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Liquid chromatography–mass spectrometry assay for quantification of Gluten Exorphin B5 in cerebrospinal fluid

Giuseppe Fanciulli [a,*], Emanuela Azara [b], Troy D. Wood [c], Giuseppe Delitala [a], Mauro Marchetti [b]

[a] *Dipartimento-Struttura Clinica Medica-Patologia Speciale Medica, University of Sassari, Viale San Pietro 8, 07100 Sassari, Italy*
[b] *Istituto di Chimica Biomolecolare, National Research Council (CNR), Sezione di Sassari, Regione Baldinca Li Punti, 07100 Sassari, Italy*
[c] *Department of Chemistry, University at Buffalo, Natural Sciences Complex 417, State University of New York, Buffalo, NY 14260-3000, USA*

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Abstract

A sensitive, precise and accurate method for the quantification of the alimentary opioid peptide Gluten Exorphin B5 (GE-B5, Tyr-Gly-Gly-Trp-Leu) in cerebrospinal fluid (CSF) was developed using liquid chromatography–mass spectrometry (LC–MS). Aliquots (10 μL) of sheep CSF were injected into a LC–MS instrument equipped with a reversed-phase C12 column at a flow rate of 250 μL/min. The mobile phase consisted of Eluent A water with 0.01% acetic acid as an ion-pairing reagent, and Eluent B acetonitrile. The LC–MS system was programmed to divert column flow to waste for 3.5 min after injection, after which time flow was directed into the mass spectrometer that operated in positive ion mode. DADLE (Tyr-D-Ala-Gly-Phe-D-Leu) was used as Internal Standard. No significant interfering peaks were detected at the retention times of GE-B5 in CSF blanks. The calibration curves were linear in the range of 0.39–78.00 ng/mL. The lower limit of detection and the lower limit of quantitation values for GE-B5 in CSF were established at 0.30 and 0.78 ng/mL, respectively. The intra-day and inter-day precision values were <12% relative standard deviation. The intra-day and inter-day accuracy were 99.46–100.86% and 98.95–100.02%, respectively. Recovery of GE-B5 in CSF samples was greater than 80%. Stability studies indicate that GE-B5 in CSF undergoes significant degradation (>55% after 600 min), which is reduced by the addition of protease inhibitors. This is the first reported method for the quantification of GE-B5 in CSF.

Keywords: Cerebrospinal fluid; Gluten Exorphin B5; Liquid chromatography–mass spectrometry

1. Introduction

Gluten Exorphins (GEs) are a family of opioid peptides identified in enzymatic digests of wheat gluten [1,2]. It has been hypothesized that GEs are released at the intestinal level [1], penetrate abnormally permeable intestinal membranes and enter the blood plasma, eventually reaching the central nervous system (CNS) where they affect behaviour ("opioid excess theory") [3].

GE-B5 (Tyr-Gly-Gly-Trp-Leu) (Fig. 1) is probably the most biologically significant peptide among the family of GEs, due to its potent in vitro opioid activity [1] and its ability to modify neurotransmission in vivo in animal models [4,5]. Moreover, the recent detection of GE-B5 in blood samples [6] indicates that this peptide can indeed permeate, at least in subjects with compromised intestinal permeability, the intestinal barrier while remaining structurally intact. However, whether GE-B5 is able to cross also the blood-brain barrier (BBB) is not known. Studies on the ability of GE-B5 to cross the BBB will require a method for its quantitation in cerebrospinal fluid (CSF). Therefore, we have developed a liquid chromatography (LC)–mass spectrometry (MS) quantitative assay for this peptide in CSF.

2. Experimental

2.1. Samples

Twenty samples of sheep CSF (5 mL each) from a tissue bank at the National Research Council (CNR) of Sassari, Italy, were used. The samples were pooled and then stored at −20 °C.

* Corresponding author at: Istituto di Clinica Medica, University of Sassari, Viale S. Pietro 8, 07100 Sassari, Italy. Tel.: +39 079 229019; fax: +39 02 700 532 950.
E-mail address: gfanciu@uniss.it (G. Fanciulli).

