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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PORPHYRINS IN CLINICAL MATERIALS

N. EVANS, A. H. JACKSON, S. A. MATLIN and R. TOWILL
Department of Chemistry, University College, Cardiff, CF1 1XL (Great Britain)

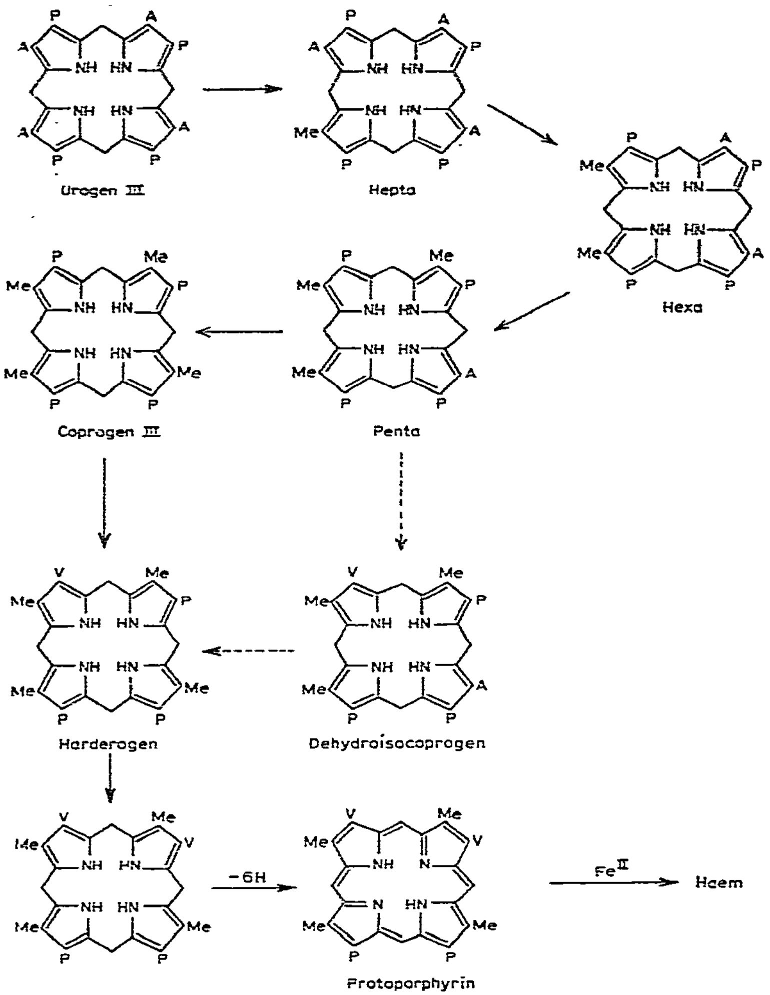
SUMMARY

Methods for the isolation of porphyrins as their methyl esters from porphyric urine and faeces as well as other biological materials are described. Quantitative analyses can be carried out by high-performance liquid chromatography (HPLC), using appropriate internal standards; hence excretion patterns in the various types of porphyria can be obtained which may facilitate clinical diagnosis more effectively than the earlier qualitative thin-layer chromatographic methods. Use of the newer microparticulate column packing materials has improved the efficiency of the HPLC analyses, and enables the more convenient isochratic elution techniques to be used (rather than gradient elution). Separations of some porphyrin isomers on these columns are also described.

INTRODUCTION

In an earlier paper[1] we described the use of high-performance liquid chromatography (HPLC) in the qualitative analysis of naturally occurring porphyrins; we also showed how field desorption mass spectrometry could be a very helpful technique in association with HPLC for the identification and characterisation of the components of a mixture. In a further development from this work, we demonstrated[2] that there is an apparently specific pathway in nature for the conversion of uroporphyrinogen-III to coproporphyrinogen-IX; a minor side-pathway from the pentacarboxylic porphyrinogen to protoporphyrinogen-IX also occurs in cases of porphyria induced by genetic defects or poisoning[3,4]. (see Scheme I).

