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A capillary liquid chromatographic/tandem mass spectrometric method for the quantification of γ-aminobutyric acid in human plasma and cerebrospinal fluid

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Abstract

A sensitive and reliable method for the determination of γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter, in human plasma and cerebrospinal fluid (CSF) has been developed. The method is based on capillary liquid chromatography (LC)/tandem mass spectrometry (MS/MS) using deuterium-labeled GABA (γ-aminobutyric acid-2,2-D_2, GABA-d_2) as internal standard. Pre-column derivatization with 7-fluoro-4-nitrobenzoxadiazole (NBD-F) was deployed, allowing both effective in-line pre-concentration and sensitive tandem MS detection of the analyte. An extraction column (10 mm × 0.25 mm, 7 μm, C_{18}) was used for preconcentrating and stacking the sample. Separation was carried out on an analytical column (50 mm × 0.25 mm, 5 μm, C_{18}). Characteristic precursor-to-product ion transitions, *m/z* 267 → 249 (for NBD-GABA) and *m/z* 269 → 251 (for NBD-GABA-d_2) were monitered for the quantification. A linear calibration curve from 10 to 250 ng/mL GABA with an r^2 value of 0.9994 was obtained. Detection limit was estimated to be 5.00 ng/mL GABA (S/N = 3). Human plasma and CSF samples were analyzed. The concentrations of GABA were found to be 98.6 ± 33.9 ng/mL (mean ± S.D., $n = 12$), and 44.3 ± 10.0 ng/mL ($n = 6$) in plasma and CSF, respectively.

Keywords: γ-Aminobutyric acid; GABA; Capillary liquid chromatography/tandem mass spectrometry; Cerebral spinal fluid; Plasma

1. Introduction

γ-Aminobutyric acid (GABA) converted from glutamic acid by glutamic acid decarboxylase is a major inhibitory neurotransmitter in the brain [1–2]. A growing number of evidence indicates that abnormal GABA levels in physiological fluids are correlated with various neurological disorders. Elevated GABA levels were observed in cerebral spinal fluid (CSF) in meningitis patients [3] and in plasma in autistic youngsters [4]. On the contrary, low levels of GABA were measured in CSF in patients with Alzheimer's disease [5] and in plasma in patients with bipolar disorder [6] and mood disorders [7]. Recently, low posttrauma GABA plasma levels have been suggested as a predictive factor in the development of acute posttraumatic stress disorder [8].

Various methods have been developed for the determination of GABA. These methods were based on techniques including HPLC [9–12], GC–MS [4,13], CE–MS [14], CE/laser induced fluorescence detection [15], electrochemical sensor [16], spectrophotometry [17]. and HPLC–MS [18]. Most of them, particularly the GC–MS based methods, required time-consuming sample pretreatments such as liquid–liquid or solid phase extraction (SPE). The CE–MS method was intended for the determination of GABA in a living rat brain using in vivo microdialysis. However, the signal was too weak to make any quantitative analysis [14]. Although the HPLC–MS method was used for the determination of GABA in rat brain tissues, it had a detection limit of 2.5 ± 0.3 μg/mL [18], Obviously, this method won't be

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sensitive enough for quantifying GABA in human plasma, in which the GABA level is consistently found to be below 150 ng/mL [4,6,19–21], HPLC coupled to mass spectrometry (MS) has become a popular analytical technique. However, the sensitivity of an HPLC–MS combination is often lackluster compared with fluorescence detection, particularly when standard-size HPLC columns (4 mm i.d.) are used. This problem can be simply caused by sample dilution within the relatively large volume comprised by the column and tubing. Therefore, capillary HPLC–MS hyphenation is gaining research interest.

The aim of this work was to develop a sensitive HPLC–ESI-MS/MS method for the determination of GABA in physiological fluid samples. Capillary HPLC columns (50 mm × 0.25 mm, 5 μm C_{18}) were used for the separation in order to achieve better ESI-MS/MS detection sensitivity. Prior to the separation, in-line pre-concentration and cleaning up of the samples derivatized with 7-fluoro-4-nitrobenzoxadiazole (NBD-F) on an extraction column (10 mm × 0.25 mm, 7 μm C_{18}) was explored.

2. Experimental

2.1. Chemicals and reagents

GABA, Glu, 7-fluoro-4-nitrolenzoxadiazole (NBD-F), trichloroacetic acid (TCA), isopropanol, formic acid, methanol (LC–MS grade), and acetonitrile (LC–MS grade) were from Sigma–Aldrich Chemicals (St. Louis, MO, USA). γ-Aminobutyric acid-2,2-D_2 (GABA-d_2) was obtained from Medical Isotopes (Pelham, NH, USA), which had a purity of 99.5% from our ESI-MS analysis. Milli-Q water was used throughout the work.

