Journal of Chromatography, 375 (1986) 57—63
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 2901

DETECTION OF δ-AMINOLEVULINIC ACID, PORPHOBILINOGEN AND PORPHYRINS RELATED TO HEME BIOSYNTHESIS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received July 18th, 1985, revised manuscript received October 3rd, 1985)

SUMMARY

Two new high-performance liquid chromatographic methods are described for the quantitative determination of porphyrins and their precursors In our method, sub-nanomole quantities of porphyrins, δ-aminolevulinic acid and porphobilinogen derivatized with o-phthalaldehyde were injected onto a C_{18} reversed-phase column and eluted with 0 1 *M* monobasic sodium phosphate—methanol—tetrahydrofuran (4 6 3) and detected with a spectrofluorometer A second reversed-phase system using methanol—tetrahydrofuran—22 m*M* acetate buffer (15 6 11) was also developed

INTRODUCTION

Naturally occurring polycarboxylic porphyrins, protoporphyrin and zinc protoporphyrin are intermediate metabolites of heme biosynthesis Two molecules of the precursor δ-aminolevulinic acid (ALA) are condensed with the monopyrrole porphobilinogen (PBG) to form metal-free cyclic tetrapyrroles (porphyrins) which are the immediate precursors of the various hemes, chlorophylls and cobalamines [1] Natural porphyrins of mammalian origin are substituted at the β-positions with methyl, ethyl, vinyl, acetate and propionate groups and occasionally by other closely related substituents Mesoporphyrin differs from protoporphyrin by having two ethyl groups in place of two vinyl groups. Free porphyrins and zinc protoporphyrin when irradiated with ultraviolet light behave similarly and exhibit strong characteristic red fluorescences which are commonly used for detection [2] and are sensitive enough to detect porphyrins at sub-nanomole concentrations. While all porphyrins involved in

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heme biosynthesis can be detected by this fluorometric method, PBG and ALA do not exhibit an appropriate fluorescence The desirability for the simultaneous detection of PBG, ALA and porphyrins prompted the development of a fluorescence method utilizing *o*-phthalaldehyde (OPA) as a derivatizing reagent [3] in the pre-column mode employed for this study

A variety of methods for the quantitation of porphyrins has been reported Several recent papers [4—9] have led to significant contributions They require gradient elution and analyze most of the intermediate metabolites in the heme biosynthetic pathway. The present paper describes two similar reversed-phase high-performance liquid chromatographic (HPLC) methods for the determination of ALA, PBG and porphyrins related to heme biosynthesis by HPLC.

EXPERIMENTAL

Materials

The dihydrochlorides of uroporphyrin I, coproporphyrin I, the penta-, hexa-, and heptamethyl esters of penta-, hexa-, and heptacarboxylporphyrin I, mesoporphyrin IX dihydrochloride, protoporphyrin IX and zinc protoporphyrin IX were purchased from Porphyrins Products (Logan, UT, U S A.) PBG, ALA, OPA and 2-mercaptoethanol were obtained from Sigma (St Louis, MO, U S.A) Absolute ethanol was purchased from Aaper Alcohol and Chemical (Shelbyville, KY, U.S A). Methanol and tetrahydrofuran (HPLC quality) were purchased from J T. Baker (Phillipsburg, NJ, U.S A.) and Burdick & Jackson Labs (Muskegon, MI, U S A), respectively All other chemicals were reagent grade

Instrumentation

Experiments were performed on a Model 5000 liquid chromatograph (Varian) equipped with a Rheodyne 7126 injector fitted with a 20-μl loop. A 10-μm Ultrasphere octadecylsilane (ODS) guard column (Whatman) connected in line with a prepared Partisil-10 ODS analytical column (25.0 × 0 46 cm, particle size 10 μm) (Whatman, NJ, U S A) and a Model SF-330 variable-wavelength spectrofluorometer (Varian) with a 40-μl flow-cell attachment All chromatograms were recorded with a Hewlett-Packard 3388A integrator All pH readings were taken on a Model 601 digital ionalyzer with a Ross combination pH electrode both from Orion Research (Cambridge, MA, U.S.A.)

