DETERMINATION OF COPROPORPHYRIN I AND III IN HUMAN URINE USING HPLC WITH FLUORESCENCE DETECTION

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The determination of selected porphyrins, especially of coproporphyrin I and III is presently the most reliable direct approach to the screening of porphyrias. An HPLC method for the simultaneous measurement of coproporphyrin I and III in urine samples has been developed and evaluated. Separation of coproporphyrin I and III from interfering substances was achieved on reversed phase HPLC with gradient elution and fluorescence detection. Analytical performance of this method is satisfactory for both coproporphyrin I and coproporphyrin III: the intra-assay and inter-assay coefficients of variation were below 10 %. Quantitative recoveries from spiked urine were between 85.0 and 101.1 %. The limit of detection was 3.0 and 2.3 μg l^{-1}, respectively. The preliminary reference ranges of coproporphyrin I and III in a group of donors are 5.0-51.6 μg l^{-1}

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(0.59-3.20 μg mmol^{-1} creatinine) and 14.9-65.4 μg L^{-1} (0.98-8.21 μg mmol^{-1} creatinine). The presented method is inexpensive and suitable for screening of porphyrias.

Introduction

Porphyrins are cyclic tetrapyrroles derived from porphin by substitution of the peripheral positions with various side-chains (Fig. 1). The measurement of porphyrin levels in urine, blood, feces and tissues is very important for the diagnosis of the porphyrias, diseases due to enzyme deficiencies in the hem biosynthetic pathway [1-4]. It is necessary to point out that abnormal porphyrin metabolism is also found in alcoholics [5], lead and chemical intoxication [6,7], iron deficiency, chronic infection, inflammation and malignancy [8]. A great number of different methods for the measurement of porphyrin levels in biological samples have been described [9]. The most powerful of published methods seems to be HPLC with fluorescence detection [10-15]. On the other hand, only a few methods use HPLC with UV/Vis [16] or electrochemical [17] detection. The porphyrins may be separated by adsorption chromatography as methyl esters [18, 19] or as free acids [20,21] by reversed-phase chromatography. Methods for the determination of methyl esters require using purification steps prior to HPLC analysis. This problem can be avoided by measuring of the porphyrins as free acids [22].

This article describes a sensitive HPLC with fluorescence detection and quantification of coproporphyrin I and III in human urine using very rapid and simple procedure of sample preparation.
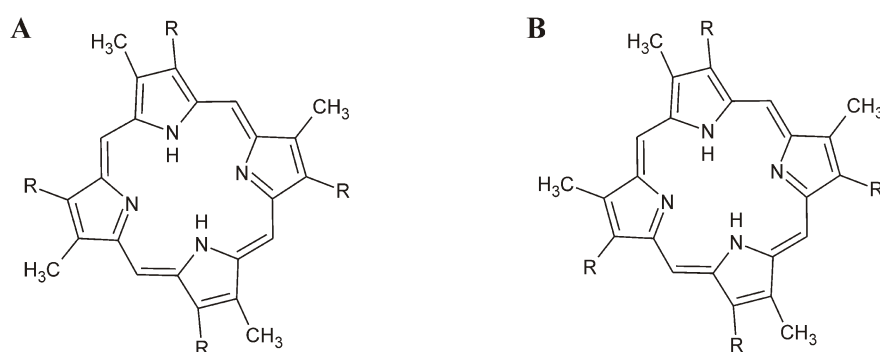

Fig. 1 Structures of coproporphyrin I (A) and coproporphyrin III (B). R: -CH$_2$-CH$_2$-COOH

Materials and Methods

Reagents and Chemicals

Uroporphyrin I dihydrochloride, uroporphyrin III octamethyl ester, coproporphyrin I dihydrochloride, coproporphyrin III tetramethyl ester, hydrochloric acid, sodium dihydrogenphosphate, sodium hydrogenphosphate, sodium acetate, and ammonium acetate were obtained from Sigma Chemical Company (St. Louis, MO, USA). HPLC-gradient grade ethanol, methanol, and acetonitrile were from Merck KgaA (Darmstadt, Germany). Lyophilized urine porphyrins controls, Level I and II, Lot 433, were from Recipe (Munich, Germany). All the other chemicals were of analytical grade.

