A Simple Method for Quantification of Five Urinary Porphyrins, Porphobilinogen and 5-Aminolevulinic Acid, Using Liquid Chromatography Tandem Mass Spectrometry

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Abstract Analysis of porphyrins and 5-aminolevulinic acid (ALA), porphobilinogen (PBG) in physiological liquids is required for diagnosis and follow-up of porphyrias. High performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC–MS) methods with higher specificity and sensitivity have been developed. The major disadvantage of those methods is that they require longer extraction times due to their matrix effects. The present study suggests a simple, fast, sensitive, and specific assay for determination of Coproporphyrin, 5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, Uroporphyrin I and ALA, PBG in urine sample by direct injection without sample pre-treatment using LC–MS. For the purposes of the present study LC–MS device was set to multiple reaction monitoring (MRM) and positive ion mode. Porphyrins and ALA, porphobilinogen were characterized by their MS/MS product ion, spectra. ALA, PBG and 5 porphyrins were detected simultaneously. Limit of detection for Coproporphyrin, 5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, Uroporphyrin I were 2 nmol/L, where it

was 5 µmol/L for ALA and 2 µmol/L for porphobilinogen. The present study suggests that the present method is very effective compared to many other available methods for it does not require pre-treatment, provides simultaneous results of ALA, PBG and 5 porphyrins quantitatively in a shorter span of time, and has suitable sensitivity and selectivity. LC–MS technique was used clinically for the determination of urine porphyrin levels.

Keywords Porphyrins · LC–MS · ALA · Porphobilinogen

Introduction

Porphyrias are metabolic diseases induced by a lack of specific enzyme involved in heme synthesis. It has been suggested that hereditary deficiencies in the enzymes involved in heme synthesis include autosomal dominant, autosomal recessive, and X-linked transmission. The interaction of environmental, physiological, and genetic factors disrupts the heme synthesis inducing the porphyrias characterized by overproduction and accumulation of porphyrin and/or porphyrin precursors [1]. Signs and symptoms appear depending upon the toxic composition of the compounds accumulated in the porphyrias [2]. While ALA and PBG, the early precursors of heme synthesis pathway, increase during the acute episodes, the increase in porphyrins, i.e. the late substrates, becomes more significant in the chronic period [3].

The first thing to do in the diagnosis and follow-up of porphyria is the determination of porphyrins and porphyrin precursors in blood, urine, and faeces [4]. Various techniques have been used until today for determination of porphyrins, including spectrofluorometry [5], second-derivative spectroscopy [6], high-performance liquid

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chromatography (HPLC) [7], thin layer chromatography [8], capillary electrophoresis [9], laser desorption/ionization time of flight mass spectrometry (LDI-TOF-MS) [10], fast atom bombardment mass spectrometry (FAB-MS) [11], and LC-MS [12]. Most of the aforementioned methods require preliminary preparation, larger sizes of samples, derivation, and a longer time span.

Increase in ALA and PBG concentrations is important for detection, diagnosis, and follow-up of acute attack types. While ALA and PBG increase is significant in autosomal dominant acute hepatic porphyria, ALA increase is significant in autosomal recessive acute hepatic porphyria. As regards the acute attack period, especially the tests on PBG increase have been conducted as the first choice. The colorimetric methods that have been frequently used in ALA and PBG measurements have lower sensitivity and specificity [13].

The present study aims to develop a method using LC-MS/MS, where spot urine can be used, ensuring simple, fast, and simultaneous determination of ALA, PBG, and 5 urine porphyrins (coproporphyrin, 5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, Uroporphyrin I), quantitative calculation, and partial differentiation of coproporphyrin I and III isomers. As a result the test would be more specific and sensitive, enable direct use of the samples without any preliminary procedure, and render results in a shorter period of time.

Experimental

Materials and Reagents

Acetonitrile and HPLC-grade water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid for analysis grade was produced by Sigma (St. Louis, MO, USA). Porphyrin acid chromatographic marker calibrator contained 10 nmol of six porphyrins (5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, uroporphyrin I, coproporphyrin, mesoporphyrin IX) and Porphyrin Recipe Chemicals (Munich, Germany). Urine control contained (Uroporphyrin I, 5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, uroporphyrin I, coproporphyrin I, and III, 5-aminolevulinic acid, porphobilinogen).

Preparation of Calibration Standards and Quality Control Samples

The chromatographic marker calibrator was dissolved in 10 mL of 6 M formic acid to obtain a stock solution. The final concentration of porphyrins was 1.0 nmol/mL. The calibration standards were diluted with 6 M formic acid to have concentrations of 0.02 and 1.0 nmol/mL (20 and

1000 nmol/L) for each porphyrin. We performed two-level quality control samples. ALA and PBG concentrations of 0.05 and 25 $\mu\text{mol/L}$ were obtained from the quality control sample.

