A CLINICAL METHOD FOR THE DETERMINATION OF URINARY PREGNANEDIOL AND PREGNANETRIOL

By

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ABSTRACT

A method suitable for the routine determination of urinary pregnanediol and pregnanetriol has been devised. It involves glucuronidase hydrolysis, extraction, and chromatography on disposable 1-g silica gel columns. The specificity of the pregnanediol determination is increased by acetylation and rechromatography on the same type of column. Sensitivity of the method is doubled by the use of sulfuric acid saturated with SO₂ gas as the chromogenic agent.

In steroid methodology as in other branches of analytical chemistry, there are ever-increasing demands for greater sensitivity and specificity. Less than two decades ago, gravimetric measurement of urinary sodium pregnanediol glucuronide sufficed to yield valuable information regarding placental production of progesterone; today, investigations of the urinary metabolites of adrenal and testicular progesterone and 17-hydroxyprogesterone excretion require far more sophisticated methods. A great refinement in methodology was the introduction by Klopper et al. (1955) of an acetylation step between two stages of chromatography on alumina. Subsequently, others have used various forms of multiple chromatography, but frequently without adequate assessment of the specificity or reliability of their methods. Wide clinical interest in enzymatic defects in adrenal function, particularly those involving 21-hydroxylation, has also led to the development of numerous methods for the estimation of pregnanetriol. Highly specific, but complex and difficult methods have been

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devised for its determination, and the problem of a suitable technique has by no means been solved. Discussions of the problems involved have been presented by Cox (1960) and Bongiovanni & Eberlein (1958).

In our experience, methods involving several stages of paper chromatography have not proved suitable for routine analysis of large numbers of samples, and column chromatography employing alumina has required frequent and cumbersome monitoring of the activity of the alumina. We have therefore developed a sensitive method for the determination of urinary pregnanediol and pregnanetriol employing commercial silica gel that requires no monitoring, and using small, disposable chromatographic columns with which 8–10 determinations (plus controls) may be carried out simultaneously.

**METHOD**

**Reagents**

Silica gel (Davison Chemical Co., grade 923, 100–200 mesh) used as packaged without any preconditioning.

Benzene: Reagent grade, washed with sulfuric acid and water; dried and redistilled.

Chloroform: Reagent grade; redistilled in the dark and stabilized with ethanol.

Ethyl acetate: Reagent grade, redistilled.

The solvent mixtures used are 5, 25, and 50 per cent (v + v) ethyl acetate in benzene.

SO₂-sulfuric acid reagent. SO₂ gas (Matheson, lecture bottle) was bubbled at the rate of 2–3 bubbles per second into 200–300 ml of reagent grade concentrated sulfuric acid for ½ hour. The reagent is stable for at least a week.

Acetic anhydride, reagent grade, fresh.

Glucuronidase (Ketodase, Warner Chilcott).

Approximately 5 N sulfuric acid.

Approximately 10 per cent and 0.1 N sodium hydroxide solutions.

Acetate buffer, pH 4.5, 1 M.

Sodium sulfate, anhydrous, reagent grade.

Pregnanediol-3a,20α, purified.

Pregnanetriol-3α,17α,20α, purified.

