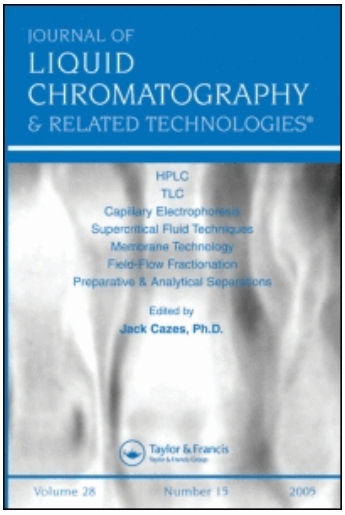


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# Quantitative Determination of Intermediary Metabolism of Heme Biosynthesis

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# QUANTITATIVE DETERMINATION OF INTERMEDIARY METABOLISM OF HEME BIOSYNTHESIS

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## ABSTRACT

A sensitive procedure has been developed for the determination of the intermediary metabolites of heme biosynthesis by high-performance liquid chromatography. The method requires a pre-treatment of the reversed phase C18 column with a binary mobile phase containing 0.1 M $NaH_2PO_4$ in methanol (18 : 30, v/v, pH 5.3 ). The separation of the intermediates including Uro-, Hepta-, Hexa-, Penta-, Copro-, Meso-, Proto- and Zinc-protoporphyrin, is complete with a ternary mobile phase containing 0.1 M $NaH_2PO_4$ in methanol and tetrahydrofuran ( 18 : 30 : 16, v/v/v, pH 5.3 ). The detection limit of the method is in the subpicomole range for the porphyrins. It is an efficient and reproducible method for the simultaneous determination of Zn-protoporphyrin and the intermediary metabolites of heme biosynthesis. The method was applied to study the intermediary metabolites of heme biosynthesis in blood .

## INTRODUCTION

Heme biosynthesis requires 8 moles of glycine and 8 moles of succinyl CoA to form the intermediate acid, ∂-aminolevulinic acid , which is then condensed to form the immediate porphyrin precursor, porphobilinogen , as shown in Figure 1. Through the continuous oxidative decarboxylation processes, intermediate metabolites characterised by the number of carboxyl groups , ranging from two to eight , on the porphyrins are formed. Protoporphyrin forms the last metabolite from which various hemes, chlorophyll and cobaltamines are formed (1-3). Disturbances of the heme biosynthetic pathway which can be brought about by an enzyme deficiency or intoxication are characterised by the elevation of intermediary metabolites in body fluids. In lead-poisoning, protoporphyrin chelates with zinc ions (4).

The analysis of the accumulated and excreted porphyrins is, therefore, important to study the heme biosynthesis. In addition, the determination of the intermediary metabolites of the heme biosynthesis and Zn-protoporhyrin provides information in diagnosis of different diseases , such as lead-poisoning, iron-deficiency and Porphyria. However, Zn-protoporphyrin which is chemically unstable in strong acidic solution dissociates to form Zn ions and protoporphyrin. But acidic medium is required for the quantitative extraction of porphyrins in body fluids. Consequently, a separate step is needed for extracting Zn-protoporphyrin in body fluids. A variety of methods for determining individual porphyrins or group of porphyrins has been reported (5-11). More specific procedures for studying the enzymatic activities of heme