Preparation of porphyrin and precursor standards

Standard solutions of copro-, meso-, hexa-, hepta-, penta-, proto- and uroporphyrins were dissolved with sonication for about 15 min in concentrated acetic acid Zinc protoporphyrin solution was prepared in ammonium hydroxide solution The stability of these standard solutions was maintained for at least five months by storing in a refrigerator at 10°C However, uroporphyrin and zinc protoporphyrin solutions had to be freshly prepared daily ALA and PBG were dissolved in 0 1 *M* hydrochloric acid solution in separate stoppered test-tubes The PBG solution was stable for a few days Borate buffer solution was prepared by dissolving 0 27 g of boric acid in 10.0 ml of deionized water The pH was adjusted to 9.5 with concentrated sodium hydroxide solution

Preparation of o-phthalaldehyde—2-mercaptoethanol buffered reagents

A total of 27 0 mg OPA was dissolved completely in 500 μl ethanol A 4.5-ml volume of 0.4 *M* sodium borate buffer (pH 9 5) was added to the solution together with 25 μl mercaptoethanol and allowed to stand for 24 h before use [10] Buffered reagents were stored at room temperature since refrigeration caused precipitation of borate buffer salts The stability of the solution was maintained by addition of 20 μl mercaptoethanol every two days

Derivatization of δ-aminolevulinic acid and porphobilinogen

A typical preparation was obtained by reacting 20 μl of each of the standard solutions with 20 μl of OPA—2-mercaptoethanol reagent After exactly 2 min, the reaction was stopped by addition of 20 μl phosphate buffer (0.1 *M* monobasic potassium phosphate, pH 4.0) A 5-μl aliquot of the mixture was injected onto the reversed-phase column with excitation wavelength and emission wavelength of the detector set at 330 and 418 nm, respectively. For the detection of porphyrins wavelengths were monitored at 405 and 630 nm, respectively

HPLC conditions

An aqueous buffer solution was prepared by dissolving 0.1 mol of monobasic potassium phosphate or 22 mmol of sodium acetate in 1 0 l of deionized water. The mobile phase for chromatography of porphyrins was prepared by mixing aliquots of sodium phosphate solution—methanol—tetrahydrofuran (4 6 3) or 22 m*M* acetate—methanol—tetrahydrofuran (11 15 6) The pH of the solution was adjusted with concentrated phosphoric acid or concentrated acetic acid to 5.2, while the mobile phase for elution of ALA and PBG was made with 0 1 *M* phosphate buffer—methanol (7 5) or 22 m*M* acetate buffer—methanol (7·5). The pH of each solution was adjusted to 3.3 with corresponding acids All mobile phases were degassed by aspiration prior to use

Reversed-phase HPLC separations of porphyrins and their precursors were performed with isocratic elution separately and the ten different compounds were chromatographed together with a binary mobile phase either with an acetate buffer or with a phosphate buffer The isocratic elution for separation of ALA, PBG and porphyrins on the C_{18} column was run at a flow-rate of 1 0 ml/min at ambient temperature. The sample volumes injected varied from 5 to 20 μl

RESULTS AND DISCUSSION

Three variables characterizing the mobile phase composition, pH, elution strength and ionic strength, have been thoroughly studied in order to optimize the separation parameters of ALA, PBG and porphyrins related to heme biosynthesis Figs 1 and 2 show the chromatograms of the separation of ALA, PBG and porphyrins with phosphate buffer When 22 m*M* acetate buffer was used in the mobile phase, similar results were obtained (Table I) The resolution between the two precursors is well defined Increasing the pH of the aqueous mobile phase from 3 0 resulted in a gradual decrease of resolution to zero at

