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SPECIFIC AND SENSITIVE METHOD FOR THE DETERMINATION OF γ-AMINOBUTYRIC ACID USING GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE OR MASS FRAGMENTOGRAPHIC DETECTION

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SUMMARY

A gas chromatographic method for the determination of γ-aminobutyric acid (GABA) in brain tissue is described. After microwave fixation, the brains were dissected and homogenized in 0.1 *N* formic acid; δ-amino-*n*-valeric acid (AVA), a homologue of GABA, was then added as an internal standard. After centrifugation, aliquots of the supernatant were treated with cation-exchange paper to adsorb the amino acids. The eluates of this paper were dried and the residues subjected to reaction with trifluoroacetic anhydride and hexafluoroisopropanol. After removal of the derivatization reagents by evaporation, the residues were dissolved in ethyl acetate and an aliquot was analysed by gas chromatography with electron-capture or mass fragmentographic detection. Quantitation can be carried out by either peak-height or peak-area measurements.

The specificity of this method has been demonstrated with brain tissue by simultaneous mass fragmentographic analysis. The sensitivity is comparable to that of mass fragmentographic methods and is in the femtomole range. The method is simple and readily automated.

INTRODUCTION

When the role of γ-aminobutyric acid (GABA) as a transmitter substance in brain tissue is studied[1], a sensitive, specific and simple analytical procedure is needed[2–8]. While specific and sensitive mass fragmentographic assays for GABA have been described[6,7], we attempted to determine GABA by less expensive gas chromatography with electron-capture detection. Such an assay has been described by Pearson and Sharman[5], who determined the trifluoroacetic–hexafluoroisopropanol derivative of GABA. Our attempts to use this method for the determination of GABA in brain tissue were not successful, as we observed interference by other compounds. Therefore, we have developed a new gas chromatographic method for the assay of GABA in brain tissue which is simple, sensitive and specific.

EXPERIMENTAL

Materials

Trifluoroacetic anhydride (TFAA) and 1,1,1,3,3,3-hexafluoroisopropano (HFIP) were obtained from Merck (Darmstadt, G.F.R.) and used without further purification. SA-2 strong acid cation-exchange paper from Reeve-Angel was purchased from Serva (Heidelberg, G.F.R.). The paper was washed several times with 1 *N* sodium hydroxide solution, followed by water and 1 *N* hydrochloric acid, and was finally converted into the H^+ form by washing with 2 *N* hydrochloric acid. The paper was then dried in air, and 1 × 5-cm strips were used. *δ*-Aminovaleric acid (AVA) was obtained from EGA Chemie (Steinheim, G.F.R.). [2,3-^{3}H(N)]*γ*-Aminobutyric acid (specific activity 38.5 Ci/mmole) was obtained from New England Nuclear (Boston, Mass., U.S.A.).

The following gas chromatographic supports were used: 1.5% OV-17 with 1.95% QF-1 on Chromosorb W HP, 80–100 mesh; 3% SE-30 on Chromosorb W HP, 80–100 mesh; 1.5% SE-52 on Chromosorb W HP, 80–100 mesh; 4% SE-52 on Chromosorb W HP, 80–100 mesh, all from Hewlett-Packard (Boeblingen, G.F.R.); 3% OV-17 on Gas-Chrom Q, 80–100 mesh, from Applied Science Labs. (Serva); and 3% Dexsil 300 GC on Supelcoport, 100–120 mesh, from Supelco (Bellefonte, Pa., U.S.A.).

Preparation of tissue extracts for gas chromatography

Swiss albino mice (weighing approximately 30 g) or Sprague Dawley rats (weighing approximately 150 g) were killed by microwave fixation for 4 sec (ref. 8) and the brains were rapidly removed and dissected. For the analysis of larger brain areas, weighing 10 mg or more, a 10% (w/v) homogenate in 0.1 *N* formic acid was prepared with a Potter-type all-glass homogenizer; smaller brain areas, weighing less than 10 mg, were homogenized by ultrasonication in 0.1 ml of the same medium. AVA, a structural homologue of GABA, was then added as internal standard.

