# A Highly Specific and Sensitive Determination of Gamma-aminobutyric Acid by Gas Chromatography Mass Spectrometry†

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**A procedure for the identification and quantification of picomole quantities of gamma-aminobutyric acid in tissue samples is given. This procedure combines the chemical specificity of dinitrophenylation with that of gas chromatography mass spectrometry to eliminate the interferences encountered with other direct derivatization procedures. Only a limited number of dinitrophenyl amino acid ethyl esters and some fatty ethyl esters are detected in the solution used for analysis. Identification is based on retention time and on the relative abundances of the three major ion fragments of the gamma-aminobutyric acid derivative. Quantitation is accomplished using isotope dilution techniques with [$^2H_2$]gamma-aminobutyric acid as an internal standard. The procedure has been successfully applied to samples of human cerebrospinal fluid and to extracts of ganglia from the mollusc, *Aplysia californica*.**

## INTRODUCTION

Gamma-aminobutyric acid (GABA), a putative inhibitory neurotransmitter, is found in high concentrations (millimolar) in mammalian central nervous systems (CNS) and in certain invertebrate preparations such as lobster ventral cord and inhibitory axons[1]. It has been suggested that changes in GABA levels in the CNS may be associated with certain neurological disorders. Decreased levels of GABA and of glutamic decarboxylase, the enzyme responsible for the synthesis of GABA, have been reported in postmortem brains from patients with Huntington's chorea.[2–6]

Glaeser *et al.*[7–9] have reported GABA levels in cerebrospinal fluid (CSF) using a liquid chromatography (LC) assay and proposed using CSF to monitor changes in GABA levels in brain. Both Enna *et al.*,[10] using a radioreceptor assay, and Huizinga *et al.*,[11] using a combined LCGCMS procedure, have confirmed these results; however, Perry and Hansen[13] were unable to find GABA in CSF using their LC procedure.

The procedure used by Huizinga required two successive LC separations prior to applying the GCMS method described by Bertilsson and Costa[12] for the determination of GABA in mammalian brain. In order to facilitate studies of GABA in human CSF and in the CSN of the mollusc, *Aplysia californica*, we investigated derivatization procedures which could be carried out directly on crude tissue samples. When direct applications of either the *N*-pentafluoropropionyl hexafluoroisopropyl ester (PFP-HFIP)[12] or TMS[14,15] derivatization procedures were attempted, interferences made the identification and quantitation of GABA impossible. However, modification of a GC procedure reported previously (Ikekawa *et al.*[16] and Aprison *et al.*[17]) has resulted in a GCMS method with sufficient specificity and sensitivity for the determination of GABA in both samples. In this procedure, GABA is converted to the *N*-dinitrophenyl ethyl ester derivative and measured by selected ion monitoring techniques. The procedure is sufficiently sensitive so that GABA can be measured quantitatively in small volumes of CSF (less than 0.5 ml) or in portions of a single *Aplysia* ganglion.

## EXPERIMENTAL

### Materials and reagents

GABA was obtained from Sigma Chemical Co., St. Louis, Missouri; [2,2-$^2H_2$]GABA from Merck & Co., Inc., Rahway, New Jersey; and [$^{15}$N]GABA from Stohler Isotope Chemicals, Waltham, Massachusetts. Dinitrofluorobenzene (DNFB) and dichloromethane (Sequanal Grade) were obtained from Pierce Chemical Co., Rockford, Illinois; ethyl acetate was purchased from Burdick–Jackson Laboratories, Muskegon, Michigan. These solvents were used without further purification.

Ethanolic HCl (1.25 N) was prepared by bubbling HCl gas into USP grade EtOH previously dried by distillation over calcium oxide. The HCl was dried by

† Abbreviations: GABA = gamma-aminobutyric acid; PFP–HFIP = *N*-pentafluoropropionyl hexafluoroisopropyl ester; DNFB = dinitrofluorobenzene; DNP–Et = dinitrophenyl ethyl.

