Direct Chemiluminescence Immunoassay of Estradiol in Saliva

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A sensitive and simple direct solid-phase chemiluminescence immunoassay is described for estradiol in saliva. In this assay, a second antibody is bound to the wells of microtiter plates. Either buffer with standards or saliva (100 µL) is incubated in these wells with monoclonal anti-estradiol antibody and with estradiol-isoluminol conjugate. Incubation time is 2 h. Chemiluminescence of the bound fraction is measured in a manually operated luminometer (Biocounter). The assay has a detection limit of 3.8 pmol/L; analytical recovery of added estradiol is 96.8% (SD 7.0%); within- and between-assay CVs range between 2.5% and 12.7%. Forty unknown saliva samples can be assayed and results calculated within 4.5 h. Results of a slightly modified procedure—with black microtiter plates and a prototype of an automated plate reader (Lumioskan)—compare well with those of the described method ($r = 0.97$). Because steroid-binding globulins have been found in saliva, the effect of displacing agents on the results of the direct chemiluminescence assay is described.

Additional Keyphrases: in vitro fertilization • ovulation cycles • manual and automated luminometers compared

Assay of estradiol ($E_2$) in serum or plasma is of great value for clinical endocrinological investigation in women. In some instances, e.g., during ovarian stimulation for ovulation induction, repeated determinations of estradiol are required. As many as 10 consecutive blood samples may be needed for an in vitro fertilization (IVF) program. This frequent blood sampling is time-consuming and may be stressful.

On several occasions it has been shown that some steroids, including estrogens, are present in saliva in concentrations that reflect the free fraction of steroids in serum or plasma ($1-4$). In addition, steroid concentrations in saliva are independent of saliva flow rate ($1, 5$), and sampling is noninvasive and stress-free. This enables frequent sampling, such as is required during an IVF program. Subsequently, saliva $E_2$ assay has been shown to be a valuable alternative to measurement of $E_2$ in blood ($6-10$). The concentrations of $E_2$ in saliva have been determined during spontaneous menstrual cycles and during cycles stimulated for ovulation induction and embryo transfer (IVF-ET). Very low concentrations have been found on both occasions, 1 to 36 pmol/L in spontaneous cycles ($4, 7, 8, 11, 12$) and 10 to 120 pmol/L in stimulated cycles ($4, 8-10$). Therefore, very sensitive assay methods are required for measuring $E_2$ in saliva, either with a direct assay ($11, 12$) or with extraction assays ($4, 7-10, 13, 14$). The amount of saliva needed for the assay varies widely: 0.5 mL ($11, 12$), 1-2 mL ($4, 14$), and 3-10 mL ($7-10, 13$). All these methods are radioimmunoassays except one, which is an assay based on enzymatic interconversion of estrone ($E_1$) and $E_2$, with signal detection by bioluminescence ($4$). It measures estrogens, $E_1 + E_2$.

Here we describe a new direct immunoassay in which the chemiluminescent molecule isoluminol is used as a label for $E_2$. The conjugate isoluminol-$E_2$ is stable and poses no known hazard for health or environment. The chemiluminescence immunoassay (CIA) is not subject to the disadvantages associated with the use of radioisotopes in RIA, but photons generated during the chemiluminescent reaction can be measured with the same sensitivity of detection as tritium decay.

Because recently sex-hormone-binding globulin and corticosteroid-binding globulin were detected in saliva ($15$), we have also compared results obtained in the absence and in the presence of displacing agents.

In the course of this work a prototype of an automated chemiluminescence plate reader (Lumioskan) became available for evaluation during a short period. Because our more recently developed CIA's use microtiter plates as a solid phase ($16-18$), we could compare the results of parallel assays by measuring chemiluminescence with both our manual plate meter (Biocounter) and the Lumioskan.

