Nonchromatographic Radioimmunoassay of Unconjugated Estriol in Plasma, with Polyethylene Glycol as Precipitant

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We describe a rapid, reliable radioimmunoassay for unconjugated estriol in plasma. Polyethylene glycol (Carbowax 6000) is used to separate antibody-bound and free steroid. The assay is sensitive (25 pg for standards), precise, and accurate. At high and low concentrations of estriol, intra-assay coefficients of variation were 7.1% and 7.6%, respectively, and inter-assay coefficients of variation were 7.2% and 10.0%, respectively. Free [3H]estriol is not precipitated by polyethylene glycol. This radioimmunoassay of estriol, with a highly specific antiserum and with polyethylene glycol as the antibody precipitant, is a reliable one-day assay that is practical both for the clinical laboratory and the obstetrician.

Additional Keyphrases: monitoring fetal well-being

A critical facet in radioimmunoassays is the separation of antibody-bound and free ligand. The original observation (1) that polyethylene glycol (PEG) was useful for this separation procedure in polypeptide hormone assays induced us to study this precipitant for steroid radioimmunoassays. Subsequently we demonstrated that use of PEG was practical for a radioimmunoassay of estradiol-17β and suggested that this method might have general applicability for steroid radioimmunoassays (2). We developed other steroid radioimmunoassays in which PEG was used to separate antibody-bound and free steroids (3, 4).

The present report demonstrates the practicability of PEG in a rapid, nonchromatographic radioimmunoassay of unconjugated estriol in plasma.

Materials and Methods

Materials

Antiserum: The specific antibody to estriol used for the assay was raised in an ewe against estriol-6-carboxymethyl-oxime/bovine serum albumin and was a gift from Dr. Uwe Goebrunsman, Department of Obstetrics and Gynecology, University of Southern California. The lyophilized antiserum (UG-R2-09) was reconstituted in distilled water and small aliquots were frozen at -45 °C. Each month, an aliquot was diluted 2000-fold with barbital/acetate/gelatin buffer (see below) and stored at 4 °C.

Steroids and estriol standards: The purity of estriol (Sigma Chemical Co., St. Louis, Mo. 63178) was assessed from its melting point. The stock solution was prepared twice a year in benzene/ethanol (9/1). From this, a working standard solution was prepared monthly containing 5 ng of estriol per milliter of the barbital/acetate buffer. All other radio-inert steroids were obtained from Steraloids, Inc., Pawling, N.Y. 12564, and from Sigma Chemical Co.

[6,7-3H]Estriol (spec. activity 53.1 kCi/mol) was purchased from New England Nuclear Corp., Boston, Mass. 02118. Its purity was tested every three to six months by chromatography on Celite, and it was used when the purity exceeded 95%.

Buffers: The stock sodium barbital/acetate was prepared by dissolving 7.357 g of sodium barbital and 4.857 g of anhydrous sodium acetate in 250 ml of distilled water. The working buffer, used in all instances except for preparing the antiserum dilution, was prepared by mixing 50 ml of this stock solution with 900 ml of sodium chloride solution (8.5 g of NaCl per liter) and adjusting the pH with hydrochloric acid (6 mol/liter) to pH 7.0. The working barbital/acetate/gelatin buffer, used for diluting the antiserum, was prepared similarly except that 3.5 g of reagent-grade gelatin (Mallinkrodt Chemical Works, St. Louis, Mo. 63160) was added per liter.

Solvents: Ethyl acetate (Mallinkrodt), n-hexane (Fisher Scientific Co., Pittsburgh, Pa. 15219), and benzene (Fisher Scientific Co.) were redistilled before use. Freshly opened diethyl ether (Mallinkrodt) and absolute ethanol (U.S. Industrial Chemicals, Anaheim, Calif. 92805) were used without distillation.
Bovine γ-globulin, fraction II, was obtained from Miles Laboratory, Elkhart, Ind. 46514.

