Improved Radioimmunoassay of Urinary Estriol

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We report a rapid double-antibody radioimmunoassay for urinary estriol. Advantages over other current methods include: (a) 30-min hydrolysis; (b) total incubation time, 55 min; (c) assay unaffected by urinary glucose; (d) no degradation of estriol evident during hydrolysis; (e) superior (85%) analytical recovery of estriol conjugates; (f) linear standard curve by logit-log extrapolation; (g) good correlation (r = 0.83) with total estrogen determination by a generally accepted colorimetric method; (h) only 20 μL of urine required; and (i) the detection range is 1.9 to 100.5 mg/24-h urine.

Additional Keyphrases: steroids · fetal status · Kober colorimetry compared

Determination of estriol, the major urinary estrogen produced by the feto-placental unit (1–7), is important in the management of high-risk pregnancies (3, 4, 8). Estriol is almost entirely conjugated and urinary excretion is the major pathway for elimination of these conjugates (9, 10). Their proportion in urine has been estimated (11) to be: estriol-16α-glucuronide, 68%; estriol-3β-glucuronide, 23%; estriol-3-sulfato-16α-glucuronide, 6.5%; and estriol-3-sulfate, 2.5%. Levitz et al. (12) determined that estriol-16α-glucuronide represents 70–80% of urinary estriol.

In the past three decades, colorimetric, fluorometric, and gas-liquid chromatographic methods have been used to determine urinary estriol (13). More recently, radioimmunoassays (RIA) have been developed for total urinary estriol (14) and even for specific estriol conjugates (15–17).

The purpose of this study was to develop an improved RIA protocol for estriol that is unaffected by glucose, with use of commercially available and easily accessible products. This protocol was designed for adoption by any clinical laboratory geared for RIA procedures, such that a skilled technologist could perform the test on 20–30 urine samples and obtain results within 4 to 5 h.

Materials and Methods

Equipment

Centrifuge. We used a refrigerated IEC Model PR-600 equipped with Model IEC-266 horizontal head and six-unit, Model IEC-1028, 28-place carriers.

Gamma scintillation counter, Packard Model CP-3, 600-tube capacity, with Western Union teletype printer.


Electrobalance, Cahn, Model 4400.

Electronic balance, Mettler, Model H.

Diluter-pipetter, Micromedic System, Inc., dual pump, Model 25004.

Eppendorff pipettes.

Dispensing bottle, NML, 2-mL delivery.

pH meter, Beckman, Zeromatic.

Reagents

All reagents were analytical grade. Estrone (E1, batch 1758) and estradiol-17β (E2, batch 1833) were obtained from Steraloids, Inc., Pawling, NY 12564. Estriol (E3, lot E-1253), sodium estriol-3β-glucuronide (lot E-2002), estriol-16α-glucuronide (lot E-1877), and human serum albumin (HSA, Fraction V, A-2386) were obtained from Sigma Chemical Co., St. Louis, MO 63178.125I-Labeled estriol (lot 72424), rabbit anti-estriol antibody (lot 1607B), and ovine anti-rabbit antibody (lot 6050E6) were from Nuclear Medical Systems, Inc., Newport Beach, CA 92660.

The water used was demineralized, filtered through charcoal, and doubly glass-distilled. Estrone, estradiol, and estriol stock standards were prepared in absolute ethanol to give a concentration of 500 mg/L. Estriol-3β-glucuronide and estriol-16α-glucuronide stock standards were prepared in phosphate-buffered saline in a concentration of 500 mg/L. The saline solution, 0.16 mol/L, pH 7.4 ± 0.1, was prepared by dissolving 5.52 g of NaH2PO4-H2O, 7.0 g of NaCl, and 1.0 g of sodium azide in 900 mL of water, adjusting the pH to 7.4, and then diluting to 1 L. All stock standards were stored at ~40 °C. In addition, we also tested our procedure with a commercial estradiol standard (TEKIT-TPE), 200 μg/vial, prepared by Oxford, Foster City, CA 94404. For hydrolysis and neutralization we used hydrochloric acid, 6 mol/L, and sodium hydroxide, 6 mol/L, respectively.

