Journal of Chromatography, 118 (1976) 395–402

CHROM. 8936

MASS FRAGMENTOGRAPHIC QUANTITATION OF GLUTAMIC ACID AND γ-AMINOBUTYRIC ACID IN CEREBELLAR NUCLEI AND SYMPATHETIC GANGLIA OF RATS

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(Received October 2nd, 1975)

SUMMARY

A method for the simultaneous quantitation of glutamic acid and γ-aminobutyric acid (GABA) in tissue by mass fragmentography has been developed. The amino and carboxylic groups of the two amino acids were in a convenient one-step reaction derivatized with pentafluoropropionic anhydride and hexafluoroisopropanol. Deuterium-labeled glutamic acid and GABA and a homologue of GABA have been used as internal standards. The usefulness of the technique has been demonstrated by measurements in parts of rat cerebellum and in the superior cervical ganglion.

INTRODUCTION

The role of γ-aminobutyric acid (GABA) as an inhibitory transmitter is now fairly well established[1,2]. The findings of a deficiency of GABA[3] and of glutamic acid decarboxylase (the enzyme that decarboxylates glutamic acid to GABA)[4,5] in the brains of patients with Huntington's chorea, have stimulated the research concerning the function of this compound.

Several methods for the analysis of GABA including enzymatic[6–8] and gas chromatographic[9] techniques have been described. In this paper we report on the simultaneous determination of GABA and its precursor glutamic acid by the sensitive and specific method of mass fragmentography. We will show that this method can be used to determine the two amino acids in small punches (about 50 μg protein) of discrete rat brain nuclei. The combination of microdissection and the analytical technique of mass fragmentography has previously proved useful for studies on other neurotransmitters at the synaptic level[10,11]. In an abstract, Cattabeni *et al.*[12] recently reported a mass fragmentographic analysis of GABA using the trimethylsilyl derivative.

EXPERIMENTAL

Reagents and reference compounds

The following compounds were commercially available: pentafluoropropionic

anhydride (PFPA, distilled before use) and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) from Pierce (Rockford, Ill., U.S.A.); L-glutamic acid and 5-amino-*n*-valeric acid (AVA) hydrochloride from K & K Labs. (Plainview, N.Y., U.S.A.); GABA from Calbiochem (Los Angeles, Calif., U.S.A.); L-[2,3,3,4,4-$^2H_5$]glutamic acid (glutamic acid-d_5) from Merck, Sharp and Dohme (Montreal, Canada); deuterium chloride (20% in deuterium oxide; 100.0 atom %D) and deuterium oxide (99.7 atom %D) from Aldrich (Milwaukee, Wisc., U.S.A.).

[4,4-$^2H_2$]Glutamic acid (glutamic acid-d_2) and γ-amino[2,2-$^2H_2$]butyric acid (GABA-d_2) were synthesized by heating glutamic acid and GABA (200 mg of each), respectively, at 130° in sealed tubes with 1 ml of 8% deuterium chloride in deuterium oxide. The solutions were reacted for three periods, each of 12 days. After each period the solvent was evaporated by a stream of nitrogen gas and replaced by new deuterium chloride solution. After the last heating period the crystals obtained were dissolved in protium water to exchange active deuterium atoms by protium. When the water was evaporated white crystals of glutamic acid-d_2 and GABA-d_2 were obtained.

Tissue preparation

Male Sprague-Dawley rats (about 150 g) were killed by exposing their heads for 2.2–2.5 sec to a focused high-intensity microwave beam as described by Guidotti *et al.*[13]. The superior cervical ganglia and the brains were dissected out and immediately frozen in dry ice. In the experiments where the concentration of glutamic acid and GABA were determined in regions of cerebellum, this part of the brain was sliced in a cryostat at −4°. From these frozen slices the deep nuclei *(n. fastigii, interpositus* and *lateralis)* and the cortex (molecular, Purkinje cell and granular layers) of cerebellum were punched out using a hollow steel tube (I.D. 0.8 mm) as described for other brain nuclei[10].