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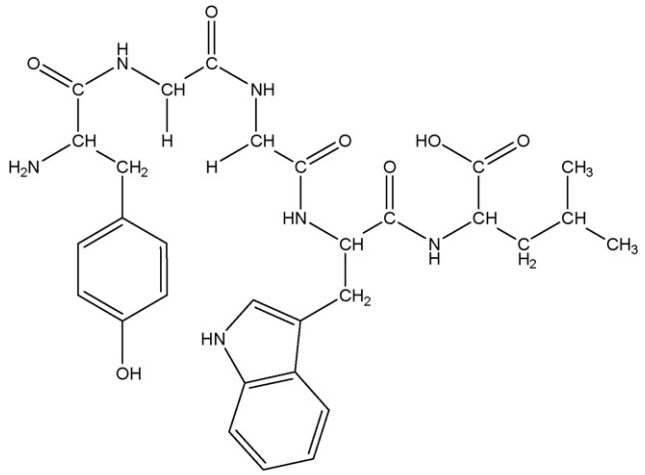

Fig. 1. Chemical structure of Gluten Exorphin B5.

2.2. Materials

GE-B5 (peptide free base, MW 594.6) and the Internal Standard (IS) DADLE (Tyr-D-Ala-Gly-Phe-D-Leu; peptide free base, MW 569.3) were obtained from Bachem Chimica (Milan, Italy). Both peptides were >99% pure. LC-grade methanol, acetonitrile, trifluoracetic acid (TFA), formic acid and acetic acid were procured from Mallinckrodt J.T. Baker (Deventer, Holland). Water was purified by a Milli-Q Academic System from Millipore (Bedford, MA, USA). Syringe filters (surfactant free cellulose acetate 0.2 μm 0.13 mm) were obtained from Nalgene Company (Rochester, NY, USA). The protease inhibitor (protease inhibitor cocktail complete, PIC) was obtained from Roche Diagnostics (Mannheim, Germany).

2.3. LC system

An Agilent Technologies (Palo Alto, CA, USA) 1100 series LC/MSD equipped with a diode-array detector and an autosampler (G1313A) was used for LC separation. A ChemStation HP A.10.02 was used for data analysis. Chromatographic separation was achieved using a Jupiter Proteo C12 (150 mm × 2.0 mm, 4 μm, 90 Å) (Phenomenex, Torrance, CA, USA) fitted with a 3 μm Jupiter Proteo C12 security guard cartridge (4 mm × 2.0 mm). The column temperature was maintained at 30 °C. The mobile phase consisted of Eluent A, water with 0.01% acetic acid, and Eluent B, acetonitrile. The solvents were filtered through a 0.45 μm membrane prior to use. The separation was performed under gradient conditions with a flow rate of 250 μL/min and a run time of 15 min, and was followed by clean-up and equilibration stage (Table 1). The injection volume was 10 μL. The guard column was replaced after 100 injections, and the LC system was rinsed daily with 10 mL 100% methanol.

2.4. MS set up

Mass spectrometric detection was performed using an Agilent G1946 (MSD 1100) single stage quadrupole instrument equipped with an electrospray atmospheric pressure ionization (ES-API) source. The system was calibrated with the procedures provided by Agilent; the mass spectrometer was optimized with an infusion of 0.39 μg/mL GE-B5 at a flow rate of 100 μL/min. The LC–MS system was programmed to divert column flow to waste for 3.5 min after injection, after which time flow was directed into the mass spectrometer that operated in positive ion mode. For quantitative measurement of GE-B5, selected ion monitoring (SIM) was employed. In the ES-API ion source, GE-B5 formed predominantly (95%) the protonated molecule $[M+H]^+$ at *m/z* 595.6. DADLE was also efficiently ionized to form its $[M+H]^+$ ion at *m/z* 570.3 (Fig. 2). The following ES-API conditions were applied: drying gas (nitrogen) heated at 350 °C at a flow rate of 9.5 L/min; nebulizer gas (nitrogen) at a pressure of 42 psi; capillary voltage in positive mode at 3600 V; fragmentor voltage at 92 V; dwell time was 460 ms.

