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The Direct Determination of Porphyrin Carboxylic Acids

HIGH-PRESSURE LIQUID CHROMATOGRAPHY WITH SOLVENT SYSTEMS CONTAINING PHASE-TRANSFER AGENTS

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A novel method for separating porphyrin polycarboxylic acids is described and illustrated by its application to the direct analysis of biological (deep-sea medusae), clinical (urine) and chemical ('haematoporphyrin derivative') samples.

Porphyrin polycarboxylic acids occur naturally, and their detection, identification and determination are of considerable interest in chemistry, in biochemistry and in medicine. The porphyrin carboxylic acids are generally rather insoluble in organic solvents, and hence are often converted into methyl esters before separation, e.g. by h.p.l.c. (e.g. see Battersby *et al.*, 1976). This intermediate esterification step, although simple enough in principle, may itself introduce problems, such as reactions at vinyl groups and the effects of differential esterification and differential extraction. Clearly it would be preferable if the free acids from the tissue could be examined directly. We now report the use of a simple solvent system containing a phase-transfer agent that facilitates both the extraction of the porphyrin polycarboxylic acids and the h.p.l.c. separation on a reversed-phase column. Phase-transfer agents (Starks, 1971) are ionic substances that are nonetheless soluble to some extent in non-polar organic solvents. They have been used to catalyse certain heterogeneous reactions (phase-transfer catalysis; Starks, 1971; Gordon & Kutina, 1977) and in various chromatographic applications (also called 'paired-ion chromatography'; Levine, 1974; Wittmer *et al.*, 1975).

Methods

A Waters M6000A pump with a UK6 injector was used, together with a Cecil CE 212 ultraviolet detector set at 400nm and a 10mV recorder. A μ Bondapak C_{18} column (3.9mm × 30cm) (Waters Associates) was used throughout. The solvent systems used were all 1mM in tetrabutylammonium dihydrogen phosphate; an appropriate amount of this salt was dissolved in water, and mixed with alkanol, as follows: solvent A, methanol/water (17:3, v/v); solvent B, methanol/water (4:1, v/v); solvent C, ethanol/water (17:3, v/v). Solvents were degassed by shaking vigorously under reduced pressure (water pump). Flow rates of 1.0–1.5ml/min were used.

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

Uroporphyrin III was isolated from touraco feathers (Nicholas & Rimington, 1951). Coproporphyrins I and III were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and from Professor A. H. Jackson and Professor A. R. Battersby. Reference samples of the porphyrin penta-, hexa- and hepta-carboxylic acids were provided by Professor T. K. With.

The method of sample preparation depended on the source. For the medusae *Atolla wyvillei* and *Periphylla periphylla* a small sample (50mg wet wt.) of the pigmented tissue preserved in formalin (R. Bonnett, E. J. H. Head & P. J. Herring, unpublished work) was homogenized with solvent system C (2ml) and filtered to remove tissue, and the filtrate was injected without further purification. Urine samples were injected neat: when dilution was necessary it was made with solvent system B. Porphyrin polycarboxylic acids were dissolved in solvent A or B.

Results and Discussion

The chromatograms from reference samples of coproporphyrin, the penta-, hexa- and hepta-carboxylic acids and uroporphyrin, and the separation of an artificial mixture of these components, are shown in Fig. 1. The polycarboxylic acids were eluted from the reverse-phase column in order of decreasing polarity, but the type isomers (e.g. coproporphyrin I and coproporphyrin III) were not separated under the conditions described. The phase-transfer agent is considered to be effective because the bulky lipo-

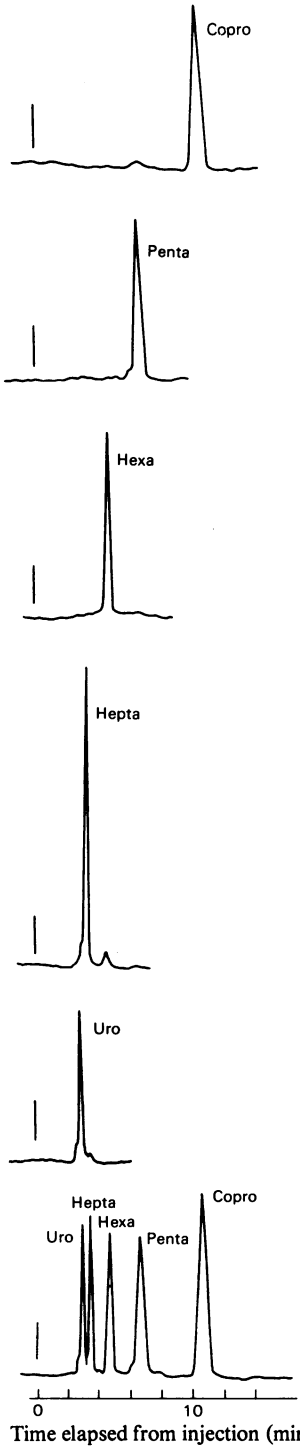

Fig. 1. *H.p.l.c. of reference samples of coproporphyrin, the penta-, hexa- and hepta-carboxylic acids and uroporphyrin, and an artificial mixture of these, with solvent B*
Experimental details are given in the text.