Procedure

The tissue was homogenized in glass homogenizer tubes (Kontes, Vineland, N.J., U.S.A.) in 80% aqueous ethanol (which has been shown to efficiently extract GABA[14]) containing the internal standards for the quantitations. Whole cerebella were homogenized in 500 μl of a solution containing 380 and 750 nmoles/ml of glutamic acid-d_5 and AVA, respectively [or, in some experiments (see Table I), 380 and 300 nmoles/ml of glutamic acid-d_2 and GABA-d_2, respectively]. Cerebellar deep nuclei and cortex were homogenized in 100 and 200 μl, respectively, of a solution containing 42 and 84 nmoles/ml of the two internal standards. Superior cervical ganglia (pooled from 2 or 3 rats) were homogenized in 150 μl of a solution of 3.3 nmoles AVA per ml.

After homogenization the tubes were centrifuged at 12,000 *g* for 5 min at −2°. The supernatant (only 50 μl from the whole cerebellum) was transferred to glass vials and evaporated to dryness by a stream of nitrogen. Fifty microlitres of HFIP and 100 μl PFPA were added, the vials were sealed, heated for 1 h at 60° and then stored at 4°. Just before the mass fragmentographic analysis, the reaction mixture was evaporated to dryness. The residue was dissolved in 10–100 μl of ethyl acetate and 1–3 μl were injected into the gas chromatograph–mass spectrometer.

When small samples of tissue were analyzed, protein was determined in the

TABLE I

MASS FRAGMENTOGRAPHIC QUANTITATION OF GLUTAMIC ACID AND GABA IN WHOLE RAT CEREBELLUM USING DIFFERENT INTERNAL STANDARDS

Concentrations are expressed in μmoles per g wet weight (mean ± standard error of the mean). Number of determinations are in parentheses.

Internal standard	*Glutamic acid*	*GABA*
Glutamic acid-d_2	11.3 ± 0.3 (5)	
Glutamic acid-d_5	12.0 ± 0.2 (5)	
GABA-d_2		1.46 ± 0.04 (5)
AVA		1.52 ± 0.04 (15)

pellet of the homogenate according to Lowry *et al.*[15]. The amount of glutamic acid and GABA was then expressed per mg of protein.

Gas chromatography–mass spectrometry

An LKB Model 9000 gas chromatograph–mass spectrometer with a multiple ion detector (LKB-Produkter, Bromma, Sweden) was used. The separations were made on a 2.5 m × 3 mm I.D. silanized glass column packed with 3% OV-17 on Gas-Chrom Q, 100–120 mesh (Applied Science Labs., State College, Pa., U.S.A.), maintained at a temperature of 115°. The temperature of the flash heater was 200° and the ion source was kept at 270°. The flow-rate of the helium carrier gas was 25 ml/min. The ionizing potential and trap current were 80 eV and 60 μA, respectively. When mass spectra of reference compounds were recorded, the column temperature was 95°.

RESULTS AND DISCUSSION

Watson *et al.*[16] have reported a procedure to derivatize simultaneously carboxylic groups with a fluorinated alcohol and phenolic groups with a fluorinated anhydride. With this in mind we have in a convenient way derivatized glutamic acid and GABA. The carboxylic groups were esterified with HFIP and the amino groups acylated with PFPA. Recently, a gas chromatographic method with electron capture detection for the determination of GABA was reported[9]. Pearson and Sharman used the same type of reagents (HFIP and trifluoroacetic anhydride) to derivatize GABA.

The mass spectra and the proposed fragmentation patterns of the PFP–HFIP derivatives of glutamic acid, GABA and the internal standards are shown in Figs. 1 and 2. The base peak at *m/e* 202 was used to monitor glutamic acid by mass fragmentography. The corresponding peaks (*m/e* 204 and 206) were used for the detection of the internal standards, di- and pentadeuterium-labeled glutamic acid. Either of these internal standards may be used for the quantitation of glutamic acid. The dideuterium-labeled species can be synthesized in each laboratory at a low cost compared to the commercially available glutamic acid-d_5. One of our goals with this methodology is to study the incorporation of stable isotopes from, *e.g.*, [^{13}C]glucose into glutamic acid and GABA *in vivo*. The formed [^{13}C]glutamic acid may interfere with the peak at *m/e* 204 if glutamic acid-d_2 is used and we have therefore chosen

