Rapid Methods for the Determination of Pregnanediol in Urine

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Pregnanediol determination in urine was performed by alumina-column chromatography followed by reaction with sulfuric acid and by gas chromatography. After enzymatic hydrolysis of the conjugated urinary steroids, there is a very good agreement between both methods.

Although many methods have been published for the determination of pregnanediol in urine, we found none of them fully satisfactory. The first gravimetric method (1), and colorimetric methods derived from this one (2,3), are rather unspecific, because, especially under pathologic conditions, fairly large amounts of similar compounds are found in the so-called pregnanediol fraction. Determination of unconjugated pregnanediol was described by several authors (4-6) and although these methods are rather useful, they are very laborious and require highly skilled technicians.

The introduction of chromatography led to many improved procedures among which the methods of Klopper et al. (7) and Goldzieher and Nakamura (8) are most widely used. In the first method (7), pregnanediol is liberated by acid hydrolysis and the toluene extract purified by alkaline and permanganate washings; after double adsorption chromatography on alumina columns and acetylation of the steroids, the final determination is done spectrophotometrically after a reaction with sulfuric acid. Quantitation requires the application of Allen's correction (9). The results are satisfying, but the method is laborious.

The second method (8), using enzymatic hydrolysis and silica-gel chromatography, is slightly more sensitive, using as a final reagent sulfuric acid saturated with sulfur dioxide.

An excellent study on the specificity of the sulfuric acid reaction was published by Henry and Thevenet (10), who also developed a detection method for pregnanetriol and pregnanediolone. This method, which is
also based on alumina-column chromatography, gave good results in our laboratory, but is still rather laborious. In order to check the specificity of this chemical procedure, we compared the method with gas chromatography, using previously published procedures (11, 12). Doing so, we developed our own gas chromatographic technic and column chromatographic spectrophotometric method, and compared these two procedures.

Materials and Methods

Hydrolysis and Extraction

The first decision we had to make was whether to submit the urine to hot acid hydrolysis (10 min. boiling in 4%, v/v, HCl under a layer of toluene) or enzymatic hydrolysis (16 hr. at 37°, buffered with acetate at pH 5.2, and addition of 1 ml. enzyme solution containing 100,000 F units β-glucuronidase and 800,000 R units sulfatase). After hydrolysis, the urine is extracted with toluene; after alkaline and water washings, the combined toluene extracts are dried over anhydrous sodium sulfate and evaporated to dryness in a rotating evaporator at 40°.

We found that during acid hydrolysis many impurities formed which interfered with the pregnanediol determination (Fig. 1A and 1B). For this reason we decided to use enzymatic hydrolysis, taking 100 ml. of urine and adding 1 ml. of enzyme solution. The urine is buffered by adding 5 ml. acetate buffer (2.0 M, pH 5.2) and adjusting the pH of the mixture to 5.2. After 16 hr. of hydrolysis at 37°, the urine is extracted once with 100 ml. toluene and once with 50 ml. toluene. The extracts are combined and washed once with 25 ml. of a 25% (w/v) NaCl solution in 1 N NaOH and twice with 25 ml. of water. The organic layer is dried over anhydrous sodium sulfate and evaporated to dryness in a rotating evaporator.

Column Chromatography

Preparation of Column

The tip of a 16-cm. glass column (I.D. 0.5 cm.) is plugged with glass wool, and the column is partly filled with benzene. Aluminum oxide, 1.5 gm., neutral, activity Grade 1 (M. Woelm, Eschwege, Germany), is added in a thin stream so that it is free from air as it settles. The height of the alumina column is approximately 7 cm.

Determination of Activity Grade of Alumina

Pregnanediol (50 μg.) in benzene is added to the column, which is then eluted with following solvent mixtures:

A. 60 ml. benzene containing 0.25% methanol
B. 50 ml. benzene containing 0.50% methanol
Then 5-ml. fractions are collected. Pregnanediol should be eluted completely in the fifth and sixth fraction of the second mixture. Detection is performed with the sulfuric acid reaction (below).

When the activity of the alumina is too low, it is raised by heating at 250° for 6 hr. and stored in a dessicator overnight. When the activity is too high, water is added to the alumina bulk, thoroughly mixed, and kept for 24 hr. in a tightly closed vessel. Once the acceptable activity is obtained, the alumina is kept in a flask with a small opening, and each time a column is to be made, the amount of the alumina is dispensed. It should be noted that each column is discarded after use.

Chromatography

To the dried extract obtained after hydrolysis and extraction of the urine, 5 ml. of benzene is added and the solution is placed on the column. Elution is carried out with Mixtures A and B. The eluate of Mixture B is collected in a round-bottom flask and evaporated to dryness in the

![Fig. 1. A. Gas chromatogram of pregnanediol diacetate from urine extract after acid hydrolysis. Sensitivity range 5 X 10⁶ ohm input resistance across electrometer grid. Attenuation 8 X. P, pregnanediol peak. B. Gas chromatogram of pregnanediol diacetate from same urine extract after enzymatic hydrolysis. Sensitivity and attenuation same as in A.](image-url)
rotating evaporator. Gas chromatography of the eluates shows that a high degree of purification is obtained in this way (Fig. 2).

**Sulfuric Acid Reaction**

An 80% (v/v) sulfuric acid solution in water (5 ml.) is added, and the reaction mixture is allowed to stand at room temperature for 30 min. Next the absorption spectrum is measured between 325 and 400 nm. The extinction is corrected (Allen’s correction, wavelengths 335, 350, and 390 nm), and the amount of pregnanediol is calculated by means of the added internal standard (pregnanediol, Sigma Chemical Co., St. Louis, Mo.).

**Gas Chromatography**

**Column Conditions**

The column was a 6-ft. siliconized glass column packed with 2% SE 30 on 80-100 mesh diatoport S. Other conditions were: column tempera-
ture, 250°; carrier gas, nitrogen; flow, ± 45 ml./min.; flame ionization detector temperature, 280°; and flash heater temperature, 280°.

**Acetylation**

The dried extract, obtained after hydrolysis and extraction of the urine, is dissolved in 1 ml. benzene and acetylated for 1 hr. at 58° by adding 1 ml. acetychloride. After acetylation, the reaction mixture is evaporated to dryness, and the remaining sample dissolved in 100 μl. cyclohexane.

**Injection**

After priming the column, 1 μl. of the sample is injected on the column.

**Calculation**

The amount of pregnanediol is calculated by comparing the peak heights of the urinary samples, with and without internal pregnanediol standard.

**Discussion**

It is clearly demonstrated by gas chromatography of the extracts of hydrolyzed urine that with enzymatic hydrolysis the possibility of producing artifacts is far less than with acid hydrolysis. When determination of both pregnanediol and pregnanetriol is desired, it seems advisable to use alumina-column chromatography followed by the sulfuric acid reaction. If only the amount of pregnanediol is to be assayed, gas chromatography is a more rapid procedure. From Table 1

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<th>Specimen No.</th>
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it can be seen that the values obtained are the same in both methods. We think it a great advantage to be able to compare, with good results, the developed gas chromatographic method with a more classic method.

References