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Gas Chromatography Study on Effect of Nitrazepam on γ-Aminobutyric Acid in Mouse Brain*

(gas-liquid chromatography/GABA/mouse brain)

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We developed a method for the determination of γ-aminobutyric acid (GABA) in brain tissue using a gas-liquid chromatograph equipped with a flame ionization detector. This method is based on the formation of a GABA derivative by treatment with isobutyloxycarbonyl chloride and diazomethane, under mild conditions. This method proved to be useful for the determination of GABA in the whole mouse brain.

We thus studied the effect of nitrazepam, one of the benzodiazepines (BDZs), on the GABA levels in the mouse brain and found that the concentration of GABA in the nitrazepam treated group was decreased markedly compared with values in the untreated controls.

γ-Aminobutyric acid (GABA) is a constituent of the mammalian central nervous system (CNS), and a putative transmitter or modulater of inhibitory nerve impulses (1). Glutamic acid decarboxylase (GAD), an enzyme that synthesizes GABA, is activated by vitamin B_6 as a coenzyme. Semicarbazide and other pyridoxal-complexing hydrazides inhibit GAD, resulting in a lowering of the GAD activity and the GABA level, in the whole brain (2—4) ; hydrazide-induced changes cause convulsions. The relationship of pyridoxine deficiency to convulsive seizures in animals (5) and in human infants (6, 7) has been established.

Among the drugs that exert their primary effects on the CNS, the benzodiazepines (BDZs) are commonly used as anticonvulsants, muscle relaxants, antianxiety agents and hypnotics. Nitrazepam is one of the most potent BDZ anticonvulsants (8, 9). The clinical actions of BDZs suggest that they enhance inhibition of excitability in the CNS and augment the action of GABA, however, the mechanisms of action are poorly understood.

When studying the role of GABA as a neurotransmitter substance in the brain, a sensitive, specific and simple analytical procedure is needed. A cyclic enzymatic method (10), amino acid analyzer (11, 12), thin-layer chromatography (13), high performance liquid chromatography (14, 15) and gas-liquid chroma-

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tography (GLC) (16—19) have all been used to determine the concentration of GABA in biological materials. However, all these methods have inherent disadvantages and are complex and/or expensive for analytical preparations.

We now report a quantitative GLC method for the determination of GABA in the whole brain, based on a modification of the procedure described by Makita *et al.* (20, 21). Application of this method to study the effect of nitrazepam on GABA in mouse brain is described.

MATERIALS AND METHODS

Reagents

GABA, 2-fluoro-β-alanine hydrochloride (FA), ε-aminocaproic acid (ACA) and an amino acid standard solution (Type H) were obtained from Wako Chemicals (Osaka, Japan). 5-Amino-n-valeric acid (AVA) was obtained from Tokyo Kasei (Tokyo, Japan). Isobutyloxycarbonyl chloride (iso-BOC-Cl) was obtained from Kanto Chemicals (Tokyo, Japan) and was used without further purification. The ion-exchange resin was Dowex 50W-X8, 100—200 mesh, from Muromachi Kagaku (Tokyo, Japan) and was washed twice in three successive preparations of 2N NaOH, 2N HCl and distilled water. Nitrazepam was from Shionogi & Co., Ltd. (Osaka, Japan). All other reagents used were of special-reagent grade.

GLC Determination

Instruments and conditions——A Shimadzu 6A gas chromatograph, equipped with a flame ionization detector, and a Shimadzu Chromatopac C-R1A [data processor] were used. The glass column (3 mm×2 m) was packed with 1% FFAP on Shimalite, 80—100 mesh. The experimental conditions were as follows : column temperature 175°C ; injection and detection temperature 250°C ; nitrogen flowrate 60 ml/min and pressure 1.1 kg/cm^2. Mass-fragmentography was performed on a Nihon Denshi D-300 gas chromatograph–mass spectrometer (GC-MS) for identification of the derivatives.

