Clinical Endocrinology (2007) **67**, 656–662 doi: 10.1111/j.1365-2265.2007.02937.x

ORIGINAL ARTICLE

Salivary testosterone: a reliable approach to the diagnosis of male hypogonadism

Alejandro L. Arregger*, Liliana N. Contreras*,†, Omar R. Tumilasci†,‡, Daniel R. Aquilano§ and Estela M. L. Cardoso*,†

*Endocrine Research Department, Instituto de Investigaciones Médicas A. Lanari, School of Medicine, University of Buenos Aires, †CONICET, ‡Laboratory of Salivary Glands, School of Medicine, University of Buenos Aires, §IABE, La Plata, Argentina

Summary

Objective This study was to demonstrate that Sal-T is a reliable biomarker of androgen status in the diagnosis of male hypogonadism.

Design In order to validate the salivary testosterone assay (Sal-T), its reproducibility, the agreement with serum free testosterone levels (Free-T), the correlation with other circulating androgen markers (bioavailable testosterone, total testosterone) and cut-off values were defined.

Patients and methods We studied 52 eugonadic (E) and 20 hypogonadic (Hy) men. Sal-T was assayed using an adapted radioimmunoassay for serum testosterone. Sal-T concentrations were compared in nine cases before and after citric acid stimulation of salivary flow rate. Free-T and bioavailable testosterone (Bio-T) were calculated by Vermeulen equation and SHBG were determined by binding assay.

Results Sal-T did not depend on salivary flow rate and morning samples from 07·00 h to 09·00 h were stable. Agreement between Sal-T and Free-T measurements was confirmed in all subjects. Sal-T levels correlated positively with all circulating androgens, showing the best correlation with Free-T in E ($r = 0{\cdot}92$) as well as in Hy ($r = 0{\cdot}97$). A cut-off value of Sal-T $\leq 0{\cdot}195$ nM showed 100% sensibility and specificity to rule out hypogonadism.

Conclusions Our data showed that Sal-T is a reliable marker of testosterone bioavailability. The results support the inclusion of this biomarker as a noninvasive approach in the diagnosis of male androgen deficiency.

(Received 3 April 2007; returned for revision 18 April 2007; finally revised 24 April 2007; accepted 25 April 2007)

Correspondence: Estela M. L. Cardoso, Endocrine Research Department, Instituto de Investigaciones Médicas A. Lanari, School of Medicine, University of Buenos Aires, Combatientes de Malvinas 3150·1427, Buenos Aires, Argentina. Tel/Fax: 54-11-45238947;
E-mail: endoexp2000@yahoo.com

Introduction

The use of saliva for monitoring testosterone levels in men has improved greatly since the first report,[1] although its clinical application remains uncertain.

In healthy adult men, 44% of the circulating testosterone is bound specifically to SHBG, 50% to albumin and 3·5% to cortisol binding globulin, while only 2% to 3% is free testosterone (Free-T).[2] Bioavailable testosterone (Bio-T) is the free circulating hormone plus the albumin bound fraction that represents the physiologically active testosterone having an extremely good correlation with Free-T.[3] Salivary testosterone (Sal-T) reflects the free fraction of plasma testosterone able to diffuse passively across the salivary glands.[4] Experimental studies performed in rats showed that salivary testosterone could be influenced by any testosterone gained or lost through metabolic conversion within the acinar cells of salivary glands.[5] A highly significant correlation between salivary and Free-T concentrations was described in healthy men[6–9] suggesting that the measurement of Sal-T may be a useful index of serum Free-T.[7,10] Recently, Morley *et al.*[11] described that Sal-T levels correlated well with serum Bio-T and Free-T in adult men showing low levels of Sal-T in subjects with symptoms of androgen deficiency. In addition Goncharov *et al.*[12] reported that patients with confirmed androgen insufficiency had lower morning Sal-T levels than eugonadic subjects. However, Sal-T measurement is not yet included in the algorithm for the study of male hypogonadism.[13]

In order to define the accuracy of the Sal-T methodology the following factors were taken into account: (1) independence of testosterone levels from saliva flow rate that was only demonstrated in the fraction of saliva drained from parotid glands;[7,14] (2) using a radioimmunoassay (RIA) that sustains the required specificity of the gold standard method;[15] (3) choosing early morning samples to assess gonadal status as testosterone secretion in men exhibits a diurnal rhythm;[16] and (4) using calculated Free-T and Bio-T validated by Vermeulen *et al.*[17] that implies the need for measuring albumin and SHBG concentrations.[3]

The purpose of this study was to establish the reproducibility of morning Sal-T samples, to validate Sal-T results against circulating Free-T in eugonadic and hypogonadic subjects, and to determine the cut-off of Sal-T and circulating testosterone values (Free-T, Bio-T, TT) in already known hypogonadic patients.

