ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
19 (1999) 847–854

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

# Enzyme inhibition XI: glutamate decarboxylase activity relationship with the reaction products as determined by the colorimetric and radioisotopic methods[1]

Manohar L. Sethi *

*Department of Pharmaceutical Sciences, College of Pharmacy, Nursing and Allied Health Sciences, Howard University, Washington, DC 20059, USA*

Received 23 January 1998; received in revised form 22 May 1998; accepted 22 May 1998

**Abstract**

The relationship of glutamate acid decarboxylase (GAD) activity with the reaction products was developed. It was based on incubating sodium glutamate substrate (S) with GAD enzyme (E) when the enzyme-substrate-complex (ES) product was obtained along with gamma aminobutyric acid (GABA) and unreacted sodium glutamate. The reaction products were separated by paper chromatography. The ES, GABA and S products were sprayed with ninhydrin reagent when ninhydrin-colored-complex (NCC) was formed on the paper chromatogram. The products were extracted with 75% ethanol containing 0.5% cupric sulfate. The NCC absorption readings of ES and S products were measured by a spectrophotometer. A standard curve was prepared by plotting absorption readings against different concentrations of sodium glutamate. This curve was the basis of determining GAD activity of *E. coli* and *C. welchii*. It was observed that NCC absorption of ES and S products was directly related with the enzyme activity. The qualities of ES and S products in the reaction mixture increased as the enzyme activity increased with the incubation time. On the other hand, some products in the reaction mixture decreased in the presence of an inhibitor of GAD activity. The relationship of reaction products with GAD activity was also established by the radioisotopic method. The results obtained by the chromatographic separation of products followed by the spectrophotometric method of determining GAD activity is a simple, safe and less expensive compared to the other methods. 

*Keywords:* GAD enzyme activity; Colorimetric method; Radio-isotopic method; Inhibition

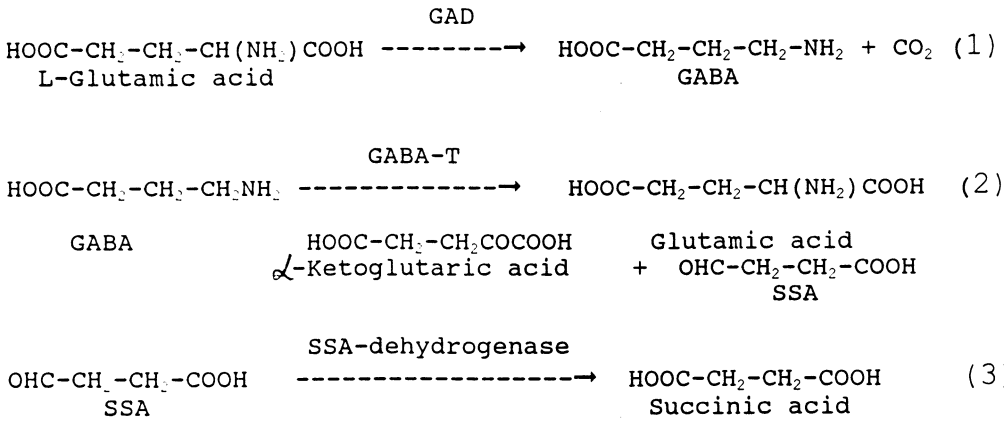
## 1. Introduction

Glutamate decarboxylase (GAD) is an enzyme that catalyzes the removal of a carboxyl group from L-glutamic acid to produce $\gamma$-aminobutyric acid (GABA) and carbon dioxide. In the mammalian central nervous system (CNS), L-glutamic

* Corresponding author. Tel.: +1 202 8066549; fax: +1 202 8064636.

[1] The work was partly presented at the International Symposium on Medicinal Chemistry held in Maastricht, The Netherlands, 8–12 September 1996.