The homogenates were centrifuged at 10,000 *g* for 5 min and 0.1-ml volumes of the supernatants were transferred to disposable polyethylene tubes containing the cation-exchange paper. The tubes were allowed to stand for about 10 min and then the paper was washed by addition of 10 ml of deionized water. After standing the tubes for 30 sec, the water was poured out and the paper was washed a second time. GABA and other amino acids retained on the cation-exchange paper were then eluted by the addition of 1 ml of 3 *N* ammonia solution. The tubes were allowed to stand for about 10 min with occasional shaking; 0.5-ml portions of the eluate were then transferred into 1-ml reaction vials and evaporated in a Büchler rotary evaporator. For the analysis of GABA in cerebrospinal fluid (CSF), purification was effected by conventional cation-exchange resin chromatography[8] with a column containing 0.5 ml of Dowex 50-X8 (H^+), 100–200 mesh.

Gas chromatography of GABA

To the dry residues 0.15 ml of a mixture of TFAA and HFIP (2:1, v/v) were added for derivatization. The tubes were stoppered and kept for 60 min at room temperature[5]. The reagents were then removed under a stream of dry nitrogen and the residues were dissolved in 1 ml of ethyl acetate. Samples from CSF with a low GABA

content were dissolved in 30 μl of ethyl acetate. The tubes were then capped with a silicone-rubber septum and the samples were ready for injection. Usually the tubes were placed in an automatic injection unit. A Hewlett-Packard HP 5715 gas chromatograph equipped with a ^{63}Ni electron-capture detector and an electronic integrator were used.

The separations were usually carried out on a 2.4 m × 2 mm silanized glass column packed with 1.5% OV-17 and 1.9% QF-1 on Chromosorb W HP (80–120 mesh). For some experiments 2.4 m × 2 mm silanized glass columns packed with 3% OV-17 on Gas-Chrom Q (80–120 mesh) or 3% SE-30 on Chromosorb W HP (80–120 mesh) were used. The temperatures used were injection port, 150°; column, 135°; and detector, 200°. Argon containing 10% of methane was used as the carrier gas at a flow-rate of 30 ml/min.

Quantification was based on the ratio of the peak areas of GABA and the internal standard AVA. Essentially the same results were obtained when quantifications were based on the ratio of the peak heights of GABA and AVA. Mass fragmentography was carried out on a Hewlett-Packard 5982-A gas chromatograph–mass spectrometer equipped with a multiple-ion detector.

The separations were effected on the OV-17/QF-1 column described above, or on a 1.8 m × 2 mm silanized glass column packed with 3% Dexsil 300 GC on Supelcoport (100–120 mesh). Helium at a flow-rate of 30 ml/min was used as the carrier gas. The gas chromatographic column was coupled to the mass spectrometer either with a membrane separator or with a single-step glass jet separator. The temperatures used were injection port, 150°; column, 135°; transfer line, 250°; ion source, 200°; and mass filter, 100°. The ionization potential was 70 eV. The fragments of GABA at *m/e* 182 and of AVA at *m/e* 196 were used for detection. Quantification was based on the calculation of the ratio of peak heights of the derivatives of GABA and AVA.

RESULTS AND DISCUSSION

The method permits the determination of GABA in very small brain samples with a sensitivity similar to that of mass fragmentographic methods[6,7]. It is based on modifications of the assay procedure described by Pearson and Sharman[5], the modifications including the use of AVA as internal standard. It is added to the homogenates and thus can compensate for the loss of GABA during all preparative steps, from centrifugation to injection into the gas chromatograph. In order to remove interfering compounds from GABA we used a different liquid phase for gas chromatography. Another modification was the use of small strips of cation-exchange paper for the purification of GABA. This was possible because only small amounts of tissue extracts were purified for the gas chromatographic analysis of GABA.