Subjects and methods

Studied population

This study included 52 eugonadic (E) men (aged 20–55 years; body mass index, BMI 24·0 ± 3·0 kg/m^2) and 20 hypogonadic (Hy) patients (aged 25–70 years; BMI 23·0 ± 2·0 kg/m^2). Primary and secondary hypogonasdism was diagnosed in 7 eugonadic and 13 hypogonadic patients, respectively. Ten patients who had been treated with testosterone enanthate (250 mg every 2–3 week i.m) were evaluated 1 month after the last injection.[13] A cut-off serum total testosterone concentration less than 10·4 nM[13] plus at least three signs/symptoms suggestive of androgen deficiency (i.e. loss of libido, erectile dysfunction, lethargy, inability to concentrate, sleep disturbance, irritability and depressed mood) were defined as hypogonadism.

Healthy subjects were recruited from the staff of the University of Buenos Aires and were not on medication at the time of the study. The protocol was approved by the Human Research Ethics Committee of the School of Medicine, University of Buenos Aires, Argentina, and all subjects gave informed consent to participate in the study.

Salivary flow rate study

Whole saliva was collected from nine randomly selected healthy subjects in fasting conditions, between 08·00 h and 09·00 h, through a soft plastic catheter connected to a suction device, placed on the floor of the mouth for 10 min during which initial (low) salivary flow rate (ml/min) was calculated as described.[18] Saliva production was then stimulated with three filter paper disks of 18 mm diameter soaked with citric acid (2%). The stimulated saliva was collected for 3 min and the reflex (high) salivary flow rate was calculated. An aliquot of the supernatants obtained by centrifugation of basal and stimulated saliva samples was stored at –20 °C until determination of salivary testosterone. On the next day, morning and evening saliva samples were collected from these nine subjects. The subjects were instructed not to brush their teeth and to rinse their mouths with tap water 2 h before saliva collection in the evening (19·00–23·00 h) to eliminate any possibility from food contamination.

Collection of saliva and serum samples

Between 07·00 h and 09·00 h, after overnight fasting and avoiding tooth brushing, all subjects collected 3·5 ml of whole saliva by directly spitting into polypropylene tubes, and simultaneous blood samples were drawn. The supernatants of saliva and serum obtained after centrifugation (1000 × *g*, 10 min) were kept at –20 °C for further analysis.

Salivary testosterone assay

Testosterone in saliva was determined using a ^{125}I double antibody test for the quantification of total testosterone in serum (DSL 4100, Diagnostic System Laboratories Inc, Webster, TX) with the modifications described by Granger *et al.*[19] Our minor adaptation of the technique consisted in increasing the antiserum volume from the suggested 20 µl to 50 µl and adjusting all other reagents accordingly (100 µl of ^{125}I labelled testosterone tracer and 1000 µl of the precipitating solution). The standard curve was prepared diluting standards provided with the kit in PBS 100-fold to give final concentrations of 1·0 pg/ml, 2·5 pg/ml, 5·0 pg/ml, 25·0 pg/ml, 100·0 pg/ml and 250·0 pg/ml. DSL internal controls (levels I and II) were diluted in PBS 10-fold to obtain values between 50·0 pg/ml and 500·0 pg/ml, respectively. Aliquots of 200 µl of saliva samples, standards and controls were processed in duplicate following the steps and incubation times specified by Granger *et al.*[19] The sensitivity of the assay, defined as the quantity of unlabelled hormone required to inhibit the binding of tracer by an amount equal to 2 SD below the mean binding observed in the absence of unlabelled hormone, was 1·0 pg/ml (3·47 pM). Low and high DSL controls were also used to determine the precision of the assay. The intra-assay and interassay coefficients of variation (CVs) were < 6·8% and < 9·5%, respectively. Method accuracy was determined by the addition of unlabelled testosterone (10·0–200·0 pg/ml) to saliva samples from hypogonadic and normogonadic men (range 20·0–200·0 pg/ml). Recoveries ranged from 85% to 105% and the average was 97%. The relation between expected and measured Sal-T levels did not significantly deviate over the concentration range studied. The antibody cross-reacted 6·6% with 5α-dihydrotestosterone, 1·8% with 11-oxotestosterone and ≤ 0·6% with other related compounds.

Serum testosterone assay

The total serum testosterone measurements of the samples were performed using a RIA kit (DSL 4100, Diagnostic System Laboratories Inc, Webster, TX) following the manufacturer's guidelines. The sensitivity of the assay was 0·08 ng/ml (0·28 nM). The intra and inter CVs were less than 6·0% and 9·0%, respectively.

SHBG assay

SHBG measurement, expressed as maximal dihydrotestosterone (DHT) binding capacity, was performed by a modification of the Scatchard analysis[2] using only one DHT concentration at saturation.[20] Briefly, 50 µl of serum were incubated with 100 µl of 0·625 mM DHT, 100 µl of 1·0 mM cortisol (to prevent DHT binding to CBG) and 100 µl of 30 000 dpm ^{3}H-DHT (s.a. 127 Ci/mmol) in a final volume of 500 µl. Free and bound fractions were separated by adding 200 µl of a charcoal–dextran suspension (6·25 mg/ml : 0·62 mg/ml). Nonspecific binding was obtained by incubating with 100 µl of 5·0 mM DHT. Maximum binding capacity was calculated from the specific dpm bound.

