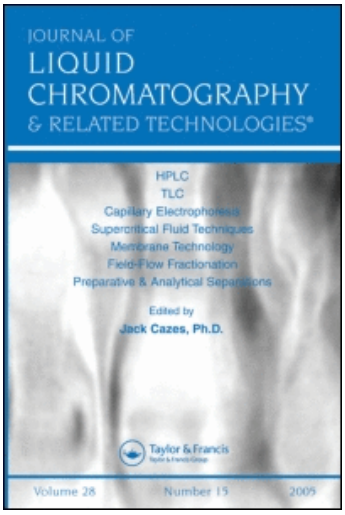

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Porphyrins in Physiological Samples

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To cite this Article Ho, John W.(1990) 'Determination of Porphyrins in Physiological Samples', Journal of Liquid Chromatography & Related Technologies, 13: 19, 3741 — 3772
To link to this Article: DOI: 10.1080/01483919008049567
URL: http://dx.doi.org/10.1080/01483919008049567

JOURNAL OF LIQUID CHROMATOGRAPHY, 13(19), 3741-3772 (1990)

Review

DETERMINATION OF PORPHYRINS IN PHYSIOLOGICAL SAMPLES

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ABSTRACT

There has been voluminous literature on the separation and analysis of different porphyrins, such as metalloporphyrins, biological, geological porphyrins and the porphyrin derivatives. The determination of these porphyrins is useful and significant in diagnosis of diseases, the study of deposition of sediments and the geology of environment. The present review reports the recent developments in the determination of porphyrins in physiological specimens by liquid chromatography.

INTRODUCTION

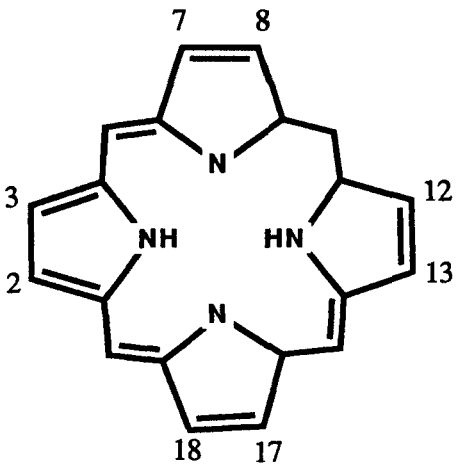
Porphyrins are the intermediate metabolites of heme biosynthesis. They are formed in the body from their precursors, δ-aminolevulinic acid and porphobilinogen. Different porphyrins represent different intermediate

metabolites in the biosynthetic pathway. Disturbance of the heme biosynthetic pathway is characterized by excess of porphyrins and their precursors in blood and other tissues. Zn-protoporphyrin is formed in the final step of heme biosynthesis and its formation is markedly increased in lead intoxication. However, other diseases , such as iron-deficiency anemia and porphyrias, present symptoms that are very similar tc those in lead poisoning. The basic metabolic abnormality most frequently encountered is an enzyme deficiency. This results in overproduction of porphyrins and their precursors. Analysis of accumulated and excreted porphyrins is important in the diagnosis of porphyrias, and it is also useful as a confirmatory test for other porphyrin-related disorders, such as lead-poisoning and iron deficiency anemia. The methods for the determination of different biological porphyrins and their precursors vary in complexity. Numerous papers on the separation and analysis of porphyrins have been reported in the literature. The present review reports the recent improvements in the determination of porphyrins in physiological specimens by liquid chromatography.

CHEMICAL PROPERTIES OF PORPHYRINS

The basic ring structures of the common , diagnostically important porphyrins are shown in Table I. In reduced form as the naturally occurring porphyrinogens, the macro ring consists of four pyrrole rings bonded by saturated carbon bridges while in the oxidized form, the tetrapyrrole ring forms a conjugated double-bond system. The porphyrin ring is essentially planar, aromatic in character, and hydrophobic with a characteristic color and considerable stability. Naturally occurring

TABLE I Structures of Porphyrins



Porphyrins	Substituents* at Position							
	2	3	7	8	12	13	17	18
Uroporphyrin	A	P	A	P	A	P	A	P
Heptaporphyrin	M	P	A	P	A	P	P	A
Hexaporphyrin	M	P	M	P	A	P	P	A
Pentaporphyrin	M	P	M	P	A	P	P	M
Coproporphyrin	M	P	M	P	M	P	M	P
Protoporphyrin	M	V	M	V	M	P	P	M
Mesoporphyrin	M	E	M	E	M	P	P	M
Zn-protoporphyrin	M	V	M	V	M	P	P	M

* M= $-CH_3$; E= $-C_2H_5$; A= $-CH_2COOH$; P= $-CH_2CH_2COOH$; V= $-CH=CH_2$

porphyrins of heme biosynthesis have beta positions substituted with groups shown in Table I. The stability of porphyrins decreases considerably in solution due to photo-oxidation. The stability also depends on the nature of the substitutents. The naturally occurring porphyrins contain from two to eight carboxylic groups , which control the solubility and acidity of the compounds. The planar aromatic tetrapyrrole ring exhibits electronic transitions in the proximity of 400 nm (Soret band) and less intense transitions in the visible range. The beta substituents affect the porphyrin absorption spectra. Free porphyrins and Zn-protoporphyrin have characteristic red fluorescence when irradiated with UV light; thus, the spectrofluorometric methods provide a sensitive method for the detection of biological porphyrins.

