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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC DETERMINATION OF GLUTAMIC ACID DECARBOXYLASE ACTIVITY IN SUBREGIONS OF RAT BRAIN

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SUMMARY

A quantitative gas chromatographic—mass spectrometric method has been developed for the determination of glutamic acid decarboxylase (GAD) activity in subregions of rat brain. The five subregions analyzed, weighing approximately 2.51 mg each, were globus pallidus, entopeduncular nucleus, ventromedial thalamus, and substantia nigra medial and lateral. The activity of the GAD enzyme has been determined indirectly by measurement of γ-aminobutyric acid (GABA) using γ-[2,2-$^2H_2$]aminobutyric acid as the internal standard. Both compounds were quantitatively converted to trimethylsilyl-GABA and trimethylsilyl-[$^2H_2$]GABA in 90 min with hexamethylchlorosilane, trimethylchlorosilane, pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide silylating agents. Using selective ion monitoring and electron impact ionization at 70 eV, the limit of detection was 15 ng GABA per mg tissue. This method is compared with a fluorimetric procedure.

INTRODUCTION

γ-Aminobutyric acid (GABA) is widely accepted as being a principal inhibitory neurotransmitter in the central nervous system of vertebrates [1]. However, GABA is distributed in glial cells as well as within neurons, while glutamic acid decarboxylase (GAD) the enzyme which 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 substantia nigra to ventromedial thalamus (VM) [4] have been reported. Decreased GAD activity has been found in the striatum and SN of post-mortem brains of humans afflicted with Parkinson's disease and Huntington's chorea [5, 6].

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A number of methods have been developed for the indirect measurement of GAD activity via measurement of GABA formation. Roberts and Simonsen [7] reported a radioenzymatic measurement of GAD activity by measurement of $^{14}CO_2$ from the decarboxylation of ^{14}C-labeled GA. The topographical distribution of GAD has been reported by Fonnum et al. [2] using a radiometric assay procedure. Lowe et al. [8] reported a fluorimetric procedure for the determination of GAD activity in neural tissue by measurement of GABA formation. Holdiness et al. [9] reported a modification of this fluorimetric method for the measurement of GAD activity in subregions of rat brain. Values reported for GAD activity were 7.91 ± 1.47 (GP), 6.87 ± 2.07 (EP), 3.83 ± 0.69 (VM), 13.80 ± 2.14 (substantia nigra medial, SN_M) and 8.23 ± 2.26 (substantia nigra lateral SN_L) μg GABA per hour per mg protein.

Recently, mass spectrometric methods have become available for measurement of GABA. Bertilsson and Costa [10] reported a gas chromatographic—mass spectrometric (GC—MS) assay method using γ-[2,2-$^2H_2$]aminobutyric acid as the internal standard with pentafluoropropionic anhydride and hexafluoroisopropanol as the derivatizing agents. Cattabeni et al. [11] developed a GC—MS procedure for GABA by forming the trimethylsilyl (TMS) derivative and used 5-aminovaleric acid as the internal standard. GABA concentration in the rat cerebellum was measured but not GAD activity.

To date only one mass fragmentographic method has been developed for the measurement of GAD activity. Cattabeni et al. [12] described a procedure in which rat cerebellum tissue homogenate was incubated with [$^2H_5$]GA and measured the formation of [$^2H_5$]GABA. It was not determined whether any isotopic discrimination occurred.

In this paper is described a GC—MS procedure for the indirect measurement of GAD activity in rat brain. In this method each tissue sample homogenate is divided into two parts. One part is inactivated with trichloroacetic acid (TCA) to serve as a blank. This gives the amount of GABA that originally existed in the tissue at the time of sampling. The other half of the sample is incubated in a substrate-buffer solution in which GABA is formed enzymatically by GAD. From the difference in GABA content of the two samples, the net GABA formation is derived and GAD activity can be calculated. Samples are collected from the discrete nuclei of GP, EP, VM, SN_M, and SN_L by a micropunch procedure [13] and a deuterated internal standard is used for quantitation.

