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Enzyme Immunoassay of Testosterone Using Nitrocellulose Discs as the Solid Phase

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Summary: An enzyme immunoassay (EIA) for the measurement of serum testosterone has been developed using nitrocellulose paper discs as the solid support. The paper discs (6 mm diameter) coated with testosterone-specific antibody were incubated with testosterone and testosterone-peroxidase conjugate in glass tubes. The amount of testosterone present in samples could be estimated from the bound peroxidase activity. The assay was validated by comparison with a microtitre plate-based ELISA and a commercial radioimmunoassay (RIA) kit. The correlation coefficient between RIA and EIA was 0.84. No significant cross reactivity was observed with other steroids, except for dihydrotestosterone. The inter- and intra-assay coefficients of variation were 4.4 and 9.6% respectively. The preservation and transport of the coated paper discs are convient and the overall cost of the method is less than that of other methods.

Introduction

Measurement of serum testosterone provides useful information of diagnostic significance in many physiological abnormalities, including hypogonadism, testicular feminization in man and virilizing disorders, hirsutism, *Stein-Leventhal* syndrome as well as ovarian and adrenal tumours in women. The first EIA for testosterone was reported by *Rajkowski* et al. who immobilized anti-testosterone antibody by copolymerization with human serum albumin or by conjugation to thyroglobulin immobilized on cellulose (1, 2). However, polystyrene tubes, beads and microtitre plates have now become standard solid phases for immobilization of antibodies and antigens in EIA (3–5), and some of these have also been used for testosterone assay (6–8). Although nitrocellulose paper has been extensively used for protein blotting, its use for immobilization of protein antigens and antibodies for EIA has been limited. A large number of nitrocellulose paper discs can be coated under uniform conditions by immersion in a protein solution, and preservation and transport of such coated discs are more convenient than microtitre plates and tubes. In this respect the use of nitrocellulose paper discs offers a definite advantage over many other solid phases.

In this report, we describe an enzyme immunoassay technique for serum testosterone using nitrocellulose discs as solid phase and horse radish peroxidase as the enzyme label. The sensitivity and the precision of the method are comparable with those using microtitre plates, and the method is suitable for the measurement of the hormone at physiological levels.

Materials and Methods

Bovine serum albumin (Cohn fraction V), horse radish peroxidase (Sigma Type VI, RZ 3.0), testosterone, 5α-dihydrotestosterone, androstenedione, dehydroepiandrosterone, 17β-oestradiol, oestriol, (aminooxy)acetic acid (carboxymethoxylamine hemihydrochloride), N-hydroxysuccinimide, dicyclohexylcarbodiimide, *o*-phenylenediamine and polyoxyethylene sorbitan monolaurate (Tween 20) were purchased from Sigma Chemical Co., USA. Nitrocellulose paper (Trans Blot) was from Bio-Rad Laboratories, California USA; flat bottomed microtitre plates were from Costar, Cambridge, Massachusetts, USA. The ra-

dioimmunoassay kit was obtained from Radioassay System Laboratories Inc., Carson, California, USA, and Sephadex G-25 and Sepharose 4B from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals and reagents used were of analytical grade.

Preparation of testosterone-3-(O-carboxymethyl)oxime

Testosterone-3-(O-carboxymethyl)oxime (9) was prepared according to the method of *Janoski* et al. (10). To 144 mg testosterone (0.5 mmol) dissolved in 2.5 ml methanol, pyrrolidine (80 µl) was added dropwise with constant stirring. A yellowish precipitate was formed and another 80 µl of the pyrrolidine was added to the mixture followed by 128 mg (1.17 mmol) of (aminooxy)acetic acid. The reaction mixture was placed in a water bath at 50 °C for 5 minutes. The precipitate dissolved and a clear solution was obtained. The methanol was removed by distillation and the residue extracted by treatment with 1 ml of 0.5 mol/l HCl and 5 ml of ethyl acetate. The ethyl acetate layer was washed with water, dried over sodium sulphate and evaporated. The residue was crystallized from benzene-petroleum ether mixture as colourless needles; yield 83%, mp. 174–176 °C (in l. c. (9): m. p. 179–181 °C). The compound was fully characterized from its NMR spectrum.

