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Measurement of 4,5-dioxovaleric acid by high-performance liquid chromatography and fluorescence detection

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Abstract

In this work we describe a sensitive method for the detection of 4,5-dioxovaleric acid (DOVA). 4,5-Dioxovaleric acid is derivatized with 2,3-diaminonaphthalene to form 3-(benzoquinoxalinyl-2)propionic acid (BZQ), a product with favorable UV absorbance and fluorescence properties. The high-performance liquid chromatographic method with UV absorbance and fluorescence detection is simple and its detection limit is approximately 100 fmol. This method was used to detect 4,5-dioxovaleric acid formation during metal-catalyzed 5-aminolevulinic acid (ALA) oxidation. Iron and ferritin were active in the formation of 4,5-dioxovaleric acid in the presence of 5-aminolevulinic acid. In addition, HPLC–MS–MS assay was used to characterize BZQ. The determination of 4,5-dioxovaleric acid is of great interest for the study of the mechanism of the metal-catalyzed damage of biomolecules by 5-aminolevulinic acid. This reaction may play a role in carcinogenesis after lead intoxication. The high frequency of liver cancer in acute intermittent porphyria patients may also be due to this reaction.

Keywords: 4,5-Dioxovaleric acid; 5-Aminolevulinic acid

1. Introduction

4,5-Dioxovaleric acid (DOVA) is the final oxidation product of 5-aminolevulinic acid (ALA), a precursor of porphyrin IX in the biosynthesis of heme (Fig. 1). It can accumulate in liver, brain, and other organs under pathological conditions such as acute intermittent porphyria (AIP), tyrosinosis [1,2] and lead poisoning [3]. It is well established that the oxidation of ALA can be catalyzed by transition metal ions like iron and that this reaction generates reactive oxygen species through an ALA enoyl radical as an intermediate [4,5]. These carbon centered radicals, together with superoxide anions, are able to release iron from ferritin during the oxidation of ALA [6] (Fig. 2). Lipid peroxidation also occurs during oxidative stress, and several other dialdehydes are formed. Among the most abundant aldehydes produced are malondialdehyde, 4-hydroxyhexenal and 4-hydroxynonenal [7]. Detection methods for these compounds have been reported [8,9].

The α-ketoaldehyde corresponding to ALA, DOVA, may form adducts with the amino groups of DNA bases. In fact, DOVA is able to add to the exocyclic amino group of the guanine ring yielding

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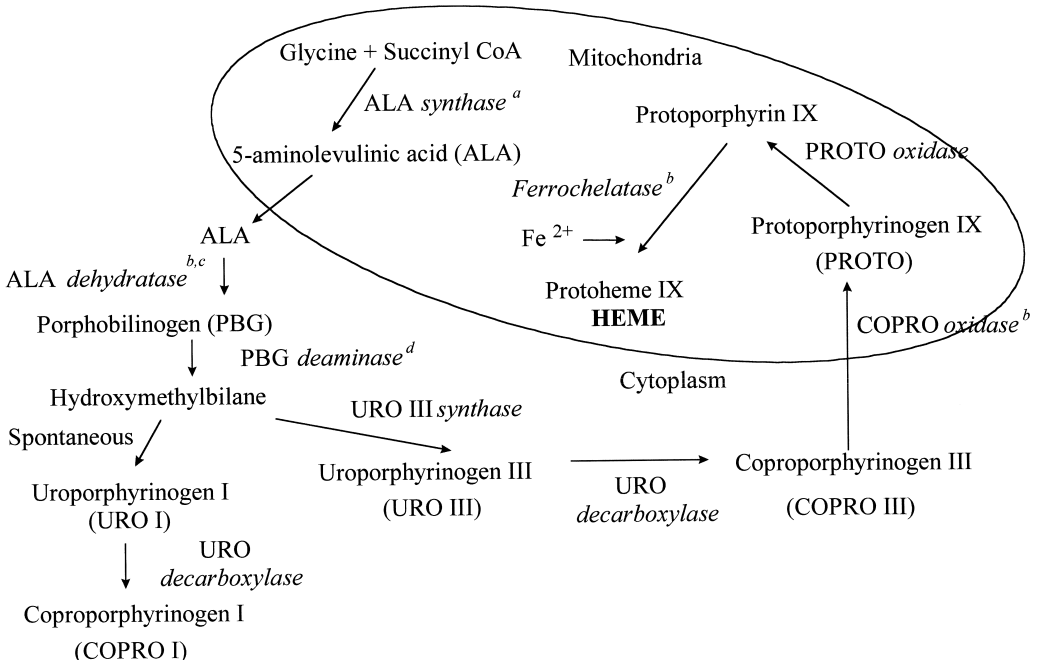