Sample Preparation

Urine samples (700 μL) were vortexed for 30 s mixed with 300 μL formic acid (6 M), and centrifuged at $10,000\times g$ for 5 min. The supernatant was transferred in a 96-well plate and 5 μL was injected for high-performance liquid chromatographic tandem mass spectrometric analysis. 10 μL mesoporphyrin was used as an internal standard.

LC-MS-MS Conditions

A Thermo Scientific (San Jose, CA) Ultimate 3000 Rapid Separation Quaternary System was used. The chromatographic separation was performed on a Thermo Scientific Synchronics C18 column (100×2.1 mm, 3 μm particle size). The gradient solvent A (0.1% formic acid) (v/v) and solvent B (90: 9.9: 0.1, acetonitrile–water–formic acid respectively) (v/v/v) was applied at a flow rate was 0.5 mL/min. The gradient program was started with 20% B for 1 min followed by a linear increase in B to 80% from 1 to 12 min. Mobile phase B was held at 80% for another 4 min and then reduced linearly 20% B until the end of run. The total run time was 24.1 min. The injection volume was 5 μL and flow-rate of column was 0.5 mL/min.

Mass analysis was performed using a Thermo Scientific TSQ access Max Triple Stage Quadrupole Mass Spectrometer (Thermo Scientific, San Jose, CA) equipped with an electrochemical ionization source in multiple reaction monitoring data acquisition mode. The temperature of the heated capillary was 300 °C, vaporizer temperature 200 °C, pump-left flow 0.5 mL/min. capillary voltage, 3.0 kV.

The mass spectrometer worked with positive electrospray ionization (ESI) in multiple reaction-monitoring-modes. Collision energies were 55 eV for porphyrins, 25 eV for porphobilinogen, and 10 eV for ALA. The masses of porphyrins, their collision energies are shown in Table 1.

Results

The LC-MS/MS results in the analyses of porphyrins and porphyrin precursors MRM transitions, cone voltages, and collision energies were optimized for both HPLC and MS. Gradient chromatographic program positive electrospray ionization mode was used for a successful differentiation of porphyrins and ALA, PBG in a short time period.

Table 1 Precursor ions and product ions of porphyrins, collision energies and polarity

Porphyrins	Precursor ion ($M + 1$) (m/z)	Major product (m/z)	Collision energy (eV)	Polarity
ALA	132	114	10	+
Porphobilinogen	209.9	122	25	+
Mesoporphyrin (IS)	567	479	55	+
Coproporphyrin I-III	655	537	55	+
Pentacarboxylporphyrin	699	595	55	+
Hexacarboxylporphyrin	743	639	55	+
Heptacarboxylporphyrin	787	683	55	+
Uroporphyrin I	831	727	55	+

The porphyrin calibrator containing Coproporphyrin (m/z 655), 5-carboxylporphyrin (m/z 699), 6-carboxylporphyrin (m/z 743), 7-carboxylporphyrin (m/z 787), and Uroporphyrin I (m/z 831) was prepared with 6 M formic acid (1 nmol/mL), scanned at MRM mode, and 6 porphyrins was determined (Fig. 1). The reaction time extended and the water phase was increased in order to differentiate the coproporphyrin I and III.

Two-level control samples and porphyrin standard were used for the repeatability studies. Figures 1, 2, 3 and 4 provides the chromatograms pertaining to the porphyrin standard, two-level control, and patient urine sample (Congenital erythropoietic porphyria) at MRM mode.

5 different porphyrin standards and two different control samples for ALA, PBG were diluted to generate standard curves between concentrations of 0.02–1.0 nmol/L and 0.05–25 μ mol/L for the determination of measuring range.

The limit of detection (LOD) at LC–MS/MS was defined as 3 times higher than the signal/noise ratio, where the lower limit of quantification (LOQ) was calculated as ten times of the signal/noise ratio. As for repeatability ($n = 10$), the lower limit of quantification was determined as 2 nmol/L for coproporphyrin, 5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, and uroporphyrin I, 5 μ mol/L for ALA, and 2 μ mol/L porphobilinogen upon analysis of the control and calibrator containing coproporphyrin, 5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, and uroporphyrin I.

Low and high level control samples were used for the intra-day and inter-day repeatability study. For intra-assay precision, aliquots were analysed on the same day ten and both the two-level control serums were measured successively in the same study for 10 days in order to show the inter-day variability. Three assays were conducted on urine

Fig. 1 LC–MS/MS chromatogram of 1 nmol/mL calibration standard coproporphyrin, 5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, uroporphyrin I, and internal standard mesoporphyrin

Fig. 2 LC-MS/MS control level 1 chromatogram ALA, PBG, coproporphyrin I- III, 5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, and Uroporphyrin I

Fig. 3 LC-MS/MS control level 2 chromatogram of ALA, PBG, coproporphyrin I-III, 5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, and uroporphyrin I

Fig. 4 Urine chromatogram for the patient with congenital erythropoietic porphyria; porphobilinogen 4.7 nmol/L, coproporphyrin I 67 nmol/L and coproporphyrin III 116 nmol/L, uroporphyrin I 34 nmol/L

samples for detection of 5 porphyrins (coproporphyrin, 5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, and uroporphyrin I), and ALA and PBG. The results are shown in Table 2.

