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17α-hydroxy-4-pregenen-3-one (17αP) assay, using acetylcholinesterase enzyme as tracer

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Abstracts: 17α-hydroxy-4-pregnen-3-one (17αP) hormone is a precursor of other steroid hormones and radioimmunoassay has already been used to measure it, but a simple and rapid "Enzyme Linked Immunosorbant Assay" (ELISA) is described and validated here. A general procedure for prepara-tion 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 10μl of plasma. The ELISA was applied to measure 17, 20α-hydroxy-4-pregnen-3-one (17, 20αP) steroid production from 17αP by 20α-hydroxy steroid dehydrogenase (20α-HSD) from sperm of roach (*Rutilus rutilus*). The results showed that cyprinid sperm contains potent and active 20α-HSD enzymes which produce 17, 20αP hormone from 17αP substrate.

Keywords: 17, α-hydroxy-4-pregnen-3-one assay, ELISA, Steroids, Acetylcholinestrase, roach

Introduction

During the last 15 years there has been a number of publications in which "Enzyme Linked ImmunoSorbent Assay" (ELISA) using microtiter plates have been described as an alternative to radioimmunoassay, for mammalian and nonmammalian steroids (Yamada *et al.*, 2002 ; Pall *et al.*, 2002 ; Dunlap, 2002 ; Rohr *et al.*, 2001 ; Melamed *et al.*, 2000 ; Manire *et al.*, 1999 ; Canario & Scott, 1991). In addition to interest in measurement of steroids in fish plasma, as an aid to aquaculture, there is increasing interest in such measurements to monitor the

effects of endocrine disrupting chemicals on their reproductive function (Wade *et al.*, 2002 ; Van Den Heuvel & Ellis, 2002 ; Wainwright *et al.*, 2001 ; Sepulveda *et al.*, 2002 ; Olsen *et al.*, 2001). A report (Tattersfield *et al.*, 1997) has highlighted the need for a simple, rapid and reliable method for the measurement of hormones specific in fish and other non-mammalian vertebrates as a basis for regulatory testing of the effects of potential endocrine disrupters on wildlife.

Recent studies have shown that in addition to the gonads, sperm itself is also capable of producing 20α or 20β reduced progestogen metabolites (Sakai *et al.*, 1989 ; Barry *et al.*, 1990 ; Koldras *et al.*, 1990 ; Koldras *et al.*, 1996). The role(s) of the 20-reductase enzymes in sperm and the function(s) of the steroids produced are still not clear (Kime, 1993). We have already shown that in *invitro* ovarian incubations of cyprinid at low substrate concentrations conjugating, 5α-reducing and 7α-hydroxylating enzymes, of high activity but low capacity, acted as scavengers to deactivate any steroids formed during the relatively low pituitary gonadotrophin secretions, which are necessary for oocyte development, but that during the prespawning gonadotrophin surge when high levels of 17αP substrate were present these enzymes are saturated and 17,20αP became the major ovarian steroid in common carp (Kime & Ebrahimi, 1997; Ebrahimi *et al.*, 1996 ; Ebrahimi *et al.*, 1995). The same shift from 11-oxygenated androgens and conjugates towards 17,20αP at high substrate concentration also have shown (Abdullah & Kime, 1994 ; Kime & Abdullah, 1994 ; Kime *et al.*, 1994).

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 (*Electrophorus electricus*) as a source of acetylcholinesterase. In this communication we extend the applicability of the methodology to the specific fish progestogen, 17αP and describe a general preparation procedure for the enzyme label which is applicable to any steroid. The method was applied to the determination of 20αHSD enzyme activity in roach (*Rutilus rutilus*) sperm.

Materials and Methods

Chemicals and equipment

Acetylthiocholine, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), acetylcholinesterase (Sigma C2888, 1000-2000 units mg^{-1}), trypsin and all steroids were obtained from Sigma (Poole, UK). The antiserum to 17,20βP (against 3-CMO-BSA in sheep) was kindly donated by Dr. D. E. Kime (Sheffield). Cross reactivities of these antiserum is described in Scott *et al.* (1980,1983).

Ninety-six well polystyrene high-binding microtiter plates (Costar Catalogue No. 3590) were used for the ELISA. Plates were sealed for storage with Anachem Sealplate sealing film. Plates were processed using a Chelsea Instruments shaker, an Anthos Model AW-1 plate washer and read with an Anthos Model HTII Plate Reader. 96-well low binding microtiter plates (Costar Cat. No. 2501) were used for sample dilution and preparation of standard curves (Cuisset *et al.*, 1994).

