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QUANTIFICATION BY SELECTED ION MONITORING OF PIPECOLIC ACID, PROLINE, γ-AMINOBUTYRIC ACID AND GLYCINE IN RAT BRAIN

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SUMMARY

A procedure for the simultaneous analysis of brain pipecolic acid, proline, γ-aminobutyric acid and glycine — amino acids with potent inhibitory actions on the central nervous system — was developed. The identification and quantification of the amino acids were performed with a gas chromatographic—mass spectrometric—computer system using deuterium-labelled amino acids as the internal standards. After separation of the amino acids by high-performance liquid chromatography, the methyl ester heptafluorobutyryl derivatives were prepared. The lower limit of quantification for this method is at the picomole level.

The usefulness of this chromatographic procedure has been demonstrated by measurement of trace amounts of pipecolic acid in rat brain.

INTRODUCTION

The role of γ-aminobutyric acid (GABA) and glycine as inhibitory transmitters in the central nervous system is now fairly well established [1, 2]. Pipecolic acid (piperidine-2-carboxylic acid), a major metabolite of lysine metabolism in rat brain [3, 4], and proline, the lower membered homologue of pipecolic acid, are alicyclic amino acids with potent neuropharmacological activity [5, 6]. In the central nervous system, pipecolic acid and proline have also been suggested to have inhibitory actions resembling those of GABA and glycine [7, 8]. Furthermore, evidence for a neuromodulatory role of pipecolic acid obtained in our laboratory and others included the occurrence [9, 10], degradation [11], distribution [12], accumulation [13], uptake and release system [14—16] in the brain.

In addition, pipecolic acid is likely to have a connection with some neuro-

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pathological diseases in infants with hyperpipecolataemia [17, 18], hyperlysinaemia [19], and the cerebrohepatorenal syndrome of Zellweger [20], which seems to be due to an inborn error in pipecolic acid or lysine metabolism. Abnormally elevated levels of pipecolic acid have been detected in the serum of young patients, and progressive mental retardation is a common symptom of these patients.

A variety of methods are available for the determination of proline, GABA and/or glycine in biological samples. However, the analytical methods for pipecolic acid that have so far been reported are too insensitive to allow convincing micro-quantification of pipecolic acid levels in biological samples [17—23], especially the brain [9, 17, 23]. In a previous study, a combination of ion-exchange column chromatography and gas chromatography—mass spectrometry (GC—MS) was used for the separation and determination of alicyclic amino acids [10].

In the present study, the selected ion monitoring technique using a gas chromatographic—mass spectrometric—computer (GC—MS—COM) system following high-performance liquid chromatographic (HPLC) pre-purification was applied to develop a new method with high specificity and sensitivity for the simultaneous quantification of pipecolic acid, proline, GABA and glycine in rat brain.

EXPERIMENTAL

Materials

L-Pipecolic acid was obtained from Kyowa Hakko (Tokyo, Japan). The following compounds were commercially available: L-proline, GABA and glycine from Kyowa Hakko; DL-[1,2,2′,3,3,4,4,5,5,6,6-$^2H_{11}$]pipecolic acid (deuterium-labelled pipecolic acid, pipecolic acid-d_{11}), L-[2,3,3,4,4,5,5-$^2H_7$]-proline (proline-d_7), γ-[2,2,3,3,4,4-$^2H_6$]aminobutyric acid (GABA-d_6) and [1,1-$^2H_2$]glycine (glycine-d_2) from Merck Sharp & Dohme (Montreal, Canada); heptafluorobutyric anhydride (HFBA), boron trifluoride in methanol complex (BF_3—CH_3OH, 14% boron trifluoride in anhydrous methanol) and formic acid from Wako (Osaka, Japan). Organic solvents were of analytical grade and were further purified by redistillation.

Apparatus

A Shin-Nihon Musen Model NJE-2601 Metabostat System microwave device was used for rapid inactivation of brain enzymes. Microwave fixation was achieved by irradiation of whole animals in an oven delivering 4.5 kW at 2450 MHz. Animals received 1.5 sec of irradiation.

