Simplified Method for Measuring Sex-Hormone Binding Globulin

Drawshan I. Fattah and T. Chard

We describe a rapid method for measurement of sex-hormone binding globulin. Serial dilutions of pregnancy serum are prepared in serum from males that has been pre-treated by heating to 60 °C for 1 h to destroy endogenous binding globulin, which is then determined by a long-used technique to yield a set of "standards." In the assay itself, a fixed amount of [3H]-labeled and unlabeled dihydrotestosterone is incubated with standard or unknown, and the bound fraction precipitated with saturated ammonium sulfate. A plot of percent of the steroid bound vs standard dilution yields a sigmoid curve, from which the results in unknowns can be read by simple extrapolation. Within-assay CVs for pools of serum from men, women, and women in late pregnancy were 6.56, 9.59, and 8.4%, respectively. Between-assay CVs for the same pools were 8.05, 9.5, and 11.5%, respectively. The correlation between results obtained by this method and those of the older technique was 0.95 for samples from non-pregnant subjects and 0.73 for those from pregnant women. Our procedure is simpler and faster than previous methods and accurately measures the differences in the globulin in sera from men, women, and pregnant women. Forty to 50 samples can be assayed in a working day.

Several methods have been described for the measurement of sex-hormone binding globulin (SHBG) (Table 1). The most commonly used are those based on the ammonium sulfate precipitation technique described by Rosner (19, 20). This technique is based on saturation of the binding sites on SHBG with 5α-dihydrotestosterone (DHT) and precipitation of the bound complex with saturated ammonium sulfate. The mass of bound DHT, assessed by including a tracer amount of 3H-DHT, gives a direct measure of SHBG binding site concentration. Although this method gives excellent results, it requires eight assay tubes of each sample and a complex calculation of the final result, thus seriously constraining throughput. We describe here a new method that involves use of artificially prepared "standards" of SHBG. This reduces the number of tubes to four, greatly simplifies the calculation, and gives results identical to those of the Rosner technique.

Materials and Methods

Reagents

5α-Dihydro[1,2,4,5,6,7(n)-3H]testosterone, 122 kCi/mol (cat. no. TRK 443; Radiochemical Centre, Amersham, U.K.) was used without further purification. 5α-Dihydrotestosterone was from Sigma Chemical Co., St. Louis, MO 63178; ammonium sulfate and all other chemicals "Analar" grade, were supplied by BDH Chemicals, Poole, U.K. The phosphate buffer used throughout was 50 mmol/L, pH 7.4, and contained, per liter, 4.5 g of sodium chloride and 2 g of sodium azide as preservative.

Table 1. Methods for the Measurement of SHBG

<table>
<thead>
<tr>
<th>Principle</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrium dialysis on Sephadex G-25</td>
<td>(1, 2)</td>
</tr>
<tr>
<td>Multiple equilibrium dialysis</td>
<td>(3)</td>
</tr>
<tr>
<td>Sephadex ultrafiltration</td>
<td>(4)</td>
</tr>
<tr>
<td>Paper electrophoresis/polyacrylamide gel electrophoresis</td>
<td>(5-7)</td>
</tr>
<tr>
<td>Adsorption on charcoal</td>
<td>(8)</td>
</tr>
<tr>
<td>Flow dialysis</td>
<td>(9, 10)</td>
</tr>
<tr>
<td>Steady-state gel filtration</td>
<td>(11-13)</td>
</tr>
<tr>
<td>Chromatography on Cibacron Blue/LH20</td>
<td>(14, 15)</td>
</tr>
<tr>
<td>Adsorption on Florisil</td>
<td>(16, 17)</td>
</tr>
<tr>
<td>Precipitation with second antibody</td>
<td>(18)</td>
</tr>
<tr>
<td>Precipitation with ammonium sulfate</td>
<td>(19-24)</td>
</tr>
</tbody>
</table>

Procedures

Measurement of radioactivity. Tritium was measured in a Model 240 β-liquid scintillation counter (Packard Instrument Co., Downers Grove, IL 60515) with 1 mL of sample and 4 mL of liquid scintillant [4 g of 2,5-diphenyloxazole (POPOP) in 600 mL of AR grade toluene and 400 mL of Triton X-100 or Triton X-114 surfactant]. The mixture of sample and scintillant is allowed to stand for at least 3 h before the radioactivity is counted, and this counting is done long enough for the counting error to be 1.5% or less.

