Sensitivity Salivary Estradiol Assay for Monitoring Ovarian Function

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Measurement of steroids in saliva has excited interest because of the numerous potential clinical applications; non-invasive, convenient sampling; and apparently accurate reflection of the concentrations of physiologically active unbound steroid in the circulation. Although assays of saliva for several steroid hormones are available and widely used, assays for salivary estradiol are not, primarily because of methodological limitations. By modifying a commercially available kit for serum estradiol, our laboratory has developed a procedure that is sensitive, highly specific, and reliable for measuring salivary estradiol. Assay sensitivity is 1.5 fmol (0.14 pg; sample concentration 1.3 pmol/L) with a mean interassay CV of 10.8% at low concentrations. Clinical studies showed that values for serum and saliva are highly correlated (P < 0.001), and demonstrated reliable detection of estradiol peaks during normal ovulatory cycles in serial samples from 15 women. Salivary estradiol peaked at 5.4 SD 1.9) pmol/L on cycle day 14.4 (SD 3.2), 1.2 (SD 0.8) days before ovulation detected by ultrasound. This assay may be particularly helpful in investigating ovarian function and free estradiol in women at various stages of the reproductive cycle.

Additional Keyphrases: menstrual cycle • reproductive disorders • monitoring postmenopausal estrogen supplementation • equilibrium radioimmunoassay

Long-term clinical monitoring of estradiol (E2) in the circulation is desirable for treatment and study of hormone-dependent carcinomas (I–3), monitoring estrogen supplementation in postmenopausal women (4, 5), and work-up of patients being treated for infertility. More short-term but intensive monitoring of E2 is also required for infertility treatments, especially in vitro fertilization (6). Recently, considerable interest has been generated in the potential of salivary steroids for tracking such conditions, both because it would represent a less-invasive method for long-term or intensive sampling and because concentrations in saliva may reflect the unbound, biologically active circulating moiety (7, 8). However, application of measurement of salivary E2 has been hampered by the lack of a sufficiently sensitive and reproducible assay (9, 10).

Previously published ranges for salivary estradiol vary considerably (9, 11–15). These inconsistencies could stem from method-related differences in antibody specificity, isotope used (gamma or beta emitters), and assay sensitivity and precision, or from variations in technique among laboratories, particularly for more complex protocols. Differences in study population, salivary collection procedures, and preparation for assay have also contributed to discrepancies among results.

Factors contributing to the reliability and improved performance of the present assay include (a) the use of an iodinated rather than a tritiated tracer; (b) separation with second antibody plus polyethylene glycol rather than charcoal; (c) double rather than direct or single extraction of saliva with anhydrous ether; (d) determination of extraction recovery for each assay tube, with correction for losses during data processing; and (e) a highly specific and sensitive antibody. The result is a more reproducible standard curve, with good slope, allowing detection at concentrations from 1.4 to 588 pmol/L.

The assay is a simple equilibrium radioimmunoassay that can be done by most laboratories that routinely assay hormones. Reagents are readily available commercially. As a result of the kit modifications, the reagent cost is about a third that of serum estradiol determinations. With batched sampling, longitudinal studies of salivary estradiol can be performed at considerably less cost than serum estradiols.

Materials and Methods

Reagents

Our protocol is a modification of the Estradiol-Quant-IVitro125I RIA Diagnostic Test Kit (Leeco Diagnostics, Inc., Southfield, MI) for the quantification of total estradiol in serum. Our minor adaptations of the kit reagents are noted in the method.

The estradiol antiserum is rabbit anti-estradiol-17ß-6-oxime–bovine serum albumin, an antibody that cross-reacts by <1.0% with estriol, estrone, testosterone, and other related compounds. We prepared the working buffer by adding 100 mg of gelatin (Knox unflavored) to 100 mL of Dulbecco's buffer (Gibco, Grand Island, NY), heating this to 45 °C to dissolve (pH 7.4). Purified E2 used in recovery studies was purchased from Sigma Chemical Co., St. Louis, MO.

