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Short Communication

Use of high-performance liquid chromatography for assay of glutamic acid decarboxylase

Its limitation in use for *post-mortem* brain

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ABSTRACT

Rat brain, obtained 10 min after death, contained high levels of endogenous γ-aminobutyric acid (GABA) and glutamic acid. Incubation of this brain homogenate at 37°C indicated decrease of GABA with time due to degradation by GABA-transaminase. Reported high-performance liquid chromatographic (HPLC) methods for glutamic acid decarboxylase (GAD) assay depend on the difference between the GABA content of the reaction mixture after and before the incubation period. None of the methods considered the degradation of GABA during incubation. Furthermore, during determination of the Michaelis constant (K_M) for the reaction none of them considered the endogenous substrate. Here we have focused on these factors which seriously affect the maximum velocity (V_{max}) and K_M values during GAD assay by the HPLC technique. By a simple and rapid HPLC technique we have measured GAD activity in *post-mortem* rat brain after removing endogenous glutamic acid by charcoal treatment and using gabaquline to prevent GABA degradation during incubation period. By this method a V_{max} value of 46±4 nmol/h/mg protein and a K_M value of 7.5±0.6 m*M* were observed for GAD activity of crude brain homogenate. For a comparative study, we have carried out radiometric assay of GAD activity from the same sample and observed a V_{max} of 48±6 nmol/h/mg protein and K_M of 6.9±0.4 m*M*.

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INTRODUCTION

Glutamic acid decarboxylase (GAD) catalyzes the decarboxylation of L-glutamic acid and is the final and rate-limiting enzyme in the synthesis of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) [1,2]. When assaying GABA metabolism and GABAnergic innervation in the brain the determination of GAD activity can be critical. Alterations of brain GAD activity has been demonstrated in different neuropsychiatric disorders [3–5] and after various neuropharmacological treatment [6].

The assay used for measuring GAD activity can be grouped into those which determined $^{14}CO_2$ liberated from L-glutamic acid and those which measure the GABA produced (see ref. 2 for a review). The later group can be further divided according to the method used to analyze GABA [2]: (i) amino acid analyzer; (ii) ion-exchange chromatography; (iii) fluorimetric method; (iv) gas chromatography–mass spectroscopy (GC–MS); and (v) high-performance liquid chromatography (HPLC) methodology [7–10]. There are three major drawbacks of the reported HPLC techniques for GAD assay. (i) GAD activity was assayed by measuring GABA content before and after the reaction; the difference between GABA content in 0 min and a certain time incubation periode gave the measure of GABA production. The major problem in this approach is that degradation of GABA during the incubation period was not taken into account. (ii) During the estimation of the Michaelis constant (K_M) of the reaction, the high level of the endogenous substrate present in the brain homogenate was not taken into account. (iii) Most of the HPLC methods used for assay of GABA have very long retention times (15–30 min) with the only exception the method of Kochhar *et al.* [10]. In this case the separation was not satisfactory.

In this communication we have tried to focus on those factors which seriously affect the results of GAD assay in brain homogenate by HPLC.

EXPERIMENTAL

For our study, enzyme was prepared by homogenizing whole rat brain (obtained 10 min after death) by sonication (5 × 5 s; setting 4 in a Vibra Cell sonicator) in 0.01 *M* 3-N-morpholinopropanesulfonic acid (MOPS) buffer, pH 7.4. The homogenate was diluted with buffer (1:1, v/v) containing 4 m*M* pyridoxal phosphate, 0.6 *M* tetraethylamine and 2 m*M* 2-aminoethyl isothiomonium (AET). The final suspension was treated with 0.4% Triton X-100 (final concentration) and centrifuged at 15 000 *g* for 5 min to obtain supernatant. Enzyme was assayed in a total of 100 μl containing 40 μl of 200 m*M* KH_2PO_4, pH 6.8, 10 μl of 5 m*M* L-glutamic acid, 5 μl of 0.2 m*M* pyridoxal 5′-phosphate, 40 μl of homogenate (2 mg protein per ml) and 1 μg of gabaquline. The reaction mixture was incubated for indicated length of time at 37°C and the reaction was stopped by the addition of 10 μl of 100% trichloroacetic acid (TCA, ice cold). The suspension

was centrifuged at 10 000 *g* for 4 min, 5 μl of supernatant were mixed with 5 μl of standard δ-aminovaleric acid (DAVA) solution and 90 μl of *o*-phthalaldehyde (OPA) solution (2 mg OPA per ml of 0.4 *M* borate buffer, pH 10.4), the mixture was allowed to react for 3–5 min, and 20 μl were injected onto an Altex Ultrasphere ODS (250 mm × 4.6 mm I.D.) 5-μm column (Rainin Instruments, Woburn, MA, USA) and eluted with buffer containing 0.2 *M* sodium acetate, 100 mg/ml EDTA, pH 3.8 and acetonitrile (40%, v/v). Fluorescence (λ_{ex} = 330 nm, λ_{em} = 440 nm) was measured using a Shimadzu RF 350 spectrofluorometer with bandpass of 20 nm.

