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Note

Separation of porphyrins on cyclodextrin-bonded phases with a novel mobile phase

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The isolation and purification of tetrapyrrole compounds, such as porphyrins, is one of the more interesting and important areas of separation in chemistry and the related disciplines. Interest in substituted porphyrins arises from their widespread occurrence in geological samples, such as petroleum, shales, coals and biological materials. Geoporphyrins which are thought to derive mainly from precursor chlorophyll[1] usually complexes with other metal ions, *e.g.* manganese and iron[2], vanadyl and nickel[3], copper and gallium[4]. The separation and analysis of geoporphyrins are important to the study of deposition of sediments and the geology of environment, whereas the determination of different porphyrins in biological tissues is useful in diagnosis of disease states and abnormal metabolism due to environmental intoxication or genetic disorders. The complexity of porphyrins has prompted the development of various chromatographic techniques for the separation and analysis of synthetic and naturally occurring porphyrins. Carboxylated porphyrins are one of the more common tetrapyrrole compounds found in biological materials (Table I). They are characterized by the number of carboxyl groups at the substituted positions. Numerous improved methods for the separation of porphyrins, which include a normal-phase chromatography using an aminopropyl-bonded silica stationary phase with a binary eluent[5], reversed-phase chromatography[6–12], and thin-layer chromatography[13], have been described earlier. More recently, reports dealing with the retention behavior of the polycarboxylic porphyrins in reversed phase chromatography[14] and the separation of individual carboxylated porphyrins on silicone polymer-coated silica gel modified with octadedyl groups[15] have appeared. Although there exists voluminous literature on the separation of porphyrins, isocratic separation of carboxylated porphyrins has not been successful. However, the previous methods have led to significant contributions to the understanding of, and improvement on the separation of porphyrins.

Cyclodextrin-bonded phases (CDs) show strong separation capability via high-performance liquid chromatography (HPLC). Separations of solutes on cyclodextrins result mainly from the formation of inclusion complexes which are formed when solute molecules enter the cavity of the CDs. The ability of the solute molecule to

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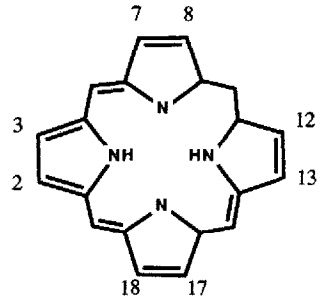
TABLE I

STRUCTURES OF PORPHYRINS



Polycarboxylic porphyrins	*Substituents[a] at position*							
	2	*3*	*7*	*8*	*12*	*13*	*17*	*18*
Uro-	A	P	A	P	A	P	A	P
Hepta-	M	P	A	P	A	P	P	A
Hexa-	M	P	M	P	A	P	P	A
Penta-	M	P	M	P	A	P	P	M
Copro-	M	P	M	P	M	P	M	P
Proto-	M	V	M	V	M	P	P	M
Meso-	M	E	M	E	M	P	P	M

[a] M = $-CH_3$; E = $-C_2H_5$; A = $-CH_2COOH$; P = $-CH_2CH_2COOH$; V = $-CH=CH_2$.

form an inclusion complex largely depends on the size, shape and chemical interactions between the solute molecule and the CDs. Other factors, such as Van der Waals forces and hydrogen bonding also affect the retention behavior of solutes. Different classes of compounds including carbohydrates and related molecules[16], optical, geometric and structural isomers[17–22], which are difficult to separate on reversed-phase columns, have been separated using CDs; thus, the separation of porphryins is attempted on the cyclodextrin bonded phases. However, the experiment is prone to some difficulty. The solubility and stability of polycarboxylic porphyrins are the major concerns in developing a chromatographic method.

Although the formation of inclusion complexes is generally accepted to be the basic property of CDs to effect the separation of different compounds, such separation mechanism is not favorable for porphyrins. The cavities of β-CDs are relatively hydrophobic and have an internal diameter of 7.8 Å (Fig. 1). This small cavity does not allow porphyrins with bulky side-chain substituents to form the inclusion complexes; thus, the separation of porphryin acids cannot be via inclusion complex formation, but may be based on adsorption of the solutes on the outside of CDs and hydrogen-bonding interaction between solutes and solvents. Recent studies of the chemical reaction of porphyrins with cyclodextrins[23] and the separation of carbohydrates on CDs[16] have demonstrated that CDs can retain compounds via adsorption rather than inclusion complex formation.

In the present study, a method for the simultaneous separation of polycarboxylic

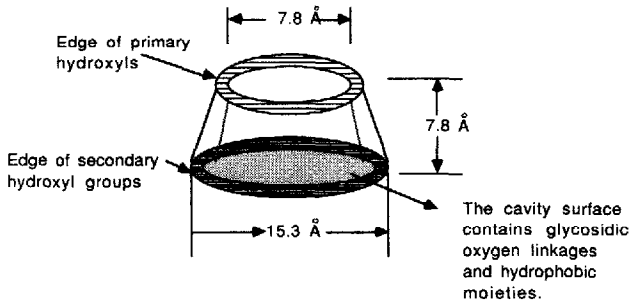


Fig. 1. The structure of β-cyclodextrin-bonded phase.

porphyrins on CDs with a novel mobile phase was described and the retention behavior of the seven porphyrins was investigated.

