Rapid Radioimmunoassay of Total Urinary Estriol

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We describe a radioimmunoassay for total urinary estriol in pregnancy. A 25-μl aliquot of the urine specimen is acid hydrolyzed, neutralized, and diluted before assay. We use rabbit antisera against estriol-6-(O-carboxymethyl)oxime/bovine serum albumin and ammonium sulfate precipitation at room temperature. Results are unaffected by glucose or methenamine mandelate, a urinary tract antiseptic. Using semi-automatic pipetting equipment, one laboratory technologist can complete 50 assays within 8 h. This technique is both reliable and convenient and should decrease the expense of routine estriol assays.

Additional Keyphrases: fetal status • normal values

For about 15 years, maternal urinary estriol (E₃) assays have been used as an indicator of fetal status in a variety of high-risk pregnancies (1). Although reliable and convenient plasma E₃ radioimmunoassays (RIA's) have been described (2), it is still unclear whether plasma E₃ measurements are clinically as useful as urinary E₃ determinations (3). Until it has been established which plasma E₃ concentrations or patterns of change indicate fetal distress requiring delivery of a potentially premature infant, many obstetricians will continue to rely upon urinary E₃ assays, for which many procedures have been published.

The few reliable methods are time consuming and costly, and the more numerous “rapid” techniques are insufficiently accurate to be of clinical value. Therefore, we have attempted to develop a rapid RIA for urinary E₃ in pregnancy that would be as reliable as the best conventional methods presently available.

This paper describes a specific RIA for E₃ in highly diluted, unextracted aliquots of urine after hot-acid hydrolysis and neutralization. In this RIA, we used rabbit antiserum to estriol-6-(O-carboxymethyl)oxime/bovine serum albumin and ammonium sulfate precipitation.

Materials and Methods

Equipment


Liquid scintillation counter (Model LS 250; Beckman Instruments, Inc., Fullerton, Calif. 92634).

Microburet (Syringe Microburet, Model SB2; Micro-Metric Instrument Co., Cleveland, Ohio 44122).

Pipettor/Dilutor (Beckman).

Reagents

All reagents were of analytical grade, except gelatin. The water used in this assay was demineralized, charcoal-filtered, and doubly glass-distilled.

Phosphate/gelatin buffer, 0.1 mol/liter, pH 6.8 ± 0.1. Prepare by dissolving 4.68 g of NaH₂PO₄, 8.66 g of NaHPO₄, 9.0 g of NaCl, 1.0 g of sodium azide, and 1.0 g of Knox unflavored gelatin in 1 liter of water.

Bicarbonate buffer, 1.7 mol/liter, pH 10.0 ± 0.5. Dissolve 80 g of NaHCO₃ in 800 ml of water and add 150 ml of NaOH (5 mol/liter), and dilute with water to 1 liter.

Saturated ammonium sulfate solution. Dissolve 300 g of (NH₄)₂SO₄ in 1 liter of water in an Erlenmeyer flask at 60 °C and allow to cool to room temperature. Use the supernatant solution.

Radioimmunoassay Components

[6,7-³H]Estriol, listed specific activity of 50 mCi/mol, was purchased from New England Nuclear, Boston, Mass. 02118. Dissolve an amount equivalent to 250 μCi of this compound in 5 ml of ethanol/benzene (7/3 by vol). Check it for radiochemical purity by chromatographing an aliquot, together with authentic E₃ (RF = 0.09), on paper in benzene/methanol/water (2/1/1 by vol). Store the rest at 4 °C. As needed, dilute 0.5 ml of this solution containing 25 μCi of tritiated E₃ with 4.5 ml of ethanol/water (7/3 by vol) and store the resulting diluted tritiated E₃ stock solution at 4 °C. To prepare a solution of 12 500 cpm of

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tritiated E₃ per 50 μl of phosphate/gelatin buffer, dilute 0.5 ml of the diluted tritiated E₃ stock solution with 10.0 ml of phosphate/gelatin buffer at the beginning of each RIA.

**Authentic estriol** was purchased from Steraloids, Wilton, N. H. 03086, and recrystallized twice before use. Prepare a stock solution of 10 mg of estriol in 100 ml of absolute ethanol and store it at -15 °C.

