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## Measurement of steroids by enzymeimmunoassay: An environmentally friendly alternative to radioimmunoassay

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### ABSTRACT

Enzymeimmunoassays (EIAs) for oestradiol-17 $\beta$  and progesterone were developed using antibody-coated microtitre plates, and steroid-horseradish peroxidase conjugates as steroid 'tracers'. The characteristics of standard curves and the precision of measurements were comparable between the EIAs and corresponding radioimmunoassays (RIAs). The applicability of the EIAs was demonstrated by using them to measure oestradiol-17 $\beta$  and progesterone concentrations in media sampled daily from cultures of bovine granulosa cells. The EIAs detected the characteristic switch in cellular steroidogenic capacity from oestradiol-17 $\beta$  to progesterone production typical of cultured granulosa cells. There was a significant linear correlation ( $P < 0.001$ ) between the EIAs and RIAs in the concentrations of steroids measured in granulosa cell culture medium by the two immunoassay formats. These findings may encourage other laboratories to consider converting "in-house" RIAs to EIAs, thereby eliminating the potential health and environmental hazards associated with the radioisotopes required for RIA.

**Keywords:** enzymeimmunoassay; radioimmunoassay; oestradiol-17 $\beta$ ; progesterone; granulosa cells.

### INTRODUCTION

Measurement of sex steroids plays an important role in investigations of reproductive physiology/endocrinology in many species. Radioimmunoassay (RIA) is generally the method of choice for measuring the low concentrations of steroids normally present in biological fluids and tissue extracts. However, RIA methodology has several disadvantages; notably its dependence on radioactive materials, which can pose potential health and environmental hazards; and the need for a specialized laboratory with suitable handling and disposal facilities. In addition, the equipment required to quantitate radioactivity at the assay end-point is sophisticated and expensive. Non-radioactive alternatives to RIA are therefore sought after. Enzymeimmunoassay (EIA) is one alternative technique in which radioisotopes are replaced by an enzyme label. Enzymeimmunoassay has several advantages over RIA including the use of non-radioactive, comparatively inexpensive reagents and a colour change assay end-point which can be easily measured by relatively simple equipment. Enzymeimmunoassays may also be readily automated, and generally assay costs per sample are low relative to RIA. Research laboratories, however, have in general been relatively slow in applying the technique of EIA to steroid measurements. The purpose of this study is to show that conversion of steroid RIAs to EIAs incorporating microtitre plates is straightforward, and can be achieved without loss of assay sensitivity or accuracy. This is demonstrated for progesterone using a monoclonal antibody, and for oestradiol-17 $\beta$  using a polyclonal antibody. Measurement of oestradiol-17 $\beta$  and progesterone concentrations in granulosa cell culture media was undertaken to demonstrate the applicability of the steroid EIAs.

### MATERIALS AND METHODS

#### Antisera

The progesterone antibody was a murine monoclonal antibody generated against 11 $\alpha$ -hydroxy progesterone hemisuccinate conjugated to bovine serum albumin (BSA). The hybridoma secreting antibody to progesterone was grown up in tissue culture, and the antibody purified from the spent culture medium by precipitation with ammonium sulphate (45%) followed by freeze-drying. Other steroids showing significant cross-reaction with the progesterone antibody were 11 $\alpha$ -hydroxy progesterone, 42%; 11 $\beta$ -hydroxy progesterone, 10%; 17 $\alpha$ -hydroxy progesterone, 8% and 5 $\alpha$ -pregnane-3,20-dione, 1%. The oestradiol-17 $\beta$  antibody was a polyclonal antibody generated in a rabbit against 17 $\beta$ -oestradiol 6-(*O*-carboxymethyl) oxime conjugated to BSA. Diluted, unfractionated serum with a high oestradiol-17 $\beta$  antibody titre was used in the immunoassays. Other steroids showing  $>0.1\%$  cross-reactivity with the oestradiol-17 $\beta$  antibody were 16-epioestriol, 1%; oestriol, 0.6% and oestradiol-17 $\alpha$ , 0.4%.

