Sensitive 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 of non-invasive, convenient sampling; and apparently accurate reflection of the concentrations of physiologically active urinary steroids 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 0.5 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 (r = <0.001), and demonstrated reliable detection of estradiol peaks during normal ovulatory cycles in serum samples from 15 women. Salivary estradiol peaked at 5.4 (SD 1.9) pmol/L on cycle days 14.4 (SD 3.2), 1.2 (SD 2.8) 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 perimenopausal estrogen supplementation · equilibrium radioimmunoassay

Long-term clinical monitoring of estradiol (E2) in the circulation is desirable for treatment and study of hormonally-dependent carcinomas (14–19). Monitoring estrogen supplementation in postmenopausal women (4, 11, 21) 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 non-invasive method for long-term or intensive samples and because concentrations in saliva may reflect the whole-body biologically active circulating moiety (7, 8). However, application to measurement of serum E2 has been hampered by the lack of a sufficiently sensitive and reproducible assay (9, 10).

Previously published ranges for salivary estradiol vary considerably (6, 11–14). These inconsistencies could stem from methodological differences in antibody specificity, in vitro used (quarrel 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.

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Factors contributing to the reliability and improved precision of the present assay include: (a) the use of an indiumated rather than a tritiated tracer; (b) separation with second antibody plus polysaccharide gelatin 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 of concentrations from 1.4 to 588 pmol/L.

The assay is a simple equilibrium radioimmunoassay that can be done in 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 estradiol.

Materials and Methods

Reagents

Our protocol is a modification of the Estradiol-Quant-IT In Vitro 125I RIA Diagnostic Test Kit (Leco 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-17β-24-oxime-bovine serum albumin, an antibody that cross-reacts by <1% with estradiol, estriol, testosterone, and other related compounds. We prepared the working buffer by adding 100 mg of gelatin (Kroh unbuffered) to 200 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 azid 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 mono- and diesters that can interfere with accurate eosinometry. On the day of the assay, thaw the samples and centrifuge (1000 × g, 10 min). Transfer the supernate to a glass tube for assay.

Control: Serum controls, supplied by Leco Diagnostics, contain estradiol in a human serum base. Saliva pools
constuted 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 100.0 μL of standards (0.1, 1.0, 10.0, 250, 500, 1000, 2000 μg/L) and serum controls and 1000 μL of saliva samples and saliva controls into 12 x 75 mm glass tubes. Dilute standard and serum control tubes to a total volume of 1.0 mL by adding 0.5 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 of 6.0% (v/v) triton to each tube. Allow to stand for 15 min. Add 0.5 mL of diluent (either lactose, 5% dextran, or 10% bovine serum albumin) to each tube. Shake 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 10 x 100 mm glass tubes and evaporate it under nitrogen in a 37°C water bath.

To the aqueous phase, add 5 μL of triton and repeat the extraction procedure to ensure no 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 extract, add 0.5 mL of working buffer and vortex-mix for 2 min. Incubate for 10 min at 37°C, then vortex-mix for 3 min. Aliquot 200.0 μL of the reconstituted volume, in duplicate, into 11 x 75 mm polypropylene tubes. Count the radioactivity in the total-count tubes and in the extracted aliquots in a gamma counter using the same model 5020 equipped with FIA-Smart and Expert QC software (Packard Instruments Co., Downers Grove, IL) for 10 min. Calculate recovery by multiplying the total radioactivity in the duplicate 200 μL aliquots by 0.25 and dividing by the average counts for the six total-count tubes.

Instrumentation: To the 200.0-μL aliquot, add 50 μL of 1% labeled estradiol and 100 μL of antiserum, prepared by dilution of the kit solution throxid with working buffer, vortex-mix, and leave at room temperature overnight. Dilute the kit-supplied polyethylene glycol-antibody solution twofold with working buffer (0.5 mL to each 11 × 75 mm tube) to give 10 mL. Vortex-mix, incubate at room temperature for 20 min, and centrifuge (Sorval Model RC-5C, Du Pont, Wilmington, DE, at 2500 rpm and 4°C to deliver a total centrifuge, effect of 20 x 10³ g). Setting force rather than time reduces inter-assay variation duration is about 1 h. Laboratory without a force-setting centrifuge option may spin the samples for 1 h at 2800 rpm and 4°C. Carefully decant the supernatant fluid, leaving the tube. Estimate the radioactivity in the precipitate (antibody-bound fraction) by gamma-counting 10 min.