Table 1
Chromatographic gradient program

Time (min)	Eluent A (%)	Eluent B (%)	Flow (μL/min)
0	87	13	250
11	65	35	250
13	40	60	250

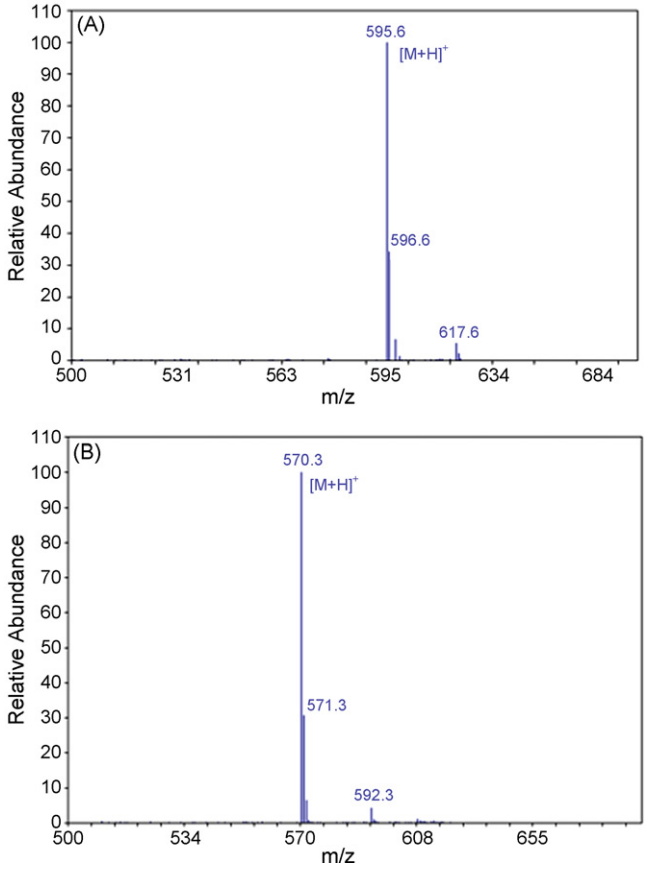

Fig. 2. Full scan mass spectra of (A) Gluten Exorphin B5 0.39 μg/mL and (B) Tyr-D-Ala-Gly-Phe-D-Leu (DADLE) 0.49 μg/mL.

2.5. Standard solutions

Stock solutions of GE-B5 were prepared by dissolving 3.9 mg of GE-B5 in 10 mL of methanol (final concentration 390 μg/mL). Stock solutions of DADLE were prepared by dissolving 4.9 mg of DADLE in 10 mL of methanol (final concentration 490 μg/mL). Stock solutions of GE-B5 and DADLE were stored at −20 °C in high-density polypropylene cryovials.

Working solutions of GE-B5 were prepared daily at the concentration of 0.78 μg/mL by diluting (1:500) aliquots of the stock solutions with the solvent system (Eluent A) and were used to spike samples. Working solutions of DADLE were prepared daily at the concentration of 0.49 μg/mL by diluting (1:1000) aliquots of the stock solutions with the solvent system and were used to spike samples. Stock solutions of PIC were prepared by dissolving one tablet in 1.5 mL Milli-Q water.

2.6. Standard curves

Six different concentrations of GE-B5 (0.39, 0.78, 1.95, 3.90, 19.50 and 78.00 ng/mL) were obtained by adding appropriate concentrations of working solutions in CSF and solvent system. These solutions were used to construct standard curves.

2.7. Quality control (QC) samples

QC samples were prepared by fortifying CSF blanks with know quantities of the GE-B5 to obtain final concentrations of GE-B5 representative of the standard curve range (0.78, 3.90, and 39.00 ng/mL), and were stored at −20 °C.

2.8. Sample preparation

Aliquots of 80 μL of CSF standards, blank, and QC samples were added to 300 μL limited volume autosampler vials, and 4 μL of IS working solution were added and the resultant solution was vortexed for 5 s. A 10 μL aliquot of the resulting solution was then injected directly into the LC–MS system.

2.9. Assay calibration

Calibration curves were produced by plotting peak area ratio of the analyte to the IS from the single ion chromatogram against the analyte's concentration ratio. The linear regression was fitted to the concentration range 0.39–78.00 ng/mL.

2.10. Sensitivity

The lower limit of detection (LLOD) and the lower limit of quantitation (LLOQ) were determined by analyzing twenty replicates of CSF blank samples. LLOD and LLOQ were, respectively, defined as three and ten times the standard deviation (S.D.) of the LC–MS baseline noise detected at the retention time of the GE-B5.

