Quantitative Measurement of Porphobilinogen in Urine by Stable-Isotope Dilution Liquid Chromatography-Tandem Mass Spectrometry

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Background: Measurement of porphobilinogen (PBG) is useful in the diagnosis of the acute neurologic porphyrias. Currently used colorimetric assays lack analytical and clinical sensitivity and specificity.

Methods: We developed a liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) method for the measurement of PBG in 1 mL of urine, using 5-(aminoethyl)-4-(carboxymethyl) 1H-2,4-[13C]pyrrole-3-propanoic acid ([2,4-13C]PBG; 2.75 μg) as internal standard. After solid-phase extraction, LC-MS/MS analysis was performed in the selected-reaction monitoring (SRM) mode. PBG and [2,4-13C]PBG were monitored through their own precursor and product ion settings (m/z 227 to 210 and m/z 229 to 212, respectively). The retention time of PBG and [2,4-13C]PBG was 1.0 min in a 2.3-min analysis.

Results: Daily calibrations (n = 6) between 0.1 and 2.0 mg/L were linear and reproducible. Inter- and intraassay CVs were 3.2–3.5% and 2.6–3.1%, respectively, at mean concentrations of 0.24, 1.18, and 2.15 mg/L. The regression equation for the comparison between an anion-exchange column method (y) and the LC-MS/MS method (x) was: y = 0.84x + 0.74 (S_y|x = 5.8 mg/24 h; r = 0.85; n = 100). In 47 volunteers, PBG excretion was 0.02–0.42 mg/24 h, lower than reported reference intervals (up to 2.0 mg/24 h) based on colorimetric methods. In 85 samples with PBG < 0.5 by LC-MS/MS, 8 (9.4%) had values > 2.0 mg/24 h by the anion-exchange method (mean ± SD, 4.3 ± 1.8 mg/24 h). In 11 patients with confirmed diagnoses of acute porphyria and increased PBG by LC-MS/MS, 2 had values within the reported reference intervals by a quantitative anion-exchange method.

Conclusions: The quantitative LC-MS/MS method for PBG measurement exhibits greater analytical specificity and improved clinical sensitivity and specificity than currently available methods.

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The porphyrias are a heterogeneous group of inherited or acquired disorders resulting from a deficiency of one of the eight enzymes that control the heme biosynthesis pathway. Clinical manifestations of specific enzyme deficiencies are attributable to the overproduction of specific heme precursors and their accumulation in tissues and body fluids. The porphyrias can be subdivided into acute and nonacute forms based on the presence of acute recurrent neurologic attacks. Acute attacks often follow exposure to precipitating factors such as porphyrinogenic drugs and chemicals, hormones, ethanol ingestion, and reduced caloric intake, which influence the rate of heme biosynthesis, leading to expression of the underlying genetic trait (1–3). Clinically, the acute porphyrias, which include acute intermittent porphyria, hereditary coproporphyria, and variegate porphyria, present intermittently with neurovisceral signs and symptoms that may be severe, possibly leading to paralysis, and in some cases can be life threatening (4, 5). Symptoms may include mental, neurologic, and abdominal manifestations (6). The signs and symptoms, however, are often variable and nonspecific, and may be confused with other medical conditions (5). Laboratory analysis is thus required to confirm or rule out acute porphyria.

Porphobilinogen (PBG)3 excretion in urine increases in acute porphyric attacks, and the measurement of PBG is particularly important in diagnosing the acute porphyrias.

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3 Nonstandard abbreviations: PBG, porphobilinogen; LC-MS/MS, liquid chromatography-electrospray ionization tandem mass spectrometry; [2,4-13C]PBG, 5-(aminoethyl)-4-(carboxymethyl) 1H-2,4-13C-pyrrole-3-propanoic acid; and SRM, selected-reaction monitoring.
(acute intermittent porphyria, hereditary coproporphyria, and variegate porphyria) during the acute stage (7, 8). Markedly increased urine PBG is diagnostic for acute porphyria (5). Neurologic symptoms without a concomitant increase in PBG excretion cannot be attributed to acute porphyria, and other causes for the symptoms should be investigated (9).

