Enzyme Immunoassay of Estriol in Pregnancy Urine

Marita K. Korhonen,1 Karl O. Juntunen,2 and Ulf-Håkan Stenman1

We describe an enzyme immunoassay for determination of total estriol in urine. Estriol covalently bound to horseradish peroxidase is used as tracer, and free and bound hormone are separated by precipitation with polyethylene glycol. The method can be used with either acid hydrolysis at 100 °C for 30 min or enzyme hydrolysis at 50 °C for 40 min; results by the former procedure are about 15% lower than results by the latter. Results were practically identical when we compared the enzyme immunoassay with a radioimmunoassay, using the same antiserum and method of hydrolysis. The day-to-day CV for three different concentrations was 10.7–12.0%, the within-series CV 6.8–8.6%. The additional time required for the enzyme reaction is compensated for by the rapid measurement of light absorbance. Thus this method is faster than radioimmunoassay when more than 25 samples are to be assayed.

Additional Keyphrases: steroids • total estriol • acid and enzyme hydrolysis • comparison with RIA • fetal status

Chemical assay of maternal urinary estriol is one of the most common indexes to fetal well-being (1–3). Radioimmunoassay (RIA) of plasma estriol is used for the same purpose (4–6), but it is still unclear which type of sample is the more useful (7). Nevertheless, because of its specificity, RIA is replacing earlier used chemical methods for urinary estriol (3, 8).

We describe an enzyme immunoassay (EIA) that combines the specificity and sensitivity of RIA with the simplicity of chemical determinations. For small laboratories the method has the advantage of not requiring expensive instrumentation. If a large series of samples is being assayed, results are obtained more rapidly than by RIA. Further advantages are: the lack of radioactive waste and the stability of the reagents. The method is based on a recently described procedure for determination of immunoreactive estriol in plasma (9).

Materials and Methods

Samples

Twenty-four-hour urine specimens were obtained from pregnant women and stored at 4 °C if assayed the same day or at −20 °C for longer periods. Ethanol, 8 mL, and chloroform, 2 mL, were added to the urine as preservatives.

Reagents

Assay buffer, tris(hydroxymethyl)methylamine (Tris), 0.2 mol/L, pH 7.0, containing bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, MO 63178), 1 g/L.

Phosphate buffer, sodium phosphate, 0.1 mol/L, pH 6.1.
Neutralizing buffer, Tris, 0.2 mol/L, pH 7.6, containing 1 g of bovine serum albumin per liter.
β-Glucuronidase (EC 3.2.1.31), from Helix pomatia (code 65602; Institute Pasteur Production, Paris, France), 200 kU/L.
Antiserum against estriol-6-carboxymethylxime–bovine serum albumin was raised in rabbits. The cross reactivity of the antiserum has been described (9). The antiserum was diluted in assay buffer to bind 60–80% of the enzyme-labeled estriol in the absence of unlabeled estriol.

Enzyme-labeled estriol was prepared by coupling 6-keto-estriol-6-carboxymethylxime to horseradish peroxidase (EC 1.11.1.7) by the mixed-anhydride method (9). The resulting complex was fractionated on Sephadex G-100 and the fractions with highest immunoreactivity and enzyme activity were used in the assay. This preparation was used at a dilution producing an absorbance at 463 nm of 0.8–1.0 when incubated with substrate under the conditions described.

Estriol standards: Stock solutions of estriol (E. Merck A.G., Darmstadt, F.R.G.) were prepared in ethanol. Final standards were diluted daily from ethanol stock solutions into the appropriate buffer. The final estriol concentrations of the standards used in EIA were: 0, 2.5, 5, 10, 20, and 50 nmol/L. Different standards are used for enzyme and acid hydrolysis. For enzyme hydrolysis the standards are diluted in substrate buffer and subjected to enzyme hydrolysis. For acid hydrolysis the standards are diluted in neutralizing buffer to which is added 120 μL of HCl (2 mol/L) per 10 mL. This makes the composition of the buffer in standards and samples identical.

Precipitant: Dissolve 150 g of polyethylene glycol 6000 (Carbowax 6000; Fluka A.G., Buchs, Switzerland) in 1 L of distilled water.

