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ENZYME IMMUNOASSAY FOR PLASMA ESTRADIOL USING A MONOCLONAL ANTIBODY

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Summary—A microtitre plate enzyme immunoassay (EIA) for plasma estradiol is described, involving competition between sample estradiol and an immobilized estradiol–bovine serum albumin complex for a monoclonal anti-estradiol antibody, followed by immobilized antibody quantitation using enzyme-labelled antiglobulins.

The assay dose–response curve covered a range of 6–1500 fmol/well. The intra- and inter-assay coefficient of variation for the assay of three plasma pools ranged from 3.1 to 4.7% and from 4.7 to 10.6% respectively. The assay showed satisfactory correlation with a standard estradiol radioimmunoassay. Pre-coated microtitre plates were stable, dried, at 4°C for up to 3 months and the anti-estradiol was stable to lyophilization and also was stable in solution at 4°C for up to 1 month.

INTRODUCTION

Much effort has been devoted to the development of alternatives to the traditional radioimmunoassay for determination of serum or plasma estradiol in women, for clinical endocrinological investigation. These alternative assays, avoiding the problems associated with the use of radioisotopes must, at least, retain the sensitivity and specificities of available radioimmunoassays. A number of steroid enzyme immunoassays (EIA) have thus been developed since the first such assay described by Van Weeman and Schuurs [1] and, more recently, the adaptation of the EIA principle using multiwell microtitre plates as the solid support has been used to great advantage for the assay of steroids [2]. The use of microtitre plates could bring greater reproducibility and an increased economy in the use of materials, thence lower costs, than tube-based assays and facilitates the rapid processing of large numbers of samples.

The aim of the present work was to develop a simple microtitre plate EIA for plasma estradiol using a high affinity monoclonal anti-estradiol antibody recently produced in this laboratory [3]. The resulting assay has been assessed by comparison with a classical radioimmunoassay. Finally, the stability to storage under different conditions of the antibody and of the immunogen-coating in microtitre plate wells has been determined.

EXPERIMENTAL

Estradiol (1,3,5(10)-estratriene-3, 17β-diol) was from Roussel Uclaf (Romainville, France) and [2,4,6,7 ^{3}H]estradiol (3.89 TBq/mmol) from Amersham Int. (U.K.). Alkaline phosphatase-conjugated mouse antiglobulins came from Biosys (Compiegne, France) and *p*-nitrophenyl phosphate (*p*-NPP) and casein (purified powder) came from Sigma. The casein was acid-washed according to the method of Livesey and Donald [4]. Microwell plates were from Nunc (Roskilde, Denmark).

Immunogen and immunosorbent

Estradiol-3-carboxymethyl–bovine serum albumin (estradiol-3CM–BSA) was synthesized by the method of Erlanger *et al.* [5]. A conjugate having 38 moles steroid per mol protein was used as immunogen and a conjugate having 14 moles steroid per mol protein was used as immunosorbent. The immunosorbent was stored lyophilized at −20°C and a substock (0.87 mg/ml) in TBS buffer (0.02 mol/l Tris, 0.5 mol/l NaCl, pH 7.5) containing 0.25 g/l sodium azide was stored at 4°C prior to use.

Monoclonal anti-estradiol antibody

The murine monoclonal antibody against estradiol 3-CM–BSA, raised in this laboratory, had an affinity of $1 \cdot 10^{10}$ l/mol for estradiol as determined by microequilibrium dialysis [6]. Its

Table 1. Cross-reactivities (%) of anti-estradiol antibody in two immunoassay systems in terms of the CR_{50}s [10]

Steroid	RIA	EIA
Estradiol-17β	100	100
Estradiol-3-glucuronide	58.1	15
Estradiol-3-sulphate	41.9	11.6
17α-Ethynyl estradiol-17β	0.9	1.2
Testosterone	0.8	0.7
Estriol	0.7	1.2
5α-Dihydrotestosterone	0.3	0.4
Estrone	0.2	0.2
Estradiol-17α	0.1	0.09
Estradiol-17-glucuronide	<0.01	<0.01
Estrone-3-sulfate	<0.01	<0.01
Progesterone	<0.01	<0.01
Aldosterone	<0.01	<0.01
Cortisol	<0.01	<0.01
Danazol	<0.01	<0.01
Dienoestrol	<0.01	<0.01
Dihydrodiethylstilboestrol	<0.01	<0.01

cross-reactions, as determined by radioimmunoassay (RIA) and enzyme immunoassay (EIA), are shown in Table 1. The ascites fluid, used without purification, was stored at −20°C diluted 10-fold in 0.05 mol/l phosphate buffer containing 0.077 mol/l NaCl, pH 7.