2.11. Assay precision and accuracy

Intra-day and inter-day precision and accuracy of the assay were assessed by performing replicate analyses of QC samples at the nominal concentrations 0.78, 3.90, and 39.00 ng/mL. Intra-day repeatability was determined by analyzing spiked samples in replicate ($n = 5$) the same day. The procedure was repeated in 5 consecutive days to determine inter-day repeatability. The precision was expressed as relative standard deviation of the samples' replicates over their mean values at each concentration. The accuracy was evaluated as [mean found concentration/nominal concentration] × 100.

2.12. Matrix effect

A recovery study was conducted to investigate possible matrix effect on this assay. Three sets of standards, within the concentration range of 0.78–39.00 ng/mL, were prepared in CSF and in the solvent system. Recovery was calculated by the following equation: % recovery = peak area slope of CSF standard curve/peak area slope of solvent system standard curve.

2.13. Stability

The stability tests were designed to cover the conditions that the samples may experience during storage and freeze-thaw. Stability of GE-B5 in CSF, CSF plus PIC (10 μL of PIC stock solution/mL of CSF), and the solvent system was evaluated in samples spiked with concentrations of 1.95 and 19.50 ng/mL. After thawing, samples were kept at room temperature and analyzed at regular intervals (120, 240, 360, 480 and 600 min). Stability of GE-B5 in CSF during three freeze-thaw cycles was also evaluated.

3. Results

3.1. ESI-MS optimization

Since mobile phase composition may strongly influence both LC separation and MS sensitivity, in the course of method development we have investigated the effects of different mobile phase additives (acetic acid 0.005%, acetic acid 0.01%, acetic acid 0.1%, formic acid 0.01%, and TFA 0.01%) on the signal suppression of the GE-B5 (Fig. 3). In order to achieve optimal MS sensitivity, acetic acid 0.01% was chosen over the other modifiers because it provided the highest MS response.

3.2. Specificity

The nominal retention times for GE-B5 and DADLE were 10.4 and 12.8 min, respectively. Throughout this study we did not detect any significant interfering peaks at the retention times of GE-B5 or the IS in twenty different CSF blanks (i.e. no false positives). Fig. 4 illustrates a typical SIM chromatogram obtained from CSF fortified with GE-B5, CSF fortified with DADLE, and CSF blank. A single column (with the above

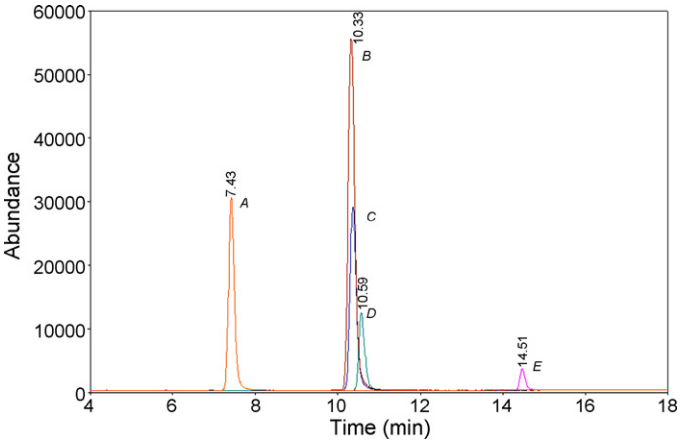

Fig. 3. Gluten Exorphin B5 peak height obtained with different organic acids: (A) acetic acid 0.005%, (B) acetic acid 0.01%, (C) acetic acid 0.1% (D) formic acid 0.01% (E) trifluoracetic acid 0.01%.

