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Short Communication

Enzyme inhibition X: colorimetric method for determining gabase activity and its comparison with a spectrophotometric method*

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Introduction

Gabase is a partially purified extract of induced cells of *Pseudomonas fluorescens* [1, 2]. It contains mainly two enzymes, γ-aminobutyrate ketoglutarate aminotransferase (GABA-T) and succinic semialdehyde dehydrogenase (SSDH). The inhibitors of these enzymes possess anticonvulsant properties. The mechanism of action of most anticonvulsants is not fully or clearly understood. However, the effect of some anticonvulsant agents is correlated in part with the increased levels of γ-aminobutyric acid (GABA) which is a neurotransmitter in the brain and other parts of the Central Nervous System (CNS) [3–5]. GABA is formed in the brain by the decarboxylation of glutamic acid (GA) with glutamic acid decarboxylase (GAD). Since GABA has been found in the brain of certain mammals, it is important to determine its level in the brain and different regions of the CNS. GAD enzyme activity has recently been determined by different methods [6].

Gabase, being a mixture of the enzyme GABA-T and SSDH, catalyses two reactions:

$$\text{GABA} + \alpha\text{-ketoglutarate} \xrightarrow[\text{SSDH}]{\text{GABA-T}} \text{succinic semialdehyde (SSA + GA)} \quad (1)$$

$$\text{SSA} + \text{NADP} + \text{H}_2\text{O} \rightarrow \text{succinate} + \text{NADPH}. \quad (2)$$

Several anticonvulsant agents inhibit GABA-T and SSDH activities in *in vitro* and *in vivo* test systems [7–11] resulting in an increase of GABA concentration in the brain. A decrease of GABA in the CNS, particularly in the brain has been implicated in epilepsy [12, 13]. Therefore, inhibitors of gabase activity play an important role in the control of different forms of epilepsy.

GABA is conventionally determined by the UV spectrophotometric method which is based on the change in absorption of compounds formed during incubation of reaction mixture at 25°C by the action of gabase on GABA. If the enzyme in the reaction mixture is not fully active, the change in absorption may be slow or scant leading to misleading results. To overcome this difficulty, a colorimetric method for determining GABA was developed using the 20 D spectronic spectrophotometer. The results obtained by the colorimetric and UV spectrophotometric methods were comparable. The colorimetric method can be used to study the inhibition of gabase activity by antiepileptic drugs.

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Experimental

Materials

Gabase was purchased from Sigma (St Louis, MO) and had a specific activity of 1.17 units/mg protein. One unit of gabase is defined as the enzyme activity to convert 1 μmole of GABA to succinic semialdehyde and then to succinate per minute with stoichiometric reduction of 1 μmole of NADP at pH 8.6 and 25°C. TLC preparative plates (2 mm F 254) were of E. Merck, Germany. GABA and GA were purchased from Sigma. All other chemicals and reagents were of analytical grade.

Amino acid isolation and assays

GA and GABA were isolated from the enzyme reaction mixture incubated at 25 and 37°C by employing preparative TLC plates and extracting the amino acids with 2 ml of 0.2 M citric acid buffer, pH 5.00. The extracts after filtration were treated with 1.5 ml of 0.2% solution of ninhydrin in ethanol in the presence of 2 ml of 0.1% aqueous solution of ascorbic acid. The solution was boiled for 30 min, cooled in ice water and Optical Density (O.D.) determined by the 20 D spectronic spectrophotometer at 575 nm. The estimations of GA and GABA were made by referring to their respective standard curves. GABA was also estimated by Jakoby's method [1] by using Varian 90 DMS UV spectrophotometer. It was based on the reduction of NADP to NADPH by coupled enzyme reactions resulting in an increase of O.D. at 340 nm. A standard curve of GABA was prepared by plotting $\Delta A340$/min versus total micrograms of GABA.

Enzyme assay

Gabase activity was determined by the method of Scott and Jakoby [2]. The reaction mixture (4 ml) contained 0.15 mM Tris-HCl buffer, pH 7.9, 0.45 mM sodium sulphate, 0.15 mM β-mercaptoethanol (0.7%), 3.9 μM NADP in 1.0 M Tris-HCl, 0.006 mM α-ketoglutarate, 10–100 μg ml^{-1} GABA and 15 mg enzyme. Appropriate quantities of inhibitors (potassium arsenate, potassium cyanide or sodium valproate) were used for enzyme inhibition studies. The control assay mixture did not contain an inhibitor.

