Direct Injection Method for Quantitation of δ-Aminolevulinic Acid in Urine by High-Performance Liquid Chromatography

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A highly sensitive and simple method for determining δ-aminolevulinic acid (ALA) in urine was established, using direct injection of urine into a high-performance liquid chromatographic column, with fluorometric detection after post-column derivatization with *o*-phthalaldehyde (OPA). The recovery of ALA was about 100% and ALA was completely separated on an ion exchange column (retention time, 38 min). The detection limit for ALA was 10 pmol ($S/N=2$). The mean levels of urinary ALA of 10 healthy volunteers, 4 patients with acute intermittent porphyria, and 2 workers occupationally exposed to lead were 0.76, 5.25, and 23.54 mg/l, respectively.

Because of its simplicity, the method is considered to be suitable for routine analysis of urinary ALA in the clinical laboratory.

Keywords δ-aminolevulinic acid; high-performance liquid chromatography; lead worker; acute intermittent porphyria; heme biosynthesis; urine

Introduction

δ-Aminolevulinic acid (ALA), the first intermediate in the pathway for heme biosynthesis, is elevated in some diseases such as acute porphyrias, hereditary tyrosinemia, iron deficiency anemia, and lead poisoning.[1] The concentration of ALA in urine has been widely used as an indicator of the biological effects of lead poisoning. Therefore, quantitative urinary ALA determination is important both in clinical diagnosis and in research on heme biosynthesis.

Urinary ALA has generally been determined by a colorimetric method based on the color reaction of ALA-pyrrole with Ehrlich's reagent,[2–4] but this method is not suitable for a routine procedure. While several high-performance liquid chromatographic (HPLC) methods for the quantitation of ALA in urine have recently been reported,[5,6] none of them allows determination of ALA by direct injection of urine into the HPLC column. In the present study, we used a direct injection of urine to the HPLC column without application of any pretreatment in order to minimize the period required and simultaneously improve the accuracy of the method.

Results

A typical chromatogram of standard ALA is shown in Fig. 2; the retention time of ALA was 38 min. Eleven consecutive injections of 20 μl of standard ALA gave good reproducibility, with a mean peak height (±S.D.) of 77.5±2.05 mm. The coefficient of variation (CV) was 2.65%. The recovery rate of ALA from normal human urine containing 0.5 nmol of ALA per 50 μl was 103±3.3% (mean±S.D.). The detection limit of the assay method at an S/N ratio of 2 was 10 pmol.

The linearity of the assay with standard ALA was examined both in terms of peak area and peak height. A linear relationship passing through the origin was found between peak area and concentration up to 60 nmol. Likewise, such a relationship passing through the origin was also observed between peak height and concentration up to 5 nmol (this range was chosen in reference to the scale of the chart paper), as represented in Fig. 3.

Chromatograms of urinary ALA obtained from 10 healthy volunteers, 4 acute intermittent porphyria (AIP) patients and 2 workers occupationally exposed to lead are shown in Fig. 4. The mean values of ALA in urine were 0.76, 5.25 and 23.54 (Table II), respectively.

