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PREPARATION, HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND CHARACTERIZATION OF HEXACARBOXYLIC PORPHYRINOGENS

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SUMMARY

A simple method for the preparation and reversed-phase high-performance liquid chromatographic separation of hexacarboxylic porphyrinogen isomers is described. Uroporphyrin I or III was partially decarboxylated in 0.5 *M* hydrochloric acid at 150°C. Unreacted uroporphyrin and the hepta-, hexa- and pentacarboxylic porphyrins formed were esterified and then group-separated by thin-layer chromatography. After hydrolysis, the porphyrins were reduced to the corresponding porphyrinogens with 3% (w/w) sodium amalgam. The hexacarboxylic porphyrinogens were separated on an ODS-Hypersil column by elution with acetonitrile–methanol–1 *M* ammonium acetate, pH 5.16 (8:12:80, v/v/v) as mobile phase. Isomers were identified by high-performance liquid chromatography of the characteristic mixture of two pentacarboxylic porphyrins formed after partial decarboxylation of individual isomers. Except for the two type I isomers, resolution of the hexacarboxylic porphyrinogens was superior to that of the corresponding porphyrins.

INTRODUCTION

The enzymic decarboxylation of uroporphyrinogen I and III to coproporphyrinogen I and III, respectively, produces a mixture of hepta-, hexa- and pentacarboxylic porphyrinogen intermediates[1]. Among these, the hexacarboxylic porphyrinogen group is the most complex, consisting of two type I and six type III isomers (Fig. 1). The preparation, separation and characterization of these compounds are important for understanding the enzymic decarboxylation process and for the isolation of pure isomers for chemical and biochemical studies. We have reported the preparation, separation and characterization of protoporphyrinogen[2], copro- and uroporphyrinogens[3], pentacarboxylic porphyrinogens[4] and heptacarboxylic porphyrinogens[5]. This paper describes a simple method for the preparation and reversed-phase high-performance liquid chromatographic (HPLC) separation and characterization of the hexacarboxylic porphyrinogen isomers.

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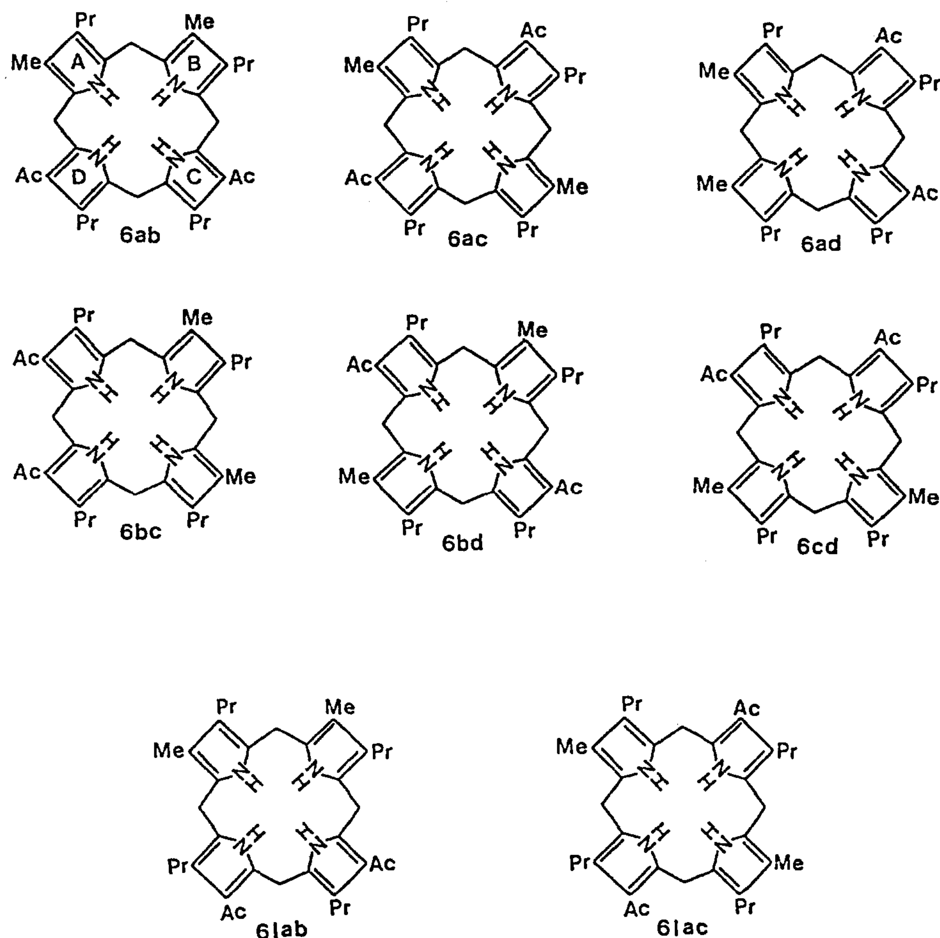

Fig. 1. Structures of hexacarboxylic porphyrinogen isomers. The letters a, b, c and d denote the positions of the methyl (Me) groups. Type I isomers are indicated with the letter I. Ac represents an acetic acid group and Pr a propionic acid group. Oxidation of the four methylene bridges to methine groups gives the corresponding porphyrins.

