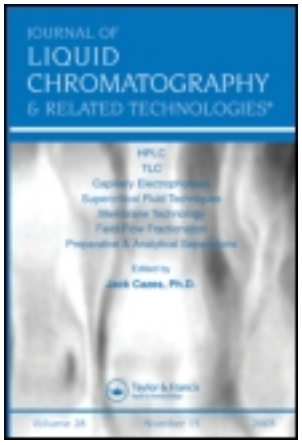

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ANALYSIS OF URINARY PORPHYRINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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□ *A high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS) method was developed for the simultaneous separation and detection of six porphyrins, namely uroporphyrin (UP), heptacarboxylic acid porphyrin (HEPTAP), hexacarboxylic acid porphyrin (HEXAP), pentacarboxylic acid porphyrin (PENTAP), coproporphyrin (CP), and mesoporphyrin (MP), in human urine samples. The recoveries of the porphyrins in urine ranged from 84 to 108%. The intra- and inter-day precisions at spiked porphyrin concentrations were 4.0–9.7% and 5.5–15%, respectively. The limits of detection (LOD) ranged from 0.2 to 3 nM. The developed method was successfully applied to the determination of endogenous porphyrins in human urine. UP, HEPTAP, and PENTAP were detected at levels of 42.4, 21.3, and 18.0 nM, respectively. CP was detected as the predominant species at 135.6 nM, wheres MP and HEXAP were not detected in the urine samples from healthy females. Significant increases of CP excretion in urine from liver disease patients were observed. This method proved potentially applicable for the routine diagnosis and treatment of porphyrin-related diseases.*

Keywords electrospray ionization, high performance liquid chromatography, human urine, mass spectrometry, porphyrins, solid-phase extraction

INTRODUCTION

Porphyrins are a ubiquitous class of naturally occurring molecules involved in a wide variety of important biological processes. The compounds are regularly and prominently investigated in all known macrocyclic ring

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systems.[1–4] Various porphyrins and related molecules are derived from the porphine framework, which contains four pyrrolic subunits linked via four methyne bridges.[2] Excessive production, accumulation, and excretion of porphyrins often result from metabolic aberration. Associated with abnormal heme biosynthesis, many diseases could be caused including porphyrias,[5] iron deficiency anemia,[6] liver disease,[7] and intoxication due to the overexposure to heavy metals,[8] environmental pollutants[9], and alcohols[10]. Therefore, monitoring the composition and contents of certain porphyrins and establishing various porphyrin metabolic patterns might provide the references for the differentiation and diagnosis of porphyrin-related diseases. On the other hand, due to the photoactivity and low dark toxicity, porphyrins and their derivatives have been successfully used as photosensitizers in photodynamic therapy treatments for malignant diseases.[11] Additionally, certain porphyrins have been reported to bring cleavage of DNA, both chemically and photochemically.[12] Additionally, based on the special molecular structures, porphyrin-based compounds are of interest in molecular electronics and supramolecular building blocks.[13,14] The involvement of porphyrins in many biological processes and the possibility to tailor their physical and chemical properties at the molecular level make porphyrins extremely versatile synthetic base materials for research projects in many disciplines of chemistry and physics.[15–18] Thus, great interest in porphyrins based on their multiple biological functions have led to broad and further investigation of porphyrins in biological, biomedical, and clinical fields.

The prevalence of porphyrins in nature at trace levels and their wide applications have promoted the fast development of reliable analytical methods and techniques for the separation and detection. In the early years, solvent fractionation methods were usually performed,[19,20] which were laborious and imprecise in the separation of porphyrins. More recently, a number of separation methods have been developed and applied for the analysis of porphyrins in biological samples, including gas chromatography (GC),[21] flow-injection analysis (FIA),[22] thin-layer chromatography (TLC),[23,24] high performance liquid chromatography (HPLC),[25–27] and capillary electrophoresis (CE).[28,29] HPLC has obtained considerable attention for the porphyrin separation[30] with various detection techniques including immunoassay,[31] inductive coupled plasma-atomic emission spectrum (ICP-AES),[32] electrochemical mode,[33,34] and the conventional ultraviolet visible (UV-Vis) detector.[25,35,36] More sensitive detection such as fluorometry[37–39] and mass spectrometry (MS)[27,40] have also been reported. The analysis of porphyrins is further facilitated with an accurate and rapid identification due to the advancements in MS technology,[27,40,41] particularly with the introduction of electrospray ionization (ESI) technique.[40,42,43]

In this work, an HPLC-ESI-MS method for the simultaneous analysis of six urinary porphyrins was presented. Solid-phase extraction (SPE) was applied to extract and clean up the porphyrins from human urine. Different metabolic patterns of porphyrins were established, in human urines collected from healthy persons and patients suffered from liver disease.

