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# DETERMINATION OF SERUM SEX HORMONE BINDING GLOBULIN (SHBG) IN PREADOLESCENT AND ADOLESCENT BOYS

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SUMMARY

The saturation assay for sex hormone binding globulin (SHBG) originally described by Rosner[1] has been modified to optimize assay conditions and the method applied to clinical studies. A set of equations is given which fully describes the assay system and helps to calculate various parameters. The use of filtration instead of centrifugation allows one to measure directly SHBG bound DHT and reduces the error due to the calculation by subtraction of nearly equal numbers in the indirect procedure of Rosner [1]. The improvement due to modification is demonstrated in detail, and a procedure is described for the assay of SHBG in human male serum. Normal values are given for boys during multiphase pubertal developments.

## INTRODUCTION

Gonadal steroids circulate in plasma mainly as protein steroid complexes bound to albumin, and a plasma protein known as sex hormone binding globulin (SHBG) [1–5]. Although the function of this latter protein is somewhat obscure, it is known that the unbound fraction of steroid hormone is in fact the active form [6–12]. Whether this function can be viewed only as a damper for steroid hormone action or a hormonal action in its own right [13] is not at all clear. In our previous studies [14–17] we demonstrated that the maturation of male reproductive organs is associated with a fall of testosterone-binding affinity which could quite possibly be due to a change in SHBG concentration. In this report we have, therefore, developed a direct method for the estimation of SHBG in human plasma and related its plasma concentration to the maturation of male reproductive process in humans.

## MATERIALS AND METHODS

*Blood samples.* Blood was obtained from 18 hospitalized but endocrinologically normal boys (see Table 3) and six male adult volunteers from the Laboratory staff. All the boys were rated for pubertal developmental stages [18].

*Reagents.* [$^3$H]-dihydrotestosterone (S.A. 60 Ci/mmol) was purchased from New England Nuclear and purified by chromatography before use. The stock solution was prepared containing 5 $\mu$Ci/ml in ethanol. All other reagents used in this study were analytical grades unless otherwise stated.

*Assay procedure.* The method was essentially derived from that used by Rosner[1] but modified in several places, especially the use of centrifugation by membrane filtration. Eight hundred $\mu$l of Sörensen phosphate buffer (0.15 M, pH 7.4) and 100 $\mu$l of Charcoal suspension (200 mg charcoal Norit A + 20 mg Dextran T 500 suspended in 100 ml of the same Sörensen phosphate buffer) were added to 100 $\mu$l serum. This mixture was shaken for 12 h at 4°C to remove endogenous steroids. After centrifugation for 10 min at 800 ***g*** at 4°C, 100 $\mu$l of the supernatant were added to 400 $\mu$l of Sörensen phosphate buffer containing 5 mg/ml of bovine serum albumin and 2 mg/ml of bovine serum $\gamma$-globulin in polystyrene tubes. One hundred $\mu$l of tracer solution were already evaporated in these tubes. All determinations, including a blank containing only 500 $\mu$l of the protein containing buffer, were run in triplicate. Blanks for samples with low protein content as derived from the isolation procedure are composed of 400 $\mu$l of protein-containing buffer and 100 ml of protein free buffer. After incubating at 37°C for 30 min, the tubes were cooled to 4°C and 500 $\mu$l of cold saturated ammonium sulphate solution were added to the tubes under continuous shaking. After 10 min 200 $\mu$l of the suspension were filtered through a membrane filter (Sartorius No. 11306) previously washed with 1 ml of half-saturated ammonium sulphate, at 4°C. The filter was dried at 100°C in an oven for 15 min and transferred to a counting vial and counted in a Packard Liquid Scintillation Spectrometer after addition of 10 ml of toluene scintillation mixture.

*Optimization of the assay system.* To find out the optimal steroid concentration in the assay the

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adopted assay procedure was examined mathematically. In this connection several equations were established which permit the following:

a. The prediction of the degree of saturation from a known concentration of SHBG and DHT; b. The calculation of the amount of total SHBG in the sample for a measured concentration of SHBG–DHT-complex.