2.2. Preparation of capillary HPLC columns

The procedure used for the preparation has been described previously [22]. Two columns were prepared: an extraction column (10 mm × 0.25 mm, 7 μm C_{18} particles) and a separation column (50 mm × 0.250 mm, 5 μm C_{18} particles). Before use, the packed column was washed by methanol and then water (20 min each) at 2500 psi using an HPLC pump run at a constant pressure mode.

2.3. Capillary HPLC–ESI-MS/MS

The capillary HPLC–ESI-MS/MS system consisted of two pumps (LC-10ADvp, Shimadzu, Kyoto, Japan), an on-line degasser (DGU-12A, Shimadzu), and an ion trap mass spectrometer with an ESI source (LCQ Deca, ThermoFinnigan, San Jose, CA). A flow splitter (75 cm × 50 μm i.d.) was used to carry approximately 97% of the mobile phase delivered by the pumps to waste. The flow rate of the mobile phase through the capillary column was measured by means of a graduated 50 μL capillary at the outlet of the ESI source and was adjusted to be at 8 μL/min by changing the pump's flow setting. Mobile phase A was water containing 0.1% formic acid, and mobile phase B was a mixture of acetonitrile and water (15:85, v/v) containing 0.1% formic acid. Isocratic elution was programmed as following: time 0.00–5.00 min, 100% mobile phase A was delivered through the extraction column to pre-concentrate and clean up the sample with the eluent directed to waste; time 5.10–12.00 min, 100% mobile phase B was delivered to elute the analytes from the extraction column and the eluent was directed onto the analytical column for separation and detection via a switching-valve; time 12.0–16.0 min, 100% mobile phase A was delivered to equilibrate the extraction column, the eluent was directed to waste; time 16.1 min, stop. Injections were performed by means of a Rheodyne 8125 injector equipped with a 20 μL sampling loop.

The mass spectrometer was equipped with an electrospray ionization (ESI) source operated in the positive ion mode. Multiple mass spectrometry (MS/MS) experiments were performed to isolate and fragment the targeted ions. The operation conditions of the MS detector were optimized with a solution of NBD-GABA (50 ng/mL GABA) and a flow rate of 10 μL/min. The signal abundance of *m/z* 267 $[M+H]^+$ was maximized as following: sheath gas flow, 40 arbitrary units; auxiliary gas flow, 0 arbitrary units; Spray voltage, 4 kV; heated capillary temperature, 250 °C. Relative collision energy of 30% was used for all MS/MS experiments with an isolation width of 1.0 u. Other parameters were optimized by the autotune program. Data were processed with Xcalibur software.

2.4. Pre-column derivatization with NBD-F

The procedure described previously [22] was followed. Briefly, a sample solution (50 μL) was spiked with the internal standard (GABA-d_2, 5 μL of 500 ng/mL aqueous solution). Borate buffer (100 mM at pH 9.0) and NBD-F solution (10 mM in acetonitrile) were added (25 μL each). The mixture was votexed and heated at 65 °C for 20 min in a dry heating block. After heating, the derivative solutions were cooled down in running tap water and kept at 5 °C till use. Portions (20 μL each) of the derivative solution were injected without further purification. The derivative solution was stable for at least 24 h at 5 °C.

2.5. Preparation of physiological fluid samples

Protocols for the use of human biological fluid samples were approved by Jackson State University Institutional Review Board and the IRB of University of Mississippi Medical Center.

2.5.1. Human CSF

CSF samples were collected at the Charity Hospital Radiology Department (New Orleans, LA, USA) during myelography using a standard protocol, which was described

previously [23]. Briefly, lumbar puncture with aseptic technique was done between 1 and 4 p.m. Cutaneous and subcutaneous injection of 1% lidocaine for local anesthesia preceded puncture with 22- or 20-gauge needle at L2–3 level. The first 3 mL of the CSF sample was sent for laboratory analysis for protein, sugar, chloride, and cells. A second 3 mL CSF sample was used for GABA analysis. Immediately after collection, the samples were placed on ice chips. Within 1 h of collection, the samples were centrifuged (2000 × *g* for 20 min), and the supernatant was stored frozen at −80 °C until analyzed. Portions (50 μL each) of a CSF sample were transferred into 0.3 mL vials. The internal standard, GABA-d_2 (5 μL of 500 ng/mL aqueous solution) was added. The solution was votexed, and then mixed with 25 μL borate buffer (100 mM at 9.0) and 25 μL NBD-F (10 mM in acetonitrile). The solution was heated at 65 °C for 20 min with a dry heating block. After cooling down to room temperature in running tap water, the derivatization solution was centrifuged at 8000 rpm for 10 min at 4 °C. Portions (20 μL each) of the supernatant were injected into the HPLC–MS/MS system.