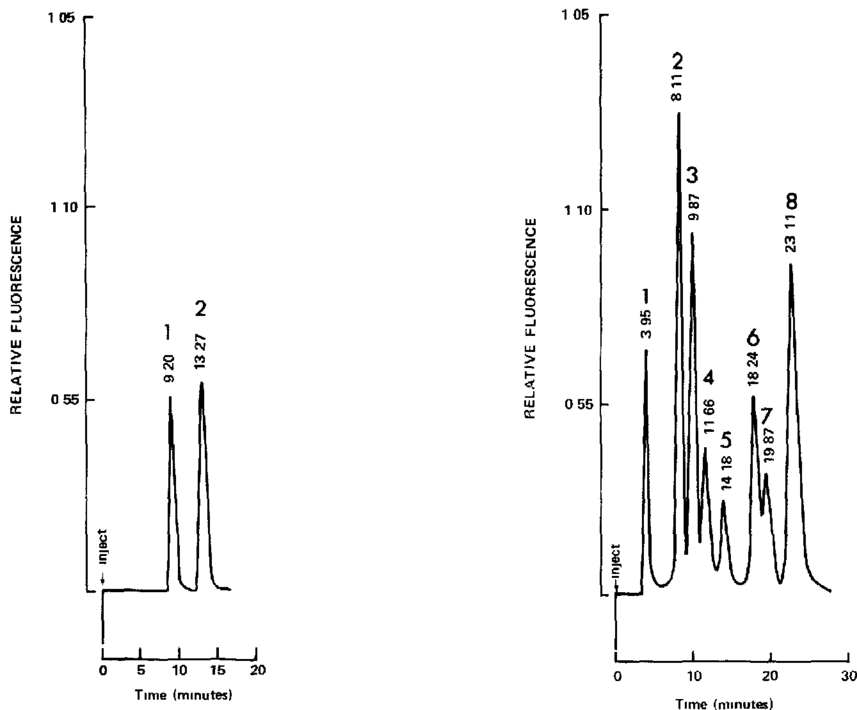

Fig 1 Chromatogram of derivatized δ-aminolevulinic acid (9 8 nmol), and porphobilinogen (0 41 nmol) Eluent methanol—0 1 *M* phosphate buffer (5 7), pH 3 3, flow-rate 1 0 ml/min Peaks 1 = δ-aminolevulinic acid, 2 = porphobilinogen

Fig 2 Chromatogram of porphyrins Eluent 0 1 *M* phosphate—methanol– tetrahydrofuran (4 6 3), flow-rate 1 0 ml/min Peaks 1 = uroporphyrin (0 36 nmol), 2 = coproporphyrin (0 16 nmol), 3 = heptaporphyrin (0 30 nmol), 4 = hexaporphyrin (0 19 nmol), 5 = pentaporphyrin (0 04 nmol), 6 = mesoporphyrin (0 40 nmol), 7 = zinc protoporphyrin (0 77 nmol), 8 = protoporphyrin (0 30 nmol)

pH 7.0. Similarly, an increase of organic component of the mobile phase decreased the resolution. The retention times of the precursors dropped significantly as the percentage of methanol was increased The effect of acetate or phosphate and methanol compositions on separations over a pH range of 3—7 were examined The capacity ratios for the two precursors under these conditions were above 2 0. Apparently, both acetate—methanol and phosphate—methanol have the same selectivity for the precursors The retention times of ALA, PBG and porphyrins obtained with these two systems were summarized in Table I

Fig 2 indicates an efficient elution profile of eight different porphyrins under isocratic conditions It is well understood that the pH and the composition of an eluent can significantly change the capacity ratio of polycarboxylic porphyrins The relative retention of the porphyrins is mainly governed by the number of alkyl groups in the molecule The retention, therefore, increases as the number of side-chain alkyl substituents increases Thus, coproporphyrins (4 COOH, 4 CH_3) were eluted before hepta- (7 $COOCH_3$, CH_3), hexa- (6 $COOCH_3$, 2 CH_3) and penta- (5 $COOCH_3$, 3 CH_3) porphyrins In