In this paper, we describe how HPLC can be used to quantify the amounts of porphyrins present in biological materials. This is of particular interest both in connection with analyses of the patterns of porphyrin excretion in porphyric patients, and in biochemical studies of porphyrin metabolism. In our earlier work it proved more convenient to convert the porphyrin free acids into the corresponding methyl esters owing to the greater solubility of the latter in organic solvents, and the ease of working with them on adsorption columns. A number of different types of packing materials have been investigated for HPLC, but in our experience reversed-phase materials have not shown very significant advantages with porphyrin esters, although it is possible that they may be more useful in the separation of isomeric porphyrin



Scheme I. Intermediates between uroporphyrin-III and protoporphyrin-IX in the biosynthesis of haem. Most of the porphyrins referred to in the text are derived from these intermediates by enzymic or aerial oxidation. Mesoporphyrin-IX is the tetrahydro analogue of protoporphyrin-IX in which the two vinyl groups are replaced by ethyl groups; other porphyrins are isomers of the naturally derived porphyrins.

free acids. Most of our work to date has been carried out with silica columns either of the pellicular type (*e.g.*, Corasil II) or microparticulate (LiChrosorb SI-60 or Partisil); the latter have given much better resolution and are now the packing materials of choice, at least for analytical work. As described below, we are now using isochratic, rather than gradient, elution techniques for most of our porphyrin work.

EXPERIMENTAL

HPLC was carried out with a system consisting of a Waters Model 6000 pump and septum injector, with $^{1}/_{8}$ or $^{1}/_{4}$-in. O.D. analytical columns of 316 stainless steel, and a Cecil variable wavelength detector fitted with a 10-μl flow cell. For maximum sensitivity in analytical work, the detector was set on the porphyrin soret band at about 400 nm. Details of the solvent systems and column packings used are described in the text or in the legends to the figures.

Gradient elution

While most of the work was carried out by isochratic elution methods, gradient elution was used for some of the urine analyses. The gradient was formed at low pressure in a simple glass mixing vessel before introduction into the pump[5]. The mixing chamber consisted of a stoppered conical flask to which three side-arms were joined, two at a low level and one at a high level. Reservoirs of two solvents (A and B) to be mixed were connected to the lower side-arms via glass taps and PTFE tubing and the reservoir was initially filled with solvent A. Solvent A was first pumped through the system, and to start the gradient tap B was opened and tap A immediately closed. The concentration of solvent B then increased exponentially with time, the precise rate depending on the volume of the mixing vessel and the flow-rate. Agreement between observed and calculated gradients was good, and the system was found to be highly reproducible.

Examples of chromatograms obtained in this way are shown in earlier papers[1,2], and full details will be described elsewhere.

Quantification

For absolute quantification of the amounts of porphyrins present in urines, mesoporphyrin-IX was used as an internal standard. Calibration graphs of molar ratio of porphyrin to mesoporphyrin *versus* area ratio of porphyrin to mesoporphyrin gave excellent straight-line plots in all cases.

Isolation of porphyrins as methyl esters

Porphyrins were extracted from urine in three different ways, as follows.

(a) Urine (50 ml) was adjusted to pH 3 ± 0.2 with concentrated hydrochloric acid and gently shaken once with ethyl acetate–*n*-butanol (1:1, v/v) (10 ml) and then with further portions of *n*-butanol (10 ml) until the organic phase showed no further fluorescence under UV light. The extracts were combined and washed with water (20 ml); the aqueous layer was then back-extracted with *n*-butanol (5-ml fractions) until no further fluorescence was observed in either layer. These *n*-butanol extracts were added to the main extracts, which were then evaporated to dryness. The product was esterified directly with 5% sulphuric acid in methanol (15 ml) for 15 h. The solution was diluted with water (100 ml) and extracted with chloroform until the extracts showed no fluorescence; the combined extracts were washed with 10% $NaHCO_3$ (30 ml) and water (2 × 30 ml) before evaporation to dryness. Residual water was removed by evaporation with methanol (2 × 5 ml). (In some cases methylation was carried out by use of diazomethane in the usual manner).

