A FILTER ASSAY FOR THE SEX STEROID BINDING PROTEIN (SBP) OF HUMAN SERUM

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1. Introduction

It is now widely accepted that the sex-steroids, 17β-estradiol, testosterone, and dihydrotestosterone, are bound to a specific β-globulin (SBP) in the plasma of various species including man (for reviews see refs. [1] and [2]). This protein was first described by Mercier et al. [3] and purification has been attempted in various laboratories [1]. The physiological function of SBP is still unknown but it has been suggested that the protein may serve a role in keeping testosterone in the circulation [4,5].

Recently we have been interested in characterizing this protein [6] in the hope of using it as a model system for the study of steroid–protein interaction in relation to intra-cellular estrogen and androgen 'receptors'. In the course of our studies, it was found that conventional methods of assay such as florisil adsorption, batchwise Sephadex equilibration [8], and ammonium sulfate precipitation [9] were not satisfactory for measuring binding activity in very diluted solutions of purified SBP [6]. Under these conditions the protein adsorbs to Sephadex, and florisil, and cannot be precipitated with ammonium sulfate (unpublished observations). Consequently, a new SBP assay was developed based on a recently reported filter paper method for measuring glucocorticoid, estrogen, and mineralocorticoid 'receptors' [10]. Our results presented in this communication indicate that the assay procedure is rapid and accurate, and is not only useful for studies on the characterization of SBP, but is also applicable for clinical investigations directed at measuring binding capacity in serum.

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2. Methods

2.1. *Reagents and materials*

5α-Dihydrotestosterone-1,2-^{3}H (44 Ci/mmole) was purchased from New England Nuclear. Radioinert dihydrotestosterone was obtained from Sigma. DEAE-cellulose filter paper discs (DE-81, 2.3 cm diameter) were purchased from Reeve Angel & Co. Human serum albumin was purchased from Sigma and defatted with charcoal–acid treatment. All other chemicals were Reagent grade.

2.2. *Serum samples*

Blood samples were collected from a pregnant woman at term, a non-pregnant woman, and an adult male. Serum was prepared by allowing the sample to clot for 1 hr at 25°C followed by centrifugation at 1500 rpm for 5 min. The sera were diluted with 10 mM Tris–Cl, pH 7.4, and used immediately.

2.3. *Assay procedure*

The principle of the method is based on the adsorption of the [^{3}H]DHT–SBP complex onto DEAE-cellulose filter paper discs due to the acidic isoelectric point of SBP [17]. 0.5 ml aliquots of human serum (pregnancy, normal male or female) diluted 50-fold with 10 mM Tris–Cl, pH 7.4, or a solution of purified SBP corresponding to about the same binding capacity are placed in test tubes containing [^{3}H]DHT. The tubes are

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allowed to remain at 25°C for 15 min and then cooled in ice. All subsequent operations are carried out in the cold room. DEAE-filter discs are equilibrated overnight with two changes of 10 mM Tris–Cl, pH 7.4, and stored at 4°C. The filter discs are placed on top of a Whatman No. 1 fil ter paper in a Buchner funnel and excess moisture removed by applying a gentle vacuum. The vacuum is removed and 100 μl or 200 μl of sample are uniformally applied to each filter paper disc with an Eppendorf pipette. After 2 min the vacuum is applied and the discs are washed 10 times with 1 ml aliquots of buffer. Excess moisture is once more removed and the filter discs are placed in vials with 10 ml of scintillant (5 g PPO, 100 g naphthalene/liter dioxane) and counted in a Beckman LS-100C scintillation counter with 34–37% efficiency.

3. Results

Fig. 1 shows the retention of radioactivity when 100 μl of diluted pregnancy serum previously equilibrated with saturating amounts of [^{3}H]DHT is applied on DEAE-filter paper discs. A total of ten 1 ml washes are required to remove all unbound and losely-bound radioactivity, less than 1% of the counts remain on the filter paper in a buffer blank. In the presence of 100-fold excess of radioinert DHT, the specifically-bound radioactivity is diluted resulting in significant loss of retained counts on the filter (fig. 1). Addition of a 1000-fold excess of radioinert DHT to either diluted serum or to a 2 mg/ml solution of human serum albumin (equivalent to the albumin concentration in the 20-fold diluted serum used in the experiment of fig. 1) does not lower the amount of radioactivity remaining on the filter after 10 washes. Consequently, this amount, probably arising from nonspecific adsorption of the steroid, must be subtracted from total bound radioactivity to the filter in order to account for specific binding. In the case of purified SBP, which is not contaminated with albumin, only 6 washes are required to remove unbound steroid [6].