EXPERIMENTAL

Materials

All porphyrin acids were purchased from Porphyrin Products (Logan, UT, U.S.A.). Methanol, tetrahydrofuran, pyridine, acetonitrile and acetone (HPLC quality) were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Potassium phosphate and 18-crown-6 were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade.

Instrumentation

Experiments were performed on a Varian 5000 liquid chromatograph equipped with a Rheodyne 7126 injector fitted with a 10-μl sample loop. A β-cyclodextrin Cyclobond I™ column (25 cm × 4.6 mm I.D.) was employed for all the experiments in this study. The column was a product from Advanced Separation Technologies (Whippany, NJ, U.S.A.). The detector was a variable-wavelength fluorescence spectrophotometer Model 650-15 (Perkin-Elmer, CT, U.S.A.) with a 12-μl flow cell attachment. All chromatograms were recorded with a Hewlett-Packard 3388A integrator,

Preparation of porphyrin acid solutions

Amounts of 20 nmol of each of the porphyrin acids were dissolved in 2 ml of 2 *M* hydrochloric acid. The dissolution was complete with sonication. The compounds were stable in solution at 4°C.

Chromatography

The mobile phase was prepared by dissolving 0.22 g of 18-crown-6 ether in 48 ml of acetone (58.5% v/v) and followed by the addition of 29 ml of pyridine (35.4%, v/v) to the solution. Subsequently, 5 ml of 0.06 *M* potassium phosphate solution were mixed with the solution. The pH of the mobile phase was adjusted to 6.37 on a digital ionalyzer with a Ross combination pH electrode from Orion Research (Cambridge, MA, U.S.A.). The separation of porphyrin acids was carried out using isocratic elution

at a flow-rate of 1.1 ml/min at ambient temperature. The injection volume was 1 μl. The excitation and emission wavelengths were set at 405 and 630 nm, respectively.

RESULTS AND DISCUSSION

The isocratic separation of the seven porphyrin acids by liquid chromatography has been studied by conventional chromatographic techniques but without success. Since CDs have shown remarkable separation capability and chiral resolution, β-CD-bonded phase is employed to study the separation of porphyrins in an attempt to resolve the seven compounds simultaneously. Although CDs have been widely used for separating different compounds in liquid chromatography, the mobile phase design for separating porphyrins on CD stationary phases is challenging and depends on the stability and the solubility of porphyrins. The porphyrin acids are soluble in strong acids. Their isoelectric points or pH values of minimal solubility are in the range 3.0–4.5. The results of the earlier studies indicated that a pH of 5.3 was necessary for the complete separation of porphyrin acids by liquid chromatography[6,14]. Common organic solvents, such as methanol, acetonitrile, ethanol, dimethyl sulfoxide and dimethylformamide in phosphate buffer, were used to develop a binary mobile phase for the isocratic separation of porphyrins on CDs. But the experiments met with a limited success. Solutes were either retained or co-eluted. As a result of the previous studies[14], it was also suggested that the addition of an organic modifier, tetrahydrofuran, was more effective to change the solvent selctivity; thus, different ternary mobile phases

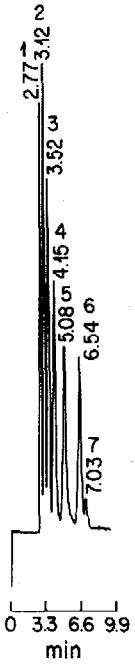


Fig. 2. Separation of porphyrin acids. Conditions were given in the Experimental section. Peaks: 1 = uroporphyrin; 2 = heptaporphyrin; 3 = hexaporphyrin; 4 = pentaporphyrin; 5 = coproporphyrin; 6 = mesoporphyrin; 7 = protoporphyrin.

were prepared from the previous methods with modifications[6,14]. Unfortunately, the ternary miobile phases produced poor retention and selectivity. Consequently, a mobile phase containing four components was developed for the isocratic separation of the porphyrin acids on the CD column. The chromatogram is shown in Fig. 2.

The influence of pyridine on retention was studied. The results showed that the capacity factors (k') decreased slightly with the increase of pyridine concentration as shown in Fig. 3. Overall retention changed only slightly for concentrations between 33 and 46% pyridine, accompanied by minor changes in selectivity. Pyridine was introduced into the mobile phase as the organic modifier to increase the solubility of porphyrin acids as well as to improve the mobile phase selectivity. It is understood that the carboxyl groups of porphyrins are strong proton donors, whereas both pyridine and the hydroxyl groups of the CDs are proton acceptors. But the hydroxyl group of CDs is a much weaker base than pyridine; thus, the hydroxyl group is not as good a proton acceptor as pyridine. Consequently, the acid–base interaction between pyridine and the porphyrin acids produced stronger hydrogen-bonding interaction, which resulted in increase in solubility, and also enhancing the preferential partition of porphyrins in the mobile phase. As a result, changes in pyridine concentration affected the elution strength of the mobile phase as observed.

