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Separation of porphyrin isomers by high-performance liquid chromatography

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A reversed-phase gradient elution system is described for the simultaneous separation of the type I and type III isomers of 8-, 7-, 6-, 5- and 4-carboxylated porphyrins and isocoproporphyrins. The method, adaptable for isocratic and stepwise separation of individual groups of isomers, is also suitable for preparative isolation of pure porphyrins. The analyses of porphyrin isomers in the urine and faeces of porphyric patients are examples of applications.

The natural porphyrins are derived from porphobilinogen by the combined action of two enzymes: hydroxymethylbilane synthase and uroporphyrinogen III synthase. The former catalyses the formation of hydroxymethylbilane from four molecules of porphobilinogen and one of water (Battersby *et al.*, 1982); the latter converts hydroxymethylbilane into uroporphyrinogen III by an intramolecular rearrangement of ring-D (Battersby *et al.*, 1981, 1982). In the absence of uroporphyrinogen III synthase, uroporphyrinogen I is formed spontaneously by the chemical ring-closure of hydroxymethylbilane.

The stepwise decarboxylation of the acetic acid side chains of uroporphyrinogens to coproporphyrinogens I and III produces heptacarboxy, hexacarboxy and pentacarboxy intermediates and is catalysed by uroporphyrinogen decarboxylase (Jackson *et al.*, 1976). In disease in which pentacarboxyporphyrinogen accumulates, such as in PCT, the 2-propionate acid group may be converted into a vinyl group before the acetic acid residue at position 5 is decarboxylated (Elder, 1975; Elder & Evans, 1978). The resulting dehydroisocoproporphyrinogen may be further converted into isocoproporphyrinogen, de-ethylisocoproporphyrinogen and hydroxyisocoproporphyrinogen by bacterial action in the gut (Elder, 1976). The porphyrinogens are transformed into porphyrins by oxidation.

The analysis of porphyrin isomers is important for the differential diagnosis and classification (Doss & Schermuly, 1976) of the porphyrias and other diseases associated with abnormal porphyrin metabolism, e.g. Dubin–Johnson syndrome (Ben-Ezzer *et al.*, 1971; Koskelo & Mustajoki, 1980), Rotor syndrome (Wolkoff *et al.*, 1976) and lead intoxication (Doss & Schermuly, 1976). In addition, effective separation of the isomers provides a means for the preparative isolation of pure compounds for chemical and biochemical studies.

We have developed an h.p.l.c. system for the resolution of coproporphyrin isomers (Wright *et al.*, 1983). The simultaneous separation of the type I and type III isomers of 8-, 7-, 6-, 5- and 4-carboxyporphyrins, however, has not been reported before. The present paper describes a simple reversed-phase gradient elution h.p.l.c. system for the rapid and effective separation of these isomers. The separation allows the isomers to be quantified without the need for the tedious and time-consuming isolation and decarboxylation of individual porphyrins for identification as coproporphyrin isomers by t.l.c. (Schermuly & Doss, 1975) or paper chromatography (Cornford & Benson, 1963).

The practical applications of the method are demonstrated by the analyses of porphyrin isomers in the urine and faeces of porphyric patients.

Abbreviations used: h.p.l.c., high-performance liquid chromatography; PCT, porphyria cutanea tarda; HC, hereditary coproporphyria; CEP, congenital erythropoietic porphyria.

Experimental

Materials and reagents

Coproporphyrin I, coproporphyrin III, uroporphyrin I octamethyl ester and uroporphyrin III octamethyl ester were from Sigma Chemical Co., Poole, Dorset, U.K. The methyl esters were hydrolysed in 25% (w/v) HCl. A mixture of type I 8-, 7-, 6-, 5- and 4-carboxyporphyrins was from Porphyrin Products, Logan, UT, U.S.A. Type III hepta- and hexa-carboxyporphyrins, a mixture of type I and

type III pentacarboxyporphyrin, and isocopro- and de-ethylisocopro-porphyrin were isolated as methyl esters from the faeces of a patient with PCT by the method of Elder (1972) and were hydrolysed in 25% (w/v) HCl.