Standard curve correction 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 unlabeled hormone required to inhibit binding of tracer by an amount equal to 25% below the mean reading observed in the absence of unlabeled hormone, is 0.3 fold (equivalent to a concentration of 1.32 pmol/L). Buffer and solvent blanks were equal to binding at zero assay.

The standard curve was highly reproducible, having a slope of -0.736 (SD 0.087), Emax of 7.04±1.56, E50 of 4.8±1.2, and E90 of 9.2±1.0 pmol/L. The NIC, range of the standard curve is from 0.3 to 500 pmol/L (0.30 to 2.58 ng/L), based on sensitivity and standard range. The precision profile yielded a CV of <10% across this range. The tube error of duplicate averaged 2.2%.

**Precision**: Intra-assay variation (CV) for a medium concentration of pooled saliva specimens measured from 1.2 to 3.4 pmol/L, mean ± SD was 18.4%; interassay variation for another medium-concentration saliva pool (172.1 ± 8.0 pmol/L) was 13.7%. The low-concentration saliva pool (44.3 ± 1.5 pmol/L) had an intra-assay variation of 10.4%, and another high-concentration pool (mean ± SD) of 8.1 ± 0.9 pmol/L had an interassay variation of 10.6%. Values for low- and medium-concentration serum controls fell within the range supplied by the manufacturer. Most coefficient variations fell in the middle of the range, with no detectable overall overall assay n = 13.

**Accuracy**: Extraction efficiency was determined for each sample, standard, and control by using 251 labeled estradiol tracer, added to each tube before extraction. Sample recovery ranged from 72% to 88%. 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 85.6 to 100% and averaged 97.9%.

**Paradigm**: Paradigm was evaluated by measuring E2 in a very high-concentration saliva sample (1273 pmol/L) diluted to medium assay range, and in a lower-concentra
tion sample (331 pmol/L diluted incrementally to the low and the range. Observed values were near those expected, across the entire range of measurement (average recovery 96%, range 88%-100%) in the absence of sample sources of significant assay interference.

**Sample preparation**: All samples were centrifuged before being divided for assay. A significant artifact was noted in matched comparisons of values obtained for centrifuged and uncentrifuged samples. However, the CV for duplicate centrifuged and uncentrifuged samples (20%) exceeded that for duplicates prepared by centrifugation.

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

<table>
<thead>
<tr>
<th>Added (pmol/L)</th>
<th>Recovered</th>
<th>Theoretical</th>
<th>Observed</th>
</tr>
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<tbody>
<tr>
<td>125 E2</td>
<td>148</td>
<td>137</td>
<td>140</td>
</tr>
<tr>
<td>75.0 E2</td>
<td>141</td>
<td>129</td>
<td>124</td>
</tr>
<tr>
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<td>140</td>
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<td>130</td>
<td>120</td>
<td>119</td>
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<tr>
<td>20.0 E2</td>
<td>125</td>
<td>119</td>
<td>119</td>
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<tr>
<td>10.0 E2</td>
<td>115</td>
<td>107</td>
<td>107</td>
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**Concentrations**

- **Nicotine (Nic)**: 250, 500, 1000, 2000 μg/L
- **Serum Controls**: 0.1, 1.0, 10.0, 250, 500, 1000, 2000 μg/L

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Clinical Evaluation

Matched serum and saliva samples were collected from women under ovulation-induction therapy with either clomiphene or gonadotropins. Samples were obtained in the late follicular, peri-ovulatory period, within 24 h after appearance of positive urinary luteinizing hormone 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 15 µmol/L (4.87 ± 5.14 µmol/L, mean ± SD, with corresponding serum values ranging from 169 to 3575 µmol/L (980 ± 743 µmol/L). Values for saliva and serum showed a strong linear relationship (r = 0.705, p < 0.001). The three saliva samples that yielded no detectable E₂ had corresponding values of 242 ± 60 µmol/L for serum. The mean ratio of salivary to serum E₂ was 0.000 ± 0.003; there was no correlation between the in-vivomurum ratio and values for total circulating E₂ (r = 0.06). Importantly, the properties 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 16 normal, untreated women who were also monitored by ultrasonography for ovulation, as described elsewhere [10]. Each woman was sampled intermittently during the early to mid-follicular phase, and then at half-day intervals (at 0900 and 2100 h) as follicular maturation peaked, until ovulation was detected by ultrasonography. 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 days 15.5 ± 3.6 (mean ± SD, range, day 12–24). For each woman, a peak salivary E₂, value was obtained and its corresponding cycle day and time to ovulation were noted (Figure 3). E₂ concentrations peaked on cycle days 14 ± 3 (SD 3.2), 1.2 ± 0.8 days before ovulation range, day -3 to 0.

