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Note

Micro assay for urinary δ-aminolevulinic acid and porphobilinogen by high-performance liquid chromatography with pre-column derivatization

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δ-Aminolevulinic acid (ALA) and porphobilinogen (PBG) are the precursors of heme biosynthesis which starts with 8 mol of glycine and 8 mol of succinyl coenzyme A to form the first intermediate acid, ALA. Two molecules of ALA condense to form PBG in the presence of the enzyme ALA dehydratase of which activity is inhibited by heavy metals. Heavy metals have diverse biochemical effects on heme biosynthesis. The determination of accumulated and excreted precursors is, therefore, important to the study of heavy-metal intoxication.

A traditional method for the determination of ALA requires a two-step derivatization [1,2]. ALA is condensed with acetylacetone to form a pyrrole which then reacts with the Ehrlich's reagent (4-N-dimethylaminobenzaldehyde) to form a red complex. The product is measured spectrophotometrically at 550 nm. However, the method is not specific because Ehrlich's reagent can react with other compounds in the excreta, such as pyrroles, indole derivatives and amino ketones [3]. In addition, the sensitivity of the method is a limiting factor. Different improved methods for the determination of ALA, such as anion-exchange chromatography [4–7] and cation-exchange chromatography with post-column derivatization [8], have been reported. Specific methods which involve the assay of ALA using ALA dehydratase and of PBG using PBG de-

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aminase have been reported as well [9,10]. However, these methods are time-consuming.

In the present work, a sensitive micromethod for the simultaneous determination of urinary ALA and PBG by high-performance liquid chromatography (HPLC) is described. The stability of the derivatized products is also discussed.

EXPERIMENTAL

Materials

PBG, ALA, *o*-phthalaldehyde (OPA) and 2-mercaptoethanol were purchased from Sigma (St. Louis, MO, U.S.A.). Methanol (HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Absolute ethanol was purchased from Aaper Alcohol and Chemical (Shelbyville, KY, U.S.A.). All other chemicals were of reagent grade.

Instrumentation

A μBondapak C_{18} column (30 cm $\times$ 0.38 cm I.D., 10 μm average particle size, Waters Assoc., Milford, MA, U.S.A.) was employed for all experiments. A Whatman guard column packed with Ultrasphere octadecylsilane (ODS) was used in-line. The HPLC system consisted of a Varian Model 5000 liquid chromatograph (Palo Alto, CA, U.S.A.) equipped with a Rheodyne 7126 injector fitted with a 50-μl sample loop together with a variable-wavelength spectrofluorimeter (Varian, Model SF-330) with a 40-μl flow-cell attachment. Chromatograms were recorded with a Hewlett-Packard 3388A integrator. The pH measurements were taken on a Model 601 digital ionalyzer with a Ross combination pH electrode, both from Orion Research (Cambridge, MA, U.S.A.).

Derivatization of ALA and PBG

The buffered solution of OPA–2-mercaptoethanol reagent was prepared 24 h prior to use according to the procedure described earlier [11].

A typical preparation of derivatized standards was carried out by mixing 10 μl each of ALA (3.9 μmol/ml) and PBG (0.41 μmol/ml) solutions with 20 μl of OPA–2-mercaptoethanol buffered solution. After exactly 2 min, the reaction was stopped by addition of 20 μl phosphate buffer (0.1 *M* potassium phosphate monobasic, pH 4.0). An aliquot (5 μl) of the solution was injected onto the HPLC column for analysis.

Likewise, an aliquot (30 μl) of urine sample was derivatized with an equal volume of OPA reagent solution as above. The reaction was stopped by addition of 20 μl phosphate buffer. An aliquot (50 μl) of the solution was injected onto the HPLC system for the analysis of ALA and PBG.

The percentage recovery of each precursor was studied with another aliquot (30 μl) of the urine sample spiked with 10 μl of ALA and PBG standard solu-

tions. The spiked urine sample was treated exactly the same way as described above before analysis by HPLC.

Chromatography

The mobile phase for the separation of derivatized ALA and PBG was 0.1 *M* phosphate buffer–methanol (65:55, v/v, pH 5.0). The mobile phase was degassed by aspiration prior to use. The chromatography of derivatized ALA and PBG was carried out using isocratic elution at a flow-rate of 1.0 ml/min at ambient temperature. The wavelengths of the detector were set at 330 and 418 nm for excitation and emission, respectively.

RESULTS AND DISCUSSION

The isocratic separation of the precursor standards is shown in Fig. 1A. The baseline resolution between the derivatized ALA and PBG is well defined. Although the separation took about 15 min to complete, the solvent strength could be adjusted to change the overall retention time by either increasing or decreasing the volume percentage of methanol in the binary mobile phase depending on the matrix interferences and the separation requirement. The baseline resolution between ALA and PBG could, however, be maintained when the methanol content was as high as 65% (v/v). OPA reacts, in the presence of 2-mercaptoethanol, with primary amines and α-amino acids to form fluorescent adducts, thio-substituted isoindoles [12–14]. ALA and PBG also bear the primary amine groups and, therefore, react to form the expected N-substituted isoindoles as shown in Fig. 2. As a result, there are interfering amino acids coeluting with ALA and PBG. However, the elution strength of the bi-

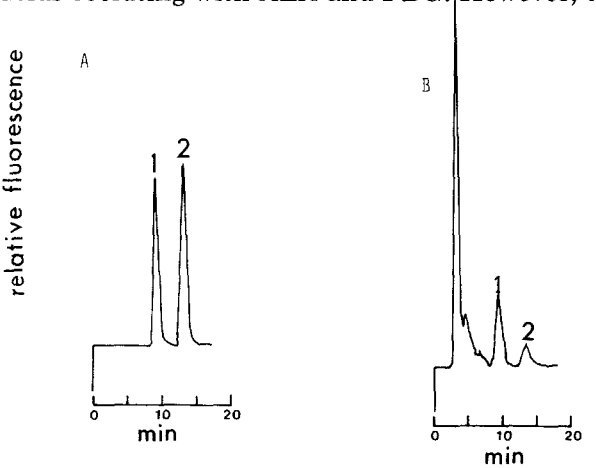

Fig. 1. (A) Chromatogram of derivatized ALA and PBG standards. (B) Chromatogram of urinary ALA and PBG from a normal individual. Eluent: 0.1 *M* phosphate-methanol (65:55, v/v, pH 5.0). See text for detailed experimental conditions. Peaks: 1 = ALA; 2 = PBG.