Since porphyrins are light sensitive, precautions were taken to minimize exposure of standard and urine samples to daylight by using either amber plastic tubes or plastic tubes protected with aluminum foil. Stock solutions of selected porphyrins were prepared in methanol (about 100 mg l^{-1}), evaporated to dryness under nitrogen (Linde Gas, Prague, the Czech Republic), and stored at -80 °C until used.

Instrumentation

The chromatographic analysis was performed with a liquid chromatograph (Shimadzu, Kyoto, Japan), LC-10ADvp solvent delivery system, SIL-10ADvp autosampler, CTO-10ASvp column oven, RF-10Axl fluorescence detector and SCL-10Avp system controller. Data were collected digitally with Clarity chromatography software (DataApex, Prague, the Czech Republic).

Subject and Samples

Samples of urine were obtained from a group of donors ($n = 40$, 20 women in the age 21-41 years, mean age 33 years, and 20 men in the age 21-53 years, mean age 39 years). None of the studied subjects exhibited renal, hepatic, gastrointestinal, pulmonary or oncological diseases. A written informed consent was obtained from all participants before starting the protocol and the Hospital Committee on Human Research (Regional Hospital of Pardubice, the Czech Republic) approved the study.

Sample Preparation

Dry residues of porphyrin stock solutions were dissolved in 200 μl hydrochloric acid (6.0 mol l^{-1}) and incubated at 25 °C for 24 h in dark. The concentrations of individual porphyrins were verified on a Shimadzu UV-1700 PharmaSpec spectrophotometer (Shimadzu, Kyoto, Japan), using molar absorptivities at 406 nm (1 cm path-length) of 4.2×10^5 l mol^{-1} cm^{-1} for uroporphyrins and at 401 nm of 4.7×10^5 l mol^{-1} cm^{-1} for coproporphyrins.

Twenty-four-hour urine samples were protected against daylight. To 500 μl urine sample or standard, 50 μl concentrated hydrochloric acid was carefully added. The mixture was then filtered through a 0.20 μm nylon filter, 4 mm diameter (Supelco, Bellefonte, PA, USA) and transferred into 0.2 ml cramped amber vial.
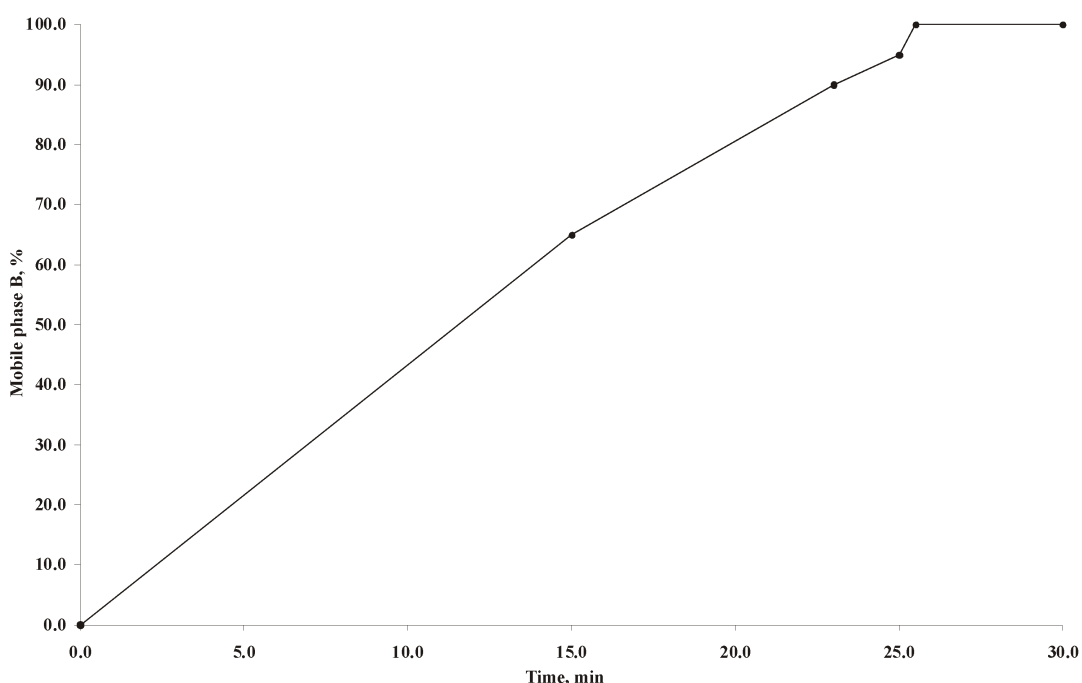

