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Short communication

Determination of glutamate decarboxylase by high-performance liquid chromatography

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Abstract

An improved method for the determination of glutamate decarboxylase (GAD) activity is described. The enzyme was evaluated by incubation with glutamic acid (L-Glu) in the presence of pyridoxal 5′-phosphate (PLP): the γ-aminobutyric acid (GABA) formed was derivatized to PTC-GABA; the latter was subsequently separated and assayed by isocratic HPLC (LiChrospher RP-18 column; isocratic elution with pH 5.8 acetate buffer in acetonitrile–water) with UV absorbance detection at 254 nm. The method described is a sensitive, reproducible and specific assay useful for following variations of GAD activity in vitro; this assay was subsequently used for the evaluation of GAD activity variations after irradiation with low doses of He–Ne laser radiation.

Keywords: Glutamate decarboxylase; Enzymes

1. Introduction

γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system, is synthesized by decarboxylation of L-glutamate (L-Glu), catalyzed by glutamate decarboxylase (GAD) [1]. GAD has been shown to play an important role in the regulation of brain excitability through the synthesis of GABA [2] and is considered a specific marker for GABAergic neurons and their processes [3]. The evaluation of GAD activity is therefore very important in neurochemical research.

In an investigation into the effects of low doses of He–Ne laser radiation on the brain metabolism of rats [4–7], we started a study with the purpose of elucidating the variations in GAD activity induced in vitro and in vivo by irradiation with low doses of He–Ne laser radiation. In the first phase of the research we developed an improved method of evaluation of GAD activity in which the enzyme was incubated with glutamic acid and PLP; the GABA formed was determined, after precolumn derivatization to PTC-GABA, by isocratic HPLC with UV absorbance detection (254 nm).

This improved method was subsequently applied to the evaluation of the variations of GAD activity induced by in vitro irradiation with a 5 mW He–Ne laser at doses between 0.1 and 0.5 J.

2. Experimental

2.1. Chemicals

HPLC-grade solvents and all other reagents were purchased from Sigma (Milan, Italy); HPLC-grade

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water was prepared using a Model Milli-Q RG apparatus (Millipore, Molsheim, France).

2.2. Enzyme source

A GAD (EC 4.1.2.15) preparation purified from *Escherichia coli* was obtained from Sigma.

2.3. Decarboxylation of Glu catalyzed by GAD

Decarboxylation of Glu was performed under the conditions described by Allen and Griffiths [8]. To 500 μl of GAD solution (0.005–0.02 μg GAD/μl) in 200 m*M* potassium phosphate buffer, pH 6.8, 15 μl 50 m*M* L-Glu and 55 μl 0.2 m*M* pyridoxal 5′-phosphate (PLP) were added. After incubation for 20 min at 37°C, 3 ml absolute ethanol was added at −20°C to terminate the reaction. The suspension was centrifuged at 1500 *g* (10 min, 0°C).

2.4. Derivatization of GABA and standard curve

GABA present in the supernatant or in a standard solution of GABA of convenient concentration was derivatized to phenylthiocarbamyl-GABA (PTC-GABA) following the indications of Gunawan et al. [9]. A 100-μl aliquot of supernatant (or of standard solution of GABA) was dried under vacuum. The residue was dissolved in 20 μl of ethanol–water–triethylamine (2:2:1) and evaporated to dryness under vacuum. A 30-μl volume of ethanol–water–triethylamine–PITC (7:1:1:1) was added to the residue and allowed to react for 20 min at room temperature to form PTC-GABA. Excess reagent was then removed under vacuum.

The standard curve for GABA was determined by applying the same procedure to four standard solutions of GABA (0.125, 0.25, 0.50 and 0.75 m*M* GABA).

2.5. HPLC separation and evaluation of PTC-GABA

The dry residue containing PTC-GABA was dissolved in 100 μl of the mobile phase, consisting of a mixture of 80% solution A (aqueous solution of 8.205 g sodium acetate, 0.5 ml triethylamine, 0.7 ml acetic acid and 5.0 ml acetonitrile in 1000 ml) and 20% solution B [acetonitrile–water (60:40)], adjusted to pH 5.8.

Isocratic HPLC separations were performed on a Kontron Instruments (Milan, Italy) apparatus, consisting of a model 32X pump system, a Rheodyne Model 7125 injector, with a 20-μl injection loop and a Model 332 UV detector, in conjunction with a Hewlett-Packard (Avondale, PA, USA) Model 3396 (II series) integrator.