A Waters Assoc. ALC/GPC 244 HPLC system equipped with a semipreparative μBondapak C_{18} column (8—10 μm, 30 cm × 7.8 mm) and a guard column packed with Bondapak C_{18}/Corasil (37—50 μm, 3.8 cm × 3.9 mm) was used for the separation of the amino acid fraction of the brain extracts. The detection system employed was a variable-wavelength ultraviolet (UV) detector (S-310A, Soma Kogaku, Japan).

The quantification of amino acids was performed on a GC—MS—COM system (JMS modified D-100 equipped with a JMA-2000S mass data analysis

system). Separations were made on a 200 × 2 mm I.D. glass column packed with 3% OV-1 on 80—100 mesh Gas-Chrom Q (Gasukuro Kogyo, Tokyo, Japan). The chromatographic conditions were as follows: column temperature 130°C; injection port temperature 250°C; helium flow-rate 40 ml/min. Selected ion monitoring was performed under the following conditions: ion source and separator temperatures 250°C; ionizing voltage 75 eV; trap current 300 μA. The instrument was used in the selected ion monitoring mode. The fragment ions used for monitoring were *m/z* 280.1 for pipecolic acid, *m/z* 289.2 for pipecolic acid-d_{11}, *m/z* 266.1 for proline, *m/z* 273.1 for proline-d_7, *m/z* 254.1 for GABA, *m/z* 260.2 for GABA-d_6, *m/z* 226.1 for glycine, and 228.1 for glycine-d_2. All these compounds were run as the HFB-Me-amino acid derivatives [10, 12].

Computer analysis of data

At the completion of analysis, two data files had been recorded on disc representing some eight separate ion current profiles (*m/z* 226.1, 228.1, 254.1, 260.2, 266.1, 273.1, 280.1, 289.2). A computer program received, as fixed data, the retention time of each peak and its area, together with a position where background intensity could be measured. The ratio of peak area for amino acid in the brain to deuterium-labelled amino acid as an internal standard pair was then calculated. Quantitation limits were estimated from the computer responses generated by the known concentrations of amino acids used in the preparation of the standard calibration curves [24].

Methods

Male Wistar rats (190 ± 10 g, 7 weeks' old) from Kyudo Farm (Kumamoto, Japan) were used. The animals were allowed free access to food (CREA, CE-2) and tap water. The animals were usually sacrificed at 10.00 a.m. The brain from one rat was used for each assay. The brain was removed after microwave irradiation, weighed and homogenized in 5 vols. of ice-cold 5% trichloroacetic acid (TCA) with a homogenizer (Ultra-Turrax, Jankel & Kunkel KG Ika-Werk, F.R.G.). The homogenate was centrifuged at 17,000 *g* for 30 min at 0°C. A 1.0-ml volume of the supernatant (about 10 ml in total volume) was taken up and transferred to a glass-stoppered test tube. Proline-d_7 (5 nmol), GABA-d_6 (100 nmol) and glycine-d_2 (100 nmol) were added to the supernatant taken up as internal standards. Pipecolic acid-d_{11} (5 nmol) was added to the remaining supernatant (about 9 ml).

The TCA solution was washed with an equivalent volume of ethyl acetate for 60 sec using a Vortex-Gemie mixer. The organic layer was removed by aspiration. The procedure was repeated four times. The remaining aqueous layer containing amino acids was evaporated under reduced pressure at 40°C. The residue was dissolved in 1.0 ml of water. For removal of numerous organic compounds, a Sep-Pak C_{18} cartridge (Waters Assoc.) was employed. The cartridge was activated with 5 ml of water, then flushed with 5 ml of methanol. The 1.0-ml aqueous solution containing the brain extract was passed through the cartridge and the eluate containing the amino acids was collected and evaporated. The residue was dissolved in about 250 μl of 0.1% formic acid solution (the mobile phase for HPLC).

