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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NATURALLY OCCURRING 8-, 7-, 6-, 5- AND 4-CARBOXYLIC PORPHYRIN ISOMERS

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SUMMARY

Naturally occurring 8-, 7-, 6-, 5- and 4-carboxylic porphyrin isomers are separated on C_{18} reversed-phase columns with various proportions (13–31%, v/v) of acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16) as the mobile phases. Hydrophobic interaction between the porphyrin side chain substituents and the C_{18} hydrophobic surface is the main retention mechanism. Ion-exchange behaviour is also observed, but this does not influence the relative retention of the isomers. All possible forms of the decarboxylation intermediates of uroporphyrinogen III are detected in normal and porphyric urine, and the results provide conclusive evidence for the existence of decarboxylation pathways other than the currently accepted clockwise sequence, starting at the ring D acetic acid group of uroporphyrinogen III.

INTRODUCTION

The porphyrins are intermediates of haem biosynthesis. The biochemical investigation of diseases associated with abnormal haem synthesis has mainly depended on the analysis of porphyrins in blood, urine and faeces. High-performance liquid chromatography (HPLC) is widely used for this purpose[1–5]. However, the separation of porphyrin isomers[6–8], which is important for understanding the nature of porphyrin metabolism and for the differential diagnosis of the porphyrias and various types of porphyrinurias, remains a problem. The ability to resolve porphyrin isomers also allows the detailed study of the decarboxylation products of uroporphyrinogen III. This is important for the understanding of this part of the haem biosynthetic pathway. A clockwise sequence for the decarboxylation of uroporphyrinogen III, starting at the acetic acid group of ring D and proceeding through the acetic acid groups of ring A, B and C, was proposed, because porphyrin intermediates with an intact ring D acetic acid group had never been isolated[9,10].

The present paper describes a highly efficient and reproducible HPLC system for the resolution of porphyrin isomers, in which C_{18} reversed-phase columns are used as the stationary phases and various proportions (13–31%, v/v) of acetonitrile in 1 *M* ammonium acetate buffer, pH 5.16 as the mobile phases. The method was applied to the detailed analysis of the decarboxylation intermediates of uroporphy-

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rinogen III in the urine of normal subjects and in porphyria cutanea tarda (PCT) patients, where the decarboxylation enzyme is defective and high levels of intermediate porphyrins accumulate. The results provide conclusive evidence for the presence of decarboxylation intermediates in which the ring D acetic acid group is unaffected and, thus, the existence of decarboxylation pathways other than the clockwise sequence.

The retention behaviour of the porphyrins is discussed and a simple acid-catalysed decarboxylation HPLC procedure is developed for the easy identification of hexa- and heptacarboxylic porphyrin isomers.

EXPERIMENTAL

Materials and reagents

Uroporphyrin I and III octamethyl esters were from Sigma, Poole, U.K. The esters were hydrolysed in 25% (w/v) hydrochloric acid at room temperature in the dark for 96 h. Type I and type III hepta-, hexa- and pentacarboxylic porphyrins were prepared by decarboxylation of uroporphyrin I and III, respectively.

Ammonium acetate, acetic acid, ethylenediamine tetraacetic acid (EDTA), ethyl acetate and hydrochloric acid were AnalaR grade from BDH Chemicals, Poole, U.K.

Acetonitrile was HPLC grade from Rathburn Chemicals, Walkerburn, U.K.

Extraction of porphyrins from urine

Porphyrins in urine were extracted by adsorption on talc and esterified as previously described[1]. The methyl esters were then hydrolysed in 25% (w/v) hydrochloric acid for HPLC separation.

HPLC

A Pye Unicam (Cambridge, U.K.) Model PU4010 liquid chromatograph and a Perkin-Elmer (Beaconsfield, U.K.) LS-3 fluorescence detector, set at an excitation and an emission wavelengths of 404 nm and 618 nm, respectively was used. Samples were applied by a Rheodyne 7125 injection valve, fitted with a 100-μl loop. The separation was carried out on 10 cm × 5 mm or 25 cm × 5 mm internal diameter columns, packed with Hypersil-ODS (5-μm spherical silica chemically bonded with a layer of octadecylsilyl groups; Shandon Southern, Runcorn, U.K.) or Spherisorb--ODS (Phase Separations, Clwyd, U.K.). The following mobile phases were used: 13–15% (v/v) acetonitrile in 1 *M* ammonium acetate buffer, pH 5.16, for elution of uro- and heptacarboxylic porphyrins; 16–18% acetonitrile and 20–22% acetonitrile in the same buffer for the separation of hexa- and pentacarboxylic porphyrins, respectively. The mobile phase flow-rate was 1 ml/min. EDTA (100 mg/l) was added to the mobile phase when preparative isolation of individual porphyrin was performed. This prevents the formation of metalloporphyrins due to the presence of metallic impurities in ammonium acetate[8]. The separated fraction was washed with ethyl acetate (2 × 10 ml). After adjustment of the aqueous solution to pH 3.0–3.2, the porphyrin was recovered by extraction into ethyl acetate, which was removed with a rotary evaporator.

