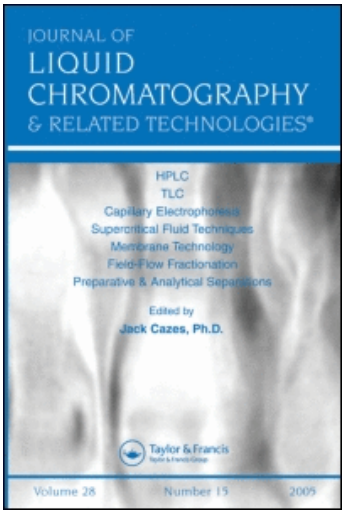

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ANALYSIS OF METALLOPORPHYRINS USING CYCLODEXTRIN STATIONARY PHASES WITH PHOTODIODE ARRAY UV DETECTION

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Abstract

A method for the high-performance liquid chromaographic separation of metalloporphyrins using cyclodextrin column with photodiode array UV detection is described. The isocratic separation of hemin, protoporphyrin IX, Mn protoporphyrin IX, cobalt protoporphyrin IX, Sn protoporphyrin IX, Zn protoporphyrin IX was achieved in less than 10 min using β-cyclodextrin stationary phases and a mobile phase consisted of a mixture of 28 ml of 5 mM ammonia solution and 90 ml of acetone (v/v, 28:90). The apparent pH of the mobile phase was 7.9. The chromatographic behavior of tin-protoporphyrin IX is markedly different from the transition metals-protoporphyrin IX in cyclodextrin column. The effects of ammonia concentration and the composition of the mobile phase were studied to optimize the separation of the compounds. The concentration of ammonia solution in the mobile phase affects the solvent strength and selectivity of the mobile phase.

Introduction

Porphyrins are the metabolites of heme biosynthesis. Different porphyrins represent different intermediate metabolites in the heme biosynthetic pathway. Zn-protoporphyrin is formed in heme biosynthesis and its concentration in body fluid is markedly different in lead poisoning and iron-deficiency anemia. Anemia is the major disorder related to low serum concentrations of vitamin B-12 (cyanocobalamin). Several

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other conditions manifest themselves as low serum vitamin B-12 content, including normal near-term pregnancy, vegetarianism, partial gastrectomy / ileal damage, oral contraception, parasitic competition, pancreatic deficiency, treated epilepsy, and advancing age (1-2). The biosynthesis of vitamin B-12, a member of the corrin family, shares the same precursors, 5-aminolevulinic acid and succinoyl CoA, of heme biosynthesis and is derived from uroporphyrinogen after a few step-wise reactions. Vitamin B-12 consists of a porphyrin-like ring system in which two of the four substituted pyrrole rings are connected directly with one another rather than through a methine bridging group (3). Other metalloporphyrins are linked to different biological functions and sources (4-5). Analysis of metalloporphyrins is useful in the diagnosis of disorders of heme biosynthesis and the other related manifestations. There are a few methods available for the determination of zinc-protoporphyrin (6-10) and other selected metalloporphyrins (11-15). The separation of metalloporphyrins by high-performance liquid chromatography (HPLC) is preferred because of its stronger separation capability. The lack of volatility of metalloporphyrins has made gas chromatographic work difficult. Studies of selected demetallated porphyrins and porphyrins in reversed-phase liquid chromatography on C_{18} columns have demonstrated its separation ability (6-10, 14-17). The demetallation procedure for metalloporphyrins yields an incomplete reaction and degradation of substituents on the porphyrin ring (15). Also, the separation of metalloporphyrins under acidic conditions results in demetallation. The present paper describes a novel HPLC method with photodiode array UV detection for the simultaneous determination of some common metalloporphyrins, namely, protoporphyrin complexes of Sn, Co, Mn, Fe, Zn together with some important porphyrins, protoporphyrin and coproporphyrin. The solvent strength is studied to characterize the separation performance .

