

Age-Associated Discrepancy between Measured and Calculated Bioavailable Testosterone in Men

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Background: Bioavailable testosterone (BT) concentration is considered the best marker for evaluating testicular function in men. The decrease of BT in older men is more pronounced than the decrease in total testosterone because of the parallel increase in sex hormone–binding globulin (SHBG) concentrations. Measurement of BT is therefore crucial for the diagnosis of hypoandrogenism in the aging male population.

Methods: We compared BT concentrations measured by a specific RIA after ammonium sulfate precipitation (BT_{meas}) with those obtained by theoretical calculations (BT_{cal}) in plasma samples from 694 young men (14 to 49 years old) and 51 older men (50 to 81 years old). We based theoretical calculations on Vermeulen's simplified mass equation using total testosterone and SHBG concentrations.

Results: BT_{cal} and BT_{meas} correlated significantly in young (Pearson $r = 0.87$) and aging ($r = 0.89$) men, but the BT_{cal}:BT_{meas} ratio differed markedly between the 2 groups (2.28 vs 3.48; $P < 0.001$).

Conclusions: In men, there is an age-associated discrepancy between calculated and measured BT concentrations. We suggest some hypotheses for the discrepancy, but additional studies will be performed to finally elucidate this difference in results and to determine the most appropriate method for BT measurements in older men.

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Aging in healthy men is generally associated with a significant decrease in the concentration of circulating androgens (1–4). There is evidence that both testicular and adrenal sources of androgens are involved in this progressive decline. In contrast to menopause, a process associated with irremediable ovarian depletion in follicles, aging of testicular function is marked by decreased capacity to produce androgens and sperm in response to gonadotropin stimuli (1). In addition, contrary to the gonadotropin status observed in postmenopausal women, aging men have no apparent increase in gonadotropin concentrations (1). Therefore, the dilemma for the clinician is to recognize clinical symptoms of mild hypogonadism, such as decreased libido, erectile dysfunction, reduced presence of facial and pubic hair, gynecomastia, reduced muscular mass, and physical and psychological asthenia, and relate them to androgen decline (5–8).

Measuring total testosterone to identify hypogonadism in older men is inappropriate, as stated in the official recommendations of the International Society for the Study of Aging Males. Indeed, according to the free hormone hypothesis, the active form of testosterone available for tissues is the fraction of circulating testosterone that is not bound to proteins (9). Equilibrium dialysis (10) and ultrafiltration (11) are reference methods for measuring free testosterone; however, they are technically unsuitable for routine assay. It has been proposed, but not experimentally demonstrated, that the concentration of testosterone not tightly bound to sex hormone–binding globulin (SHBG)⁷ could be considered the “bioavailable” fraction of circulating testosterone (12). In older men, the decrease in bioavailable testosterone (BT) concentrations is more marked than the decrease in total testosterone (T) concentrations because of the concomitant increase of SHBG concentrations with age (3, 4). A

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⁷ Nonstandard abbreviations: SHBG, sex hormone–binding globulin; BT, bioavailable testosterone; T, testosterone; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; CBG, corticosteroid-binding globulin.

reliable measurement of the concentration of BT and the establishment of an interval of values under which an individual could be considered hypogonadal are necessary for an ultimate prescription of hormonal therapies.

BT can be directly measured in a serum sample by cautious ammonium sulfate precipitation of SHBG-linked testosterone. Methods based on the preincubation of plasma samples with tritiated T measure the percentage of BT, which can then be multiplied by the total concentration of T to calculate the absolute concentration of BT (13, 14). Alternatively, BT can be directly measured in the supernatant fraction after ammonium sulfate precipitation by use of a highly specific RIA for testosterone (15). Ammonium sulfate precipitation has been used routinely in many reference laboratories, although technical concerns have limited its universal application. To overcome this difficulty, it has been proposed that BT concentrations be calculated by measuring total testosterone and SHBG and using the values in a law mass equation model (16, 17). Many laboratories, confronted with increasing demand for BT measurements, are currently using the simplified mass law equation published by Vermeulen et al. (17). This calculation assumes that T and SHBG concentrations are the main covariant of BT and assumes that albumin concentrations and affinity constants of SHBG and albumin for testosterone can be predefined. Different algorithms for the determinations of BT have been published and the results compared (18–20), showing large differences between the results of different algorithms (21).

In this study, to identify age-dependent discrepancies in BT and potential relevant mechanisms, we compared the concentrations of BT that were measured by use of a routine ammonium sulfate precipitation assay (15) to calculated BT using the Vermeulen mass action law model (17) in 2 populations of young and aging men.