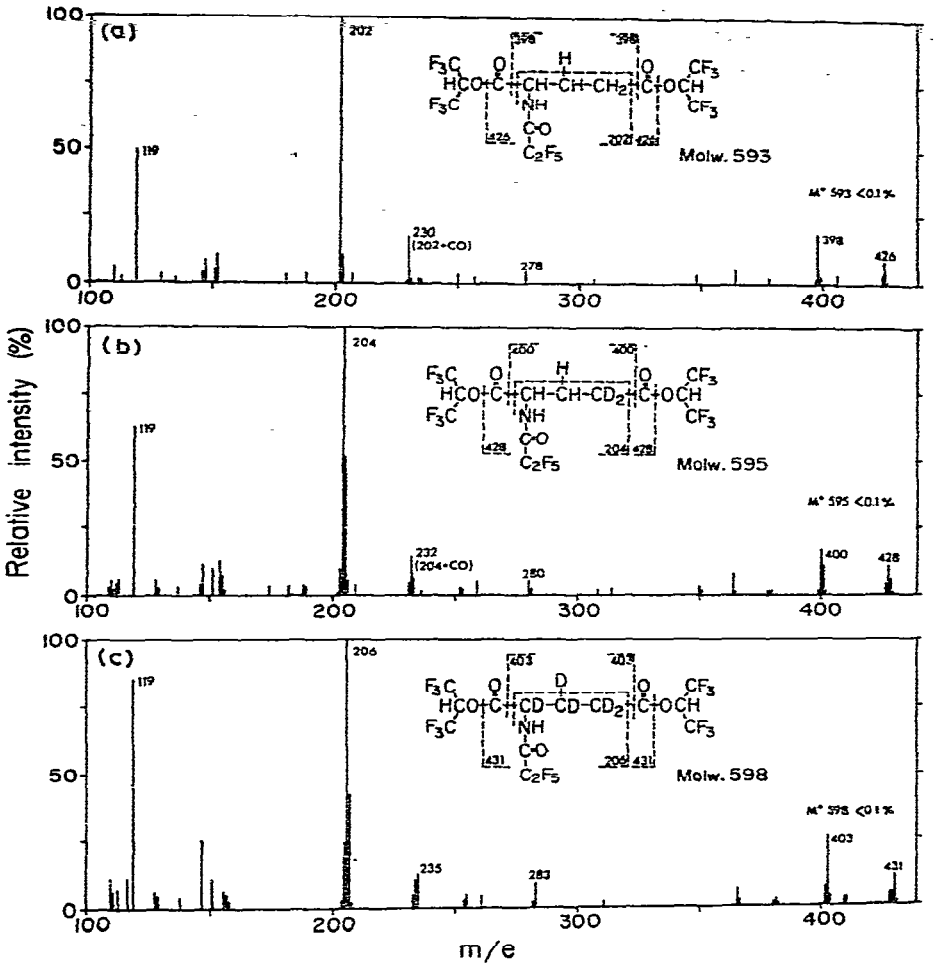

Fig. 1. Mass spectra and proposed fragmentation pattern of the HFIP–PFP derivatives of glutamic acid (a), glutamic acid-d_2 (b) and glutamic acid-d_5 (c).

glutamic acid-d_5 as an internal standard. The derivatives of GABA, GABA-d_2 and AVA have the common base peak at *m/e* 176 (Fig. 2). Thus, this fragment cannot be monitored when GABA-d_2 is used as an internal standard. As GABA and AVA have different retention times in the gas chromatographic system, the base peak can be used to record both GABA and AVA independently.

Fig. 3 shows mass fragmentograms obtained from an analysis of glutamic acid and GABA in deep cerebellar nuclei using glutamic acid-d_5 and AVA as internal standards, respectively. The retention times for the different compounds were for glutamic acid and glutamic acid-d_5 92 sec (peaks 1), for GABA 133 sec (peaks 2), and for AVA 232 sec (peaks 3).

Standard curves for the quantitation of glutamic acid and GABA were prepared by analyzing a series of standard solutions of these two compounds by the

