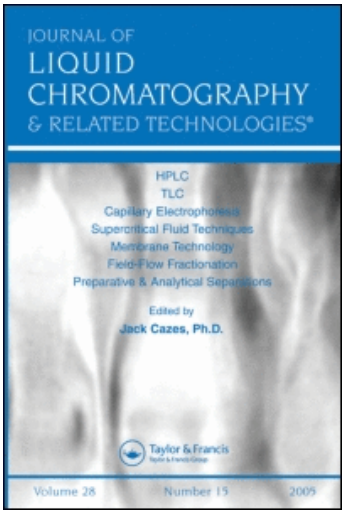


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# Determination of Glutamic Acid Decarboxylase Activity in Subregions of Rat Brain by High Pressure Liquid Chromatography

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# DETERMINATION OF GLUTAMIC ACID DECARBOXYLASE ACTIVITY IN SUBREGIONS OF RAT BRAIN BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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## ABSTRACT

A quantitative high pressure liquid chromatographic (HPLC) assay has been developed for the determination of glutamic acid decarboxylase (GAD) activity in subregions of rat brain. GAD activity was determined indirectly by measurement of gamma-aminobutyric acid (GABA). Fluorimetric detection was made possible by derivatization with ortho-phthalaldehyde and the limit of detection was 11 ng GABA.

## INTRODUCTION

Gamma-aminobutyric acid (GABA) has been identified as a principle inhibitory neurotransmitter in the central nervous system of veterbrates (1). However, GABA is distributed in glial cells as well as within neurons, while glutamic acid decarboxylase (GAD) the enzyme that decarboxylates glutamic acid (GA) to GABA,

is found only within the neurons (1). Therefore GAD activity is used as a marker of GABAergic neurons. GABA projections from neostriatum to globus pallidus (GP), entopeduncular nucleus (EP), and substantia nigra (SN) (2,3) as well as from SN to ventromedial thalamus (VM) (4) have been reported. Decreased GAD activity has been found in the striatum and SN of postmortem brains of humans afflicted with Parkinson's disease and Huntington's chorea (5,6).

A number of procedures have been developed for the indirect analysis of GAD activity via measurement of GABA formation. A radiometric method for GAD activity by measurement of $^{14}CO_2$ from the decarboxylation of $^{14}C$-labeled GA has been described by Roberts and Simonsen (7). Lowe et al (8) reported a fluorimetric procedure for the determination of GAD activity in neural tissue by measurement of GABA formation. Recently, Holdiness et al (9) reported a modification of the Lowe et al (8) method for measurement of GAD activity in subregions of rat brain. Values reported for GAD activity were 7.91 $\pm$ 1.47 (GP), 6.87 $\pm$ 2.07 (EP), 3.38 $\pm$ 0.69 (VM), 13.80 $\pm$ 2.14 ($SN_M$) and 8.23 $\pm$ 2.26 ($SN_L$) ug GABA/hour/mg protein.

In this paper is reported an HPLC method for determination of GAD activity in rat brain tissue using fluorescent detection.

## EXPERIMENTAL

### Materials

The chemicals used in this study were sodium-L-glutamate (Pfaltz and Bauer, Inc., Stamford, CN), trichloroacetic acid, gamma-aminobutyric acid and 5-aminovaleric acid (Sigma Chem. Co., St. Louis, MO). Pyridoxal 5-phosphate, triton x-100 (scintillation grade), orthophthalaldehyde and 2-mercaptoethanol were procurred from Eastman Kodac Co. (Rodchester, NY). The internal standard was prepared by dissolving 2.0 mg of 5-aminovaleric acid in 25 ml of a solution consisting of 0.50 M KCL, 0.010 M EDTA, 0.5% triton x-100 and 0.40 M sodium phosphate buffer pH 6.40.



### Instrumentation

A Waters Associates 202 HPLC with a 6UK injector was used with a stainless steel column (1 m by 4.6 mm I.D.) packed with Zipax strong cation exchange resin (DuPont, Wilmington, DE), 10 um particle size. Fluorescence was accomplished with a Perkin-Elmer MPF-4 spectrofluorimeter with an excitation wavelength of 335 nm (6 mm slit width) and as emission wavelength of 450 nm (10 mm slit width) and a dynode voltage of 500 volts. Post column derivatization was accomplished with a second Waters 6000A solvent delivery pump which connected after the column by a three way tee union. Thirty

feet of teflon tubing was required between the union and the spectrofluorimeter to allow for complete derivatization. The tubing, column and 30 ul quartz flow cell were maintained at 40°C.

Operating Procedures and Conditions

The mobile phase consisted of 0.50 M sodium acetate buffer adjusted to pH 5.0 and degassed for 30 minutes. The derivatizating agent was prepared by mixing 220 mg ortho-phthalaldehyde in a minumin amount of methanol with 200 ul of 2-mercaptoethanol dissolved in 0.50 M sodium phosphate buffer adjusted to pH 10. The flow rate was set at 2 ml/min and 0.10 ml/min for the mobile phase and derivatizating agent, respectively.



Sample Collection and Preparation

The sample collection procedure has been previously described (9). From each 1 mm thick brain tissue slice, two tissue punches were taken from symmetrical locations in the left and right hemispheres. Each set of punches was immediately transferred to a 12 ml polypropylene Eppendorf tube containing 100 ul of internal standard solution and sonicated for 10 seconds under low power sonication. After sonication, 50 ul of this solution was transferred to an identical tube containing 20 ul of 3% trichloroacetic acid (TCA). This second tube served as a blank and its concentration of GABA was

subtracted from the orginal sample. The substrate-buffer was prepared as previously described (9) and 50 ul of this solution was added to both the sample and blank tubes before they were incubated for two hours at 38°C. The enzyme was inactivated by addition of 20 ul of 3% TCA to the orginal sample tube and all tubes were centrifuged at 950 x g for 20 minutes. Injections of 40 ul of the supernate were made into the HPLC. The precipitated tissue was analyzed for protein as described by Lowery et al (10) and GAD activity has been reported in ug GABA/hour/mg protein.



## RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram of GABA and internal standard isolated from rat brain. The retention times for GABA and internal standard are 4.70 and 6.80 minutes, respectively. Positive identification was achieved by peak superimposition, i.e., by addition of GABA and 5-aminovaleric acid standards (300 ng) to the extracts and observing increased peak height at the corresponding retention times.

The lower limit of detection (2/1 signal to noise) of this procedure is 11 ng GABA. Repetitive injections of standards gave good reproducibility of retention times (standard deviation ± 2%) and peak heights (standard deviation ± 2.5%). Standard curves were

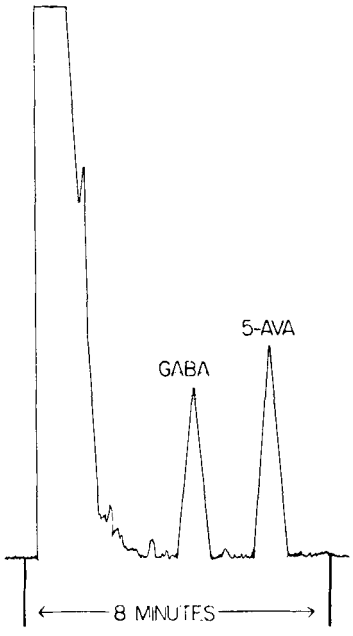


Figure 1. HPLC chromatogram of GABA and 5-aminovaleric acid (internal standard) extracted from rat brain tissue. The retention times of GABA and internal standard are 4.70 and 6.86 minutes, respectively.



linear in the range of 10 to 3000 ng and day to day reproducibility varied less than 3.1% (standard deviation). The recovery for GABA was 96 $\pm$ 6% and overall 1 to 3 fold GABA increases were observed for samples in this procedure.

The tissue punch placement is presented in Figure 2. All tissue slices are 1 mm thick and left and right hemispheric punches are combined for each nuclei. The first punch is the GP at AP 7.0 and the other locations are AP 6.0 (EP and VM) and AP 3.0 ($SN_M$ and $SN_L$) based

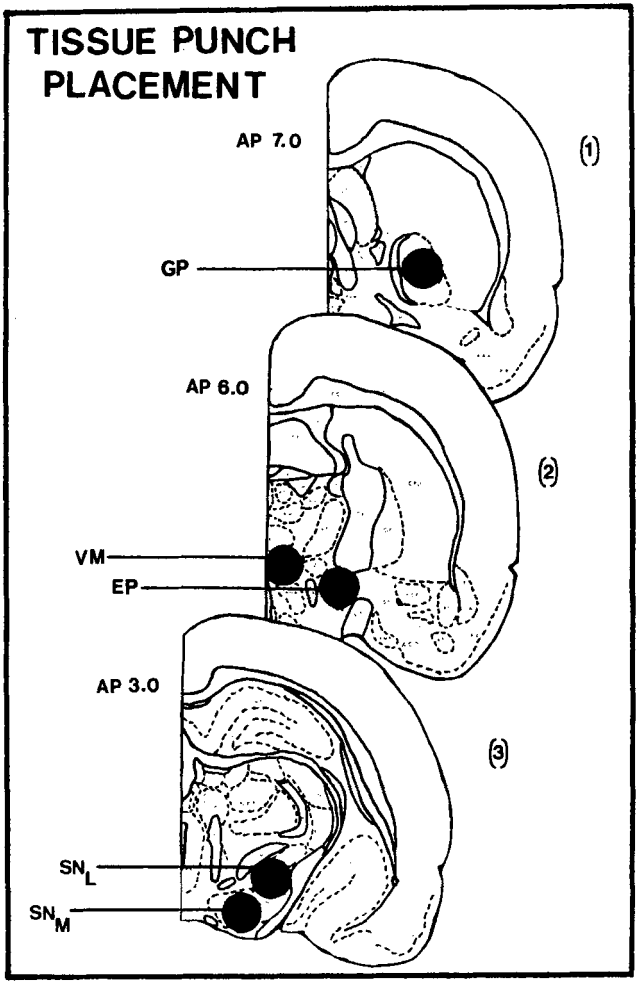


Figure 2. Tissue punch placement. The first punch was from a 1 mm thick tissue slice containing the globus pallidus (GP). The second slice contains the entopenduncular nucleus (EP) and ventromedial thalamus (VM). The third slice contains the substantia nigra medial ($SN_M$) and lateral ($SN_L$) punches. The numbers on the left refer to the anterior-posterior (AP) axis coordinates in the brain atlas of Pellegrino and Cushman (11). Punch diameter, 1.30 mm.

upon the atlas of Pellegrino and Cushman (11). An average weight of representative tissue samples from combined left and right brain punches (n = 20) is 2.30 ± 0.33 mg tissue (wet weight) with an average protein content of o.110 mg protein/mg tissue. The average GAD activity values (± standard deviation) found by this method in each brain region (n = 5) are 7.81 ± 1.08 (GP), 6.73 ± 1.58 (EP), 3.75 ± 0.71 (VM), 13.70 ± 1.75 ($SN_M$), and 8.17 ± 1.68 ($SN_L$). These values are in close agreement with the previously reported activities determined by the fluorimetric procedure (9).

In conclusion, the HPLC method described is sufficiently sensitive and specific for analysis of GAD activity in subregions of rat brain tissue.



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