EXPERIMENTAL

Chemicals and Materials

A chromatographic marker kit containing 10 nmole of each of mesoporphyrin (MP), coproporphyrin (CP), pentacarboxylic acid porphyrin (PENTAP), hexacarboxylic acid porphyrin (HEXAP), heptacarboxylic acid porphyrin (HEPTAP), and uroporphyrin (UP) was purchased from Porphyrin Products (part No. CMK–1A; Logan, UT, USA). MP dihydrochloride was obtained from the same source. The structures of the porphyrins are shown in Figure 1. Formic acid, acetic acid, ammonium acetate, and ammonia, of analytical grade, were all purchased from Baker (Deventer, Netherlands). HPLC-grade acetonitrile (ACN) was purchased from Tedia (OH, USA). Water used was produced by a Milli-Q Ultrapure water system with the water outlet operating at 18.2 MΩ (Millipore, Bedford, MA, USA).

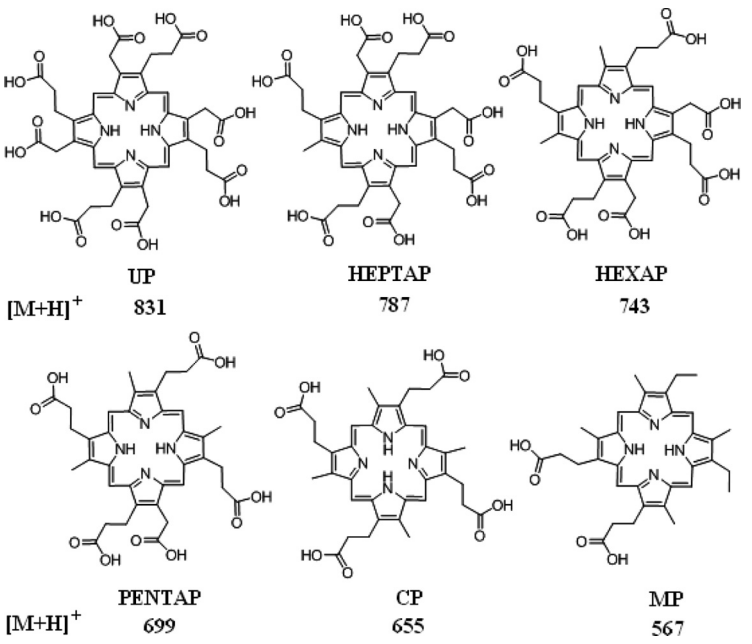

FIGURE 1 Chemical structures and values of $[M+H]^+$ ions of the targeted urinary porphyrins.

Apparatus and Conditions

An HPLC instrument (Agilent HP 1100 series) with diode array detector (DAD) was coupled with an ion-trap mass spectrometer (Bruker Esquire 4000) equipped with an ESI interface. UV-Vis detection wavelength was set at 400 nm. HPLC separation was performed by injecting 10 μL samples onto a C_8 column (Symmetry Shield RP8, 3.5 μm, 2.1 × 150 mm Column, Waters, USA). A mobile phase system consisted of two components, namely solvent A (0.1% v/v formic acid in 5 mM ammonium acetate) and solvent B (0.1% v/v formic acid in ACN). A gradient program was started from 30% B and held for 5 min, then increased linearly to 90% B within 15 min, and held for another 10 min before re-conditioning, at a flow rate of 0.20 mL min^{-1}. The effluent from the first 5 min from the LC system was diverted to waste to minimize the contamination of the ESI source.

ESI-MS analysis was performed on an Esquire 4000 ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany) with an electrospray ionization (ESI) interface. The mass spectrometer was operated in the positive ion detection mode. Nitrogen at 30 psi and 8 L min^{-1} was used as the nebulizer and drying gas, respectively. The MS inlet heated capillary temperature was set at 350°C and the entrance capillary electrospray voltage was positive 4 kV. Mass spectra were obtained by acquiring data from m/z 500–900. Tandem mass spectrometry (MS/MS) was performed in the range of m/z 400–900 with the fragment gain coefficients set at 0.65, 0.65, 0.60, 0.80, 0.85, and 0.85 for UP, HEPTAP, HEXAP, PENTAP, CP, and MP, respectively.