The reaction mixture in cuvette was incubated in the spectrophotometer for 4–5 min to achieve an equilibrium temperature of 25°C. The reaction was initiated at zero time by the addition of α-ketoglutatrate, and absorption per minute at 340 nm for 15 min was recorded. After recording absorption, the reaction mixture was incubated at 37°C for 30 min. This reaction mixture was then used for estimating GABA and GA by the colorimetric method. A blank rate of the enzyme activity was determined by observing ΔA/min from the initial linear portion of the curve. The inhibited enzyme activity was determined in presence of potassium arsenate, potassium cyanide or sodium valproate in the reaction mixture.

Calculation

From the unit definition of gabase activity in the reaction mixture, the units of gabase in the reaction mixture (U in μmoles) was determined by the formula:

$$U = \frac{A340/\text{min} \times 3.02}{6.22}, \qquad (3)$$

where 3.02 is the total volume of the reaction mixture and 6.22 is the extinction coefficient of NADP at 340 nm. The number of micrograms of GABA converted in the reaction mixture were calculated by multiplying μmole GABA by 103.1 (mol. wt GABA). The enzyme activity was expressed as micrograms of GABA or GA formed in the reaction mixture.

Results and Discussion

Spectrophotometric method

As shown by the reactions in the introduction, gabase activity is responsible for the conversion of GABA to SSA and GA and then to succinate with a reduction of NADP to NADPH. Gabase activity was, therefore, determined based on these sequence of reactions in the reaction mixture by the UV spectrophotometric method [2, 14]. GABA is also determined by radioisotopic and fluorometric methods [15, 16]. The conventional UV spectrophotometric method of GABA determination at 25°C was based on conversion of GABA to GA by GABA-T resulting in an increase of optical density. However, the change in absorption was slow which can be due to the poor activity of the enzyme or an inappropriate incubation temperature. A TLC of the reaction mixture after the UV spectrophotometric analysis of GABA was carried out with the various solvents (Table 1). The effective solvent for separating the amino acids

Table 1
TLC of reaction mixture after the UV spectrophotometric analysis of GABA

Solvent mixture	R_f Value* GA	GABA
Pyridine–methanol–water (0.4:2:8)	0.85	0.76
N-Butanol–acetic acid–water (0.4:1:1)	0.87	0.75
Alcohol–water (9:1)	0.70	0.46
N-Propanol–water (1:1)	0.80	0.63
Phenol–water (3:1)	0.87	0.70

*Ninhydrin solution was used as a spray reagent. The separation of compounds was carried out on silica gel plates, KGF (Whatman).

from the TLC plates was 90% alcohol. The reaction mixture indicated two spots, GABA and GA in which GA had a higher R_f value. However, when the reaction mixture after the UV spectrophotometric analysis was incubated at 37°C for 30 min, only one spot of GA was observed on a TLC plate. This indicated that the complete conversion of GABA to GA was obtained at the incubation temperature of 37°C instead of 25°C. The amino acids isolated from the reaction mixture by a preparative TLC formed a coloured amino acid–ninhydrin complex when treated with the ninhydrin solution. The colour absorption of this complex revealed a correlation with the quantity of

Table 2
Estimation of GABA and GA in the incubated reaction mixture

Experiment no.*	Amino acid	UV spectrophotometric method (μg) 25°C	37°C	Colorimetric method (μg) 25°C	37°C	Per cent error 25°C	35°C
1	GABA†	15.00	16.50	14.50	16.00	3.34	3.17
	GA‡	24.00	26.40	23.00	25.50	4.17	3.41
2	GABA†	20.00	23.00	19.00	22.00	5.00	4.35
	GA‡	32.00	36.80	30.75	35.50	4.91	3.54
3	GABA†	25.00	29.00	23.75	27.75	5.00	4.31
	GA‡	40.00	46.40	38.50	44.50	3.75	4.10

*Experimental procedure is given in experimental section. The results expressed under the UV spectrophotometric and colorimetric methods were the average of three assays. In the colorimetric method, the reaction mixture after UV spectrophotometric assay, was incubated at 37°C for 30 min.

†GABA was determined by the UV spectrophotometric and colorimetric methods (Experimental) from the standard curves of Figs 1 and 3, respectively.

