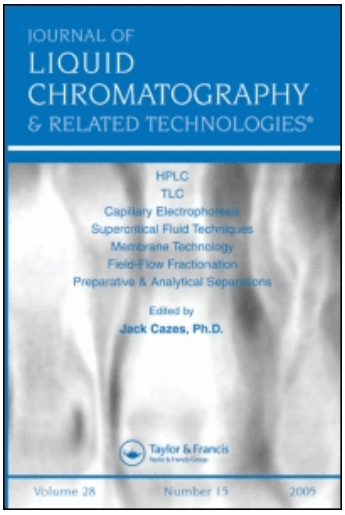

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A Study of the Solvent Composition Effects on the Separation of Seven Clinically Important Porphyrins on Cyclodextrin Bonded Phases

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A STUDY OF THE SOLVENT COMPOSITION EFFECTS ON THE SEPARATION OF SEVEN CLINICALLY IMPORTANT PORPHYRINS ON CYCLODEXTRIN BONDED PHASES

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ABSTRACT

The isocratic separation of the seven polycarboxylated porphyrins of heme biosynthesis that was previously thought to be difficult has been demonstrated using cyclodextrin bonded phases via a novel mobile phase. The multi-component mobile phase contained 0.28 g of 18-crown-6 ether in 0.06 M phosphate buffer : acetonitrile : pyridine (5 : 50 : 30 , v/v/v, pH 6.37). The seven porphyrins included mesoporphyrin, uroporphyrin, heptaporphyrin, hexaporphyrin, pentaporphyrin, coproporphyrin and protoporphyrin. The solvent composition effects on the retention characteristics of these porphyrins were studied with some common HPLC solvents . The solvent selectivity of the mobile phase was discussed. The elution order of these porphyrins on the cyclodextrin column resembled that on the reversed-phase C18 column. The retention of the porphyrins increased as the number of side-chain carboxylated substituents decreased. However, there was no correlation between the two parameters.

INTRODUCTION

Carboxylated porphyrins are the common tetrapyrrole compounds found in biological materials. Polycarboxylated porphyrins are the intermediary metabolites of heme biosynthesis . They are formed in the body from their precursors, δ-aminolevulinic acid and porphobilinogen. Through the progressive decarboxylation of the first uroporphyrin, different intermediary porphyrins containing carboxyl groups ranging from 2 to 8 are subsequently formed. Disturbances of the heme biosynthetic pathway are characterized by the excess of porphyrins in the biological tissues. Analysis of the accumulated and excreted porphyrins is important in diagnosis of porphyrias, and it is also useful as a confirmatory test for other porphyrin- related disorders such as lead-poisoning and iron-deficiency. The complexity of porphyrins has prompted the development of various analytical techniques for the analysis of these compounds in biological tissues. Among the various methods available for the determination of porphyrins, high-performance liquid chromatographic methods are the preferred techniques. However, the methods vary in complexity. Many of these methods require gradient elution. Although various methods, such as gas chromatography (1), thin layer chromatography (2-4), ion exchange chromatography (5), reversed-phase high performance liquid chromatography (6-13), and a normal-phase chromatography (14) have been described earlier, isocratic separation of the seven carboxylated porphyrins using traditional liquid chromatographic methods has not been reported. Recently, studies dealing with the retention behavior of polycarboxylic porphyrins (15), and several other separation methods (16-21) have appeared in literature. More recently, the separation of the carboxylated porphyrins using cyclodextrin bonded phases has been demonstrated (19). The method required a multi-component mobile phase to complete elution of these compounds in less than eight minutes. However, the solvent selectivity in the analysis of porphyrins in different materials by

high-performance liquid chromatography frequently needs to be changed for accurate quantification, depending on the abundance and nature of the interfering compounds in the separation process. As the solvent selectivity for porphyrins becomes better characterized, it is possible to develop different mobile phases for separating porphyrins in different materials.

The present paper describes the solvent composition effects on the separation of seven clinically important porphyrins on cyclodextrin bonded phases via a novel mobile phase containing four components. Several other common solvents were used to replace acetonitrile in the mobile phase to study the solvent selectivity.

EXPERIMENTAL

MATERIALS

Methanol, acetonitrile, tetrahydrofuran, pyridine and acetone were purchased from J.T. Baker (Phillipsburg, NJ). Potassium phosphate and 18-crown-6 ether were purchased from Sigma Chemical (St. Louis, MO, USA). All porphyrin acids were purchased from Porphyrin Products (Logan, UT, USA).

