Interference of Sex-Hormone Binding Globulin in the "Coat-A-Count" Testosterone No-Extraction Radioimmunoassay

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We evaluated a "no-extraction" assay system for serum testosterone [Diagnostic Products "Coat-A-Count" testosterone (direct)] with respect to the influence of sex-hormone binding globulin (SHBG). We conclude that the SHBG concentration strongly affects this estimation. The quantity of testosterone measured in the presence of 90 nmol of SHBG per liter (the upper reference limit of SHBG for women) was only about 40% of that measured at the lower limit of the SHBG reference interval (30 nmol/L). For SHBG concentrations <30 nmol/L one can expect the testosterone concentrations measured to be relatively too high.

The usual reason for measuring testosterone in serum is to investigate hypogonadism and hormone-replacement therapy in men (1). In the case of women, it is often used as a marker in hyperandrogenism, although 50% of all hirsute women, whether normal, oligo-, or amenorrhoeic, have normal concentrations of testosterone in their serum (2, 3). For better discrimination in hyperandrogenism, the use of the concentration of free testosterone or the ratio of testosterone to sex-hormone binding globulin (SHBG) has been proposed (4, 5). Recently Cumming and Wall (6) and Manni et al. (7), using the Pardridge model (8), hypothesized that both albumin-bound and free testosterone are readily available to cells and suggested that measuring the concentration of non-SHBG-bound testosterone might be more suitable. Currently, the discussion as to whether one could better use free testosterone or a testosterone/SHBG index is still unresolved, and the measurement of total testosterone remains important.

Direct estimation of the testosterone concentration in serum is considerably easier than measurements involving extraction, and it would be expected to yield a more precise result. An important methodological problem is how to remove binding proteins in serum, most importantly SHBG, from the analytical system. Dunn et al. (9) reported that, for normal women, the testosterone in plasma is distributed in proportions of 1:66:33 for free:SHGB-bound:albumin-bound. Ismail et al. (10) reported some outliers on decoupling the proteins in various direct assay systems, and Schade (11) had the same experience.

Consequently, in evaluating a kit for direct estimation of testosterone, the first aspect we addressed was the influence of SHBG.

Materials and Methods

Kits

In the "Coat-A-Count" no-extraction procedure for testosterone (Diagnostic Products Corp., Los Angeles, CA), 25 μL of serum and 1000 μL of phosphate buffer containing 125I-labeled testosterone are added to the antibody-coated tube. After a 3-h incubation at 37 °C the tube is washed three times with 1.5-mL portions of isotonic saline (9 g/L NaCl). The radioactivity of each tube is then counted long enough to accumulate at least 5000 counts.

The precision (CV) of the amount of antibody coated to the test tube was found to be 2.12 ± 0.48% (n = 2 × 50 tubes). We dispensed into each of 50 antibody-coated tubes 1.0 mL of 125I-labeled testosterone/buffer reagent containing 2.5 mL of serum and 100 mL of reagent. The radioactivity in each tube was counted before and after washing. The intraassay CV, determined from the duplicate results, was 12.2% in the testosterone concentration range 0 to 5 nmol/L (n = 12) and 3.6% in the 5 to 10 nmol/L range (n = 63).

Using the "Sex Hormone Binding Globulin [125I] Immuno- radiometric Assay Kit" (Farmos Diagnostica, Oklunso, Finland), we first reacted 50 μL of serum that had been diluted 100-fold with buffer with 125I-labeled mouse antibody to SHBG and rabbit antibody to SHBG. After a 1-h incubation at room temperature, we added donkey anti-rabbit antibody, fixed on magnetic particles ("Amerlex-M"; Ameraham, Cardiff, U.K.), substituting this magnetic-separation reagent for the cellulose-based separation reagent provided with the kit. After the samples stood for 5 min, the 125I-labeled-SHBG antibody complex and the free 125I-labeled mouse antibody to SHBG were separated by use of a

<p>| Table 1. Effect of SHBG Concentration on Analytical Recovery of Added Testosterone (T) |
|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>SHBG, nmol/L</th>
<th>125I, nmol/L</th>
<th>&quot;Zero&quot; kit standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.0 (20)</td>
<td>0.0 (0)</td>
</tr>
<tr>
<td>9.6</td>
<td>8.8 (42)</td>
<td>11.6 (121)</td>
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<tr>
<td>19.2</td>
<td>11.7 (46)</td>
<td>20.6 (107)</td>
</tr>
<tr>
<td>38.5</td>
<td>16.4 (35)</td>
<td>40.3 (105)</td>
</tr>
<tr>
<td>57.7</td>
<td>20.7 (31)</td>
<td>57.4 (99)</td>
</tr>
<tr>
<td>96.2</td>
<td>34.4 (33)</td>
<td>100.2 (104)</td>
</tr>
</tbody>
</table>

Fig. 1. Influence of SHBG concentration on measured testosterone (T) concentration in samples containing 25 (■) and 71 (●) nmol of testosterone per liter.
Fig. 2. Correlation between analytical recovery of added testosterone (T_{rec}) and concentration of SHBG or albumin without (●) or with (□, □) heat treatment 1 h at 60 °C

\[ T_{rec} = 58 - 0.51 \cdot [\text{SHBG}] \; \text{nmol/L} \]

\[ r = 0.90, p > 0.995 \]

side-pull magnetic separator (Corning Medical, Medford, MA), and the liquid phase was aspirated and discarded. We washed the bound phase with three 1-mL portions of water and counted its radioactivity. The intra-assay CV for SHBG, measured from the duplicates, was 4.1% for concentrations in the range 8–135 nmol/L (n = 95).

