Direct Solid-Phase Enzymoimmunoassay of Testosterone in Saliva

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This competitive, solid-phase enzymoimmunoassay for testosterone in saliva is carried out on microtiter plates and involves no chromatographic or extraction steps. With an overnight incubation the detection limit of the assay is 230 fg per well (16.1 pmol/L). There was a good correlation (correlation coefficient 0.95) between testosterone concentrations measured with and without prior extraction of the saliva samples. Repeated assay of three control saliva samples containing a range of testosterone concentrations (200–1000 pmol/L) gave within- and between-assay coefficients of variation of 5.5–13.2%. The analytical procedure is simple and closely resembles already published procedures for the determination of progesterone and estrone (with extraction) in saliva. One person can assay 200 samples in 24 h and the assay is suitable for reproductive and sports medical applications, particularly for projects involving serial sampling and yielding large numbers of samples.

Additional Keyphrases: microtiter plate · testicular function · steroids

Saliva is an attractive alternative to serum for measuring steroid hormones (1–3). It is noninvasive, and samples are easily provided in the patient’s home. Salivary testosterone measurements are used to evaluate androgen status in male infertility such as in oligoepispermic and azoospermic subjects (4), androgenic alopecia (5), prostatic carcinoma (6), and in psychological test situations (7). An important advantage of measuring testosterone in saliva is that it correlates significantly with free testosterone in serum in normal and pathological conditions (6, 8, 9). Most published methods for measuring testosterone in saliva are radioimmunoassays (RIA) and involve extraction with an organic solvent (3–5), although at least one direct RIA for salivary testosterone has been described (6). An enzymoimmunoassay (EIA) for salivary testosterone has been developed, but the procedure included an extraction step (10).

The determination of testosterone concentrations in serial saliva samples is suitable for reproductive and sports medical investigations, particularly where sampling can conveniently be performed only by the subject. The analysis of samples without extraction is appropriate when a large throughput of samples is required. Here we describe a direct EIA for testosterone in male saliva carried out on microtiter plates, and we describe its validation and clinical application.

Materials and Methods

Subjects and Sample Collection

Sixty-five healthy male volunteers, ages 15–48 years, had fasted for at least 1 h before giving 2–5 mL of saliva (unstimulated) in 5-mL polystyrene test-tubes, which were then stored at –20 °C until thawed for treatment and assay. No preliminary rinsing of the mouth was stipulated.

Reagents

Coating buffer was NaHCO3/Na2CO3 (50 mmol/L, pH 9.6). Assay buffer (pH 7.4) contained 100 mmol of Na2HPO4/NaH2PO4, 150 mmol of NaCl, 150 mmol of EDTA (tetrasodium salt), and 5 mL of Tween 20 per liter. Buffered substrate (pH 5.0) for measurement of horseradish peroxidase activity contained, per liter, 49 mmol of citric acid, 103 mmol of NaHPO4, 8.3 mmol of o-phenylene diamine, and 5.9 mmol of H2O2. Testosterone stock standard solution (69 nmol/L in ethanol) was stored at –20 °C. Working standard solutions (69 to 3467 pmol/L) were prepared in standard buffer (10 mmol of Na2HPO4/NaH2PO4, 150 mmol of NaCl, and 0.5 mL of Tween 20 per liter, pH 7.4) and stored in aliquots at –20 °C. All buffers contained 100 mg of thimerosal per liter as a preservative and were stored at 4 °C. The wash solution contained 150 mmol of NaCl and 0.5 mL of Tween 20 per liter. All solvents and buffer components were of high quality (analytical grade) and were obtained from Reidel-de-Haen, Hanover, F.R.G. Testosterone, 11-ketotestosterone, testosterone-3-carboxymethylxime, and testosterone-3-carboxymethylxime–bovine serum albumin were obtained from Steraloids, Wilton, NH. Horseradish peroxidase (EC 1.11.1.7, Type VI) and all other steroids were obtained from Sigma, Poole, Dorset, U.K.

Antiserum: Primary antiserum was raised in New Zealand White rabbits by intradermal injection of 200 μg of testosterone-3-carboxymethylxime–bovine serum albumin, emulsified in Freund’s complete adjuvant, at multiple dorsal sites. After 13 and 30 weeks, each animal received by subcutaneous injection 80 μg of the immunogen emulsified in Freund’s incomplete adjuvant. After 32 weeks, the animals were bled and the antiserum was characterized with respect to titer, sensitivity, and specificity; a suitable serum was selected.

Steroid–enzyme conjugate: Testosterone-3-carboxymethylxime was covalently linked to horseradish peroxidase by the mixed anhydride method of Erlanger et al. (11), as modified by Dawson et al. (12). The conjugate is stable for at least 18 months when stored at –20 °C in an equimolar mixture of phosphate-buffered saline and glycerol containing 1 mg of cytochrome c per milliliter. Working solution of conjugate (31.5 mg/L) may be stored for at least three months at 4 °C without loss of enzymatic or immunological activity.

Microtiter plates: We used only the inner 60 wells of 96-well immunoassay plates (code no. 5390; Costar, Broadway, MA).