EQUIPMENT

The chromatographic system consisted of a Varian 5000 Liquid Chromatograph equipped with a Rheodyne 7126 injector with a 10-μl sample loop. A β-cyclodextrin Cyclobond I column (25 cm x 4.6 mm) with a guard column packed with the same stationary phases was used for all the experiments. The analytical column was purchased from Advanced Separation Technologies Inc. (Whippany, NJ). The detector was a Perkin-

Elmer variable wavelength fluorescence spectrophotometer Model 650-15 (CT, USA) with a 12-μl flow cell attachment. All chromatograms were recorded with a Hewlett-Packard 3388A integrator. All pH readings were taken on a model 601 digital Ionalyzer with a Ross combination pH electrode purchased from Orion Research (Boston, MA).

PREPARATION OF PORPHYRIN STANDARD SOLUTIONS

The porphyrin standards were dissolved in 2 ml of 2 M HCl with sonication to complete the dissolution. The standard solutions were stable when stored at 4 ° C.

CHROMATOGRAPHIC CONDITIONS

The multi-component mobile phase was prepared by dissolving 0.28 g of 18-crown-6 ether in 50 ml of acetonitrile and followed by the addition of 30 ml of pyridine to the solution. Subsequently, 5 ml of 0.06 M of potassium phosphate solution were added to the mobile phase. Other mobile phases were prepared likewise except acetonitrile was replaced by different solvents, such as methanol , acetone or tetrahydrofuran , in separate experiments. The exact concentration of each component in the mobile phase depends upon the retention characteristics of the porphyrins. The pH of the mobile phases was adjusted to 6.37. The flow rate was set at 1.4 ml/min. The excitation and emission wavelengths were set at 405 nm and 630 nm, respectively.

RESULTS AND DISCUSSION

Although there exists voluminous literature on the separation of porphyrins, the isocratic separation of these compounds using traditional

chromatographic methods has not been successful. Consequently, cyclodextrin bonded phases were attempted to resolve the polycarboxylic porphyrins. However, the mobile phase design is difficult due to the solubility of porphyrins and the stability of the stationary phases. As a result of the previous studies (15, 17, 19), a multi-component mobile phase was developed. Some common problems related to the separation of various compounds using multi-solvent mobile phase systems in reversed phase high-performance liquid chromatography have been previously discussed (22-23). More importantly, the reproducibility of an isocratic separation depends significantly on the exact proportion of components in the mobile phase, especially a multi-component solvent system was used in this study. The reproducibility of retention of porphyrins could be enchanced by preparing the mobile phase exactly the same way each time as suggested in the 'Chromatographic Conditions.' The four components which could be mixed in different ways other than that reported in this study would result in precipitation of phosphate and 18-crown-6 ether in the mobile phase. In addition, it is necessary to equilibrate the column with the mobile phase for 10 min before running samples.

The effect of buffer concentration on the porphyrin retention was studied. The concentration of phosphate buffer in the mobile phase was limited by the solubility of phosphate in the system . A concentration of 60 mM was optimal to produce a good separation as shown in Figure 1. While a lower concentration (< 60 mM) of phosphate resulted in poor selectivity, a higher concentration of phosphate did not completely dissolve in the mobile phase. However, the presence of optimal amount of phosphate is crucial in producing a good partitioning of porphyrins , which could be achieved by optimizing the ionic strength of the buffer in the mobile phase. As related to the buffer strength, the effect of pH on the separation performance of porphyrins were studied. Changes in pH could change the solvent selectivity for porphyrins. However, at lower pH (< 6.37), coelution was a problem,

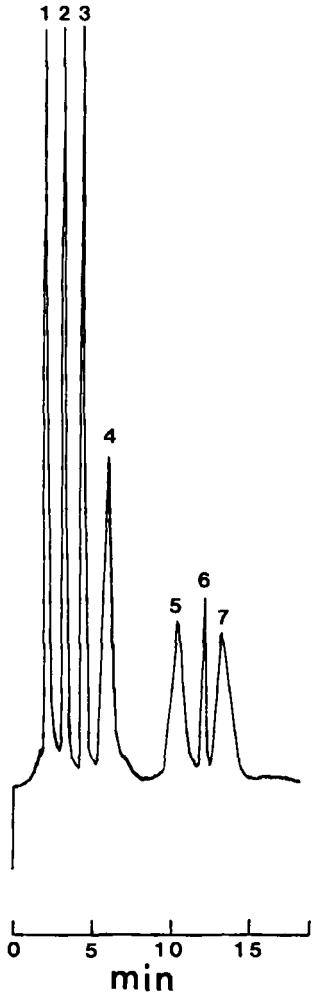

Figure 1 Chromatogram of porphyrin carboxylic acids . See ' Chromatographic Conditions ' for experimental details. Peaks : 1, uroporphyrin; 2, heptaporphyrin; 3, hexaporphyrin; 4, pentaporphyrin; 5, coproporphyrin; 6, mesoporphyrin; 7, protoporphyrin.

