Variations in Porphobilinogen and 5-Aminolevulinic Acid Concentrations in Plasma and Urine from Asymptomatic Carriers of the Acute Intermittent Porphyria Gene with Increased Porphyrin Precursor Excretion

Ylva Floderus,1 Eliane Sardh,2 Christer Mölle,3 Claes Andersson,3 Lillan Rejkjaer,3 Dan E.H. Andersson,2 and Pauline Harper1*

Background: The heme precursors porphobilinogen (PBG) and 5-aminolevulinic acid (ALA) accumulate during overt crises of acute intermittent porphyria (AIP), and high excretion of these metabolites often continues in the asymptomatic phase.

Methods: We measured concentrations of PBG and ALA and investigated the correlation between these metabolites in plasma and urine in 10 asymptomatic AIP carriers with high excretion and in 5 healthy individuals. We quantified plasma concentrations with an HPLC–mass spectrometric method and urine concentrations with ion-exchange chromatography.

Results: The mean (SD) plasma concentrations of PBG and ALA in the AIP carriers were 3.1 (1.0) and 1.7 (0.7) μmol/L, respectively. The mean 8-h urinary excretion amounts of PBG and ALA in the AIP carriers were 102 (25) and 56 (18) μmol, respectively, whereas the corresponding values for healthy individuals were 2.9 (0.7) and 9.3 (1.2) μmol. The correlations between PBG and ALA values in plasma and urine of the AIP carriers were 0.678 and 0.856, respectively. The mean PBG/ALA ratio was ~2.0 in both plasma and urine for the AIP carriers and 0.3 in urine for the healthy individuals. The renal clearance rates for PBG and ALA were 71 (15) and 70 (13) mL/min, respectively.

Conclusions: The described liquid chromatographic–mass spectrometric method enabled characterization of variations in plasma PBG and ALA in AIP carriers during an 8-h period. The renal clearances were similar for both metabolites. This method could be used to monitor AIP patients during treatment.

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Acute intermittent porphyria (AIP) is an autosomal dominant disorder caused by a metabolic error in heme biosynthesis in which the third enzyme, porphobilinogen deaminase (PBGD; EC 2.5.1.61), also called hydroxymethylbilane synthase, is deficient. The disease is characterized by acute attacks of abdominal pain, muscle weakness, and various neuropsychiatric symptoms. If not treated properly, the condition may be life threatening. Alcohol intake, infection, stress, and certain drugs and chemicals often trigger acute attacks, mainly by inducing a high rate of heme turnover (1). The high demand for heme synthesis leads to an increased flux through the deficient PBGD step, causing an accumulation of the heme precursors porphobilinogen (PBG) and 5-aminolevulinic acid (ALA). These metabolites are excreted in high amounts in urine during an acute attack (2). Many patients are “asymptomatic high excreters of PBG”, continuing to excrete high

1 Porphyria Centre Sweden, Department of Laboratory Medicine, Karolinska Institute, Karolinska University Hospital, Stockholm, Sweden.
2 Department of Internal Medicine, Karolinska Institute, Stockholm Söder Hospital, Sweden.
3 Zymenex A/S, Hillerod, Sweden, and Lidingo, Denmark.
* Address correspondence to this author at: Porphyria Centre Sweden, CMMS C2 71, Karolinska University Hospital Huddinge, SE-141 86 Stockholm, Sweden. Fax 46-8-58582760; e-mail pauline.harper@karolinska.se.
Received July 27, 2005; accepted January 24, 2006.
Previously published online at DOI: 10.1373/clinchem.2005.058198

4 Nonstandard abbreviations: AIP, acute intermittent porphyria; PBGD, porphobilinogen deaminase; PBG, porphobilinogen; ALA, 5-aminolevulinic acid; MS, mass spectrometry; PITC, phenylisothiocyanate; PTC-PBG, phenylthiocarbamyl porphobilinogen; PTC-ALA, phenylthiocarbamyl 5-aminolevulinic acid; and Mr, relative molecular mass.
concentrations of PBG even in remission (1, 3). Excretion of both PBG and ALA is higher during attacks than between attacks (2).