0731-7085/99/$ - see front matter 
*PII* S0731-7085(98)00140-X




Scheme 1. Sequence of enzyme reactions.

acid and GABA are excitatory and inhibitory neurotransmitters, respectively [1–4]. Epileptic seizures are recognized when there is a deficiency of GABA in the brain. Two major enzymes, GAD and GABA-transaminase (GABA-T), are involved in the metabolism of GABA. In sequence of enzyme reactions (1)–(3) (Scheme 1), L-glutamic acid is converted to GABA by GAD. Subsequently, GABA is degraded to succinic semi-aldehyde (SSA) by GABA-T in the presence of $\alpha$-ketoglutaric acid which is converted to glutamic acid. On further metabolism, SSA is oxidized to succinic acid by SSA-dehydrogenase. The concentration of GABA is thus dependent on chain reactions by GAD, GABA-T and SSA-dehydrogenase leading to the products formed in the CNS. Many anticonvulsant agents increase GABA levels in the brain either by mimicking, enhancing inhibitory action of GABA or by inhibiting reactions (2) and (3) [5,6]. Scheme 1

The objectives of the research were to isolate reaction products by chromatographic methods and to establish a relationship between reaction products and GAD activity. The latter activity is determined mainly by manometric, radioisotopic and fluorometric methods. But work on colorimetric and chromatographic methods of GAD activity determination is scanty. In the continued search for exploring colorimetric methods for determining GAD enzyme activity [7], the present work reports a relationship between reaction products and GAD activity based on separating sodium glutamate (S) and enzyme–substrate (ES) complex from GABA by paper chromatography (PC). These products were sprayed with ninhydrin reagent. Their ninhydrin-colored complex (NCC) absorption readings were determined colorimetrically and related to enzyme activity. Simple carboxylic acids including sodium valproate are inhibitors of GAD and GABA-T activities, as determined by manometric and radioisotopic methods [8–12]. Sodium valproate and sodium arsenate (inhibitors) were chosen so that results obtained previously by standard radio-isotopic method are compared with the colorimetric method [8]. Sodium valproate is used in the treatment of both generalized and partial seizures.

## 2. Experimental

### 2.1. Materials

Purified L-glutamate decarboxylase from *E. coli* and *C. welchii* was a commercial product of Sigma (St. Louis, MO) with a specific activity of 0.41 unit (mg protein)$^{-1}$. One unit of enzyme released 1.0 μmol of carbon dioxide per min from 0.35 μmol L-glutamic acid at 37°C. L-[$^{14}$C]Glutamic acid was obtained from New England Nuclear (MA) with a specific activity of 49.6 mCi mmol$^{-1}$. Hyamine base (benzethonium chloride) was obtained from Aldrich (Milwaukee, WI). Sodium valproate was prepared by neutraliz-

ing valproic acid (Saber Laboratories, IL) with an equivalent quantity of sodium hydroxide solution and adjusting the solution to pH 7. All other chemicals and reagents were analytical grade.

### 2.2. Colorimetric method

GAD activity and inhibitory studies were carried out by the modified method [7,13]. The reaction mixture (3 ml) contained 0.50 mM sodium acetate, pH 5.0, 0.30 M sodium chloride, 25 μM sodium glutamate and 50 μl (0.20 units) of enzyme. Following incubation at 37°C for 15 min, the reaction was stopped by cooling on ice for 15 min. After cooling, appropriate volumes of the reaction mixture were streaked on Whatman chromatography paper and developed with *N*-butanol–acetic acid–water (5:3:2) solvent. After development, the paper was dried and sprayed with ninhydrin solution (0.1%, w/v in 90% ethanol). The NCC bands of the S and ES complex were separated from GABA, cut into small pieces, and extracted with 6 ml of 75% alcohol, containing 0.05% cupric sulfate, by heating to boiling. After cooling, the absorption readings were taken by a Bausch & Lomb DB spectronic spectro-photometer at 575 nm. The standard curves were prepared by plotting the NCC absorption readings of S and ES complex obtained from the incubated reaction mixture against S concentration. A control curve was prepared from S obtained from the reaction mixture at 25°C. The absorption readings were corrected for blank readings obtained by extracting pieces of paper between bands with the same solvent. The inhibitory studies were carried out in the presence of an appropriate concentration of inhibitor by obtaining S and ES complex from the incubated reaction mixture. The NCC absorption readings of ES complex and S were referred to the standard curve and results were expressed as percent inhibition. The control reaction mixture contained all of the ingredients except an inhibitor.