A 250-μl volume of the solution was introduced on to the column via a U6K universal injector (Waters Assoc.). The flow-rate was changed after 2.6 min from 3.0 to 1.0 ml/min and was maintained at 1.0 ml/min for an additional 20 min. The detection of authentic amino acids (non-labelled and labelled amino acids) or of those in biological samples was at 205 nm. Retention times of the amino acids were 2.8 min for glycine, 4.1 min for GABA, 5.9 min for proline and 11.7 min for pipecolic acid. The elution fractions at the retention times were collected manually. For example the pipecolic acid fraction was collected between 10.2 and 14.0 min. The volume of eluate was about 3.8 ml.

The eluate was evaporated in a test tube. The residue was dissolved in 1.5 ml of distilled methanol and transferred into a Reacti-vial (Microproduct V vial with PTFE-lined cap, Wheaton Scientific). The solution in the vial was evaporated under a gentle stream of dried nitrogen. Finally, the residue was dried in a vacuum desicator (60 mmHg) over silica gel for 12 h at room temperature.

BF_3—CH_3OH reagent (100 μl) was added to the dried sample and heated for 13 min at 100°C in a Dri-Block heater (Model DB-2H, M&S Instruments, Tokyo, Japan). The vial was cooled to room temperature before being opened. After evaporation to dryness under a stream of dried nitrogen at 80°C, the trace of water was azeotropically removed twice using 100 μl of methylene chloride at 100°C [25]. The residue was dissolved in 250 μl of distilled ethyl acetate, and 100 μl of HFBA were added. The mixture in the vial was heated for 20 min at 150°C in the Dri-Block heater and then gradually cooled to room temperature. The reaction product was evaporated to dryness using a gentle stream of dried nitrogen [26]. The residue was dissolved in 50 μl of ethyl acetate and 0.1—3.0 μl were injected into the GC—MS system.

RESULTS AND DISCUSSION

There are very few studies concerning the determination of pipecolic acids, while several methods, including enzyme assay, GC and GC—MS for GABA [27, 28] and HPLC and automated amino acid analysis for proline and glycine, have been reported [29].

In the present study, selected ion monitoring using a GC—MS—COM system following HPLC was chosen as a possible method for high sensitivity and specificity. This method has some advantages over the procedure previously utilized in our laboratory [10]. The advantage includes higher purification of samples within a short time by the replacement of the ion-exchange column chromatography with a combination of crude and pre-purification using Sep-Pak C_{18} cartridges and sequential HPLC separation. In addition, the sequential HPLC operation is semiautomatic and can visually develop the peaks derived from amino acids. It has been demonstrated that selected ion monitoring is a sensitive quantitative method for pipecolic acid and other amino acids [10, 12].

Mass spectra of HFB-ME derivatives of pipecolic acid, proline, GABA, glycine and internal standards are shown in Figs. 1 and 2. The molecular and base ions of the respective derivatives were *m/z* 339.2 $[(M)^+]$ and *m/z* 280.1 $[(M - COOCH_3)^+]$ for HFB-Me-pipecolic acid and *m/z* 348.2 $[(M)^+]$ and *m/z*