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passing it through a scrubbing tower containing concentrated $H_2SO_4$. the solution was stored at 4 °C and replaced at two-month intervals. DNFB was diluted 1–10 (v/v) in dry EtOH.

All glassware was cleaned by boiling in 50% $HNO_3$ and was rinsed extensively in glass distilled $H_2O$ prior to use.

### Preparation of samples

CSF samples from hospital patients with a variety of pathological conditions were provided by Dr T. N. Chase, National Institute of Neurological and Communicative Disorders, Bethesda, Maryland and stored at −20 °C until analyzed. Aliquots (0.5 ml, containing 100 pmoles [$^2H_2$]GABA as internal standard) were freeze-dried and extracted with 0.5 ml of 80% EtOH. After centrifugation, the supernate was removed and the residue extracted a second time with 0.5 ml EtOH. The combined extracts were evaporated to dryness with a stream of dry $N_2$, and 300 $\mu$l of 0.1 M sodium carbonate buffer (pH 10) and 20 $\mu$l of DNFB reagent were added. After mixing, the tubes were capped and heated for 30 min in a 60–65 °C water bath, cooled to room temperature, and acidified with 300 $\mu$l of 4 N HCl. The solutions were extracted twice with 200 $\mu$l ethyl acetate, and the organic phases were combined and evaporated to dryness. These residues were extracted twice with 200 $\mu$l $CH_2Cl_2$, and the extracts were combined and evaporated to dryness. Ethanolic HCl (200 $\mu$l) was added and the tubes tightly capped, then heated to 60–65 °C for 60 min in a water bath. After cooling to room temperature, 200 $\mu$l $H_2O$ and 200 $\mu$l $CH_2Cl_2$ were added. After mixing, the organic phase containing the DNP-amino acid ethyl esters was removed, transferred to 300 $\mu$l vials (Reactivials, Pierce Chemical Co., Rockford, Illinois) and evaporated to dryness. This extraction was carried out twice. Just prior to analysis, the residue was dissolved in 10 $\mu$l of ethyl acetate and 1–2 $\mu$l were injected into the GCMS. The *Aplysia* ganglia were homogenized in 80% EtOH and the supernate treated in the same way as the CSF samples, except that the volumes used in preparation were smaller by a factor of 10.

Reagent blanks, as well as GABA and [$^2H_2$]GABA standards, were prepared with each series of samples. The standards were used to verify the isotope ratios for GABA and [$^2H_2$]GABA for each analysis.

### Gas chromatography mass spectrometry

A Dupont Model 321 GCMS coupled to a Model 320 data system was used for data acquisition and processing. The injection port, jet separator and ion source were all operated at 250 °C. Electron emission was 1 ma and ionizing energy was 73 eV. A signal generator (Heath–Schlumberger Model SG-1271) was substituted for the standard 321 beam modulator to improve the signal-to-noise ratio of the isotope ratio measurements. A 10 kHz triangular wave of 1.5 volts peak-to-peak was applied to the existing beam modulator plates in the mass spectrometer.

A 5 ft long × 2 mm i.d. glass column packed with 3% OV-101 on 80/100 mesh Supelcoport (Supelco, Inc., Bellefonte, Pennsylvania) was used for chromatographic separations. The helium carrier gas flow rate was 25 ml min$^{-1}$. After injection, the column temperature was programmed from 230 to 290 °C at 8 °C min$^{-1}$. When analyzing tissue samples, the column was maintained at 290 °C for approximately 5 min and then re-equilibrated to 230 °C. For quantitation, data were collected at *m/e* 252 and 254 by alternately integrating the ion current at each mass for 250 ms periods over a 1 min interval centered about the elution time of GABA.

