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Ratios of plasma and salivary testosterone throughout puberty: Production versus bioavailability

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Because diffusion of testosterone (T) into the salivary gland is thought to be largely limited to the free, biologically active fraction, salivary testosterone is expected to provide a better measure of testosterone bioavailability in the body than is plasma testosterone. Matched saliva and blood spot samples were collected from 218 Zimbabwean males (age 11–23) who were at different stages of puberty, as assessed by self-reported Tanner genital stage ratings. Testosterone concentrations in these matched samples were highly correlated (r = 0.83). Both salivary and plasma testosterone (converted from blood spot value) showed expected significant increases across puberty. However, plasma testosterone distinguished among subjects at different stages of genital development more effectively than did salivary testosterone, suggesting the former to be a better marker of testosterone bioavailability. Sex hormone-binding globulin (SHBG) levels were also measured in a subgroup of 93 of these subjects. After controlling for plasma T concentrations, we found a small but significant inverse correlation between blood spot SHBG levels and the proportion of plasma testosterone recovered in saliva, supporting the hypothesis that SHBG-related changes in T bioavailability are detectable in saliva. We conclude that salivary testosterone accurately reflects testicular production of testosterone, but that neither salivary testosterone nor plasma testosterone is clearly superior to the other as a measure of testosterone bioavailability. (Steroids **61**:374–378, 1996)

Keywords: testosterone; sex hormone-binding globulin; puberty; development; bioavailability

Introduction

Testosterone (T) concentrations in plasma reflect testicular output.[1] They also provide an estimate of the level of biologically active T in the body. They do not, however, specifically predict the amount of T that actually diffuses across cell membranes and binds to intracellular receptors in target tissues. Levels of T in saliva reflect the proportion of plasma T capable of diffusing across acinar cells lining the salivary gland,[2,3] plus or minus any T gained or lost through metabolic conversion[4–6] and/or androgen receptor binding within the acinar cells.[7,8] Because this process is analogous to the diffusion of unbound steroid across the cell membrane of target cells, salivary T concentrations have been thought to provide a better indication of the level of biologically available T in the body than do plasma T concentrations.[3,6,9]

Because genital and pubic hair development are both androgen-dependent processes, they serve as appropriate indicators of levels of T bioactivity in the body. Based on their level of development, subjects can be assigned (or can assign themselves) to one of Tanner's five stages of genital or pubic hair development. We set out to test whether either salivary or plasma testosterone was more effective in differentiating among subjects at different Tanner genital stages, and thus more accurately reflected testosterone bioavailability.

Although it has been demonstrated that T can activate intracellular second messenger pathways in target cells by binding to sex hormone-binding globulin (SHBG; bound to the SHBG receptor) on the plasma membrane,[10] SHBG-bound T is unable to freely diffuse across cell membranes.[2,3,11] There-

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fore, changes in SHBG concentration have been thought to affect T availability to intracellular receptors in target cells. If, as suggested above, the diffusion of T into the salivary ducts is analogous to the diffusion of T into target cells, then the proportion of total plasma T recovered in saliva should correlate inversely with blood levels of SHBG. We evaluated this hypothesis by calculating the ratio of salivary to plasma testosterone in the matched samples collected from each subject and testing for an association between these ratio values and subject SHBG levels.

Experimental

As part of a larger study of developmental and behavioral changes at adolescence, blood spot and saliva samples were collected simultaneously from each of 218 Zimbabwean male subjects (age 11–23). Human subjects approval was granted by the Human Subjects Committee of the University of North Carolina. Permission to collect samples was granted by the Zimbabwe Ministry of Education and headmasters of the schools acting in loco parentis. Each subject also gave oral consent for sample collection. Subjects rinsed their mouths out with water at the beginning of the sample collection procedure and were not permitted to eat or drink after this time prior to sample collection. Blood spot samples were collected first from finger pricks performed with an autolancet device. Blood drops were allowed to reach maximum size, then spotted onto preprinted circles on sample collection papers (Schleicher and Schuell no. 903). Samples were dried flat at room temperature for 4 h, then placed in a refrigerator until shipment to the United States 5 weeks later. Upon receipt, samples were stored in an airtight container at −20°C until assay. Immediately after blood spot collection, 5 mL of saliva was collected in polystyrene tubes and frozen until assay. Saliva flow was stimulated with one-half stick of Gator Gum.

