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REVIEW

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PORPHYRINS

C.K. LIM*, FAMEI LI and T.J. PETERS

Division of Clinical Cell Biology, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ (U.K.)

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CONTENTS

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LIST OF ABBREVIATIONS

AASP	Advanced automated sample processor
Ac	Acetic acid
AIP	Acute intermittent porphyria
ALA	5-Aminolaevulinic acid
ALA-D	5-Aminolaevulinic acid dehydrase
ALA-S	5-Aminolaevulinic acid synthase
CEP	Congenital erythropoietic porphyria
Copro'gen-O	Coproporphyrinogen oxidase
DMSO	Dimethyl sulphoxide
EHP	Erythrohepatic protoporphyria
Et	Ethyl
HCP	Hereditary coproporphyria
HMB-S	Hydroxymethylbilane synthase
HPLC	High-performance liquid chromatography
Me	Methyl
NMR	Nuclear magnetic resonance
PBG	Porphobilinogen
PBG-D	Porphobilinogen deaminase
PCT	Porphyria cutanea tarda
Pr	Propionic acid
Proto'gen-O	Protoporphyrinogen oxidase
TLC	Thin-layer chromatography
Uro'gen-D	Uroporphyrinogen decarboxylase
Uro'gen III-S	Uroporphyrinogen III synthase
Vi	Vinyl
VP	Variegate porphyria

1. INTRODUCTION

Porphyrins are cyclic tetrapyrroles derived from porphin by substitution of the peripheral positions (numbered 1–8) with various side-chains (Fig. 1). In naturally occurring porphyrins vinyl (Vi), ethyl (Et), methyl (Me), acetic acid (Ac) or propionic acid (Pr) groups are the usual substituents. The four pyrrole rings are represented by the letters A, B, C and D and the methine or *meso* positions are designated α, β, γ, δ (Fig. 1). The principal porphyrins, or more correctly porphyrinogens, are the haem biosynthetic intermediates, although they are also intermediates of chlorophyll and vitamin B_{12} biosynthesis. Except for protoporphyrin IX the cyclic tetrapyrroles in the haem biosynthetic pathway are the porphyrinogens in which the methine bridges of the porphyrins are reduced to the methylene groups (Fig. 1). It is at the porphyrinogen stage when the side-chain

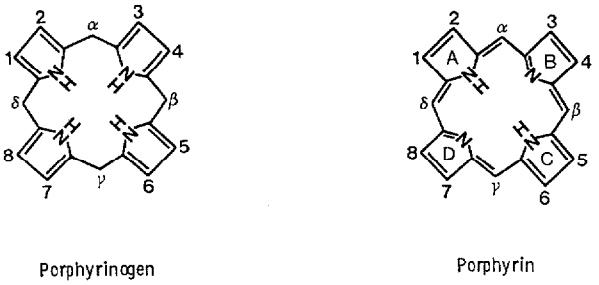


Fig. 1. Structures of porphyrin and porphyrinogen.

Ac and Pr groups are modified respectively into Me and Vi groups [1,2]. The porphyrinogens are unstable in air and are easily oxidised to the highly fluorescent porphyrins, particularly in the presence of light under acidic condition.

Analysis of porphyrins is important for the diagnosis of the porphyrias, diseases due to enzyme deficiencies in the haem biosynthetic pathway [3]. Abnormal porphyrin metabolism is also found in alcoholics [4–6], lead [7] and chemical intoxication [8], iron deficiency [9,10], chronic infection, inflammation and malignancy [10] and in cirrhosis [11].

The present review concentrates on the separation of porphyrins and porphyrinogens by high-performance liquid chromatography (HPLC) relevant to the biomedical fields. Other methods for porphyrin analysis will only be briefly discussed as detailed descriptions can be found elsewhere [12].

2. SOLVENT PARTITION AND SPECTROSCOPIC METHODS FOR PORPHYRIN ANALYSIS

Porphyrins can be determined spectrophotometrically [13–15], fluorometrically [16] or by circular dichroism [17,18] following dilution with or extraction into hydrochloric acid solutions. These methods are useful for the preliminary screening of urine and faeces for excess porphyrin production but cannot provide detailed information in the differential diagnosis of the porphyrias. Improved specificity may be achieved with second-derivative spectroscopy [19] or by solvent partioning the porphyrins into uroporphyrin, coproporphyrin and protoporphyrin fractions [14,20]. Solvent partition, however, does not separate the porphyrins completely and each fraction may be cross-contaminated with the other porphyrins.

3. COUNTER-CURRENT DISTRIBUTION OF PORPHYRINS

The solvent partition technique for separating porphyrins has been extended to the development of counter-current distribution systems for the analysis and isolation [21–24] of porphyrins. Resolution was poor and no separation of type isomers has been achieved.

4. ELECTROPHORESIS OF PORPHYRINS

This may be performed on agar gel [25,26] or other solid media but, more conveniently and rapidly, on paper [27,28]. The method of Lockwood and Davies [28] has been used for the routine analysis of urinary and faecal porphyrins. Good separation of 8-, 7-, 6-, 5- and 4-carboxylic porphyrins was achieved with mobility increased with increasing number of carboxylic groups.

5. PAPER CHROMATOGRAPHY OF PORPHYRINS

The separation of porphyrin free acids is usually carried out in an ammonia vapour-saturated system with aqueous 2,4-, 2,5- or preferably 2,6-lutidine as the developing solvent [23,29–31]. For the separation of porphyrin methyl esters, the developing solvent is usually kerosene mixed with chloroform, dioxan or 2-propanol [32,33]. The technique had been widely used for the separation of coproporphyrin [32,33] and uroporphyrin [23,34] isomers. Cross-contamination, however, may be a problem following the separation of isomeric uroporphyrin methyl esters [35,36].

6. THIN-LAYER CHROMATOGRAPHY OF PORPHYRINS

Thin-layer chromatography (TLC) is the most widely used technique for the routine analysis of porphyrins, although it is gradually being replaced by HPLC. Separation is usually carried out with methyl esters on silica gel plates [37–41] with dichloromethane–hexane, chloroform–hexane, ethyl acetate–hexane, ethyl acetate–benzene, benzene–ethyl acetate–methanol, toluene–ethyl acetate–methanol, dichloromethane–carbon tetrachloride–ethyl acetate or chloroform–kerosine–ethanol as solvent systems. TLC of porphyrin free acids on cellulose plate has also been described [42]. Two-dimensional TLC, which improved the resolution, has been used to separate isocoproporphyrin in the diagnosis of porphyria cutanea tarda (PCT) [38].

TLC has the advantage of analysing many samples simultaneously on a single plate. It is also a useful technique for the small-scale preparative isolation of porphyrin methyl esters [43,44].

7. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

7.1. HPLC of porphyrins

The development of high-resolution HPLC has greatly improved the efficiency of porphyrin separation, making identification and quantitation much more exact. It is now the technique of choice in routine and research laboratories involved in porphyrin analysis. The porphyrins may be separated by adsorption chromatography as methyl esters or as free acids by reversed-phase or reversed-phase ion-pair chromatography although partition and ion-exchange systems have also been described.

7.1.1. HPLC of porphyrin methyl esters

The potential of HPLC for porphyrin separation was first demonstrated by the separation of hardero- and isohardero-porphyrin methyl esters [24] on Corasil columns with chloroform–cyclohexane as mobile phase. The column efficiency and resolving power has greatly improved with the availability of micro-particulate packings. The column is usually silica and the eluent a mixture of organic solvents. The porphyrin methyl esters are sequentially eluted with increasing number of ester side-chains. The mobile phases most commonly employed are binary mixtures of ethyl or methyl acetate and a hydrocarbon such as hexane or *n*-heptane [45–50]. These eluents are essentially modifications of TLC solvent systems for the separation of porphyrin methyl esters. A typical example is shown in Fig. 2. Ternary [51–55] and quaternary systems [56,57] have also been reported. These more complicated systems showed no improvement in speed of analysis or resolution, over simple binary systems.

Chemically bonded phases, for example aminopropyl [58–60], cyanopropyl [61,62] and octadecyl silica [63], have also been used to separate porphyrin methyl esters. Kotal et al. [60] claimed that aminopropyl-bonded silica gave better reproducibility than silica. The variation in retention with silica sorbents is mainly due to the gradual adsorption of traces of water present in the organic mobile phases. Aminopropyl-bonded silica is less prone to water adsorption. However, good reproducibility can be achieved on silica by using "dry" organic solvents. Fig. 3 shows the separation of porphyrin methyl esters and their corresponding copper complexes on aminopropyl-bonded silica.

An obvious drawback of separating porphyrins as methyl esters is a derivatization step which may lead to undesired complications. Some porphyrin esters, particularly those with a higher number of ester groups, may be partially hydrolysed. These will not elute from the silica column and therefore remain undetected. The reaction may form mixed methyl–ethyl esters due to ethanol in the organic extractant [48]. This will lead to errors in identification and quantita-

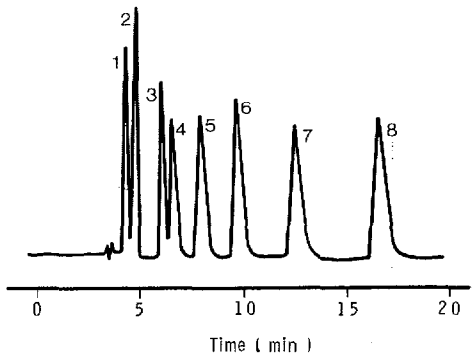


Fig. 2. HPLC of porphyrin methyl esters. Column, μPorasil (30 cm×4 mm, 10 μm particle size); eluent, *n*-heptane–methyl acetate (3:2, v/v); flow-rate, 1.5 ml/min; detector, 400 nm. Peaks: 1 = mesoporphyrin; 2 = protoporphyrin; 3 = coproporphyrin; 4 = isocoproporphyrin; 5 = pentacarboxylic porphyrin; 6 = hexacarboxylic porphyrin; 7 = heptacarboxylic porphyrin; 8 = uroporphyrin.

