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Determination of 5-aminolevulinic acid in blood plasma, tissues and cell cultures by high-performance liquid chromatography with electrochemical detection

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Abstract

A simple and sensitive method for determining 5-aminolevulinic acid (ALA) in biological samples is described. ALA is derivatized with *o*-phthaldehyde to give a compound with favorable properties for high-performance liquid chromatography with electrochemical detection. The method does not require extensive pretreatment of the samples and its detection limit is in the range of 1 pmol/20 μl injection. This method was applied to the determination of plasma ALA from normal and lead-exposed subjects, where 0.26±0.08 μ*M* (*n*=30) and 2.6±0.75 μ*M* (*n*=30), respectively were found. We also determined ALA in rat tissues, namely liver and brain, and the uptake of ALA by cultured fibroblasts and hepatocytes to illustrate the diversified applicability of the method. © 1997 Elsevier Science B.V.

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1. Introduction

The heme precursor, 5-aminolevulinic acid (ALA), accumulates in certain porphyric states, such as acute intermittent porphyria and lead poisoning, and is thought to be a neurotoxic agent [1,2]. ALA has been found to yield H_2O_2 and deleterious oxyradicals through metal-catalyzed oxidation, leading to biochemical and cellular alterations in rats, which might be relevant to the development of porphyric syndromes [3]. To associate the hypothetical neurotoxic potential and the prooxidant properties of ALA in porphyric diseases, a reliable procedure for ALA determination in biological fluids is needed. The level of ALA in human blood plasma is normally very low (0.1 μ*M*), but is markedly elevated in porphyric subjects (19 μ*M*) [2], along with an increase in urinary ALA excretion [5]. The methods generally used for measuring ALA are based on the spectrophotometric assay described by Mauzerall and Granick [4], which is relatively non-specific, or on techniques with high sensitivity and specificity but difficult applicability in clinical laboratory routine. Very sensitive methods include that described by Gorshein [5], based on the derivatization of ALA to a pyrrole derivative whose properties are suitable for gas–liquid chromatography (GLC) with electron-capture detection and that described by Minder [6],

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based on the derivatization of ALA to a dansyl derivative that is suitable for high-performance liquid chromatography (HPLC) with fluorescence detection. These two methods, however, give widely different values for the levels of ALA in plasma, and require a very extensive pretreatment of the samples, which impairs their routine use in laboratories.

The method described here involves a relatively facile derivatization of ALA with *o*-phthalaldehyde (OPA) before injection, and quantitation by reversed-phase HPLC with electrochemical detection. The reaction of OPA with amino acids in the presence of a thiol reagent to produce isoindole derivatives with electrochemical activity was first reported by Joseph and Davies [7]. Based on the electrochemical activity of indoles, they demonstrated that OPA–β-mercaptoethanol derivatives of amino acids undergo anodic oxidation at moderate potential, enabling the use of HPLC–electrochemical detection. The presence of an amino group in the structure of ALA prompted us to investigate the possibility of using this derivatization procedure in the electrochemical determination of ALA in biological samples. We have found that, besides overcoming the aforementioned negative methodological aspects, derivatization of ALA with OPA provides a specific and sensitive procedure for ALA determination in biological samples. In this study, we describe the application of this method to measuring plasma ALA in lead-exposed workers, tissue ALA in liver and brain of rats, and cell culture uptake of ALA in fibroblasts and hepatocytes.

2. Experimental

2.1. Reagents

5-Aminolevulinic acid hydrochloride (ALA–HCl), sucrose, dithiothreitol (DTT), EDTA, HEPES, penicillin, streptomycin, heparin, β-mercaptoethanol, Nonidet P40 (NP-40), RPMI cell culture medium, OPA, perchloric acid, bovine serum albumin, 5-aminolevulinic acid dehydratase and Folin Ciocalteu's reagent were purchased from Sigma (St. Louis, MO, USA). DME (Dulbecco's modified Eagle medium) cell culture medium was from Gibco (New York, USA). Fetal calf serum was from Cultilab (Campinas, Brazil). All other reagents and solvents used were of analytical grade from Merck (Darmstadt, Germany). Acetonitrile for the mobile phase was of chromatographic grade. Water was bidistilled and subsequently Milli-Q deionized.