By the application of the law of mass action the following two equations can be established which should be fulfilled simultaneously in thermodynamic equilibrium.

$$\frac{AS}{(St - AS - PS)(nA_t - AS)} = K_1 \qquad (1)$$

and

$$\frac{PS}{(St - AS - PS)(P_t - PS)} = K_2 \qquad (2)$$

where,

$AS$ = concentration of binding sites of albumin which are occupied by DHT
$PS$ = concentration of SHBG–DHT complex
$A_t$ = molar concentration of albumin
$P_t$ = molar concentration of SHBG
$S_t$ = molar concentration of DHT
$K_1$ = intrinsic association constant for the albumin–DHT complex
$K_2$ = intrinsic association constant for the SHBG–DHT complex
$n$ = number of the binding sites of albumin for DHT

The following equation emerges when the above two equations are combined:

$$AS = \frac{K_1 PS A_t n}{K_2 P_t - K_2 PS + K_1 PS}. \qquad (3)$$

Substituting equation (3) in equation (2) and rearranging, the following equation is obtained:

$$\begin{aligned} &S_t K_2^2 P_t - (K_2^2 - K_1 K_2) PS P_t S_t - K_2^2 P_t PS^2 S_t \\ &+ K_2^2 PS^2 S_t - K_1 K_2 PS^2 - K_1 K_2 PS A_t n P_t \\ &- K_2^2 PS P_t^2 + (K_2^2 - K_1 K_2) PS^2 P_t \\ &+ K_1 K_2 PS^2 A_t n + K_2^2 P_t PS^2 + (K_2 - K_1) PS^2 \\ &- (K_2^2 - K_1 K_2) PS^3 - K_2 P_t PS = 0. \end{aligned} \qquad (4)$$

Equation (4) can be solved for $S_t$ ($P_t$ and $PS$ given) for $PS$ ($S_t$ and $P_t$ given) or for $P_t$ ($PS$ and $S_t$ given).

In establishing the equation contribution of α-1-acid glycoprotein and corticosteroid binding globulin to binding of DHT was not taken into consideration for the following reasons:

1. Although α-1-acid glycoprotein has been found to bind progesterone [19], no high affinity binding of this protein has been reported.
2. Corticosteroid binding globulin has been seen to bind androgens with an association constant 100 times less than that of SHBG for DHT. Moreover only 15% of this protein is precipitated by half-saturated ammonium sulphate solution [4].

Table 1 shows the results for calculating $(PS)/(P_t)$ from $PS$ at different steroid concentrations. Since the number of binding sites of albumin for DHT is not yet known, the apparent association constant $K_{app} = nK_1$ was used. By substituting $K_1$ by $K_{app}/n$ the results for $n = 1$, $n = 25$ and $n = 100$ were obtained. Albumin concentration and sample dilution were assumed to be the same as in the assay conditions given above. It can be clearly seen that at steroid concentrations greater than $1.5 \times 10^{-8}$ mol/l either the number of binding sites or the SHBG-concentration, over at least one order of magnitude does not practically influence the degree of saturation. A steroid concentration of $2 \times 10^{-8}$ mol/l was used which resulted in degree of saturation of 81% for

Table 1. Theoretical degree of saturation for SHBG at different concentrations of SHBG and DHT