Preparation of steroid-carboxymethyloxime (CMO)

The preparation of the 3-CMO of 17,20αP was based on the method of Simpson and Wright, (1977). 17,20αP (1.1mg) and carboxymethoxylamine hemihydrochloride (1 mg; Aldrich) were incubated with sodium acetate (3mg) in 100μl methanol at 4°C overnight. Two hundred and fifty microliters of deionised water, acidified to pH2 with glacial acetic acid, was added, mixed well and left for 10 min to allow the steroid-CMO to precipitate out. The oxime was extracted with 3ml ethyl acetate, and the organic layer evaporated to give 0.5-1mg of the steroid-CMO. Completion of reaction was confirmed by the presence of a single UV absorbing area at the origin of thin-layer chromatography plates (Merck Kieselgel GF_{254}) developed in chloroform-methanol (95:5).

Generation of G4-acetylcholinesterase

Acetylcholinesterase (AChE, 1mg) in 500μl 0.1M phosphate buffer pH 7.0 was treated overnight with 25μl trypsin solution (25μg ml^{-1} in 0.1 M phosphate buffer, pH 7.0) at room temperature. The solution was dialysed against 100ml 0.1M borate buffer pH 8.5 using an 80mm length of dialysis tubing (Sigma D2272) for 24h using several changes of borate buffer. The contents of the dialysis tubing were removed and the tubing washed with clean borate buffer to give a final volume of

G4-AChE solution of 1 ml which could be stored at 4° for at least two weeks (Cuisset *et al.*, 1994).

Steroid-CMO-AChE conjugate was prepared as previously described (Cuisset *et al.*, 1994).

Milt preparation and incubation

Milt of six mature male roach (*Rutilus rutilus*) was hand stripped by gentle abdominal pressure. Twenty microliters of milt from each male fish (512±73g, mean ±SD) was incubated with gentle shaking for 3hr at 20°C in 2ml carp incubation medium (Jalabert *et al.*, 1973) containing 0, 0.1, 1 or 10μg/ml 17αP.

Steroid extraction

Steroids were extracted from incubation media with 2 X 5ml dichloromethane, the solvent evaporated and the residue redissolved in 500μl assay buffer. Twenty-five microliters of this (5μl incubation media equivalent) gave a satisfactory point on the standard curve for samples with steroid levels in the range of 0.5-10ng ml^{-1} plasma. Smaller volumes of buffer were added or serial dilutions made on the plate if lower or higher steroid levels were expected. If necessary the plasma volume was increased to 250 μl (Ebrahimi *et al.*, 1996 ; Ebrahimi *et al.*, 1995).

Plate coating

ELISA plates were coated with previously prepared anti-rabbit (Kime &Dolben, 1985). Eight microliters of polyclonal anti-rabbit IgG (affinity purified, Sigma R2004; 1mg ml^{-1}) was dissolved in 15 ml 0.05M potassium phosphate buffer pH 7.4, 150μl added to each well of the plate and incubated overnight at room temperature in a humid container. The plate was blocked by addition of a further 100μl of blocking buffer (0.1M PPBS pH 7.4 containing 3% bovine serum albumin (BSA), 1 milimolar (mM) EDTA and 25mM sodium azide) and incubation overnight at room temperature. Plates were sealed and stored at 4°C in a humid container. Plates were washed 3 times with wash buffer (100 ml PPBS and 2.5 ml Tween-20 in 5 l water) immediately before use.

Checkerboard titration

A checkerboard titration was carried out for antisera (anti-17αP and anti-17,20αP) and after each preparation of new AChE label. Serial dilutions of the

steroid antisera (from 1:1000 dilution; plate columns 1-10) were made against serial dilutions of AChE label (from 1:4; plate rows A-H) in a microtiter plate. Plates were processed as in the Standard Assay Procedure and plots made of AChE dilution against absorption for antiserum dilution to determine optimum dilutions of the two components (Diamandis & Christopoulos, 1996). For the tracers and 17αP antiserum described here, this was 1:20 for AChE tracer and 1:20,000 for antiserum to 17αP.

