We previously reported the usefulness of a fluorometric method to determine urinary \(\delta\)-aminolevulinic acid (ALA) concentrations by using post-column derivatization to monitor the effect of lead exposure. We have further improved the method by introducing pre-column derivatization by using reaction of ALA with acetylacetone and formaldehyde. Response of the hematopoietic system to lead exposure can now be easily detected at blood lead concentrations as low as 182 \(\mu\)g/L. The fluorescent ALA derivative, a new aromatic product, 2-methylideneamino-3,5-diacetyl-4,6-dimethylphenylpropionic acid, is separated on octadecyl silica column by high-performance liquid chromatography and the fluorescence intensity is detected with a fluorophotometer. Sample recoveries for 12 urine samples from workers exposed to lead and unexposed controls were 91.9–110.2%. The results obtained by the pre-column derivatization method agreed with those by the post-column derivatization method. The new method increases the sensitivity to a detection limit to 10 \(\mu\)g of \(\delta\)-aminolevulinic acid per milliliter of urine and is simple enough to be used for routine monitoring of the biological effect of exposure to low concentrations of lead.

**Additional Keyphrases:** biological monitoring • occupational hazards

Lead has various toxic effects on humans (e.g., it can cause neurological, renal, and hematological disorders) and its toxicity has been recorded since ancient history. Lead exposure can be detected by measuring \(\delta\)-aminolevulinic acid (ALA) concentrations in the urine because aminolevulinate dehydratase (EC 4.2.1.24), an enzyme in the heme-synthetic pathway, is easily inhibited by lead in vivo, causing any excess of ALA to be excreted into urine ([1]). Urinary ALA (ALA-U) concentrations, usually determined by ion-exchange column chromatographic methods, have been widely used as an indicator of lead exposure in workers ([2], [3]).

Recently, several high-performance liquid chromatographic (HPLC) methods for determining ALA-U have been reported ([4]–[6]), and they appear to be more useful for biological monitoring at low blood lead (Pb-B) concentrations than conventional colorimetric methods ([4], [7]). Previously, we reported the fluorometric determination of ALA-U by a post-column derivatization method involving reaction with formaldehyde and acetone ([5]). By this post-column derivatization method, the ALA-U concentration in workers exposed to lead was one-third of that obtained by conventional colorimetric method, 0.83 ± 0.14 vs. 2.4 ± 0.6 mg of ALA per gram of creatinine, respectively, for a low Pb-B concentration (58 ± 22 \(\mu\)g/L). A significant increase in ALA excretion was observed when Pb-B was 162 \(\mu\)g/L which is much lower than reported values (>400 \(\mu\)g/L) ([8]). The conventional colorimetric method cannot detect smaller changes in ALA-U concentrations because of overlap with other substances present in urine ([7]). Thus, the ALA-U concentration determined by our post-column derivatization method is useful for monitoring biological effects of lead exposure. However, this method cannot be applied directly to routine monitoring of occupational hazard because it requires expensive instruments. To solve this problem, we developed a fluorometric method for determining ALA concentrations by pre-column derivatization, reacting ALA with formaldehyde and acetylacetone ([6]). This method showed a sufficient sensitivity and specificity for monitoring ALA in urine. However, sample recovery was occasionally very low because of interference by urea and other urinary substances ([9]) or the lack of sufficient product stability for automated analysis. In the present study, we tried to improve our fluorometric method with pre-column derivatization to overcome these problems.

**Materials and Methods**

**Chemicals.** \(\delta\)-Aminolevulinic acid hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). Acetylacetone, formalin (containing 370 g of formaldehyde per liter), acetic acid, ethanol, and sodium chloride were obtained from Katayama Chemicals Co. (Osaka, Japan). Deuterium oxide from Merck (St. Louis, MO) was used for nuclear magnetic resonance (NMR) measurement. Other chemicals used were of the highest quality available. The water used was de-ionized and purified with a Puris system (Organo, Tokyo, Japan).

**Standard and urine samples.** A standard solution of ALA (50 mg/L) was prepared by dissolving 6.4 mg of \(\delta\)-aminolevulinic acid hydrochloride in 100 mL of water and stored at 4 °C. Urine samples from unexposed volunteers and from workers exposed to lead in a lead compound manufacturing factory were collected and stored at −80 °C until analysis.

**Analysis of samples.** We treated standard solutions and urine samples (50 \(\mu\)L) in glass tubes with a mixture of 3 mL of acetylacetone, ethanol, and water (15:10:75, by volume) containing 4 g of sodium chloride per liter, and then with 450 \(\mu\)L of aqueous formalin (85 mL of formalin per liter). We heated the solution in boiling water for 30 min and then cooled.
cooled in a water bath. Using an autosampler (Model 3CL-6A; Shimadzu, Kyoto, Japan), we injected 50 µL of samples onto an octadecyl silica column (TSK-gel 80 TM; Tosoh, Tokyo, Japan) kept at 40 °C and eluted with an aqueous solution containing 600 mL of methanol and 10 mL of acetic acid per liter at a flow rate of 0.8 mL/min by a constant-flow pump (Model LC-6A; Shimadzu). We monitored the fluorescence intensity of the eluate at 473 nm excitation wavelength, 363 nm) with a spectrofluorometer Model RF-530; Shimadzu) equipped with a 12-µL flow cell and a data processor (Model CR-3A; Shimadzu). We calculated the concentration of ALA from the peak area, based in calibration with the standard sample.