Blood spot testosterone assay

Blood spot samples were assayed for testosterone using the following method, which is a modified protocol of a commercially available kit for serum T from Binax (South Portland, ME, USA; Equate RIA Testosterone, catalog no. JPL-016).[12] The procedure is a competitive binding radioimmunoassay (RIA) with second antibody-polyethylene glycol separation. The antiserum is rabbit anti-human testosterone serum in phosphate buffer that cross-reacts 1.7% with dihydrotestosterone and <1.0% with androstenedione and other related compounds. Kit standards are human serum at testosterone concentrations of 0, 10, 50, 100, 250, 500, 1000, and 2000 ng/dL.

Standards and controls (Bio-Rad, ECS Division, Anaheim, CA, USA) are prepared for blood spot analysis by 1:2 addition of red blood cells previously washed three times with normal saline. For each standard and control, 50 μL of this mixture is dropped onto filter papers (Schleicher and Schull no. 903), allowed to dry overnight at room temperature, and stored in a sealed container at −20°C. Blood spot standard concentrations are 0, 5, 25, 50, 125, 250, 500, and 1000 ng/dL. Bio-Rad blood spot control concentrations are 46.0 ng/dL (2 SD range: 33.2–58.8) and 522 ng/dL (2 SD range: 499–645).

At the time of assay, standards, controls, and sample papers are removed from the freezer, four 1/8-inch discs are punched with a hole punch from each spot to be assayed, and the discs are transferred to 12 × 75 mm glass tubes. Three hundred microliters of protein buffer (pH 7.4) (prepared by adding 100 mg of gelatin to 100 mL of Dulbecco's buffer, Gibco, Grand Island, NY, and heating to 45°C to dissolve) is added. The tubes are rotated gently (50 rpm) for 1 h at room temperature, then eluted overnight at 4°C. Tubes are thereafter rotated gently for another hour at room temperature.

Aliquots (100 μL) of eluate are pipetted into duplicate 12 × 75 mm polypropylene assay tubes. Kit antiserum is diluted 1:4 with protein buffer used in the elution of samples. Upon addition of kit tracer (50 μL of ^{125}I-T) and diluted antiserum (100 μL), assay tubes are vortexed and incubated overnight at room temperature. Kit-provided precipitating reagent (containing goat anti-rabbit γ-globulin and polyethylene glycol) is diluted 1:2 with protein buffer, then 0.5 mL is added to the assay tubes, vortexed immediately, and incubated at room temperature for 20 min. Tubes are then centrifuged at 4°C for 60 min at 2300 × *g*. After centrifugation, the supernatant is decanted by inverting the tubes and blotting. The tubes are counted in a gamma counter for 5 min each.

Sensitivity of the modified assay is 6.3 ng/dL, defined as the dose required to reduce binding to 2 SDs below the mean of the zero standard. Intraassay coefficients of variation (CVs) are 7.6% (Bio-Rad level I, mean 52.0 ng/dL, SD = 3.98) and 7.0% (Bio-Rad level II, mean 548.2, SD = 38.2). Interassay coefficients of variation are 13.9% (Bio-Rad level I, mean 46.0, SD = 6.42) and 11.8% (Bio-Rad level II, mean 521.6, SD = 61.4). Method recoveries range from 89.9% to 109%.

Regression analysis was performed from data on 56 samples (obtained from routine admissions at Emory University Hospital) using direct plasma measures and blood spot values. Correlation of plasma and blood spot readings is high (Pearson $r = 0.9788$); it is not enhanced by correction for hematocrits on blood spot samples, and the fitted regression equation explains nearly all of the variance ($r^2 = 0.9736$). Blood spot concentrations (X) can therefore be converted to plasma equivalents (Y) with the equation $Y = 1.3X$.

