Published in final edited form as:

J Chromatogr B Analyt Technol Biomed Life Sci. 2011 August 15; 879(24): 2389–2396. doi:10.1016/j.jchromb.2011.06.034.

A LC–MS/MS method for the specific, sensitive, and simultaneous quantification of 5-aminolevulinic acid and porphobilinogen

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Abstract

Accurate determinations of 5-aminolevulinic acid (ALA) and porphobilinogen (PBG) in physiologic fluids are required for the diagnosis and therapeutic monitoring of acute porphyrias. Current colorimetric methods are insensitive and over-estimate ALA and PBG due to poor specificity, while LC–MS/MS methods increase sensitivity, but have limited matrices. An LC–MS/MS method was developed to simultaneously determine ALA and PBG concentrations in fluids or tissues which were solid phase extracted, butanol derivatized, and quantitated by selective reaction monitoring using $^{13}\text{C}_5$, ^{15}N -ALA and 2,4- $^{13}\text{C}_2$ -PBG internal standards. ALA was separated from interfering compounds on a reverse phase C8-column. For ALA and PBG, the matrix effects (87.3–105%) and process efficiencies (77.6–97.8% and 37.2–41.6%, respectively) were acceptable in plasma and urine matrices. The assay was highly sensitive for ALA and PBG (LLOQ = 0.05 μM with 25 μL urine or 100 μL plasma), and required ~4 h from extraction to results. ALA and PBG accuracy ranged from 88.2 to 110% ($n = 10$); intra- and inter-assay coefficients of variations were <10% for urine and plasma. In clinical applications, patients with mutation-confirmed acute porphyrias had normal to slightly increased urinary ALA and PBG levels when asymptomatic, and high levels during acute attacks, which decreased with hemin therapy. In AIP mice, baseline ALA and PBG levels in urine, plasma, and liver were increased after phenobarbital induction 28-/63-, 42-/266-, and 13-/316-fold, respectively. This LC–MS/MS method is rapid, specific, highly sensitive, accurate, and simultaneously measures ALA and PBG in urine, plasma, and tissues permitting porphyria clinical diagnoses, therapeutic monitoring, and research.

Keywords

5-Aminolevulinic acid; Porphobilinogen; Acute intermittent porphyria; Tandem mass spectrometry

1. Introduction

The acute porphyrias are inborn errors of heme biosynthesis, each resulting from mutations in a specific gene that impairs its encoded enzyme's synthesis or function. These disorders include autosomal dominant acute intermittent porphyria (AIP), hereditary coproporphyria

(HCP), and variegate porphyria (VP), and autosomal recessive ALA-dehydratase deficient porphyria (ADP)[1–3]. Their specific enzymatic defects, mode of inheritance, and major accumulating porphyrin precursors and porphyrins are summarized in Table 1. These disorders are characterized by life-threatening episodes of acute neurologic attacks, which are precipitated by certain drugs, hormones or diets that induce hepatic aminolevulinatase synthase (ALAS1) resulting in the increased synthesis of 5-aminolevulinic acid (ALA) and porphobilinogen (PBG). The neurovisceral attacks are characterized by abdominal pain, autonomic dysfunction, and mild, transient mental disturbances, which can progress to encephalopathy, seizures, bulbar involvement, and death. Thus, preventive efforts are focused on avoiding the precipitating factors that induce hepatic ALAS1 and cause the acute attacks [2,3].

The diagnostic hallmark of an acute attack is the marked increase of plasma and urinary ALA and PBG concentrations [2–4]. In the autosomal dominant acute porphyrias, urinary ALA and PBG levels are normal or slightly increased in asymptomatic patients, but may increase over 100–200-fold during acute attacks [1–3]. In recessive ADP, the ALA level is consistently ~25-fold elevated [5,6], and increases during acute attacks.

The recommended first biochemical test for patients with a suspected acute attack is the PBG concentration in a “spot” urine. An elevated PBG level (>20 mg/L) is diagnostic for an acute porphyric attack [2]. Urinary ALA, which is elevated in very rare ADP [5–7], lead poisoning, and hereditary tyrosinemia Type I [8,9], is not recommended as first-line test, since most laboratories require 24 h urines.

Previously, PBG concentrations were determined by the Watson–Schwartz or the Hoesch methods based on the reaction of Ehrlich’s reagent (p-dimethylaminobenzaldehyde) with PBG [10,11]. These methods are qualitative with limited sensitivity and specificity. Subsequently, the Mauzerall–Granick assay was developed using anionic and cationic exchange columns to separate ALA and PBG followed by their colorimetric determination with Ehrlich’s reagent [12]. Although this assay is useful clinically for elevated urinary ALA and/or PBG, their measurement in plasma or serum is challenging, due to their low levels (~1% of urinary ALA or PBG). In addition, the assay is not specific, since other compounds react with Ehrlich’s reagent and result in falsely higher values, particularly at low ALA or PBG concentrations [13–16]. Several modified colorimetric [17–19], fluorometric [20–22], and mass spectrometry [23–25] assays have been developed to improve ALA or PBG quantitation in plasma or urine. However, none of these methods simultaneously measured both ALA and PBG in urine, plasma, and tissues presumably due to matrix effects. Moreover, most previous methods were not sensitive enough to measure plasma levels in normal individuals and asymptomatic porphyric patients.

