Estrogen Radioimmunoassay Suitable for the Monitoring of Ovulation Induction

C. Dennis Ashby,† Hal C. Danzer,‡ and Ronald S. Swerdloff§

A rapid radioimmunoassay for estrogen in serum was characterized and validated. Analytical recoveries of estrone and estradiol were essentially 100%; estradiol was not measured because it was eliminated by sample-handling procedures. Nonspecific interference from serum extracts, which was initially reflected by positive blanks, was eliminated by a sodium hydroxide wash of the extract. Validation of the procedure included determination of a reference interval for estrogen that agreed with the sum of estradiol and estrone concentrations reported in the literature for eugonadal women. Clinical applicability of the assay was demonstrated by monitoring estrogen concentrations during the menstrual cycle and during urogenadotropin (Pergonal)-induced follicular maturation. Because of its rapid turnaround time, the assay is ideally suited for monitoring the frequency with which urogenadotropin should be administered to the infertile patient, and the dosage, thus decreasing the likelihood of multiple pregnancies or ovarian hyperstimulation.

Additional Keyphrases: monitoring therapy with urogenadotropin/chronogadotropin • responses of estrogens to treatment • steroids • gynecomastia • reference intervals • monitoring serum estrogen concentrations during the menstrual cycle

When ovulation is induced by administering urogenadotropin (human menopausal gonadotropin; "Pergonal", Serono Labs., Inc.), serial estrogen concentrations in urine or plasma should be monitored to provide data on the degree of follicular stimulation (1–5). This aids the physician in selecting the dosage and course of treatment in the infertile anovulatory patient with functional ovaries. Such monitoring can reduce multiple pregnancies and ovarian hyperstimulation with its potentially serious complications (1). Follicular maturation has been assessed by measuring estrogen in urine (5–7) and in serum (4, 8). The use of less complicated, less specific procedures for total estrogen has obviated the need for 24-h urine collection and eliminated the chromatographic step in the assay (4, 8). Good correlation between urine and serum estrogen concentrations has been demonstrated (9), as has the correlation between results of total serum estrogen assay and specific estradiol measurement (8).

We report here the characterization and validation of a serum estrogen procedure that has a turnaround time sufficiently rapid for monitoring therapy with urogenadotropin. By eliminating nonspecific interference from serum extracts, we have also made the procedure capable of assessing the sum of estrone and estradiol in the serum of normal men as well as in cycling women. The method we describe provides data on antisemur specificity, sample parallelism, and blank elimination that are not included in published work in which the same antisemur was used (10).

Materials and Methods

Reagents

Methanol, methylene chloride, toluene, and p-dioxane, all of "spectroquality," were purchased from Fisher Scientific Co., Fair Lawn, Nj, 07410. 17β-Estradiol, used as the standard, and estrone were obtained from Sigma Chemical Co., St. Louis, MO 63178. Anti-total estrogen antisemur (S-310 no. 5), produced in sheep by use of estradiol-3,16,17-β-trihemisuccinate/horse serum albumin complex, was purchased from Radioassay Systems Laboratory, Carson, CA 90746. [2,4,6,7-3H]Estradiol was purified before use (11) and was obtained from New England Nuclear, Boston, MA 02118. [6,7-3H]Estriol was also obtained from New England Nuclear. Estrone-3-sulfate, estradiol-3-sulfate, and estradiol-17-glucuronide were obtained from Steraoids, Wilton, NH 03696.

Null (estrogen-free) plasma was prepared from pooled, chelate-containing (ethylenediaminetetraacetate) plasma by treating it with charcoal (10 g per 100 mL of plasma). The null plasma contained less than 5 ng of estradiol per liter, as determined with a specific assay procedure (12).

Bound and free tritiated estradiol were separated by adsorption onto dextran-coated charcoal suspended in 50 mmol/L sodium phosphate (pH 7.4) buffer containing, per liter, 1.0 g of gelatin (Knox unflavored) and 1.0 g of sodium azide. The Dextran T-70 (Pharmacia, Uppsala, Sweden) was dissolved with slight heating and added dropwise into the activated-charcoal (Sigma Chemical Co.) suspension. The final mixture contained 625 mg of dextran and 6.25 g of charcoal per liter.