philic ion R_4N^+ forms neutral ion pairs with the porphyrin carboxylate side chains, thus:

$$P(CO_2H)_n + H_2O \rightleftarrows P(CO_2H)_{n-1}(CO_2^-) + H_3O^+$$

$$R_4N^+ \downarrow \uparrow$$

$$X^- \downarrow$$

$$[P(CO_2H)_{n-1}(CO_2^-)\cdot R_4N^+] + X^- \text{ etc.}$$

Where P is porphyrin-$[CH_2]_m^-$. This not only solubilizes the porphyrin acids in the alcoholic solvent, but also establishes an increased affinity for, and a rapid equilibrium with, the non-polar phase on the support. This produces sharp peaks, but components are still eluted in order of decreasing polarity (i.e. uroporphyrin first). It also seems likely that a second type of specific interaction, namely that between the dihydrogen phosphate anion and the central nitrogen atoms, may play some part in achieving the separations observed here.

The standard method (Rimington, 1971) for the determination of porphyrins in clinical samples involves differential acid extraction to give two main

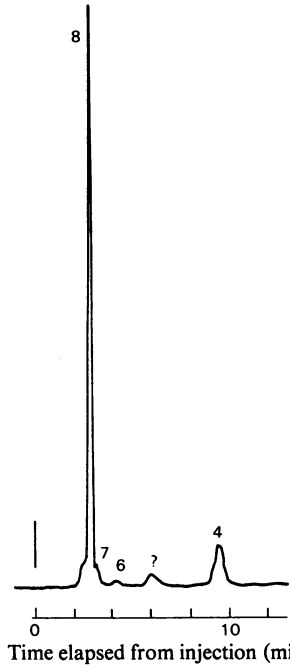

Fig. 2. *H.p.l.c. of urine sample from a patient suffering from congenital porphyria with solvent B*
Experimental details are given in the text. The following identifications were made by mixed chromatography: 8, uroporphyrin; 7, porphyrin hepta-carboxylic acid; 6, hexacarboxylic acid; 4, coproporphyrin; ?, unidentified.

fractions, namely the coproporphyrin fraction (extracted into 5% HCl) and the uroporphyrin fraction (extracted into cyclohexanone at pH 1.5). Preliminary experiments on urine samples with our system shows that the porphyrin carboxylic acids may be separated and determined by injecting urine, or urine diluted with solvent, directly on to the h.p.l.c. column. The porphyrin concentrations in normal urine are too low to be detected in this way, but Fig. 2 shows the examination of the porphyrins from the urine of a patient suffering from congenital porphyria. In this sample uroporphyrin is the main component, but smaller amounts of coproporphyrin and the hexa- and hepta-carboxylic acids are also detected (mixed chromatograms), the relative amounts decreasing in the order stated. An unidentified porphyrin is also detected.

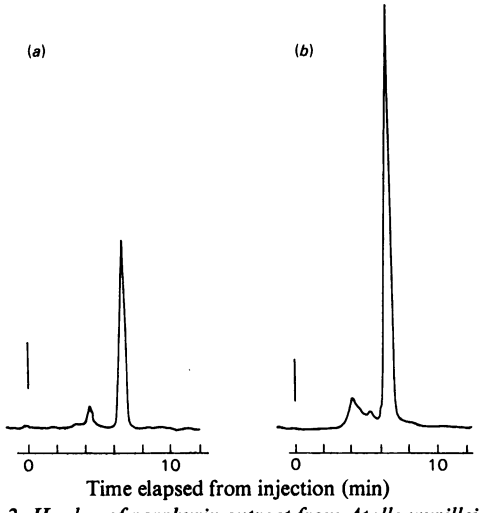

Fig. 3. *H.p.l.c. of porphyrin extract from Atolla wyvillei (a) and a mixed run with authentic protoporphyrin (b), both with solvent C*
Experimental details are given in the text.

Certain deep-sea medusae have been shown to contain free protoporphyrin (Herring, 1972; R. Bonnett, E. J. H. Head & P. J. Herring, unpublished work). Fig. 3 shows the chromatogram of an extract from the pigmented tissue of *Atolla wyvillei*, together with a mixed chromatogram with authentic protoporphyrin. The identification of the major component is confirmed; a minor amount of a second unidentified component is also detected. The pigmented tissue of the medusa *Periphylla periphylla* gives a similar result.

