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Application News

High Performance Liquid Chromatograph i-Series LC-2050C

Quantification of Six Porphyrin Biomarkers in Urine Using LC-2050C with Fluorescence Detection

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User Benefits

◆ A simple and robust HPLC method for the determination of porphyrins in urine

◆ No sample pre-treatment is required.

■ Introduction

Porphyria are a group of seven metabolic disorders of haem biosynthesis. These include aminolevulinic acid dehydratase porphyria (ADP), acute intermittent porphyria (AIP), variegate porphyria (VP), hereditary coproporphyria (HCP), porphyria cutanea tarda (PCT), erythropoietic protoporphyria (EPP) and congenital erythropoietic porphyria (CEP). People with certain types of porphyria can have a sudden life-threatening crisis. These attacks are rare and difficult to diagnose because they are non-specific. In many cases, the disease is misdiagnosed because of the rarity of some types of porphyria. For early diagnosis and identification of the type of porphyria, accurate quantification of porphyrins in urine and faeces is necessary.

This application note describes an HPLC method with fluorescence detection for the quantification of six porphyrin biomarkers; namely, uroporphyrin I, heptacarboxyporphyrin I, hexacarboxyporphyrin I, pentacarboxyporphyrin I, coproporphyrin I and coproporphyrin III in urine.

■ Experimental

A commercial lyophilized porphyrin standards kit was purchased from RECIPE Chemicals+Instruments GmbH (Munich, Germany). The kit contains a urine calibrator mixture of uroporphyrin I, heptacarboxyporphyrin I, hexacarboxyporphyrin I, pentacarboxyporphyrin I, copro-porphyrin I and coproporphyrin III of known concentration; urine control level I and level II. The calibrator and controls were reconstituted in 5 mL of Milli Q water.

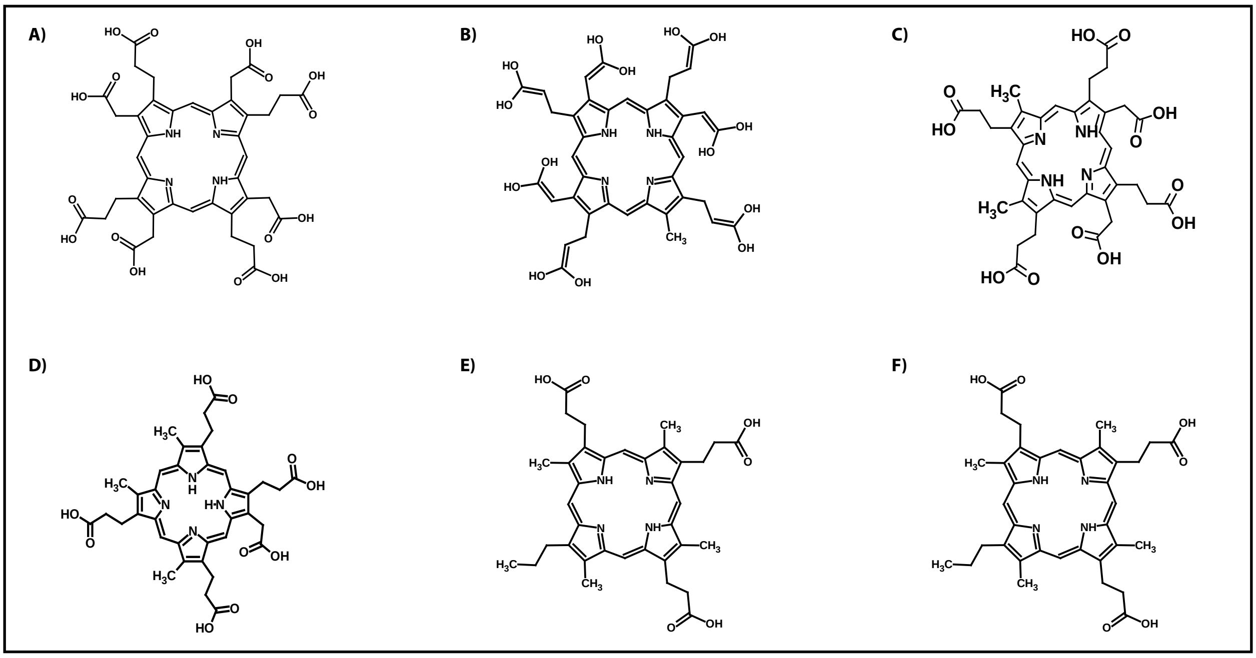

Figure 1 Structure of porphyrins - A) Uroporphyrin I, B) Heptacarboxyporphyrin I, C) Hexacarboxyporphyrin I, D) Pentacarboxyporphyrin I, E) Coproporphyrin I, and F) Coproporphyrin III.