EXPERIMENTAL

Materials

The chemicals used in this study were sodium-L-glutamate (Pflatz and Bauer, Stamford, CT, U.S.A.), trichloroacetic acid (Sigma, St. Louis, MO, U.S.A.), GABA (Aldrich, Milwaukee, WI, U.S.A.), pyridoxal 5-phosphate and Triton X-100 (Eastman Chemical Co., Rochester, NY, U.S.A.). The internal standard was synthesized by the method of Bertilsson and Costa [10]. Sylon HTP [hexamethylchlorosilane—trimethylchlorosilane—pyridine (3:1:9)] from Supelco (Bellefonte, PA, U.S.A.) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce (Rockford, IL, U.S.A.) were the derivatizing agents used

and were prepared by mixing 1000 μl Sylon HTP with 300 μl BSTFA.

Instrumentation

A Finnigan 4000 GCMS quadrupole mass analyzer spectrometer with a Model 6000 automated data system has been used in all experiments. GC analysis was performed at 140°C on a 1.8 m × 2 mm I.D. glass column with 3% OV-17 on Gas-Chrom Q (100—120 mesh), with helium flow-rate of 25 ml/min, injection port 175°C, jet separator 200°C, ion source 250°C and electron impact ionization potential of 70 eV. The ions monitored were *m/e* 304.1 and *m/e* 306.2 for GABA and [$^2H_2$]GABA, respectively. Methane was the reagent gas for all chemical ionization experiments. A Beckman Cary 118 ultraviolet—visible spectrophotometer has been used for the protein assay [14] at a wavelength of 725 nm and a Heat Systems Model W-220F sonicator for homogenization of tissue.

Sample collection

The sample collection procedure has been previously described [9]. This procedure minimizes the rapid onset of GABA formation observed in post-mortem tissue [15,16]. From each 1 mm thick brain tissue slice, two tissue punches were taken. These punches were from symmetrical locations in the left and right hemispheres. Each pair of punches was transferred immediately to a 12-ml polypropylene Eppendorf tube containing 100 μl of internal standard. The internal standard solution was prepared by addition of 2.0 mg [$^2H_2$]GABA to 25 ml of a solution consisting of 0.5 *M* potassium chloride, 0.010 *M* EDTA, 0.5% Triton X-100 and 0.4 *M* sodium phosphate buffer, pH 6.4. The tissue punches were homogenized for 10 sec under low power sonication. It has been found that sonication up to 30 sec has no effect on the enzyme's activity. After sonication, 50 μl were transferred to an identical tube containing 20 μl of 10% TCA to denature the GAD enzyme and precipitate protein. This second tube served as the blank and its concentration of GABA was subtracted from the original sample. The samples were frozen in dry ice until all had been collected and could be incubated together; however, the samples were never kept in dry ice for over 30 min. The substrate-buffer was prepared as previously described [9] and 50 μl of this solution were added to both the sample and blank tubes before they were incubated for 2 h at 38°C. Inactivation of the enzyme was completed by addition of 20 μl of 10% TCA to the original sample tube. The tubes were centrifuged at 950 *g* for 20 min and 80 μl of each solution were transferred to 1.5-ml micro test tubes and evaporated to dryness under vacuum. The material was derivatized with 50 μl of the derivatization agent at room temperature for 90 min. All samples were run immediately using 1—2 μl injections. Retention time of GABA and its deuterated analogue was 1.10 min under the conditions stated. The precipitated tissue was analyzed for protein as described by Lowry et al. [14] and activity of GAD is reported as μg GABA per hour per mg protein.

RESULTS AND DISCUSSION

A mass spectrum of the TMS derivative and deuterium labeled derivative

is presented in Fig. 1. The base peak in both spectra is *m/e* 174. The ions monitored $(M - 15)^+$ by electron impact at 70 eV were *m/e* 304.1 (GABA) and *m/e* 306.2 ($[^2H_2]$GABA). The relative abundancies of *m/e* 304.1 and *m/e* 306.2 were 22% and 20%, respectively. From mass spectra analysis, $[^2H_2]$-GABA gave a small contribution to *m/e* 304.1 of 0.22% relative abundance while GABA had an ion of 12.83% relative abundance at *m/e* 306.2. The signal intensities of the above mentioned ions were 2.5 times stronger at 70 eV than at 30 eV.

