Calculation of Bioavailable and Free Testosterone in Men: A Comparison of 5 Published Algorithms

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Background: Estimation of serum concentrations of free testosterone (FT) and bioavailable testosterone (bioT) by calculation is an inexpensive and uncomplicated method. We compared results obtained with 5 different algorithms.

Methods: We used 5 different published algorithms [described by Sodergard et al. (bioTS and FT), Vermeulen et al. (bioTV and FT), Emadi-Konjin et al. (bioTE), Morris et al. (bioTM), and Ly et al. (FT)] to estimate bioT and FT concentrations in samples obtained from 399 independently living men (ages 40–80 years) participating in a cross-sectional, single-center study.

Results: Mean bioT was highest for bioTS (10.4 nmol/L) and lowest for bioTE (3.87 nmol/L). Mean FT was highest for FT (0.41 nmol/L), followed by FT (0.35 nmol/L), and FT (0.29 nmol/L). For bioT concentrations, the Pearson correlation coefficient was highest for the association between bioTS and bioTV (r = 0.98) and lowest between bioTM and bioTE (r = 0.66). FT was significantly associated with both bioTS (r = 0.96) and bioTE (r = 0.88). The Pearson correlation coefficient for the association between bioTE and bioTM almost reached 1.0. Bland-Altman analysis showed large differences between the results of different algorithms. BioTM, bioTE, bioTV, and FT were all significantly associated with sex hormone binding globulin (SHBG) concentrations.

Conclusion: Algorithms to calculate FT and bioT must be revalidated in the local setting, otherwise over- or underestimation of FT and bioT concentrations can occur. Additionally, confounding of the results by SHBG concentrations may be introduced.

In healthy adult men, ~44% of the circulating testosterone is specifically bound to sex hormone binding globulin (SHBG), 50% is nonspecifically bound to albumin, and 3.5% is bound to cortisol-binding globulin, indicating that only 2%–3% is unbound or free (1). SHBG binding decreases the metabolic clearance rate of testosterone (2, 3) and withholds bound hormone from diffusion into the cell, although SHBG may be a necessary cofactor for cellular uptake of testosterone (4). Whether albumin-bound testosterone can dissociate sufficiently fast to enter tissues is controversial (5, 6). However, concentrations of non-SHBG-bound testosterone [bioavailable testosterone (bioT) = free + albumin-bound testosterone] and non-SHBG-nonalbumin bound testosterone [free testosterone (FT)] are extremely well correlated (7) and interchangeable in most cases.

Because concentrations of SHBG vary widely in healthy men and are related to variables such as diet (8), body mass index (BMI), insulin concentrations, and age (8–10), measurements of FT and bioT are valuable for correct assessment of the bioactive fraction of testosterone. In aging men, total testosterone concentrations tend to decrease and SHBG concentrations increase (11); therefore, measurements of FT and bioT are advocated to support the diagnosis of hypogonadism, which has nonspecific signs and symptoms in elderly men (12, 13).

BioT can be measured ex vivo with the ammoniumsulfate precipitation technique described by Tremblay and Dube (14). The gold standard for FT measurement is the dialysis method (15), although a mass spectrometry...

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based assessment of FT in ultrafiltrates was recently proposed as a candidate reference method (16). However, both ammoniumsulfate precipitation and the dialysis technique are nonautomated, time-consuming, and expensive techniques and, therefore, are not routinely used in most laboratories. Alternatively, FT concentrations can be measured with a direct RIA, but this assay has been criticized because of lack of accuracy (7, 15–17). The concentrations of FT and bioT can also be calculated by use of one of several published algorithms. The 2 most widely used equations for calculating FT and bioT are those described by Vermeulen et al. (7) and Sodergard et al. (18). These algorithms assume that when the concentrations of total testosterone, SHBG, and albumin, and the constants for the binding of testosterone to SHBG and albumin are known, FT and bioT can be calculated. These calculations depend on a proper estimation of the association constant for binding of testosterone to SHBG (kₜ) and albumin (kₘₐ). However, values for kₜ measured with various methods have been reported, including 5.97x 10⁸ (18), 10x10⁸ (7), 16x10⁸ (1), and 19x10⁸ (19).

A more pragmatic approach has been to create an algorithm based on measurement of FT or bioT and use this algorithm to predict FT or bioT in future samples on the basis of total testosterone and SHBG concentrations. Three new algorithms based on concentrations measured with a gold standard technique in large numbers of samples were recently proposed for estimating the concentrations of FT or bioT (20–22). At least 2 algorithms for calculating bioT are available on the internet (www.issam.ch and www.him-link.com).