Structurally, porphyrin consists of four pyrrole rings (five-membered closed structures containing one nitrogen and four carbon atoms) linked to each other by methine groups (—CH=). Figure 1 shows the structures of porphyrins. All porphyrin compounds absorb light intensely at or close to 410 nm. Although various methods have been developed for the analysis of urine porphyrins, the property of porphyrins to exhibit florescence at a specific wavelength make reverse-phase high-pressure liquid chromatography (HPLC) coupled with fluorescence detection the gold standard method for this purpose.

Further dilutions for the calibrator were made using mobile phase as diluent under dark conditions in amber vials, while the controls were injected without dilution. The samples were analyzed using Shimadzu i-series LC-2050C HPLC system coupled with RF-20 AXS fluorescence detector shown in Figure 2 using the analytical conditions given in Table 1.

■ Linearity of the Porphyrins

Six-point calibration curve for the porphyrin calibrator was prepared by serially diluting the calibrator mixture 5 times in diluent.

Figure 2 LC-2050C coupled with RF-20Axs detector

Table 1 Analytical conditions

HPLC system	: LC-2050 C
Column	: Shim-pack Scepter^{TM} C18-120 (250 mm x 4.6 mm, 5 μ) (P/N :227-31020-06)
Column oven temp.	: 25 ˚C
Autosampler temp.	: 4 ˚C
Mobile phases	: A- 10 mM Ammonium acetate in water (pH: 5.6) B- Acetonitrile
Flow rate	: 1.0 mL/min
Gradient program (B%)	: 0 - 17 min → 0 - 45 (%) 17 - 17.1 min → 45 - 10 (%) 25 min → STOP
Detector	: RF-20 AXS (fluorescence detector)
Wavelength	: Excitation - 395 nm Emission - 630 nm
Injection volume	: 20 µl
Diluent	: Mobile phases A / B = 90 / 10 (v/v)

A representative chromatogram of porphyrin calibrator is shown in Figure 3. The Figures 4-A to 4-F depict the calibration curve and level 1 (L1) calibrator chromatograms for uroporphyrin I, heptacarboxyporphyrin I, hexacarboxyporphyrin I, pentacarboxyporphyrin I, coproporphyrin I and coproporphyrin III, respectively.