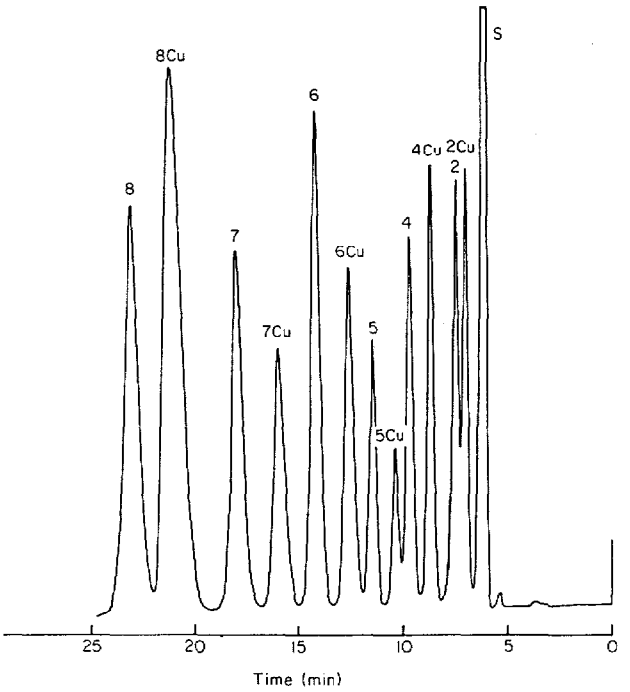


Fig. 3. HPLC of porphyrin methyl esters and their Cu complexes. Column, 30 cm×3.2 mm SEPARON SIX NH_2 (aminopropyl silica); eluent, ethyl acetate–*n*-heptane (57:43, v/v); flow-rate, 0.4 ml/min. Peaks: 2, 4, 5, 6, 7 and 8 refer to di- (proto), tetra- (copro), penta-, hexa-, hepta- and octa- (uro) carboxylic porphyrin methyl esters, respectively, and the corresponding Cu complexes are numbered 2 Cu, 4Cu, 5Cu, 6Cu, 7Cu and 8Cu; S is tetraphenylporphyrin. From ref. 60 with permission.

tion. Metal chelates are sometimes formed during the isolation of esters from tissues and deliberate conversion into copper complexes for HPLC separation has been reported [61,62]. The esterification procedures, however, result in a much cleaner porphyrin extract (particularly from faeces) for HPLC analysis.

7.1.2. HPLC of porphyrin free acids

The problems associated with the preparation and isolation of porphyrin methyl esters can be avoided by separating the porphyrins as free acids. Early attempts have been by ion-exchange HPLC which showed poor column efficiency and resolution [45] or on a silica column with 0.3% water in acetone, adjusted to pH 7.6, with tributylamine as mobile phase [64,65]. The latter system is probably also involved an ion-exchange mechanism. Gradient elution systems with acetone–dilute acetic acid [66] and acetonitrile–water containing tetraethylenepentamine [67] as solvent mixtures have been described for the separation of porphyrin free acids on silica.

The above systems have been replaced by reversed-phase systems which are much more reproducible and efficient. The first reversed-phase HPLC of porphyrin free acids was described by Adams and Vandemark [68] using an ODS column with methanol–0.01% aqueous acetic acid as eluent. Most reversed-phase

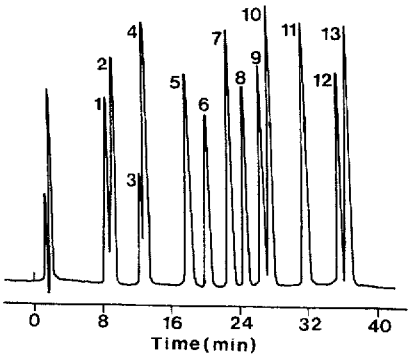


Fig. 4. Reversed-phase HPLC of porphyrin free acids. Column, 25 cm×5 mm Hypersil-SAS (C_1-bonded silica); eluents, 10% (v/v) acetonitrile in 1 *M* ammonium acetate, pH 5.16 (A) and 10% acetonitrile in methanol (B); elution, 30-min linear gradient from 0% B to 65% B followed by isocratic elution at 65% B for a further 10 min; flow-rate, 1 ml/min; detector, 404 nm. Peaks: 1 = uroporphyrin I; 2 = uroporphyrin III; 3 = heptacarboxylic porphyrin I; 4 = heptacarboxylic porphyrin III; 5 = hexacarboxylic porphyrin I; 6 = hexacarboxylic porphyrin III; 7 = pentacarboxylic porphyrin I; 8 = pentacarboxylic porphyrin III; 9 = coproporphyrin I; 10 = coproporphyrin III; 11 = isocoproporphyrin; 12 = mesoporphyrin; 13 = protoporphyrin. From ref. 83 with permission.

systems employed gradient elution which allows the separation of porphyrins with two to eight carboxylic groups. Englert and co-workers [69,70] developed a linear gradient system with increasing acetonitrile in phosphate buffer for the separation of urinary porphyrins on a C_{18} column. Similar systems were subsequently described by others [71–73]. Hill et al. [74] showed that it is possible to separate urinary porphyrins by isocratic elution on a phenyl-bonded silica column. The system is unsuitable for the analysis of faecal porphyrins as dicarboxylic porphyrins were not eluted.

Reversed-phase ion-pair chromatography was introduced by Bonnet et al. [75] for the separation of porphyrin free acids. Tetrabutylammonium phosphate was used as the ion-pairing agent. The method has been modified [76–79] and optimised [80,81]. Ion pairing is not essential for the separation of porphyrin free acids. In fact better resolution was achieved with buffers of controlled pH and molarity with acetonitrile or acetonitrile–methanol as the organic modifier [82,83]. A linear gradient elution system with 1 *M* ammonium acetate buffer, pH 5.16, and acetonitrile–methanol as the gradient mixture simultaneously separated porphyrins with from two to eight carboxylic groups, including the resolution of the type I and type III isomers (Fig. 4). Either a C_1-bonded [83] or a C_{18}-bonded silica column [84] can be used. Riboflavin, which may interfere with the uroporphyrin peak in some systems, was also well resolved [50].

7.2. Urinary and faecal porphyrin profiles by HPLC

The diagnosis of the porphyrias requires analysis of porphyrins in blood, urine and faeces. Initial screening of excess porphyrin production is best carried out by one of the spectrophotometric [14,15] or fluorometric methods [16]. If elevated

levels are detected, HPLC is then used to provide the porphyrin excretion patterns. Since each profile is characteristic for the known porphyrias, differential diagnosis is possible. Porphyrin excretion patterns have been obtained as the methyl esters [47] and more conveniently as the free acids [50,83].

The normal urinary and faecal porphyrin excretion patterns are shown in Fig. 5. Only coproporphyrin and a little uroporphyrin were detected in the urine. The main porphyrin in faeces was protoporphyrin.

In acute intermittent porphyria (AIP) the urinary and faecal porphyrin profiles are similar to that of the normal subject except during acute attack when both uro- and coproporphyrins were elevated. Small amounts of intermediate porphyrins were also detected (Fig. 6). Much of the uroporphyrins were derived from porphobilinogen (PBG) by non-enzymic condensation. Assay of urinary PBG prior to porphyrin HPLC therefore remains a very useful screen for the acute porphyrias [14,85]. Like AIP the urinary porphyrin excretion in variegate porphyria (VP) is normal in clinical remission and only elevated during acute exacerbation of the disease. VP can be clearly distinguished from AIP by the analysis of faecal porphyrins. VP characteristically produces a considerable excess of protoporphyrin accompanied by increased coproporphyrin (Fig. 7) with the former always exceeding the latter.

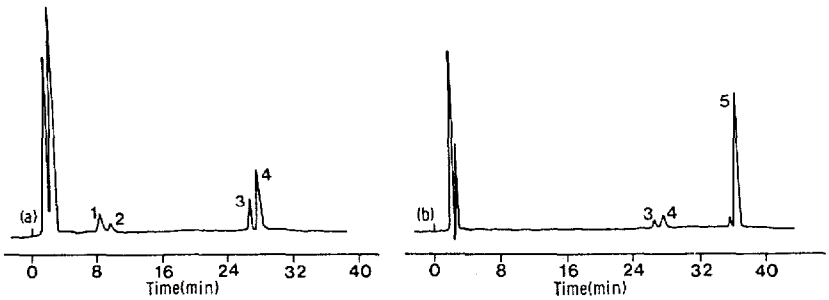


Fig. 5. HPLC of (a) urinary and (b) faecal porphyrins from a normal subject. Peaks: 1 = uroporphyrin I; 2 = uroporphyrin III; 3 = coproporphyrin I; 4 = coproporphyrin III; 5 = protoporphyrin. HPLC conditions as in Fig. 4. From ref. 83.

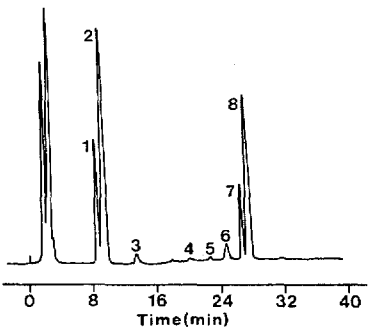


Fig. 6. HPLC of urinary porphyrins from a patient with acute intermittent porphyria during acute attack. Peaks: 1 = uroporphyrin I; 2 = uroporphyrin III; 3 = heptacarboxylic porphyrin III; 4 = hexacarboxylic porphyrin III; 5 = pentacarboxylic porphyrin I; 6 = pentacarboxylic porphyrin III; 7 = coproporphyrin I; 8 = coproporphyrin III. HPLC conditions as in Fig. 4. From ref. 83.

