Radioimmunoassay of Testosterone Not Bound to Sex-Steroid-Binding Protein in Plasma

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To measure the concentration of testosterone (T) that is not bound to sex-steroid-binding protein (SBP) in plasma, we quantified by radioimmunoassay the T in the supernates of plasma samples after precipitation with 50%-saturated ammonium sulfate. The concentrations of non-SBP-bound T, directly measured with this assay, correlated significantly (P < 0.001) with those deduced from measurement of the percentage of non-SBP-bound T determined with [3H]T as tracer or from mathematical models according to the law of mass action. It also correlated significantly with the ratio of T to SBP and with the concentration of nonbound T. As determined with this assay, the mean concentration of non-SBP-bound T in normal men was higher in young (4.67, SD 2.68 nmol/L; n = 30) than in older (>40 years) subjects (2.48, SD 1.61 nmol/L; n = 35; P < 0.001) and lower than normal in hyperthyroidism (1.61, SD 0.91 nmol/L; P < 0.01) or infertile men (3.28, SD 1.70 nmol/L; P < 0.01). In women, non-SBP-bound T was higher in hirsute patients (0.24, SD 0.11 nmol/L; P < 0.01) and was lower during pregnancy (0.09, SD 0.05 nmol/L; P < 0.05) than in normal women during the follicular phase (0.16, SD 0.07 nmol/L). We conclude that this direct measurement of non-SBP-bound T in plasma is suitable for routine use and represents a reliable index of androgenicity in human pathology, particularly when alterations of the binding capacity of SBP modify the concentrations of total T.

Additional Keyphrases: pregnancy · hirsutism · hyperthyroidism · male infertility · sex- and age-related effects

In plasma, testosterone (T) is mainly bound, with a high affinity, to a specific binding protein, sex-steroid-binding protein (SBP), also referred to as testosterone-estradiol-binding globulin or sex-hormone-binding globulin; it is also bound to albumin with a low affinity (1,2).4 Pardridge et al. (2,3) have shown that the albumin-bound T diffuses easily from circulation to tissues, and it is now believed that the non-protein-bound ("free") and the albumin-bound T circulating in plasma represent the fractions of T that are available for target cells. Therefore, measurement of the non-SBP-bound fraction of plasma T is of particular interest for the investigation of androgenicity.

Two approaches have been used to estimate the concentration of non-SBP-bound T in human plasma.

In the first, non-SBP-bound T is calculated by using mathematical models based on the law of mass action, which governs the equilibrium of T with its binding proteins. The concomitant measurement of different parameters, increasing in number as the mathematical model increases in complexity, is necessary for these methods of calculation. In the model used by Pearlman and Crépy (4), the determination of the total T concentration, the binding capacity of SBP, and the association constant of the binding of T to SBP are needed. In addition to these parameters, the model developed by Södergård et al. (5) requires determination of free T.

The second approach involves development of methods of separating the non-SBP-bound fraction of T, primarily by treating plasma samples with ammonium sulfate (6–8). The distribution of T in the pellet precipitated with ammonium sulfate (SBP-bound T) and remaining in the supernate (non-SBP-bound T) can be estimated by using [3H]T as tracer (7,9,10). This method relies on the assumption that nonspecific interactions between [3H]T and other proteins are negligible or constant. Purification of the tracer before each assay and measurement of the total T concentration are required for this assay. Ratajczak et al. (11) calculated the non-SBP-bound T concentration by subtracting the SBP-bound T, determined by radioimmunoassay in the ammonium sulfate precipitate, from the total T concentration.

In the present work, we measured T directly, by radioimmunoassay, in the supernate of plasma precipitated with 50%-saturated ammonium sulfate, to assess in one assay the non-SBP-bound T concentration. This procedure does not require concomitant determination of the percentage of non-SBP-bound T and the total concentration of T. We assessed the analytical reliability of this method by comparison with the previously described methods for non-SBP-bound T, using numerous samples exhibiting a large range of T and SBP concentrations. The clinical relevance of the non-SBP-bound T concentration was evaluated by measuring samples drawn from men and women with various physiopathological conditions known to alter concentrations of total T and (or) SBP.

