Serum Bioavailable Testosterone: Assayed or Calculated?

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Background: Bioavailable testosterone (BT), circulating testosterone not bound to sex hormone–binding globulin (SHBG), is thought to easily penetrate cells. We compared BT measurements obtained by assays with those obtained by calculation with different testosterone association constants.

Methods: We obtained sera from 2 groups of hypogonadal men [group 1 (G1), 1421 samples; group 2 (G2), 170 samples] and a group of healthy men [group 3 (G3), 109 samples]. We added minute doses of [3H]testosterone to the sera, precipitated the SHBG-bound fraction of testosterone with ammonium sulfate (50% saturation), and then assayed serum BT (ABT) as %BT × total. Calculated BT (CBT) was determined with theoretical association constants of testosterone for SHBG (Ka = 1 × 10^9 L/mol) and albumin (Ka = 3.6 × 10^4 L/mol) and paired optimal Ka and Ks values obtained by use of Microsoft Excel software.

Results: CBT calculated with theoretical constants differed from ABT by >30% in 85.7% (G1), 84.1% (G2), and 79.2% (G3) of samples, and the mean CBT/ABT ratios were 1.57 (G1), 1.85 (G2), and 1.50 (G3) in spite of fairly good correlations. CBT calculated with paired optimal Ka and Ks differed from ABT by >30% in 85.4% (G1), 87.5% (G2), and 97.5% (G3) of samples, and mean CBT/ABT ratios were 0.95–1.04.

Conclusions: To obtain CBT values as close as possible to ABT, optimal paired association constants determined for each studied population must be used instead of the theoretical association constants. Considering the uncertainty of calculating BT, however, use of the ammonium sulfate precipitation method for determining BT is advisable.

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Bioavailable testosterone (BT),7 circulating testosterone not bound to sex hormone–binding globulin (SHBG), is a biological marker of androgenicity in men. In older men, BT is related to physiologic changes, decreased muscle strength, bone density, and depressed mood (1–5). Serum BT is widely assayed in testosterone replacement programs in older hypogonadal men (6, 7). BT is usually measured after removal of SHBG from serum by ammonium sulfate precipitation (50% saturation) and calculation of the percentage of non–SHBG-bound testosterone by use of tracer-binding methods (8, 9) or direct measurement of testosterone in supernatant that contains free and albumin-bound testosterone (10). Both free testosterone and BT may be calculated by measuring total testosterone, SHBG, and albumin concentrations in serum and using the equilibrium binding constants of testosterone to SHBG and albumin given in published equations (11).

Calculation of free testosterone by use of the association constants of testosterone for albumin (Ka = 3.6 × 10^4 L/mol) and SHBG (Ka = 1 × 10^9 L/mol) produced calculated results that corresponded to those obtained by equilibrium dialysis (11), but in some cases (n = 24) led to higher calculated BT than assayed BT. Because several theoretical SHBG association constant values (range of Ks values, 0.27 × 10^9 to 1.9 × 10^9 L/mol) have been reported (11–22), we compared assayed BT (ABT) concentrations measured by 2 different laboratories for 2 large populations of hypogonadal men (n = 1421 and 170), and 120 apparently healthy men with calculated BT (CBT) concen-

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trations obtained with different testosterone association constants.