Internal standard additions were made to the urine samples subject to measurement so as to increase the endogenous concentrations of porphyrins to 50 nmol/L, ALA to 100 μ mol/L, and porphobilinogen to 25 μ mol/L. The accuracy results were found between 70 and 125% in Table 3.

20 healthy urine samples were collected for the reference studies. Accordingly the normal values of coproporphyrin I-III, 5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, uroporphyrin I, and ALA and PBG collected from healthy individuals are shown in Table 4.

Patient with congenital erythropoietic porphyria had increased concentrations of urine porphobilinogen, coproporphyrin I, and III. (Figure 4).

Table 2 Intra-assay and Inter-assay precision of ALA, PBG, coproporphyrin I-III, 5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, and uroporphyrin I concentrations

Porphyrin	Low level (n = 10)		High level (n = 10)	
	Intra-assay % CV	Inter-assay % CV	Intra-assay % CV	Inter-assay % CV
ALA (μ mol/L)	8.5	12.8	6.3	9.6
Porphobilinogen (μ mol/L)	7.2	10.8	10.9	17.9
Coproporphyrin I (nmol/L)	7.6	10.9	4.2	6.4
Coproporphyrin III (nmol/L)	6.9	11.2	4.8	7.2
Pentacarboxylporphyrin (nmol/L)	5.3	10.9	6.5	10.0
Hexacarboxylporphyrin (nmol/L)	7.1	13.6	7.1	12.1
Heptacarboxylporphyrin (nmol/L)	9.2	15.1	6.0	9.0
Uroporphyrin I (nmol/L)	8.2	12.3	1.7	2.4

Table 3 Accuracy of ALA, PBG, coproporphyrin I-III, 5-carboxylporphyrin, 6-carboxylporphyrin, 7-carboxylporphyrin, and uroporphyrin I in urine sample

Porphyrins	Spiked	Accuracy %
ALA (μmol/L)	100 μmol/L	72.1
Porphobilinogen (μmol/L)	25 μmol/L	107.6
Coproporphyrin I (nmol/L)	50 nmol/L	95.3
Coproporphyrin III (nmol/L)	50 nmol/L	106.4
Pentacarboxylporphyrin (nmol/L)	50 nmol/L	115.2
Hexacarboxylporphyrin (nmol/L)	50 nmol/L	88.7
Heptacarboxylporphyrin (nmol/L)	50 nmol/L	122.8
Uroporphyrin I (nmol/L)	50 nmol/L	98.4

Table 4 Urine ALA, PBG, coproporphyrin I, coproporphyrin III, 5-carboxyporphyrin, 6-carboxyporphyrin, 7-carboxyporphyrin, and uroporphyrin I healthy individuals

Porphyrins	Concentrations
ALA	≤ 15 μmol/L
Porphobilinogen	≤ 1.5 μmol/L
Coproporphyrin I	≤ 30 nmol/L
Coproporphyrin III	≤ 90 nmol/L
Pentacarboxylporphyrin	≤ 6 nmol/L
Hexacarboxylporphyrin	≤ 3 nmol/L
Heptacarboxylporphyrin	≤ 8 nmol/L
Uroporphyrin I	≤ 35 nmol/L

Discussion/Conclusion

Due to the differing clinical findings, utilization of biochemical assessments in distinguishing types of porphyria is very important for accurate diagnosis and treatment. Determination of porphyrins and porphobilinogen in urine helps with faster diagnosis [14].

Based on the LC–MS/MS method as developed by Bu et al. [15], the present study aimed to develop a method, which would enable simultaneous determination of porphyrin, ALA, and porphobilinogen in human urine without any preliminary procedure and in a comparatively shorter period of time. In addition to the porphyrin measurements in dog urine in the study by Bu et al., we included the study by Benton et al. [16] on ALA and porphobilinogen. Since simultaneous measurement of porphyrin and ALA, porphobilinogen was aimed; the procedure was extended to 24 min for a better differentiation. As suggested by Lim and Peter [17], determination of porphyrin I and III isomers is very important for differential diagnosis of CEP, PCT, and HCPs. We increased the water phase similar to the 60-min method by Danton et al. [18], by which the

coproporphyrin I and III isomers could have been determined, and we could have differentiated coproporphyrin I and III isomers, albeit partially. We were unable to observe differentiation in I and III since the available calibrator and control samples had uroporphyrin I.

