneously in multiple sites at the back of each animal. Booster injections with the same amount of antigen in Freund's incomplete adjuvant were administered after 2 and 4 weeks and thereafter at monthly intervals. After checking the antibody titer by Ouchterlony immunodiffusion in gel, blood was collected by cardiac puncture. The sera were then separated and the antibody purified by repeated precipitation with ammonium sulfate (50% saturation) followed by dialysis against 0.025M phosphate buffer, pH 7.3 containing 0.9% NaCl. It was further purified by passing through BSA-Sepharose 4B immunoabsorbent column for removal of anti-BSA antibodies and stored as 1 ml aliquots at -20° C.

Preparation of estradiol-enzyme conjugates - Estradiol-3-O-carboxymethyl ether was coupled to lysozyme and G6PDH by the activated ester method of Mattox and Nelson (6). The products were purified by dialysis followed by chromatography over Sephadex G-50. The protein fractions were pooled and lyophilised. The degree of conjugation of estradiol-3-O-carboxy methyl ether to lysozyme and glucose 6-phosphate dehydrogenase was 4 and 12 as determined by TNBS method (8).

Enzyme assay - Lysozyme assays were carried out in a total volume of 1 mL. To the substrate suspension a suitable volume of enzyme conjugate was added and the contents mixed by inversion. The initial linear rate of clearance of turbidity was measured by recording the decrease of OD at 450 nm with a Pyeunicam SP-300 double beam spectrophotometer. The amount of enzyme conjugate used was chosen to give an initial rate of about 0.1 absorbance unit/min for the free enzyme conjugate.

The G6PDH assays were carried out in a total volume of 1.5 mL. To a suitable concentration of enzyme conjugate, the substrate solution was added and the increase in absorbance was recorded at 340 nm against a blank containing everything except the substrate. The activity of the enzyme was determined from the initial linear portion of the curve.

Determination of optimum antibody dilution - To determine the suitable concentration of antibody to be used in enzyme immunoassay, 50 µL of enzyme conjugate was incubated at 4°C with 100 µL of antibody serially diluted with assay buffer for 1.5 h. The enzyme activities in the tubes were then measured. The lowest amount of antibody which showed maximum inhibition of enzyme activity was selected for immunoassays.

Enzyme immunoassay procedure - With lysozyme. In a series of Eppendrof tubes 100 µL of estradiol solution (standard or sample) was taken, 100 µL of antibody in assay buffer was added and the mixture incubated at 37°C for 2 h. Then the tubes were allowed to come to room temperature and 50 µL of enzyme conjugate was added to each tube and incubated again for another 2 h at 4°C. From the above mixture 125 µL was used for assay.

With G6PDH : In a series of Eppendrof tubes, 50 µL of antibody was incubated for 2 h with 100 µL of estradiol solution at 37°C. Then 50 µL of enzyme conjugate was added and incubated for another 30 min at 4°C and 100 µL of this mixture was used for assay.

The standard curves were prepared by plotting the enzyme activity against estradiol concentration on semi-log paper.

RESULTS AND DISCUSSION

Estradiol - 3-O-carboxymethyl ether has been successfully used to generate anti-estradiol antibody of high specificity (5). However, the reported method of its preparation involving refluxing of estradiol with sodium chloroacetate, sodium ethoxide and ethanol for 22 h, conversion of acid obtained to methyl ester followed by its purification by chromatography and then hydrolysis to estradiol - 3-O-carboxymethyl ether in overall 5% yield is highly unsatisfactory. The method described by us is a new procedure for the preparation of estradiol-3-O-carboxymethyl ether by simple one-step reaction within 2 h in 90% yield.

The enzyme activity recovered in the G6PDH-labeled estradiol was approximately 80% of that of the original enzyme before conjugation. In the case of lysozyme no significant loss of enzyme activity was observed. Addition of estradiol antibody to enzyme conjugates resulted in inhibition of enzyme activity. All control experiments suggested that inhibition was due to antigen-antibody reaction. No inhibition was observed with normal rabbit IgG. Our results of the effect of antibody dilution on inhibition of enzyme activity (Table 1) indicates that activity of lysozyme is strongly inhibited. Low inhibition of G6PDH activity may be due to the presence of significant proportion of unconjugated G6PDH. All efforts towards its removal by affinity chromato-