For the cation-exchange paper method, the recovery of [^{3}H]GABA added to the homogenates remained fairly constant at about 80%, even when 1 ml of tissue extracts was used for purification (Table I). Analysis of the derivatized eluate from the cation-exchange paper showed that no new peaks in the gas chromatogram originate from the resin-coated paper. Hence the use of the cation-exchange paper instead of a cation-exchange column permits a much easier and faster purification of tissue extracts. The derivatization reagents TFAA and HFIP and also the derivative

TABLE I

RECOVERY OF GABA AFTER PURIFICATION OF BRAIN EXTRACTS WITH CATION-EXCHANGE PAPER

Mice were killed by microwave fixation and aliquots of their brains were homogenized in 10 volumes of 0.1 *N* formic acid. Known amounts of [^{3}H]GABA were added and the homogenates were centrifuged. Between 0.1 and 1 ml of the supernatants were subjected to ion-exchange paper chromatography as described. The recovery of [^{3}H]GABA in the eluates is expressed as a percentage of the total added. Results are means ± S.E.M. ($n = 4$).

Volume of supernatant subjected to ion-exchange paper chromatography (ml)	*Recovery of [^{3}H]GABA (%)*
0.1	80.48 ± 1.26
0.2	80.43 ± 1.26
0.5	81.13 ± 1.11
0.7	80.00 ± 0.83
1.0	75.85 ± 0.91

of GABA are highly volatile, so that considerable amounts of the GABA derivative are lost during the removal of the reagents in the nitrogen stream. Therefore, an internal standard is necessary to compensate for this loss of GABA.

Fig. 1 shows the losses of the TFA–HFIP derivatives of GABA during evaporation of the reagents. Thus, even before all reagents are completely removed, about 40% of the derivatives of GABA and AVA are lost during evaporation and there is further loss from the dry residues when evaporation is continued. While the absolute amounts of the derivatives of GABA and AVA decline, the ratio of their peak heights or peak areas remains stable (Fig. 1), suggesting that AVA is a suitable internal standard.

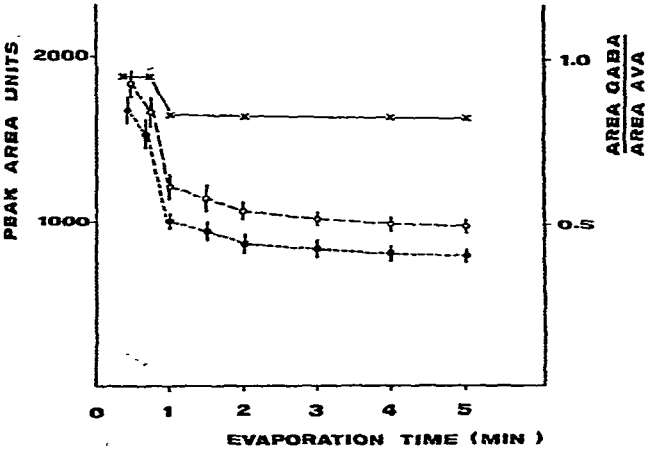

Fig. 1. Volatility of derivatives of GABA and AVA. Equal amounts of GABA and AVA were derivatized as described under Experimental. The reaction products were then evaporated under a stream of dry nitrogen for the indicated times. The derivatives were dissolved in 1 ml ethyl acetate for injection into the gas chromatograph. Results are expressed as the mean ± S.E.M. ($n = 4$) of absolute peak areas. ○, GABA; ●, AVA; ×, ratio of GABA to AVA peaks.

In confirmation of the results of Pearson and Sharman[5], we found that the TFAA–HFIP derivative of GABA has excellent gas chromatographic properties. Fig. 2 shows the mass spectral characteristics of the TFA–HFIP derivatives of GABA and AVA, and indicates that these derivatives are formed under the conditions of our assay. These derivatives of GABA and AVA have excellent stability and we were able to re-analyze samples after one month of standing.

