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Study on measurement of δ-aminolevulinic acid in plasma by high-performance liquid chromatography

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Abstract

A method for the determination of δ-aminolevulinic acid in plasma of lead-exposed workers by high-performance liquid chromatography with fluorescence detection of a fluorescent δ-aminolevulinic acid derivative (2-methylidineamino-3,5-diacetyl-4,6-dimethylpropionic acid) was established. The detection limit of δ-aminolevulinic acid in plasma was 0.01 μg/ml at a signal-to-noise ratio of 5:1. A linear correlation was obtained between the amounts of δ-aminolevulinic acid injected from 0.01 to 0.5 μg/ml ($r = 0.999$). The recovery of 0.05 and 0.1 μg/ml of δ-aminolevulinic acid added to plasma with various concentrations of δ-aminolevulinic acid in plasma ranged from 80.0 to 100.8%. This method, combined with the use of an automatic sampler, should facilitate the routine measurement of δ-aminolevulinic acid in plasma.

1. Introduction

Lead exposure can cause excessive production of δ-aminolevulinic acid (ALA), which has an inhibitory effect on haem synthesis. The increase in ALA in urine (ALAU) has been used as an index of lead exposure. ALA measurement has been conventionally done by spectrophotometry following ion-exchange column chromatographic separation [1]. However, spectrophotometric methods cannot exclude the interference of coexisting substances in urine, *e.g.*, aminoacetone [2]. In order to exclude the interference and measure ALAU more accurately, Okayama [3] and Okayama *et al.* [4] developed a method using high-performance liquid chromatography (HPLC). However, ALAU values must be corrected for the urinary creatinine concentration or urinary gravity because of seasonal fluctuations and individual differences.

To address these problems, Hosoda *et al.* [5] and Takebayashi *et al.* [6] measured ALA in whole blood (ALAB) based on Okayama's method for measuring ALAU. ALA in blood was found in the plasma or serum (less than 20%) and in the erythrocytes (more than 80%) by Chiba and Kikuchi [7]. In their work, ALA in plasma was determined by a spectrophotometric method which was affected by the interference of coexisting substances in urine. The ALA in

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plasma or serum is rapidly transferred to the urine, but not the ALA in erythrocytes as it is within the cell membrane. This would mean that ALAB does not quickly follow fluctuations in lead exposure levels. However, the concentrations of the plasma components, except in cases of anaemia, display only minor seasonal and individual fluctuations, unlike the data for urine. Hence ALA in plasma (ALAP) can be a sensitive indicator of lead exposure, especially fluctuating lead exposure. However, none of the reported HPLC methods for ALAP excludes the interference of coexisting substances in plasma.

Recently, Okayama *et al.* [8] developed a new HPLC method for measuring ALAU in which the ALA was derivatized into a highly fluorescent product. However, in the spectrophotometric measurement of ALAP, which requires deproteinization of plasma before injection into the HPLC system, Chiba and Kikuchi [7] reported that the concentration of ALAP was much lower than that of ALAU.

In this study, we aimed to establish a method for measuring ALAP by HPLC more accurately than with spectrophotometric detection. The HPLC method for ALAU of Okayama *et al.* [8] was applied to the measurement of ALAP with minor a modification of the chromatographic conditions, because the low concentration of ALAP required the separation of the ALA peak from coexisting substances.

2. Experimental

2.1. Chemicals

δ-Aminolevulinic acid hydrochloride was purchased from Sigma (St. Louis, MO, USA), iodoacetamide and formaldehyde from Wako (Osaka, Japan), acetylacetone, methanol and trichloroacetic acid from Ishizu Seiyaku (Osaka, Japan) and acetic acid from Kanto Chemical (Tokyo, Japan). The water used was deionized and distilled with a Model HS-1 distiller (Shiraimatsu, Osaka, Japan).

2.2. Preparation of standard solution

A standard solution of ALA (50 μg/ml) was prepared by dissolving 6.4 mg of δ-aminolevulinic acid hydrochloride in 100 ml of distilled water and storing it at 4°C.

2.3. Sample collection

Blood samples were obtained from three workers engaged in producing lead-glass-based paints, three workers engaged in casting lead–copper metals (lead exposed), two groups of unexposed workers (office workers, $n = 28$; worksite workers in a chemical plant, $n = 12$) and one of the authors. Blood samples were taken by venepuncture using disposable syringes and put into heparinized polypropylene tubes that had been rinsed with nitric acid and purified water. Blood samples were centrifuged at 1600 *g* for 10 min and the plasma was pipetted into polypropylene tubes and stored at −30°C until analysis. Because of the difficulty of obtaining large amounts of plasma from individual subjects, plasma samples of 28 and 12 unexposed workers were combined as two lots of control plasma (controls A and B, respectively). A large amount of plasma was obtained from one of the authors (pooled plasma). ALA standard was added to the control plasma and pooled plasma, which were then utilized as standard calibration solutions, and to examine the stability, recovery and reproducibility of ALAP.

