SIMPLE ASSAY OF PLASMA TESTOSTERONE USING A COVALENT PROTEIN–SEPHAROSE COMPLEX

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1. Introduction

In previous communications, we [1] described the use of Sepharose-linked binding proteins for a convenient and precise assay of *cyclo*-3′,5′-adenosine monophosphate (*c*AMP) according to the principle of competitive protein binding (CPB) between unlabelled and labelled *c*AMP. The method had the advantage of allowing a simple and rapid separation of "bound" from "free" radioactive *c*AMP by mere filtration of the "immobilized" protein linked to the Sepharose. It avoided all the difficulties arising with soluble *c*AMP binding proteins that had been in use hitherto (for references see [1]).

It occurred to us that similar improvements could possibly be achieved for the CPB assays of plasma testosterone. In a collaborative effort, we succeeded in devising an equally convenient and precise testosterone assay using Sepharose-linked testosterone binding proteins from human late pregnancy serum.

2. Materials and methods

Water was deionized and twice distilled before use. For measuring aqueous solutions, we used Oxford, for solutions in organic solvents, glass pipettes. Buffer I: 0.1 N $NaHCO_3$/0.1 N NaCl; buffer II: 0.05 M Tris-HCl, pH 7.4.

2.1. *Sepharose-linked testosterone binding proteins*

3 g of Sepharose activated with cyanogen bromide (purchased from Pharmacia or prepared according to [1]) were suspended in 10 ml of buffer I and kept for 20 min at room temp. The gel was transferred to a porous glass filtration funnel and washed for not more than 15 min with approx. 200 ml of 0.001 N HCl to remove soluble impurities. The material on the filter was then washed with approx. 200 ml of buffer I, sucked almost dry, and transferred to a solution of 2 ml of human late pregnancy serum (third trimester) in 5 ml of buffer I contained in a 100 ml round bottomed flask. The mixture was gently stirred by slow rotation of the flask at 4° for 15 hr. The protein-modified Sepharose was collected on a porous glass filter and washed with 250–500 ml of buffer I, followed by 250–500 ml of buffer II. The gel was then suspended in 200 ml of buffer II and stored until used at approx. 1–4° in a beaker sealed with "Parafilm".

2.2. *Assay of "testosterone-like substances" in blood plasma*

2.2.1. Preparation of plasma extracts and blanks

The heparinized blood was immediately centrifuged and the plasma stored at –20° in plastic vials. 1.0 ml of the plasma (or a smaller amount diluted to 1.0 ml with water) was placed, together with 5.0 ml of spectroscopic grade dichloromethane, into a glass-

stoppered vial and agitated for 1 min. The aqueous phase was pipetted off and replaced with 0.5 ml 1 N Na_2CO_3 solution. After agitation (30 sec) and centrifugation, the aqueous phase was removed, and the organic phase washed neutral with two to three successive 0.75 ml volumes of water (test with pH paper). Four 0.50 ml aliquots of the organic phase were pipetted into four Eppendorf vials and kept in a vacuum desiccator for at least 1 hr at approx. 10 Torr. For the estimation of blank values, 1.0 ml of water was treated in exactly the same manner as the plasma samples.

2.2.2. Calibration

For every assay (double estimations of the unknowns) a calibration curve was constructed, using 50 μl each of ethanolic solutions containing in this volume 5.0, 1.25, 0.32, 0.008, and zero ng of testosterone in Eppendorf vials. The solvent was removed as in sect. 2.2.1.

2.2.3. Binding assay

100 μl of [1,2-^{3}H]testosterone (Amersham) in water (= 10 nCi) were added to each of the desiccated vials (mechanical shaking for 30 sec dissolved the dried extract or blank). The protein-modified Sepharose in the storage beaker was evenly suspended by *slow* magnetic stirring, and 200 μl each of the suspension pipetted into the vials. After mechanical agitation for 5 sec, equilibration was achieved by standing at room temp. for unspecified times between 20 and 120 min. The contents of the vials were then pipetted onto 35 μm polyester nets (Müllereigaze Estal mono PE 35, No. 19176, Schweizerische Seidegazefabrik AG., Thal, Switzerland) contained in suction filter funnels. The vials were rinsed with 1 ml each of buffer II, and the materials on the filters washed once or twice with approx. 1 ml of the same buffer (volumes aren't critical). The nets were transferred into the counting vials and covered with 5 ml of the scintillant solution (Insta-Gel, Packard). After agitation, the radioactivity was determined by scintillation counting (Packard Tri-Carb instrument). It was usually expressed in percentage of a 100% value (= 100 μl of the above [1,2-^{3}H]testosterone solution added to the scintillant).

3. Results

3.1. *Properties of the "Sepharose-linked testosterone binding proteins"*

The mean association constant for [1,2-^{3}H]testosterone in 33 mM Tris-HCl buffer, pH 7.4, at 20°, and the Hill coefficients, n, were determined according to the method described in [1]: $K_{ass} = 9.87 \times 10^8$ l/mole ($K_{diss} = 1.01 \times 10^{-9}$ mole/l); $n = 1$. The non-specific adsorption of [1,2-^{3}H]testosterone to Sepharose and to the polyester net is less than 1% of the total tracer concentration added.

3.2. *Water blank*

The water blank is considerably lower with the present method ($\bar{x} = 6.8$ ng/100 ml, $S_{\bar{x}} = 2.2$, $n = 5$) than with Florisil 2 ($\bar{x} = 64.6$ ng/100 ml, $S_{\bar{x}} = 4.6$, $n = 13$). In both cases, the extraction was carried out with dichloromethane, the solvent giving the lowest "reagent blank" [3].