Electrospray ionization (ESI) tandem mass spectrometry provides quantitative analyses of various physiologic compounds in clinical laboratories. However, ion suppression frequently reduces sensitivity, partially explaining the difficulty to develop a method to simultaneously measure ALA and PBG in different matrices. Here, a sensitive, specific, and rapid LC–MS/MS method is described that simultaneously determines ALA and PBG concentrations in urine, plasma, or tissues. The method uses front end SPE purification, butylation, and positive ESI-MS/MS, and requires only 25 μ L of urine or 100 μ L of plasma with a lower limit of quantification (LLOQ) of 0.05 μ M. The ion suppression in different matrices and procedure efficiency were evaluated to optimize the assay for urine, plasma and tissues. Samples from mutation-confirmed AIP patients and normal individuals were analyzed to validate the method’s clinical utility. In addition, the ALA and PBG levels were assayed in urine, plasma, and tissues from wild-type and AIP mice [26] before and after phenobarbital induction of an acute attack.

2. Material and methods

2.1. Materials

ALA, PBG and ALA- $^{13}\text{C}_5$, ^{15}N hydrochloride ($^{13}\text{C}_5$, ^{15}N -ALA) were from Sigma Aldrich (St. Louis, MO). Another set of ALA and PBG were from Frontier Scientific (Logan, UT). 2,4- $^{13}\text{C}_2$ -PBG was synthesized by Frontier Scientific from 5-aminolevulinic acid-4- ^{13}C obtained from Cambridge Isotope Laboratories (Andover, MA). Ammonium hydroxide and ortho-phosphoric acid were from Sigma Aldrich. Formic acid and HPLC grade methanol was from Fisher Scientific (Fairlawn, NJ). Human serum (defibrinated, delipidated, and double charcoal stripped) for generating serum and plasma calibrators and quality controls (QCs) was obtained from Monobind, Inc. (Lake Forest, California). Oasis MCX solid phase extraction columns and MCX mElution plates were from Waters Corporation (Milford, MA). The ALA/PBG reagent kit for the double column spectrophotometric method was from Bio-Rad (Hercules, CA).

2.2. Preparations of calibrators and QCs

Two separate sets of 10 mM ALA and 2 mM PBG stock solutions were prepared in deionized H_2O for making calibrators and quality control (QC) samples. Urine calibrators were prepared by spiking ALA and PBG stock solutions into a pre-tested normal urine sample, which had very low endogenous ALA and PBG levels. The following ALA and PBG concentrations were added: 8, 16, 32, 64, 128, and 256 μM . Serum calibrators were generated by spiking ALA and PBG stock solutions into the charcoal-filtered serum which had a trace amount of ALA and no detectable PBG; ALA and PBG concentrations added to the serum were 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 μM . The same urine and serum samples and ALA and PBG stock solutions (for QC) were used to generate three level urine and serum QC samples at 10, 50, and 200 μM and at 0.4, 2.5, and 10 μM , respectively.

2.3. Specimens

2.3.1. Patients—Urine and heparinized plasma residual samples were from seven mutation-confirmed AIP, VP, or HCP patients. In addition, de-identified urine and plasma samples from 60 healthy individuals over 12 yr old and 30 healthy individuals less than 12 yr old were used to establish normative ALA and PBG values.

2.3.2. Mice—Samples were from male wild-type and AIP mice [26], 2–3 months old. Phenobarbital induction of their hepatic ALAS1 activity was performed as described, except an increased dose of 110, 120, and 130 mg/kg/day was administered [26,27]; twenty-four hour urine samples were collected at baseline and after phenobarbital induction from 10 AIP mice. Following avertin anesthesia (250 mg/kg, ip), blood was collected from the retro-orbital plexus 9 h after the last phenobarbital injection. All animal procedures were approved by the Mount Sinai Institutional Animal Care and Use Committee.

2.4. Sample preparation

2.4.1. Urine—Urine samples were processed with ALA and PBG calibrators and three level QCs in the urine matrix. Urine (25 μL), calibrators, and QCs were mixed with 25 μL of the 20 μM internal standard mixture of $^{13}\text{C}_5$, ^{15}N -ALA and 2,4- $^{13}\text{C}_2$ -PBG. Samples were purified with Waters Oasis MCX-SPE columns (1 mL, 30 mg packing) according to the manufacturer's instructions. Eluent fractions (100 μL) were derivatized with 3 N hydrochloric acid in butanol as described [28,29] and reconstituted with 100 μL of 20% methanol for mass spectrometry.

2.4.2. Plasma and tissues—Plasma or tissue homogenates were processed with the ALA and PBG calibrators and the three level QCs in the plasma matrix. Plasma (100 μ L), calibrators, and QCs were mixed with 100 μ L of the 2 μ M internal standard mixture. Samples were prepared by the urine assay protocol. For tissues, 25 μ L of tissue homogenate was used and the SPE procedure was performed on the MCX μ Elution plate, followed by the same preparation process.

2.4.3. Double column spectrophotometric method—For comparison, urinary ALA and PBG were quantified using the Bio-Rad ALA/PBG column kit, according to the manufacturer's protocol. ALA and PBG were individually absorbed and eluted from cation and anion exchange columns, respectively, and reacted with Ehrlich's reagent. The ALA and PBG concentrations were spectrophotometrically determined at 553 nm.