Materials and Methods

Subjects and Sampling

Unstimulated saliva was obtained from several different subjects: (a) a male volunteer, sampled between 0800 and 1130 h on several occasions; (b) six normal healthy volunteers with regular menstrual cycles, sampled between 0700 and 1100 h at regular intervals during the cycle [five women also delivered four to five blood samples so we could date the cycle and monitor luteal function; one woman collected saliva almost daily between 0700 and 0730 h during three consecutive cycles (cycle length 27 days) and kept a record of her basal body temperature changes]; (c) women stimulated for ovulation induction, sampled daily between 0800 and 1200 h. This last group included women enrolled in an IVF-ET program. They were stimulated either with clomiphene and human menopausal gonadotropins (hMG) or with a gonadotropin-releasing hormone (GnRH) agonist (nasal spray of buserelin, "Suprefact," or depot injection of goserelin, "Zoladex") and hMG. From these stimulated women we obtained time-matched blood
specimens by venipuncture within 10 min after saliva sampling.

Subjects received instructions to rinse their mouths several times with tap water and to start collecting 2–5 mL of saliva in a plastic vial about 5 min later. Samples were frozen within 5 min of collection and stored at −20 °C. Before assay, the samples were thawed and centrifuged (15 min, 3000 × g), and duplicate aliquots of the clear supernates were used for assay. The rest of each sample was frozen again and stored at −20 °C until eventual re-assay.

Blood was centrifuged and serum was used for the determination of E₂ with a direct RIA kit, "E₂-RIA-CT" (code 3006200; Medenix Diagnostics, Fleurus, B-6220, Belgium).

Apparatus

Biocounter. For measurement of the antibody-bound fraction in routine chemiluminescence immunoassay we used manually operated luminometers Model M 2000 and Model M 2010 Biocounter (Lumac Systems, Basel, Switzerland). A Lumacuvet containing the alkali-treated E₂-isoluminol (see Procedures) is inserted into the measuring chamber. A button is pressed and 100 μL of microperoxidases and 100 μL of hydrogen peroxide are injected into the cuvet. Light generated during the chemiluminescent reaction is integrated for 10 s. After the alkali treatment ~70 min is needed for pipetting, measuring of chemiluminescence, and data reduction. Results are expressed as "photon counts."

Luminoskan. For three weeks, we had the use of a prototype, fully computer-operated luminescence plate reader, Titertek Luminoskan (ICN Flow Laboratories, 1730 Asee-Relgem, Belgium). After alkali treatment (150 μL of 2 mol/L NaOH solution) of the wells’ contents, the plate is inserted into the light-tight measuring chamber. Built-in dispensers inject microperoxidase (25 μL of a 40 mg/L solution) and hydrogen peroxide (25 μL of an 8 g/L solution) into each well, and the light generated is integrated for 10 s by a photomultiplier tube positioned above the well. After the alkali treatment, 45 min is needed for reading and data reduction. Results are given in "relative light units."

Chemicals

Steroids were purchased from Steraloids, Wilton, NH 03866, and from Sigma Chemical Co., St Louis, MO 63178. Two steroidal compounds were obtained as a gift: danazol (17α-pregna-2,4-dien-20-yno-[2,3-d]-isoxazol-17-ol) from Winthrop Labs., Brussels, Belgium, and mesterolone (17β-hydroxy-1α-methyl-5α-androstan-3-one) from Schering N.V., Machelon, Belgium. Black microparticle plates ("Maxisorb with certificate") from Nunc AS, Kampstrup, Denmark. Other products were as described previously (16).

Reagent solutions. Doubly distilled water was used for all solutions. Assay buffer, coating buffer, wash solution, microperoxidase, and oxidant solutions were as previously described (16, 19).

Procedures

Preparation of chemiluminescent marker conjugate and antibody to estradiol. Estradiol-6-carboxymethyl oxime-aminobutylethyl-isoluminol (E₂-ABEI) and monoclonal anti-E₂ antibody 2F9 were prepared and used as described (16, 20, 21).