The cause of ALA accumulation is not clear. The enzyme ALA dehydratase, which catalyzes the condensation of 2 molecules of ALA to build PBG, is not a rate-limiting step in the heme biosynthetic pathway; therefore, an inhibitory effect of PBG on the enzyme has been hypothesized (4).

Monitoring PBG and ALA variations during periods of active disease or remission is usually based on measurements of the metabolites in urine by ion-exchange chromatography, a method that has been available for more than 3 decades. There have been only a few reports on measurement of PBG and/or ALA in plasma in acute porphyria (5, 6). Recently, methods have been developed for analyzing serum ALA by use of fluorometry (7) and urinary PBG and/or ALA by mass spectrometry (8–10). We used a method based on reversed-phase HPLC with mass spectrometric detection (HPLC-MS) for measuring plasma PBG and ALA and compared plasma values with urine measurements in asymptomatic carriers of the AIP gene and in healthy individuals.

**Materials and Methods**

**STUDY PARTICIPANTS**

AIP carriers with a known genetic defect of the PBGD gene (11) were chosen to participate in the study (n = 10; 5 men and 5 women). The mean (SD) age was 49 (15) years for men and 37 (10) years for women. The study patients were known to be in a clinically latent phase of the disease but with increased urinary excretion of PBG and ALA for several years (Porphyria Centre Sweden).

We also recruited 5 healthy persons: 3 men and 2 women [mean age, 24 (4) years]. Written informed consent was obtained from all participants, and the study was approved by the local Ethics Committee of the Karolinska Institute (Dnr 69/01), Stockholm.

**SELECTION CRITERIA**

Inclusion criteria for AIP carriers were (a) urinary PBG concentrations ≥4 times the upper reference limit, i.e., ≥4.8 mmol/mol creatinine (upper reference limit, 1.2 mmol/mol creatinine); (b) at least 30 days without AIP symptoms (e.g., abdominal or muscle pain), heme therapy, or other AIP-specific treatments; (c) negative pregnancy test; and (d) no clinical history or laboratory signs of liver disease or alcohol abuse. The inclusion concentration of urinary PBG was arbitrarily determined to also ensure high plasma concentrations of PBG.

The 5 healthy individuals included in the study had undergone medical examinations and routine laboratory screening that showed no abnormalities. The measures of urinary excretion of PBG and ALA for these individuals, plus erythrocyte PBGD activity, were all within reference values.

**PLASMA AND URINE SAMPLING**

Each study participant was admitted to the hospital early in the morning (day 1). A short catheter was inserted percutaneously into an antecubital vein in one arm and was flushed with saline. Heparinized venous samples were obtained each hour from 0800 until 1200, and then samples were obtained at 1400 and 1600. Thus, a total of 7 blood samples were obtained during 8 h. Blood samples were immediately centrifuged at 1600 g for 10 min, and plasma was stored at −80 °C until analyzed.

Participants emptied their urinary bladders just before 0800 in the morning. After that, during an 8-h period in the hospital, we obtained 6 urine samples at 1-h intervals, until 1200, then at 2-h intervals until 1600. Participants were asked to empty their urinary bladders completely at each sampling time point, and the time was recorded to the nearest minute. Participants left the hospital at 1600 and continued to collect urine until 0800 on day 2, when they delivered it to the hospital; thus, a 24-h urine collection was carried out. The volumes of the urine portions were measured, and aliquots were stored at −80 °C until analyzed. Participants were also instructed to bring a portion of the first morning urine on day 1 (random urine sample). All samples were kept protected from light. Participants had free access to food and beverages during the trial period.