Samples

The concentration of estriol was determined concurrently by colorimetry and RIA in urine from 112 women in their third trimester of pregnancy. Pooled urine from 10 normal men was used as diluent in preparation of standards, to simulate the matrix of the patients’ samples.

Procedure

Hydrolysis. We placed 20 μL of a well-mixed 24-h urine specimen in a 16 × 125 mm Pyrex glass tube, followed by 2 mL of phosphate-buffered saline and 1 mL of HCl, 6 mol/L. The contents of all the tubes were vortex-mixed and their tops sealed with double-layered Parafilm. Then, we pricked a pinhole into the Parafilm and placed all the tubes in a 100 °C water bath for 30 min, then in an ice bath for 2–5 min. We removed the Parafilm caps, neutralized the contents of each with 1.0 mL NaOH solution (6 mol/L), vortex-mixed the contents of each tube, and diluted a 0.1-mL aliquot from each to 1.0 mL with phosphate-buffered saline.

Radioimmunoassay. Table 1 summarizes the assay protocol. We performed all assays in duplicate and established a standard curve with each assay. We subtracted the average count for the blank from the counts for all the other assays.

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2 Received June 22, 1978; accepted Oct. 20, 1978.
Table 1. Protocol for RIA of Urinary Estriol

<table>
<thead>
<tr>
<th>Assay tube</th>
<th>Std. Hydrolyzed sample or control</th>
<th>PBS b</th>
<th>L HSA c 5 g/L</th>
<th>Anti-estriol antibody</th>
<th>125I Estriol</th>
<th>Pptg. antibody</th>
<th>Cold dist. water mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>—</td>
<td>100</td>
<td>—</td>
<td>2.0</td>
</tr>
<tr>
<td>Maximal binding</td>
<td>—</td>
<td>—</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>2.0</td>
</tr>
<tr>
<td>Total count</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>— I</td>
<td>100 II</td>
<td>200 III</td>
<td>IV</td>
</tr>
<tr>
<td>Standards</td>
<td>100</td>
<td>—</td>
<td>—</td>
<td>100</td>
<td>100</td>
<td>200</td>
<td>2.0</td>
</tr>
<tr>
<td>Controls</td>
<td>—</td>
<td>100</td>
<td>—</td>
<td>100</td>
<td>100</td>
<td>200</td>
<td>2.0</td>
</tr>
<tr>
<td>Patients</td>
<td>—</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>200</td>
<td>2.0</td>
</tr>
</tbody>
</table>

a All assay tubes in ice bath. b Phosphate-buffered saline. c Human serum albumin.

At I, vortex all tubes & incubate for 10 min at 37 °C, then transfer to ice bath. At II, vortex all tubes & incubate for 30 min at 37 °C, then return to ice bath. At III, vortex all tubes & incubate for 10 min at 37 °C, then return to ice bath for 5 min. At IV centrifuge (2500 × g, 10 min). Aspirate supernate & count precipitate for at least 1 min/tube.

tubes, to compensate for the nonspecific binding, and reduced the raw data with a programmable calculator, using a logit-log linear transformation program. We plotted the standard curve as fraction bound/maximal binding (B/B0) vs. log concentration on logit-log paper (semi-log paper is optional for those lacking a computerized system). The dilution factor of a hydrolyzed specimen is 2010:

\[
\text{dilution factor} = \frac{\text{total volume of hydrolysis mixture}}{\text{volume of hydrolyzed sample}} \times \text{dilution before assay}
\]

\[
= \frac{4020}{20} \times 10 = 2010 \text{ fold}
\]