Fig. 2 Gradient of mobile phase: mobile phase B percent *vs.* time (offset 2.2 min to allow for gradient front reaching the mixer of the column). Mobile phase A, mixture of 100 mmol l^{-1} ammonium acetate-methanol (90:10, v/v), pH 6.5 ± 0.1; mobile phase B − 100% methanol

Chromatography Analysis

Selected porphyrins were separated using a LiChroCART 125×4 mm i.d., Purospher STAR RP-18e, 5 μm analytical column fitted a LiChroCART 4×4 mm i.d., Purospher STAR RP-18e, 5 μm guard column (Merck KgaA, Darmstadt, Germany). All separations were performed at 37 °C using the gradient shown in Fig. 2. Mobile phase A was a mixture of 100 mmol l^{-1} ammonium

acetate-methanol (90:10, v/v), pH 6.5 ± 0.1. Mobile phase B was 100 % methanol. Prior to use, both phases were vacuum filtered and degassed using ultrasound. The flow rate was kept constant at 0.5 ml min^{-1}. The optimum response of measured porphyrins was observed when the excitation and emission wavelengths were 394 nm and 624 nm, respectively. The amount of porphyrins was quantified from the corresponding peak area using Clarity chromatography software (DataApex). The concentration of porphyrins in the urine samples was determined from the calibration curve.

Additional Analyses

Creatinine in the urine was measured with the set Creatinine Flex® by standard procedures using an automatic biochemistry analyzer Dimension® RxL Max® (Siemens Healthcare Diagnostic Ltd., Deerfield, IL, USA).

Statistical Analysis

The data are presented as mean values ± *S.D*. Differences between women and men were analyzed with the use of the Mann-Whitney Rank Sum Test and analysis of correlation was carried out using Spearman Rank Order Correlation (software QCexpert, Trilobyte, Pardubice, the Czech Republic and software SigmaStat, Systat Software, Inc., Point Richmond, CA, USA). A $p < 0.05$ value was considered statistically significant.

Results and Discussion

We describe here an HPLC assay for selected porphyrins. This work is focused on describing our efforts at optimizing sample preparation with regard to sample stability and recovery of the analytes. We found that porphyrins are optimally measured by using HPLC with fluorescence detection after acidification with concentrated hydrochloric acid.