Assay procedures

Microtitre plate wells were coated overnight at 4°C with 25 ng estradiol-3CM–BSA in 150 μl TBS buffer/well. Remaining active sites in wells were saturated for 15 min at room temperature with 250 μl acid-washed casein (30 g/l in TBS buffer). Wells were washed 3 times with 250 μl acid-washed casein (1 g/l in TBS buffer) (assay buffer). This washing procedure was carried out between each step in the assay.

The antibody dilution curve was obtained by a 2 h incubation at room temperature of 150 μl aliquots of ascites fluid, serially diluted in assay buffer, in coated wells. The liquid was then discarded and the wells washed and 150 μl alkaline phosphatase-conjugated mouse anti-globulins diluted 2000-fold in assay buffer were added. Following a 2 h incubation at room temperature the wells were emptied, washed and assayed for alkaline phosphatase activity with 150 μl substrate solution: *p*-nitrophenyl phosphate (2.5 g/l), diethanolamine (1 mol/l), $MgCl_2$ (0.5 mmol/l) pH 9.8. Plates were incubated at 37°C, the reaction was stopped with 50 μl of ethylenediaminetetra-acetic acid 0.2 mol/l and absorbances were read at 405 nm with a Dynatech MR 700 microplate reader.

Estradiol assays were carried out with the same total reagent volume (150 μl):100 μl standard estradiol or plasma extract and 50 μl anti-estradiol immunoglobulins. The latter were diluted to give the final dilution yielding 40–45% of the maximal bound alkaline phosphatase activity in the dilution curve. The final enzyme assay incubation was for 30 min.

Radioimmunoassay

Radioimmunoassay was carried out, in 0.05 mol/l phosphate buffer pH 7 containing 0.077 mol/l NaCl, 0.5 g/l sodium azide and 1 g/l gelatin, with [2,4,6,7^{3}H] estradiol. After 3 h incubation at 4°C, free/bound antigen separation was carried out using dextran–charcoal [7].

Sample preparation

Female plasma samples were extracted with ethyl acetate:cycloexane (1:1 v/v) (4 ml/ml plasma) [8] and evaporated to dryness. Extracts, to be assayed by enzyme or radioimmunoassay, were taken up with the corresponding assay buffer at a volume equal to half that of the original plasma sample. In cases where high steroid levels were clinically expected (pregnancy, stimulation of ovulation) a further volume of buffer was added. Estradiol recoveries with this method, as determined with tritiated tracer estradiol, were 94 ± 8%.

Coated plate storage

The stability of coated plates as a function of time at 4°C was tested using 3 different storage conditions:

—plates with 150 μl immunosorbent coating solution in wells.
—following coating and washing with 3 × 250 μl distilled water, the plates were dried with or without prior site saturation with casein.

RESULTS

The anti-estradiol antibody dilution curve is shown in Fig. 1. A final ascites fluid dilution of 1/150,000 gave 40–45% maximal bound alkaline phosphatase activity. This dilution was used for the assay.

The assay standard curve is shown in Fig. 2. The sensitivity of the assay in terms of the lowest detectable dose of estradiol (twice the standard deviation at zero dose) was 3 pg (11 fmol)/well (equivalent to 110 pmol/l in a plasma sample). The mid-point sensitivity of the curve was 45 pg (165 fmol)/well (equivalent to 1650 pmol/l in a plasma sample), (the estradiol concentration in normal female plasma is 280–600 pmol/l [9]). A logit/log representation

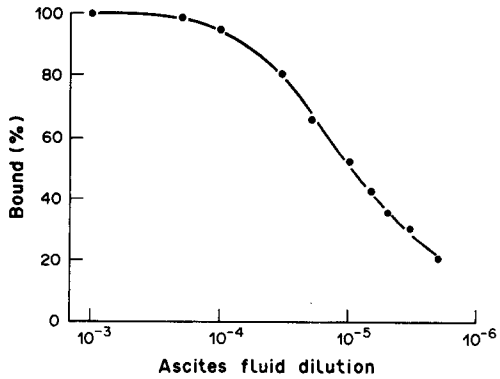

Fig. 1. Antibody dilution curve. The percentage binding of serially diluted ascites fluid (anti-estradiol immunoglobulins) to the immobilized estradiol relative to the maximum binding obtainable with the same quantity of immobilized estradiol.

