Simultaneous Determination of Urinary Estriol, Pregnanediol, and Pregnanolone by Gas-Liquid Chromatography

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A simplified method for following the urinary excretion of estriol, Pregnanediol, and Pregnanolone during the course of pregnancy is presented. The procedure has been used successfully in a large hospital laboratory. An important advantage is that the estrogen and progesterone metabolites are measured simultaneously, and separate extraction and gas-liquid chromatography is not required.

The urine specimen is hydrolyzed enzymatically, extracted, and the steroids are converted to trimethylsilyl ethers. Temperature-programmed gas-liquid chromatography is carried out on a 2% n-eopentyl glycol succinate column using a hydrogen flame ionization detector.

The correlation between urinary excretion of estrogens and fetal viability was first described in 1933 (1). Since then, numerous articles confirming this observation have been published (2-9). Similarly, a correlation between urinary pregnanediol and pregnanolone excretion, and the viability of pregnancy has been reported (9-13). Other authors, however, state that pregnanediol and pregnanolone levels reflect placental function, but not necessarily fetal condition (9, 13).

Quantitative methods using preliminary separation or fractionation of crude urinary steroid mixtures by column or thin-layer chromatography, followed by gas-liquid chromatography (GLC) have been developed (19). Due to their complexity, these procedures are unsuitable for most clinical laboratories. Other methods, which require separate hydrolysis of estriol and pregnanediol (20), or separation of estriol from pregnanediol prior to GLC (9), have similar disadvantages.

The method to be described permits determination of estriol, preg-
nanediol, and pregnanolone by GLC without the necessity of prior separation. The resulting simplification enables the laboratory to determine levels of these important metabolites during pregnancy on large numbers of samples.

**Analytic Method**

This method involves enzymatic hydrolysis of the urinary steroid conjugates, extraction with organic solvent, trimethylsilyl (TMSi) ether derivative formation, and gas-liquid chromatography.

**Reagents**

*Glusulase*  
Available from Endo Laboratories, Garden City, N. Y.

*Sodium acetate buffer, 0.2 M, pH 5.2*

*Concentrated hydrochloric acid, A.C.S. grade*

*Diethyl ether, peroxide-free, A.C.S. grade*

*Sodium bicarbonate solution, 9% (w/v), A.C.S. grade*

*Acetone, A.C.S. grade*

*Bis-(trimethylsilyl)-acetamide (BSA)*  
Available from Pierce Chemicals, Rockford, Ill.

*Trimethylchlorosilane (TMCS)*  
Available from Pierce Chemicals, Rockford, Ill.

*Estriol, pregnanediol, and pregnanolone*  
Available from Mann Research Laboratories, New York, N. Y.

*Pyridine, Fisher spectranalyzed, anhydrous, A.C.S. grade*

*Chloroform, Fisher spectranalyzed, anhydrous, A.C.S. grade*

**Procedure**

**Hydrolysis**

A 50-ml aliquot from a 24-hr. urine specimen is filtered and brought to pH 5.2 by addition of glacial acetic acid or 10 N sodium hydroxide. Following this, 10 ml of 0.2 M sodium acetate buffer and 0.5 ml of Glusulase is added, and the solution is incubated in a shaking water bath for 18–24 hr. at 37° (9).

**Extraction**

The hydrolyzed urine is cooled to room temperature and 8 ml of conc. HCl is added. The steroids are extracted with three successive volumes each 75, 75, and 50 ml of ether. Emulsions develop at times, and it may be necessary to let the separated ether phase stand until additional water has settled out.

The ether extract is washed twice with 60-ml portions of 9% sodium bicarbonate (to remove traces of HCl and acid chromogens) and is
dried over sodium sulfate. The extractions and washings are done using a mechanical shaker (Extractomatic, Virtis Research Equipment, Gardiner, N. Y.) which holds six separatory funnels. This has proved to be most advantageous.

The ether is evaporated to dryness using a rotary evaporator in vacuo at 50°. The residue is dissolved in small aliquots of acetone and quantitatively transferred to a 50-ml round-bottom flask and evaporated under rotating vacuum to complete dryness.

**Derivative Formation**

The dried extract from above is dissolved in 1 ml of pyridine and 0.5 ml BSA; 0.025 ml TMCS is added (18). The TMSi derivatives of the steroids are formed in 1/2 hr. at room temperature. Next, the sample is taken to dryness under vacuum and the flask tightly stoppered to protect the TMSi derivatives from moisture.

Near the time of completion of the work reported here, Tallent and Kleiman (22) published a method for injecting a mixture of pyridine, silylated-free fatty acids, and the silylating agent directly onto the column. This is adapted readily to steroid GLC by adding 0.5 ml. of a previously prepared mixture of pyridine, BSA, and TMCS (40:20:1) to the dried urine extract. The reaction is complete in 1/2 hr., and a portion of the reaction mixture is injected directly onto the column without solvent removal.