EXPERIMENTAL

Materials and reagents

Uroporphyrin I and III octamethyl esters were obtained from Sigma (Poole, U.K.) The esters were hydrolysed in 25% (w/v) hydrochloric acid for 96 h at room temperature in the dark.

Acetonitrile and methanol were of HPLC grade from Rathburn (Walkerburn, U.K.). Ammonium acetate, glacial acetic acid, carbon tetrachloride, chloroform, ethyl acetate and ethylenediaminetetraacetic acid (EDTA) were of AnalaR grade from BHD (Poole, U .K.).

Preparation of type I and type III hexacarboxylic porphyrinogen isomers

The method is based on that described for the preparation of penta- and hepta-carboxylic porphyrinogens[4,5]. Uroporphyrin I or III was dissolved in 0.5 *M* hydrochloric acid in a Pyrex tube and heated at 150°C for 1 h in the dark. The solution was adjusted to pH 3.5 with aqueous ammonia after cooling and then extracted with ethyl acetate. The organic layer was evaporated to dryness and the residue was dissolved in 5 ml of methanol–concentrated sulphuric acid (19:1, v/v) and left to stand overnight in the dark. The resulting porphyrin methyl esters were extracted into chloroform and separated by preparative thin-layer chromatography (TLC), as previously described[5]. The band corresponding to hexacarboxylic porphyrin methyl esters, containing two type I or six type III isomers in almost equal proportions with a total yield of 30%, was collected. The reaction also produced about 25% hepta- and 20% pentacarboxylic porphyrin methyl esters. The esters were hydrolysed and extracted into ethyl acetate, as described above for uroporphyrins. Ethyl acetate was removed by evaporation and the residue was dissolved in 250 μl of 0.01 *M* potassium hydroxide solution. The solution was shaken vigorously with freshly prepared 3% (w/w) sodium amalgam until no fluorescence was detectable under a UV lamp. The prophyrinogen solution was transferred into a clean vial, flushed with nitrogen and kept on ice in the dark.

High-performance liquid chromatography

A Varian (Walnut Creek, CA, U.S.A.) Model 5000 liquid chromatograph was used. The separation was carried out on a 25 cm × 5 mm I.D. ODS-Hypersil (5 μm particle size) column (Shandon Southern, Runcorn, U.K.) with acetonitrile–methanol–1 *M* ammonium acetate, pH 5.16 (8:12:80, v/v/v), containing 0.27 m*M* EDTA, as eluent at a flow-rate of 1 ml/min. The mobile phase was thoroughly degassed with a stream of helium before use. The sample (20–100 μl, depending on concentrations) was injected via a Rheodyne 7125 injector (Cotati, CA, U.S.A.), fitted with a 100-μl loop. An LCA-15 electrochemical detector (EDT Research, London, U.K.) set at an operating potential of +0.70 V and a detector sensitivity of 30 nA was used for solute detection in analytical separation. The detector is of the wall-jet type, employing a glassy carbon working electrode and a silver–silver chloride reference electrode. For the small-scale preparative separation of isomers, a UV detector set at 240 nm was used. The purified porphyrinogens may be oxidized to the corresponding porphyrins simply by leaving the eluates on the bench for about 1 h under low-intensity white light.

Peak identification

Individual hexacarboxyl porphyrinogen isomers were isolated by small-scale preparative HPLC and oxidized to the corresponding porphyrins. Each porphyrin was partialy decarboxylated in 0.5 *M* hydrochloric acid at 150°C for 1 h. As each hexacarboxylic porphyrin gave a characteristic pair of pentacarboxylic porphyrin isomers on partial decarboxylation, analysis of these two isomers by the established HPLC system for pentacarboxylic porphyrin isomers[6] allows the positive identification of each hexacarboxylic porphyrinogen peaks. The pentacarboxylic porphyrins were separated on an ODS-Hypersil column with acetonitrile–1 *M* ammonium acetate, pH 5.16 (20:80, v/v) as mobile phase.

