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High-performance liquid chromatography of type-III heptocarboxylic porphyrinogen isomers

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A reversed-phase h.p.l.c. system is described for the separation of the four type-III heptacarboxylic porphyrinogen isomers. The effects of buffer concentration, pH and type and proportion of organic modifier in the mobile phase on retention and resolution of isomers were studied. Optimum separation on an ODS-Hypersil column was by elution with a ternary mobile phase of acetonitrile, methanol and 1 M-ammonium acetate, pH 5.16 (7:3:90, by vol.). Isomer identification was based on a comparison of their retention times with those of authentic standards, and was further confirmed by h.p.l.c. analysis of the characteristic mixture of three pentacarboxylic porphyrins formed after partial decarboxylation of individual isomers in 0.3 M-HCl at 160 °C.

INTRODUCTION

The heptacarboxylic porphyrinogens are the first intermediates in the stepwise decarboxylation of uroporphyrinogen III to coproporphyrinogen III (Jackson *et al.*, 1976) catalysed by uroporphyrinogen decarboxylase (EC 4.1.1.37). There are four possible type-III heptacarboxylic porphyrinogens (Fig. 1) that can be formed by enzymic decarboxylation of uroporphyrinogen III, and the isomer with the ring-D acetic acid group decarboxylated to a methyl group (**7d**) has been shown to be the preferred natural isomer (Jackson *et al.*, 1976). Type-III penta- and hexa-carboxylic porphyrin isomers have been separated by h.p.l.c. (Jackson *et al.*, 1976; Smith *et al.*, 1980; Lim *et al.*, 1983), and, although type-I and a mixture of type-III heptacarboxylic porphyrins can also be easily separated (Lim *et al.*, 1983), resolution of the four type-III heptacarboxylic porphyrins has never been achieved. We have fairly recently demonstrated that separation of porphyrinogen isomers is superior to separation of the corresponding porphyrins (Lim *et al.*, 1986; Li *et al.*, 1987). On the basis of these observations, the present paper describes the development of a reversed-phase h.p.l.c. system for the complete resolution of the four type-III heptacarboxylic porphyrinogen isomers.

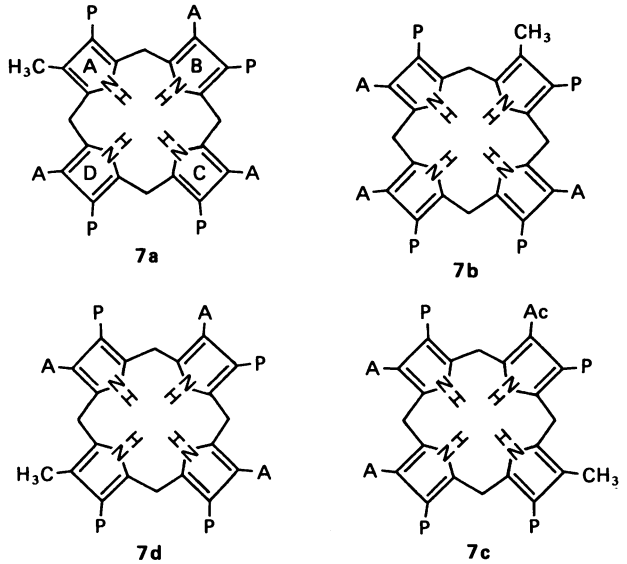

Fig. 1. Structures of type-III heptacarboxylic porphyrinogen isomers

The letters **a**, **b**, **c** and **d** denote the position on which the acetic acid group on ring A, B, C and D respectively has been decarboxylated to a methyl group (Jackson *et al.*, 1976). A represents an acetic acid group and B a propionic acid group.

EXPERIMENTAL

Materials and reagents

Uroporphyrin octamethyl ester was from Sigma Chemical Co. (Poole, Dorset, U.K.). Type-III heptacarboxylic porphyrin methyl esters [oxidized forms of **7a**, **7b** and **7c** (Fig. 1)] were gifts from Professor A. H. Jackson (University College, Cardiff, Wales, U.K.). The isomer **7d** was isolated as the methyl ester from the faeces of rats treated with hexachlorobenzene as previously described (Li *et al.*, 1987). The porphyrin methyl esters were hydrolysed in 25% (w/v) HCl for 96 h at room temperature in the dark. The porphyrins were recovered by solvent extraction into ethyl acetate, after adjusting the pH to 3.5 with aqueous NH_3 (Li *et al.*, 1987).