Consequently, we developed a method by which porphyrins, ALA and porphobilinogen in urine could be simultaneously determined by LC–MS/MS method for the diagnosis and follow-up of porphyrias. Compared to a range of methods in the literature, the resultant method was simple, enabling determination of 5 porphyrins, ALA, and porphobilinogen, with easy sample preparation and high sensitivity and specificity due to the MRM mode of the mass detection system. ALA, porphobilinogen occurs at rather low concentrations in urine yet it is important for detection of acute attacks. Therefore the present method, which uses spot urine, proves to be effective in obtaining faster results.

Considering that LC–MS/MS technique, the method would be very useful in differential diagnosis and follow-up due to ability of rendering fast and quantitative results in patients with porphyrias. Given the partial differentiation of the coproporphyrin isomers, the method should be further developed for better differentiation of the coproporphyrin isomers.

References

1. Porphyrias Kauppinen R. The Lancet. 2005;365(9455):241–52.
2. Puy H, Gouya L, Deybach J-C. Porphyrias. The Lancet. 2010;375(9718):924–37.
3. Benton CM, Lim CK. Liquid chromatography and mass spectrometry of haem biosynthetic intermediates: a review. Biomed Chromatogr. 2012;26(8):1009–23.
4. Floderus Y, Sardh E, Möller C, Andersson C, Rejckjaer L, Andersson DE, et al. 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. Clin Chem. 2006;52(4):701–7.
5. Westerlund J, Pudek M, Schreiber W. A rapid and accurate spectrofluorometric method for quantification and screening of urinary porphyrins. Clin Chem. 1988;34(2):345–51.
6. Van de Giessen A, Van Wijk E. An improved method for quantitative determination of urinary porphyrins by use of second-derivative spectroscopy. Clin Chem Lab Med. 1990;28(9):605–10.
7. Hahn ME, Gasiewicz TA. Determination of individual porphyrins in rodent urine using high-performance liquid chromatography following clean-up by anion-exchange chromatography. J Chromatogr B Biomed Sci Appl. 1991;563(2):363–8.
8. Smith S. The use of thin layer chromatography in the separation of free porphyrins and porphyrin methyl esters. Br J Dermatol. 1975;93(3):291–5.
9. Weinberger R, Sapp E, Moring S. Capillary electrophoresis of urinary porphyrins with absorbance and fluorescence detection. J Chromatogr A. 1990;516(1):271–85.

10. Jones RM, Lamb JH, Lim CK. Urinary porphyrin profiles by laser desorption/ionization time-of-flight mass spectrometry without the use of classical matrices. *Rapid Commun Mass Spectrom*. 1995;9(10):921–3.
11. Luo J, Lamb JH, Lim C. Analysis of urinary and faecal porphyrin excretion patterns in human porphyrias by fast atom bombardment mass spectrometry. *J Pharm Biomed Anal*. 1997;15(9):1289–94.
12. Li J, Cai Z, Xu S, Liao C, Song X, Chen L. Analysis of urinary porphyrins by high performance liquid chromatography-electrospray ionization mass spectrometry. *J Liq Chromatogr Relat Technol*. 2011;34(15):1578–93.
13. Zhang J, Yasuda M, Desnick RJ, Balwani M, Bishop D, Yu C. A LC–MS/MS method for the specific, sensitive, and simultaneous quantification of 5-aminolevulinic acid and porphobilinogen. *J Chromatogr B*. 2011;879(24):2389–96.
14. Marsden JT, Rees DC. Urinary excretion of porphyrins, porphobilinogen and δ -aminolaevulinic acid following an attack of acute intermittent porphyria. *J Clin Pathol*. 2014;67(1):60–5.
15. Bu W, Myers N, McCarty JD, O'Neill T, Hollar S, Stetson PL, et al. Simultaneous determination of six urinary porphyrins using liquid chromatography–tandem mass spectrometry. *J Chromatogr B*. 2003;783(2):411–23.
16. Benton CM, Couchman L, Marsden JT, Rees DC, Moniz C, Lim CK. Direct and simultaneous quantitation of 5-aminolaevulinic acid and porphobilinogen in human serum or plasma by hydrophilic interaction liquid chromatography–atmospheric pressure chemical ionization/tandem mass spectrometry. *Biomed Chromatogr*. 2013;27(2):267–72.
17. Lim C, Peters T. Urine and faecal porphyrin profiles by reversed-phase high-performance liquid chromatography in the porphyrias. *Clin Chim Acta*. 1984;139(1):55–63.
18. Danton M, Lim CK. Porphyrin profiles in blood, urine and faeces by HPLC/electrospray ionization tandem mass spectrometry. *Biomed Chromatogr*. 2006;20(6–7):612–21.