Determination of Placental Estriol in Urine by Gas–Liquid Chromatography, with Equilenin as Internal Standard

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Placental estriol in urine can be determined by rapid enzymatic hydrolysis, extraction with neutral XAD-2 resin, and quantitation by gas–liquid chromatography, with equilenin as the internal standard. Glucose, methenamine mandelate, hydrochlorothiazide, phenolphthalein, or salicylate do not interfere. Analytical recovery of estriol added to urine was 92–108%. Day-to-day precision (CV), established by 15 replicate analyses at a mean concentration of 21.3 mg/liter, was 6.6%.

The assessment of urinary placental estriol (E3) in pregnancy, as a measure of fetal well being, is well documented (1, 2). Numerous gas–liquid chromatographic (GLC) methods for urinary E3 determinations have been reported (3–8). Most of these methods lack true internal standardization; hence there is no correction for analytical variables—the internal standard is used only for peak quantitation. Smith and Stitch (8) used phenolphthalein as a true internal standard, but phenolphthalein is a commonly used laxative, and thus may be present in maternal urine.

Interference is reportedly caused by glucose (9, 10), methenamine mandelate (11), hydrochlorothiazide (12), and phenolphthalein (9) in the determination of urinary E3 after acid hydrolysis. Interference by salicylate (13) has also been reported when enzymatic hydrolysis is used.

We describe a method that obviates many of these difficulties.

Instrumentation

We used a Model 1200 gas chromatograph equipped with a flame-ionization detector (Varian Aerograph, Walnut Creek, Calif. 94598). The 2-meter glass-coil column, 2 mm i.d. was packed with 3% OV-17 on Chromosorb W H/P, 100/200 mesh size (Varian Aerograph).

Gas flow rates (ml/min) were: hydrogen 20, air 360, and nitrogen 30. Injector and detector temperatures were maintained at 295 °C. Column temperature was maintained at 280 °C.

A Varian strip-chart recorder, Model 2000, was used. Chart speed was 2.5 cm/min and full-scale response was 1 mV.

Reagents

Except as noted, the following compounds were obtained from Sigma Chemical Co., St. Louis, Mo. 63178.

β-Glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31), bacterial powder, derived from Escherichia coli.

Internal standard (d-equilenin, 3-hydroxyestra-1,3,5(10),6,8-pentaen-17-one). We used a working standard of 250 mg/liter in methanol.

Estriol (estra-1,3,5(10)-triene-3,16α,17β-triol).
Estrone (estra-1,3,5(10)-triene-3-ol-17-one).
Estradiol (estra-1,3,5(10)-triene-3,17β-diol).
16-Epiestriol (estra-1,3,5(10)-triene-3,16β,17β-triol).
17-Epiestriol (estra-1,3,5(10)-triene-3,16α,17α-triol).
16,17-Epiestriol (estra-1,3,5(10)-triene-3,16β,17α-triol).
6α-Hydroxyestriol (estra-1,3,5(10)-triene-3,6α,16α,17β-tetrol).

Standard. We used a working standard of 250 mg each of estriol and equilenin per liter of methanol.
N.O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), Pierce Chemical Co., Rockford, Ill. 61105.
Columns containing Amberlite XAD-2 resin (Rohm and Haas Co., Philadelphia, Pa. 19105). We used pre-
filled columns, 1 cm x 7 cm (Brinkman Instruments, Westbury, N. Y. 11590).
Polypropylene tubes, 50-ml centrifuge tube with screw cap (No. 25330, Corning Glass Works, N. Y.
14830).
Petroleum ether (ligroin, bp 30-60 °C), Mallinc-
rodt Inc., St. Louis, Mo. 63160.
Buffered enzyme. We used 1800 kU of β-glucuroni-
dase per liter of 1.0 mol/liter phosphate buffer, pH
7.0.