Fig. 1. Heme biosynthetic pathway and disorders associated with 5-aminolevulinic acid overload. (a) Negative feedback inhibition by heme in AIP activated by certain drugs and metabolites. (b) Inhibition by lead (lead poisoning). (c) Inhibition by succinyl acetone (tyrosinosis). (d) Deficient biosynthesis in AIP.

two predominant diastereoisomers as the result of the formation of a Schiff's base [10]. Aldehydes adducts are known to be mutagenic and are efficient alkylating agents [11,12]. A high incidence of primary liver cancer (PLC) has been reported in AIP [13–15], and is associated with the frequent acute attacks of symptoms when the ALA plasma level rises about 100-fold [16].

The formation of quinoxaline derivatives is a common choice for alfa dialdehydes analyses. Different diamino compounds [17–19] have been used to achieve this objective [20]. In this work, the benzoquinoxaline derivative is formed by the condensation of 2,3-diaminonaphthalene (DAN) with DOVA [21], followed by HPLC with UV absorbance and fluorescence detection. The reaction is quantified by the help of a calibration curve established with authentic DOVA-DAN. In this study, we describe a method for the measurement of the DOVA level in samples from in vitro experiments.

In this context it is of great interest to measure the formation of DOVA during the metal-catalyzed oxidation of ALA. We investigated this reaction under the catalysis of iron and isolated ferritin. It is interesting to note that iron deposits were found in human liver biopsies of porphyria carriers [22]. The method described here may be used to evaluate the amount of DOVA in symptomatic AIP carriers where prevalent PLC occurs.

2. Experimental

2.1. Reagents

2,3-Diaminonaphthalene was obtained from Merck (Darmstadt, Germany) and recrystallized three times from hot ethanol. 4,5-Dioxovaleric acid was synthesized by the hydrogenation of the ozonide of benzilidene levulinic acid as described by Dörnemann and Senger [21]. All other reagents were purchased from Sigma (St. Louis, MO, USA). The water was purified by a NANOpure system from Barnstead Co. (IA, USA).

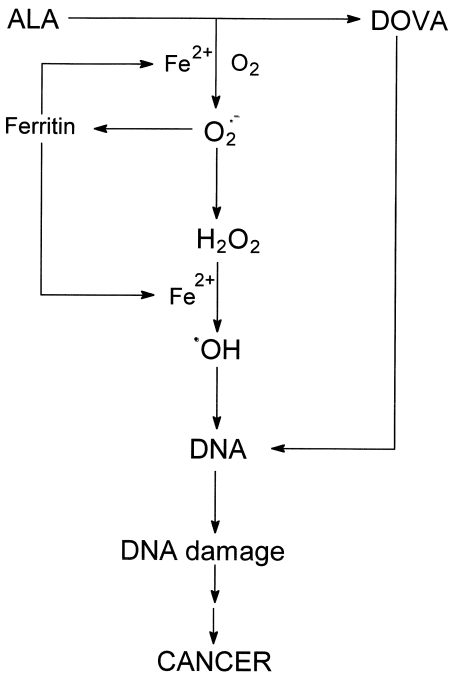

Fig. 2. Putative mechanism for the action of ferritin on the oxidation of 5-aminolevulinic acid. This reaction is proposed to produce reactive oxygen species and 4,5-dioxovaleric acid which subsequently damage DNA.

