



Considerations in the Measurement of Testosterone in Saliva and Serum Using ELISA Procedures

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Background: ELISA procedures are widely available and used for the measurement of saliva and blood (serum and plasma) testosterone. Suggestions for strong correlations between these two fluids have been made but differences in assay format, as well as in the collection procedures, storage, and processing of samples can influence results.

Methods: The present study compared saliva and serum free testosterone concentrations in 20 healthy men (31.0±11.0 years; mean ± SD) using ELISA procedures. Men provided both a saliva and blood sample on the same day in the morning hours following an overnight fast. Special care was taken in the collection, storage, and processing of samples. Following complete thawing and mixing of samples, both fluids were analyzed in duplicate using commercially available ELISA kits, both prior to and following centrifugation.

Results: Saliva testosterone values were 440.6±238.2 pg·mL⁻¹ and 348.8±210.0 pg·mL⁻¹ without and with centrifugation, respectively. Serum testosterone values were 9.0±4.2 pg·mL⁻¹ and 8.3±3.7 pg·mL⁻¹ without and with centrifugation, respectively.

Conclusion: If utilizing ELISA procedures, saliva and serum testosterone values cannot be used interchangeably, at least when utilizing the ELISA procedures employed in the present study. This is evidenced by the approximate 10-100 times higher testosterone concentration in saliva as compared to serum. Moreover, processing samples via centrifugation leads to a significant (~23%) loss in testosterone in saliva, with a much smaller loss (~7%) in serum. Investigators and

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clinicians should take note of these findings if planning to measure saliva or serum testosterone using ELISA procedures.

Keywords: Steroids; hormones; salivary; blood; immunoassay.

1. INTRODUCTION

Testosterone is a hormone responsible for multiple physiological effects, including muscle maintenance and growth, sexual desire, as well as improved energy levels [1]. Testosterone is routinely measured in clinical and research settings using blood samples. This typically involves the collection of blood via venipuncture, something that can produce anxiety and discomfort in some individuals [2], making serial sample collection challenging.

While testosterone was once measured almost exclusively using radioimmunoassay (RIA) procedures, other procedures are available including gas chromatography-mass spectrometry (GC-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and luminescence enzyme immunoassays (LIA). Recently, enzyme-linked immunosorbent assay (ELISA) procedures have gained popularity, as these are relatively inexpensive and easy to perform. Unfortunately, certain immunoassay procedures may not correlate well to more traditional analytical approaches, at least in the measure of total testosterone [3,4].

Beyond the analytical technique used in sample analysis, increased attention is now being given to measurement procedures which use human saliva rather than human blood samples, both within clinical settings and within the research community [5]. Indeed, the use of saliva makes repeated sampling less burdensome due to the noninvasive manner. As such, the use of saliva has increased in certain areas of research, such as within the exercise sciences [6].

Specific substances that circulate in the blood can enter into the salivary ducts by passive filtration through the membrane barrier. In relation to steroid hormones, saliva contains free testosterone, which has been indicated in some studies to correlate well with circulating free testosterone [3,7,8]. Unfortunately, not all studies use the same assay procedures to measure salivary testosterone and this may be problematic when attempting to make comparisons across studies. Likewise, differences in sample collection procedures, storage, and processing of saliva can influence

the testosterone concentration [9,10]. For example, Durdiaková and colleagues recently reported that a simple 5-minute centrifugation step prior to the analysis of saliva samples for testosterone using ELISA procedures resulted in a 47% loss of testosterone [10]. However, only five men were included in this study and samples were analyzed *fresh* and not following a period of freezing. Within research settings, in particular those that involve serial collections, samples are typically frozen and then thawed prior to analysis so that they can be analyzed in batch format. Therefore, knowing the potential influence of centrifugation on frozen and subsequently thawed saliva samples may be of interest to investigators. Moreover, knowing the potential influence of centrifugation on testosterone concentrations in serum samples may be of value, as many investigators routinely thaw, mix, and centrifuge samples prior to analysis in an attempt to obtain a more clear and purified supernatant to be used in the assay. Finally, a direct comparison between saliva and serum testosterone concentrations using commonly utilized ELISA procedures is timely and would provide evidence for or against the interchangeable use of these two body fluids in future clinical or research work.