**PROCEDURE**

*Hydrolysis and extraction of urine*

A 20 ml sample of urine is pipetted into a commercial 80 ml glass stoppered centrifuge bottle or preferably into a custom-made, heavy-wall 35 × 180 mm centrifuge bottle. The urine is adjusted approximately to pH 4.5 with 5 N sulfuric acid or 10 per cent sodium hydroxide, 2 ml of pH 4.5 acetate buffer and 6,000 U of β-glucuronidase (Ketodase) are added, and incubation carried out at 37°C for 18–24 hours. After the addition of 20 ml chloroform, the bottle is stoppered and shaken gently for 2 minutes. The phases are allowed to separate, using centrifugation for 15 minutes at 2500 rpm.
if an emulsion forms. The chloroform is aspirated with a suitable pipette and Pro-
pipette and transferred to a 125 ml Erlenmeyer flask. A second 20 ml portion of
chloroform is added to the urine and shaken as before. After separation of the phases
the urine is removed and discarded by suction with a glass capillary siphon con-
ected to water pump or house vacuum. The first chloroform extract is poured back
into the centrifuge bottle and the Erlenmeyer flask and pipette are rinsed with a small
volume of chloroform from a polyethylene squeeze bottle. The combined chloroform
extracts are washed once with 15 ml 0.1 N sodium hydroxide (which is siphoned off)
and once with 15 ml of distilled water (also siphoned off). A suitable amount of
anhydrous sodium sulfate (usually about 10 g) is added and allowed to stand with
occasional swirling for at least 15 minutes. The dry chloroform is filtered into a
125 ml Erlenmeyer flask with the aid of a funnel and Whatman No. 1 filter paper.
The centrifuge bottle and sodium sulfate are rinsed three times with small volumes
of chloroform. The chloroform is evaporated with a stream of filtered air on a water-
bath or hot plate at 45°C. The walls of the flask are washed down several times with
a small volume of chloroform during evaporation. The residue is transferred quantita-
tively to a 10 ml volumetric flask with chloroform, which is then evaporated. This is
made up to volume with benzene.

Preparation of chromatographic columns

Previously acid-washed 8 mm O.D. borosilicate glass tubing is cut into 35 cm
lengths. A piece of thick glass rod is heated to redness and fused to one end (un-
heated) of the glass tubing. After heating a portion of the tubing 2–3 cm above this
joint, it is drawn out to a long fine capillary tip. The tip is sealed off in the flame
about 10 cm from its origin (Fig. 1).

A row of such columns is held by clips (A. N. Thomas clamps 3237 K sizes 2 and 5,
holders 3237 J 4 and 3237 G) on a stand. About 2 ml of benzene is added to each
column and a small cotton plug is tamped into the bottom of the column with a glass
rod so that its upper surface is flat and its height is about 4–5 mm. All residual air
bubbles must be expelled from the cotton. 1.0 g silica gel is weighed out and placed
in a short test tube containing about 3 ml benzene. A dropper bulb attached to a
pipette with a wide orifice is used to mix the silica gel thoroughly and expel trapped
air. The silica gel and benzene are then transferred with the same pipette into the
column. A small additional volume of benzene may be used to suck up and transfer
all the gel from the test tube. The chromatographic column is filled to the top with
benzene and the gel settled by tapping the column gently with a glass rod. The tip
of the capillary is broken off to produce a flow of about 1 drop every second. The
supernatant benzene in the column is drawn off and discarded.

First chromatography

An aliquot not exceeding 5 ml of the benzene solution containing the assay sample
is transferred quantitatively to the column without disturbing the surface of the silica
gel. Additional benzene (the final volume not to exceed 6 ml) is used to rinse the
sample onto the column. The eluate from this fraction is discarded.

Next, a 1/2–1 ml portion of the second phase (10 ml of 25 per cent ethyl acetate in
benzene) is run through the column (a dropper bulb pipette is convenient for these
transfers) and this is followed by the remainder of the second phase. This eluate is
also discarded. The third phase, added in the same fashion, consists of 7 ml 50 per cent
ethyl acetate in benzene and elutes the pregnanediol fraction. The final phase is 10 ml
ethyl acetate; this elutes the pregnanetriol fraction.

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Acetylation

The pregnanediol fraction, collected in a large test tube, is evaporated to dryness with an air stream and gentle (45°C) warming. To each tube 1.0 ml of fresh acetic anhydride is added and rolled around the walls to dissolve the steroid residue. The tubes are then inclined on a hot plate or preferably in an aluminum block or sand bath kept at about 100°C; the exposed sides of the tubes are covered with a sheet of aluminum foil to maintain the temperature. If the acetic anhydride has not vaporized completely after 1 hour, 0.5 ml of ethanol is added and a gentle stream of air is used to complete the evaporation. (At high altitudes, e.g., Mexico City, it has been found advisable to add a drop of pyridine and let the tubes stand overnight before evaporation).
Second (acetate) chromatography

Silica gel columns are prepared in the same manner as before. The residue in the acetylation tubes is transferred quantitatively to the column with benzene, using a total of 5.0 ml. The eluates from this and from the succeeding phase (2 ml 5 per cent ethyl acetate in benzene) are discarded. The third fraction consists of a further 5 ml 5 per cent ethyl acetate in benzene; this elutes the pregnanediol diacetate quantitatively. The solvent is evaporated from this fraction in the manner described previously.

