Evaluation of a Radioimmunoassay for Estradiol in Unextracted Serum

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We describe the performance of a commercial (Steranti/EIR) RIA reagent kit for measuring 17β-estradiol directly in serum. Day-to-day precision data for control sera were as follows: mean = 102.8 ng/L, CV = 6.8%, n = 20; mean = 231.1 ng/L, CV = 5.3%, n = 21; mean = 747.7 ng/L, CV = 9.4%, n = 21. Analytical recovery of added estradiol from seven different serum pools from men, to which three different concentrations of estradiol had been added, was (mean ± SD): 98.6 ± 7.0% at 107.5 ng/L added; 98.8 ± 4.7% at 322.5 ng/L added; 108.2 ± 4.8% at 645 ng/L added. Overall recovery of estradiol in these experiments (mean ± SD for 21 determinations) averaged 101.9 ± 7.0%. Assay of 32 serum specimens from women by both the direct (y) and an extraction method (x) gave the following linear regression statistics: y = 1.12x − 1.3, r = 0.998, Syx = 30.2 ng/L, mean y = 438.2 ng/L, mean x = 391.4 ng/L. Hemoglobin, bilirubin, and moderate lipemia do not interfere. Sensitivity of the direct assay was 2.6 ng/L. Compared with the extraction assay, the direct estradiol assay has advantages of speed and simplicity.

Additional Keyphrases: hormones • "kit" methods • displacement of estradiol by dihydrotestosterone

The importance of sequential estradiol [1,3,5(10)-estratriene-3,17β-diol] measurements for monitoring ovulation induction therapy, particularly in "in vitro" fertilization programs, has emphasized the need for a reliable, rapid assay method for estradiol in serum (1). Whereas initial RIAs for estradiol included chromatographic purification steps, more recent assays have required only a relatively simple organic solvent extraction because of improved antibody specificity (2, 3). Over the past several years, direct assays of estradiol in unextracted serum have been proposed, and commercial reagent kits incorporating this approach are now available.

Here we describe our experience with one such commercial kit. Estradiol is displaced from serum-binding proteins by dihydrotestosterone (4). The kit incorporates an 125I-labeled estradiol tracer, and free and antibody-bound tracer are separated by a solid-phase-bound second antibody.

Materials and Methods

Materials

Equipment. We used a Model 20/20 10-well gamma counter (Iso-Data, Inc., Palatine, IL 60067). Standard curves were graphed as logit % B/B₀ vs log dose, and the points were connected by using the French-curve-fit data analysis program. For ultracentrifugation, we used an Airfuge ultracentrifuge (Beckman Instruments, Spinco Division, Palo Alto, CA 94304).

Reagents. 125I-Estradiol Direct (non-extraction) RIA kit (Code: ER-155) was supplied by Steranti Research Ltd., St. Albans, Harts. AL1 1TA, U.K. The kit is manufactured by EIR Radio Isotope Service, Isotopen produktion, Wurenlingen, Switzerland. The antiserum, prepared by injection of rabbits with estradiol-6-carboxymethyl oxime–bovine serum albumin, is supplied as lyophilized powder, and the 125I-labeled estradiol as a concentrated solution in ethanol. Reconstitution of antiserum and dilution of isotope are performed with the gelatin/phosphate buffer, pH 7.4, containing sodium oxide, as supplied in the kit. Goat anti-rabbit gamma-globulin covalently attached to globular particles is supplied lyophilized, and is reconstituted with the gelatin/phosphate buffer. Human serum-based standards (0, 10, 40, 100, 400, 1000 ng/L) are supplied in liquid form. Stored at 2–8 °C, all components are stable for about four weeks after reconstitution. Cross-reactivity data for 50% inhibition of tracer binding, as supplied by the manufacturer, are as follows: estrone and estriol, 2%; ethinylestradiol, progesterone, testosterone, androstenediol, <0.1%; estradiol-3-glucoronide, 0.04%; estradiol-17-glucoronide, 0.07%; estradiol, 100%. RIA kit with extraction, which we used in the comparison studies, was from Pantex, Santa Monica, CA 90404 (cat. no. 047). The stock standard solution consists of estradiol dissolved in alcohol. Subsequent dilutions of the standard are made with the supplied buffer.

Ethyl acetate and hexane used in the extraction method were obtained from Burdick and Jackson Laboratories, Muskegon, MI 49442.

Controls. The control material used was DPC Three Level RIA Control Serum (Diagnostic Products Corp., Los Angeles, CA 90045).

Recovery Studies

Estradiol (Sigma Chemical Co., St. Louis, MO 63178) was dissolved in methanol and subsequently diluted in phosphate buffer, 10 mmol/L, pH 7.4, containing 10 g of bovine serum albumin per liter. Various amounts were then added to serum pools from men to obtain appropriate concentrations of estradiol. The supplemented serum pools were diluted less than 5% with the buffered estradiol solution. Endogenous estradiol was measured in an aliquot of each serum pool to which had been added a volume of estradiol-free buffer equal to the volume of buffer used for adding the estradiol. Analytical recovery of estradiol was calculated as [(measured − endogenous)/added] × 100.

Methods

Direct method. Add 50 µL of standards and patients' samples to labeled tubes and 50 µL of the "0" standard to each nonspecific binding tube. Add 100 µL of 125I-labeled estradiol tracer to all tubes and 100 µL of primary antiserum to all but the nonspecific binding tubes. To the nonspecific binding tubes, add 100 µL of buffer. Vortex-mix briefly and incubate at 37 °C for 2 h. Add the solid-phase second antibody (500 µL) and incubate for 30 min at 37 °C. Separate the free from the bound tracer by centrifugation (10 min at 2000 × g, 22 °C), then decant. Measure the radioactivity in the pellet.

