H.p.l.c. analysis of di- and tri-carboxylic porphyrins in porphyric patients

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New h.p.l.c. methods have been developed for the quantitative determination of di- and tri-carboxylic porphyrin methyl esters, and applied to the analysis of faecal extracts from patients with four different types of porphyria.

In normal metabolism, haem is biosynthesized from uroporphyrinogen-III (1a) by decarboxylation to coproporphyrinogen-III (3a), followed by oxidative decarboxylation of the latter to protoporphyrinogen-IX (11a), dehydrogenation and insertion of iron (Scheme 1) (Bogorad, 1979). However, in patients with porphyria cutanea tarda and hepatoerythrocytic porphyria, intermediates between uroporphyrinogen-III and coproporphyrinogen-III may accumulate as a result of an impairment of the uroporphyrinogen decarboxylase enzyme; part of the excess of pentacarboxylic porphyrinogen (2a) is oxidatively decarboxylated to dehydroisocoproporphyrinogen (4a) by coproporphyrinogen oxidative decarboxylase (before the final acetic acid residue is decarboxylated) (Elder, 1972, 1975; Jackson *et al.*, 1980b). The dehydroisocoproporphyrinogen (4a) then undergoes further transformations of the vinyl group, and dehydrogenation of the macrocycle, before excretion as a mixture of four porphyrins: dehydroisocoproporphyrin (4b), isocoproporphyrin (5b), desethylisocoproporphyrin (6b) and the

hydroxyethyl porphyrin (7b) (Elder, 1972, 1975; Stoll *et al.*, 1973).

Dehydroisocoproporphyrinogen (4a), isocoproporphyrinogen (5a) and desethylisocoproporphyrinogen (6a) are all metabolized *in vitro* by chicken erythrocyte haemolysates to the corresponding dicarboxylic porphyrins (11b), (12b) and (13b) respectively via the tricarboxylic porphyrinogens (8a), (9a) and (10a) (Jackson *et al.*, 1980b). It is therefore possible that in porphyric patients an abnormal metabolic pathway may exist between the pentacarboxylic porphyrinogen (2a) and protoporphyrinogen-IX (11a) via dehydroisocoproporphyrinogen (4a). Our earlier studies showed that isoharderoporphyrinogen (14a) is not an intermediate *in vivo*, even though it can be metabolized by coproporphyrinogen oxidative decarboxylase to protoporphyrinogen-IX (11a) *in vitro* by crude enzyme preparations (Elder *et al.*, 1978; Jackson *et al.*, 1978, 1980a). We have therefore now developed h.p.l.c. methods for analysing the di- and tri-carboxylic porphyrins that might be formed in Nature from coproporphyrinogen (3a) or dehydro-

Scheme 1. Biosynthesis of haem from uroporphyrinogen-III
The structures of the porphyrinogens are shown in Fig. 1.

Fig. 1. Structures of porphyrinogens (suffix a)

In the text the corresponding porphyrins are denoted by suffix b, and their methyl esters by suffix c. A = CH₂CO₂H; P = CH₂CH₂CO₂H; V = CH=CH₂.

isocoproporphyrinogen (4a) and applied them to the analysis of faecal extracts.

Materials

Pemptoporphyrin, isopemptoporphyrin, harderoporphyrin and isoharderoporphyrin methyl esters (13c), (15c), (8c) and (14c) respectively were all available from previous synthetic studies. Protoporphyrin dimethyl ester (11c) was prepared by toluene-*p*-sulphonic acid-catalysed dehydration of haematoporphyrin (16b) in boiling chlorobenzene, followed by esterification with methanol containing 5% (v/v) H₂SO₄. Dihydroprotoporphyrin methyl ester (12c) was prepared from protoporphyrin dimethyl ester (11c) by partial synthesis from protoporphyrin (A. H. Jackson & D. M. Jones, unpublished work), by conversion into the

monohydroxyethylmonovinyl analogue (21c) (cf. Kenner *et al.*, 1976) followed by reduction of the vinyl group to an ethyl group, and transformation of the hydroxyethyl side chain back to vinyl via the bromoethylporphyrin (22c). Deuteroporphyrin-IX, desvinylharderoporphyrin and desvinylisoharderoporphyrin methyl esters (17c), (10c) and (18c) were prepared from protoporphyrin-IX, harderoporphyrin and isoharderoporphyrin methyl esters (11c), (8c) and (14c) by devinylation in a resorcinol melt (cf. Bonnett *et al.*, 1977; DiNello & Dolphin, 1981).

