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SIMPLE AND RAPID MASS FRAGMENTOGRAPHIC METHOD FOR THE DETERMINATION OF GLYCINE IN BRAIN TISSUE*

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SUMMARY

A method for the sensitive and selective determination of glycine in brain tissue has been developed. Small samples of brain tissue were homogenized by sonication in 0.7 *N* formic acid, and [1,2-$^{13}C_2$, ^{15}N]glycine was then added as internal standard. After centrifugation, aliquots of the supernatants were dried and the resulting residues were derivatized in a single step with heptafluorobutyric anhydride and hexafluoroisopropanol. After removal of the derivatization reagents by evaporation the residues were dissolved in ethyl acetate and an aliquot was analyzed by mass fragmentography. Quantification was performed by comparing the ratio of peak areas of glycine and its internal standard.

INTRODUCTION

There is evidence that glycine (Gly), an ubiquitous amino acid, has a role as an inhibitory neurotransmitter in spinal cord and medulla oblongata[1–4]. The distribution of Gly in the gray matter of spinal cord appears to be related to the presence of inhibitory interneurons[5]. For the measurement of Gly in discrete areas of the central nervous system, a method is required which permits the specific and sensitive determination of this amino acid. Several methods, developed for the analysis of a broad range of amino acids, fulfil these criteria but have the disadvantage of being too time consuming. We therefore developed a new method for the rapid determination of Gly in small samples of nervous tissue, which has the specificity and sensitivity offered by mass fragmentography.

EXPERIMENTAL

Reagents

Heptafluorobutyric anhydride (HFBA) was obtained from Pierce (Rockford, Ill., U.S.A.). 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP), glycine (analytical grade) and

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ethyl acetate were supplied by Merck (Darmstadt, G.F.R.). The internal standard, [1,2-$^{13}C_2$, ^{15}N]glycine, was obtained from Prochem (London, Great Britain).

Preparation of tissue extracts

Sprague Dawley rats (weighing *ca.* 150 g) were killed by decapitation and the brains were rapidly removed and dissected. Tissue was suspended in 100 volumes of 0.7 *N* formic acid and homogenized by sonication at 70 W for 30 sec, using a Branson Model B-12 sonifier. A constant amount of [1,2-$^{13}C_2$, ^{15}N]glycine (between 50 and 150 ng/mg tissue) was added as internal standard. The homogenates were then centrifuged at 10,000 *g* for 20 min and 10 μl of the clear supernatant (corresponding to 100 μg of tissue wet weight) were transferred to "2 ml HP-reacti vials" and evaporated to dryness.

Derivatization

Volumes of 50 μl of HFBA and 20 μl of HFIP were added to the dry residues and the vials were sealed and kept at 80° for 40 min. The reagents were then removed by a stream of dry nitrogen and the residues were dissolved in 20 μl of ethyl acetate. A 1- to 3-μl volume of this solution was injected into the gas chromatograph–mass spectrometer.

Apparatus and gas chromatographic conditions

A Hewlett-Packard HP 5992 mass spectrometer, equipped with a single step jet separator was used. Separations were performed 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) (Supelco, Bellefonte, Pa., U.S.A.). Helium was used as a carrier gas with a flow-rate of 30 ml/min. Temperatures of the injection port and column were 160° and 115°, respectively. Ionization energy was 70 eV. For detection of Gly and the internal standard [1,2-$^{13}C_2$, ^{15}N]glycine, fragments at *m/e* 224 and *m/e* 228 were monitored, respectively. Quantification was carried out by comparing the ratio of peak areas of these fragments.

RESULTS AND DISCUSSION

For the derivatization of Gly, the procedure described by Watson *et al.*[6] was modified, where compounds containing carboxyl and amino groups are derivatized by a perfluorinated alcohol and acid anhydride. Gly was derivatized in a single step procedure, using a mixture of HFBA and HFIP as reagents. HFIP was used since it reacts preferentially with ω-amino acids, where the reacting carboxylic group is not hindered by a neighbouring α-amino group[7,8]. Since glycine exhibits properties of an α- and of an ω-amino acid, it can therefore be derivatized with HFIP in preference to other α-amino acids. HFBA has been found to be a more appropriate reagent for the derivatization of the amino group of Gly than trifluoroacetic acid anhydride or pentafluoropropionic acid anhydride, since (i) the HFBA derivative has a higher molecular weight and yields fragments with higher *m/e* values, and (ii) the HFBA–HFIP derivative of Gly has a relatively low volatility and the losses during the removal of derivatization reagent are minimized. The HFBA–HFIP derivative of Gly is stable for several days.

