Radioimmunoassay of Progesterone in Saliva: Application to the Assessment of Ovarian Function

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We report a specific radioimmunoassay that has the required sensitivity (7 pg per assay tube) for determining progesterone concentrations in 400 μL of mixed saliva collected from normal women. The assay is precise: intra- and inter-assay variation (CV) never exceeded 11.0 and 8.0 %, respectively. The assay was used to determine progesterone in saliva samples collected daily for not less than 28 days by normal women and by patients having abnormal ovarian function. Four normal women provided matched saliva and plasma samples for accurate dating of the menstrual cycle by plasma progesterone, estradiol, lutropin, and follitropin. Nine further subjects collected saliva samples only, and from these data a provisional "normal range" was established. Progesterone concentrations in saliva during the follicular phase of the cycle were low (<100 pmol/L) but rose beginning on day 12 to reach peak values of 230–550 pmol/L on day 21. Thereafter, progesterone concentrations in saliva declined to values generally <170 pmol/L at the commencement of menses. Saliva samples from three patients attending an infertility clinic were also studied to assess ovarian function.

Additional Keyphrases: cyclic changes in progesterone in saliva • infertility assessment • functional capacity of corpus luteum

The determination of biologically active compounds in saliva is a particularly attractive alternative to their determination in plasma, especially when serial samples are required. Earlier studies (1, 2) have indicated the value of data on salivary cortisol for assessing adrenal function and salivary 17a-hydroxyprogesterone for monitoring treatment of children with congenital adrenal hyperplasia. This would suggest that the functional capacity of the corpus luteum could be investigated by determining concentrations of progesterone in saliva rather than plasma. This test would be a considerable advantage in studies of infertile patients because daily saliva samples can be easily collected at home by the patients themselves. A specific radioimmunoassay (RIA) having the sensitivity required for measuring the low concentrations of progesterone expected in saliva has therefore been developed. We used the RIA to determine this steroid in mixed (i.e., whole) saliva samples collected daily for not less than 28 days by normal women and patients having abnormal ovarian function. All of the normal women participating in this study gave informed consent for the collection of saliva samples, but only four could be induced to provide blood samples for conventional dating of the cycle by plasma progesterone, estradiol, and protein hormone determinations.

Materials and Methods

Subjects

Normal women. Nine healthy women, ages 21 to 41 years (28.3 ± 8.7, mean ± SD), collected 2-mL saliva samples each day for the duration of one complete menstrual cycle. These women had taken no oral contraceptive for at least six months and all had a history of regular menstrual cycles ranging from 26 to 30 days (28 ± 1.4, mean ± SD). Four of these volunteers supplied matched samples of plasma and saliva, which allowed dating of the cycle by conventional procedures. All plasma samples were assayed for progesterone, estradiol, lutropin (luteinizing hormone), and follitropin (follicle-stimulating hormone). One of these normal subjects volunteered to collect saliva samples at two-hourly intervals during waking hours on two consecutive days during the periovulatory (day 14 and 15) and luteal (day 21 and 22) phase of her cycle.

Patients. Three patients who were attending an infertility clinic (I.C., L.D., and P.D.) collected saliva samples for at least one month.

Sample Collection

Saliva samples. At the commencement of this study, all women were given the very simple instructions for collecting mixed whole saliva as described in an earlier communication (1). They were also given the requisite number of disposable glass capped tubes (75 × 12 mm) set in a small polystyrene container suitable for storage in the freezer compartment of a domestic refrigerator or deep freeze. The volunteers named and dated all tubes after sample collection between 0800 and 1000 hours. The samples were stored frozen at home, and at the end of the collection period were brought to the laboratory by the participants and stored at −20 °C until assayed; saliva samples so stored remain stable for periods exceeding six months.

Progesterone concentrations were determined in all samples of saliva. Plasma samples. Blood was collected in tubes containing lithium heparin as anticoagulant, and the plasma obtained on centrifugation was stored at −20 °C before assay of lutropin, follitropin, estradiol, and progesterone by previously published procedures (3–5).

