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AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR PLASMA TESTOSTERONE

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Summary—A rapid, single extraction ELISA for testosterone in plasma is described, using a standard 96 well microtitre plate. Testosterone is covalently bonded to bovine thyroglobulin and passively adsorbed in guanidine hydrochloride to the ELISA plate, giving an immobilised antigen approach which simplifies subsequent assay standardisation for steroid hormone assays. The addition of standard, sample and first antibody (rabbit anti-testosterone), which is unique for each different assay, is followed by a general procedure which includes washing, addition of peroxidase labelled goat antirabbit IgG, further washing and finally, addition of *o*-phenylenediamine substrate with colour development and reading of the plate at 492 nm on an automatic ELISA processor. The ELISA assay is compared to a testosterone RIA with ^{125}I-label and has similar specificity and precision to the latter with a quicker processing time, and is more cost effective. The added advantages that ELISA assays confer over RIAs in terms of isotope purchase and disposal make this an ideal procedure for use in a routine steroid laboratory.

INTRODUCTION

The measurement of testosterone in plasma is necessary for the diagnosis and management of a number of endocrine disorders, including hypogonadism, Klinefelter's Syndrome, and testicular feminisation in man; hirsutism, Stein–Leventhal Syndrome, and testosterone producing tumors, both adrenal and ovarian in women. Over the last two decades, procedures for all steroid assays, including testosterone, have been simplified with a number of different techniques being investigated, including gas–liquid chromatography (GLC) [1–2], high performance liquid chromatography (HPLC) [3–4], radioimmunoassay (RIA) [5–6], and enzyme immunoassays (EIA) [7, 8]. In the latter two methods, high affinity antibodies have eliminated most of the tedious purification steps leading to fast, efficient and sensitive assays with large throughput of samples.

A number of sensitive fluoro- and enzyme-immunoassays have been published on steroid hormones [9–12], and as a logical extension to this, we have embarked on the development of enzyme-linked immunosorbent assays (ELISA) which can be completed in a 96 well microtitre plate [13]. The use of ELISA techniques to assay steroids has only recently started to appear in the literature [14, 15] and although a testosterone ELISA has been published [16], this paper describes to our knowledge, the first simple, rapid and sensitive assay of testosterone in which the complete reaction is carried out in a 96 well microtitre plate.

EXPERIMENTAL

A testosterone standard of 200 pg/100 μl was prepared in assay buffer from a stock solution of 1 mg/ml in ethanol and aliquots were frozen at −70°C. The standard curve was derived by serial dilution of this aliquot with fresh buffer on the day of assay.

Testosterone 3-(*O*-carboxymethyloxime) was prepared from testosterone (Sigma) and carboxymethoxylamine hemihydrochloride (Aldrich) using the method of Erlanger[17] and a pure product obtained by preparative TLC in toluene–methanol–acetone (1:1:1, by vol). A trace amount of [1,2,6,7-^{3}H]testosterone (Amersham) was incorporated for estimation of yield in subsequent reactions.

Phosphate buffered saline (PBS) containing 0.1% Tween 20 (v/v), pH 7.4 was used for washing the microtitre plates while assay buffer consisted of PBS with 0.1% gelatine added.

Synthesis of testosterone-3CMO-thyroglobulin conjugate

Testosterone-3CMO 10 mg was solubilised in 1 ml of dimethylsulphoxide (DMSO)–dioxane (1:1, v/v) followed by 1 ml of H_2O and combined with a solution of bovine thyroglobulin 10 mg (Sigma) in 1 ml of H_2O followed by 1 ml of DMSO–dioxane (both analar reagents). The total mixture was acidified to pH 5–6 by careful addition of 0.1 M HCl and stirred. *N*-ethyl-*N'*-(3 dimethylaminopropyl)-carbodiimide hydrochloride (Sigma) 100 mg was dissolved in 2 ml of H_2O, pH 5, and carefully added to the stirred mixture of steroid derivative and thyroglobulin. The reaction was left overnight to stir at 4°C and then dialysed against PBS containing 0.1% sodium azide, w/v. The final solution of testosterone-3CMO thyroglobulin (Tg-T) was stored at a protein concentration of 1 mg/ml with addition of thiomersal and sodium azide as preservative. A final ratio of 60–70 moles of testosterone per mole of thyroglobulin was determined by ^{3}H counting of the

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conjugate solution before and after dialysis, which compared well with the result obtained by RIA.