| DHT (total) (mol/l) | | $1 \times 10^{-8}$ | | | $2 \times 10^{-8}$ | | | $5 \times 10^{-8}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SHBG–DHT-complex concentration | | Degree of saturation in respect to number of binding sites (presumed) | | | | | | | | |
| serum (mg/l) | incubation mixture (mol/l) | 1 | 25 | 100 | 1 | 25 | 100 | 1 | 25 | 100 |
| 2.0 | $3.48 \times 10^{-10}$ | 68.20 | 68.20 | 68.20 | 81.37 | 81.37 | 81.37 | 91.69 | 91.69 | 91.69 |
| 4.0 | $6.96 \times 10^{-10}$ | 67.40 | 67.40 | 67.40 | 81.01 | 81.01 | 81.01 | 91.63 | 91.64 | 91.64 |
| 6.0 | $1.04 \times 10^{-11}$ | 66.56 | 66.56 | 66.56 | 80.81 | 80.81 | 80.82 | 91.58 | 91.58 | 91.58 |
| 8.0 | $1.39 \times 10^{-11}$ | 65.67 | 65.67 | 65.67 | 80.53 | 80.53 | 80.53 | 91.53 | 91.53 | 91.53 |
| 10.0 | $1.74 \times 10^{-11}$ | 64.73 | 64.73 | 64.73 | 80.23 | 80.23 | 80.23 | 91.47 | 91.47 | 91.47 |

*Note*: The degree of saturation in percent ($PS/P_t \times 100$) was calculated for five concentrations of the SHBG–DHT-complex (the parameter measured by the assay) at three steroid concentrations. The unknown intrinsic association constant of albumin for DHT was substituted by $K_{app}/n$, whereby the number of binding sites was arbitrarily set equal to 1, 25, 100.

Further constants used for the calculations:

$$K_{app} = 5 \times 10^4 \text{mol/l}, \; K_2 = 1 \times 10^9 \text{ mol/l}, \; A_t = 7 \times 10^{-5} \text{ mol/l}.$$

The fifty-fold dilution of the serum in the incubation mixture was taken into account. The DHT-concentration in our assay is $2 \times 10^{-10}$ mol/l (second column). For further explanations see text.

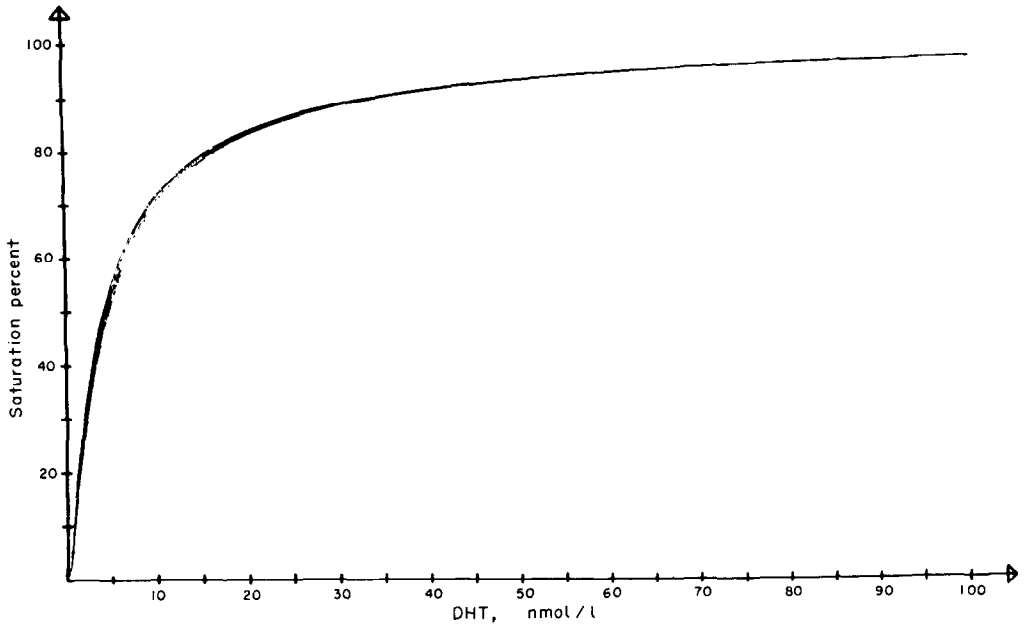


Fig. 1. Computer plot of $PS/P_t \times 100$ versus $S_t$ for five SHBG concentrations in serum: 2, 4, 6, 8 and 10 mg/l. $A_t$ = 5 mg/ml, sample dilution tenfold, $K_2$ = 1.E09, $K_{app}$ = 5.E04, $n$ = 25. For further explanation see text.

