Solid-Phase Enzymoimmunoassay of Estrone in Saliva

John Folan, James P. Goeling, Martha F. Finn, and Patrick F. Fottrell

In this solid-phase enzymoimmunoassay for estrone in saliva, microtiter plates are used after extraction of the sample with diethyl ether. No chromatographic step is involved. The detection limit of the assay is 450 fg per well (33 pmol/L). Intra- and interassay coefficients of variation for the assay of low, medium, and high concentrations of estrone in saliva were respectively 4.2, 12.7; 5.2, 8.7; and 2.7, 5.8%. Using this assay, we found a highly significant correlation ($P < 0.001$) between estrone concentrations in time-matched serum and saliva samples. The analytical procedure is rapid and relatively simple. One person can assay 50–60 saliva samples during a normal working day. We conclude that the assay is very suitable, even in small laboratories, for saliva estrone measurements, which, in facilitating serial sampling, enables dynamic observations of estrone concentrations and ovarian activity to be made easily.

Additional Keyphrases: ovarian function, reference interval, effects of treatment with clomiphene, serum estrone, fertility studies, postmenopausal women

Saliva sampling is a non-invasive, stress-free procedure. When combined with suitable assays for the determination of relevant steroid hormones, it facilitates the monitoring of ovarian or adrenal function (1, 2). Use of saliva samples instead of blood fluids allows multiple sampling away from all laboratory facilities, which is advantageous, for example, when changes in progesterone or estrone concentrations during the follicular or luteal phases of the menstrual cycle are being investigated. Also, unconjugated steroid concentrations in saliva closely approximate the corresponding unbound-steroid concentration in plasma, which is believed to be the biologically active fraction (3, 4).

Estrone in the blood is derived both from ovarian secretion and from the action of adipose tissue aromatase on circulating androstenedione. The latter source is the major one after the menopause, when estrone is quantitatively the most important unconjugated estrogen (5). During the normal menstrual cycle, blood estrone concentrations increase with the progression of both follicular and luteal development (6). The unbound fraction of estrone has been estimated to constitute about 4% of the total concentration in plasma (7). Estrone concentrations in blood are not very high in non-pregnant women (about 0.2–1.0 nmol/L), so the corresponding concentrations in saliva are very low, and few data are available concerning estrone in saliva, most such data coming from Luisi et al. (8), who used radioimmunoassay.

Here we describe a solid-phase microtiter plate enzymoimmunoassay for estrone (9), validated for use with salvia. The clinical applications of the assay are demonstrated by measurements of estrone in saliva during complete normal menstrual cycles and for a cycle in a woman who was receiving treatment with clomiphene citrate to stimulate ovarian activity.

Materials and Methods

Subjects

Healthy women, ages 21–40 y, with regular menstrual cycles (mean 29, SD 3 days) and not taking oral contraceptives, provided a saliva sample almost daily during complete menstrual cycles. Fifty-four similar volunteers provided one or more matched blood and saliva samples. Daily saliva samples were also provided by an infertile patient who was receiving therapy with clomiphene citrate to induce ovulation. Where appropriate, the day of ovulation was determined by real-time ultrasonography (Model Combison 111S; Kretz Technik, Sussex, U.K.) with use of full-bladder technique. Monitoring was commenced on day 9 or 10 of the cycle and continued on alternate days until the mean diameter of the dominant ovarian follicle measured 14 mm. Thereafter, daily examinations were performed and the reference day of ovulation was the day of maximum ovarian follicular diameter (at least 18–25 mm) before a change in size, shape, and acoustic density.

Sample collection: Written instructions explaining the method for providing a saliva sample were given to each volunteer at the beginning of the study. After an overnight fast, and between 0700 and 1000 hours, volunteers were asked to rinse their mouths with water, rest for 5 min, and then collect 2–5 mL of saliva (unstimulated) into a 5-mL polystyrene tube. After collection, the samples were stored in the volunteers' home freezers until a full cycle was completed. In the laboratory, samples were thawed at room temperature, centrifuged (3000 × g, 15 min), and the supernates stored at −20 °C until assayed. The freezing and thawing greatly decreases the viscosity of saliva and makes it easy to pipette. Each woman recorded the first days of her menses and gave daily samples for at least one complete menstrual cycle.

Serum obtained from blood samples, time-matched with saliva samples, was stored at −20 °C until analysis and assayed for estrone as described previously (9).

Reagents

All buffers, chemicals, steroids, radiolabeled steroids, enzymes, general reagents, and materials were as described previously (9).