Antisera

Antiserum to testosterone was raised in a female American Chinchilla rabbit using testosterone-3CMO-BSA (Steraloids). The rabbit was immunised by initial intradermal injection followed by subcutaneous injections monthly in the nape and flanks, giving a final titre of 1 in 200,000 at final working dilution.

Affinity purified peroxidase labelled goat-antirabbit IgG was purchased from Tago Inc. U.S.A., and used at a final dilution of 1 in 3000 in the assay.

Enzyme substrate was freshly prepared before each final workup [13].

Adsorption of testosterone-thyroglobulin to the microtitre plate

ELISA microtitre plates (Nunc-Immuno Plate I, Nunc, Denmark) were activated by the addition of 200 μl of conjugate solution/well and incubated overnight at room temperature. The conjugate solution was prepared by addition of 40 μl of T-Tg to 20 ml of 6 M guanadine hydrochloride [18]. A Behring ELISA Processor M (BEPM) automatic instrument removed the unadsorbed protein by a 4 × 200 μl wash/well and blocked any further active binding sites by the addition of assay buffer, 200 μl/well, the plate then being left to incubate for at least 2 h at room temperature.

ELISA Procedure

An aliquot of plasma, male (100 μl) or female (500 μl), was extracted with 2 × 3 ml of diethyl ether which was subsequently dried under nitrogen, before the addition of 1 ml of assay buffer with Vortex mixing to ensure complete reconstitution of extracted steroid.

The plates were aspirated dry after the addition of the blocking solution by the BEPM and then 50 μl of patient sample or standard curve sample dispensed into the appropriate wells followed by 50 μl of antibody. The plate was left to incubate overnight at 4°C. Automatic washing (4 × 200 μl/well) and aspiration followed by the addition of goat-antirabbit IgG (peroxidase labelled) is undertaken by the BEPM before allowing the plate to incubate for a further 2 h at room temperature. The plate is finally washed again (4 × 200 μl/well), 100 μl of enzyme substrate added and the colour development allowed to proceed in the dark for 15 min where upon 100 μl of 1.25 M H_2SO_4 is added to terminate the enzymatic reaction. The absorbance is read at 492 nm with a reference wavelength of 600 nm on the BEPM. All standards and samples are measured in duplicate or quadruplicate and the results interpolated from the standard curve.

Specificity and accuracy

Antibody specificity was determined by assay of potentially crossreacting steroids. The percentage crossreactivity was determined by Abraham's method [19]. Pools of plasma with low testosterone levels were spiked with various amounts of testosterone. These were assayed to allow measurement of their recoveries. In addition, patient samples measured by ELISA were compared with an RIA method used in this laboratory for the last 4 years.

Precision

The between-batch precision was determined by repeated assay of three plasma pools with low, medium and high concentrations of testosterone. Within-batch variation was calculated from the differences between pairs of duplicates in the low, medium and high ranges.

RESULTS

Standard curve

Figure 1 shows a typical standard curve. The sensitivity calculated at two standard deviations from zero was less than 2 pg/tube or 0.3 nmol/l.

Specificity

Cross reactivities of steroids tested of 50% displacement are shown in Table 1 and were obtained over

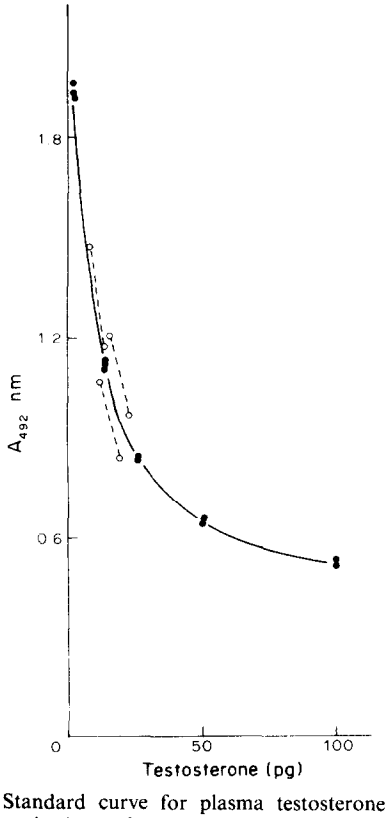

Fig. 1. Standard curve for plasma testosterone ELISA. Parallelism is shown for different volumes of pathological samples.

