PICOGRAM ORDER ENZYME IMMUNOASSAY OF OESTRADIOL+

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1. Introduction

Radioimmunoassay (RIA) methods have been widely used for determination of endogenous and exogenous substances in the biological fluids. In recent years enzyme immunoassay (EIA) which possesses several advantageous features has been developed as an alternative to RIA [1]. This method, however, is still unsatisfactory in respect of the sensitivity and versatility. Although several papers dealing with EIA of steroid hormones have been published [2–5], an application of this method to human plasma has not yet been reported. The purpose of this communication is to describe the development of an enzyme immunoassay method for oestradiol at levels ranging from 50 pg using the antibody–acrylamine glass conjugate, horse radish peroxidase (HRP)-labelled oestradiol and fluorogenic substrate.

2. Materials and methods

2.1. *Reagents*

Oestrone 17-(*O*-carboxymethyl)oxime and 6-oxoestradiol 6-(*O*-carboxymethyl)oxime were synthesized by the known methods [6,7]. HRP, Type II, R.Z. 1.2 and bovine serum albumin (BSA) from Sigma Chemical Co. (St Louis, Mo.), homovanillic acid, Rivanol (6,9-diamino-2-ethoxyacridine lactate) and 25% glutaraldehyde were purchased from Nakarai Chemicals, Ltd (Kyoto, Japan). *p*-Arylamine glass/CPG-550 (177–840 μm in diameter) was kindly donated from Corning Biological Products Department (Medfield, Mass.). [6,7-^{3}H]Oestradiol (spec. act. 42 Ci/mmol) was supplied from the Radiochemical Centre (Amersham, England).

2.2. *Preparation of antisera*

The anti-oestradiol antisera raised against 7α-hydroxyoestradiol 7-hemisuccinate–BSA conjugate were prepared in these laboratories [8]. Oestrone 17-(*O*-carboxymethyl)oxime was coupled to BSA by the mixed anhydride method employing isobutyl chlorocarbonate and tri-*n*-butylamine [6] and the resulting conjugate was used for immunization of rabbits as an antigen in the manner as described previously [8]. The antisera obtained from immunized rabbits were treated with Rivanol to separate a crude globulin fraction according to the procedure recommended by Abraham [9].

2.3. *Preparation of HRP-labelled oestradiol and antibody–glass conjugate*

Oestradiol 6-(*O*-carboxymethyloxime)–HRP conjugate was prepared as an enzyme-labelled antigen by the mixed anhydride method [6]. The reaction mixture was dialyzed against distilled water and then chromatographed on Sephadex G-100. The enzyme activity recovered in the HRP-labelled oestradiol fraction was approx. 10% of the initial one. The crude globulin was covalently linked to finely divided *p*-arylamine glass particles by the glutaraldehyde method [10]. The immobilized antibody suspended uniformly into 0.05 M phosphate buffer, pH 7.0, was highly stable at 4°C.

2.4. *RIA of oestradiol*

RIA of oestradiol was carried out employing antisera raised against oestradiol-7α-hemisuccinyl–BSA conjugate for comparison with the results by the

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newly developed enzyme immunoassay method. Reliability of the RIA procedure has been confirmed in our previous work [8].

2.5. *EIA of oestradiol*

A sample solution (0.2 ml) was incubated overnight at 4°C with 0.2 ml of a diluted suspension (1:40) of immobilized antibody. After addition of 0.1 ml of the oestradiol–HRP conjugate in 0.05% BSA solution approx. 1×10^{-5} PU of enzyme activity), the mixture was again incubated for 1 h at 4°C. The incubation mixture was centrifuged at 3000 × *g* for 15 min and the supernatant was submitted to measurement of the peroxidase activity [11]. A 0.2 ml aliquot of the supernatant was added to 0.1 ml 0.25% homovanillic acid aqueous solution, 0.1 ml 0.05% hydrogen peroxide and 2.5 ml 0.1 M Tris–HCl buffer, pH 8.5 and the mixed solution was incubated at 25°C for 1 h. The fluorescence intensity was measured on a Hitachi Model MPF-2A spectrofluorometer where 320 nm and 420 nm were chosen as excitation and emission wavelengths, respectively.

