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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DICARBOXYLIC PORPHYRINS AND METALLOPORPHYRINS: RETENTION BEHAVIOUR AND BIOMEDICAL APPLICATIONS

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## SUMMARY

The retention behaviour of the main dicarboxylic porphyrins, haemato-, deutero-, meso- and protoporphyrins, together with the Fe, Co, Cu and Zn complexes of meso- and protoporphyrins have been systematically studied. Hydrophobic chromatography with methanol–ammomium acetate buffer systems on reversed-phase columns provided the best selectivity, efficiency and resolution.

The retention of the porphyrins is controlled by the relative hydrophobicity of the porphyrin side-chain substituents. The insertion of a metal ion into the porphyrin macrocycle, however, completely alters the electronic environment around the central nitrogen atoms of the porphyrins. The retention is then greatly influenced by the species of inserted metal ion, to accept axial ligands from the mobile phase, although hydrophobic interaction of the side-chain substituents with the stationary phase surface is still an important factor. The retention behaviour can be precisely controlled by adjusting the pH, buffer concentrations and types and proportions of organic solvents in the mobile phase. The analysis of porphyrins and metalloporphyrins in blood, and the determination of ferrochelatase in bone marrow are examples of biomedical applications.

## INTRODUCTION

The dicarboxylic porphyrins are the immediate precursors of the most important naturally occurring metalloporphyrins. Iron complexes form the prosthetic groups of the various haemoproteins, magnesium complexes are found in the chlorophylls, and vitamin $B_{12}$ is a cobalt complex.

The retention behaviour of porphyrins with higher numbers of side-chain carboxylic acid substituents has been reported[1–4] but the behaviour of dicarboxylic porphyrins and metalloporphyrins in reversed-phase chromatography had not been studied. However, separations of some dicarboxylic porphyrins and metalloporphyrins have been described[5–7].

We are interested in developinmg simple high-performance liquid chromatographic (HPLC) methods for the analysis of dicarboxylic acid porphyrins and me-

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talloporphyrins in clinical specimens and for assaying ferrochelatase by using mesoporphyrin and cobalt as the enzyme substrates. The retention behaviour of the dicarboxylic porphyrins, haemato-, deutero-, meso- and protoporphyrins (Fig. 1), together with Co, Fe, Zn and Cu complexes of meso- and protoporphyrins were therefore systematically studied in order to provide optimization of a particular separation according to the nature of the application required.

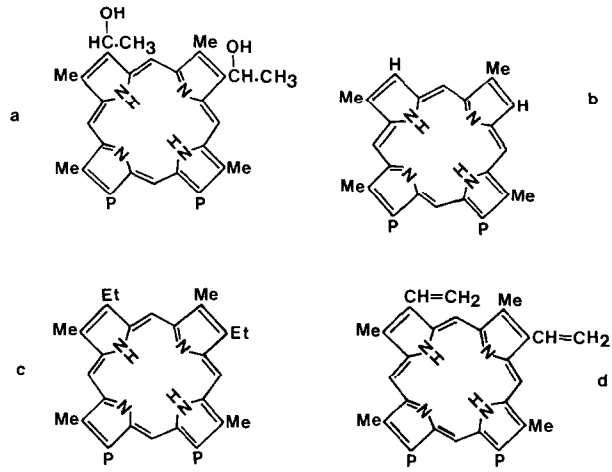


Fig. 1. Structures of dicarboxylic porphyrins. (a) Haematoporphyrin, (b) deuteroporphyrin, (c) mesoporphyrin and (d) protoporphyrin. P = $CH_2CH_2COOH$; Me = $CH_3$.

## EXPERIMENTAL

### *Materials and reagents*

Haemin, haemato-, deutero-, meso- and protoporphyrins were from Sigma (Poole, U.K.). The metal omplexes were prepared according to published procedures[8].