**ANALYSES OF PBG AND ALA IN URINE**

We measured PBG and ALA in urine by ion-exchange chromatography (12, 13) with the Bio-Rad PBG/ALA-Test. The concentrations of urinary PBG and ALA are reported as micromoles excreted per time unit or as micromoles per mole of creatinine, i.e., normalized to the creatinine concentration of the specimen (14). Urinary creatinine analysis was performed by the Jaffe method (15). The interassay variations (as CVs) for the ion-exchange chromatography method, expressed per liter, were 2.3% for PBG, 3.3% for ALA, and 2.7% for urinary creatinine. The variations for PBG and ALA, expressed per mole of creatinine, were 3.5% and 4.3%, respectively. Detection limits were 1.9 μmol/L for PBG and 1.8 μmol/L for ALA.

**ANALYSES OF PBG AND ALA IN PLASMA**

We determined plasma PBG and ALA concentrations by an HPLC-MS method. Before analysis, we deproteinized the plasma by mixing 100 μL of cold acetonitrile (Merck LiChrosolv®) with 50 μL of sample. After centrifugation of this mixture at 10,000 g for 8 min, we transferred 50 μL of the supernatant to a clean test tube and mixed it with 50 μL of ethanol (950 mL/L) and 25 μL of triethylamine (Pierce; final volume ratio, 2:2:1). The samples were dried under reduced pressure. We derivatized the free amino groups of PBG and ALA with phenylisothiocyanate (PTC; Pierce) by adding 50 μL of PTC reagent (ethanol–water–triethylamine–PTC, 7:1:1:1 by volume) to each dried sample to form phenylthiocarbamyl-PBG (PTC-
PBG) and phenylthiocarbamyl-ALA (PTC-ALA), respectively. The reaction was allowed to proceed for 30 min at room temperature. The samples were again dried under reduced pressure and then were dissolved in 62.5 μL of 200 mL/L ethanol and centrifuged at 10,000g for 5 min. The supernatants were transferred to HPLC glass vials and kept at 4 °C until analysis. We then injected 25–40 μL of the supernatant on a Zorbax SB-C18 column [pore size, 80 Å; bead size, 5 μm;15 cm × 2.1 mm (i.d.); Rockland Technologies Inc.] with a Zorbax SB-C8 guard column (pore size, 300 Å; Rockland Technologies). Both columns had been equilibrated with a 95%–5% mixture of 25 mmol/L formic acid (Merck; buffer A) and 25 mmol/L formic acid in acetonitrile (Merck LiChrosolv®; buffer B). The PTC-PBG and PTC-ALA derivatives were eluted with a 2-component gradient as follows: 2-min of 95% buffer A–5% buffer B, then via linear gradient to 40% buffer A–60% buffer B by 17 min after sample injection. This concentration ratio was maintained for 1 min before buffer A was decreased to 20% during 2 min and finally returned to the starting point (5%) after an additional 2 min. The flow rate was 0.2 mL/min. The ALA derivative elution at 15–16 min for and the PBG derivative at 18–19 min.

Sample detection was performed by selected-ion monitoring at positive polarity with an electrospray quadrupole mass spectrometer (Agilent/Hewlett Packard 1100 HPLC-MSD) equipped with a binary pump system, autoinjector, and diode array detector controlled by HP-Chemstation software. The drying gas temperature was 320 °C, at a flow rate of 10.0 L/min; nebulizer pressure was 25 psig; and capillary voltage was set to 4000 V. The PTC-PBG and PTC-ALA ions were monitored at relative molecular mass ratios of 189 100 and 249 200, and PTC-PBG ions at M\textsubscript{r} 210 200 and 362 200 (fragmentor voltages were set to 130 and 40 V, respectively). Calibration curves were constructed each time by analysis of PBG (Sigma) and ALA (Bio-Rad) calibrators prepared by adding increasing amounts of both PBG and ALA (1.0, 2.0, 5.0, 10, 15, 20, and 30 pmol) to a human plasma sample with no detectable endogenous PBG and only trace amounts of ALA. The calibration curves for both PBG and ALA were linear in this range (R\textsuperscript{2} >0.97). The unknown samples, analyzed in duplicate, were compared with the calibration curves, and the PBG and ALA concentrations were expressed as micromoles per liter. The lower limit of detection, which represented a signal that was at least 3 SD above the background, was 1 pmol (corresponding to 0.12–0.2 μmol/L of plasma, depending on the injection volume) for both ALA and PBG. The intraassay variation (CV), computed the Pearson correlation coefficient with a confidence interval. P values were calculated by use of the t-approximation, and a P value <0.05 was considered statistically significant. All tests were two-tailed.