Variation of retention times was encountered when columns, packed with dif-

ferent batches of supposedly identical materials, were used. An adjustment of the acetonitrile content in the eluent was therefore necessary to achieve similar resolution.

RESULTS AND DISCUSSION

Retention behaviour of porphyrins in reversed-phase chromatography

Hydrophobic interaction between the porphyrin side chain substituents and the non-polar hydrophobic hydrocarbonaceous functions of the stationary phase surface is the main retention mechanism. The relative hydrophobicity of the side chain substituents of the porphyrins studied is $C_2H_5 > CH_3 > H > CH_2CH_2COOH > CH_2COOH$. The relative retention of the porphyrins is therefore dominated by the number of alkyl groups present in the molecule. The retention increases with increasing number of side-chain alkyl substituents, and the following order of elution was observed[8]: uroporphyrin (8 COOH), heptacarboxylic porphyrin (7 COOH, 1 CH_3), hexacarboxylic porphyrin (6 COOH, 2 CH_3), pentacarboxylic porphyrin (5 COOH, 3 CH_3), coproporphyrin (4 COOH, 4 CH_3) and isocoproporphyrin (4 COOH, 3 CH_3, 1 C_2H_5).

The retention behaviour of individual groups of isomers is discussed below.

Uroporphyrins (eight COOH)

Fig. 1 shows the structures of uroporphyrin I and III, the two naturally occurring isomers. These two compounds are without a side chain alkyl substituent. The relative retention is therefore determined by the CH_2CH_2COOH group, which is relatively more hydrophobic than the CH_2COOH group.

Fig. 2a shows the separation of uroporphyrin I and III isomers on a 10 cm × 5 mm Spherisorb-ODS column, eluted with 13% (v/v) acetonitrile in 1 *M* ammonium acetate (pH 5.16). Uroporphyrin III has two adjacent CH_2CH_2COOH groups, which impart a stronger hydrophobic interaction with the column than the symmetrical uroporphyrin I, having no adjacent CH_2CH_2COOH groups. Uroporphyrin III was thus retained longer than the type I isomer.

In earlier studies[6,7] we have shown that increasing the ionic concentration of the mobile phase buffer decreases the retention times of the porphyrins. This phenomenon is only observed in ion-exchange chromatography; in hydrophobic chromatography[11] or ion-pair chromatography[12] the reverse is true. Thus ion-exchange mechanism also operates in the present system. This is most likely to be due to the extraction of NH_4^+ from ammonium acetate onto the residual silanol groups of the

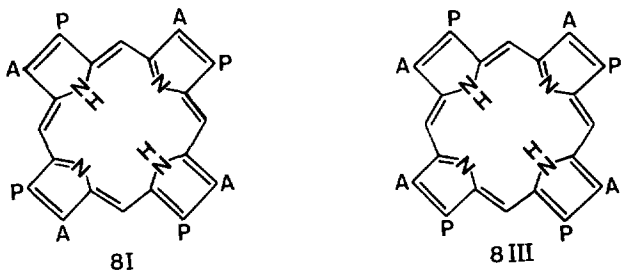

Fig. 1. Structures of uroporphyrin I and III isomers. A = CH_2COOH; P = CH_2CH_2COOH.

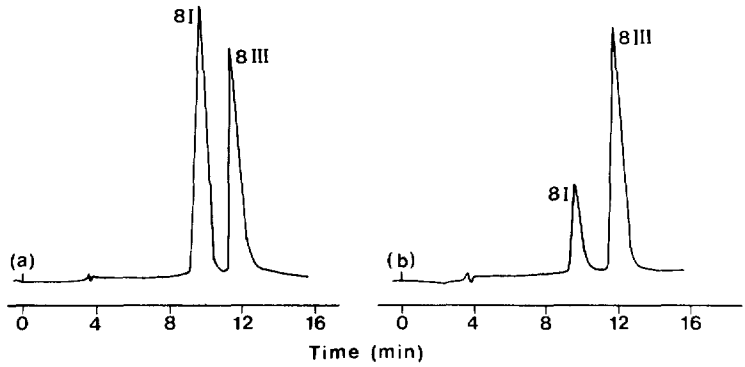

Fig. 2. Separation of uroporphyrin I and III isomers. (a) Standard mixture; (b) in the incubation mixture used for the determination of uroporphyrinogen III synthase. Column, Spherisorb-ODS (10 cm × 5 mm); eluent, 13% (v/v) acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16); flow-rate, 1 ml/min. For peak identification see Fig. 1.

stationary phase, which then act as ion exchangers. We have observed that ammonium acetate is a good masking agent for residual silanol groups[6,7]. However, the ion-exchange mechanism is not the dominant one and has no influence on the relative retention of the porphyrins. This is better demonstrated by the retention behaviour of porphyrins with alkyl substituents (see below).