Materials and Methods

Materials

Testosterone and 17β-estradiol were purchased from Steraloids, Inc., Wilton, NH; ammonium sulfate, Grade I, from Sigma Chemical Co., St. Louis, MO; Celite for chromatography (particle size 0.15–0.20 mm; 80–120 mesh) from BDH Chemicals Ltd., Poole, U.K.; Norit A charcoal from Serva, Heidelberg, F.R.G.; Dextran T70 from Pharmacia Fine Chemicals AB, Uppsala, Sweden; 1,2-propylene glycol, ethylene glycol, ethyl acetate, and isooctane from Merck AG, Darmstadt, F.R.G.; and diethyl ether from Gifrer Barbezat, Decines, France.

[1,2,6,7-3H]T-testosterone ([3H]T; specific activity: 3.0–3.9 PBq/mol) was purchased from Amersham International, Amersham, U.K. The anti-testosterone antibody was raised in our laboratory by immunization of rabbit with testosterone-3-(O-carboxymethyl)oxime-bovine serum albumin. The
antibody cross-reacted by 58% with 5α-dihydrotestosterone; 0.8% with androstenedione; <0.1% with dehydroepiandrosterone, androstosterone, or etiocholanolone; and <0.01% with cortisol, progesterone, 17α- and 17β-estradiol, or estrone.

Aqualyte™ Plus scintillation fluid was purchased from J. T. Baker Chemicals BV, Deventer, Netherlands.

For the T radioimmunoassay, we used phosphate buffer (0.1 mol/L, pH 7.4) containing 1 g of bovine serum albumin per liter. Saturated ammonium sulfate solution was stored at 4°C and filtered before use. The dextran-coated charcoal (DCC) suspension contained 2.7 g of Norit A and 0.27 g of Dextran T70 per liter.

Samples

Blood (5 mL) was collected by venipuncture into glass tubes containing lithium heparin. After centrifugation, plasma was removed and stored at −20°C.

Plasma samples were drawn from 238 men and 113 women, providing a large range of concentrations of T (0.33–81.4 nmol/mL in men, 0.10–5.98 nmol/mL in women) and SBP (12.5–183.4 nmol/mL in men, 13.9–236.4 nmol/mL in women). Men ages 18 to 40 years (n = 30) and 40 to 83 years (n = 35) and premenopausal women during the early follicular phase (n = 16) and during pregnancy (n = 9) were considered, after clinical examination, to be healthy normal controls; they were taking no medications.

The following patients were also investigated: 64 men referred to the Clinic for infertility, ages 40 years or less, taking no medications and presenting with azoospermia or oligoasthenoterminia of unknown origin; 18 hyperthyroid men, ages 26–79 years, with a free-thyroxin concentration >24 pmol/L and thyrotropin <0.01 mili-int. unit/mL; and 38 women with hirsutism according to Lorenzo’s classification and with normal or above-normal concentrations of androgens in blood. Each patient in this part of the study was examined by the clinicians involved in this work.

Procedures

Ammonium sulfate precipitation. We added dropwise, at 4°C, 1 mL of saturated ammonium sulfate solution to 1 mL of plasma, with continuous gentle shaking. We then centrifuged the samples (4°C, 3000 x g, 30 min) and aspirated the supernate.