Coating of microtiter plates. Using an automated pipet (we used a Titertek Multistepper, 50–200 μL, cat. no. 77-942-00), pipet 200 μL of second antibody, diluted 4800-fold in coating buffer, into each well of the clear Maxisorb plate. Cover the plate with sealing tape and incubate overnight at 4 °C. Wash each well three times with wash solution to remove unbound antibodies. Pipet 200 μL of assay buffer into each well and incubate 1 h at room temperature. Empty the plate by inversion and carefully drain it onto absorbent paper for 5–10 min. Cover the plate with sealing tape and either use it that same day or store it at 4 °C for no more than 10 days. After storage, wash the plate once with wash solution before use.

Immunoassay. Make up all solutions and dilutions in assay buffer. Prepare standards containing, per milliliter, 0 and 2.5 to 200 pg of estradiol. Prepare a solution of displacing agents: per milliliter, 16 ng each of mesterolone, danazol, and cortisol. Pipet, in duplicate, 100 μL of standards or unknown saliva samples, 25 μL of the mixture of displacing agents, and 50 μL of 54 000-fold-diluted monoclonal anti-E₂ antibody 2F9. Incubate the plate for 90 min at room temperature on a horizontal plate shaker (170 cycles/min). Prepare a 1 μg/L solution of E₂-ABEI in assay buffer, add 25 μL of this to each well and continue incubation for 30 min. Wash the plate three times, drain it and free the antibody-bound ligand marker by treatment with alkali: add 0.2 mL of a 2 mol/L solution of NaOH to all wells and incubate for 30 min at 60 °C in a water bath. Mix the contents of each well with a pipette and transfer 150 μL to Luminoskets for use with the Biocounter. Measure the light emitted during the chemiluminescent reaction. The total assay time for one plate (i.e., for 40 saliva samples), including light measurement, is approximately 4.5 h.

In some experiments, we performed incubations without displacing agents. Incubation volume was kept constant by adding 25 μL of assay buffer to all wells.

In another series of experiments, we prepared duplicate assays on two plates, a clear polystyrene one and a black one. Each plate was filled in the same way with standards and unknown saliva samples or solutions of cross-reacting steroids in buffer. After incubation and NaOH treatment we transferred the contents of the wells of the clear plate to Luminoskets and measured light manually with the Biocounter. The black plate was placed in the fully automated Luminoskan for light measurement. We compared the results obtained with each luminescence reader.

Note: As described above, the walls of the clear Maxisorb plate are filled to a final volume of 200 μL. This includes (a) coating of second antibody, (b) incubation for the immunoassay, and (c) NaOH treatment. A fraction (150 μL) of the NaOH reagent is then transferred to Luminoskets and 100 μL of reagents is injected twice for the chemiluminescent reaction. Because the Luminoskan measures chemiluminescence directly in the microtiter plate well, the volume of NaOH is limited to 150 μL and 25 μL of reagents is injected in the meter twice. Most reliable results are obtained when the wells of black microparticle plates are filled to a final volume of 150 μL. For coating we incubated the plates with 150 μL of 3600-fold-diluted second antibody per well (overnight, 4 °C), followed by 150 μL of assay buffer (1 h, room temperature). For sample incubation we added 100 μL of standard in buffer or saliva, followed by 25 μL of
buffer containing anti-E₂ antibody (diluted 27,000-fold) and the displacing agents as described. Finally 25 μL of E₂-ABEI is added. Total incubation time is 90 + 30 min.

**Calculation of results.** Nonspecific blanks were subtracted from all counts and B/B₀ was calculated and plotted vs log dose of the standards. Readings from the calibration curve were in picomoles of E₂ per liter. Correlation coefficients were calculated with the Spearman rank correlation test.