Ammonium acetate and glacial acetic acid were AnalaR grade from BDH (Poole, U.K.). Methanol and acetonitrile were HPLC grade from Rathburn Chemicals (Walkerburn, U.K.).

### *HPLC*

A Varian Assoc. (Walnut Creek, CA, U.S.A.) Model 5000 liquid chromatograph was used with a Varian UV-100 variable-wavelength detector, set at 404 nm, or a Perkin-Elmer (Beaconsfield, U.K.) LS-3 fluorescence detector, set at an excitation and an emission wavelength of 404 and 618 nm, respectively. A Rheodyne (Berkeley, CA, U.S.A.) 7125 injector was used for sample injection.

The columns (15 cm × 5 mm or 25 cm × 5 mm I.D.) were SAS-Hypersil (5-$\mu$m spherical silica, chemically bonded with a monolayer of trimethylsilyl groups) from Shandon Southern Products (Runcorn, U.K.).

The mobile phases used in the present study were 0.1–1 *M* ammonium acetate (pH 4.6–6.85), containing various proportions of methanol or acetonitrile. The flow-rate was 1 ml/min.

## RESULTS AND DISCUSSIONS

### *HPLC of dicarboxylic porphyrins*

The separation of the dicarboxylic porphyrins on a 15 cm × 5 mm SAS-column ($C_1$) with 70% methanol in 1 *M* ammonium acetate pH 5.16 as the eluent is shown in Fig. 2. The elution order of haemato-, deutero-, meso- and protoporphyrins is that expected for hydrophobic interaction between the side-chain substituents and the hydrocarbonaceous stationary phase surface with the relatively hydrophilic haematoporphyrin, the first, and the hydrophobic protoporphyrin, the last to be eluted.

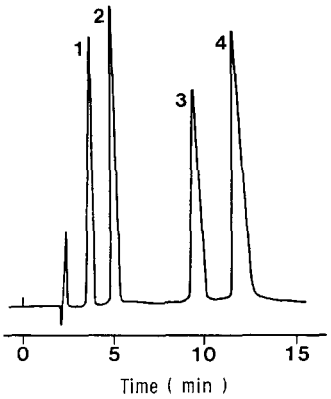


Fig. 2. Separation of dicarboxylic porphyrins. Column, 15 cm × 5 mm SAS-Hypersil ($C_1$); eluent, 70% methanol in 1 *M* ammonium acetate (pH 5.16); detector, UV 404 nm. Peaks: 1 = haematoporphyrin; 2 = deuteroporphyrin; 3 = mesoporphyrin; 4 = protoporphyrin.

The relative hydrophobicity of porphyrins can also be expressed in terms of the acid (HCl) numbers[9]. The HCl number of a porphyrin is defined as that concentration hydrochloric acid (%, w/v) which, from an equal volume of an ether solution of the porphyrin, extracts two thirds of the porphyrin. These numbers are a measure of the dissociation of the porphyrins as a base and the ether–water partition coefficient, and therefore, of the hydrophobicity of the porphyrins.

The HCl numbers of haemato-, deutero, meso- and protoporphyrins are 0.1, 0.3, 0.5 and 2.5 respectively[9]. Fig. 3 shows the relationships between the HCl numbers of the dicarboxylic porphyrins and the capacity ratio ($k'$) values. The plot is useful, since HCl numbers are extensively used for the selective extraction of porphyrins from biological materials. The graph should allow reasonable prediction of HCl numbers and hydrophobicity of unknown porphyrins, important for their isolation and characterization.

### *HPLC of metallo-mesoporphyrin and metallo-protoporphyrin*

The metalloporphyrins are formed by the coordination of metal ions to the two lone electron pairs on the pyrrolenine nitrogen atoms of free acid porphyrins with the concurrent replacement of two hydrogen atoms of the pyrrole nitrogen atoms (Fig. 4). Divalent metals are used in this study. The metal ions act as Lewis acids.