Standard assay procedure

Serial dilutions of standard steroid (400 to 0.78pg) in 100 μl assay buffer were made in the first 10 wells of rows A and B of the coated plate. The remaining 2 wells in these rows were reserved for B_0 (0pg) and non-specific bound (NSB). Twenty-five microliters of the extracted steroids from the samples (5μl incubation media equivalent) was pipetted into the remaining six rows in duplicate. Twenty-five microliters of the diluted steroid label was added to all wells, and 25μl antiserum to all wells except for the NSB at the dilutions determined by the checkerboard titration. All wells were made up to 150μl in steroid assay buffer; the plates sealed and incubated for 2h at room temperature in a humid chamber. Plates were then washed three times with wash buffer. Two hundreds microliter per well of Ellmans reagent was added, the plates incubated overnight in the dark at room temperature and read at 405 nm. Picograms per well were calculated for the samples from the standard curve using Stingray software (Dazdaq, Ringmer, UK). The same procedure was used to measure 17,20αP product.

Assay validation

A pool of female common carp (*Cyprinus carpio*) plasma (500μl) was treated with activated charcoal as described by Cuisset *et al.* (1994) and extracted twice with 5ml dichloromethane. The combined extracts were evaporated and re-constituted in 3 ml assay buffer. One hundred microliter aliquots were pipetted in duplicate into wells on two rows a microtiter plate. 17αP 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 17,20αP production by roach sperm and SPSS 6 for Windows software (SPSS Inc., 444 N. Michigan Avenue, Chicago, Illinois 60611, USA) was used for the statistical analysis of 20αHSD activity in milt of control fish (0μg/ml 17P) with other groups.

Results

Cross-reactivity of antiserum

The antiserum to 17αP cross-reacted with 17,20αP (5%), progesterone (21%), 5α-androstane-3α,17β-diol(3.7%),11β-hydroxy-testosterone(3.3%),5α-androstane-3β, 17β-diol (2.7%), 5β-androstane-3α,17β-diol (2.5%), 11-ketotestosterone (0.85%), estradiol (0.54%), 4-androstenedione (0.47%), 4-androstenetrione (0.31%) and 17,20βP (11%) at the 50% displacement level.

Standard curves and assay sensitivity

Typical standard curves for 17αP steroids showed a workable range from 0.8 to 400 pg/well (Fig. 1). The sensitivities of the assay, taken as the concentration of steroid that induced 90% of B/B_0, was 1.5pg.

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 eight replicates of each concentration were 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.

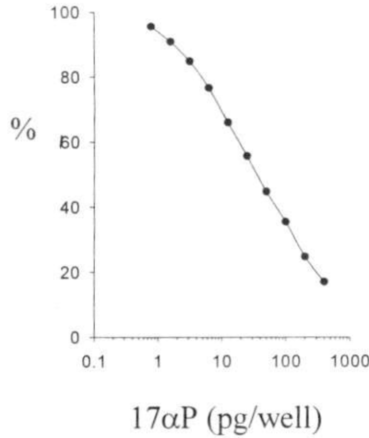

Figure 1. Typical standard curves for ELISA of 17αP. Values are means of duplicate assays.

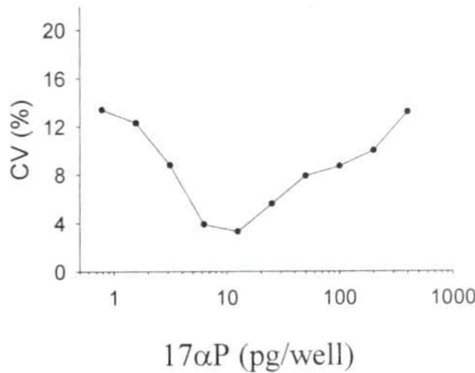

Figure 2: Intra-assay coefficient of variance (CV) for ELISA of 17αP determined from a standard curve with 10 replicates at each concentration.

Assay accuracy

There was a strong positive correlation (r>0.999) between the amount of steroid added to plasma and the amount found (Fig. 3). Application to measuring 17αP and its product17,20αP by roach sperm

17αP was measured in duplicate by ELISA using 10μl of incubation media equivalent. The amounts of 17,20αP detected by this method were 1, 4.2, 13.2 and 18.66μg/ml for 0, 0.1, 1 and 10μg/ml 17αP added to milt incubations respectively.

A clear shift in 17,20αP production was found with increase in substrate concentration in all milt incubations.