Reagents

Antiserum to progesterone was raised in rabbits to a progesterone-11α-hemiasuccinate/bovine serum albumin conjugate. It was diluted 100-fold in assay buffer and stored in 0.5-mL aliquots at −20 °C.
Radioligand [1,2,6,7,-3H]progesterone (sp. act., 81 kCi/mol) obtained from the Radiochemical Centre, Amersham, Bucks, U.K., was diluted on receipt in benzene/methanol (9/1 by vol) and stored at 4°C for not longer than four months.

Common solvents and reagents used in this assay procedure have been listed in an earlier publication (7).

The following reagents, previously described for use in a testosterone RIA procedure (6) were used without modification.

Phosphate-buffered saline. Assay buffer, pH 7.4. Phosphate-buffered isotonic saline containing 1 g of gelatin per liter.

Dextran-coated charcoal suspension. Assay buffer containing dextran, 0.25 g/L, and charcoal, 2.59 g/L.

Progesterone stock standard solution, 100 mg/L, was prepared by dissolving 1 mg of progesterone in 10 mL of absolute ethanol and stored at 4°C for not longer than four months. Ethanol standard solutions for use in the dose-response curve contained 10, 20, 30, 40, 60, 80, and 100 pg/10 μL (1–10 μg/L) and were prepared by appropriate dilution of the stock standard solution just before use.

Tritiated progesterone solution for use in assay was prepared when required by drying a 100-μL aliquot of the stock tritiated-ligand solution under nitrogen and redissolving the residue in 28 mL of assay buffer. The 100-μL aliquots of this solution used in the assay contained 30 pg of progesterone.

Assay Procedure

Add duplicate 400-μL aliquots of mixed saliva to labeled disposable 100 × 12 mm glass tubes. Add 3 mL of petroleum ether and place the stopped tubes in a vortex-type shaker for 10 min, then centrifuge the tubes for 5 min at 4°C to break the slight emulsion that may form in some of them. Freeze the aqueous layer by placing tubes in an acetone/solid CO2 mixture and decant the organic phase into clean, labeled glass 75 × 12 mm assay tubes. Transfer duplicate 10-μL aliquots of the ethanolic standards used in the dose-response curve to similar tubes, and add 3 mL of petroleum ether. Evaporate the solvent under nitrogen at 30°C.

Add 100 μL of antiserum, vortex-mix briefly, and incubate at 30°C for 1 h. Add 100 μL of tritiated progesterone containing 16 200 DPM to all tubes and equilibrate at 30°C for 1 h. Transfer the tubes to an ice bath for 15 min. Add 500 μL of well-stirred, ice-cold, dextran-coated charcoal suspension to all tubes, to separate antibody-bound and free steroid. Stand the tubes in iced water for a further 12 min and centrifuge at 2500 rpm for 10 min at 4°C. Transfer 500-μL aliquots of the supernates to scintillation vials, add 6 mL of scintillant, and count the radioactivity associated with the antibody-bound steroid.

The dose–response curve was obtained and the concentration of progesterone in the samples was calculated by using the four-parameter fit model of Rodbard and Hutt (7).

Results

The standard curve is shown in Figure 1. Replicate assays (n = 16) established that precision in the standard curve was satisfactory at all fixed points. Log-logit transformation gave an apparently linear response to progesterone from 10 to 100 pg, with a correlation coefficient of –0.95.

Specificity of the antiserum was assessed by the criteria of Abraham (8) and the cross reactivities of steroids structurally related to progesterone are presented in Table 1.

Sensitivity, defined here according to Kaiser and Specker (9) as the least amount distinguishable from zero at the 95% confidence level, was 7 pg per assay tube. This corresponded

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross reactivity, %</th>
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<tbody>
<tr>
<td>Progesterone</td>
<td>100</td>
</tr>
<tr>
<td>11β-Hydroxyprogesterone</td>
<td>83</td>
</tr>
<tr>
<td>5α-Pregnane-3β,20α-dione</td>
<td>12</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>10.7</td>
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<tr>
<td>11-Oxo-progesterone</td>
<td>10.3</td>
</tr>
<tr>
<td>20α-Dihydromprogesterone</td>
<td>1.4</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>1.3</td>
</tr>
<tr>
<td>Corticosterone</td>
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</tr>
<tr>
<td>11-Deoxy cortisolone</td>
<td>0.7</td>
</tr>
<tr>
<td>11-Deoxy cortisol</td>
<td>0.2</td>
</tr>
<tr>
<td>Cortisol</td>
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<tr>
<td>17α-Hydroxypregnenolone</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Pregnanetriol</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>20α-Dihydrompregnenolone</td>
<td>&lt;0.1</td>
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to progesterone concentrations in whole saliva of 56 pmol/L.