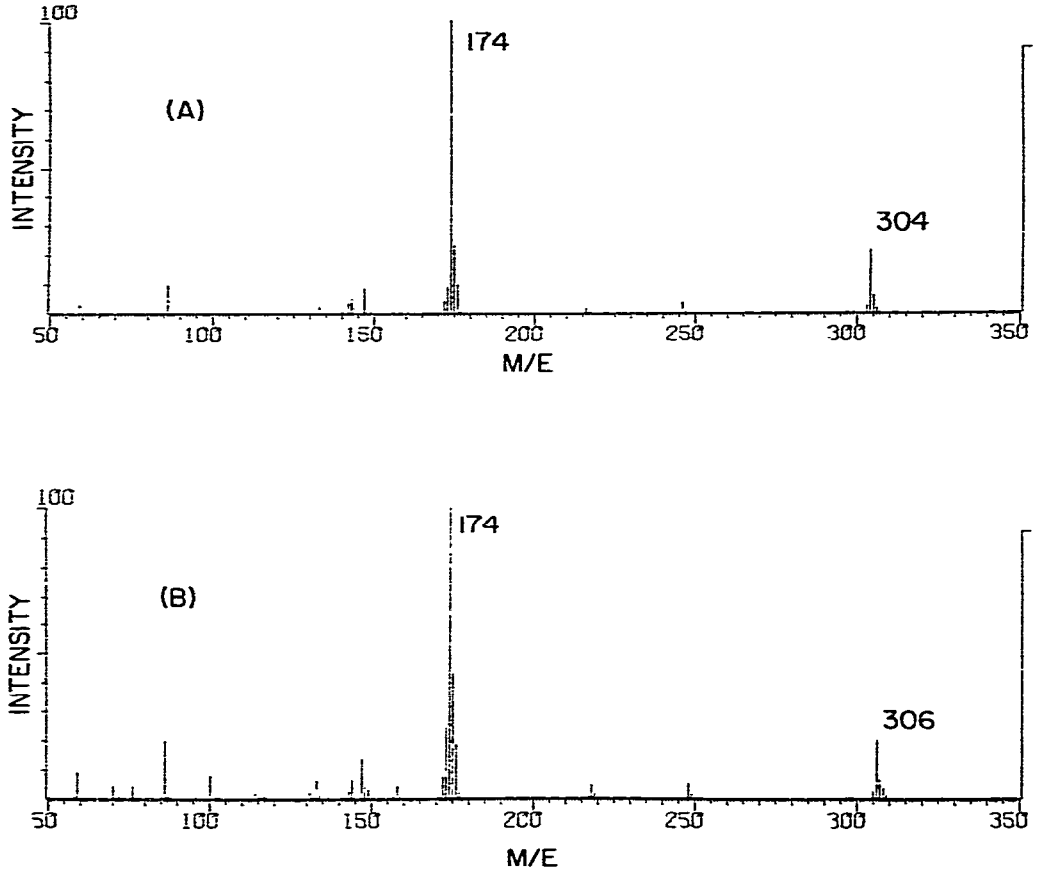

Fig. 1. Mass spectra of the trimethylsilyl derivatives of (A) GABA and (B) $[^2H_2]$GABA at 70 eV.

To test whether greater sensitivity could be achieved, spectra of pure standards of each compound were obtained using chemical ionization with methane as reagent gas. In both samples the base peak was *m/e* 174. The $(MH)^+$ ions of GABA (*m/e* 320) and $[^2H_2]$GABA (*m/e* 322) were both present at 7% relative abundance. At 70 eV the $(M - 15)^+$ relative abundancies of *m/e* 304.1 (GABA) and *m/e* 306.2 ($[^2H_2]$GABA) were 75% and 73%, respectively. It was found that the chemical ionization signal intensities of these two ions were 4.25 times less than the signal intensities at 70 eV by electron impact. Electron impact at 70 eV was therefore used in the GABA measurement.

The high sensitivity of the present method enables quantitative analysis of GABA down to 15 ng GABA per mg tissue with a 2:1 signal-to-noise ratio. The minimum detectable amount with a 2:1 signal-to-noise ratio of pure standards was 0.9 ng GABA (*m/e* 304.1) and 1.2 ng [$^2H_2$]GABA (*m/e* 306.2). The recovery of GABA for this GC—MS procedure was 95% ± 4.2% and the recovery of GABA for the fluorimetric procedure was 94% ± 6.3%.

