

The analog free testosterone assay: are the results in men clinically useful?

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Men with low testosterone concentrations are usually hypogonadal. However, because variations in the testosterone transport protein, sex hormone-binding globulin (SHBG), directly influence the total testosterone concentration, confirmation of a low testosterone with a measurement of free testosterone or "bioavailable" testosterone (BAT) is recommended. In the present study, we examined the relationship of SHBG with free testosterone (Coat-A-Count assay, Diagnostic Products) and with BAT in men ($n = 29$) and women ($n = 28$) who participated in a study of the metabolic determinants of body composition. As expected, total testosterone was strongly positively correlated with SHBG among men ($r = 0.68$; $P < 0.01$). Although the BAT was independent of SHBG in men ($r = 0.02$), SHBG was an important predictor of free testosterone ($r = 0.62$; $P < 0.01$). In contrast, in women serum concentrations of total testosterone ($r = -0.26$; $P = 0.17$), free testosterone ($r = -0.30$; $P = 0.17$), and BAT ($r = -0.46$; $P = 0.013$) all tended to be lower with increasing SHBG. Free testosterone was nearly perfectly positively correlated with total testosterone ($r = 0.97$) in men, among whom free testosterone represented a relatively constant percentage of the total testosterone (0.5–0.65%), and the percentage of free testosterone was unrelated to SHBG. Thus the Coat-A-Count free testosterone concentration in men, like the total testosterone concentration, is determined in part by plasma SHBG. Accordingly, androgen deficiency may be misclassified with this assay in men with low SHBG. Moreover, the previous findings of reduced free testosterone concentrations with hypertension or hyperinsulinemia or as a risk factor for developing type 2 diabetes,

conditions in which SHBG is reduced, may have been methodology-related.

When men present with symptoms or have clinical findings suggesting hypogonadism, the clinician generally begins the diagnostic evaluation by measuring serum total testosterone. Because variations in the serum concentration of the testosterone transport protein, sex hormone-binding globulin (SHBG), directly influence the total testosterone concentration (1), either free testosterone or the concentration of testosterone not bound to SHBG [non-SHBG-bound testosterone (non-SHBG-T)] is measured to confirm the diagnosis of hypogonadism when the total testosterone value is borderline or when the clinical findings and the plasma total testosterone concentration do not agree. Some physicians measure free testosterone routinely. This non-SHBG-T has also been called "bioavailable" testosterone (2). The non-SHBG-T is usually determined by separating the SHBG-bound testosterone from the free and albumin-bound testosterone by ammonium sulfate precipitation and then multiplying the total testosterone concentration by the percentage not bound to SHBG. The non-SHBG-T assay is technically simple to perform but is a two-step procedure.

The Coat-A-Count free testosterone assay (Diagnostics Products Corp.) is a popular single-step non-extraction method in which an ^{125}I -labeled testosterone analog competes with free testosterone in plasma for binding to a testosterone-specific antiserum immobilized to a polypropylene tube. Neither SHBG nor albumin are thought to influence the free testosterone concentration measured by analog assay. However, for eugonadal men the free testosterone values obtained with this method as a percentage of the total testosterone (0.2–0.64%) are much lower than the 1.5–4.0% determined by calculation (3), equilibrium dialysis (4), or ultrafiltration (5, 6). To explore this difference and the discrepancies between the Diagnostics Products kit result and other methods that have been noted by other investigators (7, 8), we examined the relationship of SHBG to the free and non-SHBG-T concentrations in thin and obese men and women who

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participated in a study of the metabolic determinants of body composition.

Materials and Methods

SUBJECTS

Blood samples were obtained after an overnight fast from 28 healthy women, ages 25–45 years, and 29 healthy men, ages 24–47 years, who were recruited by advertisement for a study approved by the Health Sciences Institutional Review Board of the University of Pittsburgh. The body mass index ranged from 19.6 to 39.6 kg/m² among the women and from 21.3 to 41.0 kg/m² among the men. There were 9 African-Americans, 1 Asian-American, and 37 Caucasians in the study population.

ANALYTICAL METHODS

Total testosterone was measured with the Coat-A-Count total testosterone solid phase RIA kit (Diagnostic Products). The within-assay CV (n = 4 replicates) was 4.6% at 13.1 nmol/L and 8.5% at 2.4 nmol/L.

Free testosterone was measured with the Coat-A-Count free testosterone solid phase RIA kit (Diagnostic Products). The within-assay CV (n = 4 replicates) was 1.1% at 0.56 pmol/L and 6.0% at 0.84 pmol/L.