Preparation of Standard Sample Solutions and Mobile Phases

Stock solutions of six porphyrin standards were prepared by dissolving one vial of standard mixture in 1.0 mL of 2 M formic acid. Sample solutions were prepared by diluting the stock solutions with appropriate amounts of 2 M formic acid. A 10-μL aliquot of the sample solutions was injected for the LC-MS analyses. Stock and sample solutions were stored at 4°C in darkness prior to the analysis. Solvent A and B were both filtered through 0.45 μm membranes followed by degassing using ultrasonication for 20 min. All aqueous solutions were prepared in Milli-Q water.

Urine Sample Preparation

The 24 hr pooled urine samples were collected. The urine was kept in the dark at 4°C immediately after collection and stored at −20°C until analysis. For spiked urines, appropriate amounts of porphyrin standards were added into 4 mL of urines after filtered through 0.45 μm membranes.

The urines were vortex-mixed for 15 s with ACN (1:2, v/v) and centrifuged at 12,000 rpm at 4°C for 5 min. The supernatants were subjected to SPE using a C18 Sep-Pak cartridge (360 mg, Waters) connected to a vacuum manifold. The cartridge was conditioned and equilibrated with 4 mL of ACN, 4 mL of water and 4 mL of formic acid solution (0.1% v/v). The urines (4 mL) were then loaded onto the cartridge and washed sequentially with 4 mL of water, 4 mL of formic acid solution (0.1% v/v), 4 mL of ACN/water (5:95, v/v), and 3 mL of ACN. The extracted solution was concentrated nearly to dryness under nitrogen flow (Zymark). The residues were redissolved in 100 μL of 2 M formic acid and 10 μL aliquot was injected directly into the chromatographic system. Urines obtained from the volunteers suffered from proven liver disease without porphyria were treated as described a previous section.

The study subjects included 42 healthy persons and 22 patients suffered from common liver diseases, respectively. The healthy volunteer subjects comprised 21 men (age 24 to 64 with the mean of 38) and 21 women (age 28 to 65 with the mean of 36). None of them had known or suspected porphyria, liver, pancreas, or kidney disease. The routine liver function tests were all normal and the hepatitis virus tests were seronegative. The volunteers were informed about the objectives of the study and they gave no concern for participation in the study. The patients including 12 men (age 38 to 62 with the mean of 46) and 10 female patients (age 36 to 65 with the mean of 48) were seen in the Department of Surgery of Shenzhen Nanshan People's Hospital and the Department of Heptic Disease of Yantai Yuhuangding Hospital (China). None was related to persons with known or suspected liver diseases. The patients were all suffered from common liver diseases confirmed by the liver biopsy pathology, as well as in accordance with clinical observations and laboratory examinations.

Recovery, Quantitation and Linearity Studies

The recoveries were calculated by comparing the peak areas obtained from the spiked urine with those obtained by direct HPLC injection of pure standard solutions at the same concentrations; they were determined at three different concentrations of each porphyrin. Calibration curves of UP, HEPTAP, HEXAP, PENTAP, and CP determined from urine were obtained according to the peak area ratio of analyte to MP (200 nM) as an internal standard (IS). For the curve of MP, CP (200 nM) was used as an IS. IS was added to the filtered urine sample through 0.45 μm membranes. The calibration curve was conducted by plotting peak area ratio of analyte to IS against injected amounts of analyte. The intra- and inter-day precision and accuracy of the method were evaluated by performing several

replicate determinations of each porphyrin spiked in urine against a calibration curve. To determine intra-day precision and accuracy, we performed the replicate analyses ($n = 11$) at three concentrations on the same day. The procedure was repeated on different days ($n = 11$) at the same concentrations within a period of four weeks to determine the inter-day precision and accuracy. The accuracy was expressed as the percentage recovery and the precision was given by the intra- and inter-day relative standard deviations (RSD). The limit of detection (LOD) was determined at a signal-to-noise ratio of 3.