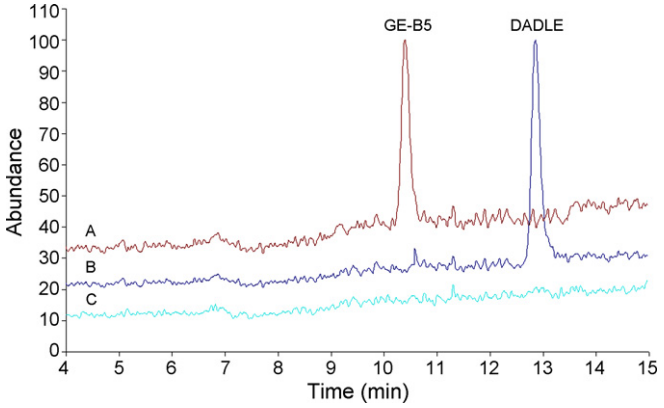

Fig. 4. Selected ion monitoring chromatogram obtained from (A) Gluten Exorphin B5 (GE-B5) 0.78 ng/mL in cerebrospinal fluid (CSF), (B) Tyr-D-Ala-Gly-Phe-D-Leu (DADLE) 0.49 ng/mL in CSF, and (C) CSF blank.

noted guard column replacements and daily methanol rinses) was employed for the duration of this study (6 months).

3.3. Linearity and sensitivity

Regression was linear over the tested concentration range (0.39–78.00 ng/mL), with an average correlation coefficient, R^2, of 0.998 (0.001 S.D.), calculated from five calibration curves. The average slope and intercept were 1.043 (0.039 S.D.) and −0.704 (0.135 S.D.), respectively. The "lack-of-fit" test showed no significant deviation from linearity ($p = 0.13$). The LLOD and LLOQ values for GE-B5 in CSF were established at 0.30 and 0.78 ng/mL, respectively, and the absolute sensitivity of GE-B5 injected on column were 3.0 and 7.8 pg. These absolute values for GE-B5 are approximately of the same order of magnitude of those observed for Gluten Exorphin A5 (GE-A5) [7].

Table 2
Intra-day and inter-day precision and accuracy results for Gluten Exorphin B5 (GE-B5) ($n = 5$)

Nominal concentrations of GE-B5 (ng/mL)	Precision (%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.78	1.11	1.07	99.46	98.95
3.90	7.99	6.30	100.86	99.23
39.00	8.91	11.61	100.08	100.02

Table 3
Recovery of Gluten Exorphin B5 (GE-B5) in cerebrospinal fluid ($n = 15$)

Nominal concentrations of GE-B5 (ng/mL)	Recovery (%) mean ± S.D.
GE-B5 0.78	80.14 ± 0.01
GE-B5 3.90	83.46 ± 0.09
GE-B5 39.00	84.35 ± 0.04

S.D. = standard deviation.

3.4. Assay precision and accuracy

Intra-day and inter-day precision and accuracy results are presented in Table 2. Intra-day and inter-day precision values were <9 and 12%, respectively. Intra-day and inter-day accuracy ranged from 99.46 to 100.86% and from 98.95 to 100.02%, respectively.

3.5. Matrix effect

Results of the comparison of solvent system standards versus CSF standards indicated that the recovery of GE-B5 in CSF

Table 4
Stability of Gluten Exorphin B5 (GE-B5) ($n = 4$)

Nominal concentrations of GE-B5	Concentration at time 0	Concentration at time 120	Concentration at time 240	Concentration at time 360	Concentration at time 480	Concentration at time 600
Stability in CSF						
I Level (1.95 ng/mL)	1.95 ± 0.02	1.70 ± 0.11	1.38 ± 0.14	1.07 ± 0.08	0.89 ± 0.09	0.78 ± 0.10
II Level (19.50 ng/mL)	19.50 ± 0.06	17.05 ± 0.75	15.95 ± 0.18	10.71 ± 0.66	9.61 ± 0.92	8.20 ± 0.49
Stability in CSF (with PIC)						
I Level (1.95 ng/mL)	1.95 ± 0.01	1.84 ± 0.01	1.83 ± 0.03	1.84 ± 0.03	1.83 ± 0.03	1.86 ± 0.01
II Level (19.50 ng/mL)	19.41 ± 0.08	18.74 ± 0.09	18.27 ± 0.10	18.13 ± 0.13	17.75 ± 0.10	17.96 ± 0.13
Stability in solvents						
I Level (1.95 ng/mL)	1.94 ± 0.01	1.96 ± 0.01	1.95 ± 0.02	1.96 ± 0.03	1.94 ± 0.03	1.97 ± 0.01
II Level (19.50 ng/mL)	19.54 ± 0.34	19.74 ± 0.38	19.07 ± 0.12	19.36 ± 0.28	19.57 ± 0.71	19.66 ± 0.38

Concentrations of GE-B5 at the different times are expressed as mean ± standard deviation; CSF = cerebrospinal fluid; PIC = protease inhibitor cocktail.

samples was greater than 80% (Table 3), similar to recovery of GE-A5 reported previously [7].