Preparation of immunogen

Testosterone-3-(O-carboxymethyl)oxime was conjugated to bovine serum albumin by the method of *Mattox* et al. (11). A solution of 3 mg of the oxime in 1 ml of dimethylformamide was treated with 5 mg of dicyclohexylcarbodiimide and 2 mg of N-hydroxysuccinimide overnight at 4 °C. The activated ester solution was allowed to react with 10 mg of bovine serum albumin in 1 ml sodium bicarbonate (0.13 mol/l) solution for 2 h. The reaction mixture was then dialysed against phosphate buffer, 25 mmol/l, pH 7.5, and purified by column chromatography on Sephadex G-25 (1.5 × 40 cm) using the same buffer as the mobile phase. Protein fractions were pooled, lyophilised and stored at −20 °C.

Immunisation procedure

Antibody was raised in New Zealand white rabbits following the same protocol as described earlier for oestradiol (12). Satisfactory antibody titres were obtained after 5 months. Blood was collected by cardiac puncture and the antibody was precipitated from the serum with ammonium sulphate (50% saturation). The precipitation step was repeated three times and the final precipitate was dissolved in 25 mmol/l sodium phosphate buffer (pH 6.8), dialysed against the same buffer containing sodium chloride (9 g/l) at 4 °C and then passed through a bovine serum albumin-Sepharose 4B immunoadsorbent column (1.3 × 4 cm for 20 ml serum) to remove anti-bovine serum albumin antibodies. The IgG solution was then stirred with activated charcoal (20 g/l) for 2 hours at room temperature to remove endogenous bound steroids, and the supernatant was stored at −20 °C in 1 ml aliquots.

Preparation of the enzyme conjugate

Horse radish peroxidase (5 mg) was conjugated with testosterone-3-(O-carboxymethyl)oxime (2 mg) by the activated ester method as described for bovine serum albumin. The enzyme conjugate was dialysed against phosphate buffer, 25 mmol/l, pH 7.5 and submitted to column chromatography on Sephadex G-25 (1.5 × 40 cm) using the same buffer. Working solutions of the enzyme conjugate were prepared by dilution with phosphate buffer, 50 mmol/l, pH 7.5 containing bovine serum albumin (1 g/l) and thiomersal (0.1 g/l).

The substrate for horse radish peroxidase assay was citrate-phosphate buffer (0.1 mol/l) pH 6.0, containing *o*-phenylenediamine hydrochloride (3.87 mmol/l) and H_2O_2 (0.2 mmol/l). The enzyme reaction was stopped by addition of 50 µl of 2 mol/l H_2SO_4, and the colour measured at 492 nm, either with a microplate reader (Bio-Rad) or a spectrophotometer.

Determination of steroid : protein ratio

The molar ratio of steroid to protein in the enzyme conjugate was determined from the ratio of absorbances at 255 and 403 nm of the conjugate, using a standard curve (fig. 1) prepared by plotting the A_{255}/A_{403} ratios against the steroid/protein ratios of solutions containing known amounts of the steroid derivative and the enzyme in different proportions. For the albumin conjugate the ratio A_{255}/A_{280} was used.

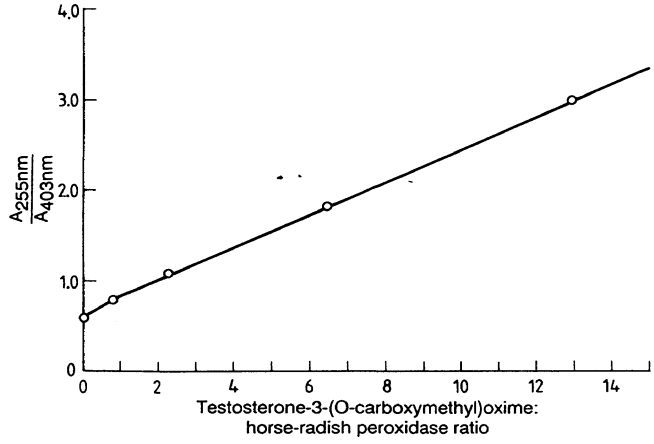

Fig. 1. Standard curve for determination of degree of conjugation in testosterone-horse radish peroxidase conjugate.

Preparation of coated plates

Microtitre plates were incubated with antibody (200 µl/well) diluted in phosphate buffer, 50 mmol/l, pH 7.5, at room temperature for 2 h and then at 4 °C overnight. The plate was washed three times with wash buffer and the bovine serum albumin solution (200 µl/well) was added and incubated at room temperature for 1 h. The plate was then dried by inversion on a blotting paper. The sealed plates could be preserved at 4 °C up to six months without significant loss of immunoreactivity.