Sample Preparation for HPLC of Porphyrins and Stability

A suitable sample preparation technique is essential for accurate analysis of porphyrins. Uroporphyrins and coproporphyrins can easily be measured by direct injection of cleaned urine. Then a guard column has to be used to protect the analytical column. Moreover precipitates, such as calcium salts, tend to adsorb

porphyrins. With a view to dissolve the precipitated material we have added the concentrated hydrochloric acid to urine. Acid treatment also prevents the formation of metalloporphyrins. With regard to very low levels of other porphyrins as are hepta-, hexa-, and pentacarboxyl porphyrins in human urine and the presence of other metabolites with similar structures and properties, it is necessary to use purification steps prior to their determination. These steps have been based on either SPE or liquid-liquid extraction. This techniques, however, does not give good recovery of porphyrins [19,22,23]. The stability of the selected porphyrins was studied under various conditions during 12 hours. We found out that uroporphyrins and coproporphyrins in the samples of acidified urine were stable on a cooled autosampler (4 °C, dark) for at least 12 hours.
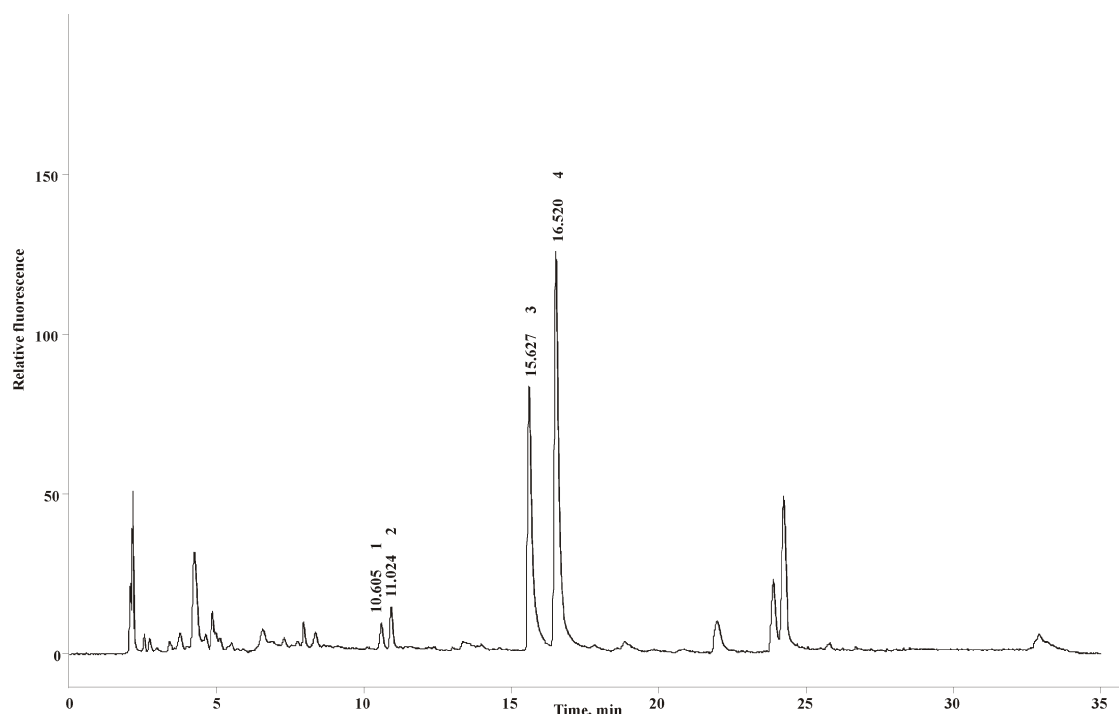

Fig. 3 An HPLC chromatogram of uroporphyrin I (2.4 μg l^{-1}), uroporphyrin III (3.3 μg l^{-1}), coproporphyrin I (40.1 μg l^{-1}) and coproporphyrin III (58.4 μg l^{-1}) in human urine. Peaks: (1) uroporphyrin I, (2) uroporphyrin III, (3) coproporphyrin I and (4) coproporphyrin III. HPLC conditions: a gradient elution [mobile phase A: mixture of 100 mmol l^{-1} ammonium acetate-methanol (90:10, v/v), pH 6.5 ± 0.1; mobile phase B: 100 % methanol]; stationary phases were a column LiChroCART 125×4 mm i.d., Purospher STAR RP-18e, 5 μm and a guard column LiChroCART 4×4 mm i.d., Purosper STAR RP-18e, 5 μm. The flow rate was kept constant at 0.5 ml min^{-1}, separation ran at 37 °C and porphyrins were monitored at excitation and emission wavelengths of 394 nm and 624 nm, respectively