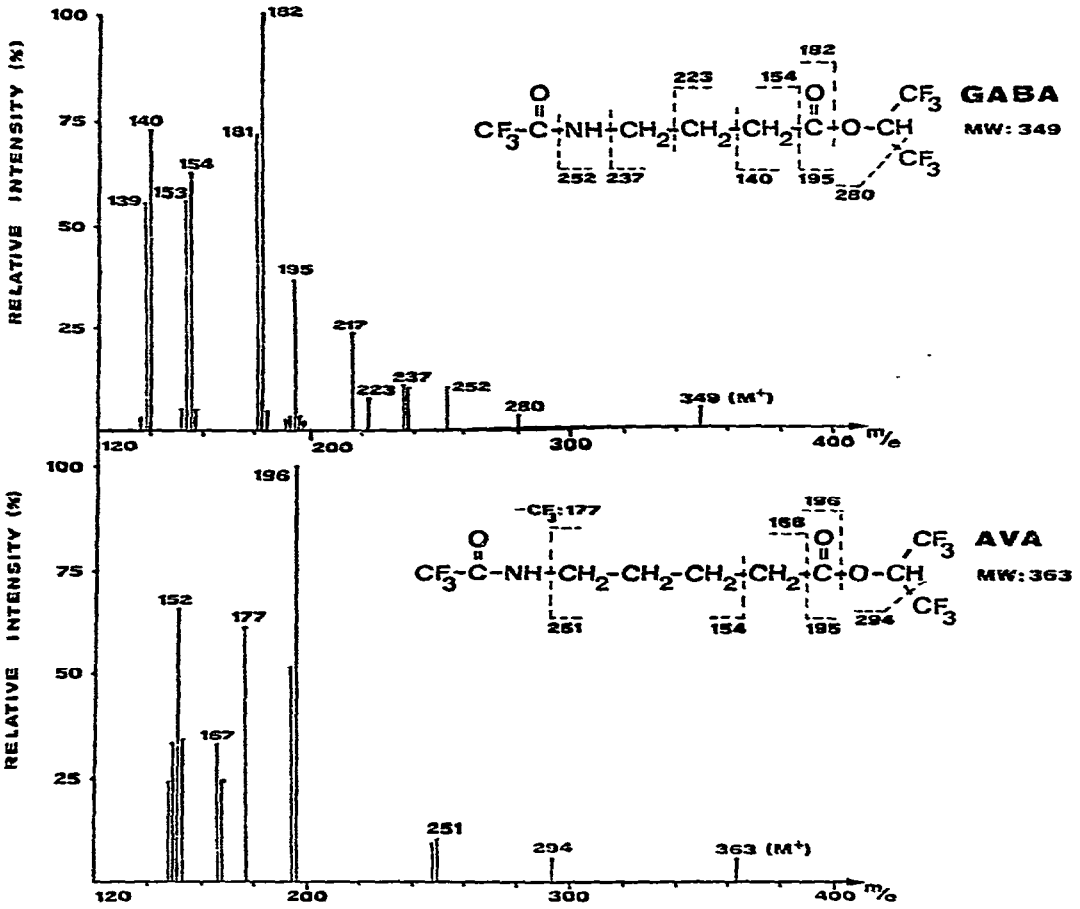

Fig. 2. Mass spectra and proposed fragmentation pattern of the HFIP–TFA derivatives of GABA and AVA.

Except for the GABA and AVA peaks, only a few other minor peaks appear on the gas chromatograms of tissue extracts (Fig. 3), although many amino acids and other compounds must be present in the purified tissue extracts. We therefore examined many substances as potential interferents in the gas chromatographic determination of GABA. Several compounds were derivatized and were found to give no electron-capture response on the gas chromatogram: dopamine, noradrenaline, serotonin, tryptamine, choline, aminooxyacetic acid, *p*-chlorophenyl-β-(γ-amino)-

butyric acid (Lioresal), γ-butyrolactone, δ-valerolactone, δ-valerolactam, aspartic acid and L-dopa. Other compounds that were derivatized and had a detector response but a different retention time from the derivatives of GABA or AVA are alanine, β-alanine, γ-amino-β-hydroxybutyric acid, norleucine, δ-aminolaevulinic acid, L-methionine and *trans*-4-aminocrotonic acid.

