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Note

Determination of 5-aminolaevulinic acid dehydrase activity in erythrocytes by high-performance liquid chromatography

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5-Aminolaevulinic acid dehydrase (ALA dehydrase, ALA-D), the second enzyme of the haem biosynthetic pathway, catalyses the condensation of two molecules of ALA to form the monopyrrole, porphobilinogen (PBG). This enzyme is inhibited by heavy metals [1—8] and by ethanol [9, 10]. The determination of ALA-D is used to differentiate between heavy metal poisoning and iron-deficiency anaemia, to study the effect of chemicals [11—13], drugs [14], and hormones [15] and to investigate disorders associated with haem biosynthesis such as the porphyrias and various types of anaemia.

This paper describes a high-performance liquid chromatographic (HPLC) method for the determination of ALA-D activity in human erythrocytes. ALA is used as the substrate and the product (PBG) formed is determined by reversed-phase ion-pair chromatography on Hypersil-SAS (short-chain alkylsilica) with methanol—water (22:78, v/v) in the presence of PIC B7 (0.005 *M* 1-heptanesulphonic acid buffered at pH 3.5) as the mobile phase. PBG is separated effectively from ALA and the internal standard 2-methyl-3-carbmethoxy-4-(3-propionic acid)-pyrrole. The method has been used to establish a reference range for ALA-D activity in normal subjects.

EXPERIMENTAL

Materials and reagents

ALA hydrochloride and PBG were obtained from Sigma (London, Great Britain). Glacial acetic acid, methanol, sodium acetate trihydrate, sodium dihydrogen phosphate dihydrate, disodium hydrogen orthophosphate dodecahydrate, trichloroacetic acid and methylacetoacetate were from BDH (Poole,

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Great Britain). Except for methylacetoacetate, all reagents were of AnalaR grade. The ion-pair reagent PIC-B7 (1-heptanesulphonic acid) was purchased from Waters Assoc. (Milford, MA, U.S.A.) and was prepared by diluting one bottle (20 ml) of the reagent to 1 l with water.

Acetate buffer, pH 4.6 was made by diluting 57 ml of glacial acetic acid with 700 ml of water. Sodium acetate trihydrate (136 g) was added and the mixture was made up to 1 l with water.

Phosphate buffer, 0.2 *M*, pH 6.8 was prepared by mixing sodium dihydrogen phosphate dihydrate and di-sodium hydrogen orthophosphate dodecahydrate. A pH of 6.7—6.8 appears to be optimal for the measurement of ALA-D activity by PBG production [16] rather than pH 6.4 which is used in the European Standard Method [17]. ALA substrate (20 m*M*) was made by dissolving 335.2 mg of ALA—HCl in 100 ml of water. The final ALA concentration in the reaction mixture was 4 m*M*, which is optimal or greater by the method of Bonsignore et al. [18] and is the recommended final ALA concentration in the European Standard Method [17].

Preparation of 2-methyl-3-carbmethoxy-4-(3-propionic acid)-pyrrole (internal standard)

ALA-HCl (2.0 mg) was dissolved in 10 ml water to which 10 ml of acetate buffer was added. Methylacetoacetate (1 ml) was added and the mixture was heated in a water bath at 100°C for 20 min. Aliquots (1.0 ml) of the mixture were dried under reduced pressure, stored at 4°C, and redissolved in 10 ml of water prior to use.

Construction of calibration curve

A standard solution of PBG was made up by weight to approximately 100 μM and the exact concentration measured by the method of Mauzerall and Granick [19]. This solution was diluted to give a range of PBG solutions of concentrations 5—50 μM. A 0.5-ml aliquot of each PBG solution was mixed with 0.1 ml of the internal standard and 20 μl were injected into the chromatograph. The calibration curve was constructed by plotting PBG concentration against the ratio of the peak heights of PBG:internal standard. The curve was linear over the range tested.

Preparation of enzyme solution and incubation procedure

ALA-D was measured on blood collected into lithium heparin. Packed cell volume (PCV) was measured using a Coulter counter. The blood samples were used immediately or stored in an ice bath at 1—2°C until the enzyme activities were measured (within 2 h). The whole blood (0.5 ml) was haemolysed in water (3.25 ml) and 1.5 ml of the haemolysate (corresponding to 0.2 ml of whole blood) was used immediately as the enzyme solution. ALA substrate (0.5 ml) and phosphate buffer (0.5 ml) were added to each of three 10-ml test tubes. Trichloroacetic acid (100 g/l) was added to one of the tubes to act as a blank. All the tubes were placed in a constant-temperature water bath at 37°C for 5 min and the haemolysate (1.5 ml) was then added to each of the tubes and mixed thoroughly. The mixture was incubated for 1 h at 37°C and the reaction was terminated by adding 1 ml trichloroacetic acid solu-

tion. After centrifugation for 5 min at 1500 *g*, 0.5 ml of the supernatant was withdrawn into a vial containing 0.1 ml of the internal standard and 20 μl of the well mixed solution were injected into the chromatograph.

High-performance liquid chromatography

A Shandon Southern (Runcorn, Great Britain) liquid chromatograph with a variable-wavelength UV detector set at 240 nm and 0.02 a.u.f.s. was used. Injection was via a Rheodyne 7120 injection valve fitted with a 20-μl loop. A 5-μm particle Hypersil-SAS (C_1) column (10 cm × 5 mm I.D.) was used with methanol—PIC B7 in water (22:78, v/v) as the eluent. The pressure was set at 80 bar and the flow-rate was 1.2 ml/min.