Ammonium acetate, acetic acid, conc. HCl, and diethyl ether were AnalaR grade from BDH Chemicals, Poole, Dorset, U.K.

Acetonitrile was h.p.l.c. grade from Rathburn Chemicals, Walkerburn, Peebleshire, U.K.

Sample preparation

Urine (100 μl) was injected directly on to the column without sample pretreatment. For faeces, 0.5 g was triturated with 2 ml of conc. HCl and the mixture was vortex-mixed wtih 6 ml of diethyl ether. Water (6 ml) was then added and the mixture was again vortex-mixed. After centrifugation at 1000 *g* for 10 min, 100 μl of the aq. acid solution was injected.

H.p.l.c.

A Varian Associates (Walnut Creek, CA, U.S.A.) model 5000 liquid chromatograph was used. Injection was via a Rheodyne 7125 injector fitted with a 100 μl loop. A variable-wavelength u.v. detector (Varian UV-50) set at 404 nm or an LS-3 fluorescence detector (Perkin–Elmer, Beaconsfield, Bucks., U.K.) set at an excitation and an emission wavelength of 404 and 618 nm respectively was used for detection.

The separation was carried out on columns of dimensions 10 cm × 5 mm and 25 cm × 5 mm internal diameter packed with ODS-Hypersil [5 μm spherical silica chemically bonded with octadecylsilyl groups (Shandon Southern, Runcorn, Cheshire, U.K.)].

The solvents for the gradient elution were acetonitrile and 1 M-ammonium acetate buffer, pH 5.16. The pH was adjusted with 1 M-acetic acid. A 15-min linear gradient from 13% (v/v) acetonitrile to 30% acetonitrile in the ammonium acetate buffer, followed by an isocratic elution at 30% acetonitrile for a further 15 min was used for separations performed on the 25 cm × 5 mm column. For separations carried out on the 10 cm × 5 mm column a 15-min

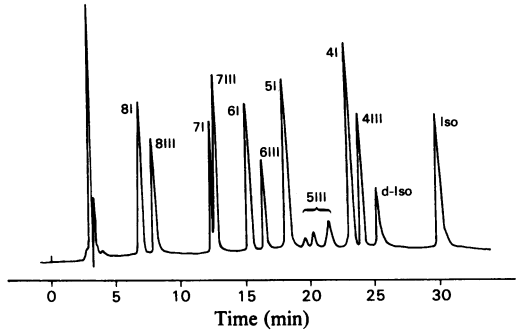

Fig. 1. *Separation of uroporphyrin I (8I), uroporphyrin III (8III), heptacarboxyporphyrin I (7I), heptacarboxyporphyrin III (7III), hexacarboxyporphyrin I (6I), hexacarboxyporphyrin III (6III), pentacarboxyporphyrin I (5I), pentacarboxyporphyrin III (5III), coproporphyrin I (4I), coproporphyrin III (4III), de-ethylisocoproporphyrin (d-Iso) and isocoproporphyrin (Iso)* Column, ODS-Hypersil (25 cm × 5 mm); mobile phase, 15-min gradient elution from 13% acetonitrile (in 1 M-ammonium acetate, pH 5.16) to 30% acetonitrile, then isocratic elution at 30% acetonitrile for 15 min; flow rate, 1 ml/min; detection, u.v. 404 nm.

