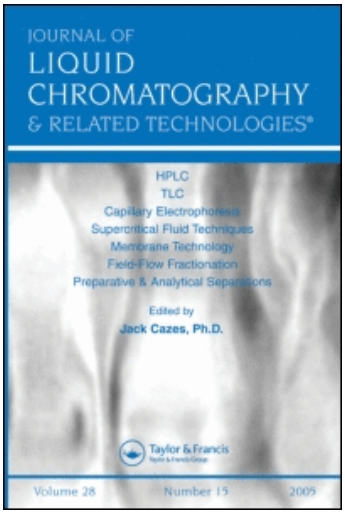


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# Effects of Injection Solvents on the Separation of Porphyrin and Metalloporphyrin in Reversed-Phase Liquid Chromatography

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# EFFECTS OF INJECTION SOLVENTS ON THE SEPARATION OF PORPHYRIN AND METALLOPORPHYRIN IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

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## ABSTRACT

This study reports differences in the chromatographic behavior of protoporphyrin and zinc protoporphyrin in reversed-phase liquid chromatography. The retention parameters of individual porphyrins using different mobile phases and a reversed-phase column were compared. Various solvents were used to dissolve protoporphyrin and zinc protoporphyrin. The solvent effects on the retention characteristics of the porphyrin and metalloporphyrin were studied. The elution order of porphyrins on the reversed-phase column was predictable. In contrast, the elution of zinc protoporphyrin could be significantly changed or reversed with respect to protoporphyrin peak, when a strong base was used as an injection solvent. Also, the retention times of protoporphyrin and zinc protoporphyrin were shifted significantly with different injection solvents. The shifting in retention time of zinc protoporphyrin is more noticeable, and the protoporphyrin peak is remarkably broadened with more basic solvents. The solubility of protoporphyrin and zinc protoporphyrin varies considerably with the injection solvents tested.

## INTRODUCTION

Porphyrins are the common tetrapyrrole compounds found in biological materials. Polycarboxylated porphyrins are the intermediary metabolites of heme biosynthesis.

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Through the continuous decarboxylation of the first uroporphyrinogen III, different porphyrins are formed. Cobyrinic acid, a known precursor of vitamin $B_{12}$, is biosynthesized from uroporphyrinogen III by a multistep process (1). Protoporphyrinogen IX which is formed by decarboxylation of coproporphyrinogen III. Chelates with iron (II) form heme or hematin if the iron is in the ferric state. Heme and hematin are the most important molecules biochemically. Heme serves as coenzyme for proteins involved in the transfer of oxygen from one site in the organism to another, for enzymes catalyzing a variety of oxidation reactions, and for enzymes catalyzing the cleavage of peroxides. An example includes human hemoglobins, a class of proteins of distinct molecular structure that perform the important function of transporting oxygen from the lungs to various tissues in the body in which oxidative metabolism occurs. Porphyrins also form metal chelates with a variety of metal ions, including those of magnesium, iron, zinc, nickel, cobalt, copper and silver. In such chelates, the metal ion lies in the center of the porphyrin nucleus, four of its ligand sites being occupied by the pyrrole nitrogen atoms. For example, chlorophyll, a magnesium chelate of a substituted porphyrin, is intimately involved in the photosynthetic process. In human lead poisoning and iron-deficiency anemia, protoporphyrin chelates with zinc ions and is present as zinc protoporphyrin in biological tissues (2-3). Analysis of excreted intermediary metabolites of heme biosynthesis and of zinc protoporphyrin is useful as a test for lead poisoning and iron deficiency anemia, and to determine the effect of environmental intoxification on heme biosynthesis.

Determinations of different porphyrins have been reported. In recent years, high performance liquid chromatography (HPLC) has become the preferred technique. In particular, reversed-phase HPLC is more efficient and allows quantitative determination of porphyrins. Three variables characterizing the mobile phase composition, pH, elution

strength and ionic strength for the separation of porphyrins using reversed-phase column, have been studied previously (4-5). The chromatographic behavior of metal-free porphyrins is well characterized. The retention increases as the number of side-chain alkyl substituents increases. Thus, the elution order of porphyrins follows the polarity in reversed-phase liquid chromatography. On the other hand, the chromatographic behavior of metalloporphyrin is not fully understood. Although the determination of metalloporphyrins have been reported previously (6-7), the effects of injection solvents on porphyrins have not been studied.



