γ-AMINOBUTYRIC ACID IN THE MURINE BRAIN: 
MASS FRAGMENTOGRAPHIC ASSAY METHOD AND 
POST-MORTEM CHANGES

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Abstract—An improved mass fragmentographic assay method for the determination of γ-aminobutyric acid (GABA) in the brain is described. Applicability of the method was examined in a study of the effect of semicarbazide on GABA levels and in a separate study to confirm post-mortem increase in GABA. The method itself is based on Cattabeni’s procedure in which GABA is assayed as trimethylsilyl derivative. Three improvements were made: a) application of a more suitable mass spectrometry system for GABA determination; b) use of 6-aminocaproic acid as the internal standard; c) selection of a high intensive ion (m/z 174) for mass fragmentographic analysis. The mass spectrometer used is accurate to as little as 25 pg. GABA levels after semicarbazide treatment decreased 54.4% in rat whole brain and 44.2% in the dorsal hippocampus. Rapid post-mortem increases in GABA levels were confirmed by application of the improved assay method; decreases were most clearly observable following microwave irradiation. Post-mortem changes in GABA were observed within 3 min after death, as reported by other researchers.

Several improved mass fragmentographic assay methods for the determination of brain γ-aminobutyric acid (GABA) have been developed (1–3). Among these, the method of Cattabeni et al. (2) is most readily applicable to laboratory conditions because of its simplicity. In this method, 5-aminovaleric acid (5-AVA) is used as the internal standard and the extracted GABA and 5-AVA are simultaneously derivatized with trimethylsilylating (TMS) reagents. In their report (2), prominent peaks were at m/z 174 (base peak) for both GABA and 5-AVA (CH$_3$=N$^+$ (TMS)$_2$) and 304 (22%) and 318 (17.2%) for GABA and 5-AVA, respectively (molecular ions minus a methyl group). Peaks 304 and 318 were used for the assay of GABA and 5-AVA. One possible reason for not selecting the high intensity peak at m/z 174 (100%) may have been to avoid overlapping with the same ions derived from other endogenous amino acids. In an attempt to eliminate the problems of overlap at m/z 174, 20 endogenous amino acids were assayed with 6-aminocaproic acid (6-ACA) rather than 5-AVA as the internal standard. A gas chromatography/quadrupole mass spectrometer (GC/QMS) was used to isolate plural ions from the total range of fragments. Extracted compounds were identified by comparison of the ion intensity ratio of two fragments simultaneously by single injection.
quantitation. These improvements made possible the use of the m/z 174 base peak ion for more sensitive GABA determination.

The usefulness of this improved method was examined during a study of the effects of semicarbazide, an inhibitor of GABA synthesis in rat brain. Applicability of the method was also studied to confirm the rapid increase of GABA levels in rat whole brain after death (4-7). Microwave irradiation of brain tissue revealed significantly altered post-mortem GABA levels by rapidly and irreversibly inactivating amine catalyzing enzymes (8-11).

MATERIALS AND METHODS

Chemicals: GABA, 6-ACA and glutamine were purchased from the Tokyo Kasei, Co., Tokyo, Japan and 5-AVA was obtained from the Aldrich Co., Milwaukee, U.S.A. The amino acids were obtained from the Takara Koshan Co., Tokyo, Japan.

Animals: Mice of the ICR strain (20-30 g) and rats of Sprague Dawley strain (120-150 g) were obtained from Nihon-Clea Co. Animals were provided Purina Labena (Purina Laboratories, U.S.A.) with tap water ad libitum. Food and water consumption related data were recorded. Lights were turned on daily at 0800 hours for 12 hours. A constant temperature of 21±1°C and a humidity of 55±2% were maintained in animal rooms. Animals were sacrificed in all experiments at approximately 2:00 p.m.

Gas chromatography-mass spectrometry: A JEOL Model Q10A gas chromatography-chemical ionization quadrupole mass spectrometer was used. The device was coupled with a basic JMA-980A computer system and a high speed graphic output system consisting of a KSR-733 silent printer and multiple ion detector. Separation was made on a round shaped 2 m×2 mm I.D. silanized glass coil packed with 3% OV17 on Gas Chrom Q, 100-120 mesh (Applied Science Labs., State College, Pa., U.S.A.) and maintained at a temperature of 146°C. The flow-rate of the helium carrier gas was 25 ml/min. The ionizing potential and current were 80 eV and 300 μA, respectively.