Pretreatment and derivatization——The mice were killed by cervical dislocation and the entire brain was immediately placed on an ice-cooled plate. The brain was then weighed, homogenized in 2 ml of 0.1N HCl, the homogenate poured into a tube and the homogenizer washed with 1 ml of distilled water which was added to the tube of homogenate. The homogenates were deproteinized with 0.5 ml of 50% trichloroacetic acid and centrifuged at 1,000×g for 10 min. These so-obtained supernatants were transferred to another tube and 0.5 ml of ACA solution (1 μmol/ml) was added as the internal standard. The mixed solutions were applied to 1.5 ml of Dowex 50W-X8 in water. After adsorption of the liquid layer to the resin, the columns were washed with 10 ml of distilled water and the amino acids were then eluted with 8 ml of 2N NH_4OH directly into a reaction tube with a stopper. These eluents were condensed to a volume of 1 ml under reduced pressure and warming.

These solutions were alkalinized with 0.5 ml of 10% sodium carbonate solution, and 0.2 ml of iso-BOC-Cl was added. The tubes were stoppered and shaken for 15 min at room temperature. The contents of the tube were extracted twice with 3 ml of diethyl ether, and the ether extracts were discarded. An adequate amount of sodium chloride was then added for salting-out. The solutions were acidified with 1 ml of 10% phosphoric acid and extracted twice with 3 ml of diethyl ether. The ether layers were dehydrated with anhydrous sodium sulfate and the drying agent was removed by decantation. The solutions were evaporated to dryness under reduced pressure and the dried residues were dissolved in 0.6 ml of diazomethane-ether solution. The reaction tubes were tightly closed and left for 1 hour at room temperature. The solutions were then ready for injection onto the GLC.

Calibration curve, recovery and detection limit —— For the quantitative determination of GABA in brain tissue, standard curves were prepared by adding GABA (0.1—1.1 μmol) and the internal standards, ACA (0.5 μmol) and FA (0.5 μmol), to distilled water. The samples were prepared and chromatographed as described above, and the peak ratios were plotted against the concentration of GABA.

The recoveries of GABA after derivatization were obtained by adding a known amount of GABA (0.2—0.8 μmol) to the homogenized brain of guinea pigs, and then treating these preparations in the same way as the calibration standards.

For estimation of the limits of detection, the standard solutions of GABA (0.5—50.0 ng) were prepared and analyzed in exactly the same way as the calibration standards.

Animal Study

The animals used were male ICR mice (6 weeks of age) weighing 14.3—27.9 g and were divided into nitrazepam-administered and control groups. After 24 hours of fasting, five mice in each group were given 10 mg of nitrazepam per kg of body weight (suspension in 1% sodium carboxymethylcellulose ; CMC solution) or the CMC solution, by gavage. The mice were treated again with the same dose of nitrazepam or CMC solution 24 hours after the first administration. These mice were used 2 hours after this second administration.

RESULTS

As indicated in Fig. 1, GABA, FA and ACA were completely separated from the amino acid standard solution, which contained 17 amino acids. Fig. 2 shows typical chromatograms obtained with extracts of mouse brain. GABA, aspartic acid and glutamic acid were efficiently extracted from the tissue. The derivative of GABA was identified as iso-BOC-GABA methyl ester from the GC-MS spectrum shown in Fig. 3. For use as the internal standards, AVA, FA and ACA were examined. The peak of AVA overlapped the peak of aspartic acid. The peak of FA was completely separated from the peaks