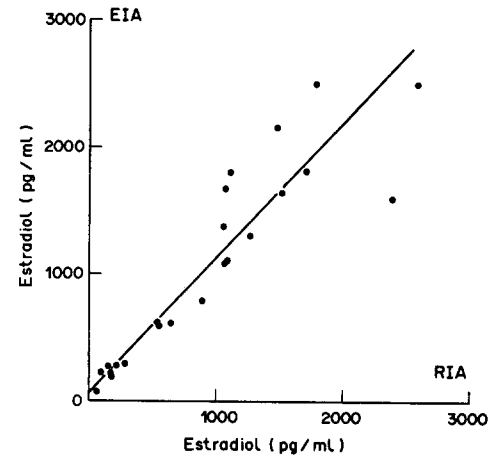

Fig. 3. Comparison of plasma estradiol concentrations as determined by a classical radioimmunoassay and by enzyme immunoassay ($n = 23$).

of the results was linear from 1.6 to 400 pg/well. The intra-assay coefficients of variation of the B/B_0 values on this standard curve ($n = 5$) ranged from 1.6 to 8.9% (Fig. 2).

The specificity of the antibody used, as assessed by enzyme and radioimmunoassay, for a range of steroids is shown in Table 1. Only the cross reactivities with estradiol-3-glucuronide and estradiol-3-sulfate were significant (15 and 11.6% by EIA; 58.1 and 41.9% by RIA respectively).

Figure 3 shows the correlation between the assay of 23 plasma samples by enzyme and radioimmunoassay (using the same antibodies). The regression line, weighted by the inverse of the mean values of each point, was: EIA = 1.07 RIA + 63 pg/ml plasma (6.3 pg/well) ($r = 0.980$).

The reproducibility of the enzyme immunoassay was determined by repetitive assay of 3 plasma pools. Intra-assay and inter-assay coefficients of variation were determined with 8 and 7 replicate determinations respectively and are shown in Table 2.

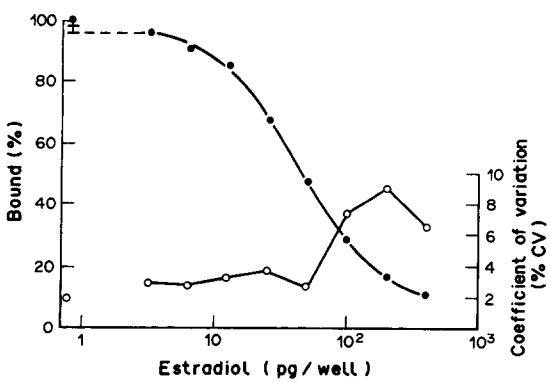

Fig. 2. Standard dose–response curve for enzyme immunoassay of estradiol showing the lower limit of detection as defined by twice the standard deviation of the blank. Intra-assay coefficients of variation of each point on the dose–response curve ($n = 5$).

The stability of coated plates to storage under different conditions was determined over a period of 2 months for plates stored with immunosorbent coating solution in wells and of 3 months for plates stored dry. When assessed in terms of the slope of the standard curve at the mid-point (an indicator of changes in the form of the curve) no significant change was observed for all storage conditions. The results of assessment in terms of the coefficient of variation at each point on the curve ($n = 6$) (an indicator of the proportion of wells giving aberrant results) are shown in Table 3.

The stability of ascites fluid stored at 4°C, lyophilized or in solution diluted 20-fold in 0.05 mol/l phosphate buffer containing 0.077 mol/l NaCl, 0.5 g/l gelatin and 0.25 g/l sodium azide, pH 7 was determined from the mid-point slope and mid-point sensitivity of standard assay curves and compared with ascites fluid stored at −20°C, diluted 10-fold in the same buffer but without gelatine and sodium azide. No change was found with lyophilized ascites fluid for up to 4 months and the solution stored at 4°C was stable for up to 1 month.

