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Fluorimetric determination of hepatic δ-aminolevulinic acid synthase activity by high-performance liquid chromatography

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ABSTRACT

A fluorimetric method for measuring the activity of δ-aminolevulinic acid synthase (ALAS) in the liver of mice has been developed. The liver homogenate was used as the enzyme source. The final concentration of glycine (substrate) used for the assay was 100 m*M*. The δ-aminolevulinic acid (ALA) formed during incubation was converted into a highly fluorescent derivative by condensation with acetylacetone and formaldehyde (application of the Hantzsch reaction). This derivative was completely separated from other fluorescent substances in the reaction medium, and it was determined using a high-performance liquid chromatograph equipped with a fluorescence monitor (370/460 nm). The activity of ALAS was expressed as nmol ALA formed per gram liver per hour.

INTRODUCTION

δ-Aminolevulinic acid synthase (EC 2.3.1.37, ALAS) catalyses the condensation of glycine and succinyl CoA to form δ-aminolevulinic acid (ALA). It is the rate-limiting enzyme of haem biosynthesis, as well as one of the mitochondrial enzymes [1]. The activity of this enzyme controls not only the rate of haem synthesis in the liver but also the rate of haemoglobin formation in erythrocytes.

The determination of ALAS activity has been performed by colorimetric methods [2–4]. These methods involve the following steps: incubation, isolation of the product ALA by ion-exchange column chromatography or solvent extraction, condensation of ALA with acetylacetone or ethyl acetoacetate to form ALA-pyrrole, colour reaction of ALA-pyrrole with Ehrlich's reagent and spectrophotometric determination. Therefore, these colorimetric methods are time-consuming. A radiochemical method [5] based on the incorporation of [2,3-^{14}C]succinate into ALA is more sensitive than the colorimetric method, but it has some limitations in handling a radioisotope. A fluorimetric coupled-enzyme assay [6] for hepatic ALAS, based on the fluorimetric determination of the oxidized uroporphyrin I, is less useful for routine analysis, because it requires two purified enzymes: δ-aminolevulinic acid dehydratase (EC 4.2.1.24, ALAD) and porphobilinogen (PBG) deaminase for ALAS assay.

We have recently reported a fluorimetric high-performance liquid chroma-

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tographic (HPLC) method for determining urinary ALA [7]. ALA was converted into a fluorescent derivative by condensation with acetylacetone and formaldehyde and it was determined fluorimetrically by HPLC. This paper describes the application of this method to the determination of ALAS activity in liver homogenates.

EXPERIMENTAL

Animals

Male ddY mice weighing 50–55 g were obtained from Kyudo (Kumamoto, Japan). The mice were killed under ether anaesthesia, and the livers were removed immediately and washed in ice-cold 0.9% NaCl solution.

Apparatus

An incubator (Personal-10Dx) was used for the enzyme assay, and a dry thermo unit with an aluminium block (TAH-2) was used for ALA derivatization (both from Taiyo Scientific Industrial, Tokyo, Japan). The Model LC-6A chromatograph was equipped with a fluorescence HPLC monitor (RF-535) and a data processor (Chromatopac C-R3A), all from Shimadzu (Kyoto, Japan).

Reagents

All chemicals were of analytical grade. ALA hydrochloride was purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were obtained from Wako (Osaka, Japan). Methanol for the mobile phase was chromatographic grade. A formaldehyde solution (10%), prepared by 3.7-fold dilution of formalin (37%) with distilled water, was stored in the dark. Tris HCl buffer (0.15 *M*, pH 7.2), disodium EDTA solution (0.2 *M*), glycine substrate (0.5 *M*) and trichloroacetic acid (TCA) solution (25%) were prepared in the laboratory. The stock solution of ALA (100 mg/l) was prepared by dissolving 12.8 mg of ALA hydrochloride in distilled water and diluting to 100 ml. This stock solution was stable for more than three months at 4°C. The working standard of ALA (1 mg/l, 7.63 μM) was made by dilution of the stock solution with distilled water.

Procedure

The livers (*ca.* 2 g) were homogenized in three volumes of ice-cold 0.9% NaCl solution. To 20-ml Erlenmeyer flask were added 1 ml of Tris–HCl buffer, 0.1 ml of 0.2 *M* disodium EDTA solution and 0.5 ml of the liver homogenate. The solution was preincubated by shaking for 5 min at 37°C. To this flask was added 0.4 ml of 0.5 *M* glycine solution, and it was incubated by shaking for 30 min at 37°C. The Erlenmeyer flask was useful for homogenization of the incubation medium by shaking. The total volume of the incubation medium was 2 ml. The reaction was terminated by adding 0.5 ml of 25% TCA. The reaction medium was transferred to a test-tube, and this tube was centrifuged at 1000 *g* for 5 min.