Serum albumin

Albumin concentration was measured using a Technicon RA500 analyser (Technicon Instruments Corporation, New York, NY) following the manufacturer's recommended protocol.

Circulating free–testosterone and bioavailable testosterone concentrations

They were both calculated as described by Vermeulen[17] using a second-order equation based on SHBG, total testosterone and albumin

concentrations (algorithms are available at http://www.issam.ch[3]). All hypogonadic patients demonstrated SHBG (27·2 ± 10·6 nM) and albumin (4·2 ± 0·21 g/dl) levels not different from those found in the 52 eugonadic subjects (30·9 ± 9·6 nM and 4·3 ± 0·38 g/dl; $P = 0·153$).

Statistical analysis

Data are expressed as mean ± SD. Variance components and intra-class correlations (ICC) were estimated by random-effects ANOVA model using the Statistical Package for the Social Sciences (SPSS 11·5, SPSS Inc., Chicago, IL). The ICC is an index of reproducibility for a test, ideal being close to 1·0 (close to100% of the observed variation explained by between-individual variation). Bland–Altman analysis based on evaluation of the difference between the methods (Sal-T concentration – serum Free-T levels) against their mean [(Sal-T concentration + Free-T levels / 2)] was used to assess the between-assay difference. The limits of agreement (estimated by the mean difference ± SD of the differences [δ]) and the percentages of outliers were also calculated. When two methods are identical, the expectation is that 95% of differences will be within 0 ± 1·96 δ.[21] In order to estimate the cut-off values, we used receiver operating characteristic (ROC) analysis for all diagnostic settings, which were optimized for sensitivity. Correlations between serum and saliva measurements were calculated by Spearman rank order correlation test. $P < 0·05$ was considered statistically significant.

Results

Checking the influence of saliva flow rate and choosing the right sampling time to optimize Sal-T measurement

Individual Sal-T levels at baseline and after flow rate stimulation are shown in Fig. 1.

Saliva flow rate (0·5 ± 0·07 ml/min) significantly increased after citric acid stimulation (1·14 ± 0·25 ml/min, $P = 0·0001$), yet mean Sal-T levels did not change (0·410 ± 0·208 nM *vs.* 0·417 ± 0·194 nM, $P = 0·942$). This observation means that Sal-T levels in whole saliva are independent of saliva flow rate. Sal-T levels along the intervals from 07·00 h to 09·00 h and between 19·00 h and 23·00 h did not show significant differences ($P \geq 0·151$ in both cases). Mean Sal-T levels were higher in the morning than in the evening (Fig. 2 $P = 0·0001$). Thus we chose morning saliva samples (07·00–09·00 h) to assess gonadal status.

Validation of salivary testosterone assay in eugonadic and hypogonadic subjects

Reproducibility. Two morning saliva and serum samples simultaneously obtained in quick succession from 20 eugonadic (E) and 20 hypogonadic subjects (Hy) were used to assess Sal-T and Free-T, respectively (Fig. 3). In E, Sal-T and Free-T levels obtained from the fisrt sample (0·453 ± 0·19 nM and 0·428 ± 0·135 nM, respectively) did not differ from the second (0·464 ± 0·194 nM and 0·444 ± 0·154 nM, respectively; $P \geq 0·700$). The calculated ICC of SAL-T and Free-T were 0·99 and 0·95, respectively (this means that 99% and 95% variation is explained by between-subjects variation and 5% or less by within-subjects variation). In Hy, first and second values of Sal-T (0·117 ± 0·056 nM and 0·111 ± 0·055 nM, respectively) and serum Free-T (0·139 ± 0·065 nM and 0·139 ± 0·059 nM, respectively) were not different ($P > 0·700$). The ICC was 0·91 for both (Sal-T and Free-T), showing a high reproducibility (less than 10% within-subject variation).

