High-performance liquid chromatography of uroporphyrinogen and coproporphyrinogen isomers with amperometric detection

Chang Kee LIM,* Famei LI and Timothy J. PETERS

Division of Clinical Cell Biology, M.R.C. Clinical Research Centre, Watford Road, Harrow, Middx. HA1 3UJ, U.K.

A reversed-phase h.p.l.c. system, with an ODS-Hypersil column with acetonitrile or methanol in ammonium acetate buffer as mobile phase, is described for the separation of uro- and copro-porphyrinogen isomers. The porphyrinogens are detected amperometrically with sensitivity comparable with that of the fluorescent detection of porphyrins. The effects of pH, buffer concentration and organic modifiers on retention and resolution were studied. The method is suitable for both analytical and preparative separation of porphyrinogens.

INTRODUCTION

Uroporphyrinogen III is the universal precursor of haem, chlorophyll and vitamin B-12. It is formed by the condensation of four molecules of porphobilinogen in a two-step reaction catalysed by hydroxymethylbilane synthase and uroporphyrinogen III synthase (Battersby *et al.*, 1982). The former catalyses the formation of hydroxymethylbilane, and the latter converts hydroxymethylbilane into uroporphyrinogen III (Battersby *et al.*, 1981, 1982). In the absence of uroporphyrinogen III synthase, uroporphyrinogen I is formed spontaneously by the chemical ring closure of hydroxymethylbilane. In diseases in which uroporphyrinogen III synthase is defective, as in congenital erythropoietic porphyria, a large quantity of uroporphyrinogen I is produced. The uroporphyrinogens I and III are converted into coproporphyrinogens I and III respectively by uroporphyrinogen decarboxylase (Jackson *et al.*, 1976). This enzyme is non-specific and will also catalyse the decarboxylation of the synthetic uroporphyrinogens II and IV into coproporphyrinogens II and IV isomers (Fig. 1).

The separation of porphyrinogens has not been reported before, although h.p.l.c. is widely used for the analysis of porphyrins (Evans *et al.*, 1976; Bonnett *et al.*, 1978; Meyer *et al.*, 1980; Lim & Peters, 1984). The

present paper describes a rapid and effective reversed-phase system for the separation of uro- and copro-porphyrinogen isomers. The porphyrinogens are detected amperometrically at high sensitivity. The method is particularly useful for studying the enzymes of the haem-biosynthetic pathways, where the ability to separate and detect the porphyrinogens is an important advantage. It is also expected to be widely applicable in the relevant fields of biochemistry, chemistry and biomedical analysis. In addition, the method is adaptable for the preparative isolation of pure isomers for biochemical and chemical studies.

EXPERIMENTAL

Materials and reagents

Uro- and copro-porphyrins I and III were from Sigma Chemical Co., Poole, Dorset, U.K. A mixture of uroporphyrins I, II, III and IV isomers was synthesized by polymerization of porphobilinogen in 1 M-HCl (Mauzerall, 1960). Coproporphyrins I, II, III and IV isomers were obtained by decarboxylation of the uroporphyrin isomers (With, 1974) and were identified by h.p.l.c. (Wright *et al.*, 1983). H.p.l.c. was used to isolate pure uroporphyrin II (Rideout *et al.*, 1983) and

Fig. 1. Structures of uro- and copro-porphyrinogen I, II, III and IV isomers

For uroporphyrinogens $R^1 = R^2 = R^3 = R^4 = \text{CH}_2\text{CO}_2\text{H}$, and for coproporphyrinogens $R^1 = R^2 = R^3 = R^4 = \text{CH}_3$; $P = \text{CH}_2\text{CH}_2\text{CO}_2\text{H}$.

* To whom correspondence should be addressed.

coproporphyrin II and IV isomers (Wright *et al.*, 1983). Porphyrinogens were prepared by reducing the corresponding porphyrins dissolved in 0.1 M-KOH with 3% (w/w) sodium amalgam.