**Participants and Methods**

Total testosterone, BT, and SHBG were assayed in fasting blood samples drawn from untreated and testosterone patch–treated hypogonadal men (group G1; 1421 samples) in laboratory 1 (Emi INSERM 03-37, Centre de Recherches Chirurgicales, CHU Henri Mondor, Créteil, France), from untreated hypogonadal men (group G2; 170 samples) in laboratory 1, and from untreated healthy men (group G3; 109 samples) in laboratory 1. The healthy participants were recruited in a health center (IRSA, Tours, France). The procedure was in accordance with the Helsinki Declaration, and each participant gave informed consent. Albumin was measured only in group G1. In groups G1 and G3, we performed total testosterone assays by time-resolved fluoroimmunoassay (TR-FIA) after extraction and then chromatography on Celite (20, 21). Interassay CVs were 4.9%, 5.1%, 4.2%, 4.6%, 3.6%, and 2.2%, respectively, for the following concentrations of serum controls: 2.60 (75), 5.20 (150), 8.66 (250), 10.40 (300), 13.86 (400), and 20.80 (600) nmol/L (ng/dL). Compared with gas chromatography–mass spectrometry (GCMS), the correlation coefficient (r) was 0.99, and the equation for the regression curve was as follows: TR-FIA = 1.0644(GCMS) – 0.0358. Nonparametric paired comparative tests showed no significant difference. In group G2, total testosterone was assayed with a commercial reagent set (Beckman Immunotech; Ref. IM 1087) after solvent extraction. The highest cross-reactivity of the anti-testosterone antibody (used in the reagent set) was 10% with dihydrotestosterone. Interassay CVs were 5%–10%. To measure BT in serum samples from the 3 groups, we added minute doses of freshly purified tritiated testosterone to serum at 37 °C; we then precipitated the SHBG by adding a saturated ammonium sulfate solution previously equilibrated at 37 °C (8, 9). Samples were immediately centrifuged for 15 min at 3000 g in a Jouan KR 422 centrifuge previously equilibrated at 37 °C. Duplicate 0.2-mL amounts were taken from the supernatant and placed in minivials. Ultima Gold scintillating fluid (Packard) for radioactivity counting (Tricard 2300 TR; Packard) was added to all vials. We deducted the percentage of non–SHBG-bound tritiated testosterone, or BT, from the radioactivity measurements and calculated the concentrations of serum BT by multiplying %BT by the serum total testosterone concentrations. The interassay CVs of %BT calculated from 3 serum pools measured in each assay run were 4.5%, 3.8%, and 10%, respectively, for mean serum %BT values of 48.9%, 30.4%, and 18.9% (n = 50 runs). SHBG was measured in all sera by an RIA method (SHBG-RIACT reagent set) purchased from Cisbio International/Schering. Interassay CVs were 7.6%, 8.5%, and 5.9% for serum SHBG at mean concentrations of 13, 38, and 72 nmol/L, respectively (n = 45 runs).

We compared results obtained with the SHBG-RIACT reagent set with those obtained with the DELFIA SHBG (Ref. A070-101; Wallac) and the SHBG IRMA ORION (Ref. 68563; Orion Diagnostica). The correlation coefficients (r) were 0.983 and 0.985, respectively (n = 40 in duplicate for each comparison). There was no significant difference between the paired SHBG-RIACT and DELFIA SHBG results or between paired SHBG-RIACT and ORION IRMA results. Moreover, although BT measurement with ammonium sulfate precipitation is widely used, we checked the efficiency of the separation of albumin from SHBG after SHBG precipitation with 50% saturated ammonium sulfate. We assayed albumin concentrations in the supernatant after SHBG precipitation and centrifugation and in the serum sample. We performed the assays with a nephelometric method with a kinetic reaction on the Array Beckman Analyzer. The albumin concentrations in the supernatant (obtained by adding 1 volume of saturated ammonium sulfate to 1 volume of serum) were one half the concentrations of albumin in the pure serum samples, indicating that no albumin had been precipitated in the assay conditions. The assays were carried out on 12 samples.

We also assayed SHBG concentrations (IRMA; Cisbio International/Schering) in the supernatant after adding saturated ammonium sulfate to the serum samples and performing centrifugation at 37 °C for 15 min at 3000g. The assays were performed on 12 samples from group G1, whose SHBG concentrations were 8.5–39 nmol/L. We found no detectable SHBG in the supernatants of the 12 samples. In addition, an assay was performed on the serum of a diethylstilbestrol-treated patient with prostate adenocarcinoma whose SHBG concentration was very high (310 nmol/L). After addition of the saturated ammonium sulfate and centrifugation, we measured the SHBG concentration in the undiluted supernatant and in 1:2, 1:4, 1:8, 1:16, and 1:32 dilutions of the supernatant. SHBG was undetectable in the undiluted and diluted supernatant samples (the detection limit of the method was <0.5 nmol/L), indicating that no SHBG was present and that, consequently, all the SHBG had been precipitated by ammonium sulfate. We assayed albumin in samples from group G1 with the bromcresol green dye-binding method on a Hitachi 911 automated analyzer, whereas we considered albumin concentrations in groups G2 and G3 to be constant and equal to 43 g/L in each serum sample.

