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Article

Bioanalytical Studies of Porphyric Disorders Using HPLC with Fluorescence Detection

Michele G. S. C. Cypriani,[a] Atecla N. L. Alves,[a] Etelvino J. H. Bechara[a,b] and Nilson A. Assunção*[,a]

[a] Departamento de Ciências Exatas e da Terra, Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo (Unifesp), Campus Diadema, R. Prof. Artur Riedel, 275, Jardim Eldorado, 09972-270 Diadema-SP, Brazil

[b] Departamento de Química Fundamental, Instituto de Química, Universidade de São Paulo (USP), Av. Prof. Lineu Prestes, 748, 05508-000 São Paulo-SP, Brazil

Neste artigo, desenvolvemos, validamos e aplicamos um método para separação e quantificação de porfirinas precursoras do grupo heme na urina de portadores de porfirias. Os isômeros coproporfirinogenio I e III (COPRO I e III), uroporfirinogenio I (URO I), heptacarboxilporfirinogenio I (HEPTA I), pentacarboxilporfirinogenio I (PENTA I) e hexacarboxilporfirinogenio (HEXA I) foram determinados em amostras coletadas de 24 pacientes de porfiria aguda intermitente e de porfiria cutânea tarda. Utilizou-se cromatografia líquida de alta eficiência (HPLC) e detector de fluorescência. As concentrações de porfirinas foram determinadas com precisão inter e intra-dias (< 5%) e exatidão dentro da faixa 95-99%. Os limites de detecção e quantificação das porfirinas, expressos em nmol L^{-1}, foram os seguintes: URO I, 0,62 e 2,05; HEPTA I, 0,59 e 1,96; HEXA I, 0,54 e 1,81; PENTA I, 0,52 e 1,73; COPRO I, 2,03 e 6,77; e COPRO III, 0,43 e 1,44. O método descrito aqui obedece a parâmetros analíticos satisfatórios, com excelente relação custo-benefício, e foi aplicado a amostras de urina de portadores assintomáticos e pacientes de porfirias. Este método foi validado analiticamente e mostrou potencial para diagnóstico de portadores de diferentes tipos de porfirias, imediatamente antes ou durante crises, e até mesmo para monitorar um tratamento farmacológico.

We describe here the development, validation, quantification and application of a method for determination of heme porphyrin precursors in the urine of porphyric patients. The isomers coproporphyrinogen I and III (COPRO I and III), uroporphyrinogen I (URO I), heptacarboxylporphyrinogen I (HEPTA I), pentacarboxylporphyrinogen (PENTA I), and hexacarboxylporphyrinogen I (HEXA I) were analyzed. These six urinary heme precursors were determined in urine samples collected from 24 patients by high-performance liquid chromatography (HPLC) equipped with a fluorescence detector. The inter- and intra-day precision (coefficient of variation < 5%) and accuracy (95-99%) were evaluated. The limits of detection and of quantification of the porphyrins, expressed in nmol L^{-1}, were as follows: URO I, 0.62 and 2.05; HEPTA I, 0.59 and 1.96; HEXA I, 0.54 and 1.81; PENTA I, 0.52 and 1.73; COPRO I, 2.03 and 6.77; and COPRO III, 0.43 and 1.44. The method described here satisfactorily results in an acceptable cost-benefit ratio, precision and speed for determining the concentrations of heme precursors in the urine of latent or symptomatic acute intermittent porphyria individuals or porphyria cutanea tarda carriers. Since it was analytically validated, this method may be used for accurate and reliable diagnostic reports to follow-up the onset of acute crisis in porphyria carriers to adopt preventive pharmacological treatment.

Keywords: porphyrins, heme biosynthesis, acute intermittent porphyria, porphyria cutanea tarda, porphyria diagnosis, HPLC analysis of porphyrinogens

*e-mail: nilson.assuncao@unifesp.br

Introduction

Porphyrias are a group of genetic and acquired rare disorders caused by enzymatic deficiencies in the heme biosynthetic pathway attributed to abnormally low enzymatic activities or inhibition by xenobiotics leading to the accumulation or deficit of heme precursors (Table 1).[1,2] Acute porphyria occurs in all ethnic groups. In most European countries the estimated prevalence is 1-2 *per* 100,000 inhabitants, with acute intermittent porphyria (AIP) being the most common type of disorder. However, there is a much higher prevalence in the psychiatric population (210 *per* 100,000 in the United States),[3] which has been attributed to misdiagnoses due to neuropsychiatric symptoms of porphyria, such as insomnia, abdominal pain, palpitations, and pain in the extremities, back, chest, neck or head.[4] Other neuropsychiatric symptoms of porphyria also include seizures, agitation, hallucinations and other psychiatric symptoms, such as psychosis similar to schizophrenia.[5,6] Late diagnosis can cause serious neurological and mental damage, and may lead to mortality, especially in the differential diagnosis of psychiatric patients.[7,8]

