Direct Radioimmunoassay for Serum Estriol during Pregnancy

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I describe an assay for serum estriol during pregnancy, which does not require prior extraction or chromatography of the serum. Fifty microliters of 20-fold diluted serum can be assayed to detect as little as 20 pg of estriol. For a 20 μg/liter serum estriol concentration the CV was 16%; for 100 μg/liter concentration it was 5%. There was no significant interference by serum "binding" proteins. The assay measures unconjugated estriol, estriol conjugated with either sulfate or glucuronide at the 3' position, and a portion of the estriol 16α-(β-D-glucuronide). Serum estriol concentrations increase during gestation from a low at 20 weeks of <10 μg/liter to a high of 100–150 μg/liter at term. Serum estriol values correlate well with those for urinary total estrogens and with values for human placental lactogen in serum at various gestational ages.

Additional Keyphrases: fetal status • changes during pregnancy • human placental lactogen • urinary estrogens • cesarean section

Determination of total urinary estrogens has been used in evaluating fetal stress during the third trimester of pregnancy; the amount of estrogens in the urine continually increases during normal gestation; declining values indicate fetal stress. Problems of collecting accurate serial 24-h urines have prompted the development of assays for serum estrogens during gestation, particularly since the usefulness of such information in predicting fetal stress has been demonstrated (1–3). Apparently all estrogens of fetal and placental origin (including estrone, estrone sulfate, estradiol, and estriol) increase during gestation (1, 4) and the concentrations of any or all (5) of these in serum could be measured to monitor fetal status.

The methods used to measure plasma estrogens during pregnancy have included extraction followed by gas chromatography (6, 7); extraction and column chromatography followed by a competitive protein-binding technique, with a “rabbit uterine cytosol” as the binding agent (8, 9); and extraction and column chromatography followed by radioimmunoassay, with use of specific antibodies raised against estrogen/albumin conjugates (10). All of these methods require considerable sample preparation and laboratory analysis time. Owing to the serious clinical implications of low and declining estriol values, there is a need for a relatively rapid and sensitive serum estrogen assay.

This report describes an assay for serum estriol that does not require extraction, is relatively rapid, is specific for estriol and its conjugates, and provides information that is useful for monitoring fetal stress during the third trimester of gestation.

Materials

Phosphate buffer, pH 7.4, 10 mmol/liter. Dissolve 1.39 g of K$_2$HPO$_4$, 0.28 g of NaH$_2$O, 7.9 g of NaCl, 5.0 g of bovine serum albumin, and 3.8 g of disodium ethylenediaminetetraacetate in 900 ml of de-ionized water. Adjust the pH to 7.4 and the volume to 1 liter.

Glutamate buffer, pH 3.3, 40 mmol/liter. Dissolve 1.2 g of L-glutamic acid and 0.4 mg of KCN in 180 ml of de-ionized water, adjust the pH to 3.3 and the final volume to 200 ml. Stable for one month at 4 °C.

Buffered dextran-coated charcoal. Dissolve 1.47 g of sodium barbital, 0.97 g of sodium acetate, and 7.9 g of NaCl in 900 ml of de-ionized water. Adjust the pH to 7.4 with 1 mmol/liter HCl and the volume to 1 liter with water. To 100 ml of this barbital buffer, add and dissolve 0.5 g of dextran (No. D-4751, clinical grade; Sigma Chemical Co., St. Louis, Mo. 63178). To a separate 100-ml portion, add and mix 5.0 g of activated charcoal (No. C-5385, Sigma). Add the charcoal suspension to the dextran solution and mix well. This suspension is stable at 4 °C for one month and must be mixed well to resuspend the charcoal immediately before each use.

Antiserum. An estriol antiserum, produced in rabbits from an estriol-6-oxime/bovine serum albumin conjugate, was purchased in lyophilized form (Endocrine Sciences, Tarzana, Calif. 91356). Reconstitute this antiserum according to the supplied instructions, and store 100-μl portions at -20 °C. Dilute each aliquot 50-fold with phosphate buffer; this can be stored at 4 °C as a "working" antibody solution for at least two months. The final dilution of the reconstituted antiserum in the reaction mix is 150-fold, and at this dilution about 60–80 pg of estriol is bound. The affinity constant of this antiserum for estriol is about 8 x 10$^9$ mol$^{-1}$.

Radiolabeled estriol. [2,4-3H]$^3$ Estriol (38 kCi/mol) was purchased from Amersham/Searle Corp., Arling-
ton Heights, Ill. 60006, divided into 20-μl portions, and is stable at -20 °C for at least six months. For the assay, dilute aliquots with phosphate buffer to a concentration of about 1500 pg/ml, a mixture that is stable at 4 °C for at least four months.

