
Improved Determination of Estriol-16α-Glucuronide in Pregnancy Urine by Direct Liquid Chromatography with Fluorescence Detection

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In this relatively simple, rapid assay of estriol-16α-glucuronide in pregnancy urine, the urine sample is diluted 20-fold with phosphate buffer (pH 5.2) containing 360 mL of acetonitrile and 2 g of cetyltrimethylammonium bromide per liter, then directly injected into the chromatograph. A sample can be assayed within 14 min. Day-to-day CVs ranged from 2.3% at 45 mg/L to 2.9% at 4.8 mg/L. The limit of sensitivity is 0.4 mg/L. Results by the present method (y) correlated and compared very well with those by a method involving fractionation of estrogen conjugates and gas chromatography (x) for 24 samples of pregnancy urine (y = 1.09x + 0.303; r = 0.947). This assay is inexpensive and suitable for complete automation.

Additional Keyphrases: monitoring pregnancy • ion-pair chromatography • estrogen conjugates • steroids • fetal status

The advantages of measuring urinary estriol-16α-glucuronide (E1-16α-G) instead of total estriol for the purpose of monitoring fetal well-being have been recognized (1–4).
Various direct radioimmunoassay (RIA) procedures have been developed for monitoring E1-16α-G in pregnancy urine (4–9); but some of these methods lack specificity, involve time-consuming incubation and phase-separation steps, and depend upon the availability of a suitable radiolabeled ligand. Alternatively, chemiluminescence assays have been proposed (10, 11), which only partly satisfy the requirements of rapidity and simplicity.

The feasibility of "high-performance" liquid chromatography (HPLC) for direct measurement of E1-16α-G in pregnancy urine has been investigated. However, one proposed method involves a relatively nonselective detector and an unsuitable mobile phase for the elution of the estriol metabolite (12), and another involves derivatization of the estriol conjugate before quantification by fluorimetry (13).

Sharp, untailored peaks for estrogen conjugates have been achieved by adding to the mobile phase suitable ion-pair forming agents, such as tetraalkylammonium salts (14). Moreover, this expedient further increases the flexibility of reversed-phase liquid chromatography, because additive parameters, such as the concentration and the nature of the tetraalkylammonium salt, can be varied to optimize the separation of ionogenic compounds (15, 16).

We describe here a direct HPLC procedure with fluorimetric detection, which exploits ion-pair, reversed-phase chromatography and the native fluorescence of E1-16α-G. This assay is extremely simple, rapid, and completely suitable for automation.

Materials and Methods

HPLC apparatus. We used a Series 1 liquid chromatograph equipped with a Model 650-10S fluorescence detector having a 20-μL flow cell and a Rheodyne Model 7125 injector with a 20-μL loop (all from Perkin-Elmer Corp.,
Norwalk, CT 06856). We used a 4.6 mm × 25 cm column filled with 5-μm (av. particle size) C18 reversed-phase packing and a guard column containing "Pelliguard," both from Supelco Inc., Bellefonte, PA 16823. As mobile phase, we used acetonitrile/phosphoric buffer (20 mmol/L; pH 5.2), 36/64 by vol, in which we dissolved 2.0 g of cetyltrimethylammonium bromide per liter of mobile phase. The flow rate was 1.5 mL/min. We monitored E2-16α-G fluorometrically, with excitation and emission wavelengths of the detector set at 280 and 308 nm, respectively, and 12-nm slit widths.

Reagents. "Chromatography-grade" acetonitrile was from Carlo Erba, Milano, Italy. Water was distilled in a glass system in the presence of permanganate. Cetyltrimethylammonium bromide was from Merck, Darmstadt, F.R.G. Working phosphate buffer, pH 5.2, was prepared by dissolving 2.76 g of Na2HPO4·H2O in 1 L of freshly distilled water and adjusting the pH to 5.2 with NaOH, 0.1 mol/L. Estradiol-3-sulfate-16α-glucuronide was synthesized from E2-16α-G according to Levitz et al. (17). E3-16α-G and the other estrogen conjugates were from Sigma Chemical Co., St. Louis, MO 63178. Stock E3-16α-G standard was dissolved in methanol at 1 g/L and later diluted with methanol to give 10 μg/L working standard.