Calculated FT and bioT concentrations, most often obtained with the Vermeulen (15,23–28) and Sodergard (24,29–33) methods, are used widely in the endocrinology literature. In most publications no arguments are given for the choice of a particular method for calculating FT or bioT although, as described above, choosing a particular set of constants will obviously influence results of calculated free and bioavailable hormone concentrations and thus might influence results of analyses. Moreover, it is doubtful whether algorithms composed and validated in one laboratory can be applied to samples from an unrelated laboratory that uses different assay techniques. The aim of this study was to compare the results of 5 published algorithms to calculate FT and bioT.

**Participants and Methods**

The study was a cross-sectional, single-center study of 399 independently living men ages 40 to 80 years. Participant recruitment methods and details of their baseline characteristics, lifestyle, and health, have been described extensively (34). All participants gave written informed consent before enrollment, and the institutional review board of the Utrecht University Medical Centre approved the study.

**Laboratory Measurements and Calculations of FT and bioT**

We obtained fasting blood samples by venipuncture. Cell-free serum was immediately stored at −20°C. We measured testosterone after performing diethyl ether extraction with an in house RIA using a polyclonal antitestosterone–antibody (AZG 3290; a gift from Dr. J.J. Pratt, Groningen, the Netherlands). The lower limit of detection of the assay was 0.24 nmol/L, and interassay variation rates were 6.0%, 5.4%, and 8.6% at 2.1; 5.6, and 23 nmol/L, respectively. We measured SHBG with an immunometric technique on an Immulite 2000 Analyzer (Diagnostic Products Corporation). The lower limit of detection was 5 nmol/L, and interassay variation rates were 6.1%, 4.9%, and 6.9% at 11.6; 36, and 93 nmol/L, respectively.

Using the equations summarized in Table 1, we calculated bioT at a fixed plasma albumin concentration of 43 g/L. The affinity constants for the binding of testosterone to SHBG or albumin were 5.97x 10⁸ L/mol and 4.06x 10⁴ L/mol, as proposed by Sodergard et al. (18) (BioTv); 10x10⁸ L/mol and 3.6x 10⁴ L/mol, as described by Vermeulen et al. (7) (BiTv); or 1.4x 10⁹ L/mol and 1.3x 10⁴ L/mol, as described by Emadi-Konjin et al. (20) (BioTₘ; Table 1A). We also calculated bioT according to the algorithm presented by Morris et al. (21) (BioTₘ; Table 1B) and calculated FT with the equations of Vermeulen (FTV), Sodergard (FTS; Table 1A), and Ly et al. (22) (FTₘ; Table 1C).

To evaluate the impact of varying albumin concentrations on bioT, we used the Vermeulen, Sodergard, and Emadi-Konjin algorithms to recalculate bioT with a fixed plasma albumin concentration of 33 g/L instead of 43 g/L and used the Student t-test for paired comparisons to test differences between estimates.

**Data Analysis**

The associations between the calculation results for all possible pairs of algorithms were assessed and expressed as Pearson correlation coefficients. The differences between the results of the calculations were quantified as Pearson correlation coefficients. The differences between the results of the calculations were quantified as Pearson correlation coefficients.

**Table 1. Equations for the calculation of FT and bioT.**

<table>
<thead>
<tr>
<th>Equation Type</th>
<th>Formula</th>
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<tbody>
<tr>
<td>A. According to Vermeulen et al. (7), Sodergard et al. (18), and Emadi-Konjin et al. (20)</td>
<td>bioT (mol/L) ( \frac{(k_{sa} \times [albumin] \times [FT] / (1 + k_{sa} \times [FT])) + [FT]}{(-b + \sqrt{b^2 + 4a[FT])/2a}} ), in which ( a = k_{sa} + k_{s} + (k_{sa} \times k_{s})[SHBG] + [albumin] - [T] ) and ( b = 1 + k_{sa}[SHBG] + k_{sa}[albumin] - (k_{sa} + k_{s})[T] )</td>
</tr>
<tr>
<td>B. Equation for the calculation of bioavailable testosterone according to Emadi-Konjin et al. (20)</td>
<td>bioT (nmol/L) ( e^{-0.266 + 0.955 \times \ln[TT] - 0.228 \times \ln[SHBG]} )</td>
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<tr>
<td>C. Equation for the calculation of bioavailable testosterone according to Ly et al. (22)</td>
<td>free T (pmol/L) ( -52.65 + 24.4[TT] - 0.704[SHBG] - 0.0782[TT] \times [SHBG] - 0.0854[TT]^2 )</td>
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</table>
Results

Mean bioT and FT concentrations calculated by the different algorithms are presented in Table 2, A and B. As anticipated from the different association constants for the binding of testosterone to SHBG, mean bioT was highest, followed by mean bioTV and mean bioTE. The result for mean bioTM was between those for bioTV and bioTE. The range (%) for bioTM covers only 15% points, indicating that the impact of varying SHBG concentrations on the concentration of bioTM is limited, as confirmed by the strong correlation between concentrations of total testosterone and bioTM (Table 3). The ranges for bioT(%), calculated using the other algorithms, varied between 41% and 47%. For FT, FT5 was highest, followed by FTV and FTL.

