Journal of Chromatography, 336 (1984) 385—391
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2326

Note

Reversed-phase high-performance liquid chromatographic method for determination of brain glutamate decarboxylase suitable for use in kinetic studies

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(First received May 18th, 1984; revised manuscript received June 19th, 1984)

γ-Aminobutyric acid (GABA) is well established as a major inhibitory neurotransmitter in the central nervous system [1], thus any disturbance of GABA metabolism which lead to altered levels of GABA may result in neurological dysfunction. The major synthetic pathway for GABA is by decarboxylation of L-glutamate catalyzed by the pyridoxal 5'-phosphate-dependent enzyme, L-glutamic acid-1-carboxylyase (GAD; EC 4.1.1.15) and it has been shown that inhibition of this enzyme can result in the appearance of convulsive episodes [2]. The interest in our laboratory involves a study of the mechanism of homocysteine-induced seizures which has application both in the study of the genetic metabolic disorder, homocystinuria and in human epilepsy.

Homocysteine has been shown to inhibit a number of pyridoxal 5'-phosphate-dependent enzymes [3, 4] and preliminary studies indicate that it may interfere with GAD activity. In order to investigate this, a sensitive and specific assay, suitable for the determination of initial velocities is required so that inhibition of the enzyme can be characterised kinetically. A number of methods for assaying GAD activity have been reported (for reviews, see refs. 5 and 6) but many of these have the disadvantage of indirectly measuring the product and so may be subject to artefacts. Methods have been described where the product, GABA, is isolated by liquid chromatography [6] although none of the reports demonstrate their suitability for use in kinetic analysis.

We have therefore developed a method based on the reversed-phase high-performance liquid chromatographic (HPLC) separation of the *o*-phthalaldehyde (OPA) derivative of GABA and its subsequent fluorimetric detection,

0378-4347/84/$03.00

which optmizes both the sensitivity and rate of separation while being eminently suitable for use in kinetic analysis.

EXPERIMENTAL

Chemicals

HPLC-grade methanol was obtained from Fisons (Loughborough, U.K.). Potassium acetate, glacial acetic acid and boric acid of AnalaR grade, and potassium dihydrogen orthophosphate of GPR grade were obtained from BDH (Dorset, U.K.). GABA was obtained from Koch-Light (Berkshire, U.K.). Amino acid standards, OPA, L-glutamic acid, triethanolamine, pyridoxal 5′-phosphate and aminoethylisothiouronium bromide (AET) were purchased from Sigma (Dorset, U.K.). Reagent-grade water was prepared by running tap water through a Milli R/Q water purification system (Millipore, U.K.) followed by filtration through a 0.2-μm membrane filter.

Enzyme source

Crude enzyme preparations were obtained by homogenisation of whole mouse brains in 10 vols. of 0.3 *M* triethanolamine buffer (pH 6.8), 1 m*M* AET and 2 m*M* pyridoxal 5′-phosphate. The homogenate was exposed to 30-sec periods of ultrasonication four times followed by centrifugation at 15,000 *g* for 20 min. The resulting supernatant was used as a source of GAD.

Enzyme assays

The assay was carried out in a total volume of 100 μl (pH 6.8) containing: 200 m*M* potassium dihydrogen orthophosphate, 5 m*M* L-glutamic acid and 0.2 m*M* pyridoxal 5′-phosphate. Each assay was started by the addition of enzyme in a 30-μl aliquot (typically 5 mg/ml protein) and incubation was carried out at 37°C. The reaction was terminated by the addition of 1 ml of ice-cold ethanol followed by centrifugation in an Eppendorf 5413 microcentrifuge at 8800 *g* for 5 min. A 5-μl aliquot of the supernatant was derivatized as described.

Preparation of amino acid standards

Individual amino acids were prepared in aqueous solutions at concentrations of 100 μ*M* and filtered through a 0.45-μ*m* membrane filter.

Derivatization

OPA (10 mg) was dissolved in 500 μl absolute ethanol, then 500 μl of 2-mercaptoethanol were added followed by dilution to 10 ml with 0.4 *M* boric acid (adjusted to pH 10.4). To maintain the reagent strength, 50 μl of 2-mercaptoethanol were added every two days while the solution was stored at 4°C. An aliquot (5 μl) of amino acid standard or reaction supernatant was mixed with 10 μl of OPA in a 1.4-ml Eppendorf tube. The contents were mixed and after 90 sec at room temperature a 5-μl aliquot was used for analysis.

Apparatus

A high-performance liquid chromatograph (Gilson International) consisting

of two Model 303 pumps with a Model 802 manometric module and a Model 702 gradient manager was used. Samples were loaded via a Rheodyne Model 7125 syringe loading sample injector fitted with a 20-μl sample loop. The detection system consisted of a Gilson International Model 121 fluorescence detector equipped with a Corning 7-60 excitation filter and a Corning 3-73 emission filter utilizing a flow-cell of 9 μl volume. Peak areas were quantified by the method of area normalization using a Shimadzu Chromatopac C-RIB data processor. A μBondapak C_{18} (10 μm particle diameter) reversed-phase column, 30 cm × 3.9 mm I.D. (Waters Assoc.) was used in conjunction with a CSK guard column, 6 cm × 2.5 mm I.D., packed with ODS Co:Pell (Whatman).