Enzyme substrate (9): Dissolve 100 mg of 5-aminosalicylic acid (Fluka A.G.) in 150 mL of phosphate buffer and add 10 μL of hydrogen peroxide (8.8 mol/L).

Dextran-coated charcoal: In the RIA method, coated charcoal is used for separation. Add 3 g of washed and dried Norit A charcoal and 300 mg of Dextran T 70 (Pharmacia) to 1 L of phosphate-buffered (0.02 mol/L, pH 7.4) saline (0.1 mol/L).

Assay Procedures

Enzyme hydrolysis: In glass test tubes, dilute standards and urine samples, in duplicate, 500-fold with phosphate buffer. Add 10 μL of properly diluted β-gluconidase to 2 mL of diluted urine samples and standards, then incubate at 50 °C for 40 min. Dilute hydrolyzed samples 10-fold with assay buffer and use 0.2 mL for the assay.

Assay of β-glucuronidase: The working dilution of β-gluconidase should be determined for each batch of enzyme. Gluconidase diluted twice- to 20-fold is used to hydrolyze diluted late pregnancy urine. (We tested 10–, 20–, 30–, 40–, and 60-min hydrolysis intervals.) The amount of estriol released is determined by EIA. Use the highest dilution giving maximal hydrolysis at 50°C in 30 min.

Acid hydrolysis: Fifty microliters of urine is diluted to 3 mL.
with 2 mol/L HCl in glass test tubes and incubated in a water bath at 100 °C for 30 min (3). Thirty microliters of the hydrolyzed sample is further diluted to 2.5 mL with neutralizing buffer, and 0.2 mL of the solution is used for the assay.

**EIA conditions:** Add 200 μL of hydrolyzed samples and standards, 0.1 mL of estriol-peroxidase conjugate, and 0.1 mL of antibody solution into 12 × 70 mm disposable polystyrene test tubes. Include a blank containing buffer instead of antiserum in each test series. After a 60-min incubation at room temperature, add 50 μL of human serum and 1.0 mL of polyethylene glycol solution. Mix the contents of the tubes on a vortex-type mixer and centrifuge the tubes for 20 min at 2000 × g at room temperature or at 4 °C. The latter temperature results in a heavier precipitate and somewhat higher absorbance readings. Decant the supernatant fluid and dissolve the precipitate in 0.4 mL of isotonic saline, add 1 mL of enzyme substrate, mix the contents, and incubate the tubes for 60 min at room temperature. Stop the reaction by adding 0.2 mL of 1 mol/L NaOH. Measure the absorbance at 463 nm vs the blank (we used an automatic LKB 2074 photometer). Construct standard curves by plotting the absorbance at 463 nm vs the concentration of estriol in the standards (Figure 1). Use separate standard curves for enzyme and for acid hydrolysis.

**RIA:** The RIA method of Anderson and Goebelsmann (3) is used with slight modifications. The antiserum is the same as used in the enzyme immunoassay. In this assay urine samples are hydrolyzed 200-fold in 2 mol/L HCl. Fifty microliters of neutralized sample at a final dilution of 2500-fold is incubated with 50 μL of tritiated estriol (The Radiochemical Centre, Amersham, U.K.) and 100 μL of antiserum (3). Instead of using ammonium sulfate, we separate bound and free antigen with 1 mL of dextran-coated charcoal.

**Results**

**Hydrolysis conditions:** Two different batches of glucuronidase were used. One gave maximal hydrolysis in 30 min at a 10-fold dilution, the other batch could be diluted only fourfold. The enzyme hydrolysis had a noticeable effect on results of the enzyme immunoassay. When the standards were subjected to enzyme hydrolysis, absorbance readings were lower than when the standards were simply diluted in the same buffer as the sample. We did not investigate this effect thoroughly, but it was mainly due to interference by the glucuronidase preparation with the activity of the peroxidase.

### Table 1. Coefficients of Variation for EIA and RIA

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<th>EIA, acid hydrolysis</th>
<th>RIA, acid hydrolysis</th>
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*Three urine samples with estriol concentrations of (A) 12, (B) 35, and (C) 100 μmol/L were assayed. The CV was calculated from results obtained by hydrolyzing and assaying 10 aliquots of each sample.