Radioimmunoassay of testosterone. To determine the analytical recovery of T, we added [3H]T (2000 counts/min) to the supernate, then extracted with 6 mL of diethyl ether. After agitation for 5 min, we froze the aqueous phase, then removed the organic phase and evaporated the solvent. We dissolved the residue in 1 mL of isooctane, then performed the chromatographic step according to the procedure of Abraham et al. (12) with minor modifications: we used a Celite column (15 x 0.66 cm, containing 1.6 mL of Celite) with ethylene glycol:propylene glycol (1:1 by vol) as stationary phase (1 mL for 2 g of Celite), and eluted steroids successively with 4 mL of isooctane (fraction 1), 4 mL of ethyl acetate:isooctane (13:100 by vol) (fraction 2), and 5.5 mL of ethyl acetate:isooctane (20:100 by vol) (fraction 3). Preliminary experiments showed that [3H]T was eluted in fraction 3. Fractions 1 and 2 were discarded. Fraction 3, containing T, was evaporated and redissolved in 0.5 mL of the phosphate-albumin buffer. The recovery of the extraction and chromatographic steps was calculated by counting the radioactivity in a 200-μL aliquot of redissolved fraction 3. We quantified T in duplicate samples by RIA as follows:

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T = \frac{K_A \cdot \text{Alb} \cdot fT}{1 + (K_A^T \cdot fT)} + \frac{K_4^T \cdot fT}{1 + (K_4^T \cdot fT)}
\]

where T: total T concentration (mol/L)
K: the association constant of the binding of T to SBP (assumed to be 10^6 L/mol in this work)
SBP: the binding capacity of SBP (mol/L)
fT: concentration of free T (mol/L)
K_A: association constant of T binding to album (L/mol)
K_4^T: association constant of SBP binding to album (L/mol)
SBP: total binding capacity of SBP (mol/L)
T: total T concentration (mol/L)
K_S: association constant of T binding to SBP (L/mol)

Non-SBP-bound T (mol/L) =

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fT + K_A^T \cdot \text{Alb} \cdot fT = T - K_S^T \cdot SBP \cdot fT
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Other indexes of protein-unbound testosterone. (a) Equilibrium dialysis: we measured the percentage of protein-unbound T by equilibrium dialysis (13), calculating the
concentration of free T by multiplying the total T concentration by the percentage of dialyzable T. (b) Commercial kit: the free-T concentration was also directly measured with the “Coat-A-Count” free-T kit (Diagnostic Products Corp., Los Angeles, CA), kindly provided by Behring Diagnostik AG (Marburg, F.R.G.). (c) Index of free T: T/SPB was calculated as an index of free T as previously proposed (14, 15).

Measurements of the testosterone-binding proteins. The binding capacity of SBP was determined in plasma by a solid-phase-binding assay, with use of concanavalin A-Sepharose 4B to adsorb SBP from plasma (16). This binding assay was also used to measure SBP in the supernates of ammonium-sulfate-precipitated plasma samples, after dialysis, to avoid potential interference from high concentrations of ammonium sulfate. We measured albumin concentration in the supernates of ammonium-sulfate-precipitated plasma samples, after dialysis, by radial immunodiffusion on Norpartigen plates purchased from Behring or by immunonephelometry.

Statistical analysis. Results are given as mean ± SD. For statistical analysis we used Student’s t-test; a P value <0.05 was considered significant.

Results

Reliability of the precipitation of testosterone-binding proteins by ammonium sulfate. In the supernates of eight plasma samples treated by ammonium sulfate at 50% saturation, SBP was undetectable (<3.5 nmol/L), whereas albumin recovery was almost absolute (96.3% ± 7.2% of the albumin concentration present in the plasma before precipitation was accounted for). The concentrations of albumin and SBP measured in the plasma supernates are shown as a function of increasing concentrations of ammonium sulfate in Figure 1. SBF was completely precipitated by ammonium sulfate at 50% saturation (final), whereas the albumin concentration in the supernate was unchanged. The concentration of T in the supernate displayed a three-step pattern: from 0% to 30% saturation with ammonium sulfate, the concentration of T corresponded to total T, because none of the binding proteins were precipitated; from 45% to 55% saturation, 20% of total T was not precipitated, which corresponded to the non-SBP-bound T fraction, because no SBP remained in the supernatant; finally, beyond 70–80% saturation, when albumin was totally precipitated, the remaining T concentration was virtually completely free (non-protein-bound) T.