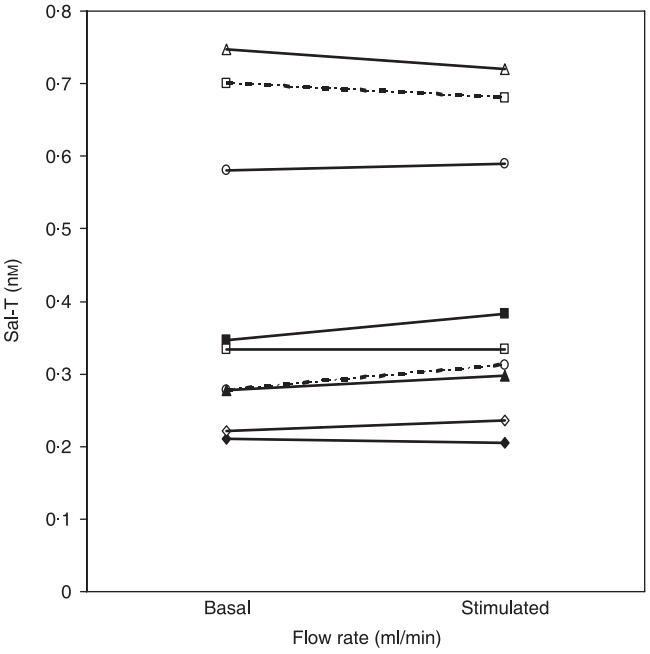

Fig. 1 Whole saliva testosterone concentrations (Sal-T) at baseline and after citric stimulation in eugonadic subjects.

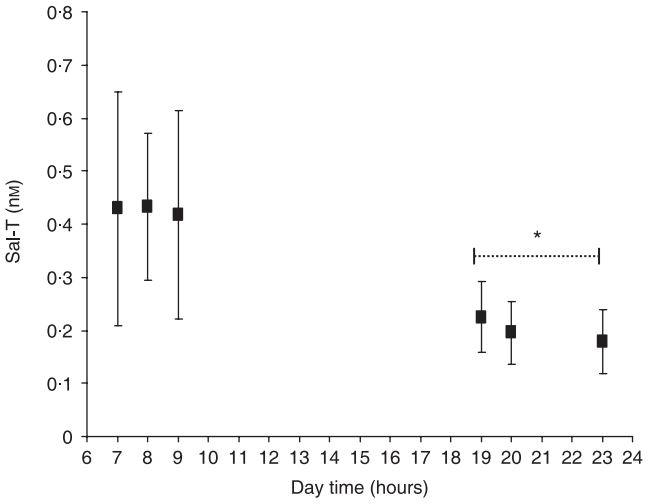

Fig. 2 Morning and evening salivary testosterone concentrations (Sal-T) in nine eugonadic subjects. Data are expressed as mean ± SD. Mean morning *vs.* mean evening levels *$P = 0·0001$.

Agreement between Sal-T and circulating Free-T. Simultaneous morning saliva and serum samples for Sal-T and Free-T determinations were obtained from 52 eugonadic subjects (E) and 20 Hy

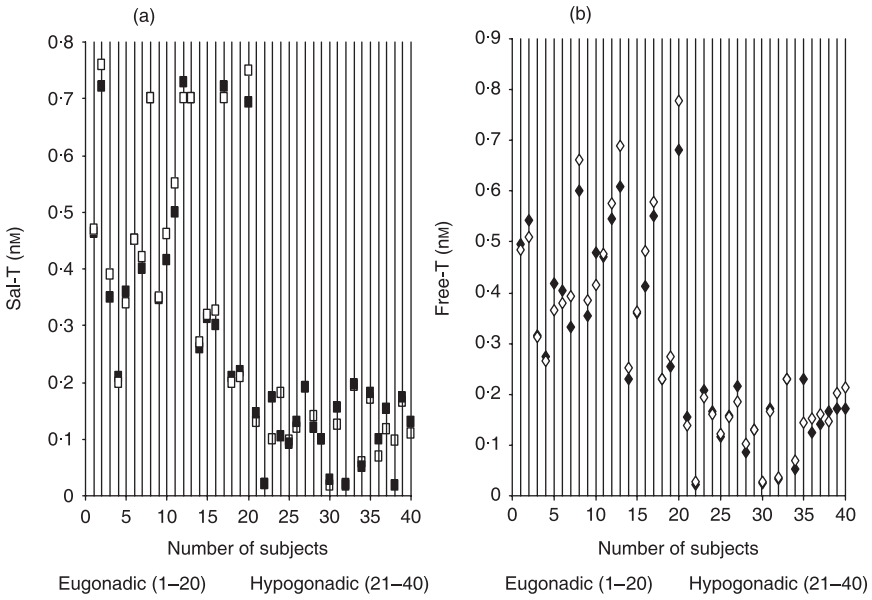

Fig. 3 (a) Morning salivary testosterone and (b) free testosterone levels in samples obtained twice in quick succession from eugonadic and hypogonadic subjects.

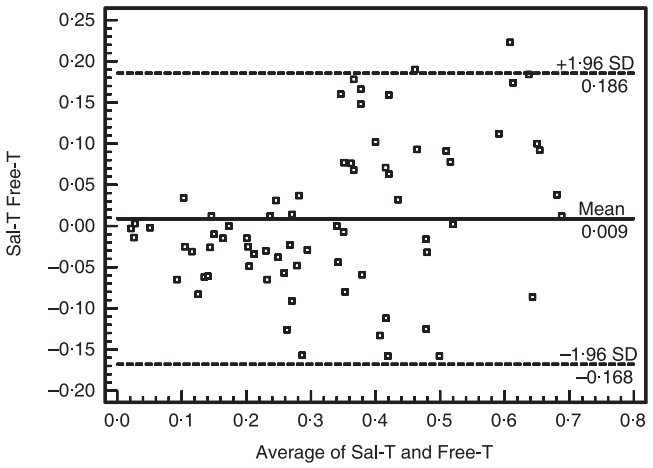

Fig. 4 Plotting differences (Sal-T – Free-T) against average of Sal-T and Free-T according to Bland and Altman.[21] Horizontal unbroken line indicates calculated mean. Broken line indicates 95% limits of agreement (mean ± 1·96 SD).