We prepared liquid scintillation cocktail by mixing 500 mL of p-dioxane and 126 mL of Spectrafluor PPO,POPOP concentrate (Amersham Corp., Arlington Heights, IL 60005) with 2.5 L of toluene. The resulting solution contained 4 g of 2,5-diphenyloxazole (POPOP) and 50 mg of p-bis[2-(5-phenyloxazolyl)]benzene (POPPOP) per liter.

Procedure

In 16 × 125 mm screw-top tubes, incubate 0.5 mL of serum at 37 °C for 20 min with 0.1 mL of tritiated estradiol (about 1000 counts/min) prepared in sodium phosphate buffer (0.1 mol/L, pH 7.4, containing 1.0 g of sodium azide per liter). Next add 5 mL of methylene chloride, cap the tubes, and shake them for 5 min on a mechanical shaker. Centrifuge the tubes at 1100 × g for 5 min and aspirate and discard the top layer. Add 0.5 mL of 0.1 mol/L sodium hydroxide to the extract, shake the tube for 5 min, centrifuge, and remove the top layer as before. Evaporate the washed extract in a 13 × 100 mm test tube under a stream of nitrogen, at 37 °C.

Reconstitute the residue with 2.0 mL of the above sodium phosphate/sodium azide buffer, and then heat at 37 °C for 15 min. Count the radioactivity of a 0.4-mL aliquot, to account for losses incurred during sample preparation.
Table 1. Elimination of Nonspecific Serum Effects in an Estrogen Radioimmunoassay

<table>
<thead>
<tr>
<th>Treatment of extract</th>
<th>Apparent estrogen %</th>
<th>B/Bo Assayed, pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>81</td>
<td>8.2</td>
</tr>
<tr>
<td>2. Water wash</td>
<td>85</td>
<td>6.5</td>
</tr>
<tr>
<td>3. 0.1 mol/L HCl wash</td>
<td>80</td>
<td>8.6</td>
</tr>
<tr>
<td>4. 0.1 mol/L NaOH wash</td>
<td>101</td>
<td>0</td>
</tr>
<tr>
<td>5. Successive wash with 3, 4, and 2</td>
<td>92</td>
<td>3.2</td>
</tr>
</tbody>
</table>

For the radioimmunoassay, pipet 0.4-mL duplicate aliquots of the reconstituted sample into 12 × 75 mm test tubes. Prepare estradiol standards in methanol and pipet 0.1 mL of each into 12 × 75 mm test tubes in amounts of 5, 10, 20, 40, 80, 160, and 320 pg per tube. Use 0.1 mL of methanol for nonspecific-binding tubes and for initial-binding tubes. To account for solvent effects, if any, add 1.0 mL of methylene chloride to the methanol added above. Evaporate the contents of the tubes under nitrogen at 37 °C and reconstitute the residue with 0.4 mL of the sodium phosphate/sodium azide buffer. Add 0.2 mL (about 7000 counts/min) of tritiated 17β-estradiol to each tube. Then add sufficient antibody (0.1 mL) to all but the nonspecific-binding tubes, to achieve an initial binding of about 50%. The final antibody dilution is approximately 800 000-fold. Prepare tracer and antibody in a buffer (pH 7.4) containing, per liter, 2.3 g of gelatin, 1.0 g of sodium azide, and 50 mmol of sodium phosphate; use this gelatin-containing buffer for the nonspecific-binding tubes. Incubate all the tubes in an ice bath for 60 min, then pipet 0.2 mL of dextran-coated charcoal into each tube, continuously stirring the charcoal suspension in an ice bath during the pipetting. Eight minutes after adding the dextran-coated charcoal to the last tube, centrifuge the tubes (1100 × g, 10 min). Add 10 mL of liquid scintillation cocktail to 0.7 mL of the supernatant fluid from each tube, and count the radioactivity of all the tubes.

Use logit-log transformation to linearize the standard curve (10). Read the sample results off the standard curve and correct for recovery losses.

Results

Although methylene chloride extracts of 0.5 mL of water typically resulted in values for apparent estrogen in blanks of less than 3 pg, null plasma extracted similarly resulted in assay blank values as high as 20 pg. The difference could not be accounted for on the basis of estrogen or other steroid content. Similar difficulties with blanks were reported by CerCEO and ElLosO, who used the same antiserum that we did (10). Table 1 summarizes results of an experiment designed to explore the possibility that the interfering material could be removed by washing the extract. Aliquots of null plasma were extracted with 5 mL of methylene chloride, and the plasma layer was removed after centrifugation. After adding 0.5 mL of either water, 0.1 mol/L hydrochloric acid, or 0.1 mol/L sodium hydroxide, we shook the tubes for 5 min, centrifuged them, and removed the aqueous layer. For treatment 5 of Table 1 we repeated this process but washed successively with hydrochloric acid, sodium hydroxide, and water. The dried extracts were reconstituted and assayed as described in Materials and Methods. In the untreated extract the assay blank reading was equivalent to 8.2 pg of estrogen and did not change appreciably after washes with water or acid. In contrast, with the sodium hydroxide wash or with the successive washes including the sodium hydroxide, the blank reading was decreased either to zero or to a value less than that of the lowest standard (5 pg).