**Results**

**Choice of Displacing Agents**

Several mixtures, each containing two or three different displacing agents, can reduce to ≤5% the binding of [³H]estradiol by steroid-binding globulins in serum. For 50 μL of serum this is obtained with (e.g.) combinations of 10.5 ng each of danazol and 5α-dihydrotestosterone plus 0.25 ng of mesterolone (16) or 10 ng each of danazol, cortisol, and mesterolone. Both combinations can be used in direct immunoassays of serum estradiol without adverse effect on antibody 2F9 and assay performance. Because the concentrations of sex-hormone-binding globulin and corticosteroid-binding globulin in saliva represent ~0.1% of concentrations in their plasma (15), the amount of each displacing agent required per 100 μL of saliva would be 20 pg. We tested higher concentrations of displacing agents, up to 8% of those used with 50 μL of serum, and observed no effect on the performance of the present assay with 400 pg each of danazol, cortisol, and mesterolone in all wells of the microtiter plate. We therefore decided to use the above combination, containing sufficient amounts of displacing agents, in the present assay.

**Effect of Displacing Agents in the CIA**

In six experiments we performed parallel incubations on the same microtiter plate. On one half of the plate, calibrators and saliva samples were incubated in the absence of displacing agents (y) and on the other half of the plate in their presence (x). Saliva samples were from women enrolled in an IVF-ET program (n = 40), from pregnant women at five to 24 weeks of gestation (n = 29), and from a man (n = 5). The presence of displacing agents did not influence the position and slope of the calibration curve. Comparison of six pairs of curves gave a correlation coefficient r = 0.979 and a regression line of y = 0.993 x + 0.009 B/B₀.

The E₂ concentrations in 72 saliva samples (varying between 1 and 365 pmol/L) determined in both ways, i.e., in the presence and in the absence of displacing agents, also correlated well: r = 0.949, y = 0.943 x + 3.00 pmol/L (Figure 1). Samples shown with estradiol concentrations <150 pmol/L were from patients in the IVF-ET program; those >150 pmol/L were from pregnant women.

Although the correlation and the linear-regression equation are indicative of close agreement of results obtained in the absence and in the presence of displacing agents, we decided to include these agents with the standards and saliva samples in the direct CIA.

**Analytical Variables**

**Calibration curve.** Figure 2 shows two dose-response curves, illustrating results for the direct CIA performed with clear microtiter plates and the Lumac Biocounter and performed with black microtiter plates and the ICN Flow Luminoskan. Each time a curve was run on a black plate, a comparable curve was run in parallel on a clear plate, with use of reagents made up at the same time. The relative binding (B/B₀) at all standard concentrations (0.25 to 20 pg/well) of the six curves obtained via Biocounter readings (x) or via Luminoskan readings (y) was similar: y = 0.894x + 0.036 B/B₀ (r = 0.941).

The detection limit of the direct CIA, calculated as the least amount of E₂ significantly different from zero at 95% confidence limits (i.e., mean for zero - 2 SD), was 3.8 pmol/L (206 fg/well or 1.03 ng/L). This compared well with published detection limits of 0.2 to 5 pg/tube for RIAs of E₂ in extracts of saliva (7–10, 14). At 0.5 B/B₀, the E₂ read from the calibration curve was 80.9 pmol/L (4.4 pg/well or 22 ng/L).

**Specificity.** The cross-reactivities of the monoclonal anti-E₂ antibody 2F9 in the direct CIA are listed in Table 1. **Analytical recovery.** To aliquots of six different saliva extracts, tributyl phosphate was added to a final concentration of 0.5% (v/v).

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**Fig. 1.** Comparison of results obtained in the direct CIA without and with the addition of displacing agents to standards and to saliva samples.

**Fig. 2.** Calibration curve of the direct CIA for estradiol: (—) mean B/B₀ ± SD of 15 consecutive curves of the direct CIA performed with clear microtiter plates and with light measurement in cuvettes by the manual Biocounter, (---) mean B/B₀ ± SD of six consecutive curves of the CIA performed with black microtiter plates and the Luminoskan plate reader.
samples with endogenous E2 concentrations between 0 and 139 pmol/L we added two to six different amounts of E2, ranging from 9.2 to 367 pmol/L. The mean analytical recovery from these saliva samples was 96.8% (SD 7.0%, range 84.1–111.2%, n = 31).