**Apparatus and Supplies**

For all samples we counted radioactivity with a Tracerlab Gamma-set 500 (Medilab-Select, Mechelen, Belgium), equipped with a 8.6-cm well-type NaI(Tl) crystal. For gamma spectrometry we used a Model S-30 multichannel analyzer (Canberra Industries Inc., Meridan, CT). To fit calibration-curve data we used a weighted logit/log curve-fitting algorithm (12, 13). An aliquot of each serum sample was stored at −20 °C until analysis. To obtain a "null serum" we filtered a pooled specimen to serum and treated it with activated charcoal (Norit SX-Plus; Norit, Amsterdam, The Netherlands), 50 g per liter of serum.

For analytical-recovery experiments we used testosterone (no. T 1500; Sigma Chemical Co., St. Louis, MO) dissolved in ethanol. To prepare a standard curve in the absence of SHBG, we used various concentrations of testosterone dissolved in a solution containing 10 g of human albumin (CLB, Amsterdam, The Netherlands) per liter of phosphate-buffered saline (0.11 mol/L, pH 7.2).

**Results and Discussion**

The influence of SHBG on the measurement of testosterone is demonstrated in the next experiments. We supplemented aliquots of a pool of serum from pregnant women and of a "zero" kit standard with various amounts of a 20 g/L ethanolic solution of testosterone. For the supplemented "zero" kit standard, we found a recovery of about 100%; however, for the supplemented pregnancy pool serum the mean averaged 37% (Table 1). In pregnancy the SHBG concentration is three to six times the upper limit for non-pregnant women; however, the analytical recovery of testosterone is not influenced only at high SHBG concentrations, as illustrated in Figure 1. This Figure presents results of an experiment wherein various dilutions were prepared from a high-concentration pool containing 338 nmol of SHBG per liter with a pool containing only 41 nmol of SHBG per liter. Aliquots of these mixtures were supplemented to contain 25 and 71 nmol of testosterone per liter. A strong correlation was found between recovery of testosterone and SHBG concentration over the entire reference range of SHBG concentrations.

To confirm that this "SHBG-effect" is not unique to pregnancy sera, we assayed 16 samples from non-pregnant patients, samples selected to span a range of SHBG concentrations. An aliquot of each was supplemented with 840 nmol of testosterone per liter of "null" serum. The expected recovery was 42 nmol of testosterone per liter. The measured recoveries are plotted vs the SHBG concentrations in Figure 2a. A significant correlation was found (\( r = 0.90, p > 0.995 \)). The fitted relation indicates a recovery of 43 nmol/L at an SHBG concentration of 30 nmol/L and a recovery of 12 nmol/L at 90 nmol of SHBG per liter.

To investigate the possibility that denaturation of serum can remove any SHBG interactions with the kit, we heated aliquots of the previous samples for 1 h at 60 °C. After this heating the degree of recovery was unchanged.

![Fig. 3. Relation between amount of testosterone standards weighed into human serum albumin/phosphate-buffered saline solution (HSA/PBS) and testosterone concentration as measured with Count-A-Count kit (T{sub}C-A-C) in triplicate](image-url)
treatment SHBG was undetectable. The testosterone data in the SHBG-denatured samples were calculated against standard testosterone concentrations, to eliminate the SHBG influence of the supplier's assay standard containing 22 nmol of SHBG per liter (Figure 3). The calculated recoveries of the heat-treated samples are plotted against the original SHBG concentration in Figure 2b. As can be seen, the SHBG dependence has disappeared. Also in these heat-treated samples no influence of albumin is found (Figure 2c).

The "SHBG effect" is demonstrable through the whole testosterone concentration range. The data points show more scatter in the lower concentration range (<5 nmol of testosterone per liter), which can be largely explained by the CV for the assay in this range (Figure 4). A consequence of the "SHBG effect" is that the observed value for a measurement of testosterone in the presence of 90 nmol of SHBG per liter (the upper reference limit for women) is only about 40% of the value measured at the lower limit of the SHBG reference interval, 30 nmol/L. At lower SHBG concentrations a relatively too-high testosterone concentration is measured as compared with the same quantity of testosterone in a sample containing 30 nmol of SHBG per liter.

![Graph showing the ratio of testosterone concentrations measured after SHBG is denatured by heating at 60 °C (TN) and without denaturation of SHBG (T), in relation to original SHBG concentration.](image)

Fig. 4. Ratio of testosterone concentrations measured after SHBG is denatured by heating at 60 °C (TN) and without denaturation of SHBG (T), in relation to original SHBG concentration

- ●: TN < t60
- ▲: 8 < TN < 40
- ■: TN > 40 nmol/L

In conclusion: over the whole concentration range for testosterone, the SHBG concentration strongly influences estimation of testosterone with this "Coat-A-Count" direct testosterone assay.

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References