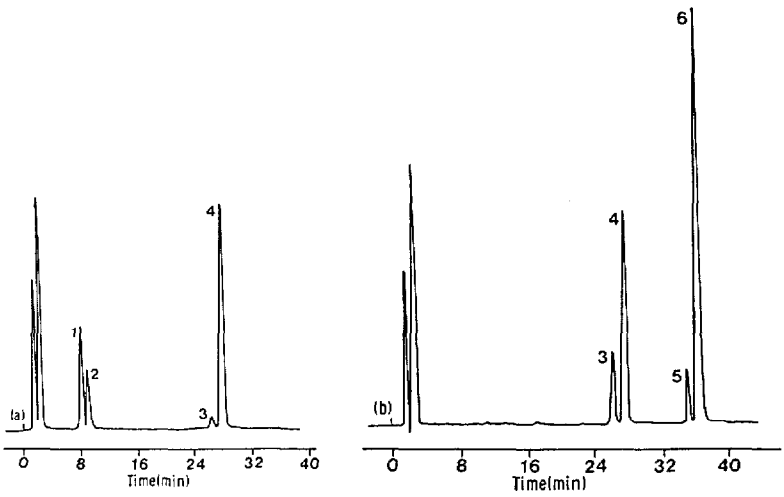


Fig. 7. HPLC of (a) urinary and (b) faecal porphyrins from a patient with variegate porphyria during acute attack. Peaks: 1 = uroporphyrin I; 2 = uroporphyrin III; 3 = coproporphyrin I; 4 = coproporphyrin III; 5 = mesoporphyrin; 6 = protoporphyrin. HPLC conditions as in Fig. 4. From ref. 83.

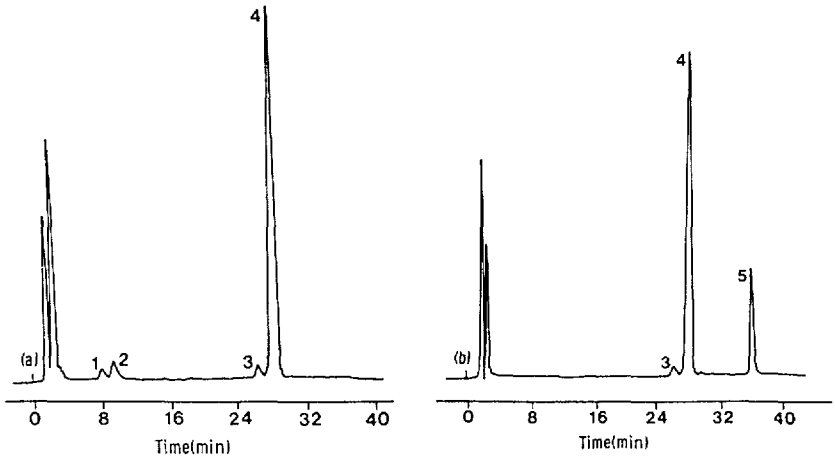


Fig. 8. HPLC of (a) urinary and (b) faecal porphyrins from a patient with hereditary coproporphyria during clinical remission. Peaks: 1 = uroporphyrin I; 2 = uroporphyrin III; 3 = coproporphyrin I; 4 = coproporphyrin III; 5 = protoporphyrin. HPLC conditions as in Fig. 4. From ref. 83.

The urinary and faecal porphyrin profiles in hereditary coproporphyria (HCP) have highly elevated coproporphyrin with the other porphyrin excretion usually being normal except during acute attack (Fig. 8). The coproporphyrin is approximately 95% type III. High levels of coproporphyrin are also found in the urine and faeces of patients with congenital erythropoietic porphyria (CEP). The coproporphyrin, however, is predominantly type I, in contrast to HCP. Analysis of type isomers is clearly useful as without isomer separation HCP may be confused with late-onset CEP, especially when solvent partition methods are used. The urine of CEP also contains a large quantity of uroporphyrin I (Fig. 9).

Erythrohepatic protoporphyria (EHP) is characterised by the excessive excre-

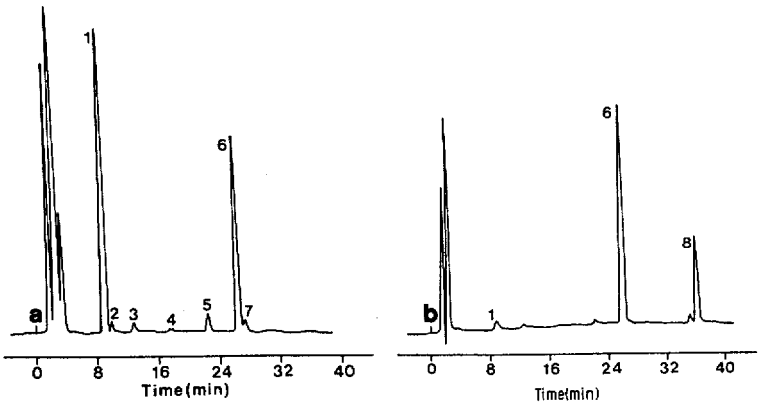


Fig. 9. HPLC of (a) urinary and (b) faecal porphyrins from a congenital erythropoietic porphyric. Peaks: 1 = uroporphyrin I; 2 = uroporphyrin III; 3 = heptacarboxylic porphyrin I; 4 = hexacarboxylic porphyrin I; 5 = pentacarboxylic porphyrin I; 6 = coproporphyrin I; 7 = coproporphyrin III; 8 = protoporphyrin. HPLC conditions as in Fig. 4. From ref. 83.

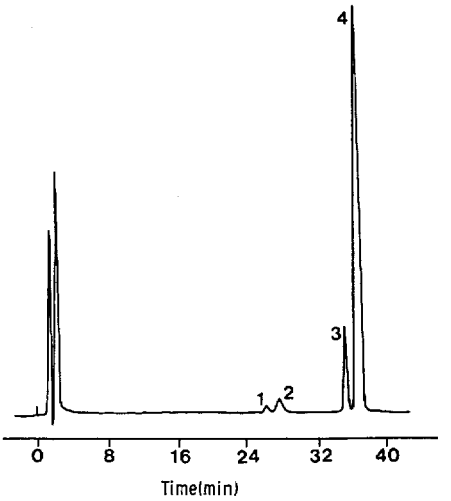


Fig. 10. HPLC of faecal porphyrins from erythrohepatic protoporphyria. Peaks: 1 = coproporphyrin I; 2 = coproporphyrin III; 3 = mesoporphyrin; 4 = protoporphyrin. HPLC conditions as in Fig. 4. From ref. 83.

tion of protoporphyrin in the faeces (Fig. 10) and high concentration of protoporphyrin in the red blood cells. The urinary porphyrin excretion is normal.

The faecal porphyrin profile of PCT is the most complex and characteristic (Fig. 11a), with elevation of every porphyrin except for protoporphyrin and the presence of isocoproporphyrin [43]. PCT urine typically contained high concentrations of uroporphyrin I and III and heptacarboxylic porphyrin III (Fig. 11b).

7.3. Determination of haem biosynthetic enzyme activities by HPLC

The rapid and effective reversed-phase HPLC systems available for the separation of porphyrins and their precursors have led to the development of simple, specific and relatively reliable assays for the haem biosynthetic enzymes.

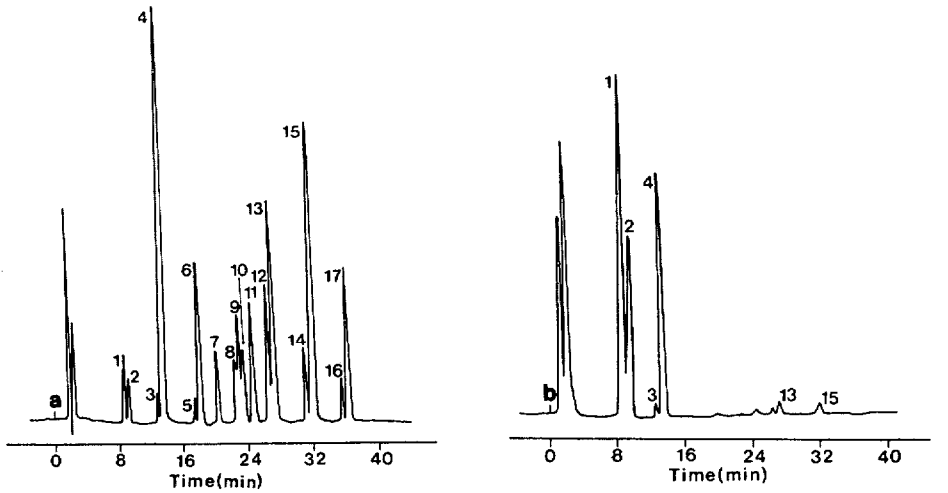


Fig. 11. HPLC of (a) faecal and (b) urinary porphyrins from porphyria cutanea tarda. Peaks: 1 = uroporphyrin I; 2 = uroporphyrin III; 3 = heptacarboxylic porphyrin I; 4 = heptacarboxylic porphyrin III; 5 and 6 = mixture of hexacarboxylic porphyrin I and III; 7 = hexacarboxylic porphyrin III with the acetic acid groups at rings A and D decarboxylated; 8 = pentacarboxylic porphyrin III (rings B, C and D decarboxylated); 9 = pentacarboxylic porphyrin III (rings A, B and C decarboxylated) and pentacarboxylic porphyrin I; 10 = pentacarboxylic porphyrin III (rings A, C and D decarboxylated); 11 = pentacarboxylic porphyrin III (rings A, B and D decarboxylated); 12 = coproporphyrin I; 13 = coproporphyrin III; 14 = deethylisocoproporphyrin; 15 = isocoproporphyrin; 16 = mesoporphyrin; 17 = protoporphyrin. HPLC conditions as in Fig. 4. From ref. 83.

5-Aminolaevulinic acid synthase (ALA-S, EC 2.3.1.37) has been assayed in human bone marrow using radio-HPLC [86]. The enzymatically formed [^{14}C]ALA was converted to a pyrrole derivative and isolated by reversed-phase ion-pair HPLC. The procedure may possibly be simplified by direct separation of [^{14}C]ALA.

The separation of ALA and PBG [87,88] was applied to the determination of 5-aminolaevulinic acid dehydrase (ALA-D, EC 4.2.1.24) activity in erythrocytes [88]. The enzyme reaction product, PBG, was separated and quantitated by reversed-phase ion-pair HPLC (Fig. 12).