Procedure. Dilute 10 μL of 24-h urine with 190 μL of the solution used as mobile phase and inject 25 to 30 μL into the chromatograph. Calculate the E2-16α-G concentration of patient’s sample by comparing the height of the peak produced by the E2-16α-G in the sample with that of a standard of the steroid. The latter is prepared for chromatography by evaporating 10 μL of the E2-16α-G working standard and reconstituting the residue with 200 μL of mobile phase. The response of the fluorimetric detector is linearly related to amounts of injected E2-16α-G within the range 0.4 to 80 ng.

Results
The excitation and emission spectra of the native molar fluorescence of the E3-16α-G were recorded and compared with those of the estradiol. We observed that introduction of a glucuronide group in position 16 does not substantially modify the native fluorescence of the estradiol molecule.

Interferences. Under our experimental chromatographic conditions, we tested several naturally occurring estrogen conjugates as potential sources of interference with the procedure. The results (Table 1) show that none interfered. Neither the 16- and 17-epiestriol conjugate standards nor 16α-hydroxyestrone-3-glucuronide was available for retention-time studies. Under the chromatographic conditions chosen, only 16α-E2-16α-G-glucuronide and 17α-E2-16α-G-glucuronide might interfere with the E2-16α-G, but they are practically absent from pregnancy urine (18). Finally, it is reasonable to assume that 16α-hydroxyestrone-3-glucuronide, analogously to estrone and its derivatives, is not fluorescent under the detection conditions selected.

We evaluated the extent of background interference eluting at the same time as E2-16α-G by analyzing urine samples from 18 nonpregnant women. The background was <0.1 mg/L of urine, and this does not interfere.

As a final check on the specificity of the assay, we ascertained the absence of interferences from any other minor-abundance estrogen conjugates by analyzing 25 samples of late-pregnancy urine. For any sample, we collected from the column outlet an appropriate volume fraction of the mobile phase supposed to contain all the chromatographic band of the E3-16α-G. The solution was evaporated, the residue was submitted to enzymic hydrolysis, and the estradiol liberated was analyzed according to a procedure reported elsewhere (19). On expressing data for E3-16α-G as estradiol, results of the linear regression analysis of the two methods were: slope = 1.03 (SD 0.26), intercept = 0.971 (SD 0.006) mg/L, r = 0.993 (y, direct method; x, nondirect method).

Sensitivity. The limit of sensitivity (signal/noise ratio = 3) was 0.4 mg/L of urine. For this concentration the CV was 5.2%.

Precision. Seven selected urine samples were each assayed six times during a month. The day-to-day CVs ranged from 2.3% at 45 mg/L to 2.9% at 4.8 mg/L. Figure 1 shows typical chromatograms obtained by this procedure.

Method comparison. We compared results by our method with these by a previously reported method (18), which involves a preliminary separation of different groups of steroid conjugates coupled with column capillary gas-liquid chromatography. Linear regression analysis of data obtained by our direct HPLC procedure (y) and the comparison method (x) for 24 urine samples from pregnant women in their last trimester of pregnancy gave the following results: slope = 1.09, intercept = 0.363 mg/L, r = 0.947. The mean of HPLC values was 25.8 (SD 11.1) mg/24 h; the mean of the comparison-method values was 23.4 (SD 10.3) mg/24 h.

Patient studies. We measured the excretion of E2-16α-G and of total estradiol in urine sampled from 24 patients who had normal pregnancies. The 24-h values, expressed as milligrams of estradiol, showed that the mean urinary excretion of E2-16α-G was 16 mg/24 h, representing 67.5% of the mean total estradiol excretion. This value agrees well with those reported by other authors (19-21).

We assessed the day-to-day variation of E2-16α-G urinary excretion by analyzing 24 pairs of 24-h urine samples collected on two consecutive days from 24 apparently normal, ambulant women in their third trimester pregnancy. The CV was 8.3%. This finding gives additional evidence of the low day-to-day fluctuations in E2-16α-G excretion (4). Interestingly, the day-to-day variations of urinary E2-16α-G we measured are significantly lower than those relative to both unconjugated and total estradiol in pregnancy plasma (23).

Discussion
Outstanding features of this assay are its rapidity and simplicity. A single assay can be completed within 14 min of its receipt, and sample manipulation is reduced to a minimum, thus eliminating almost at all possibilities of errors in a clinical laboratory. In addition, this procedure can be easily adapted to automatic sampling and data-reduction devices now commercially available, thus making it even simpler.

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Fig. 1. Chromatograms of diluted 24-h urine from a nonpregnant woman (left) and from a pregnant woman at 32 weeks of gestation (right). Arrows indicate elution time for E3-16α-G. Figure cropped at top, to save space.

References