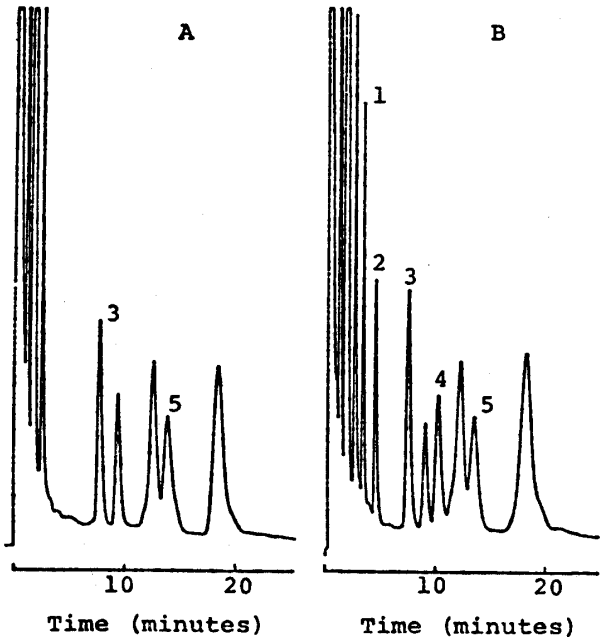

Fig. 1. Gas chromatograms of : A) Amino acid standard solution ; B) Amino acid standard solution containing GABA and internal standards.
1. 2-Fluoro-β-alanine (FA), 2. GABA, 3. Aspartic acid,
4. ε-Aminocaproic acid (ACA), 5. Glutamic acid.
Conditions : Column, FFAP 1% 3 mm×2 m.
Column temp., 175°C.
Inj. det. temp., 250°C.
Carrier gas, N_2 1.1 kg/cm^2 60 ml/min.

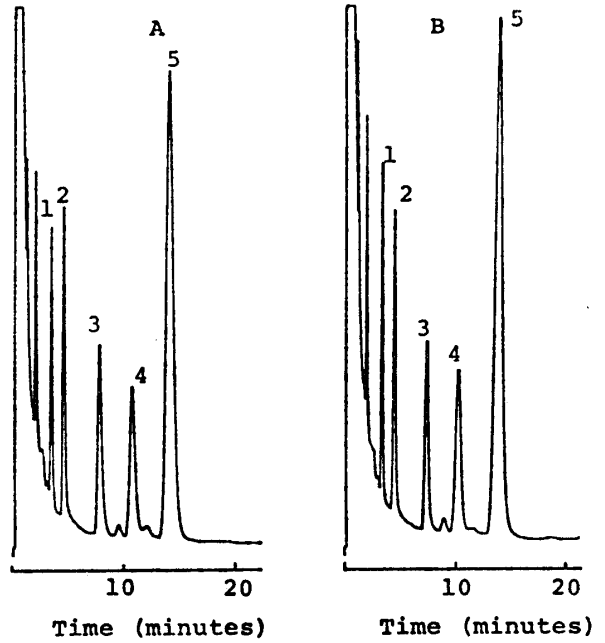

Fig. 2. Typical gas chromatograms of extract of mouse brain : A) Normal mouse ; B) after treatment with nitrazepam.
1. 2-Fluoro-β-alanine (FA), 2. GABA, 3. Aspartic acid,
4. ε-Aminocaproic acid (ACA), 5. Glutamic acid.
Conditions : Column, FFAP 1% 3 mm×2 m.
Column temp., 175°C.
Inj. det. temp., 250°C.
Carrier gas, N_2 1.1 kg/cm^2 60 ml/min.

of GABA and the other amino acids, but the peak area ratio of GABA to FA varied considerably. Therefore, AVA and FA cannot serve as internal standards. The peak derived from ACA showed complete separation, and the peak area ratio of GABA to ACA remained constant. ACA is thus a suitable internal standard.