3.3. *Plasma testosterone values*

Results obtained with the present method from samples of normal subjects and patients with various

Table 1
Plasma testosterone values in normal pubertal and adult males.

Subject No.	Age (yr)	Plasma testosterone (ng/100 ml)
1	15	644
2	16	680
3	16	685
4	17	410
		1000*
		1025*
		1300*
5	17	465
6	17	470
7	19	530
8	20	800
9	21	455
10	54	980
Mean value ± s		612 ± 172

* = 2, 4 and 6 days after administration of human chorionic gonadoptropin (5000 U per square meter of body surface area).

Table 2
Plasma testosterone values in patients with various disorders.

Subject No.	Age (yr)	Sex	Diagnosis	Plasma testosterone (ng/100 ml)
High values				
1	3	M	Idiopathic precocious puberty, untreated	750
2	6	M	Idiopathic precious puberty untreated	1140
			Under treatment with cyproterone acetate	410
3	19	M	Testicular teratoma, untreated	600
			After surgery	235
4	23	F	Idiopathic hirsutism	210
5	23	F	Idiopathic hirsutism	380
6	30	F	Idiopathic hirsutism	450
7	27	M	Congenital adrenal hyperplasia (21-hydroxylase deficiency), untreated	1265
8	30	F	Virilizing adenoma of the left adrenal:	
			Left adrenal venous plasma	6500
			Right adrenal venous plasma	1600
Low values				
9	0.6	M	Tall stature within normal limits	33
10	4	M	Hereditary defect of testosterone biosynthesis (17,20-desmolase deficiency)	20
11	6	M	Congenital anorchia	10
12	52	M	Castrate, untreated	25
13	16	M	Delayed puberty	105
14	16	M	Delayed puberty	210
15	17	M	Delayed puberty	260
16	17	M	Delayed puberty	345
17	18	M	Delayed puberty	130
18	19	M	Delayed puberty	171
19	17	M	Klinefelter's syndrome	165
20	35	M	Hypogonadism	120
21	40	M	Hypogonadism	175

endocrine disorders are displayed in tables 1 and 2. They agree well with those reported by others using different techniques [4, 5]. The mean value obtained in normal pubertal and adult males was 612 ± 172 ng/100 ml (n=10), while a compiled average from the literature [5] is 670 ± 230 ng/100 ml. The highest figure was obtained for adrenal venous blood from a patient with virilizing adrenal adenoma (6500 ng/100 ml, patient No. 8, table 2). High values were also found in untreated patients suffering from congential adrenal hyperplasia due to a 21-hydroxylase deficiency and from idiopathic precocious puberty. In the latter condition, there was, in one case studied, a definite reduction of the level during treatment with cyproterone acetate. Low values were obtained in an adult, unsubstituted castrate male, in a boy with congenital anorchia, and in one child with a congenital defect of testosterone biosynthesis (17,20-desmolase deficiency, proven by incubation of testicular tissue with testosterone precursors [6]). Moderately low levels were found in cases with benign delayed puberty, Klinefelter's syndrome, and hypogonadism, as one would expect.

4. Discussion

The competitive protein binding technique for the assay of hormones in biological fluids was introduced by Ekins (1960) for thyroxin [7]. Subsequently, Murphy [8, 9] adapted the principle for the estimation of steroids. A large number of methods for the determination of various steroids using CPB has since been published (for references and discussion see [10]. These techniques have the advantage of relative simplicity and high sensitivity. Their specificity, however, depends on the prepurification steps applied rather than on the CPB itself.

Among other difficulties, one of the major problems of CPB techniques is the separation of the free from the protein bound steroid fraction. For this purpose, various methods have been used, including gel filtration (Sephadex G25 [11], dextrane coated charcoal [12], ammonium sulfate for protein precipitation [13], and Florisil, p.e. [2, 14, 15]). We have, in the Dept. of Pediatrics, been previously using a modification of the relatively simple and non-specific method of Anderson [2] for the determination of "testosterone-like" substances in plasma, where no prepurification is carried out and the separation of the free from the bound fraction is done with Florisil. Our modifications consisted of extraction with methylene chloride instead of ether (lower non-specific "reagent" blank, cf. [3]), of a different CPB step, cf. [16], and of minor changes of shaking.

Although for clinical use, the results obtained with this previous method agreed well with those of urinary steroid determinations in the same subjects by highly specific gas chromatographic techniques (Zachmann [17]), the separation step with Florisil was a constant source of difficulties and did impair the reproducibility of the calibration curves due to its sensitivity to even minimal changes of shaking time and other methodological factors. In addition, in our experience and in that of others [9, 15], too high testosterone values were obtained with Florisil in plasma samples from prepubertal children and females, and the water blanks were relatively high.

Similar difficulties had been encountered in the CPB assays of *c*AMP and had been overcome by the use of relatively very stable, Sepharose-linked *c*AMP binding proteins [1]. The results presented in this paper show that analogous improvements can be achieved for steroid assays with the help of immobilized binding proteins. The advantages consist in: i) rapid and facile separation of bound and free steroid fractions by simple filtration, ii) the constant equilibrium between 20 min and at least 3 hr, resulting in improved reproducibility of the standard curves and relative insensitivity to changes in handling times, iii) reduction of the water blank, iv) no observable desorption of testosterone during filtration and washing procedures, v) the negligible nonspecific adsorption of testosterone to Sepharose and polyester net, and vi) the stability of the Sepharose-linked protein.

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