Table 1 Effect of antibody dilution on the activities of $E_2$3CME-lysozyme and $E_2$3CME-G6PDH conjugates

$E_2$3CME-lysozyme		$E_2$3CME-G6PDH	
Antibody dilution	% inhibition	Antibody dilution	% inhibition
1:80	44	1:64	10
1:40	47	1:32	20
1:20	53	1:16	27
1:10	59	1: 8	37
1: 5	65	1: 4	50
1:2.5	72	1: 2	53

graphy against estrone antibody (1) proved unsuccessful as it led to deactivation of the enzyme. Finally, lysozyme as label was selected as it meets the essential requirement for a practical enzyme immunoassay. The standard curve constructed with various concentration of estradiol and a constant volume of lysozyme conjugate with antibody diluted 10-fold and 20-fold is shown in Fig.1. The use of higher antibody dilution increased the sensitivity to 500 pg but the steepness of standard curve is reduced and the reproducibility decreased. With GPDH the standard curve has the range from 500 pg to 50 ng per tube.

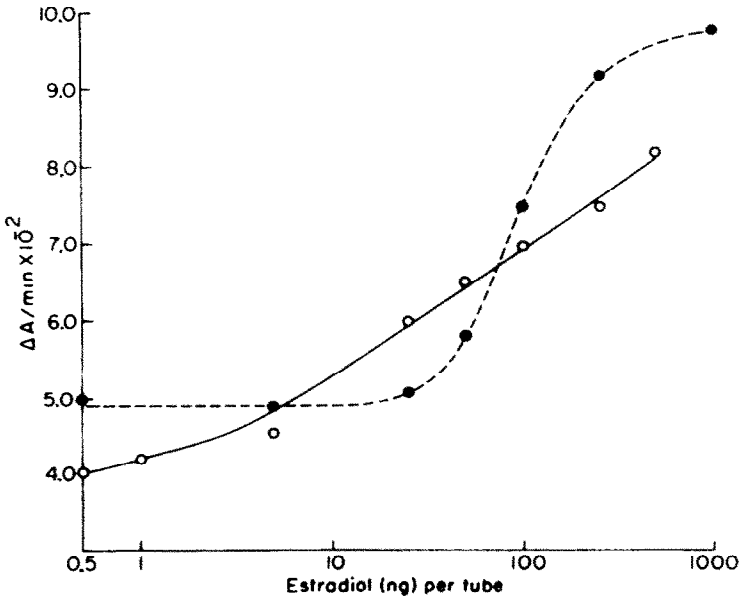

Figure 1. Standard curve for estimation of estradiol using estradiol-lysozyme conjugate, —●— , antibody dilution 1:10; —○— antibody dilution 1:20.

The measurement of cross-reactivity of analogous steroids at 50% reversal of enzyme activity was difficult due to low solubility of most of the steroids in the buffer used and the cross-reactivities of estriol and estrone were estimated from curves extrapolated beyond concentrations of 5 µg per tube. Under these conditions both the estrogens showed cross-reactivities of less than 0.01%. The cross-reactivity of testosterone and progesterone were also negligible.+

The reliability and reproducibility of the assay were evaluated by measuring the co-efficient of variation of estradiol measured at three levels. It is evident from the data shown in Table 2 that the method has satisfactory accuracy and reproducibility. The low sensitivity of the assay is obviously due to the low sensitivity of the turbidimetric assay of lysozyme and not due to low affinity of the antibody.+

Table 2 Replicate analysis of authentic estradiol dissolved in deionized distilled water

Estradiol (ng/tube) Added	Measured	n	Coefficient of variation
200	211 ± 11.8	6	5.6
50	50.4 ± 2.5	6	4.9
10	9.3 ± 0.57	6	6.1

NOTE

+Personal communication from Prof. N. R. Moudgal, Dept. of Biochemistry, Indian Institute of Science, Bangalore, India, who has used this antibody for radioimmunoassay of estradiol at a dilution of 1:50,000 and found the cross-reactivity with testosterone and progesterone to be 0.07 and 0.001%, respectively.

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APPENDIX

Trivial Name

Estrone 3-Hydroxyestra-1,3,5,(10)-trien-17-one.

Estradiol 1,3,5,(10)-Estratriene-3, 17β-diol.

Estriol 1,3,5,(10)-Estratriene-3, 16α,17β-triol.