Enzyme Linked Immunosorbant Assay for 4-pregnene-3, 20-dione Hormone Using Acetylcholinesterase Enzyme as Tracer

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Background: Enzyme Linked ImmunoSorbent Assay (ELISA) has been described as an alternative to radioimmunoassay for the mammalian and nonmammalian steroids detection. In this study, a simple and rapid ELISA is described and validated for 4-pregnen-3,20, dione (progesterone).

Materials and Methods: A general procedure for preparation of the acetylcholinesterase labelled steroid is described which is applicable to any steroid. Use of acetylcholinesterase tracer increased the sensitivity of assay so that reliable measurements of each steroid could be achieved with only 10 µl of plasma.

Results: Typical standard curves for progesterone steroids showed a workable range (detection limit) from 0.8 to 400 pg/well and the sensitivity of the assay taken as the concentration of steroid that induced 90% of B/B_0, was 1.5 pg. Inter-assay variations that gave approximately 50% displacement was 9.2% for 10 replicates and intra-assay co-efficient of variation was less than 10% over the central part of the standard curve between 3 and 200 pg/well. There was a strong positive correlation ($r>0.999$) between the amount of steroid added to plasma and the amount measured.

Conclusion: Method described here was applied to measure progesterone in plasma and this methodology could be of great interest to researchers measuring steroid hormones.

Key Words: Immunoassay, ELISA, Steroids

Introduction

Radioimmunoassay has been used as a standard method for measurement of steroids and other hormones in plasma for over 20 years (Idler 1972; Kime 1993), However, it has a number of disadvantages that restrict its application, such as disposal of the radiolabeled tracer which is an increasing problem in many countries where it is restricted to sites approved by national regulatory authorities. Radioimmunoassay requires expensive and sophisticated equipment for measurement. In addition, radioactive counting of large numbers of samples is time-consuming and requires large amounts of scintillation fluid. Finally, the availability of the radiolabeled steroids from commercial sources is very limited and severely restricts the range of steroids that can be measured, especially in non-mammalian vertebrates in which the reproductive hormones differ from those in mammals (Kime 1993; Cuisset, *et al*. 1994; Mayer *et al*. 2002; Weltzien *et al*. 2002).

During the last 15 years, there have been a number of publications in which ELISA have been described as an alternative to radioimmunoassay for the mammalian and nonmammalian steroids detection (Kime and Hews 1982; Canario and Scott 1991; Mayer *et al*. 1998; Manire *et al*. 1999; Melamed *et al*. 2000; Rohr *et al*. 2001; Dunlap 2002; Pall *et al*. 2002; Yamada *et al*. 2002) In addition to interest in measurement of steroids in plasma as an aid to clinical diagnostics, there is increasing interest in such measurements to monitor the effects of endocrine disrupting chemicals on reproductive function (MacLatchy and Van Der Kraak 1995; Olsen *et al*. 2001; Wainwright *et al*. 2001; Sepulveda *et al*. 2002; van den Heuvel and Ellis 2002; Wade *et al*. 2002). It has already highlighted the need for a simple, rapid and reliable method for the measurement of hormones as the basis for regulatory testing of the effects of potential endocrine disturbers on human and wildlife (Tattersfield *et al*., 1997).

Progesterone is one of the steroid hormones which is secreted by the corpus luteum after ovulation and by the placenta. It is responsible for preparing the body for pregnancy, and if pregnancy occurs, maintaining it until birth. Immediately after the LH surge (or hCG administration), serum

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progesterone levels rapidly increase within 30 min (Christenson and Devoto 2003).

Horseradish peroxidase (HRP) enzyme have already been used as a suitable label in previous studies by ELISA for steroid hormones measurement (Basu and Shrivastav 2000; Nishi *et al*. 2002). HRP has a major disadvantage that the enzymatic reaction must be irreversibly stopped before photometric measurement can be made (Pradelles *et al*. 1985; Cuisset *et al*. 1994). A major advantage of the use of acetylcholinesterase is that such an arrest is unnecessary and the sensitivity can be greatly increased by using even smaller concentrations of enzyme label and allowing the reaction to continue for several days until sufficient color has developed (Cuisset *et al*. 1994). The higher turnover rate of acetylcholinesterase compared to horseradish peroxidase enzyme label also gives a potentially more sensitive assay (Pradelles *et al*. 1985).

Cuisset *et al*. (1994) have described a simple and very sensitive ELISA method for the assay of 11-ketotestosterone using acetylcholinesterase as tracer, but the applicability was limited by use of electric eel as a source of acetylcholinesterase. In this communication, the applicability of the methodology to measure the specific progestogene is described along with general preparation procedure for the enzyme label which is applicable to any steroid hormone. The method was validated for progesterone measurement in research and clinical setting.