Table 1
Chromatography of the reaction mixture[a]

| Solvent mixture | $R_f$ value | | |
|---|---|---|---|
| | ES complex | S | GABA |
| Paper | | | |
| Methanol–toluene (9.5:0.5) | 0.25 | 0.30 | 0.45 |
| Methanol–chloroform (9:1) | 0.15 | 0.20 | 0.35 |
| *N*-Butanol–acetic acid–water (5:3:2) | 0.50 | 0.60 | 0.85 |
| TLC | | | |
| Methanol–chloroform (9.5:0.5) | 0.35 | 0.42 | 0.20 |
| Ethyl alcohol–water (9:1) | 0.65 | 0.75 | 0.50 |
| *N*-Propanol–water (1:1) | 0.75 | 0.85 | 0.57 |

[a] Ninhydrin (0.1%, w/v) in 90% ethanol was used as a spray reagent. Whatman paper and Merck KGF silica gel plates were used for chromatography. GABA spot was revealed from the incubated reaction mixture only. ES complex and S represent enzyme–substrate complex and sodium glutamate, respectively.

Table 2
Absorption of reaction products[a]

| Product | Ninhydrin-colored complex absorption reading | |
|---|---|---|
| | 37°C | 25°C |
| ES complex and S | 0.21 | 0.13 |
| GABA[c] | 0.05 | —[b] |
| | Percent loss of S | |
| Standard error[d] | 8.00 | 7.00 |

[a] The composition of the reaction mixture is described in the Section 2. For preparative PC, 200 μl of the reaction mixture containing 282 μg of S was streaked on the paper. The readings were an average of five experimental assays. ES complex and S represent enzyme–substrate complex and sodium glutamate, respectively. Their combined absorption readings were referred to Fig. 1 for corresponding S quantity.
[b] ES complex and GABA were not detected at this temperature.
[c] GABA was a minor quantity. Therefore, no standard curve was prepared for corresponding S quantity.
[d] A standard error was expressed as the percent loss of S from the applied and recovered S quantity.

### 2.3. Radioisotopic method

GAD activity and inhibitory studies were carried out by modifying the published method [10]. Essentially, the reaction mixture contained 0.05 M

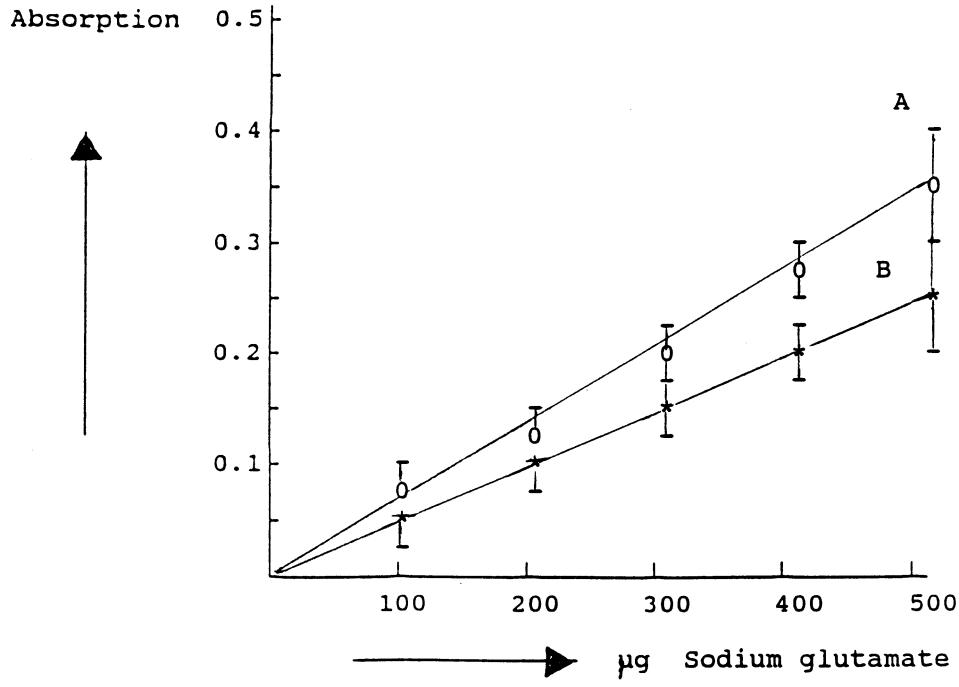



Fig. 1. Standard (A) and control (B) curves. After paper chromatography, the NCC (ninhydrin-colored complex) abrorption readings of sodium glutamate and ES (enzyme–sustrate) complex from the reaction mixture at 37°C (A) and sodium glutamate from the reaction mixture at 25°C (B) were plotted against the quantity of sodium glutamate. Data are mean values of standard error less than 10%.