**Antiserum.** Rabbit anti-estriol-6-(O-carboxymethyl)oxime/bovine serum albumin serum was obtained as previously described (4). The batch used in this study (R2-09) cross-reacted 69% with 6-oestriol, 6.8% with 16-epi-estriol, 2.7% with 2-hydroxyestriol, 0.2% with estradiol-17β, and <0.01% with estrone, 16α-hydroxyestriol, 16β-hydroxyestriol, or 16-oxoestriol-17β. The antiserum was stored undiluted in 0.5-ml aliquots at -15 °C. One 0.5-ml aliquot was thawed as needed, diluted 100-fold by adding 49.5 ml of phosphate/gelatin buffer, divided into ten 5-ml aliquots and stored at -15 °C. One 5-ml aliquot of the 100-fold diluted antiserum was thawed as needed and divided into fifteen 300-μl aliquots, which were stored in liquid-scintillation counting vials at -15 °C. At the beginning of each RIA, one 300-μl aliquot of the 100-fold diluted antiserum was further diluted by adding 15.6 ml of phosphate/gelatin buffer, to give a final 5300-fold dilution. This dilution was used in all assays. The unused portion of this diluted antiserum was discarded each day because it was noted that freezing and thawing of diluted antiserum resulted in rapidly decreasing titers.

**Scintillation fluid.** Forty-two milliliters of “Liquifluor” (2,5-diphenyloxazole/p-bis[2-(5-phenyloxazolyl)]benzene/toluene mixture, available from New England Nuclear, Boston, Mass. 02118) was added to 1 liter of toluene. The counting efficiency was 55.3% of the tritiated E₃ present in 1.1 ml of the mixture of phosphate/gelatin buffer–ammonium sulfate solution decanted after completion of the RIA into 10 ml of scintillation fluid and extracted into the latter by mixing with a vortex-type mixer.

**Procedure**

Dispense 25-μl aliquots of urine, together with 0.50 ml of distilled water, with the pipettor/dilutor, and add 1.0 ml of HCl (3.0 mol/liter) to the diluted urine aliquots. Mix with a vortex-type mixer. Cover each of the 16 × 150 mm tubes with a clean glass marble, place them for 30 min in a metal-block heater at 110 °C, and then chill them in ice water. Add 2.0 ml of bicarbonate buffer to neutralize the hydrolysat es to pH 6.8–7.0. Dispense 50 μl of each hydrolysate with a third pipettor/dilutor, together with 200 μl of distilled water, into 13 × 100 mm disposable test tubes. To this, add 800 μl of absolute ethanol and mix the contents on a vortex-type mixer.

Dispense 25-μl aliquots of this hydrolyzed, neutralized, and ethanol-diluted urine, together with 200 μl of phosphate/gelatin buffer, in triplicate, into 10 × 75 mm disposable test tubes for RIA. Mix the contents of the 10 × 75 mm RIA tubes on a vortex-type mixer. With a microburet, add 50 μl of phosphate/gelatin buffer containing 12 500 cpm of tritiated E₃ and 100 μl of appropriately diluted antiserum.

Mix the contents of all RIA tubes on a vortex-type mixer and incubate the tubes for 60 min in a water bath at 32 °C. Add 0.80 ml of a saturated (NH₄)₂SO₄ solution to all RIA tubes at room temperature, allow the tubes to stand for 2 to 5 min, and then gently agitate the tubes and centrifuge them for 10 min at 3600 × g. Decant the supernatant fluid into scintillation vials containing 10 ml of scintillation fluid. Mix the contents of each scintillation vial for 5 s on a vortex-type mixer and allow the scintillation vials to equilibrate for 30 min in the liquid scintillation counter before counting is begun.

Run a standard curve with each assay. Dispense, in triplicate, 25-μl aliquots of standard solutions containing 0, 0.78, 1.56, 3.13, 6.25, 12.5, 25, and 50 ng of E₃ per milliliter of ethanol/water (7/3 by vol) into 10 × 75 mm RIA tubes. Add 200 μl of phosphate/gelatin buffer, 12 500 cpm of tritiated E₃ dissolved in 50 μl of phosphate/gelatin buffer, and 100 μl appropriate diluted antiserum to all tubes and mix the contents on a vortex-type mixer. The total number of cpm of tritiated E₃ present in the supernate after ammonium sulfate precipitation in the absence of antiserum (T), representing total cpm of tritiated E₃ that is not subject to unspecific precipitation by ammonium sulfate, is determined as follows: dispense, in triplicate, 25 μl of ethanol/water (7/3 by vol), 300 μl of phosphate/gelatin buffer and 12 500 cpm of tritiated E₃ dissolved in 50 μl of phosphate/gelatin buffer into 10 × 75 mm RIA tubes. Mix the contents of all tubes on a vortex-type mixer. Do the incubation, ammonium sulfate precipitation, centrifugation, and liquid scintillation counting of all standard curve samples together with and in the same way as that of all unknown samples.