ANALYSIS OF THE PRECURSORS OF PORPHYRIN

The first porphyrins are formed from the condensation reaction between molecules of δ-aminolevulinic acid and the subsequent product, porphobilinogen, condenses to form porphyrins. Porphyrins are cyclic tetrapyrroles and the immediate precursors of intermediate metabolites of heme biosynthesis, and the precursors of the various hemes, chlorophylls and cobalamines. The biosynthetic pathway can be inhibited by heavy metals and other chemical intoxicants. The determination of accumulated and excreted precursors is useful and important in the study of chemical intoxication. A traditional method for the determination of δ-aminolevulinic acid requires a two-step derivatization with the Ehrlich's reagent and the product is measured spectrophotometrically. However, the sensitivity and

specificity of the method is a limiting consideration (3). A variety of methods for the determination of the precursors , such as anion-exchange chromatography (4-7) and cation-exchange chromatography (8), has been reported. Enzymatic assays for the precursors have also been reported (9-10). More sensitive methods for assays of the precursors in blood (9) and urine (10) by HPLC have recently been described. The precursors were derivatized with o-phthaldialdehyde after simple extraction of the compounds from physiological samples. The adducts were separated by HPLC and measured fluorometrically as shown in Figure 1. The method was applied to determining the precursors in human urine (Figure 2). The high intensity of the fluorescent adducts produces a detection limit in sub-picomole range ; thus, the method is suitable for microassay of the precursors in minute quantity. The HPLC method could be modified to determine the precursors from other biological samples by changing the volume ratio of the binary mobile phase.

CHROMATOGRAPHY OF PORPHYRIN ESTERS

Naturally occurring porphyrin carboxylic acids can be separated from body fluids and other biological tissues according to the number of carboxylic groups (11-12). However, the porphyrin acids could be derivatized (13) to facilitate an alternative method for determining individual porphyrin acids from different specimens. The determination of the derivatized porphyrins as esters is common and reliable due to their increased stability and solubility in solution. Different methods, such as ion-pair gradient reversed-phase chromatography (14-15), normal phase

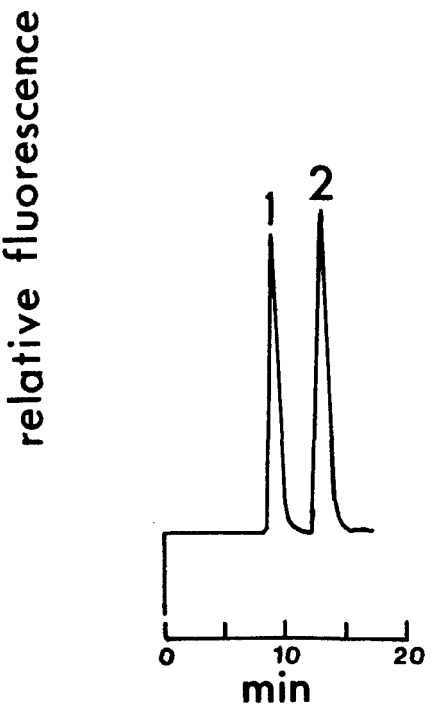


Figure 1. Chromatogram of derivatized δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) standards. Peaks: 1, ALA ; 2, PBG. (Adapted from ref.10 with permission)

chromatography (16), thin layer chromatography (13 , 17) and the reversed-phase chromatography (18-20), for the determination of methyl esters of porphyrins have been reported. More recently, a four-component mobile phase containing n-heptane, ethyl acetate, chloroform and methanol was developed to separate the common porphyrin acids in hamster Harderian glands as methyl esters on a silica column (21). However, the methods for the analysis of porphyrin esters vary in complexity; the

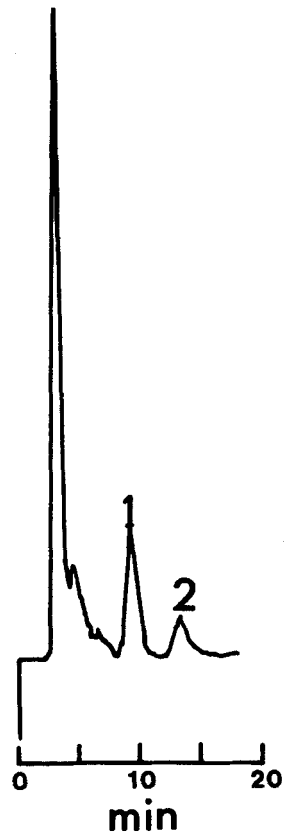


Figure. 2. Chromatogram of urinary ALA and PBG from a normal individual. See Figure 1 for labels. (Adapted from ref.10 with permission)

derivatization procedure is time-consuming. Nevertheless, the esterification of porphyrin carboxylic acids leads to a cleaner extraction of porphyrins from biological samples.