Ammonium acetate, acetic acid, KOH, HCl and EDTA were AnalaR grade from BDH Chemicals, Poole, Dorset, U.K. Acetonitrile and methanol were h.p.l.c. grade from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K.

Apparatus

A Pye-Unicam (Cambridge, U.K.) LC3-XP pump and an EDT Research (London N.W.10, U.K.) LCA-15 electrochemical detector were used. The detector, which is of the wall jet type, employed a glassy carbon working electrode and an Ag/AgCl reference electrode. Alternatively, a Varian Associates (Walton-on-Thames, Surrey, U.K.) model 5000 liquid chromatograph and a Varian UV-100 variable-wavelength detector were used. The chromatographic column (25 cm $\times$ 5 mm) was slurry-packed with ODS-Hypersil (5 μ m spherical silica chemically bonded with a monolayer of octadecylsilyl groups) from Shandon Southern Products, Runcorn, Cheshire, U.K. Sample injection was via a Rheodyne 7125 loop-valve injector fitted with a 50 μ l loop.

Chromatographic conditions

The mobile phases were various proportions of acetonitrile or methanol in ammonium acetate/acetic acid buffer containing EDTA (0.27 mM). Buffers of different molarity (0.25, 0.5, 0.75 and 1 M) and pH (4.6, 5.16 and 6.9) were investigated. The solvent was continuously degassed with a small stream of He during chromatography. The mobile-phase flow rate was 1 ml/min at ambient temperature. The peaks were detected amperometrically at a detector sensitivity in the range 10–30 nA, or spectrophotometrically at 240 nm.

RESULTS AND DISCUSSION

H.p.l.c. of uroporphyrinogen I, II, III and IV isomers

Fig. 2 shows the separation of uroporphyrinogen isomers on an ODS-Hypersil column with 4% (v/v) acetonitrile in 1 M-ammonium acetate buffer, pH 4.6, as eluent. The elution order of uroporphyrinogen isomers II, III+IV and I was the reverse of that of uroporphyrin isomers (Rideout *et al.*, 1983). In both cases, however, separation of the III and IV isomers was not achieved. The relative retention of porphyrin isomers can be explained by the relative strength of their hydrophobic interaction with the hydrocarbonaceous stationary-phase surface (Lim *et al.*, 1983). The much lower molecular rigidity of porphyrinogens compared with porphyrins probably altered the relative hydrophobicity of the isomers and consequently their elution order. This may arise through intramolecular hydrogen-bonding, which is much easier in flexible molecules.

Effect of pH on the retention and resolution of uroporphyrinogen isomers

The resolution of II, III+IV and I isomers was most effective in the range pH 4.6–4.9 (Fig. 3). At pH 5 and above separation of isomers II, III and IV was lost, but resolution of the natural I and III isomers was maintained (Fig. 4).

The porphyrinogens are zwitterions. Their state of

Fig. 2. Separation of uroporphyrinogen II, III+IV and I isomers

Separation was on an ODS-Hypersil column (25 cm $\times$ 5 mm), with as mobile phase 4% (v/v) acetonitrile in 1 M-ammonium acetate buffer, pH 4.6, at a flow rate of 1 ml/min; detection was amperometric at +0.75V.

Fig. 3. Effect of pH on the retention and resolution of uroporphyrinogen isomers

Separation was on an ODS-Hypersil column (25 cm $\times$ 5 mm), with as mobile phase 6% (v/v) acetonitrile in 1 M-ammonium acetate buffer at a flow rate of 1 ml/min.

ionization and hence the hydrophobicity is controlled by the pH of the solvent. At lower pH the pyrrole nitrogen atoms are protonated, leading to decreasing capacity ratio (k') values as hydrophobicity of the molecules decreased. Similarly, ionization of the carboxylic acid groups at higher pH decreased the k' values. The longest retention occurred at pH 4.8–5.0, where the net ionization of the pyrrole nitrogen atoms and carboxylic acid groups is minimum, allowing for maximum hydrophobic interaction.

