Direct Radioimmunoassay for Unconjugated Estriol in Pregnancy Serum, with Use of a Radioiodinated Derivative of Estriol

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We describe a rapid and direct $^{125}$I-based radioimmunoassay for quantification of unconjugated (free) estriol in pregnancy serum. Estriol in serum is adsorbed onto a small column of Sephadex, thereby allowing its separation from proteins and interfering materials. A radioiodinated derivative of estriol (6-ketoestriol-6(o-carboxymethyl)oxime-$[^{125}$I]tyrosine methyl ester) is added to the column, followed by a limiting amount of antiserum. After incubation, the antibody-bound hormone is separated by a buffer wash. The assay exhibits satisfactory recovery and parallelism, and the intra- and inter-assay coefficients of variation are <10%. The values obtained by using the assay correlate well ($r = 0.97$) with those from a comparison method in which solvent extraction and chromatographic purification are used.

Additional Keyphrases: $^{125}$I-based procedure • Sephadex • monitoring fetal status • hormones

According to experimental and clinical evidence, the feto-placental unit is responsible for the synthesis of estriol. In uncomplicated pregnancies, the production of estriol increases steadily throughout the last trimester; in pregnancies complicated by "placental insufficiency," the synthesis of estriol decreases rapidly (1). The most commonly used method for monitoring estriol synthesis (as an index to assess fetal stress) has been to measure estriol and estriol conjugates in a 24-h urine specimen. However, changes in renal clearance (2, 3) can make the results of those determinations suspect. Additionally, the sampling and handling of urine specimens and the 24-h delay in feto-placental assessment point to other serious disadvantages of this method. In recent years, investigators have evaluated the usefulness of measuring unconjugated estriol in pregnancy plasma as an alternative to the urinary assay. Sample collection is simplified and any deterioration in fetal status is more rapidly reflected in serial plasma specimens than in 24-h urines (4). In addition, unconjugated estriol in plasma is not affected by diurnal fluctuations or by the renal or positional changes that affect urinary estriol values (5, 6).

The reported (7–11) assays for unconjugated estriol in plasma are rather cumbersome and time-consuming. In all, solvent extraction is used, and most require chromatographic separation of estriol before assay.

Recently this laboratory reported a novel approach to the separation and assay of free estriol (12). Here a column containing Sephadex is used to adsorb and separate estriol from plasma proteins and interfering materials. A radioimmunoassay in which tritiated estriol is used is then conducted in the column to quantify the hormone. Although this approach was shown to simplify the plasma estriol assay, the use of a tritium-labeled radioligand limits this assay to only those laboratories possessing liquid scintillation equipment.

To expand the application of the method, we modified the assay to allow use of a radio-iodinated derivative of estriol, 6-ketoestriol-6(o-carboxymethyl)oxime-$[^{125}$I]tyrosine methyl ester ($^{125}$I-TME-estriol). The new assay is acceptably sensitive, precise, and accurate for measuring unconjugated estriol in plasma of women in the third trimester of pregnancy.

**Materials and Methods**

**Materials**

Unlabeled steroids. Unlabeled steroids1 were obtained from the following sources: estriol, 16-epiestriol, estradiol-17β, estradiol-17α, estrone, progesterone, testosterone, and DHA (Schwarz/Mann, Orangeburg, N. Y. 10962); estetrol (15α-hydroxyestriol) and 16α-hydroxyestrenone (Storaloids, Inc., Pawling, N. Y.); sodium salt of estriol-3-sulfate (Sigma Chemical Co., St. Louis, Mo. 63178); estriol 16α-glucosiduronate (Steroid Reference Collection, Hempeast, London, England). The purity of estradiol-17α and estriol 16α-glucosiduronate was determined by thin-layer chromatography on Silica Gel Plates F-254 (EM Labs, Inc., Elmsford, N. Y. 10523) with ethanol as the developing solvent. The chromatograms were exposed to iodine vapors to make the separated compounds visible.

Progesterone and testosterone were not evaluated for purity. The purity of the remaining steroids was determined by thin-layer chromatography on both Eastman Chromogram Sheets (No. 13181, Silica Gel with Fluorescent Indi-

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1 Systematic names corresponding to the trivial names given in the text are as follows: estriol, 1,3,5(10)-estratrien-3,16a,17β-triol; 16-epiestriol, 1,3,5(10)-estratrien-3,16β,17β-triol; estradiol-17β, 1,3,5(10)-estratrien-3,17β-diol; estradiol-17α, 1,3,5(10)-estratrien-3,17α-diol; estrone, 1,3,5(10)-estratrien-3,17β-diol; progesterone, 4-pregnen-3,20-dione; testosterone, 4-androstren-3β,17β-diol; DHA, 5-androstren-3β-diol; estetrol, 1,3,5(10)-estratrien-3,15α,16α,17β-tetrol; 16α-hydroxyestrone, 1,3,5(10)-estratrien-3,16α-diol-17-one; sodium salt of estriol-3-sulfate, 1,3,5(10)-estratrien-3,15α,17β-triol-3-sodium salt; estriol 16-glucosiduronate, 1,3,5(10)-estratrien-3,16α,17β-triol-16-glucosiduronate.