ANALYSIS OF ERYTHROCYTE AND URINARY PORPHYRIN FREE ACIDS

Most biochemical diagnosis of porphyrin-related diseases involve analysis of porphyrins in urine, blood and stool. Information on changes in

the erythrocyte porphyrins and the urinary excretion pattern of porphyrins are especially useful in diagnosis of disorders of heme biosynthesis. Analysis of excreted and accumulated erythrocyte intermediary metabolites of heme biosynthesis is useful as a confirmatory test for distinguishing lead-poisoning from iron deficiency anemia and porphyrias. A variety of methods is available for the determination of porphyrins as the naturally occurring porphyrin carboxylic acids. Lim and his coworkers have made significant contributions to this area of study earlier. In addition, there are numerous papers which have made significant contributions to the separation and analysis of porphyrins (11, 22-34). Some earlier methods employed reversed-phase ion-pair chromatography using tetrabutylammonium phosphate as the ion-pairing agent (31-34). Other methods required gradient elution on silica stationary phases using binary solvent systems containing traditional solvents, such as acetonitrile,acetone, water and acetic acid (35-36). More efficient gradient elution systems, also developed for the separation of porphyrins with two to eight carboxylic groups, were modified from the previous methods using reversed-phase C18 columns (37-38), and the other method employing a phenyl-bonded silica column with isocratic elution was also described (27) . However, the isocratic elution of porphyrin acids on the phenyl-bonded silica stationary phases was incomplete. The less polar porphyrins were retained. Recently, a two-step gradient elution method , for the separation of the seven porphyrin carboxylic acids and meso-porphyrin as the internal standard on a reversed-phase C18 column, was reported (39). The method required a pre-treatment of the column with a two-component mobile phase containing 0.1 M sodium phosphate in acetonitrile . Subsequently, the separation was completed isocratically by

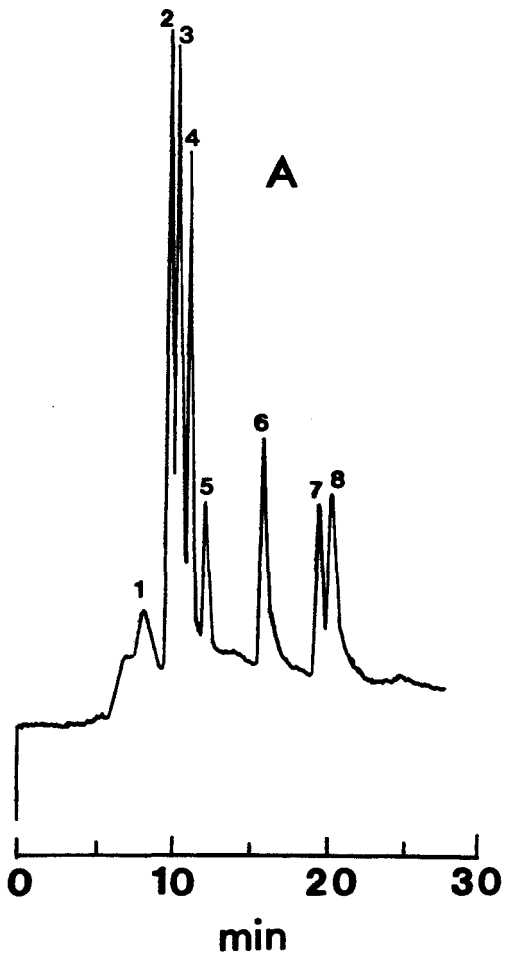


Figure 3. Chromatogram of porphyrin carboxylic acid and zine-protoporphyrin standards. Peaks: 1, uroporphyrin; 2, heptaporphyrin; 3, hexaporphyrin; 4, pentaporphyrin; 5, coproporphyrin; 6, zinc-protoporphyrin; 7, mesoporphyrin; 8, protoporphyrin. (Adapted from ref.39 with permission)

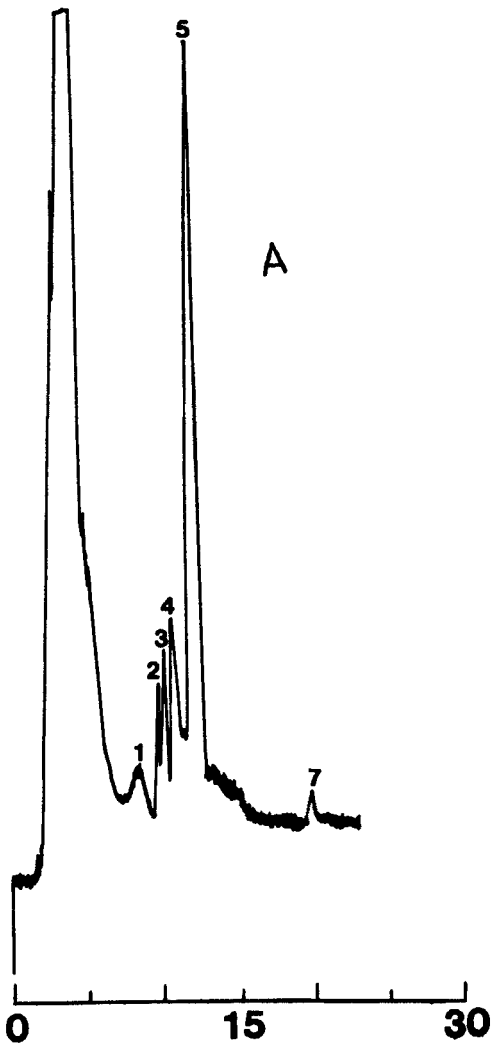


Figure 4. Chromatograms A and B of urine samples from two normal individuals. See Figure 3 for labels. (Adapted from ref.39 with permission)