Quality-control saliva: We pooled male saliva with low

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Received May 22, 1989; accepted July 5, 1989.

2044 CLINICAL CHEMISTRY, Vol. 35, No. 10, 1989
contents of endogenous testosterone and formed three quality-control pools by adding 0, 217, and 754 pmol of testosterone per liter of saliva.

Procedure

Heat-treatment of saliva: Thaw samples, heat in an oven at 56°C for 2 h, then centrifuge at 3000 × g for 15 min. Assay supernatant immediately or store in aliquots at −20°C until assayed. This heating procedure improves the correlation between direct and extraction-based salivary steroid assays (13).

Coating of microtiter plates: Coat wells with whole anti-testosterone antiserum by adding 100 μL of 2000-fold diluted antiserum in coating buffer. Cover the plate with plastic film and incubate for 90 min at 37°C. Wash each well three times with 300-μL portions of wash solution. Coated plates are stable for at least one week at 4°C.

Testosterone EIA: With a diluter dispenser (Hamilton; Microlab M, Bonaduz, Switzerland) add 50 μL of standards, control saliva, or unknown saliva samples to the plate wells, with the simultaneous addition of 150 μL of testosterone-peroxidase conjugate (8.6 μg/L). Cover the plate with plastic film, mix the solutions in the wells gently, and incubate overnight (16–20 h) at 4°C. Wash again as above. Add 150 μL of buffered enzyme substrate solution to each well in sequence and incubate the plate at ambient temperature (16–20°C) for 60 min. Stop the reaction by adding 50 μL of 4 mol/L sulfuric acid to each well in the same sequence as the substrate solution was added. Gently mix the solutions in the wells and measure the absorbances at 490 nm with a microtitre plate reader (we used a Model EL307; Biotek Instruments Inc., Burlington, VT).

The plate reader is connected on-line to a BBC Model B microcomputer (Acorn Computers Ltd., Cambridge, U.K.). Software written in BASIC and prepared by one of us (J.P.G.) is used for the analysis of the data. Standard results (bound enzyme activity at each concentration divided by bound activity at zero standard (B/Bo) vs log picograms of testosterone added) are fitted to a four-parameter model allowing interpolation of control and unknown concentrations. The standard curve, detailed assay statistics, and the control and unknown results are immediately available for printing.

Extraction of saliva: Pipette 0.3 mL of saliva into disposable glass 16 x 100 mm culture tubes and add 1.5 mL of diethyl ether. Shake the contents of the tubes for 5 min on a multivortex-type mixer (SMI Model 2001). Allow the phases to separate, and transfer 1.0 mL of the solvent to a clean test-tube with a positive-displacement pipette. Evaporate the solvent at 40°C in a stream of air and add 0.2 mL of standard buffer to each tube. To resublubize the extracted material, vortex-mix as before for 2 min, incubate at 37°C for 15 min, and again vortex-mix for 2 min. Extraction efficiency, determined with tritiated testosterone, is 101% (SD 5%).

Results

Standard curve: The dose-response curve shown in Figure 1 represents the mean of 10 consecutive standard curves, each obtained with duplicate determinations of standards. The CVs for the B/Bo ratio for each standard ranged from 3.2% to 11.0%, indicating that the precision and stability of the standard curves were satisfactory. The detection limit, calculated as the smallest amount of testosterone significantly different from zero at 95% confidence limits, was 16.1 pmol/L (230 fg/well) or 4.6 ng/L. This compared well with published detection limits of 0.4–4 pg/tube for immunoassays used for the determination of testosterone in saliva (6, 10, 14). An alternative indication of the sensitivity of the assay is given by the ED50, defined as the standard concentration equivalent to a B/Bo value of 0.5, which was 407 pmol/L (5.86 pg/well or 11.7 ng/L) in this assay.

Specificity: The specificity of the antiserum used in the assay was assessed for a range of steroids related structurally to testosterone, according to the procedure recommended by Abraham (15). As Table 1 indicates, the cross-reactivity of most steroids tested was negligible except for 5α-dihydrotestosterone, which had a cross-reaction of 31.4%. This interference was not considered very important, however, because the concentration of 5α-dihydrotestosterone in saliva or plasma from males is one tenth that of testosterone (16, 17).

As a further check on specificity, we serially diluted six samples of saliva, from males, with testosterone concentrations ranging from 375 to 830 pmol/L, then assayed. Regression analysis of the observed vs the expected values yielded a correlation coefficient >0.993 in all cases. Thus, the amount of testosterone detected was independent of the volume of saliva assayed over the range examined (10–50 μL).

Table 1. Specificity of Antiserum Used

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross-reaction, %</th>
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</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>100</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>31.4</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0.7</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>0.6</td>
</tr>
<tr>
<td>11-Ketotestosterone</td>
<td>1.1</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Estradiol</td>
<td>&lt;0.2</td>
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<tr>
<td>Cortisol</td>
<td>&lt;0.2</td>
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<tr>
<td>Progesterone</td>
<td>&lt;0.2</td>
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Analytical recovery: The ability of the assay to accurately quantify testosterone in saliva samples was also tested by determining the concentration of testosterone in three saliva samples containing different endogenous concentrations of testosterone (102, 441, and 552 pmol/L), to portions of each of which 217, 433, and 693 pmol of testosterone was added per liter. The mean analytical recovery from these saliva samples was 89.3% (SD 5.7%, range 82.4%–99.4%, n = 9).