Standards. Dissolve estriol, estriol-3-sulfate, estriol-3-(β-d-glucuronide), estriol-16α-(β-d-glucuronide), estriol-17β-(β-d-glucuronide), estrone, estriol-3-sulfate, and estradiol (Sigma Chemical Co.) in ethanol and store at -20 °C. Prepare dilutions of the estriol standard in pooled serum from men to produce concentrations ranging from 0 to 200 μg/liter. Similarly dilute the other estrogen standards to produce concentrations ranging from 0 to 2000 μg/liter. The dilutions of these standards in serum are stable at -20 °C for at least six months.

Methods

Human placental lactogen assay. We assayed human placental lactogen according to the instructions included in the kit by the manufacturer (Amer- sham/Searle).

Urinary total estrogen assay. We assayed urinary estrogens according to the instructions included with the reagents supplied by the manufacturer (Stanbio Laboratory, Inc., San Antonio, Tex. 78202).

Estriol assay. Dilute all sera (standards and patients) 20-fold with phosphate buffer and pipet 50-μl aliquots of the dilutions into 12 × 75 glass disposable test tubes. Add phosphate buffer (100 μl), antiserum (100 μl), and H3-labeled estriol (100 μl, 20000 cpmp/150 pg). Nonspecific binding is measured by including tubes that contain buffer and H3-labeled estriol, but no antiserum. Mix all ingredients on a vortex-type mixer and place the tubes in an ice bath for 15 min. Add dextran-coated charcoal (250 μl) and, after 5 min in the ice bath, centrifuge the tubes at 1000 × g and 4 °C. Decant the supernate into scintillation vials containing 10 ml of scintillation fluid (Amer- sham/Searle) and measure the radioactivity in a liquid-scintillation counter (we used one supplied by Searle Analytic Inc., Des Plaines, Ill. 60018).

Incubation with β-glucuronidase. Dilute sera 20-fold with phosphate buffer containing 50 U of β-glucuronidase (Sigma) per milliliter and incubate at 37 °C for 18 h. Then assay the diluted sera for estriol by the described procedure.

Results

Precision. I assessed the precision of this assay by averaging and converting to a logit plot the data from five separate standard curves generated by this assay protocol (Figure 1). The resulting standard curve had a slope of -1.335, intercept of 2.707, and the precision coefficient, λ (11), ranged from 0.028 to 0.084, which compares well with those reported for other radioimmunoassays (12). Figure 1 also shows results obtained for assays of dilutions of serum containing 110 μg of estriol per liter; the resulting curve does not differ significantly from the standard curve.

The precision of this assay was also evaluated by multiple analyses of two estriol-supplemented sera (Table 1). The precision for low and high estriol concentrations compares well with that reported for a direct radioimmunoassay of estradiol in serum (13).

Sensitivity. The sensitivity of this assay is expressed by using criteria recently suggested by Boutwell (14). The mean and standard deviation for 20 sera negative for estriol was 0 ± 3.8 μg/liter. With twice the SD as a "noise" level, the least detectable concentration in undiluted serum would be about 8.0 μg/liter, or 20 pg per 50 μl of serum diluted 20-fold for assay. The within-run "analytical standard deviation" (14) for the assay is expressed (Table 1) by the mean ± 1 SD for 20 assays at low and high estriol concentrations.

The incubation time and temperature necessary to achieve equilibrium are indicated in Figure 2. The reaction reaches equilibrium quickly at room temperature or at 4 °C; however, the binding is about 10–15% higher at 25 °C.

Serum estriol determined by this assay was compared to total urinary estrogens in 10 patients sampled at various times during the third trimester. This comparison (Figure 3) yields a regression equation of y = 0.287x + 1.2, with a sample correlation coefficient of 0.871.
In directly assaying serum for estriol, one must exclude interference by serum proteins that may compete with the antibody during estriol binding. One approach to eliminating such "serum binding" is to heat the serum at low pH to denature and thus inactivate the binding protein (15). Serum was diluted 20-fold with glutamate buffer, heated at 90 °C for 15 min, and assayed for estriol. There was no significant difference (P = .01) between estriol values for the samples that were heated and duplicate sera that were analyzed directly.

In another test of interference by "serum binding" proteins, 20 pregnancy sera were assayed for estriol before and after adding 100 µl of serum from either men or women to the assay tubes. There was no significant differences in the values (P < .05).

The reactivity of the estriol antibody with estrone, estrone-3-sulfate and estradiol is shown in Figure 4. There is about 2% cross-reaction with each of these estrogens (measured at B/B0 = .5). Because the total concentration of estrone and estradiol in pregnancy serum is about half that of total estriol (1), the contribution of these estrogens to estriol assayed by this method would be 1% or less.

Several other estriol conjugates that are present in pregnancy serum also react with this estriol antibody (Figure 5). The approximate cross-reactivity for the 3-glucuronide derivative is 100%; for the 3-sulfate conjugate, 70%; for the 16α-(β-D-glucuronide), 20%; and for the synthetic derivative, 17-β-(β-D-glucuronide), 5%. Because this method does not measure total serum estriol, it is important to establish (a) that estriol determined by this assay is proportional to total serum estriol and (b) that the measured serum estriol reflects fetal status.