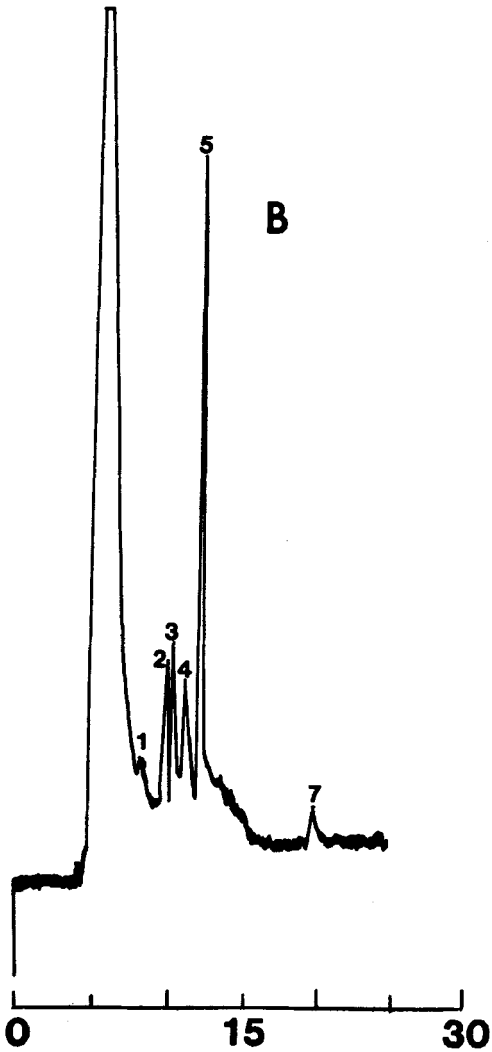


Figure 4 (continued).

increasing the concentration of acetonitrile in the mobile phase as shown in Figure 3. The method was applied to determining zinc-protoporphyrin and the porphyrin carboxylic acids in urine from two normal individuals (Figures 4A-4B). The chromatograms clearly show the excretion patterns of the porphyrin carboxylic acids in urine. Also, the influence of mobile phase pH, ionic strength, buffer composition, and organic modifier on selectivity in separation of polycarboxylic porphyrins was investigated (40). The study sheds some light on the separation performance of porphyrin carboxylic acids on the reversed-phase system. With the advent of the column technology, different chromatographic methods for the separation and analysis of porphyrins have been developed . The stationary phases of reversed-phase columns were modified to facilitate the separation of the porphyrin carboxylic acids (23, 41). Different methods for the separation of the seven common porphyrin carboxylic acids on a C1-bonded (29) or a C18 bonded silica column (42), using linear gradient elution systems with acetonitrile-methanol in ammonium acetate buffer, were reported. These methods allow a better resolution between the interferring peak, riboflavin and uroporphyrin, and facilitate a good separation between some porphyrin isomers, namely, types I and III. More recently, the seven common porphyrin carboxylic acids were separated isocratically on a β-cyclodextrin column using a novel mobile phase containing phosphate buffer, 18-crown-6 ether, pyridine and acetone (43). The novelty of the method (43) allows an isocratic separation of the common porphyrin carboxylic acids under eight minutes (Figure 5).

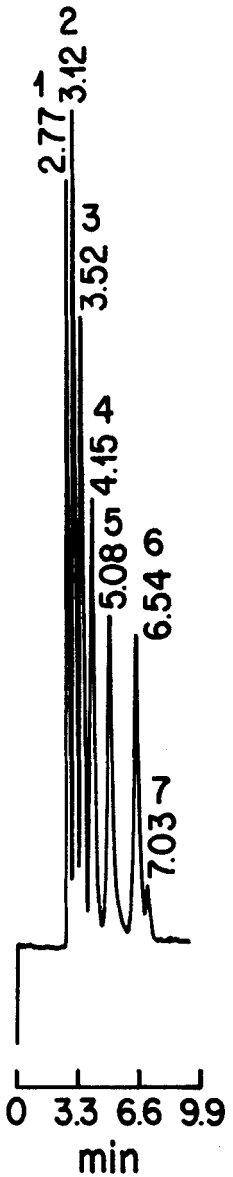


Figure 5. Chromatogram of porphyrin carboxylic acid standards. Peaks: 1, uroporphyrin; 2, heptaporphyrin; 3, hexaporphyrin; 4, pentaporphyrin; 5, coproporphyrin; 6, mesoporphyrin; 7, protoporphyrin. (Adapted from ref.43 with permission)

CHROMATOGRAPHY OF PORPHYRIN ISOMERS IN BIOLOGICAL SYSTEMS

The porphyrias are inherited and acquired disorders in which there are partial defects in enzymes of heme biosynthesis. These diseases are classified as either hepatic or erythroid in origin depending on the principal site of expression of the gene defect in each disorder. In all of the inherited forms of human porphyrias, environmental factors play a vital role in determining clinical expression of the gene abnormality. The porphyrias demonstrate an example of disease in which both the environmental and genetic factors are attributed to the pathogenesis. The determination of the porphyrin isomers is important in the diagnosis of porphyrias, and also of great significance in the study of the chemistry and biochemistry of porphyrins. Porphyrin acids, such as uroporphyrin and coproporphyrin, can be present as different positional isomers in biological systems. The determination of accumulated or excreted porphyrin isomers provides valuable information for the differential diagnosis of porphyrias.