The effect of eliminating the nonspecific blank with a 0.1 mol/L sodium hydroxide wash is shown in the parallelism study presented in Figure 1. Different volumes of pooled sera were extracted in quadruplicate, and duplicate extracts were either washed or not washed with sodium hydroxide. Although there was a linear relationship between the volumes extracted in both cases, the sample extracts not treated with the base had a y-intercept of 13.6 pg when the data were examined by linear regression analysis. With the sodium hydroxide wash, the intercept was negligible, 0.1 pg. Our attempts to eliminate
Table 2. Fate of Estriol in the Estrogen Procedure

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Estriol radioactivity Counts/min</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Add 0.5 mL of serum + 0.1 mL of [3H] estriol</td>
<td>8846</td>
<td>100</td>
</tr>
<tr>
<td>2. Extract with 10:1 methylene chloride</td>
<td>3627</td>
<td>41</td>
</tr>
<tr>
<td>3. Wash with 0.5 mL of 0.1 mol/L NaOH</td>
<td>176</td>
<td>2</td>
</tr>
<tr>
<td>4. Reconstitute with 0.5 mL of sodium phosphate buffer (0.1 mol/L, pH 7.4)</td>
<td>195</td>
<td>2</td>
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the nonspecific blank by using solvents other than methylene chloride for extraction were unsuccessful. The solvents investigated were ethyl acetate/hexane (3/2 by vol), diethyl ether, and acetone.

Specificity studies with the antiserum essentially confirmed the results reported by Abraham et al. (14). In studies of cross reactivity at 50% displacement with 17β-estradiol, estrone had 93% and estriol 63% of the activity of estradiol (average of three experiments for each). The curves were essentially parallel. Estrone-3-sulfate and estradiol-3-sulfate exhibited minimal cross reactivities (<0.2%), which supports the hypothesis that the antiserum does not recognize 3-position estrogen conjugates. Estradiol-17β-glucuronide, on the other hand, cross reacted by 36%. It is unlikely that any of the estrogen conjugates would be present at the radioimmunoassay stage of the procedure, however, because they would not be readily extracted with methylene chloride and they would be removed with the sodium hydroxide wash.

We performed analytical recovery studies to assess the accuracy of the procedure—including the extraction, the wash, and the radioimmunoassay steps. Null plasma was supplemented with estrone, estradiol, or estriol dissolved in sodium phosphate buffer (0.1 mol/L, pH 7.4). A tracer amount of tritiated estradiol was added to monitor the manipulation losses and the samples were processed as described in Materials and Methods. Figure 2 depicts the results for estradiol and estrone. In both cases the intercepts were near zero, supporting the absence of a nonspecific serum blank. The slope of 1.00 for estradiol and 0.95 for estrone agrees favorably with the expected recoveries based on antiserum cross reactivity alone. This was not the case for the analytical recovery of estriol, however, for which recovery was essentially zero. Table 2 presents the results of an experiment designed to trace estriol through the sample-handling stages of the procedure. Of the approximately 9000 counts/min of estriol added to the pooled serum, only 41% was extracted into methylene chloride. The sodium hydroxide wash removed about 95% of the material that had been extracted, leaving only 2% of the total for radioimmunoassay. Each step was monitored with triplicate tubes. The sample-preparation procedure that is appropriate for the assay of estradiol plus estrone clearly is inappropriate for the assay of estriol.

To further validate the procedure, we compared it with a specific assay for 17β-estradiol. Serum extracts from urogonadotropin-treated and eugonadal women were chromatographed on Celite columns to ensure the specificity of the assay for estradiol (11). Radioimmunoassay of estradiol was by the procedure currently in use at Nichols Institute (12). The correlation coefficient of 0.981 that we found for 45 samples (Figure 3) demonstrates the usefulness of our less-specific, more-rapid estrogen assay applied to samples from normal women or from women treated with urogonadotropin.