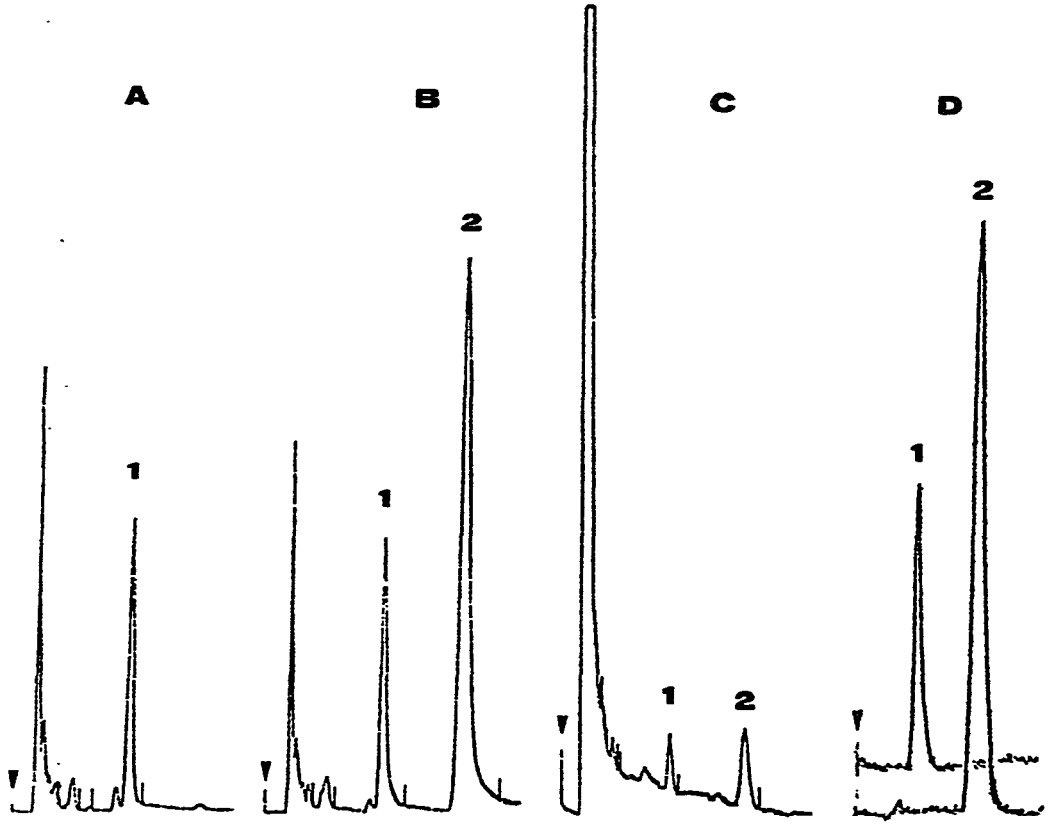

Fig. 3. Gas chromatogram and mass fragmentogram of GABA and AVA in tissue extracts. Derivatization and gas chromatographic conditions as described under Experimental. Peaks: 1, GABA; 2, AVA. (A) Gas chromatogram of endogenous GABA in extracts of whole brain. (B) Same sample with AVA as the internal standard. (C) Gas chromatogram of a hypothalamic tissue extract. The amount of endogenous GABA injected was 10 fmole. (D) Same sample as (B) with mass fragmentographic detection. Fragments used for detection were *m/e* 182 and *m/e* 196 for GABA and AVA, respectively.