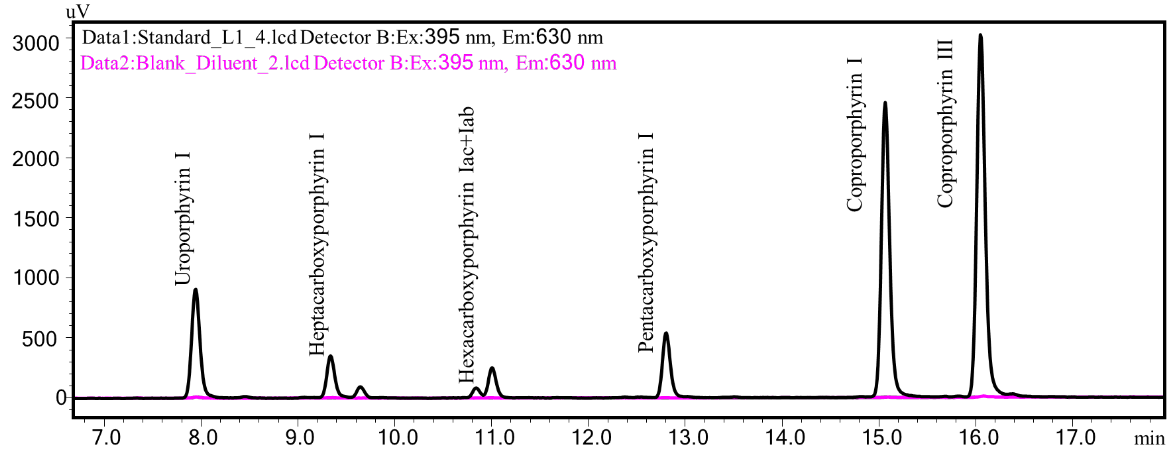

Figure 3 Representative chromatogram of Porphyrin Calibrator

Uroporphyrin I

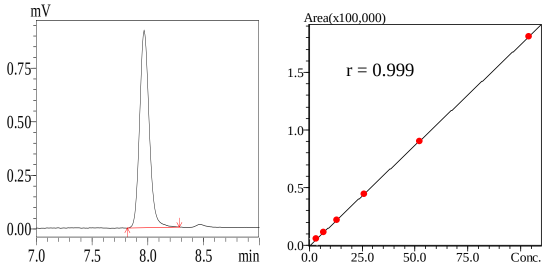

Figure 4-A L1 calibrator chromatogram and calibration curve for uroporphyrin

Heptacarboxyporphyrin I

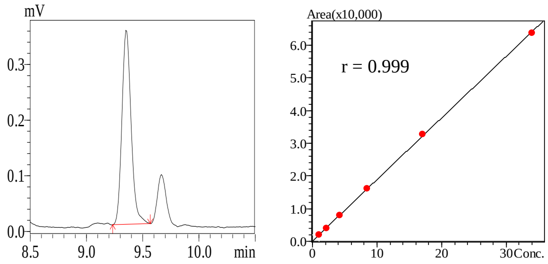

Figure 4-B L 1 calibrator chromatogram and calibration curve for heptacarboxyporphyrin I

Hexacarboxyporphyrin I

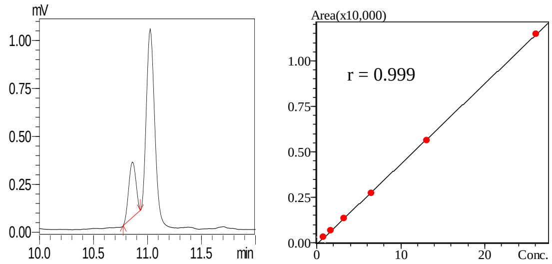

Figure 4-C L1 calibrator chromatogram and calibration curve for hexacarboxyporphyrin I

Pentacarboxyporphyrin I

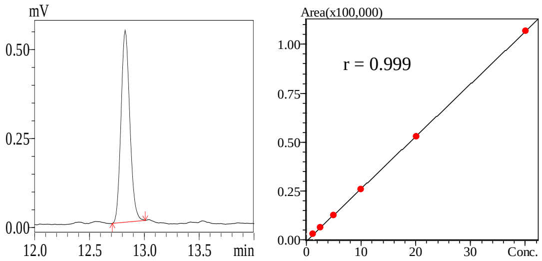

Figure 4-D L1 calibrator chromatogram and calibration curve for pentacarboxyporphyrin I

Coproporphyrin I

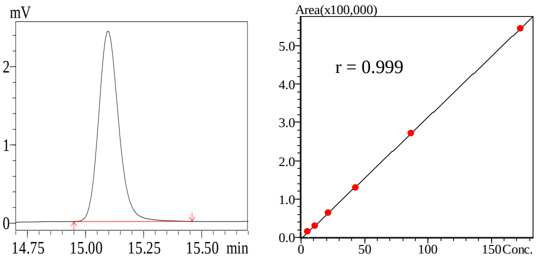

Figure 4-E L1 calibrator chromatogram and calibration curve for coproporphyrin I

Coproporphyrin III

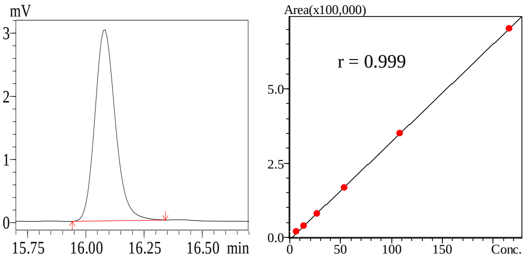

Figure 4-F L1 calibrator chromatogram and calibration curve for coproporphyrin III

Table 2 Concentration and accuracy results of porphyrins (n=3)