Materials and methods

Protoporphyrin, coproporphyrin, hemin and metalloporphyrins (Sn, Co, Mn, Fe and Zn ion complexes) were purchased from Porphyrin Products, Inc. (Logan, UT). Acetone (HPLC quality) was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Ammonia solution was obtained from Sigma Chemical Co. (St. Louis, Mo). All other reagents were of analytical grade.

Apparatus

A modular HPLC system equipped with a Rheodyne 7125 injector fitted with a 20-μl sample loop was used. Separations were made on a β-cyclodextrin cyclobond I™ column (25 cm x 4.6 mm I.D.). The column was a product from Advanced Separation Technologies (Whippany, NJ, USA) The detection system included a Waters Model 990 photodiode array detector equipped with a 8 μl flow cell attachment. All the measurements were recorded with the Waters 990 data processing system.

Preparation of standards

An amount of 150 nmol of each of the porphyrin and metalloporphyrins of Fe, Co, Zn, Sn and Mn ions were dissolved in 1 ml of 1 M ammonia solution. The dissolution was complete with sonication. The compounds were stable under refrigeration.

Chromatographic conditions

The mobile phase consisted of a mixture of 23.7% of 5mM ammonia solution and 76.3% of acetone by volume. The pH of the mobile phase was 7.9. The separation of metalloporphyrins and the demetallated porphyrins was carried out using isocratic elution

at a flow rate of 1 ml/min at ambient temperature. The injection volume was 2 μl. The UV absorbance of the elution profile of analytes was recorded at 400 nm for all measurements.

Results and discussion

The simultaneous determination of five metalloporphyrins has not been reported before. The present study reports the isocratic separation of five metalloporphyrins and the related porphyrins by liquid chromatography. The retention behavior of the metalloporphyrins on β-cyclodextrin column was studied with a wide range of solvent-based selectivity. Various combinations and compositions of traditional HPLC solvents, such as tetrahydrofuran, methanol, acetonitrile, acetone, ethanol, propanol, pyridine, with different aqueous buffer solutions including phosphate and acetate solutions, were used to develop the HPLC method but with little success. A β-cyclodextrin column was used with modifications of the earlier method for the separation of porphyrins (18). Although the elution strength and selectivity of the mobile phase is strong for demetallated porphyrins, metalloporphyrins are retained for a long period of time without practically any resolution.

Stability of β-cyclodextrin stationary phases and metalloporphyrins, as well as the solubility of porphyrins are of important considerations for developing the HPLC methods. Among the solvents tested, pyridine, 1M ammonia solution or 1M NaOH solution could readily dissolve the porphyrins. Acidic solution dissociates the metallated complex to form the corresponding metal ions and porphyrin ligand. Fortunately, the three alkaline solvents mixed with other HPLC solvents apparently show little effects on the stability of β-cyclodextrin stationary phases. After a series of experiments, we found that a mixture of acetone and pyridine as the mobile phase displayed a weak solvent

strength on the metallated porphyrins and other porphyrins on β-cyclodextrin column, and there was practical no separation among metalloporphyrins. Subsequently, NaOH solution was used in place of pyridine. The overall resolution among metalloporphyrins was improved but the selectivity of the mobile phase was dissatisfactory.

In a similar approach, NH_4OH was used in the mobile phase. Ammonia hydroxide solution allowed a good dissolution of the porphyrins and the pH of the resulting mobile phase was readily changed to the desired value. The variation of NH_4OH concentration in the mobile phase significantly affects the solvent selectivity (Figure 1). Due to the weak absorptivity, a larger amount of Fe-protoporphyrin IX was required to produce an equivalent peak size. The solvent selectivity of the mobile phase for Mn-, Co- protoporphyrin and coproporphyrin is limited at higher concentration of NH_4OH. And the resolution among coproporphyrin, cobalt protoporphyrin IX and manganese protoporphyrin IX were practically unaffected by the change of NH_4OH solution. The elution strength of the mobile phase for the three analytes was apparantly the same. However, the mobile phase selectivity for other metallated porphyrins (Zn, Fe) was improved at lower concentration of ammonia hydroxide solution and peaks of metalloporphyrins were better resolved at lower concentration of NH_4OH (< 26%). The retention time of the two analytes was significantly changed with the concentration of NH_4OH in practice.