Considering the above, the purpose of this study was to directly compare saliva and serum free testosterone measures from healthy men using ELISA procedures, both with and without prior centrifugation of samples. The results may guide future clinical and research studies requiring the routine measure of testosterone using simple ELISA techniques.

2. METHODOLOGY

2.1 Subjects

A total of 20 healthy men (age range: 19-51 years) were recruited to participate in this single laboratory visit study. Subjects were not current smokers and were not using any medication or dietary supplement thought to influence testosterone. Health history, medication and dietary supplement usage questionnaires were completed by all subjects to determine eligibility. Subjects' height, weight, and waist and hip

circumference were measured for descriptive purposes, as well their resting heart rate and blood pressure. Subject characteristics are provided in (Table 1).

Table 1. Characteristics of 20 healthy men

Variable	Value
Age (years)	31.0±11.0
Height (cm)	177.1±6.4
Body Weight (kg)	87.9±15.2
Body Mass Index (kg·m ⁻²)	27.9±4.2
Waist Circumference (cm)	89.0±11.0
Hip Circumference (cm)	105.2±9.4
Waist:Hip	0.85±0.09
Heart Rate (bpm)	72.6±10.4
Systolic Blood Pressure (mm Hg)	117.5±19
Diastolic Blood Pressure (mm Hg)	73.1±13.2

Values are mean ± SD

Prior to participation, each subject was informed of all procedures, potential risks, and benefits associated with the study through both verbal and written form in accordance with the procedures approved by the University Institutional Review Board for Human Subjects Research. Subjects provided written informed consent.

2.2 Procedures

Subjects reported to the lab one time only, between the hours of 6:30 and 9:30am. They were instructed to report to the lab following an overnight fast of at least 10 hours. Upon arrival, subjects completed the consent form and the health history, medication and dietary supplement usage questionnaires. They had their height, weight, and circumference measures taken. Their resting heart rate and blood pressure was recorded after they sat quietly in a chair for 5 minutes. A blood sample (approximately 5mL) was collected from a forearm vein into a Vacutainer® tube containing no additive, allowed to clot at room temperature for approximately 30 minutes, then centrifuged for 15 minutes at 4°C to obtain serum. Serum samples were immediately stored at -20° until analysis.

Subjects also provided a saliva sample (approximately 1.5mL) by continuous, unstimulated passive drool into a special polypropylene collection tube (SaliCap: IBL International, Germany). A mirror was provided to subjects to assist in the filling of the tube and

the time taken to fill the tube was approximately two minutes. Subjects were not allowed to brush their teeth, to chew gum or mints, or to drink anything but water within one hour of providing the saliva sample. Five minutes before the saliva collection, subjects were asked to rinse their mouth with water. Following the collection of saliva, samples were immediately stored at -20° until analysis.

Free testosterone was measured in both saliva and serum within two weeks of collection, in an attempt to preserve testosterone values [11]. All samples were removed from the freezer, allowed to thaw completely at room temperature, and then mixed thoroughly. One aliquot of each sample was then used directly in the ELISA procedure. Specifically, the serum samples were directly loaded onto the ELISA plate. The mixed saliva samples were placed into a tube rack and allowed to settle for approximately 20 minutes, in order to maintain a clear fluid in which to draw into the pipette tip before being loaded onto the ELISA plate.