Materials and Methods

STUDY PARTICIPANTS

We recruited a population of 694 men 14 to 49 years old and 51 older men, 50 to 81 years old, who were referred to the Department of Reproductive Medicine (Hospices Civils de Lyon, France) for infertility or endocrinology aging evaluation between 1990 and 2004. For each patient, concentrations of total testosterone, SHBG, and BT were measured in plasma from the blood samples collected during their visit to the hospital.

In a complementary study, T, SHBG, BT, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEAS) were measured in a single run in plasma samples from 14 patients younger than 35 and 13 patients older than 60.

LABORATORY MEASUREMENTS

We measured total T with an in-house RIA after organic extraction and diatomaceous earth (Celite) chromatography (22). Mean intrabatch CVs were 4.4%, 3.3%, 2.2%,

3.7%, 4.3%, and 3.7% for concentrations of 0.17, 1.0, 2.6, 6.9, 15.6, and 26.0 nmol/L, respectively, with interbatch CVs of 7.1%, 7.9%, and 7.1% for T concentrations of 1.8, 3.5, and 21.3 nmol/L, respectively. This method has been validated by gas chromatography/mass spectrometry measurements (23).

We measured SHBG with a commercially available immunoradiometric assay (SHBG-IRMA; Cis-Bio International), with mean intra- and interbatch CVs <6%.

We measured BT as described (15). In brief, we treated plasma samples with ammonium sulfate (50% of saturation) at 4 °C, and after centrifugation, we measured testosterone in supernatant by RIA after organic extraction and diatomaceous earth (Celite) purification. Interassay CVs were 11.7%, 8.7%, 8.9%, 7.0%, 8.7%, and 9.4% for BT concentrations of 0.13, 0.90, 1.6, 4.8, 7.8, and 14.4 nmol/L, respectively.

We measured DHEA and DHEAS with RIA using ³H-DHEA and ³H-DHEAS as radioactive markers. We separated free and bound antigen by use of dextran-coated charcoal. We performed DHEAS measurements directly on diluted plasma, whereas we measured DHEA by RIA after liquid extraction and diatomaceous earth (Celite) chromatography.

THEORETICAL CALCULATIONS

The following equation was used to calculate BT, as suggested by Vermeulen et al. (17):

$$fT = (T - [N \times fT]) / K_s (SHBG - T + [N \times fT])$$

from which we obtained

$$BT = fT + AT$$

where T = molar concentration of total T, fT = molar concentration of free T, BT = molar concentration of BT, $SHBG$ = molar concentration of SHBG, K_a = affinity constant of albumin for T ($= 3.6 \times 10^4$ L/mol), K_s = affinity constant of SHBG for T (1.0×10^9 L/mol), $N = K_a \times Ca + 1$ (where Ca = albumin concentration), and AT = molar concentration of albumin-bound T ($\approx K_a \times Ca \times fT$).

Concentrations of BT obtained by use of this calculation were compared to those obtained by the Vermeulen equation (available at <http://www.issam.ch/freetesto.htm>).

SENSITIVITY ANALYSIS

We performed a first comparison between calculated BT (BT_{cal}) and measured BT (BT_{meas}) by comparing BT_{cal} vs BT_{meas} values of 745 men while varying the respective affinity constants of SHBG and albumin for binding T (from 0.6 to 2.0×10^9 L/mol for SHBG and from 0.5 to 5.0×10^4 L/mol for albumin) in the theoretical calculations.

STATISTICS

We performed t tests on mean concentrations and calculations of Pearson correlation coefficients between BT_{cal}

and BT_{meas} with Microsoft Excel. P values <0.05 were considered statistically significant.

Results

The mean concentrations of T, SHBG, BT_{meas} , and BT_{cal} and mean ratios of $BT_{cal}:BT_{meas}$ for the 745 men who were part of the study are presented in Table 1. Concentrations of BT obtained by theoretical calculations were substantially higher than those obtained by our ammonium sulfate precipitation assay, with a $BT_{cal}:BT_{meas}$ ratio >2 . The difference in concentrations of BT_{cal} between younger (age <50 years) and older (age >50 years) men was of borderline significance ($P = 0.08$) because of a very mild nonsignificant decrease in T concentrations and a significant increase in SHBG concentrations. In contrast, the concentrations of BT_{meas} were much higher in men <50 years old than in men >50 , and the difference was highly significant ($P <0.001$). Interestingly, the $BT_{cal}:BT_{meas}$ ratio decreased significantly ($P <0.001$) from 3.48 for men >50 to 2.28 for men <50 years old. Pearson correlation coefficients, comparing BT_{cal} vs BT_{meas} in men <50 ($r = 0.87$) and >50 ($r = 0.89$), were highly significant ($P <0.001$). However, the slope of the correlations between BT_{cal} and BT_{meas} in the 2 populations was different ($y = 1.49x + 2.13$ in men <50 and $y = 1.94x + 2.24$ in men >50 years old; Fig. 1).