2.5.2. Human plasma

Blood samples were drawn between 8 and 9 a.m. from healthy people, and placed in polypropylene tubes containing heparin as anticoagulant. Samples were spun down within 1 h of sampling for 10 min at 1500 × *g* to separate plasma. The plasma was then transferred to polypropylene tubes and stored frozen at −80 °C until analysis. Portions (200 μL each) of a plasma sample were transferred into 0.3 mL microcentrifuge vials. The internal standard, GABA-d_2 (10 μL of 2 μg/mL aqueous solution) was added. The mixture was votexed thoroughly. TCA (60 μL, 30%, w/v aqueous solution) was added to deprotein. The solution was let stand on ice for 30 min, and then centrifuged at 8000 rpm for 10 min. Supernatant (50 μL) was transferred to a 0.3 mL vial, and derivatized as described above.

3. Results and discussion

3.1. Loading and preconcentration of the sample

Sample loading is a challenge with capillary or nano-HPLC–MS systems because the flow rate of mobile phases is very low (typically in the range from 500 nL to 10 μL/min). It takes several minutes to load a 20 μL sample, which is problematic if the analytes cannot be stacked on the column. One can overcome this problem by reducing the sample volume. Various nanoliter sample injection valves are commercially available. However, the method sensitivity is significantly compromised with such nanoliter sampling loops. GABA is highly hydrophilic, and it is difficult to retain on a reversed phase column. Precolumn derivatization of GABA with an appropriate reagent can increases its hydrophobicity and molecular size, thus strengthening its retention on the column. *o*-Phthalaldehyde (OPA), a widely used derivatizing reagent for amines and amino acids, was evaluated for the pre-column derivatization in this work. It was found, however, that CID fragmentation of OPA-GABA produced no predominant product ions, which impaired MS/MS detection. Another tagging reagent widely used in HPLC procedures, NBD-F, was then investigated. NBD-F reacted readily with GABA at elevated temperatures. The resultant NBD-GABA derivative was effectively ionized in the ESI source, and CID fragmentation of the $[M+H]^+$ ion *m*/*z* 267 produced one predominant characteristic ion *m*/*z* 249, allowing sensitive MS/MS detection. From the ESI-MS analysis of the derivative solution (Fig. 1A), only the mono-substituted derivative was detected in the *m*/*z* range from 200 to 1000. The structure of NBD-GABA is illustrated in Fig. 1B, which was confirmed by the full-scan MS/MS spectrum of *m*/*z* 267 (Fig. 1C).

To retain effectively NBD-GABA on the extraction column, the composition of the mobile phase for sample loading was investigated. An NBD-GABA derivative solution (20 μL) was injected into the system with mobile phase A as the carrier and eluted out with mobile phase B. The MS detector monitored the eluent during the entire process. Fig. 2 shows the elution profiles. When water was used as the mobile phase A, the retention of NBD-GABA on the column was very weak and thus it was eluted out continuously forming a broad peak (Fig. 2A). Fortunately, it was found that the addition of formic acid (0.1%, v/v) into the mobile phase A greatly improved the retention. This was likely because an increase in solution acidity reduced the acid dissociation of NBD-GABA and thus its water solubility. As can be seen from Fig. 2B, NBD-GABA was well stacked on the extraction column and effectively eluted out by mobile phase B resulting in a narrow and symmetric chromatogram peak. The loading capacity of the extraction column was not fully investigated. However, it was proven to be sufficient for loading a 20 μL sample containing up to 250 ng/mL GABA as described below. In this work, formic acid was selected as an additive in the mobile phases among many commonly used acids such as trifluoroacetic acid in order to avoid ion suppression often encountered in ESI [24]. In an ESI-MS/MS study, two diluted NBD-GABA solutions were prepared by diluting a NBD-GABA derivative solution with water or mobile phase B. The diluted solutions were then infused into the ESI-MS/MS system to measure the signal abundance for the ion transition, *m*/*z* 267 → 249. There were no observable differences in the MS signal intensities obtained from the two solutions.