Pearson correlation coefficients for the relationships between the results of the different algorithms are summarized in Table 3. For the calculations of FTV and FT5, we used an albumin concentration of 43 g/L, which led to a fixed relation between FT and bioT. Thus, associations between bioTS and bioTE and other variables were similar for FT5 and FTV, respectively. BioTV and bioTE appeared to be strongly correlated. The relationship between bioTM and bioTE was relatively weak. Whereas the relationship between bioTM and total testosterone was very strong; it was weak for bioTE. There was also a striking difference between these 2 estimates and their association with SHBG concentrations. BioTM was positively associated and bioTE was negatively associated, whereas bioTV and bioTE were not or were only weakly related to the concentrations of SHBG. FTL was strongly related to bioTM, was also strongly associated with total testosterone, and was positively associated with SHBG concentrations.

For the Vermeulen, Sodergard, and Emadi-Konjin algorithms, results were recalculated with a standard albumin concentration of 33 g/L (instead of 43 g/L) to evaluate the impact of varying albumin concentrations on the results. For all algorithms, mean bioT concentrations calculated at the lower albumin concentration were slightly but significantly lower (P < 0.001 for the differences between results obtained using 33 or 43 g/L for all algorithms). The impact of varying albumin concentrations appeared to be stronger when the fractional bioT concentration was lower. Because the highest results for mean bioT fraction were calculated with the Sodergard algorithm, this algorithm was least sensitive to variations in albumin concentration (percentage difference = 7.2% of mean bioT, range 3.2%–10.9%) followed by Vermeulen (9.1%, range 3.6%–13.2%), and Emadi-Konjin (11.8%, range 4.2%–14.9%; P < 0.001 for the difference between algorithms).

In Figs. 1 and 2, Bland-Altman plots are presented in which the results of the calculations using all pairs of algorithms are compared. Although differences between the results of the calculations were large, they were smallest when bioTS and bioTV were compared. BioTS was estimated systematically higher compared with bioTV, with a mean difference of +27% of the mean bioT concentration. Both the Vermeulen and Sodergard algorithms provided systematically higher estimates compared with the Emadi and Morris algorithms (mean difference 94% for bioTV vs bioTE, 63% for bioTS vs bioTM, 72% for bioTV vs bioTM, and 38% for bioTV vs bioTM). The mean difference between bioTM vs bioTE was limited to 36%; however, the difference between the methods for individual samples ranged between −35% and +92%.

Both FT5 and FTV gave higher estimates than did FTL (mean difference 21% for FTV vs FTL and 35% for FT5 vs FTL), but the ranges were much smaller for FT5 vs FTL. BioTM and FTL were extremely well correlated, and the plots of their differences with bioTV and bioTS or FTV and FT5, respectively, look similar, although not identical (Figs. 1 and 2).

Discussion

The Vermeulen and Sodergard algorithms on the one hand and the Emadi-Konjin, Ly, and Morris calculations on the other hand were developed from fundamentally different approaches. The Vermeulen and Sodergard equations are based on the law of mass action, in which estimates of total testosterone, SHBG, and the values for the association constants for the binding of testosterone to albumin and SHBG are used to calculate bioT and FT. The association constants for these algorithms were obtained
experimentally and were validated by comparing the results of the calculations with a gold standard technique (7, 16, 36). The consistency between the association constants and the results obtained using these constants adds to the credibility of these algorithms, as indicated by the results of van Uytfanghe et al. (16).

Emadi-Konjin et al. (20) and Morris et al. (21) first measured bioT by ammonium-sulfate precipitation and used these results to create an algorithm to predict bioT concentrations in other samples by use of the concentration of total testosterone and SHBG.

To obtain optimal correlation with their results, Emadi-Konjin et al. (20) modified the equation used by Vermeulen and Sodergard by altering the binding constants for testosterone binding to albumin and SHBG. Morris et al. (21) created a regression equation based on their measurements. Ly et al. (22) first used an ultrafiltration assay to estimate FT in ~4000 samples. Then they composed 2 regression models to predict FT in these samples: 1 equation for total testosterone concentrations <5 nmol/L and 1 for concentrations above this concentration.