It is worth noting that Tin-protoporphyrin IX behaved quite differently from the other metalloporphyrins in the β-cyclodextrin column. Its chromatographic behavior was quite predictable in a linear manner at a higher concentration of NH_4OH (> 26%). The compound was retained for relatively longer period of time when NH_4OH concentration was < 26%. When NH_4OH concentration went below 24%, Sn-protoporphyrin IX was

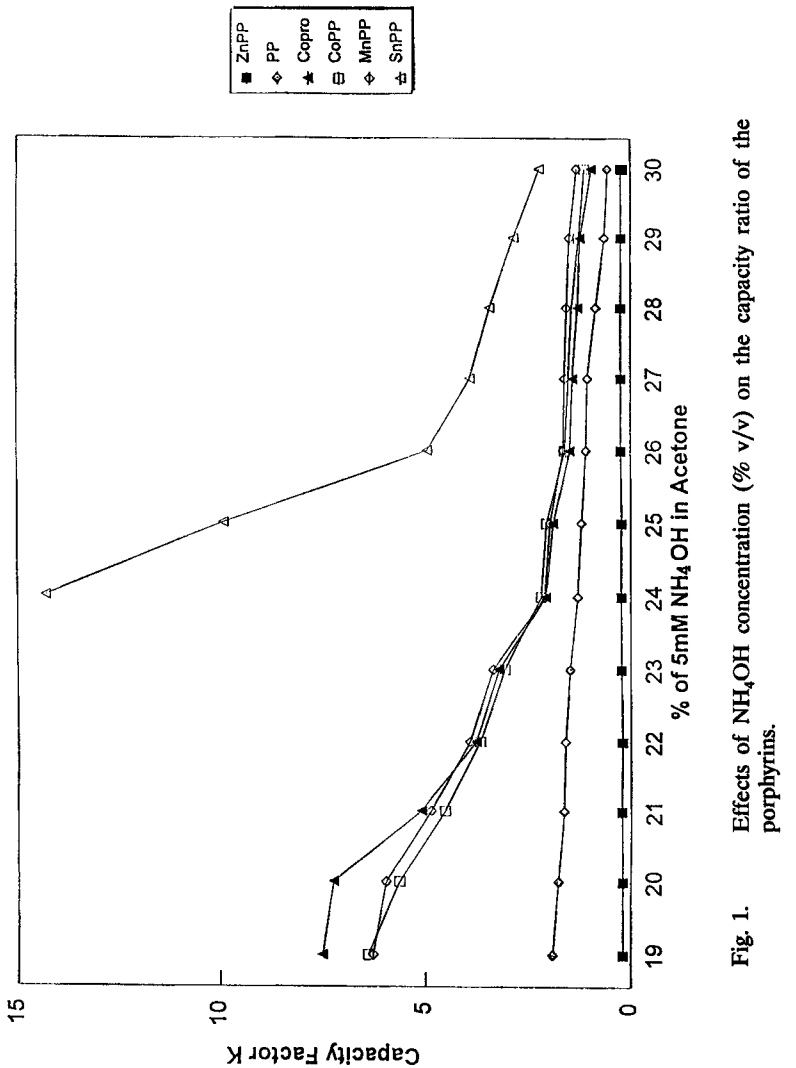

Fig. 1. Effects of NH_4OH concentration (% v/v) on the capacity ratio of the porphyrins.