**Gas-Liquid Chromatography**

The gas chromatograph used is a Hewlett-Packard (F & M Scientific) Model 402, equipped with a hydrogen flame ionization detector and temperature programmer. A 6-ft. glass U-column with an internal diameter of 3 mm. is used. The column packing is 2% neopentyl glycol succinate (NGS) on 80–100 mesh Chromosorb G. Operating conditions are as follows: (1) gas flow rates: helium 60 ml./min., hydrogen 35 ml./min., air 300 ml./min.; (2) initial temperatures: column 210°, flash heater 270°, flame detector 265°; (3) temperature programming: 10 min. After the sample is injected, the column temperature is raised at the rate of 0.5°/min.

The dried TMSi ether derivatives of the steroids are dissolved in 500 μl of chloroform. Sample injections of 5 μl are made, using a 10-μl Hamilton syringe with a Chaney adaptor.

**Standards**

TMSi ether derivatives are prepared so that 5 μl of the final chloroform solution contains 1 μg of each steroid. Estriol, pregnanediol, and pregnanolone (5 mg each) are dissolved in 2.0 ml of pyridine with
gentle warming. BSA (1 ml) and TMCS (0.05 ml) are added, and the reaction carried out for 1/2 hr. at room temperature. The solvents are removed as previously described, and the residue is taken up in 25 ml of chloroform. If protected from moisture this solution is stable—it has been used for as long as 2 months. The standard (5 µl) is injected at the beginning and end of each set of analyses. Comparison of peak areas with the corresponding unknown peak areas provides the basis for quantitating the urinary steroids.

Calculations

The concentration of the individual steroids is determined by calculating the area of the peaks on the chromatogram by the half-width method. The following formula is used to convert these areas to concentration values:

$$\frac{\text{Area}_U}{\text{Area}_S} \times \frac{\text{RAF}_U}{\text{RAF}_S} \times \frac{\text{Vol}}{500} = \text{mg/24 hr.}$$

where:

- $\text{Area}_U$ and $\text{Area}_S$ = Areas of unknown and standard, respectively
- $\text{RAF}_U$ and $\text{RAF}_S$ = range-attenuation factors of unknown and standard, respectively
- $\text{Vol.} = 24$-hr. urine volume in milliliters

Results

Figure 1 demonstrates the separation characteristics of a standard mixture of TMSi ether derivatives of estriol, pregnanediol, and pregnanolone. Figure 2 shows the separation of the derivatized steroids extracted from pregnancy urine by the method described above. Generally, the peaks all emerge within 30 min. and are separated sufficiently to permit accurate quantitation.

Analyses of 20 different pregnancy urine specimens were carried out in duplicate, and precision was calculated for the three metabolites concerned. The standard deviation (S.D.) was calculated according to the following formula:

$$\text{S.D.} = \sqrt{\frac{\sum(d)^2}{2N}}$$

where $d$ is the difference between the paired ($N$) results.

The following precision (1 S.D.) for the measurements was attained; estriol, ±0.55 mg at the 15–30 mg/24 hr. level; pregnanediol, ±1.28 mg at the 35–60 mg/24 hr. level; pregnanolone, ±0.31 mg at the 5–15 mg/24 hr. level.

To determine the recovery efficiency of the method, 0.1 mg each of the three steroids was added to 50 ml of "male" urine and run through
the procedure. On the average, 85–95% of the added amount was recovered. Addition of the conjugated steroids instead of free steroids represents a better recovery study; however, these steroids are difficult to obtain. It was assumed hydrolysis was complete, as Wakabayashi and Fishman (24) have shown enzyme hydrolysis of the conjugated steroids to be complete after 24 hr. of incubation using the conditions described here.

**Discussion**

Enzymatic hydrolysis of the steroid conjugates was used, since there is some evidence that a portion of the total pregnanediol is destroyed by mineral acid hydrolysis (14). We observed losses as high as 50%. Destruction of pregnanediol by acid hydrolysis may be prevented by hydrolysis-extraction in the presence of toluene (14). Although toluene quantitatively extracts pregnanediol, estriol is extracted only partially. This necessitates separate hydrolysis and extraction to determine the estriol level.

Another difficulty with acid hydrolysis is that the recovery of estriol from urine is lowered drastically in the presence of elevated amounts of glucose or other sugars. Certain drugs, such as Mandelamine, have
a similar effect (21). These problems are not encountered with enzymatic hydrolysis.

Other authors have prepared acetate derivatives of the steroids and applied these to columns coated with a nonselective liquid phase, such as SE-30. This procedure yields good separation of estriol from the other steroids, but measurement of pregnanediol and pregnanolone is not reliable owing to the similar retention times of their isomers (15). However, good separation of the 5α,β isomers of pregnanediol and pregnanolone is achieved by forming TMSi ether derivatives of the steroids and using a selective liquid phase, such as NGS. This liquid phase also differentiates between compounds with different degrees of unsaturation, resulting in good separation of estriol from pregnanediol and pregnanolone (16, 17).

During the past 9 months, more than 400 determinations of estriol in pregnancy urine have been performed. Although correlation of the clinical findings and estriol levels are not complete, it is apparent that estriol values below 10 mg/24 hr., after Week 35 of pregnancy, indicate probable fetal distress. Values below 4 mg/24 hr. indicate severe fetal distress or fetal death. Not enough data have been accumulated to state normal and abnormal levels of pregnanediol and pregnanolone during pregnancy, but preliminary data indicate that these measurements may have diagnostic value also for certain special clinical problems.

References