Chromatography

Anhydrous potassium acetate (0.98 g) was dissolved in 900 ml of reagent-grade water and the pH adjusted to 5.6 with glacial acetic acid. The resulting solution was diluted to a total volume of 1 l to make a 0.1 *M* potassium acetate solution. The potassium acetate buffer and methanol were filtered through a 0.2-μm membrane filter (Millipore) before use. Each of the mobile phases was degassed daily by ultrasonic treatment and under vacuum. The mobile phase gradient was run from 20% to 70% methanol in a single 20-min step at a flow-rate of 1.5 ml/min. The elution programme was followed by a 10-min isocratic washing step prior to equilibration of the column with 20% methanol.

Protein determination

Protein determination was carried out using the dye-binding method of Bradford [7] utilizing bovine serum albumin as a standard.

RESULTS

The results shown in Fig. 1A illustrate a typical chromatogram of OPA-derivatized GABA and L-glutamic acid standards. It is clear that separation of these compounds was achieved and the calculated elution times were within ± 1—2%. L-Glutamic acid (GLU) elutes at 9.83 min with GABA eluting at 17.64 min. Using this method we have been able to detect less than 1.0 pmol of GABA. In our hands, when analysed a variety of commercially available GABA standards, described as approx. 98—99% pure, were separated into a number of peaks, the largest representing no more than 75% of the total peak area. Such an extent of impurity was unsatisfactory for calibration purposes. However, the source of GABA employed in this study gave a major peak representing 99% of the total peak area. Construction of a calibration curve showed that the peak areas were directly proportional to the concentration of the OPA-derivatized GABA in the range 0—100 μM, the concentration of GABA (μM) being equal to $9.2 \cdot 10^{-4}$ times the relative peak area.

Endogenous levels of GABA in the brain extract were obtained by OPA derivatization of a replicate incubate in which the reaction was terminated at time zero. Fig. 1B shows the chromatographic separation of substrate, L-glutamic acid (retention time 9.46 min) from endogenous GABA (retention time 17.51 min). Endogenous GABA concentrations were routinely subtracted from the total amount of GABA formed under assay. In Fig. 1C, a chromato-

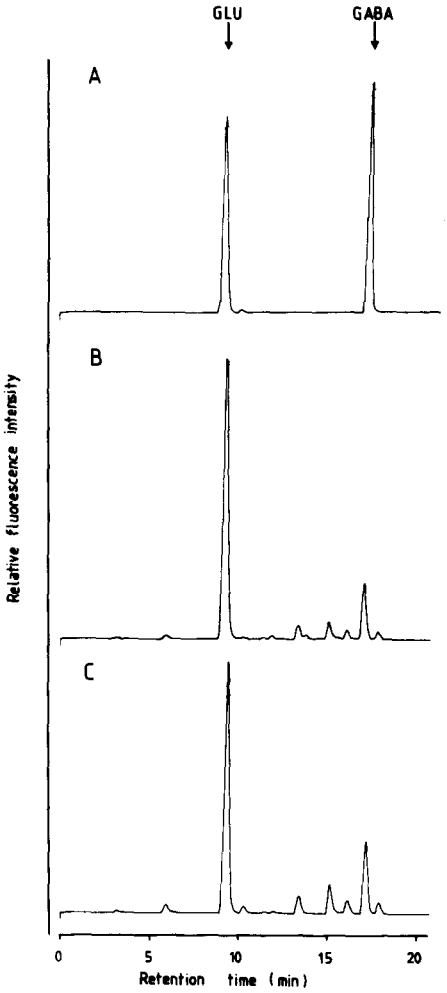

Fig. 1. (A) Chromatogram illustrating the separation of OPA-derivatized authentic L-glutamic acid (GLU) and GABA (160 pmol of each). The standards were separated on a μBondapak C_{18} reversed-phase column, elution being effected with a mobile phase of 20—70% methanol—potassium acetate, pH 5.6, in a single 20-min run at a flow-rate of 1.5 ml/min. The retention times of L-glutamic acid and GABA were 9.83 min and 17.64 min, respectively. (B and C) Chromatographic analysis of reaction mixtures, under GAD assay conditions, as a function of time. (B) Time zero showing substrate peak, L-glutamic acid (retention time 9.46 min) and the second major peak, endogenous GABA (retention time 17.51 min). (C) Time 1 min, showing separation of GABA (endogenous + enzymically formed) with a retention time of 17.67 min from L-glutamic substrate (retention time 9.58 min). Assays were carried out using 150 μg rat brain enzyme protein extract plus assay mixture which included 5 m*M* L-glutamic acid. For full details of assay see Experimental.

graphic separation of enzymically formed GABA from L-glutamic acid is shown, following incubation with brain extract for 1 min. Even after this short incubation, the proportion of the peak area representative of enzymically formed GABA was 4.5% greater than that of the endogenous level, in spite of the relatively low V_{max} (92 pmol GABA formed per mg per min) of the enzyme. It should be noted that the change in the peak size of L-glutamic acid

will be negligible due to the high initial substrate concentration required in the kinetic assay. The smaller peaks observed in the chromatogram of Fig. 1C are representative of endogenous metabolites in the brain extract, which do not interfere with quantitation of GABA. Under the assay conditions described, the separation remained highly reproducible with insignificant fluctuation in peak elution time.

