High-performance liquid chromatography of coproporphyrin isomers

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A reversed-phase system is described for the simultaneous isocratic separation of coproporphyrin I, II, III and IV isomers. The retention behaviour of coproporphyrin I and III is studied in detail. The method is suitable for both analytical and semi-preparative separation.

The naturally occurring coproporphyrinogens I and III are decarboxylation products of the corresponding uroporphyrinogens catalysed by uroporphyrinogen decarboxylase (Mauzerall & Granick, 1958). Coproporphyrinogen III is an intermediate in the biosynthesis of haem. Uroporphyrinogen decarboxylase is a non-specific enzyme and will also convert the synthetic uroporphyrinogen II and IV to coproporphyrinogen II and IV respectively (Mauzerall & Granick, 1958; Frydman *et al.*, 1972). The porphyrinogens are converted into porphyrins by oxidation. Coproporphyrins can also be prepared by heating uroporphyrins in acid solution (With, 1974) or by heating the solid (With, 1975).

The separation and determination of coproporphyrin I and III isomers is clinically important. It has been used for the differential diagnosis of the porphyrias (Doss & Schermuly, 1976) and for distinguishing the Dubin–Johnson Syndrome from the Rotor Syndrome (Ben-Ezzer *et al.*, 1971; Wolkoff *et al.*, 1976). In addition the analysis and preparative isolation of pure isomers is important in the fields of porphyrin chemistry and biochemistry.

The separation of coproporphyrin isomers has been by paper chromatography of the free acids (Falk *et al.*, 1956; Eriksen, 1958) or by t.l.c. of the methyl esters (Schermuly & Doss, 1975). More recently h.p.l.c. has also been used (Battersby *et al.*, 1976; Meyer *et al.*, 1980; Lim & Chan, 1982). The resolution of most of the h.p.l.c. methods, however, was unsatisfactory.

The simultaneous separation of coproporphyrin I, II, III and IV isomers has not been described before. Battersby *et al.* (1976) separated the isomers as tetraethyl esters into three fractions by recycling the peaks on two 30 cm μ-Bondapak C_{18} reversed-phase columns in series. The unresolved III and IV isomers

Abbreviation used: h.p.l.c., high-performance liquid chromatography.
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fraction was transesterified to a mixture of tetramethyl esters and this was separated on two 30 cm μ-Porasil (silica) columns in series. A partial separation was achieved after 10–12 recycles.

We describe here a rapid and effective method for the simultaneous separation of all four coproporphyrin isomers. The parameters controlling the retention and resolution of the two naturally occurring isomers, coproporphyrin I and III, have been studied in detail. The system is suitable for both analytical and semi-preparative separation of the porphyrins.

Experimental

Materials

Porphobilinogen, coproporphyrin I and coproporphyrin III were from Sigma Chemical Co., Poole, Dorset, U.K.. A mixture of coproporphyrin I, II. III and IV isomers was prepared by decarboxylation (With, 1974) of a sample containing the statistical proportion of uroporphyrin I, II, III and IV isomers synthesized by polymerization of porphobilinogen in acid (Mauzerall, 1960).

Ammonium acetate and acetic acid were AnalaR grade from BDH Chemicals, Poole, Dorset, U.K. Acetonitrile was h.p.l.c. grade from Rathburn Chemicals, Walkerburn, Peebleshire, U.K.

H.p.l.c.

A Varian (Walnut Creek, CA, U.S.A.) model 5000 liquid chromatograph was used with a Varian UV-50 variable wavelength detector set at 406 nm. A Rheodyne 7125 injector was used for sample (100 μl) injection.

A separation was carried out on a column (25 cm $\times$ 5 mm) packed with ODS-Hypersil (5 μm spherical silica chemically bonded with· octadecylsilyl groups) (Shandon Southern Products, Runcorn, Cheshire, U.K.). The mobile phase for the

separation of coproporphyrins type I, II, III and IV was 26% (v/v) acetonitrile in 1 M-ammonium acetate solution adjusted to pH 5.15–5.16 with 1 M-acetic acid. Eluents of different ammonium acetate molarity, pH and acetonitrile contents were used to study the retention behaviour of coproporphyrin I and III isomers. All solvents were degassed by ultra-sonication before use. The flow rate was maintained at 1 ml/min throughout.