The present paper describes a reversed-phase HPLC method with a photodiode array UV detector for the study of the chromatography of protoporphyrin and zinc-protoporphyrin. The mobile phase composition and various injection solvents are used to examine the separation performance of protoporphyrin and zinc protoporphyrin.

## Experimental

### Materials

Protoporphyrin IX and zinc protoporphyrin were purchased from Sigma Chemical Company (St. Louis, MO, USA) and Porphyrin Products, Inc. (Logan, UT), respectively. Tetrahydrofuran and methanol (HPLC quality) were purchased from Sigma Chemicals (MO, USA). All other reagents were of analytical grade.

### Apparatus

Experiments were performed on a modular Waters HPLC system equipped with a Rheodyne 7125 injector fitted with a 20-$\mu$l sample loop. Separations were made on a Silica gel C18 analytical column (Isco, 4.6 mm x 25 cm). The eluent was monitored with a photodiode array detector (Waters Model 990) with a variable wavelength ranging

from 190 nm to 600 nm equipped with a 8 μl flow cell attachment. All the measurements were recorded and integrated by the Waters 990 data processing system.

Preparation of protoporphyrin IX and zinc protoporphyrin solutions

Protoporphyrin (1.65 μmol) and zinc-protoporphyrin (1.49 μmol) were separately dissolved in 1 ml of each of the following injection solvents : pyridine, $NH_4OH$ (0.5 M), $NH_4NO_3$, methyl isobutyl ketone, the mobile phases, ethyl acetate, chloroform and sodium hydroxide. The dissolution was made with sonication.



Chromatographic Conditions

Two mobile phases using either acetate or phosphate as buffer were prepared. The first reversed-phase system contained methanol-tetraphydrofuran - 0.1M phosphate buffer (18:30:20, V/V/V), the apparent pH is 5.38, and the second mobile phase system consisted of 22 mM acetate buffer, tetrahydrofuran and methanol (11:15:6, V/V/V), the apparent pH is 6.74. All the pH values were measured by a digital ionalyzer with a pH electrode (Corning, USA). The isocratic method was employed for all the experiments at a flow-rate of 1 ml/min at ambient temperature. The injection volume was 20 μl. The absorbance was monitored at 400 nm during the whole experiment.

Results and discussion

The injection solvent effects on the separation performance of protoporphyrin and zinc protoporphyrin were investigated in the present study. The common variables characterizing the mobile phase composition, pH, elution strength and ionic strength have been studied previously (4). The retention of porphyrins is predictable with respect to the elution order when reversed-phase liquid chromatography is employed for the

separation. Thus, it is known that the retention is governed by the number of carboxyl groups of porphyrins. Protoporphyrin is among the least carboxylated porphyrin. Therefore, it is usually retained for a longer period of time under reversed-phase condition. On the other hand, the retention of metalloporphyrin has not been studied. Much attention is paid to the chemistry and chromatography of other metal-free porphyrins. Zinc protoporphyrin is one of the more important biological molecules and could be used an indicator for clinical test and confirmative diagnosis of porphyrin-related diseases and the abnormality of heme biosynthesis. The previous studies have reported the unusual chromatographic behaviour of zinc protoporphyrin (6-7). However, the retention of metalloporphyrins is not understood.



Zinc protoporphyrin is protoporphyrin chelated with zinc metal ion in the center of the porphyrin nucleus. The retention of zinc protoporphyrin is, therefore, expected to be similar to protoporphyrin chromatographically. However, under reversed-phase condition, metal-free protoporphyrin peak broadening and tailing is noticeable. The elution profile of metallopoorphyrin shows a sharp and symmetrical peak under the same reversed-phase chromatographic conditions (4,6). The smaller particle size (5 $\mu$m) of column packings improved the peak shape of protoporphyrin and zinc protoporphyrin. A typical chromatogram is shown in Figure 1. Both mobile phases used in this study, phosphate or acetate buffer, affect the elution profile or peak shape by about the same extent.