Figure 2 shows the influence of the incubation time of plasma with ammonium sulfate at 50% final saturation. For incubation <15 min, the non-SBP-bound T concentration decreased by <8% of the value obtained with rapid centrifugation. Non-SBP-bound T concentration progressively decreased when the incubation time was longer than 15 min, reaching 30% after 3 h. During this prolonged incubation, the concentration of albumin in the supernate was unchanged. The effect of increasing incubation times on non-SBP-bound T was investigated by assaying plasma samples that had been previously overloaded with various concentrations of 17β-estradiol. The decrease in non-SBP-bound T was progressively blunted and disappeared in the presence of high 17β-estradiol concentrations (Figure 3).

Reliability of the non-SBP-bound testosterone assay. The detection limit of the assay was 45 pmol/L (>2SD of the B₀ value in the standard curve of the radioimmunoassay for T). The intra-assay (n = 8) and interassay (n = 10) coefficients of variation (CVs) were respectively 14.0%, 8.3%, 6.5%, and 6.5% and 16.1%, 13.9%, 8.0%, and 10.1% for mean non-SBP-bound T concentrations of 0.16, 0.80, 2.42, and 8.15 nmol/L.

Table 1 compares the concentrations of non-SBP-bound T as measured by this method with those obtained by the method of Tremblay and Dube (9) or by the theoretical determinations of Pearman and Crépy (4) and Södergard et al. (5). Also shown are the T/SPB ratio and the concentration of protein-unbound T measured by equilibrium dialysis and by Coat-A-Count kit. The concentrations of non-SBP-bound T measured by the present direct assay correlated highly significantly with those measured by each method tested. By contrast, the correlation with the concentration of total T was rather low.

In the male subjects of this study, the concentrations of non-SBP-bound T obtained with the direct assay were not different from those obtained with the method of Tremblay and Dube (9) or that of Pearman and Crépy (4). However, the values obtained with the method of Södergard et al. (5)
were higher. In the women, the values given by the method of Tremblay and Dubé (9) were slightly higher than those measured by the direct assay. Lower values were obtained by the method of Pearlman and Crepy (4) and higher values by the method of Södergard et al. (5) (Figure 4).

Non-SBP-bound T in pathophysiological situations. In Table 2 we compare the concentrations of non-SBP-bound T in physiological and pathological situations. In normal men, the concentrations of non-SBP-bound T decreased significantly with age. In hyperthyroid men, a situation where both total T and SBP are increased, non-SBP-bound T was lower than in age-matched controls. In infertile men, the mean non-SBP-bound T was lower than in age-matched controls.

During normal pregnancy, a dramatic increase in the binding capacity of SBP was associated with an increased total T level; in this circumstance the non-SBP-bound T was slightly decreased. Conversely, in women with hirsutism, SBP binding capacity was significantly (P <0.05) lower than normal, and the mean non-SBP-bound T concentration was higher (P <0.01) than in non-hirsute women.

Discussion

The measurement of non-SBP-bound T in plasma usually requires the determination of at least two parameters of the binding equilibrium of T with its binding proteins in plasma: T, percentage free T, and (or) SBP-binding capacity (4, 5, 9–11). A direct assay for non-SBP-bound T is described from the measurement of T by classical RIA in the supernate of plasma samples after precipitation by 50% saturation with ammonium sulfate.