Correlation with and without extraction: To determine whether an organic solvent extraction step was necessary, we assayed 32 different saliva samples with and without prior extraction with diethyl ether. Regression analysis of the results (Figure 2) yielded a line with a slope of 0.91, a y-intercept of −8.2 pmol/L, and a correlation coefficient of 0.96. This indicated that a direct nonextraction assay of testosterone in male saliva was feasible.

Precision: Repeated assay in one run of three control saliva samples, containing different testosterone concentrations, yielded the intra-assay CVs listed in Table 2. The interassay CVs were determined by including in duplicate, three more control samples in 16 or 23 routine assay runs. The variation in every instance was <14% (Table 2).

Clinical applications: Salivary testosterone concentrations, in males, determined by our direct EIA for specimens selected without conscious bias, ranged from 100 to 680 pmol/L (mean = 291 pmol/L, n = 65), which are similar to published values (18). In addition, because salivary testosterone concentrations show circadian variation (18), we obtained samples from three volunteers regularly throughout the day and measured testosterone concentrations. A broadly similar pattern was observed in all cases (Figure 3), with the testosterone concentrations being greatest in each subject on waking and decreasing within 2–4 h.

Discussion

This EIA for salivary testosterone is convenient and has a relatively high throughput compared with other published immunoassays (6, 10). The peroxidase–testosterone conjugate is stable, more convenient than an 125I label, and there are no problems with disposal of radioactive waste. Another advantage of this assay is its ability to measure testosterone in saliva samples without prior extraction with organic solvent. The use of a microtiter plate, solid-phase system dispenses with the need for centrifugation steps and greatly facilitates the determination of the endpoints. Consequently, one person can assay 200 samples in 24 h, including the overnight (16–20 h) incubation.

The detection limit of the assay (230 fg/well) is much lower than that of a published direct RIA for salivary testosterone (1.15 pg/tube) (6) and the accuracy and precision of the assay compares favorably with published methods (6, 10). Values for male salivary testosterone measured with this assay are similar to published results obtained by RIA and EIA (3, 6, 10). Likewise, patterns of diurnal variation for salivary testosterone were similar to published results (3, 18).

Many studies have shown an excellent correlation between concentrations of salivary testosterone and of total testosterone in serum (3, 6, 9), supporting the replacement of assays of serum testosterone by salivary testosterone analysis. However, the correlations may be influenced by certain pathological conditions or by medication (19, 20). Most of the testosterone in blood is protein-bound, either to sex-hormone-binding globulin or to albumin (17), whereas salivary testosterone is believed to reflect more closely the unbound testosterone fraction in plasma (9, 19). However, in one study (21) the actual testosterone concentration in saliva exceeded the concentration of free testosterone in plasma: 2% and 0.6% of total plasma values, respectively. The presence of low concentrations of the binding globulin

| Table 2. Intra- and Interassay Variation in Quality-Control Saliva Samples |
|-----------------------------|---|---|---|
| Testosterone concn          | Low | Medium | High |
| Intra-assay variation        |    |       |     |
| n                           | 17 | 18    | 20  |
| Mean, pmol/L                | 205| 495   | 984 |
| SD, pmol/L                  | 20.1| 27.2  | 75.8|
| CV, %                       | 7.7| 9.8   | 5.5 |
| Interassay variation        |    |       |     |
| n                           | 23 | 16    | 23  |
| Mean pmol/L                 | 203| 517   | 929 |
| SD, pmol/L                  | 35.5| 68.2  | 106.7|
| CV, %                       | 12.1| 13.2  | 11.7|

Fig. 2. Correlation of testosterone concentrations in 32 serum samples as determined by enzymoimmunoassay with (x) and without (y) a solvent-extraction step.

Fig. 3. Concentrations of salivary testosterone throughout the day in three normal male subjects.
and albumin in saliva has been demonstrated (22), probably from contamination with gingival fluid, which may constitute up to 0.5% of the volume of saliva in healthy subjects (23). Thus, some testosterone in saliva may be protein-bound. Nevertheless, except in cases of severe gum disease, salivary testosterone measurement should provide a useful and convenient index of the biologically-active plasma free testosterone concentration.

We have previously developed and published two other microtiter plate-based EIAs validated for the determination of reproductive steroids in human saliva (1, 2, 13). Given the validation of the assay described here, progesterone, estrone (with an extraction step), and testosterone can now be conveniently determined in saliva samples by using procedures and reagents that are very similar or equivalent. These assays can greatly facilitate investigations of reproductive function that necessitate serial sampling or involve large numbers of samples—and at no sacrifice of assay precision or sensitivity.

We are grateful to Wajdi Abdul-Ahad and Daniel Collins for help, to Leslie Lyons and Dara Keys for assistance with obtaining some of the samples, and to the Irish Health Research Board and to Bioresearch Ireland for grants.

References