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Note

Fluorimetric determination of urinary δ-aminolevulinic acid by high-performance liquid chromatography and post-column derivatization

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Lead has diverse biochemical effects, all of which are deleterious. In haem biosynthesis, δ-aminolevulinic acid dehydratase (ALA-D, EC 4.2.1.24), which converts δ-aminolevulinic acid (ALA) into porphobilinogen, is markedly inhibited by exposure to lead, following which a large amount of ALA is excreted in the urine [1]. Thus, urinary excretion of ALA has been widely used as an indicator of lead exposure, with the determination based on ion-exchange column chromatographic methods [2–5]. However, these methods are time-consuming, and the interference of other substances in urine is not negligible [4]. Recently, some reports have appeared on the determination of ALA by high-performance liquid chromatography (HPLC). An octadecyl silica (ODS) column was used in the fluorimetric determination of an ALA derivative by *o*-phthalaldehyde (OPA) in a standard mixture [3]. However, the fluorescent intensity of the ALA derivative was not sufficient for the measurement of the urinary level of ALA. Witting et al. [4] reported a colorimetric method for the determination of ALA using post-column derivatization, and they observed lower values for urinary levels of ALA than those obtained by the conventional method. However, the concentrations of urinary ALA from normal controls are sometimes below their detection limit (unpublished data). In an earlier study, we found a highly fluorescent derivative of ALA, and reported the fluorimetric determination of ALA using pre-column derivatization of ALA with formaldehyde and acetylacetone [6,7].

In the study reported here, we improved our fluorimetric determination of urinary ALA by post-column derivatization. With simple sample preparation, we were able to measure various concentrations of ALA in urine samples.

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EXPERIMENTAL

Chemicals

δ-Aminolevulinic acid hydrochloride was purchased from Sigma (St. Louis, MO, U.S.A.). Acetylacetone, formalin, acetic acid, ethanol, sodium dihydrogenphosphate dihydrate, disodium hydrogenphosphate dodecahydrate and trichloroacetic acid were from Katayama Chemicals (Osaka, Japan). Other chemicals used were of the highest quality available. The water used was deionized and purified by a Puric-R system (Organo, Tokyo, Japan).

Standard and urine samples

A standard solution of ALA (50 μg/ml) was prepared by dissolving 6.4 mg of δ-aminolevulinic acid hydrochloride in 100 ml of 1% acetic acid solution (v/v) and stored at 4°C. Urine samples from normal volunteers and workers exposed to lead in a factory making lead compounds were collected and stored at −80°C until analysis.

Mobile phase and reaction mixtures

The following solutions were used: (A) 0.01 *M* sodium phosphate buffer (pH 3.0); (B) 0.01 *M* disodium hydrogenphosphate in 0.05 *M* sodium hydroxide; (C) 18.5% formaldehyde solution in 2 *M* acetic acid; (D) water–ethanol–acetylacetone (1:1:2, v/v).

Chromatographic procedure

Experiments were conducted with a Model LC-6A liquid chromatograph (Shimadzu, Kyoto, Japan) (Fig. 1). Urine samples (1 ml) were mixed with 0.5 ml of 10% trichloroacetic acid and centrifuged for 5 min at 12 000 *g*. An aliquot of the resultant supernatant (10 μl) was injected onto a cation-exchange column (TSK-GEL SCX, 150 mm×6 mm I.D., 5-μm particles, Toyo Soda, Tokyo, Japan) with an autosampler (Model SCL-6A, Shimadzu). A stepwise gradient elution (Fig. 2) was carried out by mixing mobile phases A and B with a system controller (Model SIL-6A, Shimadzu) and constant-flow pumps A and B (Model LC-6A, Shimadzu) at a total flow-rate of 1.0 ml/min. The column eluate was mixed with solvent C at a flow-rate of 0.1 ml/min using pump C and then led into a reaction coil (10 m×0.25 mm I.D.) at 95°C and mixed with solvent D at a flow-rate of 0.4 ml/min with mixing in the tubing (20 m×0.4 mm I.D.) at 95°C. The fluorescence intensity was measured at 473 nm with excitation at 363 nm in a spectrofluorometer (Model RF-530, Shimadzu) equipped with a 12-μl flow-cell and a data processor (Model CR-3A, Shimadzu). The concentration of ALA was calculated from the peak area.

