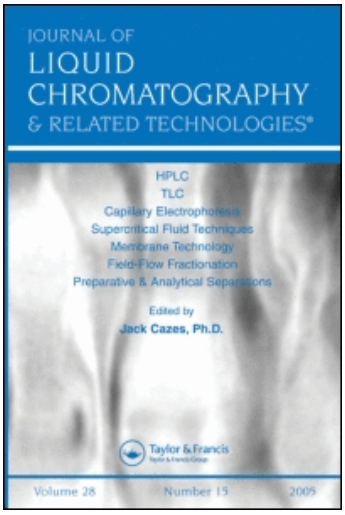

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Lin, M. and Huie, C. W.(1997) 'Determination of Porphyrins by High Performance Liquid Chromatography with Chemiluminescence Detection', Journal of Liquid Chromatography & Related Technologies, 20: 5, 681 — 691
To link to this Article: DOI: 10.1080/10826079708014134
URL: http://dx.doi.org/10.1080/10826079708014134

J. LIQ. CHROM. & REL. TECHNOL., 20(5), 681–691 (1997)

DETERMINATION OF PORPHYRINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH CHEMILUMINESCENCE DETECTION

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ABSTRACT

The peroxyoxalate chemiluminescence reaction, based on the oxidation of bis (2,4 dinitrophenyl) oxalate with hydrogen peroxide, was developed as a new detection method for the determination of porphyrins after separation by reverse phase high performance liquid chromatography. To optimize signal to noise ratio, experimental parameters such as design of the detection flow-cell and the effects of pH and organic solvents were investigated. Significant enhancements in detectability, selectivity and linearity were demonstrated for the determination of various hydrophilic and hydrophobic porphyrins when compared to absorbance and fluorescence methods (e.g., the detection limits (S/N = 3) for uroporphyrin and coprophorphyrin were about 40 and 5 fmol, respectively). Application of this method for the sensitive and selective determination of urinary porphyrins was also demonstrated.

INTRODUCTION

Porphyrins are naturally occurring tetrapyrrole derivatives with a porphine structure and play a key role in biological processes.[1] Disorders arising from inherited or acquired defects in biosynthetic pathway of heme in the bone marrow and liver are known as porphyrias.[2] Measurement of porphyrins present in biological materials is important for the diagnoses of these diseases.[3] Earlier methods of determination included liquid extraction,[4] ion exchange[5] and column chromatography,[6] followed by UV absorbance or fluorimetric determination. These methods were laborious and lacked sensitivity and selectivity. Thin layer chromatography (TLC) has improved the separation and quantitation of porphyrins[7] and the technique of high performance liquid chromatography (HPLC) has further enhanced the analysis speed, sensitivity and selectivity for the determination of porphyrins.[8]

Although UV-Vis absorbance detection has been widely used for HPLC, its detection limit was inadequate for the determination of minor porphyrin components.[9-11] Several authors have used fluorescence detection to improve sensitivity; however only a few publications have appeared in the literature so far[9-14] and background interferences from sample matrices appear to be a problem for the quantitation of various porphyrins present in real samples.

Peroxyoxalate chemiluminescence(PO-CL) has been demonstrated to be a highly sensitive and selective detection technique for the detection of biological molecules.[15] Albrecht et al.[16] have applied PO-CL for the screening of total porphyrin content in urine and the detection limit was about 250 μg/L. Recently, determination of porphyrins with PO-CL by coupling with TLC,[17] cloud point extraction,[18] and flow injection analysis[19] have been reported. In the present work, we further establish PO-CL as a new detection method for porphyrin determination by combining with HPLC separation.

EXPERIMENTAL

Pure standards of uro-, copro-, hemato-, proto-, zinc proto-porphyrins and hexa-, penta-carboxylic acids were obtained from Porphyrin Products (Logan, UT USA). HPLC grade acetonitrile, hydrogen peroxide (30%) were purchased from Fisher. High purity of bis(2,4 dinitrophenyl) oxalate (DNPO) was purchased from Fluka (Ronkonkoma, NY). All other reagents were of analytical grade obtained from Fisher and Aldrich. Doubly distilled deionized water was used.

The porphyrin standards were dissolved in a small amount of 1 M NaOH and were then diluted with doubly deionized water. Except for proto- and zinc proto-porphyrins, all other porphyrin standard solutions were stable for at least one week when kept in the dark. Proto- and zinc proto-porphyrin standard solutions have to be prepared just before use. DNPO with concentrations of 1.0-4.0 mmol/L in ethyl acetate and H_2O_2(3%-5%) in acetonitrile were used respectively and were made up just before each experiment and used immediately.