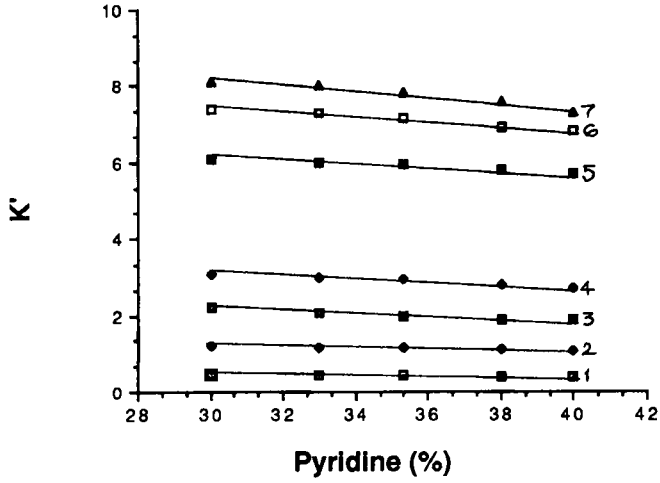

Figure 2 Effect of pyridine concentration on the capacity factors. Experimental conditions and labels are the same as in Figure 1.

while at higher pH, the solvent selectivity decreased. Peak tailing of porphyrin peaks was noticeable. However, the problem could be reduced by lowering the pH. Consequently, the separation performance was significantly improved. A pH of 6.37 appeared to be optimal for a good separation of the compounds (Figure 1).

The effect of pyridine on the selectivity was also studied. The partition efficiency of porphyrins could be increased by improving its solubility in the mobile phase. Pyridine was a very good solvent for porphyrins ; thus, it was used as an organic modifier in the solvent system to improve the separation performance. As predicted, the separation efficiency as measured by the capacity factor decreased as the concentration of pyridine concentration increased (Figure 2). Also, the increase in pyridine concentration resulted in poor selectivity, and coelution of porphyrins occured. An optimal pyridine concentration was 35.3 % by volume in both solvent systems with either acetone or acetonitrile as the main component (Table I).

TABLE I The Separation Performance of the Two Different Solvent Systems As Measured By the Selectivity (α), Capacity Factor (K') and Resolution (R).

Porphyrins	Acetone[a]			Acetonitrile[b]		
	K'	α	R	K'	α	R
Uroporphyrin	0.14			0.43		
		2.00	1.20		2.75	1.13
Heptaporphyrin	0.29			1.17		
		1.57	1.04		1.68	1.31
Hexaporphyrin	0.45			1.97		
		1.57	0.88		1.51	1.06
Pentaporphyrin	0.71			2.97		
		1.53	0.87		2.00	2.00
Coproporphyrin	1.10			5.92		
		1.54	1.17		1.20	1.09
Mesoporphyrin	1.70			7.13		
		1.12	0.80		1.10	0.75
Protoporphyrin	1.90			7.81		

a acetone : pyridine : phosphate : ether (48 : 29 : 5, v/v/v, 0.22 g ether).
b acetonitrile : pyridine : phosphate : ether (50 : 30 : 5, v/v/v, 0.28 g ether).

In addition, the effect of 18-crown-6 ether on the chromatographic performance was studied. The results (Figure 3) showed that the capacity factors decreased slightly with the increase of ether concentration. However, the elution strength of the mobile phase was practically unaffected by increasing the ether concentration. The solubility of 18-crown-6 ether in the solvent system is a limiting factor. Nevertheless, a suitable amount of 18-crown-6 ether was required to produce a good solvent selectivity. There was no separation between porphyrins without the addition of 18-crown-6 ether in the mobile phase. The presence of crown ether is essential to