Table 1. Range percentiles for serum and salivary testosterone in eugonadic and hypogonadic subjects

	Range percentiles*							
	1%	5%	10%	25%	75%	90%	95%	99%
Eugonadic group								
TT(nM)	10·40	11·00	11·80	13·17	21·50	26·00	37·00	33·00
Bio-T (nM)	5·41	5·44	5·82	6·99	11·70	14·30	16·00	17·10
Free-T (nM)	0·23	0·25	0·27	0·30	0·50	0·58	0·66	0·69
Sal-T (nM)	0·20	0·21	0·23	0·29	0·52	0·70	0·70	0·73
Hypogonadic group								
TT (nM)	1·74	1·82	1·98	4·80	8·10	9·45	10·05	10·10
Bio-T (nM)	0·48	0·53	0·68	2·15	3·99	5·04	5·17	5·30
Free-T (nM)	0·02	0·02	0·03	0·10	0·17	0·22	0·23	0·23
Sal-T (nM)	0·02	0·02	0·02	0·07	0·16	0·19	0·19	0·20

*Data were obtained from morning samples (07·00–09·00 h) from 52 eugonadic and 20 hypogonadic subjects.
TT, serum total testosterone; Bio-T, serum bioavailable testosterone defined by Vermeulen; Free-T, serum free testosterone calculated as Vermeulen equation; Sal-T, salivary testosterone.

patients (Fig. 5). In E, the concentration ranges for Sal-T and Free-T were 0·200–0·729 nM and 0·230–0·686 nM, respectively; while in Hy they were 0·019–0·195 nM and 0·023–0·229 nM, respectively. In E and Hy the standardized kurtosis for Sal-T and Free-T were –1·09 and –0 78, and –0·80 and –0·57, respectively. As all standardized kurtosis were within the range [–2, +2], Sal-T and Free-T from E and Hy subjects demonstrated a normal distribution. This condition allowed us to apply Bland–Altman analysis to define agreement between methods. Bland–Altman plot (Fig. 4) showed that 97·3% of the differences were within 0·009 ± 1·96 × 0·0903 (mean ± 1·96 of the differences), suggesting that both methods agree (because most of the observations lie within the 95% limits of agreement). Moreover, a positive and significant correlation ($r = 0{\cdot}97$, $P = 0{\cdot}0001$) was found between Sal-T and Free-T levels (Fig. 6).

Testosterone cut-offs. Range percentiles for serum TT, Bio-T, Free-T and Sal-T from morning samples obtained in E and Hy subjects are represented in Table 1. Mean TT (17·98 ± 5·4 nM), Bio-T (9·48 ± 3·17 nM), Free-T (0·402 ± 0·125 nM) and Sal-T (0·423 ± 0·157 nM) were significantly higher in E than Hy (6·32 ± 2·53 nM, 3·15 ± 1·50 nM, 0·139 ± 0·065 nM and 0·115 ± 0·05 nM, respectively; $P = 0{\cdot}0001$). The quality of the different tests for the biochemical diagnosis of hypogonadism was estimated by ROC analysis and expressed as AUC_{ROC} (Table 2). Estimating the cut-off values of TT, Bio-T, FreeT and Sal-T by ROC, optimized for sensitivity, we found a test specific cut-off of 10·1 nM, 5·3 nM, 0·229 nM and 0·195 nM appropriate to