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The following solvent systems were used: ethyl acetate/iso-octane, 5/20, 10/15, and 15/10 by vol. Chromatograms were examined for quenching under an ultraviolet light (250 nm) and were developed with iodine vapors, or \( \text{H}_2\text{SO}_4 / \text{ethanol} \) (equal volumes). Estriol 16α-glucosiduronate contained several impurities, including an estimated 2% unconjugated estriol; 16-epiestriol contained at least 10% unconjugated estriol; estriol contained at least 5–10% unconjugated estriol. The remaining steroids were judged to be free of contamination with estriol.

Radionuclide: An estriol derivative, 6-ketoestriol 6-(o-carboxymethyl)oxime-tyrosine methyl ester was labeled with \( ^{125}\text{I} \) by Nuclear Medical Systems, Inc., Newport Beach, Calif. 92660. The material exhibited two major migrating radioactive components on the Silica Gel F-254 Plates. The specific activity of the material was estimated to be 55 kCi/mol. The radionuclide preparation was used without purification. Tritiated estriol [(6,7-\( ^3\text{H} \)) estriol] was purchased from New England Nuclear, Boston, Mass. 02118. It was >94% pure, as determined by thin-layer chromatography on Silica Gel G Plates in three solvent systems \( (12) \). The specific activity of this material was estimated at 51.4 kCi/mol.

Antisera: Anti-estriol antisera were prepared in our laboratory by immunization of three rabbits with an estriol/bovine serum albumin conjugate prepared according to published procedures \( (13) \). Although antisera from all rabbits were evaluated for their anti-estriol activity, the antisera from only one rabbit was used in the development of this assay.

Assay buffer: A gelatin/phosphate buffer (0.11 mol/liter, 1 g of gelatin and 500 mg of \( \text{NaN}_3 \) per liter, pH 7.4) was used to dilute the antisera, label, sample, and standards.

**Clinical specimens**. Serum specimens were obtained from normal and high-risk subjects in their third trimester of pregnancy. Frozen aliquots of these specimens were shipped in solid \( \text{CO}_2 \) to Dr. Uwe Goebelsmann, Dept. of Obstetrics and Gynecology, Women's Hospital, Los Angeles, Calif. for comparison assay \( (11) \).

**Assay Procedure**

The Sephadex G-10 columns and stock standards were prepared as previously described \( (12) \).
Calculations

Results were calculated in terms of fraction bound (FB) or logit FB, by the following formulas:

\[
FB = \frac{\text{cpm in sample or standard}}{\text{cpm in absence of added estradiol}}
\]

\[
\text{Logit } FB = \ln \left( \frac{FB}{1 - FB} \right)
\]

Standard curves were obtained by plotting the logit values for standards vs. the log concentration of standards (in ng/ml, or \(\mu g/\text{liter}\)). The values for the clinical samples were obtained from the logit standard curves and corrected for dilution.

Results and Discussion

During the preliminary phase of this study, we tried to simply replace the tritiated estradiol radioligand with the radiodinated derivative of estradiol. However, as shown in Figure 1, the dose/response curve developed with this iodinated radioligand was less sensitive than the dose/response curve developed with the tritiated radioligand. However, when sodium chloride was removed from the buffer used in the assay, the dose/response curve became almost superimposable upon the dose/response curve generated with the tritiated estradiol radioligand (Figure 1). The effect was interpreted to be the net result of both a weakening in the attractive forces of Sephadex G-10 for the radiodinated estradiol derivative and a concomitant increase in the binding of antibody with radioligand. Such an interpretation is supported by the observations and predictions of Eaker and Porath (14) concerning the increased adsorption of aromatic residues, such as the tyrosine moiety of the estradiol derivative, to Sephadex G-10 in the presence of NaCl.
Table 2. Cross Reactivity of Anti-Estriol Antiserum with Steroids (2-m1 Water Wash)

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross reactivity*%</th>
<th>Reported clinical ratio**</th>
<th>Apparent increase of estradiol concn***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estriol</td>
<td>100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estetrol</td>
<td>19.7d</td>
<td>.1--.2 (15)</td>
<td>1--2</td>
</tr>
<tr>
<td>16-Epiestriol</td>
<td>14.3e</td>
<td>2.5 (16, 17)</td>
<td>14.5</td>
</tr>
<tr>
<td>16α-Hydroxyestrone</td>
<td>1.1(NR)f</td>
<td>1 (17)</td>
<td>none</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>&lt;0.3f</td>
<td>.05---.1 (16)</td>
<td>none</td>
</tr>
<tr>
<td>Estrone</td>
<td>&lt;0.1f</td>
<td>3 (16)</td>
<td>none</td>
</tr>
<tr>
<td>17α-Estradiol</td>
<td>&lt;0.1f</td>
<td>not referenced</td>
<td>none</td>
</tr>
<tr>
<td>Testosterone</td>
<td>NR</td>
<td>not referenced</td>
<td>none</td>
</tr>
<tr>
<td>Progesterone</td>
<td>NR</td>
<td>not referenced</td>
<td>none</td>
</tr>
<tr>
<td>DHA</td>
<td>NR</td>
<td>not referenced</td>
<td>none</td>
</tr>
</tbody>
</table>

* Cross reactivities are determined at logit FB = 0 (FB = 0.5) where
% cross reactivity = mass (ng) estriol / mass (ng) of test compound × 100.
** Ratio to estradiol as unity in term pregnancy sera.
*** Apparent % increase is reported with correction for estradiol contamination.

