High-pressure-liquid-chromatographic analysis of tetracarboxylic porphyrins in hepatic porphyrias

Anthony H. JACKSON,* K. R. Nagaraja RAO* and Sydney G. SMITH†

*Department of Chemistry, University College, Cardiff CF1 1XL, and †Department of Medical Biochemistry, Welsh National School of Medicine, Heath Park, Cardiff CF4 4XN, Wales, U.K.

(Received 3 August 1982/Accepted 23 September 1982)

New high-pressure-liquid-chromatographic methods have been developed for the quantitative analysis of mixtures of coproporphyrins I and III, and of isocoproporphyrin, dehydroisocoproporphyrin and de-ethylisocoproporphyrin.

Significant quantities of tetracarboxylic porphyrins are excreted in the urine and faeces of patients with porphyria cutanea tarda or hepatoerythrocytic porphyria, and in the urine and faeces of rats poisoned with hexachlorobenzene. These porphyrins consist largely of coproporphyrin I (1a), coproporphyrin III (2a), isocoproporphyrin (3a) and de-ethylisocoproporphyrin (4a), together with smaller amounts of dehydroisocoproporphyrin (5a) and the hydroxyethyl analogue (6a) of isocoproporphyrin (Elder, 1972, 1974; Stoll *et al.*, 1973). Isocoproporphyrin (3a) and its congeners (4a) and (6a) are thought to be formed by reduction, devinylation and hydration respectively of the dehydroisocoproporphyrin (5a); the dehydroisocoproporphyrin is formed from the corresponding porphyrinogen, which in turn arises by action of coproporphyrinogen oxidative decarboxylase on the normal pentacarboxylic porphyrinogen precursor (7b) of coproporphyrinogen III (2b). Evidence for this suggestion has been provided by the demonstration that isocoproporphyrinogen (3b), de-ethylisocoproporphyrinogen (4b) and dehydroisocoproporphyrinogen (5b) (as well as coproporphyrinogen-III) are all substrates for the mixed enzyme system in chicken haemolysates (uroporphyrinogen decarboxylase and coproporphyrinogen oxidative decarboxylase) (Jackson *et al.*, 1980) and give rise ultimately to the corresponding dicarboxylic porphyrins (8a), (9a) and (10a). We also suggested that when overproduction of the pentacarboxylic porphyrinogen (7a) occurs (as in certain types of porphyria) it might be degraded by an alternative pathway via dehydroisocoproporphyrinogen (5b) to protoporphyrin IX (10a) as well as by the normal route via coproporphyrinogen III (2a) (Jackson

et al., 1980). In the present paper we describe further detailed studies of the tetracarboxylic porphyrins formed *in vivo* in porphyric patients, and rats, by using h.p.l.c. methods.

Materials

Coproporphyrin I and coproporphyrin III tetramethyl esters were synthesized by established procedures (cf. Jackson *et al.*, 1972) and we also acknowledge gifts of these materials generously supplied by Dr. S. F. MacDonald (National Research Council, Ottawa, Ont., Canada). Isocoproporphyrin, de-ethylisocoproporphyrin and dehydroisocoproporphyrin were all synthesized (A. H. Jackson, T. D. Lash & D. J. Ryder, unpublished work) by the *b*-oxobilane route (cf. Jackson & Smith, 1973; Clezy & Jackson, 1978).

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

Experimental

Porphyrins were isolated from the urine and faeces of porphyric patients and rats, as their methyl esters, by following established methods (With, 1958; Smith, 1975, 1977). Thus the urinary porphyrins were adsorbed on talc, and then converted into their methyl esters by treatment of the talc with methanol/conc. H₂SO₄ (19:1, v/v). Faecal porphyrins were methylated directly by stirring the faeces with the methanol/H₂SO₄ overnight in the dark; faecal residues were then removed by centrifugation. The porphyrin esters from both the urinary

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

(4) R = H

(5) R = CH=CH₂

(6) R = CH(OH)-CH₃

(7) R = A

(9) R = H

(10) R = CH=CH₂

P = CH₂-CH₂-CO₂H

A = CH₂-CO₂H

Structures of porphyrins discussed in the present paper. The porphyrins are numbered (1a)–(10a), the corresponding porphyrinogens (1b)–(10b) and the porphyrin methyl esters (1c)–(10c) in the text.

and faecal sources were isolated by dilution of the methanol/H₂SO₄ extracts with distilled water followed by extraction with chloroform. The extracts in each case were washed with dilute NaHCO₃ solution and water before being dried (over MgSO₄) and evaporated to dryness.