TABLE I

RETENTION TIMES OF ALA, PBG AND PORPHYRINS

Compound	Retention time (min)	
	Phosphate buffer*	Acetate buffer**
ALA	9 20	7 97
PBG	13 27	10 09
Uroporphyrin	3 95	3 95
Coproporphyrin	8 11	7 67
Heptaporphyrin	9 87	9 61
Hexaporphyrin	11 66	11 33
Pentaporphyrin	14 18	14 63
Mesoporphyrin	18 24	19 85
Zinc protoporphyrin	19 87	21 60
Protoporphyrin	23 11	25 98

*Eluents 0 1 *M* phosphate—methanol (7 5), pH 3 3, for ALA and PBG, 0 1 *M* phosphate—methanol—tetrahydrofuran (4 6 3), pH 5 2, for porphyrins

**Eluents 22 m*M* acetate–methanol (7 5), pH 3 3, for ALA and PBG, 22 m*M* acetate—methanol—tetrahydrofuran (11 15 6), pH 5 2, for porphyrins

our experiments, the pH was adjusted to about 5.2 to maximize the resolutions between porphyrins and to maintain the capacity ratio of uroporphyrin close to one. Also, the stability of zinc protoporphyrin could be maintained throughout the elution There was incomplete separation of all porphyrins at or near neutral pH. A reasonable resolution between porphyrins was achieved at a pH as low as 3.0. However, the chelating metal from zinc protoporphyrin dissociated to a significant extent and unexpected protoporphyrin was accumulated The elution strength of acetate—methanol—tetrahydrofuran was stronger than the phosphate ternary mobile phase as indicated by the better resolution between mesoporphyrin and zinc protoporphyrin. Ionic strengths as high as 0 1 *M* acetate buffer or as low as 0.08 *M* phosphate buffer did not seem to significantly influence the resolution of porphyrins. However, the separation and the retention times are affected considerably by small volume changes of organic components in the mobile phases. A slight increase in the amount of methanol (as little as 3%) compressed the porphyrin peaks and resulted in poor separation Similarly, effects on retention behavior of the porphyrins were predictable with variation of acetate or phosphate buffer in the mobile phase To facilitate the separation of ALA, PBG and porphyrins related to the heme biosynthesis (ten different intermediate metabolites) in the same samples of blood or urine, a mixture of derivatized precursors and different standard porphyrins were chromatographed simultaneously The results are shown in Figs. 3 and 4 In order to separate and detect the ten standard compounds in a single run, the wavelengths were initially set at 330 nm for excitation and 418 nm for detection of ALA and PBG After exactly 9 min or 12 min of elution with the binary mobile phase acetate—methanol or phosphate—methanol, respectively, the ternary mobile phase (phosphate— or acetate—methanol—tetrahydrofuran) was introduced for the remainder of the run. After the porphobilinogen was eluted the detector wavelengths