Methods currently used to measure PBG in urine require derivatization of PBG, typically with Ehrlich reagent, to form a chromogen that is quantified colorimetrically. The Watson–Schwartz method is a screening test that is still widely used today (10). However, the method is not quantitative and has poor sensitivity and specificity. Other qualitative and quantitative methods have been reported (11–14), many of which include prepurification of PBG with anion-exchange resins, but all rely on the derivatization of PBG with Ehrlich-type reagent, which is not specific for PBG, and thus have the potential for false positives. We have developed an alternative method for PBG analysis that takes advantage of the versatility, sensitivity, and specificity of liquid chromatography-electrospray ionization tandem mass spectrometry (LC-MS/MS) using stable-isotope dilution, which does not require derivatization of PBG.

**Materials and Methods**

**MATERIALS**
PBG monohydrate was purchased from Frontier Scientific. The labeled PBG internal standard [5-(aminoethyl)-4-(carboxymethyl) 1H-2,4-13C-pyrrole-3-propanoic acid ([2,4-13C]PBG)] was synthesized by Frontier Scientific (Logan, UT) from 5-amino-4-13C-oxopentanoic acid (4-13C-aminolevulinic acid), which was custom made by the Organic Synthesis Core Facility, Mayo Clinic Jacksonville (Jacksonville, FL). All other reagents were of the highest purity available from commercial sources and used without purification. Oasis HLB® solid-phase extraction columns were obtained from Waters Corporation.

**SAMPLE PREPARATION**

Urine (5 mL) was acidified with 6 mol/L HCl to pH 2.0. One milliliter of the acidified urine was placed in a 10 × 75-mm glass culture tube and mixed with 100 μL of internal standard solution (2.75 mg/L). Extraction was performed on a Gilson ASPEC® automated SPE sample processor. Oasis HLB columns (1 mL, containing 30 mg of packing) were preconditioned with 1 mL of 1 mol/L glacial acetic acid and 1 mL of reverse osmosis water. One milliliter of sample was added to the column, followed by a 500-μL water wash. PBG and [2,4-13C]PBG were then eluted with 1 mL of 1 mol/L glacial acetic acid. The eluate was transferred to a sealed, amber glass autosampler vial for injection onto the LC/MS/MS instrument. Calibrators were prepared in urine of known PBG concentration (blank) by the addition of a 100 mg/L working solution to produce PBG concentrations of 0.1, 0.2, 0.5, 1.0, and 2.0 mg/L. Calibrators were treated and extracted like samples, and the endogenous PBG concentration of the blank sample was subtracted from the calibrators after visual verification of results.

**METHODS**

An API 2000 LC-MS/MS triple quadrupole mass spectrometer (Perkin-Elmer Sciex) with a TurbolonSpray ionization probe source (operated at 5800 V) was used. Peripherals included a Perkin-Elmer Series 200 pump and an autosampler. PBG and [2,4-13C]PBG were separated from the bulk of the specimen matrix on a short HPLC column [Supelcosil LC-18; 3.3 cm × 4.6 mm (i.d.); 3-μm bead size; Supelco] to enhance the stability of the signal. Autosampler injections of 10 μL were made using a mobile phase composed of 300 mL/L acetonitrile in 1.4 g/L formic acid in water. The flow rate was 1.0 mL/min. The column was directly connected to the TurbolonSpray ionization probe operating with the turbo gas on (6 L/min; sensor temperature, 250 °C) with the LC column effluent flow-splitting set at 1:5. PBG and [2,4-13C]PBG eluted apart from the bulk of the specimen matrix at a retention time of ~1.0 min. Total instrument acquisition cycle time was 2.3 min/sample.

All results were generated in positive-ion mode with the orifice voltage set at 6 V, automatically optimized using the protonated PBG ion. Mass calibration and resolution adjustments (at 0.7 atomic mass units, full width at half-height) on both the resolving quadrupoles were automatically optimized using a 1 × 10−4 mol/L poly(propylene)glycol solution introduced via the built-in infusion pump on the API/2000. Collisionally activated decomposition MS/MS was performed through the closed-design Q1 collision cell, operating with nitrogen at 0.06 kPa as collision gas. The 15-eV (lab frame) collision energy was adjusted automatically by the AutoTune algorithm.