A calibration curve was constructed in which known amounts of GABA (0—10 μg) were added to tubes containing a fixed amount of [$^2H_2$]GABA (2 μg) and the mixture carried through the incubation and derivatizing procedure. A least-squares fit (r = 0.997) of these data gave a linear relationship between peak area ratio and GABA concentration from which the following equation resulted:

$$\mu g\ GABA = 0.987 \frac{\text{Area GABA}}{\text{Area } [^2H_2]\text{GABA}} + 0.007\ \mu g\ GABA \qquad (1)$$

Eqn. 1 was used to calculate the GABA concentration of the tissue samples.

The tissue punch placement is presented in Fig. 2. All tissue slices are 1 mm thick and the left and right hemispheric punches are combined for each nucleus. 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 upon the atlas of Pellegrino and Cushman [17]. An average weight of representative tissue samples from combined left and right brain punches (n=10) is 2.51 ± 0.16 mg tissue (wet weight) with an average protein content of 0.105 mg protein per mg tissue.

After incubation and derivatization, the concentration of GABA was measured and the corresponding blank subtracted from each sample to obtain the net GABA formation. Overall 1—3 fold GABA increases were observed for samples in this procedure. By the fluorimetric method, 1—3 fold GABA increases over the blank GABA concentration were also noted.

In Fig. 3, a mass fragmentogram from one of the tissue samples is presented. The chromatographic retention time of 1.10 min of the endogenous compound is identical to that of its deuterated analogue. The average GAD activity values (± S.D.) found by this method in each brain region (n = 5) are 7.75 ± 0.93 (GP), 6.70 ± 1.02 (EP), 3.68 ± 0.70 (VM), 13.66 ± 1.06 (SN_M), and 8.12 ± 0.95 (SN_L). These values agree closely with the fluorimetric data obtained in this laboratory for GAD activity in these subregions of rat brain.

Previous studies have been made by radioisotopic methods to determine GAD activity in rat brain tissue. Nagy et al. [3] recorded activities of 9.07 μg GABA per hour per mg protein for middle GP and activities for whole dissected regions of 22.66 (GP), 12.57 (EP) and 31.93 (SN). Tappaz et al. [18] reported activities of 47.48 (GP), 23.28 (nucleus ventralis thalamus), 49.85 (SN-compacta), and 114.3 (SN-reticulata) while Walaas and Fonnum [1] reported dry weight activities of 38.72 μg GABA per hour per mg tissue (GP) and 20.01 (EP). However, it has been observed that the measurement of the evolution of $^{14}CO_2$ is not a true estimate of GAD activity in crude homogenates because of alternative routes of CO_2 production from glutamate [19—21]. Wilson et al. [20] measured the production of $^{14}CO_2$ and [^{14}C]GABA in cultured neural cells from [^{14}C]glutamate and reported GAD activities by [^{14}C]GABA forma-