Using the assay results for total testosterone, SHBG, and albumin, we determined the CBT according to the formulas of Vermeulen et al. (11), applying various association constants (K) between 0.6 × 10^9 L/mol and 2 × 10^9 L/mol and various K values. We then compared the CBT with the ABT concentrations.

We based our comparison of ABT and CBT on calculation of the correlation coefficients between CBT and ABT, the CBT/ABT ratio for each sample, and the number of samples for which CBT differed from ABT by less than 10%, 20%, and 30%. For this purpose, we determined the
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*Shown are the slopes of the regression curves between ABT and CBT, the correlation coefficients (r), the mean (SD) CBT/ABT ratios, and the numbers and percentages of individuals with absolute RDs < 0.1, < 0.2, and < 0.3.
(CBT-ABT)/ABT ratio (negative, positive, and absolute ratio), termed the relative difference (RD). These comparisons were carried out with Microsoft Excel software. We also performed global variance analysis and post-ANOVA Bonferroni/Dunn tests to compare ABT and CBT results.

**Results**

We compared CBT concentrations calculated with the theoretical association constants $K_a = 1 \times 10^9$ L/mol and $K_a = 3.6 \times 10^4$ L/mol (11) with ABT concentrations. In group G1 (1421 samples), the correlation coefficient between CBT and ABT was 0.9720 and the mean (SD) CBT/ABT ratio was 1.576 (0.292), indicating that the CBT was higher than the ABT. The number of samples displaying absolute RDs $<0.10$, $<0.20$, and $<0.30$ were only 45, 107, and 204, respectively. Thus, 1217 (1421 - 204) samples (85.6%) exhibited an absolute RD between CBT and ABT greater than 0.30. These results are reported in Table 1. We obtained similar results for group G2 (170 samples): $r = 0.9575$; CBT/ABT = 1.85. The number of samples with an absolute RD $<0.10$, $<0.20$, and $<0.30$ were 8, 14, and 27, respectively. In the group of 109 healthy persons (G3), the correlation coefficient was 0.9307, the CBT/ABT ratio was 1.51, and the number of samples with an absolute RD $<0.10$, $<0.20$, and $<0.30$ were 0, 9, and 25, respectively. For all 3 groups, CBT values obtained with the theoretical association constants were higher than the ABT values.

For $K_a = 3.6 \times 10^4$ L/mol, the number of CBT values that were nearly identical to the ABT values increased when the $K_a$ value increased (Fig. 1). For $K_a = 2.9 \times 10^9$ L/mol, the absolute RDs were $<0.10$, $<0.20$, and $<0.30$, respectively, for 560, 995, and 1243 samples, correspondingly to 39.4%, 70%, and 87.4% of the 1421 samples. The absolute RD decreased for higher $K_a$ values, and there were paired optimal $K_a$ (2.9 $\times$ 10⁹) and $K_a$ (3.6 $\times$ 10⁴) values for which close correspondence of CBT to ABT values was maximal. In group G2, the optimal $K_a$ was 3 $\times$ 10⁴ L/mol for a $K_a$ of 3.6 $\times$ 10⁴ L/mol. With these paired optimal $K_a$ and $K_a$ values, 40%, 68%, and 85% of the 170 samples from group G2 had an absolute RD $<0.10$, $<0.20$, and $<0.30$, respectively.

For $K_a = 1 \times 10^9$ L/mol in group G1, the number of CBT values nearly identical to the ABT values increased when the $K_a$ increased, and an optimal value of $K_a$ was reached for $K_a = 1.1 \times 10^4$ L/mol (Fig. 2). For $K_a$ values $>1.1 \times 10^4$ L/mol, the number of CBT values nearly identical to the ABT values decreased.

