Direct Solid-Phase Enzyme Immunoassay of Progesterone in Saliva

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We have developed a rapid enzyme immunoassay for progesterone in saliva. This solid-phase assay is carried out on microtitre plates with no extraction or centrifugation steps. The detection limit of the assay is 200 fg per well (3.2 pmol/L). Intra- and interassay coefficients of variation for low, medium, and high concentrations of progesterone were 7.5, 16.0; 9.1, 8.3; and 8.7, 6.7%, respectively. Correlation between total plasma progesterone (assayed by enzyme immunoassay with extraction) and salivary progesterone concentrations was good ($r = 0.848$, $p < 0.001$, $n = 56$). We found the assay useful for monitoring ovarian function. The analytical procedure is convenient, and one person can assay more than 200 saliva samples per working day. The turnaround time for 36 samples is 2 h, including 1.5 h of incubation time, when previously coated plates are used. We conclude that such assays are very suitable for measuring progesterone in serial saliva samples and could become the preferred method for monitoring ovarian function.

Additional Keyphrases: results for plasma and saliva compared - monitoring ovarian function - fertility studies - pregnancy

The measurement of progesterone in samples of serum or plasma is a recognized method for investigating luteal function and monitoring ovulation. An attractive alternative is the measurement of progesterone in saliva, because sampling is noninvasive and practically stress-free. Furthermore, use of saliva facilitates multiple sampling, which is necessary for accurate monitoring of ovarian function (1). Published methods for the determination of progesterone in saliva are based on radioimmunoassay procedures and involve an extraction step (2–4). Such assay procedures are relatively costly with respect to operator time and equipment and are not suitable for smaller laboratories; direct radioimmunoassays offer advantages in time over indirect assays (5). An enzyme immunoassay for plasma and salivary progesterone has also been described (6).

Here we describe a sensitive, rapid, and cost-effective enzyme immunoassay for the determination of progesterone in saliva without extraction or centrifugation. The solid phase consists of antibody coated onto 96-well microtitre plates. The detection limit of the assay is 200 fg and the assay incubation steps total less than 2 h. The application of the assay in the assessment of ovarian function is demonstrated for a control group of individuals whose menstrual cycles are regular. We also show a progesterone concentration profile for a woman during late pregnancy and postpartum.

Materials and Methods

Subjects

Healthy volunteer women, ages 21 to 40 years, with regular menstrual cycles (29 ± 3 days, mean ± SD) and not taking oral contraceptives, gave samples of saliva nearly every day while under observation. Eleven of these volunteers supplied matched blood and saliva samples on one to three occasions per week while they were giving saliva samples. Cycles were dated from the first day of menstruation; basal body temperature was recorded in some cases. Daily saliva samples were also obtained from a 25-year-old primigravida volunteer, who delivered spontaneously at term, from her 37th week of gestation until three months postpartum.

Sample collection: Clearly written instructions were given to each volunteer at the beginning of the study. Each woman recorded the date and number of days since commencement of menses in the current cycle. After an overnight fast, and between 07:00 and 10:00 hours, volunteers were asked to rinse their mouths with water, rest for 5 min, then collect 2 to 5 mL of saliva (unstimulated) into a 50 × 14 mm polystyrene tube. To ensure stability, they stored the saliva samples in their home freezers as soon as possible after collection. After the samples were brought to the laboratory we thawed them at room temperature, centrifuged at 3000 × g for 15 min, and stored the supernates at −20 °C until assay. Saliva samples so treated lose their viscosity and are easily pipetted when they have been frozen and thawed.

Time-matched blood samples were collected into lithium heparin-containing tubes and the plasma was stored at −20 °C until assayed for progesterone.

Reagents

The key reagents—progesterone–enzyme conjugate, antiprogesterone antiserum, and anti-rabbit IgG—were supplied by Nocotech Ltd., Dublin, Ireland.

Antiserum: The total immunoglobulin (IgG) fraction of rabbit antiserum raised against 11α-hemisuccinate–bovine serum albumin conjugate was supplied in sodium borate buffer (0.35 mol/L, pH 8.4), which we diluted 8000-fold before use. A second antiserum, donkey anti-rabbit IgG, was also supplied as the purified IgG fraction (7).

Conjugate: Horseradish peroxidase (EC 1.11.1.7; Sigma, London, U.K.) was coupled to the 11α-hydroxyhemisuccinate derivative of progesterone by a modification of a mixed anhydride method (8). Unreacted material was separated from the conjugate by chromatography on Sephadex G-25. The conjugate is very stable, and these preparations have not lost enzymic or immunological activity after storage at 4 °C over four years. Working solutions (1 mg/L) may be kept at 4 °C for up to 12 months.