SHBG was measured using the Active SHBG two-site immunoradiometric assay (Diagnostic System Laboratories). The within-assay CV (n = 4 replicates) was 13.5% at 29 nmol/L and 9.7% at 107 nmol/L.

The determination of non-SHBG-T was based on the separation, by 500 g/L ammonium sulfate precipitation, of serum SHBG-bound testosterone after incubation with ³H-testosterone at 23 C (9). The non-SHBG-T concentration was calculated by multiplying the percentage of tracer in the supernatant (not bound to SHBG) by the total testosterone concentration. The between-assay CV from a pool of serum from healthy women was 4.9%.

Samples were analyzed in one immunoassay kit to eliminate any effect caused by between-assay variation. The percentage of bioavailable testosterone was determined in two separate assays

STATISTICAL ANALYSIS

Data are presented as the mean ± SE. Linear regression was performed using Systat for Windows, Ver. 5 (Systat).

Results

The hormone profiles of the study groups are presented in Table 1. Serum concentrations of total and free testosterone were within the manufacturer's 95% central confidence range, and non-SHBG-T concentrations were

within reference values in all 28 men. In one woman, free testosterone was increased, and non-SHBG-T was increased in 8 of the 29 women, of whom 2 also had an increased total testosterone. These eight women tended to be heavier (body mass index, 33.3 ± 1.9 vs 29.9 ± 1.2 kg/m²; P = 0.23), and to have lower plasma SHBG (66 ± 16 vs 108 ± 13 nmol/L; P = 0.093) than the remaining 21 women. Although there was no formal assessment of menstrual function in this study, one of these eight women described oligomenorrhea.

Fig. 1 shows the relation between SHBG and total, free, and bioavailable testosterone in men. As expected because it is a high affinity testosterone-binding protein, SHBG was an important predictor of the plasma total testosterone concentration (r = 0.68; P < 0.01). By contrast, the non-SHBG-T was independent of plasma SHBG (r = 0.02). Unexpectedly, SHBG and free testosterone were highly positively correlated (r = 0.62; P < 0.01) among men. Among women on the other hand, total (r = -0.26; P = 0.17), bioavailable (r = -0.46; P = 0.013), and free testosterone (r = -0.30; P = 0.17) were each inversely correlated with SHBG (data not shown).

Among the explanations for the unexpected positive correlation between SHBG and free testosterone only in men is that SHBG binds the ¹²⁵I-labeled testosterone analog, creating a reservoir with less tracer available to compete with unlabeled testosterone for binding to the solid phase antiserum. Because the concentration of unoccupied SHBG steroid binding sites is quite variable among men, the measured free testosterone concentration increases as the concentration of SHBG in the sample increases. This effect would not be expected in plasma samples from women, among whom the molar concentration of SHBG far exceeds that of total testosterone, creating a surplus of unoccupied SHBG sites in all samples (3). If this hypothesis is correct, adding SHBG to male plasma would increase the measured free testosterone concentration. To test this notion, plasma from one man was supplemented with SHBG in pregnancy plasma as shown in Table 2. Contrary to our hypothesis, however, the measured free testosterone was lower than the expected value as SHBG was increased in the sample, with a recovery of 68% at a dilution of 3:1 (male plasma: pregnancy plasma).

We also tested whether the ¹²⁵I-testosterone analog binds to SHBG by selective adsorption with Concanavalin A-Sepharose, which is known to bind SHBG (10). Table 3 reveals that minimal quantities of the tracer were selectively adsorbed by Concanavalin A and that adsorption

Table 1. Hormonal characteristics of the study group.

Sex	Age, years	BMI, ^a kg/m ²	Total testosterone, nmol/L	SHBG, nmol/L	Bioavailable testosterone, nmol/L	Free testosterone, pmol/L
Female	36.5 ± 1.3	30.8 ± 1.1	0.98 ± 0.08	96.3 ± 10.8	0.32 ± 0.04	5.7 ± 0.6
Male	36.4 ± 1.1	31.2 ± 1.2	17.3 ± 1.26	58.6 ± 4.51	8.70 ± 0.80	96.7 ± 6.9

^a Body mass index.