Numerous procedures have been described for the determination of porphyrin isomers. However, HPLC is the preferred technique. An example includes an HPLC method for the separation of coproporphyrin types I and III isomers as methyl esters on a silica column with a binary mobile phase containing dichloromethane and acetone (44). In addition, there are quite a number of reversed-phase chromatographic methods (29, 31, 42, 45-47) which allow a better resolution between isomers of some common porphyrins with improved efficiency. Several of the improved methods employing reversed-phase ion-pair chromatography for the simultaneous isocratic separation of the four structural isomers of

coproporphyrin were reported (31, 48-49). The other ion-pair HPLC method enabled the determination of uroporphyrin and coporporphyrin types II and IV isomers (45). An alternative approach to isomer resolution was to use thin-layer chromatography. A thin-layer chromatography method for the separation of coproporphyrin types I and III isomers and isocoproporphyrin together with other porphyrin acid standards was reported earlier (28). But the method required tedious sample preparation and pre-concentration of porphyrin carboxylic acids from biological specimens. Recently, an HPLC method for the separation of the free acids of uroporphyrin types I and III, and copro- porphyrin types I and III isomers from the type I isomers of some other porphyrin carboxylic acids, using a micro octadecylsilyl 3 cm long column, has been reported (50). The method allows a rapid microassay of urinary and hepatic porphyrins with excellent sensitivity. However, a gradient elution procedure with fluorescence detection was employed.

ANALYSIS OF METALLOPORPHYRINS

While information on changes in the excretion pattern of and the accumulated porphyrin carboxylic acids are useful in diagnosis of disorders of heme biosynthesis, measurement of accumulated zinc protoporphyrin is a good indicator of lead poisoning (51). There are numerous methods available for the separation and analysis of metalloporphyrins and porphyrins (9, 20, 23, 25-26, 33, 39, 52-60).

Quantitative assays of zinc-protoporphyrin and erythrocyte protoporphyrin by direct fluorometric measurement following extraction

have been reported (55, 58, 60). But the direct fluorometric methods are not without drawbacks. The limitation of hematofluorimetry is the quality control of standardization. Also, the accuracy of the method is affected by interferences from bilirubin and protoporphyrin in the extract. Moreover, the overlapping of the fluorescence spectra of protoporphyrin and zinc-protoporphyrin is a limiting factor. However, a modified extraction procedure coupled with the computer-assisted fluorimetric analysis of the second derivative fluorescence spectra of protoporphyrin and zinc-protoporphyrin (55) has improved the quantitative assay of the compounds.

Another common technique that has been widely used for determination of metalloporphyrins is liquid chromatography. Several HPLC methods have been developed to simultaneously measure zinc-protoporphyrin and protoporphyrin (23, 57, 59) , and together with coproporphyrin (33) in biological samples with excellent efficiency. The HPLC method for determining erythrocyte protoporphyrin and zinc-protoporphyrin as described by Sakai and his coworkers (23) has considerable advantages over most of the other methods reported earlier due to its simplified extraction procedures and the improved extraction efficiency. However, the earlier HPLC method (33) allows a simultaneous determination of zinc- protoporphyrin, protoporphyrin and coproporphyrin with better efficiency. But an obvious drawback of the method (33) is that a new column is needed after 150 sample injections while the column used in Sakai's method apparently remains in good condition after 1500 injections of samples as reported (23).

Thin-layer chromatography was also employed for the analysis of metalloporphyrins and porphyrins (52-54). However, few studies dealing

with porphyrins and metalloporphyrins in biological systems have been reported. Recently, different methods for the simultaneous determination of zinc-protoporphyrin and intermediary metabolism of heme biosynthesis in physiological samples have been reported (9, 20, 25, 39, 56). The methods allow an improved analysis of accumulated and the excreted porphyrin acids, and the resulting elution profiles provide more information for diagnosis of porphyrin-related disorders by determining the intermediate metabolites of heme biosynthesis together with zinc-protoporphyrin in a single experiment . The HPLC method (39) for the separation of the intermediate metabolites and zinc-protoporphyrin with fluorescence detection required a pre-treatment of the reversed-phase C18 column with a binary mobile phase containing acetonitrile and phosphate buffer. The pre-treatment process served two purposes as to ensure good resolution between peaks and eliminate the major interferring metabolites in urine (Figure 4). The other methods for the determination of porphyrin carboxylic acids and zinc-protoporphyrin (56), together with the precursors of porphyrin (9, 25) in whole blood and dried blood were also described (Figure 6). The chromatogram in Figure 6 shows the base-line resolution of some common porphyrins and their precursors in a single experiment. The methods for the analysis of erythrocyte porphyrins and their precursors required simple extraction procedures. However, a pre-treatment of the column and different ternary mobile phases for optimized separation performance were needed for the described HPLC methods. The elution profile of some common erythrocyte porphyrins from a normal individual is shown in Figure 7. The method provides high sensitivity for determining zinc-protoporphyrin and porphyrin acids in two 0.64 cm discs from dried blood specimen. The ternary mobile phase composition , which was studied