Results

The mean (SD) plasma PBG and ALA concentrations for the 10 AIP carriers were 3.1 (1.0) μmol/L (range, 1.7–5.1 μmol/L) and 1.7 (0.7) μmol/L (range, 0.9–3.6 μmol/L), respectively. The corresponding values for the excreted amounts of urinary PBG and ALA were 102 (25) μmol/8 h (range, 68–146 μmol/8 h) and 56 (18) μmol/8 h (range, 32–91 μmol/8 h). In the 5 healthy individuals, plasma PBG concentrations were below the detection limit for the method (<0.12 μmol/L), and the mean value for plasma ALA was 0.38 (0.03) μmol/L (range, 0.36–0.41 μmol/L). The mean values for urinary PBG and ALA in the healthy individuals were 2.9 (0.7) μmol/8 h (range, 2.3–4.1 μmol/8 h) and 9.3 (1.2) μmol/8 h (range, 7.8–10.5 μmol/8 h), respectively. The individual concentrations and variations for plasma and urine PBG and ALA for the AIP carriers and healthy individuals are presented in Table 1. The individual patterns in one AIP carrier with low and in another with high intraindividual variation are illustrated in Fig. 1.

The renal clearance values for PBG and ALA during 8 h are also shown in Table 1. Because of the low concentrations of PBG in plasma in the healthy individuals, we could not calculate their PBG clearance. There was
Table 1. Individual PBG and ALA concentrations and ratios in urine and plasma, total excretion in urine, and renal clearance in 10 asymptomatic but biochemically active AIP carriers and in 5 healthy individuals.

<table>
<thead>
<tr>
<th>Study participant</th>
<th>Concentration in random morning urine sample, mmol/mol creatinine</th>
<th>Mean (SD) concentration in 7 plasmas, μmol/L</th>
<th>Plasma PBG/ALA ratio</th>
<th>Mean (SD) concentration in 6 urine samples, mmol/mol creatinine</th>
<th>Urine PBG/ALA ratio</th>
<th>Total urinary excretion in 8 h, μmol</th>
<th>Urinary clearance (8 h), mL/min</th>
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<tr>
<td><strong>AIP carriers</strong></td>
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<td>1.9 (0.3)</td>
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<td>1.9 (0.3)</td>
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<td>7.4</td>
<td>5.1 (0.6)</td>
<td>1.7 (0.3)</td>
<td>26.0 (2.6)</td>
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<td>2.8 (0.4)</td>
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<td>9.0</td>
<td>3.6 (0.3)</td>
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<td><strong>Mean (SD)</strong></td>
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<td><strong>Healthy individuals</strong></td>
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<td>1</td>
<td>0.3</td>
<td>1.8</td>
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<td>0.36 (0.05)</td>
<td>0.37 (0.08)</td>
<td>1.64 (0.17)</td>
<td>0.23 (0.06)</td>
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<td>2</td>
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<td>1.4</td>
<td>&lt;0.12</td>
<td>0.38 (0.06)</td>
<td>0.60 (0.13)</td>
<td>1.46 (0.21)</td>
<td>0.41 (0.07)</td>
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<tr>
<td>3</td>
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<td>1.1</td>
<td>&lt;0.12</td>
<td>0.41 (0.05)</td>
<td>0.45 (0.10)</td>
<td>1.31 (0.11)</td>
<td>0.34 (0.06)</td>
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<tr>
<td>4</td>
<td>0.3</td>
<td>1.6</td>
<td>&lt;0.12</td>
<td>0.38 (0.07)</td>
<td>1.28 (0.50)</td>
<td>2.07 (0.13)</td>
<td>0.34 (0.08)</td>
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<tr>
<td>5</td>
<td>0.4</td>
<td>1.4</td>
<td>&lt;0.12</td>
<td>0.37 (0.09)</td>
<td>0.58 (0.22)</td>
<td>2.07 (0.29)</td>
<td>0.28 (0.11)</td>
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<td><strong>Mean (SD)</strong></td>
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<sup>a</sup>The detection limit of the assay is 0.12 μmol/L.
no statistically significant difference between the values for renal clearance of PBG and ALA in the AIP carriers ($P = 0.730$, paired $t$-test). The mean (SD) values for creatinine clearance for the AIP carriers were 90.4 (12.0) mL/min (range, 74–113 mL/min) for 8 h. The corresponding values for the healthy participants were 149 (25.8) mL/min (range, 119–176 mL/min). The reference interval is 90–150 mL/min for individuals up to 50 years of age. We found no correlation between creatinine clearance and PBG or ALA clearance.