Materials and Methods

All steroids and chemicals were obtained from Sigma Chemical Company (Poole, UK) and antiserum to progesterone (against 3-CMO-BSA in sheep) was kindly donated by Dr. D. E. Kime (Sheffield U.K.). Ninety-six well polystyrene high-binding microtiter plates (Costar Catalogue No. 3590) were used for the ELISA. Progesterone-carboxymethyloxime (CMO) preparation was done as described by Cuisset, *et al*. (1994).

Acetylcholinesterase (AChE, 1 mg) in 500 μl 0.1M phosphate buffer pH 7.0 was treated overnight with 25 μl trypsin solution (25μg ml^{-1} in 0.1 M phosphate buffer, pH 7.0) at room temperature. The solution was dialysed against 100 ml 0.1M borate buffer pH 8.5 using an 80 mm length of dialysis tubing (Sigma D2272) for 24 h using several changes of borate buffer. The contents of the dialysis tubing were removed and the tubing was washed with clean borate buffer to give a final volume of G4-AChE solution 1 ml which could be stored at 4° for at least two weeks. The activity of enzyme checked by Ellmans reagent as will describe later.

Preparation of steroid-CMO-AChE conjugate
Four hundred nmol (174 μg) of the steroid-CMO in 38μl of freshly made N-hydroxysuccinimide solution (1 mg ml^{-1} in anhydrous dimethylformamide) was treated overnight with 32 μl freshly made N,N'-dicyclohexylcarbodiimide solution (2 mg ml^{-1} in anhydrous dimethylformamide) in the dark (Cuisset *et al.,* 1994). Progesterone was extracted from 50 μl rainbow trout plasma with 5 ml dichloromethane. The solvent was evaporated and the residue was redissolved in 500 μl ethanol. ELISA plates were coated with anti-rabbit because a competition method between progesterone labelled with ACHE enzyme and progesterone available in serum used here. Eight microliters of polyclonal anti-rabbit IgG (affinity purified, Sigma R2004; 1 mg ml^{-1}) was dissolved in 15 ml 0.05M potassium phosphate buffer pH 7.4 and, 150μl of this was added to each well of the plate and incubated overnight at room temperature in a humid container. The plate was blocked by adding 100 μl of blocking buffer (0.1M PPBS pH 7.4 containing 3% bovine serum albumin (BSA), 1 mM EDTA and 25 mM sodium azide) and incubating overnight at room temperature. Plates were sealed and stored at 4° in a humid container. Immediately before use, plates were washed 3 times with washing buffer (100 ml PBS and 2.5 ml Tween-20 in 5 l water).

Standard assay procedure
Serial dilutions of standard steroid (400 to 0.78 pg) in 100 μl assay buffer were made with B0 (0 pg) and non-specific bound (NSB) wells. Twenty-five microliters of the extracted steroids from the samples (5 μl incubation media equivalent) was pipetted into the remaining 6 rows in duplicate. Twenty-five microliters of the diluted steroid label was added to all wells, and 25 μl antiserum to all wells except for the NSB at the dilutions determined by the checkerboard titration. All wells were made up to 150 μl by steroid assay buffer; the plates sealed and incubated 2 h at room temperature in a humid chamber. Plates were then washed three times with wash buffer. Ellmans reagent was added 200 μl per well, the plates were incubated overnight in the dark at room temperature and then were read at 405 nm. Picograms per well were calculated for the samples from the standard curve using Stingray software (Dazdaq, Ringmer, UK).