Methods

Collection of saliva: Prepare saliva-collection tubes by adding one drop of a solution of sodium azide (200 mg in 10 mL of de-ionized water) to 16 × 100 mm borosilicate glass tubes, evaporate to dryness, cap the tubes with polyethylene closures, and mark a 5-mL fill line on the tube.

Subjects are instructed to rinse their mouths with water, wait 5 min, and then collect 4–5 mL of saliva into one of the prepared tubes. (Collection takes about 10 min.) With the sodium azide added as a preservative, the samples are stable for longer than a week at room temperature. However, when possible, we freeze the samples soon after collection. No samples are processed fresh; instead, all are frozen and thawed before being divided into aliquots, so as to break down the mucus polysaccharides that can interfere with accurate pipetting. On the day of the assay, thaw the samples and centrifuge (1000 × g, 10 min). Transfer the supernate to a glass tube for assay.

Controls: Serum controls, supplied by Leeco Diagnostics, contain estradiol in a human serum base. Saliva pools
consisted of samples collected as specified from both men and women, combined and aliquoted to make two pools, one of low and one of medium concentration.

**Extraction procedure:** Pipet 100 μL of standards (0, 10, 50, 100, 250, 500, 1000, 2000 ng/L) and serum controls and 1000 μL of saliva samples and saliva controls into 18 × 150 mm glass tubes. Dilute standard and serum control tubes to a total volume of 1.0 mL by adding 0.9 mL of working buffer. (For convenience, buffer rather than saliva is used, because either yields equivalent binding.) To assess the efficiency of extraction, add 5 μL (0.86 pg) of tracer to each tube. Also, pipet 5 μL of tracer into three 12 × 75 mm polypropylene tubes at the beginning and end of tracer addition, giving six tubes to be used for total count measurement.

Vortex-mix the contents of all tubes on a multi-tube vortex-type mixer (Baxter/Scientific Products, Stone Mountain, GA) for 1 min. Incubate at 37 °C for 30 min and vortex-mix again for 1 min. Add 5 mL of diethyl ether (anhydrous analytical-reagent grade; Mallinckrodt, St. Louis, MO; use within seven days of opening) to all tubes and vortex-mix vigorously for 4 min. Allow the layers to settle for 10 min at room temperature, then freeze the aqueous phase by immersing in a solid carbon dioxide/methanol bath or by placing the tube in a −20 °C freezer for 20 min. Carefully decant the upper solvent phase into 16 × 100 mm glass tubes and evaporate it under nitrogen in a 37 °C water bath.

To the aqueous phase, add 5 mL of ether and repeat the extraction procedure to ensure more complete extraction of steroid into the solvent phase. (Nine samples we evaluated gave results 8.4% lower after single extraction than after double extraction.) To the dried extracts, add 0.5 mL of working buffer and vortex-mix for 2 min. Incubate for 10 min at 37 °C, then vortex-mix for 1 min. Aliquot 200 μL of the reconstituted volume, in duplicate, into 12 × 75 mm polypropylene tubes. Count the radioactivity in the total-count tubes and in the extracted aliquots in a gamma counter (we used a Cobra Model 5005 equipped with RIA-Smart and Expert QC software; Packard Instrument Co., Downers Grove, IL) for 10 min. Calculate recovery by multiplying the average counts from the duplicate 200-μL aliquots by 2.5 and dividing by the average counts for the six total-count tubes.

**Radioimmunoassay:** To the 200-μL aliquot, add 50 μL of 125I-labeled estradiol and 100 μL of antiserum, prepared by diluting the kit solution fourfold with working buffer; vortex-mix, and incubate overnight at room temperature. Dilute the kit-supplied polyethylene glycol–second antibody solution twofold with working buffer and add 0.5 mL to each assay tube. Vortex-mix, incubate at room temperature for 20 min, and centrifuge (Sorvall Model RC-3C; Du Pont, Wilmington, DE) at 2800 rpm and 4 °C to deliver a total centrifugal effect of 29 × 10⁶ rad/s². Setting force rather than time reduces inter-spin variation (duration is about 1 h). Laboratories without a force-setting centrifuge option may spin the samples for 1 h at 2800 rpm and 4 °C. Carefully decant the supernatant fluid, blotting the tube rims. Estimate radioactivity in the precipitates (antibody-bound fraction) by gamma-counting 10 min.