The total urine excretion of PBG in 24 h was 244 (51.4) μmol in AIP carriers and 6.5 (1.1) μmol in healthy individuals. For ALA, the corresponding values were 136 (39.4) and 25.8 (3.7) μmol, respectively. In both AIP carriers and healthy individuals, the amount of PBG and ALA excreted during the 8-h study period (Table 1) was thus ~40% of the total amount in the 24-h collection.

AIP carriers showed no statistically significant difference between urinary PBG concentrations in the random morning sample compared with samples collected during 8 or 24 h (normalized to creatinine; $P = 0.257$ and 0.426, respectively). The same was true for urinary ALA concentration ($P = 0.203$ and 0.229, respectively). The results remained unchanged when the samples obtained from healthy individuals were included.

The concentrations of both plasma and urine PBG in the AIP carriers were twice as high as those for plasma and urine ALA. The mean (SD) PBG/ALA ratio was 2.0 (0.8) in plasma (range, 1.2–3.3) and 2.0 (0.5) in urine (range, 1.3–3.1). In healthy individuals, the corresponding mean (SD) ratio in urine PBG/ALA was 0.32 (0.07), and the range was 0.23–0.41. This ratio is in the same range as the range we found for AIP carriers in the clinically and biochemically latent phase (data not shown).

The correlation between mean plasma PBG concentration and the total amount of urine PBG excreted in 8 h was significant at the 5% level ($r = 0.678$; 95% confidence interval, 0.09–0.92; $P = 0.031$), and between plasma and urine ALA, it was significant at the 1% level ($r = 0.856$; 95% confidence interval, 0.49–0.97; $P = 0.0016$; Fig. 2). The healthy individuals were not included because their plasma values were below the limit of detection for the method (PBG) or were very low (ALA).

To study the general variation patterns for plasma PBG and ALA (μmol/L) during the 8 h of observation time, we calculated each single determination as a percentage of the mean value for the 7 plasma determinations for each AIP carrier. We performed the same procedure for the 6 urine determinations (expressed as μmol/min) in each AIP carrier. The plasma PBG concentration was significantly higher in the morning sample (drawn at 0800) than in the last sample drawn at 1600 ($P < 0.001$, paired $t$-test; Fig. 3). This was also true when urinary PBG concentrations measured in samples collected between 0800 and 0900 were compared with those collected between 1400 and 1600 ($P < 0.002$). We observed no differences in plasma or urine ALA concentrations when we compared the morning and afternoon samples.

**Discussion**

In the asymptomatic AIP carriers, the plasma concentrations of PBG and ALA quantified by the described
HPLC-MS method showed high correlation over time with the urinary concentrations for both mean values (Fig. 2) and individual values (Table 1 and Fig. 1). The fact that the PBG/ALA ratio in plasma was ~2 for AIP carriers, the same as found for urine, also confirms the accuracy of the described method for plasma.