The importance of isomer separation is best demonstrated by the development of an HPLC method for the simultaneous determination of hydroxymethylbilane synthase (HMB-S, EC 4.3.1.8) or porphobilinogen deaminase (PBG-D), and uroporphyrinogen III synthase (Uro'gen III-S, EC 4.2.1.75) activities in human erythrocytes [89]. The assay is based on the fact that HMB-S is heat-stable, while Uro'gen III-S is heat-labile and can be heat-denatured [90]. In this method, a known volume of red blood cells was heated to inactivate Uro'gen III-S. Following addition and incubation with unheated red blood cells, the reaction products were oxidised to uroporphyrin I and III for separation and quantitation by HPLC. The total uroporphyrins (I + III) formed are a measure of HMB-S activity in the total red blood cells (heated plus unheated) while the uroporphyrin III produced reflects Uro'gen III-S activity in the unheated red blood cells. Fig. 13 shows the separation of uroporphyrin I and III isomers in the incubation mixture for the

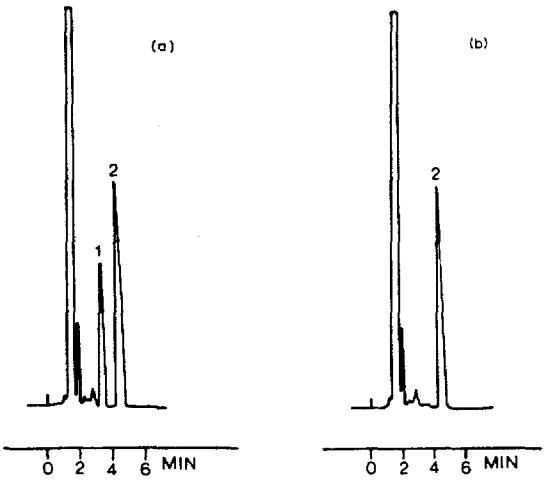


Fig. 12. Determination of ALA-D in human erythrocytes by HPLC. (a) test; (b) blank. Column, Hypersil-SAS; eluent, 22% (v/v) methanol in water containing PIC-B7 (heptanesulphonic acid buffered at pH 3.5); flow-rate, 1.2 ml/min; detector, 240 nm. Peaks: 1 = PBG; 2 = internal standard. From ref. 88 with permission.

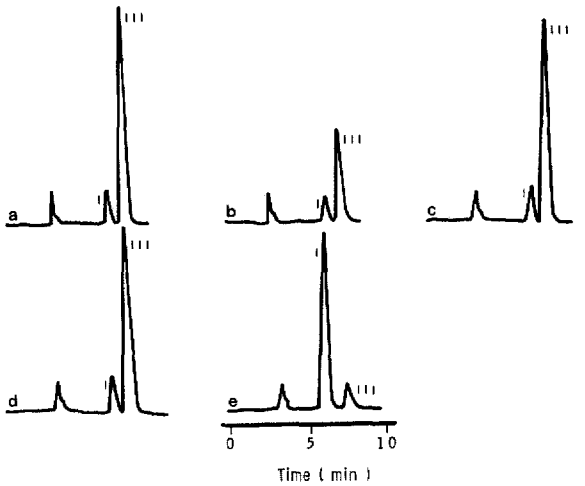


Fig. 13. Simultaneous determination of HMB-S and Uro'gen III-S in erythrocytes by HPLC. (a) Normal subject; (b) acute intermittent porphyria; (c) variegate porphyria; (d) coproporphyria; (e) congenital erythropoietic porphyria. Column, Hypersil-ODS; eluent, 13% (v/v) acetonitrile in 1 *M* ammonium acetate, pH 5.16; flow-rate, 1 ml/min; detector, 400 nm. Peaks: I = uroporphyrin I; III = uroporphyrin III. The apparently low uroporphyrinogen III formed in (b) was due to HMB-S deficiency resulting in insufficient hydroxymethylbilane produced for the subsequent Uro'gen III-S reaction.

simultaneous deterination of HMB-S and Uro'gen III-S. Without the ability to separate the two isomers it is obvious that Uro'gen III-S cannot be assayed directly. The method has been modified to measure Uro'gen III-S activity alone [91].

Several HPLC methods have been described for the assay of uroporphyrinogen

decarboxylase (Uro'gen-D, EC 4.1.1.37) activity based on separation of porphyrins [92,93] or their methyl esters [94,95]. Either uroporphyrinogen or pentacarboxylic porphyrinogen may be used as the enzyme substrate. Methods with pentacarboxylic porphyrinogen as substrate [93,94] are preferred as only coproporphyrin will be formed, thus, the separation and quantitation is simplified. With uroporphyrinogen as substrate, separation and quantitation of all decarboxylation intermediates and of coproporphyrin are necessary.

The separation of dicarboxylic porphyrins and metalloporphyrins [96,97] indicated the possibility of HPLC methods for measuring coproporphyrinogen oxidase (Copro'gen-O, EC 1.3.3.3), protoporphyrinogen oxidase (Proto'gen-O, EC 1.3.3.4) and ferrochelatase (EC 4.99.1.1), or haem synthase activities. Assay of the former two enzymes involved simple separation and quantitation of protoporphyrin by reversed-phase chromatography [98,99]. In the assay of Copro'gen-O, the protoporphyrinogen formed was completely converted into protoporphyrin by the endogenous Proto'gen-O. A typical chromatogram is shown in Fig. 14.

A number of bivalent metal ions (Fe^{2+}, Co^{2+}, Zn^{2+}) and dicarboxylic porphyrins (protoporphyrin, mesoporphyrin, deuteroporphyrin) may be used as substrates for ferrochelatase. Zn^{2+} is the preferred metal substrate because the Zn-porphyrin enzyme reaction product formed is fluorescent. This allows highly sensitive assay suitable for clinical applications to be developed [100]. Fig. 15 shows the separation of Zn-mesoporphyrin from mesoporphyrin (substrate) and Zn-deuteroporphyrin (internal standard) in the incubation mixture used for ferrochelatase assay. In situations where endogenous haem is present, this can also be resolved by adjusting the organic modifier content in the mobile phase (Fig. 16). HPLC methods using Fe^{2+} [96] or Co^{2+} [101] as substrate are of little use because they lack the necessary sensitivity.

The activities of all eight enzymes of the haem biosynthetic pathway have now

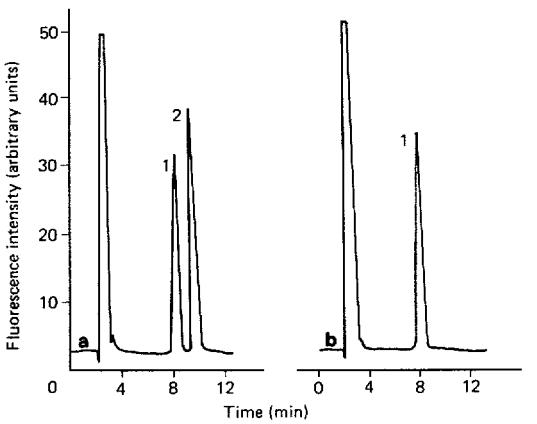


Fig. 14. Determination of coproporphyrinogen oxidase in human liver biopsy by HPLC. (a) Enzyme incubation mixture; (b) zero-incubation time blank. Column, Hypersil-ODS; eluent, 88% methanol in 1 *M* ammonium acetate, pH 5.16; flow-rate, 1.5 ml/min; detector, fluorescence excitation at 400 nm and emission at 618 nm. Peaks: 1 = mesoporphyrin; 2 = protoporphyrin.

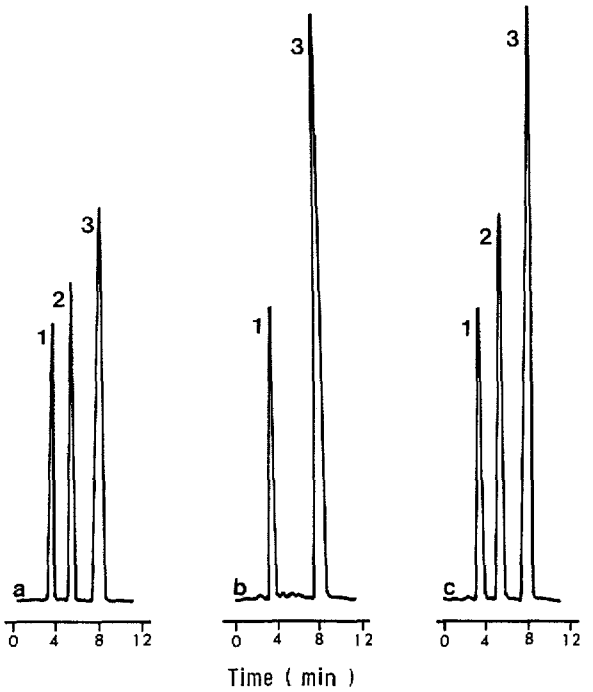


Fig. 15. Determination of ferrochelatase in rat liver mitochondria. (a) Standard mixture; (b) blank; (c) rat liver mitochondria incubation mixture. Column, Hypersil-ODS; eluent, 88% methanol in 1 *M* ammonium acetate, pH 5.16; flow-rate, 1.5 ml/min; detector, UV 400 nm. Peaks: 1 = Zn-deuteroporphyrin; 2 = Zn-mesoporphyrin; 3 = mesoporphyrin. From ref. 100 with permission.

been measured by HPLC. These methods are superior to spectrophotometric, fluorometric and radioactivity measurement procedures in terms of speed, sensitivity and specificity.

7.4. HPLC separation of type isomers of porphyrins and porphyrinogens

The separation of type I and III isomers of uro- and coproporphyrins is important for the accurate diagnosis of some forms of porphyrias. In order to understand fully the nature of the enzymic decarboxylation process, it is also necessary to analyse the isomeric decarboxylation intermediates of uroporphyrinogen III. Isomer resolution provides a mean for the isolation of pure porphyrins from biological materials or from chemical synthesis.