RESULTS AND DISCUSSION

LC-MS Analysis of Porphyrin Standards

A full-scan MS analysis of the porphyrin standard solution dissolved in 2 M formic acid displayed the dominant molecular ions $[M+H]^+$ for all six porphyrins (Figure 2). The porphyrin molecules are easily protonated in acidic solutions,[27,40,44] due to the occurrence of two imino nitrogen atoms. The porphyrins containing 2, 4, to 8 carboxylic groups were well separated chromatographically (Figure 3a and 3b). The gradient solvent program allowed the sequent elution of UP, HEPTAP HEXAP, PENTAP, CP, and MP within 30 min. Similar UV absorbance (Figure 3a) and significantly different ESI-MS intensity (Figure 3b) were observed for the

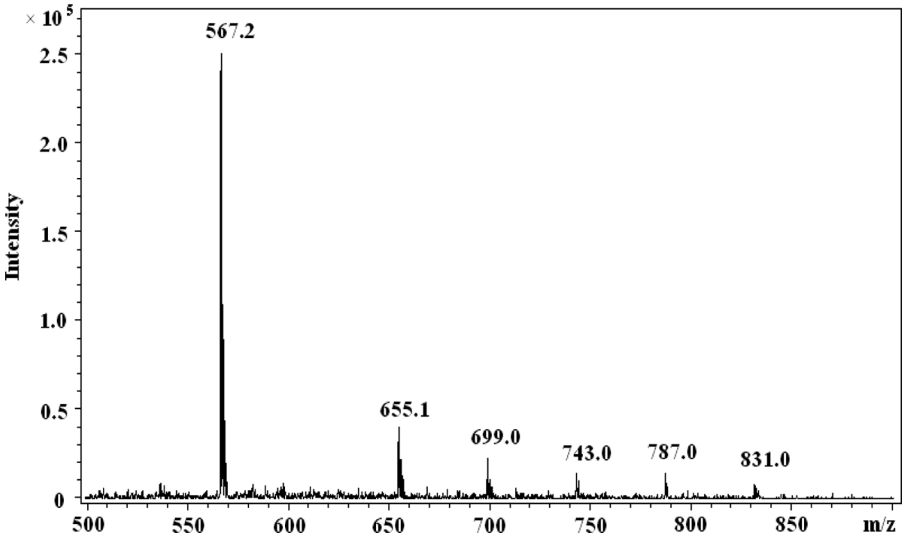

FIGURE 2 ESI-MS determination of $[M+H]^+$ ions of the urinary porphyrins. UP (m/z 831), HEPTAP (m/z 787), HEXAP (m/z 743), PENTAP (m/z 699), CP (m/z 655), and MP (m/z 567).

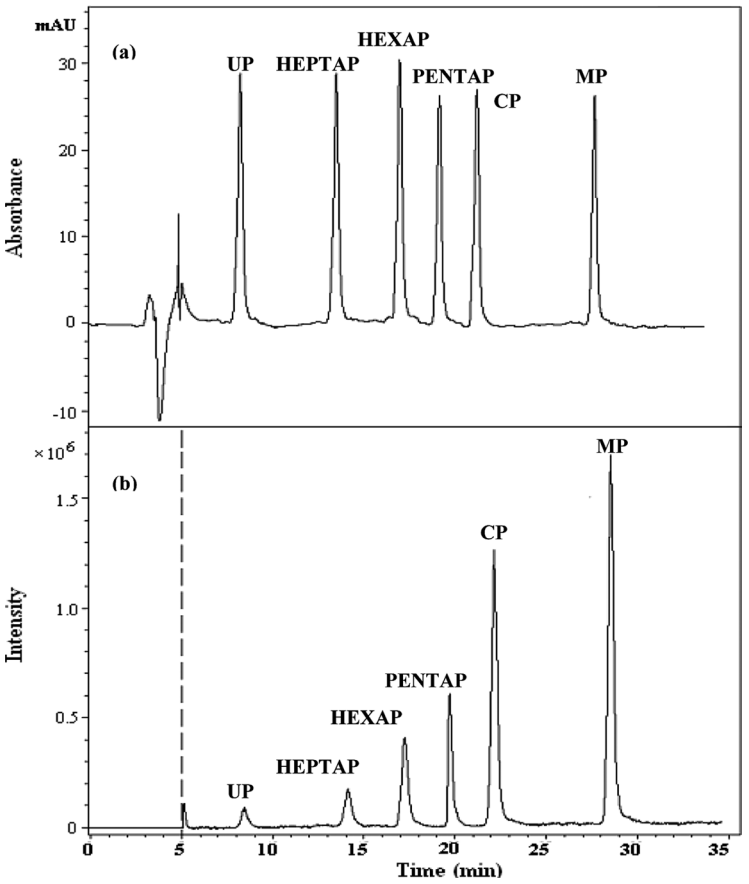

FIGURE 3 LC-MS chromatograms of 0.5 μmol L^{-1} standard porphyrins with the detections of (a) UV and (b) MS.

