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High-performance liquid chromatographic determination of D-amino acid oxidase activity

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ABSTRACT

A new procedure for the assay of D-amino acid oxidase activity has been developed. α-Ketoisovaleric acid, derived from D-valine, was estimated by high-performance liquid chromatography after reaction with *o*-phenylenediamine to give the corresponding quinoxalinol derivative. α-Ketovaleric acid was used as an internal standard to ensure the reproducibility of the method. As an example of application, kidney cortex homogenates were analyzed for their D-amino acid oxidase activity. The advantages of the presented procedure for the determination of the enzymatic activity in biological samples compared with previously reported procedures are discussed.

INTRODUCTION

D-Amino acid oxidase [D-amino-acid:oxygen oxidoreductase (deaminating), EC 1.4.3.3; DAAO] is a well known enzyme which catalyzes the oxidative deamination of D-amino acids to the corresponding α-keto acids, ammonia and hydrogen peroxide. Its availability in large amounts has allowed extensive studies on its mechanism of action. Until now, the estimation of DAAO activity has been based on (a) the spectrophotometric analysis of H_2O_2 produced [1] or (b) the electrochemical measurement of O_2 used [2] or (c) the spectrophotometric estimation of the α-keto acid produced from the corresponding D-amino acid, either directly, using D-phenylglycine as a substrate [3], or by the lactate dehydrogenase/NADH-coupled reaction, using D-alanine as a substrate [4].

In this paper we report an alternative method for the determination of DAAO activity with the purpose of avoiding most of the interferences usually encountered when biological samples are assayed. The new assay procedure is based on the high-performance liquid chromatographic (HPLC) analysis of α-keto acids following their derivatization with 1,2-diaminobenzene (*o*-phenylenediamine, OPD). The latter reagent was chosen because of its well established handling conditions [5–7], which have often been evaluated for biological samples [8,9]. Quantitative analysis was based on internal standardization. As an example of the applicability of the method, estimations of DAAO activities both in commercial preparations and in beef kidney extracts were performed.

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EXPERIMENTAL

Materials

DAAO type II from porcine kidney, purchased from Sigma (St. Louis, MO, U.S.A.), was extensively dialyzed and brought to a final concentration of 0.1 mg/ml with 0.1 *M* pyrophosphate buffer, pH 7.4, containing 10 μM flavin-adenine dinucleotide (FAD). Beef kidney extract was obtained by dissecting and homogenizing the cortex in an ice bath after the addition of 2.5 volumes of 0.1 *M* pyrophosphate buffer, pH 8.5, containing 10 μM FAD; the suspension was then centrifuged at 27 000 *g* for 30 min and the supernatant stored at −20°C. OPD was obtained from Fluka (Buchs, Switzerland) and purified by using Sep-Pak C_{18} cartridges (Water, Milford, MA, U.S.A.), according to the procedure described by Kieber and Mopper [8], giving a solution of about 0.44 *M* in 2 *M* HCl, containing 5 μl/ml mercaptoethanol according to Livesey and Edwards [9].

α-Keto acids [pyruvic, ketobutyric, ketovaleric (KVA) and ketoisovaleric (KIVA) acids] and D-valine (D-Val) were purchased from Aldrich (Steinheim, Germany) and other reagents and solvents from Merck (Darmstadt, Germany).

Apparatus and chromatographic conditions

A Jasco HPLC system equipped with a Familic 300 S pump and an FP-210 fluorimetric detector was used. The separations were performed on a 250 mm × 4 mm I.D. column packed with LiChrospher 100 RP-8 (5 μm) from Merck; a mixture of 0.35 *M* ammonium acetate aqueous solution and acetonitrile (60:40, v/v) was used as eluent, and the eluate was monitored by setting the excitation and emission wavelengths of the fluorimetric detector at 340 and 420 nm, respectively.