HPLC Analysis of Coproporphyrin I and III

An HPLC chromatogram of selected porphyrins in a urine sample is shown in Fig. 3. The analytical parameters of coproporphyrin I and III analysis are shown in Table I and II. To determine the within-day precision, the urine sample was analyzed 10 times on the same day under the same conditions. Similarly, the between-day precision obtained on the same urine sample was analyzed on 10 different days. Precision and recovery studies were carried out using normal adult urine for coproporphyrin I and III, aliquoted and stored at –20 °C. Sample of this urine was spiked with coproporphyrin I and III to obtain levels of these porphyrins in pathological range. Coefficients of variation were below 10 %. The spike recoveries ranged between 85.0 and 101.1 %. The calibration curve (13-point for a determination of analytical parameters and 8-point for a routine analysis was linear in the whole range tested: 5.0-500.0 μg l^{-1} (Fig. 4 and 5). The

Table I   Precision and recovery of coproporphyrin I for urine samples analysis. *Mean of triplicate assays is recorded

A) Precision (within-day)		
n	Mean ± *S.D.* μg l^{-1}	*CV* %
10	44.1 ± 2.1	4.8
10	214.5 ± 4.9	2.3
B) Precision (between-day)		
n	Mean ± S.D. μg l^{-1}	*CV* %
10	46.8 ± 3.4	7.3
C) Recovery		
Added, μg l^{-1}	Observed μg l^{-1}*	Recovery %
0.0	44.3 ± 2.0	
12.7	55.1 ± 2.1	85.0
63.7	100.5 ± 3.6	88.2
127.0	163.6 ± 5.6	93.9
317.5	365.3 ± 11.3	101.1
		Mean 92.1 ± 7.1
		CV 7.7

calibration curve values obtained from the combination of five standard curves are shown in Table III. The lowest concentration that can be quantified with acceptable accuracy and precision was 5.0 μg l^{-1} for both coproporphyrin I and coproporphyrin III. Furthermore, the limit of detection for coproporphyrin I and III, defined as a signal-to-noise (*S/N*) ratio of 3:1, was 3.0 and 2.3 μg l^{-1}.

Porphyrins were separated on a reverse-phase column using a gradient system of methanol and ammonium acetate. The mobile phase was optimized in order to obtain the best separation of the analytes in the shortest time. Standard solutions of porphyrins as well pooled urine were used for a study of the mobile

Table II   Precision and recovery of coproporphyrin III for urine samples analysis. *Mean of triplicate assays is recorded

A) Precision (within-day)		
n	Mean ± *S.D.* μg l^{-1}	*CV* %
10	48.2 ± 2.9	6
10	252.2 ± 6.8	2.7
B) Precision (between-day)		
n	Mean ± *S.D.* μg l^{-1}	*CV* %
10	51.5 ± 4.0	7.8
C) Recovery		
Added, μg l^{-1}	Observed μg l^{-1}*	Recovery %
0	51.4 ± 2.2	
11.3	61.3 ± 2.6	87.6
56.5	104.3 ± 4.1	93.6
113	160.2 ± 5.8	96.3
282.5	337.0 ± 9.8	101.1
		mean 94.7 ± 5.6
		CV 5.9

phase composition and a gradient program. Several mobile phases (namely phosphate and acetate buffers containing methanol, ethanol and acetonitrile) and several gradients were assayed and the best results were obtained for the conditions described in the experimental. The retention behavior was studied in

Table III   Calibration curve values for the HPLC method. *Thirteen-point for a determination of analytical parameters and eight-point for a routine analysis