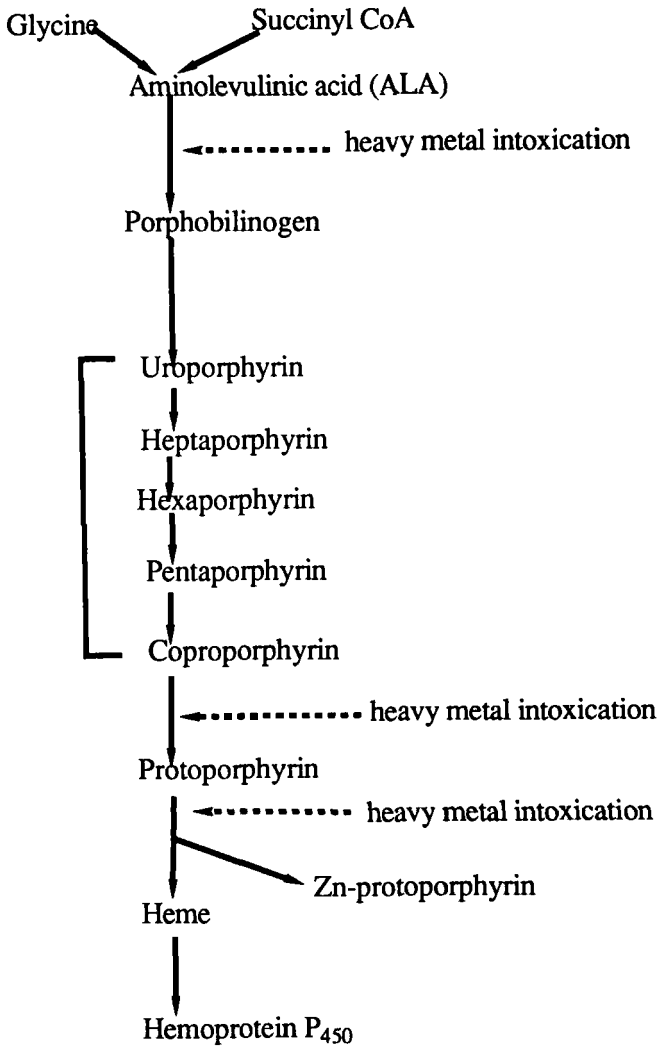


Figure 1 Heme Biosynthetic pathway

biosynthesis have also been described (12-15). However, the previous methods are not without drawbacks.

In the present study, a micro assay for the simultaneous determination of the intermediary metabolism of heme biosynthesis together with Zn-protoporphyrin by high performance liquid chromatography is described . The method is applied to determining the compounds in human blood.

## EXPERIMENTAL



### Materials

Porphyrin acid standards, protoporphyrin IX and Zn-protoporphyrin were purchased from Porphyrin Products, Inc. ( Logan, Utah ). Methanol and tetrahydrofuran (HPLC quality) were purchased from J.T. Baker Chemical Co. ( Phillipsburg, NJ ). All other reagents are of analytical grade.

### Apparatus

The HPLC system consists of a Varian Model 5000 Liquid Chromatograph ( Calif. USA ) equipped with a Rheodyne 7126 injector with a 300-µl sample loop and a Perkin-Elmer Model 650-15 variable wavelength spectrofluorometer ( CT, USA ) with a 12 µl flow cell attachment ). The analytical column ( 3.9 mm x 30 cm ) was a reversed phase µBondapak C18 from Waters Associates ( MA, USA ). A Whatman guard column was connected in line. A Hewlett-Packard 3388A integrator was used for all measurements. The pH measurements were taken on a model 601 digital ionalyzer with a Ross combination pH electrode, both

from Orion Research ( MA, USA ). For the detection of porphyrins the excitation and emission wavelengths were set at 405 nm and 630 nm, respectively.

Preparation of standards

Porphyrin acid standards were dissolved in 1 N HCl. The dissolution was complete with sonication. The Zn-protoporphyrin and protoporphyrin IX standards were dissolved in a minimum amount of pyridine and diluted to desired volumes with methanol. All standard solutions were kept in a refrigerator at 10 °C.



Extraction of erythrocyte porphyrins

An aliquot (250 µl) of acidified methanol ( 1.5 M HCl-methanol, 1:2, v/v ) was added to 40 µl of blood . The extraction was performed with vortex-mixing for 2 min and sonication in a water-bath for 5 min. The mixture was then spun down at 2500 g on a table-top centrifuge for 5 min. An aliquot (150 µl) of the supernatant was used for the analysis of porphyrins by HPLC.