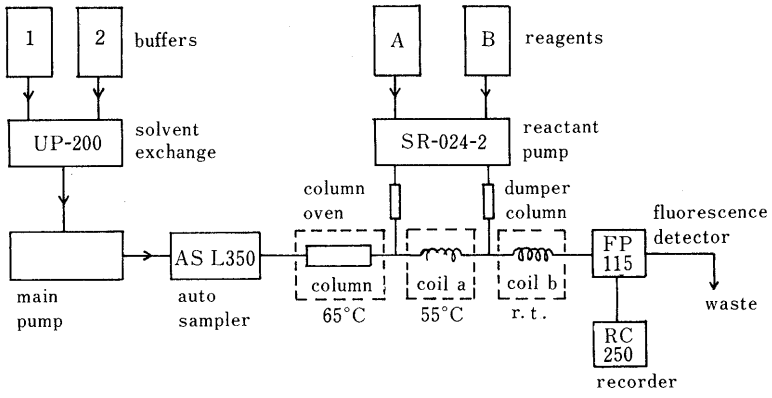

Fig. 1. Arrangement and Flow Diagram of the Continuously Operating System

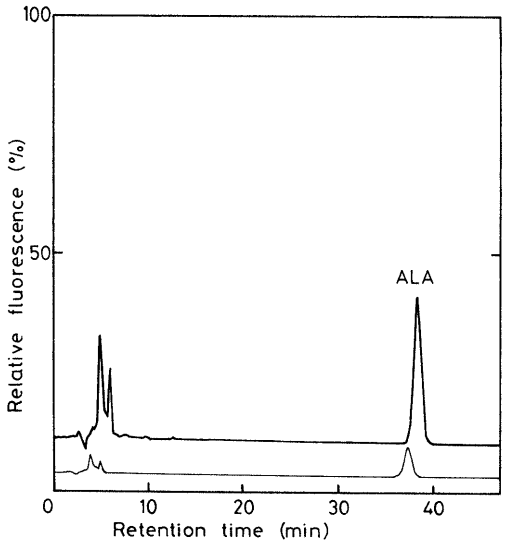

Fig. 2. Typical High-Performance Liquid Chromatogram of Standard ALA

Standard sample volume injected was 20 μl (1 nmol).

TABLE I. Analytical Conditions for HPLC Measurement of Urinary ALA

Chromatograph	Trirotar-V (JASCO)
Column	AA-pak Li (JASCO) 100 mm × 6.0 mm i.d., 5 μm particle size strongly acidic (sulfonated type) cation exchange resin (Li type)
Column temperature	65 °C
Detector	Fluorescence HPLC monitor (JASCO FP-115) Excitation wavelength 350 nm Emission wavelength 440 nm
Mobile phase	I: 0.1 N Li-citrate (pH 4.90) II: 0.3 N LiOH (for regeneration)
Flow rate of mobile phase	0.6 ml/min
Reagent pump	SP-024 (JASCO)
Reaction reagents	A: Sodium hypochloryte solution (0.1 mg/l)/0.4 M potassium borate buffer, pH 10.5 B: OPA/0.4 M potassium borate buffer, pH 10.5 (1.6 mg OPA/1)
Reaction temperature	A: 55 °C, B: room temperature
Reaction tube	A: 0.5 mm i.d. × 1 m stainless tube B: 0.5 mm i.d. × 2 m stainless tube
Flow rate of reagents	0.4 ml/min (each)

TABLE II. ALA in Porphyrinuria

Patient	Diagnosis	Sex	ALA (mg/l)
1	AIP	F	4.32
2	AIP	F	7.60
3	AIP	M	5.58
4	AIP	F	3.48
5	Lead poisoning	M	32.25
6	Lead poisoning	M	14.83
Control ($n=10$)	Mean ± S.D.		0.76 ± 0.48
	Range		0.18 − 1.76

The mean values of ALA in urine from 10 subjects (4 healthy volunteers, 4 AIP patients and 2 workers exposed to lead) obtained by the colorimetric method[3] and by our present method were 9.74 ± 11.34 and 7.91 ± 10.12 (mean ± S.D.), respectively, and the correlation coefficient, r, was 0.979 (Fig. 5).

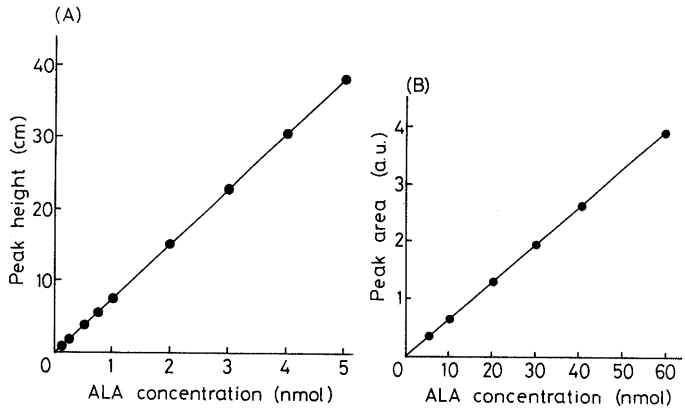

Fig. 3. Relationship between the Amount of ALA Injected and Height (A), and Area (B) of the Detected Fluorescent Peak

