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Determination of Testosterone in Human Blood Plasma by Using 3β, 17-β-Hydroxysteroid Dehydrogenase

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An enzymatic procedure for the determination of testosterone in human blood plasma is described. The testosterone from blood plasma, after extraction, is measured, by the rate of transformation of NAD in NADH by comparison to the slope-curve of different testosterone concentrations. Specificity, sensivity and nature occurring inhibitors are analysed.

The determinations of testosterone as a tool to assess the functional status of the Leydig cell is becoming a common procedure in many laboratories, in which the routine load is an important aspect of their activities. The electron «capture» gas liquid chromatography (2), coupled to the isotope displacement (9) methods, seem to be the most sensitive and broadly applied procedures (3). However some laboratories have to face the reality of poor instrumental facilities and need to give reliable results.

The purpose of this paper is to describe a procedure that can be used for routine clinical purposes.

Materials and Methods

The blood plasma testosterone determinations were performed on 31, apparent normal human males, as well as in many patients with human fertility problems. Dried power of 3β, 17-β-hydroxysteroid dehydrogenase (1.1.1.51, 3β, 17-β-hydroxysteroid:NAD oxidoreductase), from induced *Pseudomonas testosteroni* (11), with an specific activity of 0.038 units/mg protein toward testosterone and 0.043 units/mg protein toward androsterone, from Worthington (Freehold, New Jersey, U.S.A.) was bought. Testosterone, androsterone, Δ^4-androstene-3,17-dione, androstane-3,17-dione, NAD and NADP, 17-β-stradiol were obtained from Sigma, St. Louis, Missouri (U.S.A.) Spectrophotometric readings in a Beckman D U 2 spectrophotometer were done. Rest of reagents were the highest pure grade commercially available.

Enzymatic assays. The 3β, 17-β-hydroxysteroid dehydrogenase activity was measured by determining the rate of transformation of NAD to NADH, by using the 6.6 value as the extinction molar coefficient (1). Incubation mixtures contained 100 μmoles of Tris-ClH (*p*H 8.9), 0.5 mg of the purified 3β, 17-β-hydroxysteroid dehydrogenase solution, 0.5 μmoles of NAD and sample to be tested, in a total volume of 3 ml. Reactions were run at room temperature for, usually, 5 minutes and taking the 340 mμ absorption readings every 15 seconds. Testosterone and sample was omitted in the blank. The assays for the calibration curve, were performed by replacing the sample for increasing amounts of testosterone, ranging from 5 to 100 mμ moles. Slope values corresponding to different testosterone concentrations were plotted (Fig. 1) toward the final testosterone concentration.

Extraction procedure. Samples corresponding to 50 ml of uncoagulated blood, by adding, 1.1 ml of 20 % sodium diethylamintetracetic acid (*p*H 7.4) were centrifuged at 5,000 × g for 10 minutes. The supernatant, after being extracted with equal volumes of ether-chloroform 3:1 (v/v) is succesively washed, with the same volumes of 1 M sodium hydroxyde, saturated solution of sodium bicarbonate and then water. Finally, the ether-chloroform extracts are evaporated to dryness at 40° C and low pressure. The dry residue is dissolved in 1 ml of water and then transferred to the spectrophotometer cuvettes, when the enzymatic activity was tested.

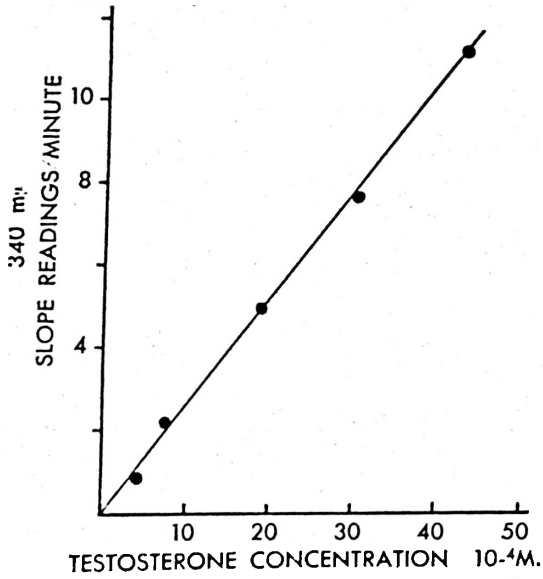

Fig. 1. *Typical calibration curve.* Enzymatic assays were performed as described in «Materials» and Methods». The increasing slope/minute readings are plotted towards the testosterone final concentration.