Calculation

The PBG concentration in the incubation mixture was read off the calibration curve and the enzyme activity expressed as μmol of PBG formed per min per l of erythrocytes (RBC) at 37°C. Thus

$$\text{ALA-D activity} = \frac{X \times 3.5}{60 \times 0.2} \cdot \frac{100}{\text{PCV}}$$

where X is the concentration of PBG, 3.5/0.2 is the dilution factor and PCV is the percentage packed cell volume.

RESULTS AND DISCUSSION

HPLC has been used for the separation of ALA and PBG [20]. The present system, using ion-pair chromatography on a short-chain alkylsilica (C_1) column, improves the resolution and shortens the analysis time. With methanol—water containing the pairing ion 1-heptanesulphonic acid as the eluent effective separation of ALA, PBG and the internal standard is achieved (Fig. 1). The system is also satisfactory for the resolution of PBG and the internal standard from the endogenous impurities (Fig. 2a, b). When treated with Ehrlich's reagent (*p*-dimethyl-aminobenzaldehyde) the peak corresponding to PBG isolated from the incubation mixture by HPLC gave a typical absorption spectrum with an absorption maximum at 553 nm. This confirms the identity of

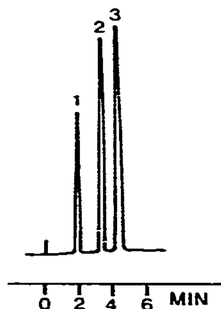

Fig. 1. Separation of ALA (1), PBG (2) and internal standard (3). Stationary phase, Hypersil-SAS; mobile phase, methanol—PIC-B7 in water (22:78, v/v); pressure, 80 bar; flow-rate, 1.2 ml/min; detection, 240 nm.

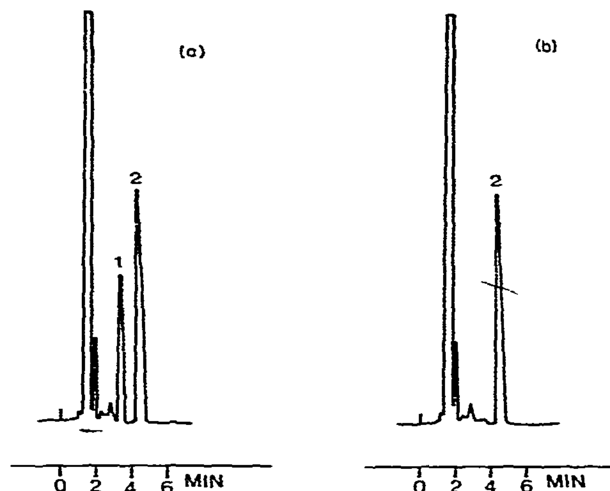

Fig. 2. Separation of PBG (1) and internal standard (2) in incubation mixture. (a) Test; (b) blank. Chromatographic conditions as Fig. 1.

the PBG peak in addition to evidence from capacity ratio measurement. This peak is absent (Fig. 2a) in the incubation mixture pretreated with trichloroacetic acid.

Using this method measured PBG production in 25 normal volunteers was found to be 17.50 ± 3.84 μmol/l RBC/min (Fig. 3). This is almost identical to the values given by Bonsignore et al. [18] (16.7 ± 3.3 μmol/l RBC/min after conversion from enzyme units) and by Tomokuni [4] using the method of Bonsignore et al. [18] (17.4 ± 3.2 μmol/l RBC/min), but is lower than that obtained by Burch and Siegal [21] using a different buffer system (29.3 ± 7.2 μmol/l RBC/min after conversion from enzyme units). The reproducibility of the method was shown by determination of ALA-D activity in one subject on six different occasions. This gave values of 19.25 ± 0.39 (S.D.) μmol PBG/l RBC/min (C.V. 2.03%).

The published methods for measuring ALA-D in terms or PBG production have not attempted to make any correction for loss of PBG, although this compound is unstable on incubation. It is obviously difficult and time consuming to perform an accurate correction for PBG loss in individual cases since in an unknown sample it is not known what concentration of PBG will be achieved in the incubation mixture and the rate of loss will depend at least partly on this. The European Standard Method [17] converts directly from measured PBG production to ALA consumption by multiplying by 2; in the present case this would give a mean of 35.0 ± 7.7 μmol ALA consumed/l RBC/min (Fig. 3).

In two alcoholic patients PBG production was markedly reduced, giving ALA-D activities of 4.9 and 5.4 μmol/l RBC/min, confirming that the present method is able to detect activity of the enzyme.

CONCLUSION

ALA-D activity in erythrocytes can be satisfactorily determined by mea-

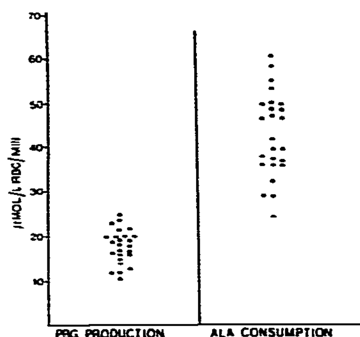

Fig. 3. ALA-D activity in 25 normal subjects determined by HPLC. Results are expressed as μmol/l RBC/min of PBG produced and ALA consumed.

suring the amount of PBG formed on a reversed-phase column with methanol—aqueous 1-heptanesulphonic acid (22:78, v/v) as the mobile phase. ALA was used as the enzyme substrate and 2-methyl-3-carbmethoxy-4-(3-propionic acid)-pyrrole was used as the internal standard. The mean value obtained by this method in 25 normal subjects was found to be 17.50 μmol/PBG produced/l RBC/min (S.D. ± 3.84, observed range 10.3—25.0). This was well correlated with existing methods for ALA-D determination.

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