Glutamic acid was found to form three derivatives with a moderate detector response (Fig. 4). One of these glutamic acid derivatives cannot be separated from the GABA derivative under the gas chromatographic conditions described by Pearson and Sharman[5] (Fig. 4). Although the detector response of this glutamic acid derivative is much smaller than that of the GABA derivative (Fig. 4), the high concentration of glutamic acid in brain tissue makes it likely that this derivative would interfere in the gas chromatographic determination of GABA. This evidence was strengthened by

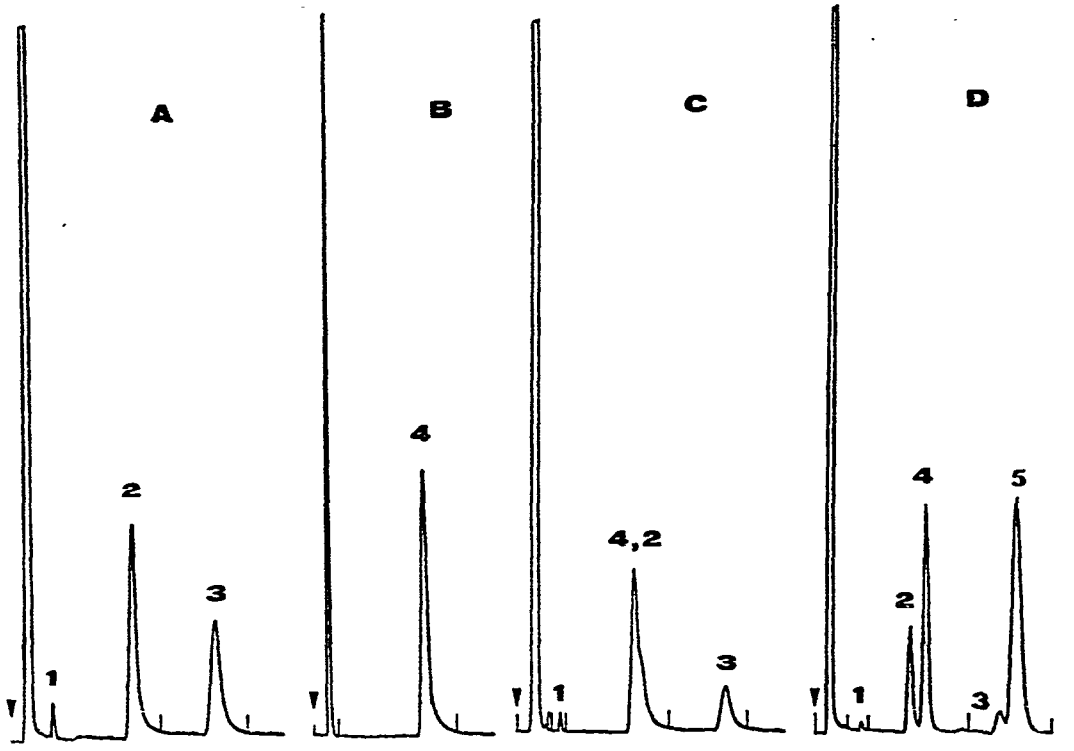

Fig. 4. Gas chromatograms obtained from the TFA–HFIP derivatives of 50 ng of glutamic acid (peaks 1–3), 5 ng of GABA (peak 4) and 5 ng of AVA (peak 5). A 1.5-m column of 4% SE-52 (A, B, C) or a 2.4-m column of 1.5% OV-17 and 1.9% QF-1 (D) was used for separation. (A) Glutamic acid; (B) GABA; (C) glutamic acid and GABA; (D) glutamic acid, GABA and AVA.

the results of a simultaneous analysis of GABA levels by gas chromatography and by mass fragmentography. We found that GABA levels obtained by gas chromatographic analysis were about 20% higher than those obtained by mass fragmentography. Thus, we conclude that an interfering compound, probably a derivative of glutamic acid, is not separated from the GABA derivative when a 3% SE-52 column is used for separation[5]. Attempts to separate this interfering derivative of glutamic acid from the GABA derivative with columns packed with 3% OV-17 or 3% SE-30 failed. When we tried other packing materials we found that the peak shape deteriorated when more polar phases were used. Therefore, we resorted to the use of a mixed-phase column packed with 1.5% OV-17 and 1.9% QF-1. Under these conditions, a sufficient separation of the derivative of GABA and glutamic acid and a good peak shape could be obtained (Fig. 4).