2.2. Equipment

The HPLC system consisted of a LC 10AD pump (Shimadzu, Tokyo, Japan) connected to a 7125 Rheodyne injector (Cotati, CA, USA). For analytical purposes, the system was equipped with a 250×4.6 mm I.D. (particle size 5 μm) Spherex 5 C_{18} column (Phenomenex, Torrance, CA, USA) reversed-phase and with a pre-column Spherex C_{18} guard, 30×4.6 mm I.D. (particle size 5 μm) connected to either a Shimadzu SPD-M10AV diode-array or Shimadzu SPD-10A UV spectrometer set to 268 nm (Shimadzu), and a RF-551 fluorescence detector (Shimadzu) set to 350 nm excitation and to 540 nm emission wavelength, high sensitivity, gain×16 and range=1. The isocratic eluent was a 50 m*M* solution of sodium phosphate, pH 7.0, containing 20% acetonitrile. The flow-rate was 1.0 ml/min. Data acquisition was performed using the software Shimadzu CLASS-LC10, version 1.6 (Shimadzu). Electrospray ionization (ESI) mass spectrometry analyses in the positive mode were performed in a Platform II mass spectrometer (Micromass, Altricham, UK). A Waters 501 pump (Millipore, Bedford, MA, USA) using a splitter was used to pump the eluent, 1:1 mixture of water and acetonitrile, at a final flow-rate of 5–10 μl/min. The samples (0.8 μg/μl) were injected through a 50 μl Rheodyne loop into the mobile phase consisting of an aqueous solution of 0.2% formic acid. The source temperature was maintained at 70°C, and flow-rates of drying and nebulizing gas (nitrogen) were optimized at 200 and 29 l/h, respectively. The cone voltage was maintained at 23 V, and the capillary and HV electrode potentials were at 3.50 and 0.69 kV, respectively. Full-scan data were acquired over a mass range of 115–400 Da. The data were processed and transformed into values of molecular masses on mass scale using the Mass Lynx NT™ data system 2.22 version (Micromass).

2.3. Procedures

2.3.1. Derivatization of 4,5-dioxovaleric acid

The formation of benzoquinoxaline-2-propionic acid, the condensation product of DOVA with DAN, was carried out as described by Porra et al. [23]. Briefly, DOVA (50 μg/ml) and DAN (100 μg/ml) were condensed by heating at 60°C for 1 h in the dark in 10 m*M* Tris-HCl buffer, pH 8.0, with 25% ethanol (v/v) to maintain the diamino compound in solution. This sample was analyzed by mass spectrometry and HPLC system with UV absorbance and fluorescence detection.

2.3.2. Incubation of ALA in the presence of iron

5-Aminolevulinic acid (100 μ*M*) and Fe^{2+} (20 μ*M*) were incubated at 37°C for 2 h. An aliquot of 100 μl was collected and DAN (317 μ*M*) was added. The sample was incubated at 60°C for 1 h in 10 m*M* Tris–HCl buffer, pH 8.0. After this, an aliquot of 100 μl was injected into the HPLC system.

2.3.3. Incubation of ALA in the presence of ferritin

Horse spleen ferritin was pre-purified to remove loosely bound iron. Thus, it was incubated for 1 h at 4°C in 20 m*M* Tris–HCl buffer, pH 7.4, containing 140 m*M* NaCl and 10 m*M* EDTA and then purified

on Sephadex G-25, equilibrated with 20 m*M* Tris–HCl buffer, pH 7.4, with 140 m*M* NaCl. The concentration of purified ferritin was quantified by the Bradford method [24]. For the test, 5-aminolevulinic acid (1 m*M*) was incubated with 17 μg of ferritin in 150 μl of Tris–HCl buffer 0.1 M, pH 8.0, at 37°C for 1 h. The ferritin was then precipitated by vigorous shaking with the same volume of chloroform and centrifuged at 6600 *g* for 2 min. Then, DAN were added at a final concentration of 317 μ*M* and the reaction mixture incubated for 1 h at 60°C. Finally, 20 μl aliquots of the supernatant were injected into the HPLC system.