Paper chromatography. Descending paper chromatography was run in a Panglass-Shandon tank. The above mentioned dry residues were extracted with 20 ml of petroleum ether (60°) and then, overnight freezing at —24° C, the precipitate by centrifugation was removed. The methyl-alcohol extract is washed with 30 ml of 70 % methyl-alcohol. After three times extracted with 20 ml of bencene. The combined bencene extracts are once washed, with 20 ml of water and finally reduced to dryness. The residues, after being dissolved, in 1 ml of methyl-alcohol, are spotted on Whatman n.° 1 papers, follow by the development on the ligroin-propanediol (v/v) system, for 72 hours. The testosterone region was identified by the U.V. absorption.

Miscellaneous determinations. The commercial 3β, 17-β-hydroxysteroid dehydrogenase was purified according to TALALAY *et al.* (6). Protein determinations by the biuret method (5) were done.

Results

Enzyme specificity. The purified enzyme, as above described, was free of 3-α-hydroxysteroid dehydrogenase. However the purified enzyme was able to catalyze, at low rate, the reactions in which the androsterone was used as a substrate.

Stability, pH optimum, and Michaelis kinetics. The dry extract of the purified enzyme was stable over a year when kept at —20° C, however enzyme activity was slowly lost at room temperature. Purified and diluted enzyme, up to 50 mg/ml, was stable several months, when stored in the freezer. An optimum *p*H of 9.1 was found (Fig. 2), as well as a testosterone K_m (8) of 3.2×10^{-4} M. The K_i (10) for the stradiol was 0.5×10^{-5} M. However no other inhibitors were tested. The stradiol inhibition kinetics were straight competitive type (4).

Table I. *Enzymatic specificity.*
Enzymatic assays, in a 3 ml final volume, contained 100 μmoles of Tris-ClH buffer (*p*H 8.9), 15 μmoles of Testosterone 0.5 μmoles of NAD and 0.5 mg enzyme, having a specific activity of 4.2 μmoles/min/mg protein. Testosterone was replaced by androsterone, hydrocortisone and deoxycorticosterone.

Substrates	Crude extract	Purified enzyme
Testosterone	30	100
Androsterone	26	6
Hydrocortisone	1.2	0.02
Deoxycorticosterone	0.1	0.1

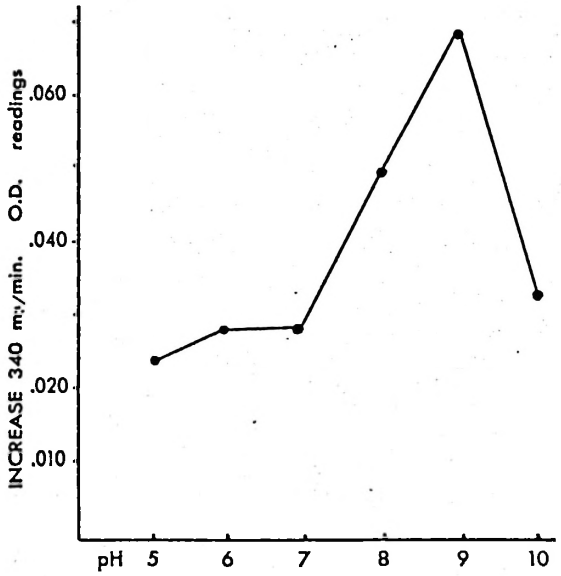

Fig. 2. *Enzyme pH optimum.*
Enzymatic assays were performed as described in Table I, changing the pHs of the buffer.

Testosterone blood plasma levels. Mean values of testosterone blood plasma levels in 31 apparent normal adult males were: 1.1-2 μg %. Testosterone traces in eight adult normal women were found. The method, did not show reliable results, when the testosterone blood plasma levels were determined prior and after gonadotrophin stimulation.

Discussion

Enzymatic procedures as a tool to test different pathways of the metabolism (7) is becoming a common event of many laboratories. Specially those, in which the endocrinological and metabolic activities are a quite important aspect of the routine work.

Two major points or apparent loopholes deserve a brief comment. They are concerned to the enzymatic specificity, when the testosterone is used as substrate and the sensitivity of the method to furnish reliable levels of blood plasma testosterone. Data showed in this paper, seem to indicate that the purified 3β, 17-β-hydroxysteroid dehydrogenase is highly specific toward testosterone. On the other hand, the low K_i for the stradiol may suggest the absence of inhibition interferences, on the way of assay performances. The sensitivity of the method gives similar blood plasma levels to other broadly used methods. The testing of testosterone in pathological individuals points out the 0.85 μg% level as the turning point for the Leydig cells functional deficiency.

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