Microwave irradiation technique: A model NJE 2601 Metabostat was used for microwave irradiation. The output of the device is adjustable from 0 to 5 kw at 2.45 MHz. A stabilized power supply maintains a constant power output from a 2M12 magnetron produced by Shin-Nihon Musen (Saitama, Japan) even when input voltages vary 10% from 200 V AC (60 Hz, rms). The magnetron is water-cooled and equipped with a thermal switch which prevents temperatures exceeding permitted values. Duration of irradiation can be set from 0.1 to 9.9 sec in 0.1 sec units with high accuracy. The animal holder for the device is made of a transparent plastic and is designed so that the head is held firmly for precise centering of irradiation. Rats and mice sacrificed by microwave irradiation were exposed to 5 kw for 1.5 sec and 0.3 sec, respectively. The temperature of the midbrain was elevated to higher than 85°C in all animals.

Extraction of GABA: Rats and mice were sacrificed by microwave irradiation. Rat whole brain was removed from the skull and GABA extraction was carried out with 10 ml (4 ml for mice) of 80% aqueous ethanol containing 0.5 mg of 6-ACA using a Polytron homogenizer (Kinematica Co., Luzern CH, Switzerland). The supernatant obtained by centrifugation at 15,000 g for 15 min was transferred to a brown vial. An amount of hexane equal to that of the supernatant was added and the vial was shaken for a few seconds on a shaker. After centrifugation at 1,300 g for 10 min, 1 ml of the aqueous layer was transferred to another vial and evaporated to dryness with a rotary evaporator at 40°C.

Silylation for GABA, 6-ACA and other amino acids: The method employed is
based on the procedure of Cattabeni et al. (2). Standard amino acids and samples prepared by the method described in the previous section on extraction were treated with a mixture of hexamethyldisilazane, trimethyl dichlorosilane, and N,N,0-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) in dry pyridine (3:1:3:9). The solution was allowed to stand at room temperature for 90 min and a few microliters of the sample were injected into the GC/MS.

**Tissue preparation for testing the GABA inhibitor:** Twenty rats were divided into two groups including ten control animals. Half the number of rats were pretreated with a single intravenous administration of semicarbazide (300 mg/kg) and were decapitated 2 hours later. Five were used for measuring GABA levels in the whole brain by the previously described GABA extraction method. Another five rats were used for the assay of amine levels in the dorsal hippocampus. The area analyzed was excised and placed on a glass plate cooled with dry ice until assay. Ten control animals were treated in the same manner as the group given semicarbazide.

**Assay of post-mortem GABA increase in mouse whole brain:** To assess the rapid change in GABA levels after death, GABA concentrations following various fixation methods were determined: a) decapitation, removal and freezing of brain in liquid nitrogen for 60 sec; b) decapitation and placement in liquid nitrogen for 60 sec; c) whole body immersion in liquid nitrogen for 60 sec; d) microwave irradiation at 5.0 kw for 0.3 sec. Also, following sacrifice by microwave irradiation and/or exsanguination, some animals were left at room temperature for 0, 2 and 30 min before the brain was homogenized. Mice were sacrificed by bleeding rather than by decapitating to maintain efficiency of microwave absorption of the head after death by bleeding the body intact. On bleeding, most animals appeared to die within 10 sec.

**RESULTS**

**Formation of trimethylsilyl derivatives and mass spectra of GABA, 5-AVA and 6-ACA:** Under the reaction conditions described in Materials and methods Section, formation of N,N,0-Tris-Trimethylsilyl derivatives of GABA and 5-AVA was confirmed by gas chromatography. A single peak for 6-ACA (internal standard) was also obtained separately. Mass spectra of these derivatives are shown in Fig. 1. Fragmentation patterns for GABA and 5-AVA were similar to those reported by Cattabeni et al. (2). The presence of molecular ions at m/z 319, 333 and 347 for GABA, 5-AVA and 6-ACA, respectively, showed that two trimethylsilyl groups were introduced into the amino group and one into the carboxylic group. Prominent base peaks at m/z 174 (C\textsubscript{2}H\textsubscript{2}=N+(TMS)\textsubscript{2}) appeared for all three compounds. Other peaks were identified as m/z 304 (48.9%) for GABA, m/z 318 (20.1%) for 5-AVA and m/z 332 (40.6%) for 6-ACA by subtracting 15 from their respective molecular ions. As shown in Fig. 2, a large unknown peak overlapped with the 5-AVA peak when the base peak at m/z 174 was used to monitor endogenous GABA. For this reason 6-ACA was chosen as the internal standard.

**Identification and standard curve:** Sharp, single peaks of the two fragments m/z 174 and 304 of GABA were observed when the compounds were derivatized with a mixture of silylating reagents (Fig. 3). Gas chromatographic and mass spectrometric inspection of the extracted samples revealed that the GABA peak was free from any impurities, but it was deemed necessary to identify GABA further using other methods. Identification of GABA based on the ion intensity ratio of m/z 174 and 304 gave satisfactory results. This ratio is 0.186 for
the authentic GABA and 0.185 for brain GABA. The agreement between the two values shows that the peak of brain GABA is free from impurities. The standard curve of silylated GABA is shown in Fig. 4. There was an excellent positive correlation between the peak height ratios and compound quantities.