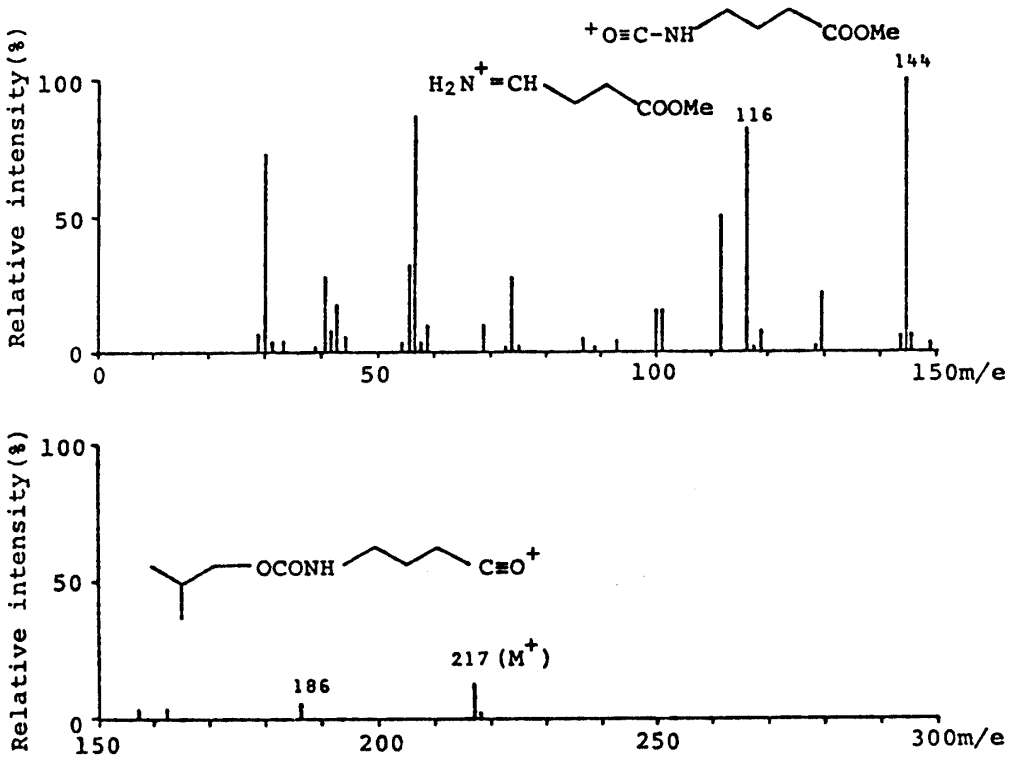

Fig. 3. Mass spectrum of iso-BOC-GABA Me-ester.
Conditions : IV, 24V. IC, 300 μA. CT, 250°C.

Fig. 4 shows the calibration curve. There was a linear relationship between the concentration of GABA and the ratio of the peak area given by the derivatives of GABA and ACA. Thus, the peak area method can be used to estimate GABA in tissue extracts. The recovery of authentic GABA (0.2 −0.8 μmol) added to the tissue homogenate was 87.1±4.8% (mean±S. D.). The detection limit of this method was 5 nmol of GABA ; this corresponds to the endogenous GABA level of about 0.01 μmol/g of fresh tissue.

The results of the animal study are shown in Table I. In the treated group, the level of GABA in the whole brain was lower than that in the control and the difference is statistically significant at $p<0.05$.

DISCUSSION

Analyses of GABA in biological samples by GLC and electron capture detection have been reported (16−19). These procedures include the following characteristic processes : 1) tissue extracts were evaporated to dryness under reduced pressure with prolonged heating (1−2 days) ; 2) the derivative of