One of the most important clinical applications of the separation of uroporphyrin I and III isomers is the development of an assay for the enzyme uroporphyrinogen III synthase[13], where it is necessary to resolve the isomers. Fig. 2b shows a typical separation of the isomers in the incubation mixture used for the determination of uroporphyrinogen III synthase in erythrocytes.

Heptacarboxylic porphyrins (seven COOH)

The structures of the seven-COOH porphyrins are shown in Fig. 3. The presence of a single CH_3 group, which dominates the retention, made the capacity ratio values (k') of the isomers very similar. The separation of the four type III isomers

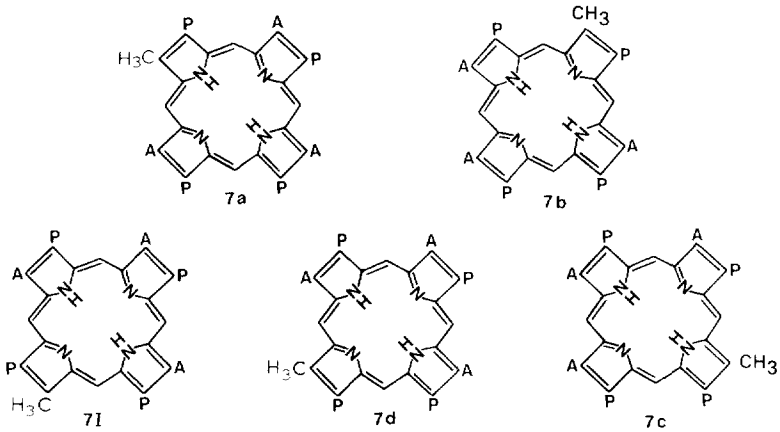

Fig. 3. Structures of heptacarboxylic porphyrin isomers.

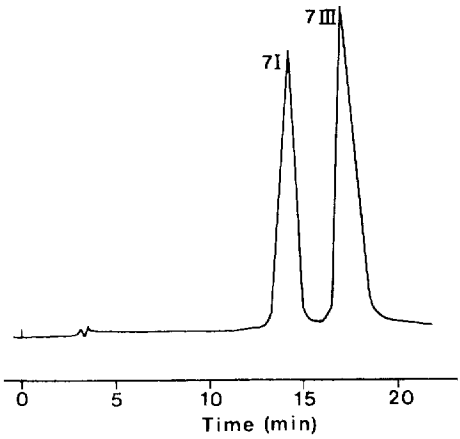

Fig. 4. Separation of heptacarboxylic porphyrin isomers. Column, Spherisorb-ODS (10 cm × 5 mm); eluent, 15% (v/v) acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16); flow-rate, 1 ml/min. Peaks: 7I = type I heptacarboxylic porphyrin; 7III = mixture of type III heptacarboxylic porphyrins.

has not been achieved, although they separated completely from the type I isomer (Fig. 4) on a 10 cm × 5 mm Spherisorb-ODS column with 15% (v/v) acetonitrile in 1 *M* ammonium acetate (pH 5.16) as mobile phase. However, the partial decarboxylation of heptacarboxylic porphyrins produce characteristic hexa- and pentacarboxylic porphyrin patterns (Table I), which can be identified by HPLC (see separation of six- and five-COOH porphyrins below). This technique was therefore employed for the characterisation of heptacarboxylic porphyrins.

The heptacarboxylic porphyrin isolated from PCT urine gave 6ad, 6bd, 6cd (Fig. 5a), 5abd, 5acd and 5bcd (Fig. 6) on partial decarboxylation. Therefore, it is pure 7d (Fig. 3), as reported by Jackson *et al.*[9].

The heptacarboxylic porphyrin isolated from normal urine, on the other hand, produced all six type III hexacarboxylic porphyrins (Fig. 5a) and all four pentacarboxylic porphyrins (Fig. 6). It is thus a mixture of heptacarboxylic porphyrins. However, the proportion of each isomer was difficult to ascertain.