Like many hereditary diseases, porphyrias have an uneven distribution. For example, porphyria cutaneous tarda has a prevalence of 1:10,000,[9] which varies between countries; the prevalence is 1:25,000 in North America, 1:5,000 in Czechoslovakia, and 2-5 cases *per* million *per* year in the UK. There is no reported data for Brazilian AIP prevalence. Although porphyria cutanea tarda (PCT) is probably the most common of all porphyrias, its prevalence has not been well estimated, and no reliable data have been published so far. It is estimated that the prevalence of PCT in Spain is approximately 1:1,000 inhabitants. In the case of AIP, for example, the ratio is 1:20,000 inhabitants, while in northern Sweden it is 1:10,000. Porphyria variegata (PV) is particularly common among Afrikaners (white South Africans, especially Dutch descendants), with an incidence of 1:250. In South Africa, the prevalence of PV is 1:400. However, it is difficult to estimate the incidence of porphyria because many individuals remain asymptomatic throughout life.[10] For early diagnosis and identification of the type of porphyria, samples of urine, blood and stool should be carefully examined by a laboratory with expertise in diagnosis of porphyria.

Heme occupies a critical position in the aerobic metabolic map. It is the prosthetic group of oxygen carrier proteins (hemoglobin, myoglobin), cytochromes in electron transport chains, and peroxidases, such as catalase and tryptophan-2,3-dioxygenase. In AIP, failure in 5-aminolevulinic acid dehydratase (ALA-D) biosynthesis results in intracellular accumulation of the first and second heme precursors,[11] namely, ALA and porphobilinogen (PBG), whereas in lead poisoning both ALA-D and ferrochelatase are inhibited by Pb^{2+} ion, ultimately preventing iron incorporation into the heme molecule, and release of the intermediates into the blood.[12,13] Accumulation of specific heme precursors in some tissues and organs[1-12] triggers so-called "acute hepatic", "hepatic cutaneous" and "erythropoietic cutaneous" clinical manifestations,[14] which are accompanied by altered urinary and fecal excretion of heme intermediates.[15]

Table 1. Types of porphyrias and the corresponding altered urinary porphyrin (adapted from Alves *et al.*[20])

Porphyria	Clinical expression	Enzymes altered	Urinary porphyrin altered
Erythropoietic			
CEP	Photosensitivity	URO III synthase	URO I, COPRO I
EEP	Photosensitivity	ferrochelatase	COPRO
Hepatic			
Acute			
ADP	Visceral and neurologic	ALA-D	COPRO
AIP	Visceral	PBG-D	URO, COPRO I
HCP	Visceral, neurologic and photosensitivity	Uroporphyrinogen decarboxylase	COPRO III
VP	Visceral and neurologic	Protoporphyrinogen IX oxidase	COPRO I
Chronic			
PCT	Photosensitivity	URO-D	URO, HEPTA
HEP	Visceral, neurologic and photosensitivity	URO-D	URO, HEPTA

CEP: congenital erythropoietic porphyria; EEP: erythropoietic protoporphyria; ADP: acute ALA-D deficiency porphyria; AIP: acute intermittent porphyria; HCP: hereditary coproporphyria; VP: variegate porphyria; PCT: porphyria cutanea tarda; HEP: hepatic erythropoietic porphyria; ALA: delta-aminolevulinic acid; ALA-D: aminolevulinate dehydratase; PBG-D: porphobilinogen deaminase; URO: uroporphyrin; COPRO: coproporphyrin; URO-D: uroporphyrinogen decarboxylase.

Heme biosynthesis is initiated in the mitochondria from the reaction of glycine with succinyl-CoA to form ALA, which migrates into the cytosol where PBG is formed under the action of ALA-D.[16,17] Porphyrin derivatives are formed by the condensation of the pyrrole rings of four molecules of PBG, yielding uroporphyrins (URO), which contain eight carboxyl groups, and are successively decarboxylated to the less polar intermediates[14] heptacarboxyl porphyrins (HEPTA), hexacarboxyl porphyrins (HEXA), pentacarboxyl porphyrins (PENTA), and coproporphyrins (COPRO), that contain four carboxyl groups. Porphyrin derivatives originating from PBG can be formed in two ways, spontaneously or enzymatically, and are denominated URO I, HEPTA I, HEXA I, PENTA I or COPRO I or URO III, HEPTA III, HEXA III, PENTA III or COPRO III. Porphyrins produced enzymatically in the cytosol are ultimately converted into protoporphyrinogen IX (PROTO IX) and protoporphyrin IX (PP IX), which migrate to the mitochondrial matrix where they coordinate with an iron ion in a reaction catalyzed by ferrochelatase to produce the heme molecule.