The polyethylene glycol reagent, used as a precipitant, was prepared as a 300 g/kg solution of polyethylene glycol ("Carbowax 6000"; Union Carbide Corp., San Francisco, Calif. 94138) in the buffer.

Radioactivity was determined by using a modified Bray's solution (5), lacking methanol and ethylene glycol.

Procedure

Plasma extraction: Duplicate 0.5-ml aliquots of plasma were incubated for 30 min at room temperature with 1000 dpm (2.5 pg) of [6,7,3H]estriol and then extracted with 3 ml of ethyl acetate in n-hexane (10/90 by vol). The organic phase was discarded, and the aqueous phase was extracted with 3 ml of ethyl acetate in n-hexane (40/60 by vol). The organic phase was evaporated under nitrogen and the residue was dissolved in 0.5 ml of buffer. For estimating recoveries, 0.15-ml aliquots were transferred to scintillation vials to which 10 ml of Bray's solution was added. For assay purposes, 0.15-ml sample aliquots were used.

Radioimmunoassay: The standard curve was prepared in triplicate by adding 0, 10, 20, 40, 70, 120, 200, 300, and 400 μl of the 5 ng/ml estriol standard. After adding buffer to all samples to give a total volume of 0.4 ml, 40 000 dpm (100 pg) of [6,7,3H]estriol in 0.1 ml of buffer was added to all tubes, which were shaken gently. Then 0.2 ml of the antiserum, diluted 2000-fold to produce an initial 70% antibody-binding of [3H]estriol in the absence of standard, was added to all tubes except those used to calculate the total radioactivity. The contents of the tubes were gently mixed and allowed to equilibrate for 2 h at 0 °C.

After the addition of 1 mg of bovine γ-globulin in 50 μl of buffer and 0.75 ml of the polyethylene glycol solution, the contents of the tubes were mixed on a vortex-type stirrer, returned to an ice-water bath, and centrifuged (1750 × g, 30 min, 4 °C). The supernate was decanted into scintillation vials, 10 ml of Bray's solution added, and the radioactivity in the mixture was then counted.

Estriol concentrations in the analyzed samples were determined from the constructed standard curves, and plasma concentrations were calculated as follows:

\[
\text{Estriol (μg/liter)} = \left( \frac{100 \times X \times V}{R \times P \times B} \right)
\]

where \( X = \text{pg from standard curve, } V = \text{total volume of buffer used for redissolving residue (ml), } R = \text{% recovery, } P = \text{volume of plasma assayed (ml), and } B = \text{volume of buffer aliquot assayed (ml). When 0.5 ml of plasma is extracted, 0.5 ml of buffer is used to redissolve the residue, and 0.15 ml of this solution is assayed and counted for recovery (as is described for our typical assay), then the calculations are further simplified as follows:}

\[
\text{Estriol (μg/liter)} = \frac{X}{r}
\]

Results

Separation of Antibody-Bound and Free Estriol

Polyethylene glycol concentration: Figure 1 illustrates the complete solubility of [3H]estriol. Even at 200 g/kg concentrations of PEG the [3H]estriol remains in the supernate. The concentration of PEG is important for achieving maximum precipitation of antibody-bound estriol, but the optimum range for maximum precipitation is very wide. We used 150 g of PEG per kilogram for all subsequent work.

Protein concentration: In the absence of antibody the [3H]estriol was not precipitated nonspecifically by the bovine γ-globulin (Figure 2). The presence of γ-globulin, however, did facilitate the precipitation of antibody-bound [3H]estriol, because less antibody-bound [3H]estriol precipitated in its absence. About 0.6 mg of γ-globulin was necessary for maximum precipitation; we chose to use 1 mg of γ-globulin, to ensure optimum conditions.

Precipitation time: We studied two timing requirements for the separation of antibody-bound and free estriol. First, standard curves were set up as usual, and, after the incubation at 0 °C, PEG was added.