Because all of our study participants had total testosterone concentrations >5 nmol/L, we used only the latter equation. In contrast to the Vermeulen and Sodergard methods, the Morris, Ly, and Emadi-Konjin algorithms rely solely on the accuracy of their measurements.

Calculation of FT and bioT with the Vermeulen and Sodergard equations is accurate only when competition for the binding sites by other steroid hormones is limited. For example, in the presence of supraphysiologic concentrations of estradiol or dihydrotestosterone, calculation with either method will underestimate the concentration of bioT. In clinical practice, however, this problem is rarely encountered. In addition, these methods assume that the association constants for $k_t$ and $k_a$ are known, but the fact that the Sodergard and Vermeulen equations use different constants shows that this is not the case.

With either algorithm, the results of the calculation of FT and bioT depend on a proper determination of the concentration of total testosterone (also necessary for measurement of FT and bioT concentrations with the dialysis, ultrafiltration, or ammoniumsulfate precipitation...
Results differ considerably for commercially available assays for testosterone (37–39) and SHBG (15, 40). Because most commercial automated testosterone assays perform inaccurately in women and children (38, 39), calculation of FT and bioT with these results will also be inaccurate. Differences in the estimation of the SHBG concentration will influence the result of the calculated but not the measured FT or bioT concentration. In this respect, it should be noted that commercial SHBG assays were traditionally standardized against a measurement of binding capacity, whereas more recently several assays were calibrated on the basis of SHBG mass, which may lead to important differences related to the number of steroid binding sites in the SHBG homodimers (41, 42).

The Vermeulen and Sodergard methods appear to be used rather indiscriminately, and proper validation of the calculated results is rarely described. In some studies the calculation as proposed by Sodergard is used and validated by referencing to validation experiments in which the Vermeulen calculation is used (24, 31, 43).

Our study shows that results differ considerably for calculations based on different algorithms applied to testosterone and SHBG concentrations measured in an unrelated laboratory. BioT_M and bioT_E concentrations actually measured with ammonium-sulfate precipitation were much lower than calculated bioT_S and bioT_V concentration estimates, possibly indicating that these widely used algorithms systematically overestimate bioT concentrations. This possibility of overestimation is supported by validation experiments in which measured FT concentrations were lower than estimates calculated with the Vermeulen equation (16, 22, 36, 37). With their ultrafiltration technique, Ly et al. (22) measured FT concentrations that were lower than concentrations calculated with the Vermeulen or Sodergard algorithm.

Although both bioT_M and bioT_E estimates are based on actual measurements of bioT, the correlation between the results of the adapted algorithms is only 0.66 (Pearson’s r), and the difference between the estimates ranges between −35% and +92% of the mean bioT concentration. As stated above, algorithms depend on the methods used to measure total testosterone and SHBG and thus are not directly transferable to other users. However, as shown in Table 4, the reference ranges of the assays used in the Morris and Emadi-Konjin studies were not very different from the results in our study, which makes it unlikely that differences between assays can explain all of the variabili-

![Graph showing percentage difference in calculated FT concentrations against the average of the 2 applied algorithms.](image)

Fig. 2. Plots of the percentage differences in calculated FT concentrations against the average of the 2 applied algorithms. The solid line represents 0%, the dotted lines the 2 SD of the mean percentage difference.

<table>
<thead>
<tr>
<th>Assay Type</th>
<th>Emadi-Konjin</th>
<th>Morris</th>
<th>Ly</th>
<th>This study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total testosterone (nmol/L)</td>
<td>Roche Elecsys 2010</td>
<td>DRG Instruments ELISA</td>
<td>DPC Immulite</td>
<td>In-house RIA&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>7.3–28.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.9–23.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5–240&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.20–39.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>DPC Immulite</td>
<td>DRG Instruments ELISA</td>
<td>DPC Immulite</td>
<td>DPC Immulite</td>
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<td></td>
<td>13–71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15–100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3–329&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12–91&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reference intervals as supplied by manufacturer.

<sup>b</sup> Range in studied population.

<sup>c</sup> This method yields results comparable to those obtained with the DPC Coat-a-Count RIA (11).
ity presented here. The lack of uniformity between the results of the Morris and Emadi-Konjin algorithms suggests that either one or both methods are flawed. This finding is not entirely surprising because ammonium sulfate precipitation measurement of bioT is a demanding procedure that relies on selective and reproducible precipitation of all SHBG without precipitating albumin and requires a critical concentration of ammonium sulfate (44). The high correlation of bioT with FT, the results of which are based on a different gold standard technique, argues in favor of the Morris algorithm. A positive finding of our study is that the 2 most widely used algorithms, those described by Vermeulen and by Sodergard, are fairly concordant, although the Sodergard algorithm provides systematically higher estimates.