3.2. GABA-d_2 as internal standard

A stable isotope-labeled internal standard is generally advantageous in any quantitative mass spectrometric methods. It is usually considered to be essential in order to correct for matrix effects. In this work, GABA-d_2 (MW = 105) was chosen as the internal standard because it was commercially available and had been used as internal standard for GABA determination in a GC–MS method [4]. GABA-d_2 and

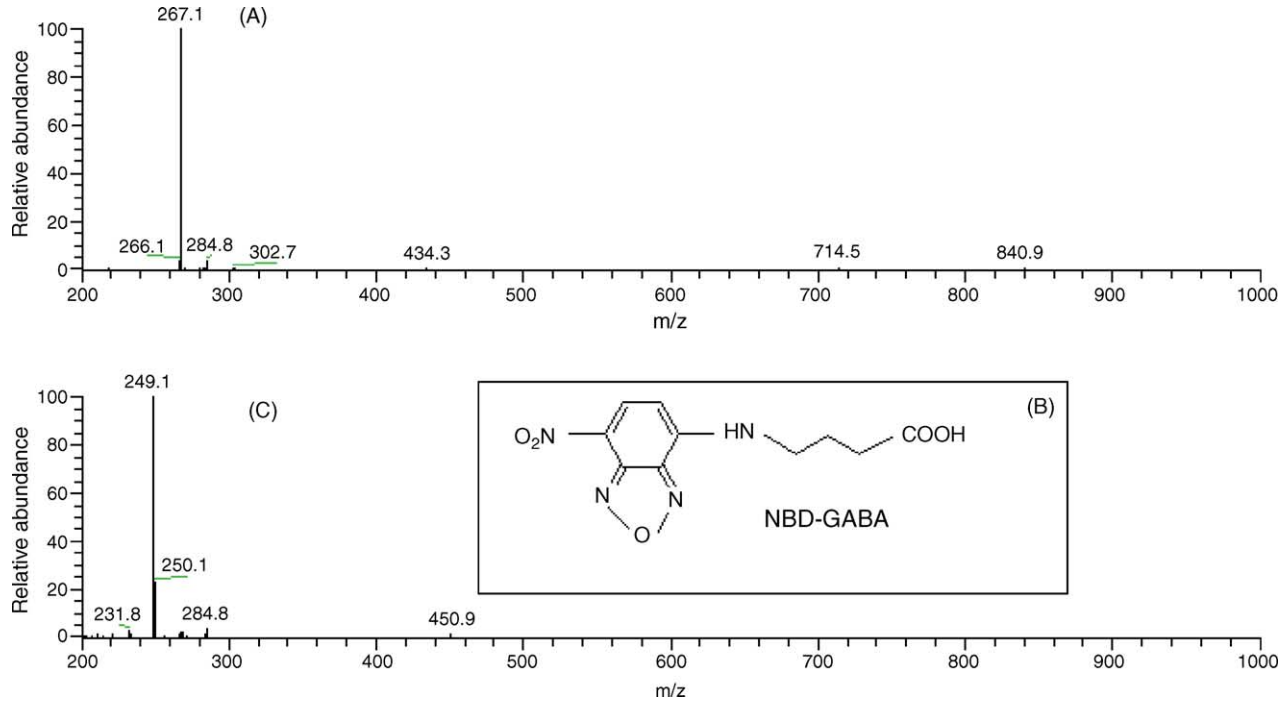

Fig. 1. ESI-MS/MS study of NBD-GABA: (A) ESI mass spectrum of an NBD-GABA derivative solution (100 ng/mL GABA); (B) the chemical structure of NBD-GABA derivative; and (C) full-scan MS/MS spectrum of *m/z* 267 with a collision energy of 30%.

GABA are identical in terms of many chemical properties. They co-elute in the HPLC separation, and have essentially the same ESI characteristics. Therefore, the ratio of signal intensities for analyte/internal standard is independent of the recoveries from chemical processes and the degree of ionization, thus providing a reliable basis for quantitation. This is illustrated by the chromatograms (Fig. 3) obtained from separating a 50 ng/mL GABA standard solution spiked with GABA-d_2 at 50 ng/mL. From Fig. 3A, it can be seen that GABA and GABA-d_2 were co-eluted. The MS/MS detection had very similar response factor to the transitions *m/z* 267–249 for NBD-GABA and *m/z* 269 → 251 for NBD-GABA-d_2 as shown in Fig. 3B and C. The MS/MS spectra (Fig. 3D and E) confirmed the peak identifications for GABA and GABA-d_2, respectively. Ideally, when a stable isotope labeled internal standard is used, it should be more than 3 mass units higher than the analyte to avoid natural isotopic contribution from the unlabeled analyte. However, in this work the internal standard (i.e. GABA-d_2) is only 2 mass units higher than the analyte (i.e. GABA). Therefore, a close examination