In order to estimate whether or not the [^{3}H]DHT–SBP complex is completely retained by the filter during the washing procedure, the assay was done under non-saturating conditions with respect to SBP. Fig. 2 shows that the amount of bound DHT increases linearly with DHT concentration to the extent that 75% of the [^{3}H]DHT–SBP complexes are retained by the filter as calculated from the slope of the line. This value is a direct measure of the 'efficiency' of the assay and should be used as a correction factor to obtain the

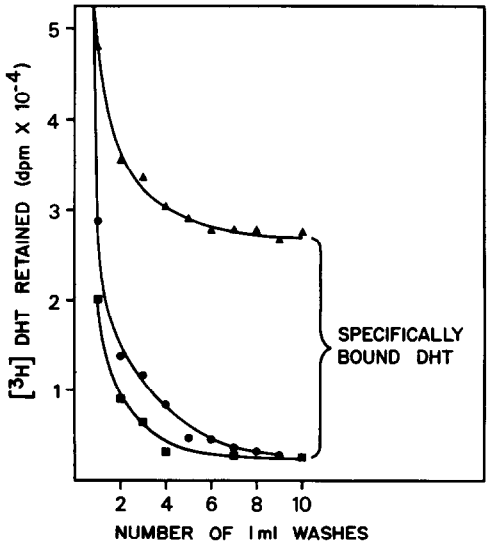

Fig. 1. [^{3}H]DHT retained by DEAE-cellulose filters at 4°C. 0.5 ml of solution incubated with 5 × 10^5 dpm (S.A. = 5 × 10^4 dpm/ng). 100 μl applied to each filter. (▲——▲) Human pregnancy serum diluted 20-fold with 10 mM Tris–Cl, pH 7.4; (●——●) same diluted serum in the presence of 1 μg radioinert DHT (100-fold excess); (■——■) 2 mg/ml human serum albumin.

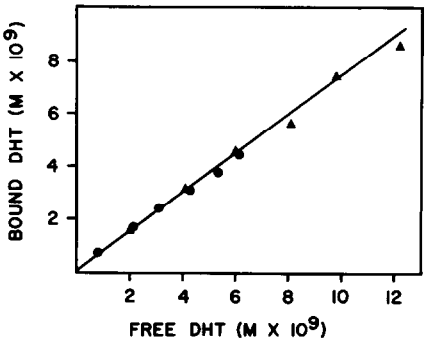

Fig. 2. Adsorption of DHT–SBP complex to DEAE-cellulose filters at low steroid concentrations. [^{3}H]DHT, S.A. = 9.77 × 10^7 dpm/nmole. Ten 1 ml washes at 4°C. (▲——▲) 20-fold diluted pregnancy serum; (●——●) 50-fold diluted pregnancy serum.

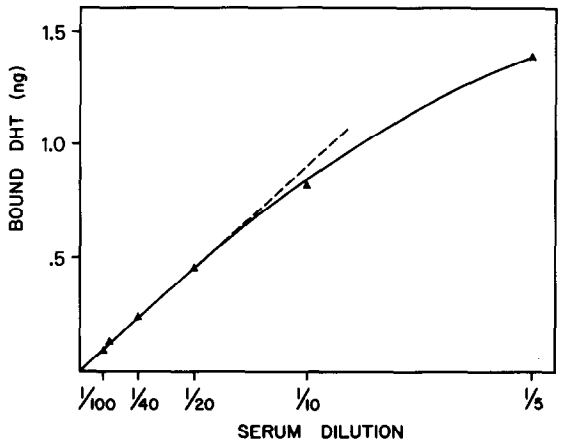

Fig. 3. Adsorption of DHT–SBP complex to DEAE-cellulose filters as a function of pregnancy serum dilution. 0.5 ml diluted serum incubated with 1.73×10^6 dpm DHT (S.A. = 1.73×10^5 dpm/ng). 100 μl applied to each filter. Ten 1 ml washes at 4°C. Data were corrected for 25% loss of complex ('efficiency' correction, see fig. 2), represent ng DHT bound per filter.

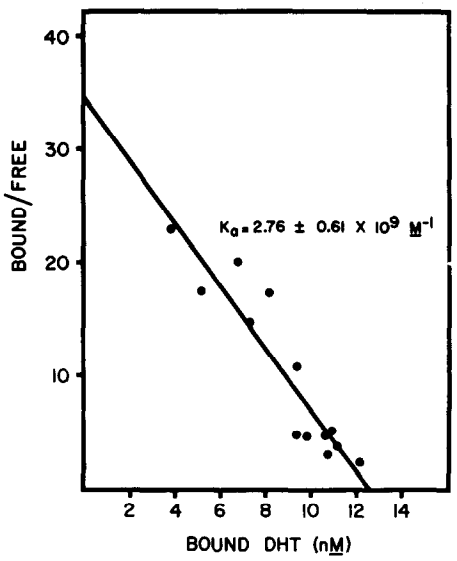

Fig. 4. Determination of equilibrium constant of association of DHT binding to SBP at 4°C by Scatchard analysis. 0.5 ml of 50-fold diluted pregnancy serum were incubated with [^{3}H]DHT (S.A. = 9.77×10^7 dpm/nmole). 200 μl applied to each filter. Ten 1 ml washes were used. Data were corrected for 25% loss of complex ('efficiency' correction; see fig. 2) and nonspecific binding. The free steroid was determined by subtracting the total DHT added from that bound to the filter.