### Quantitation

Quantitation was achieved using [$^2H_2$]GABA as an internal standard. The isotopic variant was selected in preference to a GABA homolog because of accuracy and precision considerations.[18] The 252/254 isotope ratios were computed from areas integrated using the 320 data system. The quantitative calculation was done according to the equation

$$x = y\frac{(R_y - R_m)(R_x + 1)}{(R_m - R_x)(R_y + 1)}$$

where: $x$ = moles of GABA in unknown, $y$ = moles of [$^2H_2$]GABA added to unknown, $R_x$ = isotope ratio for pure GABA, $R_y$ = isotope ratio for the [$^2H_2$]GABA added and $R_m$ = isotope ratio for the sample containing the [$^2H_2$]GABA internal standard.

This equation was derived by solving the simultaneous equations given by Jenden.[19] The [$^2H_2$]GABA internal standard was calibrated against a known GABA standard to normalize for its isotopic purity and any significant isotope effects.

## RESULTS

The dinitrophenyl ethyl ester of GABA (GABA-DNP-Et) eluted at 6.7 min under the conditions described and was well separated from neighboring peaks resulting from the isoleucine (Ileu) and aspartic (Asp) derivatives (retention times = 5.9 and 7.2 min, respectively). Both of these compounds were present in the samples analyzed at concentrations much greater than that of GABA. The base peak of Ileu-DNP-Et occurs at *m/e* 252, so its chromatographic tail had to be minimized in order to reduce background noise during data acquisition. The Asp-DNP-Et also produced ion fragments at *m/e* 252 and 254 and, although these fragments were of low relative abundance, the ability to obtain precise and accurate measurements for GABA-DNP-Et required complete separation.

A chromatogram showing separation of the DNP ethyl ester derivatives of the amino acids detected in CSF is given in Fig. 1. It was produced on an analog recorder monitoring the ion current amplifier in parallel with the data system. During the first and last portions of the chromatogram, the mass spectrum from 85–250 amu was displayed on an oscilloscope. The darkened area in the record was produced by the data collection process when the mass spectrometer was operated in the selected ion monitoring mode rather

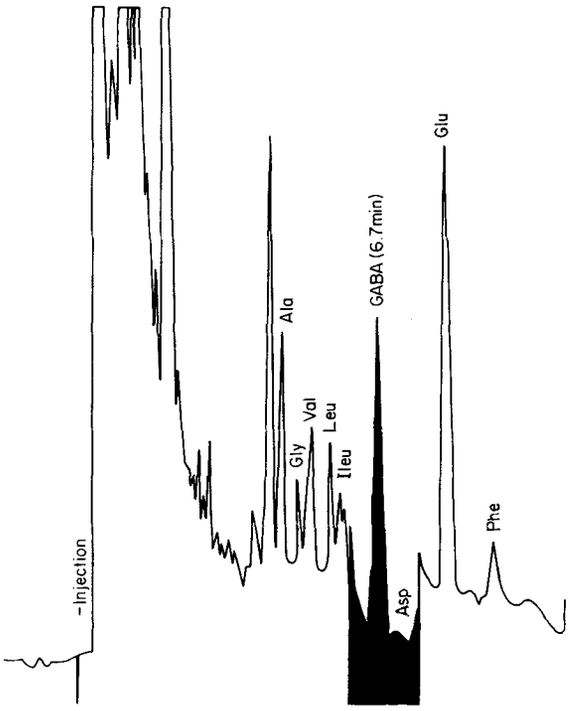


**Figure 1.** Analog gas chromatogram for a CSF sample with the *darkened area indicating the period where data were collected.*

than in the scanning mode. This also accounts for the apparent change in recorder sensitivity during this portion of the chromatogram. The data system was engaged just before GABA was about to elute and stopped approximately 1 min later. In this way, storage space in the data system was used only for the data of interest, and many more sample runs could be stored on a single magnetic disc than would otherwise have been possible. The early eluting peaks in the chromatogram include a variety of fatty acid ethyl esters, derivatizing reagents and phthalate esters. The major amino acid derivatives could be identified by their mass spectra on the oscilloscope as they eluted.