Standard	Regression equation	Mean slope 95 % confidence interval	Intercept[**] μmol l^{-1} 95 % confidence interval	Correlation coefficient
COP-I*	$y = 20.535\ x - 19.488$	20.535 (20.155 - 20.915)	0.9 (0.5-1.1)	0.9998
COP-III*	$y = 22.201\ x - 16.341$	22.201 (21.877-22.525)	0,7 (0.4-1.0)	0.9999

[**] x-intercept (μmol l^{-1}) is point at which the line crosses x-axis (where y value equals 0)
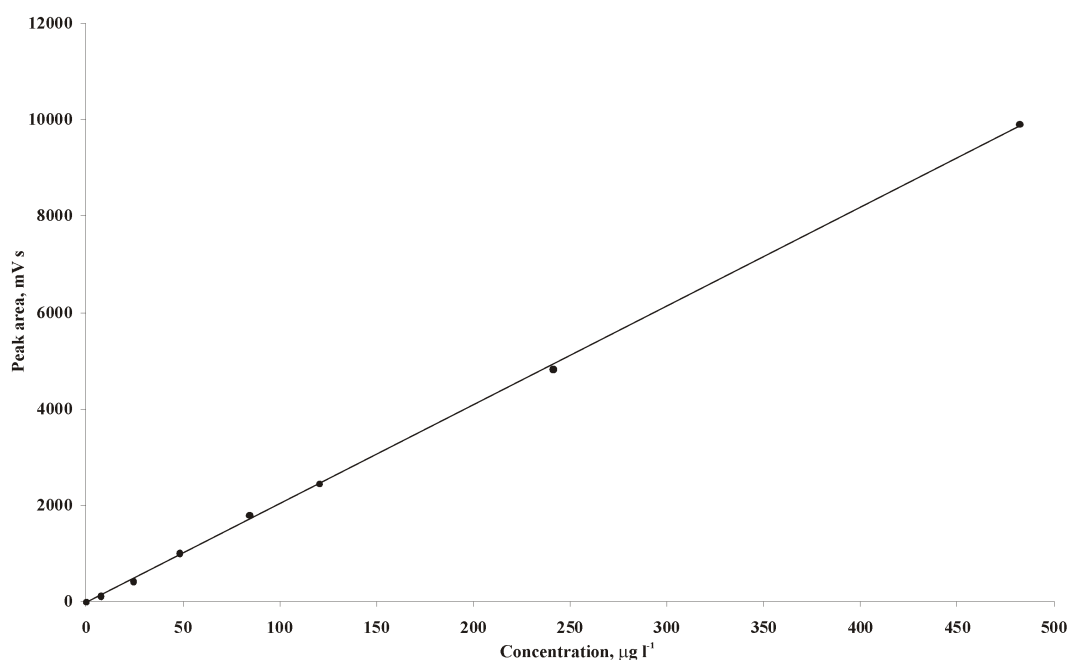

Fig. 4   Typical standard curve for HPLC quantification of coproporphyrin I Regression equation: $y = 20.535x - 19.488$; $R = 0.9998$; $A = 20.535$ (*S.D.* = 0.155); $B = -19.488$ (*S.D.* = 30.803)