2.6. *Separation of oestradiol in blood plasma*

A plasma sample obtained from normal pregnant women was diluted with 2.5 vol. distilled water, a 0.1 ml aliquot of which was extracted with 2 ml ether. The extract was chromatographed on a Sephadex LH-20 column using benzene–methanol (85:15) as an eluent and the desired oestradiol fraction was separated [12]. A known amount of oestradiol in the range of 100 pg to 2 ng added to 0.1 ml of human plasma was recovered at a rate of 76.7 ± 4.9%.

3. Results and discussion

A preliminary experiment revealed that the enzyme assay method by fluorometry with homovanillic acid as a substrate was approximately two-hundred times as sensitive as that by spectrophotometry with 5-aminosalicylic acid. A dose–response curve was constructed by plotting the percentage of immunoreactive enzyme bound with antibody against the amount of non-labelled oestradiol. Using the standard curve, oestradiol could be determined in the range of 50 pg to 1 ng/tube. The cross-reactivities of this assay system were found to be 15.4% for oestrone and 9.5% for oestriol. Accordingly,

Table 1
Replicate analyses of authentic oestradiol added to deionized distilled water

Oestradiol		Coefficient of variation	
Added (pg/0.1 ml)	Estimated (pg/0.1 ml)	(*n*)	(%)
70	83.8 ± 7.5	4	8.9
400	381 ± 39.8	4	10.4
600	625 ± 77.0	4	12.3

separation of oestradiol was required prior to EIA. Oestradiol added to deioinized distilled water was chromatographically separated and assayed in the manner as described above. It is evident from the data in table 1 that the known amounts of oestradiol at three levels could be estimated with satisfactory accuracy and precision. The reliability of the present method was then evaluated by comparison with the results by RIA with peripheral plasma samples from pregnant women (31–41 weeks). The mean value of oestradiol determined by EIA was 14.2 ± 5.2 ng/ml, while that by RIA was 12.5 ± 4.3 ng/ml. As illustrated in fig.1, a satisfactory correlation is observed between the results obtained by EIA and RIA methods.

It has been suggested that the sensitivity of EIA is considerably improved when a slightly heterologous combination of the oestrogen–enzyme conjugate and

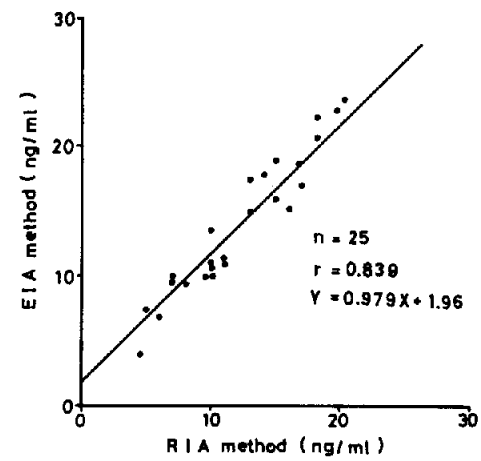

Fig.1. Correlation between the results on oestradiol level in late pregnancy plasma obtained by EIA and RIA methods.

antibody is employed [3]. In this experiment heterology was brought about by utilizing the different positions on the steroid nucleus, namely C-6 and C-17, through which enzyme and carrier protein were linked to estrogen, respectively. The choice of the insoluble support for immobilizing antibody is also important to enhance the sensitivity in heterogeneous EIA. It has been previously reported that the use of Sephadex or Sepharose for this purpose resulted in a significant decrease in the sensitivity of the assay system and immunoreactivity of antibody [13] and an extremely slow rate of the antigen–antibody reaction [14]. In addition, the activated cellulose derivative is so unstable that the suitable antibody–support conjugate is not always obtained. These difficulties could be overcome and also nonspecific binding of the steroid to the support could be avoided when finely pulverized arylamine–glass was employed [10].

It is to be noted that a highly sensitive immunoassay method for determination of oestradiol in plasma has been established with success by the use of acrylamine glass as an insoluble support, heterologous combination of antibody and oestradiol–HRP conjugate and fluorometric measurement of enzyme activity. This type of EIA may be applicable extensively for quantitation of various steroid hormones in the biological materials.

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