A LiChrospher 100 RP-18 column (250×4 mm I.D., particle size 5 μm; Merck, Darmstadt, Germany) was used, together with a LiChrocart RP-18 (4×4 mm I.D., particle size 5 μm) guard column. The mobile phase for isocratic elution was pumped at 0.6 ml/min, at room temperature; detection was at 254 nm.

2.6. In vitro irradiation of GAD

The enzyme solution (10 μg GAD in 2 ml 0.2 m*M* KH_2PO_4 in the presence of 0.2 m*M* PLP) was irradiated, under dim light, in a disposable cuvette, on the whole surface (1 cm^2), with a He–Ne laser, at a distance of 15 cm, at room temperature. Irradiation times were 20, 40, 60, 80 and 100 s, and the corresponding radiation doses were 0.1, 0.2, 0.3, 0.4 and 0.5 J. The irradiations were performed in triplicate; for each irradiated sample the HPLC runs were performed in duplicate (the duplicate data were mediated).

Enzyme activity was determined as described above immediately after irradiation.

The statistical significance of the differences between groups was evaluated by variance analysis (ANOVA); differences were considered significant for $P \leq 0.05$ and highly significant for $P \leq 0.01$.

3. Results and discussion

3.1. HPLC separation and quantitation of PTC-GABA

HPLC separation of a standard mixture of PTC-GABA and PTC-Glu, under the conditions described above, gave two well-resolved and symmetrical peaks; the retention times (3.46 min for PTC-Glu and 12.98 min for PTC-GABA) were reproducible

when temperature variations were ≤1°C. Peak area values showed good reproducibility, with relative standard deviation (R.S.D.) values between 0.30 and 11.50%.

The calibration curve for PTC-GABA was calculated by linear least-squares regression. The regression equation was $y=4068x-3009$ ($r=0.9961$). The data showed a linear relationship between peak area and PTC-GABA concentration in the range 5–20 pmol (i.e., in the range of the best response of the apparatus). The limit of quantitation (the lowest concentration that can be assayed with R.S.D.≤10%) was below 0.25 pmol/μl.

3.2. GAD activity determination

In the chromatogram obtained after the derivatization of GABA formed from L-Glu in the GAD activity assay, the PTC-GABA peak was well separated from the PTC-Glu peak and from the smaller peaks of secondary metabolites (Fig. 1). Retention time variations at constant temperature were insignificant; R.S.D. for peak-area values ranged from 2.6 to 11.5%.

As in the case of standard GABA, the calibration curve showed a linear relationship between GAD concentration (between 1 and 6 ng GAD, i.e., in the range of the best response of the apparatus) and peak area, the regression equation being $y=9817x-2958$; $r=0.9918$.

3.3. GAD activity variations following He–Ne laser irradiation

As can be seen in Table 1 and Fig. 2, upon irradiation of the enzyme solution under the above conditions, GAD activity showed, with respect to control, highly significant increases upon irradiation with doses of 0.2, 0.3, 0.4 and 0.5 J, while a dose of 0.1 J caused a non-significant variation.

Effects of irradiation at doses higher than 0.5 J have not been studied, since under such conditions laser irradiation causes thermal effects which do not allow discrimination between effects of irradiation and effects of temperature increase.

The method described in this study is an improvement of the assay described by Allen and Griffiths [8], since the gradient elution is substituted by the more simple and reproducible isocratic elution and OPA is substituted by PITC as derivatizing agent. Derivatization with PITC provides stable amino-acid derivatives which absorb at 254 nm and can be evaluated by means of the more versatile UV–VIS