SHBG concentration in the range from 1–10 mg/l serum.

Figure 1 shows a computer plot of $PS/P_t \times 100$ versus $S_t$ at five SHBG-concentrations: 2, 4, 6, 8 and 10 mg/l serum; the 50-fold dilution in the incubation mixture was taken in account. The negligible influence of the SHBG-concentration on the degree of saturation is again demonstrated.

Figure 2 shows a plot from an experiment in which a serially diluted serum was tested with our assay system for its SHBG-content. The theoretically predicted linear dependence of the counts on the filter from the SHBG-concentration is obtained with a regression coefficient of 0.996.

*Calculation of SHBG-concentration in the sample.* Under the assay conditions given above, the degree of saturation is independent of the SHBG-concentration in the range of interest; its theoretical value is 81% (see above). The SHBG-concentration in the sample is therefore proportional to the counts of the filter and can be calculated from the simple relation:

$$\text{SHBG}\,(\mu\text{g/ml}) = \frac{(CPM_s - CMP_b) \times 50 \times 1.15 \times 10^{-8} \times f_d \times 1.0 \times 10^{-4}}{\text{S.A.} \times \text{Effi.} \times \text{Sat.}}$$

$CPM_s$ = sample counts, $CPM_b$ = blank counts, $1.15 \times 10^{-8}$ = molar weight (mg) of SHBG (obtained from gel filtration experiments in our laboratory ($f_d$ = sample dilution, S.A. = specific activity of the tracer, Effi. = counting efficiency, Sat. = degree of saturation (0.81); the other numbers are volume correction factors.

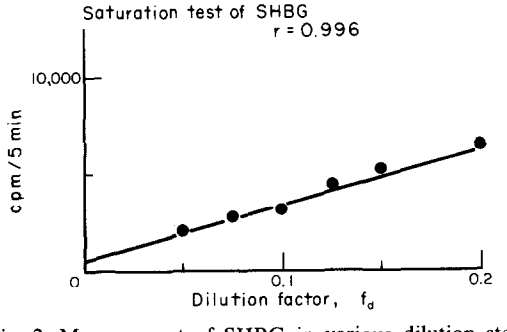


Fig. 2. Measurement of SHBG in various dilution steps of a single serum sample.

## RESULTS

### 1. *Steroid adsorption on the tubes and effect of albumin*

In the procedure of Rosner[4] unspecific binding of tracer by the walls of the assay tubes may considerably simulate specific binding of DHT to SHBG. To check this possibility, a fixed amount of [$^3$H]-DHT was incubated with protein free phosphate buffer and an equal vol. of saturated ammonium sulphate solution was added. The adsorption by glass or polysterene was evaluated by counting the tracer amount remained in solution. As shown in Table 2, a considerable amount of tracer was unspecifically bound by the walls of the tubes. Furthermore, the percentage of tracer bound was found to be time-dependent. To avoid unspecific binding, 5% BSA was added to the incubation mixture. When the experiment was repeated under these conditions, the recovery of the tracer was nearly 100%.

### 2. *Precipitation with ammonium sulphate and effect of α-globulin*

In incubation mixtures with low globulin content, the precipitation with ammonium sulphate may not

Table 2. Adsorption of DHT to the walls of the test tubes in protein free buffer

| | Glass tubes | Polystyrene tubes |
|---|---|---|
| DHT total (c.p.m.) | 327.400 c.p.m. | 327.400 c.p.m. |
| DHT in solution (c.p.m.) after addition of ammonium sulphate | 138.400 c.p.m. | 176.200 c.p.m. |
| Adsorption (%) | 57.7% | 47% |

be quantitative [20]. To check this possibility, the SHBG content was measured in a 20-fold diluted serum sample using a buffer containing either 5% BSA or 5% BSA plus 2% bovine γ-globulin. The average c.p.m. in the precipitate was 2695 ± 342 ($n = 6$) without γ-globulin and 3583 ± 385 ($n = 6$) with γ-globulin. This shows that the precipitation is quantitative only if the γ-globulin content at low SHGB concentrations is 2 mg/ml.