(b) Urine (100 ml) was shaken with successive portions of talc until all of the porphyrin was adsorbed, and fresh portions of talc did not fluoresce under UV light. The porphyrins were converted into their methyl esters by elution and esterification by direct treatment of the talc with methanol–sulphuric acid as described above.

(c) For urines with relatively high concentrations of porphyrin (>1 mg/l), direct esterification could be carried out. Urine (10 ml) was mixed with 5% sulphuric acid in methanol (100 ml) and left for 36 h at room temperature in the dark and under nitrogen. Isolation of the porphyrin methyl esters was carried out as described above, prior to HPLC.

Porphyrins were extracted from wet faeces (10 g) by direct esterification with 5% sulphuric acid in methanol (100 ml) for 24–36 h at 20°. The mixture was stirred with Celite (25 g) to facilitate filtration and filtered. The residue was washed with small portions of methanolic sulphuric acid until the extracts showed no fluorescence. The porphyrin esters were then isolated as described above.

For HPLC, the porphyrin esters were dissolved in chloroform (1 ml) and 10-µl portions were injected into the chromatograph. The attenuation of the UV detector was varied in an appropriate fashion with each sample, depending on the porphyrin concentration.

RESULTS AND DISCUSSION

Initially we showed that porphyrin esters could be separated on pellicular columns using isochratic elution techniques, the retention times increasing as the number of ester side-chains increased. The later peaks were, however, rather broad, leading to an effective loss in sensitivity on analytical runs and this effect could not be entirely obviated even by increasing the flow-rate. Thus in our structural studies[2] of the porphyrins present in the faeces of poisoned rats we turned to gradient elution techniques and, to minimize expense, we formed the gradient at low pressure before pumping the solvent mixture on to the column (see Experimental). The solvent system found to be most useful was cyclohexane–ethyl acetate (or light petroleum–ethyl acetate) and it is interesting that a similar system (isooctane–methyl acetate) has been independently developed by other workers[6]. We have, in general, preferred to avoid the use of chloroform or methylene chloride, because of the variable amounts of stabilizing ethanol which they may contain, and because of the possibility of corrosion of the stainless-steel column tubing. Early examples of the types of separations we have achieved are described in previous papers[1,2].

The recent availability of microparticulate packing materials and hence of columns with much higher resolving power and efficiency has led us to re-investigate the

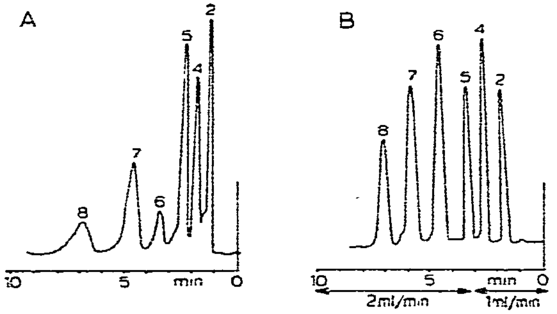


Fig. 1. HPLC separations of standard mixtures of porphyrin with 2–8 carboxylic ester side-chains, on 5-µm Partisil columns (20 × 0.4 cm) in ethyl acetate–cyclohexane (60:40). (A) Flow-rate 1.5 ml/min; (B) flow programmed, 1.0 and 2.0 ml/min.