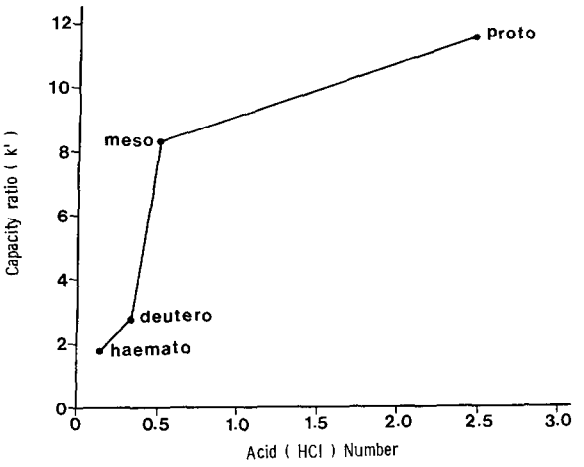


Fig. 3. Relationships between capacity ratios ($k'$) of dicarboxylic porphyrins and acid (HCl) numbers.

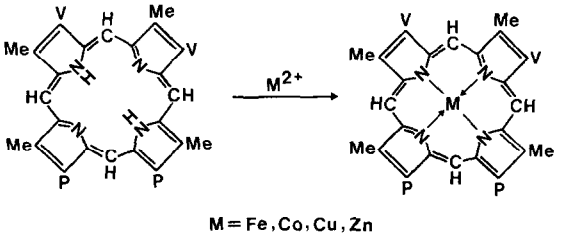


Fig. 4. Formation of the equatorial coordination groups of a metalloporphyrin. V = Vinyl; P = $CH_2CH_2COOH$.

The insertion of a metal ion which completely occupies the centre of the porphyrin hole significantly altered the electronic environment around the central nitrogen atoms[10]. The retention of the metalloporphyrins is therefore largely dependent on the ability and thus the species of the inserted metal ion to accept axial ligands from the mobile phase (Fig. 5). The axial ligands in the present mobile phase are probably $OH^-$ or $CH_3COO^-$ ions. The hydrophobic interaction of the side-chain substituents with the hydrocarbonaceous functions is, however, still important for metalloporphyrins which do not accept axial ligands.

The separation of the Co, Fe, Zn and Cu complexes of meso- and protoporphyrins is shown in Fig. 6. Elution, in the order of Co, Fe, Zn and Cu complexes,

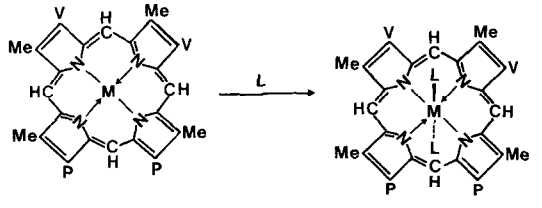


Fig. 5. Formation of the axial coordination groups of a metalloporphyrin. L = $OH^-$ or $CH_3COO^-$.

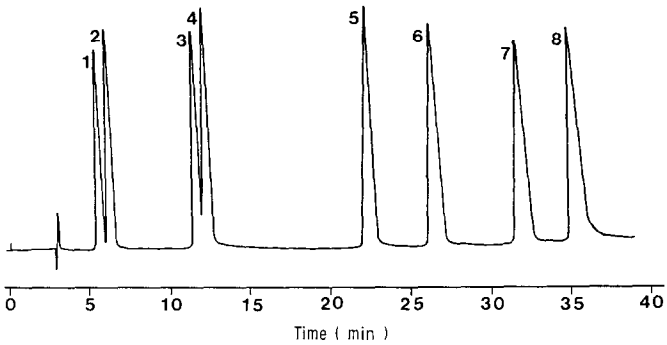


Fig. 6. Separation of metalloporphyrins. Column, 25 cm × 5 mm SAS-Hypersil ($C_1$); eluent, 65% methanol in 1 *M* ammonium acetate (pH 4.6) for 27 min, then 80% methanol in the buffer; detector, UV 404 nm. Peaks: 1 = Co (proto); 2 = Co (meso); 3 = (Cl) Fe (proto); 4 = (Cl) Fe (meso); 5 = Zn (meso); 6 = Zn (proto); 7 = Cu (meso); 8 = Cu (proto).