We calculated the estriol concentration of a 24-h specimen as follows:

\[
\text{Conc.}, \frac{\mu g}{L} = \frac{\text{Concentration} \times \text{dilution factor}}{\text{dilution before assay}}
\]

\[
\times \frac{1000 \mu g}{\text{mg}} \times \frac{1}{1000} \text{mL} = \frac{\text{mg}}{24 \text{ h}}
\]

**Results**

**Accuracy of standard.** We diluted the estriol stock standard in phosphate-buffed saline to concentrations of 30.0, 15.0, 7.5, 3.75, 1.88, and 0.94 μg/L, which extrapolates favorably in comparison with a commercially available standard (Oxford TEKIT-TPE) diluted in phosphate-buffed saline to final concentrations of 20.0, 10.0, 5.0, 2.5, and 1.25 μg/L (Figure 1).

**Precision.** We assessed the intra-assay coefficient of variation (≤5.01%) by assaying 14 aliquots from each of three urine control samples, and determined the inter-assay coefficient of variation (≤5.22%) by assaying the same urine control samples on seven different days (Table 2).

**Sensitivity.** With use of a 20-μL aliquot of urine diluted as described, the minimal and maximal concentrations of estriol detected were 1.9 mg/24 h and 100.5 mg/24 h, respectively.

**Effect of hydrolysis on estriol.** We prepared two sets of estriol in normal male urine to concentrations of 60.3, 30.15, 15.075, 7.538, 3.769, and 1.884 mg/L and took one set through the hydrolysis process; the other was diluted in phosphate-buffered saline in an equivalent fashion so as to give final estriol concentrations of 30.0, 15.0, 7.5, 3.75, 1.88, and 0.94 μg/L. We detected no evidence of estriol destruction by the hydrolytic procedure (Figure 2).

**Effect of glucose.** To check the possible effect of glucose on this RIA protocol, we prepared estriol standards in pooled normal male urine with and without 100 g/liter glucose to give the following estriol concentrations: 40.2, 20.1, 10.05, 5.025, and 2.5125 mg of estriol per liter. We took these samples through the hydrolysis procedure to give a final estriol concentration of 20, 10, 5, 2.5, and 1.25 μg/L. The recoveries (Table 3) indicated no significant difference in the two systems. To increase the size of the residual antigen–double antibody complex, we conducted the same experiment in the

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Table 2. Mean, SD, and CV, Inter-assay and Intra-assay, for Three Control Samples

<table>
<thead>
<tr>
<th>Control no.</th>
<th>Mean ± SD, μg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.99 ± 0.20</td>
<td>5.01</td>
</tr>
<tr>
<td>2</td>
<td>8.48 ± 0.30</td>
<td>&lt;5.00</td>
</tr>
<tr>
<td>3</td>
<td>18.94 ± 0.42</td>
<td>&lt;5.00</td>
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<tr>
<td>Inter-assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.02 ± 0.21</td>
<td>5.22</td>
</tr>
<tr>
<td>2</td>
<td>8.51 ± 0.35</td>
<td>&lt;5.00</td>
</tr>
<tr>
<td>3</td>
<td>18.75 ± 0.65</td>
<td>&lt;5.00</td>
</tr>
</tbody>
</table>

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Fig. 1. Comparison of assay estriol standard and the Oxford TEKIT-TPE standard

Fig. 2. Effect of acid hydrolysis on estriol
<table>
<thead>
<tr>
<th>Table 3. Fraction of Hydrolyzed Urinary Estriol Standards Bound in Presence and Absence of Glucose, 100 g/L, and Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Estriol, µg/L</strong></td>
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<tr>
<td></td>
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<td></td>
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<tr>
<td>1.25</td>
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<tr>
<td>2.50</td>
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<tr>
<td>5.00</td>
</tr>
<tr>
<td>10.00</td>
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<tr>
<td>20.00</td>
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<td></td>
</tr>
<tr>
<td><strong>Glucose plus albumin, 5 g/L</strong></td>
</tr>
<tr>
<td>1.25</td>
</tr>
<tr>
<td>2.50</td>
</tr>
<tr>
<td>5.00</td>
</tr>
<tr>
<td>10.00</td>
</tr>
<tr>
<td>20.00</td>
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<td></td>
</tr>
</tbody>
</table>