During the substrate kinetic study, we removed the endogenous glutamic acid by treating the homogenate with activated charcoal (10 mg charcoal per 800 μl of homogenate containing 0.4% Triton X-100) for 30 min at 37°C and then by centrifuging at 10 000 *g* for 4 min.

The radiometric assay of GAD activity was measured according to Roberts and Simonsen [11] by trapping the liberated $^{14}CO_2$ during the reaction from [^{14}C]glutamic acid.

RESULTS AND DISCUSSION

The HPLC technique used for assay of GABA in this study is simple and rapid with retention times for GABA and DAVA of 3.9 and 4.9 min, respectively. A typical chromatogram is shown in Fig. 1. Glutamic acid was eluted within 1.6 min and did not interfere with the GABA and DAVA separation. The detection limit

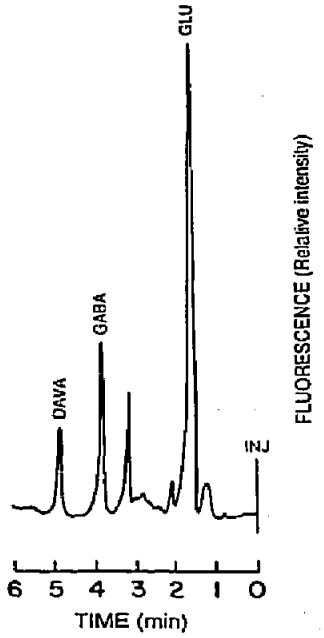

Fig. 1. Chromatogram illustating the separation of OPA derivatives of glutamic acid, GABA and DAVA (internal standard) in crude brain homogenate. Analysis was performed before incubation; endogenous levels for the samples shown were 5 and 3 μg/ml protein for GABA and DAVA, respectively.

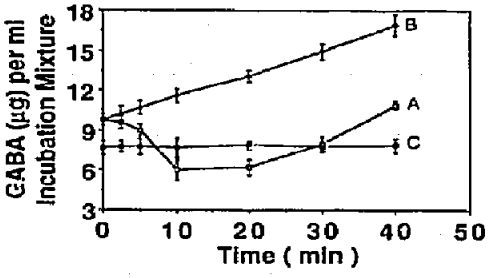

Fig. 2. Changes in the GABA content of rat brain homogenate during incubation period for GAD assay. (A) Untreated homogenate; (B) homogenate in presence of gabaquline; (C) charcoal-treated homogenate in presence of gabaquline. Data are means ± S.D. of three triplicate experiments.

by this method was 0.9 ± 0.2 pmol of GABA. Sources of variability were held to be a minimum by using internal standard and improved stability of the OPA derivatives. The intra- and inter-assay coefficients of variation were 4.5 and 8.2%, respectively.

When rat brain homogenates were incubated at 37°C, there was an initial decrease in the GABA content with time up to 10 min [which contains a very high level of endogenous GABA (47 ± 8 nmol/mg protein)] and thereafter some rise in GABA content was observed (Fig. 2, curve A). The initial loss of GABA was due to its degradation, and later increase in the GABA content was due to rapid synthesis of GABA with time from the endogenous substrate. In order to solve the problem we have used gabaquline (10 µg/ml) which inhibits the breakdown of GABA by GABA-transaminase. The results indicate that there was no loss of GABA with time but it started rising from the beginning of the incubation due to synthesis of GABA from endogenous glutamate (Fig. 2, curve B). When we treated the homogenate with activated charcoal before the incubation to remove endogenous glutamic acid, we found a constant value for the GABA content of the homogenate even up to 40 min (Fig. 2, curve C) (there was about 30% removal of the GABA also from the homogenate due to charcoal treatment).

After overcoming the problem of GABA degradation during incubation using gabaquline we have assayed GAD in rat brain homogenate. Time kinetic study (Fig. 3) indicated that we could obtain a linear increase in GABA production for

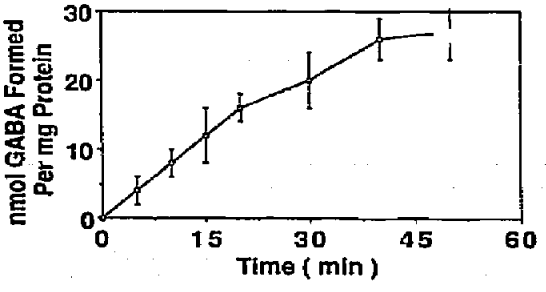

Fig. 3. Time kinetic study of GABA production by GAD in rat brain homogenate. Homogenate (80 µg of protein) was incubated for the specified lengths of time (0–48 min), the GABA formed was determined by HPLC as described in the Experimental section. Data are means ± S.D. of three triplicate experiments.