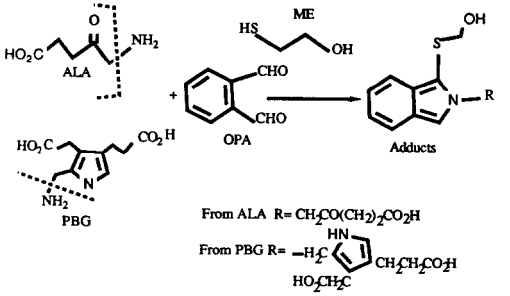

Fig. 2. Derivatization reaction of amines with OPA in the presence of 2-mercaptoethanol.

TABLE I

COEFFICIENTS OF THE CALIBRATION GRAPHS AND THE RECOVERY STUDY

Precursors	Peak-height ratio	Peak-area ratio	Recovery[a] (%)
ALA	0.98	0.95	97
PBG	0.92	0.88	95

[a]Average value from duplicate run of urine sample spiked with ALA and PBG standards.

nary mobile phase was adjusted to clearly separate the ALA and PBG derivatives from interfering products derived from other amines in urine (Fig. 1B). The clean separation was also enhanced by setting the excitation and emission wavelengths at 330 and 418 nm, respectively. Most of the derivatized amino acids were eluted at the beginning of the run. There were no observable peaks after the complete elution of PBG despite the continuous elution for about 40 min.

The quantification of the urinary ALA and PBG was done by plotting the peak height versus the amounts (pmol) of each standard. Calibration factors for the standards were calculated by the detector response factor which was determined as the ratio of either the peak area or the peak height to the calibrated amount of standards. Each calibration line was calculated using the least-squares method. The results are summarized in Table I. The results suggest that peak-height ratio produces better accuracy.

The percentage recovery for the precursors was determined as shown in Table I. The results show that the determination of ALA and PBG is accurate. Although the urine sample was directly derivatized with OPA solution without prior treatment, the determination of urinary ALA and PBG by the HPLC method developed in this study is simple and direct without the matrix interferences. However, day-to-day analysis of the urine sample indicated that the concentrations of ALA and PBG decreased gradually due to their instability in urine especially under acidic conditions. Nevertheless, the stability of uri-

nary ALA and PBG can be maintained by neutralizing the urine sample before storing it at −80°C. In addition, the stability of the ALA and PBG derivatives was examined over a period of time (Fig. 3). The results indicate that the fluorescence intensity of the derivatized ALA and PBG decreases noticeably after about 15 min. The decrease in intensity is more significant for PBG. Nonetheless, the instability of the fluorescent adducts did not affect the accuracy of the determination of urinary ALA and PBG in this study. A modified procedure [15] is available for preparing the stable fluorescent products. However, the method requires high temperature (97°C) and takes 3 h to complete. It is not, therefore, an efficient method. Even though the excess OPA reacts with 2-mercaptoethanol to form hemimercaptal [16], the amount of the by-product is insignificant and does not affect the separation of the major adducts. In addition, hemimercaptal does not exhibit the same fluorescence at the excitation and emission wavelengths as the derivatized ALA and PBG.

The method for determining urinary ALA and PBG was applied to five normal individuals. The results are summarized in Table II. Although sporadic urine samples were used in this study, the results correlate with values for

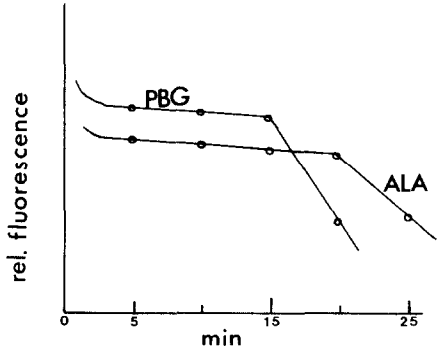

Fig. 3. Time study of the stability of the fluorescent adducts of ALA and PBG.

TABLE II

URINARY ALA AND PBG LEVELS FROM FIVE NORMAL INDIVIDUALS

Subject No.	Concentration (mg/l)	
	ALA	PBG
1	12.5	0.64
2	7.2	Not detected
3	0.9	Not detected
4	10.2	0.2
5	5.5	Not detected

normal individuals as reported previously [1,2,4]. However, for a more accurate determination, a 24-h urine sample is recommended.

In conclusion, a micro assay for urinary ALA and PBG by HPLC has been developed. The method is sensitive and accurate for determining ALA and PBG in fresh and stored urine samples. The high intensity of the fluorescent adducts produces a detection limit in a fraction of picomole. The simplicity of sample preparation coupled with the high sensitivity of the method allows a broad application of the technique to simultaneously analyse ALA and PBG in different biological materials. The quantification of accumulated ALA and PBG in urine is important in detecting abnormalities of heme biosynthesis and can be useful to the study of heavy metals intoxication.

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