Finally, a chemical example is given. There has recently been a growing interest in photochemotherapy as an approach to the management of cancer. This development depends on the preferential absorption of certain photosensitizers by cancer cells: on irradiation, preferential degradation of the cancer is reported to occur (Kelly *et al.*, 1975; Dougherty *et al.*, 1976). Possibly a singlet-oxygen reaction is involved, although this is by no means established. A sensitizer that has been commonly used is a preparation called 'haematoporphyrin derivative', which is prepared by the action of acetic acid/H_2SO_4 on haematoporphyrin (Lipson & Baldes,

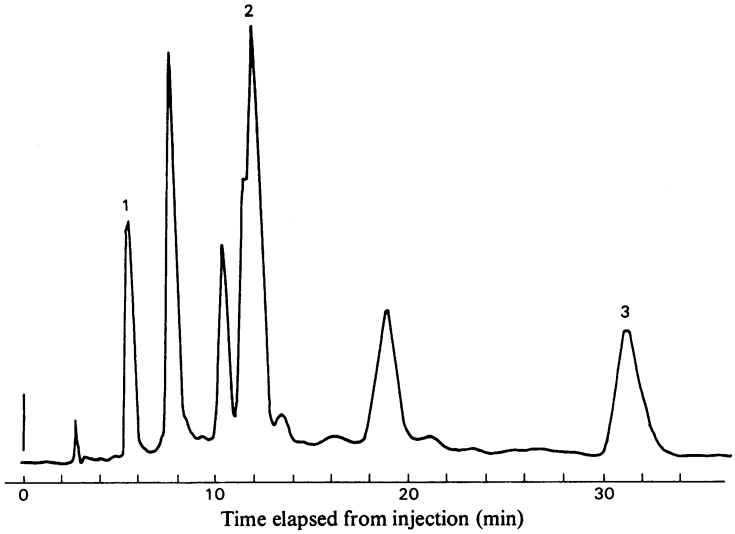

Fig. 4. *H.p.l.c of haematoporphyrin derivative with solvent A*
Experimental details are given in the text. Identifications of major components: 1, haematoporphyrin; 2, 3,8-di-(1-acetoxyethyl)deuteroporphyrin; (*OO'*-diacetylhaematoporphyrin); 3, protoporphyrin.

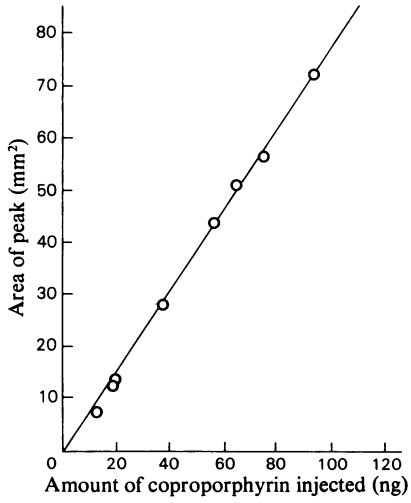

Fig. 5. *Dose–response curve for coproporphyrin*
Experimental details are given in the text. Eight samples containing between 10 and 100ng of coproporphyrin I were used with solvent B.

1.'60). 'Haematoporphyrin derivative' is known to be a mixture of somewhat variable composition containing, among other things, protoporphyrin, haematoporphyrin and mono- and di-acetoxy derivatives of haematoporphyrin (M. C. Berenbaum, R. Bonnett, R. J. Ridge & M. E. Snell, unpublished work). We find that it is readily assayed by our h.p.l.c. system, and Fig. 4 shows a typical chromatogram. Clearly this suggests itself as the basis of a separation method leading to the identification of the minor components.

In order to use the h.p.l.c. technique described here as an analytical tool it is necessary to establish the shape of the dose–response curve. We have done this for coproporphyrin and find that, for injected samples between 20 and 100ng, there is a linear relationship between the amount of porphyrin and the peak area (Fig. 5). It appears that the method has many advantages in the direct determination of porphyrin polycarboxylic acids.

Some of the work described here has depended on collaborative efforts with Dr. P. J. Herring (Institute of Oceanographic Sciences) and Dr. M. C. Berenbaum and Mr. M. C. Snell (St. Mary's Hospital Medical School), whom we thank for their advice and help. We thank Professor A. R. Battersby, Professor A. H. Jackson and Professor T. K. With for the gifts of standard porphyrin samples, Dr. D. C. Nicholson for the porphyric urine sample, and the Medical Research Council for their support.

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