The separation of 7I and 7III isomers, like that of uroporphyrin isomers, is probably due to the relative distances between the side chain CH_2CH_2COOH groups. The CH_2CH_2COOH groups become a factor in influencing the relative retention, because the CH_3 groups are virtually identical in these compounds. Type I hepta-

TABLE I

HEXA- AND PENTACARBOXYLIC PORPHYRINS FORMED BY HEATING HEPTACARBOXYLIC PORPHYRINS IN 0.3 *M* HYDROCHLORIC ACID AT 160°C FOR 2 h

Hepta	*Hexa*	*Penta*
7a	6ab, 6ac, 6ad	5abc, 5abd, 5acd
7b	6ab, 6bc, 6bd	5abc, 5abd, 5bcd
7c	6ac, 6bc, 6cd	5abc, 5acd, 5bcd
7d	6ad, 6bd, 6cd	5abd, 5acd, 5bcd

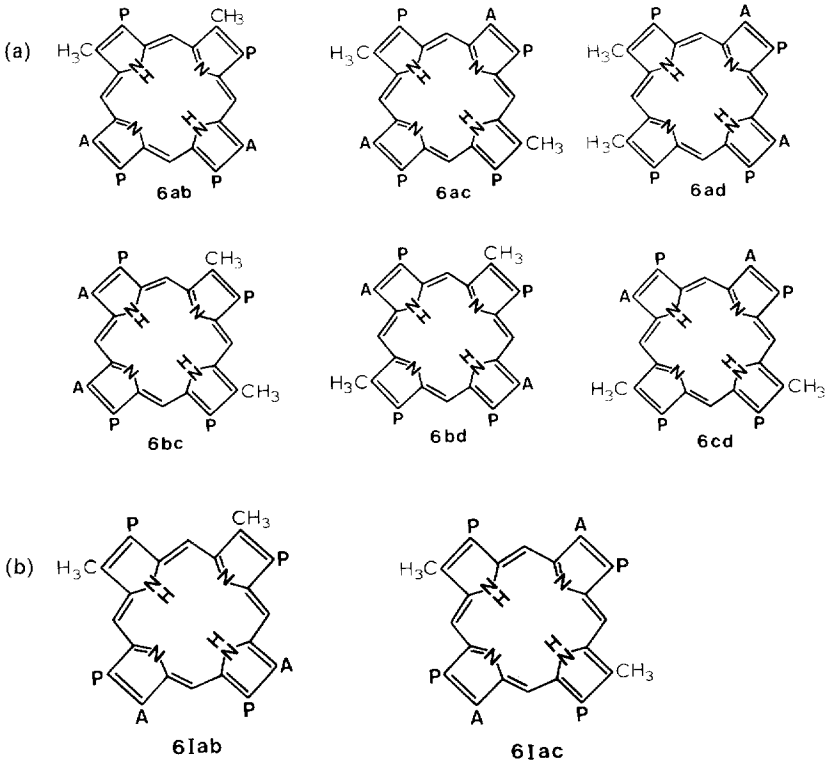

Fig. 5. Structures of hexacarboxylic porphyrin isomers. (a) Type III; (b) type I.

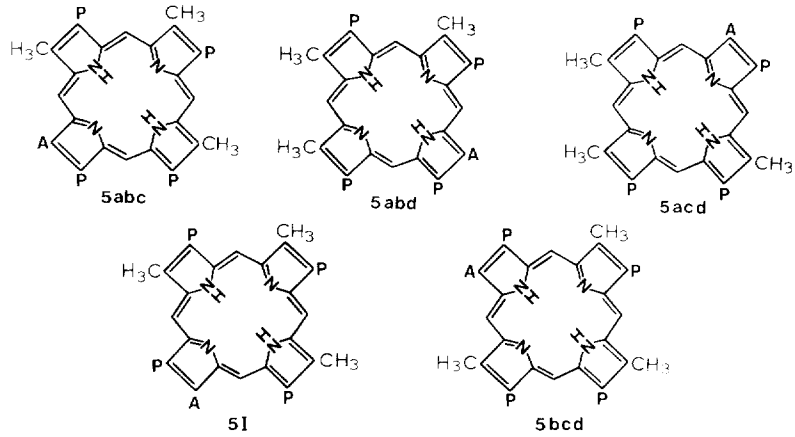

Fig. 6. Structures of pentacarboxylic porphyrin isomers.

carboxylic porphyrin was therefore eluted before the type III isomers, because of the lack of adjacent CH_2CH_2COOH groups.

Hexacarboxylic porphyrins (six COOH)

There are six type III (Fig. 5a) and two type I (Fig. 5b) hexacarboxylic porphyrins. With 16% (v/v) acetonitrile in 1 *M* ammonium acetate (pH 5.16) as eluent on a 25 cm × 5 mm Hypersil-ODS column, the type I isomers could be separated from the type III isomers, but the separation of 6ab and 6bc, 6ac and 6bd was not achieved (Fig. 7a). However, the porphyrins can be positively identified by chromatography after admixture of pure standards. In addition, they were isolated by small-scale preparative HPLC and gave on partial decarboxylation, characteristic pentacarboxylic porphyrin products (Table II).

The main hexacarboxylic porphyrin in normal and PCT urine was 6ad, being about 50–80% and 80–90% of the total hexacarboxylic porphyrin formed respectively (Fig. 7b, c). Individual variations in the percentage of 6ad excreted were observed. The two possible type I isomers were not detected in these urine.