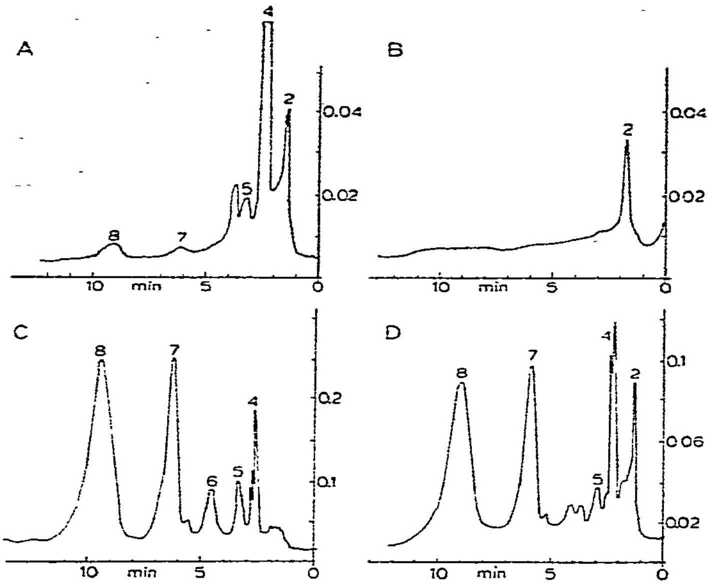


Fig. 2. HPLC separations of porphyrin methyl esters obtained from porphyric urines by direct esterification with 5% sulphuric acid in methanol, showing some of the different types of excretion patterns observed. Each injection corresponds to 1 ml of the original urine; column and solvent system as in Fig. 1. Total porphyrin recovered: (A) 0.42; (B) <0.1; (C) 3.36; (D) 0.75 μmole/l.

use of isochratic elution techniques for analytical separations. Thus we can now obtain complete resolution of mixtures of porphyrin esters with 2–8 carboxylic ester side-chains in an isochratic run without appreciable loss of sensitivity in less than 10 min (Fig. 1); this method is particularly convenient experimentally in enabling repeated determinations to be carried out in rapid sequence as the column does not need reconditioning between runs as with gradient separations.

A series of porphyric urines has been investigated in this way and their porphyrin contents determined; a selection of the results is summarized in Fig. 2,

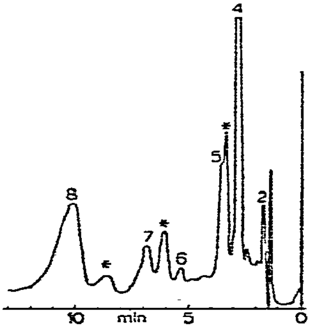


Fig. 3. HPLC analysis of porphyrin esters from a bulk sample of normal urine (collected in the Departmental urinal). Conditions as in Fig. 1. Total porphyrin content 0.13 μmole/l. Note the presence of small amounts of metal complexes (peaks with asterisks) which run slightly ahead of the corresponding uncomplexed porphyrins; these are always observed with low initial porphyrin levels (see text).

showing some of the different excretion patterns which may be observed. For comparative purposes, the results obtained with a normal urine specimen are shown in Fig. 3. The results were reasonably reproducible, repeated injections of the same sample varying only by 5% in intensity of the various peaks. However, the main problem in this type of work is not the sensitivity, or reproducibility, of the HPLC method but the initial preparation of the sample. The sensitivity is adequate even to deal with the relatively small amounts of porphyrin present in normal human urine, and 100-ml samples of urine are adequate for analysis.

As described under Experimental, three different types of extraction procedure have been used to extract porphyrins from urine in the present studies (a) solvent extraction, following a modification of the Fernandez method[7], (b) adsorption on to talc[8–10], or (c) direct esterification of the urinary porphyrin without prior separation. As can be seen from Table I, which shows a comparison of the three methods with two porphyric urines, reasonable agreement was obtained. The direct esterification procedure is probably the simplest of the three methods, but is only suitable for porphyric urines that contain more than 1 mg/l of total porphyrin. For normal urines in which the total porphyrin content may be less than 100 μg/l, the amounts of esterifying agent required become prohibitive and preliminary concentration of porphyrins is best achieved by the talc method.

TABLE I

COMPARISON OF THREE DIFFERENT METHODS OF ISOLATING PORPHYRINS FROM PORPHYRIC URINES

Patient	*Porphyrin (No. of carboxyl groups)*	*Relative molar percentages of porphyrins*		
		Solvent extraction	*Talc adsorption*	*Direct esterification*
Patient X: total porphyrin* 1.6 μmole/l	8	63	50	53
	7	28	24	26
	6	3	6	5
	5	2	4	6
	4	4	16	10
Patient Y: total porphyrin* 0.49 μmole/l	8	54	42	35
	7	31	33	32
	6	2	4	7
	5	5	8	12
	4	8	13	14

* Derived from direct esterification procedure.

