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Neuberg DIAGNOSTICS CENTER FOR PROTEOMICS & METABOLOMICS

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Comprehensive and Sensitive UHPLC-MS/MS Analysis for Urinary Porphyrins

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1. Introduction

Porphyrins (Figure 1) are a ubiquitous class of naturally occurring compounds some of which are critical biomolecules involved in heme biosynthesis. Their altered urinary excretion patterns indicate metabolic disorders like porphyria. These disorders arise from enzymatic deficiencies that disrupt heme synthesis, leading to porphyrin accumulation.[1] Existing analytical techniques, including spectrofluorometry, HPLC, and mass spectrometry, often require extensive sample preparation, large volumes, or derivatization.[2] We developed and validated a robust UHPLC-MS/MS method for selective porphyrin quantification in urine, using Shimadzu LCMS-8045 (Figure 2). The streamlined one-step extraction and reversed-phase liquid chromatography method enables precise differentiation of porphyrin profiles in healthy individuals and patients with liver disease, facilitating biochemical and clinical research.

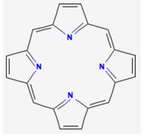

Figure. 1 Structure of porphyrins

Figure. 2 Nexera™ X3 UHPLC coupled with an LCMS-8045

2. Experimental

Calibrator and quality control (QC) standards were provided by RECIPE Chemicals. LCMS methanol and acetonitrile were purchased from Honeywell. LCMS grade formic acid was purchased from Sigma Aldrich. All calibrator standards and QC samples were reconstituted in 5 mL of LCMS grade water and mixed for 15 min. The calibrator and QC concentrations are mentioned in Table 1. Precursor ion selection, MRM optimization at different collision energies (CE) and voltages was done using Shimadzu's "Optimization for method" tool. Optimized MRMs for all 8 porphyrins were developed using optimized voltages and CE. An LC method (Table 2) was developed using HPLC column (Shim-pack GIST C18) to separate the 8 porphyrins. Using the developed LC method and optimized MRM (Table 3), a calibration curve covering the clinically relevant concentrations was plotted.

Table 1. Calibrator and QC levels of porphyrins.

Sr. No.	Compounds	Calibrator Level (µg/L)	QC Level (µg/L) Level-1	Level-2
1	Uroporphyrin I	104.00	16.70	194.00
2	Heptacarboxyporphyrin I	34.00	6.36	56.10
3	Hexacarboxyporphyrin I	26.10	4.67	45.10
4	Pentacarboxyporphyrin I	40.10	6.94	58.90
5	Coproporphyrin I	173.00	34.70	295.00
6	Coproporphyrin III	216.00	71.90	356.00
7	5-Aminolevulinic Acid	11.30	4.06	18.20
8	Porphobilinogen	2.52	1.54	6.23

Table 2. Analytical conditions

UHPLC condition (Nexera™ X3)	
Column	Shim-pack™ GIST C18, 3 µm, 75 × 4.6 mm (P/N: 227-30009-04)
Mobile phase A	0.1% formic acid in acetonitrile : water (90:10 v/v)
Mobile phase B	0.1 % formic acid in water
Flow rate	0.5 mL/min
Elution mode	Gradient
Column temp	40 ℃
Autosampler temp	10 ± 5 °C
Injection Volume	20 µL
Acquisition time	20 min
MS parameters (LCMS-8045)	
MS interface	Electro Spray Ionization (ESI)
Polarity	Positive
Nebulizing gas flow	3 L/min
Drying gas flow	10 L/min
Interface temp.	300 °C
DL temp.	250 °C
Acquisition mode	MRM

For samples, 75 µL of urine were taken in 2 mL micro centrifuge tubes. To this 30 µL of 6.0 M formic acid was added and samples were vortexed for 60 s. The samples were then centrifuged for 10 mins at 13000 rpm. The supernatant was taken in auto sampler vial and injected into LC-MS/MS system.

Table 3. MRM Transitions for porphyrins

Sr No.	Compound Name	MRM Transition	CE
1	Uroporphyrin I	831.0 > 831.0	-15
2	Heptacarboxyporphyrin I	787.4 > 787.2	-20
3	Hexacarboxyporphyrin I	743.2 > 743.0	-12
4	Pentacarboxyporphyrin I	699.3 > 699.0	-20
5	Coproporphyrin I	655.3 > 655.0	-20
6	Coproporphyrin III	655.3 > 655.0	-15
7	5-Aminolevulinic Acid	132.1 > 132.0	-20
8	Porphobilinogen	227.0 > 226.2	-16

3. Results

The optimized UHPLC-MS/MS method demonstrated excellent chromatographic resolution (Figure 3), with enhanced porphyrin ionization in positive electrospray mode. Method validation showed strong linearity ($R^2 > 0.99$) across clinically relevant concentrations as shown in Figure 4.

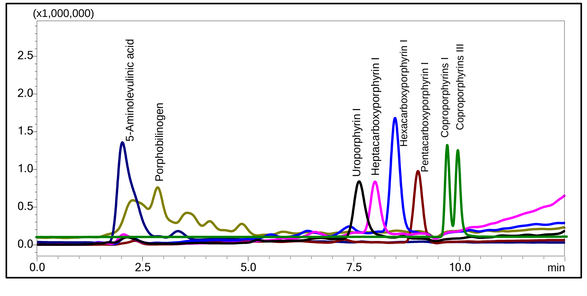

Figure. 3 MS Chromatogram of 8 porphyrin compounds

The results of the QC samples as shown in Table 4. Control sample analysis confirmed accuracy within specified QC ranges for all 8 porphyrins.

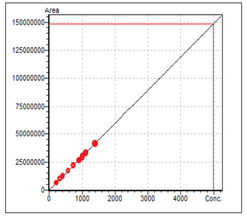

Figure. 5 Calibration curve for 8 porphyrin compounds

Table 4. Results of RECIPE QC samples

Sr. No.	Compounds	Level-1 (µg/L) Conc.	Range	Level-2 (µg/L) Conc.	Range
1	Uroporphyrin I	15.32	12.5-20.9	192.54	155-233
2	Heptacarboxyporphyrin I	6.01	4.46-8.27	54.93	44.9-67.3
3	Hexacarboxyporphyrin I	4.30	3.27-6.07	43.27	36.0-54.1
4	Pentacarboxyporphyrin I	5.98	4.86-9.03	59.10	47.1-70.6
5	Coproporphyrin I	36.90	27.7-41.6	298.35	236-354
6	Coproporphyrin III	72.61	57.5-86.3	361.09	285-427
7	5-Aminolevulinic Acid	3.65	3.25-4.87	17.64	14.6-21.9
8	Porphobilinogen	1.45	1.08-2.01	6.13	4.67-7.79

4. Conclusion

- A high-throughput UPLC-MS/MS method was developed, validated and applied for analysis of simultaneous determination of porphyrin from urine samples.
- The method gave good accuracy, precision and repeatability for the porphyrin analysis.
- This method has significant advantages in terms of reproducibility and simple and clean extraction procedure.

References:
1) Quantification of six Porphyrin biomarkers in urine using LC-2050C with fluorescence detection (2023) 06-SAIP-LC-054-EN.
2) Simultaneous determination of six urinary porphyrins using liquid chromatography–tandem mass spectrometry (2003) Journal of Chromatography B 783.2: 411-423.Bu, Wei, et al.