Instrumentation

The HPLC system consisted of a Varian 5500 liquid chromatograph(Houston, TX, USA), a model 7125 sample injector with a 10 μL injection loop (Rheodyne, Cotati, CA, USA), and a Perkin-Elmer model 610-S fluorescence detector (Norwalk, CT, USA) equipped with a 10 μL flow cell. Fluorescence excitation was performed at 400 nm with a bandpass of 20 nm and emission was collected at 620 nm with a bandpass of 20 nm. Chromatograms were recorded on a Perkin Elmer LCI-100 integrator or on a strip-chart recorder.

A model 7611-00 Cole-Parmer Ismatec Schlauch pump (Chicago, IL, USA) was used to deliver the chemiluminescence reagents for postcolumn reaction. The detection system consisting of a 10 μL quartz-tube micro flow-cell, a EMI-Gencom INC. model RFI/B-258B 211 photomultiplier (Plainview, NY, USA), a current amplifier, a high-voltage supply and a Perkin Elmer LCI-100 Integrator.

The HPLC analytical column was a 5 μm Econosphere C18 (150 x 4.6 mm I.D.), (Alltech, Deerfield, IL, USA). This column was protected by a 3 cm refillable pellicular C18 guard column (Alltech, Deerfield, IL, USA).

Chromatographic Conditions

HPLC grade acetonitrile and doubly-distilled deionized water were vacuum filtered through a 0.45 μm filter. Eluent A was a mixture of 95% acetonitrile with 5% 0.02 M phosphate solution (pH = 7.0). Aqueous mobile phase (eluent B) was prepared by first dissolving sodium phosphate monobasic in water to give a 0.02 M solution and adjusted to pH 3.5 with phosphoric acid. Acetonitrile was added to give a 5% mixture (V/V). Both eluents were degassed further by sonication for five minutes. Table 1 shows the linear gradient conditions employed for the separation of various porphyrins.

Table 1

Linear Gradient Conditions

Time (min)	Eluent A*	Eluent B**
0.00	20	80
5.00	60	40
10.0	62	38
10.5	100	0
17.0	100	0
17.5	20	80
20.0	20	80

* Eluent A was 95% CH_3CN, 5% 20 mM phosphate buffer (pH = 7.00).

**Eluent B was 5% CH_3CN, 95 % 20 mM phosphate buffer (pH = 3.50).

Sample Preparation

Urine samples were collected from healthy adults over a twenty four hour period and were preserved with sodium carbonate (2g/L urine) and ethylenediaminetetraacetate (3g/L urine) and stored under refrigeration in the dark at 4°C. Before injection, urine was filtered through a 0.20 mm filter membrane and readjusted to its previous pH (~6.8).

RESULTS AND DISCUSSION

Design of Post-Column Detection System

Although TCPO is a more stable oxalate reagent and is widely used in PO-CL HPLC systems, it does not offer sufficient sensitivity for the determination of minor porphyrin components in urine without preconcentration.[18] On the other hand, DNPO could offer much higher sensitivity as a result of shorter CL half-life when compared to TCPO, provided that the detection system is capable of exploiting the fast kinetics of the DNPO system. Using a FIA system[19] we have demonstrated that detection limits in the 10^{-9} M range can be achieved for the DNPO-CL detection of various porphyrins under optimized conditions; in the present HPLC system, a slight modification of the FIA detection flow-cell was carried out to maximize S/N.

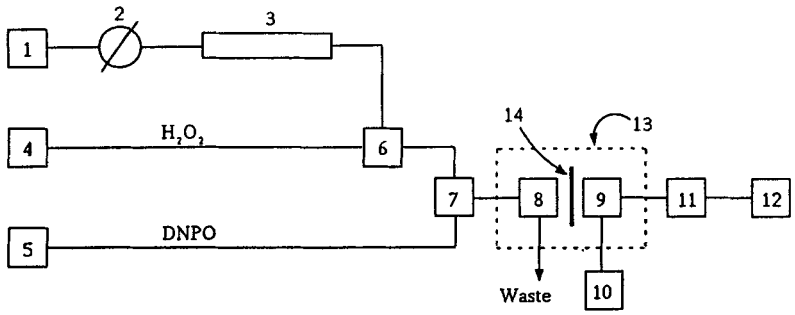

Figure 1. Schematic diagram of HPLC system with postcolumn chemiluminescence detection of porphyrins. 1 - LC pump; 2 - injection valve; 3 - HPLC column; 4,5-pumps for DNPO and H_2O_2; 6,7 - mixing fee; 8 - detection flow-cell; 9 - photomultiplier tube; 10 - high voltage power supply; 11 - current amplifier; 12 - integrator or recorder; 13 - dark box; 14 - 570 nm cut-off filter.

Figure 1 shows a schematic diagram of the postcolumn PO-CL detection system for porphyrins. The eluent from the HPLC column first mixed with H_2O_2 within a low dead-volume stainless-steel tee and then the solution mixed with DNPO inside another tee that was connected to the detection flow-cell via a PTFE tubing (see Fig. 2). The distance between the point at which the eluent, H_2O_2 and DNPO converged (mixing point) and the detection window was found to be critical in maximizing the CL signal intensity.