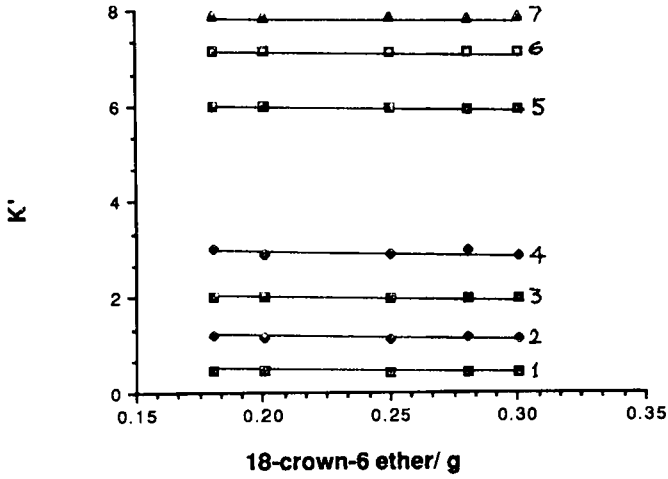

Figure 3 Effect of 18-crown-6 ether concentration on the capacity factors. Experimental conditions and labels are the same as in Figure 1.

facilitate an efficient partition of porphyrins in the mobile phase due to its stronger interactions with solutes. The interactions of crown ether significantly attribute to its ion complexing properties. Consequently, crown ether could more efficiently solubilize phosphate buffer by forming crown ether-phosphate ionophores in the mobile phase.

Finally, the effects of other common solvents on the separation performance were also studied (Figure 4). While phosphate buffer , crown ether and pyridine are the essential elements in the mobile phase, acetonitrile could be substituted by acetone to produce an acceptable solvent selectivity. Other solvents, such as tetrahydrofuran, methanol , were also used in the mobile phase. But the resulting solvent systems with either tetrahydrofuran or methanol led to coelution of porphyrins, and long retention time over an hour. The resulting solvent system containing acetone : pyridine : phosphate : crown ether (48 : 29 : 5, v/v/v, with 0.22 g crown

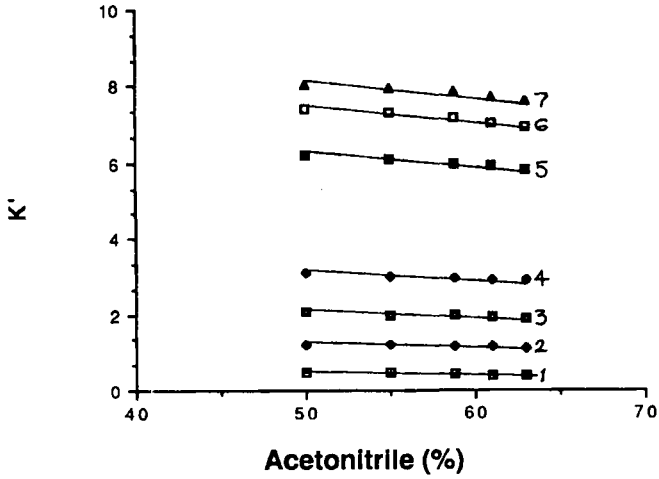

Figure 4 Effect of acetonitrile concentration on the capacity factors. Experimental conditions and labels are the same as in Figure 1.

ether) yielded the strongest elution strength. More significant changes in retention and selectivity could be brought about by a relative smaller changes in the volume composition of acetone than acetonitrile in the respective solvent systems (Table I). Acetone is more effective in changing the elution strength possibly due to its stronger interaction with porphyrins. Although acetonitrile could be used in place of acetone in the mobile phase , but at a different composition, to produce the base-line separation of porphyrins, it took much longer time (14 min) to complete the elution. Different flow rates and mobile phase composition with acetonitrile were experimented. The optimal separation performance with acetonitrile was obtained as shown in Figure 1. However, the mobile phase containing acetonitrile yielded the maximum changes in solvent selectivity and resolution between peaks. This allows us to achieve the necessary change in solvent selectivity without sacrificing resolution. It is especially useful for determining porphyrins in biological materials when interfering metabolites

become significant. Nevertheless, an alternative, more convenient approach is simply to use acetone in the mobile phase without trying the various changes in solvent composition, when there are no detectable interfering compounds. Although the mobile phase with acetonitrile took longer time to complete the elution, the different resolutions between porphyrin peaks allowed a wider range of selectivity changes for improving resolution. The acetonitrile-solvent system gives larger separation selectivity (α) values for a better separation and resolution between peaks.

In conclusion, the isocratic separation of seven clinically important porphyrins on cyclodextrin bonded phases via a multi-component solvent system has been demonstrated. The solvent selectivity could be controlled by changing the volume composition of either acetone or acetonitrile in the respective solvent systems. The retention of porphyrins is apparently governed by the number of carboxyl groups. But there is no direct correlation between the two parameters. Although the separation of porphyrins on cyclodextrin bonded phases is intrigued by different interactions between solutes and solvents, the separation performance could be improved when needed.