Table 3 gives the normal values obtained with this procedure; they are consistent with the sum of normal values reported for estrone and estradiol assayed by specific radioligand procedures (15–17). About a third of the samples from apparently normal men gave values of <70 ng/L, which is the typical sensitivity of the procedure. The values for cycling women are lower than those reported by Cerceo and Elloso (10), probably because of the elimination of nonspecific serum effects.

This assay can be successfully used to monitor urogonadotropin/chorigonadotropin-induced ovulation in patients with pituitary insufficiency. Seven different series of uro-

![Fig. 3. Comparison of estrogen concentrations (ordinate) and specific estradiol concentrations (abscissa) in 45 serum samples.](image-url)

Table 3. Reference Intervals for Estrogen Values in Normal Men and Women

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean (2 SD) ng/L</th>
<th>Range used</th>
</tr>
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<tbody>
<tr>
<td>Women:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early follicular phase (−21 to −6)*</td>
<td>78</td>
<td>182 (167)</td>
<td>70 to 400</td>
</tr>
<tr>
<td>Late follicular phase (−5 to 0)</td>
<td>42</td>
<td>402 (379)</td>
<td>100 to 900</td>
</tr>
<tr>
<td>Luteal phase (+1 to +13)</td>
<td>82</td>
<td>325 (239)</td>
<td>70 to 700</td>
</tr>
<tr>
<td>Pregnancy (second trimester)</td>
<td>11</td>
<td>11 728 (8435)</td>
<td>6000 to 20000</td>
</tr>
<tr>
<td>Pregnancy (third trimester)</td>
<td>30</td>
<td>25 040 (30 692)</td>
<td>6000 to 70000</td>
</tr>
<tr>
<td>Men:</td>
<td>77</td>
<td></td>
<td>less than 130</td>
</tr>
</tbody>
</table>

* Days from peak lutropin concentration in serum.
nadotropin injections given to five patients were monitored with the estrogen procedure. Two of the seven had estrogen concentrations of <400 ng/L and seemed not to respond to urogonadotropin/choriogonadotropin administration (as was also judged to be the case from basal body temperature and the quantity and quality of cervical mucus). The remaining five series were considered to be ovulatory because progesterone concentrations >4 μg/L were noted four to seven days after chorionadotropin injection. Figure 4 shows the estrogen values for two patients who responded to therapy. Twins were detected by ultrasound in the patient with the higher estrogen values; both women delivered subsequently without complications.

Discussion

Interference by nonspecific materials extracted from relatively large volumes of serum has long plagued steroid radioimmunoassay procedures. The resolution of this problem has classically been achieved by introducing a chromatographic step before radioimmunoassay, but this slows the assay undesirably. A brief washing of the extract with 0.1 mol/L sodium hydroxide eliminates the positive blank, perhaps as a result of removing nonesterified fatty acids, which reportedly interfere with steroid radioimmunoassay procedures (18).

Chemical validation of the estrogen procedure included accuracy or analytical-recovery studies with estrone and estradiol. The high recovery of estrone supports the conclusions that tritiated estradiol, which is used to monitor manipulation losses, does reflect estrone losses as well as those of estradiol, and that estradiol is an appropriate standard to use for both estrone and estradiol measurement. Parallel displacement curves were observed with increasing amounts of estrone and estradiol standard and with serial dilutions of serum from eugonadal and urogonadotropin-treated women. This assay cannot be classified as a “total” estrogen assay because estriol is removed during the extraction and sodium hydroxide wash steps.

This assay has been successfully used to monitor urogonadotropin/choriogonadotropin-induced ovulation in patients with pituitary insufficiency, and it is ideally suited for this purpose. While not yet tested, the assay may prove to be helpful in the evaluation of men with gynecomastia.

The procedure is not appropriate when specific measurement of estradiol or estrone is required. Moreover, because the assay is relatively insensitive (the level of detectability is approximately 70 ng/L), it is not useful when low concentrations of estrogens are suspected, as in prepubertal children and postmenopausal women.

In conclusion: we have described an estrogen (estrone plus estradiol) radioimmunoassay with a rapid turnaround time (5 to 6 h) that has been successfully used to monitor ovulation induction. Results of the procedure correlate well with increases in serum estradiol produced by urogonadotropin administration, and therefore the procedure is valuable for monitoring the estrogen responses of patients on such treatment.

References