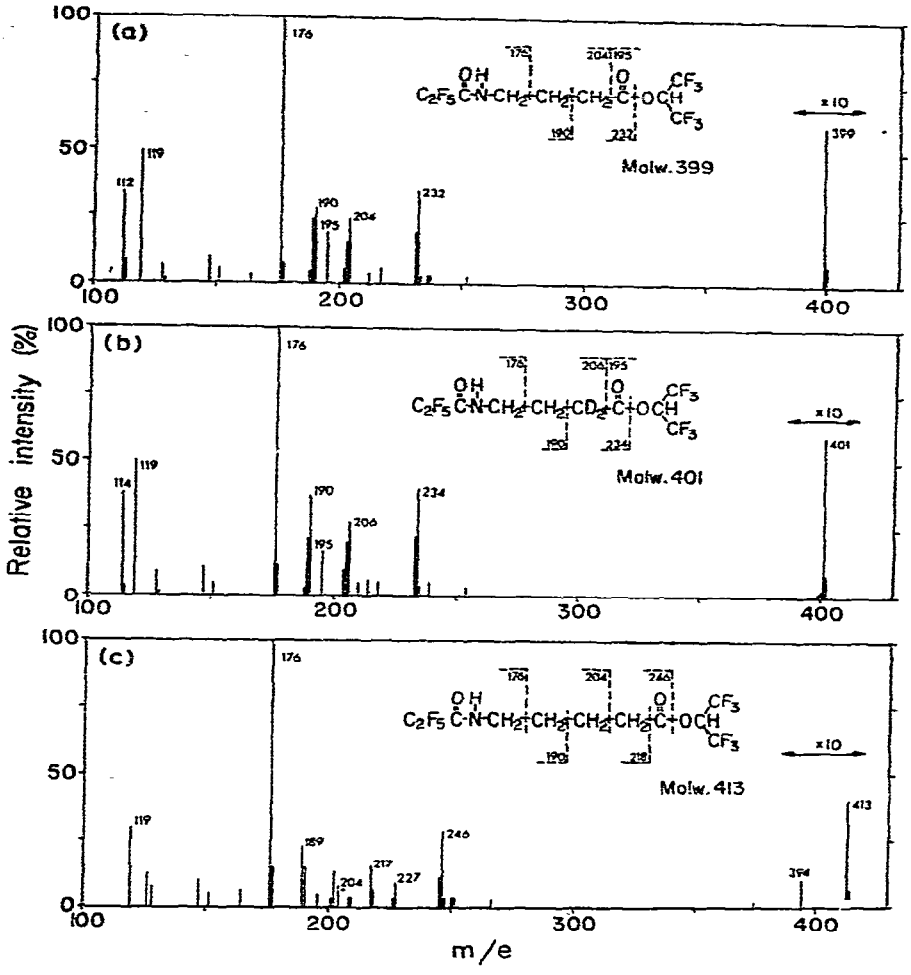

Fig. 2. Mass spectra and proposed fragmentation pattern of the HFIP–PFP derivatives of GABA (a), GABA-d_2 (b) and AVA (c). The intensity of the ions in the region of *m/e* 400 is magnified 10 times.

procedure described in Experimental. The peak height ratio between glutamic acid or GABA and the internal standard was plotted against the known concentration of the actual compound. Table I shows the results from the determination of glutamic acid and GABA in whole rat cerebellum. When the two different deuterium-labeled glutamic acids were used as internal standards, similar concentrations of glutamic acid were obtained. The concentrations of GABA determined with either GABA-d_2 or AVA as internal standard were almost identical (1.46 ± 0.04 and 1.52 ± 0.04 μmoles/g wet weight, respectively). This shows that AVA can be used as an internal standard for quantitation of GABA. The concentrations of glutamic acid and GABA in the whole rat cerebellum obtained here are similar to those reported by some other investigators[14,17].