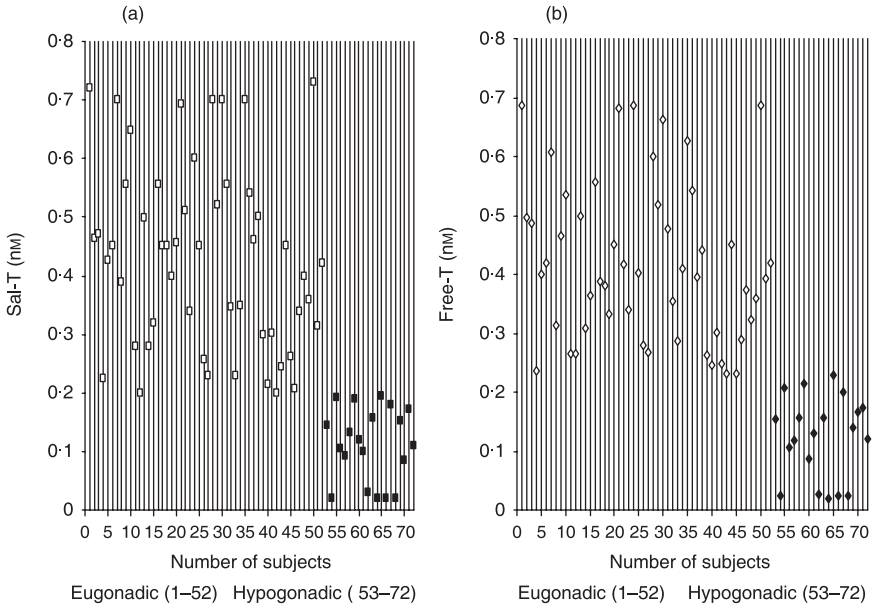

Fig. 5 (a) Simultaneous morning salivary testosterone and (b) serum free testosterone levels in samples from 52 eugonadic and 20 hypogonadic subjects.

Table 2. Comparison of the different test performances applied to define the hypogonadic status through testosterone levels

Test	Sensitivity [% (95% CI)]	Specificity [% (95% CI)]	AUC_{ROC} (95% CI)
TT (≤ 10·1 nM)	100 (83–100)	100 (93·1–100)	1 (0·95–1·00)
Bio-T (≤ 5·3 nM)	100 (83–100)	100 (93·1–100)	1 (0·95–1·00)
Free-T (≤ 0·229 nM)	100 (83–100)	100 (93·1–100)	1 (0·95–1·00)
Sal-T (≤ 0·195 nM)	100 (83–100)	100 (93·1–100)	1 (0·95–1·00)

TT, serum total testosterone; Bio-T, serum bioavailable testosterone calculated from Vermeulen equation; Free-T, serum free testosterone calculated from Vermeulen equation; Sal-T, salivary free testosterone; and CI, confidence interval.
TT, Bio-T, Free-T and Sal-T showed similar diagnostic performance in the screening of hypogonadism (the AUC_{ROC} were not significantly different). The cut-off values are estimated by ROC analysis and optimized for sensitivity. Eugonadic subjects ($N = 52$, aged 20–55 years) were used as negative control group; hypogonadic patients ($N = 20$) were used as positive group (samples were obtained at 07·00–09·00 h).

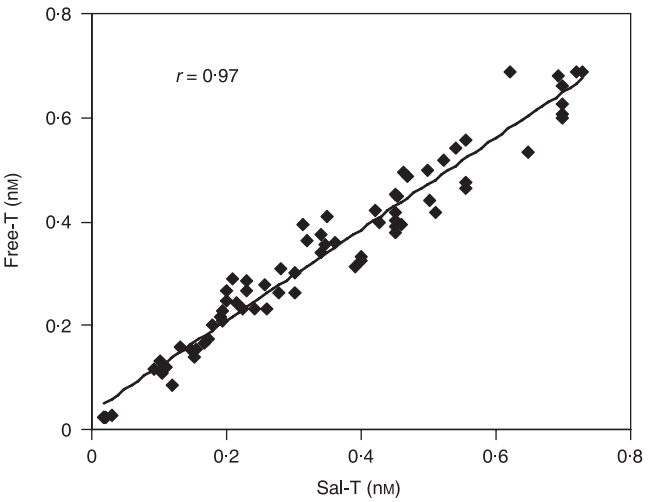

Fig. 6 Significant correlation ($P = 0·0001$) between salivary testosterone (Sal-T) and serum free-testosterone (Free-T) levels was observed in 72 male subjects.

distinguish between hypogonadic patients and eugonadic subjects, with a sensitivity and specificity of 100%.

Correlation between Sal-T and serum testosterone concentrations. Sal-T correlates positively and significantly with TT($r = 0·74$), Bio-T ($r = 0·72$) and Free-T ($r = 0·92$) in E as in Hy patients ($r = 0·76$, $r = 0·86$ and $r = 0·97$, respectively; $P = 0·0001$ in all the cases).

Discussion

The present study demonstrates that morning Sal-T levels are useful to differentiate hypogonadic from eugonadic patients. Sal-T is independent of salivary flow rate and morning samples (from 07·00 to 09·00 h) are stable. Agreement between Sal-T and Free-T measurements was confirmed in E and Hy subjects. The best correlation was found between Sal-T and Free-T in both E and Hy subjects ($r = 0·92$ and $r = 0·97$, respectively). Clear cut-off values estimated for Sal-T as well as for serum Free-T, Bio-T and TT showed 100% of sensibility and specificity to rule out hypogonadism. These data suggest that either Sal-T, Free-T or Bio-T are reliable markers of testosterone bioavailability in subjects with normal levels of circulating binding proteins.