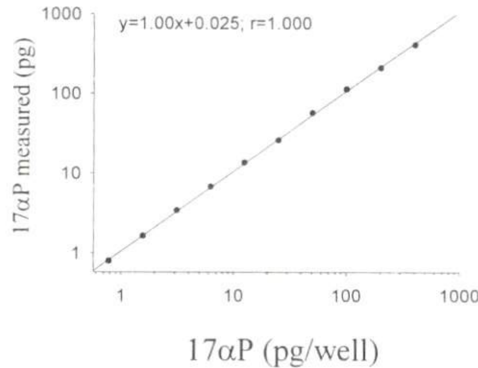

Figure 3: Assay accuracy for 17,2 0αP in carp plasma. A known amount of the steroid was added to an aliquot of a steroid free extract of pooled female carp plasma and 2 x serial dilutions made with the extract. Assays were performed in duplicate.

Discussion

Here 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 0pg is just over 1 pg for 17αP. Using identical conditions for label preparation and assay we also obtained a sensitivity of 0.39pg for hormone, comparable to that obtained 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. So in this study, we used the antiserum that were available to us but have little reason to expect that any antiserum which gives good results with RIA will not give similar results to those that we describe for ELISA.

Previous reports of ELISA for steroid hormones have generally used horseradish peroxidase as the enzyme label (Karawajew, *et al.*, 1988 ; Madersbacher, *et al.*, 1992 ; Bolton, *et al.*, 1999 ; Basu & Shrivastav, 2000 ; Nishi, *et al.*, 2002). This has

a major disadvantage in that the enzymatic reaction must be irreversibly stopped before photometric measurement can be made (Cuisset, *et al.*, 1994 ; Pradelles, *et al.*, 1985). 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. 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 paper a method that is sufficiently sensitive for the rapid measurement of steroid hormones in common fish species such as cyprinids has been described which also could be used to measure other steroid hormones.

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 RIA are frequently 15-20% even when measured at the steepest part of the curve. For the assays reported here we obtain intra-assay CVs around 5% in the steep part of the curve, rising to 15-20% at the flatter end regions, 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 carp and carp plasma. 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.

Although a major problem with the assay of steroids in plasma of non-mammalian vertebrates is the availability of tracer (Kime, 1993), but 17αP can be purchased from providers. Availability of commercial enzymatically labelled steroids, including acetylcholinesterase labels (Cayman Chemical Company, Ann Arbor), 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 communication we describe a simple procedure for making enzyme label from commercially available acetylcholinesterase which

requires no facility more complicated than a simple chromatographic column. Using such a procedure we have made acetylcholinesterase labelled 17αP 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 as that used to link it to the bovine serum albumin to stimulate the immunological reaction in the host animal, it is possible to make an enzyme label for any steroid for which it is possible to raise an antiserum. In addition to the steroid described in this paper, acetylcholinesterase label and obtained standard curves of similar sensitivity estradial, testosterone, 11-ketotestosterone, 17,20β-dihydroxy-4-pregnen-3-one (17,20βP), 11-deoxycortisol, 17,20β,21-trihydroxy-4-pregnen-3-one (20βS), cortisol and 17,20αP have been prepared previously which demonstrates the widespread applicability of the methodology. This greatly expands the numbers of steroids that are possible to measure by ELISA, since only the labels for testosterone and estradiol are available commercially. Such flexibility is particularly important in fish in which a large number of steroids are produced by gonadal tissue (Kime, 1993) which cannot be measured by radioimmunoassay due to lack of availability of radiolabel, except 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 progestogene, 20α-, 20β-, 5α- and 5β-reduced steroids in fish. As with radioimmunoassay it is essential that ELISA be fully validated for each new species treated. 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 salmonid and cyprinid fish.

To demonstrate an application of the ELISA method we have measured the steroid 17αP in roach milt incubations. A clear shift in enzyme activity was found when the concentration of substrate (17P) increased. The same shift has been also reported in milt (Kime & Ebrahimi, 1997), gonadal (Ebrahimi *et al.*, 1995 ; Kime & Ebrahimi, 1997 ; Kime & Abdullah, 1994 ; Kime *et al.*, 1994 ; Lee *et al.*, 1998 ;

Manning & Kime, 1984 ; Abdullah & Kime, 1994) and non-gonadal organs (Ebrahimi *et al.*, 1996).

In this communication, we present the data from a single fish species milt incubation as an illustration of the additional information (even for 17αP measurement in *in* vivo plasma) that can be obtained using the higher sensitivity of ELISA using acetylcholinesterase as enzyme label compared with radioimmuno-assay.

It has been also shown that this method can be readily used for measurement of other steroids by using a low sample volume from fish plasma (especially at this time of the year when steroid levels are naturally 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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