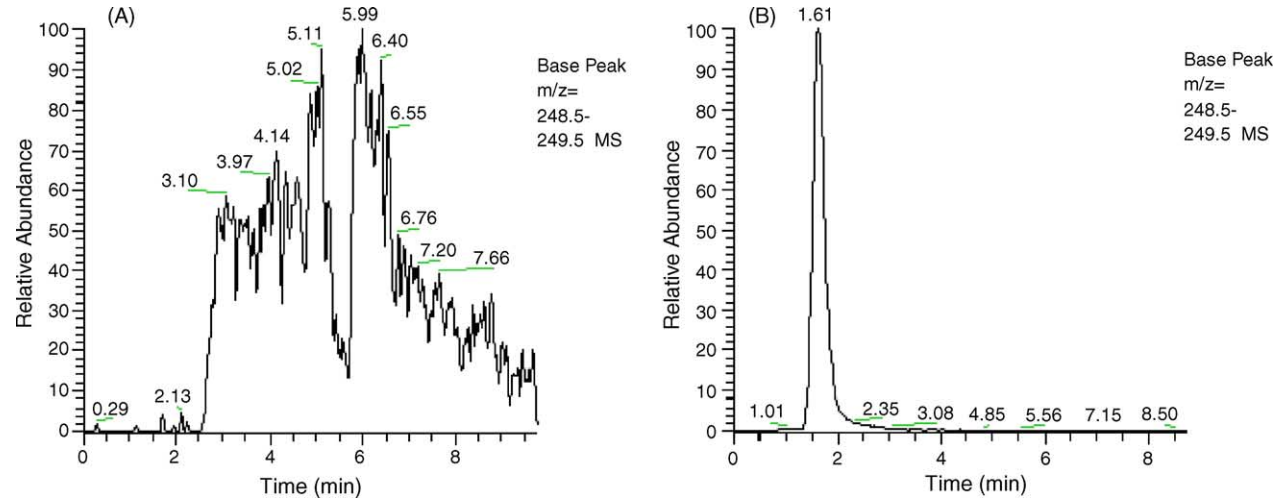

Fig. 2. Stacking NBD-GABA on the extraction column: (A) water was used as mobile phase A for sample loading and (B) water containing 0.1% formic acid was used.