MS/MS spectra were collected in continuous flow mode by connecting the built-in infusion pump directly to the TurbolonSpray probe. For MS/MS optimization, solutions of 20 μmol/L PBG and [2,4-13C]PBG were prepared in 10 mL of 1 g/L formic acid and infused separately at a rate of 10 μL/min. In SRM mode, the instrument was optimized by the built-in algorithm to monitor the m/z 227 to 210 and m/z 229 to 212 transitions for PBG and [2,4-13C]PBG, respectively. Data were acquired and processed using the Mass-Chrom software (Ver 1.1.1; Perkin-Elmer Sciex) including Multiview, Ver. 1.4, for chromatographic and spectral interpretation and Turboquan for Windows NT (Ver 1.0; Perkin-Elmer Sciex) for the quantitative processing. To calculate sample PBG concentration, the ratio of the area of the PBG analyte to the area of the internal standard was compared to area ratios of calibrators with known PBG concentrations. PBG values were reported in units of mg/24 h.

LC/MS/MS results were compared with a modified Watson-Schwartz screening method and with quantitative anion-exchange PBG analysis. Briefly, the screening
The method involved addition of 1 mL of urine or PBG calibrator (3.0 mg/L) to 1 mL of Ehrlich reagent (5.6 g; p-dimethyl-amino-benzaldehyde in 1000 mL of concentrated HCl and 800 mL of water). After mixing, 2 mL of chloroform was added, and the resulting solution was centrifuged for 5 min at 2000 g. The upper aqueous layer was added to 2 mL of n-butanol, mixed, and centrifuged for 5 min at 2000 g. The upper butanol layer was discarded, and the absorbance (555 nm) of the aqueous layer was measured using a Beckman DU 650 spectrophotometer. If the absorbance of the sample was greater than that of the calibrator, the sample was considered positive for the presence of PBG. If the calibrator was less than that of the calibrator, PBG quantification by anion-exchange column was accomplished using the ALA/PBG Column Test from Bio-Rad Laboratories, which is based on the method of Mauzerall and Granick (12). The manufacturer provided instructions for analysis as well as reagents and supplies.

**Results**

The MS/MS spectrum obtained by infusion of 20 μmol/L PBG is shown in Fig. 1A. This spectrum was acquired by transmitting the protonated molecular ion via Q1 and scanning the second resolving quadrupole, Q3, for products resulting from fragmentation in the collision cell. The autotune algorithm provided in the system software was used to optimize the instrument for transmission of the protonated molecular ion and for maximum intensity of the selected fragment. These results were used to design the SRM experiment to sequentially transmit the m/z 227 protonated molecular ion and the m/z 210 fragment via Q1 and Q3, respectively. Fig. 1B shows the MS/MS spectrum obtained by infusion of 20 μmol/L [2,4-13C]PBG, which was optimized for the transmission of the m/z 229 protonated molecular ion and the m/z 212 fragment.

![Fig. 1. MS/MS product ion scans of standard protonated ions. (A), PBG optimized to favor the m/z 227 to 210 transition. (B), [2,4-13C]PBG optimized to favor the m/z 229 to 212 transition.](image)

The extracted SRM chromatogram obtained from a specimen in which the calculated PBG concentration was 0.10 mg/24 h is shown in Fig. 2A. The extracted SRM chromatogram obtained from a specimen from a patient with acute intermittent porphyria in which the calculated PBG concentration was 110 mg/24 h is shown in Fig. 2B (this sample was diluted 1:100 before analysis).

**LINEARITY**

Three urine samples with high PBG concentrations obtained from patients with acute porphyria were serially diluted to test assay linearity. In the range of 0.05–3.0 mg/L, the coefficients of linear regression (r²) were 0.9987, 0.9996, and 0.9999. Calibrators with PBG concentrations of 0.1, 0.2, 0.5, 1.0, and 2.0 mg/L prepared in urine matrix were assayed on 6 different days. The mean slope and r² were 1.07 and 0.9978, respectively. The CVs for the slope and r² of the PBG calibration curves were 4.2% and 0.2%, respectively. For routine sample analysis, samples with PBG concentrations above the highest calibrator (2 mg/L) were diluted to produce values that fell within the range of the calibration curve. The SRM-extracted chromatogram signal-to-noise ratios for two specimens with calculated PBG concentrations of 0.10 and 0.06 mg/L were 34.8 and 21.5, respectively. There was no appreciable carryover of PBG on the HPLC column until the PBG concentrations exceeded 5.0 mg/L in the final solutions. Specimens analyzed immediately after samples with PBG concentration >5.0 mg/L were reinjected to ensure accurate quantification.

