Gas–Liquid Chromatographic Method for Determining Urinary Estriol, with 6α-Hydroxyestriol as Internal Standard

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I describe a chromatographic procedure for quantification of urinary estriol in late pregnancy, with 6α-hydroxyestriol used as internal standard. The hydrolyzed sample is successively passed through a Bond Elut C₁₈ column and a DEAE-Sephadex column. The estrogens are then completely resolved as silyl ethers on a packed gas–liquid chromatographic (GLC) column in isothermal mode. Analytical recoveries of conjugated estriol ranged between 96 and 102%. Between-day precision (CV) was 4.4% at a mean concentration of 85.1 μmol/L. Specificity was assessed by comparing results obtained with a capillary GLC column.

Additional Keyphrases: fetal status · pregnancy · estrogens

The importance of measuring urinary estriol excretion as an index of fetal well-being is well documented (1–5). When gas–liquid chromatographic (GLC) methods have been used (4–7), the most commonly used technique for sample preparation is liquid–liquid extraction (4, 5). Thin-layer (6) or anion-exchange chromatography (7) or adsorption on Sep-Pak cartridges (8, 9) or graphitized carbon black (10) have also been used. Inadequate sample preparation makes it necessary to use GLC capillary columns or temperature programming.

Here I present a GLC method for quantification of urinary estriol in late pregnancy by use of a combination of sorption and ion-exchange chromatography for purification of samples. After enzymatic hydrolysis of the urine sample the non-polar compounds are adsorbed onto a disposable reversed-phase C₁₈ column. Neutral and acid estrogens are separated on a short ion-exchange column packed with DEAE (OH⁻)-Sephadex. The eluted weakly acid estrogens are silylated and these compounds in urine are completely resolved on a packed GLC column in isothermal mode. The internal standard 6α-hydroxyestriol is added before any sample-preparation step.

Materials and Methods

Apparatus. A Model 5830A gas chromatograph with a flame-ionization detector was used with a 180-cm glass column packed with 3% SE-30 on Chromosorb W, AW-DMCS, 100–200 mesh (all from Hewlett Packard, Palo Alto, CA 94304). The instrument was equipped with a combined integrator–controller and printer–plotter.

Column temperature was 250°C, injector and detector temperature was 275°C. Flow rates were: nitrogen 40, hydrogen 40, and air 240 mL/min, respectively.

"Bond Elut" C₁₈ columns (500 mg of sorbent per 6-mL reservoir) were used as supplied, together with a "Vac-Elut" apparatus (Analytichem International Inc., Harbor City, CA 90710). I used disposable polystyrene columns (8 × 102 mm, 6-mL volume) supplied complete with porous polyethylene diaks, stoppers, and end caps (cat. no. 29920) by Pierce Eurochemie, Beijerland, Holland.

Reagents. Estriol, estriol-16α-glucuronide, and 6α-hydroxyestriol (from Sigma Chemical Co., St. Louis, MO 63178) were dried over phosphorus pentoxide under reduced pressure before use. I prepared a stock 1 mmol/L estriol solution in methanol; for working standards I dissolved

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evaporated aliquots in urine from a male. The 82 μmol/L 6α-hydroxyestriol internal standard solution was prepared in methanol.

*Helix pomatia* juice was from Reactifs IBF, 92390 Ville-neuve-La-Garenne, France. *N*-O-Bis(trimethylsilyl)trifluoroacetamide containing 10 g of trimethyl chlorosilane per liter (BSTFA:TMCS) was from Analytical Standards, Kungsbacka, Sweden. All other chemicals were of analytical grade, from Merck, Darmstadt, F.R.G.

I prepared methanolic carbonic acid by bubbling carbon dioxide for 5 min through a Pasteur pipette into 100 mL of methanol. The solution, prepared freshly each week, was stored in a capped bottle at room temperature.

DEAE-Sephadex A-25 was from Pharmacia, Uppsala, Sweden. I washed it successively with 250-, 500-, and 750-mL/L aqueous methanol and stored it at 4 °C. Before use it was converted to its OH⁻-form with five bed volumes of 0.5 mol/L sodium hydroxide in 750 mL/L aqueous methanol. The gel was then washed with 750 mL/L aqueous methanol until neutral, then with undiluted methanol to remove water. The disposable polypropylene columns from Pierce were packed with approximately 1 mL of gel in methanol to give a bed height of 20 to 22 mm, then capped at both ends.

**Sample collection.** I used 24-h urine specimens collected in plastic bottles containing no preservatives and stored aliquots at −18 °C until analysis.

**Procedure.** To a 1.0-mL aliquot of urine in a 10-mL tube, add 200 μL of internal standard solution, 4 mL of acetate buffer (0.2 mol/L, pH 4.6), and 200 μL of *Helix pomatia* juice. Incubate at 60 °C for 1 h in a heating block. Cool the tubes and centrifuge (1500 × g, 5 min).

Mount up to 10 Bond Elut columns in the VacElut and activate them by treatment with 3 to 5 mL of methanol and 5 to 10 mL of water. They should not be allowed to dry out. Transfer the supernatant fluid from the 10-mL tubes to the columns and aspirate through during approximately 2 min. Wash out polar substances with 10 mL of water and aspirate out excess water. Mount the conical collecting tubes in the VacElut and elute the non-polar estrogens with 3 mL of methanol.