Buffers and standards: The general assay buffer (PBS/BSA) contained, per liter, 10 mmol of sodium phos-

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phate, 150 mmol of sodium chloride, 15 μmol of bovine serum albumin, and 0.1 g of thimerosal (pH 7.4). The buffer used to coat the wells of the microtitre plate (see below) was sodium carbonate, 50 mmol/L, pH 9.6, with 0.1 g of thimerosal per liter. The enzyme assay buffer used was 5.9 mmol of hydrogen peroxide and 5.5 mmol of o-phenylenediamine per liter of sodium acetate buffer, 20 mmol/L, pH 6.0. Microtitre plate wells were washed with 0.15 mol/L sodium chloride solution containing 0.5 mL of Tween 20 per liter. Progesterone stock standard solution (1.0 mmol/L in ethanol) was stored at 4 °C. Working solutions (20 to 1000 pg/mL) were prepared in PBS/BSA and stored at 4 °C.

Quality-control saliva: Unstimulated saliva from men was treated with activated charcoal and filtered through diatomaceous earth. Four quality-control pools were then prepared by adding 79, 159, 477, and 795 fmol (25, 50, 150, and 250 pg) of progesterone per milliliter of treated saliva.

Procedures

Extraction of plasma and saliva samples: Pipet 300 μL of saliva or 150 μL of plasma into a disposable glass culture tube (16 × 100 mm) and add 1.5 mL of petroleum ether (40–60 °C boiling range, high purity grade). Shake the tubes and contents on a multivortex-type mixer (SMI Model 2601) for 4 min, allow the phases to separate, then use a positive-displacement pipette to transfer 1.0 mL of the organic phase to a clean culture tube. Evaporate the solvent at 40 °C under a stream of air and add PBS/BSA to each tube (0.2 mL to saliva samples and 0.5 mL to plasma). Shake the tubes for 30 s as above, incubate at 37 °C for 10 min, and mix again for 30 s. Experiments with tritiated progesterone tracer show that about 88% of the progesterone is recovered in this extraction procedure.

Coating of microtitre plates: First, coat each well with second antibody by adding 200 μL of IgG (20 mg/L) in coating buffer. Cover the plate with plastic film and incubate at 37 °C for 90 min. Wash each well three times with 300 μL of wash buffer to remove unbound antibodies. Add to each well IgG from progesterone antiserum in 100 μL of PBS/BSA (8000-fold dilution of IgG made up in PBS/BSA to a volume equivalent to the volume of serum from which it was prepared); cover the plate and incubate at 37 °C for 1 h. Again, wash the wells three times as described above. Coated plates are stable for at least two weeks at 4 °C.

We investigated the well-to-well variation in the efficiency of antibody absorption to the solid phase by assaying plates with zero standard in all wells (described later). We calculated the variation for all wells on the plate, for the wells inside the peripheral wells, and for each row and each column of wells. Although overall variation from well to well was relatively low (CV = 1–3%) on the microtitre plates used (Immunoplate 1 with certificate, Nunc AS, Kamstrup, Denmark), the actual value appeared to be batch dependent. This degree of variation is lower than reported by others (9). Higher values for overall well variation (CV = 4–6%) could be greatly reduced by omitting results from the perimeter wells. Therefore, we decided during this study to use only the inner 60 wells on each 96-well plate.

Enzyme immunoassay: Using a diluter-dispenser (Compu-diluter-100, General Diagnostics), add 50 μL of standards, control saliva, and unknown saliva samples to individual wells plus 150 μL of progesterone-peroxidase conjugate (6.6 μg/L). Cover the plate with plastic film and, after mixing the solutions in the wells, incubate at 37 °C for 1 h. Empty the wells by sudden inversion and wash the plate three times as above. Add 150 μL of buffered enzyme substrate solution to each well in order, and incubate the plate at room temperature (16–20 °C) in the dark. After 30 min, stop the enzyme reaction by adding 50 μL of sulfuric acid (4 mol/L) to each well in the same order and at the same rate as the substrate solution was added. Mix the solutions in the wells, then measure absorbance at 492 nm with a microtitre plate reader. We used a Model EL307 plate reader (Bio-Tek Instruments Inc., Burlington, VT 05401), interfaced to a B.B.C. Model B microcomputer (Acorn Computers Ltd., Cambridge, U.K.).