**RECOVERY, PRECISION, AND STABILITY**

The recovery and precision data for the method are summarized in Table 1. These experiments were conducted using a urine matrix (blank). We added PBG to aliquots of the blank urine to produce final PBG concentrations of 0.26, 1.20, and 2.15 mg/L. Six aliquots of each
sample were analyzed on each of 6 consecutive days. The method demonstrated quantitative recovery and CVs <4%.

The stability of PBG in 10 urine samples was determined after the addition of 2.5 mg of PBG per liter of sample. Aliquots of each sample were stored at room temperature, refrigerated at 4 °C, and frozen at −70 °C. Sodium carbonate (final concentration, 0.05 mol/L) preservative was added to a portion of each of the 10 PBG-enriched urine samples, and aliquots were stored refrigerated. The pH of the unpreserved urine samples with added PBG varied from 5.2 to 7.8. Fig. 3 depicts the mean PBG concentration in the 10 samples after 1, 3, and 7 days of storage. A substantial loss of PBG was observed in many samples within 24 h of storage at room temperature. Minimal loss of PBG was observed in unpreserved samples stored refrigerated, with a mean loss of 11% at 7 days. PBG was stable in samples stored frozen and in samples preserved with sodium carbonate and stored refrigerated.

The stability of extracted specimens (in 1 mol/L glacial acetic acid) stored in amber glass vials at room temperature was tested by repeat injection of 15 extracted urine samples (PBG concentration range, 0.02–4.77 mg/24 h) at 5 h and 24 h after baseline measurement. Mean recovery of PBG was 97.7% and 100.4% of baseline at 5 and 24 h, respectively.

**Table 1. Precision and recovery of the LC-MS/MS PBG method.**

<table>
<thead>
<tr>
<th>No. of aliquots</th>
<th>Added, mg/L</th>
<th>Detected, mg/L</th>
<th>Recovery, %</th>
<th>CV, % (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intraassay</td>
<td>Interassay</td>
</tr>
<tr>
<td>6</td>
<td>0.00</td>
<td>0.02 ± 0.001</td>
<td>91.7</td>
<td>5.6</td>
</tr>
<tr>
<td>6</td>
<td>0.24</td>
<td>0.24 ± 0.01</td>
<td>98.3</td>
<td>2.9</td>
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<tr>
<td>6</td>
<td>1.18</td>
<td>1.18 ± 0.01</td>
<td>100.0</td>
<td>3.1</td>
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<tr>
<td>6</td>
<td>2.13</td>
<td>2.15 ± 0.07</td>
<td>98.3</td>
<td>2.6</td>
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</table>

*Mean ± SD.

**METHOD COMPARISON**

The LC-MS/MS PBG method was compared with both a qualitative modified Watson–Schwartz method (n = 146) and a quantitative anion-exchange column method from Bio-Rad Laboratories (n = 100) based on the method of Mauzerall and Granick (12). Included in both comparisons were samples from 11 patients with confirmed diagnoses of acute porphyria. The regression equation for the LC-MS/MS (x) method and the anion column method (y) was: \( y = 0.84x + 0.74 \) mg/24 h \( (r = 0.85, S_{yx} = 5.8 \) mg/24 h). The distribution of the mean vs the difference of paired PBG values (Bland–Altman plot) is shown in Fig. 4.

For 135 samples with PBG <0.5 by LC-MS/MS, 6 (4.4%) screened positive by the Watson–Schwartz method. Of 85 specimens with PBG <0.5 by LC-MS/MS, 8 (9.4%) had values >2.0 mg/24 h by the quantitative anion-exchange method (mean ± SD, 4.3 ± 1.8 mg/24 h). Table 2 shows PBG concentrations obtained by the LC-MS/MS, anion-exchange, and Watson–Schwartz methods in 11 patients with confirmed diagnoses of acute porphyria. Three (27%) screened negative by the Watson–Schwartz method, and two (18%) had values within the reported reference intervals (≥2.0 mg/24 h) by the anion-exchange method. These comparative data indicate the improved sensitivity and specificity of the LC-MS/MS method over currently used colorimetric methods.

**REFERENCE VALUES**

A reference value study was performed using 24-h urine collections from 47 active and retired healthcare workers with no previous history of renal or hepatic disease. Mean ± SD PBG excretion was 0.10 ± 0.10 mg/24 h (range, 0.02–0.42 mg/24 h). We report the reference interval for the LC-MS/MS method as 0–0.5 mg/24 h. PBG was also determined in random urine collections from 60 active and retired healthcare workers. Mean ± SD PBG was 0.09 ± 0.05 μg/mg of creatinine (range, 0.04–0.45 μg/mg of creatinine).