The consequence of varying values of the binding affinity constants of albumin (K_a) and SHBG (K_s) for testosterone, within the interval of values published in the literature, is shown in Fig. 2. When K_s and K_a were 1.6×10^9 and 1.0×10^4 L/mol, respectively, the concentrations of BT_{cal} and BT_{meas} were virtually identical and $BT_{cal}:BT_{meas}$ was close to 1.

Mean testosterone, SHBG, DHEA, and DHEAS concentrations in 14 men <35 years old and 13 men >60 years old are given in Table 2. In older men, mean testosterone concentrations were significantly lower and mean SHBG concentrations significantly higher than in younger men. In addition, older men had much lower mean DHEA and DHEAS concentrations than younger men. As expected, BT_{cal} and BT_{meas} were higher in younger than in older men. The $BT_{cal}:BT_{meas}$ ratio in older men was twice as high as in younger men (4.70 vs 2.09; $P <0.001$). In addition, $BT_{cal}:BT_{meas}$ was inversely correlated with the concentration of DHEA (Pearson $r = -0.503$) and DHEAS ($r = -0.626$).

Discussion

This study shows that measuring BT or calculating it by use of mass law equations achieves different results. Using the ammonium sulfate precipitation assay (15), we found that BT_{meas} and BT_{cal} concentrations had very high Pearson correlation coefficients. As reported in other studies (17, 18, 24), however, BT_{cal} gives values that are generally twice as high as those of BT_{meas} , and these are even higher in young men compared with older men. In our study of 745 patients, men >50 years old had $BT_{cal}:BT_{meas}$ ratios 2-fold higher than younger men (3.48 vs 2.28; $P <0.001$). We also observed this difference in our complementary study on 27 men, in which older men had ratios of $BT_{cal}:BT_{meas}$ 2-fold higher than younger men. The results of our complementary study, although preliminary, are of interest because we measured samples from both young and old men within the same analytical batch, underlining the fact that the differences observed in absolute concentrations between BT_{cal} and BT_{meas} in the 2 different populations are real and not an artifact of measurement imprecision or a change in the methodology over time.

Some hypotheses may explain the discrepancy in absolute concentrations between BT_{cal} and BT_{meas} . Limitations may exist in the accuracy of measuring BT by serum ammonium sulfate precipitation. Indeed, the association constant of SHBG for T is slightly higher than at 37 °C (25), and because the precipitation of the SHBG-bound T complex by ammonium sulfate was performed at 4 °C, this may cause a decrease in the fraction of BT. Moreover, during this step, a fraction of albumin can be precipitated by ammonium sulfate, which might decrease the concentration of BT. Because the testosterone binding to corticosteroid-binding globulin (CBG) is not negligible, as emphasized by Nisula et al. (26), the small part of CBG-bound T that is precipitated by ammonium sulfate might also contribute to the decrease in the final result of measured BT. During the precipitation step, T could partially dissociate from albumin, and get fixed on the precipitate, contributing as well to an overall decrease in BT_{meas} . These methodological deficiencies have been carefully checked previously (15), however, and are unlikely to explain a 2-fold difference between measured and calculated BT.

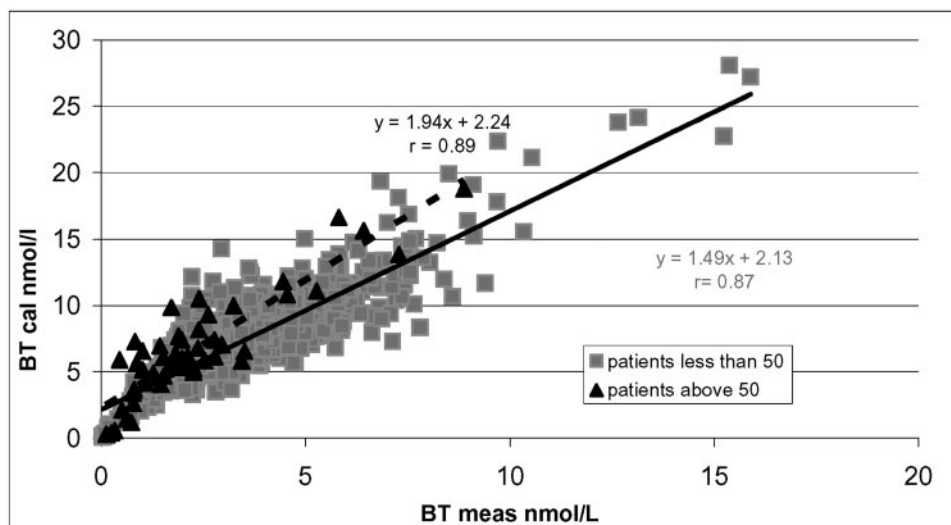
When BT is calculated from total testosterone and SHBG concentrations, several assumptions are made. The binding affinity constants for testosterone used by Ver-