porphyrins. During the gradient elution, the apolarity of the mobile phase increased with increasing the content of organic solvent. In the apolar mobile phase as nebulizing solvents, the ionization efficiency of more polar UP was much lower than that for apolar porphyrins under the same ESI-MS conditions. The following investigation also indicated that ESI-MS provided substantially better sensitivity than UV for the detection of porphyrins.

Notably, ammonium acetate in solvent A was used as buffer to supply appropriate ion strength and support LC separation and MS detection. The related justification has been demonstrated in our previous work.[44] Briefly, better analytical performance could be obtained when ammonium acetate was used as buffer than ammonium formate (data not shown). The variation of the MS intensity was also affected by the concentrations of ammonium acetate. Better sensitivity was obtained at lower buffer concentrations, due to the low ion suppression in ESI.

The detection of the porphyrins was confirmed from the MS/MS analysis of the respective $[M+H]^+$ ions. An intensive product ion of UP was observed at m/z 767 resulted from the loss of carboxylic group and water molecule. For HEPTAP, HEXAP and PENTAP, significant signals of product ions were seen from the loss of an acetyl group and a carboxylic group (i.e., loss of 104 Da) from their precursor ions. The MS/MS fragmentation pattern of CP was dominated by the ion of m/z 596 with the loss of acetyl group or 59 Da. MP gave a significant product ion at m/z 479 from the loss of a propionyl group and a methyl group. The m/z values of the precursor ions and product ions obtained from the analyses of the porphyrins are listed in Table 1.

Method Performance

Direct sample injection analysis of biological fluids without sample pretreatment is often not feasible for LC-MS analysis, especially considering that nonvolatile salt ions may impair ESI-MS analysis and decrease sensitivity. Solid-phase extraction (SPE) has been proved to possess various advantages for the pretreatment of complex sample matrices[45]. By using the C18 SPE procedure, the interfering substances in the urine samples were removed and the porphyrins were retained on the cartridge and then eluted with 95% ACN. The recovery test on the SPE was carried out by spiking known amounts of porphyrin standards into 4 mL of human urine collected from healthy persons. Recoveries of the porphyrins measured with the porphyrins added at low, medium, and high concentrations ranged from 84 ± 7.3 to $108 \pm 5.3\%$ (Table 2).

Calibration curves were obtained from the analysis of the spiked urine matrix with porphyrin standards at various concentration ranges. Table 3 lists the regression equations, correlation coefficients (R^2), linear range, and LODs for the porphyrins. Good linearity was obtained with the calibration range over three orders of magnitude for HEXAP, PENTAP, CP, and MP. The precision for each calibration point was less than 3.2% ($n = 6$). The LODs of the porphyrins in the 4-mL urine samples were

TABLE 1 LC-MS/MS Precursor Ions and Product Ions of the Urinary Porphyrins

Porphyrins	Precursor Ion $[M+H]^+$ (m/z)	Major Product Ions (m/z)	Other Product Ions (m/z)
UP	831	767	813, 785, 767, 749, 727, 667
HEPTAP	787	683	769, 741, 728, 656, 622, 577
HEXAP	743	639	725, 683, 625, 579
PENTAP	699	595	639, 581, 566, 522
CP	655	596	637, 582, 551, 537, 523
MP	567	479	549, 538, 507, 493, 449

TABLE 2 Method Recoveries and Precisions for the Determination of Porphyrins in Spiked Healthy Human Urine Samples ($n = 8$)

Porphyrins	Added (nM)	Recovery (%)	RSD[a] (%)
UP	6	88	8.2
	100	95	3.2
	400	96	2.3
HEPTAP	5	84	7.3
	100	92	5.9
	400	94	2.4
HEXAP	3	84	8.2
	100	101	6.3
	400	89	4.7
PENTAP	3	85	4.2
	100	103	2.6
	400	96	2.3
CP	0.4	108	5.3
	100	93	3.3
	400	104	2.0
MP	0.3	93	2.2
	100	96	2.1
	400	102	1.7

[a]Relative standard deviation.