Porphyrias can be genetically transmitted by autosomal dominant inheritance, with the exception of congenital erythropoietic porphyria (autosomal recessive),[18] or can be chemically acquired by exposure of individuals to xenobiotics, such as lead, a condition referred to as lead poisoning, plumbism or saturnism.[14] The excretion patterns of heme intermediates enable the classification of a case of porphyria and the establishment of the most suitable strategies for drug therapy and patient monitoring[19] (Table 1). Many spectrofluorometric techniques are available to detect porphyrin excretion in urinary fractions; however, gradient reversed-phase high-performance liquid chromatography (RP-HPLC) using C18 columns with fluorescence detection is an accessible methodology for most laboratories and is sensitive enough to detect at least five fractions.[20] Here, we used a modified HPLC methodology to identify COPRO I and COPRO III isomers, which may also be applied to the diagnosis of other diseases that are based on alterations of normal heme.[21]

Recently, Balwani and Desnic published a comprehensive review on the diagnosis and treatment of the eight genetically distinguished porphyrias.[1] Moreover, Danton and Lim discussed the reported methods for analyzing the heme biosynthetic metabolites and their oxidation products by HPLC/mass spectrometry (MS), which may eventually result in the characterization of the type of porphyria carried by a patient.[22] Alternatively, several hypotheses for biochemically correlating the data on heme intermediates present in urine and blood with the clinical porphyric data have been discussed in the literature. Among them, a free radical- and oxidative stress-based hypothesis based on the accumulation of pro-oxidant ALA in tissue has been reinforced by biochemical data obtained from *in vitro* and *in vivo* studies with cell cultures and animal and human organs that are ALA-rich endogenously or due to experimental interventions.[14,23]

Laboratory diagnosis is achieved by the quantification of these metabolites present in the heme biosynthetic pathway and subsequent measurement of the enzyme activities related to the metabolites. The current accepted values for random urinary porphyrins in healthy individuals are as follows: URO: ≤ 30 nmol L^{-1}; HEPTA: ≤ 7 nmol L^{-1}; HEXA: ≤ 2 nmol L^{-1}; PENTA: ≤ 5 nmol L^{-1} and total COPRO (I + III) ≤ 110 nmol L^{-1}.[24]

We propose here a rapid, cheap and sensitive HPLC method to identify urinary porphyrin metabolites in patients in public hospital networks that include the distinction between COPRO I and III. Previous reports stress the importance of determining COPRO I and III isomer levels as biomarkers of arsenic poisoning,[25] as exposed groups have been found to exhibit significantly higher COPRO I and III levels compared with those in the control group. Alterations in porphyrin concentrations are determined in urine samples from clinically identified porphyric patients compared to samples obtained from healthy individuals, thereby demonstrating the reliability of this method to identify the class of porphyria.

Experimental

HPLC method

Urinary porphyrins were analyzed using a Shimadzu HPLC chromatograph (Kyoto, Japan) composed of the following parts: 1 RF 20A fluorescence fetector; 1 DGU-20A5 degasser; 2 LC-20 AT liquid chromatography pumps; 1 SIL-20 HT auto sampler; 1 CTO-20A column oven, and 1 CBM-20A communications bus module, programmed for excitation at 400 nm and emission at 620 nm, and one integrator (CR7A PLUS). The column used was a reversed phase C18 HPLC column Phenomemex® (Torrance, CA, USA), model LiChrospher, 125×4.00 mm, attached to a pre-column, 4.0 cm $\times$ 4.0 mm, both 5 mm RP-18, 100 Å, 5-μm particles, with a flow of 1 mL min^{-1} and a 100 μL sample injection size.