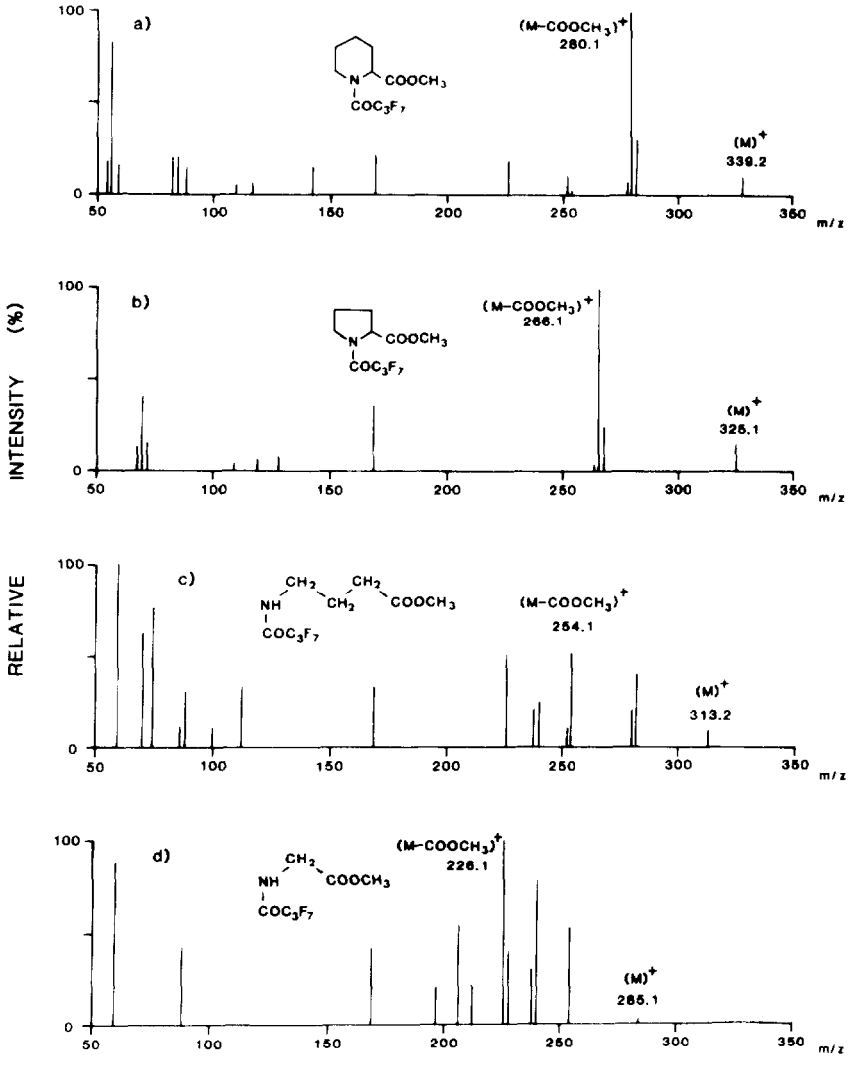

Fig. 1. Mass spectra of authentic HFB-Me derivatives of (a) pipecolic acid, (b) proline, (c) GABA and (d) glycine.

289.2 $[(M - COOCH_3)^+]$ for HFB-Me-pipecolic acid-d_9; *m/z* 325.1 $[(M)^+]$ and *m/z* 266.1 $[(M - COOCH_3)^+]$ for HFB-Me-proline and *m/z* 332.1 $[(M)^+]$ and *m/z* 273.1 $[(M - COOCH_3)^+]$ for HFB-Me-proline-d_7; *m/z* 285.1 $[(M)^+]$ and *m/z* 226.1 $[(M - COOCH_3)^+]$ for HFB-Me-glycine and *m/z* 287.1 $[(M)^+]$ and *m/z* 228.1 $[(M - COOCH_3)^+]$ for HFB-Me-glycine-d_2.

Because the base peak ions described above had the characteristics of the parent compound moieties, they were used for selected ion monitoring. The derivatives of GABA and GABA-d_6 have the molecular peak ion $[(M)^+]$ at *m/z* 313.2 and 319.2, the common base peak ions $[(COOCH_3)^+]$ at *m/z* 59.0, and the other intensive peak ions $[(M - COOCH_3)^+]$ at *m/z* 254.1 and 260.2, respectively. Thus the common base peak ion derived from GABA and GABA-d_6 had a very low molecular weight and did not have the specificity of the