The CV was calculated from the mean values obtained for the same urine sample in 10 different assays. The samples are the same as those used for the within-assay evaluation.
Therefore we routinely subjected the standards to the same enzyme hydrolysis as the samples.

For acid hydrolysis we adopted the procedure of Anderson and Goebelsmann (3). The standards were not subjected to hydrolysis, but they were made up in a buffer that had the same final salt concentration as the sample.

Comparison of EIA and RIA: We assayed 89 samples from women at different stages of gestation by EIA and RIA, using acid hydrolysis in both assays (Figure 2). The correlation coefficient was 0.96, and $y_{\text{RIA}} = 0.99 x_{\text{EIA}} - 1.87 \mu\text{mol/L}$.

Comparison of enzyme and acid hydrolysis: We assayed 47 samples by EIA with enzyme hydrolysis and RIA with acid hydrolysis (Figure 3). The correlation coefficient was 0.95, and $y_{\text{RIA}} = 1.18 x_{\text{RIA}} - 0.76 \mu\text{mol/L}$.

Intra-assay variation: The within-assay variation (Table 1) was studied by determining the CV for 10 aliquots, each at three concentrations: 12, 35, and 100 $\mu\text{mol/L}$. No significant differences between the different assays were observed.

Inter-assay variation: The between-assay CV was calculated by determining aliquots of the same three samples on different days. As evident from Table 1, all assays gave acceptable results.

Assay range: The EIA was optimized for assay of urinary estriol in pregnancy. Therefore the range of the standard curve was restricted to 2.5–50 $\mu\text{mol/L}$, corresponding to urinary concentrations of 12.5–250 $\mu\text{mol/L}$. However, this does not represent the maximum sensitivity of the EIA method (9).

Efficiency: In the estriol EIA, the steps up to the separation are very similar to those of RIA. In the EIA an additional 1-h is required, for the enzyme reaction. Therefore the measurement of radioactivity in the RIA can be started about 1 h before measurement of the absorbance in EIA. If a counting time of 1 min is used, the total assay time for the RIA will be similar to that of the EIA when 20–25 samples are assayed. With more samples or a longer counting time the EIA will be more rapid.

Discussion

EIA has earlier been used to determine total estrogens in urine (10) and plasma (11) and immunoreactive estriol in plasma (9). In some EIA methods (10, 11) several washings have been considered necessary, to eliminate nonspecific interference from the sample. This was not a problem with the present EIA method, as evidenced by the very close correlation with the RIA method. Apparently the high dilution used eliminated interference by urinary constituents.

Acid hydrolysis is known to cause destruction of estriol when the urine contains methenamine mandelate or above-normal concentrations of glucose. This problem can be eliminated by diluting the urine before hydrolysis (3, 8). We adopted this method of acid hydrolysis. Thus we expected to get the same results by enzyme and acid hydrolysis. However, the results obtained by acid hydrolysis were somewhat unexpectedly about 15% lower than those obtained by enzyme hydrolysis. We carefully excluded the possibility that this difference was due to the observed interference by the gluconidase with the EIA. The same difference between enzyme and acid hydrolysis was observed when quantitation was performed by RIA (data not shown). This may indicate that estriol is incompletely recovered from urinary estriol conjugates by acid hydrolysis. This interpretation is supported by data of Jawad et al. (8). They obtained 85% recovery when hydrolyzing a mixture of estriol-3-sulfate and estriol-16-gluconide with acid, the same recovery we observed in our study.

Our data seem to indicate that enzyme hydrolysis gives the most accurate results. However, results obtained by RIA in combination with acid hydrolysis correlate well with results obtained by earlier used chemical methods (3, 8). This is true for the EIA method, too, because it gives the same results as the RIA method, when acid hydrolysis is used.

Thus the choice between RIA and EIA will in most cases depend on the equipment available. If an automated photometer is used, the EIA is more rapid than the RIA, when more than 25–30 samples are being processed daily. In laboratories lacking isotope equipment the EIA offers the advantage of simplicity, sensitivity, and specificity not obtained by chemical methods.

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References