Another aliquot of each sample was placed into a refrigerated centrifuge (4°C) for 10 minutes at 2000g in an attempt to produce a clean supernatant. This is the recommended duration and speed of centrifugation for saliva samples, as suggested by the ELISA manufacture (IBL International, Germany). Samples were analyzed in 50µL of saliva (IBL International, Germany: catalog # RE52631). Serum samples were analyzed in 25µL using an ELISA kit purchased from Cal Biotech (Spring Valley, CA: catalog # FT178S). Certificates of quality control were provided for both ELISA kits. The cross reactivity for testosterone was noted by the manufacturers to be 100% for both ELISA kits. For the saliva assays, the coefficient of variation was 5.5%, while the range of detection (as determined by the manufacturer) was 2.0 - 760 pg·mL⁻¹. For the serum assays, the coefficient of variation was 5.1%, while the range of detection (as determined by the manufacturer) was 0.25 - 100 pg·mL⁻¹. All samples were analyzed in duplicate on first thaw.

2.3 Statistical Analysis

Free testosterone data were analyzed for mean and SD. Correlation analyses were performed between saliva and serum samples, with and without centrifugation. Additional pairwise correlations were made between testosterone values and subject characteristics (e.g., age,

body weight). Analyses were performed using JMP statistical software (version 4.0.3, SAS Institute; Cary, NC).

3. RESULTS

Values for saliva were not obtainable for two subjects, as they fell outside of the high end of the standard curve. Values for saliva and serum free testosterone are provided in (Table 2). Centrifugation resulted in a significant (~23%) loss in testosterone in saliva, with a much smaller loss (~7%) in serum. For saliva, 17 of the 18 samples analyzed were noted to be lower following centrifugation (Fig. 1A), while 17 of the 20 samples analyzed in serum were noted to be lower following centrifugation (Fig. 1B).

Table 2. Saliva and serum free testosterone values with and without centrifugation of 20 men

Fluid type	Free testosterone (pg·mL ⁻¹)	Decrease with centrifugation
Saliva	440.6±238.2	
Saliva with centrifugation	348.8±210.0	23.5%
Serum	9.0±4.2	
Serum with centrifugation	8.3±3.7	7.6%

Values are mean ± SD

A strong correlation was noted between saliva testosterone values with and without centrifugation ($r = 0.98$, $p < 0.00001$), as well as between serum testosterone values with and without centrifugation ($r = 0.98$, $p < 0.00001$). No significant correlations were noted between saliva and serum testosterone measures. When compared without centrifugation, values for saliva testosterone were 54.0 ± 34.4 times higher than for serum (range: 12.1 - 121.5). When compared with centrifugation, values for saliva testosterone were 45.9 ± 31.3 times higher than for serum (range: 12.3 - 103.7).

Negative correlations were noted between saliva testosterone (both with and without centrifugation) and age ($r = -0.60$, $p = 0.008$), as well as waist:hip ($r = -0.52$, $p = 0.03$).

4. DISCUSSION

The main findings of this study are as follows: 1) If utilizing certain commercially available ELISA kits, saliva and serum testosterone values cannot

be used interchangeably, as values for saliva are on the order of 10-100 times higher than serum and 2) Processing samples via centrifugation for 10 minutes as a speed of 2000g leads to a significant (~23%) loss in testosterone in saliva, with a much smaller loss (~7%) in serum. Investigators and clinicians who are planning to measure saliva or serum testosterone using ELISA procedures should take note of these findings and plan accordingly.

The results presented here demonstrate clearly that saliva and serum samples result in dramatically different values of free testosterone when analyzed using two commercially available ELISA procedures. As such, those interested in the measurement of free testosterone should identify one method and maintain that method throughout all testing. Although all but two values obtained for serum free testosterone were within the expected range indicated by the kit manufacturer (5-30 pg·mL⁻¹), all values for saliva testosterone except for one were higher than the manufacturer's noted expected range (30-143 pg·mL⁻¹).