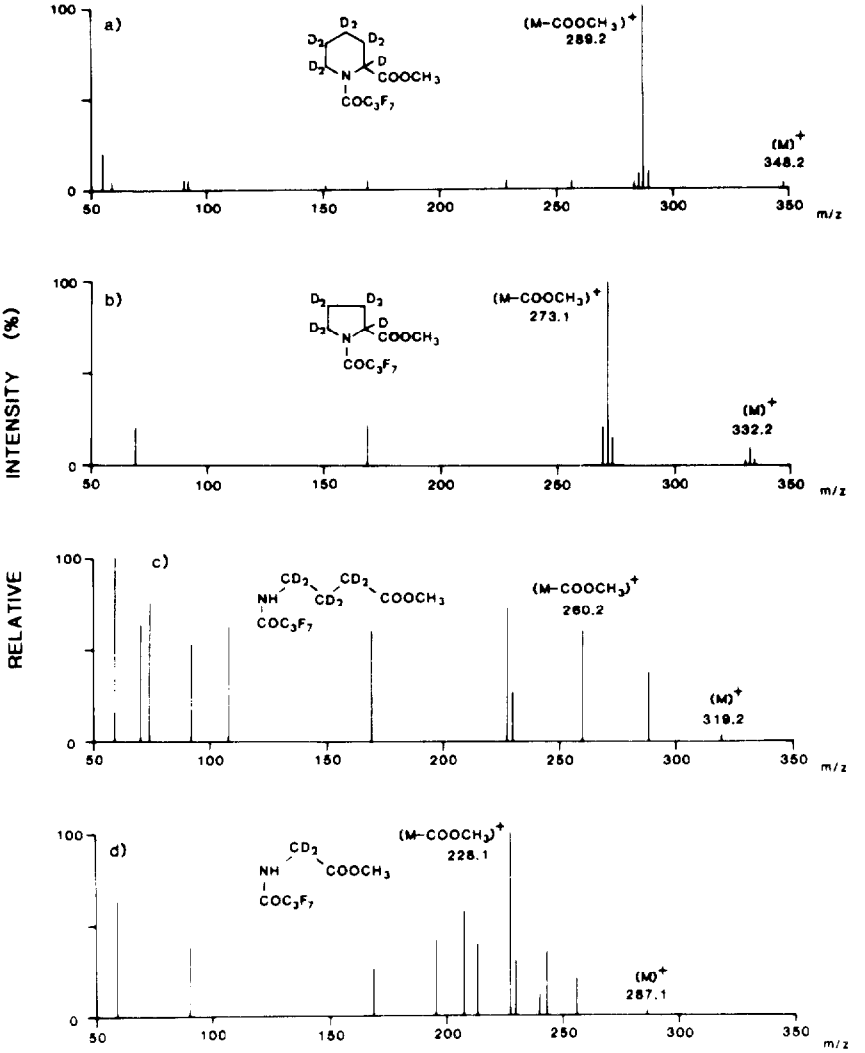

Fig. 2. Mass spectra of authentic HFB-Me derivatives of (a) pipecolic acid-d_{11}, (b) proline-d_7, (c) GABA-d_6 and (d) glycine-d_2.

parent compound moieties. Accordingly, intensive ions with *m/z* 254.1 and 260.2 which had GABA moieties were applied to GABA determination for selected ion monitoring.

Amino acids in brain extract were identified by the selected ion monitoring technique; that is, the ratios of peak area of the base or intensive ions to the molecular ions of HFB-Me-amino acids were measured using the standards and the brain extract samples, respectively. The ratios of *m/z* 280.1 to 339.2 for pipecolic acid were 10.7 ± 0.85 (standard) and 10.8 ± 0.64 (brain); *m/z* 266.1 to 325.1 for proline were 7.87 ± 0.44 (standard) and 7.75 ± 0.76 (brain); *m/z* 226.1 to 285.1 for glycine were 40.0 ± 1.06 (standard) and 38.7 ± 1.88 (brain); *m/z* 254.1 to 313.2 for GABA were 2.52 ± 0.22 (standard) and 2.57 ± 0.39 (brain) (six determinations each). Thus, the ratios were almost the same for the

standard and the brain extract, demonstrating the specificity of this method.

Fig. 3 shows selected ion monitoring recordings obtained from an analysis of pipecolic acid and other amino acids in rat brain extract containing the deuterium-labelled amino acids as the internal standards. The retention times for the HFB-Me derivatives of each amino acid and internal standard were 1.0 min for glycine, 2.4 min for GABA, 3.0 min for proline and 4.0 min for pipecolic acid.