2.5. LC–MS/MS analysis

An Agilent QQQ 6460 mass spectrometer with a jet stream electrospray ion source and an Agilent 1200 series fast resolution LC system (Wilmington, DE) was employed. MassHunter software was used for system control, data acquisition, and data processing. LC separation was performed on an Agilent Zorbax Eclipse Plus C8 reverse phase column (50 mm $\times$ 3.0 mm i.d., 1.8 μ m particle size, Santa Clara, CA) maintained at 40 $^{\circ}$ C with a gradient program at a flow rate of 0.5 ml/min. The mobile phase A consisted of 100% HPLC grade water with 0.1% formic acid and mobile phase B consisted of 100% HPLC grade methanol with 0.1% formic acid. The gradient started with 20% solvent B and increased to 75% B in 3.2 min, with a further increase to 95% B in 4 min, and then was held at 95% B for 2 additional minutes. The column was re-equilibrated with 20% B for 5 min. Total run time was 13 min with a 5 μ L injection volume. The mass spectrometer was operated in positive jet stream ESI mode. Nitrogen was used as nebulizer, turbo (heater) gas, curtain, and collision-activated dissociation gas. The capillary voltage was +4000 V and the nozzle voltage was +1000 V. The ion source gas and jet stream gas temperatures were 325 $^{\circ}$ C with flows of 10 L/min. ALA and PBG were measured by selective reaction monitoring (SRM). Table 2 lists the optimal mass spectrometric settings (fragmentor and collision energy) for each quantifying and qualifying transition.

3. Results

3.1. Development of LC–MS/MS method

ALA is small, very polar, barely retained on a reverse phase column, and is isobaric to isoleucine (Ile), leucine (Leu), 4-hydroxyproline (OH-Pro), and creatine (Cre). Thus, ALA poses an analytical separation challenge for liquid chromatography and assay selectivity. However, butylated ALA-(Bu-ALA) is more hydrophobic and is retained on a C8 column. Ile, Leu, and OH-Pro were completely separated from Bu-ALA on transition 188/86 and from creatine on 188/114 and 188/86 transitions (Fig. 1). Chemical structures for Bu-ALA and (Bu)₂-PBG and their predicted precursor and product ions are shown in Fig. 2. The qualifier ratios for Bu-ALA and (Bu)₂-PBG were monitored for selectivity in daily batch assays. In different plasma samples, the mean qualifier ratio of Bu-ALA (m/z 188/68) and (Bu)₂-PBG (m/z 322/222) were 14.5% (CV = 3.5%, n = 22), and 13.5% (CV = 5.5%, n = 15), respectively. Similarly, consistent qualifier ratios were obtained for urine and internal standards (Table 2). Chromatographic separation and consistent ALA and PBG qualifier ratios demonstrated that this method was selective and specific for ALA and PBG.

3.2. Matrix effects and ion suppression

Matrix effects (ME) and ion suppression are the major analytical challenges in quantifying ALA and PBG in different biological matrices by LC–MS/MS. To date, no mass

spectrometry method has simultaneously analyzed ALA and PBG in urine and plasma [24,30]. During method development, different procedures, including SPE, methanol deproteinization, and liquid–liquid extraction with or without chemical derivatization, were assessed. ME, the major cause of ESI signal loss and process efficiency (PE) were evaluated using the Matuszewski method [31]. Samples included Set A, a neat standard mix containing 10 μM ALA and PBG; Set B, the extracted plasma or urine spiked with 10 μM of ALA and PBG; and Set C, plasmas spiked with 10 μM of ALA and PBG. $\text{ME}\% = \text{peak area B/A} \times 100$ and $\text{PE}\% = \text{C/A} \times 100$. All experiments were performed in triplicate.

The SPE method alone, without chemical derivatization, did not achieve acceptable ME or PE as both were $<1\%$ (data not shown). When methanol precipitation followed by butylation was used, the ME and PE both increased 20–30%, but the method was not satisfactory for sensitive analyses (Fig. 3). With SPE followed by butylation, the mean ME for ALA was $105 \pm 5.0\%$ and $96.9 \pm 1.6\%$ from plasma and urine matrices, respectively, indicating minimal matrix effects. The mean PE for ALA in plasma and urine matrices was $77.6 \pm 4.7\%$ and $97.8 \pm 10.9\%$, respectively. An additional 27% loss was observed in plasmas, presumably due to the extraction and derivatization processes. The mean ME for PBG from plasma and urine matrices were $87.3 \pm 3.3\%$ and $103 \pm 4.3\%$, respectively, indicating minimal matrix effects. In the plasma and urine matrices, the mean PE for PBG was $37.2 \pm 2.3\%$ and $41.6 \pm 10.4\%$, respectively, which were lower than the PE for ALA. This is the first method that systematically evaluated the ME for simultaneous ALA and PBG analyses. The SPE step and butylation were necessary to achieve a satisfactory ME and PE, and high sensitivity for ALA and PBG determinations.

3.3. Analytical performance

3.3.1. Linearity—Assay linearity was assessed using the ALA and PBG standard mixture at 0, 0.05, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, and 256 μM . Experiments were performed in triplicate. The lower and upper limits of quantification (LLOQ and ULOQ) were defined where the percent bias (%bias) of accuracy and precision were within $\pm 20\%$. LLOQ for ALA and PBG were both 0.05 μM ; ULOQ values were at least 256 μM , which is beyond the levels in acute attacks. Linearity was consistent in urine and plasma spiked calibrators, except at the LLOQ due to variable levels of endogenous analytes. ALA and PBG linearity in the plasma calibration was from 0.4 to 12.8 μM .