While the retention of protoporphyrin can be predicted under a given condition, the retention of metalloporphyrin is not easily followed. The effects of injection solvents on metal-free porphyrins have not been observed, but the retention of metalloporphyrin was significantly changed (6); thus, various solvents were used to prepare protoporphyrin and zinc protoporphyrin solutions for the study. About 20 $\mu$l of each of the solutions

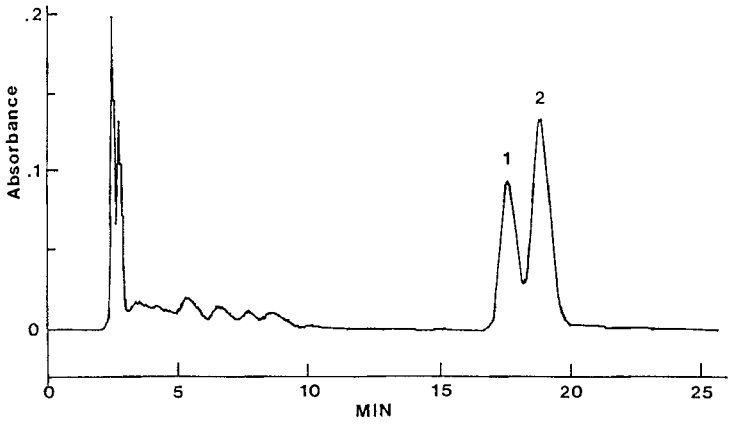


Figure 1 Chromatogram of protoporphyrin (1) and zinc protoporphyrin (2) standards. See chromatographic conditions for experimental details. The injection solvent is pyridine.



were injected onto the HPLC column for analysis. The retention times are summaried in Table I. The solubility of the two compounds varies remarkably. Protoporphyrin is more soluble than zinc protoporphyrin in the various solvents tested. Zinc protoporphyrin was sparingly soluble in ammonium nitrate, methyl isobutyl ketone or ethyl acetate. Hence, there are no retention data available with these solvents.

The influence of ionic strength using sodium phosphate or acetate buffer on retention was studied in the pH range which minimizes the dissociation of zinc protoporphyrin. Two phosphate buffers at different concentrations and one acetate buffer were used to study the retention of protoporphyrin and zinc protoporphyrin. The concentration of the organic modifier, THF, was slightly elevated to facilitate a convenient retention time when 0.1 M phosphate buffer was used. However, the change of THF concentration did not affect the elution order of the compounds. The effects of ionic strength on the retention of protoporphyrin have been reported earlier (6). The retention increases with the ionic strength. The back pressure also increases considerably

Table I

Retention times of protoporphyrin and zinc protoporphyrin

| Injection solvents | Retention time (min) phosphate buffer (a) | | phosphate buffer (b) | | acetate buffer (c) | |
|---|---|---|---|---|---|---|
| | P | Z | P | Z | P | Z |
| Pyridine | 5.60 | 5.09 | 16.98 | 18.18* | 6.05 | 4.68 |
| $NH_4OH$ | 5.23 | 5.86 | 18.22 | 19.62 | 5.60 | 6.08 |
| $NH_4NO_3$ | 6.27 | -[e] | 17.06 | -[e] | 6.76 | -[e] |
| Methyl isobutyl Ketone | 5.50 | -[e] | 18.92 | -[e] | 7.59 | -[e] |
| Ethylacetate | 5.71 | -[e] | -[e] | -[e] | 6.49 | -[e] |
| Chloroform | 5.70 | 5.60 | --[f] | --[f] | 8.06 | 5.76 |
| NaOH | 4.98 | 3.67 | 15.55 | 18.03 | 4.44 | 5.06 |
| Mobile phases[d] | 5.90 | 5.40 | 16.44 | 18.56** | 7.54 | 6.64 |

[a] Eluents : 0.05 M phosphate-methanol-THF (18:30:16).