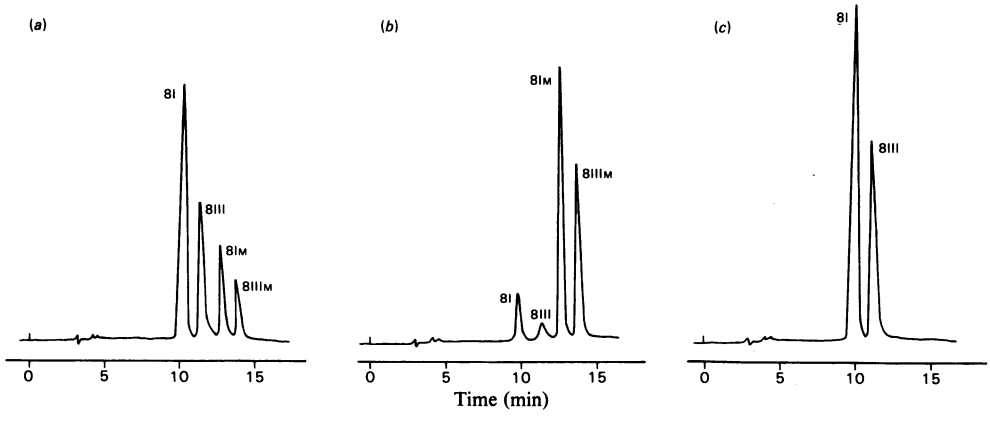

Fig. 2. *Effect of ammonium acetate buffer on metalloporphyrin formation*
(*a*) 2 h after contact; (*b*) 24 h after contact; (*c*) 24 h after contact in the presence of EDTA. Column, ODS-Hypersil (25 cm × 5 mm); mobile phase, 13% acetonitrile in 1 M-ammonium acetate buffer, pH 5.16; flow rate, 1 ml/min; detection, u.v. 404 nm. Peaks: uroporphyrin I (8I), uroporphyrin III (8III), uroporphyrin I–metal complex (8IM), uroporphyrin III–metal complex (8IIIM).

linear gradient from 10% (v/v) acetonitrile to 30% acetonitrile in ammonium acetate buffer, followed by isocratic elution at 30% acetonitrile for another 10 min was used. The mobile-phase flow rate was 1 ml/min.

Results and discussion

The separation of a standard mixture of type I and type III porphyrin isomers and isocoproporphyrins is shown in Fig. 1. The complete resolution of the isomers on a 25 cm × 5 mm ODS-Hypersil column required just 30 min. Adequate separation of the isomers can be achieved on a 10 cm × 5 mm column in less than 25 min with minor adjustment of the gradient system (see under 'H.p.l.c.).

In our study of the separation of coproporphyrin isomers (Wright *et al.*, 1983), we have shown that a high molar concentration (1 M) of ammonium acetate buffer and careful control of pH (5.15–5.20) were important for achieving separation. These conditions were also essential for the separation of uroporphyrin and the intermediate porphyrins. The use of a high molar concentration of buffer is also an advantage, as it allows acid solutions of porphyrins to be injected without damaging the reversed-phase column. Strong acids such as the 25% (w/v) HCl solution used to hydrolyse the porphyrin methyl esters can be diluted with the ammonium acetate buffer before sample injection. The ammonium acetate buffer for the dilution, however, must contain EDTA to prevent the formation of metalloporphyrins on being left, owing to the presence of metallic impurities (Cu, Fe, Mg) in ammonium acetate.

The gradual conversion of porphyrins into metalloporphyrins is demonstrated in Fig. 2. After contact with 1 M-ammonium acetate buffer, pH 5.16, for 2 h an acid solution of uroporphyrin I and III isomers was partially transformed into the metal complexes (Fig. 2*a*). On being left overnight, the porphyrins were almost completely converted into metalloporphyrins (Fig. 2*b*). In the presence of EDTA, however, no metalloporphyrins were detected (Fig. 2*c*).

Table 1. *Mobile phases for isocratic and stepwise elution of individual groups of porphyrin isomers on 25 cm × 5 mm columns of ODS-Hypersil*

Porphyrins	Acetonitrile in 1 M-ammonium acetate buffer, pH 5.16 (%)
Uro	13
Heptacarboxy	15–16
Hexacarboxy	19–20
Pentacarboxy	22–24
Copro	28–30
Isocopro	30

The formation of metal complexes was not observed during h.p.l.c. separation of the porphyrins, probably because of the relatively short