We determined the optimal $K_a$ values in group G1 for several published $K_a$ values (0.6 $\times$ 10⁶ to 1.9 $\times$ 10⁷ L/mol; Table 1) and the absolute, negative, and positive RDs, the $r$ values, and the CBT/ABT ratios [mean (SD)] for each optimal pair of $K_a$ and $K_a$ association constants. The optimal correlation coefficient ($r$) was 0.9790–0.9792, the CBT/ABT ratio was 1.0136–1.0404, and the number of samples with an absolute RD $<0.10$, $<0.20$, and $<0.30$ was maximal and nearly the same regardless of the optimal paired $K_a$ and $K_a$ values (Table 1). Thus, for an absolute RD $<0.30$, the number of CBT values differing by $<30\%$ from ABT values was 1242–1245. In comparison, with the theoretical association constants, the number of samples with CBT values that differed by $<30\%$ from the ABT values was only 204 (Table 1). Moreover, for the same samples, the CBT values did not change regardless of the optimal paired association constants $K_a$ and $K_a$ used for calculation.

$$K_a = 3.6 \times 10^4 \text{ L/mol}$$

![Fig. 1. Variation in the number of samples (in group G1: 1421 samples) with an absolute RD $<0.10$ (□), $<0.20$ (□□), and $<0.30$ (□□□) for $K_a = 3.6 \times 10^4$ L/mol with increasing $K_a$ (from $0.6 \times 10^9$ L/mol to $4.40 \times 10^9$ L/mol).](image-url)
We obtained similar results for group G2 with optimal paired $K_s$ and $K_a$ values ($K_s = 0.6 \times 10^9$ to $1.9 \times 10^9$ L/mol, corresponding to a $K_a$ of $0.5 \times 10^4$ to $2 \times 10^4$ L/mol). The percentages of samples with an absolute RD $<0.10$, $<0.20$, and $<0.30$ were $35.2\%$–$37.6\%$, $66.4\%$–$69.4\%$, and $86.4\%$–$87.5\%$, respectively. These percentages are close to those reported for group G1. For $K_s = 1 \times 10^9$ L/mol, the optimal $K_a$ was $1.1 \times 10^4$ L/mol, as in group G1 ($r = 0.9597$–$0.9610$; CBT/ABT ratios $= 0.96$–$1.0$).

On the basis of the correlation coefficients, optimal $K_s/K_a$ pairs were those yielding the greatest correlation coefficient ($r = 0.9793$). For this correlation coefficient, in group G1, the optimal paired $K_s$ and $K_a$ values were $0.6 \times 10^9$ L/mol and $0.8 \times 10^4$ L/mol, $0.8 \times 10^9$ L/mol and $1 \times 10^4$ L/mol, $1 \times 10^9$ L/mol and $1.4 \times 10^4$ L/mol, $1.2 \times 10^9$ L/mol and $1.6 \times 10^4$ L/mol, $1.8 \times 10^9$ L/mol and $2.4 \times 10^4$ L/mol, and $1.9 \times 10^9$ L/mol and $2.6 \times 10^4$ L/mol. Although these optimal $K_s$ and $K_a$ pairs were not exactly the same as those obtained from the RD determination (Table 1), this optimization approach led to practically the same results as those obtained by counting samples with absolute RD values $<0.10$, $<0.20$, and $<0.30$ in group G1, the optimal $K_s/K_a$ pairs that yielded a CBT/ABT ratio as close as possible to 1 were $0.6 \times 10^9$ L/mol and $0.6 \times 10^4$ L/mol, $0.8 \times 10^9$ L/mol and $0.8 \times 10^4$ L/mol, $1 \times 10^9$ L/mol and $1.1 \times 10^4$ L/mol, $1.2 \times 10^9$ L/mol and $1.3 \times 10^4$ L/mol, $1.4 \times 10^9$ L/mol and $1.6 \times 10^4$ L/mol, $1.6 \times 10^9$ L/mol and $1.8 \times 10^4$ L/mol, $1.8 \times 10^9$ L/mol and $2 \times 10^4$ L/mol, and $1.9 \times 10^9$ L/mol and $2.2 \times 10^4$ L/mol, which were very similar to those reported in Table 1.

Whatever the mode of optimization, we found that use of optimal paired $K_s$ and $K_a$ values yielded a greater number of samples with the CBT close to the ABT, in contrast to the results obtained with the theoretical $K_s = 1 \times 10^9$ L/mol and $K_a = 3.6 \times 10^4$ L/mol, as illustrated in Fig. 3.