3. *Dose response*

When the optimum assay conditions were used, a linear relationship was found over at least one order of magnitude. In Fig. 2 the radioactivity on the filter is plotted against the SHBG-concentration in a dilution series of serum.

4. *Reproducibility*

The average intra-assay coefficient of variation for triplicate determinations was 5.1% ($n = 20$). The inter-assay variation coefficient as measured by determinations in the same plasma pool was 5.9% ($n = 10$). It was not possible to determine the absolute accuracy as an active SHBG which could fully bind was not available. However, the linear dose response curve demonstrates the internal accuracy of the assay and the validity of our theoretical assumptions. The method of Rosner *et al.* [1] has a coefficient of variation between 5.5 and 9.7%, whereas the current method provides similar coefficients of variation.

5. *Detection limits*

The detection limit is given by the smallest significant difference from the blank value. For a blank value of 60000 counts/5 min ± 3000 S.D. ($n = 5$) and for triplicate sample determinations this difference was calculated by the one-sided Student's '$t$'-test. For a confidence limit of 5% the detection limit was 0.74 mg/l and for a confidence limit of 1% it was 1.1 mg/l.

Table 3. SHBG concentration in boys and men

| Boys Puberty stage [18] | Number ($n$) | Serum SHBG concentration (μg/ml ± SD) |
|---|---|---|
| I | 4 | 7.7 ± 1.5 |
| II | 3 | 5.6 ± 1.2 |
| III | 4 | 4.8 ± 0.9 |
| IV | 4 | 3.0 ± 0.9 |
| V | 3 | 3.1 ± 0.5 |
| Adult men | 6 | 1.9 ± 0.7 |

6. *Reference values*

Reference values during male pubertal development are given in Table 3. SHBG concentration significantly (using two-sided Student's '$t$'-test) dropped from puberty stage 1 to stage 2 and from stage 3 to stage 4. A further significant drop was observed after the end of puberty.

## DISCUSSION

The purpose of this work was to develop an assay method for serum SHBG which could be utilized in clinical studies. Such a method must be simple, rapid and at the same time be acceptably accurate and reliable. Procedures like equilibrium dialysis [2], steady state disc electrophoresis [17, 21], differential dissociation [23], cross immune electrophoresis [24] are technically too complicated for this purpose.

The principle of the saturation assay as devised by Rosner *et al.*[1] was found to be the most suitable for our study. Apart from some minor modifications the major modification was the replacement of the centrifugation step by filtration on membrane filters. Using centrifugation to separate bound and unbound DHT, SHBG concentration in the sample must be calculated from a small difference between two large numbers of counts. An excess of DHT greater than 20-fold, which is necessary to achieve saturation, brings the difference down to 5% of total DHT, while a difference of 50% between DHT total and unbound implies only a 2-fold excess of tracer, which is insufficient for saturation. Using filtration on membrane filters one can directly measure the radioactivity bound to SHBG. Further, the use of protein containing buffer makes it possible to determine SHBG in very diluted albumin free samples, whereas the method of Rosner *et al.*[1] suffers from incomplete precipitation and adsorption artefacts. This makes the current assay especially useful for determining SHBG during isolation procedure.

The SHBG concentrations in serum measured by the present method in normal males agree well with those previously reported by Rosner[1, 4] and are comparable on a molar basis with the values found by Ritzén and Hansson[25].

In a recently published paper Tulchinsky and Chopra[26] used a competitive ligand binding assay for

quantitative measurement of SHBG in serum. In their investigation they found that mean values for SHBG concentration were lower in prepubertal boys when compared to newborn males, but higher when compared to adult males.

Our previous studies [14, 15] on testosterone-binding affinity and the present data indicate that in peripheral plasma the androgens are quantitatively more bound in the prepubertal males than in the adults. It is possible that binding is the limiting factor in the rate at which sex steroid hormones have access to cells, depending on cell characteristics; and with the process of male sexual maturation more hormones are available.

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