Preparation of coated nitrocellulose discs

Round discs (6 mm diameter) of nitrocellulose paper were cut by a hand paper puncher. The discs were gently shaken for 3 h at room temperature in a petri dish containing suitably diluted testosterone antibody. Then the papers were washed twice with wash buffer and incubated with the blocking solution for 1 h at room temperature with gentle shaking. After washing the paper discs three times with the wash buffer, they were stored moist at 4 °C in a sealed glass container.

Extraction of testosterone from serum

To 100 µl of serum in a 12 mm × 75 mm glass test tube was added an equal amount of distilled methanol followed by 1 ml of distilled ethyl acetate, and the mixture was vortexed and centrifuged at 300 min^{-1} for 5 minutes. The clear supernatant was decanted into a 10 ml conical flask. To the residue, 0.5 ml of ethyl acetate was added and centrifuged again and the

supernatant was collected in the same flask. The combined ethyl acetate extract was evaporated on a steam bath. The dried material was dissolved in 400 µl of bovine serum albumin solution and this solution was used for assay.

Wash buffer

Sodium potassium phosphate buffer, 58 mol/l, pH 7.2, containing Tween-20 (0.5 ml/l).

Bovine serum albumin solution

Phosphate-buffered saline containing 1 g/l bovine serum albumin and 0.1 g/l thiomersal. This solution was used for blocking nitrocellulose papers and microtitre plates, and also for the preparation of testosterone standards and samples.

Testosterone standards

A stock solution of testosterone (1 g/l) was prepared in 90% ethanol. Working standards were prepared by dilution with the bovine serum albumin solution.

Radioimmunoassay

Radioimmunoassay of testosterone in serum sample was carried out by the direct method without extraction, using a ^{125}I-labelled testosterone kit according to the manufacturer's protocol. Briefly, to 50 µl of the serum or standard were sequentially added, 0.1 ml of steroid binding globulin inhibitor solution, 0.5 ml of [^{125}I]testosterone and 0.5 ml of antitestosterone antibody solutions. The samples were vortexed and incubated at 37 °C for 120 minutes. After addition of 0.1 ml of second antibody, the mixture was incubated at 37 °C for a further 60 minutes. After centrifugation the bound radioactivity of the precipitates was counted.

Protocol for testosterone assay

1. To antibody-coated plate or discs add 100 µl of standard/sample and incubate for 2 h at 37 °C.
2. Add 50 µl of enzyme conjugate and incubate for 1 h at room temperature and wash three times.
3. Add substrate (200 µl/well or 1 ml/tube) and incubate for 45 min at room temperature.
4. Add 2 mol/l H_2SO_4, (50 µl/well or 500 µl/tube) and read absorbance at 492 nm.

Results and Discussion

Because of the low level of the hormone in females, any testosterone assay must be highly sensitive. As the hormone levels are different in male and female, the assay should also cover a wide range of hormone concentrations. The present investigation was undertaken to ascertain the suitability of nitro-cellulose paper as a solid phase for such a demanding assay.

Testosterone-3-(O-carboxymethyl)oxime was coupled to bovine serum albumin and horse radish peroxidase by the activated ester method and the steroid : protein ratios in the conjugates were found to be 22 : 1 and 5.5 : 1 respectively.

No significant difference was observed when phosphate buffer (50 mmol/l, pH 7.5) and carbonate buffer (50 mmol/l, pH 9.6) were used for coating either the microtitre plates or the nitrocellulose discs; phosphate buffer was used in the present work.

Sample preparation

Direct assay of testosterone in serum diluted with phosphate-buffered saline containing 1 g/l bovine serum albumin gave analytical values for the steroid that were erroneously low. This was not unexpected as testosterone is known to bind strongly to several plasma proteins (13, 14); addition of 8-anilino-1-naphthalene sulphonic acid (0.4 g/l) did not release the steroid from the binding proteins. The extraction procedure, however gave satisfactory results. The recovery of the steroids as determined by assay of the extracted steroid added to charcoal-stripped serum, and comparison with values obtained by dissolving the same amount of steroid in the assay buffer showed that the recovery was almost complete (87–94%).

Sensitivity

The standard curves obtained for both the solid phases, using the optimized antibody dilution of 1 : 4000 and the enzyme conjugate dilution 1 : 1000 are shown in figure 2. The two standard curves are almost identical. The lowest amount measurable is 5 pg per tube or well, corresponding to 0.2 µg/l of serum.

