Rapid Gas Chromatographic Assay for Pregnanolone in Pregnancy Urine

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In 3.8 N HCl at 100°, pregnanediol is selectively destroyed in the presence of pregnanolone, and urinary pregnanolone hydrolysis is complete within 5 min. A rapid gas chromatographic assay for pregnanolone in pregnancy urine is described. As many as 30 samples may be prepared for injection in 1.5 hr. Four women in the third trimester of normal pregnancy excreted 10–29 mg. of pregnanolone per 24 hr.

Gas chromatograms of chloroform extracts of hydrolyzed pregnancy urine have a characteristic appearance (1). Three major peaks appear regularly and are thought to be associated with a group of 11-desoxy-17-oxosteroids, with pregnanolone, and with pregnanediol, respectively.

During a study of the effect of acid concentration on the rate of hydrolysis of urinary pregnanediol glucuronide, it was noted that in some circumstances the pregnanediol peak disappeared. The phenomenon suggested the possibility of using the “rather harsh” conditions of acid hydrolysis to destroy selectively the pregnanediol in pregnancy urine and thus permit the measurement of pregnanolone in crude extracts of hydrolyzed urine. This paper establishes the feasibility of such an assay and makes a preliminary report on its use.

Materials and Methods

Reagents and Apparatus

- Pregnanolone (5β-pregnan-3α-ol-20-one) Make up as 100 μg./ml. in ethanol (AR).
- Progesterone (4-pregnen-3,20-dione) in chloroform, internal standard A concentration of 25 μg./ml. is convenient for use with urine specimens containing between 6 and 25 mg. pregnanolone per liter.
- Hydrochloric acid (AR) Concentrated and diluted 1:1 (v/v) with water.

From the Medical Unit, The Princess Margaret Hospital, Christchurch, New Zealand.
Supported by a grant from the New Zealand Medical Research Council.
Thanks are due to Dr. A. C. Arcus for continuous assistance with instrumentation.
Received for publication Apr. 30, 1968; accepted for publication June 23, 1968.
**Chloroform, AR or anesthetic grade**
**NaOH, 1 N**

**Stationary phase, neopentylglycol adipate polyester (NPGA; Cambridge Industries, Mass.)**

**Coated support**  
Gas Chrom Z (12 gm.), 80-100 mesh, (Applied Science Laboratories) is slurried with gentle degassing in 100 ml. of a 0.5 gm./100 ml. solution of NPGA in acetone (AR). After 15 min. the support is filtered, left to dry for 1 hr. at room temperature, and for a further hour at 90° (2).

**Columns**  
Glass columns (length 150 cm., internal diameter 3 mm.) are packed with hot coated support under gentle suction, care being taken not to damage the particle surfaces. Before use, the columns are conditioned for 32 hr. in a slow stream of nitrogen at 235°.

**Trimethylsilyl ether formation**  
Dry steroid residues are heated for 20 min. at 65° in 0.2 ml. hexamethyldisilazane plus 2 drops trimethylchlorosilane. The reacted solutions are taken to dryness and redissolved in 50 µl. chloroform for gas chromatography at 197°.

**Procedure**

Filtered urine (2.0 ml.*) is hydrolyzed for 15 min. at 100° with 4.0 ml. diluted HCl. Blanks (2.0 ml. water) and standards (0.25 ml. pregnanolone standard and 2.0 ml. water) are treated similarly. The cooled hydrolyzate is shaken vigorously for 1 min. with 4 ml. chloroform plus 0.50 ml. internal standard. The aqueous phase is discarded. The organic phase, washed with 2 ml. 1 N NaOH (shaken 1 min., and the aqueous phase discarded), a portion is transferred by syringe into a pointed tube and dried at 100°. The residue is dissolved in 50 µl. chloroform for gas chromatography (flame ionization detector, column temperature 235°, column nitrogen flow 50-70 ml./min. at 50-52 psi). The ratio of the "areas" of the pregnanolone and progesterone peaks is measured (the area is the peak height times the peak width at half height) and the result expressed in terms of the standard.

**Results**

A typical pregnancy urine chromatogram is shown in Fig. 1B. The second peak has the same retention time as pregnanolone, and the third the same as pregnanediol and 5α-pregnanediol. When a pregnancy urine extract was trimethylsilylated, the resultant chromatogram (Fig. 2) had three major peaks whose retention times were identical with

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*If a pregnanolone concentration of less than 6 mg./L. is expected, 4.0 ml. urine is hydrolyzed with 2.0 ml. conc. HCl.
those of the trimethylsilyl ethers of pregnanolone, pregnanediol, and 5α-pregnanediol. This was confirmatory evidence that the central peak in pregnancy urine chromatograms is due to pregnanolone. The peak is only seen in association with high concentrations of pregnanediol.

Considerable changes occur when pregnancy urine is hydrolyzed at 100° in 3.8 N HCl (Fig. 1). Firstly, large amounts of pregnanediol and pregnanolone are released. Then, as heating continues, the pregnanediol peak gradually disappears, until after 10 min. it is barely visible. During this time the pregnanolone peak appears relatively unchanged.