7.4.1. HPLC of coproporphyrin(ogen) isomers

The structures of the four type isomers of coproporphyrinogen is shown in Fig. 17. Oxidation of the methylene bridges to methine groups gives the corresponding porphyrin structures. The first reported separation of coproporphyrin isomers was by recycling HPLC [63]. The porphyrins were resolved as ethyl esters into I, III + IV and II after two recycles on two 30-cm μBondapak C_{18} columns. The III + IV isomers were transesterified to a mixture of methyl esters and then sep-

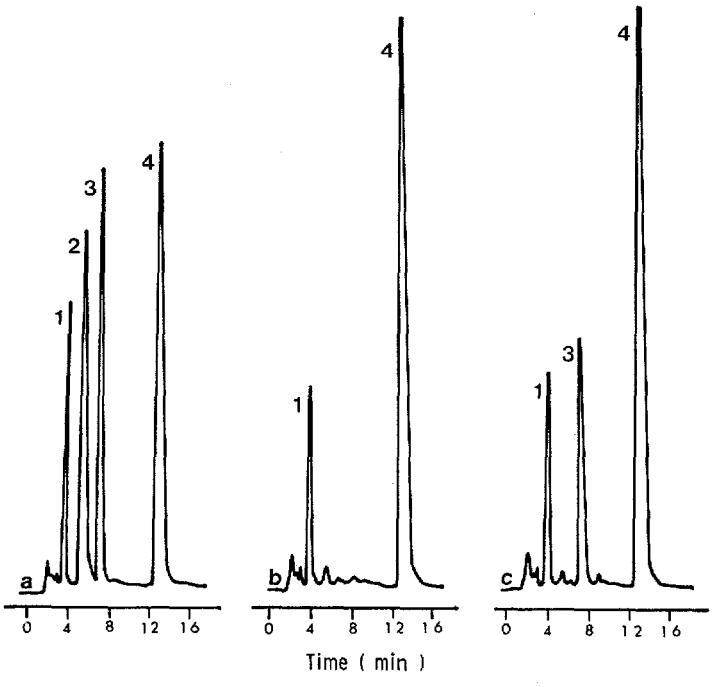


Fig. 16. Chromatograms for determination of ferrochelatase activity in rat liver homogenate. (a) Standard mixture; (b) blank; (c) rat liver homogenate incubation mixture. Column, Hypersil-ODS; eluent, 86% methanol in 1 *M* ammonium acetate, pH 5.16; flow-rate 1.5 ml/min; detector, UV 410 nm. Peaks: 1 = Zn-deuteroporphyrin; 2 = haemin; 3 = Zn-protoporphyrin; 4 = protoporphyrin. From ref. 100 with permission.

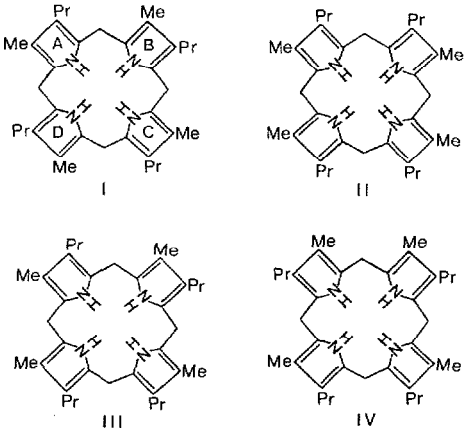


Fig. 17. Structures of coproporphyrinogen I, II, III and IV isomers.

arated on two 30-cm μPorasil columns after ten recycles. This method is obviously too complicated and time-consuming for the separation of all four isomers but nevertheless is relatively rapid for the resolution of the two natural type I and III isomers.

Coproporphyrin I and III methyl esters can also be separated on silica with 1,2-dichloromethane–acetone (24:1, v/v) as eluent [102]. This system also separated the coproporphyrins from isocopro-, deethylisocopro- and dehydroisocoproporphyrins but resolution was inadequate.

Coproporphyrin I and III isomers are better resolved by reversed-phase [70,82–84,103–105] or reversed-phase ion-pair chromatography [81]. The simultaneous isocratic separation of all four isomers is also possible [81,104]. The reversed-phase system with acetonitrile and ammonium acetate buffer as mobile phase on ODS-Hypersil [104] allows retention and resolution to be precisely controlled by manipulation of pH, buffer concentration and organic modifier content in the mobile phase. Optimal resolution was achieved with 26% acetonitrile in 1 *M* ammonium acetate, pH 5.16, as eluent (Fig. 18). Using this system, it can be shown that the retention of porphyrins is dominated by hydrophobic interaction between the most hydrophobic side-chain substituents (usually Me groups) and the hydrocarbonaceous stationary phase [105]. This behaviour can be used with high degree of accuracy to predict the relative retention of porphyrin isomers.

The relative hydrophobicity of the four coproporphyrin isomers is determined by the arrangements of the four peripheral Me groups. The closer these groups the larger the hydrophobic surface available for interaction and the stronger the compound is retained. Thus coproporphyrin II with two pairs of adjacent Me groups (Fig. 17) is the most hydrophobic and longest retained (Fig. 18, peak II) and the symmetrical coproporphyrin I with no adjacent Me groups the least hydrophobic and fastest eluting isomer (Fig. 18, peak I). Coproporphyrin III and IV each have a pair of adjacent Me groups (Fig. 17). In this situation the relative distance between the adjacent pair and the remaining two non-adjacent Me groups becomes an important factor in determining relative hydrophobicity. In coproporphyrin IV, each of the adjacent pair Me group is five bonds away from their nearest non-adjacent Me group. In coproporphyrin III these are five and six bonds apart, respectively. The slightly longer distance between one of the adjacent pair Me groups and its nearest non-adjacent Me group (six instead of five bonds apart) is sufficient to make coproporphyrin III (peak III) less hydrophobic and therefore eluted before coproporphyrin IV (peak IV). The elution order of I, III, IV, II

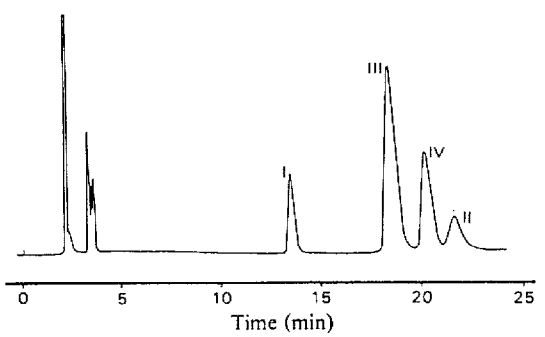


Fig. 18. Separation of coproporphyrin I, II, III and IV isomers. Column, Hypersil-ODS; eluent, 26% acetonitrile in 1 *M* ammonium acetate, pH 5.16; flow-rate, 1 ml/min; detector, 400 nm. From ref. 104 with permission.

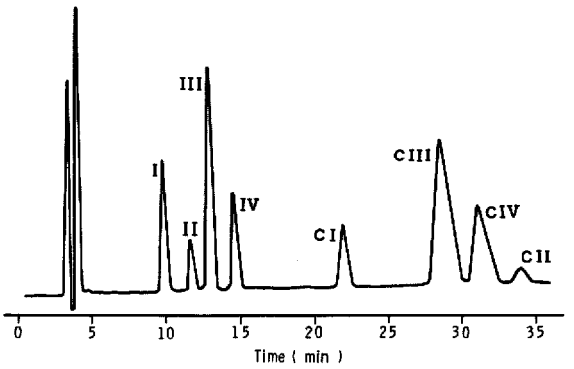


Fig. 19. Simultaneous separation of coproporphyrin (CI, CII, CIII, CIV) and coproporphyrinogen (I, II, III, IV) isomers. HPLC conditions as in Fig. 18 except detection of coproporphyrinogens was at 240 nm. From ref. 106 with permission.

was identical to that reported for the separation of coproporphyrin methyl esters on ODS [63], further confirming the hydrophobic interaction hypothesis.

The prediction of relative retention based on the distances between side-chain hydrophobic (Me) groups and stationary phase is less applicable to the separation of porphyrinogens. An elution order of I, II, III, IV was observed when coproporphyrinogen isomers were chromatographed [106]. The relative retention of the I, III and IV isomers can be similarly explained as for the porphyrins. Coproporphyrinogen II, however, eluted before the III and IV isomers, indicating a weaker hydrophobic interaction. An additional "Me group shielding" or steric factor affecting the availability of Me groups for interaction was proposed [106]. This is possible since reduction of the rigid methine bridges of porphyrin to the methylene groups resulted in a flexible porphyrinogen molecule (Fig. 1). In such a molecule the smaller peripheral Me groups may be subjected to varying degrees of steric hindrance or shielding by the larger carboxylic acid groups. It is probable that some of the Me groups in coproporphyrinogen II are shielded to some extent, reducing its ability to interact with the stationary phase and, therefore, altering its retention.

The separation of coproporphyrinogens is superior to that of the corresponding porphyrins and simultaneous separation of these two groups of compounds (Fig. 19) has been achieved [106].

7.4.2. HPLC of pentacarboxylic porphyrin(ogen)s

The pentacarboxylic porphyrinogens (Fig. 20) are the ultimate intermediates in the decarboxylation of uroporphyrinogen I and III to coproporphyrinogen I and III. The pentacarboxylic porphyrins have been partially separated as methyl esters [107] and as the free acids [105] on an ODS column with 22% acetonitrile in 1 *M* ammonium acetate, pH 5.16, as eluent (Fig. 21). As with the separation of coproporphyrins the relative retention of the isomers can be precisely predicted by considering the relative distances between the three Me groups. These are furthest apart in 5bcd and it was therefore first to be eluted (Fig. 21, peak 1). The distances between the Me groups of 5I and 5abc are identical. These two com-

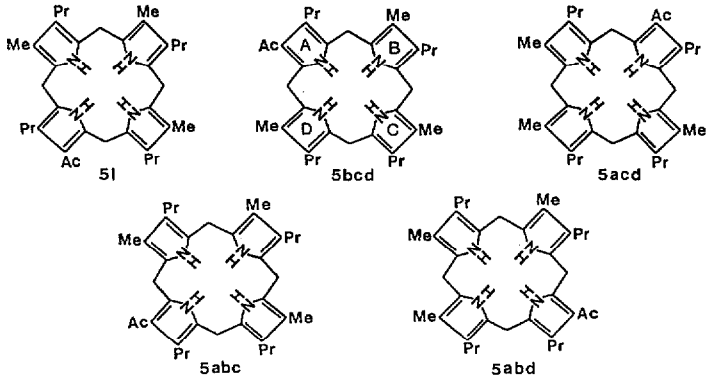


Fig. 20. Structures of pentacarboxylic porphyrinogen isomers. The letters a, b, c and d denote the position on which the acetic acid group on ring A, B, C and D, respectively, has been decarboxylated to a methyl group.