The effect of crown ether on the retention of porphyrins was also studied. The results showed that the k' values decreased noticeably with the increase in crown ether concentration (Fig. 4). Although the elution strength was not significantly changed over the concentration range between 7 and 14 mM, the addition of crown ether in the mobile phase is essential to produce an acceptable solvent selectivity in this study. Crown ether is especially effective in that it contains both polar groups and the hydrophobic moieties. The presence of crown ether presumably facilitates a more efficient partition of porphyrins in the mobile phase due to its hydrophobic and polar interactions with the solutes. Furthermore, the addition of crown ether is necessary because earlier studies[6,14] have suggested that phosphate buffer is important in effecting the solubility and partitioning or porphyrins in the mobile phase. But phosphate buffer is only slightly soluble in the organic phase developed in this study. Nevertheless, crown ether can solubilize potassium phosphate in the non-polar organic phase by forming specific complexes with potassium cations.

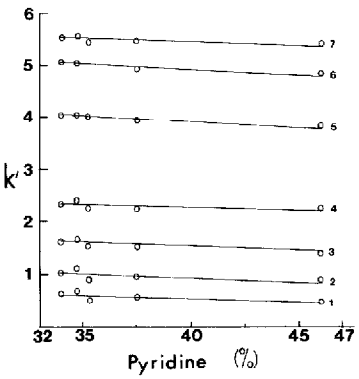


Fig. 3. Effects of pyridine content in the mobile phase on the capacity factors. Experimental conditions and labels are the same as in Fig. 2.

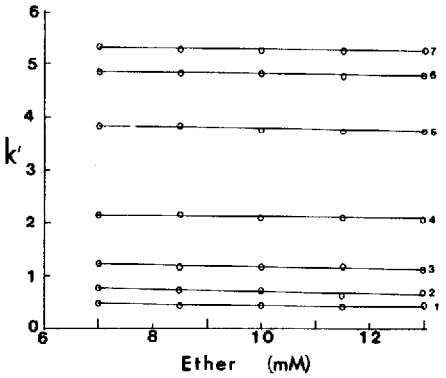


Fig. 4. Effects of crown ether (18-crown-6) in the mobile phase on the capacity factors. Experimental conditions and labels are the same as in Fig. 2.

As related to the formation of potassium cation–crown ether complexes, the influence of phosphate content on the retention and selectivity was also studied. The results showed that 0.06 *M* of phosphate solution was the optimal concentration to produce a good retention and selectivity as evidenced by the separation of porphyrins shown in Fig. 2. Higher phosphate concentration (> 0.06 *M*) would, however, produce precipitate in the mobile phase, whereas lower phosphate concentration would result in significant decrease in resolution among porphyrins.

In addition, the effect of acetone concentration on retention was studied (Fig. 5). The results indicated that the k' values showed marked but similar changes as the proportion of either acetone or pyridine was changed. However, significant changes in retention and selectivity could be obtained by altering the volume composition of acetone of the mobile phase. The results suggested that acetone has a greater affinity than porphyrins for the adsorption site. Consequently, an increase in acetone concentration reduces the interaction between solutes and CDs; thus, the k' values decrease as the volume composition of acetone increases. In addition, the present study showed that a relative smaller change in acetone concentration in the mobile phase resulted in greater changes in the k' values, suggesting that acetone was more effective in changing the elution strength of the mobile phase. The different porphyrin retentions appear to be based on the adsorption process through the acetone interactions with the carboxyl groups of porphyrins and CDs.

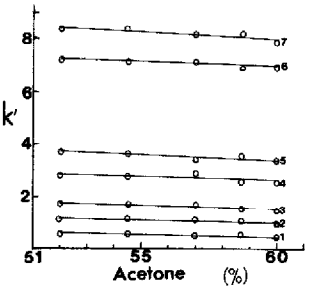


Fig. 5. Effects of acetone content in the mobile phase on the capacity factors. Experimental conditions and labels are the same as in Fig. 2.

CONCLUSIONS

The isocratic separation of porphyrin acids on β-CD-bonded phases with a novel mobile phase has been demonstrated. As the solvent selectivity for solutes becomes better characterized, it is possible to develop novel mobile phases for separating different compounds especially macromolecules. The results of this study have also shown that compounds with similar structures, such as mesoporphyrin and protoporphyrin bearing identical substituents except an additional double bond at the position three (Table I), can be easily separated. The retention of porphyrins is reasonably believed to be governed by an adsorption process on the outside of CDs. More polar porphyrins eluted before the less polar ones. It appears that the separation of porphyrins is closely related to the number of carboxyl groups on porphyrins. Although the separation of porphyrins on CDs is intrigued by various interactions between solutes and solvents, the results of the present study have demonstrated one other possible technique to resolve some macrocycles using CDs via adsorption process.

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