Experimental

For isolation of porphyrins, faecal samples were collected from patients with the clinically diagnosed symptoms of porphyria cutanea tarda,

hepatoerythrocytic porphyria, variegata porphyria and erythropoietic protoporphyria. After being freeze-dried the samples were treated with methanol containing 5% (v/v) H_2SO_4 and kept in the dark for 24 h at 20°C. The porphyrin methyl esters were then isolated as described previously (Smith, 1977) and separated into di- and tri-carboxylic fractions by semi-preparative t.l.c. on Merck silica gel 60G eluted with toluene/ethyl acetate/ethanol (80:15:3, by vol.) (Smith, 1975).

H.p.l.c. analyses of these porphyrins were carried out (with the solvent systems described below) on a Hypersil (particle size 5 μm) column (250 mm $\times$ 4.5 mm internal diam.), solvent being delivered by a Waters 6000A pump. The eluent was monitored with a Cecil 272 variable-wavelength detector set at 400 nm, and with an absorbance-unit full-scale sensitivity of 0.1. Approx. 10 μg porphyrin samples were injected on to the column for all the analyses reported (Fig. 2). All solvents were of analytical-reagent grade purchased from BDH Chemicals, and were used without further purification.

Results and discussion

In earlier studies of the h.p.l.c. separation of porphyrin esters on silica gel (Evans *et al.*, 1976; Smith *et al.*, 1979; Jackson *et al.*, 1980c, 1982a,b) we have found that mixtures of ethyl acetate with light petroleum, hexane or cyclohexane are among the most convenient solvent systems to use, higher proportions of ethyl acetate being used with the more-polar porphyrins containing larger numbers of carboxylic ester side chains.

Further experiments with this type of solvent system have now enabled us to separate protoporphyrin, dihydroporphyrin, mesoporphyrin, deuteroporphyrin, pemptoporphyrin and isopemptoporphyrin methyl esters by isocratic elution with ethyl acetate/light petroleum (3:17, v/v), as shown in Fig. 2(a)(i). The dicarboxylic porphyrin fractions obtained from the faecal extracts from porphyrin patients were analysed with this system and the results are shown in Figs. 2(a)(ii)–(v). The faecal extracts from the patients with porphyria cutanea tarda give the most complex pattern, variable amounts of mesoporphyrin, deuteroporphyrin, pemptoporphyrin and protoporphyrin being found, and possibly also a small amount of dihydroporphyrin. With the patients with variegata porphyria and erythropoietic protoporphyria the major band corresponded to protoporphyrin, although small bands corresponding to deuteroporphyrin, mesoporphyrin and dihydroporphyrin were also observed. Interestingly deuteroporphyrin was the

major dicarboxylic porphyrin present in the faecal extracts from the patients with hepatoerythrocytic porphyria, together with a small amount of protoporphyrin; the absence of mesoporphyrin or dihydroporphyrin suggests that in these patients the enzymes in the faecal bacteria lack the ability to reduce vinyl side chains, or that devinylation is a more rapid process.

H.p.l.c. analysis of the tricarboxylic porphyrin esters (8c), (9c) and (10c) was carried out with a slightly more-polar solvent system by using 20 or 25% (v/v) ethyl acetate in light petroleum, and Fig. 2(b)(i) shows the separation achieved. Analyses of the faecal tricarboxylic porphyrins obtained from porphyric patients are shown in Figs. 2(b)(ii)–(v); the patterns observed were quite complex, except for the extract from the patients with erythropoietic protoporphyria, which showed only one major peak. This peak was also observed in the other three extracts and its retention time was quite close to that of isoharderoporphyrin trimethyl ester. As our previous studies have shown that the pathway between coproporphyrinogen-III and protoporphyrin-IX is regiospecific (Elder *et al.*, 1978; Jackson *et al.*, 1980a), involving harderoporphyrinogen only (but not its isomer), we collected the anomalous fraction. Its h.p.l.c. characteristics and visible-absorption spectrum were compatible with a chlorophyll degradation product, and h.p.l.c. comparisons confirmed that it was methyl phaeophorbide-*a* (little or no porphyrin being present).