Blood spot SHBG assay

Blood spot samples were assayed for SHBG in 93 of the 218 subjects from whom blood spot samples were collected. Initially, 100 subjects were selected for SHBG analysis, such that they would be distributed equally across the five stages of genital development. Unfortunately, there were only four subjects in stage 1, two of whom had adequate sample remaining for the SHBG assay procedure. Five of the remaining 100 subjects also had inadequate samples, resulting in a total n of 93. The assay used is a modified protocol of a commercially available kit for serum SHBG from Wallac, Inc. (Gaithersburg, MD, USA) (DELFIA). The procedure is a fluoroimmunoassay based on the direct sandwich technique employing two monoclonal antibodies directed against different sites on the SHBG molecule. The method is described in detail in another article in the process of being submitted for publication. Performance characteristics of the assay are as follows. Assay sensitivity, defined as the dose required to reduce binding 2 SDs below the mean of the zero standard, is 0.2 nmol/L. The intraassay CV is 13.2% (Bio-Rad level 1, 29.7 ± 3.92 nmol/L, mean ± SD). The interassay CV is 14.5% (Bio-Rad level 1, 30.0 ± 4.36 nmol/L, mean ± SD). The assay has no significant cross-reactivity with human IgG, human IgA, fibrinogen, α-antitrypsin, transferrin, haptoglobin, glycoprotein α-1 acid, and plasminogen. Recoveries range from 98% to 111%.

Regression analysis was performed on 21 samples using direct plasma measures and blood spot values. Correlation of plasma and blood spot readings is high ($r = 0.93$), even without correction for variability in hematocrits on blood spot samples. The fitted regression equation explains 87% of the variance. Plasma concentrations can therefore be estimated from blood spot concentrations using the equation $Y = 2.0X - 10.2$, where Y is the estimated plasma SHBG concentration and X is the blood spot SHBG concentration.

Salivary testosterone assay

Saliva samples were assayed for testosterone by RIA using a previously published method.[13] The method is a modification of the

Leeco Diagnostic, Inc. double-antibody ^{125}I radioimmunoassay kit for serum testosterone. Based on previous work in our laboratory, the sensitivity of the modified assay, defined as the dose required to reduce binding 2 SDs below the mean of the zero standard, is 0.1 ng/dL. For the samples analyzed in this study, the estimated sensitivity of the assay, based on the average concentration at 95% B/B_0, is 0.46 ng/dL. Intraassay CVs ranged from 7.9% to 12.9% across the standard curve. Interassay CVs ranged from 10.2% to 15.6%. Interassay CV for the low control pool (mean = 4.44 ng/dL) was 11.0%, whereas that for the high control (mean = 51.1 ng/dL) was 6.6%. Cross-reactivity with dihydrotestosterone was 1.7%. There was no significant cross-reactivity with other related steroids.

Measure of pubertal development

Tanner stages of genital development were self-reported by subjects based on a standard series of line drawings representative of each stage of development.[14]

Results

For all subjects combined, the matched salivary and plasma testosterone concentrations (converted from blood spot values) correlated at $r = 0.83$ ($n = 218$; $r^2 = 0.682$). The strength of the association was significantly greater for subjects in the early stage (stage 2; $r = 0.96$) compared to the adult stage of genital development (stage 5; $r = 0.79$; $Z = 2.73$, $P < 0.01$). Salivary and plasma testosterone increased and SHBG decreased with advancing genital development (Figure 1). Analysis of variance revealed that plasma testosterone was more effective than salivary testosterone in differentiating among subjects at different stages of genital development. The F-test for difference among Tanner genital stage groups was highly significant for plasma testosterone ($F = 4.53$, $P < 0.05$) and only marginally so for salivary testosterone ($F = 2.26$, $0.05 < P < 0.10$). In addition, whereas plasma testosterone levels could distinguish subjects in stage 5 from those in each of the other four stages, salivary testosterone levels could only distinguish stage 5 subjects from subjects in stages 1 or 2, but not stages 3 or 4.