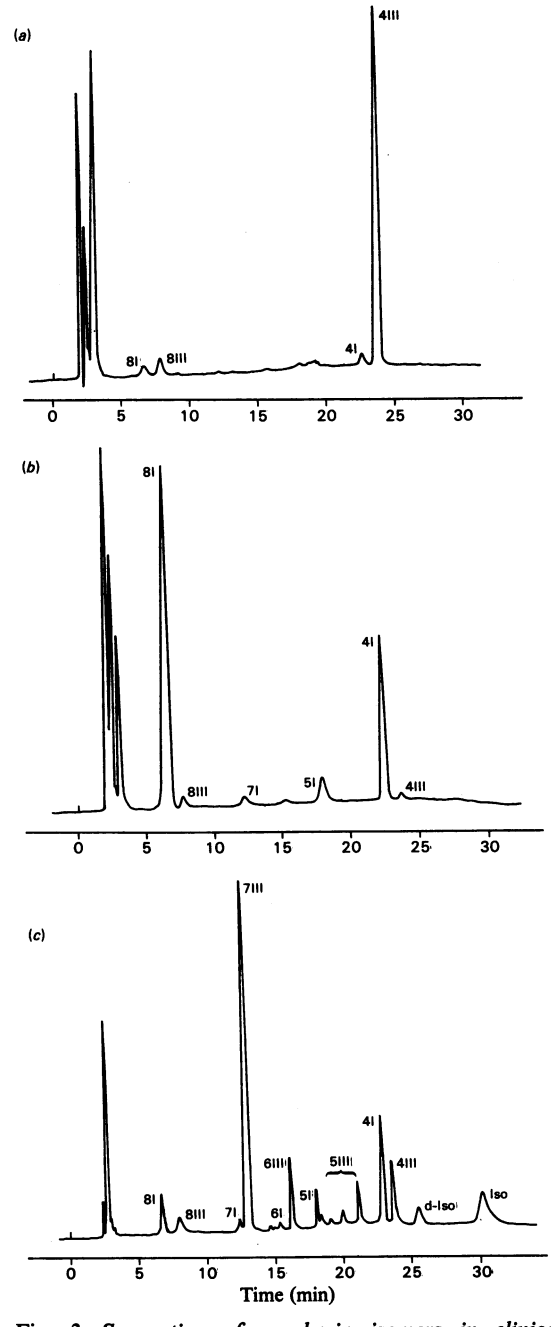

Fig. 3. *Separation of porphyrin isomers in clinical materials*

(*a*) HC subject (urine); (*b*) CEP subject (urine); (*c*) PCT subject (faeces). H.p.l.c. conditions and peak identification were as described in the legend to Fig. 1.

time of contact between the metals and the porphyrins.

The h.p.l.c. system is adaptable for the preparative isolation of porphyrins. EDTA (10mg/100ml of mobile phase) can be added to the mobile phase without affecting the retention or resolution of the porphyrins and the isolated porphyrins are free of metalloporphyrin contamination.

In laboratories where gradient elution facilities are not available Table 1 shows the mobile phases suitable for the separation of the individual group of isomers by isocratic elution or for separation of all the isomers by stepwise elution.

The analyses of porphyrin isomers in the urine of HC and CEP subjects and in the faeces of PCT subjects are examples of practical applications.

The urine of HC subjects contained a large amount of coproporphyrins, 98% of which was type III isomer (Fig. 3*a*). The urine of CEP subjects contained excessive uroporphyrins and coproporphyrins, together with a moderate amount of pentacarboxyporphyrins (Fig. 3*b*). The porphyrins were almost entirely of type I isomers. The faecal porphyrin excretion of PCT subjects is complex, being characterized by the presence of excess heptacarboxyporphyrin (type III) and copro- and isocopro-porphyrins (Fig. 3*c*), together with smaller amounts of uroporphyrins (65% type III, 35% type I), hexacarboxy- (98% type III) and pentacarboxy-porphyrins. The pentacarboxy fraction consisted of the type I isomers and three other detectable isomers, presumably the different type III isomers present in the urine of PCT patients as reported by Smith *et al.*, 1980).

The above examples clearly demonstrated the ability of the h.p.l.c. system to accurately analyse the ratios of the porphyrin isomers, even in situations where one isomer is present in large excess of the other.

Conclusion

Type I and type III isomers of uro-, hepta-carboxy-, hexacarboxy-, pentacarboxy- and copro- and isocopro-porphyrins can be separated by reversed-phase chromatography by isocratic, stepwise or gradient elution with mixtures of acetonitrile and 1M-ammonium acetate buffer, pH5.16, as the eluents. The method is suitable for the detailed analysis of porphyrin isomers in clinical materials and for the preparative isolation of pure isomers in chemical and biochemical studies.

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