As GABA is an important transmitter of cerebellar Purkinje, Golgi, basket

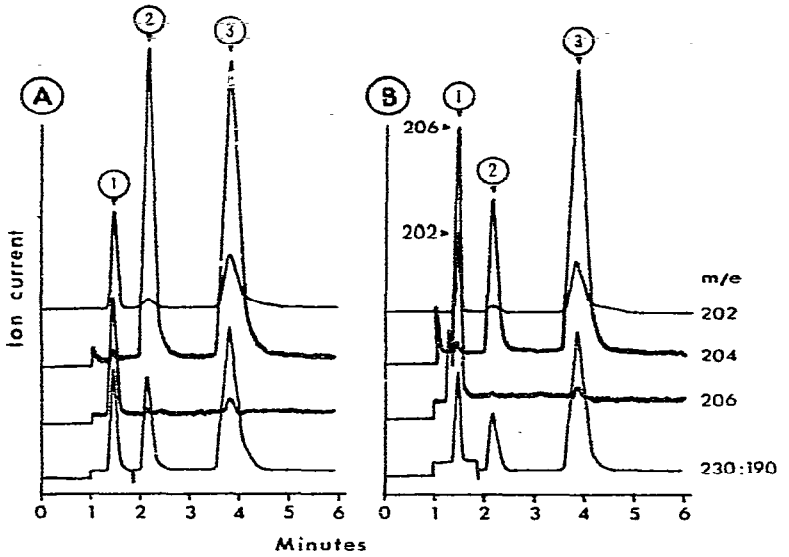

Fig. 3. Mass fragmentograms obtained from HFIP–PFP derivatives of (A), reference glutamic acid and glutamic acid-d_5 (peaks 1), GABA (peaks 2) and AVA (peaks 3); and (B) material obtained from rat cerebellar deep nuclei homogenized in an 80% aqueous ethanol solution containing glutamic acid-d_5 and AVA as internal standards. The mass spectrometer was in this case set to detect *m/e* 202 (glutamic acid), 204 (GABA and AVA) and 206 (glutamic acid-d_5). The fourth channel was initially used to detect a second fragment of glutamic acid (*m/e* 230) and 110 sec after injection this channel was changed to detect *m/e* 190, a fragment of both GABA and AVA. The relative sensitivities on the four channels used to detect *m/e* 202, 204, 206 and 230 (190) were 1:10:10:3.3.

and stellate cells we have started our investigations on the regulation of GABA-ergic mechanisms in this part of the central nervous system. The concentration of GABA was higher in deep cerebellar nuclei than in the cortex ($P < 0.01$; Table II). This finding is in line with a three-fold higher activity of glutamic acid decarboxylase in *n. interpositus* than in the cerebellar cortex[19]. The cerebellar nuclei contain nerve terminals of the Purkinje cells, which have their cell bodies in the cortex. Interestingly, we found higher levels of glutamic acid in the cortex than in the deep cerebellar nuclei

TABLE II

MASS FRAGMENTOGRAPHY OF GLUTAMIC ACID AND GABA IN CEREBELLAR REGIONS OF RAT

Values are mean ± S.E.M. Numbers of determinations are in parentheses.

Sample	*Glutamic acid*	*GABA*
Quantitation (μmoles per mg protein)		
Deep nuclei	84 ± 7 (5)	24.2 ± 1.6 (5)
Cortex	120 ± 6 (5)	14.2 ± 1.5 (5)
*Identification**		
Standards	0.321 ± 0.003 (8)	1.07 ± 0.02 (7)
Deep nuclei	0.316 ± 0.003 (5)	1.08 ± 0.02 (5)
Cortex	0.310 ± 0.003 (5)	1.05 ± 0.01 (5)

* Glutamic acid: *m/e* 230/202; GABA: *m/e* 204/190.

TABLE III

MASS FRAGMENTOGRAPHY OF GABA IN SUPERIOR CERVICAL SYMPATHETIC GANGLIA OF RAT

Quantitation: 309 ± 22 pmoles per mg protein (12 determinations). Values are mean ± S.E.M. Number of determinations are in parentheses.

Sample	*m/e 190/176*	*m/e 204/176*
Standards	0.285 ± 0.003 (14)	0.167 ± 0.002 (15)
Ganglia	0.287 ± 0.004 (11)	0.165 ± 0.003 (12)

($P < 0.01$; Table II). Glutamic acid and GABA were each identified by the ratio of two characteristic fragments (Table II and Fig. 3).

We believe that in studies of GABA the use of microwave to sacrifice the rats is mandatory. When rats are killed with a high-intensity microwave beam, the glutamic acid decarboxylase is promptly inactivated; thus, the GABA content is stabilized to a level close to that existing *in vivo*. The "punching" of nuclei from brain slices allows studies of the regulation of GABA with a direct reference to the synaptic organization of the tissue under study. By mass fragmentography both GABA and its precursor glutamic acid can be measured simultaneously with high sensitivity and specificity. Moreover, by labeling the glutamic acid pool with stable isotopes (*e.g.*, infusion of [^{13}C]glucose) the turnover rate of these two amino acids may be determined in brain nuclei.

The presence of endogenous GABA in the rat superior cervical ganglion has previously not been shown. Nagata *et al.*[20] reported that the GABA concentration in this ganglion was less than 1 nmole/mg protein; this was the lower limit of sensitivity of their method. Using the mass fragmentographic technique described in the present report, GABA was identified by the ratio between three fragments (*m/e* 176, 190 and 204; see Table III) at the gas chromatographic retention time of GABA. The concentration of GABA was 309 ± 22 pmoles/mg protein, which is only a few percent of the level found in rat brain. As the superior cervical ganglion contains fairly high concentrations of glutamic acid[20], this compound was not further investigated.

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