3.3.2. Accuracy and precision—Intra- and inter-assay precision tests were conducted using plasma QC samples at 0.4, 2.5, and 10 μM and urine QC samples at 10, 50, 200 μM (Table 3). For intra-assay precision, eight aliquots were analyzed the same day, and for inter-assay precision and accuracy, one aliquot was analyzed on each of ten days. The coefficient of variance (%CV) and accuracy $\leq \pm 20\%$ at the LLOQ and $\leq \pm 15\%$ at other concentrations were considered acceptable. For the urine assay, the ALA and PBG intra- and inter-assay precisions at low, medium and high concentrations were between 1.9–3.2%, and 3.1–9.3%, respectively, with the accuracy or recovery percent being 94.1–108%. Similarly, the plasma ALA and PBG intra- and inter-assay precisions were 1.2–7.3% and 4.7–8.7%, respectively, with an accuracy of 88.2–110% (Table 3).

3.3.3. Reference intervals—The normal reference ranges for endogenous urine and plasma ALA and PBG were established with 60 healthy individuals (age > 12 years old) (Table 4). Urinary ALA and PBG values were 0.09–3.0 mmol/mol creatinine (0.9 ± 0.6) and 0–1.1 mmol/mol creatinine (0.07 ± 0.1), respectively. In individuals < 12 yr old, ALA and PBG were 0.3–2.5 mmol/mol creatinine (1.1 ± 0.6) and 0–0.5 mmol/mol creatinine (0.2 ± 0.2), respectively. There were no significant differences between the two groups, although the mean value of PBG in pediatric group was slightly higher. Of note, the urinary ALA and

PBG concentrations were lower than those determined by the column method [24,25,32], probably due to the low LLOQ and high selectivity of our method. Plasma ALA values ranged from 0.07 to 0.2 μM (0.1 ± 0.01) regardless of age. Normal plasma PBG concentrations were below the quantification limit of 0.05 μM (Table 4).

3.4. Method comparison

The LC–MS/MS method for determining ALA and PBG levels was compared to the Bio-Rad column method using colorimetric detection with Ehrlich's reagent [12]. Pure ALA and PBG standard mixtures up to 256 μM were compared for sensitivity and accuracy without the sample matrix. Using 1 mL of standard solution, the column method accurately and precisely measured ALA from 8.0 to 256 μM and PBG from 2.0 to 256 μM . However, the LLOQs were much higher than those of the LC–MS/MS method, despite the larger sample volumes used (1 mL in the column method vs 25 μL in the LC–MS/MS method).

Normal and porphyric human and murine urines were assayed by both methods. Overall, the two data sets had similar results with correlation coefficients of 0.95 and 0.97 for ALA and PBG, respectively. However, both human and murine urines had higher ALA and PBG values when assayed by the column method; the mean column/MS ratios (mean $\pm$ 1 std) for ALA and PBG for human normal ($n = 5$) and porphyric urines ($n = 5$), and for urines of AIP mice ($n = 10$) prior to and after phenobarbital induction, were 1.68 ± 0.22 and 5.78 ± 2.17 , 1.23 ± 0.31 and 1.50 ± 1.41 , 1.24 ± 0.45 and 1.42 ± 0.27 , and 0.81 ± 0.17 and 0.98 ± 0.12 , respectively (Table 5). Note that the column method had significantly higher levels of ALA and PBG, particularly for normal individuals (approximately 2- and 6-fold higher, respectively) (Table 5), presumably due to the presence of other compounds in the samples that reacted with Ehrlich's reagent [14,15]. When ALA and PBG concentrations were extremely high as in post-phenobarbital induced AIP mice, the effect of interfering compounds was less.

3.5. ALA and PBG levels in porphyric patients and mice

3.5.1. Acute porphyria patients—The ALA and PBG concentrations were determined in plasmas and urines from mutation-confirmed AIP, HCP, and VP patients when asymptomatic or during acute attacks. In asymptomatic patient (H-VP-2), urinary ALA and PBG levels were normal. In symptomatic patients (H-AIP-1, 2 and 4), urinary ALA and PBG were 5.0–5.6 and 0.4–9.7 mmol/mol creatinine, respectively. During acute attacks (H-AIP-3, H-VP-1, and H-HCP-1), urinary ALA and PBG levels were 18.5–34.6 and 8.0–33.6 mmol/mol creatinine, respectively. In an AIP patient (H-AIP-1) after an attack, urinary PBG levels approached baseline after hemin therapy (Table 4). The ALA and PBG levels in human plasmas from normal individuals were $<0.2 \mu\text{M}$ and $<0.05 \mu\text{M}$, respectively. In acute porphyria patients during or after acute attacks, the plasma ALA was variably increased (5- to 43-fold) while PBG was increased at least 2 to 8-fold (Table 4).

3.5.2. AIP mice—The mean urinary, plasma, and hepatic ALA levels in wild-type mice were 6.25 ± 1.12 mmol/mol creatinine ($n = 4$), $0.15 \pm 0.05 \mu\text{M}$ ($n = 4$), and 10.1 ± 2.56 nmol/g protein ($n = 5$), respectively (Table 6). In contrast, the mean baseline urinary, plasma, and hepatic ALA levels in the AIP mice were 29.2 ± 20.7 mmol/mol creatinine ($n = 10$), $0.36 \pm 0.0007 \mu\text{M}$ ($n = 2$), and 28.1 ± 9.19 nmol/g protein ($n = 3$), respectively. After phenobarbital induction, the mean urinary, plasma, and hepatic ALA values increased to 985 ± 762 mmol/mol creatinine ($n = 10$), $15.0 \pm 0.77 \mu\text{M}$ ($n = 4$), and 374 ± 98.2 nmol/g protein ($n = 3$), respectively. Similarly, the mean baseline urinary and hepatic PBG values in the AIP mice were 7.8 ± 5.2 mmol/mol creatinine ($n = 10$), and 21.1 ± 19.4 nmol/g protein ($n = 3$), respectively. After phenobarbital induction, the urinary and hepatic values increased to 599 ± 428 mmol/mol creatinine ($n = 10$) and 6670 ± 604 nmol/g protein ($n = 3$),