Main assay reagents: Estrone-peroxidase conjugate, anti-estrone IgG, and all buffers were prepared as described previously (9).

Quality-control saliva: Larger volumes of whole saliva were obtained by pooling and mixing a large number of individual samples from healthy male volunteers. This saliva was treated by freezing, thawing, and centrifuging as described above. Three controls were prepared by adding 25, 100, and 225 fmol (6.8, 27, 61 fg) of estrone per milliliter of

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saliva. This gave three pools with mean estrone concentrations of 46, 138, and 265 pmol/L for low, medium, and high controls, respectively.

Procedures

**Extraction of saliva:** Pipette 1.0 mL of saliva into disposable glass 16 × 100 mm culture tubes and add 5.0 mL of diethyl ether (analytical grade; Reidel de Haen, Hanover, F.R.G.). Vortex-mix the contents of the tubes for 2 min. Allow the phases to separate with the tube in crushed ice and transfer 4.0 mL of the solvent phase to a clean culture tube with a positive-displacement pipette. Evaporate the solvent at 40 °C in a stream of air and add to each tube 0.2 mL of phosphate-buffered saline containing bovine serum albumin (9). Vortex-mix as before for 3 min and incubate at 37 °C for 15 min. Leave the tubes at room temperature (16–20 °C) for at least 1 h, then again vortex-mix for 2 min. [Completeness of extraction, as determined with the use of tritiated estrone, was 90 (SD 5) %, and we took this recovery factor into account in calculating final results.] The ether used for extraction must be of high quality if blank values are to be low. Include a separate ether blank in every assay run, and aliquot standard solutions into culture tubes before use, so that both samples and standards are exposed to the same environment.

**Estrone enzymoimmunoassay:** The procedure for assaying estrone in sample extracts is similar to that described previously for serum samples (9) but is outlined here for completeness. After coating the plate wells with antibody (9), add, in duplicate, 50 μL of standards, control, or unknown saliva sample extracts. Simultaneously, add 150 μL of estrone–peroxidase conjugate (5 μg/L). Gently mix the solutions in the wells, and incubate for 4 h at ambient temperature (16–20 °C). Wash the wells, add 150 μL of buffered enzyme substrate solution to each well in sequence, and incubate the plate at ambient temperature (16–20 °C). After 30 min, stop the enzyme reaction by adding 50 μL of 4 mol/L sulfuric acid. Mix again and measure the absorbance at 490 nm with a microtiter plate reader (we used a Model EL307, Bio-Tek Instruments Inc., Burlington, VT). The plate reader is connected on-line to a microcomputer and the standard curve, detailed assay statistics, and the control and unknown results are immediately available for printing.

**Results**

**Analytical Variables**

**Standard curve:** The standard curve in Figure 1 represents the mean curve obtained from 10 consecutive assay runs, each with duplicate standards. The total CV's for the B/B₀ ratios for each standard point ranged from 4.2% to 5.3%, indicating that the stability and precision of the standard curves are satisfactory. The detection limit, defined as the sample concentration of estrone needed to give a B/B₀ value equivalent to the point where B is equal to B₀ minus 2 × the SD for B₀, was 33 pmol/L (450 fg per well).

**Specificity:** The specificity is as described for the serum estrone assay (9), because the same antiserum (R21/73) was used.

**Analytical recovery:** We assessed how well the assay quantified estrone in saliva samples by determining the concentrations of estrone in three saliva samples containing different concentrations of estrone (43, 58, and 87 pmol/L) to portions of each of which 37, 155, and 370 pmol of estrone was added per liter. Analytical recovery of steroid from all nine saliva samples was 99.0% (SD 8.0%, range 88.4–113.7%).

**Independence of volume:** Another measure of assay validity is to serially dilute a saliva sample and measure the amount of estrone in each dilution. The amount measured should decrease by the appropriate amount in each fraction if the assay does not suffer any matrix effects or other bias. Figure 2 shows the results obtained of such an experiment with four individual saliva samples.

**Precision:** Intra- and interassay CV's were determined by including, in duplicate, three control samples containing different estrone concentrations in each of 20 assay runs. The CV's obtained ranged from 2.7% to 12.7% (Table 1).