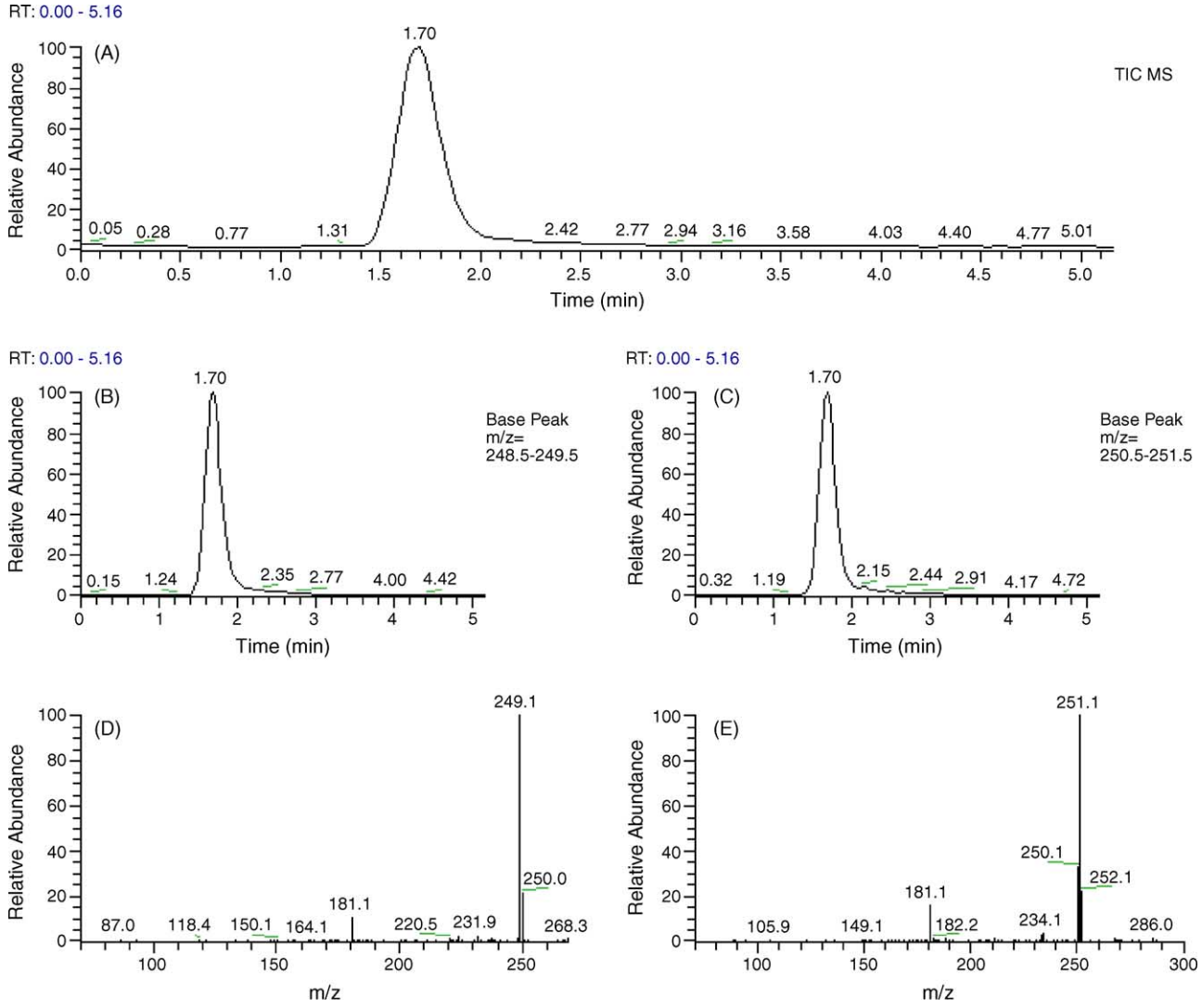

Fig. 3. Evaluation of GABA-d_2 as internal standard: (A) TIC of *m/z* 267 and *m/z* 269 from separating a mixture of GABA and GABA-d_2; (B) extracted mass chromatogram of *m/z* 267 → 249 from (A); (C) extracted mass chromatogram of *m/z* 269 → 251 from (A); (D) full-scan MS/MS spectrum of *m/z* 267; and (E) full-scan MS/MS spectrum of *m/z* 269.

on the contribution of GABA to the GABA-d_2 signal or vice versa was made. Six NBD-GABA/NBD-GABA-d_2 mixtures containing 0, 25, 50, 100, 250, 500 ng/mL GABA, respectively, and 100 ng/mL GABA-d_2 were prepared with mobile phase B (15% acetonitrile in water with 0.1% formic acid). These solutions were infused into the MS detector at 5 μL/min with a syringe pump. The peak heights for the ion transition, *m/z* 269 → 251 of NBD-GABA-d_2 were measured from each solution. The measurements were repeated five times. Results were evaluated using *Paired t*-tests. It was found that there were no significant differences among the means of peak heights at a confidence level of 99%, which suggested that the contribution of GABA to the GABA-d_2 signal was insignificant under the selected MS detection conditions. This result was further confirmed by the very good linearity of the calibration curve ($r^2 = 0.9994$) described below.

3.3. Analytical figures of merit

Using the *m/z* 267 → 249 for NBD-GABA and *m/z* 269 → 251 for NBD-GABA-d_2, GABA was quantified by means of the signal ratio of analyte to internal standard. A 5-point calibration curve was prepared with authentic GABA solutions at concentrations ranging from 10 ng/mL to 250 ng/mL while keeping GABA-d_2 cone, constant at 50 ng/mL. Peak heights were used for the quantification. Linear regression analysis of the results yielded the following equation:

$$Y = 0.0237X - 0.189, \qquad r^2 = 0.9994$$

where Y is the ratio of the peak height for GABA to that for GABA-d_2, X is the GABA concentration in ng/mL, and r^2 is the correlation coefficient. Interday (5 days) precisions of the slope and intercept of the calibration curve were found

Table 1
Method precision and accuracy

GABA added (ng/mL)	Measured mean (ng/mL)	Accuracy (%)	Precision (%RSD)
Intraday ($n = 6$)			
Pooled plasma			
0	97.3		3.6
25	121.3	96	4.2
50	148.6	102.6	2.3
100	197.5	100.2	1.8
200	295.1	98.9	2.7
Pooled CSF			
0	44.2		2.9
25	68.7	98	2.2
50	93.5	98.6	3.1
100	144.0	99.8	1.5
200	247.7	101.8	2.0
Interday ($n = 6$ in 5 days)			
Pooled plasma			
0	96.0		5.6
25	123.1	108.4	3.6
50	147.4	102.8	7.2
100	193.2	97.2	6.1
200	299.0	101.5	4.7
Pooled CSF			
0	46.8		4.3
25	71.4	98.4	6.2
50	98.1	102.6	3.9
100	149.9	103.1	5.5
200	241.6	97.4	2.8

to be 1.7 and 3.4% (R.S.D., $n = 5$), respectively. From the calibration curve, the limit of detection was estimated to be 5 ng/mL GABA (signal/noise = 3).