2.2. Apparatus

Isocratic liquid determinations of the ALA–OPA derivative were performed using a HPLC system that consisted of an LC10AD pump coupled to an LECD 6A electrochemical detector from Shimadzu (Kyoto, Japan). The detector's working electrode (glassy carbon) was maintained at $\cong +0.45$ V vs. Ag/AgCl (at 3 *M* KCl) and its signal was delivered to a 386 ASA computer with data collection and handling provided by Scientific Software (San Ramon, CA, USA). All chromatography was performed with Waters Associates (Milford, MA, USA) 4.0 μm C_{18} columns (15.0 cm×3.9 mm I.D.) and with 0.05 *M* phosphate buffer, pH 7.0, containing 10% acetonitrile and 2.4 m*M* EDTA as the mobile phase. The samples were introduced into a 7125-055 injector equipped with a 20-μl external loop from Rheodyne (Cotati, CA, USA) and eluted with the mobile phase circulated at 1.0 ml/min.

2.3. Procedures

2.3.1. Derivatization

OPA reagent was made up in the proportions described by Lindroth and Mopper [8] by dissolving 27 mg of OPA in 500 μl of methanol, followed by the addition of 5.0 ml of 0.1 *M* sodium tetraborate and 20 μl of mercaptoethanol. A 10-μl volume of sample (standard solution or biological material) was placed in a small conical reaction vessel, to which 5 μl of OPA reagent (36 m*M*) and 35 μl of water were added. The sample was then incubated for 1 min at room temperature and a 20-μl aliquot μl was injected into the HPLC system. Standard ALA solutions (30.0 μ*M*) were prepared in water.

2.3.2. Preparation of biological samples

Blood samples were collected from normal subjects and lead-exposed workers, under informed consent, in plastic tubes containing heparin and were immediately centrifuged at 800 *g*. The separated plasma was kept at −20°C until assayed. On the day

of assay, the plasma was deproteinized using $HClO_4$ at a final concentration of 0.8 *M* and the supernatant was neutralized by the addition of $NaHCO_3$ crystals to ca. pH 7.6. Tissue samples were collected from male six-month old Wistar rats maintained at 23±2°C and 50±10% relative humidity, and fed a normal commercial diet and water ad libitum. Animals were quickly anesthetized with ether and their livers and brains were dissected. The tissues were then promptly rinsed, sliced and homogenized in 10 m*M* HEPES buffer, pH 7.2, containing 0.25 *M* sucrose and 0.5 *M* EDTA, at 1:3 (w/v). The homogenate obtained was then centrifuged at 800 *g* for 5 min and the supernatant was maintained at −20°C until the assay of ALA. Cell samples from an established line of V79 Chinese hamster ovary fibroblasts or from BRL cells, were grown in DME, pH 7.0, supplemented with 10% (v/v) fetal calf serum, 472 units of penicillin/ml and 94 mg of streptomycin/ml. Cells were incubated in a humidified CO_2–air atmosphere (1:19) at 37°C. Cell were treated by addition of 1.0 m*M* ALA to the cell medium at different times. To obtain cell extracts, $1 \cdot 10^6$ cells were washed twice with phosphate buffered saline (137 m*M* NaCl, 2.68 m*M* KCl, 8.1 m*M* Na_2PO_4, 1.47 m*M* KH_2PO_4, pH 7.2), detached from the flask by the addition of 1 ml of 1 m*M* EDTA in the same buffer and were collected in a 1.5 ml eppendorf tube. The cell pellet, obtained by centrifugation at 12 000 *g* for 5 min, was resuspended with 20 μl of Munro's buffer (10 m*M* HEPES, pH 7.2, 2 m*M* $MgCl_2$, 40 m*M* KCl, 5% glycerol and 1 m*M* DTT) supplemented with 0.2% NP-40 and homogenized by resuspension using a micropipet. After adding another 40 μl of Munro's buffer, the mixture was centrifuged at 12 000 *g* for 15 min. For determinations of ALA, the supernatant was assayed immediately, or after storage for one day at −20°C. The protein content of tissues and cell samples was determined by the method of Lowry et al. [9].