An additional difference between the tritiated and iodinated radioligands necessitated further revision of the original Sephadex protocol. Whereas a water wash subsequent to the simultaneous addition of tracer and sample effectively removed cross-reacting water-soluble steroids from the sample in the tritium-based assay (12), it also effectively eluted the radiodinated estriol derivative (Figure 2). Consequently, the assay protocol was revised to include a delayed addition of the radioligand, and the gel bed was mixed before the antiserum was added, to distribute the specimen and radioligand uniformly throughout the column for subsequent interaction with the antiserum. With this procedure, estradiol standards in plasma were shown to react like those prepared in buffer (Figure 3).

Comparison of Sephadex Estril Methods

To establish that the modifications made in the scheme produced an assay comparable to the tritium-based system, we compared results of these two assays by evaluating the estradiol concentrations in clinical specimens. As shown in Figure 4, the procedure utilizing the iodinated radioligand gave values for 34 clinical specimens close (r = 0.91) to those obtained with the tritiated Sephadex-column procedure. Although we did not use the same volume of water to eliminate cross-reactants in each of these studies, the modifications we made in order to use the iodinated estriol radioligand had evidently not adversely affected the adsorption of endogenous estril to the Sephadex. The new radioimmunoassay was therefore evaluated for its sensitivity, specificity, and accuracy.

Sensitivity. The sensitivity of this assay is defined as the smallest concentration of estril that gives a response significantly different from the response given by a specimen containing no estril. At the 95% confidence limit the sensitivity of this assay is about 20 ng/liter; thus, the lowest detectable concentration in a specimen diluted 11-fold is about 220 ng/liter, a sensitivity adequate for monitoring unconjugated estril in the serum of women in the third trimester of pregnancy (15).

Precision. Figure 5 shows a mean standard curve (±2 SD) derived from 10 curves run on separate days. The intra- and inter-assay coefficients of variation over the range 0.50--4.04 μg/liter are less than 10% (Table 1).
**Fig. 6. Comparison of estriol values for 22 clinical specimens obtained with the 125I-TME-estriol column radioimmunoassay and a comparison radioimmunoassay**

**Specificity.** The specificity of the 125I-column RIA for serum estriol was evaluated by determining how much cross reactivity with endogenous steroids similar in structure to estriol there was in the assay. The specificity of this assay depends on the use of both a relatively specific anti-serum and a water-wash step to remove possible interfering compounds such as water-soluble conjugated derivatives of estriol from the reaction column. Table 2 shows the cross reactivity found with various steroids when a water wash of only 2.0 ml was used. Only estetrol and estriol-3-sulfate reacted to any extent, and only the latter could cause a substantial apparent increase in the estriol concentration of a serum from a term pregnancy. Because increased volumes of water have little effect upon the retention of estriol to Sephadex, a larger volume of water (8.0 ml) was used to ensure removal of cross-reactants for actual test specimens.

To further test the specificity of the assay, we did a parallelism study. Samples were diluted to various extents and assayed, to determine if they would yield the same result when multiplied by the dilution factor. (An assay that gives parallel results suggests that various components of serum, including binding proteins and potentially cross-reactive steroids, do not interfere in the test.) As shown in Table 3, acceptable results were obtained over a five- to 44-fold dilution range.

**Accuracy.** The accuracy of the assay was evaluated by determining the analytical recovery of exogenous estriol added to clinical specimens. Specimens were assayed before and after exogenous estriol was added in various amounts, and the amount of estriol analytically accounted for was calculated. The specimens included two pooled plasma samples from term pregnancies as well as individual plasma and serum specimens from diabetic and toxemic patients in the last trimester of pregnancy. The results (Table 4) indicate acceptable recovery.

The accuracy of the assay was also tested by a comparative analysis of 22 clinical specimens. The samples were assayed by the 125I-procedure and also by a method in which solvent extraction and chromatographic purification are used before assay (11). Figure 6 gives a comparison of the results from the two assays. The correspondence between the two sets of data is very satisfactory, the correlation coefficient being 0.97 and the standard error of estimate being 1.32 mg/liter.

These results demonstrate that the 125I-based column radioimmunoassay is a valid method for measuring unconjugated estriol in pregnancy serum. This procedure eliminates the need for solvent extraction and the attendant corrections for losses, for chromatographic purifications, and for liquid scintillation counting equipment. In addition, because most of the operations are done in a single reaction chamber—the column—transfers are minimized and the assay thus simplified.

We are grateful to Dr. Uwe Goebelmann of the Department of Obstetrics and Gynecology, Women's Hospital, Los Angeles, Calif., for his assistance in supplying comparison values for the clinical specimens.

**References**