To determine precision and accuracy, a pooled human plasma and a CFS sample ($n = 4$ in both cases) were spiked with GABA at varying concentrations. Six replicate analyses were carried out on these samples. The results are summarized in Table 1. Intraday precision (R.S.D.) ranged from 1.8 to 4.2% for plasma assay and 1.5 to 3.1% for CSF assay, respectively. Intraday accuracy (percent of expected values) ranged from 96 to 103% for plasma assay and 98 to 102% for CSF assay, respectively. Between-run (5 days) precision was found to be in the range from 3.6 to 7.2% (plasma assay) and 2.8 to 6.2% (CSF assay). The accuracy was in the range from 97 to 108% for plasma assay and 98 to 103% for CSF assay, respectively. Recovery of GABA from human plasma and CSF was also investigated. GABA-d_2 was added to a pooled plasma and a CSF sample ($n = 4$), and water at three concentrations, i.e. 25, 100, and 200 ng/mL. The samples were then derivatized and analyzed as described above. Recovery was calculated by comparing the GABA-d_2 peak height obtained from a plasma or CSF sample with the peak height from the corresponding GABA-d_2 water solution. The recoveries were found to be $20.1 \pm 0.5\%$ and $57.7 \pm 1.4\%$ from plasma and CSF, respectively. They were very low, indicating the sample matrix effects were sever. Therefore, stable isotope dilution must be used in this work. From the results of method accuracy studies shown above, the matrix effects have been satisfactorily taken care of by using GABA-d_2 as an internal standard.

3.4. Determination of GABA in human plasma and cerebrospinal fluid samples

GABA level in human plasma has been extensively studied, particularly in psychiatry-related researches. However, the results reported so far are inconsistent likely due to the distinct performances of analytical methods used for the studies. For example, plasma GABA level in healthy human subjects was found to be in the range from 12.5 to 18.8 ng/mL by using an HPLC method with fluorescence detection [19,21]. However, in a recent study the level was reported to be 126.9 ng/mL with a modified HPLC/fluorescence detection method [6]. The huge difference was noted by the authors, but not explained. Using the present capillary HPLC–MS/MS method, twelve plasma samples taken from healthy subjects were analyzed. Typical chromatograms from such an analysis are shown in Fig. 4A–C. The peaks corresponding to GABA and GABA-d_2 were well defined. GABA in the plasma sample could be reliably determined based on the peak height ratio of these two peaks. The analytical results are summarized in Table 2. From these analyses, plasma GABA level in healthy subjects was found to be 98.6 ± 33.9 ng/mL (mean $\pm$ S.D., $n = 12$), which is statistically in accordance with the result reported recently by Shiah et al. [6] (126.9 ± 63.8 ng/mL, mean $\pm$ S.D., $n = 10$). However, this number is much higher than some other literature values (12.5–18.8 ng/mL) [19–21]. After carefully reading these papers, we found that the measurements of plasma GABA in all of these works were carried out following an HPLC/fluorescence procedure described by Hare and Maynam [25]. It is our postulation that the low GABA results were due to a low recovery of GABA from plasma matrix, which was not corrected for in these measurements. Six human CSF samples were also analyzed using the present method. There was no need to remove proteins from these samples for the analysis. Therefore, the CSF samples were directly derivatized with

Table 2
Analytical results of GABA in human plasma and CSF samples

Sample name	GABA content[a] (ng/mL)	Sample name	GABA content[a] (ng/mL)
Plasma #1	127.2	Plasma #10	127.2
Plasma #2	100.9	Plasma #11	67.3
Plasma #3	83.6	Plasma #12	61.8
Plasma #4	116.3	CSF#1	45.1
Plasma #5	149.0	CSF#2	35.8
Plasma #6	68.2	CSF#3	56.2
Plasma #7	149.1	CSF#4	47.4
Plasma #8	60.9	CSF#5	51.7
Plasma #9	71.8	CSF#6	29.3

[a] Mean of two measurements.