**Heat stability of GABA:** Presentation of data on heat stability of the target compound following microwave irradiation is essential. To our knowledge, no similar data have been reported. To determine the stability of GABA during microwave fixation, the effect of temperature on GABA concentration was examined by showing the per cent decomposition during 30 min incubation at a specified temperature in pH 6.7 distilled water. As shown in Table 1, GABA was very stable at elevated temperatures. A GABA solution was heated to 90°C for 30 min with only 4.30 percent decomposition. This suggested that negligible amounts of endogenous GABA are decomposed during microwave irradiation.

**Effect of semicarbazide on central GABA levels:** Brain levels of GABA after treatment with semicarbazide, a biosynthesis inhibitor,
are shown in Table 2 and may be compared to normal levels. Semicarbazide treatment produced a 54.4% decrease in central GABA levels in whole brain and a 44.2% decrease in dorsal hippocampus. For the study of the effect of benzodiazepines on GABAergic recurrent inhibition in rat hippocampus, the area was used for GABA analysis. Control GABA levels in rat whole brain are in agreement with those reported by Lovell et al. (4) and Minard and Mushahwar (6). It is well known that semicarbazide, a convulsant, lowers GABA concentration in the brain (12–16). The percentage of decrease in whole brain GABA levels was approximately equal to that in mouse brain (14). Precise data on GABA concentration in the hippocampus is limited (17, 18), especially for semicarbazide.

Post-mortem GABA levels: Data in Table 3 show that there was no significant change in GABA levels in mouse whole brain frozen by three different methods. However, values following decapitation and removal of brain and placement into liquid nitrogen were significantly different from those following microwave irradiation. Assayed GABA levels in mouse brain following microwave irradiation did not vary significantly from those reported by Knieriem et al. (10).

A comparison of GABA concentration at zero-times in the bleeding and microwave irradiation groups showed that GABA levels

![Fig. 2. Mass fragmentogram of GABA and 6-ACA in tissue extract. Derivatization and GC-MS conditions as described under Materials and methods Section. Peaks: 1, GABA; 2, endogenous impurities; 3, 6-ACA. Fragment used for detection was m/z 174.](image)

![Fig. 3. Ratios of peak heights of the two fragments 174 and 304 of GABA. Identification of GABA based on the ion intensity ratios of m/z 174 and 304 was positive. The ratios are described in the Result and Discussion Sections.](image)

![Fig. 4. Standard curve for the analysis of GABA by mass fragmentography. The compound was derivatized with a mixture of silylating reagents as described in the Method Section.](image)
Table 1. Effects of temperature on the stability of GABA

| Incubation temperature (°C) | GABA concentration (ng/ml) | Percent decomposed GABA |
|-----------------------------|---------------------------|-------------------------|
| 37                          | 20.00                     | 0±0                     |
| 60                          | 20.00                     | 0±0                     |
| 70                          | 20.00                     | 0±0                     |
| 80                          | 19.56                     | 2.20±0.05               |
| 90                          | 19.14                     | 4.30±0.20               |

Values for percent decomposed GABA following 30 min incubation at specified temperatures in pH 6.7 distilled water. Each value represents the mean±S.E. of 3 determinations.

Table 2. Effects of semicarbazide on the concentration of GABA in whole brain and dorsal hippocampus

| Treatment                  | μmol GABA/g tissue | Whole brain | Hippocampus |
|----------------------------|--------------------|-------------|-------------|
| Control (saline)           | 2.41±0.31          | (100.0)     | (100.0)     |
| Semicarbazide (300 mg/kg i.v.) | 1.10±0.14    | (45.6)      | (55.8)      |

Values show the mean for mol/g brain tissue±S.E. from 5 determinations and percent restoration of normal levels in parenthesis.

Table 3. GABA levels in mouse whole brain following various methods of inactivation

| Fixation method                  | Number of animals | GABA μmoles/g Wt. S.E. |
|----------------------------------|-------------------|------------------------|
| Decapitation, removal of brain into liquid N₂ | 6                 | 2.47±0.15**            |
| Decapitation into liquid N₂      | 6                 | 2.20±0.07**            |
| In situ, into liquid N₂          | 6                 | 2.06±0.10*             |
| MWR 5.0 kw – 0.3                 | 6                 | 1.77±0.10              |

*P<0.05 differs from microwave-treated. **P<0.01 differs from microwave-treated.