Table 1. Mean (SD) concentrations of total T, SHBG, BT_{meas} , BT_{cal} , and $BT_{cal}:BT_{meas}$ according to age in men.^a

	n	T, nmol/L	SHBG, nmol/L	BT_{meas} , nmol/L	BT_{cal} , nmol/L	$BT_{cal}:BT_{meas}$
All patients	745	14.48 (6.92)	29.28 (14.52)	3.60 (2.06)	7.60 (3.55)	2.37 (0.92)
Men <50 years old	694	14.52 (6.82)	29.00 (14.25)	3.70 (2.04)	7.67 (3.51)	2.28 (0.72)
Men ≥ 50 years old	51	14.00 (8.23)	33.72 (17.35)	2.27 (1.86)	6.65 (4.02)	3.48 (1.99)
P		0.61	0.02	<0.001	0.08	<0.001

^a P values compare men <50 with men ≥ 50 years old.

Fig. 1. Correlation between calculated and measured BT in the study population.



meulen et al. (17) were 1.0×10^9 L/mol for SHBG (K_s) and 3.6×10^4 L/mol for albumin (K_a), although a broad range of affinity constants have been reported (18). Calculating BT using higher K_s values and lower K_a values reconciled BT_{cal} vs BT_{meas} , with a $BT_{cal}:BT_{meas}$ ratio that tends to 1. Our calculation shows that $BT_{cal}:BT_{meas}$ is highly sensitive to the effect of protein binding affinity constants for T. Although exact values of K_s and K_a are difficult to establish, we might speculate that the K_s and K_a used in the equation of Vermeulen tend to overestimate the true value of BT. Indeed, different theoretical calculations can give different results depending on the algorithm used (21). For example, when using the algorithm by Morris et al. (19), the mean BT_{cal} on the study population was 4.58 nmol/L, whereas it was 3.60 for BT_{meas} and 7.60 when BT_{cal} was estimated by using the algorithm by Vermeulen et al. (17) (results not shown).

Our study shows that $BT_{cal}:BT_{meas}$ had a tendency to increase and eventually double when young and older men were compared. This discrepancy is of clinical relevance, because with similar concentrations of total T and SHBG, BT_{meas} is lower in old men than in young men, whereas BT_{cal} is similar. This difference in $BT_{cal}:BT_{meas}$ between young and old men in our study population was observed when BT_{cal} was calculated by using the algorithm by Vermeulen as well as when using the algorithm by Morris et al. (21), with a significant ($P < 0.001$) decrease in $BT_{cal}:BT_{meas}$ from 2.38 for men older than 50 to 1.44 for men younger than 50 years. Although an increase in K_s with age has been suggested (27) and some mutations in SHBG have been reported (28), they are unlikely to account for such a large discrepancy.

Because we did not measure albumin concentration in our patients, we cannot exclude the possibility that a

Fig. 2. Sensitivity analyses of $BT_{cal}:BT_{meas}$ while changing K_a and K_s between 0.5 and 5×10^4 and 0.6 and 2.0×10^9 L/mol, respectively.