0.2–3 nM for the LC-MS analysis. LODs obtained from the LC-UV analysis were 31 nM for UP, 28 nM for HEPTAP, 24 nM for HEXAP, 21 nM for PENTAP, 17 nM for CP, and 16 nM for MP, which were approximately 10–86 times higher than those of LC-MS.

The method precision and accuracy were determined by analyzing spiked urine samples at three different concentrations of porphyrins. The intra-day precisions were less than 8.6, 7.5, 9.7, 9.7, 7.7, and 8.4% for UP, HEPTAP, HEXAP, PENTAP, CP, and MP, respectively. Within a period of four weeks, the inter-day precisions were less than 15, 15, 15, 14, 12, and 10%, respectively. The method accuracy was in the range 92–107% and 91–105%, respectively (Table 4).

TABLE 3 Regression Equations and Detection Limits for the Porphyrins from Healthy Human Urine

Porphyrins	Equation[a]	R^2	Linear Range (nM)	LOD (nM)
UP	$y = 0.00042x + 0.0039$	0.9933	5–500	3
HEPTAP	$y = 0.00093x + 0.0059$	0.9953	4–500	3
HEXAP	$y = 0.00054x + 0.0014$	0.9961	2–500	1
PENTAP	$y = 0.00081x + 0.0016$	0.9946	2–500	1
CP	$y = 0.0087x + 0.0085$	0.9973	0.3–500	0.2
MP	$y = 0.012x + 0.047$	0.9982	0.3–500	0.2

[a]The data were subjected to linear analysis of peak area ratios (y) of analyte to IS against the spiking analyte concentrations (x).

TABLE 4 Method Precision and Accuracy for Porphyrins Determination in Spiked Healthy Human Urine ($n = 11$)

		Intra-Day		Inter-Day	
Porphyrins	Added (nM)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
UP	6	8.6	92	15.0	91
	100	4.5	98	11.0	97
	400	4.0	98	9.9	102
HEPTAP	5	7.5	95	15.0	99
	100	4.2	101	13.0	96
	400	5.8	97	8.5	105
HEXAP	3	9.7	94	15.0	92
	100	7.8	98	11.0	103
	400	5.1	107	7.4	104
PENTAP	3	9.7	95	14.0	94
	100	6.9	99	9.6	105
	400	4.2	102	6.9	99
CP	0.4	7.7	95	12.0	101
	100	5.4	103	9.3	99
	400	5.3	98	6.5	97
MP	0.3	8.4	96	9.9	93
	100	4.3	98	6.1	105
	400	4.0	99	5.5	102

Determination of Porphyrins in Human Urine

The method was applied for the determination of endogenous porphyrins in human urine. The urine samples from 42 healthy adults were analyzed. Typical extracted ion chromatograms (EIC) obtained from analysis of a healthy human urine sample extracts are shown in Figure 4. MP (200 nM) as IS was added to the samples because it was not present in human urine.[27–29,40] The peaks of UP, HEPTAP, PENTAP, and CP in the human urine samples were identified with the detection of the corresponding molecular ions and with the comparison of chromatographic retention times with those of the authentic standards shown in Figure 3. HEXAP was not detected in the urine samples from the healthy human. Other porphyrins were quantified by using the internal standard method. Each urine sample was analyzed six times and the averaged results are listed in Table 5. The measured concentrations of porphyrins represented the normal levels of porphyrins in human urine because the healthy subjects were not treated with or exposed to any drugs or chemicals before the urine samples were collected. No significant differences in the urinary porphyrin excretion levels were observed between the healthy female and male subjects (Table 5). LC-UV determination gave similar results for CP and UP. The concentrations of CP in the healthy human urine samples were 122 ± 18.3 nM for female and 111 ± 15.4 nM for male, which was less