Table 4. Post-mortem GABA increase in whole mouse brain

| Method of sacrifice     | Zero-time* | μmol GABA/g tissue Post-mortem |
|-------------------------|------------|---------------------------------|
|                         | 2-min      | 30-min                          |
| Microwave (5.0 kw –300 msc) | 1.77±0.10 (6) | 1.85±0.13 (6) | 1.86±0.15 (6) |
| Exanguination           | 1.76±0.10 (6) | 2.61±0.21 (6)* | 3.59±0.22 (8)* |

* Significantly different from zero-time microwave irradiation, P<0.01 (Student's-t-test).
* Zero-time indicates removal and homogenization of the brain within 60 sec.
in the microwave group were almost the same as those in the bled group (Table 4). In both groups, the brain was removed and homogenized within 60 sec after death. However, when the brain was kept at room temperature for 2 min and 30 min prior to homogenization, GABA levels increased by 47.5% and 100% in the bled group, respectively, while zero-time values did not significantly increase. This percent increase is relatively higher than those reported by others (8, 10), however, the results clearly show that exposure to 300 msec of microwave irradiation prevented post-mortem elevation of GABA levels.

Long-term post-mortem GABA increases were also investigated. Mice were left at room temperature for 2, 4 and 8 hours following sacrifice by exsanguination prior to microwave irradiation. Microwave fixation after death was carried out to eliminate the effects of reversible enzyme activity during the extraction procedure. GABA concentrations were 2.38 (2 hr), 2.69 (4 hr) and 3.45 (8 hr) times higher than concentrations at zero-time with microwave treatment. No significant changes were observed in groups left at room temperature for 2, 4 and 8 hours after microwave irradiation. Lovell et al. (4), also reported that GABA levels increased as much as 100% in brains kept at room temperature for 1 hr.

**DISCUSSION**

Identification of GABA was confirmed by assay of the ion intensity ratio of the two fragments at m/z 174 and 304. The ratio was 0.186±0.011 for authentic GABA and 0.185±0.003 for endogenous GABA as shown in Fig. 3. Application of the GC/QMS Computer System made feasible identification of endogenous GABA and simultaneous quantitation. The usefulness of m/z 174 was also confirmed by examination of contamination of the same ion and GC retention time for twenty silylated amino acids compared to those of GABA and 6-ACA. Amino acids responded to gas chromatography with an ion of m/z 174 were GABA (retention time, min: 3.6), 5-AVA (6.6), 6-ACA (10.5), Aspartic acid (4.8), Glutamic acid (6.8) and Glycine (1.8). No Arginine, Cystine, Histidine, Lysine and Tyrosine peaks appeared on gas chromatograms after treatment with silylating reagent, under the conditions described in the Materials and Methods Section. Trimethylsilyl derivatives which gave clear peaks on gas chromatograms but presented no intensity at m/z 174 on mass spectra were Alanine (0.8), Hydroxyproline (3.6), Leucine (1.2), Isoleucine (1.6), Methionine (4.3), Phenylamine (7.5), Proline (1.6), Serine (1.8), Threonine (1.8) and Valine (1.1). GABA retention time (3.6) overlapped that of Hydroxyproline (3.6), however, it had no fragment at m/z 174.

With the improved method, GABA was clearly separated and measured. Single peaks for the compounds, including the internal standard, were observed on mass fragmentograms within 10 min when monitoring m/z 174. The positive correlation between the peak height ratio and the compound quantities was good (Y=0.0006x−0.003, r=0.9997). A single extraction with 80% aqueous ethanol containing the internal standard gave 99.0 percent recovery of GABA. GABA was determined to the level of 25 pg.

Various methods for preventing post-mortem increases in GABA have been developed. Recent progress in microwave inactivation of brain tissue has confirmed the significance of this innovation, which is free of the various defects of the freezing methods as a result of the rapid and irreversible inactivation of brain enzymes. This device was used with the mass fragmentographic method and enzymatic assay method developed by Jakoby and Scott.
(19), and Scott and Jakoby (20), to determine the usefulness of the device for studying rapid post-mortem increases in GABA levels in mouse whole brain. Brain temperature increased more than 85°C following microwave irradiation (21) as explained in the Material and Methods Section. This temperature is reportedly sufficient for the inactivation of synthetic and degradative enzymes of GABA (9). Post-mortem changes in rat brain with time following freezing under various conditions have been documented by Alderman and Shellenberger (22) and they suggested that the 60 sec prior to freezing of the tissue is a critical period for preventing post-mortem GABA increases.

Determination of GABA levels in brain areas is now under investigation using the microwave irradiation method.

In summary, we designed an improved method for the determination of brain GABA by the mass fragmentographic technique and its usefulness was confirmed in a study on the effect of semicarbazide on central GABA levels. The method was also effectively used for the study of rapid post-mortem changes in amino acid levels.

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