2.4. Sample preparation

ALAP was determined based on the method of Okayama *et al.* [8]. The control and lead-exposed plasma samples (0.25 ml) were mixed with 15 μl of 0.06 *M* iodoacetamide solution and, after 2 min, 60 μl of 35% (w/v) trichloroacetic acid solution to prevent thiol formation and deproteinize the plasma, respectively, followed by centrifugation for 10 min at 1600 *g*. In order to synthesize 2-methylideneamino-3,5-diacetyl-4,6-dimethylpropionic acid (Fig. 1), which is a fluorescent ALA derivative, a 0.1-ml

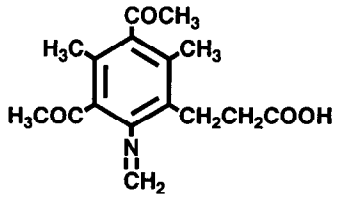

Fig. 1. Structural formula of 2-methylideneamino-3,5-diacetyl-4,6-dimethylpropionic acid.

portion of the supernatant was mixed with 0.4 ml of acetylacetone, 2.5 ml of 2% acetic acid and 1.0 ml of 10% formaldehyde in a glass tube, and this mixture was heated at 100°C for 10 min. After cooling, 0.1 ml of the mixture was injected into the HPLC system.

2.5. Chromatography

The HPLC system consisted a Model CCPD pump (Tosoh, Tokyo, Japan), Model F-1050 spectrofluorometer (Hitachi, Tokyo, Japan), Model D-2500 chromato-integrator (Hitachi) and Model 655A 52 column oven (Hitachi). The analytical conditions for HPLC were as follows: mobile phase, methanol–water (45:55); analytical column, LiChrospher 100 RP-18(e) (125 × 4 mm I.D., 5-μm particle diameter) (Merck, Darmstadt, Germany), protected by a LiChrospher 100 RP-18 guard column (4 × 4 mm I.D.); flow-rate, 0.7 ml/min; column temperature, 30°C; and injection volume, 100 μl. The excitation and emission wavelengths were set at 373 and 463 nm, respectively.

3. Results

3.1. Chromatographic separation

Typical chromatograms of the ALA derivative (0.1 μg/ml ALA standard solution) (left), a plasma sample to which ALA has been added to prepare 0.1 μg/ml of ALA in pooled plasma (middle) and a plasma sample from a lead-exposed worker (right) are shown in Fig. 2. In a chromatographic analysis taking 20 min, the

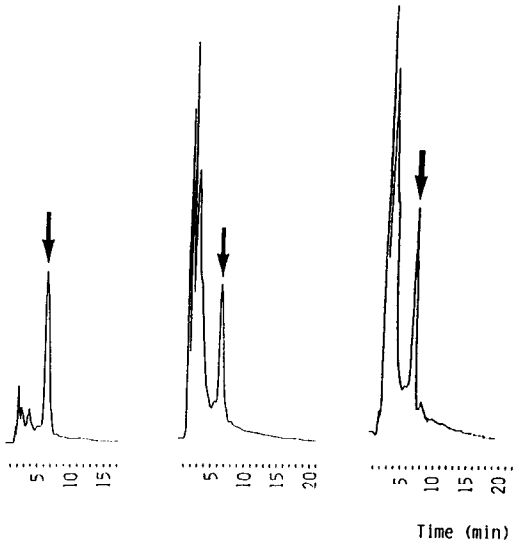

Fig. 2. Chromatogram of δ-aminolevulinic acid (ALA) in standard solution (0.1 μg/ml, left), pooled plasma + ALA (prepared at 0.1 μg/ml, middle) and plasma sample from lead-exposed worker (0.1444 μg/ml, right). Arrow = peak of ALA derivative.

ALA derivative was eluted at 6.5 min, showing one large, sharp peak. Baseline correction was applied for the measurement.

3.2. Calibration

Statistical calculation at the 99% confidence interval showed that the slopes of the calibration graphs that were obtained after combining the standard solutions of ALA with an equal volume of control plasma or water were different. Therefore, mixed solutions of the standard solutions and plasma must be used as standard calibration solutions. Amounts of ALA (x) from 0.01 to 0.5 μg/ml in standard calibration solutions were linearly related to the fluorescence intensities expressed as peak areas (y) with a correlation coefficient (r) of 0.999 ($y = 2.22\ x + 2.51$).