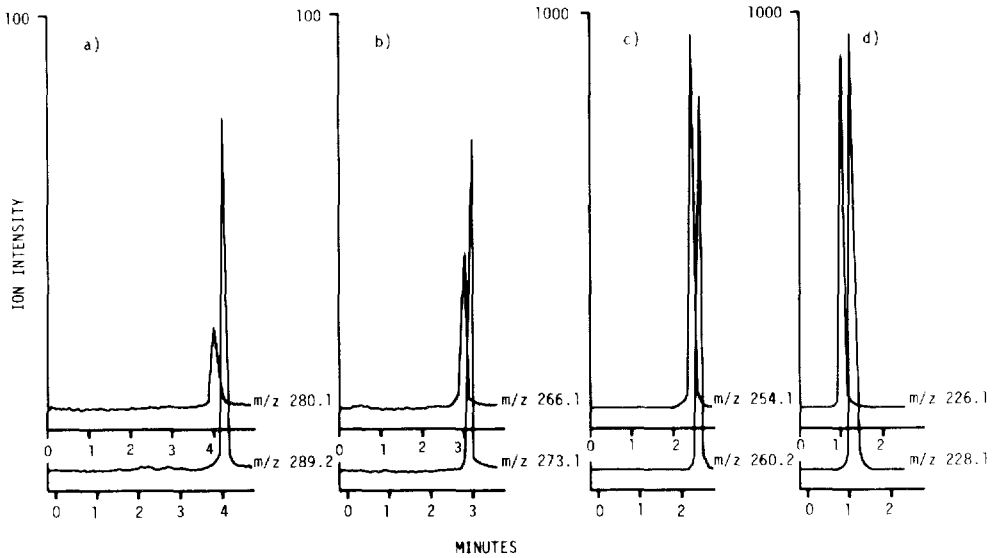

Fig. 3. Selected ion monitoring records of the extract from rat brain with added internal standards: (a) HFB-Me-pipecolic acid (*m/z* 280.1) and HFB-Me-pipecolic acid-d_9 (*m/z* 289.2); (b) HFB-Me-proline (*m/z* 266.1) and HFB-Me-proline-d_7 (*m/z* 273.1); (c) HFB-Me-GABA (*m/z* 254.1) and HFB-Me-GABA-d_6 (*m/z* 260.2); (d) HFB-Me-glycine (*m/z* 226.1) and HFB-Me-glycine-d_2 (*m/z* 228.1).

The amino acid concentrations in rat brain were calculated from the ratio of peak areas of *m/z* 280.1 to 289.2 for pipecolic acid, *m/z* 266.1 to 273.1 for proline, *m/z* 254.1 to 260.2 for GABA and *m/z* 226.1 to 228.1 for glycine using the computer system. Linear calibration curves (r = 0.97—0.99) were obtained with amounts in the nanomole range. The quantitative limits of the amino acids were as low as 5—10 pmol (signal-to-noise ratio = 2—4). The detection limit of the amino acids was about 0.5 pmol.

Pipecolic acid, proline, GABA and glycine levels in rat brain were as follows (mean ± standard error of ten determinations): 0.69 ± 0.04 nmol/g for pipecolic acid, 77.8 ± 13.7 nmol/g for proline, 1.29 ± 0.04 μmol/g for GABA and 1.44 ± 0.07 μmol/g for glycine. The proline, GABA and glycine levels in whole rat brain were similar to those reported by some other investigators [29].

Previously, pipecolic acid had been reported to be present only in the blood [17—22] and urine [17, 19]. However, recent preliminary studies in our laboratory [10, 12] and also by Gatfield et al. [17] and Schmidt-Glenewinkel et al. [23] have indicated that pipecolic acid is present in human and rat brain. The concentration of pipecolic acid in rat brain determined by this study was

one-twentieth that given by Schmidt-Glenewinkel et al. Accordingly, insufficient specificity may be one explanation for the tendency to high pipecolic acid concentrations found in the published papers. Since the chemical behaviour of other alicyclic amino acids (nipecotic acid, isonipecotic acid and hydroxypipecolic acids, etc.) such as the elution pattern in HPLC, column and gas chromatography resembles that of pipecolic acid, other alicyclic amino acids and other contaminants would increase the apparent value of pipecolic acid in tissues.

Thus the present paper describes a highly sensitive and specific method for the detection and assay of pipecolic acid. Furthermore, the method seems to be easily adapted to the simultaneous analysis of physiological, pathological and pharmacologically induced variations of these amino acids especially pipecolic acid in the brain regions, and such studies appear to be useful for investigating their possible physiological and pathological roles.

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