presence of human serum albumin, 5 g/L (100 µL per tube). This decreased the relative binding (B/B0) and B/B0, giving an increase of the sensitivity of the logit-log extrapolation. The results (Table 3) demonstrate that the assay is free of glucose interference.

**Cross reactivity of the anti-estriol antibody with estrone and estriadiol.** We diluted estrone and estriadiol stock solutions to give concentrations of 100.5, 50.25, 25.125, 12.563, 6.281, and 3.109 mg/L in normal male urine, and the final concentrations after hydrolysis: 50.0, 25.0, 12.5, 6.25, 3.12, and 1.56 mg/L. The anti-estriol antibody shows direct specificity for estriol with negligible (<0.1%) affinity for the other two major urinary estrogens, estrone and estriadiol (Figure 3).

**Cross reactivity of the anti-estriol antibody with major estriol conjugates before and after hydrolysis.** Estriol 16α-glucuronide and 3β-glucuronide together constitute about 90% of the conjugated estriol in urine. We designed this experiment to check the need for hydrolysis and the analytical recovery of these conjugates. We prepared estriol and the above-mentioned glucuronides in normal male urine to give the following concentrations: 60.3, 30.15, 15.75, 7.54, 3.77, and 1.88 mg/L. Of each, we prepared two tests; one we took through the hydrolysis protocol; the other was similarly diluted in phosphate-buffered saline to the final estriol molar equivalence summarized in Table 4.

The non-hydrolyzed 16α-glucuronide showed negligible (<0.1%) cross reactivity with the anti-estriol antibody, while the hydrolyzed conjugate exhibited 85% cross reactivity, demonstrating the necessity for hydrolysis (Figure 4). The estriol 3β-glucuronide showed 47% cross reactivity with the antibody before hydrolysis, 85% after (Figure 5).

**Method comparison.** We subjected to our RIA protocol 112 urine samples routinely assayed by the Oxford TEKIT-TPE kit method that utilized the modified (18) Kober reaction (Figure 6). The urinary estriol determined by RIA correlated directly with that determined colorimetrically.

**Discussion**

Our data show our RIA to be accurate, precise, sensitive, and specific—all important factors in monitoring feto-placental function. The anti-estriol antibody must distinguish estriol from other urinary estrogens such as estradiol and estrone whose precursors are not necessarily of fetal origin. The sensitivity of this assay is well below the established criteria for estriol excretion during normal and jeopardized pregnancy (1).

The principal feature of our RIA is that it can be done rapidly without compromising its accuracy, precision, specificity, and sensitivity. A properly trained technician, using an appropriate automatic or semi-automatic pipetting apparatus and an adequate data-reduction system, can usually complete assays of 20–30 specimens and obtain results within 4 to 5 h. Because of the specificity of the primary antibody, an extraction step is not required. Hydrolysis of the estriol
Protocols for estriol-16α-glucuronide have been developed, but they assume the uniform presence of this glucuronide in all urine samples (15–17). The spectrum of the relative concentration of these glucuronides may vary throughout the third trimester of pregnancy (17). Furthermore, in the case of some diabetic pregnancies, the ratios of one conjugate to another are drastically altered (21). We evaluated only the 3β-glucuronide and the 16α-glucuronide conjugates of estriol because they comprise more than 90% of the estriol conjugates in the urine of pregnant patients. The antibody used is probably directed to the D ring of the estriol molecule, explaining the discrepancy between the affinities for non-hydrolyzed estriol-3β-glucuronide and estriol-16α-glucuronide.

References