3. Results and discussion

Due to the high reactivity of α-ketoaldehydes, the reaction between DOVA and DAN leads to the formation of the BZQ (Fig. 3) which can be separated by reversed-phase HPLC (Fig. 4). With our method we can isolate the benzoquinoxaline without pre-purification from the excess DAN, before it is subjected to the spectrophotometric assays [23]. The qualitative determination of the DOVA derivative was performed with a UV absorbance and a fluorescence detector in series, taking advantage of the UV and fluorescence characteristics of the BZQ. The formation of BZQ was confirmed by the identification of the molecular ion in the mass spectra analysis (Fig. 5) and by the UV spectra (Fig. 4 inset) which

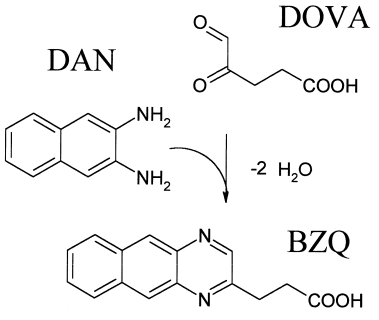

Fig. 3. Structure and formation of benzoquinoxaline derivatives by the condensation of 2,3-diaminonaphthalene with 4,5-dioxovaleric acid resulting from 5-aminolevulinic acid oxidation.

was similar to that obtained by Dörnemann and Senger [21].

3.1. UV absorbance and fluorescence detection and mass spectrometric analysis

Our first methodological aim was the optimization of the HPLC conditions for the measurement of the DOVA-DAN derivative, starting with the incubation conditions for the analysis as described by Dörnemann and Senger [21]. The peak corresponding to the reaction product, BZQ, was observed in the UV elution profile at a retention time of 9.8 min, whereas the excess of DAN eluted at 26.6 min (Fig. 4A). At the chosen excitation (λ=350 nm) and emission (λ=540 nm) wavelengths for fluorescence detection, only the BZQ was identified at a retention time of 10.4 min in fluorescence (Fig. 4).

The fluorescence calibration curve for standard derivatized DOVA can be represented by equation $y=62.1\times10^4\ x$, and the UV curve by $y=3.8\times10^4\ x$. The linearity of the curves evaluated by correlation coefficients for the UV and fluorescence curves was 0.993 and 0.998, respectively, within the range of 5–60 μ*M*. The detection limit for authentic DOVA in Tris-HCl was 100 fmol/injection, based on a signal-to-noise ratio of 3. The coefficients of variation for replicate reaction mixtures (n=5) for these equations were 2 and 15% for peak area and 0.2% for retention time.

The UV spectra of BZQ was similar that obtained by Dörnemann and Senger [21] with a maximum absorbance in 268 nm (Fig. 4 inset).

The ESI mass spectrum of the BZQ derivative by the condensation DAN with DOVA in the positive mode (Fig. 5) exhibits a pseudomolecular ion M+H at m/z 253. 2,3-Diaminonaphthalene showed a peak at m/z 159 and the m/z 122 peak corresponds to the Tris–HCl buffer.

3.2. Detection of DOVA after the ALA oxidation in the presence of iron and ferritin

The development of this method enabled us to follow the formation of DOVA as a final product of ALA oxidation in the presence of iron and ferritin.