Colorimetry

Exactly 4.0 ml of the SO₂-sulfuric acid reagent is added to each tube from a 100 ml burette. The reagent is rolled around the tube walls to dissolve the steroid residue. The tubes are stoppered lightly (glass stoppers are convenient), heated in a boiling water bath for exactly 4 minutes, and cooled in ice water. The resulting colour is very stable. Both the pregnanediol diacetate and pregnanetriol fractions are developed in the same manner. Readings are carried out in a Beckman DU or DB spectrophotometer, with measurements at 395, 430 and 465 mµ for pregnanediol and 400, 435 and 470 mµ for pregnanetriol. A blank derived from solvents run through the column and standards of 50 µg (pregnanetriol) and 25 µg (pregnanediol) are used. The correction equations

$$\text{Corr. O.D.} = 2 \times \text{O.D.}_{430} - (\text{O.D.}_{395} + \text{O.D.}_{405})$$

and

$$2 \times \text{O.D.}_{435} = (\text{O.D.}_{400} + \text{O.D.}_{470})$$

have been found to be valid by the method of O’Sullivan (1958).

RESULTS

Accuracy

Recoveries were studied by the addition of 25 µg quantities of pregnanediol and/or pregnanetriol to the residues from chloroform extracts of hydrolyzed urine. In 32 experiments with pregnanediol the average recovery was 99.3 ± 8.6 (S. D.) per cent and in 17 experiments with pregnanetriol, the recovery was 101.2 ± 3.9 per cent.

Precision

Reliability of the method was evaluated by determinations carried out on duplicate aliquots of enzyme-hydrolyzed urine. There were 18 urines in which the pregnanediol excretion ranged from 0.03 to 1.00 mg/day. The mean difference between duplicates was 0.12 ± 0.107 (S. D.) mg/day, with the standard deviation calculated from the formula

$$s = (\Sigma d^2 / 2N)^{1/2}.$$  

In a group of 14 urines where the pregnanediol excretion ranged from 1.7 to 10.9 mg/day, the mean difference between duplicate determinations averaged 0.15 ± 0.16 mg/day. Similar estimations were carried out for the determination of pregnanetriol. In 10 urines where the excretion ranged from 0.10 to 0.85 mg/day, the mean difference between duplicate determinations was 0.04 ± 0.01 mg/day, and for 9 urines where the excretion ranged from 1.1 to 3.5 mg/day, the mean difference between duplicates was 0.12 ± 0.09 mg/day.
Sensitivity

This parameter, defined as the smallest quantity which can be distinguished from zero with a certainty of \( P = 0.01 \), was calculated from the formula \( S = ts/N^{1/2} \), where \( t = 2.572 \) for \( P = 0.01 \), \( s \) = the standard deviation of the mean difference between duplicates (see precision, above) and \( N = 2 \) for duplicate determinations. On this basis, the sensitivity of the method for pregnanediol, based on 18 urines with low pregnanediol content (0.03 to 1.00 mg/day) was 0.19 mg/day. The sensitivity for pregnanetriol, based on 10 urines with low pregnanetriol content (0.10 to 0.85 mg/day) was 0.02 mg/day.