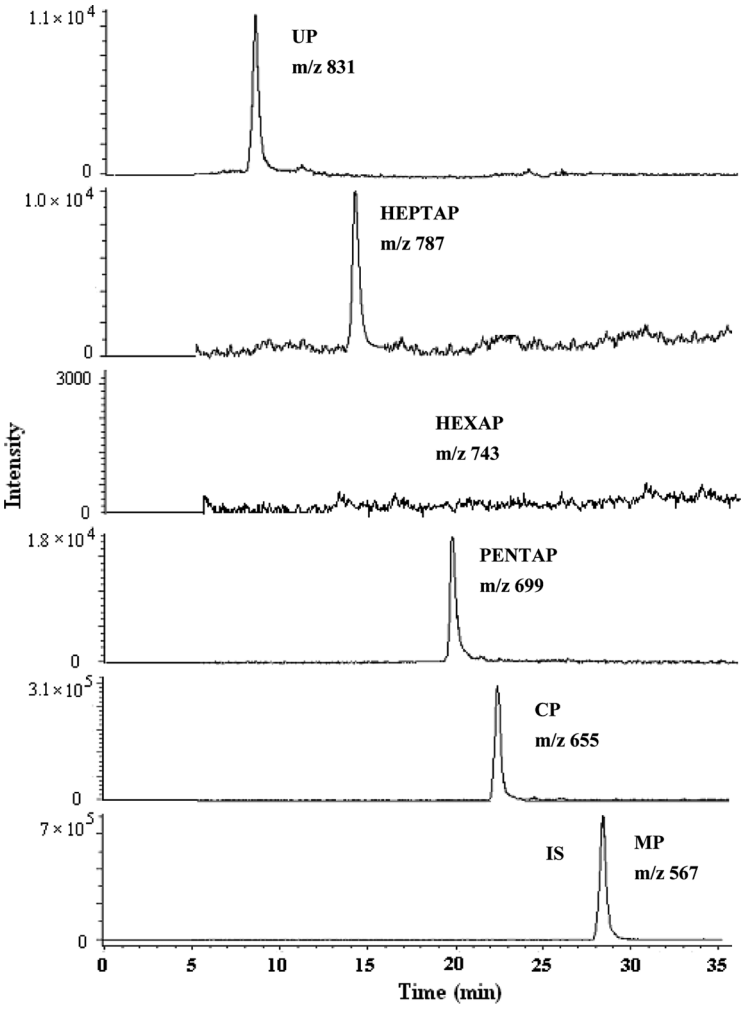

FIGURE 4 Extracted ion chromatograms for the porphyrins in urine extract from a healthy human. MP was used as internal standard (200 nM).

TABLE 5 Porphyrin Concentrations (nM) in Human Urine Determined by LC-MS

Urine source	Number	MP	CP	PENTAP	HEXAP	HEPTAP	UP
Healthy female	21	ND[a]	135.6 ± 20.3	18.0 ± 3.3	ND	21.3 ± 6.4	42.4 ± 11.1
Healthy male	21	ND	126.4 ± 22.5	16.6 ± 4.2	ND	25.4 ± 5.3	40.6 ± 13.4
Liver female	10	ND	384 ± 76.5	51.6 ± 8.5	19.6 ± 6.4	35.6 ± 8.9	38.6 ± 10.4
Liver male	12	ND	367 ± 80.5	59.2 ± 9.8	18.8 ± 5.2	40.5 ± 6.4	41.5 ± 11.2

[a]Not detected.

than 10% different from the LC-MS results. The differences between UV and MS determinations for UP were 12.4% for female and 10.8% for male samples. HEPTAP and PENTAP were not detected by LC-UV because of the lower sensitivity of UV than MS detection. The obtained porphyrin excretion patterns, for example, the dominance of CP in the urine samples, were in agreement with the published data for healthy individuals.[27,29,40] The averaged concentrations of endogenous CP and UP in normal urine samples were reported as 113.0 nM for CP and 19.8 nM for UP from CE