[b] Eluents : 0.1 M phosphate-methanol-THF (18:30:20).

[c] Eluents : 22 mM acetate-methanol-THF (11:15:6).

[d] The corresponding phosphate or acetate-containing mobile phases

[e] Zinc protoporphyrin is insoluble

[f] not available

P = protoporphyrin

Z = Zinc protoporphyrin

* Pyridine-meOH - 22.51 (Z); 19.36 (P)

** $OAc^-$ - Mobile phase : 16.83 min

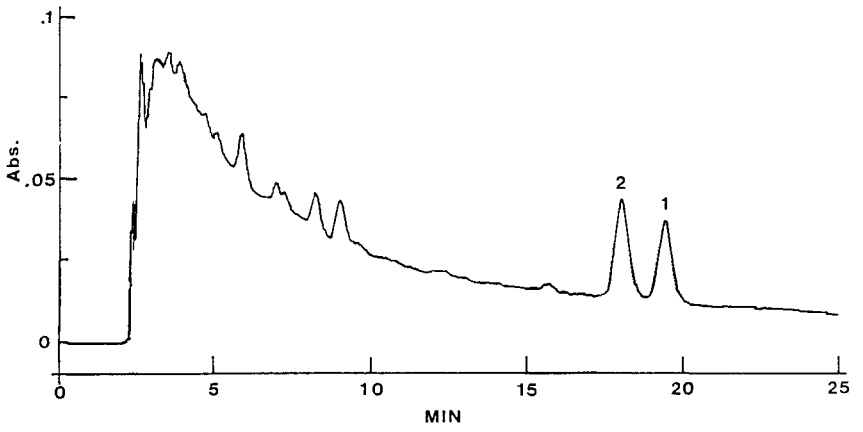


Figure 2 Chromatogram of protoporphyrin (1) and zinc protoporphyrin (2) standards. See chromatographic conditions for experimental details. The injection solvent is $NH_4OH$.



at higher ionic strength. The protoporphyrin peak is broadened more noticeably than that of zinc protoporphyrin. The result suggests a more efficient partition of zinc protoporyhyrin in the mobile phase. The more pronounced effect of ionic strength is shown on the retention of zinc protoporphyrin.

Among the injection solvents tested, NaOH and $NH_4OH$ show an unusual influence on the retention of zinc protoporphyrin. Essentially, the elution order of zinc protoporphyrin and protoporphyrin was reversed with a strong base as an injection solvent. In contrast, protoporphyrin came off the column first before zinc protoporphyrin with the any other injection solvents tested in the study. Nevertheless, when the ionic strength increases, the shifting in retention of zinc protoporphyrin becomes less affected. Consequently, the elution order of protoporphyrin and zinc protoporphyrin remained the same with phosphate buffer at concentration of about 0.1 M. The reversal of elution

order is only effective at lower ionic strength with buffer concentration < 0.05 M and with $NH_4OH$ or NaOH as the injection solvent. Similarly, the mobile phase with acetate buffer also affects the elution order when a strong base is used as an injection solvent (Table 1). Furthermore, NaOH produced a high background during the elution (Figure 2). Therefore, it is not recommanded as an injection solvent for real sample analysis.

## Conclusions

There is a significant effect of injection solvent on the retention of zinc protoporphyrin when a strong base, such as ammonium hydroxide and sodium hydroxide, is used as an injection solvent. Moreover, the ionic strength of a buffer at a lower concentration (< 0.05M) coupled with a strong base as an injection solvent would retain zinc protoporphyrin for a shorter period of time than metal-free protoporphyrin. Consequently, the elution order of protoporphyrin and zinc protoporphyrin appeared to be reversed under this condition. The solvent effect is an important consideration for clinical testing or analysis of metalloporphyrin, which requires an alkaline solvent media for an efficient extraction. Although the mobile phases tested in this study could be used as injection solvents, the solubility of porphyrins is a consideration. Therefore, a weak base (solvent) and an appropriate ionic strength of a buffer solution should be used for the determination of zinc protoporphyrin and protoporphyrin. Also, the retention of metalloporphyrins is worth further study.

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