The detection of isomers with the ring D acetic acid group unaffected by enzymic decarboxylation indicated clearly that the decarboxylation of uroporphyrinogen III does not always begin at ring D and proceed through ring A, B and C in a clockwise manner, as proposed by Jackson *et al.*[9]. Fig. 7d shows the separation of six-COOH porphyrins in the urine of a patient with congenital erythropoietic porphyria, a condition characterized by the excretion of large quantities of type I isomers. The detection of both 6Iab and 6Iac and all type III isomers again indicated that the enzyme uroporphyrinogen decarboxylase is non-specific and is able to start the decarboxylation sequence from the acetic acid group of either ring A, B, C or D of the uroporphyrinogens.

The relative retention of the six-COOH porphyrins is dominated by the two CH_3 groups, and the elution order of the isomers can be predicted simply by counting the number of bonds between these groups. There being two possible ways of counting the bonds between two CH_3 groups, the shorter distance was used.

Of the six type III isomers only 6ad has two adjacent CH_3 groups, which are four bonds apart, the lowest number among these isomers. It was therefore the longest retained compound (Fig. 7a and Table III). The closer the CH_3 groups, the stronger the hydrophobic interaction that is possible with the C_{18} stationary phase and, hence the larger is the k' value. A weaker hydrophobic interaction and a smaller k' value (Table III) was observed for isomers with the two CH_3 groups ten and nine bonds apart as in 6ac and 6bd. These two isomers were the most rapidly eluted type III six-COOH porphyrins (Fig. 7a). If it were possible to separate 6 ac from 6 bd, the former is expected to be eluted before the latter. The porphyrins with intermediate number of bonds between the two CH_3 groups, *i.e.* 6cd, 6ab and 6bc, had intermediate k' values (Table III), and 6cd with a 6-bond gap was eluted before 6ab and 6bc, both having the CH_3 groups five bonds apart.

The above general rules concerning the relative retention can be tested by considering the retention behaviours of the two type I six-COOH porphyrins, bearing in mind that, all other substituents being equal, porphyrins with symmetrical CH_2CH_2COOH groups around the macrocycle are eluted before those with non-symmetrical arrangements (see sections on uro- and heptacarboxylic porphyrins). It