RESULTS AND DISCUSSION

A typical chromatogram of a 10-μl ALA standard solution (0.5 mg/l) is shown in Fig. 2. The standard mixture was injected onto a column 2 min after the gradient programme was started, and ALA was eluted at 17.6 min. One analysis could

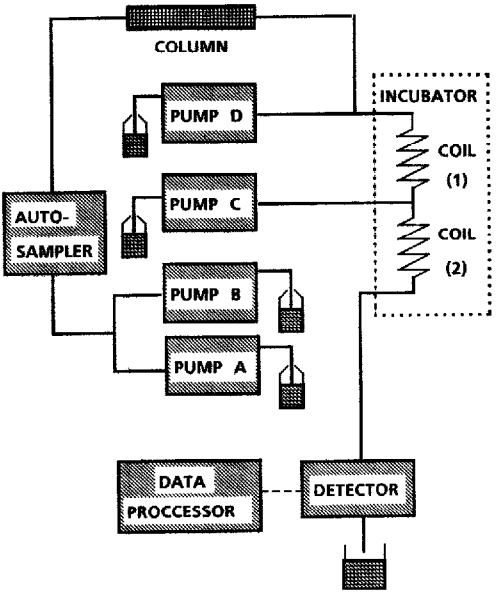

Fig. 1. Schematic diagram of the post-column reaction detection system.

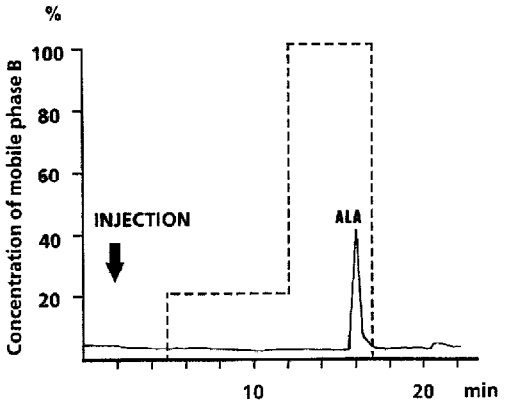

Fig. 2. Schematic diagram of the stepwise elution programme (dashed line) and a chromatogram of 10 μl of ALA standard solution (0.5 mg/l). The standard was injected after 2 min and ALA was eluted at 17.6 min. For other chromatographic conditions, see Experimental.

be completed within 22 min. A good linear correlation was obtained between the elution profiles and concentrations of ALA at various concentrations, and then amounts of ALA injected in the range 0.25–50 mg/l were linearly related to the fluorescent intensities expressed as peak areas. The linear regression equation was $y=x+1.9\cdot10^{-4}$ with a correlation coefficient (r) of 0.9999. The within-day and between-day coefficients of variation were 1.2% ($n=10$) and 2.5% ($n=2$ for two days) for 200 ng of ALA. The detection limit for injected ALA was 100 pg at a signal-to-noise ratio of 5. A typical elution pattern for a urine sample from a normal control is shown in Fig. 3. The peak of ALA (0.62 mg/l) was separated

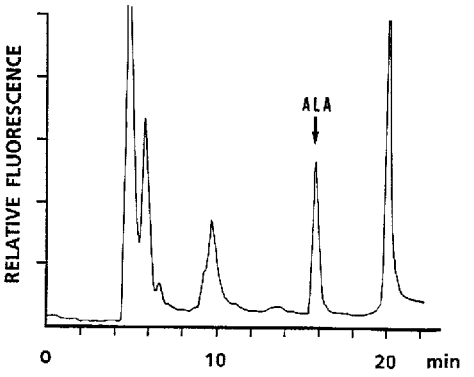

Fig. 3. Chromatogram of 10 μl of a urine sample from a normal control (0.62 mg/l). ALA is eluted at 17.6 min. Chromatographic conditions are given in the text.

TABLE I

RECOVERIES FOR VARIOUS CONCENTRATIONS OF URINARY ALA

Samples 1–5 and 6–12 are from workers exposed to lead and from normal controls, respectively.

Urine sample No.	Concentration (mg/l)			Recovery (%)
	Urine	Standard (added)	Found	
1	6.48	17.2	24.77	105.9
2	7.61	17.2	24.07	95.5
3	8.97	17.2	25.57	96.1
4	21.09	17.2	39.67	107.6
5	21.93	17.2	39.49	101.8
6	0.62	1.72	2.38	102.1
7	0.53	1.72	2.13	92.4
8	1.17	1.72	2.84	96.6
9	0.87	1.72	2.53	96.1
10	0.41	0.30	0.75	112.0
11	0.24	0.30	0.52	91.9
12	0.30	0.30	0.62	104.9

from other peaks. The overall recoveries of ALA added to various urine samples are shown in Table I. For the assays, 0.5-ml urine samples were mixed with 0.5 ml of the standard mixture and with 0.5 ml of water. Recoveries were calculated by comprising the peak areas of the spiked samples with those of the standards. The mean recovery was found to be $100.2 \pm 6.4\%$.

Our method allows the processing of more than seventy samples in one day with an autosampler. Good recoveries were obtained for various concentrations of ALA in urine samples. A detection limit of 100 pg is sufficient to monitor urine levels of ALA by injecting only 10 μl of the sample solution.

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