Optimized Liquid-Chromatographic Method for Fluorometric Determination of Urinary δ-Aminolevulinic Acid in Workers Exposed to Lead

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We developed a fluorometric method for determining δ-aminoolevulinic acid (ALA) in urine of lead workers. A high-performance liquid chromatograph (HPLC) equipped with a fluorescence HPLC monitor is used. The detection limit for aqueous ALA is 20 μg/L (15 pmol of ALA in the 100-μL sample). The working linear range of urinary ALA concentration was 0.1 to 100 mg/L. In 25 lead-exposed workers, ALA values by the present method for urine correlated well with those obtained by a conventional colorimetric method (r = 0.996). The advantage of the present method for microdetermination of urinary ALA is its high sensitivity.

Additional Keyphrases: monitoring toxicity · environmental hazards · colorimetry compared

The concentration of δ-aminoolevulinic acid (ALA) in urine has been widely used as a measure of the biological effect of lead, in workers occupationally exposed to lead. Generally, urinary ALA has been determined by colorimetric methods based on the color reaction of ALA-pyrrrole with Ehrlich's reagent (1-5). Although these conventional methods are useful in large-scale routine analysis, the analytical sensitivity is low. Recently, fluorometry of ALA after "high-performance" liquid chromatography (HPLC) has been reported by several investigators (6-10). These methods are much more sensitive than the colorimetric methods. We re-examined comparatively the procedures for fluorescence-producing derivatization for ALA used in these methods. We found that the derivatization with acetylacetone (2,4-pentanedione) and formaldehyde (application of the Hantzsch reaction) used by Okayama et al. (10) was most convenient for fluorometric HPLC analysis for ALA in urine. Here we describe our study of the HPLC procedure of Okayama et al. (10) with regard to analytical conditions. Our findings led to modifications in the procedure, which are described here.

Materials and Methods

Urine specimens. Untimed urine specimens to be tested for lead exposure were obtained from 25 men whose occupational exposure to lead had lasted one to 23 years. Their blood lead concentrations ranged from 200 to 960 μg/L.

Apparatus. We used a Model LC-6A HPLC equipped with a fluorescence HPLC monitor (RF-535) and a data processor (Chromatopac C-R3A), all from Shimadzu, Ltd., Kyoto, Japan. For comparison with the conventional method, we also used a Model 100-40 spectrophotometer (Hitachi, Ltd., Tokyo, Japan). For heating the test tubes, we used an aluminum block (107 mm wide, 107 mm long, 80 mm high, with 25 holes, each 70 mm deep and 16.5 mm in diameter) on a hot plate (type TAH-1; Taiyo Scientific Industrial Co., LTD., Tokyo, Japan). We recorded the fluorescence spectrum of the ALA derivative with a Model RF-503 recording spectrofluorophotometer (Shimadzu). For blood lead analysis, we used a Model AA-646 flameless atomic absorption spectrophotometer equipped with a deuterium background corrector (Shimadzu).

Reagents. All chemicals were of analytical grade. ALA hydrochloride was purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were obtained from Wako Pure Chemicals, Osaka, Japan. Methanol for the mobile phase was chromatographic grade. A formaldehyde solution (100 g/L), prepared by 3.7-fold dilution of the chemical reagent (370 g/L) with distilled water, was stored in the dark.

Artificial urine. Artificial urine was prepared by dissolving 11.6 g of sodium chloride, 2 g of dibasic ammonium phosphate, and 15 g of urea in distilled water and diluting to 1 L.

Procedure. Dilute the urine specimen fivefold with distilled water to keep the matrix interference to a minimum.

To prepare the fluorescent derivative of ALA, mix 0.1 mL of this sample, 2.5 mL of dilute (20 mL/L) acetic acid, 0.4 mL of acetylacetone, and 1 mL of the formaldehyde solution with a vibrator mixer for about 5 s, and heat this mixture for 10 min at 100 °C on the aluminum block. Then quickly place the test tube in an ice-cold bath (0 °C) until analysis. Use a 1 or 5 mg/L aqueous solution of ALA as the working standard.

Perform the HPLC analysis under the analytical conditions shown in Table 1. Calculate the area of the ALA peak on the chromatogram by using the data processor (Chromatopac).

Our comparison method was the colorimetric method of Tomokuni and Ogata (5), which is widely used in Japan. Results obtained with this conventional method correlate well with those measured by the original method of Mauzerall and Granick (1).