3. Results and discussion

3.1. Electrochemical detection and validation

The first step to optimize the chromatographic conditions for the assay of the ALA–OPA derivative was to determine the optimum electrochemical detection potential. As shown in Fig. 1, the ALA–OPA derivative has a relatively low half-wave potential ($E_{1/2} \cong +0.45$ V vs. Ag/AgCl). This potential is compatible with that obtained for amino acid–OPA derivatives and highlights one of the advantages of using this kind of derivatization, that is, to allow the electrochemical detection of compounds in the presence of lower background current (1–3 nA). As observed for amino acid–OPA derivatives, the ALA–OPA derivative shows a less steeply increasing electrochemical activity at the working potential than do simpler compounds (e.g., catechols, indoles), probably due the interaction of multiple oxidisable groups in the OPA derivatives [7].

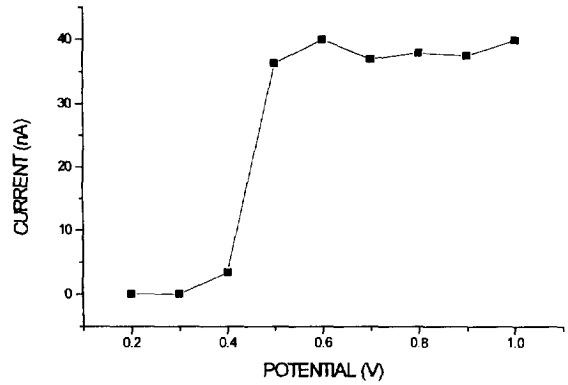

Fig. 1. Potential profile for the ALA–OPA derivative. The mobile phase was 0.05 *M* phosphate buffer, pH, 7.0 containing 2.39 m*M* EDTA and 10% acetonitrile. The sample (20 μl) injected contained 5 μl of standard ALA solution (30 μ*M*), 5 μl of OPA stock solution (36 m*M*) and 40 μl of deionized water. The elution was isocratic with a flow-rate of 1.0 ml/min and the potential of the working electrode varied from 0.1 to 1.0 V.

Fig. 2 represents the electrochemical response as a function of reaction time for the ALA–OPA derivative. Separate reaction mixtures were incubated for predetermined times; the resulting derivative peak heights were plotted against the reaction time. Derivatization was maximal until 5 min of incubation, and so injections were made at 2 min, for convenience. The calibration curve for standard derivatized ALA can be represented by equation $y=0.45+9.80x$, and that of the ALA–OPA derivative in plasma spiked with authentic ALA by the equation $y=0.65+23.90x$. The linearity of the curves evaluated by the correlation coefficients obtained (0.99882/0.99967) was within the range of 2–200

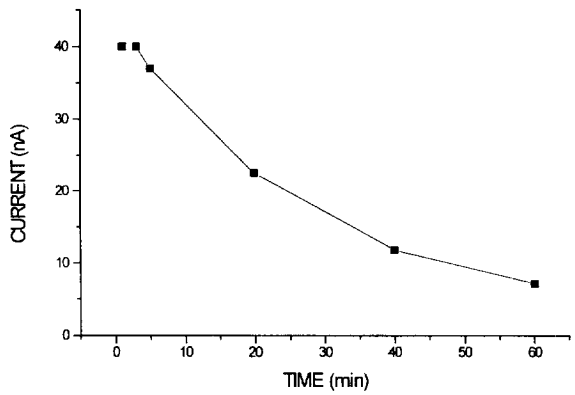

Fig. 2. Electrochemical response of the ALA–OPA derivative as a function of reaction time. The same reaction mixture as described in Fig. 1 and the chromatographic conditions described in Section 2 were used to analyze the stability of the ALA–OPA derivative. The incubation time varied from 1.0 to 60.0 min.

pmol/20 μl injection. The coefficients of variation for replicate reaction mixtures ($n=5$) for these equations were 2 and 15%, respectively, for peak area and 0.2% for retention time for all points. The detection limit for authentic ALA in water and biological samples by this method was 1.0 pmol/injection, based on a signal-to-noise ratio of 3. The recuperation was determined by comparing the slopes of the aforementioned equations. The slopes would be identical for 100% recovery. Considering all calibration curves obtained in this study, the average recovery of ALA obtained was 100±1.8%.