#### Radioimmunoassays

The progesterone and oestradiol-17 $\beta$  RIAs were performed using standard methodology. Samples, steroid standards, antibodies and radioactive steroid tracers were prepared in assay buffer (0.1M phosphate buffer containing 0.9% sodium chloride, 0.1% gelatin and 0.1% sodium azide, pH 7.0). Aliquots of 0.1 ml of sample/standard, 0.1 ml antibody (at a dilution which bound 50% of the added tracer) and 0.1 ml of tritiated steroid tracer ( $\sim 10,000$  cpm of either [2, 4, 6, 7- $^3$ H] oestradiol-17 $\beta$  or [1 $\alpha$ , 2 $\alpha$  (n)- $^3$ H] progesterone) were pipetted into glass tubes and incubated together overnight at 4 $^{\circ}$ C. One ml of dextran-coated charcoal (0.075% charcoal, Norit A and 0.025% dextran T-70 in assay buffer) at 4 $^{\circ}$ C was added, and the tubes centrifuged for 10 min at 3000 g, after 15 min

incubation at 4°C. The supernatant fraction containing antibody bound counts was decanted into scintillation vials and scintillation fluid added. The vials were counted in a liquid scintillation spectrometer. Standard curves were generated by plotting percentage 'bound' versus the logarithm of the progesterone or oestradiol-17 $\beta$  standard concentration, where 'bound' is defined as the mean cpm associated with a steroid standard/mean cpm associated with the zero steroid standard. Steroid concentrations of samples were calculated by interpolation from the standard curves.

### Enzymeimmunoassays

Steroid-enzyme tracers were prepared by conjugating 17 $\beta$ -oestradiol 6-(0-carboxymethyl) oxime (E) or progesterone 3-(0-carboxymethyl) oxime (P) to horseradish peroxidase (HRP) using an active ester method (Henderson *et al.*, 1994). The progesterone EIA was performed as follows. Wells of microtitre plates (Maxisorp C12, Nunc, Kamstrup, Denmark) were coated by overnight incubation at 4°C with 0.1 ml of progesterone antibody, appropriately diluted in coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6). The wells were emptied by inversion, and remaining active sites saturated by incubation for 30 min. at room temperature with 0.25 ml blocking buffer (coating buffer containing 0.5% BSA). Wells were emptied and washed 3x with washing buffer (10 mM phosphate buffer containing 0.8% NaCl and 0.05% Tween 20, pH 7.4). Replicate aliquots of progesterone standard, or sample diluted in assay buffer (washing buffer containing 0.1% BSA and 0.01% thimerosal, pH 7.4) were added to the wells followed by P-HRP enzyme-tracer to a total volume of 0.2 ml. (A dilution of P-HRP in assay buffer was selected so that the assay end-point absorbance reading of the zero standard was between 0.5 and 1.0). The plates were incubated for 2 h at 37°C to equilibrate. The plates were again emptied and washed 3x with washing buffer. Finally, the peroxidase activity in each well was quantitated by the addition of 0.1 ml/well of substrate-chromogen solution (50 ml of 0.05M phosphate-citrate buffer, pH 5.0, containing 20 mg o-phenylenediamine and 0.02 ml 30% H<sub>2</sub>O<sub>2</sub>). The plates were incubated for 15 to 30 mins at 37°C before the enzymic reaction was terminated by adding 0.05 ml/well of 2M H<sub>2</sub>SO<sub>4</sub>. Colour formation was quantitated by reading the absorbance of each well at 490 nm using a Bio-Tek EL311 microplate autoreader. Standard curves were generated by plotting percentage 'bound' versus the logarithm of the progesterone standard concentration where 'bound' is defined as the mean absorbance reading of the progesterone standard/mean absorbance reading of the zero progesterone standard. The progesterone concentration of samples was then calculated by interpolation.

The oestradiol-17 $\beta$  EIA was performed in a similar manner except for the following modifications. Gelatin replaced BSA in the blocking and assay buffers. Instead of coating wells directly with oestradiol-17 $\beta$  antibody, the wells were coated by overnight incubation at 4°C with goat antibody to rabbit IgG (1  $\mu$ g/well). After blocking and washing, 0.1 ml of an appropriate dilution of the rabbit polyclonal oestradiol-17 $\beta$  antibody in assay buffer was added and the plates incubated for 2 h at 37°C. After emptying the wells and

washing, replicate aliquots of oestradiol-17 $\beta$  standard or sample were added to the wells followed by E-HRP enzyme-tracer to a total volume of 0.2 ml in assay buffer. The plates were incubated at 4°C for 24 h to equilibrate before being emptied and washed. Quantitation of peroxidase activity etc. was then performed as for the progesterone EIA.