respectively. In the AIP mice, the mean baseline plasma PBG level was within the normal range ($<0.05 \mu\text{M}$), while after phenobarbital induction the mean level was $13.3 \pm 0.72 \mu\text{M}$ ($n = 4$) (Table 6). More pronounced PBG increases occurred in the liver after phenobarbital induction (Table 6). Instead of being undetectable as in previous studies [33], the baseline liver ALA and PBG concentrations in normal and AIP mice could be accurately measured by the more sensitive LC–MS/MS method (Table 6).

4. Discussion

The LC–MS/MS method for the simultaneous quantitation of ALA and PBG in fluids and tissues employed solid phase extraction (SPE) by an MCX system and butylation of ALA and PBG for front-end sample purification. The derivatization efficiency and reduced ion suppression in various matrices was dramatically improved for urine, plasma, and tissue homogenates. Without the SPE process, the ALA and PBG butylation reactions were largely inhibited, probably due to hydrolysis of the complex matrix, as ALA and PBG esterification required acidic and water-free conditions. Systematic evaluation of the ME and PE with different procedures demonstrated that both were essential for sensitive ALA and PBG measurements in different samples. In addition, ALA-butylation increased its hydrophobicity, thereby improving its retention on reverse phase chromatography, as well as separating it from isobaric amino acids.

This assay was highly sensitive; only 25 μL of urine or 100 μL of plasma were required. The “spot” urine is a great advantage in the clinical setting, particularly when a quick diagnostic answer is needed to confirm an acute attack. LLOQ values for ALA and PBG were as low as $0.05 \mu\text{M}$ compared to 2–8 μM by the column method. Such sensitivity made plasma and tissue sample analyses possible. Sensitivity could be further improved if micro-elution plates were used for smaller sample volume, as was done here for the tissue samples.

The assay is highly selective and specific. Multiple criteria were used to unambiguously define ALA and PBG peaks by evaluating: (1) specific ion fragments, (2) retention times, (3) quantifier ion/qualifier ion ratios, (4) co-elution with the stable isotope-labeled ALA and PBG internal standards, and (5) separation of ALA from confounding isobaric compounds. Inclusion of $^{13}\text{C}_5$, ^{15}N -ALA and $^{13}\text{C}_2$ -PBG standards was essential to normalize differences in recovery caused by the sample preparation, injection, column separation steps, and potential ion suppression from the sample matrix.

Compared to the column method, the normal ranges for both urinary ALA and PBG were lower than previously reported [24,32]. Moreover, the column method had higher ALA and PBG concentrations than those obtained by mass spectrometry for normal and disease samples, particularly at low concentrations, albeit the column method accurately measured the pure standards. The overestimations of ALA and PBG by the column method were not evident when their concentrations were markedly elevated, as in the AIP mice after phenobarbital induction. These findings indicate that the LC–MS/MS method is more specific, as it is free of interference from substances that react with Ehrlich’s reagent and cause falsely elevated results, which occurs with the column method [13–16]. Moreover, the LC–MS/MS method permitted the rapid, sensitive, and specific measurement of ALA and PBG in plasmas, which were difficult if not impossible by the column method.

The ability to measure plasma ALA and PBG concentrations provides a new approach to rapidly and specifically monitor the exacerbation, evolution, treatment and remission of an acute attack. The AIP mice permit studies of the pathophysiologic events underlying an acute attack. In particular, the ability to measure ALA and PBG in tissues, including the liver and brain, should increase our understanding of the pathogenic role of these porphyrin

precursors in the acute attacks. In addition, the assay will readily permit the evaluation of the porphyrinogenicity of drugs in the AIP mice.

In conclusion, this LC–MS/MS method, which simultaneously measures ALA and PBG levels in various sources, provides improved diagnosis and monitoring of acute porphyria patients in clinical settings, as well as facilitates research into the pathogenesis of the acute neurologic attacks.

Acknowledgments

The authors acknowledge the expert technical assistance of Lin Gan, B.S. for performing the ion exchange column assays and the expert technical advice of M.-P. George, M.S. and Andre Szczesniowski, M.S., Agilent Technology, Inc., for the development of the Mass Spectrometry method and James Mullins, M.S., Waters Corporation, for the optimization of Solid Phase Extraction procedures.