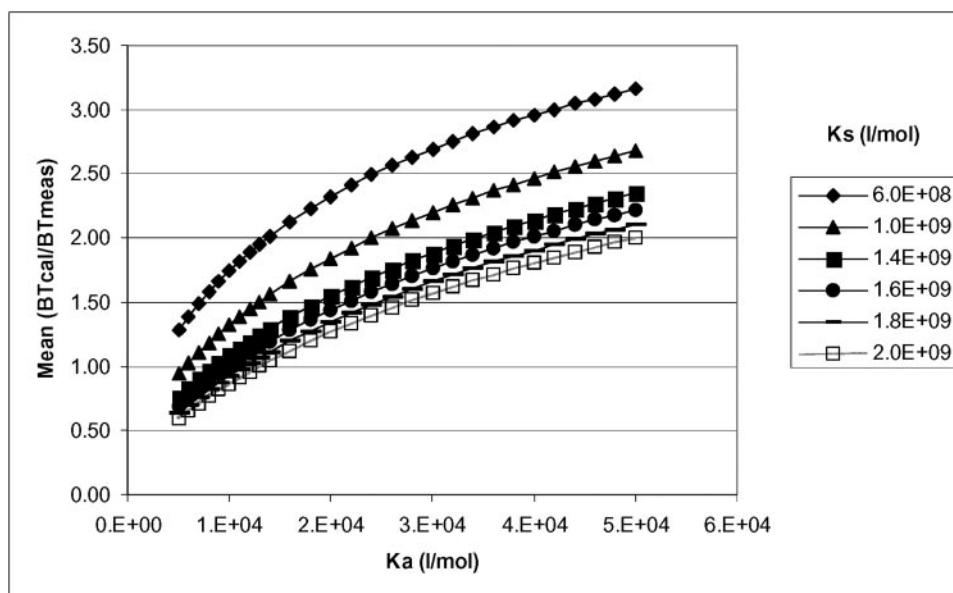


Table 2. Mean (SD) concentrations of T, SHBG, BT_{meas}, BT_{cal}, BT_{cal}:BT_{meas}, DHEA, and DHEAS in 27 men.

	n	T, nmol/L	SHBG, nmol/L	BT _{meas} , nmol/L	BT _{cal} , nmol/L	BT _{cal} :BT _{meas}	DHEA, nmol/L	DHEAS, μmol/L
Men <35 years old	14	17.02 (5.66)	30.58 (13.11)	4.26 (0.82)	8.62 (1.65)	2.09 (0.58)	19.96 (8.58)	9.55 (3.65)
Men ≥60 years old	13	12.66 (5.98)	49.62 (15.06)	1.07 (0.47)	4.55 (1.96)	4.70 (1.75)	4.67 (2.05)	2.40 (1.72)
<i>P</i> (t-test)		0.63	1.88×10^{-3}	4.36×10^{-12}	5.40×10^{-6}	1.73×10^{-5}	1.05×10^{-5}	1.01×10^{-6}

decrease in albumin concentration in older men may contribute to lower BT, although Vermeulen (29) recently reported that for a decrease in albumin concentrations from 43 to 35 g/L, the BT_{cal} would diminish by only 10%.

Interestingly, Cooke et al. (30) reported some discrepancy between BT_{cal} and BT_{meas} concentrations according to cortisol concentrations and suggested that lipids or other molecules could interact with albumin to decrease the number, affinity, or disposal of albumin steroid-binding sites. In this hypothesis, testosterone would be displaced from CBG to SHBG, inducing an increase of the ratio BT_{cal}:BT_{meas}.

An increase in various T-binding proteins of weak affinity constant (CBG, orosomucoid), because they may be partially precipitated by ammonium sulfate, might also contribute to discordant BT_{cal} and BT_{meas} in older individuals and therefore contribute to the decrease of the BT_{meas} concentrations.

It has also been suggested that an important cause for the decline in BT_{meas} vs BT_{cal} concentration in aging men is the lowering of adrenal androgens that bind significantly to SHBG. Because the affinity constants of SHBG for DHEA and Δ5-androstenediol are not negligible (66×10^6 and 1.5×10^9 L/mol, respectively), it has been predicted that more than 10% of the SHBG binding sites are occupied by these adrenal androgens in young men (31). In agreement with previous studies, we observed a highly significant decline in DHEA/DHEAS in older men (1–4). Therefore, in older men, some of these sites on SHBG may be available for binding T, consequently decreasing the concentration of BT_{meas}, whereas the concentration of BT_{cal} would remain unchanged. In our study, by including DHEA in the mass action equation, we found that BT_{cal} increased with increasing concentrations of DHEA. This influence of adrenal androgen, however, was observed for Δ5-androstenediol using concentrations reported in the literature (data not shown). This influence of adrenal androgens is further supported by the results of our complementary analysis in which the ratio BT_{cal}:BT_{meas} was inversely correlated with the concentration of DHEA.

In summary, there is substantial discordance between absolute concentrations of BT_{meas} and BT_{cal}, suggesting that a simplified law of mass action cannot predict the variations in steroid hormone distribution in serum. Further investigation should explore their physiological relevance in aging men and contribute to a consensual approach for BT measurement as a tool for therapeutic decisions.

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