Fig. 1. Solubility of [3H]estriol with various final concentrations (g/100 g) of polyethylene glycol in buffer

Open symbols, without antibody; closed symbols, with antibody

Fig. 2. Precipitation of antibody-bound estriol as a function of protein concentration

Open circles, without antibody; closed circles, with antibody
added in the usual manner. Then the tubes were centrifuged after various time intervals. A decrease of the initial percent of antibody-bound estriol was observed when centrifugation was delayed (Table 1). This decrease has not been observed in our previously reported steroid hormone assays (2, 3). Therefore, a prolonged delay in centrifugation after adding PEG could result in erroneously low values. To eliminate this possibility, we investigated the timing for our routine assay. Less than 10 min is required for complete addition of PEG to 50–60 tubes. During this period we could not detect a significant change in the standard curve. To magnify the effect, we centrifuged the tubes for three standard curves at 10-min intervals, 1 h after adding PEG. A slight decline in the standard curve could be detected after 20 min, but not after 10 min, indicating that under the prescribed conditions of our assay, the separation procedure is valid. To further ensure the validity of this method, the assays should be set up such that PEG is added in the following order: standard curve, patents’ samples and controls, standard curve, patients’ samples and controls, standard curve. Any significant decline in the percent bound caused by an inadvertent delay will then be recognized.

The second time period studied was the time delay after centrifugation and before decantation. There was no time dependence in this step; the initial percent bound remained constant for 6 h (Table 1).

Analytical Variables

Sensitivity, blank, recovery, and precision: The lowest amount of estriol that routinely was significantly different from zero (no standard estriol present) was 25 pg. Sensitivity could be improved by increasing the antiserum dilution, but the range of the standard curve was narrowed and the increased sensitivity was not considered advantageous. The blank ranged from 0–70 pg for 17 assays and was not subtracted from the sample values, because it represented less than 3% of the standard-curve range (13). Since 55 to 65% of the estriol was recovered in the ethyl acetate/hexane extract, the lowest measurable dosage, in the absence of blank, is 0.3 μg/liter. Because plasma concentrations are vastly higher in serum during pregnancy, this sensitivity is more than adequate, even with the small blank.

The precision of the method was studied by performing repetitive determinations on pooled plasma from pregnant women with high and low concentrations of estriol, in replicate and from day to day. The means (±SD) for two samples, assayed in replicate (n = 10), were 16.9 ± 1.2 μg/liter and 4.6 ± 0.35 μg/liter, respectively, with corresponding coefficients of variation of 7.1% and 7.6%. The means (±SD) for two different samples assayed from day to day were 14.4 ± 1.04 μg/liter (n = 17) and 4.44 ± 0.44 μg/liter (n = 14), with respective coefficients of variation of 7.2% and 10.0%.

Accuracy. The accuracy of the method was studied in three ways. The cross-reactivity of steroids with the antiserum was determined by the method of Abraham (6). With this antiserum the 6-oxo-derivative of estriol and estradiol-17β demonstrated 100% and 10% cross reactivity, respectively (Table 2). The 16-epiestriol, but not the 17-epiestriol, also demonstrated significant cross reactivity. Less than 1% cross reactivity was observed for the other steroids.

For the second method of assessing accuracy, we added various amounts of radio-inert estriol to plasma from men, and the plasma was subsequently assayed. The amount of estriol observed was close to the expected results (Figure 3) and ranged from 94 to 106% of that added.

Finally, various volumes of pooled plasma from women were assayed. Linear regression analysis calculated by the least-squares method resulted in a
straight line with a correlation coefficient of 0.996 and y-intercept of -0.216 ng (Figure 4).

Normal Values

One hundred twenty-two samples were obtained from healthy women at various times in the last trimester of pregnancy (Table 3). The values are similar to those reported by other investigators (7–11).

Discussion

We have demonstrated for the first time that PEG can be used in a sensitive, precise, and accurate radioimmunoassay of unconjugated estriol in plasma. Other reported radioimmunoassays have utilized solid-phase (6), dextran-coated charcoal (7–14), gel filtration (15), and ammonium sulfate (16–18) techniques for separating antibody-bound and free estriol.