3.3. Precision, recovery and detection limit

To examine the reproducibility of ALAP measurement, ALA standard was added to pooled

Table 1
Precision of assay using pooled plasma with added δ-aminolevulinic acid (ALA) standard ($n = 3$)

Added ALA concentration (μg/ml)	Mean assayed concentration (μg/ml)	C.V. (%)
0.010	0.00949	5.4
0.025	0.0255	8.9
0.050	0.0546	2.3
0.100	0.0910	1.8
0.200	0.1977	2.8
0.500	0.4983	3.6

Table 3
Stability of δ-aminolevulinic acid (ALA) derivative kept at 4°C for 20 h

Concentration (mean ± S.D.) (μg/ml)	n	C.V. (%)
Before		
0.0283 ± 0.001	5	3.6
0.0447 ± 0.0021	9	4.7
0.0635 ± 0.014	5	2.1
After 20 h		
0.0309 ± 0.001	5	3.2
0.0473 ± 0.0023	9	4.8
0.0668 ± 0.0014	5	2.1

plasma. The coefficients of variation (C.V.) of ALAP ranged from 1.8 to 8.9% (Table 1). The recoveries of 0.05 and 0.1 μg/ml of ALA added to the control plasma and that from three lead-exposed workers with various concentrations of ALAP ranged from 80.0 to 100.8% (Table 2). Using our method, the ALAP levels of the unexposed and lead-exposed workers were found to be 0.0178 and 0.0215 μg/ml for the former group (controls A and B, respectively) but ranged from 0.0283 to 0.1415 μg/ml for the six lead-exposed workers. The detection limit was 0.01 μg/ml of ALA at a signal-to-noise ratio of 5:1.

3.4. Stability

In order to test the stability of the ALA derivative, we examined plasma samples from three other lead-exposed workers with different concentration levels (0.0283, 0.0447 and 0.0635 μg/ml, five samples each). After 20 h at 4°C, the mean value for the lowest level sample was 0.0309 μg/ml (S.D. 0.001 μg/ml, C.V. 3.2%) and that for the highest level was 0.0668 μg/ml (S.D. 0.0014 μg/ml, C.V. 2.1%) (Table 3). To test the stability of ALA in plasma, we examined

Table 2
Recoveries of δ-aminolevulinic acid (ALA) standard added to plasma samples

Original concentration of sample (μg/ml)	Added standard: 0.05 μg/ml		Added standard: 0.1 μg/ml	
	Mean ($n = 3$) measured value (μg/ml)	Mean recovery (%)	Mean ($n = 3$) measured value (μg/ml)	Mean recovery (%)
0.0178 (control A)	0.0678	100.0	0.1086	90.8
0.0215 (control B)	0.0719	100.8	0.1219	100.4
0.0587 (exposed)	0.1060	94.5	0.1451	86.4
0.0603 (exposed)	0.1040	85.3	0.1425	82.2
0.1415 (exposed)	0.1861	89.2	0.2215	80.0

Controls A and B = plasma samples from office workers and worksite workers not exposed to lead. Exposed = plasma samples from lead-exposed workers.

Table 4
Stability of δ-aminolevulinic acid (ALA) added to plasma samples of control A kept at −30°C for 1 week

Added standard (μg/ml)	Measured (mean ± S.D., $n = 3$) (μg/ml)	Preserved ALA standard
0.05	0.0685 ± 0.0031	0.0507 ± 0.0031
0.1	0.1139 ± 0.0039	0.0961 ± 0.0038

Concentration of ALA in plasma = 0.0178 μg/ml.

the control plasma (control A, 0.0178 μg/ml ALAP) to which 0.05 or 00.1 μg/ml of ALA standard had been added. After storage for 1 week at −30°C, only minor changes in the ALA concentration levels were obtained (Table 4).

4. Discussion

The ALAP measurement showed high reproducibility and a relatively high recovery (although slightly less at high ALAP concentrations), as can be seen in Tables 1 and 2. The ALA in plasma remained stable even after storage for 1 week, suggesting the possibility of the routine application of the method.

As seasonal and individual fluctuations in urine are unavoidable, ALAP may be a better indicator than ALAU of lead exposure. From the practical viewpoint, only one venepuncture is needed for blood lead (PbB) and ALAP measurements in health examinations for lead-exposed workers, while both venepuncture and urinary sampling are needed for PbB and ALAU measurements. This reduces the time and labour for both subjects and health-care workers. Also, a more direct correlation can be obtained with PbB or lead in plasma (PbP) especially PbP, as ALAP originates from the same matrix.

Hence ALAP should be useful for monitoring and investigating the effect of lead on haem synthesis. However, the procedure for ALAP measurement is more complex than that for ALAU. We suggest that employing an automatic sampler for HPLC can help resolve the problem and increase the efficiency of ALAP measurement.

5. References

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