Analysis and separation of natural and synthetic mixtures of uroporphyrins by high-pressure liquid chromatography

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A new method for the quantitative analysis of mixtures of the methyl esters of uroporphyrins I and III was developed; this can be applied both to the analysis of naturally occurring uroporphyrins and also to their semi-preparative isolation.

Porphobilinogen (1), the monopyrrolic precursor of naturally occurring porphyrins, is converted by a combination of two enzyme systems, porphobilinogen deaminase and uroporphyrinogen co-synthetase, into the unsymmetrical uroporphyrinogen III (4a). The overall process involves formation of an open-chain tetrapyrrole, which subsequently cyclizes with inversion of the terminal pyrrole ring (Battersby *et al.*, 1980). In certain types of porphyria, however, uroporphyrinogen I (2a) may also be formed, presumably owing to a defect in the co-synthetase enzyme. It is therefore desirable, both for enzyme assay and in the diagnosis of porphyrias, to be able to separate and quantify the isomeric uroporphyrins produced by oxidation of the porphyrinogens. Furthermore, our own interest in the pathways of enzymic decarboxylation of uroporphyrinogen isomers (Jackson *et al.*, 1976, 1980) was an added stimulus to develop methods for isolation of pure materials by h.p.l.c. on a semi-preparative scale, from both natural and synthetic sources.

The first (partial) separations of uroporphyrin isomers (as their methyl esters) were reported by Bommer *et al.* (1977) with the use of a μ -Porasil

h.p.l.c. column with recycling (five times), and each analysis took 2 h. An improved procedure was reported by Nordlov *et al.* (1980) with the use of two μ -Porasil columns in series, but the analysis took some 3 h; we had difficulty in repeating this procedure with a number of commercially available packing materials such as Spherosorb, Hypersil and Partisil, and the mobile-phase composition appeared to be too polar. Reversed-phase methods for separating the uroporphyrin I and III free acids have also been described by two groups of workers (Wayne *et al.*, 1979; Walker *et al.*, 1980); these

(1)

A = CH₂-CO₂H; P = CH₂-CH₂CO₂H

(2a)

(3a)

(4a)

(5a)

Structures of uroporphyrinogens I, II, III and IV [the corresponding porphyrin octamethyl esters are numbered (2b)–(5b) in the text]

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

methods, however, though adequate for analytical purposes, are unsuitable for semi-preparative work owing to the low capacity of reversed-phase h.p.l.c. columns and their relatively high cost.

In continuation of our earlier studies (Evans *et al.*, 1976; Smith *et al.*, 1979) we now describe a simple but reliable method for separating uroporphyrin I and III methyl esters (2b) and (4b) by h.p.l.c., which we have applied to routine assay of samples obtained from both human and animal porphyrins; it can also be adapted to the isolation of the individual isomers.

Materials

Uroporphyrin octamethyl esters I, II, III and VI (2b, 3b, 4b and 5b) were synthesized by established procedures, and we are also very glad to acknowledge gifts of these materials generously supplied by Dr. S. F. MacDonald (National Research Council, Ottawa, Canada). Uroporphyrins were also isolated from the urine and faeces of patients with porphyria cutanea tarda and congenital erythropoietic porphyria and converted into their methyl esters by use of methanol/5% (v/v) H_2SO_4 .

Analytically pure solvents [methanol, toluene, ethyl acetate, hexane and light petroleum (b.p. 60–80°C)] were purchased from BDH Chemicals and used directly without further purification. Hypersil and Spherosorb were obtained from Phase Separations Ltd. (Deeside, Clwyd, Wales, U.K.) and from Shandon Southern Ltd. (Runcorn, Cheshire, U.K.).

Experimental

Analytical h.p.l.c. separations were performed on 200 mm $\times$ 4.5 mm (internal diam.) columns packed with 3 μm or 5 μm Hypersil or with 5 μm Spherosorb; semi-preparative separations were performed with a 500 mm $\times$ 12 mm (internal diam.) column packed with 5 μm Hypersil. The packing materials were stirred in methanol (250 ml) and packed at a constant pressure of 45 MPa (6500 lbf/in²) by the upward-packing method. The columns were conditioned by pumping through dry toluene (500 ml) and were later equilibrated with the appropriate mobile phase.

Solvent was delivered by a Waters 6000 pump operating at 1 ml/min for analytical columns, and at 4–5 ml/min for semi-preparative columns. A Cecil 212 variable-wavelength detector set at 400 nm with a 8 μl flow cell (path length 1 cm) was used for analytical work. For semi-preparative work the detector was set at 500 nm and a 20 μl flow cell (path length 0.2 mm) was used.

Results and discussion

During the last few years we have experimented with most of the commercially available h.p.l.c.

packing materials and have found that the spherically shaped Hypersil and Spherosorb give the best separations of uroporphyrin methyl esters that we have been able to achieve. Fig. 1(a) illustrates the type of analytical separation that can be obtained with a mixture of uroporphyrins obtained from the faeces of a porphyric patient, with a Spherosorb column and ethyl acetate/hexane (3:1, v/v) as eluent. This system can be used in the same run both for the analysis of the range of porphyrins with two to eight carboxylic side chains and for the quantification of the uroporphyrin methyl esters I and III (2b) and (4b) (Fig. 1b).

It was relatively easy to scale up this process and to isolate milligram quantities of both uroporphyrin isomers. Figs. 2(a) and 2(b) show how 5 mg samples of mixtures of uroporphyrin I and III methyl esters (2b) and (4b) obtained from porphyric urine and faecal extracts could be virtually completely sepa-

Fig. 1. H.p.l.c. analysis of porphyrin methyl esters on a 3 μm Hypersil column (200 mm $\times$ 4.5 mm) eluted with hexane/ethyl acetate (1:1, v/v) at a flow rate of 0.8 ml/min

(a) Separation of the octamethyl esters of uroporphyrins I (2b) and III (4b). (b) Separation of the methyl esters of a mixture of porphyrins isolated from the faeces of a porphyric patient. (The numbers indicate the number of carboxylic side chains.)

Fig. 2. Semi-preparative separation and isolation of the octamethyl esters of uroporphyrin isomers on a 5 μ m Hypersil column (500 mm $\times$ 12 mm) with hexane/ethyl acetate (1:3, v/v) as eluent

(a) Uroporphyrin fraction from the faeces of a porphyric patient. (b) Uroporphyrin fraction from the urine of a porphyric patient. (c) Synthetic mixture of uroporphyrins I, II, III and IV.

rated in one pass on the semi-preparative Hypersil column. Loss of resolution was not observed even after 30 repeated injections, but if quantities as large as 10 mg were injected the peaks overlapped. This was thought to be due essentially to overloading of the column, but it is possible that another factor might be the association of porphyrins in more concentrated solutions. The homogeneity of the materials isolated by use of the semi-preparative column was checked by analytical h.p.l.c., and by conversion into the corresponding coproporphyrins (by acid-catalysed decarboxylation), which were compared chromatographically with authentic synthetic standards.

Attempts to perform semi-preparative separations of all four isomeric uroporphyrin methyl esters were frustrated by co-elution of the type III and IV isomers (4b) and (5b) (Fig. 2c), and even on an analytical scale these two isomers were only partially resolved.

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