Procedure
Add 1.0 ml of buffered enzyme to 5.0 ml of urine. Add 2 drops of chloroform, gently mix, and incubate at 60 °C for 30 min. Pass the cooled hydrolysate through a column containing hydrated XAD-2 resin, rinse the hy-
drolysate tube with 5.0 ml of distilled water, and pass the rinsing through the column. After the column has completely drained, expel the XAD-2 resin into a polypropylene tube with gentle air pressure and rinse the column with 10 ml of diethyl ether. Into the tube add diethyl ether to a total volume of 25 ml, 2 drops of concentrated hydrochloric acid, and 0.5 ml of the working internal standard. After shaking the mixture for 10 min, pour the ether layer into a glass centrifuge tube and evaporate. To the dried residue add 5.0 ml of sodium hydroxide solution (100 g/liter) and 15 ml of diethyl ether/petroleum ether (1/3 by vol). Shake for 5 min and aspirate the ether layer. Add 1.5 ml of con-
centrated hydrochloric acid to the sodium hydroxide layer, cool the tube, and add 15 ml of diethyl ether. Shake the tube for 5 min, remove the ether layer, dry it with sodium sulfate, and evaporate it to dryness. Evaporate a 0.4-ml aliquot of the standards. Add 50 μl of dimethylformamide and 120 μl of N,O-bis(trimethyl-
silyl)-trifluoroacetamide to the residues and incubate the tubes at 70 °C for 30 min. Inject 1-2 μl of the sily-
lated products into the gas chromatograph for analysis.

Results
Analytical Variables
Hydrolysis. We established the optimal conditions for the enzymatic hydrolysis by adding various con-
centrations of the enzyme β-glucuronidase to 5.0-ml aliquots of pregnancy urine buffered with 1.0 ml of phos-
phosphate buffer (1 mol/liter, pH 7.0). For each enzyme concentration, incubation time was varied from 15 to
60 min and incubation temperature from 25 to 70 °C.

Hydrolysis was complete when 1800 units of β-glucu-
ronidase was used at 60 °C for 30 min.
A comparison between β-glucuronidase and an en-
zyme preparation containing both β-glucuronidase and sulfatase as described by Van de Calseyde et al. (14)
revealed that the two methods of hydrolysis were comparable. Results of the linear regression analysis of the
two methods of hydrolysis were: slope = 1.059, in-
tercept = -0.92, r = 0.95, n = 20 (x, present method; y,
other method).
XAD-2 resin extraction. The XAD-2 resin columns
used in this study will bind up to 0.25 mg of E3 (equi-
alent to a 5.0 ml aliquot of a 50 mg/liter concentration of E3). At E3 concentrations above 50 mg/liter the col-
umn effluents contained significant amounts of E3.

Purification of estrogens. The estrogens were sepa-
rated from the neutral steroids by washing the aqueous
sodium hydroxide layer with diethyl ether/petroleum
ether (1/3 by vol). Figure 1 shows a resulting chroma-
matogram of a maternal urine carried through the
procedure.

Interfering Compounds
Chromatographic. Other naturally-occurring estro-
gens in pregnancy urine that could potentially interfere
are estrone, estradiol, 16-epiestriol, 17-epiestriol, 16,17-epiestriol, and 16α-hydroxyestradiol. Estrone, es-
stradiol, and 16,17-epiestriol eluted well before E3 and
the internal standard. 17-Epiestriol and 16-epiestriol, eluted as a front and back shoulder, respectively, on the
E3 peak. 6α-Hydroxyestradiol co-eluted with 16-epiestriol.
These are present in late pregnancy urines in average
concentrations of 0.8 mg/liter for 16 epiestriol, 0.1
mg/liter for 17-epiestriol, and 1.0 mg/liter for 6α-hy-
droxyestradiol (15), concentrations insignificant as com-
pared to the E3 concentration in late pregnancy urines.
Other Interferences

As previously noted, glucose, hydrochlorothiazide, phenolphthalein, methenamine mandelate, and salicylate have been reported to interfere with the hydrolysis of estrogen conjugates.

**Glucose.** Glucose was added to a normal pregnancy urine in concentrations up to 20 g/liter. There was no interference with the E₃ determination with added glucose.

**Hydrochlorothiazide.** Hydrochlorothiazide was added to pregnancy urine up to a concentration of 100 mg/liter. No interference was noted.

**Phenolphthalein.** Phenolphthalein is poorly absorbed through the intestinal tract, and only 15% of the average 100 mg/day dose is excreted in the urine. Phenolphthalein is excreted as a glucuronide and this conjugate may interfere with enzymatic hydrolysis by substrate competition. Phenolphthalein glucuronide was added to a pregnancy urine in concentrations up to 100 mg/liter. A 50% decrease in the apparent E₃ concentration was noted at a concentration of 100 mg/liter of phenolphthalein glucuronide. However, there was no interference with the E₃ determination up to a concentration of 50 mg of phenolphthalein glucuronide per liter. Greater concentrations of phenolphthalein glucuronide in the urine are unlikely. Phenolphthalein is carried through the extraction procedure and forms a silyl derivative that elutes as a peak in the chromatogram. However, this peak does not interfere with either the E₃ or the internal standard peak.