The mass spectrum of GABA-DNP-Et prepared as described above and the structures of its major ion fragments (*m/e* 196, 206 and 252) are given in Fig. 2(a). The [$^2H_2$]GABA-DNP-Et spectrum [Fig. 2(b)] showed a similar fragmentation pattern, but with major peaks at *m/e* 208 and 254 resulting from the two deuterium atoms attached to the carbon skeleton. A $^{15}$N-GABA derivative was prepared and its mass spectrum obtained to confirm the assignment of ion fragment structures.

In CSF and in *Aplysia* ganglia, the levels of GABA were too low to obtain complete mass spectra of interpretable quality. Therefore, identifications were based on retention times and on the relative abundances of the three major ion fragments in the GABA-DNP-Et spectrum. The relative abundances were computed from the areas under the *m/e* 196, 206 and 252 selected ion profiles as integrated with the data system (Fig. 3). GABA standards in the appropriate concentration range and sample extracts were analyzed in replicate, and the means and standard deviations of the relative abundances were calculated. If the relative abundances for 'GABA' in the sample fell within one standard deviation of the mean relative abundances of the

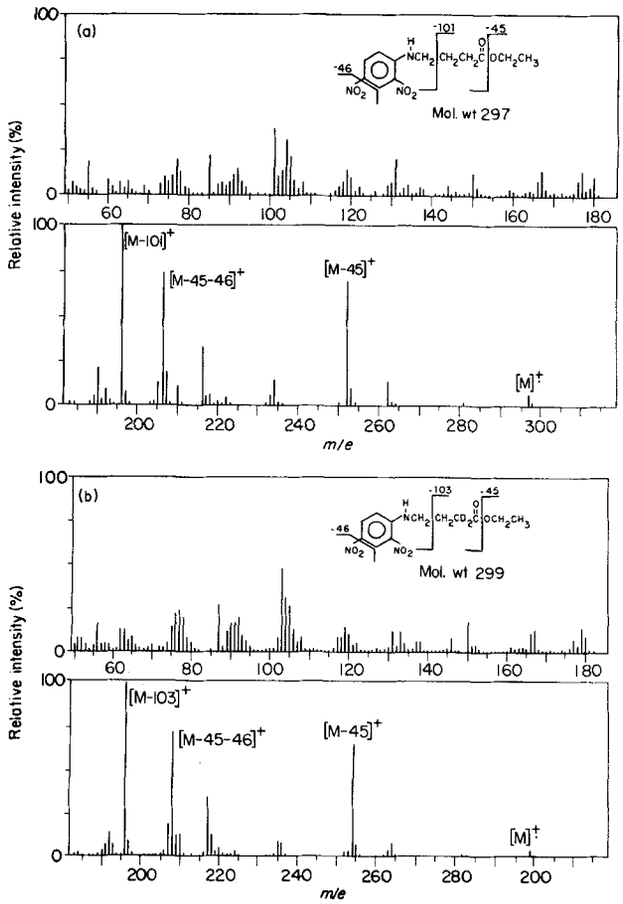


**Figure 2.** Mass spectra of the *N*-dinitrophenyl ethyl esters of (a) GABA; (b) [$^2H_2$]GABA.

GABA standard, identification was considered confirmed at an acceptable level of confidence. Table 1 shows the relative abundances for GABA identified in the CSF sample illustrated in Fig. 3, and Table 2 shows a similar set of data for an *Aplysia* ganglion sample. The relative abundances tend to vary from day to day due to factors which affect the ionization process, such as ion source cleanliness, instrument tuning, and changes in ion source pressure and temperature. However, over the short periods (8 h or less), the rela-

**Table 1. Relative abundances of GABA-DNP-Et in cerebrospinal fluid**

| | Pure standard | | Cerebrospinal fluid | | |
|---|---|---|---|---|---|
| *m/e* | Relative abundance | Standard deviation | Area | Relative abundance | Error |
| 196 | 0.371 | 0.003 | 66064 | 0.371 | 0.000 |
| 206 | 0.359 | 0.004 | 64428 | 0.362 | 0.003 |
| 252 | 0.270 | 0.003 | 47620 | 0.267 | −0.003 |