Standard concentrations are corrected for recovery by multiplying each recovery value by the pre-extracted standard concentration. Sample and control values are each corrected for recovery after interpolation from the standard curve. Data are reduced by the spline-function method.

### Results

**Assay Evaluation/Assay Performance**

**Sensitivity:** The sensitivity of the assay, defined as the quantity of unlabelled hormone required to inhibit binding of tracer by an amount equal to 2SD below the mean binding observed in the absence of unlabelled hormone, is 0.5 fmol (equivalent to a concentration of 1.32 pmol/L). Buffer and solvent blanks were equivalent to binding a zero dose.

The standard curve was highly reproducible, having a slope of −0.736 (SD 0.05), ED₅₀ of 7.34 (SD 1.5), ED₅₀ of 48.8 (SD 5.1), and ED₂₀ of 392.7 (SD 31.9) pmol/L. The readable range of the standard curve is from 1.3 to 57 pmol/L (0.36 to 156 ng/L), based on sensitivity and standard range. (The precision profile yielded a CV of <10% across this range.) Tube error of duplicates averaged 2.5%.

**Precision:** Intra-assay variation (CV) for a medium concentration of pooled saliva specimens (87.7 ± 5.5 pmol/L mean ± SD) was 6.5%; interassay variation for another medium-concentration saliva pool (77.1 ± 5.5 pmol/L) was 7.4%. The low-concentration saliva pool (14.3 ± 1.5 pmol/L) had an intra-assay variation of 10.7%, and another low concentration pool (mean 8.1 ± 0.9 pmol/L) had an interassay variation of 10.8%. Values for low- and medium-concentration serum controls all fell within the range supplied by the manufacturer. Most control readings fell in the middle of the range, with no detectable overall skewness (assay n = 13).

**Accuracy:** Extraction efficiency was determined for each sample, standard, and control by using 125I-labeled estradiol tracer, added to each tube before extraction. Sample recovery ranged from 73% to 85%. Method accuracy was determined from known amounts of unlabeled E₂ added to saliva samples containing various endogenous concentrations, as shown in Table 1. Recoveries ranged from 85% to 105% and averaged 97%.

**Parallelism:** Parallelism was evaluated by measuring E₂ in a very high-concentration saliva sample (273 pmol/L) diluted to medium assay range, and in a lower-concentration sample (131 pmol/L), diluted incrementally to the low end of the range. Observed values were near those expected across the entire range of measurement (average recovery 94%; range 88–100%), suggesting the absence of sample sources of significant assay interference.

**Sample preparation:** All samples were centrifuged before being divided for assay; we found no significant difference or trend in matched comparisons of values obtained for centrifuged and uncentrifuged samples. However, the CV for duplicate centrifuged and uncentrifuged samples (23%) exceeded that for duplicates prepared by centrifugation.

### Table 1. Analytical Recovery of Estradiol Added to Saliva with Low and High Concentrations

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<th>Added Conc., pmol/L</th>
<th>Endogenous</th>
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<th>Recovery, %</th>
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alone (see Precision). Uncentrifuged samples require more effort to achieve complete homogenization and to minimize pipetting errors.

Clinical Evaluation

**Matched serum and saliva samples**: Eighteen matched serum and saliva samples were collected from women under ovulation-induction therapy with either clomiphene or gonadotropin. Samples were obtained in the late follicular, peri-ovulatory period, within 24 h after appearance of positive urinary lutropin readings from a self-administered test kit. The relationship of concentrations in serum and saliva is plotted in Figure 1. Salivary E₂ concentrations ranged between 0 and 19.5 pmol/L (4.67 ± 5.14 pmol/L, mean ± SD), with corresponding serum values ranging from 169 to 3157 pmol/L (930 ± 743 pmol/L). Values for saliva and serum showed a strong linear relationship (r = 0.765, P < 0.001). The three saliva samples that yielded no detectable E₂ had corresponding values of 242 ± 66 pmol/L for serum. The mean ratio of salivary to serum E₂ was 0.005 ± 0.003; there was no correlation between the salivary:serum ratio and values for total circulating E₂ (r = 0.06). Inferentially, the proportion of free circulating E₂ did not appear to systematically vary with values for total E₂ in the circulation.