When standard curves from tissue extracts were then subjected to simultaneous gas chromatographic and mass fragmentographic analysis, the same results were obtained with both methods (Fig. 5). Therefore, we conclude that the proposed method permits the specific determination of GABA in brain tissue. However, the resolution of the packed gas chromatographic column is not sufficient to permit a gas chromatographic assay of GABA under conditions where very high glutamic acid levels and low GABA levels occur. Thus, with extracts of tissues where the ratio of glutamic

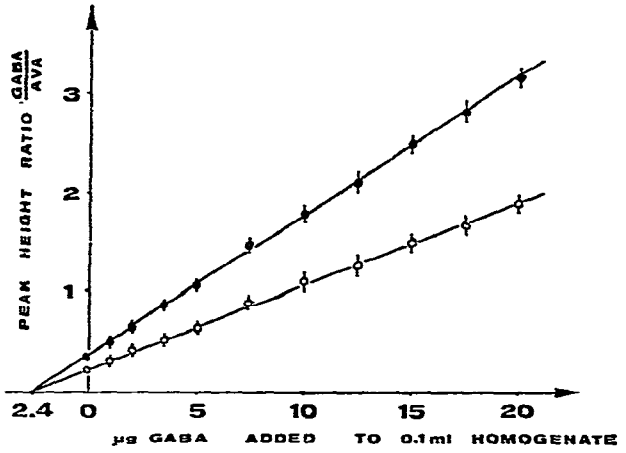

Fig. 5. Standard plots of the ratio of peak heights of GABA and AVA *versus* amount of GABA added to whole rat brain extracts before processing. ○, Gas chromatographic detection; ●, mass fragmentographic detection. The fragments used for detection were *m/e* 182 and *m/e* 196 for GABA and AVA, respectively. Mean values ± S.E.M. of four determinations are given.

acid to GABA levels is much higher than in brain tissue, this gas chromatographic method would probably not be applicable. Such a problem was found to occur in CSF, in which we currently measure GABA levels by mass fragmentographic detection. We have not yet tested whether capillary columns might permit the gas chromatographic determination of GABA in such samples.

The sensitivity of the method is shown in Fig. 3, where about 10 fmole of GABA, corresponding to the endogenous GABA level of 0.01 μg of wet tissue, were injected into the gas chromatograph. The method is very simple, rapid and reproducible. We analyzed GABA in mouse and rat brain by gas chromatography and compared our results with those of Balcom *et al.*[8], who studied GABA levels in animals killed by microwave fixation and analysed GABA by a fluorimetric method. Almost identical results were obtained with each method (Table II). Thus, we conclude that

TABLE II

REGIONAL GABA LEVELS IN RAT BRAIN

Regional GABA levels in rat brain were determined by either gas chromatography or mass fragmentography as described, or by an enzymatic fluorimetric method described by Balcom *et al.*[8]. Results are mean ± S.E.M. ($n = 4$–8). Animals pre-treated with aminooxyacetic acid (AOAA) received a dose of 30 mg kg^{-1} intraperitoneally 3 h before death.

Region	*Amount in tissue (nmole/mg)*		
	Gas chromatography	*Mass fragmentography*	*Enzymatic fluorimetric method*[8]
Hypothalamus	4.19 ± 0.23	4.79 ± 0.12	3.76 ± 0.37
Striatum	2.46 ± 0.15	2.39 ± 0.12	2.21 ± 0.20
Frontal cortex	1.52 ± 0.06	1.51 ± 0.01	1.25 ± 0.10
Cerebellum	1.16 ± 0.21	1.34 ± 0.17	1.31 ± 0.24
Cerebellum + AOAA	8.52 ± 0.36	8.34 ± 0.48	— — —

for the assay of GABA in brain tissue gas chromatography with electron capture detection is sufficient and that expensive mass fragmentographic equipment is unnecessary.

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