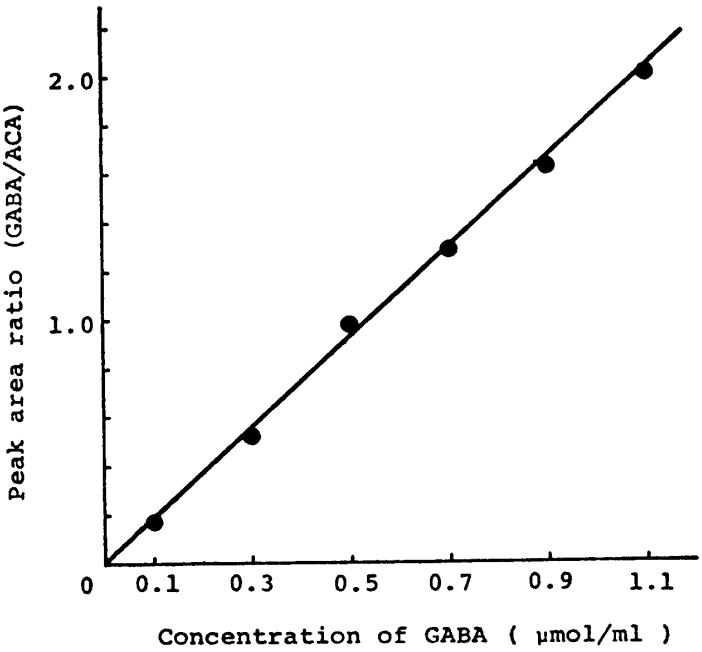

Fig. 4. Calibration curve for GABA in mouse brain.

TABLE I. *Effect of Oral Administration of Nitrazepam on GABA Levels in Mouse Brain*

Treatment*	Body weight (g)	Brain weight (g of fresh tissue)	GABA levels (/ mol/g of brain)
	14.3	0.41	3.12
	21.0	0.40	2.98
Control	20.8	0.41	2.72
	23.0	0.40	2.79
	26.1	0.43	2.86
		Mean±SD	2.89±0.16
	26.6	0.48	2.86
	25.8	0.46	2.53
Nitrazepam	26.9	0.45	2.44
	17.0	0.41	2.42
	17.6	0.41	2.66
		Mean±SD	2.58±0.18**

* Nitrazepam in doses of 10 mg per kg of body weight were given by gavage to 6 wk-old ICR male mice after a 24 hr fast.
** Comparison with control, $p<0.05$.

GABA was prepared by heating under anhydrous and acidic conditions ; 3) excess reagents were removed by evaporation under a dry nitrogen stream while heating. However, each of these steps is disadvantageous in relation to the GLC technique for the following reasons : 1) removal of the water is time consuming ; 2) GABA is unstable under anhydrous and acidic conditions, and dehydration of GABA could occur, and 3) the derivative of GABA would be volatile, and would therefore be lost during removal of the reagents under the nitrogen stream (17).

We developed the following procedures to avoid the problems associated with the above processes : 1) tissue extracts were directly and rapidly prepared by acylation with iso-BOC-Cl in an aqueous medium at room temperature ; 2) the derivatization processes of acylation and methylation were performed under mild conditions at room temperature ; 3) ether solutions of the methylated samples were directly injected onto the GLC, without further evaporation or redissolution.

Under the conditions used for the extraction and gas chromatography, we found that GABA, aspartic acid and glutamic acid were clearly separated on the chromatogram of an extract of mouse brain. Thus, our method enables quantitative measurement of the brain levels of these amino acids, which are possibly neurotransmitters.

The concentration of GABA in the control mouse brain in this study was 2.89 ± 0.16 μmol/g of fresh tissue. This is in excellent agreement with the value of 2.83 ± 0.21 μmol/g of fresh tissue reported by Battistin *et al.* (22) who studied the GABA levels in mouse brain using a Technicon Auto-Analyzer. The effect of nitrazepam on the GABA level in the normal brain is summarized in Table I. The results show a clear decrease in the concentration of GABA when mice are treated with nitrazepam. Previously, Saad (23) reported that diazepam increased the GABA level in the cerebral hemisphere of mouse brain and it has been suggested that these levels might be related to the anticonvulsant and antiepileptic actions of diazepam.

Fuxe *et al.* (24) observed that the level of GABA in the whole brain of the rat was increased by inhibition of GABA transaminase with amino-oxyacetic acid, and that the accumulation of GABA was reduced by diazepam. They suggested that the reduction in GABA turnover could be the result of activation of a feedback inhibition of GABA synthesis due to increased GABA receptor activity caused by diazepam. These mechanisms may partially contribute to the reduction in GABA by nitrazepam, but cannot completely explain our observations.

The present method is a simple, rapid and reliable technique for the determination of GABA in brain tissue. The effects of various doses of nitrazepam on GABA concentration in mouse brain are now being investigated.

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