Specificity

A number of steroids, particularly those closely related to pregnanediol and pregnanetriol, or those likely to interfere in certain clinical situations, have been studied for their behaviour on the silica gel chromatograms both before and after acetylation. The results of a number of determinations with each steroid are shown in Table 1. Pregnanediol-3α,20β is seen to be slightly less polar than the α,α isomer, and a small amount is usually lost in the 25 per cent ethyl acetate/benzene preceding the pregnanediol fraction of column 1. It is also slightly less chromogenic (94.3 per cent) microgram for microgram, than the α,α isomer. Thus, with an 83.5 per cent overall recovery and a 94.3 per cent relative chromogenicity, this isomer will register only 78.5 per cent of the value of an equivalent quantity of the α,α isomer. The allopregnanediols have about 50 per cent of the net chromogenic value of pregnanediol-3α,20α. These compounds will therefore interfere in an attempt to measure only the major metabolite of progesterone, but will yield an underestimate if an index of all the dihydroxy metabolites of progesterone is desired. In the same connection, it is noteworthy that pregnanolone and allopregnanolone are well separated from the pregnanediol fraction. As metabolites of progesterone (especially in pregnancy), their inclusion might be desirable under some circumstances. We therefore investigated the possibility of using sodium borohydride to reduce these compounds to diols, which would cause them to appear in the desired fraction. However, this procedure also reduced the urinary 17-ketosteroids to diols, and these exhibited a prohibitive interference in the final colorimetric determination. Other avenues will therefore have to be explored for the development of a method which includes all the major metabolites of progesterone. Of the dihydroxy monoketones tested, only allopregnan-3β,17α-diol-20-one passed through the two stages of chromatography and acetylation, with a net recovery of only 5 per cent. The chromogenicity of this compound is quite low, however, so that it yields in the final result only 2 per cent of the colour of an equivalent quantity of the reference pregnanediol-3α,20α. The diketones and triols tested are well separated from pregnanediol, as are most of the highly oxygenated steroids of the corticoid group.
Reichstein's compound S, tetrahydro-S and cortolone are present to an appreciable degree in the pregnanediol fraction of the first column, and after acetylation to a minor extent in the pregnanediol diacetate fraction of the second column. The chromogenicity of these compounds is very low, however, and they do not interfere to a significant extent. Of the major urinary 17-keto-steroids, 11-ketoaetiocholanolone and aetiocholanolone appear in the pregnanediol fraction of the first column. Acetylation and rechromatography eliminates the former steroid completely, while the latter yields a colour equivalent to only 1.2 per cent of the reference compound. It would therefore seem that the specificity of the method for steroid diols is satisfactory.

The specificity of the method for pregnanetriol is of course much less since it involves only one stage of chromatography and no change in polarity by acetylation. However, it will be seen that further isolation of pregnanetriol is generally unnecessary. The 5α isomer, allopregnane-3β,17α,20β has the same chromogenic potency as the reference compound, but a sharply different mobility, hence it has a net chromogenic value of only 32 per cent of that of pregnanetriol-3α,17α,20α. The other major metabolites of 17-hydroxyprogesterone such as the 3,17-dihydroxy-20-ketone, are well separated. Furthermore, the 11-keto derivative of pregnanetriol has a markedly different absorption spectrum, so that it shows little interference in spite of the fact 72 per cent of the compound is eluted in the pregnanetriol fraction. The pregn-5-enetriol is less polar than the pregnanetriols and develops only 15.2 per cent as much colour as the reference compound, hence an equivalent quantity interferes only to the extent of 8 per cent. A number of the corticosteroid metabolites appear in the pregnanetriol fraction as well, but their absorption spectra are such that they interfere to a negligible degree; tetrahydrocortisone, for example, yields only 5.7 per cent as much colour as an equivalent quantity of pregnanetriol.

Sodium sulfite or metabisulfite have been used by others to enhance the colour developed in the reaction between the steroids and sulfuric acid. This reagent has proved most unsatisfactory in our hands, since it is usually somewhat opalescent and precipitates crystals on slight cooling. Saturation of sulfuric acid with SO₂ also has the effect of doubling the absorbance of the steroid chromogens, but yields a stable, crystal-clear reagent.