For comparison, ALA concentrations were determined by the same specimens by a post-column derivatization method (5).

**Purification and identification of fluorescent ALA derivative.** Dissolve 500 mg of δ-aminolevulinic acid dihydrochloride in 260 mL of boiling water, then add 120 mL of acetylated, and follow immediately with 20 mL of formalin. Heat the mixture for 15 min, cool in water, and then adjust to pH 7.5 with sodium hydroxide, 5 mol/L. Mix the solution vigorously two times with 200 mL of ethyl acetate and then discard the ethyl acetate layer. Adjust the aqueous layer to pH 4.5, and then extract twice with 300 mL of ethyl acetate. Evaporate the ethyl acetate layer to dryness under reduced pressure. Suspend the residue in dichloromethane (20 mL), filter it through a 10-µm (pore size) filter, wash it twice with 5 mL of dichloromethane, dry it, and then dissolve it in 5 mL of 50 mmol/L disodium hydrogen phosphate solution. Adjust the solution to pH 3.0 by slowly adding 1 mol/L aqueous phosphoric acid. Crystallize the ALA derivative, filter it through a 0.45-µm (pore size) filter, and lyophilize. This procedure gave us 430 mg of ALA derivative.

The 13C NMR spectrum of the ALA derivative was obtained at 50.3 MHz on a Varian XL 200 spectrometer. The derivative was dissolved in deuterium oxide and adjusted to pH 7.0 with a diluted sodium deuterioxide solution. The sample temperature cited is that of the probe temperature at the bottom of the sample tube. The mass spectrum was obtained by electron impact excitation with a Model D-300 instrument (JEOL, Tokyo, Japan) by using direct insertion.

**Results**

Figure 1A shows a typical chromatogram of 50 µL of ALA standard solution (0.5 mg/L). ALA was eluted at 7.6 min. The amounts of ALA injected in the range of 0.25–50 mg/L were linearly related to the fluorescence intensities expressed as peak areas. The linear-regression equation was \( y = 1.0x + 1.9 \times 10^{-4} \), with a correlation coefficient of \( r = 0.999 \). The detection limit for ALA in urine was 10 µg/L (signal-to-noise ratio = 5). A typical elution pattern for a urine sample from an unexposed control is shown in Figure 1B. The ALA peak (0.62 mg/L) was clearly separated from other peaks. One analysis can be completed within 10 min. The overall recoveries of ALA added to various urine samples are shown in Table 1. Sample recoveries ranged from 91.9% to 112.0%. The fluorescence intensity of the ALA derivative did not change significantly for 24 h at an ambient temperature (1.04 of initial intensity). Data obtained by this pre-column derivatization method agreed well with those from the post-column derivatization method (Figure 2; \( r = 0.986 \)).

The mass spectrum for the purified ALA derivative is shown in Figure 3. The molecular mass of the derivative was 289 Da (C16H16O4N) because the [M + 1]+ peak was observed at 290 Da. The derivative seems to combine two molecules of acetylated, one of formaldehyde, and one of δ-aminolevulinic acid, with deletion of four molecules of water. The NMR spectrum of this substance is shown in Figure 4. A non-decoupled spectrum (Figure 4B) gives four quartet signals, three triplet signals, and nine singlet signals, two of which are spaced closely at 141.2 and 141.3 parts per million (ppm). Chemical shift values of the singlet signals suggest that this substance has six aromatic carbons (six peaks in the region of 120–140 ppm), one carboxyl carbon (a peak at 186.4 ppm), and two carbonyl carbons (two peaks in the low field, below 200 ppm). These results show that this derivative is a new material (2-methylideneamino-3,5-diacyl-4,6-dimethylphenylpropionic acid) produced by the reaction shown in Figure 5.

**Discussion**

Although ALA concentration offers a good criterion for lead exposure, a conventional colorimetric method (3) is not efficient for monitoring lead exposure at low concentrations because endogenous substances in urine conceal the slight changes in ALA excretion in urine (7). Figure 6 shows the dose–response relationship between Pb-B (an index of lead exposure) and ALA-U concentrations determined by this

| Table 1. Analytical Recoveries for Various Concentrations of Urinary ALA |
|---------------------------|-----------------|-----------------|------------------|
|                          | Sample          | Standard mg/L  | Total mg/L       | Recovery of added standard, % |
| Exposed to lead           |                 |                 |                  |                               |
| 4                         | 8.48            | 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            | 38.67            | 107.6                         |
| 5                         | 21.93           | 17.2            | 39.49            | 101.8                         |
| Unexposed control         |                 |                 |                  |                               |
| 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                         |