is observed for both meso- and protoporphyrins. This is not totally unexpected and is consistent with the fact that Co and Fe complexes are particularly sensitive to axial ligand formation in solution and may add two extra ligands; the Zn complex can add one extra ligand, while further coordination of the Cu complex is only possible under special conditions[10,11]. The addition of axial ligands (usually $OH^-$ ions) leads to a decrease in hydrophobicity and therefore in $k'$ values. Although divalent metals are used, it should be pointed out that Co(II) and especially Fe(II) complexes are easily autooxidised to Co(III) and Fe(III) complexes. The Fe(II) complex is undoubtedly converted to Fe(III). The oxidation state of the Co complex is less certain but is also more likely to be Co(III). Haemin may be converted to haematin in the presence of $OH^-$ ions.

The retention behaviour of the Zn and Cu complexes of meso- and protoporphyrins is the same as that of the corresponding free acid porphyrins, *i.e.*, protoporphyrin is retained longer than mesoporphyrin (Fig. 2). These metalloporphyrins do not accept axial ligands readily. Their relative retention is thus still governed by the relative hydrophobicity of the porphyrin side-chain substituents.

The retention order is completely reversed with the Co and Fe complexes, which are excellent axial ligand acceptors. The reversal in elution order, with the protoporphyrin complexes eluted before the mesoporphyrin complexes, is attributed to the decreased electron density at the ring nitrogens due to the vinyl groups of protoporphyrin. This is reflected in the chelated metals, leading to increased affinity for the donor electrons of the extra ligands[11] and decreased hydrophobicity.

*HPLC of dicarboxylic porphyrins and metalloporphyrins*

The metalloporphyrins are generally much more hydrophobic than the porphyrins. They are expected to be strongly retained and eluted after the parent porphyrins. This is indeed the case for Cu complexes. However, the ability to accept axial ligands in aqueous solution renders the metalloporphyrins more solvophilic. Metalloporphyrins with this ability are therefore eluted before the free acid porphyrins, as shown in Fig. 7.

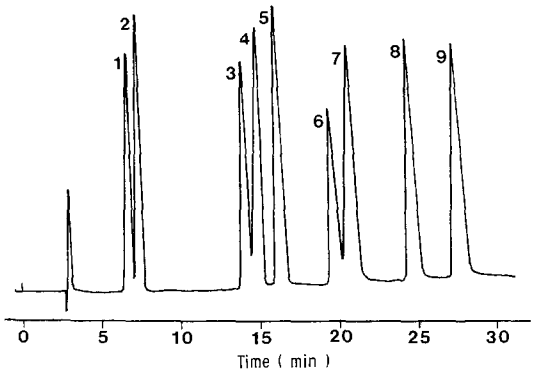


Fig. 7. Separations of dicarboxylic porphyrins and metalloporphyrins. Column, 25 cm × 5 mm SAS-Hypersil ($C_1$); eluent, methanol (A) and 1 *M* ammonium acetate (pH 4.6) (B); elution, 62% (A) for 6 min, then linear gradient to 70% (A) from 6.1 to 13 min followed by isocratic elution at 75% (A); detector, UV 404 nm. Peaks: 1 = Co (proto); 2 = Co (meso); 3 = (Cl) Fe (proto); 4 = (Cl) Fe (meso); 5 = deuteroporphyrin; 6 = Zn (meso); 7 = Zn (proto); 8 = mesoporphyrin; 9 = protoporphyrin.