BioT<sub>ft</sub>, bioT<sub>mp</sub>, and FT<sub>1</sub> were significantly associated with SHBG concentrations (Table 3). In healthy men neither a positive nor a negative relationship between SHBG and bioT is expected, because the pituitary is assumed to increase luteinizing hormone stimulation of the testes to compensate for higher SHBG concentrations, and bioT and FT are assumed to truly represent the bioactive fraction of total testosterone. Therefore, a strong association between FT or bioT concentrations and SHBG not only raises questions regarding the reliability of the estimates but also introduces fundamental problems in interpreting these estimates. Confounding of these results by SHBG cannot be solved by establishing method-specific reference ranges. Aging men, for instance, who generally have higher concentrations of SHBG than do young men, will be more prone to have low calculated estimates of bioT concentrations when the Emani-Konjin equation is used and higher concentrations when the Morris or Ly equation is used. The dependency of these estimates on SHBG concentrations is particularly undesirable in older men, in whom accurate estimations of FT and bioT are needed to support a clinical diagnosis of hypogonadism. Not Morris, Ly, or Emadi-Konjin described a coefficient of correlation for the relation between bioT or FT concentrations and SHBG in their studies, whereas the absence of such a relation would favor the validity of their results. In our study, bioT<sub>3</sub> and bioT<sub>V</sub> were not or were only weakly related to SHBG concentrations, possibly indicating that these estimates are closer to actual bioT concentrations than are estimates from other algorithms.

Application of the Morris or Ly equations yields a narrow range of (fractional) bioT or FT concentration estimates, suggesting that, over a wide range of SHBG concentrations, SHBG has little effect on absolute and fractional bioT<sub>mp</sub> or FT<sub>1</sub> concentrations. This conclusion is also reflected by the very high association between bioT<sub>mp</sub> or FT<sub>1</sub> and total testosterone concentrations, an association not found in the original study by Morris et al. (21) and not described by Ly et al. (22). With such a high association, it is questionable whether the calculation of bioT and FT concentrations is any more valuable than the measurement of total testosterone.

As shown in this study, variable albumin concentrations entered in the Sodergard, Vermeulen, and Emadi-Konjin algorithms gain importance when fractional bioT concentrations are lower. Therefore, the actual albumin concentration should be added to the algorithm when gross abnormalities of the albumin concentration are expected (as in nephrotic syndrome or severe malnutrition) or when SHBG concentrations are high (as in hyperthyroidism, women, or aging men).

Although the Morris, Ly, and Emadi-Konjin algorithms were based primarily on measurements in men (percentage males: Morris 100%, Ly 87%, and Emadi 92%), we repeated the analyses in 415 premenopausal women patients at a tertiary fertility outpatient clinic and obtained Bland-Altman plots similar to those for men (data not shown). In women, the differences between the results of the algorithms were larger for all comparisons except for Morris/Vermeulen and Morris/Sodergard. These results are in line with extrapolation in the very low bioFT or FT range of the results for men (Figs. 1 and 2). Differences between the results of the Sodergard, Vermeulen, and Emadi-Konjin algorithms were larger because of the higher mean concentrations of SHBG in women; the difference between the results of these algorithms was strongly associated with SHBG concentrations.

Although we observed appreciable differences between the results of several published algorithms for calculating bioT and FT, we are not able to recommend one algorithm over another because we did not compare our results with an accepted reference method for measuring bioT or FT. Even if we had done so, however, we would have been reluctant to disqualify any of the evaluated algorithms. Also, our measurements depended on SHBG and total testosterone assay characteristics and may have been distorted by technical flaws.

We conclude that algorithms to calculate bioT and FT are not simply transferable to samples from other laboratories unless careful revalidation in the local setting has been performed. Otherwise, over- or underestimation of bioT or FT and confounding of results by SHBG concentrations can occur. The latter can be evaluated by quantifying the relationship between SHBG and calculated bioT or FT concentrations or by adjusting the relationship between any variable and calculated bioT or FT for SHBG.

Although these conclusions seem obvious, calculated concentrations of bioT or FT are widely described in scientific journals and probably used in clinical practice, mostly without prior in-house validation of the calculated results. Without in-house validation, the use of these algorithms is highly questionable.

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