Assay procedure

A mixture of 20 μmol of D-Val, 10 nmol of FAD, 10 μg of catalase and 2 nmol of KVA, used as internal standard, was added to 0.1 *M* pyrophosphate buffer, pH 8.5 (total volume 1 ml), at 25°C. The enzymatic reaction was started by the addition of different volumes of either a 0.1 mg/ml solution of commercial DAAO (10–100 μl) or the beef kidney homogenate prepared as above (10–100 μl). At different time intervals, 200-μl aliquots of the mixture were withdrawn and added to 100 μl of ice-cooled 6 *M* HCl directly in polytetrafluoroethylene (PTFE)-lined screw-capped test tubes. When homogenates were analyzed, centrifugation (15 000 *g*) using an Eppendorf 5414 centrifuge was performed at this stage in order to remove denatured proteins. A 200-μl aliquot of the purified OPD solution was then added and the mixtures were flushed with nitrogen and kept for 15 min at 80°C in a sand bath. After cooling, 0.3 ml of 6 *M* sodium acetate aqueous solution were added and 20-μl aliquots were injected into the HPLC system.

Quantitative analysis

Different amounts of KIVA (0.5–50 nmol) and a constant amount of KVA (2 nmol) were dissolved in 200 μl of 0.1 *M* pyrophosphate buffer, pH 8.5, and treated with HCl, OPD and sodium acetate according to the procedure described above. From the chromatographic profiles, the ratios (R_h) of the peak heights of KIVA and KVA for each different mixture were determined and the data used to set up a calibration curve, plotting the R_h values against the amounts of treated KIVA. The DAAO activity of a sample was calculated from the amount of KIVA produced by the enzymatic reaction. A DAAO unit is defined as the amount of enzyme which produces 1 μmol/min KIVA under the conditions described.

RESULTS

Fig. 1 shows the chromatographic profiles obtained from the analysis of a standard mixture of KIVA and KVA and a beef kidney homogenate either treated according to the described procedure or without the addition of substrate and internal standard. The KIVA/KVA R_h dependence on the amounts of KIVA in the presence of 2 nmol of KVA was linear in the range 0.5–50 nmol according to the following relationship:

$$R_h = 0.176 \times \text{nmol KIVA} + 0.081 \quad (r = 0.998)$$

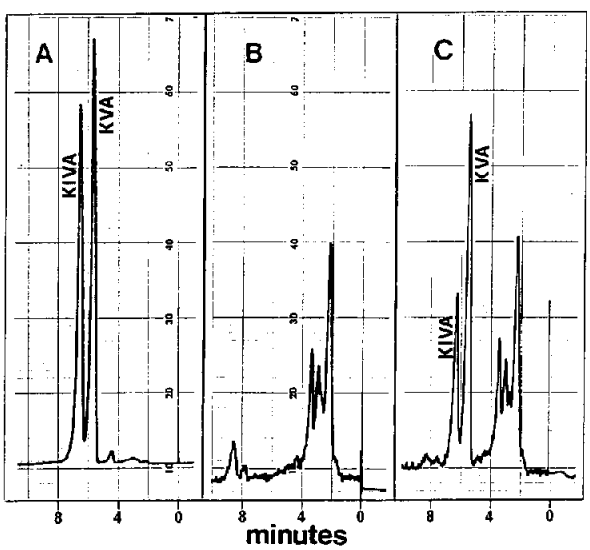
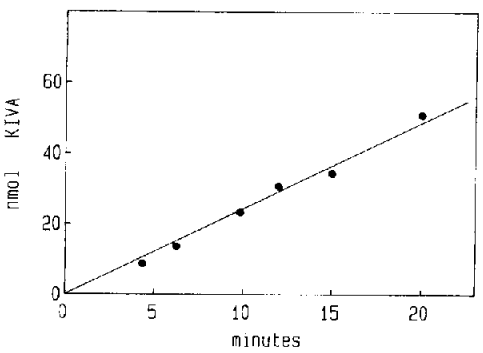


Fig. 1. Typical chromatographic profiles derived from the analysis of (A) a standard mixture of KIVA and KVA, (B) a beef kidney homogenate (10 μl, corresponding to 4 mg of tissue) treated according to the described procedure except that D-Val and KVA were not added, and (C) the same beef kidney homogenate treated according to the described procedure (10 min incubation time). DAAO content in this sample was 21.80 mU/ml.



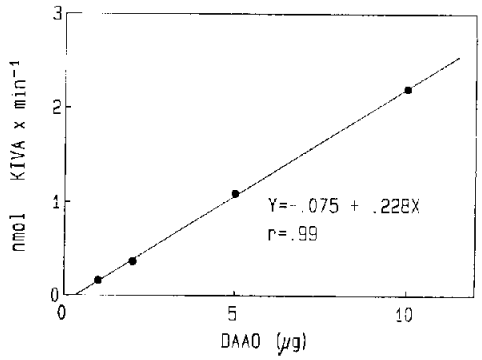


Fig. 2. Time dependence of the appearance of KIVA in the presence of 10 μg/ml DAAO and 20 m*M* D-Val. Specific activity of the commercial DAAO preparation used was 238 mU/mg.