3.2. Applications

Fig. 3 shows the chromatographic profiles of ALA in rat liver extract, the plasma of an individual not exposed to lead and the plasma of a lead-exposed subject. Confirmation that the ALA–OPA derivative did not co-chromatograph with other components of the biological sample was obtained by treating the sample with ALA dehydratase (5 μl, giving 0.01 unit of activity) for 30 min at 37°C and observing the complete disappearance of the ALA peak in the HPLC trace. We also evaluated the optimum concentration for the OPA reagent and selected the one yielding maximal derivatizing ability and minimum noise due the presence of extraneous peaks (data not shown).

Plasma ALA concentrations were measured in 30 subjects not exposed to lead and in 30 subjects exposed to lead concentrations higher than 30 μg/dl. The average values obtained for these groups were 0.26±0.08 and 2.58±0.75 μ*M*, respectively. These data agree well with those obtained using GLC analysis by Gorshein and Webber [10]. Using this method, the level of ALA in plasma of normal subjects was found to range from 0.024 to 0.27 μ*M*, which agrees well with the average value obtained herein. However, the GLC method, although extremely sensitive, is excessively laborious, hindering its routine use in a laboratory [5].

The level of plasma ALA observed by HPLC–fluorescence detection in normal subjects [6] was ten times higher than that reported here by us. The current method has greater applicability in routine laboratory use, since the HPLC method associated with fluorescence detection, like the GLC method, requires extensive pretreatment of the biological samples. Thus, the HPLC method reported herein allows the relatively fast, simple, accurate and sensitive measurement of plasma ALA, which might be useful for evaluating and monitoring patients with acute hepatic porphyrias and plumbism.

Using the HPLC–electrochemical detection method, we have also characterized the basal levels of ALA in liver (10.1±0.9 nmol/mg protein) and total brain (1.1±0.15 nmol/mg protein) homogenates of rats. These values agree with the data reported by McGillion et al. [11] when studying tissue uptake of ALA in rats. Furthermore, the possible utility of the method for determining ALA in cell culture is illustrated in Fig. 4 for ALA uptake in fibroblasts and hepatocytes. The level of ALA in ALA-treated fibroblasts (A) is greater than that found in hepatocytes (B), but shows a less homogeneous profile of uptake than that found in the hepatocytes (Fig. 4).

3.3. Conclusions

The method described here allows the quantification of ALA with the same accuracy and precision as found for other methods [5,6], however, it is much less laborious, which makes it more useful as a routing laboratory test. It may be useful for monitoring ALA overload in porphyric patients [1], in model studies of ALA-treated animals, and in tumor