There are two reasons why structural analogues of glycine are not well suited as internal standards for the gas chromatographic determination of glycine. First, homologues such as β-alanine and 4-aminobutyric acid are natural constituents of brain[9] and therefore cannot be used as internal standards. In addition, because Gly is the smallest amino acid, even the HFBA–HFIP derivative of glycine exhibits some volatility. This volatility is diminished by the use of larger structural analogues as internal standards. Thus, these compounds do not accurately compensate for the loss of the glycine derivative during the evaporation procedure[8]. Thus, the use of an isotopically labelled glycine as internal standard and mass spectrometric detection appears to be a prerequisite for the quantitative gas chromatographic determination of glycine. In initial experiments we attempted to use [2,2-$^2H_2$]glycine as internal standard which was prepared by a base catalyzed exchange reaction. However, this compound is not suitable as an internal standard because of a high rate of deuterium–protium reexchange which occurs during derivatization (data not shown). Therefore, in all further experiments the stable [1,2-$^{13}C_2$, ^{15}N] glycine has been used as internal standard.

The mass spectra and the proposed fragmentation pattern of the HFBA–HFIP derivatives of Gly and its internal standard are shown in Fig. 1. They indicate that for the mass fragmentographic analysis of Gly the fragments at *m/e* 224 (Gly) and *m/e* 228 (internal standard) can be used.

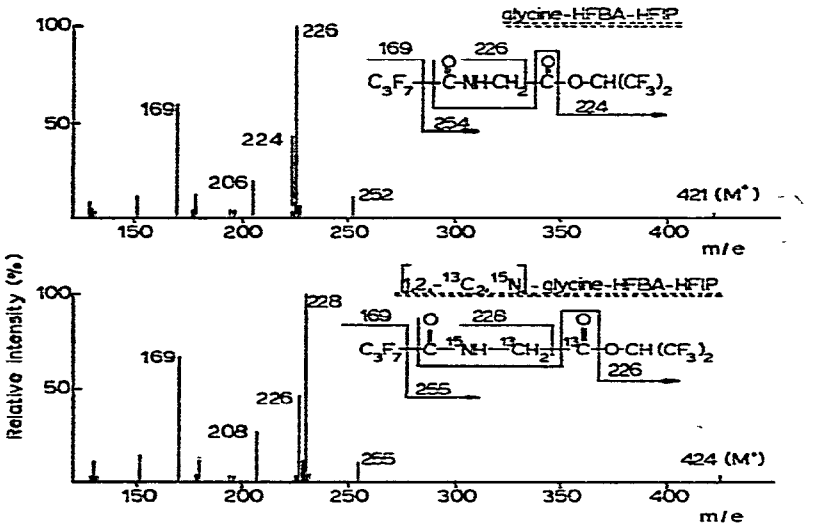

Fig. 1. Mass spectra and proposed fragmentation pattern of the HFBA–HFIP derivatives of Gly and of the internal standard [1,2-$^{13}C_2$, ^{15}N]glycine.

In Fig. 2 the mass fragmentograms of the derivatives of synthetic Gly, of the internal standard [1,2-$^{13}C_2$, ^{15}N]glycine and of a tissue extract to which the internal standard has been added are shown. Evidence that the ion *m/e* 224 with a retention time of 1.6 min in biological samples is derived from Gly was obtained by simultaneous measurement of other fragments derived from Gly such as *m/e* 226 and *m/e* 252, which were eluted at the same time with the same relative abundance in biological