Effect of ammonium acetate concentration on the retention and resolution of uroporphyrinogen isomers

By using uroporphyrinogen I and III as examples, it can be shown that increasing the ammonium acetate

Fig. 4. Separation of uroporphyrinogen I and III isomers

Separation was on an ODS-Hypersil column (25 cm $\times$ 5 mm) with as mobile phase 6% (v/v) acetonitrile in 1 M-ammonium acetate buffer, pH 5.16, at a flow rate of 1 ml/min; detection was amperometric at +0.75V.

Fig. 5. Effect of ammonium acetate buffer concentration on the retention and resolution of uroporphyrinogen isomers

Separation was on an ODS-Hypersil column (25 cm $\times$ 5 mm) with as mobile phase 6% (v/v) acetonitrile in 1 M-ammonium acetate buffer, pH 4.6, at a flow rate of 1 ml/min.

concentration decreased the retention (Fig. 5). This behaviour is similar to that observed for the porphyrins (Lim *et al.*, 1983), and was attributed to the adsorption of NH_4^+ ions from the buffer on the stationary phase, which then act as ion-exchangers. Decreasing the k' by

Fig. 6. Separation of coproporphyrinogen I, II, III and IV isomers

Separation was on an ODS-Hypersil column (25 cm $\times$ 5 mm) with as mobile phase 50% (v/v) methanol in 1 M-ammonium acetate buffer, pH 5.15, at a flow rate of 1 ml/min; detection was amperometric at +0.75 V.

increasing the ammonium acetate concentration had little effect on the resolution. It is therefore an advantage to use a higher molar concentration of buffer to achieve rapid separation of the I and III isomers.

Effect of organic modifier on the retention and resolution of uroporphyrinogen isomers

The effect is that expected for reversed-phase chromatography: increasing the organic modifier content in the mobile phase decreases the retention. Methanol, which causes excessive retention of uroporphyrins as a result of hydrogen-bonding between the porphyrin side-chain carboxylic acid groups and methanol adsorbed on the stationary phase (Lim & Peters, 1984), can be used for the separation of uroporphyrinogens. Uroporphyrinogens I and III can be completely separated with 10% (v/v) methanol in 1 M-ammonium acetate buffer, pH 5.16. This is not surprising, since the flexible uroporphyrinogens are more likely to form intramolecular than intermolecular hydrogen bonds with the adsorbed methanol. They were therefore unaffected by the hydrogen-bonding effect in the presence of methanol.

H.p.l.c. of coproporphyrinogen I, II, III and IV isomers

The four coproporphyrinogen isomers were completely separated on an ODS-Hypersil column with 25% (v/v) acetonitrile or 50% (v/v) methanol in 1 M-ammonium acetate buffer, pH 5.16, as the eluent (Fig. 6). The elution order of the isomers, found by co-chromatography with standards, was I, II, III and IV. This is different from the order (I, III, IV and II) reported for the separation of coproporphyrin isomers (Wright *et al.*, 1983). Although resolution of uroporphyrinogen isomers showed no improvement over uroporphyrin isomers, the separation of the four coproporphyrinogen isomers was much better than that of coproporphyrin isomers in terms of speed and resolution. The simultaneous separation of coproporphyrinogens and coproporphyrins is also possible, as shown in Fig. 7.

Fig. 7. Simultaneous separation of coproporphyrinogen (I, II, III and IV) and coproporphyrin (CI, CII, CIII and CIV) isomers

Separation was on an ODS-Hypersil column (25 cm $\times$ 5 mm) with as mobile phase 25% (v/v) acetonitrile in 1 M-ammonium acetate buffer, pH 5.16, at a flow rate of 1 ml/min; detection was by u.v. absorption at 240 nm for coproporphyrinogens and then by absorption at 404 nm for coproporphyrins.

Fig. 8. Effect of pH on the retention and resolution of coproporphyrinogen I, II, III and IV isomers

Separation was on an ODS-Hypersil column (25 cm $\times$ 5 mm) with as mobile phase 50% (v/v) methanol in 1 M-ammonium acetate buffer at a flow rate of 1 ml/min.

