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Short communication

Rapid assay for γ-aminobutyric acid in mouse brain synaptosomes using gas chromatography–mass spectrometry

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Abstract

A sensitive and efficient assay for γ-aminobutyric acid (GABA) was applied to fresh mouse whole brain synaptosomes where the extracted GABA was analyzed as its di(*tert.*-butyl(dimethylsilyl)) derivative by gas chromatography–mass spectrometry (GC–MS) using GABA-d_6 as an internal standard. Endogenous levels of 20.01 ± 0.75 nmol GABA/mg protein were found. The method is characterized by a detection limit of about 10 fmol injected GABA derivative and coefficients of intra-day and inter-day variation of 0.95% and 7.7%, respectively. The rate of synaptosomal GABA synthesis was used to determine the activity of glutamate decarboxylase (GAD) as 314.9 ± 9.0 nmol GABA/mg protein/h. Both GABA levels and GAD activity were significantly elevated by therapeutic doses of the antiepileptic drug valproic acid.

1. Introduction

Neurotransmission involving γ-aminobutyric acid (GABA), the principal inhibitory neurotransmitter in the mammalian brain, is the target of several classes of drugs, notably benzodiazepines and anticonvulsants. GABA is synthesized from glutamate by glutamate decarboxylase (GAD) in neuronal nerve terminals where it is released and subsequently taken up by neurons and glia prior to degradation. For our research with analogues of the antiepileptic drug valproic acid (VPA), we required a simple but sensitive method for evaluating how therapeutic doses of these drugs affect the neurotransmitter pool of GABA located in the nerve terminals. Monitoring drug-induced changes in GABA levels in nerve terminals, rather than whole tissue, has the advantage of reducing interference from metabolic pools of GABA which do not participate in seizure protection, and thus provides a better reflection of the pharmacodynamics of the drug [1]. This paper describes our isolation of the synaptosomal (nerve terminal) fraction of mouse whole brain homogenate followed by the determination of GABA levels and GAD activity, as measured by the rate of GABA production, using GC–MS.

There are numerous reports in the literature describing the analysis of GABA by GC–MS (for example, see Refs. [2,3] and references cited therein), the most common of which is based on the derivatization of GABA with pentafluoropropionic anhydride and hexafluoroisopropanol developed by Bertilsson and Costa [4] to assay for GABA and glutamate in rat cerebellum.

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Although its sensitivity is good (detection limit about 24 fmol injected GABA derivative [2]), this method employs fairly low molecular mass (m/z 204) ions for selected-ion monitoring (SIM) in the electron ionization mode, which increases the risk of interference from other species. Furthermore, the length of the derivatization procedure, the low stability of the product [5] and the formation of acidic by-products, which may degrade the column's stationary phase, make this approach less than ideal for large numbers of samples.

An alternative derivatization procedure is silylation of GABA to N,N,O-tris(trimethylsilyl)-GABA [6]. This derivatization and associated GC–MS assay overcomes the drawbacks of the Bertilsson and Costa method although the detection limit (60 fmol) is inferior. The silylation protocol has subsequently been modified by the use of the versatile reagent N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) to afford N,O-bis((*tert.*-butyl)dimethylsilyl)-GABA [7–9]. However, these procedures used either aqueous standards or tissue homogenates and did not fully evaluate the recovery, variability and detection limit. In this paper, we present our version of this approach as applied to the analysis of synaptosomal GABA, to both determine its endogenous level and as a measure of the activity of neuronal GAD.

2. Experimental

2.1. Materials

N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) were purchased from Pierce (Rockford, IL, USA). Mature male CD-1 mice were obtained from Animal Services of the University of British Columbia (Vancouver, B.C., Canada). GABA-d_6 was purchased from MSD Isotopes (Montreal, Que., Canada). 3-Mercaptopropionic acid and pyridoxal 5-phosphate (PLP) were purchased from Sigma (St. Louis, MO, USA). GABA and *tert.*-butyldimethylsilyl chloride (*tert.*-BDMSCl) were purchased from Aldrich (Milwaukee, WI, USA). VPA was purchased from Aldrich and redistilled before use. Acetonitrile was purchased from BDH (Toronto, Ont., Canada) and redistilled from calcium hydride under nitrogen prior to use.