A standard commercial lyophilized porphyrin kit was purchased from Sigma-Aldrich (St. Louis, MO, USA), which contains URO I dihydrochloride, synthetic COPRO I dihydrochloride, and COPRO III tetramethyl ester. In addition, a CMK-1A porphyrin acid chromatographic marker kit containing URO I dihydrochloride, HEPTA I

heptamethyl ester, HEXA I hexamethyl ester, PENTA I pentamethyl ester, and COPRO I tetramethyl ester was acquired from Frontier Scientific (Logan, UT, USA). Standard solutions of porphyrins suspended in HCl from Merck KGaA (Darmstadt, Germany) were prepared at concentrations of 6.0 and/or 3.0 nmol L^{-1}. For the porphyrin analysis, 100 µL porphyrin aliquots were injected into the chromatograph at a flow rate of 1.0 mL min^{-1} with a total analysis time of approximately 43 min. Phase A was prepared using 1.0 mol L^{-1} ammonium acetate buffers (Synth, São Paulo, Brazil) with pH values of 4.0, 4.3, 4.6, 4.9, 5.2, 5.4, 5.7 and 6.0, adjusted with 99% glacial acetic acid (Sigma-Aldrich) containing 10% acetonitrile. Phase B was prepared from 90% HPLC grade methanol and 10% acetonitrile (Merck KGaA). Elution was performed using the gradient program, starting with 100% phase A and 0% phase B, followed by 10 min with 35% phase A at the selected pH and 65% phase B. Twenty minutes after separation of a given sample, the column was cleaned and stabilized. These sets of chromatographic variables allowed the separation of the porphyrin fractions due to clear-cut polarity differences based on the distinct number of porphyrin carboxyl groups. The choice of composition and separation program for the HPLC analysis of urinary porphyrins to attain optimization was guided by varying phase A. This study also intended to promote the separation of COPRO isomers I and III, of enzymatic and non-enzymatic origins, respectively, and 10% acetonitrile was added in both phases to improve the resolution of analyte separation. The retention time, the area of the metabolite peaks and the number of theoretical plates were evaluated for phase A at different pH values.

Bioanalytical validation and statistical analysis

The analytical validation of the method described here complies with the procedures and parameters recommended by the US Food Drug Administration (FDA).[26] The following analytical parameters were evaluated here: specificity, linearity, precision, sensitivity, limit of detection, limit of quantification and accuracy. The calibration curves were determined using spiked urine samples containing six known concentrations of each analyte: 5, 50, 100, 500, 1,000 and 5,000 nmol L^{-1} for URO, HEPTA, HEXA, PENTA, and COPRO I; and 7, 70, 700, 1,700, 3,500, and 7,000 nmol L^{-1} for COPRO III.

The evaluation of the accuracy of the method was used to estimate the analyte recovery parameter, which can be defined as the ability of the method to approach the true analyte concentration in the real sample. Therefore, analyses were performed using three samples, with three different concentrations of each analyte and five replicates of each trial.

The precision of the method was evaluated for each analyte at three different concentrations performed on the same day (intra-analysis precision) and by analysis of the same solutions on five different days (inter-analysis precision).

The sensitivity of the method was assessed by calculating the limits of detection and of quantification. The limit of quantification (LOQ) is defined as the smallest quantity in the sample that can be assayed under experimental conditions with a defined accuracy, with a coefficient of variation (CV) lower than 15%.

Patients studied

The study was approved by the institutional (Unifesp) ethics committee, according to the protocol number 1964/10. Questionnaires were answered by 40 patients with a suspected or confirmed diagnosis of porphyria, referred by the Brazilian Porphyria Association (Associação Brasileira de Porfiria, ABRAPO). A kit for collecting random urine samples was mailed to the patients in a foam box, which contained two sterile, light-protected bottles containing 5 g L^{-1} sodium bicarbonate (Synth). The terms of consent and the collection guidelines were included in the kit. Twenty four urine samples were obtained from patients between ages 17 and 70 years.

Storage and preparation of urine samples

Patient urine samples were collected in the morning, protected from light, kept on ice and delivered to the laboratory on the same day. Upon receipt, the samples were aliquoted and kept frozen at –80 °C until use. Then, 2 mL of each urine sample were thawed and acidified with 70 µL of HCl (Merck KGaA) to obtain a pH of ca. 2.0. After homogenization, the samples were filtered through a 0.22 µm membrane (Millipore Corporation, Billerica, MA, USA) and sonicated (Elma, Ultrasonic Digital S, Manchester, UK) for 20 min. All determinations were performed in parallel with authentic porphyrin samples.