Table 1. Cross-reactivity of testosterone antiserum

Compound	Cross reaction %
Testosterone	100
5α Dihydrotestosterone	33.0
5β Dihydrotestosterone	14.0
Epitestosterone	1.6
5α Androstene 3α 17 diol	17.0
5α Androstene 3α ol-17 one	0.3
Δ^4 Androstene 3, 17 dione	3.3
Δ^4 Androstene 17β,19 diol 3 one	1.3
Δ^5 Androstene 3β17β diol	2.5
Dehydroepiandrosterone	0.9
Etiocholanolone	0.5
Progesterone	<0.001
Estriol	<0.001
Cortisol	<0.01
17α Hydroxy progesterone	<0.001

the range 0–1 μg of testosterone. A comparison of plasma testosterone levels by ELISA and RIA is shown in Fig. 2.

Precision

The precision was determined by analyses of 3 pools of plasma. Intra-assay variation for 6 sets of duplicates from the 3 pools with mean results of 4.3, 13.8 and 22.7 nmol/l was 7.6, 9.8 and 12.0% respectively, with inter-assay variations not exceeding 12.5% for the 3 pools.

Recoveries

Recoveries of exogenously added testosterone ranged from >90% to <110% within the range of 2–25 nmol/l.

DISCUSSION

The analysis of hormone concentrations has primarily been undertaken by RIA and the use of enzyme labels, widely used in immunology and other fields, has only recently been used for the determination of steroids. There are advantages in EIA over RIA and a number of reviews have been published on these aspects [20]. To date very few steroid ELISA assays have been reported [13-16]. This assay has been developed along similar lines to a reported ELISA assay for cortisol for a number of reasons [13]. The use of immobilised antigen instead of the usual immobilised antibody [15] procedure allows maximum usage of antibody without wastage and also allows the use of a common second antibody: goat-antirabbit IgG, for all assays developed using this technique. As a result, all the steroid hormone assays in the laboratory can be automated after the addition of the unique first antibody step.

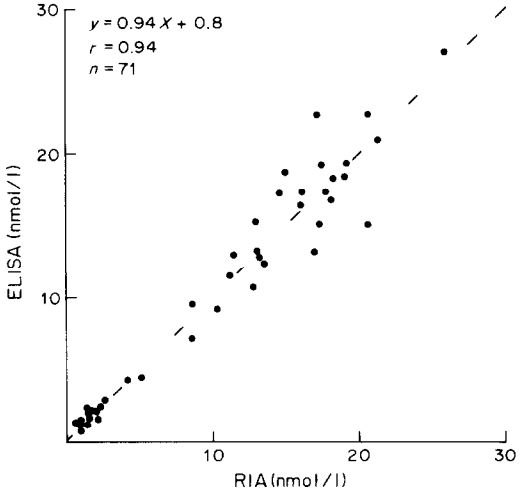

Fig. 2. Comparison between ELISA and RIA for normal and pathological testosterone samples.

A second advantage is that the critical step of attaching an enzyme label to the steroid hapten without grossly altering the enzyme activity is avoided. A recent publication on ELISA testosterone by Joshi *et al.*[16] highlights this problem where only 40% of the enzyme was recovered and only 40% of that recovered enzyme was immunoreactive, an analogous situation to that of low specific activity tracer in RIA.

The use of an automated ELISA processor and dispenser (Behring ELISA Processor M) has meant that a high degree of precision is dispensing aliquots of reagent into the microtitre wells is now possible (CV of <1%). At present, however, only the steps subsequent to the addition of first antibody can be efficiently carried out by the processor due to inbuilt programming inflexibility.

This assay compares well with RIA and has been validated in the important areas of specificity, parallelism, precision and accuracy. The overnight incubation step, while not mandatory, allows assay workload to be spread evenly. A rapid result, if desired, can be generated within 6 h.

Finally, in contrast to Joshi *et al.*[16], who transferred aliquots from the ELISA plate for optical density measurements, this assay is completed in a 96 well microtitre plate. With the updating of the Behring ELISA microprocessor memory it will be possible to process an assay from the initial antigen

coating to the final absorbance reading, with the exception of patient sample loading, on a single machine.