2.2. Preparation of synaptosomes

Synaptosomes from mouse whole brain were prepared by a modification of the method described by Dodd et al. [10]. All steps were carried out at 0–4°C. For the assay of GABA, all sucrose solutions were 1 m*M* in sodium 3-mercaptopropionate to inhibit the large post-mortem GABA increase [11]. Ten mice (five per GABA assay and five per GAD assay) were injected with 4 ml/kg of either 0.9% saline or VPA as an aqueous solution of its sodium salt (0.83 mmol/kg), decapitated at 15 min postdose into 0.9% saline, the brains were quickly removed, homogenized in 0.32 *M* sucrose (4 ml), diluted with sucrose (2 ml) and centrifuged at 1000 *g* for 10 min. The supernatant was layered onto 1.2 *M* sucrose (9.5 ml) and then centrifuged (220 000 *g*, $\omega^2 t = 1.6 \cdot 10^{10}$ rad^2/s): the interface was collected by pipet, made up to 10 ml, layered onto 0.8 *M* sucrose (9.5 ml) and centrifuged as before. The supernatant was removed and the pellet resuspended in either water (GAD assay, 5 ml) or buffer (GABA assay, 5 ml: 0.5 *M* KCl, 0.4 *M* sodium phosphate, 10 m*M* EDTA, 0.5% Triton X-100, 47.4 nmol GABA-d_6, pH 6.4) [12]. The protein content of the synaptosomes was 19.6 ± 2.1 mg/g tissue for the GABA assay ($n = 10$) and 16.3 ± 2.0 mg/g tissue for the GAD assay ($n = 10$), using a modified Lowry assay [13]. Samples were stored at −78°C until further analysis.

2.3. GABA and GAD assays

In the GAD assay, each synaptosome sample (50 μl) was incubated in triplicate for 1 h at 37°C with 50 μl of a solution containing glutamate (250 nmol) and PLP (25 nmol) in 0.1 *M* pH 6.4 sodium phosphate buffer [11]. For the blank (run

in duplicate), the glutamate/PLP mixture was replaced with 50 μl of buffer. The reaction was quenched by the addition of 1.7% trichloroacetic acid (53 μl) containing GABA-d_6 (660 pmol), the precipitated material separated by centrifugation (13 600 *g*, 10 min) and the supernatant lyophilized and derivatized with an acetonitrile solution (300 μl, containing 30 μl MTBSTFA and 0.3 μl *tert.*-BDMSCl [7]) at 65°C for 1 h prior to GC–MS analysis. GAD activity was defined as the amount of GABA in the sample incubated with glutamate and PLP less the amount obtained in the blank. Both the glutamate ($n = 3$) and PLP ($n = 2$) were omitted in separate control experiments.

In the brain GABA assay, run in triplicate for each synaptosome preparation, resuspended synaptosome samples (50 μl) were acidified with 1.7% trichloroacetic acid (60 μl) and centrifuged (13 600 *g*, 10 min). An aliquot (80 μl) of the supernatant was then removed, lyophilized and derivatized as described above.

2.4. Instrumental conditions

The GABA contents of the above assays were analyzed using a Hewlett-Packard (Palo Alto, CA, USA) HP 5890 gas chromatograph interfaced to a HP 5989A mass spectrometer. GC: 34.5 kPa helium head pressure, Hewlett-Packard HP-1 capillary column: 12 m × 0.2 mm I.D., 0.33 μm crosslinked methyl silicone. Oven: 50°C initial, increasing to 130°C at 30°/min, then to 200°C at 10°/min and finally to 280°C at 30°/min, with a final hold time of 2 min. Equilibration time: 1 min. Injection: 1 μl. MS: electron impact ionization potential 70 eV, SIM for *m/z* 274 and 280 (dwell time 75 ms), source 200°C, analyzer 100°C. The tune was optimized for *m/z* 264 with the high-energy dynode set at 10 kV.

Individual standard curves were prepared for the GABA and GAD assays using aqueous solutions of GABA and GABA-d_6 containing the appropriate amounts of buffer and trichloroacetic acid. These solutions were evaporated to dryness and derivatized as above. For the GAD blank and the GABA assay, a range of 400–1900 pmol GABA was used, while a range of 3–20 nmol was employed for the GAD assay.