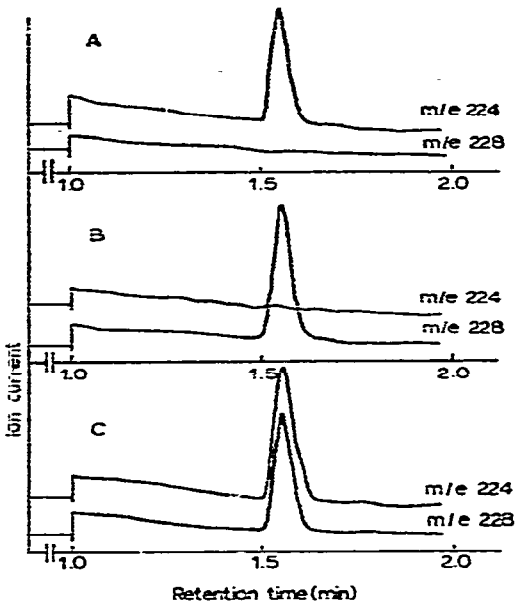

Fig. 2. Analysis of glycine in extracts of rat cerebellum. The fragments at *m/e* 224 and *m/e* 228 were used to monitor Gly and its internal standard [1,2-$^{13}C_2$, ^{15}N]glycine respectively. A, Tissue extract without internal standard; B, internal standard; C, tissue extract with internal standard.

samples and in synthetic standards (data not shown). Furthermore, monitoring of the total ion current obtained from brain samples revealed a single peak at the retention time of glycine which had a mass spectrum identical so that of synthetic glycine.

Standard curves were prepared by addition of increasing amounts of glycine to aliquots of biological extracts containing a known amount of internal standard. In Fig. 3 it is shown that the peak area ratio of *m/e* 224 over *m/e* 228 permits the accurate

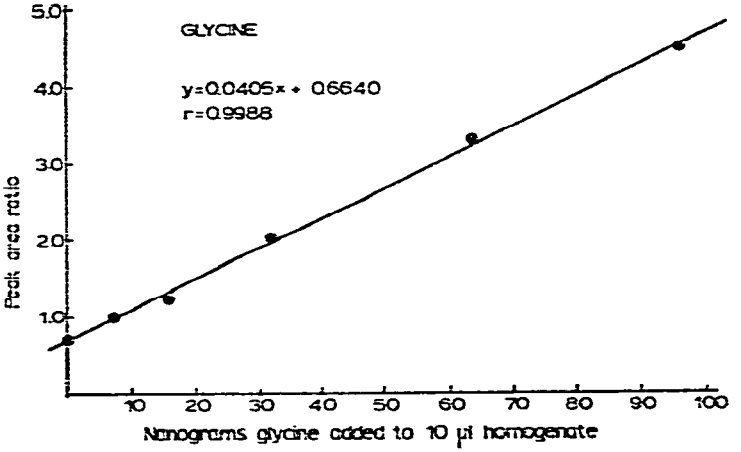

Fig. 3. Calibration curve for the analysis of Gly, prepared by addition of increasing amounts of exogenous unlabelled Gly to aliquots of tissue extracts containing a constant amount of the internal standard [1,2-$^{13}C_2$, ^{15}N]glycine.

quantitative determination of Gly, although the fragments are derived from different structural parts of the Gly molecule (Fig. 1). With this new method less than 1 ng Gly can be measured, which corresponds to the glycine endogenously present in less than 20 μg brain tissue wet weight.

Using this method we determined glycine in different regions of the rat brain (Table I) and found, in agreement with previous reports, enhanced levels in those areas where Gly is considered to have a role as an inhibitory transmitter.

TABLE I

DETERMINATION OF Gly IN SEVERAL REGIONS OF THE RAT BRAIN

Results are expressed as μmol/mg tissue wet weight $\pm$ S.E.M. ($n = 4$).

Region	*Gly content*
Cerebral cortex	0.97 $\pm$ 0.10
Cerebellum	0.88 $\pm$ 0.10
Medulla oblongata	2.90 $\pm$ 0.09
Spinal cord	
cervical enlargement	2.30 $\pm$ 0.09
mid thoracic	2.22 $\pm$ 0.18
lumbal enlargement	2.53 $\pm$ 0.09
sacral	3.58 $\pm$ 0.53

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