**Discussion**

The commercial availability of bench-top LC-MS/MS systems and the reliability of the electrospray ionization interface (15) have led to the utilization of this technology in the clinical laboratory for routine analysis of both small
and large molecules. Our laboratory recently described LC-MS/MS methods for total homocysteine (16), methylmalonic acid (17), and homovanillic acid (18) as well as for glycosylated transferrin (19), which are currently being used in the clinical laboratory. Here we describe the development of a LC-MS/MS method for PBG quantification and compare it with two colorimetric methods for assessment of acute porphyria.

The LC-MS/MS method has an advantage over a modified Watson–Schwartz screening method and the quantitative anion-exchange column method in that it measures PBG in its native form without derivatization. Substances such as urobilinogen, phenothiazines, indoles, and methyldopa, which are known to cause false-positive results with colorimetric methods (20, 21), do not interfere with the LC-MS/MS method, which quantifies based on the monitoring of a specific fragment ion of PBG in ratio to a labeled PBG internal standard. The labeled [2,4,13C]PBG internal standard corrects not only for minor variations in individual sample preparation but also for the effect of sample matrix on ion formation. It is important to note that even with the preanalytical sample clean-up and separation of PBG from the majority of the sample matrix by on-line reversed-phase HPLC, substantial differences in the peak area of the added internal standard were observed from one sample to the next. This emphasizes the necessity of internal standardization when measuring PBG by LC-MS/MS.

The LC-MS/MS method does require solid-phase extraction of urine before analysis of PBG to remove components in the urine matrix that suppress the ion signal. This solid-phase extraction is, however, performed in an automated fashion using a Gilson ASPEC Work Station, producing a net time savings compared with the Bio-Rad Laboratories anion-exchange column method. Because this method produces highly consistent calibration curves, calibration is required only when system changes, such as a new column or preparation of a new internal standard stock solution, take place. High and low quality-control samples are analyzed daily to assure assay performance, and calibration curves are generated on a monthly basis.

Studies of the stability of PBG in urine samples indicate that when stored frozen at −70 °C, PBG is stable for at least 7 days. This indicates that unpreserved random urine samples, frozen immediately, can be used for analysis of PBG. A 24-h urine collection is required for

<table>
<thead>
<tr>
<th>Patient</th>
<th>LC-MS/MS, mg/24 h</th>
<th>Anion column, mg/24 h</th>
<th>Watson–Schwartz, ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63.0</td>
<td>75.1</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>54.0</td>
<td>28.2</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>44.2</td>
<td>7.0</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>39.0</td>
<td>55.0</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>31.0</td>
<td>52.1</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>22.8</td>
<td>13.1</td>
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<td>7</td>
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<td>11</td>
<td>5.7</td>
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<td>−</td>
</tr>
</tbody>
</table>

Figure 4. Bland–Altman comparison of PBG by LC-MS/MS and Bio-Rad methods. The inset is a magnification of the plot showing the low-end data; mean PBG range, 0–4.0 mg/24 h.
assessment of overall PBG excretion rate. Because PBG is not stable in urine stored at room temperature and because some loss of PBG is observed in samples stored refrigerated for extended periods of time, we recommend preserving the 24-h urine by adding 5 g of sodium carbonate to the collection container before sample collection. This will typically produce a urine pH of 7.5–9.5, at which PBG is most stable.

The reference value studies using LC-MS/MS indicate that previous reference intervals (typically 0–2.0 mg/24 h or higher) for PBG excretion established by colorimetric methods are overestimated, likely because of the inability to completely remove interfering substances from the sample before complexation with Ehrlich reagent. Our studies indicate that the reference interval for PBG excretion in nonporphyric patients is 0–0.5 mg/24 h.

In conclusion, the LC-MS/MS method provides a novel approach to the analysis of PBG excretion in urine that can be readily performed in the routine clinical laboratory. Sample preparation requires a simple manual procedure and automated solid-phase extraction. The method does not require derivatization and uses a stable-isotope-labeled internal standard with chromatographic behavior identical to that of native PBG to control both the solid-phase extraction and sample matrix effects. The increased clinical sensitivity and specificity of the LC-MS/MS method may allow more reliable biochemical diagnosis of the often difficult to diagnose acute porphyrias.

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