L-glutamic acid, 0.012 μmol pyridoxal phosphate, 0.013 μmol L-[$^{14}$C]glutamic acid, 0.08 μmol sodium chloride, 0.05 M sodium acetate buffer, pH 5.0, and 100 μl of enzyme (0.3–0.5 units). Following incubation at 37°C for 1 h, the reaction was stopped by adding 0.20 ml of 75% trichloroacetic acid and the mixture was cooled on ice for 15 min. The filter paper soaked in hyamine base (benzethonium chloride) absorbed radiolabeled carbon dioxide. The radioactivity was determined in a toluene-based scintillation fluid. The results were calculated by comparing with the control enzyme activity. The inhibitory studies were carried out in the presence of an inhibitor.

## 3. Results and discussion

In establishing a relationship between GAD activity and the reaction products using the radioisotopic and colorimetric methods, preliminary chromatography of the incubated reaction mixture indicated products consisting of a major spot corresponding to ES complex and a minor spot corresponding to S and GABA. Similar spots were observed in determining GABAase activity [7]. Table 12223 lists solvents, two types of chromatographic techniques and $R_f$ values of ES complex, S and GABA. The $R_f$ value of ES complex and S were generally higher on TLC than on paper. On the other hand, the $R_f$ value of GABA was higher than ES complex or S on paper than on TLC plate by using various solvents. The magnitude and relative order of $R_f$ vales of ES, S and GABA on thin-layer and paper chromatographies is unknown; but it may be due to the chemical affinity of glutamate decarboxylase for the carboxyl group of glutamic acid and GABA relative to physical adsorption and lipophilicity of

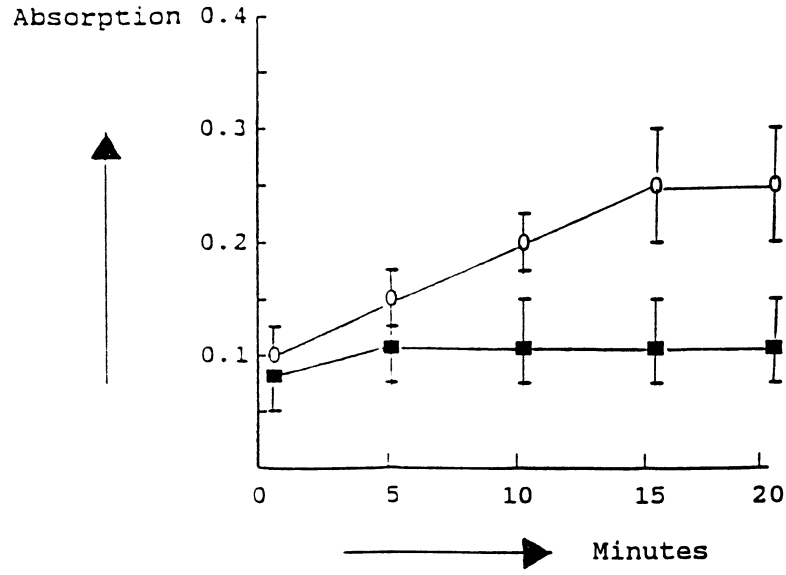



Fig. 2. Kinetics of enzyme reaction mixture. After chromatography, the NCC (ninhydrin-colored complex) absorption readings of sodium glutamate and ES (enzyme–substrate) complex from the reaction mixture at 37°C (○) and sodium glutamate from the reaction mixture at 25°C (■) were plotted against incubation time. Vertical bars indicate mean values of standard error less than 10%.