Using standard mixtures of meso-, copro- and uroporphyrins, we estimate the recoveries of porphyrin from urine to be about 75–80%; it has not been possible to determine the precise reasons for the losses, but they tend to be greater with low concentrations of porphyrin. In all cases purified solvents were used, and urines were treated with EDTA prior to extraction to minimise contamination by metal ions (which could have led to metal complex formation). The talc used for adsorption was also treated with EDTA solution prior to use.

The analysis of faecal porphyrins is best carried out by direct esterification of raw faeces with methanol–5% sulphuric acid; alternatively, the material can be freeze dried before esterification and this is often preferable for aesthetic reasons. Most of our biosynthetic and metabolic studies have been carried out with haemolyzates of red blood cells[11,12], and in this work it has usually proved useful to extract the buffered product of the reactions exhaustively with solvent, prior to esterification and HPLC analysis.

In another series of experiments in which we were attempting to determine the usefulness of these methods at very low porphyrin levels, a diluted chloroform solution of a standard mixture of meso-, copro- and uroporphyrin methyl esters was kept for 3 days and analyzed for porphyrin from time to time by HPLC. The initial analysis corresponded well with that expected but within a few hours (Fig. 4) additional peaks were observed near the meso- and coproporphyrin ester peaks; after 3 days these had increased considerably and almost all of the original mesoporphyrin ester had disappeared. Field desorption mass spectrometry[1] of the additional peaks showed that they were due to the corresponding copper complexes. This occurred in spite of the use of vigorously purified solvents and care to keep the solution away from metallic contamination; presumably trace amounts of acid formed in the chloroform may have caused dissolution of trace amounts of copper in the flasks, or HPLC system, which then complexed with the porphyrins preferentially with the meso- and coproporphyrin esters, but hardly at all with the uroporphyrin ester. Trace amounts of metal porphyrins have been observed in extracts from porphyric urines, but these were generally insignificant in relation to the parent metal-free porphyrins; only at very low concentration of porphyrin was metal complex formation a problem (see Fig. 3).

Quantification of the amounts of porphyrin present in porphyric materials is best carried out by addition of a standard porphyrin (free acid) in known amounts. In most of our work we have used mesoporphyrin-IX as a standard, partly because it is not often found in natural sources. However, with very rapid isochratic elution techniques it may not always be resolved from protoporphyrin-IX; furthermore,

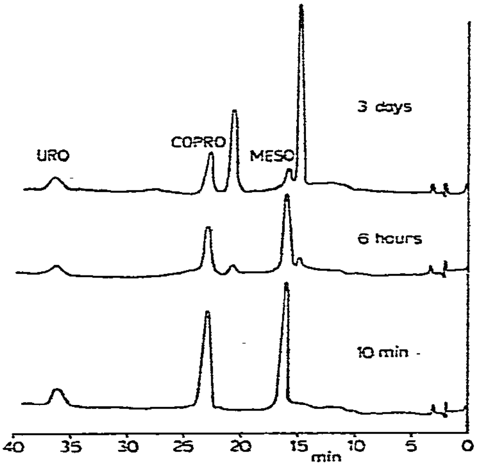


Fig. 4. Conversion of porphyrin esters into copper complexes on standing in chloroform solution. HPLC conditions: Corasil-II, 1 m × 2 mm; exponential gradient, cyclohexane to 70% ethyl acetate–cyclohexane.

uroporphyrins, because of the greater number of carboxylic acid side-chains, are not so rapidly esterified as mesoporphyrin or protoporphyrin and may require up to 36 h for complete esterification conditions under which protoporphyrin may start to decompose unless precautions are taken to exclude light and air. Thus for proper quantification across the range of porphyrins normally found in nature (*i.e.*, with 2–8 carboxyl groups) it may be desirable to add a standard mixture of say three porphyrins *e.g.*, meso- (two carboxyls), copro- (four carboxyls) and uroporphyrin (eight carboxyls). Determinations of the amounts of porphyrins present (after extraction, esterification and HPLC) with and without added standard would then allow for any differences due to incomplete esterification, as well as any losses in the overall procedure.