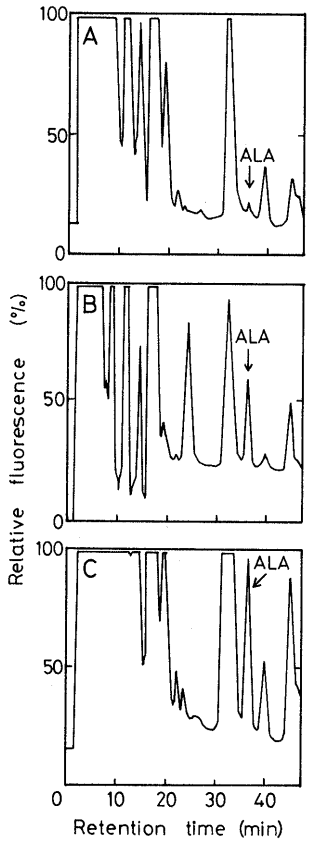

Fig. 4. Chromatogram of Urinary ALA

A, urine of healthy volunteer. B, urine of a patient with acute intermittent porphyria. C, urine of a worker occupationally exposed to lead. Urine sample volumes were 50 μl (A), 20 μl (B), and 20 μl (C). The estimated urinary concentrations of ALA in A, B, and C were 0.45, 7.60, and 14.83 mg/l, respectively.

Discussion

A direct injection method for the determination of ALA in urine has been developed. The experimental procedure does not entail pretreatment of urine, affording an approximately 100% recovery, and permits accurate measurement of ALA in urine. The most commonly used means of quantifying of ALA is colorimetry after column

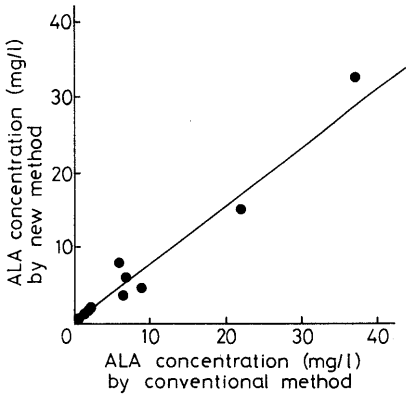

Fig. 5. Comparison of Results Obtained by the Colorimetric Method[3] and the HPLC Method for Determining ALA in Urine Specimens Obtained from 4 Healthy Volunteers, 4 AIP Patients and 2 Workers Occupationally Exposed to Lead

chromatography, and values of urinary ALA concentration obtained by our new method were lower, especially in normal value, [3-7] than those found with this conventional method. This difference is considered to be due to the formation of several of Ehrlich's positive substances besides the free ALA pyrrole in the conventional method, which is converted from ALA by the chemical condensation reaction used.

Experimental

Chemicals Most of the chemicals used were of the highest grade commercially available, and were used without further purification. *o*-Phthalaldehyde (OPA) and 2-mercaptoethanol were purchased from E. Merck. δ-ALA was from Daiichi Pure Chemical Company, Tokyo. Other chemicals were from Wako Pure Chemicals Ltd., Tokyo.

Urine Specimens Urine samples used in this study were collected from 10 healthy volunteers, from 4 patients with AIP, and from 2 workers occupationally exposed to lead. The urine samples were stored in the dark at $-20\,^{\circ}C$ for no longer than one week before analysis.

Apparatus The flow system is illustrated schematically in Fig. 1. A microcomputer-controlled HPLC (JASCO Trirotar-V) equipped with a JASCO AApak-Li column (100 mm × 60 mm i.d.) packed with 5 μm ion exchange resin, was used. An additional reciprocal pump (JASCO, SP-024-2) with two plunger units, each of which could be operated separately, was used to deliver NaClO and OPA reagents into the eluate through a dumper column (44—88 μm glass beads, 2.0 × 250 mm). A fluorometric detector (JASCO, FP-115 filter type) was used with excitation at 350 nm and emission at 440 nm.

Procedure HPLC analysis was carried out under the analytical conditions shown in Table I. Urine samples (20—50 μl) were directly injected. The results were compared with those obtained by the colorimetric method of Kondo *et al.*[7] Results obtained with this conventional method have been shown to correlate well with those measured by the method of Urata and Granick.[3]

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