Pregnanediol-3α-Glucuronide Measured in Diluted Urine by Mass Spectrometry with Fast Atom Bombardment/Negative-Ion Ionization

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We describe a mass-spectrometric method based on the fast atom bombardment ionization technique in the negative-ion mode for measuring pregnanediol-3α-glucuronide in diluted urine from women. The procedure requires addition of testosterone-17β-d-glucuronide (2.5 μg/25 μL) to the urine sample as internal standard, and the sample is added directly to the fast atom bombardment target with no further manipulation. We have assessed and evaluated the method by the traditional criteria of reliability.

Additional Keyphrases: steroids · luteal function · fertility studies · monoclonal antibodies

For two reasons, measurement of urinary pregnanediol-3α-glucuronide (Pd-gl), the major metabolite of progesterone appearing in urine, has not been widely used for assessing luteal function: (a) determinations of hormones in urine usually require a 24-h urine specimen; and (b) available methods for urinary Pd-gl based on colorimetry (1), gas chromatography (2), or mass spectrometry (3, 4) require previous enzymic or acid hydrolysis of the Pd-gl and subsequent extraction of the free steroid—steps that are time-consuming and hydrolysis procedures that are not always reliable (5).

Recent developments, however, have improved the practical and methodological prospects for determination of this analyte.

The availability of specific monoclonal and polyclonal antibodies to glucuronide steroids has allowed direct measurement of these metabolites, even in dilute urine. Immunoadsorbents for pregnanediol-3α-glucuronide (6, 7), estrone-3α-glucuronide (8, 9), estriol-16α-glucuronide (10, 11), estradiol-3α-glucuronide (12), and testosterone-17β-glucuronide (13), with use of radioactive (6, 8, 10, 12) or chemiluminescent (7, 9, 11, 13) tracers have been already described.

In addition, several investigators (14–16) have demonstrated the adequacy of measuring the concentration of Pd-gl in specimens of first morning urine instead of 24-h urines for studying luteal function. This approach increases the applicability of the test by facilitating sample collection.

With the development and commercial availability of mass spectrometers equipped with more sophisticated ionization systems, such as the fast atom bombardment (FAB) ionization technique, one now can analyze compounds directly that previously could not be easily assayed because of their high polarity, thermolability, or low vapor pressure (17, 19). Regarding urinary steroid metabolites (glucuronides and sulfates), Shackleton et al. (20, 21) have described mass-spectrometric methods based on FAB ionization technique in the negative-ion mode that are suitable for detection of Pd-gl and urinary metabolites of other steroids.

The main advantages of this novel approach are that one can measure steroid glucuronides, which, because they are highly polar, are not ionized by traditional techniques (i.e., electron impact ionization) without derivatization. Moreover, the FAB ionization technique in the negative-ion mode almost exclusively produces (M–H)– ions in the spectra without co-production of fragment ions, and this results in high detection sensitivity (20).

The approach of Shackleton et al. (20) thus offers substantial advantages in comparison with the method of Spiegelhalder et al. (22), which requires preparation of trimethylsilyl ether esters of the steroid glucuronides. However, the procedure described by Shackleton et al. requires concentration and pre-purification of the urine sample (20) and gives only a qualitative profile of conjugate human urinary steroids (23). Moreover, this approach is not suitable for assessment of the luteal phase, where a faster and quantitative determination of Pd-gl is required (for assay of 25 to 30 samples per menstrual cycle).

In our laboratory, we have developed a FAB/negative-ion mass-spectrometric method for the quantification of Pd-gl in diluted urine samples from women. Testosterone-17β-d-glucuronide (T-G) is used as the internal standard. In an attempt to evaluate its potential capability in clinical diagnosis, we have evaluated the method for reliability and practicability, and we compared the results obtained with those by a conventional gas–liquid chromatographic (GLC) method and by a chemiluminescent immunoassay (LIA) method.

Materials and Methods

Mass spectrometry. Pd-gl (5β-pregn-3α,20α-diol-3α-glucopyranosiduronate) and T-G (17β-hydroxy-3-oxo-4-androstene-3-glucopyranosiduronate) were obtained commercially (Sigma Chemical Co., St. Louis, MO) and used without further purification.