Results and discussion

Separation of coproporphyrin I, II, III and IV isomers

The separation of the four coproporphyrin isomers is shown in Fig. 1. The isomers were eluted in the order I, III, IV and II. Coproporphyrin I and III were positively identified by co-chromatography with pure standards. Since the mixture used for this separation was prepared by decarboxylation of uroporphyrins synthesized by polymerization of porphobilinogen in aq. HCl it contained 25% type IV and 12.5% type II isomers (Mauzerall, 1960). These two isomers can therefore be identified by their respective peak height or peak area. The separation is the first direct and complete resolution of the coproporphyrin isomers. An important feature of the present system is the ease with which retention and resolution can be precisely controlled by manipulation of pH, buffer concentration and organic modifier content in the mobile phase.

Control of retention by pH adjustment

The porphyrins are zwitterions and their state of ionization is pH-dependent. In reversed-phase h.p.l.c. without the use of ion-pairing agents, complete io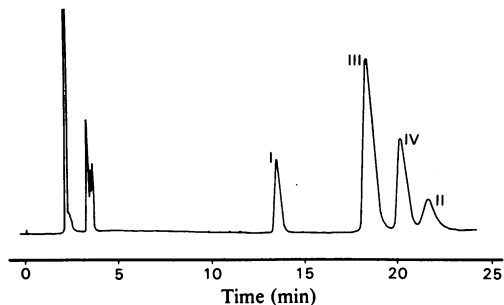nization of a compound results in no retention. By controlling the ionization with a buffer solution at appropriate pH values, these compounds can be chromatographed satisfactorily on reversed-phase columns.

The effect of pH on the retention and resolution of coproporphyrin I and III is shown in Fig. 2, a plot of the pH of the eluent against the capacity ratios (k') of the isomers. The retention and resolution decreased with increasing pH and no separation is possible at pH above 5.7. It is reasonable to assume that at higher pH some or all the propionic acid side chains were ionized and the porphyrins are therefore not retained. The optimum pH was between 5.1 and 5.3 for a rapid analytical separation of the isomers in 1 M-ammonium acetate buffer containing 28–30% (v/v) acetonitrile.

Retention control by altering buffer concentration

The effect of buffer concentration on the retention and selectivity of coproporphyrin I and III have been studied by using 28% acetonitrile in 0.5, 0.75, 1.0 and 1.5 M-ammonium acetate buffer, pH 5.16, as the mobile phases. The results are shown in Fig. 3. Increasing the molar concentration of the buffer decreases the k' values of both isomers but adequate resolution was maintained up to 1.5 M. For a fast separation 1 M is chosen.
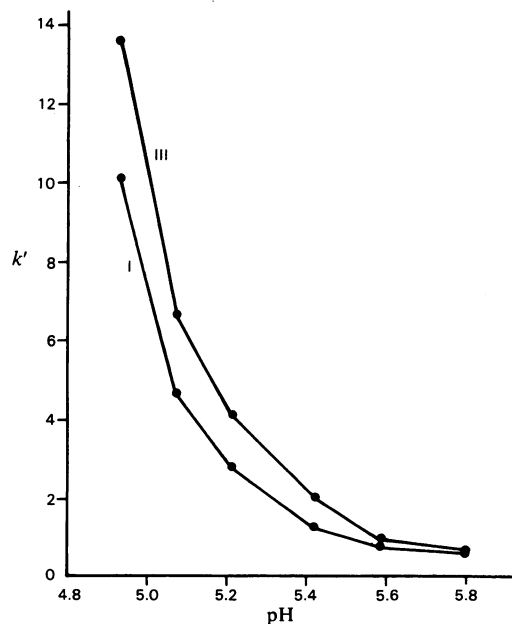

Fig. 1. *Separation of coproporphyrin I, II, III and IV isomers*
Column (25 cm × 5 mm) ODS-Hypersil; mobile phase, 26% acetonitrile in 1 M-ammonium acetate buffer, pH 5.16; flow rate, 1 ml/min; detection, u.v. 406 nm.