We have shown that PEG is a useful and convenient method of separating antibody-bound and free steroid for other radioimmunoassays (2–4). When we described those steroid radioimmunoassays in which PEG is used, we suggested that the method might be applicable for all steroid radioimmunoassays, but it would be necessary to demonstrate this for each new assay. Our original method for estradiol-17β (2) and subsequent assay for a less polar steroid, testosterone (3), had the significant advantage over the commonly used dextran-charcoal method in that there was complete lack of time dependence in the separation procedure. This was not observed for the estradiol radioimmunoassay described here.

The conditions of the different assays were quite similar. Therefore, the explanation for this phenomenon appeared related to either the antisera or the steroid characteristics. Our present data (unpublished) suggest that the apparent time-dependent dissociation of the antibody–estriol complex is due, at least in part, to the characteristics of the antisera.

Using PEG, we have found sensitivities for a variety of antisteroid antisera that agree with those reported by other investigators using dextran-coated charcoal for separation. In addition, our precision for several radioimmunoassays, including that for estriol, has consistently equaled or bettered that reported by other investigators. Although we cannot conclude that PEG separation technique improves the precision of the method, we have demonstrated that with this method we consistently achieve a between-day coefficient of variation between 5 and 10%. This includes radioimmunoassays of estradiol-17β (2), testosterone (3), and estriol. We have previously pointed out that gelatin did improve the precision of our assay for estradiol-17β with use of PEG (2), and we have continued to use gelatin in all our steroid radioimmunoassays.

The measurement of urinary estriol is a well-accepted procedure for monitoring fetal well-being during the last trimester of pregnancy (19–21). Both gas-chromatographic (22) and fluorometric (23) assays were originally used. However, a 24-h urine specimen is required and the difficulties in accurate collection and the 24-h delay have prompted several investigators to develop plasma assays to avoid these disadvantages.

Recently radio receptor assays (24, 25) and radioimmunoassays have been developed for measuring plasma estriol. An unspecific antiserum for estriol and its conjugates was originally used by Gurpide et al. (16). High specificity for unconjugated estriol in plasma when moderately specific antisera are used has been achieved with either column chromatography on Celite (7, 11, 13) or LH-20 Sephadex (8, 12, 14).

Recently, highly specific antisera have been prepared against estriol conjugated to a protein via either the A (10, 26–28) or the B (9, 10, 29–33) ring. The antisera produced from the latter antigen (the 6-carboxymethylxime protein derivative) has had higher cross reactivity with the estriol sulfate and glucuronide conjugates. These conjugates obviously do not interfere in an assay with a simple solvent-extraction step. The 6-oxo derivatives also significantly cross react with the latter antisera, but the concentrations of these steroids are too low for significant interference (10). Using either type of antisera, accurate and rapid radioimmunoassay of unconjugated estriol is now possible (9, 10, 28). The method is therefore attractive for routine use in clinical laboratories.

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<th>Table 3. Plasma Estriol in Last Trimester of Pregnancy</th>
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Fig. 4. Estriol concentration measured as a function of sample plasma volume.
The clinical utility of plasma estriol assays in various conditions of pregnancy is presently under investigation in several laboratories. By developing the nonchromatographic method with PEG described here, we have been able to analyze hundreds of samples in a practical manner at relatively low cost. The results of our study comparing plasma and urinary estriol concentrations are presently being evaluated.

The high specificity of the antisera permitted us to eliminate an additional separation procedure to remove interfering steroids. Unfortunately, antisera prepared against the 6-carboxymethylloxime or the 4-carboxyphenylazo derivatives of estriol have not been made generally available commercially. However, if the plasma estriol assay proves to be as valid clinically as the urinary estriol procedure, then commercial companies will be strongly encouraged to make these highly specific antisera available for all clinical laboratories.

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References