Parallelism. We diluted two- to ninefold with assay buffer 25 saliva samples with endogenous E2 concentrations between 95 and 749 pmol/L. Assay of 40 dilutions showed a linear correlation between the expected (x) and observed (y) values of E2: y = 1.036x + 0.26 pmol/L (r = 0.977, n = 40).

Precision. Within-assay precision was assessed from replicate measurements in one CIA and between-assay precision from repeated analysis in consecutive CIAs (Table 2).

### Table 1. Cross-Reactivity (%) of Anti-Estradiol Antibody Clone 2F9

<table>
<thead>
<tr>
<th>Steroid</th>
<th>CIAa</th>
<th>CIAb</th>
<th>RIAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol-17 β</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Estradiol-17 α</td>
<td>1.56</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>Estriol</td>
<td>0.40</td>
<td>0.003</td>
<td>0.1</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.32</td>
<td>0.001</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Progesterone</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>nd</td>
</tr>
</tbody>
</table>

a Present method. Antibody 2F9 in solution, added to the incubation mixture at the beginning of the CIA, binds to the solid-phase second antibody during the 2-h incubation.

b Direct CIA for E2 in serum (16). Antibody 2F9 is bound to the solid-phase second antibody at least one day before the immunoassay. Immunoassay is in two steps (2 × 30 min) with a washing step in between.

Antibody 2F9 is in solution; phase separation is by use of dextran-coated charcoal (21).

### Table 2. Precision of the Direct CIA for Estradiol in Saliva

<table>
<thead>
<tr>
<th>Samplesa</th>
<th>No. of duplicates</th>
<th>Mean (and SD) E2 conc, pmol/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-assay variation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>9</td>
<td>20.2 (2.31)</td>
<td>11.4</td>
</tr>
<tr>
<td>S1</td>
<td>9</td>
<td>46.1 (3.91)</td>
<td>8.5</td>
</tr>
<tr>
<td>P2</td>
<td>9</td>
<td>77.1 (6.19)</td>
<td>8.0</td>
</tr>
<tr>
<td>S2</td>
<td>10</td>
<td>116.2 (5.78)</td>
<td>5.0</td>
</tr>
<tr>
<td>P3</td>
<td>9</td>
<td>354.3 (8.98)</td>
<td>2.5</td>
</tr>
<tr>
<td>P4</td>
<td>9</td>
<td>499.0 (31.1)</td>
<td>6.2</td>
</tr>
<tr>
<td>Between-assay variation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td>10</td>
<td>26.9 (3.43)</td>
<td>12.7</td>
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<tr>
<td>S4</td>
<td>9</td>
<td>41.2 (3.60)</td>
<td>8.7</td>
</tr>
<tr>
<td>S5</td>
<td>8</td>
<td>86.1 (6.97)</td>
<td>8.1</td>
</tr>
<tr>
<td>S6</td>
<td>8</td>
<td>101.4 (7.71)</td>
<td>7.6</td>
</tr>
<tr>
<td>S7</td>
<td>9</td>
<td>347.7 (35.45)</td>
<td>10.2</td>
</tr>
</tbody>
</table>

a S, saliva samples; P, pooled saliva.

Fig. 3. Estradiol concentrations in saliva throughout the normal menstrual cycle
Mean and SD of three consecutive cycles, length 27 days, of the same woman. At days 1, 5, 10, and 24 only one saliva sample was available.

Saliva concentrations of E2 during ovulation induction for IVF-ET ranged between 125 and 130 pmol/L (median 38.4 pmol/L). These concentrations compared well with those found by others using extraction RIA (8-10). Concentrations of E2 in serum ranged from 140 to 10 282 pmol/L (median 3176 pmol/L). E2 concentrations in saliva (y) and serum (x) correlated well: y = 0.00972x + 1.8 pmol/L (r = 0.858, n = 80). The mean ratio of saliva:serum concentrations calculated from time-matched pairs was 1.1% (SD 0.51%, n = 80). Figure 4 illustrates the correlation between saliva and serum concentrations of E2 during ovulation induction for IVF-ET.