RESULTS AND DISCUSSION

Synthesis of hexacarboxylic porphyrinogens

The direct synthesis of porphyrinogens from pyrromethanes is impractical, because porphyrinogens are unstable to oxidation by air. The corresponding porphyrins were therefore prepared and then reduced to the porphyrinogens with sodium amalgam or sodium borohydride. The hexacarboxylic porphyrins have been synthesized by condensation of appropriate dipyrromethanes[1]. However, a mixture of isomeric hexacarboxylic porphyrins can also be easily and conveniently prepared by partial decarboxylation of uroporphyrin in 0.5 *M* hydrochloric acid at 150°C. As the reaction also produced penta- and heptacarboxylic porphyrins, preliminary group separation by TLC was performed[4,5] in order to simplify the subsequent HPLC purification. The isolated hexacarboxylic porphyrins were reduced to the porphyrinogens for HPLC separation. They were stable for at least 3 h when kept on ice in the dark.

High-performance liquid chromatography

The separation of a mixture containing two type I and six type III hexacarboxylic porphyrinogen isomers (see Fig. 1 for structures) is shown in Fig. 2. The ternary mobile phase acetonitrile–methanol–1 *M* ammonium acetate, pH 5.16 (8:12:80, v/v/v) was used, as it provided better resolution than binary systems with either acetonitrile or methanol as the organic modifier. The retention and resolution of the isomers were significantly influenced by the pH and the concentration of the ammonium acetate buffer used. Increasing the pH decreased the retention and consequently the resolution, the optimal pH being between 5.1 and 5.2. As with the separation of other porphyrinogens[2–5], a 1 *M* buffer solution was chosen for rapid separation without sacrificing resolution. Decreasing the buffer concentration increased the retention of all isomers without a significant improvement in resolution. Despite optimization of the mobile phase, only seven peaks were detected (Fig. 2), indicating that two of the isomers must have remained unresolved.

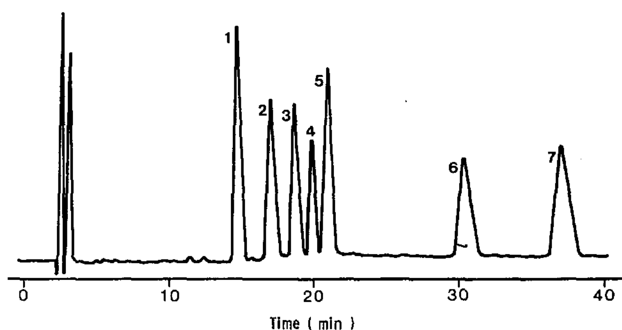

Fig. 2. HPLC separation of hexacarboxylic porphyrinogen isomers. The separation was carried out on a 25 cm × 5 mm I.D. ODS-Hypersil column with acetonitrile–methanol–1 *M* ammonium acetate, pH 5.16 (8:12:80, v/v/v) as mobile phase at a flow-rate of 1 ml/min; detection was amperometric at + 0.70 V. Peaks: 1 = isomer 6ab; 2 = isomer 6 cd; 3 = isomer 6bc; 4 = isomers 6Iab + 6Iac; 5 = isomer 6ac; 6 = isomer 6bd; 7 = isomer 6ad (see Fig. 1).

Peak identification

The two type I isomers (6Iab and 6Iac) were easily identified, as the corresponding porphyrins were available. The porphyrins have been prepared by partial decarboxylation of uroporphyrin I and then isolated by HPLC[6]. Sodium amalgam reduction followed by HPLC separation showed that 6ab and 6ac had identical retention times, corresponding to peak 4 in Fig. 2. These two isomers had therefore been eluted together under the HPLC conditions used. They were, however, completely separated from the six type III isomers.

For the identification of the type III isomers, a small-scale preparative HPLC isolation of isomers was performed. The purified isomers were oxidized to the corresponding porphyrins and then partially decarboxylated to pentacarboxylic porphyrins. As each type III hexacarboxylic porphyrin isomers can only give two type III pentacarboxylic porphyrins with fixed structures, analysis of these isomers allows the positive identification of individual hexacarboxylic porphyrins. The pentacarboxylic porphyrin pairs derived from each peak and the peak assignments are summarized in Table I. The elution order was 6ab, 6cd, 6bc, 6Iab + 6Iac, 6ac, 6bd and 6ad.

Comparison of the separation of hexacarboxylic porphyrins and porphyrinogens

Fig. 3 shows the separation of a standard mixture containing the eight hexacarboxylic porphyrin isomers using the reversed-phase system previously described[6]. Compared with the separation achieved in Fig. 2, it is obvious that the resolution of the porphyrinogens is superior to that of the porphyrins. The same trend has been observed for copro-[3], pentacarboxylic[4], and heptacarboxylic porphyrinogens[5]. In order to obtain pure isomers of the porphyrins, it is therefore necessary to reduce them to the porphyrinogens for purification. The purified porphyrinogens are then oxidized back to the porphyrins.