Fig. 2. *Effect of pH on capacity ratios of coproporphyrin I and III*
Column, ODS-Hypersil; mobile phase, 28% acetonitrile in 1 M-ammonium acetate; the pH was adjusted with acetic acid.
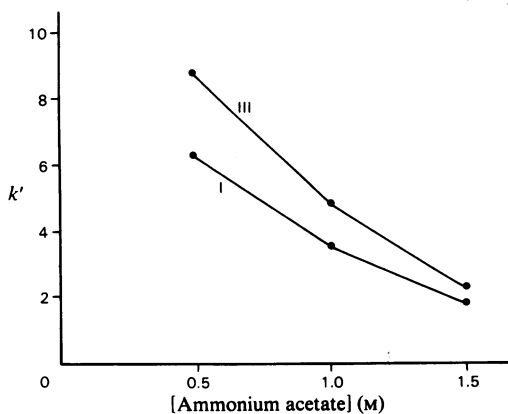
Fig. 3. *Effect of ammonium acetate buffer concentration on the retention of coproporphyrin I and III*
Column, ODS-Hypersil; Eluent, 28% acetonitrile in ammonium acetate buffer, pH 5.16.

Fig. 4. *Relationship between capacity ratios of coproporphyrin I and III and percentage acetonitrile content of the mobile phase*
Column, ODS-Hypersil; eluents, acetonitrile in 1 M-ammonium acetate buffer, pH 5.16.

We have used ammonium acetate as part of a reversed-phase solvent system for many years and our experience has shown that it is a good masking agent for residue silanol groups of reversed-phase packings. It may also compete with solutes containing amino groups for extraction on to the hydrophobic surface. The efficient separation of the porphyrin isomers with solvents containing a high molar concentration of ammonium acetate is a result of these properties, as replacing it with sodium acetate led to a loss of column efficiency. With the ionization of the propionic acid side chains suppressed the separation mechanism is likely to be dominated by interactions of the −NH groups of the porphyrin macrocycle with the stationary phase modified by ammonium acetate.

Control of retention by adjusting the acetonitrile content

The effect of acetonitrile concentration on the retention of coproporphyrin I and III is that expected for reversed-phase h.p.l.c. The k' values increased with decreasing acetonitrile content (Fig. 4). This parameter is particularly useful for controlling the degree of resolution, depending on whether an analytical or a semi-preparative separation is required.

Conclusion

Coproporphyrin I, II, III and IV isomers can be separated isocratically by reversed-phase chromatography with 26% acetonitrile in 1 M-ammonium acetate buffer, pH 5.16. The retention behaviour of coproporphyrin I and III has been studied in detail and it was concluded that for rapid analytical separation a mobile phase of 28–30%
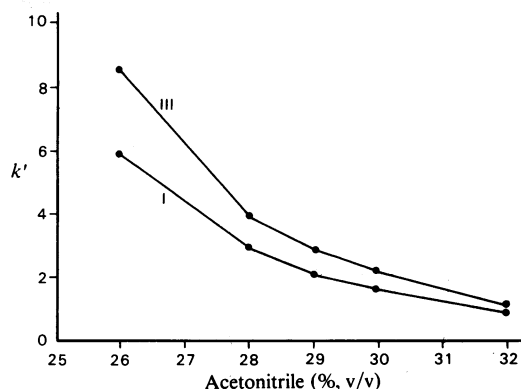
(v/v) acetonitrile in 1 M-ammonium acetate buffer, pH 5.0–5.2, is required. The method can be conveniently adapted for semi-preparative separation, as the degree of separation can be precisely controlled.

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