Fluorometric Determination of Erythrocyte δ -Aminolevulinate Dehydratase Activity by High-Performance Liquid Chromatography

Key words: Erythrocyte— δ -Aminolevulinate dehydratase—Normal levels—High-performance liquid chromatography—Fluorometry

δ -Aminolevulinate dehydratase (EC 4.2.1.24, ALA-D), which catalyzes the condensation of two molecules of δ -aminolevulinic acid (ALA) to produce porphobilinogen (PBG), is largely contained in circulating erythrocytes.

It has been demonstrated that the ALA-D activity of erythrocytes is strongly inhibited by lead and the degree of inhibition closely correlates with the concentration of lead in blood.^{1–6)}

In particular, the determination of erythrocyte ALA-D activity is a practical screening test for low-level exposure to lead.

In routine measurement, ALA-D assay is commonly done according to the method of Bonsignore *et al.*⁷⁾ or a modification^{8–10)} based on original assays developed by Gibson *et al.*¹¹⁾ and Granick and Mauzerall.¹²⁾ In these methods, the amount of PBG formed during incubation is determined spectrophotometrically by a color reaction with Ehrlich's reagent. However, the use of this colorimetric method could lead to erroneous values if the experimental conditions for enzyme assay are not strictly controlled, judging from the fact that PBG is sensitive to light and air oxidation and that the color reaction of PBG with Ehrlich's reagent is time-dependent.

On the other hand, it is known that PBG in an acidic medium is converted to uroporphyrin (URO), which is stable and highly fluorescent, by heating in a boiling water bath.¹³⁾ Previously, in an application of this reaction, fluorometric methods for determining erythrocyte ALA-D activity have been developed.^{14,15)} However, these methods require a blank tube to take off the fluorescence of porphyrins other than URO in the reaction mixture.

In the present study, we performed a separate determination of URO in the reaction medium using a high-performance liquid chromatograph (HPLC) equipped with a spectrofluorometer and applied it to the measurement of erythrocyte ALA-D activity.

Blood specimens

Heparinized whole blood was obtained from 58 healthy adults with no history of occupational lead exposure. ALA-D assay was performed within 5 h after blood collection.

Apparatus

A Shimadzu LC-6A HPLC equipped with a fluorescence HPLC monitor (RF-530) as a detector (Shimadzu, Ltd., Kyoto, Japan) and a Hitachi Model 100-40 spectrophotometer (Hitachi, Ltd., Tokyo, Japan) were used in this experiment.

Reagents

All reagents used were of analytical grade. ALA hydrochloride, coproporphyrin I (CP) and uroporphyrin I (URO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan). Ehrlich's reagent was prepared by dissolving 2 g of *p*-dimethylaminobenzaldehyde in about 60 ml of glacial acetic acid and adding 32 ml of 70% perchloric acid to a final volume of 100 ml. The CP standard (500 $\mu\text{g/l}$) was made by dissolving 5 μg of CP (in vial) in 10 ml of glacial acetic acid. This solution was stable for more than 6 months at -20°C in the dark. URO was also dissolved in glacial acetic acid at appropriate concentrations.

Procedure for HPLC

The enzyme assay was performed as follows: 0.1 ml of whole blood was well hemolyzed with 1.4 ml of distilled water, then 0.5 ml of phosphate buffer (0.2 mol/l, pH 6.8) was added. This was placed in a thermo-regulated water bath at 37°C for 5 min. After adding 0.5 ml of ALA (20 mmol/l) substrate, the mixture was incubated for 1 h at 37°C . The enzyme reaction was stopped by adding 1 ml of 10% trichloroacetic acid (TCA) and mixing well, after rapid cooling of the incubation tubes in a melting ice bath to obtain a clear supernatant was pipetted into another test tube. The tube was heated for 15 min in a boiling water bath to convert PBG to URO, then cooled in a melting ice bath. The analytical conditions for HPLC are shown in Table 1. ALA-D activity, calculated by the following formula, was expressed in $\mu\text{mol URO/h/l RBC}$:

Table 1. Analytical conditions for HPLC

Apparatus	Shimadzu LC-6A HPLC
Column	Shim-pack CLC-ODS (Shimadzu) 150 $\times$ 6.0 mm, 5 μm particle size Reversed-phase column
Detector	Fluorescence HPLC monitor (Shimadzu RF-530) Excitation wavelength 400 nm (band width 18 nm) Emission wavelength 620 nm (band width 22 nm)
Mobile phase	Acetonitrile/aqueous KH_2PO_4 (10 mmol/l)/ glacial acetic acid (500/500/10 by volume)
Flow rate	1.5 ml/min
Column temp.	Room temp. (20–25 $^{\circ}\text{C}$)
Chart speed	5 mm/min
Injection volume	10 μl

$$\frac{F_s}{F_c} \times \frac{500}{655} \times \frac{3.5}{0.1} \times \frac{100}{H_t} = \frac{F_s}{F_c} \times \frac{100}{H_t} \times 26.7$$

where F_s =fluorescence of sample, F_c =fluorescence of CP standard, 655=molecular weight of CP, H_t =hematocrit (%).

As the fluorescent peak corresponding to URO was not detected in the blank tube without ALA substrate, the blank tube was omitted from the enzyme assay.

Procedure for colorimetry

One ml of the supernatant obtained from the above assay was mixed with 2 ml of Ehrlich's reagent. After 10 min, the absorbance was measured at 555 nm. ALA-D activity, calculated according to the following formula, was expressed in $\mu\text{mol PBG/h/l RBC}$:

$$\text{Absorbance} \times \frac{100}{H_t} \times \frac{3.5}{0.1} \times \frac{3}{64} \times 10^3 = \frac{\text{Absorbance}}{H_t} \times 164 \times 10^3$$

where 6.4×10^4 was used as the molecular extinction coefficient of PBG.

Under the present analytical conditions for HPLC, the retention time of authentic URO was 1.6 min, the same as that of URO formed from PBG by heating. The CP standard was eluted at 4.6 min. When compared at the same micromolar concentration, the fluorescence intensity of the URO standard was equal to that of the CP standard under the present analytical conditions for HPLC. Therefore, the CP standard was used to calculate the activity instead of URO, because a 5- μg vial of CP is inexpensive and convenient. The relationship between fluorescence intensity and quantity of CP injected was linear up to 5,000 $\mu\text{g/l}$.

Figure 1 shows the relationship between fluorescence intensity of URO produced from PBG and the duration of heating. The optimum conversion to URO was obtained by heating the acidic solution (approx. pH 2) of PBG for 15–20 min in the boiling water bath. The conversion rate, which was estimated to be almost constant for each sample, was low (30–35%). When the sample for HPLC was allowed to stand for 16 h at room temperature (20–23°C), loss of fluorescence intensity for URO was about 7%.

Figure 2 compares the values of erythrocyte ALA-D activity as measured by the present HPLC method and the colorimetric method in blood specimens from 58 healthy adults. The correlation between the values obtained with the two different methods was excellent ($r=0.971$). However, contrary to expectation, the regression line obtained did not pass through zero. Therefore, a re-examination of ALA-D measurements in both control and lead-exposed blood is necessary.

The normal levels for erythrocyte ALA-D activity obtained in this study were 63 ± 29 (mean $\pm$ SD) $\mu\text{mol URO/h/l RBC}$ by the present HPLC method and 814 ± 228 (mean $\pm$ SD) $\mu\text{mol PBG/h/l RBC}$ by the conventional colorimetric method.

In conclusion, the present HPLC method is available for ALA-D measurement

Fig. 1. Effect of heating time at 100°C on uroporphyrin (URO) formation from porphobilinogen (PBG), in the measurement of ALA-D activity. Each point shows the mean value of triplicate analyses.

Fig. 2. Relationship between erythrocyte δ -aminolevulinic acid dehydratase (ALA-D) activity by the HPLC method (Y) and colorimetric method (X).

in normal persons and workers exposed to low levels of lead, and it has the advantage of omitting the use of a blank tube for each sample and chemical reagents such as Ehrlich's reagent, which is more irritating to the skin.

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