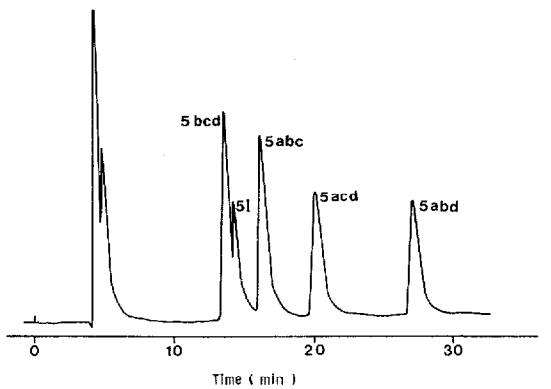


Fig. 21. Separation of pentacarboxylic porphyrin isomers. Column, Hypersil-ODS; eluent, 22% acetonitrile in 1 *M* ammonium acetate, pH 5.16; flow-rate, 1 ml/min; detector, 400 nm. From ref. 105 with permission.

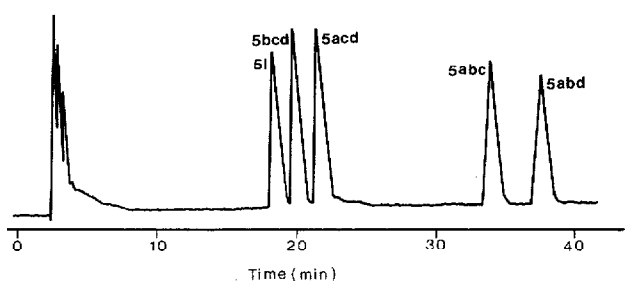


Fig. 22. Separation of pentacarboxylic porphyrinogen isomers. Column, Hypersil-ODS; eluent, 40% methanol in 1 *M* ammonium acetate, pH 5.16; flow-rate, 1 ml/min; detector, electrochemical +0.70 V. From ref. 108 with permission.

pounds were only partially resolved with 5I (peak 2) eluted before 5abc (peak 3) because the Pr groups, which are the next most hydrophobic groups are symmetrically arranged in 5I. The strongest hydrophobic interactions were observed in

5acd and 5abd, both with adjacent Me groups. 5abd (peak 5) was retained longer than 5acd (peak 4) since the third Me group is five bonds away from the nearest adjacent Me group and this is six bonds apart in 5acd (Fig. 20).

Reduction of pentacarboxylic porphyrins to the porphyrinogens resulted in superior resolution of isomers. The five isomers were completely separated [108] on ODS-Hypersil with 40% methanol in 1 *M* ammonium acetate, pH 5.16, as mobile phase (Fig. 22). The variability of the Me groups in these flexible molecules may have contributed to the improved separation but also made prediction of retention order more difficult.

7.4.3. HPLC of hexacarboxylic porphyrin(ogen)s

The most complex group among the decarboxylation intermediates of uroporphyrinogens is the hexacarboxylic porphyrinogens. There are six possible type III and two type I isomers (Fig. 23). The separation of hexacarboxylic porphyrinogens has not yet been reported, although partial separations of the porphyrins have been described [2,105]. Reversed-phase chromatography with 16% acetonitrile in 1 *M* ammonium acetate, pH 5.16, as eluent on ODS-Hypersil [105] separated the porphyrins into six peaks (Fig. 24). The separations of 6ab and 6bc, 6ac and 6bd have not been achieved.

The relative retention is dominated by the number of bonds between the two Me groups and there are two possible ways of assessing these; the shorter distance was used [105]. Of the six type III isomers, only 6ad has adjacent Me groups (four bonds apart) which is the closest of these isomers. Since the closer the Me groups the stronger the hydrophobic interaction with stationary phase, 6ad was therefore the longest retained compound (Fig. 24, peak 6). The weakest hydrophobic interaction and the fastest eluting type III compounds were observed for isomers with the two Me groups ten and nine bonds apart as in 6ac and 6bd (Fig. 24, peak 2). Had it been possible to separate 6ac from 6bd, the former (ten bonds apart) would have been expected to elute before the latter (nine bonds apart). The type III isomers with intermediate number of bonds between the two Me groups, 6cd, 6ab and 6bc, had intermediate capacity ratio (k') values, and 6cd (peak 3) with a six-bond gap was eluted before 6ab and 6bc (peak 5), both having the Me groups five bonds apart. The above general rules also applied to the two type I isomers. 6Iac (peak 1) with ten bonds between the Me groups was eluted before 6Iab (peak 4) with the two Me groups five bonds apart. In the original paper [105] the elution order of these two compounds was incorrectly labelled. The distance between the Me groups in 6Iab, 6ab and 6bc are identical; 6Iab had shorter retention time because the Pr groups are symmetrically arranged [105].

7.4.4. HPLC of heptacarboxylic porphyrin(ogen)s

Heptacarboxylic porphyrinogens are the first intermediates in the decarboxylation of uroporphyrinogen and there are four possible type III and one type I isomers (Fig. 25). Type I and III porphyrin isomers have been separated as methyl esters [2,56] and as free acids [82,83,105]. In reversed-phase chromatography, the type I isomer with the Pr groups symmetrically arranged eluted before the non-symmetrical type III isomer (Fig. 26). The separation of the four type III

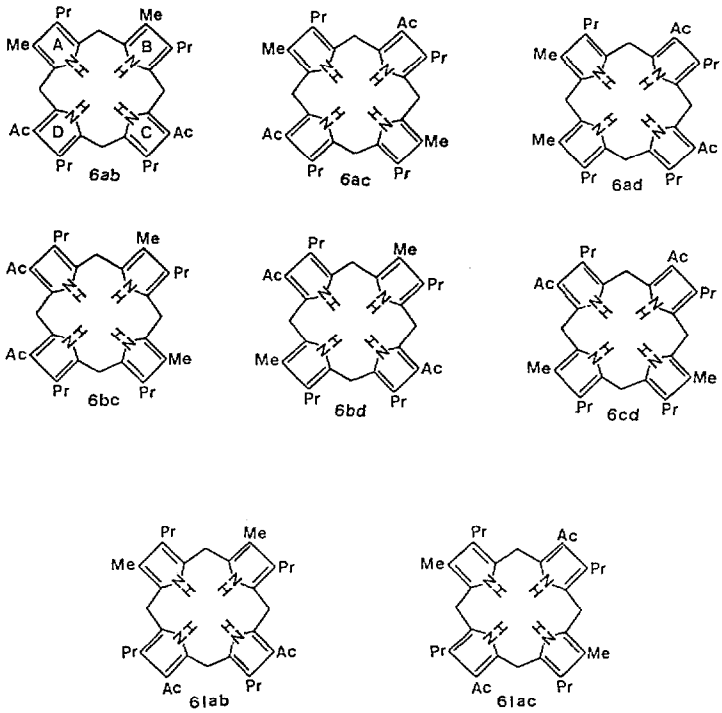


Fig. 23. Structures of hexacarboxylic porphyrinogen isomers.

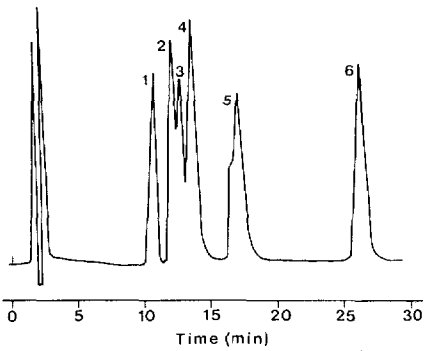


Fig. 24. Separation of hexacarboxylic porphyrin isomers. Column, Hypersil-ODS; eluent, 16% acetonitrile in 1 *M* ammonium acetate, pH 5.16; flow-rate, 1 ml/min; detector, 400 nm. Peaks: 1 = 6Iac; 2 = 6ac + 6bd; 3 = 6cd; 4 = 6Iab; 5 = 6ab + 6bc; 6 = 6ad. From ref. 105 with permission. In the original paper peaks 1 and 4 were wrongly labelled as 6Iab and 6Iac respectively.

heptacarboxylic porphyrins has never been achieved despite numerous attempts. A probable explanation is that each of these isomers has only one hydrophobic Me group dominating the interaction with the stationary phase. They are therefore virtually identical in hydrophobicity and are thus difficult to separate.

The reduction of the four methine bridges in the rigid porphyrin macrocycles to the flexible heptacarboxylic porphyrinogens resulted in complete resolution of the type I and all four type III isomers (Fig. 27). The separation was achieved on ODS-Hypersil with acetonitrile–methanol–1 *M* ammonium acetate, pH 5.16

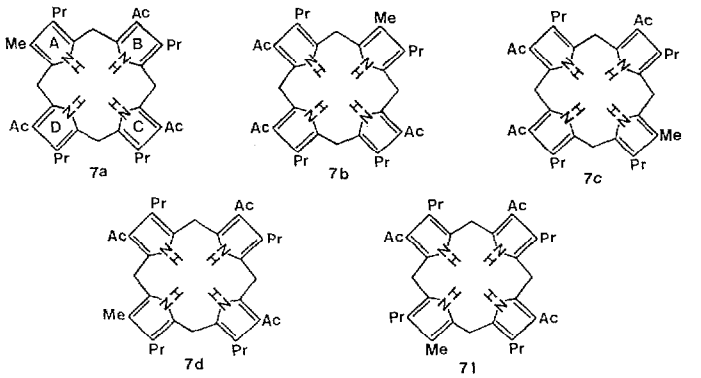


Fig. 25. Structures of heptacarboxylic porphyrinogen isomers.