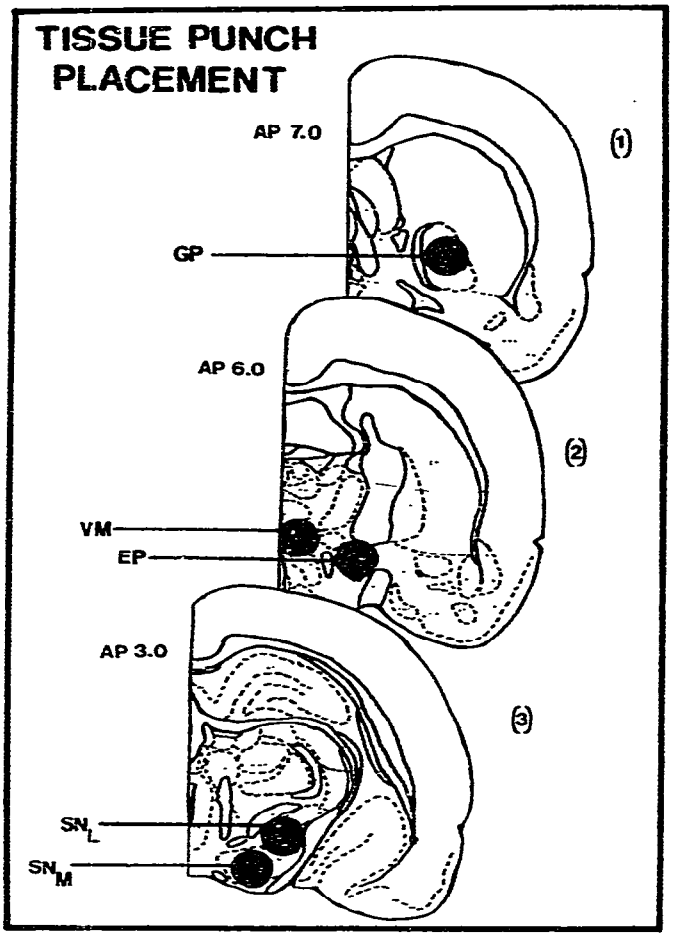

Fig. 2. Tissue punch placement. The first punch was from a 1 mm thick slice containing the globus pallidus (GP). The second tissue slice contained the entopeduncular nucleus (EP) and ventromedial thalamus (VM). The third slice contained the substantia nigra medial (SN_M) and lateral (SN_L) punches. The numbers on the left refer to the anterior–posterior (AP) axis coordinates in the brain atlas of Pellegrino and Cushman [17]. Punch diameter, 1.35 mm.

tion 10% less than those measured by $^{14}CO_2$ production. Drummond and Phillips [19] obtained similar results from non-neural tissue and indicated that the increased $^{14}CO_2$ formation from alternative pathways in crude homogenates may be due to the coupled reactions of glutamic acid dehydrogenase and α-ketoglutarate dehydrogenase. By measurement of a characteristic ion of GABA and comparison of its retention time to that of a deuterated internal standard, this assay is more specific than the measurement of $^{14}CO_2$ production from [^{14}C]glutamate in crude homogenates.

The highest GAD activity found in the brain is in the SN [1] and such was the case for the subregions tested in this study. The activities in the GP, EP, and SN are in agreement with the pattern of activities found by Nagy et al. [3] and Walaas and Fonnum [1].

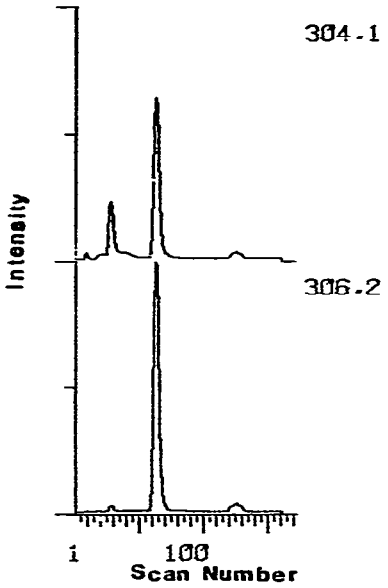

Fig. 3. Mass fragmentogram of *m/e* 304.1 of GABA and *m/e* 306.2 of [$^2H_2$]GABA from tissue sample.

A comparison of regional GAD activities was made with our data and the previously cited literature. Due to the different ways activity values are reported (i.e. per mg tissue, mg protein, dry weight, wet weight), ratios of activities between regions were used for comparison. Although a direct comparison of these ratios is not ideal, the ratios should be relatively consistent from method to method. The ratio of GP to EP obtained by this GC—MS method is 1.16, which is comparable with the results of Nagy et al. [3] (GP:EP = 1.80) and Walaas and Fonnum [1] (GP:EP = 1.93) while the GP to VM ratio of 2.11 obtained by this assay procedure is comparable with that of Tappaz et al. [18] (GP:VM = 2.03). Other possible discrepancies among these activity values could be due to extraneous $^{14}CO_2$ production from alternative pathways, variations in anterior—posterior axis locations of the brain regions, differences in protein content among regions, comparison of whole dissected regions as opposed to micropunches and differences in the strains of rats tested.

CONCLUSION

The GC—MS method described in this paper is useful for determination of GAD activity in subregions of rat brain. Similar activity values are obtained by the GC—MS method and by the fluorescence method. The regional GAD activity ratios of GP to EP and GP to VM obtained in this study are comparable with the ratios of values in the literature.

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