Discussion

Our observation that salivary E₂ concentrations correlate highly with serum for stimulated cycles contrasts with an earlier study [12] and supports others that have found good correlation (23, 25), especially in stimulated cycles (27, 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 mean by cycle served peak E₂ concentrations averaged 6.4 (SD 1.9) µmol/L, range 1.5–10.0 µmol/L. To facilitate comparison with other reports, we calculated average ± SD values for follicular (more than three days before ovulation) (6.6 ± 1.6 µmol/L) and peri-ovulatory (day -3 to 0.5) (3.4 ± 2.3 µmol/L) periods.

Discussion

Our observation that salivary E₂ concentrations correlate highly with serum for stimulated cycles contrasts with an earlier study [22] and supports others that have found good correlation (23, 25), especially in stimulated cycles (27, 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 mean by cycle.
phase, we made a rough estimate based on concentrations graphed by day cycle. The period before onset of the pre-ovulatory increase in $E_2$ 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 triestral- and ovine-based radioassays should be drawn with caution, however. We note that findings from two reports (13, 20) 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 estradiol, estriol, 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 antiserum 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 antiserum and tracer was used (13, 15, 22). Using a similarly sensitive but very different assay system based on enzymatic reaction and bioimmunoassay, Pincuk et al. (21) found values for free $E_2$ in plasma and estriol-$E_2$ in saliva to be the same as the salivary $E_2$ values we report here. The kit assay that we modified reportedly yields consistently lower values for serum than other commercial preparations; this is reflected by our finding of increased $770 \pm 783$ pmol/L, but markedly lower than previously published (17), concentrations of $E_2$ at midpoint in stimulated cycles. Finally, we note that a report based on the broad sampling of populations (19) yielded average values quite close to ours.

A similarity to our observation of lower salivary $E_2$ concentrations is the finding of unusually low saliva-serum $E_2$ ratios, 0.006 < 0.000. This contrasts with reports of saliva-serum ratios between 0.51 and 6.02 (4, 13, 17, 18). Findings of similar proportions of free $E_2$ in serum (19, 22) have been taken as supportive of the notion that salivary concentrations mirror the free circulating $E_2$ concentrations. Values measured for serum at which concentrations in the corresponding saliva fell below detectability in this assay (0.92 ± 66 pmol/L) corresponded closely with the concentrations at which 0.5% free $E_2$ would lie below assay sensitivity (1.22 pmol/L). Unlike one recent study (13), we saw no inverse relationship between the salivary 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 assemblable to sample preparation, for which significantly higher values were yielded by uncentrifuged, frozen plasma samples (23). We centrifuge all samples before extraction, because we observe less variance in replications with this preparation.

An $E_2$ peak, demarcated with ascending and descending segments, was discernible in all cycles studied. However, there was considerable inter-individual difference in overall $E_2$ concentrations achieved. Further, variation in timing of peak $E_2$ relative to ovulation blurs individual observed peaks when averages are calculated relative to time of ovulation. Alignment of data with respect to time of peak $E_2$ provides better resolution of actual peak shape (Figure 3) in agreement with a recent report (19).

We conclude that, with this sensitive assay, monitoring salivary $E_2$ can detect the presence or absence of an active follicle in a normal cycle. Onset of production and midcycle 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 $E_2$ assay in in vitro fertilization and other fertility treatments. Salivary $E_2$ and the salivary serotone ratio $E_2$, having been demonstrated to be higher by stimulated conception cycles during the follicular and ovulatory phases as well as the luteal phase (15, 22), indicating the importance of the free form. Data reported here show that $E_2$ measured in serum at midcycle in stimulated patients does not correlate with the salivary serum ratio, which presumably reflects the percentage of free circulating $E_2$. Therefore, a clinically significant question that could be investigated by using this assay is the relationship between salivary estradiol and free steroids, particularly, the percentage that is free and successfully taken to unstimulated as well as stimulated patients. Thus, monitoring $E_2$ concentrations in saliva may provide a useful diagnostic tool that allows better resolution of

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