3. Results

3.1. Comparison with MSTFA

Our initial treatment of GABA with MSTFA afforded the N,O-bis(trimethylsilyl) GABA derivative. The compound showed sharp peaks and good sensitivity with the *m/z* 232 ion, corresponding to a species having lost one methyl group presumably from the amino substituent [7]. When MSTFA was replaced with its *tert.*-butyl analogue, MTBSTFA (containing 1% *tert.*-BDMSCl), GABA was derivatized to the N,O-bis((*tert.*-butyl)dimethylsilyl) compound: sensitivity improved approximately ten-fold using the analogous ion *m/z* 274 (molecular ion less a *tert.*-butyl substituent) which appeared as the base peak. It was found that a 10% solution of the MTBSTFA reagent in acetonitrile was adequate for complete derivatization, in contrast to the high concentrations reported previously [8].

3.2. Assay characteristics

The calibration curves were linear ($r^2 > 0.99$). Coefficients of inter-day variation for the GC–MS portion of the method were 7.7% and 7.9% for the GABA and GAD assays ($n = 7$), respectively, calculated from their respective calibration curves. The coefficients of intra-day variation were 0.95% (five replicates of five samples) and 1.9% (three replicates of ten samples) for the GABA and GAD assays, respectively. The response was linear from 0.1 to at least 20 nmol GABA.

For a 3:1 signal-to-noise ratio, the detection limit was about 10 fmol of injected GABA derivative, corresponding to about 6 pmol GABA per 50 μl synaptosome aliquot. The calibration curve ($y = 0.00424 + 0.8182x$) for this range (6.7–107 fmol GABA per injection) was linear with $r^2 = 0.9998$. This sensitivity was more than adequate for the GABA and GAD assays

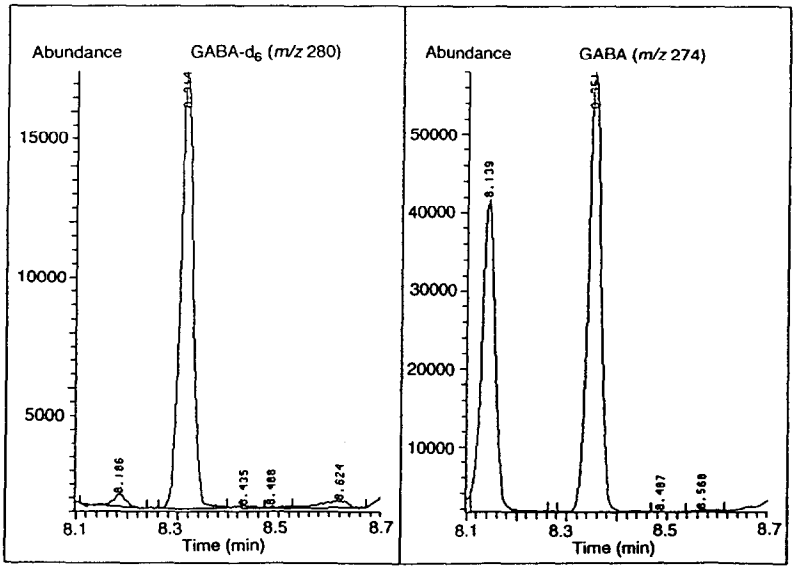

Fig. 1. SIM chromatograms from GABA synaptosome assay. A 50-μl aliquot of the resuspended synaptosomes, containing 474 pmol GABA-d_6, was combined with 1.7% trichloroacetic acid (60 μl) and centrifuged (13 600 g, 10 min). A portion (80 μl) of the supernatant was lyophilized and derivatized with MTBSTFA solution (300 μl in acetonitrile, containing 10% MTBSTFA and 0.1% *tert.*-BDMSCl, v/v) at 65°C for 1 h. The sample was analyzed by GC–MS using the following conditions. GC: 34.5 kPa helium head pressure, Hewlett-Packard HP-1 capillary column (12 m × 0.2 mm I.D., 0.33-μm crosslinked methyl silicone). Oven: 50°C initial, increasing to 130°C at 30°/min, then to 200°C at 10°/min and finally to 280°C at 30°/min, with a final hold time of 2 min. Injection: 1 μl. MS: electron-impact ionization potential 70 eV, SIM for m/z 274 and 280 (dwell time 75 ms), source 200°C, analyzer 100°C. The tune was optimized for m/z 264 with the high-energy dynode set at 10 kV.

since the average synaptosome sample in either assay contained at least 1 nmol GABA. There were no interferences for either the GABA or GABA-d_6 peaks.