Results and Discussion

The study of the pH effect of eluent A on the separation efficiency by fluorescence detection is summarized in Table 2. Standard samples of 100 nmol L^{-1} URO, HEPTA, HEXA, PENTA or COPRO I and 700 nmol L^{-1} COPRO III were injected into the HPLC equipment. The data presented in Table 2 and Figure 1 indicate that although the pH value

Table 2. Retention times, peak areas and theoretical plates of the HPLC separation of six porphyrins obtained at pH levels varying from 4.0 to 6.0

	pH 4.0		pH 4.3		pH 4.6		pH 4.9		pH 5.2		pH 5.4		pH 5.7		pH 6.0	
	t_R	TP	t_R	TP	t_R	TP	t_R	TP	t_R	TP	t_R	TP	t_R	TP	t_R	TP
URO	2.1	32.8	6.3	298.7	6.4	857.9	3.2	105.3	4.1	111.8	4.4	162.8	2.0	94.1	1.7	60.1
HEPTA	5.0	58.8	9.0	3153.1	9.0	598.5	7.3	1407.6	7.8	477.1	7.9	449.7	5.5	211.6	2.8	72.9
HEXA	9.5	314.4	11.5	847.2	11.4	1071.3	10.2	737.9	10.5	1674.6	10.6	2312.1	8.7	2335.8	7.2	1037.7
PENTA	12.7	2164.5	13.6	1341.7	13.5	1602.9	12.7	1404.0	12.9	2053.4	12.9	4652.3	11.2	5702.0	10.0	2164.8
COPRO I	15.9	809.9	16.1	1658.2	15.8	5803.3	15.1	11606.9	15.1	5801.9	15.1	10958.1	13.5	12718.0	12.4	4185.2
COPRO III			16.5	838.6	16.3	951.3	15.5	1073.1	15.6	5889.7	15.6	1177.0	14.1	1199.3	13.2	4735.5

t_R: retention time; TP: theoretical plates.

of 4.0 provides the largest numbers of theoretical plates, separation of COPRO I and III is not attained. However, by increasing the pH of the chromatographic phase, a good resolution of the chromatographic peaks of COPRO I and III and considerable amounts of theoretical plates were obtained. The best resolution was achieved with ammonium acetate buffer at pH 5.7 as eluent A (Table 2 and Figure 1). Partial separation of COPRO I and III was reached using these conditions and is consistent with the chromatograms obtained from both standard porphyrin solutions and a urine sample obtained from a porphyric patient (Figure 2).

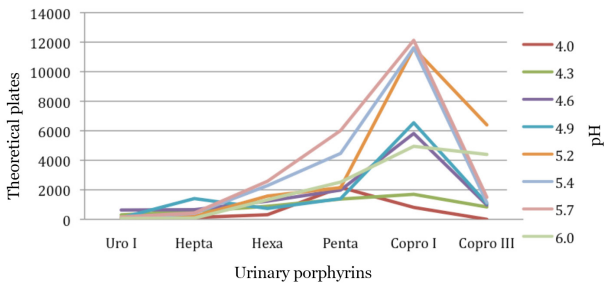

Figure 1. Theoretical plates for six urinary porphyrins upon the variation of pHs values. Porphyrin concentration was 500 nmol L^{-1}.

The gradient started from 0% of eluent B, which made possible to separate the fractions based on their differences in polarity and helped eliminate polar interferences present in the samples. The HPLC separation parameters were also evaluated by spiking acidified urine samples with URO, HEPTA, HEXA, PENTA, COPRO I, and COPRO III standards.

Student *t*-test was used to verify the linearity of deviations from the calibration curves obtained with the analytes. The experimental porphyrin concentration values (T) found by HPLC (n = 6) coincided with the calculated, nominal values with a confidence level of 95%. Calibration curves were constructed and analyzed statistically using a Student *t*-test for URO, HEPTA, HEXA, PENTA, COPRO I

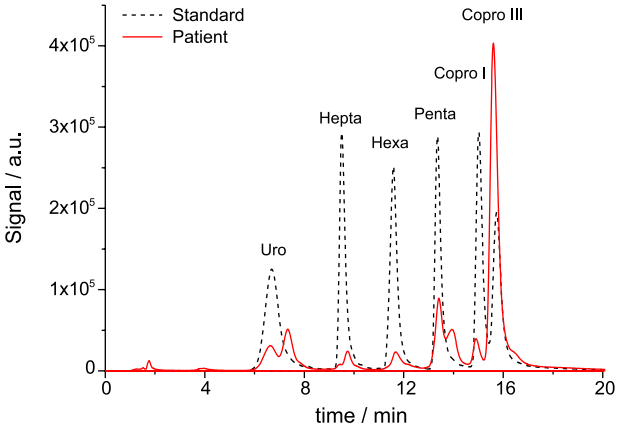

Figure 2. Comparative HPLC/fluorescence traces obtained for a standard mixture of porphyrins (dashed line) and the urine of a porphyric patient (solid line). Eluent A: 1.0 mol L^{-1} ammonium acetate buffer containing 10% acetonitrile at pH 5.7. Eluent B: methanol:acetonitrile (9:1, v/v). Patient 2 was clinically diagnosed as an acute intermittent porphyria carrier. Porphyrin concentration was 500 nmol L^{-1}.