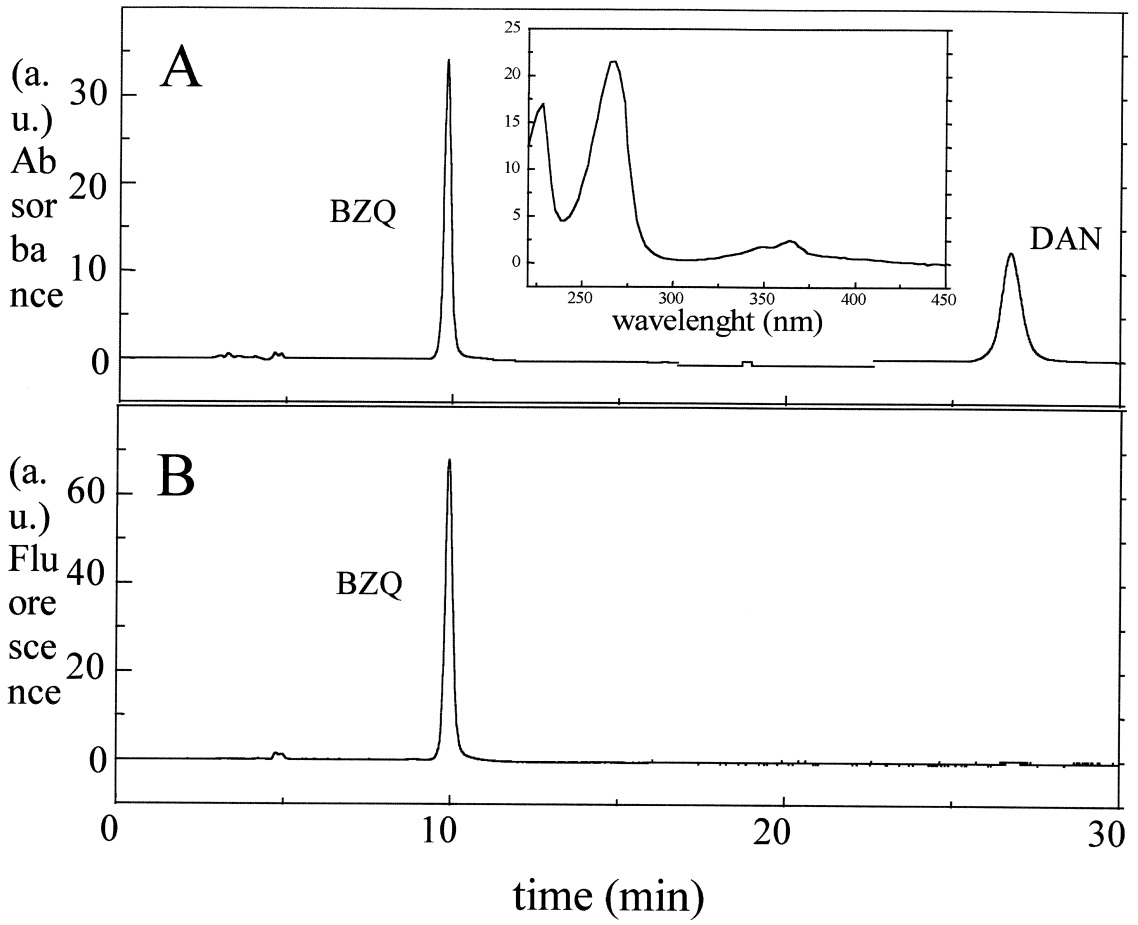

Fig. 4. HPLC elution profiles of the benzoquinoxaline derivative of 4,5-dioxovaleric acid resulting from the oxidation of 5-aminolevulinic acid (100 μ*M*) in the presence of Fe^{+2} (20 μ*M*) performed by UV absorbance (A) and fluorescence (B) detection. Inset A shows the BZQ UV spectrum. The same results were obtained in the presence of ferritin.

This determination is important since DOVA can react with DNA bases [10], which can then be involved in the mechanisms of the carcinogenesis process in AIP patients. The incubation of ALA in the presence of iron leads to the formation of DOVA that reacts with DAN resulting in the BZQ derivative which was detected by UV absorbance and fluorescence HPLC detection system (Fig. 4A and B). 5-Aminolevulinic acid (10 μ*M*) in the presence of iron (20 μ*M*) leads to the formation of DOVA (8 μ*M*), measured by the integration of BZQ peak area. The incubation with ferritin showed similar results (data not shown).

4. Conclusions

In this work we used a sensitive method to detect DOVA that is generated during ALA oxidation mediated by iron and ferritin.