REFERENCES

1. Marriott, P.J., Gill,J.P., Evershed, R.P., Eglinton, G., and Maxwell, J.R., Capillary GC and GC-MS of porphyrins and metalloporphyrins, Chromatographia, 16, 304, 1982.

2. Friedmann, H.C. and Baldwin, E.T., Reverse-phase purification and silica gel TLC of porphyrin carboxylic acids, Anal. Biochem., 137, 473, 1984.

3. Petryka, Z.J., Watson,C.J., Separation and spectrodensitometric quantitation of porphyrin esters on thin-layer chromatograms, J. Chromatogr., 179, 143, 1979.

4. Huie, C.W. and Williams, W.R. , Laser fluorometric detection of porphyrin methyl esters for high-performance TLC, Anal. Chem., 61, 2292, 1989.

5. Schreiber, W.E., Raisys, V.A. and Labbe, R.F., Liquid-chromatographic profiles of urinary porphyrins, Clin. Chem. 29(3), 527, 1983.

6. Ho, J.W., Guthrie , R. and Tieckelmann, H., Detection of δ-aminolevulinic acid, porphobilinogen and porphyrins by HPLC with fluorometric measurement, J. Chromatogr., 375, 57, 1986.

7. Rideout, .M., Wright , D.J. and Lim, C.K., HPLC of uroporphyrin isomers, J. Liq. Chromatogr., 6(2), 383, 1983.

8. Meyer, H.D., Vogt, W. and Jacob, K., Improved separation and detection of free porphyrins by HPLC, J. Chromatogr., 290, 207, 1984.

9. Crowne, H., Lim, C.K. and Samson, D., Determination of 5-aminolaevulinic acid acid dehydrase activity in erythrocytes by HPLC, J. Chromatogr., 223, 421, 1981.

10. Lim, C.K. , Li , F. and Peters, T.J., HPLC of uroporphyrinogen and coproporphyrinogen isomers with amperometric detection, Biochem. J., 234, 629, 1986.

11. Ford, R.E. , Ou, C.N. and Ellefson, R.D., Liquid-chromatographic analysis for urinary porphyrins, Clin. Chem. 27(3), 397, 1981.

12. Hill, R.H.Jr., Bailey , S.L. and Needham, L.L., Development and utilization of a procedure for measuring urinary porphyrins by HPLC, J. Chromatogr., 232, 251, 1982.

13. Bizet, S., Delgado , O. and Brault, D., Purification of haematoporphyrin IX and its acetylated derivatives, J. Chromatogr., 283, 157, 1984.

14. Kotal, P., Porsch, B., Jirsa, M. , Kordac, V., Rapid isocratic HPLC of porphyrin esters on aminopropyl-bonded silica, J. Chromatogr., 333, 141, 1985.

15. Ho, J.W., Effects of mobile phase pH, ionic strength, and buffer composition on the separation of porphyrins by HPLC, LC.GC, 7 (4), 348, 1989.

16. Abe, K. and Konaka, R., Quantification of urinary porphyrins by liquid chromatography after oxidation of porphyrinogens, Clin. Chem. 35(8), 1619, 1989.

17. Ho, J.W., High-performance chromatographic determination of zinc-protoporphyrin and porphyrin carboxylic acids in urine, Anal. Biochem., 183, 134, 1989.

18. Gunter, E.W., Turner, W.E. and Huff, D.L., Investigation of protophyrin IX standard materials used in acid-extraction methods and a proposed correction for the millimolar absorptivity of protoporhyrin IX, Clin. Chem., 35(8), 1601, 1989.

19. Ho, J.W., Separation of porphyrins on cyclodextrin bonded phases with a novel mobile phase, J. Chromatogr. (in press).

20. Kennedy, S.W. and Maslen, A.L., Separation of porphyrin isomers by HPLC, J. Chromatogr., 493, 53, 1989.

21. Ho, J.W., Separation of zinc-protoporphyrin and porphyrin methyl esters by HPLC, J. Liq. Chromatogr., 12 (13), 2611, 1989.

22. Dolan, J.W., Mobile phase proportioning problems : A case study , LC.GC, 7(1), 18, 1989.

23. Coenegracht, P.M.J., Metting, H.J., Shilde, A.K. and Coenergracht-Lamers, P.J.M., A chronometric investigation of the selectivity of multisolvent mobile phase systems in RP-HPLC, Chromatographia, 27, 135, 1989.

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