**Calculation of Results**

The cpm of tritiated E₃ present in the total tubes (T) to which no antiserum was added, and the cpm of free (F) tritiated E₃ were measured in the supernate of all standard and unknown sample tubes. The ratio of free (F) to bound (B) tritiated E₃ was calculated according to the formula F/B = F/(T-F). Plotting F/B ratios vs. concentrations of E₃ standards, linear standard curves were obtained between 0.78 ng/ml and 50 ng/ml, with correlation coefficients that invariably exceeded 0.99. With a Compucorp Model 265E electronic calculator, the linear regression line for the standard curve was calculated and used to determine the E₃ concentration in all unknown samples. The difference between the results for the zero tube (no E₃ added to the tritiated E₃/antiserum incubation mixture) and the water blank (25 μl of distilled water processed instead of urine) was subtracted from each unknown sample. The raw values thus obtained for the unknown samples, read as nano-
grams of E₃ per milliliter, were converted to milligrams of E₃ per milliliter of urine by multiplying the raw values with the factor of 2.961 × 10⁻³ by which we accounted for sample volume as well as dilutions. Multiplication by the total 24-h urine volume (in milliliters) yielded the entire 24-h urinary E₃ excretion in mg per 24 h. Three 24-h control urines obtained from pregnant women containing 10, 25, and 50 mg of E₃, respectively, as determined by our colorimetric urinary E₃ assay, were analyzed in each RIA. The results for all unknown samples in each RIA were normalized by multiplication with a correction factor determined by dividing the expected control urine values by their actual results observed in that particular RIA. The average correction factor was 1.

Results

Standard Curve

About 60 to 65% of the tritiated E₃ incubated was bound to the antibody when no unlabeled E₃ was added to the tritiated E₃/antiserum incubation mixture (B₀) and about 10% of the tritiated E₃ was bound when 25 μl of an E₃ standard solution was added that contained 50 ng of E₃ per milliliter. Standard curves were prepared by plotting the ratio of F/B vs. E₃ concentration (Figure 1). All standard curves were linear between 0.78 and 50 ng of E₃ per milliliter with correlation coefficients greater than 0.99. When logit transformation (5) was used to construct standard curves, plots of logit B/B₀ vs. log E₃ concentration were also linear between 0.78 and 50 ng of E₃ per milliliter (Figure 2). At the dilutions used in this procedure, E₃ concentrations ranging from 2.3 to 148 mg/liter of urine can be measured without altering the urine aliquot used for hydrolysis.

Analytical Variables

Precision. The intra-assay coefficient of variation was determined by assaying 10 aliquots from each of three urine samples averaging 7.1, 27.6, and 49.9 mg of E₃ per liter. The intra-assay coefficients of variation calculated from the results of these determinations were 8.2, 6.2, and 6.7%, respectively. Day-to-day precision was assessed by assaying, on each of two days, aliquots of 100 different urines from women found to excrete 6.2 to 81 mg of E₃ per 24 h. The inter-assay coefficient of variation for these 100 duplicate measurements, done in 17 different RIA’s by four different technologists, was 9.98%.

Accuracy. This was assessed by assaying 100 urine samples containing between 5 and 65 mg per 24 h volume by this RIA procedure as well as by our colorimetric procedure, which was shown to be highly accurate (6). Comparison of urinary E₃ determined by colorimetry (x) with that assayed by RIA (y) yielded the regression equation y = 0.992x + 1.28 and a correlation coefficient r = 0.953 (Figure 3). These data indicate that the results obtained with this RIA for urinary E₃ are virtually identical with those measured by our colorimetric technique.

Specificity. The specificity of this RIA depends on the specificity of the antiserum used; ours cross-reacted as described under Antiserum. We saw no cross-reaction with neutral steroids. As both estriol-3-sulfate and estriol-3-glucosiduronate are hydrolyzed to E₃, neither one of these two cross-reacting E₃ conjugates will interfere.

Sensitivity. Using the 25-μl urine aliquot and the subsequent dilutions suggested in this RIA procedure, we could measure 2.3 mg of E₃ per liter of urine reliably. Owing to the relative and absolute abun-
dance of $E_3$ in pregnancy urine, sensitivity does not appear to be at all a limiting factor.

Interfering substances. Glucose added to urine aliquots to concentrations of 20 g per liter had no influence on results of this RIA.

The effect of methenamine mandelate, a urinary tract antiseptic, on this procedure was assessed as follows. A male volunteer ingested 8 g of methenamine mandelate daily (twice the recommended dose) for three days and collected urine during the third day. Urine aliquots from seven women in late pregnancy were diluted with equal volumes of urine collected from the male volunteer before and on the third day of methenamine mandelate intake. All samples were assayed by RIA as well as by our colorimetric procedure. As depicted in Table 1, the RIA results were essentially unchanged whether or not the added male volunteer’s urine contained methenamine mandelate. However, colorimetric $E_3$ determination produced virtually zero readings and 0% recovery of the tritiated estriol-16-glucosiduronate used as internal standard (6) when the urinary tract antiseptic was present. These data indicate that methenamine mandelate does not interfere in this RIA.