Ammonium acetate, acetic acid, concentrated HCl, concentrated H_2SO_4, $NaHCO_3$, KOH, EDTA (disodium salt), metallic sodium, mercury, chloroform and ethyl acetate were AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.). Acetonitrile and methanol were h.p.l.c. grade from Rathburn Chemicals (Walkerburn, Peebles-shire, Scotland, U.K.).

Preparation of a standard mixture of isomers 7a, 7b, 7c, and 7d

Uroporphyrin III (1 mg) in 0.5 M-HCl (10 ml) in a Pyrex tube was thoroughly flushed with N_2. The tube

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was capped and then heated for 1 h at 150 °C in the dark. After cooling to room temperature the solution was adjusted to pH 3.5 with aqueous NH_3 and extracted with ethyl acetate. The organic layer was evaporated to dryness at 50 °C under reduced pressure. The residue was dissolved in 5 ml of methanol/conc. H_2SO_4 (19:1, v/v) and kept for 24 h in the dark. The solution was then poured into 10 ml of chloroform in a separating funnel. Water (50 ml) was added and the mixture was shaken vigorously with occasional release of pressure. The chloroform layer was collected and washed successively with 50 ml of saturated $NaHCO_3$ solution and twice with 50 ml of water. The organic layer was then filtered through a filter paper (Whatman no. 1) pre-wetted with chloroform, and the filtrate was evaporated to dryness at 50 °C under reduced pressure. The residue was redissolved in 200 μl of chloroform for preparative t.l.c. separation as described previously (Li *et al.*, 1987). The band corresponding to heptacarboxylic porphyrin methyl ester was isolated, hydrolysed to the porphyrins and extracted into ethyl acetate as described above. This contained a mixture of oxidized isomers **7a**, **7b**, **7c** and **7d** in almost equal proportions with a total yield of about 25%. The porphyrins were dissolved in 200 μl of 0.01 M-KOH and the solution was shaken vigorously with 3% (w/w) sodium amalgam until no fluorescence was detectable under a u.v. lamp. The porphyrinogen solution was transferred into a clean vial, flushed with N_2 and capped. It was stable for at least 3 h when kept on ice in the dark.

H.p.l.c. of heptacarboxylic porphyrinogens

A Varian Associates (Walnut Creek, CA, U.S.A.) model 5000 solvent-delivery system was used. Injection was via a Rheodyne (Cotati, CA, U.S.A.) 7125 injector fitted with a 100 μl loop. Depending on sample concentrations, 20–100 μl was injected. The separation was carried out on a 25 cm × 5 mm ODS-Hypersil (5 μm particle size) column (Shandon Southern, Runcorn, Cheshire, U.K.), with acetonitrile/methanol/1 M-ammonium acetate, pH 5.16 (7:3:90, by vol.), containing 0.27 mM-EDTA as eluent at a flow rate of 1 ml/min. Mobile phases containing various proportions of acetonitrile and/or methanol in buffers of different pH (adjusted with acetic acid) and molarity were also investigated for the separation. The mobile phase was thoroughly degassed with He before use. An LCA-15 electrochemical detector (EDT Research, London N.W.10, U.K.) set at an operating potential of +0.070 V with a detector sensitivity of 30 nA was used for solute detection. For small-scale preparative separation of isomers, a u.v. detector set at 240 nm was employed. The purified isomers were oxidized into the corresponding porphyrins by leaving the eluates on the bench for 1 h under low-intensity white light.

RESULTS AND DISCUSSION

H.p.l.c. of isomers 7a, 7b, 7c and 7d

The separation of a standard mixture containing **7a**, **7b**, **7c** and **7d** on an ODS-Hypersil column with acetonitrile/methanol/1 M-ammonium acetate, pH 5.16 (7:3:90, by vol.) as mobile phase is shown in Fig. 2. In previous studies we have shown that retention and separation of porphyrins in reversed-phase h.p.l.c. is dominated by hydrophobic interaction between the most hydrophobic group (usually methyl) of the porphyrin side-chain substituents and the hydrocarbonaceous stationary-phase surface (Lim *et al.*, 1983). The different arrangements of the methyl groups around the peripheral of coproporphyrin, pentacarboxylic porphyrin and hexacarboxylic porphyrin isomers confer different hydrophobicity to these molecules, thus leading to their separation. The four type-III hepatacarboxylic porphyrins, however, have only one methyl group each. They are therefore virtually identical in hydrophobicity, and attempts to resolve these isomers have all ended in failure. The reduction of the four methine bridges in the rigid porphyrin macrocycle to the methylene groups