To optimize this particular distance, the length of the PTFE tubing which connected the second mixing tee and the detection flow-cell was varied and a distance of ~5 cm was found to provide the best S/N.

Optimization of PO-CL Conditions

The effect of flow rate on the CL intensity were investigated and the relative CL intensity was found to reach a maximum and then decreased gradually when the flow rate was increased from 0.5 to 2.5 mL/min. In the range of 0.75-1.50 mL/min, relatively high CL signals, low noise, and reproducible peaks were observed, and the CL signal decreased about 20% when the flow rate exceeded 2 mL/min. We chose 1.25 mL/min as the optimal flow rate for our experiments, since it provided the best S/N while minimizing peak broadening.

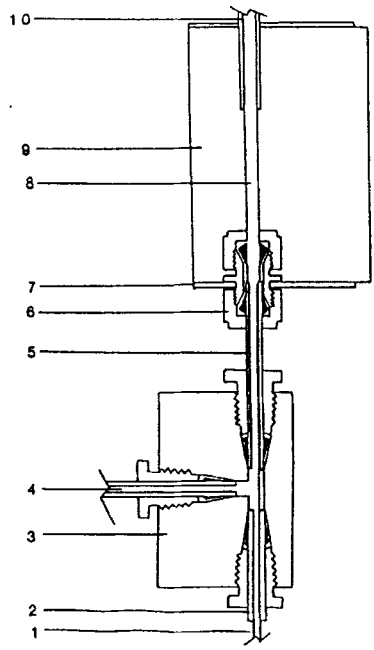

Figure 2. Diagram of detection flow-cell interfaced to the mixing-tee. 1 - 0.32 mm I.D. capillary column for DNPO; 2 - 0.4 mm I.D. PTFE tubing; 3 - mixing tee; 4 - 0.32 mm I.D. PTFE tubing for HPLC eluent mixed with H_2O_2; 5 - flow-cell connecting tubing (0.32 mm I.D. PTFE tubing); 6 - 1/16 in. swagelok union; 7 - light seal (darkened metal plate); 8 - location of the detection window; 9 - flow cell holder; 10 - waste outlet.

The effect of DNPO concentration was also examined. The CL signal increased with an increase in DNPO concentration; however, from 2 mmol/L to 4 mmoll/L, the signal intensity increased only about 10%, and at higher concentrations, the reaction rates appeared to occur too fast for the detection system to capture and gave poorer S/N noise. So, the optimum DNPO concentrations fall in the range of 1.5 mmol/L to 2.0 mmol/L.

Effect of pH and Organic Solvent

An important consideration in adapting PO-CL detection to HPLC is that the pH and solvents used for efficient/selective HPLC separation should be compatible with those employed for providing optimum CL detection.[20]

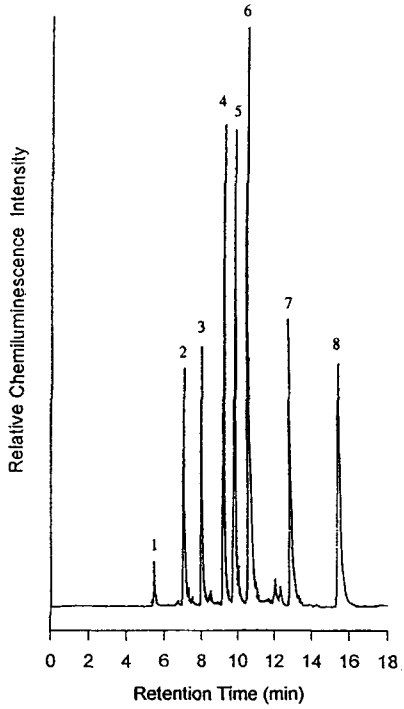

Figure 3. Chromatogram of porphyrin standards (each at 250 μg/L). u = uroporphyrin; 6: hexacarboxylic acid porphyrin; 5: pentacarboxylic acid porphyrin; C1: coproporphyrin I; C3: coproporphyrin III; HP: hematoporphyrin IX; Znpp: zinc protoporphyrin and PP: protoporphyrin.

Based on our previous studies,[19] DNPO can be used in a solvent mixture of up to 35% H_2O with no significant effect on the CL intensity for most porphyrins. Thus, it is possible to use a mobile phase consisting of a relatively high percentage of an aqueous buffer while maintaining optimum conditions for PO-CL detection of porphyrins. Also, based on our earlier work,[19] we found that the optimum pHs for performing CL detection are as follows: uroporphyrin (pH = 4 to 4.5); copro- and other porphyrins (pH = 5 to 7), and fortunately, these pHs happened to fall within the pH range of mobile phases employed for efficient/selective porphyrin separations in HPLC.[9-14] Table 1 shows the linear gradient conditions employed in our experiments for achieving optimum HPLC separation and PO-CL detection of porphyrins.