An aliquot of the supernatant was diluted five-fold with distilled water, and this solution was used as the sample for ALA analysis.

To prepare the fluorescent derivative of ALA, 0.1 ml of this sample, 2.5 ml of distilled water, 0.4 ml of acetylacetone and 1 ml of formaldehyde solution were mixed with a vibration mixer for *ca.* 3 s, and this mixture was heated for 10 min at 100°C on the aluminium block. Then the test-tube was quickly placed in an ice-cold bath until analysis. The HPLC analysis was performed under the analytical conditions shown in Table I.

TABLE I

ANALYTICAL CONDITIONS FOR HPLC MEASUREMENT OF ALA FORMED BY ALAS ASSAY

Column	Unisil NQ C_{18} (Gasukuro Kogyo), 250 mm × 4.6 mm I.D., particle size 5 μm
Detector	Fluorescence HPLC monitor (Shimadzu RF-535), excitation wavelength 370 nm, emission wavelength 460 nm, range × 4, sensitivity high
Mobile phase	Methanol–water glacial acetic acid (600:400:10, v/v)
Flow-rate	0.5 ml/min
Pressure	90 kg/cm
Column temperature	Ambient (22–25°C)
Chart speed	5 mm/min
Injection volume	20 μl

Calculation

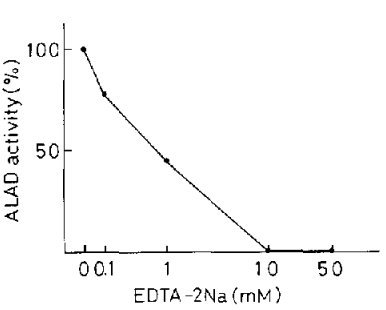
The hepatic ALAS activity (nmol ALA per g liver per h) was calculated according to the following formula:

$F_u \times 5/F_{st} \times 0.1 \times 7.63 \times 4/0.5 \times 60/30 = F_u \times 5/F_{st} \times 12.2$ where F_u is the fluorescence intensity of sample, and F_{st} is the fluorescence intensity of ALA standard (1 mg/l, 7.63 μM).

RESULTS AND DISCUSSION

The fluorimetric determination of ALA based on the derivatization with acetylacetone and formaldehyde is *ca.* 50 times more sensitive than the colorimetric determination based on the condensation of ALA-pyrrole with Ehrlich's reagent (unpublished data).

ALAD is the enzyme that catalyses the conversion of ALA into PBG. To investigate the relationship between the inhibition of ALAD activity in the liver homogenates and the concentration of disodium EDTA added to the incubation



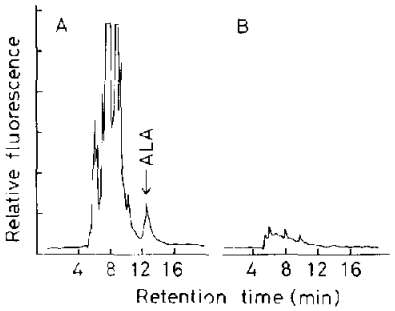


Fig. 1. Effect of addition of disodium EDTA on the activity of ALAD in the liver homogenate. Several solutions of disodium EDTA (0, 0.002, 0.02, 0.2 and 1.0 *M*) were used for this experiment. The concentration of disodium EDTA is a final concentration in the incubation mixture (2 ml).

Fig. 2. Chromatograms of the ALA product: (A) from a five-fold dilution of the supernatant after enzyme reaction; (B) from a five-fold dilution of the supernatant obtained with no addition of substrate glycine. The analytical conditions for HPLC are shown in Table 1.

medium, the hepatic ALAD assay was performed according to a modification [8] of the method of Nikkanen *et al.* [9].

Fig. 1 indicates that hepatic ALAD activity is completely inhibited by the addition of disodium EDTA (10–50 m*M*) to the incubation medium. This finding demonstrates that the enzymic conversion of ALA into PBG can be stopped if 10 m*M* disodium EDTA is added to the assay medium for ALAS. Therefore we chose a 10 m*M* concentration of disodium EDTA in the present study.

Fig. 2A shows that the fluorescence product of ALA is completely separated from other fluorescent substances in the reaction mixture. Comparison of the chromatograms A and B in Fig. 2 shows that most of the fluorescent substances other than the ALA derivative seem to result from the substrate glycine. In the fluorescence derivatization of ALA produced during incubation, it is necessary to dilute the supernatant five-fold with distilled water. No dilution of the supernatant leads to incomplete separation of the ALA derivative on the chromatogram, because of the high concentration of glycine present in the supernatant.