dependence of pH value of the mobile phase A in the range of 4.0-7.0. The optimal pH 6.5 was chosen for the best separation and detection of selected porphyrins. Column temperature was adjusted from 25 to 45 °C. Optimal temperature interval was from 35 to 40 °C. The criteria were the resolution, the stability of the fluorescence intensity and the analysis speed. Pursuant to records, we can establish that presented method for the determination of coproporphyrin I and III in human urine is highly robust.
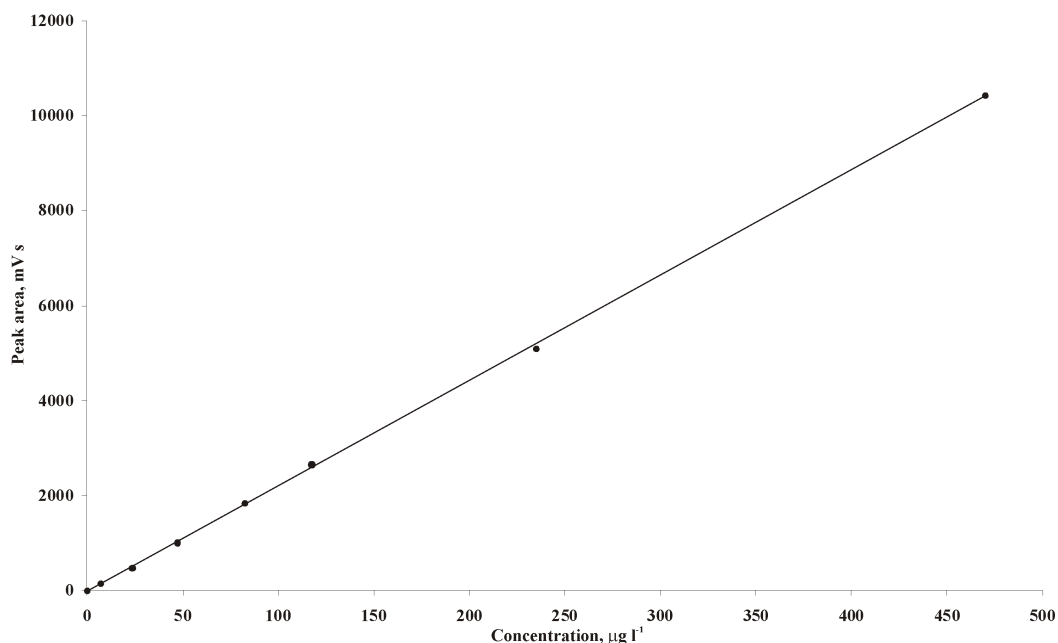
Fig. 5   Typical standard curve for HPLC quantification of coproporphyrin III .Regression equation: $y = 22.201x - 16.341$; $R = 0.9999$; $A = 22.201$ (*S.D.* $= 0.132$); $B = -16.341$ (*S.D.* $= 25.568$)

Determination of Coproporphyrin I and III in Human Urine of Donors

The preliminary reference ranges of coproporphyrins I and III in a group of donors are 5.0-51.6 µg l^{-1} (0.59-3.20 µg mmol^{-1} creatinine) and 14.9-65.4 µg l^{-1} (0.98-8.21 µg mmol^{-1} creatinine). Our normal reference intervals for urine coproporphyrins are similar to those of others [20,24,25]. We found no significant differences in both coproporphyrin I and coproporphyrin III concentration between women and men (0.95 ± 0.27 µg mmol^{-1} creatinine *vs.* 1.02 ± 0.23 µg mmol^{-1} creatinine, $p = 0.389$ and 1.82 ± 0.83 µg mmol^{-1} creatinine *vs.* 1.91 ± 0.89 µg mmol^{-1} of creatinine, $p = 0.411$, respectively). We observed no significant correlation between age and concentration ($R = 0.142$, $p = 0.695$).

As coproporphyrins predominate both in normals and patients with porphyrias (especially in acute intermittent porphyria, porphyria cutanea tarda, porphyria variegata, hereditary coproporphyria, and erythropoetic protoporphyria) [24], we have measured only them. In contrast to uroporphyrin I and III, coproporphyrin I and III were identified in all donors.

Conclusion

We developed a rapid, simple, and very selective HPLC method with fluorescence detection for the determination of coproporphyrin I and III in human urine. This method provides an inexpensive alternative to those using time-consuming purification steps prior to HPLC analysis. It affords excellent sensitivity, precision, and accuracy in follow-up samples and is suitable for both research and clinical trials, especially within porphyrias screening.

Abbreviations used: SPE – solid phase extraction

Acknowledgements

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