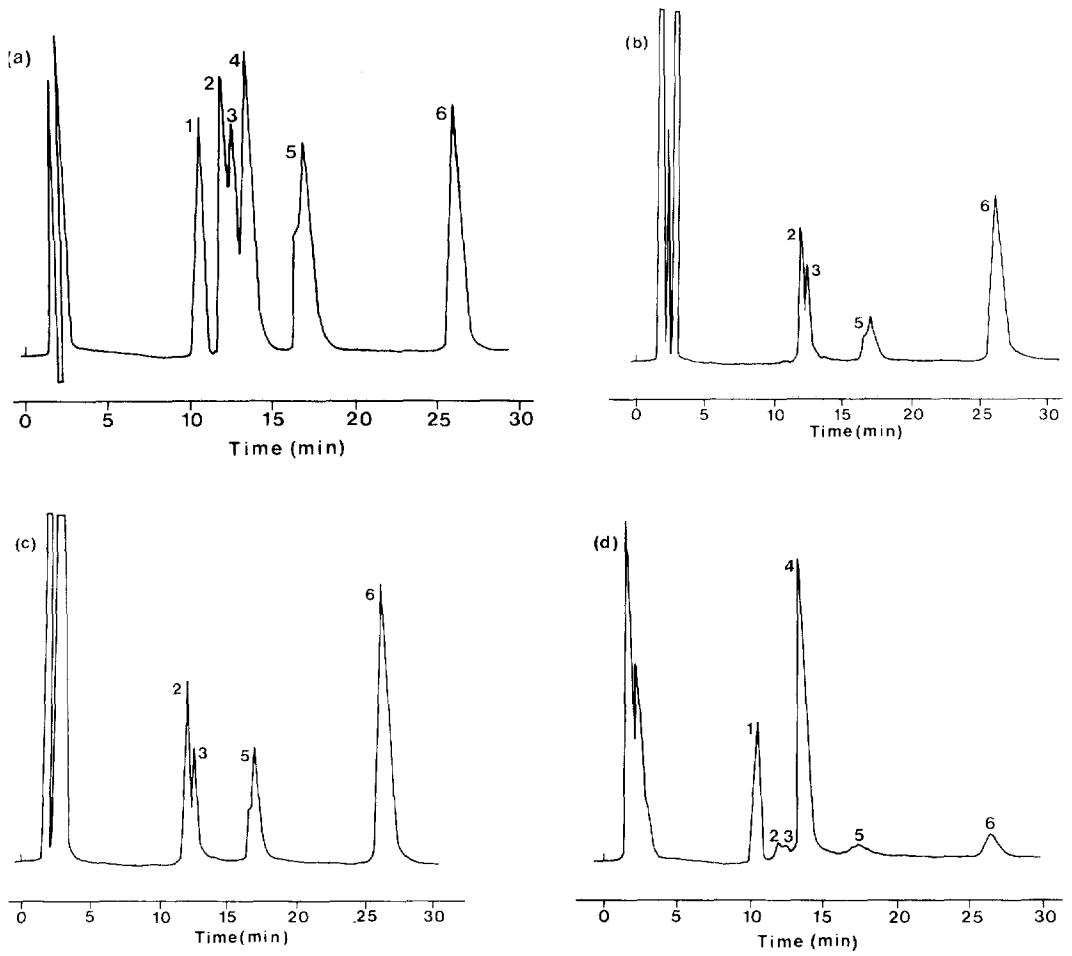

Fig. 7. Separation of hexacarboxylic porphyrin isomers. (a) Standard mixture; (b) normal urine; (c) PCT urine and (d) congenital erythropoietic porphyria urine. Column, Hypersil-ODS (25 cm × 5 mm); eluent, 16% (v/v) acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16); flow-rate, 1 ml/min. Peaks: 1 = 6Iab; 2 = 6ac + 6bd; 3 = 6cd; 4 = 6Iac; 5 = 6ab + 6bc; 6 = 6ad. For compound structure identification see Fig. 5.

is therefore predicted that the symmetrical 6Iab with ten bonds between the CH_3 groups should be eluted before 6ac, also having ten bonds between the CH_3 groups but having non-symmetrical CH_2CH_2COOH groups. And, for the same reason, 6ab and 6bc should be retained longer than the symmetrical 6Iac. Fig. 7a and Table I clearly demonstrate that the retention behaviour is as expected for the compounds with their particular arrangements of the side chain substituents. The rules postulated for the prediction of relative retention are therefore valid.

Pentacarboxylic porphyrins (five COOH)

There are four possible type III pentacarboxylic porphyrins (Fig. 6) that can be produced by decarboxylation of uroporphyrinogen III, and all were found in the

TABLE II

PENTACARBOXYLIC PORPHYRINS FORMED BY HEATING HEXACARBOXYLIC PORPHYRINS IN 0.3 *M* HYDROCHLORIC ACID AT 160°C FOR 30 min

Hexacarboxylic porphyrin	*Pentacarboxylic porphyrins*
6ab	5abc, 5abd
6ac	5abc, 5acd
6ad	5abd, 5acd
6bc	5abc, 5bcd
6bd	5abd, 5bcd
6cd	5acd, 5bcd

urine of normal subjects and PCT patients (Fig. 8b, c). Previous studies in support of the clockwise decarboxylation hypothesis[9,10] had concluded that pentacarboxylic porphyrin with the ring D acetic acid group intact, *i.e.* 5abc (Fig. 6), was not a natural product. This was clearly due to the inadequate separation obtained with the HPLC system used[10]. The separation and identification of 5abc, which was a significant fraction (about 30%) of the total type III pentacarboxylation production in both normal and PCT urine (Fig. 8b, c), clearly demonstrated the existence of decarboxylation pathways other than the "natural" clockwise sequence. There were minor individual variations in the proportion of each isomer excreted in normal urine but the general patterns were similar.

The porphyrins were characterised by their chromatographic behaviour, which was identical with that of the authentic compounds, by conversion to coproporphyrin III on decarboxylation and by their typical electronic absorption spectra.

The rules used to predict the retention of six-COOH porphyrins are also applicable to the five-COOH porphyrins. The following elution order was observed on Hypersil-ODS with 20% (v/v) acetonitrile in 1 *M* ammonium acetate (pH 5.16) as the eluent: 5bcd, 5I, 5abc, 5acd and 5abd (Fig. 8a and Table IV). The three CH_3 groups are farthest apart in 5bcd. It was therefore first to be eluted. The distance

TABLE III

CAPACITY RATIOS (k') OF HEXACARBOXYLIC PORPHYRIN ISOMERS

Column, Hypersil-ODS (25 cm × 5 mm); eluent, 16% (v/v) acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16).