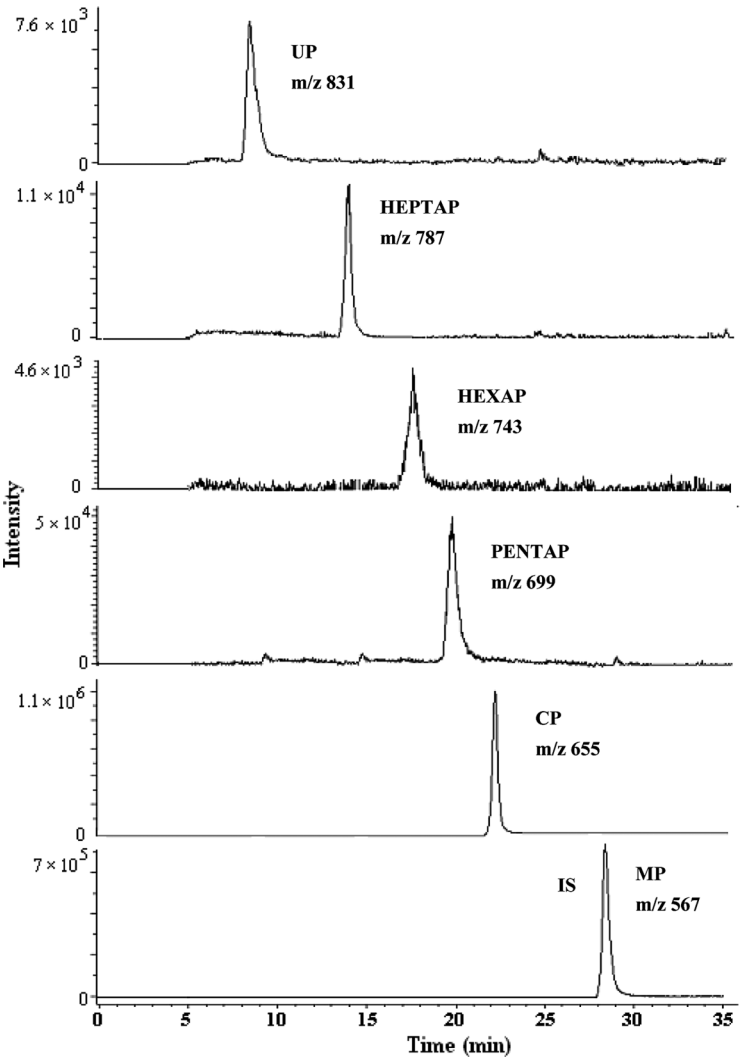

FIGURE 5 Extracted ion chromatograms for the porphyrins in urine extract from a patient suffered from liver disease. MP was used as internal standard (200 nM).

analysis.[29] A LC-MS-MS method without sample pretreatment provided the contents of CP, PENTAP, HEPTAP, and UP at 126, 18.7, 32.4, and 58.1 nM in healthy female, respectively.[27] The pattern of CP dominance in healthy human urine was also shown by using LC-MS method with porphyrin esterification and extraction from urine.[40]

Patients suffered from liver diseases have been found to excrete elevated levels of CP[7]. Figure 5 shows the EIC of porphyrins detected in one urine sample from a female patient with liver disease. The porphyrin excretion contents from the patients are also depicted in Figure 5. Six portions of the 24-hour urine were collected from each of the healthy male, healthy female and patients, respectively, within two months ($n = 6$). Levels of CP in the patient urines were observed about two times higher than those of healthy persons. The significant increase of CP in patient urine was consistent with that previously reported for patients with liver disease,[46] indicating that the determination of CP in human urine might be served as a diagnostic symptom of liver disease. It was also observed that the levels of HEXAP and PENTAP in the patient urine samples were significantly higher than those in healthy human urine. Because the data from the persons with liver disease were limited, however, more investigation and comparisons are needed in order to draw a clear conclusion for HEXAP and PENTAP in human urine.

It is noted that isomers of CP and UP were not separated using the present HPLC method. The analytical identification of the isomers of CP and UP is of considerable practical significance.[47] The HPLC separation of isomers requires selected gradient elution program with optimized solvents,[48] or using octadecylsilica monolithic columns,[38] and so forth. We expect to explore appropriate HPLC conditions (e.g., column, elution solvents, and elution program) for isomers of the representative porphyrins in the future.

CONCLUSIONS

An LC-MS method was developed, validated, and applied for the analysis of six porphyrins in human urine samples. The simple SPE procedure provided efficient cleanup to remove urine interference with high recoveries. The method gave good accuracy, precision, and repeatability for the porphyrins analysis. The developed method was successfully applied to the determination of the porphyrins in human urines at low-nM levels. Different metabolic patterns of porphyrins were established, in human urines collected from healthy persons and patients who suffered from liver disease. While CP was found to be predominant in all tested human urines, its levels in the patient urine were significantly higher than those detected in the urine samples from healthy human. Analysis of urine porphyrins and

establishment of the unique patterns of porphyrins excretion might assist in providing evidence for the diagnosis and treatment of liver diseases.

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