### Granulosa cell cultures

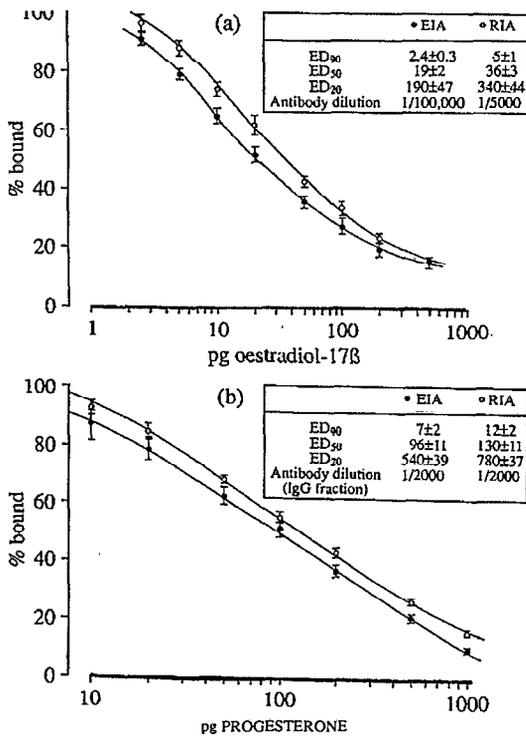
Granulosa cells obtained from cow ovarian follicles were cultured as described previously (Henderson *et al.*, 1987). Briefly, cow ovaries were obtained from a local abattoir and granulosa cells harvested and pooled from follicles  $\geq 10$  mm in diameter. The cells were washed, counted and resuspended at a concentration of  $0.5 \times 10^6$  cells/ml in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf-serum, glutamine (2 mM), nystatin (100 units/ml), gentamicin (100  $\mu$ g/ml) and testosterone (1  $\mu$ g/ml). Replicate 1 ml aliquots were pipetted into individual wells of multi-welled tissue culture dishes. The cells were cultured at 37°C, in a humidified atmosphere of 5% CO<sub>2</sub> in air, for 6 days with the medium being renewed daily. Spent medium was stored frozen at -20°C until assayed for oestradiol-17 $\beta$  and progesterone. Aliquots of culture medium were diluted with appropriate assay buffer and assayed directly in the EIAs and RIAs.

## RESULTS

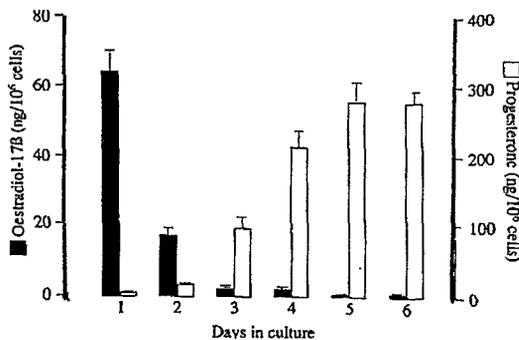
The oestradiol-17 $\beta$  and progesterone EIAs were both optimized with respect to primary antibody concentration and steroid-enzyme concentration using checkerboard titrations (Engvall, 1980). Standard curves were then generated, and these are shown in Fig. 1 together with standard curves generated by RIA. In both the oestradiol-17 $\beta$  and progesterone immunoassays there was little difference in the standard curve profiles between the EIA and RIA, though the ED<sub>90</sub>, ED<sub>50</sub> and ED<sub>20</sub> values were lower in the EIA. The oestradiol-17 $\beta$  antibody could be used at a 20-fold higher dilution in the EIA compared with the RIA. The precision of measurement was similar between the EIAs and RIAs. In the oestradiol-17 $\beta$  immunoassays the intra- and inter-assay coefficients of variation for the EIA were 6.6% and 11.8% respectively while the corresponding values for the RIA were 7.1% and 9.4% respectively. In the progesterone assays, the intra- and inter-assay coefficients of variation for the EIA were 4.5% and 8.7% respectively while the corresponding values for the RIA were 6.3% and 10.2% respectively.

The suitability of the EIAs for measuring steroid concentrations in biological fluid was examined by using the EIA to measure oestradiol-17 $\beta$  and progesterone concentrations in culture medium sampled daily from cultures of bovine granulosa cells. When cultured *in vitro*, granulosa cells undergo luteinization. This is characterized by a loss in their ability to metabolize androgen to oestradiol-17 $\beta$ , and an increase in their capacity to produce progesterone (Henderson *et al.*, 1987). This switch in steroidogenic capacity was detected by the EIAs when they were used to measure steroid production by cultured granulosa cells, as shown in Fig. 2. When samples of granulosa cell culture medium were assayed by both EIA and RIA, there was a highly

**FIGURE 1:** Standard curves for (a) oestradiol-17 $\beta$  and (b) progesterone generated by EIA (●) and RIA (○). Values are mean  $\pm$  s.e.m. of 6 separate curves.



**FIGURE 2:** Daily oestradiol-17 $\beta$  (■) and progesterone (□) production by bovine granulosa cells cultured for 6 days. Values are mean  $\pm$  s.e.m. for N=8. Steroid concentrations were measured by EIA.