**DISCUSSION**

Of the various types of well-established steroid chromatography, silica gel micro-columns appear to have decided advantages in terms of reproducibility, stability, simplicity and convenience. Except for a simple airstream evaporator arrangement and perhaps an aluminum heating block for the acetylations, no equipment is required which is not readily available in a routine laboratory. Even the measurement of the chromatographic phase volumes can be simplified.
Table 1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Column I</th>
<th>Column II</th>
<th>Net % appearing as pregnanediol diacetate</th>
<th>Chromogenicity as % of pregnanediol</th>
<th>Net chromogenicity as % of equivalent amount of pregnanediol</th>
<th>Pregnanetriol</th>
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<tr>
<td></td>
<td>Column I Fraction</td>
<td>Column II Fraction</td>
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<td></td>
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<tr>
<td>2</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>Pregnane-3α,20α-diol</td>
<td>0.7</td>
<td>95.0</td>
<td>0.6</td>
<td>94.0</td>
<td>89.3</td>
<td>(100)</td>
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<td>Pregnane-3α,20β-diol</td>
<td>9.0</td>
<td>88.1</td>
<td>94.5</td>
<td>83.5</td>
<td>94.3</td>
<td>78.5</td>
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<td>Allo pregnane-3α,20α-diol</td>
<td>21.6</td>
<td>66.2</td>
<td>3.4</td>
<td>94.8</td>
<td>62.7</td>
<td>80.7</td>
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<td>Allo pregnane-3β,20α-diol</td>
<td>12.8</td>
<td>80.8</td>
<td>98.0</td>
<td>79.1</td>
<td>79.1</td>
<td>62.7</td>
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<td>Allo pregnane-3β,20β-diol</td>
<td>46.1</td>
<td>50.4</td>
<td>97.2</td>
<td>49.0</td>
<td>94.4</td>
<td>46.7</td>
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<td>Pregnane-3α-ol-20-one</td>
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<td>Allo pregnane-3α-ol-20-one</td>
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<tr>
<td>Allo pregnane-3β,17α-diol-20-one</td>
<td>31.2</td>
<td>50.0</td>
<td>10.2</td>
<td>5.0</td>
<td>39.6</td>
<td>2.0</td>
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<td>Pregn-5-ene-3β,17α-diol-20-one</td>
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<td>43.9</td>
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<td>Pregn-4-ene-17α,20-diol-3-one</td>
<td>-</td>
<td>67.8</td>
<td>32.8</td>
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<td>0.0</td>
<td>1.0</td>
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<td>Pregnane-3α-ol-11,20-dione</td>
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<td>Pregnane-3α,17α,20α-triol</td>
<td>91.9</td>
<td></td>
<td></td>
<td></td>
<td>(100)</td>
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<td>Allo pregnane-3β,17α,20β-triol</td>
<td>73.5</td>
<td>31.5</td>
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<td>32.2</td>
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<td>Pregnane-3α,17α,20α-triol-11-one</td>
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<td>72.1</td>
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<td>5.9</td>
<td>4.3</td>
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<td>Substance</td>
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<td>4.4</td>
<td>4.2</td>
<td>1.4</td>
<td>0.06</td>
<td>3.4</td>
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<tr>
<td>Reichstein's S</td>
<td>94.2</td>
<td>3.6</td>
<td>4.4</td>
<td>4.2</td>
<td>1.4</td>
<td>0.06</td>
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<td>Tetrahydro-cortisone</td>
<td>11.5</td>
<td>85.7</td>
<td>4.3</td>
<td>0.5</td>
<td>27.3</td>
<td>0.14</td>
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<td>Tetrahydro-cortisol</td>
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<td>76.9</td>
<td>0.0</td>
<td>0.39</td>
<td>8.9</td>
<td>0.03</td>
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<td>Tetrahydro-S</td>
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<td>75.0</td>
<td>1.7</td>
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<td>0.0</td>
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<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<td>Cortolone</td>
<td>28.9</td>
<td>39.1</td>
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<td>0.0</td>
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<td>Androsterone</td>
<td>89.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td>Aetiocholanolone</td>
<td>65.1</td>
<td>25.1</td>
<td>0.0</td>
<td>47.6</td>
<td>12.0</td>
<td>10.3</td>
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<td>Dehydroepiandrosterone</td>
<td>86.6</td>
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<tr>
<td>11-ketooetiocholanolone</td>
<td>89.8</td>
<td>3.5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>-19.7</td>
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</table>
by the use of reproducing syringes or dispensing heads (Calab dispensers, California Laboratory Equipment Co., Berkeley, California) if the volume of work warrants.

The foregoing technique permits the determination of two steroids of frequent clinical interest. The pregnanediol determination, in particular, is suitable both for situations where large quantities of this metabolite are present, as in pregnancy urines, and for problems where the quantities excreted are very low, as in the study of ovulation inhibition or of adrenal pregnanediol excretion. It would be useful to have a method, based on the same equipment, for the fractionation of urinary 17-ketosteroids. Such a method has been developed and is the subject of another communication (Goldzieher et al., in press).

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REFERENCES


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