After preliminary purification by column chromatography on alumina the crude mixtures of porphyrin esters were applied to thick-layer chromatography plates (20 cm × 20 cm) and developed in the solvent system toluene/ethyl acetate/ethanol (16:3:1, by vol.). The bands corresponding to the coproporphyrin and to the isocoproporphyrin fractions (*R_F* 0.6 and 0.7 respectively) were scraped off the plates and extracted into chloroform containing small amounts of ethanol, and after filtration the solvent was removed under reduced pressure and the residues were examined by spectroscopic methods and by t.l.c. (Smith, 1977) and h.p.l.c. The field-

desorption mass spectra of the crude isocoproporphyrin preparations and of the synthetic porphyrins were obtained by using a Varian CH5D mass spectrometer with an emitter wire current of 20 μA, and the spectra were recorded on a Statos 600 data system. H.p.l.c. analyses were performed with a Waters 6000A dual reciprocating pump, a Cecil Ce272 u.v.–visible spectrophotometer set at 404 nm and a 250 mm × 4.9 mm stainless-steel column packed with 5 μm Hypersil. Details of the solvent system used are described below.

Results and discussion

The field-desorption mass spectrum of the isocoproporphyrin fraction obtained from the urine of a patient with the clinical characteristics of porphyria cutanea tarda revealed a molecular ion at *m/z* 683 corresponding to the (*M*+1)⁺ ion of de-ethyl-

Fig. 1. Field-desorption mass spectra of porphyrin tetramethyl esters derived from the 'isocoproprophyrin fractions' of (a) faeces, (b) urine and (c) bile of a patient with hepatoerythrocytic porphyria
For full experimental details see the text.

isocoproprophyrin tetramethyl ester (4c), but the ion at m/z 711 expected for isocoproprophyrin tetramethyl ester (3c) was of negligible intensity (Fig. 1). On the other hand, the isocoproprophyrin fraction from the faecal extract showed an ion at m/z 683 as the main peak, and also a peak of moderate intensity at m/z 711, indicating clearly that it contained both isocoproprophyrin and de-ethylisocoproprophyrin tetramethyl esters (3c) and (4c). The mass spectrum of a synthetic mixture consisting of equal amounts of these porphyrins again showed molecular ions at m/z 683 and 711, but their intensities were unequal; this was not unexpected, as there are often differences in the rates of desorption of different compounds from the emitter wire in field-desorption mass spectra, and this makes the technique difficult to use for quantitative estimations. The field-desorption mass spectrum of the isocoproprophyrin fraction isolated from bile surprisingly showed a molecular ion at m/z 709, indicating that this fraction was largely dehydroisocoproprophyrin tetramethyl ester (5c). In the light of these results we considered that it was worthwhile performing further quantitative investigations of the relative amounts of these porphyrins by h.p.l.c., and during this work, described below, we have developed an efficient method of separating these porphyrins. Attempts to separate isocoproprophyrin, de-ethylcoproprophyrin and dehydroisocoproprophyrin tetramethyl esters by t.l.c. methods under a variety of conditions were only partly successful, but h.p.l.c. proved to be entirely satisfactory. In earlier studies we had explored the use of hexane/ethyl acetate and similar systems for separation of these esters, and Gray *et al.* (1977) also

reported partial separations of isocoproprophyrin and de-ethylisocoproprophyrin methyl esters.

However, with a modification of this type of solvent system and with 5 μ m Hypersil as column packing we have now been able to achieve almost baseline separations of the three components in the isocoproprophyrin fraction. Fig. 2 showed typical separations obtained for the urine, faecal and bile isocoproprophyrin fractions, and comparisons with a synthetic mixture.

The isocoproprophyrin fractions obtained from a number of patients with porphyria cutanea tarda or hepatoerythrocytic porphyria, as well as from hexachlorobenzene-poisoned rats, were screened by this new h.p.l.c. technique. In accord with earlier preliminary t.l.c. studies of a patient with porphyria cutanea tarda (Smith, 1977), it was found that the isocoproprophyrin fraction obtained from urine contained essentially only de-ethylisocoproprophyrin (4a). The faecal extracts contained mainly de-ethylisocoproprophyrin (4a) with about 20% of isocoproprophyrin (4a). Very similar results were observed with extracts from all three sources, i.e. patients with porphyria as well as the poisoned rats. This pattern appears to be typical of a classic disorder, and, although we were unable to obtain many samples from bile, with the patients with porphyria cutanea tarda, at least, virtually the only porphyrin observed was dehydroisocoproprophyrin (5a). It thus seems that bile, or liver, could be the main pool in which the dehydroisocoproprophyrin (5a) [or the corresponding porphyrinogen (5b)] is formed, and that this compound is subsequently degraded by micro-organisms in the intestine to isocoproprophyrin (3a) and de-ethylisocopro-