The average ratio of salivary to plasma testosterone for all subjects combined was 0.028. For those who had completed puberty (those in Tanner stage 5), the ratio was 0.024. Ratios were not calculated for subjects with salivary T concentrations below the sensitivity limit of our assay (0.46 ng/dL). Nor were ratios calculated for subjects with plasma T concentrations of less than 20 ng/dL, since the matching salivary T concentration needed to produce a reasonable ratio (i.e., 0.024) would be below the sensitivity of our salivary assay.

Blood spot SHBG levels were not correlated with ratio values ($n = 82$, $r = 0.11$, $P > 0.05$). However, after controlling for plasma testosterone concentrations, SHBG became a significant predictor of ratio values in a multiple linear regression model ($t = -2.4$, $P < 0.05$, partial $r = -0.29$). Plasma T values were decomposed into strata (stratum 1 = 20–50 ng/dL, stratum 2 = 51–150 ng/dL, stratum 3 = 151–300 ng/dL, stratum 4 = 301–450 ng/dL, stratum 5 = >450 ng/dL) and entered into the model as an ordinal variable. This variable was a strong predictor of ratio values ($t = -5.31$, $P < 0.001$, partial $r = -0.65$). Although we expected the ratio of salivary to plasma testosterone to increase across puberty because of the pubertal decline in SHBG, the mean ratio (for subjects whose T levels did not fall below the sensitivity limit of our assay) actually decreased (Figure 2), but not significantly ($F = 2.15$, $P > 0.05$).

Discussion

The correlation between plasma equivalents (derived from blood spots) and salivary testosterone reported here ($r = 0.83$) is consistent with that found in other studies using male subjects ($r = 0.94$,[15] $r = 0.64$[16]). In our study, approximately one-third of the variance in plasma testosterone cannot be explained by salivary testosterone concentrations ($r^2 = 0.68$). Nevertheless, the relatively strong correlation between the two measures, coupled with their similar developmental profiles, suggests that salivary testosterone is a good marker of testicular production throughout puberty.

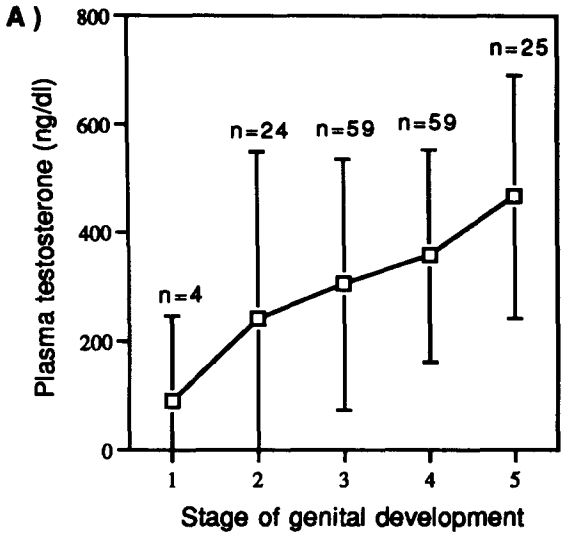
Plasma testosterone levels were more effective than salivary testosterone levels in distinguishing among subjects at various stages of genital development, suggesting the former may be a better marker of T bioavailability since genital development is testosterone-dependent. It could be argued that the reduced precision of our salivary T assay relative to our plasma T assay contributed to the lack of a significant difference in mean salivary testosterone concentrations between stage 5 and stages 3 and 4. Consistent with this argument, the sample coefficients of variation for the salivary T assay in stages 3 and 4 (88.1% and 68.2%) are higher than those for the plasma assay in stages 3 and 4 (75.7% and 54.8%). However, the P-values for the t-tests comparing mean salivary testosterone concentrations do not even approach significance (stage 5 versus 4, $P = 0.56$; stage 5 versus 3, $P = 0.27$). Therefore, the lower precision of our salivary assay is unlikely to explain these results.