up to 20 min. After 20 min, there was some loss of linearity. The 20-min incubation period was sufficient to obtain a marked increase (~ 52%) in GABA content relative to that seen at zero time (after incubation with 25 m*M* glutamate and with 80 μg of homogenate protein; the reaction was found to be linear up to 140 μg of homogenate protein). The maximum velocity (V_{max}) of the reaction was found tc be 46 ± 4 nmol/h/mg protein, which was in good aggreement with the reports of GAD activity of rat brain by three different analytical techniques, *viz.* fluorimetric, HPLC and GC–MS procedure [2]. The radiometric assay of the same sample gave a V_{max} of 48 ± 6 nmol/h/mg protein.

Though the V_{max} for GAD activity of the charcoal-treated and untreated rat brain homogenate were identical, the substrate kinetics study for GAD reaction with charcoal-untreated rat brain homogenate suffered a serious problem. Due to high endogenous glutamic acid content in the homogenate, we could not observe a significant effect of exogenous substrate on the rate of reaction: the K_M value was found to be low (1.1 ± 0.3 m*M*) under this condition which was in good agreement with the values obtained by other groups [9,12,13]. However, after removal of the endogenous glutamic acid by charcoal treatment, we observed substrate dependency of GAD activity in the homogenate (Fig. 4): the K_M calculated under these conditions was 7.5 ± 0.6 m*M*.

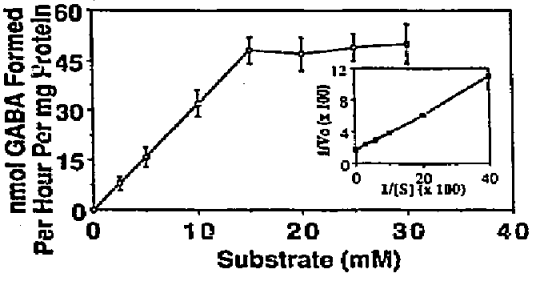

Fig. 4. Substrate kinetics of GAD assay using charcoal-treated *post-mortem* brain homogenate. Homogenate was treated with activated charcoal to remove endogenous glutamic acid. The GAD assay was then carried out with various concentrations of glutamic acid (2.5–30 m*M*). GABA formed was measured by HPLC as described in the Experimental section. Data are means ± S.D. of three triplicate experiments. Inset is the Lineweaver-Burk plot for determination of V_{max} and K_M values.

Although several HPLC methods for GAD assay have been reported [7–10], none have completely addressed the problems associated with the presence of endogenous substrate and the degradation of GABA during the incubation period. These problems can be especially troublesome when using *post-mortem* specimens with elevated GABA levels [2] and when trying to accurately determine the K_M value. The estimation of GABA by our method in HPLC is very sensitive (detection limit 0.9 ± 0.2 pmol of GABA), relatively rapid and does not require a complex gradient elution system. Moreover, the difference between peak height of GABA in 0-min and 20-min reaction mixtures typically was about 50% ($p < 0.001$).

The HPLC method for GAD assay in this communication appears to be an improvement over previous HPLC methods and should prove useful in animal studies and in *post-mortem* studies of human brain.

REFERENCES

1 M. Errera and J. P. Greenstein, *J. Biol. Chem.*, 178 (1949) 495.
2 M. R. Holdiness, *J. Chromatogr.*, 227 (1983) 1.
3 E. G. McGeer and P. L. McGeer, *J. Neurochem.*, 26 (1976) 65.
4 E.D. Bird, J. S. Gale and E. G. Spokes, *J. Neurochem.*, 29 (1977) 539.
5 E. K. Perry, P. H. Gibson, G. Blessed, R. H. Perry and N. E. Tombinson, *J. Neurol. Sci.*, 34 (1977) 247.
6 E. G. S. Spokes, *Brain*, 102 (1979) 333.
7 S. L. Pahuja, J. Albert and T. W. Reid, *J. Chromatogr.*, 225 (1981) 37.
8 M. R. Holdiness, *J. Liq. Chromatogr.*, 5 (1982) 479.
9 I. C. Allen and R. Griffiths, *J. Chromatogr.*, 336 (1984) 385.
10 S. Kochhar, P. K. Mehta and P. Christen, *Anal. Biochem.*, 179 (1989) 182.
11 E. Roberts and D. G. Simonsen, *Biochem. Pharmacol.*, 12 (1963) 113.
12 J.-Y. Wu and E. Roberts, *J. Neurochem.*, 23 (1974) 759.
13 P. V. Taberner, M. J. Pearce and J. C. Watkins, *Biochem. Pharmacol.*, 26 (1977) 345.