	Uroporphyrin I			Heptacarboxyporphyrin I			Hexacarboxyporphyrin I		
Calibration Level	**Expected conc. (µg/L)**	**Observed conc. (µg/L)**	**Accuracy**	**Expected conc. (µg/L)**	**Observed conc. (µg/L)**	**Accuracy**	**Expected conc. (µg/L)**	**Observed conc. (µg/L)**	**Accuracy [%]**
L1	3.25	3.50	108	1.0625	1.00	94	0.82	0.95	117
L2	6.5	6.69	103	2.125	2.07	98	1.63	1.71	105
L3	13	12.95	100	4.25	4.19	99	3.26	3.26	100
L4	26	25.59	98	8.5	8.46	100	6.53	6.35	97
L5	52	51.84	100	17	17.38	102	13.05	12.92	99
L6	104	104.17	100	34	33.83	100	26.10	26.20	100

	Pentacarboxyporphyrin I			Coproporphyrin I			Coproporphyrin III		
Calibration Level	**Expected conc. (µg/L)**	**Observed conc. (µg/L)**	**Accuracy**	**Expected conc. (µg/L)**	**Observed conc. (µg/L)**	**Accuracy**	**Expected conc. (µg/L)**	**Observed conc. (µg/L)**	**Accuracy [%]**
L1	1.25	1.39	111	5.41	6.07	112	6.75	7.82	116
L2	2.51	2.58	103	10.81	11.08	102	13.50	13.91	103
L3	5.01	4.97	99	21.63	21.19	98	27.00	26.41	98
L4	10.03	9.85	98	43.25	42.31	98	54.00	52.56	97
L5	20.05	19.98	100	86.50	86.88	100	108.00	108.31	100
L6	40.10	40.17	100	173.00	173.06	100	216.00	216.22	100

Table 3 Summary result of urine control I (C1) and urine control II (C2) analysis (n=6)

Compound	Observed Conc. (µg/L)		Expected Conc. (µg/L)		Control range (µg/L)		Accuracy[%]		% RSD of Retention Time		% RSD of Conc.	
	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2	C1	C2
Uroporphyrin I	16.03	190.38	16.70	194.00	12.5 to 20.9	155 to 233	96	98	0.00	0.05	0.11	0.12
Heptacarboxyporphyrin I	5.98	56.44	6.36	56.10	4.46 to 8.27	44.9 to 67.3	94	101	0.04	0.07	0.17	0.09
Hexacarboxyporphyrin I	5.03	44.52	4.67	45.10	3.27 to 6.07	36.0 to 54.1	108	99	0.06	0.04	0.62	0.52
Pentacarboxyporphyrin I	7.41	59.13	6.94	58.90	4.86 to 9.03	47.1 to 70.6	107	100	0.04	0.06	0.09	0.05
Coproporphyrin I	35.86	295.80	34.70	295.00	27.7 to 41.6	236 to 354	103	100	0.03	0.03	0.84	0.06
Coproporphyrin III	75.41	358.78	71.90	356.00	57.5 to 86.3	285 to 427	105	101	0.04	0.03	0.08	0.08

■ Results

Linearity:

The summary results for concentration and accuracy of porphyrins in urine calibrator are given in Table 2. Good linear correlations along with correlation coefficients greater than 0.999 were acquired for all porphyrins as shown in Figures 4-A to 4-F.

Obtained Controls:

Urine control level I (C1) and level II (C2) were analyzed. The summary of results of urine controls are given in Table 3. The retention time %RSD for the six porphyrins was between 0.00 to 0.07 and the concentration %RSD was between 0.05 to 0.84. The measured concentrations of both control samples were well within the control range specified.

■ Conclusion

- A home brew, simple and low-cost method for analysing urinary six porphyrins was developed on Shimadzu i-series LC-2050C HPLC system coupled with RF-20 AXS fluorescence detector which needs no sample preparation.
- Ease of operation, no sample preparation, rugged technology, reduced cost of analysis, unambiguous outcome and overall performance, will, definitely, appeal to industries for reduction of time and cost merit.
- This method may also assist in the differential analysis of various porphyria.

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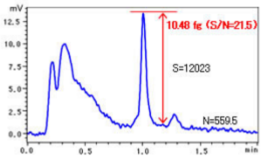

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