Table 2. Intra- and inter-assay precision of the EIA for estradiol in plasma

Sample	Mean (nmol/l)	Coefficient of variation (%) Intra-assay ($n = 8$)	Inter-assay ($n = 7$)
Pool A	768	4.6	6.3
Pool B	1827	4.7	4.7
Pool C	4743	3.1	10.6

Table 3. Stability of immunosorbent-coated plates under 3 different storage conditions

Storage time	Coefficient of variation (%) Storage conditions With coating solution	Dry without site saturation	Dry with site saturation
Initial	<5	<5	<5
15 days	ND	<5	5
1 month	6.5	6.5	<5
2 months	6	5	5
3 months	ND	5	9

Plates were stored at 4°C: (1) with coating solution in the wells; or following coating and washing with distilled water, dry with (2) or without (3) prior site saturation with casein.
The resulting standard displacement curves are described in terms of the mean of the coefficient of variation of each point on a curve ($n = 6$). ND = not determined.

DISCUSSION AND CONCLUSION

The most successful of the steroid immunoassays designed to overcome problems associated with the use of radioisotopes have been enzyme [1–11] and, more recently, chemiluminescence immunoassays [12]. The sensitivities of some of these are better than those of radioimmunoassay [13]. The use of microtitre plates for these assays also means a reduction in the quantities of reagents consumed.

The present microtitre EIA was based on the same principle as the competitive technique for the detection of small antigens outlined by Voller *et al.* [14]. The characteristics displayed by this EIA were defined by the high affinity of the monoclonal antibody ($1 \cdot 10^{10}$ l/mol). This antibody was also highly specific; the only significant cross-reactions for the steroids tested were with estradiol-3-glucuronide and estradiol-3-sulphate. This cross-reactivity was not expected to interfere with the immunoassay because these conjugates are present only at low concentrations in plasma.

The antibody did not recognize BSA, which had been used to make the immunogen, so the same protein could be used to make the protein–steroid conjugate for well coating. An estradiol-3-CM–BSA conjugate which had fewer molecules of estradiol per molecule of protein than the immunogen was used in order to avoid possible steric hindrance of antibody binding to the conjugate on a solid surface. There was 1.3 ng of estradiol in the quantity of the conjugate used for well coating, though the amount of immunoreactive estradiol actually coated was not known.

The lower limit of detection of this assay (3 pg) is typical of reported steroid enzyme immunoassays involving immobilized steroid, whose lower limits of detection are in the range 0.5–50 pg/well [3–15]. Assays in which the antibody is immobilized and an enzyme–steroid conjugate is used, appear to be slightly more sensitive: reported lower limits of detection are in the range 0.2–25 pg [16, 17]. On the other hand, problems of interference can arise in the latter assays if the enzyme and the biological sample are present simultaneously in the well [13–18]. Sequential additions of the sample and the enzyme-labeled steroid, separated by a washing step, are needed to exclude the possibility of such interference, as described for a chemiluminescence immunoassay [19], but then the assay ceases to be competitive.

The accuracy of this assay in terms of its correlation with the RIA was good ($r = 0.98$) but there was a slight estradiol overestimation (6.3 pg/well). This could not be directly ascribed to antibody specificity since the same antibody was used for the RIA. The precision (inter-assay coefficient of variation) were of the same order as those of other steroid enzyme immunoassays [13–20] and consistent with clinical needs. The assay required 4.5 h for completion (excluding plasma extraction). This time could be shortened by incubation at 37°C [19–21].

The study of antibody and coated plate storage showed that the antibody was not affected by lyophilization and thus could be stored this way over long periods. For the coated plates, the form of the standard curve calculated from means of six replicate results across a stored plate was not a sensitive indicator of the resistance of coating to storage since it did not change significantly with time. Increases in the coefficients of variation of the replicates with time indicated that the coating in some wells deteriorated before that in others. This measure therefore appears to be a better indicator of coating stability. Coated plates, before or after site blocking with casein, could be stored dry at 4°C less than 3 months for plates saturated, and for up to 3 months for plates not saturated, before a significant proportion of wells started to give aberrant results. Other authors have reported stability of coated plates with immunosorbent for up to 1 month (with or without buffer) at 4°C and less than 1 month dry at room temperature [8]. The ability to coat many plates and then store them until required may be found time- and labour-saving compared with coating each plate immediately prior to its use.

In conclusion, this assay seems to fulfil the requirements for clinical assay of plasma estradiol, uses stable reagents (antibody, coated plates) and minimizes reagent consumption.

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