Results and Discussion

Fluorescence spectrum of ALA derivative. The excitation maximum for the ALA derivative is at 375 nm, the emission maximum at 465 nm (Figure 1). The Hantzsch reaction, which involves one molecule each of formaldehyde and an amine and two molecules of acetylacetone, is useful for fluorometry of amino acids and related compounds. It was

| Table 1. Analytical Conditions for HPLC Measurement of Urinary ALA |
|------------------------|------------------|
| Column                 | Shim-pack CLC-ODS (Shimadzu) 150 × 8.0 mm, 5-μm particle size Reversed-phase column |
| Detector               | Fluorescence HPLC monitor (Shimadzu RF-535) Excitation wavelength 370 nm Emission wavelength 460 nm Range × 4, Sensitivity high |
| Mobile                 | Methanol/water/glacial acetic acid, 600/400/10 (by vol) |
| Flow rate              | 1.0 mL/min |
| Column                 | Ambient (18–21 °C) |
| temp.                  | 5 mm/min |
| Injection vol.         | 20 μL |

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first applied to the fluorometry of glycine and other amino acids (11).

Chromatography of urinary ALA. Figure 2 shows chromatograms of a 1 mg/L ALA standard and of urinary ALA from two lead workers as measured by the present method.

![Chromatograms](image)

**Fig. 1.** Fluorescence stability of ALA derivative: ○○, in an ice bath (0°C); ●●, at room temperature (18–21°C)

**Fig. 2.** Chromatograms of urinary ALA: A, from a ALA standard (1 mg/L in water); B, from a lead worker's urine (ALA 3.8 mg/L); C, from a lead worker's urine (ALA 18.3 mg/L).
The urine was diluted fivefold with distilled water before reaction.

Excitation/emission wavelengths of the detector were set at 370/460 nm. The chromatogram for the 1 mg/L ALA standard had two peaks (retention times 5.3 and 6.1 min, respectively), the areas of which were correlated with the concentration of aqueous ALA. We used the larger, later-eluting peak for routine analysis of urinary ALA. As Figure 2 (B, C) shows, urinary ALA was completely separated from other fluorescent substances in urine.

**Effect of heating time on fluorescence intensity.** When the reaction mixture was heated for 15 min at 100°C, the fluorescence intensity of ALA derivative reached a maximum, decreasing rapidly after further heating (Figure 3, left). Therefore, we routinely used 10 min as the optimum heating time.

**Effect of volume of acetylacetone added on fluorescence intensity.** When different volumes of acetylacetone were used in the reaction mixture (total reaction volume, 4 mL), the fluorescence of the ALA derivative increased gradually after being heated at 100°C (Figure 3, middle). Although the fluorescence was strongest when 0.6 mL of acetylacetone was added, this condition was unsuitable because the mixture separated into two layers after the reaction. We used 0.4 mL of acetylacetone in the present method.

**Effect of concentration of formaldehyde on fluorescence intensity.** Figure 3, (right) shows the relationship between the concentration of formaldehyde in the reaction mixture and the intensity of fluorescent ALA derivative. The optimum final concentration of formaldehyde was 25 g/L; this corresponds to using 1 mL of formaldehyde solution in the 4-mL reaction mixture.

**Fluorescence stability of ALA derivative.** Figure 4 shows the fluorescence stability of ALA derivative. When the test tube containing ALA derivative was allowed to stand in an ice-cold bath (0°C) under the dark light, the fluorescence was fairly stable, with 10% of the initial intensity being lost after 3 h. Keeping the ALA derivative at room temperature (18–21°C) without protection from light led to a rapid loss of fluorescence intensity, only half of the initial fluorescence remaining after 3 h.

**Calibration curve for ALA and analytical recovery.** The fluorescence intensity of ALA derivative was linear between 1 and 20 mg/L of ALA (Figure 5). Dissolving ALA in the artificial urine disclosed some matrix interference. The intensity of fluorescent ALA derivative in the artificial urine was about 56% of that in distilled water, even though the linearity of intensity vs concentration remained good. Perhaps urea interferes with the derivatization of ALA to a
fluorescent product. When ALA was dissolved in a fivefold-diluted solution of the artificial urine, the calibration curve of ALA was almost equal to that of aqueous ALA. The detection limit of aqueous ALA was about 20 μg/L (15 pmol of ALA in the 100-μL sample). The within-run variation (CV) of the present method was about 4% for 1 mg/L of aqueous ALA (n = 5).

Analytical recovery of ALA from urines with various densities, from apparently healthy subjects, was 65%–80%. On the other hand, when these urine specimens were diluted fivefold with distilled water, 90%–98% of the ALA was recovered.

Comparison with conventional colorimetric method. We determined urinary ALA in 25 lead workers by the present method and the colorimetric method (Figure 6). Moreover, in the 25 lead workers tested, the log of urinary ALA concentration obtained with the present method correlated with the concentration of lead in blood (r = 0.685).

In conclusion, the fluorometric HPLC method for determining urinary ALA is much more sensitive than the colorimetric method. We suggest, therefore, that it also be applied to measurement of ALA in serum or plasma.

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References

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