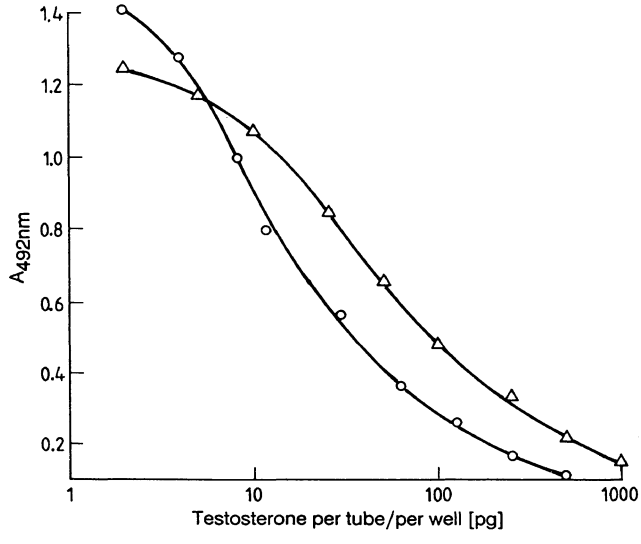

Fig. 2. Standard curves for measurement of testosterone using nitrocellulose paper discs (o–o) and microtitre plate (△–△).

Precision, reproducibility and validation of the assay

The inter- and intra-assay coefficients of variation were 4.4% (mean ± S.D.: 116.4 ± 5.2 pg/tube; n = 10) and 9.6% (mean ± S.D.: 118.0 ± 11.4 pg/tube; n = 7). The testosterone levels of 52 serum samples measured by the present method and by a commercial RIA kit showed good correlation (correlation coefficient 0.84), the regression equation being y = 0.96x + 0.24 where x = RIA value and y = EIA value (fig. 3).

Although the overall correlation of the EIA and RIA values is satisfactory, considerable discrepancies between the two methods were observed for a few samples, possibly due to handling errors during the sample preparation step for EIA.

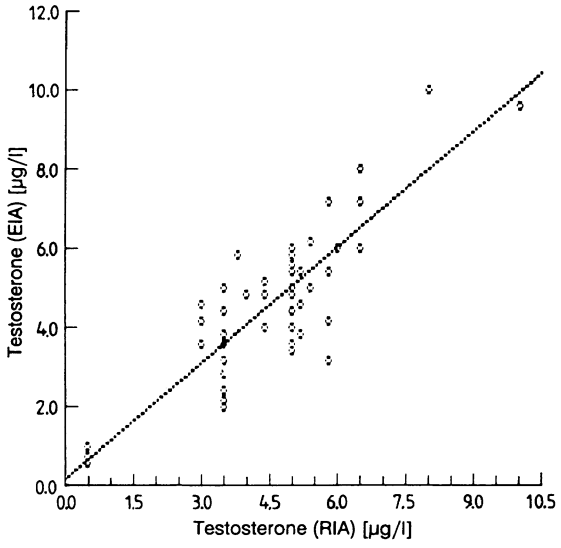

Fig. 3. Correlation of testosterone values found by RIA and by nitro-cellulose paper-based EIA in serum samples.

Cross-reactivity

The cross reactivity of the antibody towards other closely related steroids at 50% displacement level as determined by the microtitre plate method is shown in table 1. Only dihydrotestosterone showed significant cross reactivity. High cross reactivity of dihydrotestosterone towards testosterone antibody has also been reported by earlier workers (7, 8).

Tab. 1. The cross reactivity of the testosterone antibody with related steroids

Steroids	% Cross reactivity
1. Testosterone	100.00
2. 5α-Dihydrotestosterone	28.00
3. Androstenedione	0.03
4. Dehydroepiandrosterone	0.02
5. 17β-Oestradiol	0.05
6. Oestriol	0.01
7. Progesterone	0.01

Nitrocellulose paper discs have been used for the simultaneous detection in serum of IgE antibodies against several allergens using the dot method (15). It has been shown to be an excellent solid phase for radioimmunoassays, being able to bind five times more protein than standard microplate wells (16). It can be seen from data presented here that nitrocellulose paper is also a suitable solid phase for quantitative enzyme immunoassay. The coated discs showed no significant loss of immunoreactivity after storage at 4 °C for up to 6 months. Since coating, washing and preservation of such discs are easy, and an ELISA reader is not required, the method should find wider applicability.

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