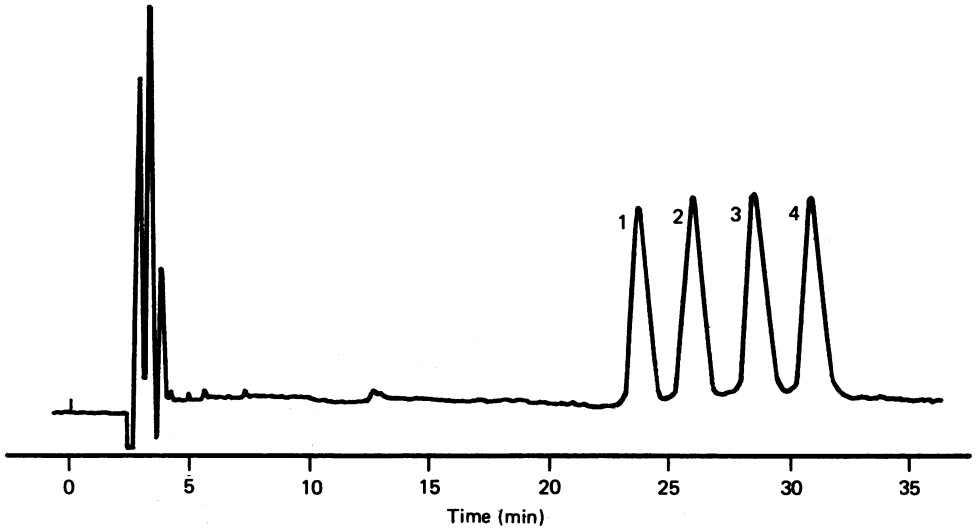

Fig. 2. H.p.l.c. separation of type-III heptacarboxylic porphyrinogen isomers

The separation was carried out on a 25 cm × 4 mm ODS-Hypersil column with acetonitrile/methanol/1 M-ammonium acetate, (7:3:90, by vol.), pH 5.16, as mobile phase, at a flow rate of 1 ml/min; detection was amperometric at +0.07 V. Peak 1, isomer **7a**; peak 2, isomer **7c**; peak 3, isomer **7b**; peak 4, isomer **7d** (see Fig. 1).

Table 1. Pentacarboxylic porphyrins formed by partial decarboxylation of heptacarboxylic porphyrin

The pentacarboxylic porphyrins were separated on an ODS-Hypersil column with 20% (v/v) acetonitrile in 1 M-ammonium acetate, pH 5.16, as mobile phase (Lim *et al.*, 1983). The letters **a**, **b**, **c** and **d** denote the positions of methyl groups, i.e. the position in which the acetic acid groups have been decarboxylated (Jackson *et al.*, 1976).

H.p.l.c. peak no. (Fig. 1)	Pentacarboxylic porphyrins	Peak assignment
1	**5abc, 5abd, 5acd**	**7a**
2	**5abc, 5acd, 5bcd**	**7c**
3	**5abc, 5abd, 5bcd**	**7b**
4	**5abd, 5acd, 5bcd**	**7d**

resulted in a relatively flexible molecules. In such a molecule the smaller peripheral methyl groups may be subjected to a variable extent of steric hindrance or shielding by the larger carboxy groups. The separation of the four type-III heptacarboxylic porphyrinogen isomers is probably due to the steric effect. The differing extents of shielding allows the methyl group in each isomer to interact differently with the hydrophobic stationary phase, thus achieving the separation. This extra factor may also explain why porphyrinogens are better resolved than the corresponding porphyrins.

Peak identification

The identification of isomers is based on a comparison of their retention times with those of authentic standards. To confirm the identities, pure isomers were isolated by small-scale preparative h.p.l.c. These were converted into the porphyrins by oxidation under low-intensity white light and then partially decarboxylated to the pentacarboxylic porphyrins as described previously (Li *et al.*, 1987). The isomeric composition of the pentacarboxylic porphyrins formed by each isomer was analysed by an established h.p.l.c. system for the separation of porphyrin isomers (Lim *et al.*, 1983). Since each heptacarboxylic porphyrin produces a characteristic mixture of three pentacarboxylic porphyrin isomers, this method allows the isomers to be characterized. The results are summarized in Table 1. The elution order was **7a**, **7c**, **7b** and **7d**.

