Improved Method for the Measurement of Pregnanetriol in Urine

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ABSTRACT

A procedure for the measurement of pregnanetriol in urine by gas chromatography is described. The internal standard is added to the standards, controls and unknowns prior to the enzymatic hydrolysis of the steroid conjugates. Next, the hydrolyzed steroids are extracted from urine and converted to the volatile trimethylsilyl ether derivatives prior to gas chromatography. The initial addition of the internal standard appears to compensate for all procedural losses in this rather complex procedure. The intra- and interassay coefficients of variation at a level of 1.3 mg per liter were 5.3 percent and 11.8 percent, respectively. The method is linear to a pregnanetriol:internal standard ratio of 0.871. The analytical recovery for the method was 103 ± 10.2 percent.

Introduction

The measurement of pregnanetriol in urine has proved to be useful in the diagnosis of adrenogenital syndrome and is valuable in following the response of these patients to glucocorticoid therapy. In addition, some workers have suggested that this test is useful in the diagnosis of Stein-Leventhal syndrome.

Initially, the method of Bongiovanni and Eberlein was used for the measurement of pregnanetriol. This method uses alumina chromatography to separate pregnanetriol from other steroids prior to a non-specific colorimetric reaction. The most difficult part of this method is the reproducible deactivation of alumina which, in a routine laboratory, proves to be a constant problem, contributing to the poor precision and specificity of this method. In view of these problems, it was decided to use a gas chromatographic method for this measurement, since this approach could provide the required sensitivity, precision and specificity.

A literature search revealed that Sanghvi et al had reported the successful resolution by gas chromatography of un-derivatized steroids. Attempts to reproduce this work, however, were unsuccessful and it was subsequently decided to concentrate on gas chromatographic
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methods using volatile steroid derivatives. Luukkainen et al reported the successful resolution of steroids as their trimethylsilyl ether derivatives.4 The method to be described uses these derivatives and is based in part on the procedure of Chattoraj for the fractionation of 17-ketosteroids.3 The modifications of the procedure are: (1) the addition of an internal standard to each sample at the beginning of the procedure and (2) the use of a working standard which is subjected to the enzymatic hydrolysis, extraction, washes, chemical derivatization and, finally, gas chromatography.

Thus, the internal standard compensates for any losses in the solvent extraction and chemical derivatization steps. The working standard compensates for any extraction efficiency differences between pregnanetriol and the internal standard. These modifications coupled with the known specificity and sensitivity of gas chromatography have yielded an accurate and precise method for the measurement of pregnanetriol.

Methods and Materials

REAGENTS

The following reagents were used as received: spectroanalyzed methylene chloride, chloroform, 2.5 M NaOH, anhydrous sodium sulfate and glacial acetic acid.* Trimethylsilyl derivatives were formed using Sil-Prep, which is a solution of hexamethyldisilazane and chloro-trimethylsilane in pyridine.† Sodium acetate buffer at pH 5 was prepared by adding 28.3 g of sodium acetate to 5.8 ml of glacial acetic acid and adjusting the volume to one liter with distilled water.‡ A 10,000 unit per ml solution of β-glucuronidase, #G-0251, type B-1 from bovine liver was prepared in the acetate buffer described previously.§ Pregnanetriol and the internal standard epicoprostanol† were prepared at concentrations of 50 and 200 mg per liter, respectively, in ethanol.¶

PROCEDURE

One ml of the epicoprostanol internal standard solution is pipetted into all tubes. Into an appropriately labeled tube, one ml of the pregnanetriol standard is pipetted and the contents of all tubes are evaporated to dryness. Ten ml of urine are transferred to each patient tube, control tube and 10 ml of distilled water are placed in the standard tube. One ml of acetate buffer and one ml of β-glucuronidase are added to each tube. The tubes are capped, mixed and incubated for 20 hr at 42° C. All tubes are extracted with 30 ml of methylene chloride, centrifuged and the aqueous upper layer is discarded. The methylene chloride extract is washed with 10 ml of 2.5 M NaOH, followed by four additional washes with distilled water. After aspiration of the last water wash, approximately two g of anhydrous sodium sulfate are added to each tube to remove residual water. The methylene chloride is decanted through filter paper into conical tubes and evaporated to dryness.

The trimethylsilyl derivatives of the steroids are formed by the addition of one ml of Sil-Prep to each tube, followed by a two hr incubation at room temperature. At the completion of the reaction, the tubes are centrifuged and the supernatant is transferred to a conical tube and evaporated to dryness. The residue is dissolved in 50 μl of chloroform and 4 μl are injected into the gas chromatograph. The concentration of pregnanetriol is calculated from either peak height or peak area ratios.