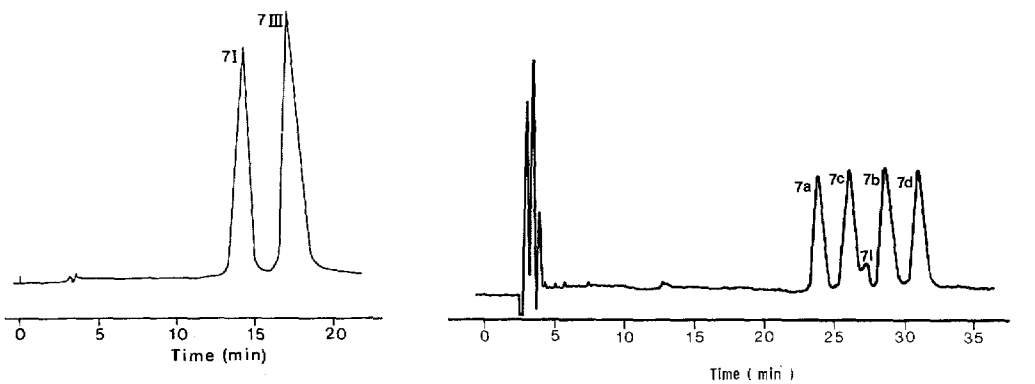


Fig. 26. Separation of heptacarboxylic porphyrin I and III isomers. Column, Spherisorb-ODS; eluent, 15% acetonitrile in 1 *M* ammonium acetate, pH 5.16; flow-rate, 1 ml/min; detector, 400 nm. Peaks: 7I, type I isomer; 7III, mixture of four type III isomers. From ref. 105 with permission.

Fig. 27. Separation of heptacarboxylic porphyrinogen isomers. Column, Hypersil-ODS; eluent, acetonitrile–methanol–1 *M* ammonium acetate, pH 5.16 (7:3:90); detector, electrochemical +0.70 V; flow-rate, 1 ml/min. From ref. 109 with permission.

(7:3:90, v/v/v) as mobile phase [109]. The separation is probably due to the varying degree of shielding of the Me group by the larger Ac and Pr groups, allowing the single Me group in each isomer to interact differently with the stationary phase. The complete resolution of the heptacarboxylic porphyrinogens provided the best evidence for the steric and conformational effects on the reversed-phase separation of porphyrinogens.

7.4.5. HPLC of uroporphyrin(ogen)s

There are four uroporphyrinogen isomers (Fig. 28) of which the type I and type III are naturally occurring. The first reported separation of uroporphyrin I and III isomers was on a silica column eluted at 40 °C with 0.3% water in acetone adjusted to pH 7.6 with *tributylamine* [64,65]. Good resolution was shown but the separation has not been repeated by others. Two very lengthy methods have

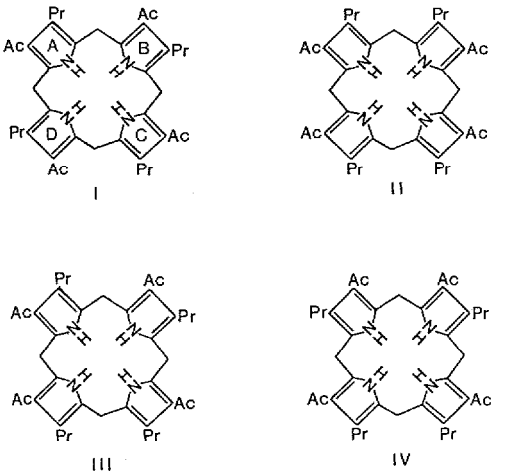
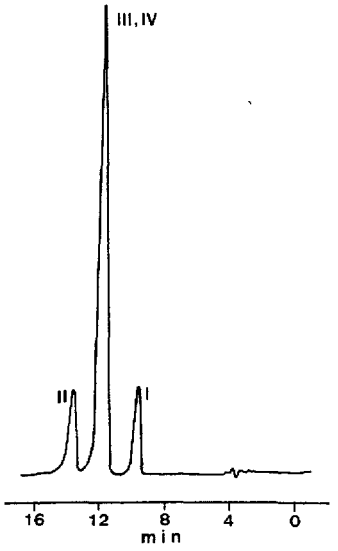


Fig. 28. Structures of uroporphyrinogen isomers.

been described for separating uroporphyrin I and III methyl esters. The first [110] was by recycling (five cycles over 2 h) and the second [111] by elution over 3 h on two 30 cm×0.46 cm μPorasil columns. Other separations of uroporphyrin I and III methyl esters involved the use of quaternary mobile phases on aminopropyl-bonded silica [58] or silica [56]. The best separation of uroporphyrin isomers as methyl esters was achieved by Jackson et al. [112] using a 3 μm particle size silica column with hexane–ethyl acetate (1:1, v/v) as eluent. The system separated the type I from the type II and type III+IV isomers.

It is generally believed that silica has a peculiar property for the separation of isomers. The separations of porphyrins and porphyrinogens have shown otherwise, with the reversed-phase columns providing the best isomer resolution. This is true for all porphyrins, including the uroporphyrins. The first described reversed-phase system [113] separated uroporphyrin I, III, IV and II isomers in that order. The separation of the III and IV isomers was partial. The column used was μBondapak C_{18} and the mobile phase was 4% acetonitrile in 0.01 *M* phosphate buffer, pH 6.95. Type I, III+V and II isomers can also be separated on ODS-Hypersil (Fig. 29) with 13% acetonitrile in 1 *M* ammonium acetate, pH 5.15, as mobile phase [114]. Similar separation, but with longer retention times, was also possible by reversed-phase ion-pair chromatography [81].

The separation of uroporphyrinogen isomers did not show improvement over the porphyrins [106]. Fig. 30 shows the separation of uroporphyrinogen isomers on ODS-Hypersil with 4% acetonitrile in 1 *M* ammonium acetate, pH 5.16, as eluent. The elution order of II, III+IV and I was the opposite of that observed for uroporphyrin isomers (Fig. 29). The relative retention of porphyrins in reversed-phase chromatography has been discussed in terms of hydrophobic interaction [105]. The hydrophobicity of the porphyrin side-chain substituents increases in the order of Ac, Pr, H, Me, Et and Vi groups. In the uroporphyrin molecule the most hydrophobic groups are the Pr groups. Uroporphyrin III, which has two adjacent Pr groups, is relatively more hydrophobic than the symmetrical



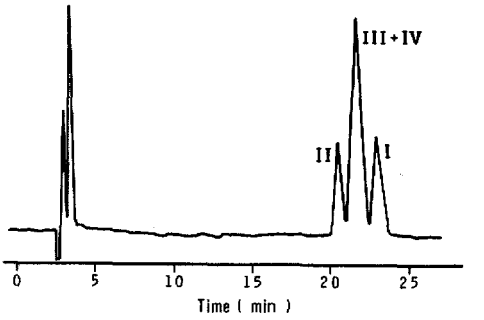


Fig. 29. Separation of uroporphyrin isomers. Column, Hypersil-ODS; eluent, 13% acetonitrile in 1 *M* ammonium acetate, pH 5.15; flow-rate, 1 ml/min; detector, 400 nm. From ref. 114 with permission.

Fig. 30. Separation of uroporphyrinogen isomers. Column, Hypersil-ODS; eluent, 4% acetonitrile in 1 *M* ammonium acetate, pH 5.16; flow-rate, 1 ml/min; detector, electrochemical +0.70 V. From ref. 106 with permission.

uroporphyrin I with no adjacent Pr groups. Uroporphyrin III therefore has a longer retention time than uroporphyrin I [105]. In a flexible uroporphyrinogen molecule, however, steric hindrance of adjacent groups and/or intramolecular interaction such as hydrogen bonding are possible. These factors may be responsible for the reversal of elution order in the separation of uroporphyrinogen isomers.

Methanol, which caused excessive retention as a result of extensive hydrogen bonding between the uroporphyrin side-chain carboxylic acid groups and a layer of methanol adsorbed on the stationary phase surface [83], may be used as an organic modifier for the separation of uroporphyrinogens. This observation confirms that the flexible uroporphyrinogen molecules may be involved in intramolecular hydrogen bonding in place of the intermolecular hydrogen bonding with adsorbed methanol.

7.5. HPLC detectors for porphyrin(ogen)s

The porphyrins have an intense absorption band near the 400-nm region (Soret band) with extinction coefficients often exceeding 200 000 [115]. The simultaneous, sensitive detection of a wide range of porphyrins in biological samples is therefore possible by setting the UV–visible detector at a compromise wavelength of 400–405 nm. A detection limit of 1 ng is commonly achieved. Much higher sensitivity of detection (pg injected) may be obtained with a fluorescence detector. The excitation and emission wavelengths used were usually 395–420 and 580–620 nm, respectively, depending on the applications. For example, ex-

citation at 417 nm and emission at 590 nm has been used for the sensitive and specific detection of Zn-protoporphyrin in human red blood cells [50].

The porphyrinogens are colourless, non-fluorescent compounds with weak UV absorption at the 200–240 nm region. The UV detector can be used when preparative separation is performed [106,108,109] but it is not sensitive enough for the detection of porphyrinogens in body fluids. The ease of porphyrinogen oxidation, however, makes these compounds ideal for highly sensitive electrochemical detection by the oxidation mode [106,108,109]. Combining HPLC with electrochemical detection thus provides a highly sensitive and specific method for porphyrinogen analysis in body fluids, although this has not yet been reported.

The electrochemical behaviour of porphyrinogens has been investigated with coproporphyrinogen I, II, III and IV isomers [116]. All four isomers were easily detectable at a potential of +0.45 V. Potentials of between +0.65 and +0.70 V were adequate for the sensitive (pg) detection of the porphyrinogens without significant detector noise level. The sensitivity is at least comparable to the fluorescence detection of porphyrins. Since electrons can be removed from the porphyrin ring system, they can also be detected electrochemically. However, the electrochemical activity of porphyrins is much lower than the porphyrinogens and a fluorescence or UV detector is preferred.