We describe a direct assay for Sal-T, employing a commercial RIA initially developed for serum T. This assay uses a small volume of whole saliva and takes minimal sample processing. Participants should avoid tooth brushing 2 h before collection because micro-injury to the oral mucosa provokes a significant rise in Sal-T levels

until 45 min after this manoeuvre.[22,23] The detection limit of the assay was 1 pg/ml in agreement with others.[19,24] To our knowledge this is the first report that verifies the independence of testosterone concentrations in whole saliva from saliva flow rate, allowing the assessment of Sal-T in patients with flow rates either as low as 0·40 ml/min or as high as 1·6 ml/min. Saliva was collected by directly spitting into polypropylene tubes because, as previously described,[25,26] cotton from collecting devices (e.g. Salivette) interfere with testosterone results.

Salivary steroids reflect the circadian rhythm found in serum. In eugonadic subjects, we detected higher Sal-T concentrations in the morning than in the evening, in accordance with previously reported data.[1,27] Sal-T samples obtained between 07·00 h and 09·00 h showed low intra subject variability, suggesting that saliva collection during this period is appropriate for clinical testing, as circulating testosterone. In addition, high stability of Sal-T was demonstrated along repetitive sampling overtime.[12]

Differences in SHBG concentrations influence the results of calculated fractions of testosterone. It should be noted that some commercial SHBG assays are calibrated on the basis of SHBG mass which lead to important differences related to the number of steroid binding sites in the SHBG homodimers and therefore against the measurement of binding capacity.[3,28] Our measurements also depend on SHBG (binding assay) and TT assay characteristics. Sal-T levels agree with Free-T concentrations in eugonadic and hypogonadic subjects, based on the diffusion of nonprotein bound steroids into peripheral tissues and their secretions.[14] By contrast, reported data showed discrepancies while comparing Sal-T and Free-T concentrations using different methodologies.[12,29]

Sal-T measurement with a cut-off value of 0·195 nM seems to be as good as Free-T (≤ 0·229 nM), Bio-T (≤ 5·3 nM) or TT (≤ 10·1 nM) to discriminate hypogonadic from eugonadic subjects. Interestingly, our Free-T and Bio-T cut-off values are close to those recently proposed by the Endocrine Society Clinical Guidelines.[13]

Sal-T-testing is harmless and useful for screening outpatients. Our results support the inclusion of this biomarker as an additional noninvasive approach in the diagnosis of male androgen deficiency.

Acknowledgements

We thank Mrs Gloria Monardes (CONICET) and Mrs Gladys Videla (UBA) for their technical assistance, and Diagnostic System Laboratories Inc, Webster, TX, USA, for providing the RIA kits used for salivary and serum testosterone measurements.

References

1 Landman, A.D., Sanford, L.M., Howland, B.E., Dawes, C. & Pritchard, E.T. (1976) Testosterone in human saliva. *Experientia*, **32**, 940–941.

2 Dunn, J.F. & Rodbard, D. (1981) Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone binding-globulin and corticosteroid-binding globulin in human plasma. *Journal of Clinical Endocrinology and Metabolism*, **53**, 58–68.

3 De Ronde, W., Van der Schouw, Y.T., Pols, H.A.P., Gooren, L.J.G., Muller, M., Grobbee, D.E. & Jong, F.H. (2006) Calculation of bioavailable and free testosterone in men: a comparison of 5 published algorithms. *Clinical Chemistry*, **52**, 1777–1784.

4 Rilling, J.K., Worthman, C.M., Campbell, B.C., Stallings, J.F. & Mbizva, M. (1996) Ratios of plasma and salivary testosterone throughout puberty: production versus bioavailability. *Steroids*, **61**, 374–378.

5 Cefalu, W.T., Pardridge, W.M., Ghaudhuri, G. & Judd, H.L. (1986) Serum bioavailability and tissue metabolism of testosterone and estradiol in rat salivary gland. *Journal of Clinical Endocrinology and Metabolism*, **63**, 20–28.

6 Smith, R.G., Besch, P.K., Dill, B. & Buttram, V.C. (1979) Saliva as a matrix for measuring free androgens: comparison with serum androgens in polycystic ovarian disease. *Fertility and Sterility*, **31**, 513–517.

7 Sannikka, E., Terho, P., Suominen, J. & Santii, R. (1983) Testosterone concentrations in human seminal plasma and saliva and its correlation with non-protein-bound and total testosterone levels in serum. *International Journal of Andrology*, **6**, 319–330.

8 Khan-Dawood, F.S., Choe, J.K. & Dawood, M.Y. (1984) Salivary and plasma bound and free testosterone in men and women. *American Journal of Obstetrics and Gynecology*, **148**, 441–445.

9 Vittek, J., L'Hommedieu, D.G., Gordon, G.G., Rappaport, S.C. & Southren, A.L. (1985) Direct radioimmunoassay (RIA) of salivary testosterone: correlation with free and total serum testosterone. *Life Sciences*, **37**, 711–716.