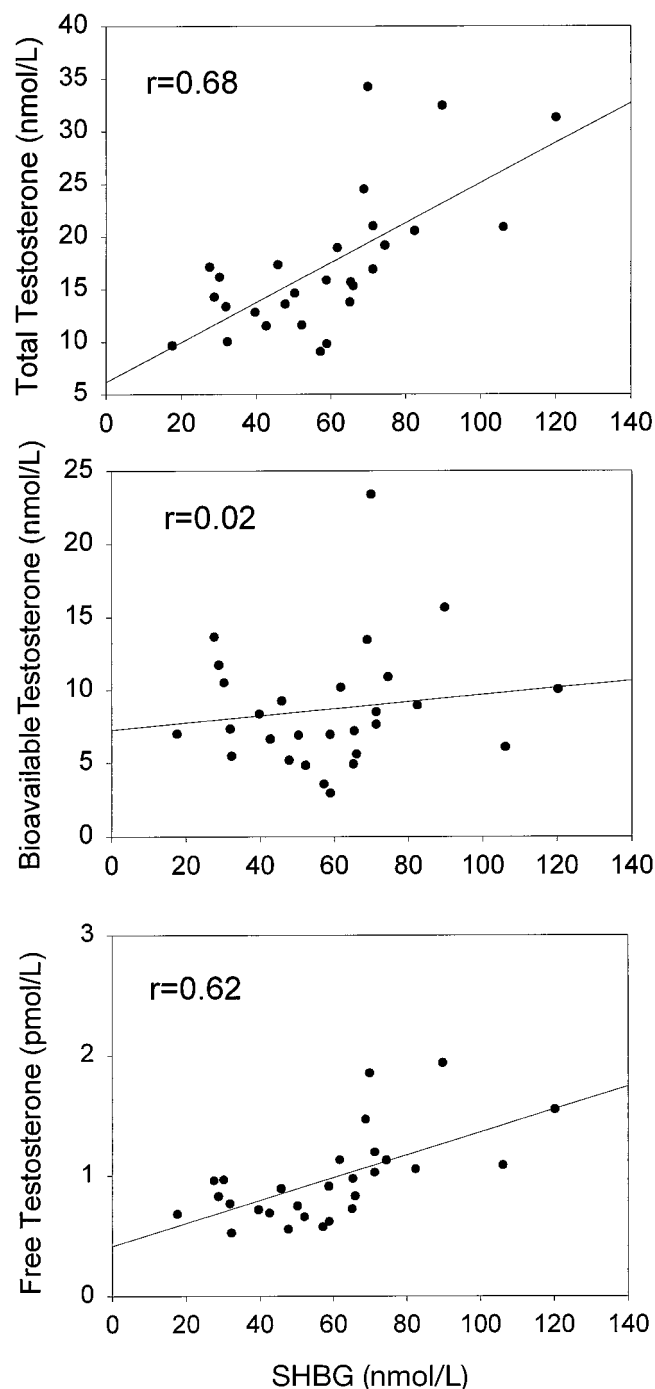


Fig. 1. Correlation between serum SHBG and total (*top*), bioavailable (*middle*), and analog free (*bottom*) testosterone concentrations in 28 healthy men.

was unrelated to the concentration of SHBG in the plasma sample.

We next calculated the percentage of free/total and bioavailable/total testosterone in the adult male plasma samples and related the result to the plasma concentration of SHBG (Fig. 2). We found that the percentage of free testosterone was quite constant (0.5–0.65%) in adult male

Table 2. Effect of added SHBG on the free testosterone concentration in adult male plasma.

Sample composition ^a	SHBG, ^b nmol/L	Free testosterone, pmol/L	
		Expected	Measured (%) ^c
100% M	52.5		61.0
100% P	212		14.1
95% M:5% P	60.5	58.5	58.9 (100)
90% M:10% P	68.4	56.2	50.9 (90)
85% M:15% P	76.4	53.9	36.9 (68)
80% M:20% P	84.4	51.5	33.0 (64)
75% M:25% P	92.4	49.2	33.3 (68)

^a Plasma from a healthy man (M) was mixed with plasma from a pregnant woman (P) in the indicated dilutions and measured in the free testosterone assay.

^b The values for SHBG in the diluted specimens are estimates.

^c The free testosterone measured value is also calculated as a percentage of the expected value.

plasma and was unrelated to the concentration of SHBG. By contrast, the percentage of non-SHBG-T was much more variable among men, ranging from 30% to 80%, and the non-SHBG-T was strongly inversely correlated with SHBG ($r = -0.66$; $P < 0.01$). Furthermore, as shown in Fig. 3, the concentrations of total and free testosterone were almost perfectly positively correlated ($r = 0.97$), whereas the concentration of non-SHBG-T was less strongly predicted by the total testosterone concentration ($r = 0.79$). Similar relationships were found among androgen concentrations in women, among whom the correlations of total with free and bioavailable testosterone concentrations were 0.94 and 0.80, respectively.