*The effect of bonded-phase chain-length on retention*

The effect is that expected for reversed-phase chromatography; increasing the bonded-phase chain-length increases the retention. The $C_{18}$-bonded phase is less suitable for the separation of dicarboxylic porphyrins and metalloporphyrins due to the strong hydrophobic interaction between the solutes and the $C_{18}$ functions. The $C_8$-bonded phase is better, but preference is for the least hydrophobic $C_1$-bonded column, which requires less organic solvent for elution. This is important, since porphyrins are more soluble in aqueous buffer than in methanol.

*The effect of mobile phase organic solvents on retention and selectivity*

The retention of dicarboxylic porphyrins and metalloporphyrins decreasess with increasing organic solvent concentration. In earlier studies[3,4] it was found that acetonitrile was essential for the elution of porphyrins with a high number of carboxylic acid groups. Methanol altered the column selectivity so that the retention of uroporphyrins was excessive. Extensive hydrogen bonding between the highly polar uroporphyrin peripheral substituents with methanol extracted onto the stationary phase is a probable explanation for this observation[12].

The dicarboxylic porphyrins and metalloporphyrins, on the other hand, require methanol as an organic solvent for effective separation; resolution was poor when acetonitrile was used. Solute–organic solvent–stationary phase interactions are clearly important for the separation of these more hydrophobic porphyrins. The hydrophobicity also necessitated the use of high organic solvent concentration for elution. Ammonium acetate is much more soluble in methanol than in acetonitrile, and since a molar buffer solution was used, methanol is preferred.

*The effect of buffer concentration on retention*

The dicarboxylic porphyrins and metalloporphyrins had a retention behaviour similar to porphyrins with four to eight carboxylic acid groups[4]; retention decreased with increasing buffer (ionic) concentration. This behaviour, observed only in ion-

exchange chromatography, is due to the extraction of $NH_4^+$ ions from ammonium acetate onto the stationary phase, which then act as ion-exchangers.

A molar buffer solution was used for the separation, because dicarboxylic porphyrins and metalloporphyrins are soluble only in relatively strong acid and alkali, respectively. A strong buffer allows these solutions to be injected without causing damage to the stationary phase.

*Retention control by altering the pH of ammonium acetate*

The pH of the buffer provides one of the best parameters for controlling the retention of the dicarboxylic porphyrins and metalloporphyrins. The relationships between the $k'$ values of the porphyrins and the pH of the buffer is shown in Fig. 8. In general, $k'$ decreased with increasing buffer pH, the exception being the Fe complexes, where $k'$ increased with increasing pH.

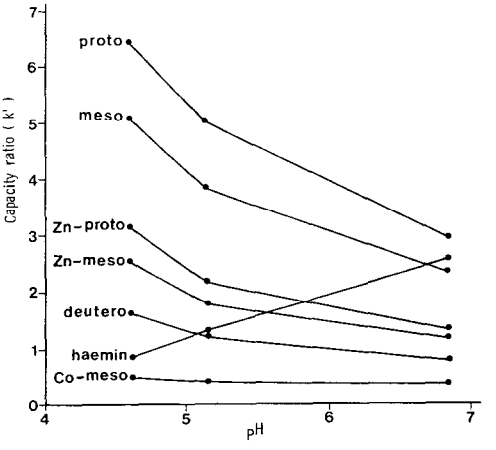


Fig. 8. Effect of pH on the capacity ratios ($k'$) of porphyrins and metalloporphyrins.

The "unusual" behaviour of Fe complexes is probably due to the gradual formation of dimers at higher pH. The demerization of Fe complexes of meso- and protoporphyrins in aqueous alkali is well established[13].

*Biomedical applications*

The analysis of dicarboxylic porphyrins and metalloporphyrins is important for the biochemical diagnosis of diseases, such as the porphyrans and heavy metal, particularly lead, intoxication. Zn(II)–protoporphyrinato-IX levels, for example, are raised in the erythrocytes of patients suffering from lead poisoning (Fig. 9). Mesoporphyrin was used as the internal standard. The intense negative peak was due to quenching by the large quantity of non-fluorescent protohaem present in erythrocytes. If only Zn(II)–protoporphyrinato-IX is measured, this peak can be shifted to higher $k'$ by adjusting the pH of the buffer.