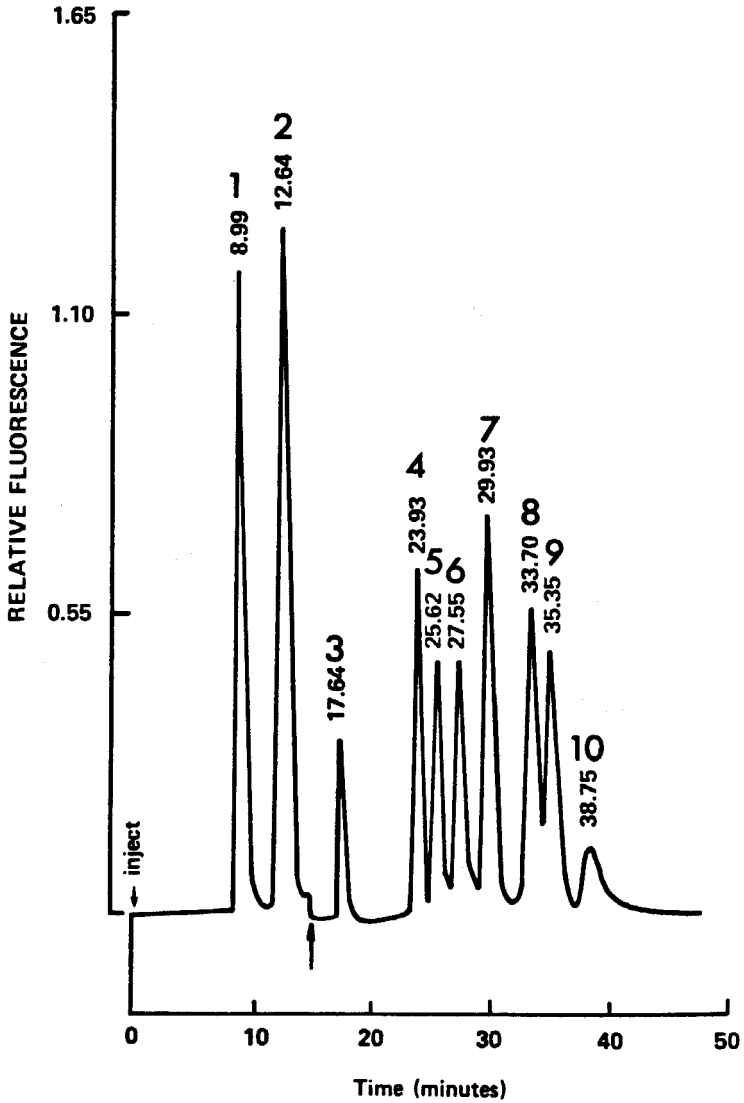


Figure 6. Chromatogram of ALA, PBG and porphyrins. Peaks: 1, ALA; 2, PBG; 3, uroporphyrin; 4, coproporphyrin; 5, heptaporphyrin; 6, hexaporphyrin; 7, pentaporphyrin; 8, mesoporphyrin; 9, zinc-protoporphyrin; 10, protoporphyrin. (Adapted from ref.25 with permission)

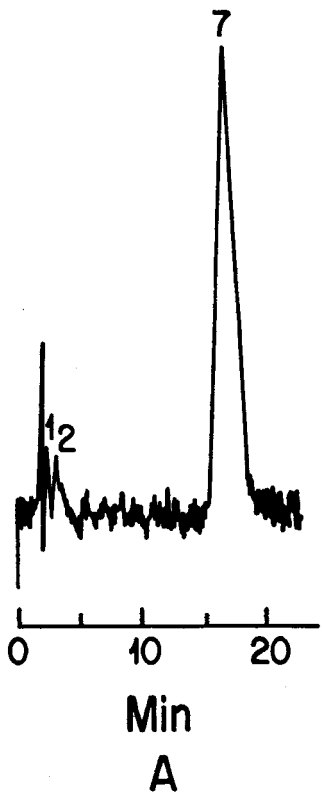


Figure 7. Chromatogram of erythrocyte porphyrins. See Figure 6 for labels. (Adapted from ref.9 with permission).

earlier (40), could be easily modified by changing the volume ratio of the organic modifier, tetrahydrofuran, for the analysis of individual porphyrins in body fluids and excreta from different biological systems. The methods are simple and efficient . Generally, the methods took less than 30 min to complete . An alternative method for determining urinary porphyrin acids as methyl esters and zinc-protoporphyrin was also

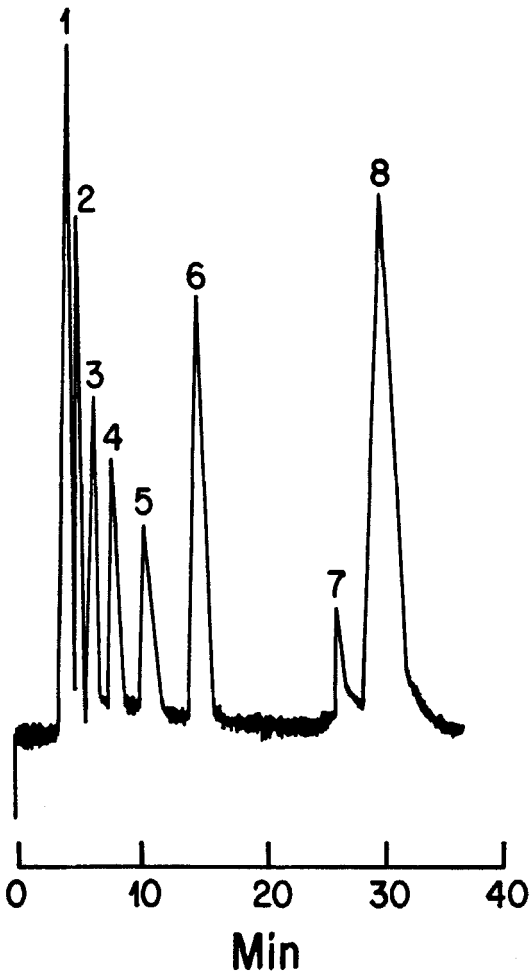


Figure 8. Chromatogram of porphyrin methyl esters and zinc-protoporphyrin standards. Peaks: 1, uroporphyrin; 2, heptaporphyrin; 3, hexaporphyrin; 4, pentaporphyrin; 5, coproporphyrin; 6, mesoporphyrin; 7, zinc-protoporphyrin; 8, protoporphyrin. (modified from ref.20)

reported (20). All the porphyrin esters including mesoporphyrin as the internal standard were well resolved by this method (Figure 8). The base-line separation of the compounds took less than 35 min (20). The method was successfully applied to determining the compounds in urine.