We prepared computer programs for immediate processing of enzyme immunoassay data. The standard data—bound enzyme activity at each standard concentration divided by bound activity at zero standard (B/Bo) vs log picograms of progesterone standard—fitted well to a simple cubic polynomial, allowing the interpolation of control and unknown concentrations. These data, detailed assay statistics, and a standard curve were all printed out by the computer.

Results

Analytical Variables

Standard curve: The dose–response curve shown in Figure 1 represents the mean of 10 curves, obtained separately, each with duplicate concentrations of standards. The CVs for the B/Bo ratios for each standard ranged from 2.2% to 5.4%, indicating that the precision and stability of the standard curves are satisfactory.

Sensitivity: The sensitivity, defined as the standard concentration equivalent to the B0 minus 2 SD of B0, was 3.2 pmol/L (200 fg per well or 1.0 ng/L), compared with previously reported detection limits of 56–125 pmol/L (2–4) for radioimmunoassays of progesterone in saliva. One enzyme
immunoassay procedure for progesterone in plasma, after extraction, had a detection limit of 105 pmol/L (10). Of greater practical relevance is the value for ED 50, defined as the standard concentration equivalent to a B/Bo of 0.5: 760 pmol/L (12 pg per well or 240 ng/L) in this direct assay.

**Specificity:** The cross reactivities of various steroids, either structurally related to progesterone or of physiological importance, were assessed without an extraction step, as recommended by Abraham (11). As Table 1 indicates, the antiserum we used was relatively specific for progesterone.

The effect of saliva volume on the amount of progesterone detected was examined by diluting with PBS/BSA three samples containing different amounts of endogenous progesterone, and assaying aliquots of each dilution. The amount of progesterone detected was independent of saliva volume over the range examined (3–50 µL).

**Analytical recovery:** We routinely monitored analytical recovery by using as quality-control saliva a pool of saliva from men, supplemented with different concentrations of standard progesterone. Allowing for a "blank" reading of 44.5 ± 10.8 pmol/L (mean ± SD, n = 6), we determined mean recoveries over 14 assays (n = 13 for 79.5 pmol/L sample) as 89, 97, 99, and 95% when progesterone concentrations of 79.5, 159, 477, and 795 pmol/L (final concentrations) were added to saliva.

**Precision:** Repeated assay (n = 14 or 15) of the quality-control saliva samples assayed in one run yielded the intraassay CVs listed in Table 2. Table 2 also lists the interassay CVs for duplicate determinations of these samples in 14 (or in one case, 13) routine assay runs, each carried out in uninterrupted sequence by one person.

**Comparison of Progesterone Concentrations in Blood and Saliva**

Matched samples of blood and saliva, collected within 20 min of each other, were obtained from 11 nonpregnant women throughout their menstrual cycle. We measured the progesterone concentrations in these samples by enzyme immunoassay directly in saliva and after extraction with petroleum ether in plasma. The concentrations in the saliva (19–954 pmol/L) were about 1% of those in plasma (0.1–20 ng/mL, 0.3–64 nmol/L), but the correlation between them was highly significant (r = 0.85, n = 56, p < 0.001) (Figure 2). Further regression analysis of concentrations of progesterone in the matched plasma and saliva samples from four subjects showed statistically significant (p < 0.01) correlations (r1 = 0.96, n1 = 7; r2 = 0.96, n2 = 6; r3 = 0.88, n3 = 9; r4 = 0.95, n4 = 8).

**Assessment of Ovarian Function**

Progesterone was measured in saliva samples from nonpregnant volunteers who had regular menstrual cycles. The concentrations detected in two such individuals are shown in Figure 3. A distinct but irregular progesterone peak, beginning on day 16 and continuing until day 26, was associated with the latter part of the cycle of one individual. This peak, typical of ovulation, coincided with an increase in basal body temperature. In the second individual the highest concentrations of progesterone occurred earlier and

**Figure 2. Correlation between salivary and plasma progesterone concentrations in samples of saliva and plasma matched for time of collection:**

**Figure 3. Concentration of progesterone in saliva collected daily during the menstrual cycle from two volunteers**

BBT, basal body temperature; M indicates onset of menses

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**Table 1. Specificity of Antiserum Used**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross reaction, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>100</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>4.6</td>
</tr>
<tr>
<td>5α-Pregnane-3,20-dione</td>
<td>5.7</td>
</tr>
<tr>
<td>Progesterolone</td>
<td>0.2</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1.0</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>1.2</td>
</tr>
<tr>
<td>11-Deoxycorticosterone</td>
<td>2.0</td>
</tr>
<tr>
<td>Estradiol-1β</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Table 2. Intra- and Interassay Variation in Quality-Control Saliva Samples**