6-COOH Porphyrin (Fig. 5)	k'
6Iab	4.83
6ac	5.75
6bd	5.75
6cd	6.08
6Iac	6.58
6ab	8.08
6bc	8.08
6ad	13.50

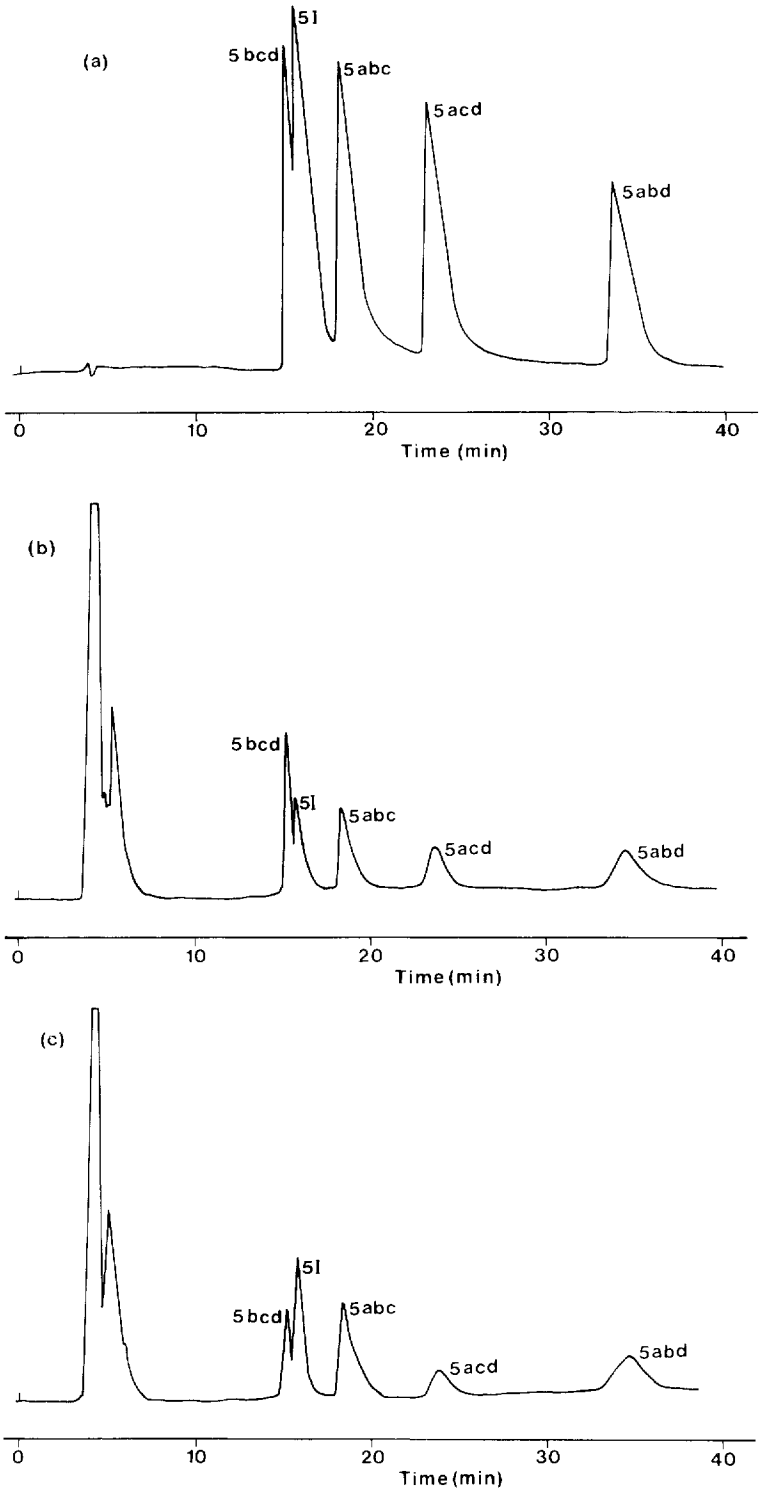

Fig. 8. Separation of pentacarboxylic porphyrin isomers. (a) Standard mixture; (b) normal urine and (c) PCT urine. Column, Hypersil-ODS (25 cm × 5 mm); eluent, 20% (v/v) acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16); flow-rate 1 ml/min. For peak identification see Fig. 7.

TABLE IV

CAPACITY RATIOS (k') OF PENTACARBOXYLIC PORPHYRIN ISOMERS

Column, Hypersil-ODS (25 cm × 5 mm); eluent, 20% (v/v) acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16).

5-COOH Porphyrin (Fig. 6)	*k'*
5bcd	3.68
5I	3.96
5abc	4.58
5acd	5.84
5abd	8.68

between the CH_3 groups of 5I and 5abc are identical, but 5I was eluted before 5abc because the CH_2CH_2COOH groups around the molecule are symmetrically arranged. The strongest hydrophobic interactions were observed in 5acd and 5abd, both with adjacent CH_3 groups, 5abd being retained longer than 5acd since the third CH_3 group is five bonds away from the nearest adjacent CH_3 group and this is six bonds apart in 5acd (Fig. 8a).