FAB mass spectra were obtained with a VG 70-70 EQ instrument (VG Analytical, Manchester, U.K.) fitted with its own standard FAB ion source, Ar atoms of 7 keV kinetic energy. We recorded complete spectra in negative-ion mode at M/ΔM 3000 mass resolution (10% valley definition) and a run speed of 20 s per decade. The data were processed by a Digital PDP8/A computer system. We added 25 μL (2.5 μg) of T-G dissolved in ethanol to 100 μL of urine. After mixing, 5 μL was added directly to 3 μL of glycerol, and the mixture was transferred onto the FAB target and inserted into the ion source, with no other manipulation. We dissolved the
samples in glycerol to obtain long-lasting and intense ion signals; the computer removed the characteristic glycerol peaks from all spectra. Figure 1 shows the resulting mass spectra for Pd-gl and T-G.

The standard curve points were determined by selected ion recording, based on the area ratio between the ions m/z 495 and 463 (the [M - H]- ions in the FAB negative-ion mass spectrum) for Pd-gl and T-G, respectively. Area integrating time was 50 s, starting 5 s after sample introduction. Dew time was 100 ms, channel-delay time 20 ms. The Pd-gl content of unknown samples was determined by comparison with the standard curve.

Comparison methods. For GLC we used the method of Adessi et al. (2). We enzymically hydrolyzed 0.5 mL of urine overnight with 2000 Fishman units of β-glucuronidase (EC 3.2.1.31; "Helicase"; Industrie Biologique Française, Villeneuve–La Garenne, France), extracted this mixture twice with 3-mL portions of chloroform, combined the extracts, and washed them with 1 mL of 2 mol/L NaOH and then 1 mL of water. After dehydrating the extracts with anhydrous Na2SO4 and evaporating them under nitrogen in the presence of 1 μg of epicoprostanol (5β-cholestan-3α-ol) as the internal standard, we formed the trimethylsilyl ether derivative of the residue and injected this onto a 25 m × 0.35 mm (i.d.) OV-1 glass-capillary column at 250°C (isothermal analysis).

The LIA we used has been previously described by Eshhar et al. (7).

Sample collection. We used samples from 24-h urine collections from normal women and first morning urines from a normal fertile 30-year-old woman. These were stored frozen (−20°C) until assay.

Results

Choice of internal standard. Quantitative analysis of chemical compounds by mass spectrometry and selected ion recording requires use of isotopic labels such as deuterium. The method described here, however, involves no extraction or chromatographic steps, so that any compound that behaves like Pd-gl in the FAB ionization technique can be used as the internal standard (24).

We suggest the use of T-G as the internal standard for three reasons:

1) T-G, a steroid glucuronide metabolite with a chemical structure similar to that of Pd-gl, is also present in women's urine in relatively low concentrations, 10 to 38 μg per day in normal fertile women (13), in comparison with the concentrations of Pd-gl that are present during various phases of the menstrual cycle (range, 0.5 to 8 mg per day).

2) In addition, the amount of T-G in 100 μL of women's urine is at most 5 ng, while 2500 ng are added as internal standard in the procedure described above. Therefore, the contribution of endogenous T-G to the ion current of T-G can be disregarded.

3) Lastly, one of the most relevant inconveniences of the FAB ionization technique for quantitative analysis is the instability of the ion current during the analysis. Therefore, it is necessary that the behavior of the internal standard and the analyte with regard to the FAB ionization procedure be closely analogous. In this case, the ratio between the two different ion signals can remain substantially constant, as is in fact the case for Pd-gl and T-G (Figure 2).

Figure 3 shows an example of mass spectra for Pd-gl in urine sampled from a fertile woman during the luteal phase (25th day), before and after addition of the internal standard, T-G (2.5 μg/25 μL).