Evaluation of the Luminoskan
To compare the Biocounter (x) with the Luminoskan (y), we examined (a) the standard points of the calibration curves, (b) results obtained for saliva samples, and (c) cross-reaction studies of the antibody. We found the following:

(a) r = 0.941, y = 0.981x + 0.036 B/B0, n = 35
(b) r = 0.962, y = 1.055x – 0.027 B/B0, n = 30
(c) r = 0.989, y = 0.979x + 0.0 B/B0, n = 25

The expression of all results, including those of saliva concentrations, as B/B0 allows the comparison of pooled data (Figure 5). Because of the good agreement of results, we conclude that the two methods are equivalent on an analytical level.

### Discussion
The present method differs considerably from published procedures. It has the advantages of using a nonradioactive, innocuous label and of being performed on unextracted saliva, of which only 200 μL is needed for duplicate measurement. Moreover, the advent of an automated plate reader makes it as easy and simple for endpoint determination as an enzyme immunoassay. Microtiter plates offer a reliable solid-phase system and allow for easy and fast washing without loss of the bound fraction (16). In addition, sample manipulation and pipetting are facilitated.
Fig. 4. Saliva and serum estradiol concentrations during ovulation induction for IVF-ET
Cycles A (Clomid/hMG) and B (Suprefact/hMG) are from the same patient; cycles C and D (Suprefact/hMG) are from different patients

The high binding affinity of the monoclonal antibody for estradiol (16), the incubation of both E₂ and antibody in solution in the wells, and the delayed addition of the E₂-ABEI label are responsible for the high sensitivity obtained at the zero dose. Indeed, the detection limit of 206 fg/well makes possible the measurement of low concentrations of E₂ in saliva during the normal menstrual cycle. Accuracy and precision are satisfactory, and E₂ concentrations measured with the direct CIA are similar to published results obtained by RIA during the normal cycle (7, 8) and during stimulation for IVF-ET (8-10). The ratio of saliva:serum E₂ concentrations, 1:1%, is close to that found by others (6, 9, 10, 12). Also the correlation between saliva and serum concentrations of E₂ confirms earlier reports (8-10, 12).

Hammond and Langley (15) identified sex-hormone-binding globulin in considerable concentrations in mixed saliva, as has been confirmed by Selby et al. (22). It is therefore surprising that in the direct CIA we found no significant difference between results obtained in the absence and in the presence of sufficient amounts of displacing agents. This comparison included saliva samples from men, pregnant women, and patients enrolled in an IVF-ET program. However, in a high-concentration pregnancy sample (not included in this study) two consecutive determinations of E₂ in saliva in the presence/absence of displacers yielded 614/536 and 623/576 pmol/L, respectively, for an average of 10% more E₂ in the presence of displacing agents.

The Luminoskan yielded results that correlate well with those of the direct CIA method performed with the Biocounter. In addition, the regression equation indicates that the Luminoskan readings are not biased. Although time was insufficient to test the plate reader more extensively, our results indicate that this apparatus can perform satisfactorily for reliable measurement of very small quantities in the wells of microtiter plates. As suggested earlier (16), there is a need for a plate reader suited for measuring the short-lived type of chemiluminescence typical of isoluminol.

The present method is a welcome addition to the microtiter-plate-based direct CIs already developed in our laboratory (16-18). Progesterone, estradiol, and estriol can now be measured directly in saliva samples. The methods are simple, reliable, and cheap and involve similar procedures and reagents. These assays can facilitate clinical endocrinological investigations in women that may necessitate frequent sampling, e.g., IVF-ET procedures. In such instances saliva can indeed by a valuable alternative to blood serum or plasma.

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References