and III at concentrations of 5, 50, 100, 500, 1,000 and 5,000 nmol L^{-1} for URO, HEPTA, HEXA and COPRO I, and 7, 70, 700, 1,700, 3,500 and 7,000 nmol L^{-1} for COPRO III. The equations found were y = 3.29403x + 3625.6; y = 3.07211x + 220841; y = 3.36552x + 118242; y = 3.33461x + 150771; y = 3.71105x + 281962; y = 1.00796x + 837120, respectively. The respective linear correlation coefficients (r^2) were all above 0.99, thus confirming the linearity of the method. The evaluation of the accuracy of the method is displayed in Table 3 and demonstrates satisfactory recovery values for all analytes at all concentrations tested. The recovery percentages range from 85 to 115%, which is required by the FDA.

The precision of the method evaluated based on the intra- and inter-assay analysis for URO, HEPTA, HEXA, PENTA, COPRO I and III determinations are in accordance with the FDA recommendations,[26] all of which exhibited CV below 15%. During the chromatographic analysis of the patients, a mixture containing six diluted metabolites in urine samples from donors was injected

Table 3. Recovery and accuracy of the HPLC method developed to determine URO, HEPTA, HEXA, PENTA, COPRO I and III concentrations in urine

Analyte	Concentration / (nmol L^{-1})	Recovery / %	Intra-assay / (CV%)	Inter-assay / (CV%)
URO	100	108.2	2.4	1.3
	500	105.1	7.6	2.6
	1,000	109.6	3.2	2.7
HEPTA	100	93.9	5.1	2.3
	500	111.2	6.2	1.3
	1,000	92.5	14.1	1.0
HEXA	100	90.9	6.4	3.2
	500	104.5	14.9	2.2
	1,000	104.8	1.9	0.9
PENTA	100	91.0	12.4	2.3
	500	114.5	11.5	0.6
	1,000	103.1	1.3	0.7
COPRO I	100	100.7	5.8	1.0
	500	109.4	7.3	2.1
	1,000	89.5	1.8	2.8
COPRO III	700	112.4	10.4	1.8
	1,700	99.2	12.5	5.1
	3,500	99.8	3.8	1.6

CV: coefficient of variation.

to confirm that the retention times and chromatographic resolution were reproduced, which was indeed observed in all analyses.

The sensitivity of the method was assessed by calculating the limits of detection and quantification expressed in nmol L^{-1} as follows, respectively: URO, 0.62 and 2.05; HEPTA, 0.59 and 1.96; HEXA, 0.54 and 1.81; PENTA, 0.52 and 1.73; COPRO I, 2.03 and 6.77; and COPRO III, 0.43 and 1.44. Although chromatograms demonstrate that there is partial resolution of COPRO I and III, validation data meet the FDA guidelines and can be used to analyze patient urine samples. These data document the usefulness of the method for detecting and quantifying porphyrin metabolites with high sensitivity. Similar results were obtained by other authors using different HPLC methodologies.[27,28] The validated method was then used to analyze random urine samples from 24 patients whose observed porphyrin concentrations are displayed in Table 4.

Healthy individuals displayed a typical urinary porphyrin excretion pattern, with 2-8 times more COPRO III than COPRO I (Table 4). To the best of our knowledge, no reference values for the COPRO porphyrins have been reported. The urinary porphyrin data suggest that patients with an AIP diagnosis (Table 4) exhibit augmented total COPRO urinary levels, even when they are not in crisis. Some of these patients belong to the same family and were clinically confirmed or suspected to be AIP carriers on the basis of symptoms, such as intense abdominal pain, but only patient No. 2 (chromatogram depicted in Figure 2) was clearly under acute crisis. This finding is important because minor changes in metabolic urinary porphyrin patterns might indicate the onset of crisis, allowing preventive medication to avoid further patient suffering.

Patient 14 exhibited symptoms characteristic of both PCT and AIP. As reported in the literature,[18,29] PCT patients exhibit skin blemishes when exposed to sunlight, which is characteristic of PCT, and abdominal pain, which is typical of AIP. Changes were detected in HEPTA, HEXA, PENTA, COPRO I and III.