Pass the eluate through a DEAE(OH⁻)-Sephadex column, then wash out the neutral steroids with 3 mL of methanol. Elute the acetoxy estrogens with 5 mL of the carboxic acid–methanol.

Evaporate the methanol under a stream of nitrogen at 60 °C and dissolve the residue in 100 μL of pyridine. Add 50 μL of BSTFA:TMCS, then incubate for 10 min at 60 °C. Evaporate the reaction mixture and dissolve the residue in 100 μL of ethylene dichloride. Inject 2 μL of the silylated estrogens into the column.

**Results and Discussion**

Figure 1 shows a typical chromatogram for a pregnancy urine containing 32.4 μmol of estriol per liter. Retention times for estriol and the internal standard are 6.1 and 10.1 min, respectively. Completion of the chromatogram requires 12 min.

**Linearity.** Figure 2 shows a calibration graph for estriol added to urine from a male. The peak-area ratio for estriol/internal standard was linearly related to all concentrations investigated up to 150 μmol/L. Each standard was assayed in duplicate. The equation for the regression line, ratio (y) vs concentration (x), was $y = -0.0135 + 0.0073x$ ($r = 0.9998$). Concentrations were routinely determined with a one-point calibration.

**Analytical recovery.** I added estriol glucuronide to urine samples from men and processed them in duplicate through the entire procedure. Analytical-recovery data are summarized in Table 1.

**Method comparison.** I processed 37 samples from pregnant women (duration of pregnancy 28 to 40 weeks) by the outlined procedure. The silylated extracts were also analyzed on a 25-m fused-silica capillary column coated with SE-30. Estriol concentrations ranged from 9 to 141 μmol/L. The regression equation for results from the capillary column (y) vs the packed column (x) is $y = 1.68 + 1.06x$ ($r = 0.9831$). The 95% confidence limits for the slope and inter-
cept are 0.99 to 1.13 and −2.11 to 5.48, respectively. The chromatograms from the capillary column indicate that the estriol peak is homogeneous.

**Precision.** I analyzed, in duplicate, a pooled specimen of urine from a pregnant woman, during four days. The mean value was 85.1 μmol/L, the overall CV 8.1%, and the within-run and between-run CVs were 6.7 and 4.4%, respectively. Other methods have generally comparable analytical imprecision (4, 11, 13).

**Method development.** I divided centrifuged and pooled urinary hydrolysates into aliquots and adsorbed them onto the same Bond Elut column. Between every sorption the column was washed with 5 mL of methanol and 5 mL of water. The eluates were further purified as outlined and the area ratio estriol/internal standard was determined. The CV for the ratio was 1.26% (n = 8). I conclude that the column has sufficient capacity to be used at least eight times before being discarded. In other reports Sep-Pak cartridges have been used five times (7, 8), with some loss of extraction efficiency. The difference in capacity is not surprising because I only used 1 mL of urine and the suprante from centrifuged hydrolysates, i.e., colloidal material, was removed. The use of an internal standard obviates the need to determine the absolute analytical recovery from the column.

The estrogens behave like weak organic acids and thus are adsorbed by DEAE(OH−)-Sephadex, as pointed out by Fotsis et al. (7). I eluted the columns with carbonic acid–methanol, which selectively elutes the weak acids and leaves no salt residue when evaporated. One hundred milliliters of methanol is saturated with carbon dioxide in less than 15 min if the described procedure is followed. The estrogens are eluted by less than 5 mL of carbonic acid–methanol, even if the method is not completely saturated. The columns are regenerated with washes of 0.1 mol/L HCl in methanol and 0.5 mol/L sodium hydroxide in methanol.

Acetylation of estriol with acetic anhydride in pyridine is a mild but slow reaction (5). I followed the silylation of estriol and 6a-hydroxyestriol at 60 °C with BSTFA:TMCS during 25 min and found that the reaction was complete in less than 2 min. Routinely the reaction mixture is incubated for 10 min at 60 °C.

Different internal or external standards have been used in GLC assays of estriol, e.g., epoprostenol, 6-oxo-estradiol, equilin, cholestanol, or estrone (11–15). In this procedure 6a-hydroxyestriol is used because of its structural resemblance to estriol. Both substances are adsorbed and eluted under the same conditions in the sample-preparation steps. The silylated product is well separated from the derivative of estriol, and only one derivative is formed. If 6-ketoestriol is used, time is lost because the methoxime has to be formed and further silylated. Because in this method the 6a-hydroxyestriol is added before any sample-preparation step and thus is a true internal standard, correction for losses is obviated.

The use of Bond Elut columns obviates the need to handle hazardous solvents. Furthermore, the Vac Elut manifold makes it possible to handle as many as 10 samples simultaneously, making sample preparation less time-consuming and cumbersome.

In some other methods, sample preparation is oversimplified, making temperature programming (5) or separation on capillary columns necessary (8, 9, 12). Peak height is measured on the slope of the solvent front rather than peak area (11, 13–15). I find that the method outlined is suitable for routine quantification of estriol in late-pregnancy urines, because the estrogens are completely resolved on a packed column in isothermal mode and thus peak areas can be reliably determined with an integrator, making automation feasible. Costs can be decreased if the sample preparation columns are used several times as suggested.

**References**


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**Table 1. Analytical Recovery of Unconjugated Estriol**

<table>
<thead>
<tr>
<th>Added (μmol/L)</th>
<th>Recovered (μmol/L)</th>
<th>Recovered, %</th>
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*Estriol glucuronide added to urine from a male.

*Mean values of duplicate.