Discussion

Our data confirm the previous finding that the total testosterone concentration in the plasma of healthy men is highly positively correlated with the plasma concentration of SHBG (11). The principal new finding from this study is that SHBG is also an important determinant of free testosterone in men when measured with the Coat-A-Count free testosterone assay but not when assessed as bioavailable testosterone. Moreover, the Coat-A-Count

Table 3. Adsorption by Concanavalin A-Sepharose of Coat-A-Count ¹²⁵I-testosterone analog in plasma.^a

Sample	SHBG, nmol/L	¹²⁵ I-Testosterone bound to Concanavalin-A, %
Male rat plasma	0	2.8
Human male plasma	52.5	6.6
Human female plasma	151	5.2
Human pregnancy plasma	278	3.6

^a Plasma samples (100 μ L) in duplicate were added to 0.3 mL of a 1:1 slurry of Concanavalin-A-Sepharose (Pharmacia) in phosphate-buffered saline for 1 h at 23 °C with frequent mixing. Then, 20 254 cpm of ¹²⁵I-testosterone analog was added for 2 h with frequent mixing. The pellet was washed three times, and the radioactivity in the pellet was counted. Under the same conditions, SHBG in human male plasma bound 39% of labeled testosterone.

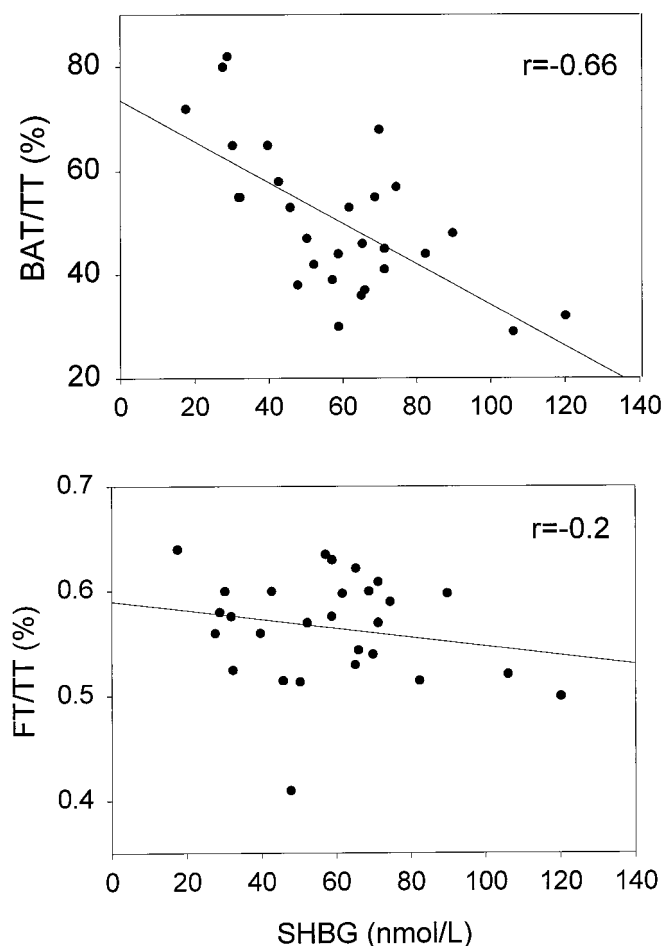


Fig. 2. Relationship between serum SHBG and the percentage of bioavailable (top) or free (bottom) testosterone in plasma samples from healthy men.

free testosterone assay appears to measure a constant fraction of the total testosterone in adult male plasma.

The purpose of the free or bioavailable testosterone determination is to correct the total testosterone concentration for the effect of variable binding by SHBG; thus, when testosterone production is within the reference range for eugonadal men but SHBG is low, the percentage of free testosterone in the sample should be increased. When SHBG is increased on the other hand, the percentage of free testosterone in the sample should be reduced. An inverse relationship between SHBG concentration and the percentage of free testosterone has been shown by computer modeling by Dunn et al. (3). In addition, the percentage of unbound testosterone in vitro is inversely related to the concentration of SHBG in the sample (12). Moreover, the percentage of free testosterone in plasma is inversely correlated with SHBG as men age (11, 13). In the present study, the percentage of non-SHBG-T was inversely related to SHBG, but the percentage of free testosterone was not.