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Fig. 2. Correlation between ALA concentrations (mg/L) by pre- and post-column derivatization methods
The regression equation between ALA values is ALA (pre-column) = 1.07 x ALA (post-column) + 0.32 mg/L (r = 0.986), n = 23

Fig. 3. Mass spectrum of the ALA derivative
Values are expressed as a relative intensity of [M + 1]* (100)

Fig. 4. 13C NMR spectra of ALA derivative at 23°C: (A) proton-decoupled 13C-NMR; (B) non-decoupled 13C-NMR

Fig. 5. Proposed reaction of ALA with formaldehyde and acetylacetone, and proposed structure of the ALA derivative

Fig. 6. Dose–response relationship between concentration of blood lead and the percentage of the urine samples exceeding the cutoff values for ALA-U [1.12 and 3.5 mg of ALA per gram of creatinine for a post-column derivatization method (‡) and a conventional colorimetric method (O), respectively] for every 100 μg/L change in Pb-B. Standard deviations of Pb-B concentrations are shown as bars. The regression curve (r = 0.96), based on a logistic equation, is redrawn from ref. 7

With the conventional method, the response of ALA-U cannot be successfully detected at blood lead concentrations below 500 μg/L. Thus, determination of ALA by fluorometric derivatization is essential for biological monitoring of low lead exposure. The post-column derivatization requirement for expensive instruments is solved by the new, simpler pre-column derivatization method introduced here.

Earlier, we reported a simple, sensitive fluorometric method of ALA determination in urine by pre-column derivatization with formaldehyde and acetylacetone (6). However, the sample recovery from concentrated urine samples was low because urinary urea interfered at physiological concentrations (9), and the changes in fluorescence intensity of ALA derivative after 24 h could not be ignored (0.75 of initial intensity). To simplify the pre-column derivatization method, we optimized the reaction conditions. The sensitivity improves as the acetylacetone concentration increases (9); because acetylacetone is not very soluble in water, we used as a reaction mixture an aqueous ethanol solution that can hold up to 150 mL of acetylacetone per liter. This improved the sensitivity five
old. Because the fluorescence is damped by substances in urine such as sodium chloride, potassium chloride, and urea, the reaction conditions were modified by requiring him to add only a small amount of sodium chloride and by prolonging the reaction period. These conditions led to negligible interference from excreting urinary substances. The fluorescence intensity of the derivative remained stable for at least one day at room temperature. Good recoveries were obtained for various concentrations of ALA in urine samples (Table 1). A detection limit of 10 μg/L in urine is sufficient to monitor ALA concentrations in urine.

Sawicki and Carnes (10) first reported a fluorometric determination of amino acids by using acetylacetone followed by formaldehyde. However, the fluorescence intensity of the ALA derivative per mole was about 0.2% of that of the other amino acid derivatives (e.g., glycine, alanine), which was not suitable for ALA at urinary concentrations. We modified reaction conditions to obtain a stronger intensity (10 ⁵ times that of Sawicki and Carnes’s method) (6). We thus assumed that the structure of the ALA derivative obtained here was different from theirs. As shown in Figure 1, the product of the proposed reaction of ALA with formaldehyde and acetylacetone differed from their derivative. Our ALA derivative is an aromatic compound.

The fluorometric method described here was optimized or the determination of ALA in urine by pre-column derivatization. The simple and sensitive method is suitable for the daily monitoring of ALA in workers exposed to low concentrations of lead.

References

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Ultrasensitive Time-Resolved Fluorescence Method for α-Fetoprotein
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We have examined the maximum sensitivity of a newly developed and optimized time-resolved fluorescence immunoassay system. The system, originally described elsewhere (Clin Biochem 1986;21:139–50), has undergone significant improvements in sensitivity through improvements of the labeled reagent used. We have chosen an α-fetoprotein (AFP) assay as a model and used mononuclear “capture” antibodies and mononuclear or polyclonal biotinylated antibodies in “sandwich-type” assay configurations. Streptavidin labeled with the europium chelator 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid was used for detection. We can measure as few as 3 × 10⁶ molecules of AFP with the optimized system. We have applied this assay to measure AFP in the serum of normal individuals after a 10-fold sample dilution. We conclude that this system is extremely sensitive and can be used in immunoassay or other applications where biotinylated reagents can be applied.

Additional Keyphrases: nonisotopic immunoassay · europium chelates · attomole detection · reference interval

Immunoassays, introduced about 30 years ago, have made an enormous impact on biomedical research and clinical practice because they combine specificity and sensitivity (1). Currently, there is considerable interest in further improving the sensitivity of this technique by using nonisotopic labeling systems (2–4). Improved sensitivity is desirable because of the potential for new applications, for example: (a) measurement of analytes at sub-normal concentration ranges (e.g., antidiuretic hormone and corticotropin); (b) measurement of new analytes in serum (e.g., the hypothalamic releasing hormones); (c) discovery of new analytes; (d) detection of very small amounts of tumor-