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REFERENCES

1. Brownie A. C., Van der Molen H. J., Nishizawa E. E. and Eik-Nes K. B.: Determination of testosterone in human peripheral blood using gas liquid chromatography with electron capture detection. *J. clin. Endocr. Metab.* **24** (1964) 1091–1102.
2. Collins P. W., Sisterton J. M., Kaulapis E. N., Mansfield M. D. and Sommerville I. F.: The evaluation of a gas liquid chromatographic method for determination of plasma testosterone using a nickel-63 electron capture detection. *J. Chromat.* **37** (1968) 33–45.
3. Satyaswaroop P. G., Lopez de la Osa E. and Gurpide E.: High pressure liquid chromatographic separation of C_{18} and C_{19} steroids. *Steroids* **30** (1977) 139–145.
4. Darney K. J. Jr, Wing T. Y. and Ewing L. L.: Simultaneous measurement of four testicular Δ^4-3 ketosteroids by isocratic high performance liquid chromatography with online ultraviolet absorbance detection. *J. Chromat.* **257** (1983) 81–90.
5. Ismail A. A. A., Niswender G. D. and Midgley A. R. Jr: Radioimmunoassay of testosterone without chromatography. *J. clin. Endocr. Metab.* **34** (1972) 177–184.
6. Williams B. M., Horth C. E. and Palmer R. F.: The measurement of testosterone in plasma. *Clin. endocr.* **3** (1974) 397–410.
7. Joshi U. M., Shah H. P. and Sudhama S. P.: A sensitive and specific enzymeimmunoassay for serum testosterone. *Steroids* **34** (1979) 35–46.
8. Tateishi K., Yamato H., Ogihara T. and Hayashi C.: Enzyme immunoassay of serum testosterone. *Steroids* **30** (1977) 25–32.
9. Al-Ansari A. A. K., Smith D. S. and Landon J.: Polarisation fluoroimmunoassay of serum and salivary cortisol. *J. steroid Biochem.* **19,** (1983) 1475–1480.
10. Arakawa H., Maeda M., Tsuji A. and Kambegawa A.: Chemiluminescence enzyme immunoassay of dehydroepiandrosterone and its sulphate using peroxidase as label. *Steroids* **38** (1981) 453–464.
11. Pal S. B.: *Enzyme Labelled Immunoassay of Hormones and Drugs.* Walter de Gruyter & Co., Berlin (1978).
12. Barnard G., Collins W. P., Kohen F. and Ludner H. R.: The measurement of urinary estriol-16-glucuronide by a solid-phase chemiluminescence immunoassay. *J. steroid Biochem.* **14** (1981) 941–948.
13. Lewis J. G. and Elder P. A.: An enzyme-linked immunosorbent assay (ELISA) for plasma cortisol. *J. steroid Biochem.* **22** (1985) 673–676.
14. Shah H. P. and Joshi U. M.: A simple, rapid and reliable enzyme-linked immunosorbent assay (ELISA) for measuring estrone-3-glucuronide in urine. *J. steroid Biochem.* **16** (1982) 283–286.
15. Munro C. and Stabenfeldt G.: Development of a microtitre plate enzyme immunoassay for the determination of progesterone. *J. Endocr.* **101** (1984) 41–49.
16. Joshi U. M., Shah P. and Sankolle G. M.: Penicillinase as a marker in enzyme-linked immunosorbent assays for steroid hormones. *J. steroid Biochem.* **19** (1983) 419–421.
17. Erlanger B. F., Borck F., Bieser S. M. and Lieberman S.: Steroid protein conjugates: Preparation and characterisation of conjugates of BSA with progesterone, deoxycorticosterone, and estrone. *J. biol. Chem.* **234** (1959) 1090–1094.
18. Wieslander J., Bygren P. and Heinegard D.: Isolation of the specific glomerular basement membrane antigen involved in Good Pasture Syndrome. *Proc. natn. Acad. Sci., U.S.A.* **81** (1984) 1544–1548.
19. Abraham G. E.: Radioimmunoassay of steroids in biological fluids. *J. steroid Biochem.* **6** (1975) 261–270.
20. O'Sullivan M. J., Bridges J. W. and Marks V.: Enzyme immunoassay: A review. *Ann. clin. Biochem.* **16** (1979) 221–240 and references quoted therein.