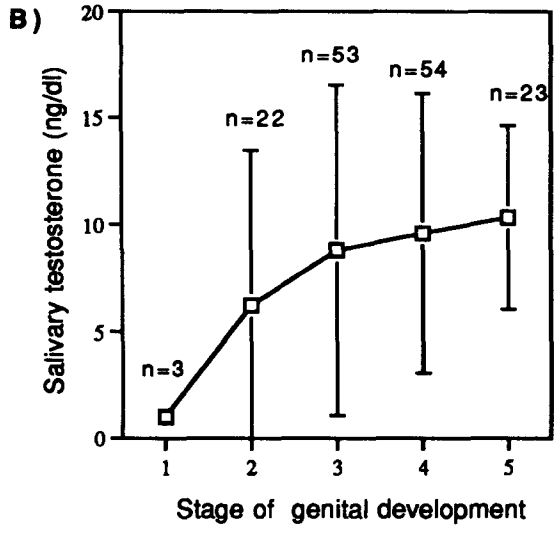
Our measure of pubertal development may be a less than ideal indicator of T bioavailability since it is based on potentially biased subject self-report. The fact that subjects who rated themselves as being more advanced in pubertal development had higher testosterone levels (Figure 1) suggests that the measure has some validity.

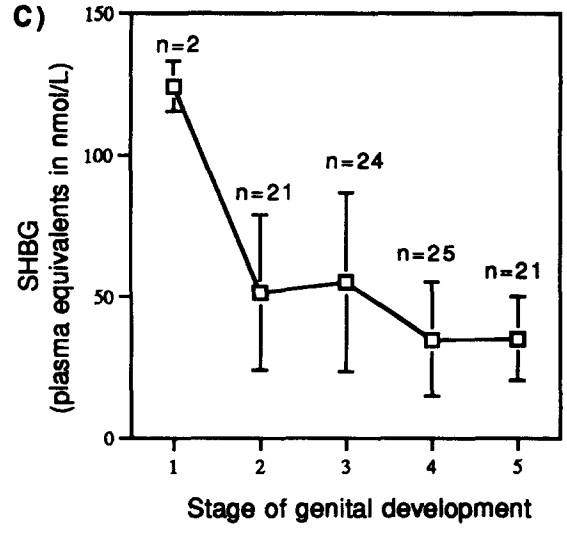
The average ratio of salivary to plasma equivalent testosterone levels of subjects in this study who had completed puberty (Tanner stage 5; 0.024) is only slightly greater than that found in two other studies in which matched saliva and plasma samples were collected from adult male subjects. (0.014[15] and 0.022[16]). This value is similar to the proportion of total circulating testosterone that is unbound to carrier proteins (2%),[17] consistent with the idea that it is primarily the unbound fraction that has access to the salivary gland.

In this study, plasma equivalent SHBG levels decreased significantly with advancing pubertal status (Figure 1). Similar declines in SHBG across puberty have been reported by several other groups.[18–20] Because testosterone inhibits SHBG production,[21] it has been suggested that the pubertal increase in testosterone may be responsible for the decline in SHBG.[19] Insulin has also been proposed as a possible mediator of the pubertal decline in SHBG.[18]

The small but significant inverse correlation between plasma equivalent SHBG levels and the proportion of total







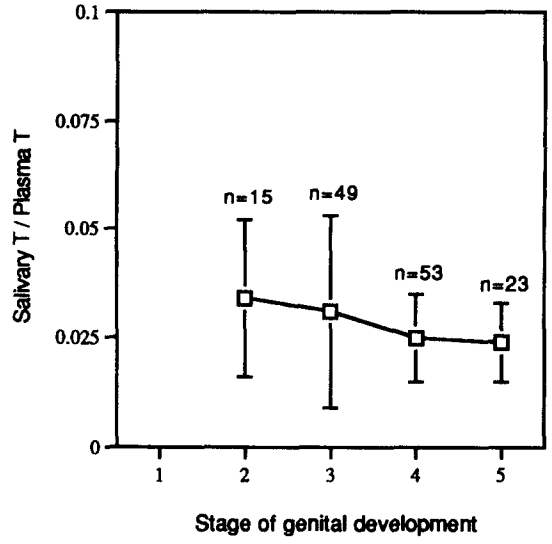


Figure 2 Mean (±SD) ratio of salivary to plasma testosterone for subjects by Tanner genital stage rating. *F*-test for difference among Tanner genital stage groups, 2.15 ($P > 0.05$). After excluding subjects with plasma testosterone values below 20 ng/dL, there were no subjects remaining in Tanner genital stage 1. Means and standard deviations for the other four stages are as follows (mean ± 1 SD): stage 2, 0.034 ± 0.018; stage 3, 0.031 ± 0.022; stage 4, 0.025 ± 0.01; and stage 5, 0.024 ± 0.009.