ANALYSIS OF FECAL PORPHYRINS

Determination of fecal porphyrins is important in the differential diagnosis of porphyrias. Traditional methods for determining the porphyrin content of feces required several steps of extraction from the fecal sample with ether and acetic acid, followed by differential extraction of the porphyrins with various concentrations of hydrochloric acid (61-63). The extracts were analysed by different chromatography techniques including thin-layer chromatography. An obvious disadvantage of these methods is a lack of efficiency and the extraction method is laborious. Recently, a rapid semi-quantitative measurement of total porphyrins in feces by magnetic circular dichroism has been described (64). The magnetic circular dichroism technique allows a rapid semi-quantitative analysis of total fecal porphyrins for screening potential disorders. On the other hand, a more accurate method for the determination of fecal porphyrins using HPLC has also been reported (29). The method allows the simultaneous separation of type I and III isomers of the common porphyrins on a trimethylsilyl-bonded silica column using a novel gradient solvent systems containing acetonitrile and methanol in ammonium acetate buffer. The method was also applied to the analysis of urinary porphyrins. More recently, a rapid method with a modified extraction procedure for fecal porphyrin assay has been reported (65). The method permits a rapid estimate of total

porphyrins. The modified extraction procedure (65) yields an extract suitable for the analysis of porphyrins by thin-layer or liquid chromatography. Also, the excretion patterns of fecal porphyrins , including coproporphyrin types I and III isomers, proto- porphyrin and its transformed products such as pempto-, deutero-, and meso- porphyrins, for normal individuals and patients were neatly established using a reversed-phase HPLC technique (66). The results of the study show that the fecal porphyrin content is affected by anaerobic bacterial metabolism which results in production of pempto-, deutero-, and meso- porphyrins. However, a most recent study of the effect of diet on excretion of fecal porphyrins by the HPLC methods (29, 66) has showed that the fecal porphyrin content can be significantly changed by ingestion of red meat or gastrointestinal bleeding (67).

PREPARATION OF PORPHYRIN CARBOXYLIC ACIDS FROM SPECIMENS

There are a vast number of methods for the isolation and extraction of porphyrins from different specimens. But the extraction methods prior to chromatographic analysis are not significantly different from each other. Traditional methods for the sample preparation require, acid extraction (68-69), neutral solvent extraction (70), the addition of detergents in the extraction solvent (71), the direct measurement of porphyrins with a minimal treatment of sample as in hematofluorimetry (72), and a direct injection of acidified urine sample without further treatment prior to the analysis (24), or aging of the urine sample at ambient temperature for 24 h to allow oxidation of porphyrinogens to porphyrins prior to the analysis (27). Acidification of samples, however,

dissociates metalloporphyrins such as zinc-protoporphyrin, and the quantification of metalloporphyrins is, therefore, measured as unchelated porphyrins, namely, free protoporphyrin. Furthermore, acidified specimens may contain interferring substances that exhibit fluorescence or absorbance similar to that of the porphyrins. Consequently, monitoring at different excitation and emission wavelengths alone does not eliminate the interferring metabolites; thus, a good method must be capable of separating the porphyrins and metalloporphyrins from interferring metabolites. Numerous methods for the preparation of porphyrins from different physiological samples to facilitate different analytical methods have been previously reported and referenced section-wise in this report. In addition, pre-concentration of porphyrins from biological samples by ion-exchange chromatography provided an approach for the preparation of porphyrins from specimens in the earlier studies (73-75). However, the methods are tedious. Recently, a variety of methods for the preparation of porphyrins from specimens using solid-phase extraction techniques has been reported (22, 28, 38, 42,76-77). The extraction procedures were facilitated by using reversed-phase bonded-phase cartridges coupled with centrifugation technique. The other commonly used technique requires derivatization of the porphyrins from specimens as their methyl esters. The experimental conditions for esterification have been recently refined to improve the product yield (78). More recently, an HPLC method for determining urinary porphyrinogens in human as iodinated porphyrins has been described (79). The method required oxidation of the porphyrinogens with iodine in acidified ethanol followed by centrifugation prior to HPLC analysis using gradient elution with a ternary mobile phase. The method

offers a different approach to sample preparation prior to the analysis of porphyrins.

ANALYSIS OF HAEMATOPORPHYRIN AND ITS DERIVATIVES

Information on changes in the therapeutic drugs, haematoporphyrin and its derivatives , are useful in the diagnosis and treatment of cancer. It is well known that there is an elevation of the porphyrins in tumor cells (80-82). The tumors can be distinguished from normal tissue by monitoring selectively the fluorescence of the porphyrins (81-82). The irradiation of cells in the presence of haematoporphyrin and its derivatives results in damage and death of cells (80, 83). The analysis and purification of these compounds, however, are difficult due to their unstability (84). The labile side-chain substituents are attributed to the unstability of these compounds. Commercial haematoporphyrin is usually contaminated with its derivatives, protoporphyrin and other polar porphyrins (85). Nevertheless, haematoporphyrin and its derivatives have been analysed by thin-layer chromatography (86-87) and HPLC (87-92) in recent studies. Also, a normal-phase HPLC method for the isocratic separation of dicarboxylic porphyrin complex containing haematoporphyrin, hydroxyethylvinyl-deuteroporphyrin and protoporphyrin along with their isomers on a silica column using mobile phase containing hydrochloric acid in acetone-ethyl acetate solution has been reported (93). It is complementary to the existing methods.