**Comparisons of salivary estradiol with date of ovulation**: Estradiol was measured in saliva samples from 15 normal, untreated women who were also monitored by ultrasound for ovulation, as described elsewhere (16). Each woman was sampled intermittently during the early to midfollicular phase, and then at half-day intervals (at 0900 and 2100 h) as follicular maturation peaked, until ovulation was detected by ultrasound. All monitored cycles were ovulatory; the number of samples from each woman averaged eight.

The daily profile of mean E₂ is shown in Figure 2, the values for each individual having been aligned with respect to time of ovulation. Ovulation occurred on cycle day 15.5 ± 3.6 (mean ± SD; range, day 11–24). For each woman, a peak salivary E₂ value was identified, and its corresponding cycle day and time to ovulation were noted (Figure 3). E₂ concentrations peaked on cycle day 14.4 (SD 3.2), 1.2 (SD 0.8) days before ovulation (range, day -3 to 0). Observed peak E₂ concentrations averaged 5.4 (SD 1.9) pmol/L (range 1.5–8.9 pmol/L). To facilitate comparison with other reports, we calculated average (± SD) values for follicular (more than three days before ovulation) (0.6 ± 1.6 pmol/L) and peri-ovulatory (day -3 to +0.5) (3.4 ± 2.3 pmol/L) periods.

**Discussion**

Our observation that salivary E₂ concentrations correlate highly with values for serum in stimulated cycles contrasts with an earlier study (12) and supports others that have found good correlation (13, 15), especially in stimulated cycles (17, 18). Our values for salivary E₂ in the first half of normal ovulatory cycles are notably low; however, the sensitivity and precision of the assay permitted reliable discrimination of periods of active E₂ production. Table 2 compares E₂ concentrations reported in the literature. Where authors did not specify means by cycle

Fig. 2. Concentrations of E₂ in saliva, mean ± SEM, by day of cycle relative to time of ovulation in individual sample series

To preserve the degree of variation among cycles in trajectory of E₂ before the time of ovulation, values have been plotted by half-days for the six days preceding ovulation.

Fig. 3. Concentrations of E₂ in saliva, mean ± SEM, plotted with respect to the time at which the E₂ concentration peaked in each woman's sample series.

Twice-daily (morning and evening) saliva sampling facilitated discrimination of the pre-ovulatory peak in E₂ production.

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**Figures and Tables**

1. Relationship of concentrations of E₂ (in pmol/L) in matched samples of saliva and serum. The values are highly correlated (r = 0.765, P < 0.0001). Omitting values -1400 pmol/L from the analysis much improves the correlation (r = 0.819).

2. Concentrations of E₂ in saliva, mean ± SEM, by day of cycle relative to time of ovulation in individual sample series.

3. Concentrations of E₂ in saliva, mean ± SEM, plotted with respect to the time at which the E₂ concentration peaked in each woman's sample series. Twice-daily (morning and evening) saliva sampling facilitated discrimination of the pre-ovulatory peak in E₂ production.
phase, we made a rough estimate based on concentrations graphed by cycle day. The period before onset of the pre-ovulatory increase in E2 was designated the follicular phase; the maximum value attained per cycle in the pre-ovulatory surge was taken as the observed peak; and the interval from one day after ovulation through the last half of the cycle was designated the luteal phase. Comparison of values from tritium- and iodine-based radioassays should be drawn with caution, however. We note that findings from two reports (13, 19) agree with those given here.