The separation of porphyrin isomers or other closely related porphyrins is a challenging problem at present, and some progress has already been made. In our earlier paper[1], we referred to the separation of hardero- and isoharderoporphyrin and this has now been improved to give a difference in retention time of at least 3 min with baseline separation (Fig. 5); this has enabled us to show that the intermediate product formed in conversion of coproporphyrinogen-III into protoporphyrin-IX is probably entirely harderoporphyrin rather than its isomer[11].

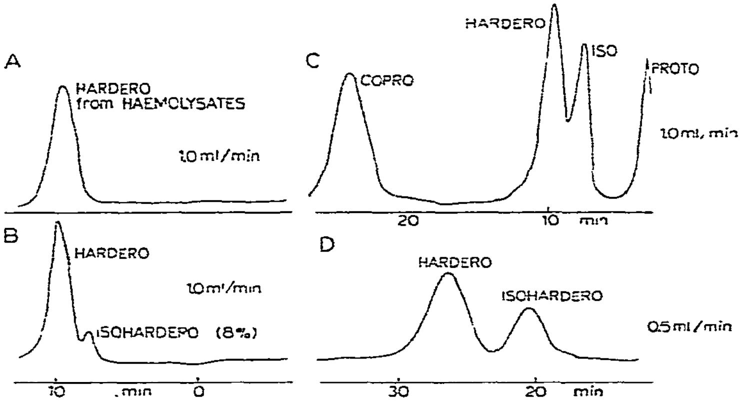


Fig. 5. HPLC separation of hardero- and isoharderoporphyrin trimethyl esters on Corasil-II (1 m × 2 mm) in ethyl acetate–cyclohexane (20:80). (A) Harderoporphyrin trimethyl ester isolated from haemolyzates showing virtual absence of the isomer; (B) a mixture of synthetic hardero- and isoharderoporphyrin trimethyl esters; (C) separation of proto-, isohardero-, hardero- and coproporphyrin methyl esters; (D) baseline separation of hardero- and isoharderoporphyrin esters at low flow-rates.

Separations of some or all of the four coproporphyrin isomers have recently been reported by three groups of workers[5,14,15]. The complete separation of all four isomers, however, required separations into two groups, a transesterification, and a further separation of the two unresolved isomers by recycling[14]. The tetramethyl esters of coproporphyrin, isocoproporphyrin and dehydroisocoproporphyrin can be separated from each other much more readily; for example, in Fig. 2c the tetracarboxylate band is resolved into three peaks and this was achieved even in a normal isochratic run; similar results have also been described by Gray *et al.*[6]. Recently we have

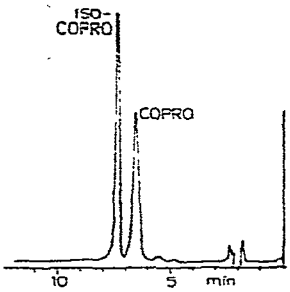


Fig. 6. HPLC separation of copro- and isocoproporphyrin tetramethyl esters on 5-μm Partisil (15 cm × 0.4 cm) in ethyl acetate–cyclohexane (40:60).

completed the synthesis of isocoproporphyrin methyl ester and shown it to be identical with natural material[16]; further careful experimentation has enabled us to achieve baseline separation from coproporphyrin ester on HPLC (Fig. 6). This should be helpful in the detailed analysis of tetracarboxylic porphyrins which are clinically significant in the study of symptomatic porphyria[3,4].

The baseline separation of protoporphyrin methyl ester from mesoporphyrin methyl ester, which can also readily be achieved (Fig. 7), has enabled us to analyze three samples of porphyrin ester running as protoporphyrin-IX dimethyl ester on thin-layer chromatography (TLC) and kindly provided by Professor M. Doss. HPLC showed that one sample was almost entirely protoporphyrin dimethyl ester, one was a mixture of proto- and mesoporphyrin esters and the third contained both of these materials and another component with a retention time intermediate between the two; it seems very likely that this third component is the monovinylmonoethyldeuteroporphyrin ester and presents a half-way stage in the intestinal bacterial reduction of protoporphyrin-IX to meso-porphyrin-IX. Unfortunately, we were unable to obtain a field desorption spectrum[1] to confirm this as insufficient material was available, but its synthesis is in progress.