Effects of pH, buffer concentration and organic modifier on the retention and resolution of coproporphyrinogens

The effect of pH on the retention and resolution of coproporphyrinogens I, II, III and IV is shown in Fig. 8. It is essentially similar to that described for the uroporphyrinogens, although the pH for optimum resolution is different.

Mobile-phase buffer concentration markedly affects the retention and resolution of coproporphyrinogens.

Fig. 9. Effect of ammonium acetate buffer concentration on the retention and resolution of coproporphyrinogen I, II, III and IV isomers

Separation was on an ODS-Hypersil column (25 cm $\times$ 5 mm) with as mobile phase 50% (v/v) methanol in ammonium acetate buffer, pH 4.6, at a flow rate of 1 ml/min.

Fig. 10. Effect of methanol content on the retention and resolution of coproporphyrinogen I, II, III and IV isomers

Separation was on an ODS-Hypersil column (25 cm $\times$ 5 mm) with as mobile phase methanol in 1 M-ammonium acetate buffer, pH 5.16, at a flow rate of 1 ml/min.

Decreasing the buffer concentration from 1.0 to 0.5 M almost doubled the k' values and greatly improved the resolution (Fig. 9).

Acetonitrile and methanol are both good organic modifiers for the separation of coproporphyrinogens. Increasing the organic modifier content in the mobile phase decreased the retention and resolution of the isomers (Fig. 10). For rapid separation of the natural I

and III isomers, 26% acetonitrile or 60% methanol is recommended.

Choice and sensitivity of detector

The porphyrins are strongly fluorescent compounds and can be detected with great sensitivity with a fluorescence detector. A u.v.-visible-region detector is much less sensitive but is adequate for the detection of porphyrins at the Soret region (approx. 400 nm). The porphyrinogens neither fluoresce nor have strong u.v. absorption, and, although they can be detected at 240 nm, the sensitivity of detection is inadequate for most biochemical or clinical analyses. These compounds, however, are sensitive to oxidation, and are therefore ideal for electrochemical detection by the oxidation mode. Useful detection begins at a potential of +0.65 V for both uro- and copro-porphyrinogens. An operation potential of +0.75 V was chosen to provide adequate sensitivity and detector stability. At higher potentials (about +0.90 V) the increased sensitivity achieved is accompanied by an increase in detector noise level. Thus +0.75 V is a compromise. At the detector sensitivity of 10 nA and an operation potential of +0.80 V, 1 nm-uro- or copro-porphyrinogen can be readily detected. The sensitivity is comparable with that of the fluorescent detection of the porphyrins.

The choice of detector depended on the application and the sensitivity required. The u.v. detector is only useful for the preparation of pure isomers. The present system is particularly good for the resolution of coproporphyrinogen isomers. To obtain highly purified coproporphyrin isomers it may be advantageous to convert them into coproporphyrinogens for preparative purification. The purified coproporphyrinogens are then oxidized back to the coproporphyrins.

The simultaneous detection of porphyrins and porphyrinogens is also possible by coupling a fluorescence detector in series with an electrochemical detector. Uro- and copro-porphyrins are not electro-active under the conditions used but can be detected fluorimetrically, whereas the non-fluorescent electro-active porphyrinogens are detected amperometrically.

CONCLUSIONS

The following conclusions can be drawn from the above study.

1. Rapid and effective separation of uro- and copro-porphyrinogen isomers can be achieved by reversed-phase h.p.l.c. on ODS-Hypersil with various proportions of acetonitrile or methanol in 0.5–1.0 M-ammonium acetate/acetic acid buffers, pH 4.6–5.2, as mobile phases.

2. The porphyrinogens can be detected amperometrically with sensitivity comparable with that of fluorescent detection of porphyrins.

3. The resolution of uroporphyrinogen isomers is similar to that of uroporphyrins, but separation of coproporphyrinogens is vastly superior to that of the coproporphyrins.

4. The system is suitable for sensitive analysis of porphyrinogens with the use of amperometric detection, and for preparative isolation of pure isomers, particularly the coproporphyrinogen isomers, with the use of u.v. detection at 240 nm.

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