The pattern of RIA-T, SBP, and albumin in the supernate of ammonium-sulfate-precipitated plasma was carefully assessed as a function of increasing concentrations of ammonium sulfate. This experiment indicated that, at 50% saturation, SBP was fully precipitated with no precipitation of albumin. The method requires that ammonium sulfate be added slowly so as to avoid macroprecipitation and that the elapsed time between the precipitation of SBP and centrifugation of the test tubes be <15 min. Under these conditions there is no significant decrease in T concentration in the supernate (Figure 2). For longer incubation times, a slow decrease in the concentration of non-precipitated T was observed. This phenomenon was not attributable to T-bound albumin adsorption onto the precipitate, because the albumin concentration was unchanged in the supernate, but rather to a slow displacement of the binding equilibrium of T from the albumin-binding sites to the SBP-binding sites. This conclusion was supported by the inhibition of the decrease in non-SBP-bound T concentration when plasma was pre-incubated with increasing concentrations of 17β-estradiol. Under such conditions, 17β-estradiol competed with the albumin-dissociated T to occupy the available SBP binding sites, obviating the increase in SBP-bound T.

The incubation time of plasma with ammonium sulfate must be short, so as not to limit the practicability of the
method. In less than 15 min, 20 samples can be run concurrently for ammonium sulfate precipitation before centrifugation.

The precision of the overall method was close to the precision of the T-RIA performed after extraction and chromatography. The detection limit sufficed to measure the non-SBP-bound T concentration in men as well as in women.

Non-SBP-bound T concentrations measured by this technique correlated significantly with those obtained with the methods of Tremblay and Dube (9), Pearlman and Crépy (4), and Södergard et al. (5) as well as with the T/SBP index and the free-T concentrations. The coefficients of correlation were lower for women than for men, probably owing to the lesser reliability of the radioimmunoassay for low concentrations of T.

In the direct assay, the measurement of total T concentration, the use of a highly purified tracer, and the use of a quenching correction for radioactivity counting are not necessary. The measurements of several parameters of the binding equilibrium of T to calculate non-SBP-bound T according to mathematical models, because of the variability of each assay for these parameters, give rise to a large variability in the finally calculated non-SBP-bound T concentration. This might explain the discrepancy observed between the different methods. Moreover, the mathematical models do not take into account the possibility of the presence of endogenous steroids or of specific or nonspecific ligands, which may interfere with the binding equilibrium of T in plasma and therefore modify the concentration of non-SBP-bound T (17).

For clinical investigations, the non-SBP-bound T evaluation appeared to be of particular interest in male patients with mild gonadal dysfunction. As in other reports, we too found a decrease in non-SBP-bound T concentration with aging in normal men (18, 19). In hyperthyroid males, the concentration of T was increased and associated with an increased binding capacity of SBP, as already well documented (20). This was explained by the reported in vitro stimulating effect of thyroid hormones on the hepatic production of SBP (21). We found that the concentration of non-SBP-bound T was significantly lower in hyperthyroid than in non-hyperthyroid men. In the infertile men of our study, the non-SBP-bound T was, on average, lower than normal. This observation is consistent with a Leydig cell dysfunction in some infertile men (22). This important issue merits further investigation.

In women, during pregnancy, high SBP binding capacity is induced by the increased concentrations of estrogens. It has been well documented that the increase of SBP decreases the metabolic clearance rate of T (23). Therefore, increased SBP binding capacity and the consequent decreased metabolic clearance rate of T explain the relatively high concentrations of total T during pregnancy. However, non-SBP-bound T is not increased, in agreement with the absence of clinical symptoms of hyperandrogenism in pregnant women. Conversely, in hirsute patients, increased T and (or) decreased SBP binding capacity are generally reported. In most of these patients, the non-SBP-bound T concentration was increased, and it was a better marker of hyperandrogenism than total T. This concords with previous reports (24, 25).

We conclude that the direct radioimmunoassay of T in ammonium-sulfate-precipitated plasma is a sensitive, reproducible method. The analytical and clinical reliability of this method is similar to the previously described methods for measurement of non-SBP-bound T. It is a very practical assay, appropriate for routine use in investigating gonadal function in humans. This assay would be useful in patients with abnormal binding of T to SBP, which may alter the distribution of T in plasma and its availability for target cells. In these circumstances, the non-SBP-bound T assay gives a correct interpretation of the concentration of total T and renders determination of SBP unnecessary.

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References

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