The formation of ALA from glycine and succinyl CoA (Fig. 1) via the classical Shemin-pathway [25], is the first step in heme biosynthesis, and is the rate limiting enzymatic reaction in this biosynthesis. Heme is the prosthetic group of a great number of enzymes and is responsible for the structure and function of various proteins [1]. 5-Aminolevulinic acid can accumulate under pathological conditions,

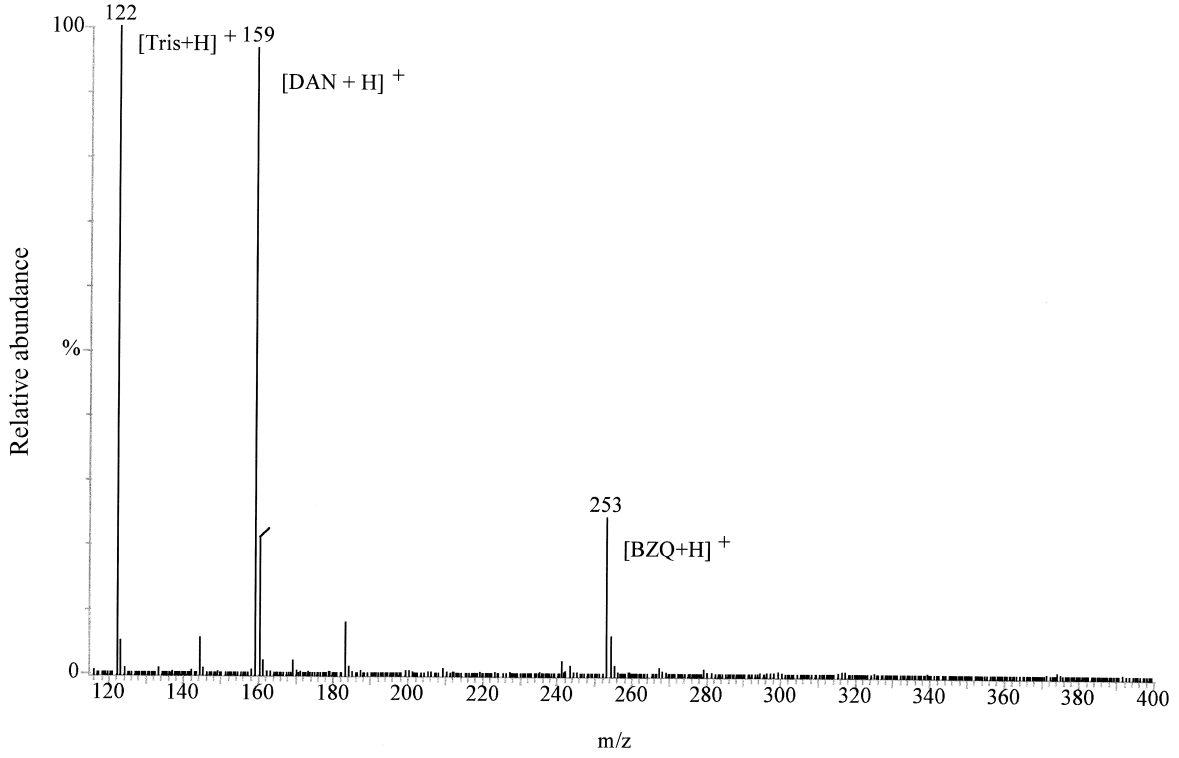

Fig. 5. Electrospray mass spectrum of the benzoquinoxaline derivative by the condensation of 2,3-diaminonaphthalene with 4,5-dioxovaleric acid. The conditions were described in Experimental.

such as lead intoxication, acute intermittent porphyria (AIP) and tyrosinosis (Fig. 1) [5]. High incidence of primary liver cancer has been associated with AIP and high ALA levels in the plasma [13–16]. It has been found that reactive oxygen species are produced during the metal-catalyzed oxidation of ALA [4]. In addition, ALA has been found to release iron from ferritin [6]. Recently, attention has been focused on the final oxidation product of ALA, 4,5-dioxovaleric acid, as a potential DNA alkylation agent [10]. It has reinforced the hypothesis that ALA overload may be involved in PLC. The HPLC method with the fluorescence detection described here allows the quantification of DOVA. This methodology which is much less laborious and faster than other methods [16], enables a large sample throughput. In addition, other sensitive and specific methods, using the promising HPLC–MS–MS assay, can be developed to determine the nature and quantity of DOVA. These methods may be useful for establishing roles of DOVA in biomolecule damage, especially in the incidence of PLC in patients of AIP, and of carcinogenesis after lead intoxication.

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