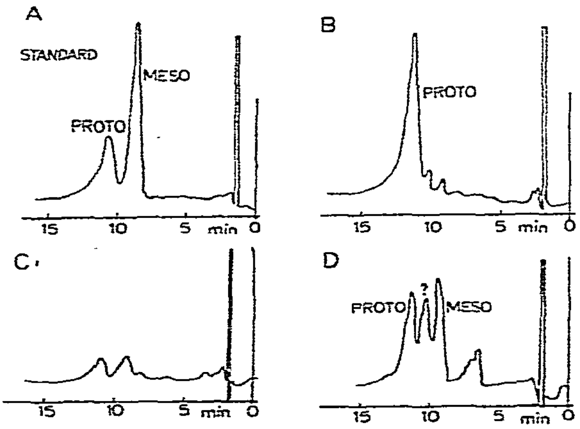


Fig. 7. HPLC separation of meso- and protoporphyrin dimethyl esters on 10-μm Merckosorb (15 cm × 0.4 cm) in ethyl acetate–cyclohexane (15:85) at a flow-rate of 0.5 ml/min. (A) Standard mixture; (B), (C) and (D), samples provided by Professor M. Doss (see text).

We have also separated the dimethyl esters of protoporphyrin-IX and -XIII recently by HPLC[17]; this is of interest in connection with the biochemical conversion of the unnatural coproporphyrinogen-IV into protoporphyrin-XIII by chicken haemolyzates[18]. The esters of protoporphyrin-IX and protoporphyrin-I can also be separated[15,17].

In conclusion, HPLC is an extremely useful technique for the quantitative analysis of porphyric materials in nature; its sensitivity is more than adequate to deal with the normal range of porphyrin concentrations in urine and faeces, and it seems likely that small amounts of tissue containing microgram amounts of porphyrins could also be analyzed in this way. Fortunately, for clinical work in porphyrias, it is not usually necessary to be able to quantitate the individual porphyrin very precisely; the excretion pattern is the most important feature, and this can be defined much more readily quantitatively by HPLC than by the earlier TLC methods[19,20]. Moreover, variations in daily urine output and the difficulties of ensuring complete collection make absolute quantification almost impossible to achieve. Fig. 8 shows in histogram form the natural variation in the porphyrin content of the urine of a symptomatic porphyric over a 2-year period; this demonstrates clearly the potential usefulness of HPLC in clinical studies for studying variations that occur naturally or as the result of treatment. The major limitations to absolute quantification are not in the HPLC methods used, but in the extraction procedures. The use of HPLC in separating isomeric porphyrins looks very promising, and further exciting developments may be expected especially in view of the much improved packing materials now available.

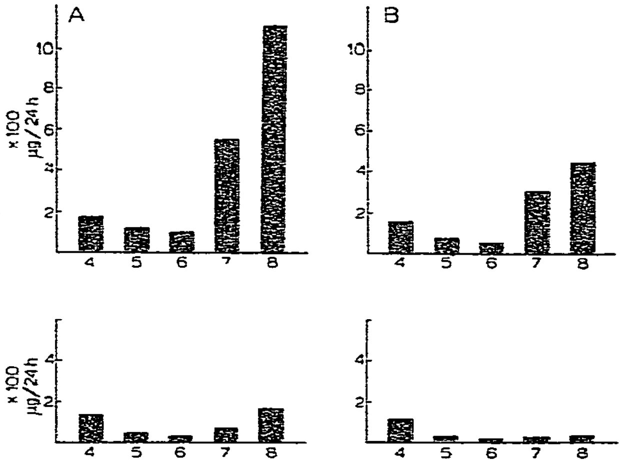


Fig. 8. Histograms showing variation of porphyrin content in the urine of a symptomatic porphyric with time. Specimens collected (A) March 1973, (B) February 1974, (C) June 1974 and (D) April 1975.

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