Assay validation
A pool plasma (500μl) was treated with activated charcoal as described by Cuisset *et al*. (1994) and extracted twice with 5 ml dichloromethane. The combined extracts were evaporated and reconstituted in 3 ml assay buffer. One hundred microliter aliquots were pipetted in duplicate into wells on two rows of a microtiter plate. Progesterone was added to one pair of wells to give a concentration of 400 pg/well, serial dilutions (x2) performed and the samples assayed according to standard procedure. The same procedure was used to assay progesterone measurement in plasma.
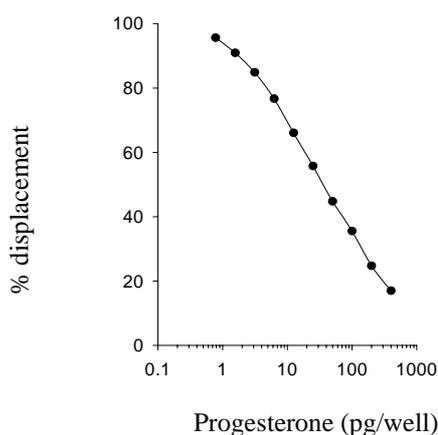
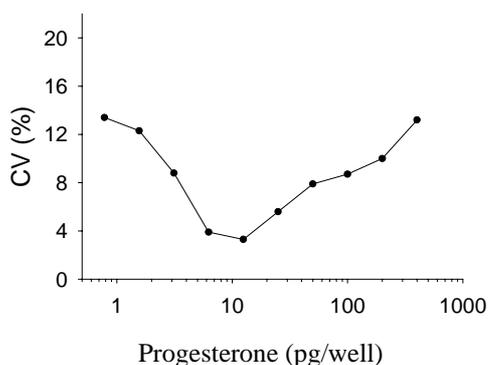**Figure 1**. Typical standard curves for ELISA of progesterone. Values are means of duplicate assays.

Figure 2. Intra-assay coefficient of variance (CV) for ELISA of progesterone determined from a standard curve with 10 replicates at each concentration. The co-efficient of variation was less than 10% over the central part of the standard curve between 3 and 200 pg/well.

Figure 3. Accuracy test for progesterone assay. A known amount of the steroid was added to an aliquot of a steroid free extract of pooled plasma and 2 x serial dilutions made with the extract. Assays were performed in duplicate. There was a strong positive correlation (r>0.999) between the amount of steroid added to plasma and the amount measured.

Results

Cross-reactivity of antiserum

The antiserum to progesterone cross-reacted with 17P (1.9%), 17,20β-dihydroxy-4-pregnen-3-one (4.3%), 5α-androstane-3α,17β-diol (3.7%), 11β-hydroxy-testosterone (0.33%), 5α-androstane-3β,17β-diol (0.27%), 5β-androstane-3α,17β-diol (0.25%), 11- ketotestosterone (0.85%), estradiol (0.54%), 4-androstenedione (0.47%), 4-androstenetrione (0.31%) and 17,20α-dihydroxy-4-pregnen-3-one(4.1%) at the 50% displacement level.

Standard curves and assay sensitivity

Typical standard curves for progesterone steroids showed a workable range (detection limit) from 0.8 to 400 pg/well (Fig. 1). The sensitivity of the assay,
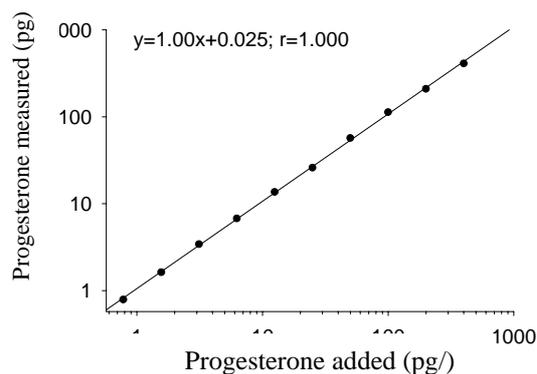
taken as the concentration of steroid that induced 90% of B/B_0, was 1.5 pg. Inter-assay variations measured on female carp plasma samples, that gave approximately 50% displacement, was 9.2% for 10 replicates. Intra-assay variations were determined from standard curves in which 10 replicates of each concentration was used (Fig. 2). The co-efficient of variation was less than 10% over the central part of the standard curve between 3 and 200 pg/well. There was a strong positive correlation (r>0.999) between the amount of steroid added to plasma and the amount measured (Fig. 3).

Discussion

The procedure described by Cuisset *et al.* (1994) for validation of the 11-ketotestosterone ELISA using the same enzyme label was closely followed. Sensitivity of the assays, which has been taken as 90% of the binding with 0 pg is just over 1 pg for progesterone. Using identical conditions for label preparation and assay, a sensitivity of 0.39 pg was obtained for hormone comparable to that reported by Cuisset *et al.,* (1994). As with radioimmunoassay, the sensitivity is a function of both the specific activity of the label and the quality of the antiserum, and will therefore vary with the material available to the user. Therefore in this study, I used the antiserum that was available but have little reason to expect that any antiserum which gives good results with radioimmunoassay will not give similar results to those that was describe for ELISA.