Fig. 2. H.p.l.c. analysis of porphyrin methyl esters obtained from the 'isocoproprophyrin fraction' from a patient with hepatoerythrocytic porphyria

The porphyrin methyl esters were run on a $5\mu\text{m}$ Hypersil column ($250\text{ mm} \times 4.5\text{ mm}$) and eluted with ethyl acetate/light petroleum (b.p. $60\text{--}80^\circ\text{C}$) ($20:3$, v/v) at a flow rate of 1 ml/min . (a) Synthetic mixture of isocoproprophyrin, de-ethylisocoproprophyrin and dehydroisocoproprophyrin tetramethyl esters (3c), (4c) and (5c) respectively. (b) Extract of the bile from the patient with porphyria. (c) Extract of the faeces from the patient with porphyria. (d) Extract of the urine from the patient with porphyria.

Fig. 3. H.p.l.c. analysis of a synthetic mixture of coproporphyrin I and coproporphyrin III tetramethyl esters (1c) and (2c) and of isocoproprophyrin, dehydroisocoproprophyrin and de-ethylisocoproprophyrin tetramethyl esters (3c), (4c) and (5c)

The porphyrin methyl esters were run on a $5\mu\text{m}$ Hypersil column ($250\text{ mm} \times 4.5\text{ mm}$) eluted with 1,2-dichloroethane/acetone ($24:1$, v/v) at a flow rate of 1 ml/min . (Note that in this solvent system the isocoproprophyrin fraction is not fully resolved.)

porphyrin (4a). Two factors may be responsible for this effect, namely (i) a rapid rate of devinylation compared with reduction of the vinyl group, and (ii) a specific enterohepatic migration of de-ethylisocoproprophyrin (4a) from the intestine into the urinary tract.

The relative amounts of coproporphyrins I and III in the 'coproporphyrin fraction' can also be analysed by h.p.l.c. with a novel solvent system 4% (v/v) acetone in dichloroethane, and some results are shown in Fig. 3. Although partial separations of the isocoproprophyrin fraction can also be obtained in this system, complete separation has only been achieved by the system shown in Fig. 2 (but this does not resolve the coproporphyrin isomers). In the urine of patients with porphyria cutanea tarda or hepatoerythrocytic porphyria the coproporphyrin fraction is usually somewhat larger than the isocoproprophyrin fraction, whereas in the faeces

the reverse pattern is observed and the isocoproporphyrin fraction is several times greater than that of coproporphyrin.

During the present work we have noted the presence of other new porphyrins on the t.l.c. plates and further work on the structures of these new porphyrins is required. Preliminary analysis (A. H. Jackson, K. R. N. Rao & S. G. Smith, unpublished work) of the dicarboxylic porphyrins obtained from these patients indicates that deuteroporphyrin is the main constituent, demonstrating that devinylation is the preferred route of microbial degradation. It is not absolutely clear whether these reactions take place at the porphyrin level of oxidation, or at the porphyrinogen level, but it seems likely that it is at the porphyrin level.

We thank the Science and Engineering Research Council for their support of this work.

References

- Clezy, P. S. & Jackson, A. H. (1978) in *The Porphyrins* (Dolphin, D., ed.), vol. 1, pp. 265–288, Academic Press, New York
- Elder, G. H. (1972) *Biochem. J.* **126**, 877–891
- Elder, G. H. (1974) *Enzyme* **17**, 61–68
- Gray, C. H., Lim, C. K. & Nicholson, D. C. (1977) *Clin. Chim. Acta* **77**, 167–178
- Jackson, A. H. & Smith, K. M. (1973) in *The Total Synthesis of Natural Products* (ApSimon, J., ed.), vol. 1, pp. 143–278, John Wiley and Sons, New York
- Jackson, A. H., Kenner, G. W. & Wass, J. (1972) *J. Chem. Soc. Perkin Trans. 1* 1475–1483
- Jackson, A. H., Lash, T. D., Ryder, D. J. & Smith, S. G. (1980) *Int. J. Biochem.* **12**, 775–780
- Smith, S. G. (1975) *Br. J. Dermatol.* **93**, 291–295
- Smith, S. G. (1977) *Biochem. Soc. Trans.* **5**, 1472–1473
- Stoll, M. S., Elder, G. J., Games, D. E., O'Hanlon, P., Jackson, A. H. & Millington, D. S. (1973) *Biochem. J.* **131**, 429–432
- With, T. K. (1958) *Biochem. J.* **68**, 717–720