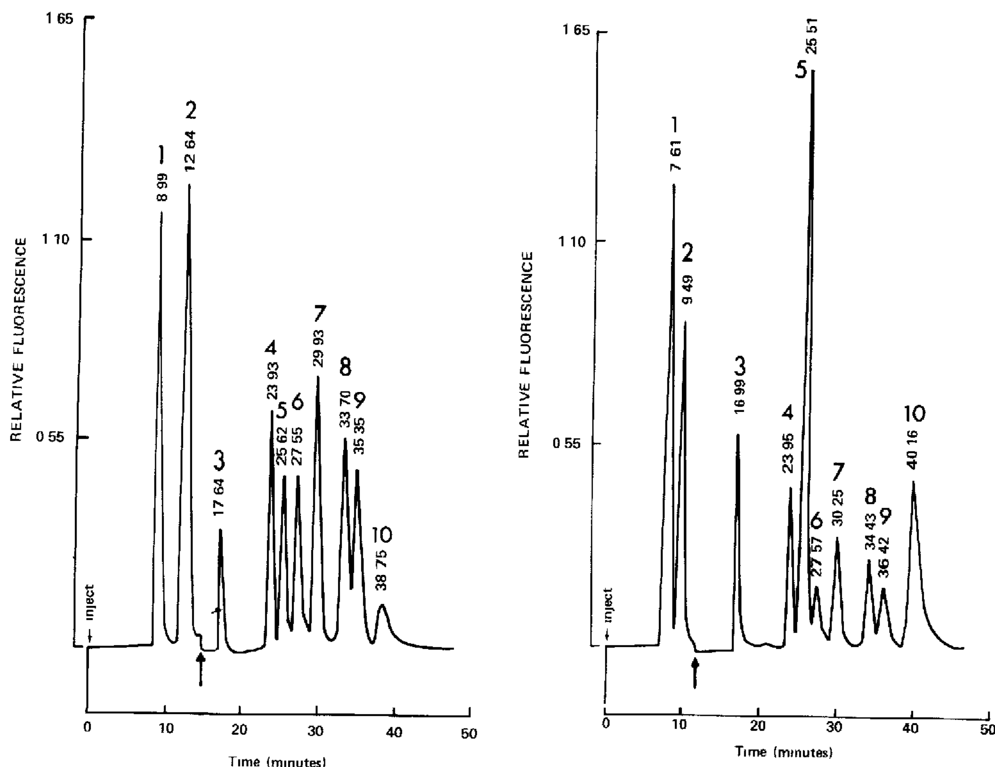

Fig 3 Chromatogram of ALA, PBG and porphyrins Eluent 0 1 *M* phosphate—methanol (7 5), pH 3 3, elution for 12 min, continuous isocratic elution with 0 1 *M* phosphate—methanol—tetrahydrofuran (4 6 3) for the rest of the run, the excitation and emission wavelengths were changed from 330 and 418 nm to 405 and 630 nm after the complete elution of porphobilinogen indicated by the arrow; flow-rate 1 0 ml/min Peaks· 1 = δ-aminolevulinic acid (20 4 nmol), 2 = porphobilinogen (0 84 nmol), 3 = uroporphyrin (0 17 nmol), 4 = coproporphyrin (0.08 nmol), 5 = heptaporphyrin (0 14 nmol), 6 = hexaporphyrin (0 21 nmol), 7 = pentaporphyrin (0 11 nmol), 8 = mesoporphyrin (0 37 nmol), 9 = zinc protoporphyrin (1 04 nmol), 10 = protoporphyrin (0 04 nmol)

Fig 4 Chromatogram of ALA, PBG and porphyrins Eluent 22 m*M* acetate—methanol (7 5), pH 3 3, elution for 9 min, a ternary mobile phase 22 m*M* acetate–methanol—tetrahydrofuran (11 15 6) was used for the rest of the run at a flow-rate of 1 0 ml/min The same wavelength changes as in Fig 3, indicated by the arrow Peaks 1 = δ-aminolevulinic acid (21 9 nmol), 2 = porphobilinogen (0 60 nmol), 3 = uroporphyrin (0 30 nmol), 4 = coproporphyrin (0 05 nmol), 5 = heptaporphyrin (0 46 nmol), 6 = hexaporphyrin (0 08 nmol), 7 = pentaporphyrin (0 04 nmol), 8 = mesoporphyrin (0 16 nmol), 9 = zinc protoporphyrin (0 37 nmol), 10 = protoporphyrin (0 16 nmol)

were monitored with the excitation set at 405 nm and emission at 630 nm. These binary isocratic conditions of elution of ALA and PBG and porphyrins were highly reproducible and it is an excellent method for separating these complex mixtures The application of these procedures to samples of patients with porphyrin-related diseases is underway

ACKNOWLEDGEMENTS

The authors thank Dr Edwin W Naylor for suggesting the problem and for

helpful discussions. This work was supported by two grants, No. MCJ360476020 and project 435 from the Bureau of Community Health Services, Department of Health and Human Services.

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