The development of an assay for ferrochelatase, the enzyme catalysing the incorporation of Fe(II) into protoporphyrin-IX to form haem, is another useful clinical application. Estimating this enzyme in bone marrow or liver biopsy is important

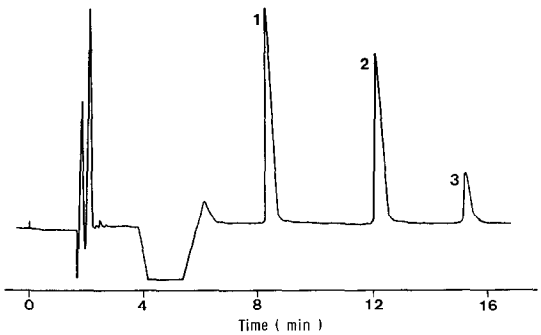


Fig. 9. Separation of erythrocyte Zn (II)–Protoporphyrin-IX and protoporphyrin. Column, 15 cm × 5 mm SAS-Hypersil ($C_1$); eluent, 65% methanol in 1 *M* ammonium acetate (pH 4.6); detector, fluorescence excitation 404 nm, emission 618 nm. Peaks: 1 = Zn (proto); 2 = mesoporphyrin; 3 = protoporphyrin.

for the diagnosis of diseasess associated with abnormal haem synthesis, such as the phorphyrias and various forms of anemias. The assay can be carried out by using the relatively stable mesoporphyrin and Co(II) as enzyme substrates. The product, Co(II)–mesoporphyrinato-IX, and the residual substrate can be easily separated (Fig. 10) and quantitated.

Apart from biomedical applications, the present study should provide the basis for applications in porphyrin and metalloporphyrin chemistry and biochemistry, either in the preparation, purification, isolation, or characterization of the compounds. The study of haematoporpohyrin derivatives[14–16], currently in progress in this laboratory, is an obvious application. The nature of these cancer-localising dicarboxylic porphyrins is still uncertain[16].

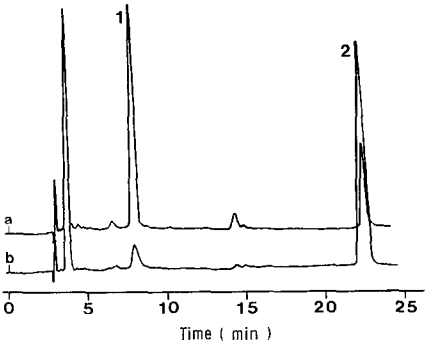


Fig. 10. Separation of Co(II)–mesoporphyrinato-IX and mesoporphyrin in the incubation mixture used for the determination of ferrochelatase. (a) Test and (b) blank. Column, 25 cm × 5 mm SAS-Hypersil ($C_1$); eluent, 62% methanol in 1 *M* ammonium acetate (pH 4.6); flow-rate, 1 ml/min for 10 min, then 1.5 ml/min; detector, UV 404 nm. Peaks: 1 = Co (meso); 2 = mesoporphyrin.

## CONCLUSIONS

The following conclusions can be drawn from the present study:

(1) Hydrophobic interaction between the porphyrin side-chain substituents and the hydrocarbonaceous stationary phase surface is the main retention mechanism.

(2) The retention of metalloporphyrins is also influenced by the ability —and therefore the species of the inserted metal ion— to accept axial ligands from the mobile phase.

(3) Ion-exchange behaviour is observed for both porphyrins and metalloporphyrins. This is due to the extraction of $NH_4^+$ ions of ammonium acetate onto the stationary phase, which then act as ion exchangers.

(4) The present study provides the basis for a wide range of chemical, biochemical, and clinical applications involving dicarboxylic porphyrin and metalloporphyrin separation.

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