The other major peak in the extract from patients with porphyria cutanea tarda corresponded to harderoporphyrin, although small amounts of other (unidentified) porphyrins were also present. The extract from patients with variegata porphyria showed a major fraction with a much longer retention time than any of three synthetic porphyrin methyl esters; further studies must await the provision of more material. Perhaps the most interesting result is the presence of substantial amounts of desvinylharderoporphyrin as well as harderoporphyrin in patients with hepatoerythrocytic porphyria; this accords with the finding of deuteroporphyrin as the only dicarboxylic porphyrin [Fig. 2(a)(v)] and suggests that devinylation occurs preferentially in this disease rather than reduction to ethyl. The differences in composition of the faecal extracts from patients with variegata porphyria and erythropoietic protoporphyria and those from patients with porphyria cutanea tarda and hepatoerythrocytic porphyria may be due to the very much larger amounts of protoporphyrin excreted in variegata porphyria and erythropoietic protoporphyria.

The results described in this paper show that closely related di- and tri-carboxylic porphyrin esters can be separated by h.p.l.c., and that

Fig. 2. *H.p.l.c. analyses of porphyrins obtained from the faeces of porphyric patients*

(a) Dicarboxylic porphyrins. The amount found is usually in the range 100–150 $\mu\text{g/g}$ dry wt. of faeces, but may increase considerably in the acute phase. (b) Tricarboxylic porphyrins. The amount found is usually in the range 1–5 $\mu\text{g/g}$ dry wt. of faeces. (i) Synthetic mixtures of porphyrins; (ii)–(v) porphyrins from patients with (ii) erythropoietic porphyria, (iii) variegate porphyria, (iv) porphyria cutanea tarda and (v) hepatoerythrocytic porphyria. Peak 1, meso, 20c; peak 2, dihydroproto, 12c and 19c; peak 3, proto, 11c; peak 4, pempto, 13c; peak 5, isopempto, 15c; peaks 6 and 7, deutero, 20c; peak 8, isohardero, 14c; peak 9, hardero, 8c; peak 10, tripropionate, 10c; *, methyl phaeophorbide-*a* dimethyl ester.

distinctive patterns are observed from faecal extracts obtained from various types of porphyric patients. Whether the reduction and/or removal of vinyl groups occurs at the porphyrin, or porphyrinogen, level of oxidation remains to be determined.

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References

- Bogorad, L. (1979) in *The Porphyrins* (Dolphin, D., ed.), vol. 6 (*Biochemistry*, part A), pp. 125–178, Academic Press, New York
- Bonnett, R., Campion-Smith, I. H. & Page A. J. (1977) *J. Chem. Soc. Perkin Trans. 1*, 68–71
- DiNello, R. K. & Dolphin, D. (1981) *J. Org. Chem.* **46**, 3498–3502
- Elder, G. H. (1972) *Biochem. J.* **126**, 887–891
- Elder, G. H. (1975) *J. Clin. Pathol.* **28**, 601–604
- Elder, G. H., Evans, J. O., Jackson, J. R. & Jackson, A. H. (1978) *Biochem. J.* **169**, 215–223
- Evans, N., Jackson, A. H., Matlin, S. A. & Towill, R. (1976) *J. Chromatogr.* **125**, 345–355
- Jackson, A. H., Elder, G. H. & Smith, S. G. (1978) *Int. J. Biochem.* **9**, 877–882
- Jackson, A. H., Jones, D. M., Philip, G., Lash, T. D., Battle, A. M. del C. & Smith, S. G. (1980a) *Int. J. Biochem.* **12**, 681–688
- Jackson, A. H., Lash, T. D., Ryder, D. J. & Smith, S. G. (1980b) *Int. J. Biochem.* **12**, 775–780
- Jackson, A. H., Rao, K. R. N. & Smith, S. G. (1980c) *Int. J. Biochem.* **12**, 1081–1084
- Jackson, A. H., Rao, K. R. N. & Smith, S. G. (1982a) *Biochem. J.* **203**, 515–517
- Jackson, A. H., Rao, K. R. N. & Smith, S. G. (1982b) *Biochem. J.* **207**, 599–603
- Kenner, G. W., Quirke, J. M. E. & Smith, K. M. (1976) *Tetrahedron* **32**, 2753–2756
- Smith, S. G. (1975) *Br. J. Dermatol.* **93**, 291–295
- Smith, S. G. (1977) *Biochem. Soc. Trans.* **5**, 1472–1473
- Smith, S. G., El-Far, M. A., Rao, K. R. N. & Jackson, A. H. (1979) *Biochem. Soc. Trans.* **7**, 1291–1293
- Stoll, M. S., Elder, G. H., Games, D. E., O'Hanlon, P., Millington, D. S. & Jackson, A. H. (1973) *Biochem. J.* **131**, 429–432