**Table 2. Relative abundances of GABA-DNP-Et in an *Aplysia* ganglion**

| | Pure standard | | Pedal ganglion | | |
|---|---|---|---|---|---|
| *m/e* | Relative abundance | Standard deviation | Area | Relative abundance | Error |
| 196 | 0.349 | 0.014 | 16240 | 0.360 | 0.011 |
| 206 | 0.386 | 0.015 | 17334 | 0.385 | −0.001 |
| 252 | 0.267 | 0.011 | 11548 | 0.256 | −0.011 |

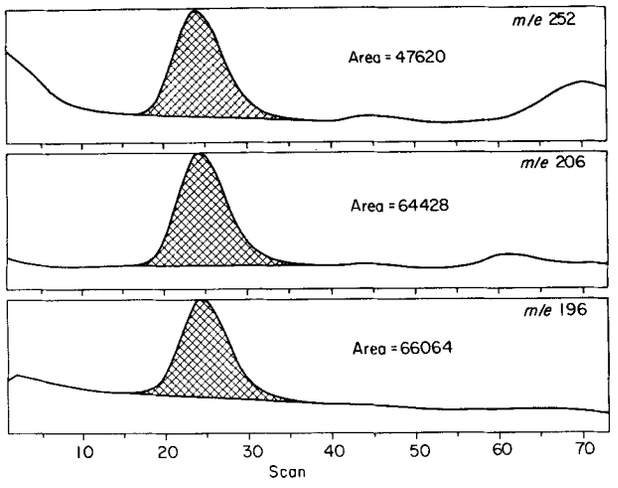


**Figure 3.** Selected ion monitoring data used to calculate the relative abundances and to identify GABA in a CSF sample.

tive abundances remain stable and can be measured with precisions of 1 to 5% standard deviation for samples in excess of 10 pmoles per injection.

The 252/254 isotope ratio was selected for quantitation since the 206/208 ratio showed interference from OV-101 column bleed. Analysis of a series of standards demonstrated that the quantitative relationship was linear from 3 to 3000 pmoles per injection (Fig. 4). Deviations over this range were ±7.6%; however, over the range used for sample analysis (10–300 pmoles per injection), the mean deviation was only ±1.6%. The 252/254 isotope ratios for sample plus internal standard in the concentration range used for quantitation were obtained with precisions of 0.5 to 1% standard deviation. Precision was better for the isotope determinations than for relative abundance determinations (1) because isotope ratios are less affected by factors which influence the ionization process, and (2) because the standard beam modulator was replaced by the function generator. When using the function generator, flat-topped rather than conventionally shaped mass peaks are produced and, as a result, precision is improved. Since the function generator could not be amplitude-modulated, it could not be used for the relative abundance determinations.