The rates of decomposition of pregnanolone and pregnanediol in hot acid are shown in Fig. 3. Pregnanediol is about six times as liable to acid damage as is pregnanolone, and in 3.8 N HCl at 100° about 90% is destroyed in the first 15 min. In similar conditions the maximum release of pregnanolone from urine occurs within 5 min. (Fig. 4). Because sufficient pregnanediol remains after 5 min. to interfere with the quantitation of pregnanolone (Fig. 1C), hydrolysis must be allowed to continue beyond this time. After 15 min. the amount of pregnanolone damaged is 10–15%, and sufficient pregnanediol has been destroyed to prevent it from interfering with the measurement of pregnanolone.

The acid decomposition products of pregnanediol do not appear on these chromatograms, and presumably travel through the column, either in the solvent front or behind progesterone; they do not interfere
with the assay. A break-down product of pregnanolone has almost the same retention time as the 11-desoxy-17-oxosteroid peak in pregnancy urine chromatograms. Pregnanolone and progesterone solutions may be safely dried at 100°. No loss occurred when the two steroids were heated at 100° for 30 min. (p > 0.4). Factors affecting the use of progesterone as an internal standard, information about the reagents used and their contribution to the blank signal, and an examination of the recommended washing procedure have already been reported (1).

Preliminary work on the application of the assay to urine indicate that it is reliable (Table 1) and meaningful (Table 2).

**Discussion**

Steroids are excreted in urine mainly as sulfates and glucuronides. Before analysis, it is customary to convert these conjugates to the free form. Fig. 2. Chromatogram of a trimethylsilylated extract of pregnancy urine. *PD*, pregnandiol; *Pone*, pregnanolone; *allo PD*, 5α-pregnanediol.
steroids either by acid or enzyme hydrolysis. Acid hydrolysis is a destructive process compared with the action of $\beta$-glucuronidase and the sulfatases, and in urinary steroid work is used only because of the rapidity with which it can be undertaken.

Fig. 3. Rates of decomposition of pregnanolone and pregnanediol in 3.8 N HCl at 100°

Fig. 4. Effect of acid concentration on the rate of hydrolysis of urinary pregnanolone at 100°.
Table 1. Recovery of Pregnanolone Added to Urine

<table>
<thead>
<tr>
<th>Added (mg./L.)</th>
<th>Found (mg./L.)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.9</td>
<td>-</td>
</tr>
<tr>
<td>6.25</td>
<td>8.7</td>
<td>109</td>
</tr>
<tr>
<td>9.4</td>
<td>12.2</td>
<td>110</td>
</tr>
<tr>
<td>12.5</td>
<td>15.5</td>
<td>109</td>
</tr>
<tr>
<td>18.75</td>
<td>21.2</td>
<td>103</td>
</tr>
<tr>
<td>25.0</td>
<td>28.0</td>
<td>104</td>
</tr>
</tbody>
</table>

Each figure is the mean of three determinations. The S.D. of all the figures from their respective means is 0.1 mg./L.

Table 2. Pregnanolone Excretion During Normal Pregnancy

<table>
<thead>
<tr>
<th>Weeks pregnant</th>
<th>Pregnanediol (mg./4 hr.)</th>
<th>Pregnanolone (mg./4 hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>33</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>34</td>
<td>50</td>
<td>21</td>
</tr>
<tr>
<td>37</td>
<td>59</td>
<td>29</td>
</tr>
</tbody>
</table>

The assay described in this paper makes use of the destructive properties of hydrochloric acid. Pregnanolone is "separated" from pregnanediol by choosing hydrolysis conditions such that major damage to pregnanediol occurs with only minor damage to pregnanolone. Hydrolysis, and a degree of purification sufficient to allow the measurement of pregnanolone in urine, are achieved together.

As a result of this procedural economy, 30 urine samples may be prepared for injection in 1.5 hr. Each gas chromatographic analysis takes 10 min. It appears that a practical pregnanolone assay exists which is technically simple, requires the minimum of preparative work, and is suitable for the analysis of large numbers of samples.

Initial measurements of the excretion of pregnanolone by 4 women in the third trimester of pregnancy (Table 2) agree with results reported in a 1963 review (3), but not with those reported by Lachèse et al. (3). The Lachèse group obtained low results compared with earlier workers and considered the disagreement due to the improved specificity of their assay, a claim which cannot, however, be confirmed in the absence of quantitative information about the conversion of pregnanolone glucuronide to pregnanolone under the conditions they used. The proof that true pregnanolone glucuronide is being measured in the method described in this paper awaits the availability of radioactive pregnanolone glucuronide. Only then will critical studies of assay losses and of the radiochemical purity of the gas chromatographic pregnanolone peak be possible.
References


Erratum


On p. 157 under Materials: Reagent 3 should read Alcoholic potassium hydroxide ... with 95% ethyl alcohol. Working solution: dilute 100 ml of stock solution to 50 ml with 95% alcohol on day of use.

On p. 157 under Materials: Reagent 6 should read Sodium arsenite, 0.5 M.

On p. 158, line 9: ... glycerol released on saponification). After exactly 10 min., the oxidation was stopped by the addition of 0.1 ml sodium arsenite. Several minutes later....