The values for subjects in the present study are higher than those reported in other work, including a recent study including young and healthy men, also using ELISA procedures [10], and there remains no firm explanation for these high values. It is possible that rapid fluctuations in saliva testosterone, unlike for serum testosterone, may be implicated in our findings, as it has been suggested that multiple saliva samples may be needed in order to best approximate serum concentrations [12]. It was indicated by the technical support staff at IBL International (the manufacturer of the salivary ELISA kit) that differences in the development of the standard curve between manufactures could result in vastly different values being obtained in sample analysis if comparing one ELISA kit to another, in particular if comparing saliva to serum. This needs to be strongly considered by those planning to measure testosterone via ELISA techniques in future experiments. In relation to the above, a limitation of this work is that only one commercially available ELISA kit was used for both saliva and serum measurement. It is possible that different kits may demonstrate greater similarity in results in terms of testosterone concentration. Future study may seek to investigate this.

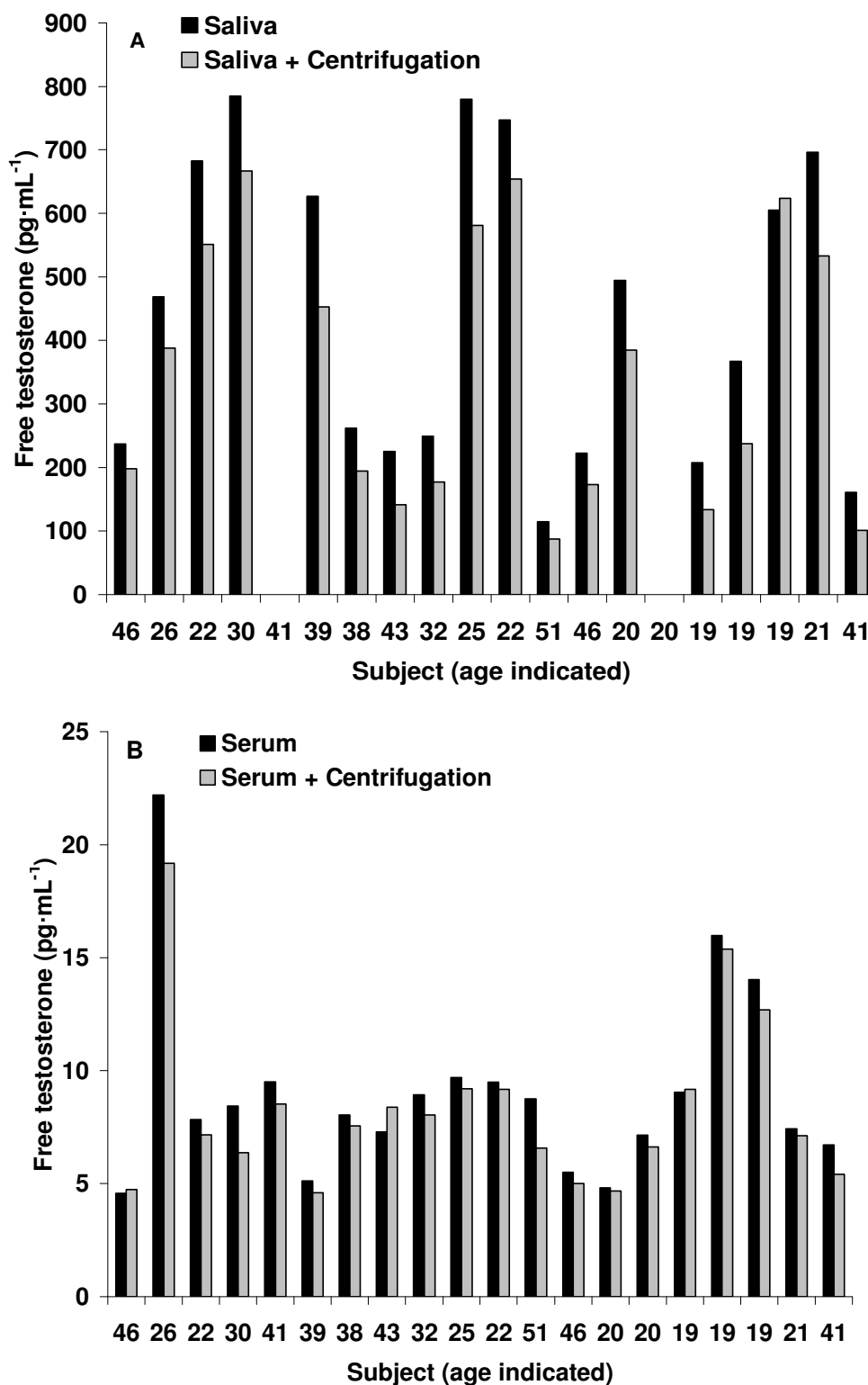


Fig. 1. Individual saliva (A) and serum (B) free testosterone values with and without centrifugation of 20 men

Note: Data are unavailable for saliva free testosterone for two subjects who exceeded the high standard value

In contrast to the present findings, prior work has demonstrated strong correlations between saliva and serum testosterone. For example, Arregger and colleagues [7] noted that saliva measures of testosterone were strongly correlated to blood measures in a sample of eugonadic ($r = 0.92$) and hypogonadic ($r = 0.97$) men. However, in this study, an adapted RIA procedure was used for the saliva testosterone assay, rather than an ELISA procedure. A study by Goncharov and coworkers [3] also noted that salivary testosterone correlated well to calculated free testosterone in blood, in healthy men ($r = 0.75$) and in patients with androgen deficiency ($r = 0.89$), although a LIA method was used for sample analysis. In contrast to these findings, Flyckt et al. [13] failed to note a significant correlation between saliva and serum testosterone measurements; although samples were obtained from postmenopausal women.