**Precision.** Three pools of saliva were obtained by mixing samples having high, medium, and low progesterone concentrations. Aliquots (n = 16) of each pool were determined in one assay and these data were used to assess intra-assay variance (Table 2). These pools were then split into aliquots, stored at −20 °C, and used as quality-control samples for subsequent routine assays, thereby providing the inter-assay variance data presented in Table 2.

**Analytical recovery.** Tritiated progesterone was added to saliva samples having progesterone concentrations approximating the lowest and highest standards used in the dose–response curve, and the samples were incubated overnight at 4 °C. Because recovery on extraction exceeded 95% in all samples, we deemed it unnecessary to monitor recovery in routine practice.

**Assessment of Ovarian Function**

The RIA procedure reported here has the sensitivity required for determining salivary progesterone concentrations during the luteal phase of the cycle. In the follicular phase, however, values were generally below the lower limit of sensitivity of the method (<56 pmol/L). The nine normal women taking part in this study were all in full-time employment. They found collection of 24-h urine samples difficult, and only four consented to venepuncture; dating of the cycle about the day of ovulation in most of these women was therefore impossible. This difficulty was resolved by numerically adjusting the cycle length in each subject to 28 days, day 1 being the first day of menses. The progesterone concentrations (mean ± SD) in combined values from consecutive two-day samples were calculated and when plotted gave the "provisional normal range" for salivary progesterone illustrated by the shaded area in Figure 2.

In these subjects, salivary progesterone concentrations from day 1 to day 12 fluctuate from values that were too low for accurate determination (<56 pmol/L) to values not exceeding 100 pmol/L. Progesterone concentrations increased rapidly from day 12 to day 21, reaching a maximum on day 21, which ranged from 230 to 550 pmol/L. Salivary progesterone concentrations declined thereafter and at the onset of menses rarely exceeded 170 pmol/L.

RIA of matched plasma and saliva samples provided by the women having regular cycles dated by accepted procedures indicated that progesterone concentrations of plasma samples in the luteal phase were accurately reflected by concentrations of progesterone in saliva. Regression analysis of concentrations of progesterone in the matched samples of saliva and plasma from four subjects showed excellent correlations ($r_1 = 0.93$, $r_2 = 0.92$, $r_3 = 0.91$, and $r_4 = 0.97$).

Figure 2 shows salivary progesterone profiles for three infertile patients. A patient with primary amenorrhoea (subject P.D.) had consistently low salivary progesterone concentrations throughout the 46 days of study. Subject L.D., stated to have "luteal phase deficiency," showed changes in salivary progesterone similar to those observed in normal women; however, from day 13 onwards the concentrations observed were consistently lower than normal. The variation in salivary progesterone values observed in subject I.C. appeared grossly abnormal, progesterone values exceeding 390 pmol/L during the early and late phases of the cycle.

Figure 3 shows the data obtained from samples of saliva provided by a normal woman at 2-h intervals on days 14, 15, 21, and 22 of her menstrual cycle. Progesterone in saliva fluctuated irregularly throughout the day in both the periovulatory and the luteal phase; however, the amplitude of these fluctuations was sufficiently small that overlap in the periovulatory and luteal ranges did not occur. This would suggest that timing of collection of saliva samples may not be critical. The possibility that untimed "spot" samples collected in an infertility clinic could be used as a screening procedure is therefore not excluded. However, the results obtained from three patients attending an infertility clinic emphasize the need for frequent sampling for the accurate assessment of luteal function. The standard practice of collecting two or three plasma samples during the latter part of the cycle for assay of progesterone would appear inadequate to define such abnormalities.

Collection of frequent saliva samples by the patients themselves would readily resolve the difficulty of timely sampling and therefore facilitate serial progesterone determinations for the accurate assessment of luteal function.

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**References**

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