Fig. 3. Relationship between the detected activity, calculated from the appearance of KIVA, and DAAO amounts used. Specific activity of the commercial DAAO preparation used was 218 mU/mg.

The time dependence of the enzymatic production of KIVA from 20 m*M* D-Val used as substrate appeared to be linear at least up to 20 min in the presence of 10 μg/ml DAAO (Fig. 2). The activity was also shown to be linear with different amounts of enzyme (Fig. 3).

The detection limit was about 0.2 nmol of KIVA, corresponding to 0.01 mU of DAAO. In order to determine the reproducibility of the procedure, a beef kidney cortex extract was analysed five times and its DAAO content was found to be 31.41 ± 2.45 mU/ml (coefficient of variation, C.V. 7.8%).

DISCUSSION

DAAO is one of the best-known peroxisomal enzymes. Therefore its activity is used as a marker for peroxisomal functionality as an alternative to catalase [10]. Thus an assay which could be a good alternative to the ones already used for the detection of DAAO in biological samples is needed. In fact, the O_2 electrode determination requires specific apparatus and is not very sensitive. With regard to determination of H_2O_2, care must be taken to avoid side-reactions resulting from the large amounts of catalase; this is usually overcome by using inhibitors of catalase (such as sodium azide), most of which, however, are also inhibitors of peroxidase. This fact results in the need to use a large amount of this latter enzyme in the assay mixture. A similar drawback applies to the assay based on the determination of pyruvic acid using the lactate dehydrogenase/NADH system; in fact the presence of considerable amounts of both endogenous pyruvic acid and other dehydrogenases which turn over the NAD^+ produced during the coupled reaction reduces the applicability of this method to tissue extracts. As concerns the method based on the UV determination of benzoylformic acid derived from D-phenylglycine, its applicability can be reduced by the presence of

material from the homogenate, which gives a large background absorption at 252 nm.

In this work we exploited the ability of α-keto acids to give, after treatment with OPD, fluorescent derivatives which are easily estimated after HPLC separation. The substrate chosen offers a good compromise between the known kinetic parameters of the different suitable D-amino acids and the known chromatographic features of the OPD derivatives of the corresponding α-keto acids. As regards the HPLC conditions, in order to increase the assay speed, this procedure employs an isocratic elution of the quinoxalinol derivative of the α-keto acid produced by the enzyme action and of the one used as internal standard, instead of the gradient elution generally used for the OPD derivatives of α-keto acids. After preliminary experiments, D-alanine and α-aminobutyric acid were discounted because of the presence of large amounts of pyruvic acid in biological samples and because of the appearance in blank samples of a peak overlapping the α-ketobutyric acid derivative. The best α-keto acid pair for a rapid HPLC assay proved to be the KIVA/KVA couple, because their k' values were clearly different from those of the other α-keto acids, under the described conditions, and because of the suitable kinetic parameters of the corresponding D-amino acids used as substrates (D-Val and D-norvaline). D-Val was finally chosen because of its higher V_{max}.

The enzymatic reaction conditions used were the same as described in the literature for the D-phenylglycine method [3]. The analysis of beef kidney extracts allowed us to test the suitability of the OPD derivatization for the estimation of DAAO in biological samples. No interfering peaks appeared in the blank with k' values corresponding to KVA and KIVA. It should be noted that the method may be used for biological samples from any source since it is feasible to chose a substrate/internal standard couple such as to avoid possible interference from peaks deriving from endogenous α-keto acids.

It should be pointed out that the sensitivity of the method could be increased in different ways. For example, the final quinoxalinols could be extracted into an organic solvent (ethyl acetate), brought to dryness and redissolved in a minimum volume to give more concentrated samples. In addition, other more expensive 1,2-diamino aromatic compounds which give derivatives with higher fluorescence response [11,12] have been introduced. However, in our experience the method presented, based on the derivatization with cheap OPD and on the isocratic separation of quinoxalinol derivatives of α-keto acids, appears to be a reliable and sensitive method of detecting DAAO activity in tissue extracts.

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