Although, different enzymes such as penicillinase (Pandey *et al*. 1990; Tomita *et al*. 1992; Malakaneh *et al*. 2001; Kumari and Dhir 2003) and alkaline phosphatase (Chen Ren *et al*. 2002; Kumari and Dhir 2003) have already been used as label in hormone assay by ELISA methods, but horseradish peroxidase is more common (Basu and Shrivastav 2000; Kumari and Dhir 2003). This has a major disadvantage in that the enzymatic reaction must be irreversibly stopped before photometric measurement can be made (Pradelles *et al*. 1985; Cuisset *et al*. 1994). A major advantage of the use of acetylcholinesterase is that such an arrest is not necessary and the sensitivity can be greatly increased by using even smaller concentrations of enzyme label and allowing the reaction to continue for several days until sufficient color develops. Plates can, in fact, be read daily until it is judged that the absorption is sufficient (Cuisset *et al*. 1994). The higher turnover rate of acetylcholinesterase compared to other enzyme labels also gives a potentially more sensitive assay (Pradelles *et al*. 1985). In this work, a method that is sensitive for the rapid measurement of steroid hormones in mammals and nonmammals and also presents an example of such an application was described.

A major advantage of ELISA is the ability to minimise errors resulting from multiple pipetting by use of multichannel pipettes. Inter- and intra-assay coefficient of variations (CV) reported in radioimmunoassay are frequently 15-20%, even when measured at the steepest part of the curve. For the assays reported here, intra-assay CVs around 5% was obtained in the steep part of the curve, rising to 15-20% at the flatter end regions (Fig. 2), while interassay variation (at 50% displacement) was around 8%, comparable to that reported by Cuisset *et al*. (1994) for 11-ketotestosterone. There was a highly significant correlation between the steroid added to and that measured in stripped plasma (Fig. 3). A further advantage of ELISA is that sample handling can also be made more rapid and reproducible by the use of low binding microtiter plates for sample storage and dispensing with multichannel pipettes.

A major problem with the assay of steroids in plasma is the availability of tracer (Kime 1993). Progesterone must be prepared chemically from other materials and must then be purified chromatographically. Such conversion of radioactive materials is beyond the expertise of many laboratories and has severely restricted measurement of these hormones in plasma. Availability of commercial enzymatically labelled steroids, including acetylcholinesterase labels, is similarly restricted to those of mammalian steroids that are routinely assayed in clinical labs. Cuisset *et al*. (1994) prepared the acetylcholinesterase from electric eel, but in this study, a simple procedure for making enzyme label from commercially available acetylcholinesterase is described which requires no facility more complicated than a simple

chromatographic column. Using such a procedure, acetylcholinesterase labelled progesterone was made which has similar sensitivity to that was described previously (Cuisset *et al*. 1994).

A major advantage of ELISA is that, since the steroid is coupled to the enzyme by exactly the same reaction that link to the bovine serum albumin to stimulate the immunological reaction, it is possible to make an enzyme label for any steroid to raise an antiserum. In addition to the steroid described in this study, acetylcholinesterase label and obtained standard curves of similar sensitivity estrodial, testosterone, 11-ketotestosterone, 17,20α-dihydroxy-4-pregnen-3-one, 11-deoxycortisol, 17,20β-dihydroxy-4-pregnen-3-one, 17,20β,21-trihydroxy-4-pregnen-3-one (20βS), cortisol and 17α-hydroxyprogesterone have been prepared in our lab which demonstrates the widespread applicability of the methodology. This greatly expands the numbers of steroids which can be possible measured by ELISA, since only the labels for testosterone and estradiol are available commercially. This flexibility is particularly important in animals in which a large number of steroids are produced by gonadal tissue (Kime 1993). These steroids either cannot be measured by radioimmunoassay due to unavailability of radiolabels or can be measured by a prohibitively expensive custom preparation involving very high levels of radioactivity.

ELISA may therefore play a major part in clarifying the role, for example, of progestogen, 20α-, 20β-, 5α- and 5β-reduced steroids. As with radioimmunoassay, it is essential that ELISA be fully validated for each new species used. This is especially true for a new method, since it is not known whether other chemicals extracted from plasma may interfere with binding or enzymatic processes. The present validation suggests that there are no such problems in this assay.

In this communication, the presented data confirmed higher sensitivity of ELISA using acetylcholinesterase as enzyme label compared with radioimmunoassay. It has been also shown that this method can be readily used for measurement of other steroids simply by using a small volume of plasma, especially when steroid levels in plasma are low (Cuisset *et al*. 1994). The rapid determination of several steroids from the very small volumes of blood allows the examination of profiles of steroids at even higher sampling frequencies or over longer periods. This methodology will therefore be of great interest to workers examining the temporal endocrine mechanisms involved in photoperiodic, circadian and other chronobiological processes.

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