Others (14, 15) have noted that free testosterone mea-

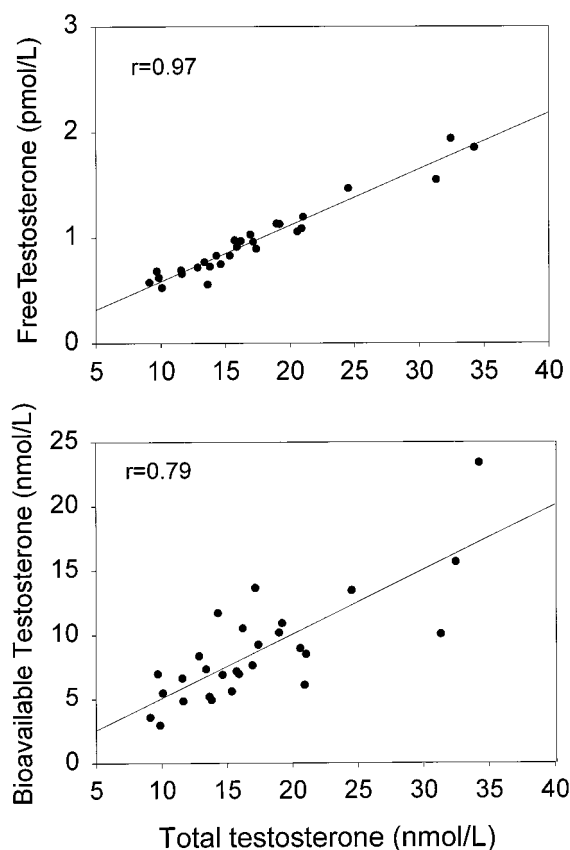


Fig. 3. Correlation between total and free (top) or bioavailable (bottom) testosterone concentrations in plasma from healthy men.

sured by the Coat-A-Count assay is influenced by the concentration of SHBG; however, the potential importance of this relationship was not emphasized. An SHBG effect on the Coat-A-Count testosterone assay result was previously reported by Slaats et al. (16). In that study the recovery of testosterone added in increasing amounts to pregnancy plasma (containing a high concentration of SHBG, although the concentration was not specified) was only 37%, indicating that a "too low value" is obtained at high SHBG concentrations. Our results using adult male plasma mixed with pregnancy plasma produced a similar result. In addition, there was no apparent binding of the ^{125}I -testosterone analog to SHBG, based on binding to Concanavalin A. Moreover, the lack of positive correlation between SHBG and free testosterone in women in our study confirms a previous study (17). Jowett et al. (7) have noted that analog tracers used in assays for the measurement of free thyroid as well as free steroid hormones do, in fact, bind to serum proteins, leading to diagnostic unreliability.

A nearly perfect correlation was found between the total and free testosterone assay values in adult male plasma, apparently with free testosterone being 0.5–0.65% of the total testosterone. By contrast, the percentage of free testosterone among men determined by calculation (3),

equilibrium dialysis (4, 18), or ultrafiltration (6) has a much broader range of ~1.5–4%. Similarly, the percentage of non-SHBG-T we observed among healthy thin and obese men ranged from 30% to 80% of the total testosterone concentration.

Epidemiological studies have concluded that total testosterone concentrations as well as free testosterone concentrations measured with the Coat-A-Count assay kit are low in men with hypertension (15), and that men with low total or free testosterone are at increased risk for developing diabetes (14, 19). The latter studies have tried to link testosterone production to central adiposity, insulin resistance, and hyperinsulinemia, each of which is associated with reduced SHBG. In light of the present results, other methods for free testosterone should be used to confirm those findings. When plasma samples contain substances that interact with SHBG and alter the distribution of testosterone, erroneous results may also occur.

One important clinical consequence of our findings relates to the evaluation of men for suspected hypogonadism who have low plasma SHBG, for example, in obesity, type 2 diabetes, or hypothyroidism (1). In clinical practice these men often present with sexual dysfunction and may have a low free testosterone value when tested with the analog method. Because testosterone production is presumed to be impaired, extensive and expensive diagnostic testing for hypothalamic-pituitary dysfunction is performed, usually with no abnormalities found (20). In addition, men with hypogonadism and increased SHBG concentrations whose total testosterone is consequently within the reference interval (21) may also be misclassified by the Coat-A-Count free testosterone assay.

Although the present findings imply that the Coat-A-Count free testosterone concentration provides essentially the same information as the total testosterone in men and fails to correct for differences in the plasma SHBG concentration, the subjects in this study were healthy men with testosterone values within the reference interval, so that confirmation of this notion will require additional data in hypogonadal men to include measurement of the testosterone production rate. Moreover, further studies are needed to thoroughly understand the differing relationship between SHBG and the distribution of plasma androgens in women and men.

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