In any kinetic study employing a discontinuous assay system, it is essential to establish that the procedure for termination of the reaction is effective. When zero-time controls were compared with controls in which the brain extract had been heat-inactivated, no difference in product (GABA) levels were observed, indicating that the addition of ice-cold ethanol terminates the reaction instantaneously.

The data shown in Fig. 2 illustrate a progress curve of GAD activity for assays carried out in triplicate. The reaction is linear for at least a 15-min incubation period during which time easy measurements of initial velocity for use in kinetic studies can be made. Experiments to establish true initial velocity are frequently neglected so that published data may represent only an extent of reaction which is erroneously utilized for determination of kinetic parameters. Bearing this in mind, assays were performed at a range of L-glutamic acid concentrations and initial rates of GABA formation determined for each. The maximum velocity (V_{max}) of 92 pmol GABA formed per mg per min and the Michaelis constant (K_M) for L-glutamic acid of 1.35 m*M* were determined by regression analysis of the data; moreover the K_M value for L-glutamic acid was in excellent agreement with values determined by other workers, for example Wu and Roberts [8] and Taberner et al. [9] who report K_M values of 0.7 m*M* and 1.8 m*M*, respectively.

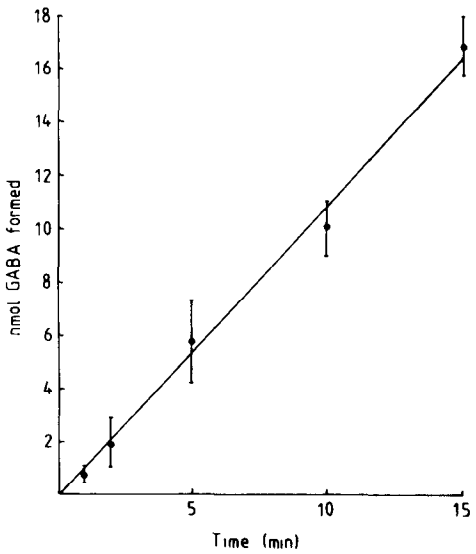

Fig. 2. Progress curve of GAD reaction plotted against time. Rat brain extract was prepared as described in the text. GAD assays were carried out for varying periods of time, at a fixed L-glutamic acid concentration, and GABA formation was measured following chromatographic separation as described in Experimental. Assays were carried out in triplicate and the data presented as the means ± S.D.

DISCUSSION

Although a number of non-chromatographic procedures are available for analysis of GAD activity [10—12], chromatographic methods are preferable owing to their specificity of analysis. The use of HPLC has the added advantages of increased resolution and sensitivity as well as being more rapid. A number of HPLC methods for determination of GABA have been reported [6] although very few were designed to specifically measure GAD activity [13—15]. Pahuja et al. [14] have applied reversed-phase HPLC to the separation of dansylated GABA from retinal cells. However, in this method, GAD activity was quantified by liquid scintillation spectrometry after separation of the dansylated derivatives by HPLC. Furthermore, the use of incubation times of at least 1 h is not commensurate with kinetic analysis, since the rate of the reaction is non-linear at this stage. A further limitation of this method is the level of detection which the authors state to be 1 nmol of GABA.

More recently, Holdiness [15] reported a fluorimetric procedure for analysis of GAD activity in sub-regions of rat brain using cation-exchange HPLC, which attained a lower limit of detection at 0.1 nmol of GABA. Although appearing more sensitive than the method of Pahuja et al. [14], supporting evidence for use in kinetic analysis was not available. To our knowledge, none of the previously reported methods of assay, which employ the advantages of HPLC, have been demonstrated as being suitable for kinetic studies. We have therefore developed a cost-effective and robust assay which specifically measures GABA formed during the reaction. Using this method, it is possible to detect < 1 pmol of enzymically formed GABA utilizing periods of incubation well within the linear portion of the progress time course.

The method for determination of GAD activity, reported herein, is an improvement over other methods and is being presently utilized in our studies on human homocystinuria and generalised epilepsy in which a possible defect in GABA metabolism may result in the appearance of seizures. In the course of our present research, we have found this assay suitable for determination of GAD activity in crude brain extracts, specific brain regions and subcellular organelles and in cultured cells.

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