Table 2

Linearity, Detection Limits and Precision Data

Paramter	Uro-	Hexa	Penta	CoproI	CoproIII	Hemato	Znpro-	Proto
Detection Limit (μg/L)	3.0	0.71	0.63	0.43	0.43	0.35	0.62	0.78
Linear Range (μg/L)	0-10^3	0-10^3	0-10^3	0-10^3	0-10^3	0-10^3	0-10^3	0-10^3
Interception	1.21	0.66	2.99	3.49	3.90	4.57	2.84	1.87
Slope	0.05	0.14	0.19	0.47	0.46	0.64	0.19	0.13
Correlation Coefficient	0.997	0.999	0.999	0.998	0.997	0.998	0.998	0.998
R.S.D. (%) (n = 6)	1.56	2.78	0.96	0.43	0.40	0.46	0.37	0.42

Figure 3 illustrates a chromatogram of porphyrin standards which consisted of 250 μg/L each of uro-, hexa-, penta-, copro-I and III, hemato-, zinc proto- and proto-porphyrins, showing complete baseline separation for all porphyrins within 18 minutes. Table 2 shows the results of calibration data, detection limits and precision. Importantly, when compared to absorbance detection,[11] an improvement factor ranging from about 50 to 100 was obtained with PO-CL detection for the porphyrins investigated in this study. When compared to fluorescence detection,[9,11,14] an improvement factor of about 5 to 10 was obtained.

Fig. 4 shows a chromatogram of a urine sample from a healthy individual using PO-CL detection. When compared to chromatograms obtained with fluorescence detection,[9-14] an important difference is that when fluorescence detection is employed, large amounts of non-porphyrin fluorescent substances are usually eluted ahead of the porphyrins and lead to the appearance of a large, broad band near the column void volume; however, such potential background interference is clearly absence from the chromatogram shown in Fig. 4, thus demonstrating the superior selectivity of PO-CL detection. By comparing with retention times of porphyrin standards as shown in Fig. 3 and chromatograms from urine samples spiked with various porphyrin standards, peak assignments in Fig. 4 can be made as follows: Peak 1 = uroporphyrin: peak 2 = hexacarboxylic acid porphyrin; peak 3 = pentacarboxylic acid porphyrin; peak 4 = coproporphyrin I and peak 5 = coproporphyrin III. It can be seen that peak 1 is actually comprised of two small

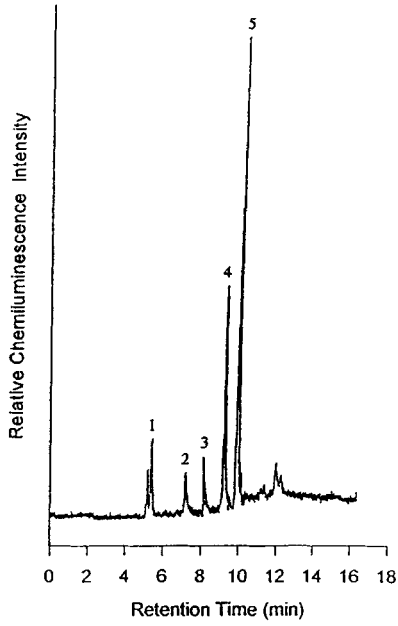

Figure 4. Chromatogram of urinary porphyrins from a healthy adult female.

peaks, arising possibly from uroporphyrin I and III isomers. Unfortunately, a standard of uroporphyrin III was not available during our experiments and the identity of the latter peak at the location of peak 1 is not certain. However, very similar peaks, in terms of relative elution order and peak area, have been reported for the HPLC separation of uroporphyrin I and III isomer standards using fluorescence detection.[14] Concentration of uroporphyrin I and coproporphyrin I and III as shown in Fig. 4 were found to be 48 μg/L, 10 μg/L and 25 μg/L, respectively, which are within the normal concentration range found in urine of healthy individuals.[1] For recovery tests, various known concentrations of porphyrin standards were spiked into urine samples and recoveries fall between 96 to 101%.

In summary, the PO-CL reaction has been successfully coupled with HPLC using DNPO as the oxalate reagent for the HPLC determination of various hydrophilic and hydrophobic porphyrins. Significant improvements in limits of detection and selectivity were demonstrated for the measurement of micro-trace amounts of porphyrins in urine samples when compared to conventional detection and selectivity methods. Such improved analytical capabilities should be valuable to clinical and biomedical scientists who engage in porphyrin research.

ACKNOWLEDGMENTS

This work was supported in part from a research grant awarded by the American Association of Clinical Chemistry.

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Received July 31, 1996
Accepted August 27, 1996
Manuscript 4257