Preparation of SHBG "standards." A 30-mL pooled specimen of serum was obtained from women at 15-30 weeks of gestation. The SHBG concentration in this pool was 309.8 (SD 10) nmol/L by the modified Rosner method (19, 20). Six serial dilutions (from 1/2.5 to 1/80) were prepared in serum from males, which had been pre-treated by heating at 60 °C for 1 h to destroy endogenous SHBG (25). Aliquots of 250 μL were frozen and stored at −20 °C. SHBG in each standard, as measured six times by the Rosner technique, was assigned values from 3.75 to 120.0 nmol/L.

Principle of the present method. In the present method a single fixed quantity of 3H-DHT and DHT is added to all sample and standards. When equilibrium is reached, the bound DHT is precipitated with saturated ammonium sulfate. The results for the samples are then read from the standard curve by simple extrapolation. However, because samples differ in their quenching of the 3H-tracer, additional tubes must be included as "quench controls" containing an excess of DHT.

Assay procedure. The assay is done in 11.5 × 75 mm disposable glass tubes. Four tubes are set up for each sample or standard. Reagents are added as follows: (1) 400 μL of sample (serum or plasma) or standard, each diluted eightfold with buffer. (2) 100 μL of a mixture of DHT and 3H-DHT in buffer: 0.75 ng of DHT, including 15 000 dpm 3H-DHT, to two assay tubes, and 100 ng of DHT, including 15 000 dpm 3H-DHT, for two quench-control tubes.

Incubate the tubes for 10 min at 4 °C, then add an equal volume of saturated ammonium sulfate (also 4°C), mix by vertical rotation, and leave for 10 min at 4 °C. [Note: this

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Fig. 1. Binding of DHT (0.75 ng DHT + 15,000 dpm $^3$H-DHT per tube) by serial dilutions of late pregnancy serum with heated serum from males

The SHBG values on the horizontal axis were assigned on the basis of multiple assays by the Rosner (19) technique.

Temperature is essential to prevent dissociation of the DHT-SHBG complex (26). Centrifuge (1500 $\times$ g, 20 min, 4 °C) and decant the supernate (free fraction) into 4.0 mL of scintillation fluid, mix, and leave for 3 h to equilibrate before counting. The percentage of DHT-bound is calculated as:

\[
\text{Counts in quench-control tube} - \text{counts in assay tube/counts in quench-control tube} \times 100
\]

The standard curve is plotted as shown in Figure 1. Results of unknowns are read from this curve.

Serum Samples for Correlation Study

Serum samples were obtained from 60 male patients and from 60 non-pregnant women patients with hirsutism, infertility, or hypo- or hyperthyroidism. A further 56 samples, obtained from pregnant women between 16 and 36 weeks of gestation, were assayed at a dilution of fivefold in the heated male serum.

Fig. 2. Binding of various labeled steroids by late pregnancy plasma, as a function of time

In each case, the bound fraction was precipitated with ammonium sulfate (600 g/L) after the incubation time shown. (●—●) pregnancy plasma with $^3$H-DHT; (——) pregnancy plasma with $^3$H-testosterone; and (Δ—Δ) pregnancy plasma with $^3$H-cortisol

Fig. 3. Effect of DHT concentration on apparent binding of DHT by late pregnancy serum

Serial dilutions of serum from late pregnancy in heated serum from males (3.75-120 nmol of SHBG per liter) were incubated for 10 min with a fixed amount of $^3$H-DHT (15,000 dpm/tube) and various amounts of unlabeled DHT per tube: $A = 7.5$ ng, $B = 3.0$ ng, $C = 1.5$ ng, $D = 0.75$ ng, and $E = 0.375$ ng).