<table>
<thead>
<tr>
<th>Progesterone concn</th>
<th>Low</th>
<th>Medium 1</th>
<th>Medium 2</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, pmol/L</td>
<td>102</td>
<td>182</td>
<td>506</td>
<td>979</td>
</tr>
<tr>
<td>SD, pmol/L</td>
<td>7.6</td>
<td>17.8</td>
<td>46.1</td>
<td>84.9</td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>14</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>CV, %</td>
<td>7.5</td>
<td>9.8</td>
<td>15.1</td>
<td>8.7</td>
</tr>
<tr>
<td><strong>Interassay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, pmol/L</td>
<td>115</td>
<td>198</td>
<td>518</td>
<td>801</td>
</tr>
<tr>
<td>SD, pmol/L</td>
<td>18.4</td>
<td>14.9</td>
<td>42.9</td>
<td>53.7</td>
</tr>
<tr>
<td>n</td>
<td>13</td>
<td>14</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>CV, %</td>
<td>16.0</td>
<td>7.6</td>
<td>8.3</td>
<td>6.7</td>
</tr>
</tbody>
</table>

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gradually decreased toward the onset of menses. These patterns of progesterone concentrations in daily saliva samples from these two individuals and from others confirm the reported fluctuations in progesterone values during the menstrual cycle (1). Progesterone in 15 individuals who gave daily saliva samples ranged from 50.8 to 235 pmol/L during the early phase of the menstrual cycle to 312 to 852 pmol/L in the latter phase, values in agreement with published data (1, 2).

In the 11 volunteers previously compared for plasma and salivary progesterone concentrations, the mean plasma to saliva ratio before day 14 of the cycle was 13.3. After day 14 and before onset of menses the mean plasma to saliva ratio was 55.9.

Monitoring Progesterone Pre- and Post-Partum

Saliva samples were collected from a pregnant woman almost daily for 18 days before parturition and for 12 weeks post-partum. Immediately post-partum she began breast feeding for six weeks. Progesterone concentrations in her saliva are shown in Figure 4. The first notable increase above the post-partum baseline was detected at 11 weeks postpartum; the volunteer's first menstrual period postpartum occurred within a week of this increase.

Discussion

One of the major advantages of measuring steroids in saliva, as we confirm here, is the relative ease and convenience of obtaining serial samples, which provide a more nearly accurate means of assessing endocrine function than does single sampling (1). Another advantage of salivary steroid assays is their potential use in the parallel investigation of unbound steroids in plasma (12, 13).

The enzyme immunoassay we describe has a relatively high throughput, 200 samples per person per working day, compared with published radioimmunoassay procedures (1–4), thus facilitating the processing of serial samples. Other advantages of a direct enzyme immunoassay include the elimination of the requirements for radioactive isotopes and extraction or separation steps. The relatively rapid turnaround time, 2 h for 36 samples, allows the complete processing of 200 samples in less than a day and is advantageous in the operation of routine assay services. It is as precise and accurate as a radioimmunoassay recommended for in vitro fertilization studies in a recent report (14) and is more sensitive and rapid.

The applications of radioimmunoassay for progesterone in plasma for the assessment of ovarian function are well established (15–18). Other applications of progesterone measurements include studies on the return of post-partum fertility (19) and, indeed, the present report demonstrates the advantages of salivary progesterone assays for this type of study. Such information might allow a woman to predict the return of ovarian function after the cessation of breast feeding and take appropriate action. A similar type of study with serial saliva samples might be able to indicate a return of ovarian function after the cessation of oral contraceptives.

The convenience of saliva sampling combined with the availability of relatively inexpensive high-throughput assays should facilitate further and more detailed study of ovarian function in females pre- and post-menarche, in female athletes, in patients with premenstrual syndrome, in pre- and post-menopausal women, and in patients with early pregnancy failure.

We are grateful to Martin Brett, Henry Bourke, and Helen Grimes for their help and to our many volunteers for their kind cooperation.

References


![Figure 4. Concentration of progesterone in daily samples of saliva collected ante- and post-partum, from one volunteer. Arrows indicate time of parturition and cessation of breastfeeding.](image-url)