APPARATUS

A Shimadzu gas chromatograph (Model 4BM PFE equipped with a hydrogen

‡ Mallinckrodt Chemical Works, St. Louis, MO 63160.
§ Sigma Chemical Co., St. Louis, MO 63170.
**TABLE I**

Precision Data of Pregnanetriol Method

<table>
<thead>
<tr>
<th></th>
<th>Within-Day Urine Pool 1</th>
<th>Within-Day Urine Pool 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>R (mg per liter)</strong></td>
<td>1.30</td>
<td>8.70</td>
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<tr>
<td><strong>S.D. (mg per liter)</strong></td>
<td>0.07</td>
<td>0.55</td>
</tr>
<tr>
<td><strong>C.V. percent</strong></td>
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<td>6.30</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Day-to-Day Urine Pool 1</th>
<th>Day-to-Day Urine Pool 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td><strong>R (mg per liter)</strong></td>
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<td>7.80</td>
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<tr>
<td><strong>S.D. (mg per liter)</strong></td>
<td>0.16</td>
<td>0.90</td>
</tr>
<tr>
<td><strong>C.V. percent</strong></td>
<td>11.80</td>
<td>11.80</td>
</tr>
</tbody>
</table>

* Shimeadzu Scientific Instrument Co., Columbia, MD 21045.
† Supelco, Inc., Bellefonte, PA 16823.

The temperature program used rose at a rate of one degree per min from an initial temperature of 255°C to a final temperature of 275°C.

**Results and Discussion**

The evaluation of the method consisted of determining the conditions for the gas chromatographic resolution of pregnanetriol from other steroids found in urine and then the characterization of the precision, linearity and analytical recovery under these conditions. In figure 1 is shown the degree of resolution attainable under the gas chromatographic conditions selected. The left panel shows the resolution of dehydroepiandrosterone (DHEA), pregnanediol, estriol, pregnanetriol and the internal standard epicoprostanol. The right panel shows the chromatogram from a typical urine specimen, demonstrating that pregnanetriol is adequately resolved from other steroids found in urine.

**Precision**

In table I are shown the within-day and day-to-day precision data. The within-day precision study was done on two human urine pools. Pool 1 contained pregnanetriol at a concentration within the reference interval. Pools 2 and 3 were at concentrations greater than the reference interval and were made from the urine of congenital adrenal hyperplasia (CAH) patients. These results demonstrate the excellent precision for a method as complex as the one under discussion in this paper.

**Linearity**

Tubes containing 25, 50, 75 and 100 μg pregnanetriol, and equal amounts (200 μg) of the internal standard were analyzed. The ratio of peak heights of pregnanetriol to internal standard was found to be linear throughout this range (range of ratios 0.093 to 0.432). Also, a urine specimen from a patient with CAH was diluted and the ratios of pregnanetriol
to internal standard were found to be linear to 0.871. Linearity cannot be expressed in terms of mg per 24 hr owing to the differences in possible 24 hr urine volumes.

**RECOVERY STUDIES**

Analytical recovery studies were performed by adding a known amount of pregnanetriol to urine specimens of varying pregnanetriol content, and then assaying these specimens as previously described in the procedure. The mean analytical recovery for 17 specimens was 103 ± 2 percent. The range of recovery values (± 10.2 percent) is within the expected day-to-day analytical variation. This demonstrates that the internal standard can adequately compensate for procedural losses and that the working standard compensates for any differences in extraction efficiency between the internal standard epicoprostanol and pregnanetriol.

**REFERENCE INTERVALS**

In figure 2 is shown the distribution of pregnanetriol values from apparently healthy pre-menopausal adult women. The distribution of pregnanetriol excretion probably reflects changes in progesterone metabolism during the menstrual cycle.

The range of values is consistent with reported reference values of less than 2.0 mg per 24 hr, which has been found with other gas chromatographic methods. Twenty-four hour urine specimens were obtained from a group of seven apparently healthy men. Pregnanetriol excretion ranges from 0.5 to 2.0 mg per 24 hr. These values also support an upper limit of 2.0 mg per 24 hr for the reference interval. Twenty-four hour urine specimens were collected from 16 children between the ages of four and 10 years. The mean pregnanetriol excretion was 0.11 and the range was from 0.05 to 0.24 mg per 24 hr. The difficulty in collecting valid 24 hr urines from children is well recognized, leading some workers to suggest that pregnanetriol, creatinine ratios on spot urines, may yield clinically valid results.1 It has been suggested that for children the upper limit for this ratio should be less than 0.5 µg pregnanetriol per mg of creatinine. Our study of specimens from 16 children is consistent with this upper limit for the pregnanetriol-creatinine ratio.

This method has been in use since November 1976 and has been performed on a rotating basis by four senior technologists. The day-to-day CV has been at approximately 11 percent over this period of time. This method is accurate, precise and well suited for use in any appropriately equipped clinical chemistry or endocrine laboratory.

**References**