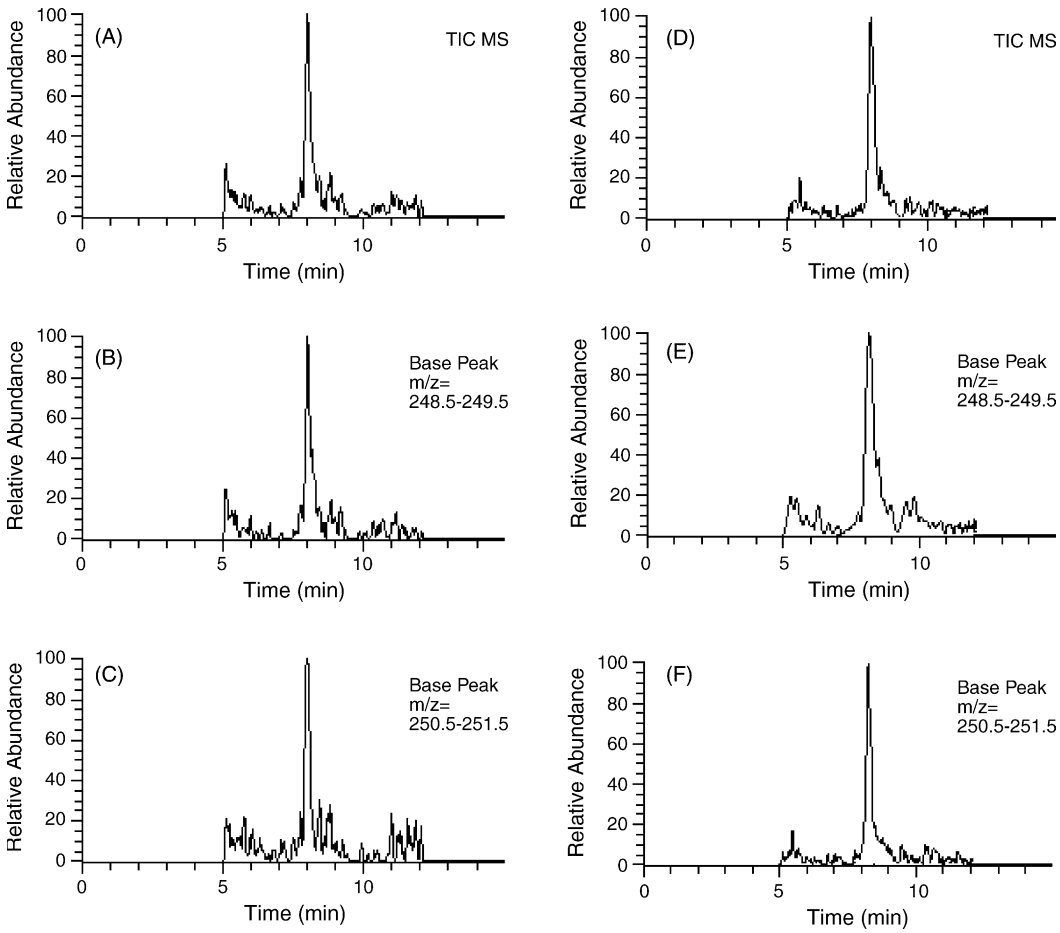

Fig. 4. Capillary HPLC–MS/MS determination of GABA in human plasma and CSF samples: (A) TIC mass chromatogram of *m*/*z* 267 and *m*/*z* 269 from an analysis of a human plasma sample; (B) extracted mass chromatogram of *m*/*z* 267 → 249 from (A); (C) extracted mass chromatogram of *m*/*z* 269 → 251 from (A); (D) TIC mass chromatogram of *m*/*z* 267 and *m*/*z* 269 from an analysis of a human CSF sample; (E) extracted mass chromatogram of *m*/*z* 267 → 249 from (D); and (F) extracted mass chromatogram of *m*/*z* 269 → 251 from (D).

NBD-F after being spiked with the internal standard. Typical chromatograms from the analysis of CSF samples were shown in Fig. 4D–F. The results of GABA content are listed in Table 2. From these analysis, GABA level in human CSF was found to be 44.3 ± 10.0 ng/mL (mean ± S.D., $n = 6$).

4. Conclusions

A sensitive and reliable capillary HPLC–MS/MS method has been developed for the determination of GABA, a major inhibitory neurotransmitter, using GABA-d_2 as internal standard. The present method is ca. 500 times more sensitive than an HPLC–MS method previously reported [18] and, therefore, well suited to quantify trace levels of GABA present in physiological fluids. GABA levels have been found to be 98.6 ± 33.9 ng/mL (mean ± S.D., $n = 12$) and 44.3 ± 10.0 ng/mL ($n = 6$) in human plasma and CSF, respectively.

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