Marver *et al.* [2] have previously demonstrated that the enzyme ALAS can be measured in rat liver homogenates using both added glycine and endogenously generated succinyl CoA, and the production of ALA in this system is greater than in the isolated mitochondrial system with exogenous succinyl CoA generation. We also used the endogenous succinyl CoA-generating system for hepatic ALAS assay in the present study. On the other hand, Bonkowsky and Pomeroy [10] have determined the human hepatic ALAS activity with and without the exogenous system for generation of succinyl CoA in the liver homogenates, and they have indicated that the liver ALAS activity measured with the exogenous generating system was 2.5 times higher than that measured without.

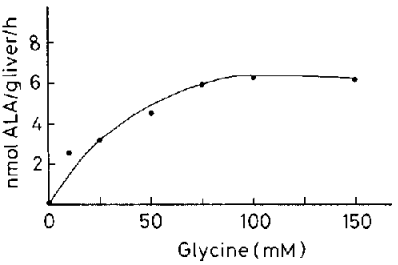


Fig. 3. Relationship between glycine (substrate) concentration in the incubation mixture and the amount of ALA formed. Several solutions of glycine (0, 0.05, 0.125, 0.25, 0.375, 0.5 and 0.75 *M*) were used for this experiment.

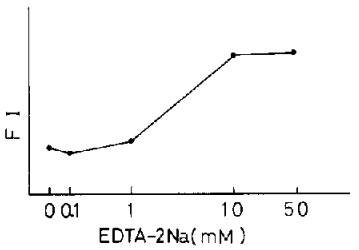


Fig. 4. Relationship between disodium EDTA concentration in the incubation mixture and the fluorescence intensity (FI) of the ALA derivative (the amount of ALA formed). The solutions of disodium EDTA used correspond to those in Fig. 1.

Fig. 3 shows the relationship between the glycine concentration in the incubation mixture and ALA production. The optimal conditions for ALAS measurement was obtained with a substrate concentration of 100 m*M* or more. The concentration of the substrate glycine used in this study was 100 m*M*.

Fig. 4 shows the relationship between the concentration of disodium EDTA in the incubation mixture and ALA formation. The amount of ALA in the reaction mixture was increased with increasing concentration of disodium EDTA, and was constant in the disodium EDTA concentration range 10–50 m*M*.

Fig. 5 shows the relationship between the amount of liver homogenates used for the enzyme assay system and the fluorescence intensity of the ALA derivative corresponding to the ALAS activity. The production of ALA (enzyme activity) was proportional to the amount of the liver homogenate present in the incubation mixture. In the present method, 0.5 ml of the liver homogenate was used as the enzyme source.

The relationship between the production of ALA during incubation and the incubation time was linear up to 60 min (data not shown).

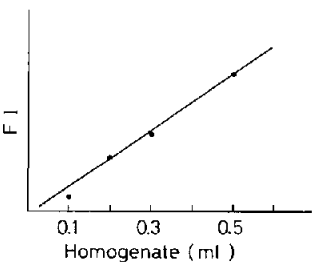


Fig. 5. Relationship between the volume of liver homogenate used for the enzyme assay and the fluorescence intensity (FI) of the ALA derivative (the amount of ALA formed).

We used the described method to measure ALAS activity in liver homogenates from normal mice. The activity values obtained ranged from 5.6 to 11.0 with a mean of 8.4 nmol ALA per g liver per h ($n = 16$). The coefficient of variation, obtained from five repeated assays, was 9%.

Marver *et al.* [2] reported that normal ALAS activity in the rat liver homogenates ranged from 4 to 15 nmol ALA per g liver per h ($n = 24$), as measured by the colorimetric method. Bishop *et al.* [6] developed a fluorimetric coupled-enzyme assay for ALAS, and they reported a value of the ALAS activity of liver homogenate from normal rats of 8.7 nmol ALA per g liver per h. Our values for ALAS were similar. This hepatic ALAS is induced by administration of a porphyrinogenic drug, such as allylisopropylacetamide (AIA). Lien and Beattie [4] reported that the hepatic ALAS activity in male rats treated with AIA was 105 ± 18 ($n = 4$) nmol ALA per g liver per h.

CONCLUSION

The fluorimetric HPLC method for measuring ALAS activity is sensitive and rapid compared with the colorimetric method, and it is applicable to the determination of ALAS activity in animal and human biopsy livers.

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