plasma T recovered in saliva suggests that SHBG-related influences on testosterone bioavailability are detected in saliva, which, in contrast to the above finding, supports the idea that salivary testosterone is a better marker of testosterone bioavailability than is plasma testosterone. The results of the regression analysis also suggest that the ratio of salivary T to plasma T decreases as plasma T increases. This fact, coupled with the failure of the ratio to increase across puberty as SHBG levels declined, suggests that changes are occurring across puberty (as T rises) that counter the SHBG effect and serve to decrease the diffusion of plasma T into the salivary ducts. What those changes are is currently unknown, but one possibility might be altered concentrations of steroid-metabolizing enzymes and/or androgen receptors in the salivary gland, both of which influence the amount of testosterone that diffuses into the salivary duct.[4-8]

Figure 1 **(A)** Mean (±SD) plasma testosterone by Tanner genital stage of subjects. *F*-test for difference among Tanner genital stage groups, 4.53; $P < 0.01$. Stage 5 mean (466.6 ± 224.5 ng/dL) was significantly greater than means at stage 1 (91.2 ± 155.7 ng/dL) ($t = 4.18$; $P < 0.01$), stage 2 (241.9 ± 308.6 ng/dL) ($t = 2.91$; $P < 0.01$), stage 3 (305.5 ± 231.2 ng/dL) ($t = 2.98$; $P < 0.01$), and stage 4 (357.9 ± 196.1 ng/dL) ($t = 2.11$; $P < 0.05$). **(B)** Mean (±SD) salivary testosterone by Tanner genital stage of subjects. *F*-test for difference among Tanner genital stage groups, 2.26; $0.05 < P < 0.10$. Stage 5 mean (10.34 ± 4.3 ng/dL) was significantly greater than means at stage 1 (1.00 ± 0.1 ng/dL) ($t = 10.39$; $P < 0.01$), and stage 2 (6.24 ± 7.2 ng/dL) ($t = 2.30$; $P < 0.05$), but not stage 3 (8.78 ± 7.74 ng/dL) ($t = 1.12$; $P > 0.05$) or stage 4 (9.60 ± 6.5 ng/dL) ($t = 0.59$; $P > 0.05$). **(C)** Mean (±SD) plasma SHBG equivalents by Tanner genital stage of subjects. *F*-test for difference among Tanner genital stage groups, 8.60; $P < 0.001$. Stage 5 mean (35.28 ± 14.81 nmol/L) was significantly less than means at stage 1 (124.5 ± 8.91 nmol/L) ($t = 12.6$; $P < 0.05$), stage 2 (51.34 ± 27.34 nmol/L) ($t = 2.37$; $P < 0.05$), and stage 3 (55.05 ± 31.60 nmol/L) ($t = 2.74$; $P < 0.05$), but not stage 4 (34.94 ± 20.13 nmol/L) ($t = 0.06$; $P > 0.05$).

Several studies have attempted to determine whether salivary testosterone, plasma testosterone, or some other measure (i.e., Free Androgen Index [total plasma T/SHBG] or plasma free testosterone) is a superior measure of T bioavailability through comparing the effectiveness of these different measures in identifying women diagnosed with hirsutism.[6,9,22] Two of these studies found salivary testosterone levels to be elevated in a greater proportion of the cases compared with plasma testosterone,[6,9] whereas the third reported opposite results.[22] Nor have the findings reported here unambiguously established either salivary or plasma testosterone as a superior marker of T bioavailability. SHBG levels suggested the former to be better, whereas Tanner genital stage ratings suggested the latter to be better. It should be emphasized that the correlation between SHBG and ratios of salivary to plasma T, although significant, was weak. More research is needed to settle this issue. However, at this time, it appears as though neither measure is overwhelmingly superior to the other as an index of T bioavailability.

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