Several features of the assay reported here may account for the observation of lower values than observed by some groups. The antibody has high specificity, having <1% cross-reactivity with estrone, estradiol, and related compounds. Comparison is difficult because most reports have omitted details of cross-reactivity; in the case of the highest published values for normal cycles, notable antisera cross-reactivity was reported as well as an uncorrected assay blank (20). Also, sensitivity is greater in this assay, approached only by that reported by two groups, in both of which the same source of antisera and tracer was used (13, 15, 21). Using a similarly sensitive but very different assay system based on enzymatic reaction and bioluminescence, Mounib et al. (18) found values for free E2 in plasma and estrone–E2 in saliva to be the same as the salivary E2 values we report here. The kit assay that we modified reportedly yields consistently lower values for serum than most other commercial preparations; this is reflected by our finding of increased (930 ± 743 pmol/L), but markedly lower than previously published (17), concentrations of E2 at midpoint in stimulated cycles. Finally, we note that a report based on the broadest sampling of populations (19) yielded average values quite close to ours.

A corollary to our observation of lower salivary E2 concentrations is the finding of unusually low saliva:serum E2 ratios: 0.005 ± 0.003. This contrasts with reports of saliva:serum ratios between 0.01 and 0.02 (9, 13, 17, 18). Findings of similar proportions of free E2 in serum (18, 22) have been taken as supportive of the notion that saliva concentrations mirror the free circulating E2 concentrations. Values measured for serum at which concentrations in the corresponding saliva fell below detectability in this assay (242 ± 66 pmol/L) corresponded closely with the concentration at which 0.5% free E2 would lie below assay sensitivity (1.32 pmol/L). Unlike one recent study (15), we saw no inverse relationship between the saliva:serum ratio and serum concentrations.

Comparison of values obtained from centrifuged and homogenized uncentrifuged saliva for nine samples, including three from pregnant women, showed no discernible consistent difference. These findings contrast with reported differences in progesterone concentration ascribable to sample preparation, for which significantly higher values are yielded by uncentrifuged luteal-phase samples (23). We centrifuge all samples before extraction, because we observe less variance in replicates with this preparation.

An E2 peak, demarcated with ascending and descending segments, was discernible in all cycles studied. However, there was considerable inter-individual difference in over all E2 concentrations achieved. Further, variation in timing of peak E2 relative to ovulation blunts individuall observed peaks when averages are calculated relative to time of ovulation. Alignment of data with respect to time of peak E2 provides better resolution of actual peak shape (Figure 3), in agreement with a recent report (15).

We conclude that, with this sensitive assay, monitoring salivary E2 can detect the presence or absence of an active follicle in a normal cycle. Onset of E2 production and mid-cycle peak can also be discriminated with this method and observed peaks correspond well with ovulation (detected by ultrasound). This and other recent studies in which sensitive assays were used indicate the usefulness of salivary E2 assay in vitro fertilization and other fertility treatments. Salivary E2 and the saliva:serum E2 ratio have been demonstrated to be higher in stimulated conception cycles during the follicular and luteal phases as well as the luteal phase (15, 21), indicating the importance of the free moiety. Data reported here show that E2 measured in serum at midcycle in stimulated patients does not correlate with the saliva:serum ratio, which presumably reflects the percentage of free circulating E2. Therefore, a clinical significant question that could be investigated by using this assay is the relationship between salivary estradiol (inferentially, the percentage that is free) and successful conception in unstimulated as well as stimulated patients. Thus, monitoring E2 concentrations in saliva may provide a useful diagnostic tool that allows better resolution.
some clinical problems than do values for serum alone. Good reproducibility of this method allows tracking of intra-individual variation in $E_2$ over weeks and months. Unlike serum, saliva can be collected by the patient at home over time, stored in the home freezer, and brought in at the time of clinic visits. Paired with any of several adequate measures of salivary progesterone (8, 24), determination of $E_2$ can allow tracking of ovarian function across intermenstrual intervals, and aid in detection of causes of reproductive failure in both stimulated and unstimulated cycles.

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References