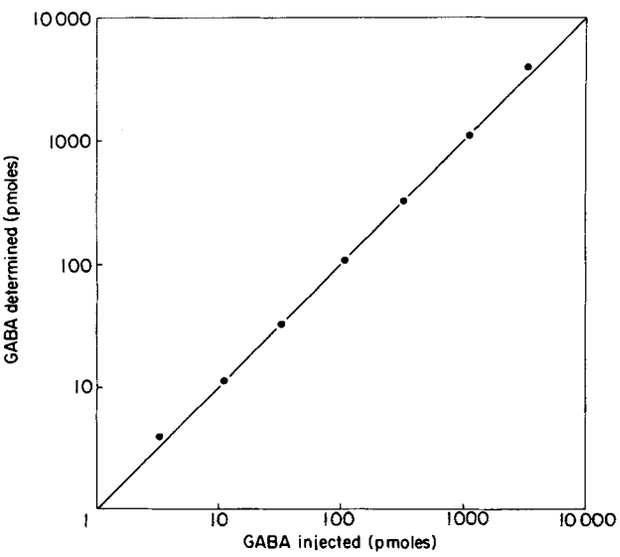


**Figure 4.** Calibration curve for GABA standards.

GABA has been identified and quantitated in nine human CSF samples. The concentrations ranged from 323–687 pmoles $ml^{-1}$ with a mean of 519 pmoles $ml^{-1}$ and standard deviation of 143 pmoles $ml^{-1}$. These data are in good agreement with values reported previously using other procedures.[8,9,11,12] Since these CSF samples were obtained from a group of unselected patients, the large deviation probably reflects their heterogeneity. Studies of GABA levels in CSF from patients with various neurological diseases, particularly Huntington's chorea, are in progress.

Among the various *Aplysia* ganglia, the quantity of GABA per ganglion varied over a sixfold range. The values found were 48, 100, 116, 291 and 326 pmoles per pleural, buccal, abdominal, pedal and cerebral ganglion, respectively. These values are based on duplicate determinations of tissue obtained from three animals. More extensive studies of the distribution of GABA in various parts of the ganglia, as well as in individual, identified neurons isolated from these ganglia, will be published elsewhere.

## DISCUSSION

GABA could be identified and quantified in low concentration tissue samples using the DNP-Et derivatization procedure. Highly reactive derivatizing reagents, such as TMS reagents, form volatile derivatives not only with amino acids, but also with many other small molecules containing 'active' hydrogens (amines, acids, alcohols, carbohydrates, phenols, etc.). Thus, in tissue extracts, the potential for interference is high. Analysis of a silylated extract of CSF revealed that an unidentified compound (probably a polyhydroxyl alcohol) and aspartic acid both co-eluted with GABA. The interference situation could be altered by changing chromatographic conditions such as temperatures, flow rates and liquid phases, but the result was only to replace one set of interferences with another. However, by using the DNP-Et derivatization procedure, GABA plus only a small number of other compounds present in the original sample were detected. GCMS analysis was simplified by this 'partial purification,' and accurate, quantitative data could be obtained.

The GC procedure described by Aprison *et al.*[17] for amino acids as DNP-methyl esters required approximately 60 min per sample to resolve GABA from the other tissue components. If analysis time was reduced to approximately 15 min in order to more effectively utilize instrument time, the GABA and aspartic acid derivatives co-eluted. By preparing the ethyl rather than the methyl esters, the carbon number of Asp was increased by two units, while that of GABA was increased by only one. As a consequence, good separation could well be achieved in conjunction with rapid sample turn-around on the GCMS. The Asp-GABA separation could be increased further by making the propyl or butyl esters; however, these derivatives elute at successively higher column temperatures.

Although the specificity of the GABA-DNP-Et procedure is much greater than that of direct TMS procedures, sensitivity is potentially greater with the latter. This is because 25–50% of the total ionization of GABA-TMS is encountered at *m/e* values suitable for quantitation.[14] With GABA-DNP-Et, however, only about 5% of the total ionization is encountered at any one of the three major ion fragments. Because of this, the detection limit for GABA-DNP-Et is 5–10 times less favorable than for GABA-TMS. Sensitivity with the PFP-HFIP derivative is intermediate between that of GABA-DNP-Et and GABA-TMS based on its fragmentation profile.[12] However, this derivative was prepared with variable yields when picomole quantities of GABA were used and, as a consequence, has not proven suitable for trace analysis. This may be due to partial hydrolysis of the derivative or to 2-pyrrolidinone ($\gamma$-butyrolactam) formation resulting from acid-catalyzed ring closure of the ester.[20]

Although the specificity of GMCS is high compared with other physical methods of chemical analysis, additional specificity may be required when complex biological sample, such as body fluids or tissues, are analyzed for compounds present at extremely low concentrations. The DNP-Et procedure described here for GABA is an example of how increased specificity can be achieved through judicious derivative selection, and how such a procedure can be used for identification and quantitation at the picomole level.

## Acknowledgment

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