The calculated CBTs and the corresponding ABTs are shown on the same axis in Fig. 3. The CBTs were calculated based on 2 paired $K_s$ and $K_a$ values: $K_s = 1 \times 10^9$ L/mol with $K_a = 1.10 \times 10^4$ L/mol (one of the optimal $K_s/K_a$ pairs; Table 1), and $K_s = 1 \times 10^9$ L/mol with $K_a = 3.6 \times 10^4$ L/mol [association constants applied by Vermeulen et al. (11)]. The results show that the CBT values

![Fig. 2. Variation in the number of samples (in group G1; 1421 samples) with absolute RD $<0.10$ (■), $<0.20$ (□), and $<0.30$ (●) for $K_s = 1 \times 10^9$ L/mol with increasing $K_a$ (from $0.10 \times 10^4$ L/mol to $3.90 \times 10^4$ L/mol).](image)

![Fig. 3. Regression curves between CBT and ABT (in group G1; 1421 samples).](image)
obtained from the association constants of Vermeulen et al. (11) were well above the CBT obtained from optimal paired $K_a$ and $K_s$ values.

Using global variance analysis and a post-ANOVA Bonferroni/Dunn test, we found a significant difference between ABT and CBT values obtained with the formulas of Vermeulen et al. (11) but no significant difference between ABT and one set of the optimal paired $K_s$ and $K_a$ values reported in Table 1.

In group G3, for the same $K_a$ the optimal $K_s$ values higher than in groups G1 and G2 (Table 2). The absolute percentage of CBT values obtained from each pair of optimal $K_s$ and $K_a$ values that differed by <30% from the ABT values was 97.5% (Table 2). In this group, the mean %BT of the 34 young men (20–39 years of age) was 39% for a mean total testosterone of 16.8 nmol/L and a mean ABT of 6.38 nmol/L.

**Discussion**

Vermeulen et al. (11) reported that free testosterone concentrations could be calculated with the association constants $K_a = 1 \times 10^9$ L/mol and $K_s = 3.6 \times 10^4$ L/mol. The authors noticed, however, that CBT concentrations were 23-fold and ABT 20-fold higher than free testosterone concentrations, indicating higher CBT than ABT in a small group of individuals ($n = 24$) (11). Using the same association constants, we also found, in a rather large sample group of 1421 samples (group G1), that the choice of these 2 numeric values ($K_a = 1 \times 10^9$ L/mol and $K_s = 3.6 \times 10^4$ L/mol) yielded CBT results that were clearly much higher than the ABT results. Even higher or similar CBT/ABT mean ratios have also been reported by others [CBT/ABT = 2.2 in 700 patients (Dechaud et al., unpublished data) and CBT/ABT = 1.50 in control and hypogonadal men (Tremblay et al., unpublished data)].

Although different values of $K_a$ have been published (11–22), we found that by recalculation of the CBT to obtain a greater number of samples with CBT results close to the ABT, a $K_a$ of $3.6 \times 10^4$ L/mol led to optimal $K_s$ values of $2.9 \times 10^9$, $3 \times 10^9$, and $2.3 \times 10^4$ L/mol for our sample groups G1, G2, and G3, respectively. The $K_s$ values of $2.9 \times 10^9$ and $3 \times 10^9$ L/mol, however, were higher than the upper value of the previously published $K_s$ value of $1.9 \times 10^9$ L/mol (11–22). On the basis of the hypothesis that $K_a = 3.6 \times 10^4$ L/mol was not the exact association constant of testosterone for albumin in serum, our recalculation of the optimal paired $K_a$ and $K_s$ values for various $K_a$ values of 0.6 to $1.9 \times 10^9$ L/mol showed that the corresponding $K_s$ values were $0.60 \times 10^4$ L/mol to $2.29 \times 10^4$ L/mol (group G1), $0.5 \times 10^4$ L/mol to $1.97 \times 10^4$ L/mol (group G2), and $0.8 \times 10^4$ L/mol to $3 \times 10^4$ L/mol (group G3), lower than the $K_s$ ($3.6 \times 10^4$ L/mol) applied by Vermeulen et al. (11).