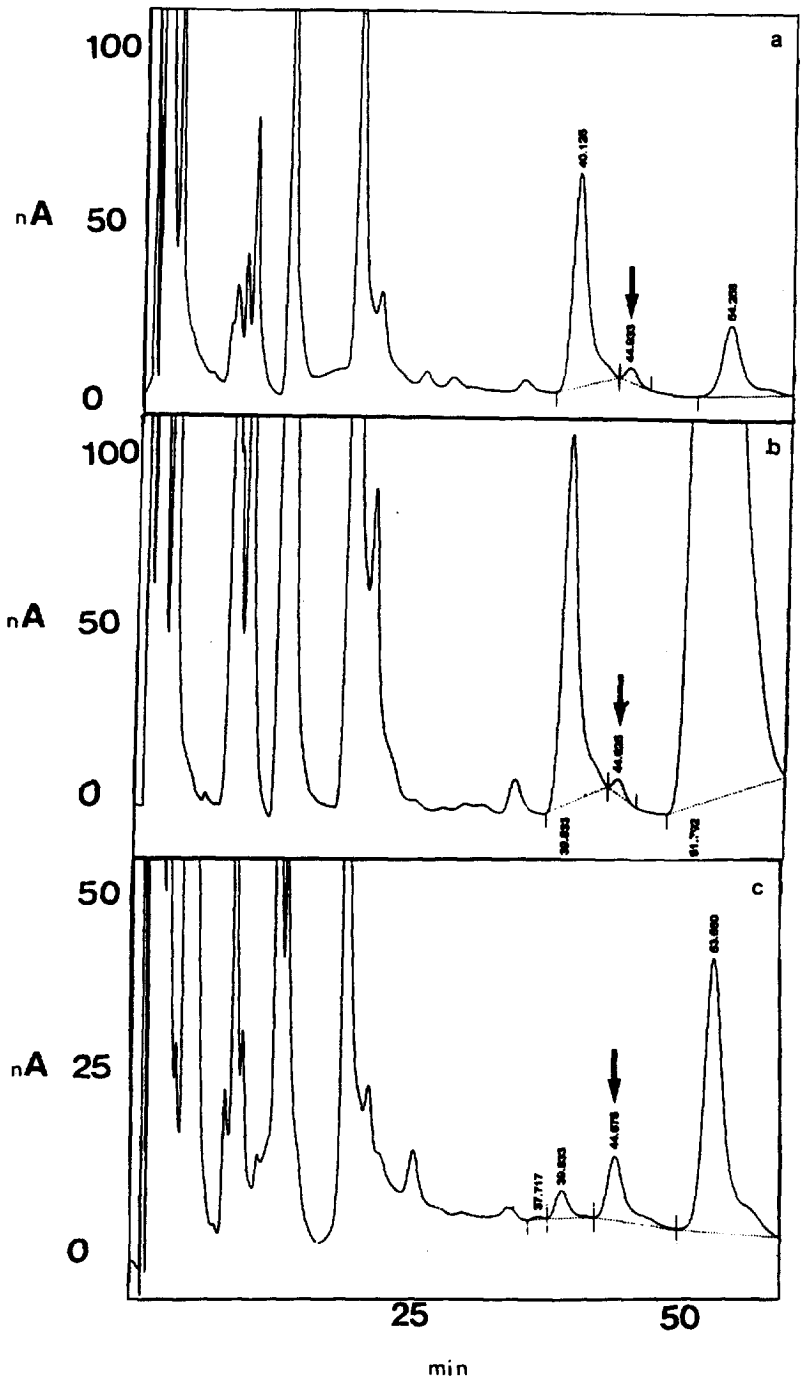

Fig. 3. Chromatograms of ALA in different biological samples. Chromatograms of ALA in rat liver extract (a), ALA in plasma of a subject exposed to lead (b) and ALA in cell cultured medium (c). The ALA peak has a retention time equivalent to 44.6 min and is represented by an arrow. The chromatographic conditions and the sample derivatization procedure are described in Section 2.

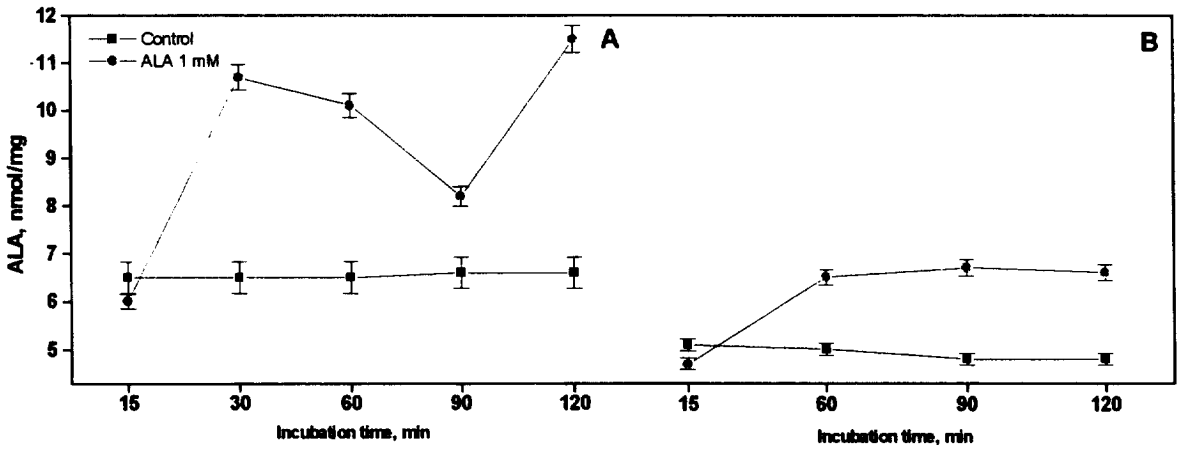

Fig. 4. ALA uptake by cells in culture. Cells were incubated in the presence of 1 m*M* ALA added to the culture medium, as described in Section 2. Intracellular ALA levels were determined at each indicated time in the cellular extracts. (A) Fibroblast cells and (B) hepatocytes. Control, untreated cells.

photodynamic therapy based on endogenous generation of porphyrins from administered ALA [12].

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