Extraction of erythrocyte Zn-protoporphyrin

Another aliquot (40 µl) of the blood sample was mixed with 250 µl of the extraction solvent containing acetone, pyridine and sterox solution ( 300 : 15 : 15, v/v/v ). The mixture was vortex-mixed for 2 min and the pellet was separated on a table-top centrifuge at 2500 g for 5 min. The supernatant (150 µl) together with the acidic porphyrin extract was simultaneously injected onto the column for the analysis of the intermediate metabolites and Zn-protoporphyrin.

For the recovery experiments, a known amount of each standard was added to the blood sample. The analysis of the metabolites was run in parallel as before.

HPLC determiantions

The reversed-phase HPLC separation of porphyrins was performed isocratically after column pre-treatment with the binary mobile phase ( 0.1 M $NaH_2PO_4$ in methanol , 18 : 30, v/v, pH 5.3 ) for six minutes and followed by simultaneous sample injection and change of solvent to the ternary mobile phase containing 0.1 M $NaH_2PO_4$ in methanol and tetrahydrofuran ( 18 : 30 : 16, v/v/v, pH 5.3 ) to complete the elution. The isocratic elution was run at 1.4 ml/min at ambient temperature.



## RESULTS AND DISCUSSION

The chromatogram of the eight porphyrin standards is shown in Figure 2. The mobile phase composition and pH were studied to produce the optimal resolution between peaks. The solvent selectivity can be increased by varying the phosphate buffer content in the ternary mobile phase depending on the separation requirement and the analysis of an individual porphyrin. However, the separation of the seven porphyrins and Zn-protoporphyrin mainly depends on the pre-treatment of the column with the binary mobile phase. Without the pre-treatment process , there is practically no separation among uro-, hepta-, hexa-, porphyrins. The six-minute pre-treatment was, therefore, carefully determined for the

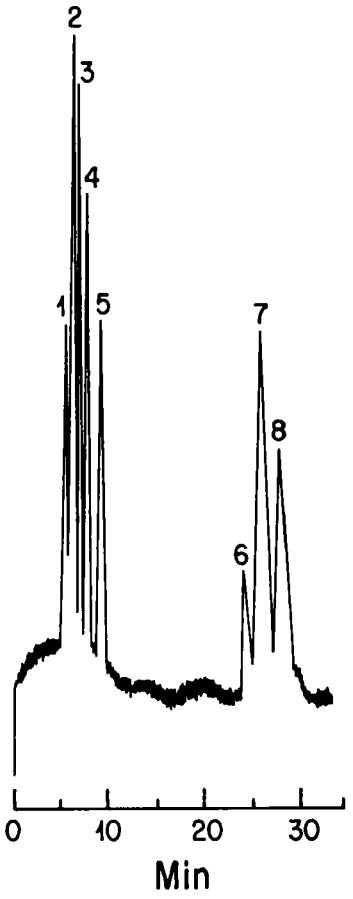


Figure 2 Chromatogram of porphyrin standards. See text for the experimental conditions. Peaks : 1 = uro- (0.02 nmole of peaks 1-6) , 2 = hepta-, 3 = hexa-, 4 = penta-, 5 = copro-, 6 = meso-, 7 = proto-porphyrin (0.03 nmole), 8 = Zn-protoporphyrin ( 0.06 nmole).

separation performance in this study. However, the change of mobile phase did not shift the stability of the baseline.

The quantification of each metabolite in blood was achieved by the addition of mesoporphyrin as an internal standard to enhance the accuracy of the determination. Calibration of the standards was done by measuring the peak height ratio of porphyrins to that of the internal standard. Each calibration line was calculated using the least-squares method. The correlation coefficients of the calibration curves are summarized in Table 1. The peak height ratio was used to determine the porphyrin concentration in blood.