Coproporphyrins (four COOH)

Coproporphyrin I, coproporphyrin III and isocoproporphyrin (Fig. 9) are the three most important four-COOH porphyrins. Their separation on a 25 cm × 5 mm Spherisorb-ODS column with 31% (v/v) acetonitrile in 1 *M* ammonium acetate (pH 5.16) as eluent is shown in Fig. 10a. The presence of a C_2H_5 group in isocoproporphyrin, especially close to the CH_3 groups, imparts a strong hydrophobic interaction between the molecule and the C_{18} stationary phase. As a result, isocoproporphyrin is strongly retained. Coproporphyrin III was eluted after coproporphyrin I because of the presence of adjacent CH_3 groups as described for the five- and six-COOH porphyrins.

Fig. 10b shows the separation of the three coproporphyrins in the faeces of a patient with PCT. The presence of isocoproporphyria is characteristic of the disease[14].

Further applications of the present HPLC system

Apart from the clinical importance of the analysis of porphyrin isomers, the ability to predict with some degree of certainty the relative retention of the isomers according to the arrangement of the side chain substituents facilitates the identifi-

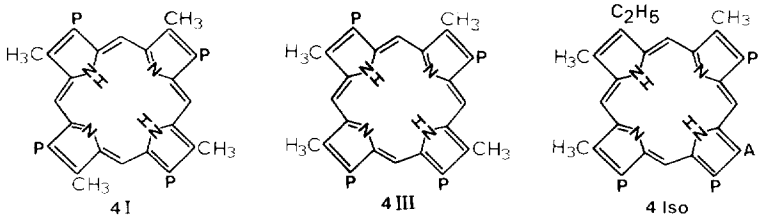

Fig. 9. Structures of coproporphyrin isomers.

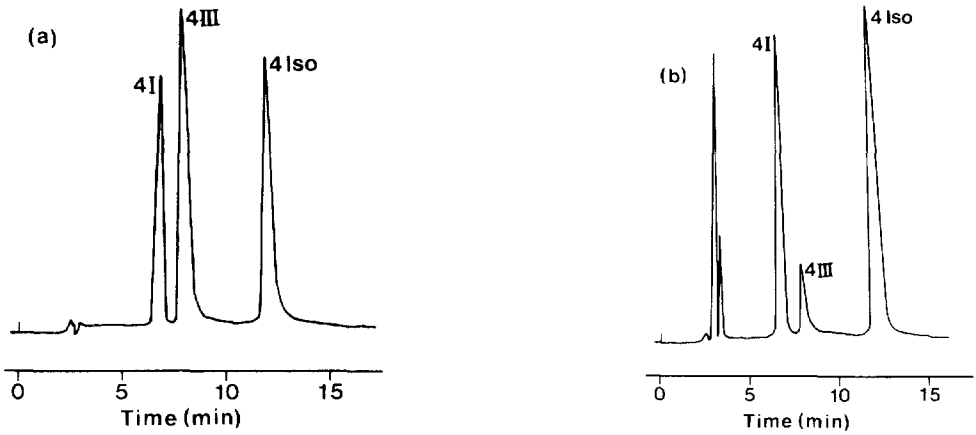

Fig. 10. Separation of coproporphyrin isomers. (a) Standard mixture; (b) in the faeces of a PCT patient. Column, Spherisorb-ODS (25 cm × 5 mm); eluent, 31% (v/v) acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16); flow-rate, 1 ml/min. For peak identification see Fig. 9.

cation of known compounds. For an unknown porphyrin with a typical k' value in relation to the known compounds it is possible to postulate the arrangements of side chain substituents and to assign a tentative structure. This makes the final structural confirmation by other physico-chemical methods easier. It is anticipated that such a technique will lead to the isolation and identification from biological materials of porphyrins not previously described or not thought to be natural products.

CONCLUSION

The separation of naturally occurring porphyrin isomers has been achieved on C_{18} reversed-phase columns with various concentrations of acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16) as mobile phases. Hydrophobic interaction between the porphyrin side chain substituents, particularly the alkyl groups, and the C_{18} hydrophobic surface is the main retention mechanism. The relative retention is determined by the relative arrangement of the side chain substituents around the porphyrin macrocycles. This can be used to identify in clinical specimens known isomers important for the diagnosis of the diseases or to assign tentative structures for unknown isomers isolated from biological materials or from chemical syntheses.

The analysis of the penta-, hexa- and heptacarboxylic porphyrins in urine proved conclusively that enzyme decarboxylation of uroporphyrinogen III does not always start at ring D and proceed through ring A, B and C in a clockwise fashion. The detection of only 7d, the main porphyrin produced in PCT, may be a characteristic feature of the disease, since other heptacarboxylic porphyrins are detected in normal urine and the hexa- and penta-carboxylic porphyrin excretion patterns are similar except for the higher levels in PCT.

The nature of enzymic decarboxylation of uroporphyrinogen III is not yet fully understood. The presence of more than one catalytic sites on uroporphyrinogen decarboxylase has been suggested[15,16]. In view of the complexity of decarboxylation intermediates detected, further investigation with pure isomers is required.

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