‡GA quantities in micrograms shown under the UV spectrophotometric method are the values obtained theoretically calculated from the reaction of GABA to GA by GABA-T. GA quantities in micrograms shown under colorimetric method were determined from the standard curve (Fig. 2). GA was isolated by a preparative TLC of the reaction mixture.

Table 3
Estimation of GABA and GA in the incubated reaction mixture in the presence of inhibitor*

Experiment no.†	Amino acid	UV spectrophotometric method (μg) 25°C	37°C	Colorimetric method (μg) 25°C	37°C	Per cent error 25°C	35°C
1	GABA‡	11.25	12.50	10.75	12.00	4.45	4.00
	GA§	18.00	20.00	17.00	19.00	5.56	5.00
2	GABA‡	15.10	16.50	14.50	16.00	4.07	3.13
	GA§	24.16	26.40	23.00	25.50	4.81	3.41
3	GABA‡	19.00	21.00	18.25	20.00	3.95	4.76
	GA§	30.40	33.60	29.00	32.00	4.61	4.76

*Potassium arsenate or potassium cyanide (100 μmole) was used to inhibit the gabase activity. Each compound inhibited approximately 25% of the enzyme activity. Thus, GABA quantities in micrograms determined by the UV spectrophotometric (Fig. 1) and colorimetric (Fig. 3) methods were correspondingly less than the quantities shown in Table 2. Under similar conditions, gabase activity was also inhibited by sodium valproate and hydroxylamine hydrochloride [unpublished data].

†Experimental procedure is given in experimental section. The results expressed under the UV spectrophotometric and colorimetric methods were the average of three assays. In the colorimetric method, the reaction mixture after the UV spectrophotometric assay, was incubated at 37°C for 30 min.

‡GABA was determined by the UV spectrophotometric and colorimetric methods (Experimental) from the standard curves of Figs 1 and 3, respectively.

§GA quantities in micrograms shown under the UV spectrophotometric method are the values obtained theoretically calculated from the reaction of GABA to GA by GABA-T. GA quantities in micrograms shown under colorimetric method were determined from the standard curve (Fig. 2). GA was isolated by a preparative TLC of the reaction mixture.

amino acids obtained from the reaction mixture incubated at 25 and 37°C (Tables 2 and 3).

A standard curve of GABA by the UV spectrophotometric method [1] was prepared as shown in Fig. 1. A linear exponential curve was obtained when the cuvette contained up to 40 μg GABA. This standard curve was also used in the determination of GABA from the reaction mixture with or without the presence of inhibitor.

Colorimetric method

The colorimetric method of GA and GABA analysis was carried out by preparing standard curves of the amino acids. Figure 2 shows a standard curve of concentration versus absorption of GA by the colorimetric method as obtained by the 20 D spectronic spectrophotometer. A standard curve of GABA concentration versus absorption by the colorimetric method is shown in Fig. 3. Both amino acids obeyed Beuguer–Lambert–Beer Laws. However, a linear exponential curve of GA and GABA by the colorimetric method was obtained when the concentration of amino

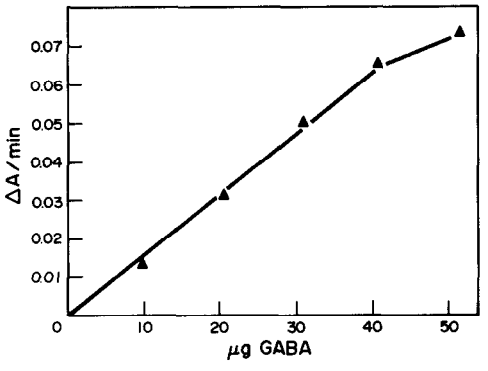

Figure 1
Standard curve of GABA (UV spectrophotometric method).

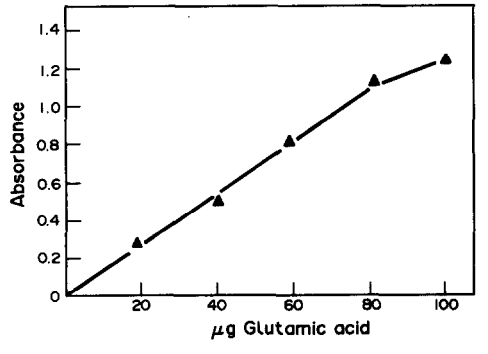

Figure 2
Standard curve of glutamic acid (colorimetric method).