10 Wang, C., Plymate, S., Nieschlag, E. & Alvin Paulsen, C. (1981) Salivary testosterone in men: further evidence of a direct correlation with free serum testosterone. *Journal of Clinical Endocrinology and Metabolism*, **53**, 1021–1024.

11 Morley, J.E., Perry, H.M. III, Patrick, P., Dollbaum, C.M. & Kells, J.M. (2006) Validation of salivary testosterone as a screening test for male hypogonadism. *Aging Male*, **9**, 165–169.

12 Goncharov, N., Katsya, G., Dobracheva, A., Nizhnik, A., Kolesnikova, G., Herbst, V. & Westermann, J. (2006) Diagnostic significance of free salivary testosterone measurement using a direct luminescence immunoassay in healthy men and in patients with disorders of androgenic status. *Aging Male*, **9**, 111–122.

13 Rosner, W., Auchus, R.J., Azziz, R., Sluss, P.M. & Raff, H. (2007) Position statement: utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. *Journal of Clinical Endocrinology and Metabolism*, **92**, 405–413.

14 Vining, R.F., McGinley, R.A. & Symons, G. (1983) Hormones in saliva: mode of entry and consequent implications for clinical interpretation. *Clinical Chemistry*, **29**, 1752–1756.

15 Gaskell, S.J., Pike, A.W. & Griffiths, K. (1980) Analysis of testosterone and dehydroepiandrosterone in saliva by gas chromatography-mass spectrometry. *Steroids*, **36**, 219–228.

16 Axelsson, J., Ingre, M., Akerstedt, T. & Holmback, U. (2005) Effects of acutely displaced sleep on testosterone. *Journal of Clinical Endocrinology and Metabolism*, **90**, 4530–4535.

17 Vermeulen, A., Verdonck, L. & Kaufman, J.M. (1999) A critical evaluation of simple methods for the estimation of free testosterone in serum. *Journal of Clinical Endocrinology and Metabolism*, **84**, 3666–3672.

18 Tumilasci, O.R., Cersosimo, M.G., Belforte, J.E., Micheli, F.E., Benarroch, E.E. & Pazo, J.H. (2006) Quantitative study of salivary secretion in Parkinson's disease. *Movement Disorders*, **21**, 660–667.

19 Granger, D.A., Schwartz, E.B., Booth, A. & Arentz, M. (1998) Salivary testosterone determination in studies of child health and development. *Hormones and Behavior*, **35**, 18–27.

20 Riesco, O., Aquilano, D.R. & Scaglia, H.E. (2002) Description of a simple method for the determination of bioavailable testosterone: critical evaluation and application in clinical endocrinology. *Acta Bioquímica Clínica Latinoamericana*, **46**, 5–26.

21 Petersen, P.H., Stöckl, D., Blaabjerg, O., Pedersen, B., Birkemose, E., Thienpont, L., Lassen, J.F. & Kjeldsen, J. (1997). Graphical interpretation of analytical data from comparison of a field method with a reference method by use of difference plots. *Clinical Chemistry*, **43**, 2039–2046.

22 Schramm, W., Smith, R.H., Craig, P.A. & Grates, H.E. (1993) Testosterone concentration is increased in whole saliva, but not in ultrafiltrate after toothbrushing. *Clinical Chemistry*, **39**, 519–521.

23 Granger, D.A., Shirtcliff, E.A., Booth, A., Kivlighan, K.T. & Schwartz, E.B. (2004) The 'trouble' with salivary testosterone. *Psychoneuroendocrinology*, **29**, 1229–1240.

24 van Anders, S.M. & Watson, N.V. (2006) Relationship status and testosterone in North American heterosexual and non-heterosexual men and women: cross-sectional and longitudinal data. *Psychoneuroendocrinology*, **31**, 715–723.

25 Shirtcliff, E.A., Granger, D.A., Schwartz, E. & Curran, M.J. (2001) Use of salivary biomarkers in biobehavioral research: cotton-based sample collection methods can interfere with salivary immunoassay results. *Psychoneuroendocrinology*, **26**, 165–173.

26 Gröschl, M. & Rauch, M. (2006) Influence of commercial collection devices for saliva on the reliability of salivary steroids analysis. *Steroids*, **71**, 1097–1100.

27 Howard, K., Kane, M., Madden, A., Gosling, J.P. & Fottrell, P.F. (1989) Direct solid-phase enzymoimmunoassay of testosterone in saliva. *Clinical Chemistry*, **35**, 2044–2047.

28 Avvakumov, G.V., Grishkovskaya, I., Muller, Y.A. & Hammond, G.L. (2001) Resolution of the human sex hormone-binding globulin dimer interface and evidence for two steroid-binding sites per homodimer. *Journal of Biological Chemistry*, **276**, 34453–34457.

29 Rey, F., Chiodoni, G., Gomez, F. & Felber, J.P. (1988) Interpretation of the discrepancy observed between plasma free and salivary testosterone levels in man. *Steroids*, **52**, 371–372.