Estriol values from sera assayed before and after incubation with β-glucuronidase (EC 3.2.1.31) at 37 °C for 18 h show a correlation coefficient of 0.75 for direct values greater than 6 µg/dl (Figure 6). For estriol values smaller than 6 µg/dl the nature of the correlation changes, and the total estriol is about two-fold higher than the directly measured estriol. Figure 7 illustrates another indication of the relationship between the directly assayed and total serum estriol; assays of serum from a pre-eclamptic patient who was sampled daily showed similar changes in both the directly assayed and total serum estriol.

Evidence that this direct serum estriol assay reflects fetal status is shown in Figure 8. Serum estriol values increase during gestation, and values for one patient who delivered a stillbirth progressively decline until they are below those for normal pregnancies.

There is correlation between directly measured serum estriol levels and the serum placental lactogen
concentrations in sera taken at various times during gestation (Figure 9). Values for both lactogen and estriol increase until placental lactogen values of 6 to 7 mg/liter are reached at gestational ages of 34–36 weeks. At later gestational ages it does not increase significantly, but the increase in estriol continues, resulting in the nonlinear relationship as shown.

Discussion

Most reported assays for estrogens in pregnancy serum have involved relatively long sample-preparation steps in order to isolate and purify estrogens before assay. The data presented here indicate that these initial steps are not necessary for accurate measurements of serum estriol. The usual reasons for such purification and concentration steps before the assay are to increase both the selectivity and sensitivity of the assay. Because all estrogens of fetal and placental origin increase in serum during gestation (1, 4) and the use of an antibody confers some degree of specificity to the estrogen assay, high selectivity is not necessarily the prime consideration in designing a direct serum estrogen assay.

The selectivity of this assay depends on the antibody specificity, which in turn is related to the nature of the pregnancy serum estriol conjugates. Geobelmann et al. (16) reported that half of serum estriol is present as the 16α-(β-D-glucuronide) and 3-sulfate-16α-(β-D-glucuronide) conjugates, the other half being unconjugated estriol, estriol 3-sulfate, and estriol 3-(β-D-glucuronide). Using this information, together with the observations that (a) the concentration of serum estriol measured by this direct assay is approximately half that reported for total serum estriol (16), (b) both the 3-sulfate and 3-glucuronide derivatives react well with the estriol antibody, while the 16α derivative reacts to a lesser degree, and (c) there is a two- to threefold increase in assayed serum estriol after hydrolysis, one may conclude that this assay primarily measures “free” estriol and estriol conjugated at the 3-position. Because the concentration of conjugated estriol is 20-fold higher than “free” estriol during the third trimester (1) and the free estriol concentration is about 10 μg/liter (1, 9), one must assume that most of the estriol being measured in this assay is conjugated at the 3′ position.
However, since 30–40% of the estriol is conjugated at the 16α position (16), this derivative also contributes to the directly measured estriol.

The values for directly assayed estriol do correlate well with gestational age and show the expected agreement with the serum placental lactogen values. Also, there is good correlation (r = 0.87) between values for serum estriol and for total estrogens in urine during the third trimester.

An additional observation that supports the clinical validity of this assay is the 10-fold increase in the directly assayed serum estriol values between 20 and 40 weeks of gestation. The magnitude of this increase agrees well with that reported for un conjugated serum estriol during the same gestational period (1, 9). Also, the directly assayed estriol values are proportional to the total serum estriol values in one patient analyzed daily, and in several patients analyzed throughout gestation. This parallelism indicates that the values obtained by this assay reflect both the “free” and conjugated serum estriol, and that they provide a valid estimate of serum estriol concentrations during gestation.

The nonlinear relationship between placental lactogen and estriol is consistent with a proportionally smaller increase in placental lactogen as compared to estriol after 34–36 weeks of gestation. After about 34 weeks of gestation, serum placental lactogen concentrations reportedly increase very little (17) or actually decrease (18). This leveling off of placental lactogen values contrasts with the continuous increase in serum estriol that is even greater after 30 weeks of gestation (4).

The sensitivity of the assay, expressed as the detection limit in undiluted serum containing 8 μg of estriol per liter, is such that abnormally low serum estriol concentrations can be detected beyond 26–28 weeks gestation. Because the clinical alternative to delivery by cesarian section becomes feasible only after about 26 weeks, the sensitivity of this direct assay is sufficient to support that clinical determination. The sensitivity, when expressed as the precision of assaying low and high estriol concentrations, is sufficient to detect a shift of greater than 32% (±2 SD) in low values and 12% (±SD) in high values assayed in daily serum samples. Based on one example of a patient who delivered a stillbirth, a decrease of greater than 100% might be expected in serial serum samples during fetal distress.

To conclude, my data support the use of a direct serum estriol assay as an indication of fetal well-being. This method provides an alternative to the more time-consuming extraction assays and allows for relatively rapid and simple daily serum estriol determinations.

References