total amount of DHT bound per ml of serum. Factors such as pH, temperature, ionic strength and buffer ion, as well as differences in methodology will contribute to variations in the efficiency value. Consequently, an experiment as illustrated in fig. 2 should be performed at the outset in order to establish the 'efficiency' of the assay. 84% 'efficiency' was obtained for purified SBP [6] and 86% for the titration of the glucocorticoid 'receptor' isolated from hepatoma cells [10].

The influence of serum dilution upon the total amount of [^{3}H]DHT bound to SBP under saturating conditions is shown in fig. 3. The assay is linear at low protein concentration where sensitivity and accuracy depends only upon the specific radioactivity of the steroid. Deviation from linearity occurring above 20-fold dilution of serum probably results from 'overloading' of the filter paper.

The determination of the equilibrium constant of association for the [^{3}H]DHT–SBP complex at 4°C by Scatchard analysis is shown in fig. 4. Each point was corrected for 'efficiency' after 10 washes. The binding constant agrees with the other published values at 4°C, 2.8×10^9 M^{-1} and 1.6×10^9 M^{-1} [12] measured under equilibrium conditions. Extrapolation of the linear plot of fig. 4 to the abscissa yields 18.2 μg DHT bound/100 ml pregnancy serum. This value is higher than that obtained in the standard filter assay at saturating steroid concentrations (table 1). The discrepancy may result from the error involved

Table 1
Reproducibility of the method*

Serum	Mean (μg DHT bound/100 ml serum)	S.E.M.	S.D.	Range
Male	1.28	0.01	0.04	1.18– 1.32
Female	2.86	0.03	0.09	2.71– 2.99
Pregnant	14.17	0.09	0.28	13.73–14.53

* The values were obtained from 10 determinations on the same serum.

Table 2
Comparison with other published values at 4°C for human serum (μg DHT bound/100 ml serum)

	Male		Female		Pregnant	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Rosner [9]	0.93	0.06	1.85	0.13	11.9	0.67
Vermeulen et al. [14]	1.33	0.12	2.15	0.23	9.41	1.35
Corvol et al. [15]	0.49	0.04	1.42	0.22	10.9	0.74
Heyns and De Moor [16]	1.07	–	1.76	–	12.5	–
Shanbhag et al. [12]	1.76	–	3.00	–	12.7	–
This work	1.28	0.01	2.86	0.03	14.17	0.09

in the determination of the binding constant by Scatchard analysis (fig. 4).

The reproducibility of the method is shown in table 1 which summarizes the results from 10 individual experiments. The total amount of SBP calculated on the basis of 52 000 daltons [13] is 2.29 mg/liter for male serum, 5.13 mg/liter for female serum, and 25.4 mg/liter for pregnancy serum. Table 2 compares our values with those determined in other laboratories using different assay methods.

4. Discussion

The data presented indicate that the filter assay can be used to determine accurately the sex steroid binding capacity of human serum. The method is reproducible, rapid, and requires very little serum (less than 1 ml was needed to carry out the entire study reported in this paper). Ten samples can be assayed in duplicate in less than 2 hr including the calculations (the vials do not need to stabilize for 6 hr before counting as in the case of the $(NH_4)_2SO_4$ method [9]). The procedure is most useful at low protein concentration for which it was originally designed to meet our research needs [6]. Under those conditions, the sensitivity depends only upon the specific radioactivity of the steroid.

The determination of SBP concentrations from the binding capacity of serum by the filter assay depends upon two main assumptions. The first is that mole/mole binding of steroid to protein occurs. At present this is not known and further work on the protein chemistry of SBP will be required to validate this assumption. The published experimental values may have to be corrected depending upon the actual stoichiometry and the refined value of the molecular weight of SBP. The second assumption is that the equilibrium concentration of steroid is not disturbed during the assay. Theoretically, the method may not yield a thermodynamically meaningful binding constant because the assay is not carried out under strict equilibrium conditions, i.e. equilibrium dialysis. However, the data in table 1 and fig. 4 agree with those obtained from experiments carried out under such conditions [12]. The rate of dissociation of the steroid–protein complex under the assay conditions is too slow to account for significant changes in the actual concentration of the complex remaining on the filter. Consequently, the method is applicable not only to measure SBP concentrations but also to study the physical chemistry of the binding process.

Finally, a DEAE-cellulose filter assay can be developed for other binding proteins in serum since most of those have acidic isoelectric points. For example, recent experiments indicate that human CBG, corticosteroid binding globulin, can be measured by this method (Schiller and Petra, to be published).

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