### Table 1. Effect of Methenamine Mandelate (MM) on Urinary Estriol Assays for Seven Subjects

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$^a$ Figures are mg estriol per 24-h urine.  
$^b$ With MM, there was no color and virtually 0% recovery of tritiated estriol-16-glucosiduronate used as internal standard.

Normal Values

We conclude from the agreement between the results measured by our colorimetric $E_3$ assay and by this RIA that the normal values obtained with this method agree with those determined by our colorimetric procedure in uncomplicated pregnancies (Figure 4).

### Discussion

The method presented in this paper has been shown to be specific, precise, and accurate. One technician using appropriate semi-automatic pipetting equipment can easily complete 50 triplicate urinary $E_3$ determinations in 8 h.

Extraction has been eliminated, but acid hydrolysis is still required, because virtually all the $E_3$ excreted in the urine is conjugated, predominantly as estriol-16-glucosiduronate and estriol-3-glucosiduronate but also as estriol-3-sulfate and estriol-3-sulfate-16-glucosiduronate (7). Only estriol-3-sulfate and estriol-3-glucosiduronate cross-react with the antiserum (4), but all four $E_3$ conjugates are readily hydrolyzed to unconjugated $E_3$ and quantitated by RIA after neutralization of the acid hydrolysate.

Davis and Loriaux (8) recently described a simple RIA for urinary $E_3$ in highly diluted, unhydrolyzed pregnancy urine. This assay is more convenient than the one described in this paper because acid hydrolysis and neutralization are eliminated. Their procedure, however, measures almost exclusively estriol-16-glucosiduronate instead of the sum of all urinary estriol conjugates determined in our assay. About two-thirds of the $E_3$ produced is excreted as estriol-16-glucosiduronate (7). The ratio of urinary estriol-16-glucosiduronate to the sum of all urinary estriol conjugates depends upon estriol metabolism and excretion. It may vary between patients, and from day-to-day in any one patient. We concluded that urinary
E₃ results might vary less if total urinary E₃ was measured instead of estriol-16-glucosiduronate.

Glucose and methenamine mandelate, which are known to interfere with acid hydrolysis in conventional urinary E₃ assays (9), have no effect in this procedure, most likely because the 25-μl urine aliquot is diluted 60-fold before acid hydrolysis. Comparison of results for urinary E₃ obtained by our RIA procedure with those observed by our colorimetric technique—in which tritiated estriol-16-glucosiduronate is used as internal standard and solvent partitions, methylation, and chromatography are used for specificity (6)—indicate that this urinary E₃ RIA is virtually accurate without correcting for losses that might occur during acid hydrolysis. This may be explained by the possibility that other estrogens present in the hydrolysate, particularly 6-oxo-estriol, may increase the RIA estimate of E₃, compensating for such losses.

In contrast to the dextran-coated charcoal separation procedure, the ammonium sulfate technique is not particularly affected by time or temperature, eliminating the need for low-temperature equilibrium, a refrigerated centrifuge, and rapid centrifugation to separate supernate and precipitate. Statistically accurate counts are obtained more rapidly when unbound tritiated E₃ in the ammonium sulfate supernate is counted instead of antibody-bound tritiated E₃ present in the supernatant fluid after dextran-coated charcoal separation. Furthermore, unbound tritiated E₃ is easily extracted from the ammonium sulfate supernate by the toluene scintillation fluid. Expensive solubilizers used to dissolve aqueous material in toluene, and which reduce counting efficiency, are not required. Under the conditions described in this paper, counting time for a ±3% counting error averaged 1 min per vial.

Utilization of ¹²⁵I-labeled instead of tritiated E₃ as radioligand would further decrease the cost of this RIA by eliminating expenses for liquid scintillation counting vials and liquid. Many laboratories which have gamma counters but no liquid scintillation counter could perform this technique if ¹²⁵I-labeled E₃ were used.

As described in this paper, calculation can be done without electronic data-handling equipment. However, use of such equipment in combination with appropriate programs utilizing logit transformation (5) would save time and eliminate miscalculation caused by human error or use of inappropriate standard curves or parts thereof. We consider it essential to use in each assay a low, medium, and high urinary E₃ pool that has been standardized by a reliable method. Deficiencies in any part of the assay, including hydrolysis and data handling, will be reflected by a deviation of the results for the pools. Normalizing all assay results by multiplication by the average actual pool result and division by the average expected pool result provided clinically reliable data in some 4500 individual urinary E₃ RIA’s performed during the past 12 months.

In summary, this method is an accurate, precise, specific, convenient and economical technique for measuring urinary E₃ in pregnancy. It is a considerable improvement of our previous urinary RIA procedure for E₃, which, because of the use of an unspecific antiserum, had to include ether extraction and solvent partition before RIA (10).

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