Optimization of the Assay

The optimal incubation interval was determined as follows. Four hundred microliters of pregnancy serum, diluted eightfold with buffer, was incubated with 0.1 mL of either $^3$H-DHT, $^3$H-cortisol, or $^3$H-testosterone at a concentration of 15,000 dpm/tube and incubated at 4 °C for 1, 5, 15, 30, 45 min and 1, 2, 3, 4, and 16 h. The bound complex was precipitated by adding an equal volume of saturated ammonium sulfate. Equilibrium was achieved in 10 min (Figure 2). Nonspecific precipitation due to cortisol binding globulin was <10%.

To establish the optimal concentration of DHT, we incubated five sets of standards with a fixed amount of $^3$H-DHT (15,000 dpm/tube) and various concentrations of unlabeled DHT (0.375-7.5 ng/tube). Three different control sera with low, medium, and high values of SHBG were included with each set of standards. On the basis of the results (Figure 3) we selected a concentration of 0.75 ng of DHT per tube.

Results

Precision. Within-assay precision was calculated from six replicate estimations on three control pools. The values were 6.56% (mean value 24.09 nmol/L), 9.95% (mean value 47.99 nmol/L), and 8.94% (mean value 290.42 nmol/L).

Between-assay precision for replicate estimations in six consecutive assays during six weeks was 8.05% (mean value 24.09 nmol/L), 9.5% (mean value 47.99 nmol/L), and 11.95% (mean value 290.42 nmol/L).

Method comparison. Results by the new method and old agreed well for both non-pregnant and pregnant subjects. For 120 non-pregnant subjects the correlation coefficient was 0.95 ($y = 4.01 + 0.89x$); for 56 pregnant subjects it was 0.73 ($y = 0.27 + 0.9x$).

Reference intervals. For 18 normal women (not on oral contraceptive agents) the mean SHBG value was 63.4 (SEM 17.4) nmol/L, the range 38.0-102.5 nmol/L. For 13 normal men the mean SHBG value was 29.7 (SEM 9.0) nmol/L, ranging from 17.0 to 50.0 nmol/L. For 20 pregnant women the mean SHBG value was 349.37 (SEM 68.93) nmol/L, ranging from 192 to 444.
Table 2. Present Method for SHBG Compared with the Rosner (19) Technique

<table>
<thead>
<tr>
<th>Present method</th>
<th>Rosner method</th>
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<tbody>
<tr>
<td>Principle: a fixed amount of $^3$H-DHT and DHT is added to all samples and standards; at equilibrium, bound DHT is precipitated with saturated ammonium sulfate</td>
<td>Principle: saturation of the binding sites on SHBG with different amounts of DHT; at equilibrium, bound DHT is precipitated with saturated ammonium sulfate</td>
</tr>
<tr>
<td>Serial dilutions of pregnancy serum used as standards</td>
<td>No standards</td>
</tr>
<tr>
<td>Four assay tubes/sample</td>
<td>Eight assay tubes/sample</td>
</tr>
<tr>
<td>Calculation by extrapolation of unknown results to standard curve</td>
<td>Complex calculation</td>
</tr>
</tbody>
</table>

Discussion

Our simplified method for measuring SHBG permits analysis of 40–50 samples during a working day. Furthermore, a wide range of values can be measured in non-pregnant subjects with a single fixed dilution of the sample. The correlation with the Rosner technique was excellent.

The present method has several points of similarity to those described by Anderson (17) and Tulchinsky and Chopra (18). However, the key feature of the technique is the use of a set of artificially prepared standards, which permits direct calculation of the results in exactly the same manner as for a radioimmunoassay. Comparability of standard and unknowns, which is essential to this approach, is achieved by preparing material containing a high concentration of SHBG (late-pregnancy serum), diluted with normal serum heated to destroy endogenous SHBG. This is equivalent to the use of hormone-free serum as a medium in other assay systems. It results in a simplified assay procedure, and a very much simplified calculation (Table 2).

The only remaining disadvantage is the need to include control tubes to correct for different rates of sample quenching. If this problem can be overcome, for example by the use of DHT labeled with a gamma-emitting isotope or a fluorophore, then the technique would have the convenience ordinarily associated with RIA.

References