References

1. Anderson, KE.; Sassa, S.; Bishop, DF.; Desnick, RJ. Metabolic and Molecular Bases of Inherited Disease. Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D.; Childs, B.; Kinzler, KW.; Volgelstein, B., editors. New York: The McGraw Hill Companies, Inc.; 2001. p. 2991
2. Anderson KE, Bloomer JR, Bonkovsky HL, Kushner JP, Pierach CA, Pimstone NR, Desnick RJ. Ann. Intern. Med. 2005; 142:439. [PubMed: 15767622]
3. Puy H, Gouya L, Deybach JC. Lancet. 2010; 375:924. [PubMed: 20226990]
4. Ventura P, Cappellini MD, Rocchi E. Intern. Emerg. Med. 2009; 4:297. [PubMed: 19479318]
5. Doss M, von Tiepermann R, Schneider J, Schmid H. Klin. Wochenschr. 1979; 57:1123. [PubMed: 513604]
6. Doss M, von Tiepermann R, Schneider J. Int. J. Biochem. 1980; 12:823. [PubMed: 7450139]
7. Plewinska M, Thunell S, Holmberg L, Wetmur JG, Desnick RJ. Am. J. Hum. Genet. 1991; 49:167. [PubMed: 2063868]
8. Sassa S, Kappas A. Trans. Assoc. Am. Physicians. 1982; 95:42. [PubMed: 7182986]
9. Fischbein A, Alvares AP, Anderson KE, Sassa S, Kappas A. Toxicol. Environ. Health. 1977; 3:431.
10. Watson CJ, Schwartz S. Proc. Soc. Exp. Biol. Med. 1941; 47:393.
11. Lamon J, With TK, Redeker AG. Clin. Chem. 1974; 20:1438. [PubMed: 4425514]
12. Mauzerall D, Granick S. Biol. Chem. 1956; 219:435.
13. Marver HS, Tschudy DP, Perloth MG, Collins A, Hunter G. Anal. Biochem. 1966; 14:53.
14. Pierach CA, Cardinal R, Bossenmaier I, Watson CJ. Clin. Chem. 1977; 23:1666. [PubMed: 890911]
15. Reio L, Wetterberg L. JAMA. 1969; 207:148. [PubMed: 5818135]
16. Shuster L. Biochem. J. 1956; 64:101. [PubMed: 13363811]
17. Doss M, Schmidt A. Klin. Chem. Klin. Biochem. 1971; 9:99.
18. Sun MW, Stein E, Gruen FW. Clin. Chem. 1969; 15:183. [PubMed: 5773268]
19. Tomokuni K, Ogata M. Clin. Chem. 1972; 18:1534. [PubMed: 4639865]
20. Lee C, Qiao X, Goeger DE, Anderson KE. Clin. Chim. Acta. 2004; 347:183. [PubMed: 15313157]
21. Oishi H, Nomiyama H, Nomiyama K, Tomokuni K. Anal. Toxicol. 1996; 20:106.
22. Sithisarankul P, Cadorette M, Davoli CT, Serwint JR, Chisolm JJ, Strickland PT. Environ. Res. 1999; 80:41. [PubMed: 9931226]
23. Felitsyn NM, Henderson GN, James MO, Stacpoole PW. Clin. Chim. Acta. 2004; 350:219. [PubMed: 15530481]
24. Floderus Y, Sardh E, Moller C, Andersson C, Rejkjaer L, Andersson DE, Harper P. Clin. Chem. 2006; 52:701. [PubMed: 16497943]
25. Ford RE, Magera MJ, Kloke KM, Chezick PA, Fauq A, McConnell JP. Clin. Chem. 2001; 47:1627. [PubMed: 11514396]

26. Lindberg RL, Porcher C, Grandchamp B, Ledermann B, Burki K, Brandner S, Aguzzi A, Meyer UA. Nat. Genet. 1996; 12:195. [PubMed: 8563760]
27. Lindberg RL, Martini R, Baumgartner M, Erne B, Borg J, Zielasek J, Ricker K, Steck A, Toyka KV, Meyer UA. Clin. Invest. 1999; 103:1127.
28. Chace DH, Hillman SL, Millington DS, Kahler SG, Roe CR, Naylor EW. Clin. Chem. 1995; 41:62. [PubMed: 7813082]
29. Rashed MS, Ozand PT, Bucknall MP, Little D. Pediatr. Res. 1995; 38:324. [PubMed: 7494654]
30. Sardh E, Harper P, Andersson DE, Floderus Y. Eur. J. Intern. Med. 2009; 20:201. [PubMed: 19327613]
31. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Anal. Chem. 2003; 75:3019. [PubMed: 12964746]
32. Hindmarsh JT, Oliveras L, Greenway DC. Clin. Biochem. 1999; 32:609. [PubMed: 10638943]
33. Johansson A, Moller C, Fogh J, Harper P. Mol. Med. 2003; 9:193. [PubMed: 15208740]

Fig. 1. The extracted ion chromatograms of urinary ALA and PBG in symptomatic AIP (A and B) and healthy (C and D) individuals. ALA and PBG quantifier ion (qualifier) transitions (grey) are 188 > 114 (188 > 86) and 322 > 222 (322 > 248), respectively, with their respective internal standards in black. OH-Pro, 4OH-proline; Cre, creatine; Ile, isoleucine; Leu, leucine.

Fig. 2.

The chemical structures of 5-aminolevulinic acid (ALA) and porphobilinogen (PBG) butyl esters, and predicted precursor and product ions of butylated ALA and PBG. Bu-ALA, ALA butyl ester; (Bu)₂-PBG, PBG di-butyl ester.

Fig. 3. Comparison of the matrix effect (ME) and process efficiency (PE) of plasma and urinary ALA and PBG processed by solid phase extraction/butylation (SPE) vs methanol extraction/butylation (MeOH).

Table 1

The genetic defects and biochemical findings in the acute porphyrias.