There are only a few previous reports on plasma PBG concentrations. To measure serum PBG and ALA in AIP carriers during different stages of the disease, Miyagi et al. (6) used a modified method based on one described by Mauzerall and Granick (13). In their study (6), the patients in clinical remission had high urinary and plasma concentrations of porphyrin precursors, in accordance with our results, and the plasma concentrations were in the same range as in our AIP carriers. The mean concentrations of serum PBG (5.3 μmol/L) were higher than the mean concentrations for ALA (3.3 μmol/L), which is the same pattern as found in our study.

The concentrations of PBG and ALA in plasma and urine differed between the AIP carriers (Table 1) but were relatively constant within each individual during the 8 h of observation, with the exception of participant 7 (Fig. 1). In this individual, the peak-like increase in the ALA concentration was followed 1 h later by increased urinary ALA. No change in plasma PBG was observed, but there was a slight and sustained increase in urinary PBG. These observations corroborate the higher correlation found between the concentrations of ALA in plasma and urine compared with the correlations between PBG in plasma and urine. Because the renal clearances for PBG and ALA were not significantly different, the different behaviors of PBG and ALA cannot be explained by different renal clearances for these metabolites.

The clearance values for PBG and ALA were ~77% of that for creatinine in the AIP carriers, and in the healthy participants the clearance of ALA was ~34% of that for creatinine. The AIP carriers had lower creatinine clearance than the healthy participants. The finding that the
renal clearance values for ALA in the AIP carriers were approximately in the same range as in the healthy individuals (Table 1) suggests that the AIP carriers had conserved enough renal function to handle the increased plasma concentration of ALA (and PBG).

In healthy participants, the PBG/ALA ratio in urine was ~0.3, which is the same ratio as found in latent AIP carriers (data not shown). In contrast, in the AIP carriers with high excretion of porphyrin precursors, the PBG/ALA ratio was ~2.0 in both plasma and urine. This finding cannot be explained by different renal handling of the metabolites (see above); it probably indicates that the deficient PBGD enzyme may be overloaded in asymptomatic patients with high PBG and ALA excretion and may cause selective accumulation of PBG, the substrate for the enzyme. The increased ALA concentrations in AIP may reflect up-regulation of the biosynthetic pathway to compensate for heme deficiency and/or a possible inhibitory effect of PBG on ALA dehydratase (1, 4). In other studies in which the patterns for PBG and ALA have been described during symptomatic periods, it has been shown that the PBG/ALA ratio returns to reference values during remission after treatment (16, 17).

When studying the general variation patterns of plasma and urinary PBG and ALA, we found a significant difference between the morning and afternoon PBG values in plasma and urine, a decrease in PBG that may reflect circadian variation (Fig. 3). Further studies in larger groups are needed to corroborate this observation. In our study, the plasma and urinary ALA concentrations within individuals were stable during the observation period, a finding that is in accordance with that of Gorchein and Webber (5), who did not find any circadian variation of ALA during 24 h of observation in healthy individuals.

Measurement of porphyrin precursors in urine has been performed for decades. The method has reliable sensitivity and is readily performed in the clinical laboratory, but urine measurements are laborious and time-consuming and affected by several factors, such as the pH of the sample, temperature, exposure to light, diuresis, and sampling errors. When creatinine was normalized, we found no significant differences in PBG and ALA concentrations in the initial random urine sample or in the 8- or 24-h collections; we thus conclude that random samples of morning urine are preferable to time samplings.

To our knowledge, this is the first report surveying the porphyrin precursor patterns over time in plasma and urine in asymptomatic but biochemically active AIP carriers. When the HPLC-MS or tandem MS technology becomes available in clinical laboratories, measurement of PBG and ALA in plasma may facilitate the monitoring of acute porphyria crises. Such monitoring could be particularly beneficial in the management of AIP patients with renal failure, particularly if they are in an anuric state. The analysis of PBG and ALA in plasma represents a new tool that may further the understanding of the pathophysiology of acute porphyria attacks and improve treatment monitoring.

References