Another finding of interest from the present work is that simple centrifugation of samples resulted in a reduction in testosterone concentration of approximately 23% in saliva and 7% in serum when analyzed using ELISA techniques. It is typically recommended that saliva samples be frozen following collection and then thawed, mixed, and centrifuged prior to analysis. The process of centrifugation allows for a clean, filtered sample to be obtained—one that does not contain any significant amount of substances that may alter antigen binding and lead to inflated values. It is possibly the omission of these substances within the centrifuged saliva samples that may be responsible for the dramatic decrease in measured testosterone values using the ELISA procedure. It is uncertain whether or not similar findings of reduced testosterone concentration would be noted if samples were centrifuged and then analyzed using an alternative assay format other than ELISA. While ELISA procedures have increased in popularity within recent years, these procedures can sometimes result in falsely high or low results, possibly due to cross reactivity with substances other than the substance of interest. The present results do not specifically indicate that individuals should or should not use ELISA procedures or centrifuge samples prior to analysis; the results should simply be considered prior to future work focused on the measurement of testosterone using saliva and serum samples, in particular when considering ELISA procedures as the assay format.

Finally, and not a prime focus of this investigation, negative correlations were noted between saliva testosterone (both with and without centrifugation) and age ($r = -0.60$, $p = 0.008$), as well as waist:hip ($r = -0.52$, $p = 0.03$). These findings confirm prior work suggesting an age-related decline in testosterone in men [14], as well as an association between testosterone and adiposity [15].

5. CONCLUSION

In conclusion, if utilizing the ELISA procedures employed in this study, values for saliva and serum testosterone should be expected to differ. Values for saliva are approximate 10-100 times higher than for serum, with more similar values obtained following centrifugation. Processing thawed samples via centrifugation leads to a significant (~23%) loss in testosterone in saliva, with a much smaller loss (~7%) in serum. Investigators and clinicians should take note of these findings if planning to measure saliva or serum testosterone using ELISA procedures, possibly performing pilot investigations using ELISA kits from a variety of manufacturers prior to deciding on a final format.

CONSENT

Prior to participation, each subject was informed of all procedures, potential risks, and benefits associated with the study through both verbal and written form. Subjects provided written informed consent.

ETHICAL APPROVAL

This study was approved by the University Institutional Review Board for Human Subjects Research (Protocol #3020) and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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