	Gene defect	Mode of inheritance	Biochemical findings
Acute intermittent porphyria (AIP)	<i>HMBS</i>	AD	PBG and ALA (urine, blood), uroporphyrin (urine)
Hereditary coproporphyria (HCP)	<i>CPOX</i>	AD	PBG and ALA (urine, blood), coproporphyrin (urine, feces)
Variegate porphyria (VP)	<i>PPOX</i>	AD	PBG and ALA (urine, blood), coproporphyrin (urine, feces), protoporphyrin (feces)
ALA dehydratase porphyria (ADP)	<i>ALAD</i>	AR	ALA (urine, blood), coproporphyrin (urine), protoporphyrin (RBC)

HMBS, hydroxymethylbilane synthase; PPOX, protoporphyrinogen oxidase; CPOX, coproporphyrinogen oxidase; ALAD, 5-aminolevulinic acid dehydratase; ALA, 5-aminolaevulinic acid; PBG, porphobilinogen; AD, autosomal dominant; AR, autosomal recessive.

Table 2

Selective reaction monitoring (SRM) transitions, mass spectrometer settings and quantifier/qualifier response ratios for Bu-ALA, (Bu)₂-PBG, and their stable isotope-labeled internal standards.

	Quantifier ion			Qualifier ion			Ratio (Qualifier/Quantifier)		
	<i>m/z</i>	<i>F</i> (v)	CE (v)	<i>m/z</i>	<i>F</i> (v)	CE (v)	Mean ± SD (<i>n</i>)	CV (%)	
Bu-ALA	188 > 114	82	14	188 > 86	82	6	Urine 49.0 ± 4.4 (60)	9.0	
							Plasma 48.7 ± 4.7 (60)	9.6	
Bu- ¹³ C ₅ , ¹⁵ N-ALA	194 > 120	82	14	194 > 91	82	6	Urine 56.6 ± 1.2 (60)	2.1	
							Plasma 56.4 ± 1.5 (60)	2.7	
Bu-ALA	188 > 114	82	14	188 > 68	82	24	Urine 14.9 ± 0.7 (20)	4.6	
							Plasma 14.5 ± 0.5 (22)	3.5	
Bu- ¹³ C ₅ , ¹⁵ N-ALA	194 > 120	82	14	194 > 73	82	24	Urine 15.1 ± 0.6 (20)	4.1	
							Plasma 14.4 ± 0.3 (22)	2.3	
(Bu) ₂ -PBG	322 > 222	128	14	322 > 248	128	6	Urine 13.2 ± 1.1 (21)	8.2	
							Plasma 13.5 ± 0.7 (15)	5.5	
(Bu) ₂ -2,4- ¹³ C ₂ -PBG	324 > 224	128	14	324 > 250	128	6	Urine 13.2 ± 0.9 (21)	6.9	
							Plasma 13.7 ± 0.7 (15)	5.1	

Bu-ALA: 5-aminolevulinic acid, butyl ester; (Bu)₂-PBG: porphobilinogen, di-butyl ester; *F*: fragmentor; CE: collision energy; SD: standard deviation; CV: coefficient of variation.

Table 3

Intra- and inter-assay precision and accuracy of ALA and PBG concentrations in urine and plasma samples at various concentrations.

ALA and PBG (μmol/L)	ALA		PBG	
	Intra-assay CV (%) (n = 8)	Inter-assay CV (%) (n = 8)	Accuracy (%) (n = 10)	Inter-assay CV (%) (n = 10)
Plasma assay				
0.4	1.2	5.6	110.5	8.7
2.5	1.9	4.8	100.0	4.7
10	1.5	5.3	97.9	5.8
Urine assay				
10	1.9	5.5	103.8	5.7
50	3.2	6.2	99.2	9.3
200	3.1	3.1	107.5	6.0
				105.0

ALA, 5-aminolevulinic acid; PBG, porphobilinogen; CV, coefficient of variation.

Table 4
Urine and plasma ALA and PBG levels in acute porphyria patients and normal individuals.

	Age (Gender)	Urine (mmol/mol creatinine)		Plasma (μmol/L)		Gene	Mutation	Clinical status
		ALA	PBG	ALA	PBG			
Acute porphyria patients ^a								
H-AIP-1	33 y (F)	5.6	0.4	0.2	<0.05	HMBS	IVS10-31A > G/+	Symptomatic, on hemin therapy
H-AIP-2	30 y (M)	5.0	9.5	2.0	2.2	HMBS	R173W/+	Symptomatic, a few weeks after an acute attack
H-AIP-3	39 y (F)	18.5	33.6	3.3	4.1	HMBS	c.107_111delACAG	During acute attack
H-AIP-4	48 y (M)	5.4	9.7	N/A	N/A	HMBS	c.107_111delACAG	Symptomatic
H-VP-1	55 y (F)	27.6	8.0	0.4	0.1	PPOX	Q189X/+	During acute attack, before hemin administration
H-VP-2	60 y (M)	0.8	0.04	N/A	N/A	PPOX	IVS11 + 4A > G	Asymptomatic
H-HCP-1 ^b	5 mo (M)	34.6	8.3	N/A	N/A	CPOX	H327R/H327R	During an acute attack
Normal individuals								
>12 years, <i>n</i> = 60 (mean ± SD) median		0.09–3.0 (0.9 ± 0.6) 0.9	0–1.1 (0.07 ± 0.1) 0.05	0.07–0.2 (0.1 ± 0.01) 0.01	0.05 0.09			

^a AIP, acute intermittent porphyria; VP, variegate porphyria; HCP, hereditary coproporphyria (Harderoporphyria); ALA, 5-aminolevulinic acid; PBG, porphobilinogen.

^b Harderoporphyria form of HCP, A. Hasanoglu et al., *J. Inherit. Metab. Dis.* 34 (2011) 225–231.