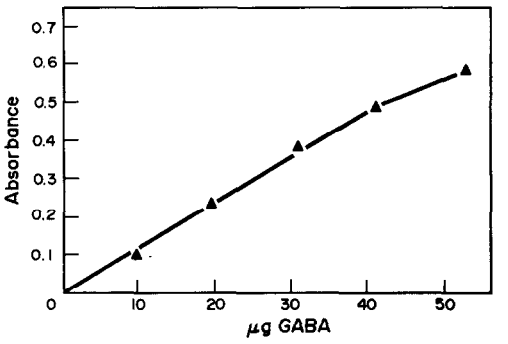

Figure 3
Standard curve of GABA (colorimetric method).

acids in the assay mixture was up to 80 and 40 μg, respectively. From the reaction mixture, GABA was estimated by the spectrophotometric and colorimetric methods by referring to Figs 1 and 3, respectively, whereas GA was determined by the colorimetric method only (Fig. 2). Table 2 shows an estimation of GABA and GA in the incubated reaction mixture at 25 and 37°C, respectively. GABA was estimated from the standard curve (Fig. 1) by the UV spectrophotometric method. After GABA determination, GA was isolated from the reaction mixture by a preparative TLC and the results were obtained by the colorimetric method. The results of these experiments indicated that quantities of GABA obtained by the UV spectrophotometric method at 25 and 37°C were 15.00, 20.00, 25.00 μg and 16.50, 23.00, 29.00 μg, respectively. With the colorimetric method, the corresponding quantities of GABA were 14.50, 19.00, 23.75 μg and 16.00, 22.00, 27.75 μg at 25 and 37°C, respectively. GA quantities determined by the colorimetric method at 25 and 37°C were 23.00, 30.75, 38.50 μg and 25.50, 35.50, 44.50 μg, respectively. Table 2 also indicates that GABA and GA determinations were approximately 10% higher when the reaction mixture was incubated at 37°C.

Comparison of spectrophotometric and colorimetric methods

A comparison of the analysis of GABA and GA by spectrophotometric and colorimetric methods indicated a 3.17–5.00% error in results at different temperatures of incubation. Table 3 shows results of three experiments of GABA and GA estimation from the incubated reaction mixture in the presence of an inhibitor. The estimated quantities of GABA

and GA in micrograms are given under the UV spectrophotometric and colorimetric methods at 25 and 37°C. GABA quantities by the UV spectrophotometric method at 25 and 37°C were 11.25, 15.10, 19.00 μg and 12.50, 16.50, 21.00 μg, respectively. The corresponding GABA quantities by the colorimetric method were 10.75, 14.50, 18.25 μg and 12.00, 16.00, 20.00 μg at 25 and 37°C, respectively. GA quantities by the colorimetric method at 25 and 37°C were 17.00, 23.00, 29.00 μg and 19.00, 25.50, 32.00 μg, respectively. These quantities were approximately 25% less than GABA and GA quantities obtained in the absence of potassium cyanide, potassium arsenate, sodium valproate or hydroxylamine hydrochloride in the reaction mixture (cf. Table 2). The results indicated that the inhibitor deactivated enzyme activity by interfering with the conversion of GABA to GA during incubation of reaction mixture at 25 or 37°C. However, GABA quantities obtained at 37°C were approximately 10% higher than at 25°C. In any case, per cent variations in results of GABA and GA determinations by the spectrophotometric and colorimetric methods remained at 3.13–5.56% both at 25 and 37°C. The data indicated that the colorimetric method of analysis of GABA was comparable to the conventional method of GABA determination by the UV spectrophotometer.

Conclusion

GA determination from the reaction mixture by the colorimetric method was reported for the first time. The method consists of incubating the reaction mixture for 30 min at 37°C, isolating GA and GABA from a preparative TLC plate, treating with ninhydrin and using a 20 D spectronic spectrophotometer for measuring colour absorbance of amino acid–ninhydrin complex. The results further indicated that the colorimetric method of estimating GABA and GA can be used for studying the inhibitory effect of anticonvulsant and/or other inhibitors on GABA-T and SSDH [unpublished data]. The colorimetric method is simple, convenient and accurate as compared to the UV spectrophotometric method which sometimes exhibits a slow or meagre increase in absorption due to low activity of the enzyme.

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