Patient 15 displayed alterations in HEPTA, HEXA, PENTA, COPRO I and III urinary concentrations and skin lesions, which suggest PCT disorder. Accordingly, his anamnesis registered psychological disturbances that are characteristic of AIP. Moreover, the patient genetic report revealed small changes in some of the enzymes in the heme biosynthetic pathway, such as alterations in activity of the heme biosynthetic enzyme URO III synthase, a deficiency in which massive porphyrin accumulation in blood cells results and is responsible for hemolytic anemia and skin photosensitivity.[30,31]

A reliable diagnosis of PCT via HPLC urinary analysis seems to be more complex because all porphyrin - URO, HEPTA, HEXA, PENTA, or total COPRO - levels do not appear to follow a reproducible pattern, but are altered. These findings also demand further clarification, as minor changes in metabolic urinary porphyrin patterns might denounce the onset of crisis and recommend preventive treatment.

Of the 24 patients referred by ABRAPO who had their samples analyzed in our study, 83.3% exhibited significant changes in one or more metabolites of the heme biosynthetic pathway, suggesting that the biomedical characterization of this rare health condition might actually be methodologically simple. Moreover, we may also consider the exposure of individuals to chemicals, such as polychlorinated biphenyls, hexachlorobenzene, pentachlorophenol, dioxins and heavy metals (arsenic, lead and mercury), which have a well-known risk of inducing porphyria and should obviously be avoided.[32,33] In addition, these factors can cause porphyria in individuals with no documented genetic predisposition as an acquired toxin-induced condition. Public policies adopted in developed countries to educate and monitor people who are at risk of lead poisoning have dramatically lowered instances of neurotoxicity in children, adolescent behavioral problems,

Table 4. Urinary porphyrin concentrations found in urine samples collected from 24 patients diagnosed as porphyria carriers

Porphyrins / (nmol L^{-1})	URO	HEPTA	HEXA	PENTA	COPRO I	COPRO III	COPRO I + III	COPRO I/III ratio	Supposed diagnosis
RV	30.0	7.0	2.0	5.0	NE	NE	110.0	NE	Healthy
P 1	14	5.1	1.1	1.2	16.3	45.4	61.7	0.36	
P 10	4.1	3.3	2.0	2.9	17.4	54.7	72.1	0.32	
P 11	5.4	2.6	1.4	1.8	8.5	59.4	67.9	0.14	
P 21	1.7	0.5	0.4	0.8	10.1	68.5	78.6	0.15	
P 16	2.9	3.9	2.8	3.6	22.9	92.7	115.6	0.25	AIP
P 2	90	83.4	63.8	207.9	94.7	3155.9	3250.6	0.03	
P 13	14.6	16.8	14.8	14.6	34.7	131.5	166.2	0.26	
P 22	5.4	0.7	0.4	1.4	21.4	124.2	145.6	0.17	
P 23	5.4	1.5	0.6	2.4	31.4	164.1	195.5	0.19	
P 4	5.6	3.2	0.6	1.9	30.3	294.7	325	0.10	
P 5	3.6	2.5	0.3	2.1	13.2	160.9	174.1	0.08	
P 6	7.8	5.0	3.0	3.9	28.8	322.3	351.1	0.09	
P 12	21.0	3.5	1.1	1.5	20.3	92.2	112.5	0.22	
P 19	7.4	0.6	1.1	1.4	45.9	209.4	255.3	0.22	
P 17	5.4	4.5	1.6	4.2	18.4	163.0	1814.4	0.11	
P 7	2.7	10.6	10.9	13.0	15.7	55.6	71.3	0.28	PCT
P 8	5.5	5.5	4.7	6.0	23.2	77.3	100.5	0.30	
P 9	0.4	5.4	2.7	4.3	50.1	136.2	186.3	0.37	
P 3	4.4	7.9	1.8	13.9	43.1	616.4	659.5	0.07	
P 24	8,1	5,1	3,3	3,5	17,4	83,4	100,8	0.21	
P 14	11.0	7.4	5.6	6.3	41.9	146.1	188.0	0.29	
P 18	170	2.1	16.5	11.4	24.3	54.6	78.9	0.45	
P 15	16.4	20.9	3.7	6.9	45.2	113.7	158.9	0.40	
P 20	17.1	6.3	1.2	24.3	26.4	347.1	373.5	0.08	

RV: reference values (healthy); P: patient; NE: not established.

and societal economic losses.[34] HPLC fluorescence detection proved to be an efficient and inexpensive (about US$ 20.00 *per* analysis) tool for the quantitative analysis of the six metabolites studied here. Of the examined patients, only one was in AIP crisis, while the remaining twenty-three patients were asymptomatic.