The recovery study (Table 1) suggests that the extraction of erythrocyte porphyrins and Zn-protoporphyrin is quantitative . The extraction efficiency of erythrocyte Zn-protoporphyrin with



TABLE I

Coefficients of Correlation of Peak Height Ratio of the Amount (pmol) of Each Porphyrin and the Recovery Study.

| Porphyrins | Peak Height Ratio | % Recovery* |
|---|---|---|
| Uroporphyrin | 0.97 | 94 |
| Heptaporphyrin | 0.95 | 93 |
| Hexaporphyrin | 0.92 | 93 |
| Pentaporphyrin | 0.92 | 94 |
| Coproporphyrin | 0.96 | 96 |
| Protoporphyrin | 0.97 | 95 |
| Zn-protoporphyrin | 0.85 | 90 |

* Average value from duplicate runs of blood samples spiked with porphyrin standards.

acetone-pyridine-sterox solution proved to be efficient. There was little amount of other porphyrins extracted in the solution. The acid extraction of free erythrocyte porphyrins is complete with acidified methanol. The method is a reliable micro-assay for studying the intermediary metabolism of heme biosynthesis. A two-step extraction procedure was necessary for the simultaneous determination of the intermediary metabolites and Zn-protoporphyrin in blood. The measured protoporphyrin concentration is, however, a measure of the total free protoporphyrin ( protoporphyrin + dissociated Zn-protoporphyrin ) in blood. Therefore, the actual protoporphyrin concentration is the difference between the total free protoporphyrin and Zn-protoporphyrin concentrations. The erythrocyte porphyrin concentrations measured in this study are summarized in Table II. Zn-protoporphyrin , hepta-, hexa-, and penta-porphyrins were not detected . The results are in good agreement with the



TABLE II

Concentrations of Erythrocyte Porphyrins from Normal Individuals.

| | Subjects | | |
|---|---|---|---|
| Porphyrins(μg/dl) | 1 | 2 | 3 |
| Uroporphyrin | 0.16 | ND | ND |
| Heptaporphyrin | ND | ND | ND |
| Hexaporphyrin | ND | ND | ND |
| Pentaporphyrin | ND | ND | ND |
| Coproporphyrin | 1.24 | 1.30 | 0.70 |
| Protoporphyrin | 17.0 | 20.1 | 18.2 |
| Zn-protoporphyrin | ND | ND | ND |

ND : Not Detected.

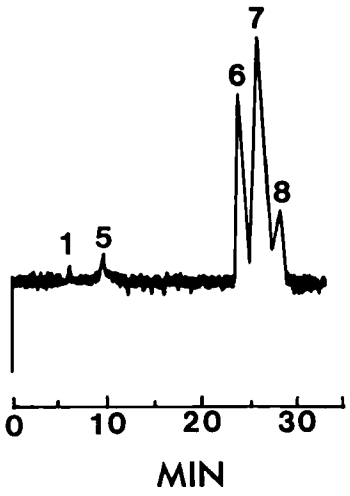


Figure 3 Chromatogram of intermediary metabolites in blood spiked with Zn-protoporphyrin standard. See Figure 2 for the experimental conditions and labels.



previous reports (5-14). Moreover, the results suggest that the method described in this study is very sensitive and efficient for analysing intermediary metabolites in blood. The sensitivity of the method was obtained in the subpicomole range for porphyrins and about 1.0 pmole for Zn-protoporphyrin.

The chromatogram of blood specimen ( Figure 3 ) indicates that there are no interfering metabolites throughout the elution .The precision study of the blood specimen spiked with Zn-protoporphyrin indicated that the concentration of Zn-protoporphyrin decreased noticeably as indicated by the simultaneous elevation of protoporphyrin concentration in the blood specimen after two weeks. However, the concentrations of other intermediate metabolites remained essentially unchanged. Thus , the

stability of Zn-protoporphyrin is the limiting factor for analysing blood specimen . The detection of porphyrins with fluorometric measurement offers better sensitivity and selectivity for quantification of erythrocyte porphyrins in 40 µl of blood. The method is rapid and reliable for clinical testing.

In conclusion, a micro-assay for determining the intermediary metabolism of heme biosynthesis together with Zn-protoporphyrin has been developed. The method can be applied to study heavy metals intoxication and other related disorders, such as iron-deficiency anemia . The method is also useful to study the effects of various chemicals on heme metabolism. In addition, the results suggest that there is a difference in distribution of the diagnostically important porphyrins and Zn-protoporphyrin.



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