Effect of pH on the retention and resolution of isomers 7a, 7b, 7c and 7d

The pH of ammonium acetate buffer had a profound effect on the retention and resolution of isomers. Fig. 3 is a plot of capacity ratio (k') against the pH of the ammonium acetate buffer. The optimum pH for isomer separation was between 5.1 and 5.2. At pH values below 4.3 or above 6.0, rapid elution with consequent loss of resolution occurred. This is consistent with the fact that, at lower pH, the pyrrole nitrogen atoms are protonated and at higher pH the carboxy groups are ionized. Ionization renders the molecule hydrophilic and therefore decreases the retention in reversed-phase h.p.l.c. The longest retention was at pH 4.7, when ionization of the pyrrole nitrogen atoms and carboxy groups is minimal, thus allowing maximum hydrophobic interaction. At this

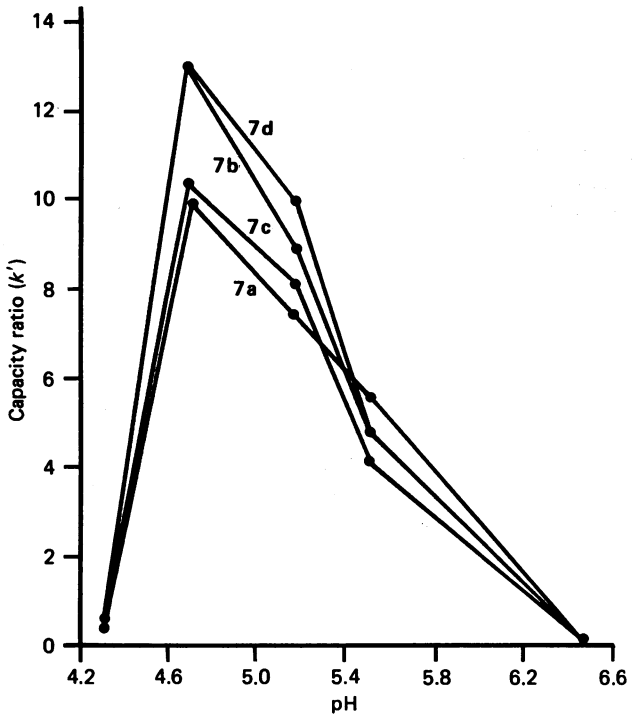

Fig. 3. Effect of pH on the retention and resolution of isomers 7a, 7b, 7c and 7d

Separation was on an ODS-Hypersil column with, as mobile phase, acetonitrile/methanol/ammonium acetate buffer (7:3:90, by vol.).

pH, however, resolution of isomers **7a** and **7c** was partial and that of isomers **7b** and **7d** was lost.

Effect of ammonium acetate concentration on the retention and resolution of isomers 7a, 7b, 7c and 7d

The effect was similar to that observed for the separation of uro- and copro-porphyrinogens (Lim *et al.*, 1986), i.e. decreasing buffer concentration increased solute retention. The k' values of heptacarboxylic porphyrinogens more than doubled when the buffer concentration was decreased from 1.0 to 0.5 M. Lower buffer concentration can be used, but the organic modifier content in the mobile phase has to be adjusted so that excessive retention is not a problem. For example, an eluent consisting of 8% (v/v) acetonitrile and 2% (v/v) methanol in 0.75 M-ammonium acetate, pH 5.16, gave similar isomer separation to a mobile phase of 7% acetonitrile and 3% methanol in 1 M-ammonium acetate, pH 5.16. The 1 M buffer is preferred because it gave sharper peaks and slightly shorter retention times.

Effects of organic modifiers on the separation of isomers 7a, 7b, 7c and 7d

The type of organic modifier in the mobile phase significantly affected the separation of the isomers. With 20% (v/v) methanol in 1 M-ammonium acetate, pH 5.16, as mobile phase, isomers **7b** and **7d** were only partially separated, whereas isomers **7a** and **7c** were completely resolved. An attempt to improve the separation of isomers **7b** and **7d** by decreasing the methanol content in the mobile phase resulted in excessive retention without significant improvement in resolution. Replacing methan-

ol with acetonitrile as the organic modifier greatly improved the separation of isomers **7b** and **7d**, and all four isomers can thus be resolved with an eluent of 8% (v/v) acetonitrile in 1 M-ammonium acetate, pH 5.16. For rapid and effective separation, however, a mixture of acetonitrile and methanol was used as the organic modifier. In such a system (Fig. 1), rapid separation of isomers **7a** and **7c** was maintained by the presence of methanol, whereas the addition of acetonitrile favoured the resolution of isomers **7b** and **7d**.

Separation of type-I and type-III heptacarboxylic porphyrinogen isomers

By using the system described in Fig. 1, type-I heptacarboxylic porphyrinogen (**7I**) was eluted just after isomer **7c** with retention times of 26.6 and 25.8 min respectively. The separation, although incomplete, is adequate for identification of isomers on the basis of retention time. Since type-I and type-III heptacarboxylic porphyrins can be easily separated (Lim *et al.*, 1983), no attempt was made to improve the resolution further.

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