Table I Absorption maximum and separation parameters of metalloporphyrins & porphyrins

Porphyrin	Retention time		Wavelength of maximum absorption	Colour of solution	Capacity Factor K	
	(90:25)*	(90:30)*			(90:25)*	(90:30)*
ZnPP	1.70	1.69	400 nm	Light Brown	0.14	0.13
PP	3.21	2.20	404 nm	Dark Brown	1.15	0.48
Copro	6.48	2.95	408 nm	Purple Red	3.35	0.98
Uro	37.13	32.10	409 nm	Bright Red	23.90	20.54
CoPP	5.52	4.04	423 nm	Bright Red	2.71	1.71
MnPP	5.46	3.80	373 nm	Dark Brown	2.66	1.55
SnPP	29.50	27.52	403 nm	Purple	18.80	17.47
FePP	10.21,38.56	4.00,27.94	400 nm	Dark Green	5.85,24.8	1.68,17.75
Air	1.49	1.49				

*Mobile phase composition (% v/v) = Acetone : 2 mM NH_3

*The composition of mobile phases cannot cleanly separate the porphyrins. There are some overlapping of peaks. However, the resolution among peaks can be improved by changing the percentage composition of NH_4OH by volume in the mobile phase.

practically retained in the column. The retention time of Sn-protoporphyrin IX was generally longer than that of any other porphyrins used in this study, and peak broadening of Sn-protoporphyrin IX was noticeable. With Sn ions, protoporphyrin IX seemingly interacts more strongly with the β-cyclodextrin stationary phases.

Effort has been made to use diluted base for dissolution of metalloporphyrins and the preparation of the mobile phase. The experimental results show that the β-cyclodextrin stationary phases are quite stable under this condition in all our experiments. Previous study has shown the effects of alkaline injection solvents on the elution order of metalloporphyrins (19). However, with the diluted base of fixed alkalinity as the injection solvent, the elution order of metalloporphyrins and porphyrins could well be predicted without incidents.

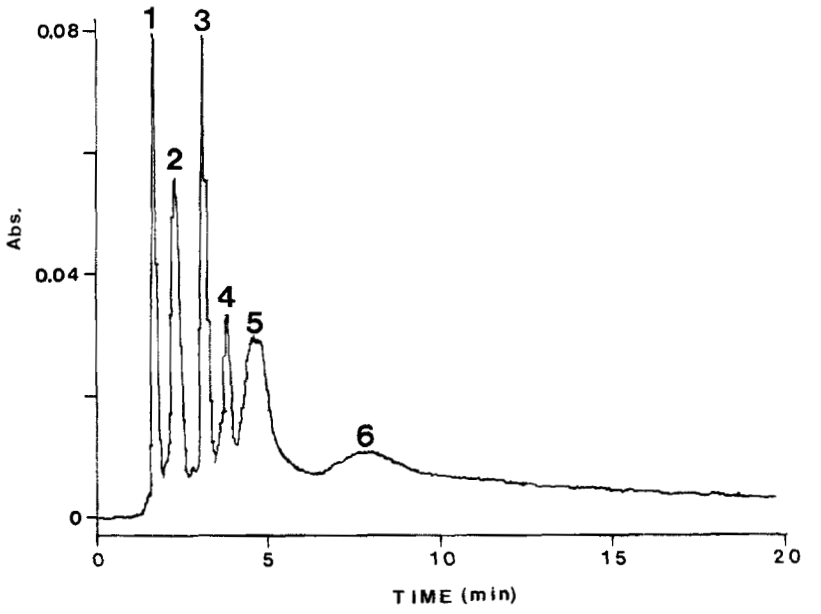

Fig. 2. Chromatogram of metalloporphyrins and porphyrins. See chromatographic conditions for experimental details. Peaks : 1, Zn-protoporphyrin IX; 2, protoporphyrin IX; 3, Coproporphyrin; 4, Co-protoporphyrin IX; 5, Mn-protoporphyrin IX; 6, Sn-Protoporphyrin IX.

The absorption characteristics of the metalloporphyrins and porphyrins used in the present study were listed in Table I. Under the present chromatographic conditions, all analytes display an absorption maximum close to 400 nm. An amount of sub-nano gram of each porphyrin, except Fe-protoporphyrin, is sufficient for quantitative analysis in each experiment. A typical chromatogram of the porphyrins and metalloporphyrins is shown in Figure 2. The HPLC method is sensitive and efficient, and it is unique in the way that a relatively strong base is used as the component in the mobile phase for isocratic separation of the porphyrins on the β-cyclodextrin column. The present method is useful for the analysis of metalloporphyrins and some important porphyrins.

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