Table 5

Comparison of ALA and PBG concentrations in human and murine urines determined by the LC-MS/MS and colorimetric column methods.^a

ID	Column ^b (μM)			MS (μM)			Ratio (Column/MS)		
	ALA	PBG		ALA	PBG		ALA	PBG	
Acute porphyria patients	H-AIP-1	62.5	11.5	35.9	2.9	1.7			4.0
	H-AIP-2 ^a	53.4	71.5	54.4	102.5	1.0			0.7
	H-AIP-2 ^b	53.2	66.6	53.4	75.3	1.0			0.9
	H-AIP-3	71.3	117.8	60.7	110.1	1.2			1.1
	H-VP-1	76.7	14.7	61.8	17.7	1.2			0.8
Normal subjects							1.23 ± 0.31		1.50 ± 1.41
	H-N-1	10.2	5.3	7.0	0.7	1.4			7.5
	H-N-2	38.9	9.5	23.5	1.2	1.7			7.9
	H-N-3	23.0	6.1	12.1	1.1	1.9			5.7
	H-N-4	21.7	2.7	14.4	1.1	1.5			2.5
Murine AIP (Baseline)	H-N-5	19.0	5.3	9.9	1.0	1.9			5.1
							1.68 ± 0.22		5.78 ± 2.17
	M-AIP-1	39.9	32.9	78.8	19.8	0.5			1.7
	M-AIP-2	112.7	34.4	119.6	27.4	0.9			1.3
	M-AIP-3	91.4	33.7	76.0	22.0	1.2			1.5
	M-AIP-4	148.5	39.2	120.3	26.9	1.2			1.5
	M-AIP-5	96.2	29.4	53.5	14.6	1.8			2.0
	M-AIP-6	93.8	22.5	58.5	17.0	1.6			1.3
	M-AIP-7	103.3	27.6	86.2	26.4	1.2			1.0
	M-AIP-8	144.6	35.7	100.5	29.0	1.4			1.2
Murine AIP (Post-phenobarbital induction)	M-AIP-9	64.1	34.0	98.4	25.8	0.7			1.3
	M-AIP-10	159.2	34.0	86.1	25.9	1.8			1.3
							1.24 ± 0.45		1.42 ± 0.27
	M-AIP-1	1080	910	1210	910	0.9			1.0
	M-AIP-2	2320	1540	3530	1600	0.7			1.0
	M-AIP-3	1820	1300	1720	1240	1.1			1.0

ID	Column ^b (μM)		MS (μM)		Ratio (Column/MS)	
	ALA	PBG	ALA	PBG	ALA	PBG
M-AIP-4	1450	1010	1770	970	0.8	1.0
M-AIP-5	925	714	978	678	0.9	1.1
M-AIP-6	1500	1430	1610	1297	0.9	1.1
M-AIP-7	1400	967	1760	1040	0.8	0.9
M-AIP-8	2340	1820	2950	1660	0.8	1.1
M-AIP-9	2700	2330	5880	3400	0.5	0.7
M-AIP-10	2300	1910	3130	2060	0.7	0.9
					0.81 ± 0.17	0.98 ± 0.12

^aThe ALA and PBG values for individual AIP mice, identified by number, M-AIP-1, 2, ..., 10, were determined at baseline and after phenobarbital induction.

^b300–1000 μL urine samples were used for the column method and 25 μL samples were used for LC–MS/MS method.

Table 6

ALA and PBG levels in plasma, urine and liver in wild-type and AIP mice before and after Phenobarbital induction.

	Mouse genotype (<i>n</i>)	ALA	PBG
Plasma (μM)	WT (<i>n</i> = 4)	0.15 ± 0.05 (0.09–0.19)	<0.05 (<0.05)
	AIP, Baseline (2)	0.36 ± 0.0007 (0.36–0.36), <i>p</i> = 3.0 × 10 ^{−3}	<0.05 (<0.05)
	AIP, Post pheno (4)	15.0 ± 0.77 (14.2–15.7), <i>p</i> = 3.9 × 10 ^{−5}	13.3 ± 0.72 (12.7–14.4)
Urine (mmol/mol creatinine)	WT (4)	6.25 ± 1.12 (4.6–7.0)	1.5 ± 0.63 (1.0–2.4)
	AIP, Baseline (10)	29.2 ± 20.7 (14.8–73.2), <i>p</i> = 3.4 × 10 ^{−3}	7.8 ± 5.2 (4.0–19.2), <i>p</i> = 2.0 × 10 ^{−3}
	AIP, Post pheno (10)	986 ± 763 (372–2850), <i>p</i> = 2.8 × 10 ^{−3}	599 ± 428 (258–1650), <i>p</i> = 1.7 × 10 ^{−3}
Liver (nmol/g protein)	WT (5)	10.1 ± 2.56 (7.4–13.1)	2.4 ± 0.9 (1.8–4.0)
	AIP, Baseline (3)	28.1 ± 9.2 (20.1–38.1), <i>p</i> = 0.08	21.1 ± 19.4 (5.9–43.0), <i>p</i> = 0.23
	AIP, Post pheno (3)	374 ± 98.2 (282–478), <i>p</i> = 0.023	6670 605 (6127–7320), <i>p</i> = 2.7 10 ^{−3}

ALA and PBG values were presented as mean ± standard deviation (range). *P* values were calculated against wild-type values using standard *t*-test. ALA, 5-aminolaevulinic acid; PBG, porphobilinogen; WT, wild-type.