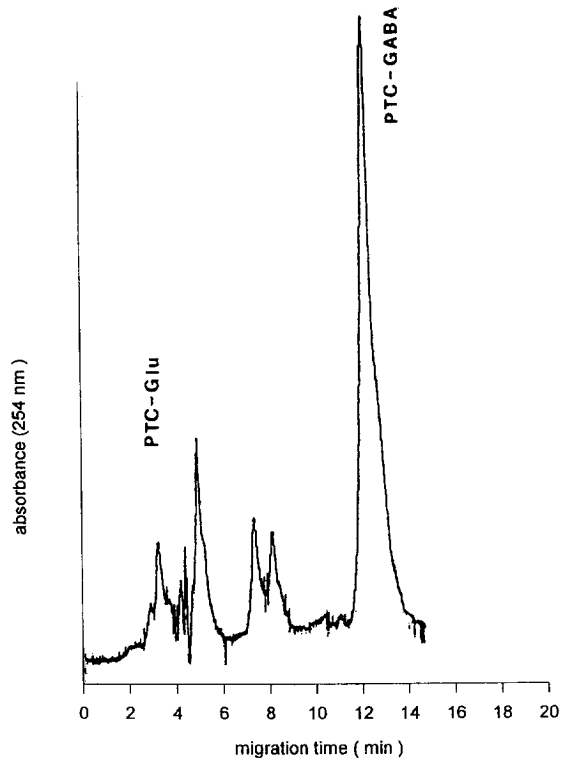

Fig. 1. HPLC separation of PTC-GABA formed in the GAD activity assay under the conditions described in Section 2.

Table 1
GAD activity variations following in vitro He–Ne laser irradiation

Dose (J)	GAD activity (μg)	Activity increases with respect to the control	Significance (*P* value)
0	10.30±0.61	–	–
0.1	10.93±0.15	0.63	N.S.
0.2	13.53±0.65	3.23	≤0.01
0.3	14.70±0.10	4.40	≤0.01
0.4	14.07±0.83	3.77	≤0.01
0.5	12.40±0.26	2.10	≤0.01

Data are means±S.D.; irradiations were performed in triplicate, HPLC runs were performed in duplicate (the duplicate data were mediated); N.S., not significant.

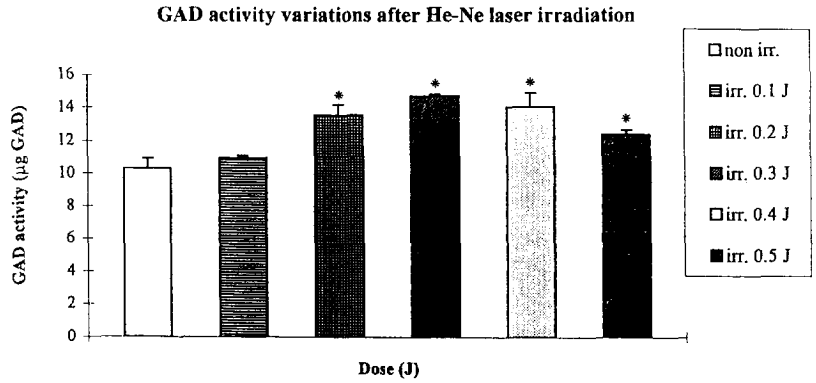

Fig. 2. Variations in GAD activity with different He–Ne laser radiation doses (data are means of 3 determinations±S.D.; *$P \leq 0.01$).

absorbance detector, while lowering the detection limit to the pmol level.

Data presented in this study indicate that the method described for GAD activity evaluation is specific and reproducible. The minor peaks, which are also present in a blank run, are well separated from the PTC-GABA peak, and therefore do not interfere with it. The asymmetry of the PTC-GABA peak, amplified in Fig. 1 by the high sensitivity and the low attenuation used, does not interfere with the reproducibility of the peak area or with the linearity of the calibration curve; the same can be said for baseline instability.

The high sensitivity and the low attenuation values were used to make the method also suitable for the evaluation of GAD activity in rat brain extracts, i.e., at very low activity levels.

The data reported in this paper show that GAD irradiation with low doses of He–Ne laser in vitro causes significant variations of GAD activity: although a dose dependence is demonstrated, the dose–activity relationship is non-linear.

GAD activity variations upon laser irradiation are in accordance with previous observations of photostimulation of other enzymes by coherent and non-coherent radiations in vitro [10–12] and in vivo [4], and in particular with the increase of GAD activity reported by Safarov and Kerimov [13] in the brain tissues of rats treated with low-frequency radiation.

Although the effects of coherent and non-coherent light on biological systems have been widely investigated [14], the mechanisms of interaction still remain to be fully established.

The significant variations of GAD activity upon irradiation might be due to the interaction of coherent electromagnetic laser radiation with the low-energy bonds at the catalytic site of the enzyme [10]. A trigger mechanism could account for the considerable variations in enzymatic activity upon irradiation with such low-energy doses [10].

Acknowledgments

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