7.6. Quantitation of porphyrins

Apart from the quantitative determination of PBG in urine [14,85] clinical porphyrin analysis usually begins with the measurement of total porphyrin excretion in urine and faeces. Total urinary porphyrins can be measured by direct spectrofluorometry of a 50-fold dilution of urine in 0.28 *M* hydrochloric acid [16] and total faecal porphyrins by extracting the porphyrin with hydrochloric acid followed by spectrophotometric scanning [15]. It may also be necessary to determine the total red blood cell porphyrins by hydrochloric acid extraction and spectrofluorometric measurement [117]. If excess porphyrins are detected in urine, faeces and/or blood, qualitative HPLC profiles (see Section 8) are sufficient for the differential diagnosis of the porphyrias.

HPLC has been used for the quantitative analysis of porphyrins in urine and faeces [50,54]. Porphyrin standards are available commercially (Porphyrin Products, Logan, UT, U.S.A.) for the construction of calibration curves either by measurement of peak heights or more frequently peak areas with an electronic integrator. Mesoporphyrin is a good internal standard for the quantitation of urinary porphyrins and tetraphenylporphyrin has been suggested as an internal standard for the quantitation of porphyrin methyl esters [60]. Mesoporphyrin has also been used as internal standard for the quantitation of protoporphyrin in the determination of coproporphyrinogen oxidase [98] and protoporphyrinogen oxidase [99] activities by HPLC.

7.7. Peak identification

The UV–visible and fluorescence spectra of porphyrins are highly characteristic of the compounds [115]. Effective HPLC separation coupled with specific

UV or fluorescence detection has shown very little interference by endogenous compounds. The porphyrins can therefore be identified by their capacity ratios or relative retention compared to standards. A set of standards containing porphyrins with two to eight carboxylic groups is available from Porphyrin Products. In most routine laboratories, however, a mixture of proto-, copro- and uroporphyrin is sufficient; the intermediates are simply identified by their elution orders.

Confirmation of peak identity, if necessary, may be obtained with a photodiode array detector or by trapping the peak in the detector flow cell using the stop-flow technique followed by scanning for the characteristic UV–visible or fluorescence spectrum. Peaks may also be collected off-line for scanning or for mass spectrometry.

Spectroscopy and mass spectrometry are not suitable for the identification of type isomers, although nuclear magnetic resonance (NMR) spectroscopy with lanthanide shift reagents has been used [2]. A much simpler technique is based on partial decarboxylation of porphyrins by heating in dilute hydrochloric acid, followed by HPLC analysis of the characteristic set of pentacarboxylic porphyrins formed from each isomer [105,108,109]. This technique has been used for the unequivocal assignment of individual hepta-, hexa- and pentacarboxylic porphyrin(ogen) isomers [105,108,109]. The characteristic patterns produced by the type III hepta- and hexacarboxylic porphyrins are shown in Tables 1 and 2, respectively.

TABLE 1

PENTACARBOXYLIC PORPHYRINS FORMED BY PARTIAL DECARBOXYLATION OF TYPE III HEPTACARBOXYLIC PORPHYRIN ISOMERS

See Figs. 20 and 25 for structures.

Heptacarboxylic porphyrin	Pentacarboxylic porphyrins
7a	5abc, 5abd, 5acd
7b	5abc, 5abd, 5bcd
7c	5abc, 5acd, 5bcd
7d	5abd, 5acd, 5bcd

TABLE 2

PENTACARBOXYLIC PORPHYRINS FORMED BY PARTIAL DECARBOXYLATION OF TYPE III HEXACARBOXYLIC PORPHYRIN ISOMERS

See Figs. 20 and 25 for structures.

Hexacarboxylic porphyrin	Pentacarboxylic porphyrins
6ab	5abc, 5abd
6ac	5abc, 5acd
6ad	5abd, 5acd
6bc	5abc, 5bcd
6bd	5abd, 5bcd
6cd	5acd, 5bcd

7.8. Sample preparation for HPLC of porphyrins

The successful application of HPLC to porphyrin analysis not only depends on efficient separation but also on a good sample preparation procedure. A good sample preparation technique is essential to minimise quantitative errors and places less demand on the chromatography resulting in better separation and faster analysis.

7.8.1. Preparation of porphyrin methyl esters

Direct esterification of clinical specimens (urine, faeces and plasma) followed by solvent extraction was originally used to prepare porphyrin methyl esters for TLC separation. The methods are also suitable for HPLC applications. The most widely used esterifying agent is 5–10% sulphuric acid in methanol although 10–14% boron trifluoride in methanol and diazomethane have also been employed [12]. Typically, 0.5 g of wet faeces or 1 ml of urine was thoroughly mixed with 5 and 10 ml of 5% sulphuric acid in methanol, respectively. The mixture was refluxed for 20 min or left standing overnight in the dark at room temperature. The resulting porphyrin methyl esters were recovered by solvent extraction with dichloromethane or chloroform. Straka and Kushner [118] suggested the use of trimethyl orthoformate to eliminate water from the esterification mixture in order to achieve a more effective esterification. Prior to HPLC separation, it is advisable to further purify the extracted mixture by passing the solution through a small glass column or pasteur pipette filled with silica or alumina (for column chromatography). The porphyrin methyl esters were recovered by eluting the column with chlorofrom–methanol (1:1, v/v). Particulate materials and strongly adsorbing compounds are retained on the column. This gives a much cleaner solution for HPLC analysis.

7.8.2. Direct injection of urine

Normal levels of uro- and coproporphyrins can be easily detected fluorometrically by direct injection of 100 μl of urine. A good UV detector set at 400 nm also has sufficient sensitivity for the detection. A small guard column may be used to protect the analytical column from contamination. Precipitates are often seen in stored urine. These are usually calcium salts which tend to adsorb porphyrins. The urine should therefore be thoroughly mixed with concentrated hydrochloric acid (40 μl per ml urine) to dissolve the precipitated material. Acid treatment also prevents the formation of metalloporphyrins.

7.8.3. Extraction of porphyrin free acids from faeces

The hydrochloric acid–diethyl ether extraction method of Lockwood et al. [15] for the spectrophotometric measurement of faecal porphyrins also conveniently provided an extract suitable for reversed-phase HPLC separation. About 25 mg of wet faeces were vortex-mixed with 1 ml of concentrated hydrochloric acid followed by 3 ml of peroxide-free ether and then 3 ml of water. After centrifugation, 100 μl of the lower aqueous acid layer were injected into the HPLC system. The

upper ether layer contained unwanted chlorophyll and carotenoid pigments. A guard column should be used to protect the analytical column.

7.8.4. Extraction of red blood cell Zn-protoporphyrin and protoporphyrin

Total red blood cell porphyrins are usually estimated by extraction with a mixture of ethyl acetate or diethyl ether and acetic acid followed by back-extraction into hydrochloric acid [117]. The final extract can only be used for the separation of free porphyrins [119] as dilute mineral acids demetallate metalloporphyrins to porphyrins. The initial ethyl acetate–acetic acid extract has been used for the HPLC analysis of Zn-protoporphyrin and protoporphyrin [120,121]. However, this contains a large amount of haemin derived from haemoglobin which apart from contaminating the column also caused a negative peak because of fluorescence quenching by haemin.

n-Propanol containing tetrabutylammonium hydroxide (pH 7.5) and Triton X-100 have successfully been used for protein precipitation and extraction of Zn-protoporphyrin and protoporphyrin in whole blood [77] for reversed-phase ion-pair chromatography. An alternative, possibly better, approach was described by Rossi and Garcia-Webb [10] who employed a mixture of acetone and dimethyl sulphoxide (DMSO) for the extraction of haemin-free Zn-protoporphyrin and proporphyrin from whole blood. After evaporation of acetone, the aqueous DMSO solution was analysed by reversed-phase HPLC.

7.8.5. Solid-phase extraction of porphyrins

The most widely used solid phase for the extraction of porphyrins is talc [12]. This, however, does not give good recovery of porphyrins. More recently, small disposable cartridges packed with various sorbents have been employed for the effective and quantitative (virtually 100% recovery) extraction of porphyrins from urine, faeces and tissues [60,72,84,122–124]. A reversed-phase cartridge (C_8- or C_{18}-bonded) is normally used. A typical procedure for extracting urinary porphyrins is as follows:

(1) Activate cartridge with 2 ml of methanol.

(2) Condition cartridge with 5 ml of a buffer solution (usually that used in the HPLC mobile phase).

(3) Load 1 ml of urine and wash cartridge with 5 ml of buffer.

(4) Elute porphyrins from cartridge with 2×2 ml of methanol or acetonitrile.

For faeces and tissues the sample is homogenised with 10–20% trichloroacetic acid–DMSO (1:1, v/v) or other extractants [124] and centrifuged. The supernatant after dilution with buffer is loaded onto the cartridge [84] and then processed as described for urine. The cartridge extraction technique has been further developed into an advanced automated sample processor (AASP) by Varian Assoc. (Walnut Creek, CA, U.S.A.) capable of automatic sample processing, injection and elution when coupled to an HPLC instrument. Porphyrin profiles in urine and faeces [84] and assays for erythrocyte haem biosynthetic enzymes [93] by AASP–HPLC have been described. The cartridges act as preconcentration columns and vastly improved the sensitivity of detection. They also act as indi-

vidual guard columns which protect the analytical column from contamination and greatly prolong the column life.

The AASP–HPLC technique may also be used to advantage in the small-scale preparative isolation of porphyrins. Using this technique relatively large amounts of porphyrins can be concentrated on the cartridge before injection and elution with the mobile phase. This avoids the direct injection of a large volume of strongly acidic or alkali solvent (used to dissolve porphyrins) which is very likely to cause irreversible damage to the column.

8. SUMMARY

Techniques for the analysis of porphyrins in the biomedical fields are reviewed. The emphasis is on high-performance liquid chromatography and its aspplications in: (1) the quantitative analysis of porphyrins in blood, urine and faeces; (2) qualitative porphyrin profiles in normal subjects and in the porhyrias; (3) assay of haem biosynthetic enzyme activities and (4) resolution of type isomers of porphyrins and porphyrinogens. Detection systems, quantitation methods, peak identification and sample preparation procedures are discussed.

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