**Salicylate.** Ingested acetylsalicylic acid is excreted as a glucuronide, which has been reported to interfere with enzymatic hydrolysis by substrate competition (13). Four normal pregnancy urines were diluted twofold with urine from a normal man, containing 2.5 g of salicylate glucuronide per liter. These same urines were diluted twofold with a pooled salicylate-free urine from normal men. An average 15% decrease in the apparent E₃ concentration was noted in the salicylate-containing urines. However, when the same salicylate-containing urines were determined once again, but using a twofold increase in the β-glucuronidase (3600 units) for hydrolysis, the apparent E₃ concentrations were comparable to those for the salicylate-free urines. Thus, urines that are known to contain salicylate could be processed by using a twofold increase in β-glucuronidase.

**Methenamine mandelate.** Methenamine mandelate is excreted in the urine as formaldehyde. Formaldehyde reportedly interferes with acid hydrolysis of estrogens (10). Formaldehyde or methenamine mandelate was added to a pregnancy urine in various concentrations up to 1000 mg/liter. Neither interfered with the E₃ determination.

**Urine background.** Fifteen pregnancy urines were processed without adding the internal standard. There was no significant background near the internal standard.

**Accuracy and Precision**

Accuracy was evaluated by determining recoveries of known amounts of free and conjugated E₃ added to urine from males. The recovery of free E₃ from 25 samples with a concentration from 10 to 50 mg/liter ranged from 92 to 103%. The recovery of estriol-16α-glucuronide from 10 samples with an estriol concentration from 5.9 to 47.2 mg/liter ranged from 92 to 110%. Richardson (7) reported recoveries of free E₃ from male urine to be approximately 80%; Smith and Stitch (8) reported recoveries averaging 99.2% from 10 samples. Within-run and day-to-day precision was evaluated by assaying replicate aliquots from a maternal urine. Table 1 shows the results of these studies.

**Linearity**

Peak height (y) and concentration (x) were linearly related over the range of 0 to 50 mg/liter estriol. The regression analysis of the five linearity curves had a slope of 0.968, and an intercept of 0.498. The CV for the slope of the lines was 6.7%.

**Discussion**

Of the many published assay methods for placental estriol, few correct for analytical losses by incorporating a valid internal standard, as we have done.

The use of Amberlite XAD-2 neutral resin is an efficient and simple method of extraction. In addition, its use eliminates the necessity of washing the ether extract with alkaline carbonate, a procedure common to many E₃ assay methods, intended to remove many strongly acidic, potentially interfering compounds. Coincidentally, the alkalinity of this carbonate solution also removes polar estrogens, such as E₃. Our method does not require centrifugation, as phase separation is rapid and complete; no emulsion is formed as it is in most acid hydrolysis procedures and some enzymatic procedures—another potential source of E₃ loss during processing.

Rapid enzymatic hydrolysis of the conjugated estrogen eliminates the decreased recovery of E₃ caused by glucose, methenamine mandelate, phenolphthalein, and hydrochlorothiazide. Hydrolysis time is comparable to that for acid hydrolysis with the added advantage that enzymatic hydrolysis is less destructive.

The purification step, which separates the phenolic estrogens from the potentially interfering neutral steroids, helps create a more specific method. The short additional time needed to accomplish this separation is well worth the effort. If this purification step is eliminated, if necessary, the method is speeded but will be less specific and less accurate.

### Table 1. Precision of Assay for Placental Estriol in Maternal Urine

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<th>Within-run</th>
<th>Day-to-day</th>
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<tr>
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<td>Range (mg/liter ± SD)</td>
<td>CV, %</td>
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<tr>
<td>n</td>
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<tr>
<td>15</td>
<td>7.0 ± 0.24</td>
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<tr>
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<td>15</td>
<td>21.3 ± 1.4</td>
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Our method is accurate, precise, and specific for urinary placental $E_2$. A single sample can be processed in about 2 h and at least 30 samples can be processed in 8 h. Use of this procedure by technologists rotating through this Division for the last four months has resulted in a day-to-day precision (CV) of 5.7% ($n = 80$).

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References