![Fig. 1. Composite standard curve calculated from the results of 10 successive enzymoimmunoassays for estrone, carried out in duplicate. The mean and standard deviation, indicated by the vertical bars, are shown for each standard.](image)

![Fig. 2. Concentrations of estrone determined in four saliva samples that were serially diluted for assay validation.](image)

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<th>Table 1. Intra- and Interassay Variation in Quality-Control Saliva Samples</th>
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<td>Estrone concentration</td>
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Comparison of Estrone Concentrations in Blood and Saliva

Matched samples of blood and saliva, collected within 15 min of each other, were obtained from non-pregnant women. The estrone concentrations in these samples were determined by enzymoimmunoassay after extraction. The average concentration in the saliva samples (12–89 pmol/L) was 8.1 (SD 3.1) % of that in the serum samples (175–1815 pmol/L). There was a highly significant correlation between them \( P < 0.001 \), Figure 3 and the fitted line had a slope of 0.035, a y-intercept of 14.5 pmol/L, and a correlation coefficient of 0.79. This is equivalent to saying that the concentration in saliva is 3.5% of that in serum, plus 14.5 pmol/L.

Clinical Applications

Estrone was measured in daily saliva samples from non-pregnant women who had regular menstrual cycles. The concentrations detected in two such individuals are shown in Figure 4. A typical biphasic pattern, also associated with estrone concentrations in serum (6), was observed in both cases. In both individuals also, the major peak observed was the pre-ovulatory surge, the highest concentration being on the day before ovulation in one case, and on the day of ovulation in the other. Also, in both individuals, we observed an increase in estrone in the mid-luteal phase of the cycle, coinciding with normal corpus luteum development. Altogether, we measured salivary estrone concentrations over the whole menstrual cycle in 10 women, finding concentrations ranging from 18 to 82 pmol/L.

Saliva samples were also collected daily from an infertile woman receiving therapy with clomiphene citrate to induce ovulation, and the estrone concentrations found are shown in Figure 5. A biphasic pattern was again observed, but with significantly higher estrone levels (30–121 pmol/L) compared with the two non-treated cycles previously shown. Treatment with clomiphene reportedly gives rise to higher estrogen concentrations in serum (9, 10).

Discussion

The measurement of steroids in saliva is an attractive alternative to plasma analysis because serial sampling is easy. As we confirm here, this appears to provide a more accurate indication of endocrine activity (1). Also, a theoretical advantage of salivary steroid assays is the parallel investigation of unbound steroids in plasma (3, 4).

The enzymoimmunoassay procedure described here is easily done, requires no radioactive isotopes, and is sensitive enough for the assay of estrone in saliva. The detection limit of the assay (450 fg per well) is better than that of a previously reported RIA, e.g., 3000 fg per tube (8). The assay also allows 50–60 samples to be assayed by one person in a normal working day.

In the treatment of infertility, estrogen measurements in serum and urine are of clinical importance in predicting the time of ovulation (10, 11). Daily serum measurements are difficult to obtain, and therefore assays have been devised for urinary estrogen metabolite measurements (12). The collection of urine and the precise quantification of estrogens in urine also presents difficulties, and the assay of estrone in saliva is presented as a possible alternative. A saliva specimen can be collected quite easily, even by children, and, once treated by freezing and thawing, is esthetically acceptable to handle and can be pipetted accu-

Fig. 4. Concentration of estrone in saliva collected daily during the menstrual cycle from two volunteers

Fig. 5. Concentration of estrone in saliva collected daily during the menstrual cycle from a volunteer receiving clomiphene citrate therapy for ovulation induction

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rately. On the other hand, there have been questions raised as to the validity of salivary steroid measurements, particularly in relation to whether or not steroids are metabolized in saliva, and to the problem of sample contamination by blood or gingival fluid. However, in vitro studies on human saliva have shown that there is no metabolic conversion of estrone in the saliva of subjects with normal gingiva and only a limited (3.6%) conversion to 17β-estradiol in the saliva of patients with chronic gingival inflammation (13).

 Estrone in blood is largely bound to binding proteins, mainly albumin (7, 14). Albumin, although not expected to be present in saliva, was recently found in trace amounts in saliva samples (15), perhaps as a result of contamination by blood or gingival fluid. Therefore, under certain circumstances, such as severe gum disease, such contamination could result in a significant overestimation of the "real" value.

 In conclusion, it would appear that measurement of estrone in saliva by enzymoimmunoassay provides a useful, non-invasive, stress-free method of monitoring the estrogen status of an individual. This method could prove useful in assessing the effect of clomiphene citrate therapy (11), in predicting ovulation in patients receiving therapy to induce ovulation, and in patients attending in vitro fertility programs. Also, this method could be used for any investigation on peri- and postmenopausal women where estrone needs to be determined in large numbers of samples.

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 References