GENERAL PROBLEMS IN QUANTITATIVE ANALYSIS OF PORPHYRINS

The current analytical methods for the analysis of porphyrins are based on chromatographic techniques, hematofluorometry and fluorometric

measurement of total free erythrocyte protoporphyrin. Preceding the porphyrin analysis by different preparation steps, such as liquid-liquid extraction followed by derivatization , solid-phase extraction, has been accepted practice. The application of chromatographic methods, spectrophotometric or spectrofluorometric techniques to the quantitative analysis of porphyrins poses several questions regarding the accuracy of the measurements and the reference levels, the use of appropriate internal standards, the purity and validation of standards and reagents, interferences, chemical states of the compounds, the resolution power and the limitation of an analytical method.

Numerous studies have addressed the questions. Gunter and his coworkers have investigated into the chemistry of the porphyrin standard materials used in acid-extraction methods and proposed a correction for the spectrophotometric measurement (94). The reference materials were characterized by different spectroscopy techniques and analytical methods in the study (94). The results of the study suggest that protoporphyrin IX dimethylester be used for the calibration of analytes in methods required acid extraction. Different internal standards, such as 2-vinyl-4-hydroxymethyl-deuteroporphyrin for the quantitation of urinary porphyrins (95), tetraphenyl-porphyrin for the quantitative analysis of porphyrin methyl esters (96), and 2,4-dihydroxymethyldeuteroporphyrin (97), have been used for the analysis of porphyrins. However, meso-porphyrin remains to be the commonly used internal standard. Nevertheless, its use is limited in the analysis of fecal porphyrins due to its formation by microbial degradation of protoporphyrin in feces (98).

Also, the effect of sample matrix and pH on the quantitation of porphyrins by HPLC with fluorometric detection was studied (99). The

results of the study suggest that the quantitative analysis of porphyrins is pH-dependent ; the loss of porphyrins from solution becomes significant at pH > 1.0 . The loss are apparently attributed to the interaction between albumin and porphyrin, which results in precipitation and aggregation of porphyrins. It was suggested that standard solutions of porphyrins for the HPLC analysis should be prepared in a urine matrix and the pH of aqueous solutions be adjusted to pH < 1 to minimize the loss of porphyrins (99). The detail of the porphyrin-albumin interaction and the binding characteristics of the porphyrins was reported earlier (100). A reason for the discrepancy in the quantitative analysis of porphyrins is believed to be a result of incomplete oxidation and different rates of the reaction of the porphyrinogens. An alternative approach to optimize the oxidation of porphyrinogens to porphyrins , in addition to the long existing and acceptable methods of acidification , photo-oxidation and esterification of the compounds, is by quantitative oxidation of porphyrinogens to porphyrins with iodine prior to the analysis (79, 101). In addition, a reference method for the quantitative analysis of porphyrins using creatinine as the reference compound has been reported (79, 101).

The detection techniques for porphyrins based on the fluorometric and absorbance measurements have also been studied. Factors affecting the fluorescence spectra of free porphyrins were examined (102). The results show that the intensity of porphyrin fluorescence depends on pH and the ionic strength. The commonly used ethyl acetate-acetic acid extraction solvent system reduces more significantly the fluorescence of porphyrins than other aqueous acidic solvents. Different corrections have been reported to improve the measurement. Study includes developing a

different solvent system for the extraction of porphyrins (69). The total porphyrin content was determined at the isosbestic point for uro- and copro- porphyrins to order to ensure that the compounds could be equally detected, and the mole fractions of these compounds were evaluated from the wavelength of the signal maximum (101). Alternatively, the application of the second-derivative synchronous fluorescence spectroscopic technique to the simultaneous and direct assay of uro- and copro- porphyrins in human has been reported (103). The method allows a rapid quantitative measurement of the compounds in the same extract without being physically separated from each other in the matrix prior to the analysis. The method (103) provides a way to enhance the spectral resolution. Similar studies for the quantitation of urinary porphyrins by use of second-derivative spectroscopy (104), and the quantitative analysis of protoporphyrin and zinc-protoporphyrin by measuring the second derivative fluorescence spectra of the compounds (55), have been reported . The technique was also applied to evaluate the first and second derivative absorption spectra for the simultaneous quantitation of bilirubin and hemoglobin (105). In addition, the determination of urinary porphyrins by multi-wavelength analysis of derivative spectra has been reported recently (106).

Finally, effect of bias in hematofluorometer measurements of protoporphyrin in mass screening programs was studied (107). Although the effects of hematofluorometer bias on the efficiency vary from place to place, the problems can be improved by re-calibration of the instruments at regular intervals with fluorescent dye as the primary standard, and testing the instrument with synthetic blood specimen (107). Alternatively,

a recently introduced hematofluorometer, the "Protofluor-Z" (Helena Laboratories, Beaumont, TX), allows an empirical determination of erythrocyte porphyrins to facilitate the establishment of a cutoff for Lead-poisoning screening at 70 micromoles of zinc-protoporphyrin per mole of heme, which helps field users minimize the frequency of false-negative results (108).

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Received: August 20, 1990
Accepted: August 29, 1990