Finally, we emphasize the potential importance of the separation of the two isomers COPRO I and III achieved here because it may help clarify the type of porphyria. In this regard, the COPRO I:COPRO I / (I + III) ratio is important in the diagnosis of Dubin-Johnson syndrome.[35,36] This syndrome is attributed to the mutation of the ABCC2 gene, which is associated with a high excretion rate of COPRO I. This gene encodes the multidrug resistance-associated protein 2 (MRP2), which is involved in the secretion of numerous drugs and endogenous substrates.[37,38] Reference values are available in the literature only as the sum of these two isomers, given that current analyses are not able to separate COPRO I and III, although the distinct enzymatic route that produces COPRO III has already been identified.

Porphyria is classified as a rare disease, and its definition is therefore dependent upon the time period and geographic area being considered.[39] However, patients with rare diseases face the challenges of a lack of scientific knowledge and competent medical professionals that can provide an accurate diagnosis, which prevents the family and patient from identifying the disease, triggering the patient's symptoms in order to prescribe him an appropriate treatment.

In Brazil, ABRAPO was founded on 07/21/2006 to provide support to patients and their families, as well as raise awareness of porphyria patients so that they are better known, diagnosed and treated. According to the Paraná State Decree 15,781 of 02/21/2008, ABRAPO was declared of public interest. The activities and webpage

(www.porfiria.org.br) of this association make it possible to exchange information, guidelines for access to diagnosis, treatment, and integration between physicians and patients from different regions of Brazil. Support, information and guidance are provided via the Internet, telephone and e-mails. In 2011, the average number of monthly e-mails sent to patient's families was 180. These e-mails contained information regarding major medical issues on hematin treatment, the list of banned drugs, and clinical tests for diagnosis, as well as general orientation. For dissemination, information and guidance, study groups, lectures, and patient meetings were also conducted, as well as participation in national and international conferences and other events. Until 12/31/2011, ABRAPO had 292 registered patients, and in May 2012, it went up to 310, which still does not represent the real number of carrier families known by the association. According to the monthly average consultation, ABRAPO hears approximately 06 patients *per* day with requests for information.

ABRAPO includes information for physicians, which has been translated into Portuguese by the American Porphyria Foundation, a result of the Global Porphyria Alliance site. The porphyria guidelines from the Brazilian Medical Association are nearing completion. Based on Google Analytics reports from 2011, the ABRAPO site received 21,413 visits, including 20,402 from Brazil (São Paulo, Rio de Janeiro, Curitiba, Belo Horizonte, Brasília, Salvador, Fortaleza, Campinas, Porto Alegre, and Recife were the 10 cities with the highest number of visits) and 1011 from other countries, with 436 from Portugal. Of these, 16,945 (79.13%) were new visitors and 4,468 (20.87%) were accessing the site again.

These data demonstrate the importance of information on rare maladies, which may have access to specialized professionals, specific medications, contact with other patients and various clarifications. It is difficult to diagnose and treat porphyria. Although not officially, laboratories and hospitals with specialties in porphyria can be found in the Brazilian Universal Health System (public and free system). Hematin is the only drug used to regulate the metabolism of heme during attacks of acute porphyria. This drug is not produced in Brazil, and the request for registration with the National Health Surveillance Agency has not been answered. Its high cost prevents access by patients unless it is required by judicial order. This further complicates the arrival of the drug in a timely manner for the patient. However, in a meeting of the Ministry of Health in Brasília, on April 26, 2011, with various supporting institutions, a working group was formed regarding people with rare diseases in the Brazilian Universal Health System.

Conclusions

The HPLC/fluorescence detection method reported here facilitates the accurate, sensitive and inexpensive separation of six heme precursors as compared to mass spectrometry, for example. This method can be an important tool in public hospitals in continental countries, such as Brazil, to screen for patients and relatives with and without porphyria symptoms. We emphasize that all patients sent samples via regular mail. The diagnosis of AIP carriers is particularly important for anticipating the manifestation of acute crises, which can be fatal, and also to therapeutically monitor patients with chronic porphyria. Moreover, it may help the diagnosis of other diseases that are based on alterations in normal heme production or differential excretion of these isomers, which reportedly occurs, for example, in Dubin-Johnson syndrome and arsenic poisoning.[28]

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