reaction products on paper cellulose or silica gel. *N*-Butanol–acetic acid–water was a better solvent because of a clear separation of ES complex and S from GABA. Preparative PC was used for obtaining reaction products, which were extracted completely from the paper but not from the TLC plate with 75% alcohol. Cupric ions are known to interact stoichiometrically with amino acids and produce deep-blue copper derivatives [14,15]. Cupric sulfate imparted deep-blue color to the NCC of amino acids, which were easily extracted with 75% alcohol. GAD activity was based on the quantities of ES complex and S, which were determined colorimetrically by their NCC absorption. Upon critical chromatographic examination of the incubated reaction mixture, it was observed that an enzyme (E) reacted with sodium glutamate (S) to form ES complex which yielded GABA (product) as shown below:

$$\underset{\text{Enzyme}}{\text{E}} + \underset{\text{Substrate}}{\text{S}} \rightarrow \underset{\text{Complex}}{\text{ES}} \rightarrow \underset{\text{Product}}{\text{GABA}} + \text{E} + \text{S}$$

Although GABA should be a major product, reaction mixture contained mainly ES complex indicating incomplete conversion of ES complex to GABA. Thus, PC revealed three spots upon spraying with ninhydrin reagent. The S spot ($R_f$ value 0.60) was blue in color. The ES complex spot was yellowish-blue ($R_f$ value 0.50) and the GABA spot was violet ($R_f$ value 0.85). In order to determine the quantity and nature of reaction products, attempts were made to separate S from ES complex with butanol–acetic acid–water (5:3:2) and other solvents, but none of the solvents showed adequate separation of ES complex from S spots because of similar polarity of the compounds.

To establish a relationship of products formed at 37 and 25°C, two portions of the reaction mixture, each containing 282 μg of S, were prepared. One portion was incubated at 37°C and the other was kept at 25°C for 15 min. Preparative PC was used to extract NCC bands of S, ES complex and GABA. The NCC absorption readings of S, ES complex and GABA obtained from the reaction mixture were 0.07, 0.14 and 0.05, respectively. Since the NCC absorption reading of S or GABA was low as compared to ES complex, the latter was present as the main product in the

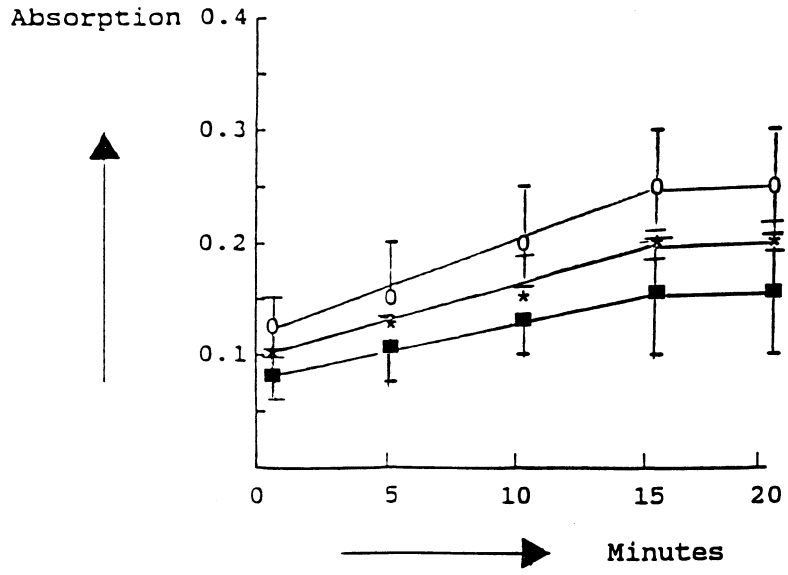



Fig. 3. Inactivation of GAD by sodium valproate. After chromatography, the NCC (ninhydrin-colored complex) absorption readings of sodium glutamate and ES (enzyme–substrate) complex from the reaction mixture at 37°C containing no drug (control, ○), sodium valproate at 100 (*) or 200 μg (■) were plotted against incubation time. Vertical bars indicate mean values of standard error less than 10%.