Reversed-phase chromatography of porphyrins has been shown to be dominated by hydrophobic interactions between the most hydrophobic side-chain substituents (methyl groups) and the hydrocarbonaceous stationary-phase surface[6]. In a

TABLE I

PENTACARBOXYLIC PORPHYRINS FORMED BY PARTIAL DECARBOXYLATION OF HEXACARBOXYLIC PORPHYRIN

The pentacarboxylic porphyrins were separated on an ODS-Hypersil column with acetonitrile in 1 *M* ammonium acetate, pH 5.16 (20:80, v/v) as mobile phase[6].

HPLC peak No. (Fig. 2)	*Pentacarboxylic porphyrins**	*Peak assignment**
1	5abc, 5abd	6ab
2	5acd, 5bcd	6cd
3	5abc, 5bcd	6bc
4	5I	6I
5	5abc, 5acd	6ac
6	5abd, 6bcd	6bd
7	5abd, 5acd	6ad

* 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[1]. Type I isomers are indicated with the letter I.

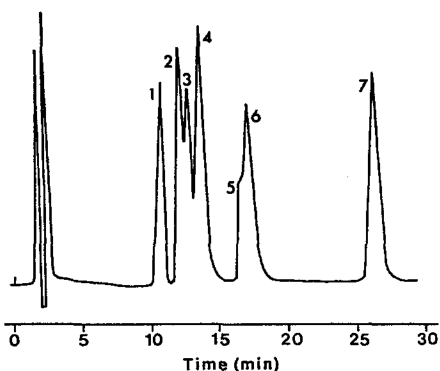

Fig. 3. HPLC separation of hexacarboxylic porphyrins. The separation was carried out on an ODS-Hypersil column with acetonitrile–1 *M* ammonium acetate, pH 5.16 (16:84, v/v) as eluent at a flow-rate of 1 ml/min; UV detection at 400 nm. Peaks: 1 = 6Iac; 2 = 6ac + 6bd; 3 = 6cd; 4 = 6Iab; 5 + 6 = 6ab + 6bc; 7 = 6ad (see Fig. 1).

hexacarboxylic porphyrin molecule there are two side-chain methyl substituents (see Fig. 1). The relative retention of the isomers is therefore governed by the distance between these two groups. The closer the methyl groups, the larger is the hydrophobic surface area available for interaction and the longer the compound is retained. The observed elution order of the hexacarboxylic porphyrin isomers (6Iac, 6ac + 6bd, 6cd, 6Iab, 6ab + 6bc and 6ad) is consistent with this hypothesis. However, the elution order of the hexacarboxylic porphyrinogen isomers deviated significantly from that predicted. This is probably due to the variable shielding of the methyl groups by the larger carboxylic acid groups in different isomers. Partial or complete shielding of one or both methyl groups will lead to a change in hydrophobicity and hence in elution order. Shielding of the methyl groups is possible in porphyrinogens because these molecules, with methylene instead of methine bridges, are flexible. The porphyrins are not affected by the shielding effect, because they are rigid molecules. Steric factors may have contributed to the improved resolution seen in the separation of porphyrinogens[3–5]. Separation of the two type I hexacarboxylic porphyrinogens, 6Iab and 6Iac, has not been achieved with the present system, although the corresponding porphyrins can be easily separated (Fig. 3, peaks 1 and 4). This is an exception to the general trend. A possible explanation is that one of the isomers adopted a conformation which resulted in an identical hydrophobicity to the other, resulting in an identical elution time.

CONCLUSIONS

Partial decarboxylation of uroporphyrin followed by reduction and HPLC separation of the hexacarboxylic porphyrinogens provided a simple and convenient route to the synthesis and isolation of pure hexacarboxylic porphyrin isomers. The porphyrinogens are easily converted to the porphyrins by oxidation. The resolution of the type III hexacarboxylic porphyrinogen isomers was superior to that of the corresponding porphyrins. The separation of the two type I isomers has not been achieved, although the corresponding porphyrins can be easily separated. The reten-

tion of the rigid hexacarboxylic porphyrins is dominated by hydrophobic interactions between the two side-chain methyl substituents and the hydrocarbonaceous stationary-phase surface. An additional important factor, partial or complete shielding of one or both of the methyl groups by the larger carboxy substituents, also affected the retention and resolution of the flexible hexacarboxylic porphyrinogen molecules.

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