3.6. Stability

In CSF samples without PIC, GE-B5 concentrations markedly decreased over time (−29.2 and −54.3% at times 240 and 480 min, respectively, in samples spiked with 1.95 ng/mL. The difference between GE-B5 concentrations at time 0 min and time 600 min (for both starting nominal concentrations of 1.95 and 19.50 ng/mL) was statistically significant ($p < 0.03$, calculated by Wilcoxon-signed rank test). In CSF samples added with PIC, GE-B5 appeared to be stable (−4.6% at time 600 min, in samples spiked with 1.95 ng/mL (Table 4). Finally, after three freeze-thaw cycles GE-B5 concentrations were 3.88 (0.06 S.D.) (starting from nominal concentrations of 3.90 ng/mL), and 38.97 (0.24 S.D.) (starting from nominal concentrations of 39.00 ng/mL).

4. Discussion

In the last 10 years, there has been increasing interest on the possible effects of alimentary opioid peptides on the CNS in animals [8,9] and humans [3,10]. Unfortunately, few analytical methods have addressed whether these peptides can be measured in biological fluids. We have recently developed a method for the quantification of a gluten-derived opioid peptide, GE-A5, in the CSF [7]. In the present work, we attempted to develop another quantitative method, for the assay of a different GE (namely GE-B5).

GE-B5 is very similar in structure to Leu-enkephalin (L-ENK, Tyr-Gly-Gly-Phe-Leu), an endogenous opioid with profound effects on CNS [11,12], differing only at the second amino acid residue. Interestingly, this variation causes, compared to L-ENK, a reduction of GE-B5 opioid activity on the μ and Δ receptors (1.2 and 4.2 times lower, respectively) and opioid affinity (1.2 and 1.7 times lower, respectively) [1]. L-ENK concentrations in mammalian CSF have been estimated to be between 1 to 5 ng/mL, using a LC–MS method [13]. There are no available data on the concentration of GE-B5 in CSF which may cause biologic effects on the CNS; for this reason, we chose our analytical range for GE-B5 (0.39–78.00 ng/mL) of the same order of magnitude to that of its structurally correlated opioid peptide L-ENK. However, the range also included higher concentrations (up to 15 times the upper limit for L-ENK), in view of possible future pharmacokinetic and toxicity studies.

As in the previous studies on GE-A5 [7], we observed a massive reduction over time of GE-B5 concentrations: 240 min after thawing at room temperature, GE-B5 levels in CSF were markedly reduced (approximately −30%); after 480 min, GE-B5 concentration were less than one half of the initial level. Also as observed for GE-A5, protease inhibitors prevented the degradation of GE-B5. These data confirm the extreme susceptibility of GEs to the action of proteases, and highlight an important pre-analytical factor affecting GEs quantification, namely the requirement of adding protease inhibitors before storage of the samples. The absence of GE-B5 in our naïve sample, therefore, could be explained by the lack of pre-treatment with protease inhibitors in the samples stored in our tissue bank. Recently, a study using LC–MS failed to detect opioid peptides in urine samples from autistic children and controls [14]. These authors concluded that the lack of detection of opioids by LC–MS did not support the opioid excess theory of autism; however, in light of these and our previous results showing rapid degradation of exorphins in the absence of protease inhibitor treatment [7], a re-examination of urines treated with protease inhibitors immediately after collection appears warranted.

Matrix effects can decrease quantitative accuracy and precision; the best way to eliminate the influence of matrix effects on the accuracy and precision of a quantitative method is by the use of isotopically labelled IS. However, limited availability and high costs of such standards limit their wide use. In our study we have employed the non-isotopically labelled synthetic opioid peptide DADLE. This peptide has been previously employed as an IS in studies on quantification of opioid peptides in CSF [7,15], and was chosen because of its high stability in biological fluids in the absence of enzymatic inhibitors [16].

5. Conclusions

This is the first method that describes the quantification of GE-B5 in CSF. The LC–MS procedure, that employs extremely small volumes of CSF, has been shown to be sensitive, precise and accurate.

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