incubated reaction mixture. Due to similar $R_f$ values and the concomitant nature of S and ES complex, it was difficult to separate these products. Table 2 shows products obtained by PC of the reaction mixture and their NCC absorption readings. The NCC absorption readings (0.21) of ES complex and S obtained from incubated reaction mixture corresponded to 300 μg S (Fig. 1A). The NCC absorption readings (0.13) of ES complex and S obtained from the reaction mixture at 25°C (control) corresponded to 250 μg S (Fig. 1B). The higher NCC absorption reading of ES complex and S from the incubated reaction mixture, as compared to control, was due to specific interaction of ninhydrin with ES complex at the incubated temperature. It was conceivable that enzyme reaction at 25°C did not proceed to a detectable product, ES complex or GABA. But in the reaction mixture at 37°C, ES complex was predominant as the enzyme activity increased upon incubation of reaction mixture at 5, 10 and 15 min (Fig. 2). In any case, NCC absorption readings of S and ES complex were employed to construct standard and control curves (Fig. 1). Although, ninhydrin forms intensely colored complexes with amino acids, this interaction is also exhibited by peptides and proteins [14,16]. The intensity of color in the complex formation varies with the type of proteins.

The Beuguer–Lambert–Beer Laws were obeyed in the colorimetric method when the NCC absorption readings of S and ES complex were plotted against S obtained from the reaction mixture at 37°C (Fig. 1A). For comparison, a control curve of S obtained from the reaction mixture kept at 25°C was also prepared (Fig. 1B). The purpose of preparing this curve was to ascertain that ES complex and S were formed in the incubated reaction mixture. Also, it was used in establishing a standard error (Table 2). The kinetics of enzyme reaction at 37°C exhibited a linear exponential line of ES complex and S when the NCC absorption readings were plotted against incubation period (Fig. 2). The reaction mixture reached equilibrium in 15 min. For determining enzyme activity, the optimum incubation time was 15 min, since the activity declined after this time period. At 25°C, there was practically no enzyme activity

Table 3
Comparison of percent inhibition of GAD activity by sodium arsenate and sodium valproate using radioisotopic and colorimetric methods[a]

| Method | Sodium arsenate (conc. μg/assay mixture) | | Sodium valproate (conc. μg/assay mixture) | |
|---|---|---|---|---|
| | 35 | 80 | 35 | 80 |
| Radioisotopic (% inhibition) | 70 | 90 | 67 | 87 |
| Colorimetric (% inhibition) | 63 | 85 | 64 | 83 |

[a] All readings were an average of five experimental assays. Methods of determining GAD activity and its inhibition by sodium arsenate or cyanide and sodium valproate are described in the Section 2. A variation of $\pm 7\%$ in results was observed in the radioisotopic and colorimetric methods.

since a nearly straight line of S was obtained. At 37°C, a linear exponential line of the NCC absorption of S and ES complex, demonstrated a relationship of enzyme activity to the products.

Preliminary inhibitory studies indicated that in the incubated reaction mixture, GAD activity decreased in the presence of sodium valproate (Fig. 3). The inhibitory effect of 100 μg sodium valproate on GAD was approximately half that exhibited by 200 μg sodium valproate in the reaction mixture. Similar results were obtained when sodium arsenate or cyanide was used as an inhibitor of enzyme activity (data not shown). Cassel and co-workers reported that GAD activity was noncompetitively inhibited by sodium cyanide as it reacted with the substrate or products in the reaction mixture [17]. Although mechanism of action of sodium valproate is not fully understood, in vitro and in vivo studies indicated that valproates increased GABA levels in whole brain and several brain regions of rodents by inhibiting GABA-T and SSA-dehydrogenase [18,19]. Other workers have reported that sodium valproate increased potassium or sodium conductance on *Aplysia* neurons, thus affecting membrane ion channels [20,21].

Radioactivity and manometric methods are also used to determine GAD activity [22,23]. The latter is based on measuring carbon dioxide released in the reaction mixture when L-glutamic acid is converted to GABA. Fluorometric and chromatographic methods involve estimation of GABA in the reaction mixture [24,25]. Our in vitro studies on inhibition of enzyme activity were based on using radioisotopic and colorimetric methods. The percent inhibition of GAD activity by sodium arsenate and sodium valproate at two different concentrations are shown in Table 3. When the radioisotopic method was used, sodium arsenate in concentrations of 35 and 80 μg/assay mixture showed 70 and 90% inhibition, whereas in the colorimetric method, inhibitions were 63 and 85%, respectively. Similarly, sodium valproate in concentrations of 35 and 80 μg/assay mixture showed 67 and 87% inhibition in the radioisotopic method. The corresponding inhibitions with the colorimetric method were 64 and 83%. The results obtained by both methods were comparable and in close agreement.