Standard curve. Data for the standard curve for Pd-gl were obtained in quadruplicate. The standards were prepared by adding 25 μL of a 100 mg/L ethanolic solution of T-G and increasing amounts of Pd-gl (range 0.312 to 10 mg/L) to 100 μL of pooled urine from children. We used children's urine as the matrix to minimize possible matrix effects between standards and unknown samples and because the endogenous Pd-gl in children's urine (<0.5 mg/L) is much less than the concentrations observed in women's urine collected during the luteal phase. Nevertheless, the basal value for Pd-gl in children's urine was automatically subtracted for all points of the calibration curve by the computer–mass spectrometer system we used.

The standard curve was linear over the range 0.312 to 10 mg/L. Its equation was: y = 0.238x + 0.020 (r = 0.994, n = 28), where y is the area ratio m/z 495/463 and x is the concentration in mg/L.

Analytical variables. We assessed the sensitivity, precision, and accuracy of the mass-spectrometric method for Pd-gl (Table 1).
The sensitivity—the smallest concentration differing significantly from zero at the 95% confidence limit—was determined from six different dose–response curves to be 380 (SD 40) μg/L.

Accuracy, as indirectly assessed by analytical recovery of Pd-gl (1 mg/L) added to previously assayed urine samples (range 0.5–10 mg/L), was 96.5% (SD 8.3%, n = 10).

Pd-gl concentrations in morning urines of a fertile woman as determined by the different assay methods. Aliquots of first morning urines collected daily by a normal woman during the menstrual cycle were assayed for Pd-gl by mass spectrometry, GLC, and LIA. The results are shown in Figure 4. The concentration of Pd-gl as measured by the mass-spectrometric method (y) and by GLC (x) gave the linear regression equation y = 1.165x − 0.03 (r = 0.948, n = 24); for results by the mass-spectrometric method (y) and by LIA (x), the linear regression equation was y = 0.993x + 0.010 (r = 0.964, n = 24).

The lower concentrations of Pd-gl measured by the GLC method are probably related to losses during the hydrolysis, extraction, and derivatization steps, which we performed without a control for recovery.

Discussion

This method for quantitative determination of Pd-gl in urine samples from women is based on the FAB/negative-ionization technique. It requires only two procedural steps: addition of the internal standard to the crude urine specimens and direct measurement of the analyte in 2 to 5 μL of urine by mass spectrometry. Quantification is by calculation of the relative-area ratio between the Pd-gl and T-G ions.

The main features of this novel approach are:

- high practicability, because no treatment of the biological sample is needed
- improved reliability, because there are no sample-pre-treatment losses

Table 1. Precision of the Mass-Spectrometric Method for Pd-gl in Urine

<table>
<thead>
<tr>
<th></th>
<th>No. of samples</th>
<th>Pd-gl concn, mg/L (mean (SD))</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>6</td>
<td>0.49 (0.05)</td>
<td>10.2</td>
</tr>
<tr>
<td>High</td>
<td>8</td>
<td>5.20 (0.33)</td>
<td>6.3</td>
</tr>
<tr>
<td>Inter-assay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>10</td>
<td>0.55 (0.07)</td>
<td>12.7</td>
</tr>
<tr>
<td>High</td>
<td>8</td>
<td>5.66 (0.64)</td>
<td>11.3</td>
</tr>
</tbody>
</table>

Fig. 2. Selected ion recording of Pd-gl and T-G in a urine sample during the 50-s acquisition time used for area integration

Fig. 3. Negative-ion FAB mass spectra of a urine sample from a fertile woman in the luteal phase before (A) and after (B) addition of the internal standard

Fig. 4. Concentration of Pd-gl in morning urine samples from a single female subject, as determined by negative-ion FAB mass spectrometry (---), LIA ( ), and GLC ( )
• satisfactory sensitivity and precision for monitoring Pd-gl concentrations during the menstrual cycle

• very high specificity

On the other hand, the proposed method requires very expensive and complicated instrumentation, which currently is available only in a few clinical chemistry laboratories. Although this makes the proposed method poorly practicable for clinical purposes, we believe that these procedures will become more widespread in the near future, as the expense of this kind of instrumentation declines. For the present the described method could be considered a candidate reference method for measurements of Pd-gl during the menstrual cycle.

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References


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