Extraction method. Extract 0.6 mL of serum with 6 mL of
ethyl acetate/hexane (3/2 by vol). Evaporate 5-mL aliquots under nitrogen at 45 °C. Add 1.25 mL of buffer to each extract, vortex-mix briefly, and incubate at 37 °C for 30 min. Pipet 500 μL of standards, and reconstituted extracts of control sera and patients' samples into labeled tubes, and add 500 μL of buffer to nonspecific binding and "0" standard tubes. Add 100 μL of 125I-labeled estradiol tracer to all tubes. Next add 100 μL of primary antiserum to all but the nonspecific binding tubes. Vortex-mix, then incubate at 37 °C for 30 min. Add 500 μL of secondary antiserum and incubate for a further 10 min at room temperature. Separate free from bound tracer by centrifugation (10 min at 2500 × g; 22 °C) and decant. Measure the radioactivity in the pellet.

Results

**Standard curve.** Figure 1 shows typical standard curves for the direct method and for the extraction method. The standard curve for the direct method exhibits adequate sensitivity (a reasonably steep slope for the standard curve) throughout the range of 10–700 ng/L. However, because of the relative flatness of the curve and the resulting higher imprecision of the assay above 700 ng/L (see Table 2), we routinely dilute specimens containing more estradiol than this. Assay sensitivity, based on 2 SD of the mean of 20 replicate tests of the zero standard, was 2.6 ng/L. For the direct method, % BV/T ranged from 51 to 62% and the nonspecific binding was less than 3.0%. Total counts for the direct assay were greater than 20 000 cpm four weeks before expiration of the kit.

**Precision.** Specimens and controls were analyzed in duplicate by the direct RIA method, and single extracts of specimens and controls were analyzed in duplicate by the extraction RIA; the results for within-run precision are shown in Table 1. Within-run precision data for the extraction method reflect only the precision of the RIA and do not assess the contribution of variable extraction efficiency to the overall within-run precision.

Day-to-day precision data for both methods, obtained with control sera, are shown in Table 2.

Six technologists performed these assays with the direct method and four technologists used the extraction method.

**Accuracy.** To assess analytical recovery, we measured estradiol in seven serum pools by both the direct and the extraction methods. As summarized in Table 3, the recovery for the direct method averaged 101.9%, while the mean recovery for the extraction method was 95%. These data were collected on two separate days with the direct method and on four separate days with the extraction method.

We measured estradiol in 32 specimens from women, by both the direct and extraction methods, on three separate days. As shown in Figure 2, results by both methods agreed very well.

Several specimens containing high concentrations of estradiol were assayed at various dilutions; typical results are shown in Table 4. Similar satisfactory results were obtained with dilutions of sample extracts assayed by the extraction RIA (data not shown).

Hemoglobin (≤3.0 g/L) and bilirubin (≤0.25 g/L) had no effect on the direct assay. To test the effect of lipemia, we prepared a pool from lipemic specimens (milk-like appearance, triglycerides = 9.7 g/L), divided the pool into two parts, and ultracentrifuged one part to obtain an essentially clear pool. To both the lipemic and clear pools, we added equivalent amounts of estradiol. The clear and lipemic pools were then blended in various proportions and assayed. We also ultracentrifuged the lipemic pool after estradiol addi-
and the very good correlation of the direct method with the extraction method suggest that the antibody used possesses the required specificity.

Because of the multiplicity of estradiol reference categories, we have not yet established our own normal reference limits, but rather are using those supplied by the manufacturer. We have measured daily estradiol concentrations in several human menopausal gonadotropin-induced cycles, as well as spontaneous cycles, and observed the expected exponential increase in estradiol (5).

The direct assay has the advantage of a slightly shorter total assay time: about 5 h vs 6 h for 12 patients' specimens. Furthermore, the direct method requires about 2.5 h less "hands-on" time.

The extraction of estradiol from serum is not only time consuming but can also be a source of serious analytical error. During our recovery and correlation studies, we occasionally (in about 3 to 5% of the specimens) observed grossly low values of estradiol; these were associated with the persistence of insoluble lipid material after addition of buffer to the dried lipid residues. In these instances, resolubilization problems were not encountered when the extraction step was repeated; the repeat estradiol values were much higher than the original results, agreeing well with the expected values in the recovery and correlation studies. This sporadic problem of incomplete resolubilization, apparently an unpredictable event not attributable to some inherent property of the individual serum sample, requires implementation of suitable quality-control procedures to avoid the reporting of a falsely low result. Use of the direct method, of course, circumvents this problem.

In summary, in comparison with an extraction assay, the direct estradiol method is more reliable analytically, is technically simpler, and saves time.

References

![Graph](image_url)

Fig. 2. Comparison of the direct method and the extraction method for estradiol in serum from women
The 95% confidence limits for the slope are 1.09 and 1.15; for the y-intercept 14.1 and -16.7 ng/L. $S_p = 30.2$. Mean $x = 391.4$ ng/L, mean $y = 438.2$ ng/L.

<table>
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<th>Dilution</th>
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Table 4. Effect of Sample Dilution on Results by the Direct Method
Estradiol, ng/L