In group G2, the optimal paired $K_a$ and $K_s$ values were slightly different from those obtained in group G1, as were the correlation coefficients and CBT/ABT ratios. These slight differences may be related to the methods...
used to assay total testosterone, which were not identical, and to the choice of the same arbitrary concentration of albumin (43 g/L) in all G2 patients for the calculation of BT. However, the percentages of samples with an RD <0.10, <0.20, and <0.30 were practically the same in groups G1 and G2. In group G3, we observed for one theoretical published $K_a$ ($1.9 \times 10^9$ L/mol) (12) that the corresponding optimal $K_a$ ($3 \times 10^4$ mol) was only a little lower than the theoretical $K_a$. It is probable that the theoretical $K_a$ ($3.6 \times 10^4$ L/mol) determined on pure human albumin (25) is higher than the true $K_a$ in serum, which could partly explain an ABT lower than the CBT. Vermeulen et al. (11) hypothesized the presence of lipids to explain why CBT was lower than ABT, and free fatty acids in serum have been reported to change albumin-bound steroids (26). It is possible that higher optimal $K_a$ determined in samples from healthy men (group G3) compared with groups G1 and G2 could be attributable to lower serum concentrations of free fatty acids. The mean percentage of BT that we found in 34 young men (20–39 years of age) among the 109 healthy men of group G3 was 39%. This value was between the extreme mean values for BT reported previously (20%–50%) in different, rather small populations of young healthy men (10, 27–32), which were obtained by similar, but not strictly identical, BT assay methods (method differences concerning incubation temperature, use or not of a tritiated testosterone tracer, and purification of tritiated testosterone, frequently not reported in the BT assay methods). However, this dispersion in the published assayed percentage of BT of healthy young men does not explain the much lower ABT we measured in the 3 populations compared with the CBT obtained with $K_a = 1 \times 10^9$ L/mol and $K_a = 3.6 \times 10^4$ L/mol.

Recently, Emadi-Konjin et al. (33), applying the formulas given by Vermeulen et al. (11), with $K_a = 1 \times 10^9$ L/mol and $K_a = 3.6 \times 10^4$ L/mol, found systematic differences between CBT and ABT in samples from a group of almost 400 men. These authors reported “implausibly” higher CBT than ABT, and most of the percentage CBT values were in the 30%–70% range, whereas the corresponding measured %BT results were in the range 10%–40%. On the basis of the best correlation coefficients, these authors empirically adjusted the $K_a$ and $K_a$ association constants and found optimal paired $K_a$ and $K_a$ values of $1.4 \times 10^8$ L/mol and $1.3 \times 10^4$ L/mol, respectively. These reported results (27) can be compared with ours: for the same $K_a$ ($1.4 \times 10^8$ L/mol), we found optimal $K_a$ values of $1.60 \times 10^4$ L/mol in group G1, $1.40 \times 10^4$ L/mol in group G2, and $2.1 \times 10^4$ L/mol in group G3. We do not think that lower concentrations of ABT compared with CBT (with the association constants $K_a = 1 \times 10^9$ L/mol and $K_a = 3.6 \times 10^4$ L/mol) that we and others (Dechaud et al. and Tremblay et al., unpublished data) have found can be explained by a methodologic problem, although differences in methodologies exist. To our knowledge, no such comparisons of CBT and ABT [except by Emadi-Konjin et al. (33)] in large numbers of patients and healthy men have been reported. The CBT largely depends on the $K_a$ and $K_a$ values chosen. Numerous theoretical $K_a$ values have been reported in the past, and the exact $K_a$ and $K_a$ values in serum are not well known. Moreover, as suggested recently (34, 35), the $K_a$ value could vary with age.

In conclusion, by calculating optimal pairs of $K_a$ and $K_a$, we were able to determine CBT values that better agreed with the ABT values than CBT values determined with theoretical association constants. Using optimal $K_a/K_a$ pairs, we found in our population of untreated and treated hypogonadal nonfasting patients that 30% of the CBT results differed from ABT by at least 20%, whereas in the population of fasting healthy men, CBT obtained with optimal $K_a/K_a$ pairs led to CBT values close to the ABT values in 97% of samples. Considering the uncertainty of calculating BT, ABT obtained with ammonium sulfate precipitation seems to be a better method than CBT. It would be wise, however, to thoroughly standardize the BT ammonium sulfate precipitation assay method and to determine BT reference values in men.

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