## 4. Conclusion

GAD activity was determined on enzyme reactions forming products, GABA, ES complex and unreacted S and GAD. The main product, ES complex concomitant with S, was isolated by preparative PC. The NCC absorption readings of ES complex and S were determined colorimetrically and related to the enzyme activity. GAD activity was also determined using a radioisotopic method. The results were compared to the colorimetric method, and it was demonstrated that that method could be used in the study of enzyme inhibition by anticonvulsant agents. The colorimetric method is simple, convenient and less expensive.

## Acknowledgements

This grant was supported by the Minority Biomedical Research Support Program (ISO6-RR08244-03).

## References

[1] E. Roberts, T.N. Chase, D.B. Tower (Eds.), GABA in Nervous System Function, Raven Press, New York, 1976.
[2] B.S. Meldrum, Int. Rev. Neurobiol. 17 (1975) 1–36.
[3] W.B. Pratt, P. Taylor, Principle of Drug Action, in: The Basis of Pharmacology, Churchill Livingstone, New York, 1990.
[4] R.H. Levy, F.E. Dreifuss et al. (Eds.), Antileptic Drugs, Raven Press, New York, 1989.
[5] L. Battistin, M. Varotto, G. Berlese, G. Roman, Neurochem. Res. 9 (1984) 225–231.
[6] A.J. Turner, S.R. Whittle, Trends Pharmacol. Sci. 1 (1980) 257–260.
[7] M.L. Sethi, J. Pharm. Biomed. Anal. 11 (1993) 613–617.
[8] M.L. Sethi, K.R. Scott, A. Acheampong, Phytochemistry 26 (1987) 3141–3144.
[9] H.W. Blume, Y. Lamour, E. Arnauld, B.S. Layton, L.P. Renaud, Brain Res. 171 (1979) 182–185.
[10] R.B. Silverman, A.J. Muztar, M.A. Levy, J.D. Hirsch, Life Sci. 32 (1983) 2717–2723.
[11] R.W. Horton, G.M. Anlezark, M.C.B. Sawaya, B.S. Meldrum, Eur. J. Pharmacol. 41 (1977) 387–397.
[12] L.J. Fowler, J. Beckford, R.A. John, Biochem. Pharmacol. 24 (1975) 1267–1270.
[13] M.L. Fonda, Arch. Biochem. Biophys. 153 (1972) 763–768.
[14] B.S. Furniss, A.J. Hannaford, P.W.G. Smith, A.R. Tatchell (Eds.), Vogel's T.B. of Practical Organic Chemistry, 5th ed., Wiley, New York, 1989.
[15] J.P. Greenstein, M. Winitz, Chemistry of the Amino Acids, Wiley, New York, 1961.
[16] K.D. Kopple (ed.), Peptides and Amino acids, W.A. Benjamin, New York, Amsterdam, 1966.
[17] G. Cassel, L. Karlsson, A. Sellstrom, Pharmacol. Toxicol. 69 (1991) 238–241.
[18] W. Löscher, Brain Res. 501 (1989) 198–203.
[19] W. Löscher, Neurochem. Res. 18 (1993) 485–502.
[20] G.E. Slater, G.D. Johnston, Epilepsia 19 (1978) 379–384.
[21] R. Davis, D.H. Peters, D. McTavish, Drugs 47 (1994) 332–372.
[22] M.L. Fonda, Biochemistry 11 (1972) 1304–1309.
[23] E. Roberts, D.G. Simonsen, Biochem. Pharmacol. 12 (1963) 113–134.
[24] W. Löscher, Arch. Int. Pharmacodyn. 243 (1980) 48–55.
[25] Y. Godin, L. Heiner, J. Mark, P. Mandel, J. Neurochem. 16 (1969) 869–873.