The derivatized samples were stable for at least several weeks at room temperature.

3.3. Application to synaptosomes

GABA levels and GAD activity for both fresh and frozen synaptosomes are shown in Table 1. As might be expected, GABA concentrations were relatively similar for both fresh and frozen samples, whereas GAD activity was markedly decreased by freezing. The values reported here compare favourably with those obtained using a virtually identical synaptosome preparation protocol but analyzed with a radioreceptor binding assay [11]. Our values (Table 1) offer a markedly higher degree of precision. The noticeably higher GABA levels recorded in our assay may be the result of the detergent in the resuspension buffer, which may have permitted a more efficient extraction of GABA from vesicle sites, although this factor was not investigated further.

The GABA values obtained in the GAD assay are at least ten-fold greater than the blank levels, indicating minimal interference from endogenous GABA. This is in contrast to the three-fold increase observed in a GC–MS assay of GAD in brain punches [12]. The enzyme reaction's strong dependence on both PLP and glutamate (Table 1) indicates that GABA was being formed by a specific enzymatic process. Our GAD assay method is thus a considerable improvement over the more common radiometric assay which measures the $^{14}CO_2$ evolved during the incubation of the sample with labelled glutamate (reviewed in Ref. [14]): unlike GABA, which is effectively

Table 1
Analysis of GABA and GAD in mouse whole brain synaptosomes[a]

Conditions	GABA (nmol/mg protein)	GAD activity (nmol GABA/mg protein/h)
Saline-dosed		
Fresh	20.01 ± 0.75	314.9 ± 9.0
Frozen	18.92 ± 0.82	228 ± 13
Frozen less Glu		26.61 ± 0.48[b]
Frozen less PLP		63.3 ± 5.7[c]
Frozen less Glu and PLP		23.48 ± 0.08
Reference [11]	16.6 ± 4.2	442 ± 134
VPA-dosed	22.17 ± 0.86[d]	351 ± 21[e]

[a] All values reported as mean ± S.D. ($n = 5$) except when noted otherwise.
[b] Mean ± S.D. ($n = 3$).
[c] Mean ± S.D. ($n = 2$).
[d] Fresh synaptosomes.
[e] Frozen synaptosomes.

produced only by GAD [15], the generation of $^{14}CO_2$ from glutamate can also proceed by pathways not involving GAD [16].

The recovery of GABA-d_6 from the resuspended synaptosomal pellet in the GABA assay was 78 ± 3% ($n = 3$), based on the ratio of the GABA-d_6 peak area obtained in the assay to the peak area recorded when this internal standard was incorporated into the MTBSTFA derivatization mixture rather than into the initial pellet resuspension buffer. Although the recovery value is modest, the associated error is apt to be minimal owing to the chemical similarity of GABA-d_6 to GABA. It is unlikely that protein binding was the cause of these losses as the recovery was still only 87 ± 4% ($n = 6$) when GABA-d_6 was added immediately prior to lyophilization rather than at the initial pellet resuspension. The determination of the recovery of GABA itself using a spiking method might not be reliable since the endogenous compound is in vesicles rather than simply in solution.

Finally, it was verified that GABA levels and GAD activity in mice were significantly elevated by the antiepileptic drug VPA at an ED50 dose (0.83 mmol/kg; Dr. Wei Tang, personal communication) based on the subcutaneous pentylenetetrazole test. These increases in GABA in response to VPA are similar to those reported previously [11].

In conclusion, we have extended a highly sensitive GC–MS method to analyze GABA levels and GAD activity in brain nerve terminals. Our values not only agree with published values but exhibit the low variability required for measuring the subtle influence of drugs acting on GABAergic neurotransmission.

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