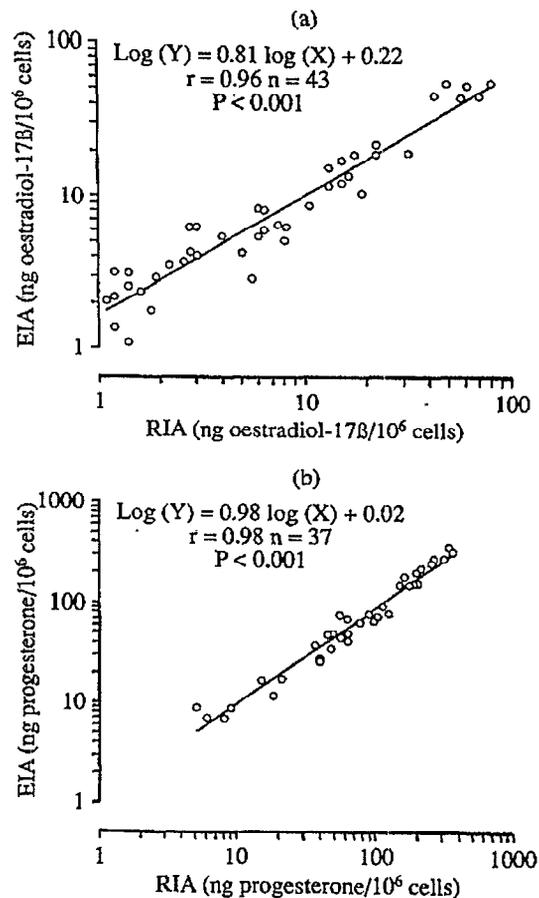


significant ( $P < 0.001$ ) linear correlation between the two immunoassay formats in the concentrations of steroids measured, as shown in Fig. 3.

**DISCUSSION**

This study demonstrates the use of EIA formats for measuring oestradiol-17 $\beta$  and progesterone, and shows that the EIAs are comparable to RIAs in terms of standard curve characteristics, assay precision and the concentrations of steroids measured in granulosa cell culture medium. The use of steroid-enzyme 'tracers' in steroid immunoassays instead of radioisotope 'tracers' eliminates the potential health and environmental hazards posed by the use of radioactive reagents. Enzyme immunoassays also have the advantages that the assay reagents are relatively inexpensive, the assay end-

**FIGURE 3:** Correlation between EIA and RIA in measuring (a) oestradiol-17 $\beta$  and (b) progesterone production by cultured bovine granulosa cells.



point is a colour change which can be measured by relatively simple equipment and there is potential for the assays to be automated. A crucial component of steroid EIAs is the appropriate steroid-enzyme 'tracers'. Like their radioactive counterparts for RIA usage, they can be purchased from a variety of commercial sources. However, they are also straightforward to synthesize "in-house", and a number of methodologies for coupling haptens to enzymes have been reported (Tijssen, 1985). In this laboratory, horseradish peroxidase (HRP) has been found to be the most suitable enzyme for use in steroid EIAs, and successful coupling of steroids to HRP is consistently achieved using an active ester methodology (Henderson *et al.*, 1994). Steroid-HRP conjugates are also very stable, and can be stored at 4°C or -20° without loss of activity for at least a year.

In antibody-coated EIA formats, the primary antibody can either be coated onto microtitre plate wells directly (as in the progesterone EIA) or indirectly by first coating an appropriate second antibody to capture the primary antibody (as in the oestradiol-17 $\beta$  EIA). Direct coating is practicable when there is ample amounts of purified antibody IgG available. If the amount of primary antibody available is limited, or it is only available in crude form, then indirect coating via an appropriate second antibody would be the method of choice. Capture second antibodies are readily available commercially.

In this study, the applicability of the steroid EIAs was demonstrated by using them to measure steroid concentrations in granulosa cell culture medium, to show the switch in steroidogenic

capacity which occurs when granulosa cells are cultured *in vitro* (Fig. 2). The EIAs would also likely be quite satisfactory for measuring steroid concentrations in other biological fluids such as blood, saliva, milk and tissue extracts. However, as with RIAs, preliminary extraction of some biological fluids to remove interfering substances, e.g. binding proteins, may be necessary before performing an EIA.

In conclusion, this study has demonstrated, using oestradiol-17 $\beta$  and progesterone assays as examples, that steroid RIAs can be readily converted to an EIA format without compromising the assays. It is hoped that other laboratories might be encouraged to consider converting "in-house" RIAs to EIAs, thereby eliminating the potential health and environmental hazards associated with the radioisotopes required for RIA.

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