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Short communication

Combined method for the determination of γ-aminobutyric and β-alanine in cerebrospinal fluid by stable isotope dilution mass spectrometry

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Abstract

A previously described method for the determination of GABA in CSF has been expanded to include both GABA and β-ALA, using a single GC–MS analysis. A stable isotope labelled internal standard for β-ALA was synthesised to achieve accurate quantification. This new combined method expands the diagnostic power compared to an isolated GABA measurement. Control values for free and total GABA and free and total β-ALA are described. Age <2 years: free GABA 0.017–0.067 μ*M*, total GABA 4.2–13.4 μ*M*; free β-ALA 0.049–0.11 μ*M*, total β-ALA 2.1–4.6 μ*M*. Age >2 years: free GABA 0.032–0.17 μ*M*, total GABA 3.3–12.2 μ*M*; free β-ALA 0.021–0.058 μ*M*, total β-ALA 0.91–3.5 μ*M*.

Keywords: Stable isotope dilution; γ-Aminobutyric acid; β-Alanine

1. Introduction

Measurement of gamma aminobutyric acid (GABA) in cerebrospinal fluid (CSF) plays an important role in the diagnosis of various neurological and mental disorders [1,2]. Next to GABA, β-alanine (β-ALA) also is an important central nervous system metabolite and increased levels are associated with Cohen's syndrome [3]. The very low levels of free GABA and free β-ALA concentrations in CSF makes a sensitive and selective analytical method absolutely necessary. Several methods are described for the detection of GABA in CSF and brain tissue. Palaty et al. [4] described a method whereby GABA was detected as its di(tertiairbutyl(dimethylsilyl) derivative. In this procedure GABA-d6 was used as internal standard. More recently Ma et al. [5] described a successful liquid chromatography–mass spectrometry (LC–MS) application for the detection of underivatised GABA in the brain of rats. The method described by Kok et al. [6] showed that it is possible to measure free and total GABA in CSF, using negative chemical ionisation mass fragmentography combined with the use of a stable isotope labelled internal standard, with high precision and accuracy. We present here an extension of this

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method that allows measurement of both GABA and β-ALA in a single analysis. Sample preparation and derivatisation is the same for both compounds. Quantitation is established using stable isotope labelled internal standards for both GABA and β-ALA.

2. Experimental

2.1. Sample collection

Lumbar CSF samples were frozen immediately after collection in dry-ice and ethanol to avoid hydrolysis of GABA and β-ALA. Prior to analysis, the samples were stored at −30°C in the dark.

2.2. Materials

Methylchloroformate was obtained from Merck (Darmstadt, Germany). Pentafluorobenzyl-bromide was purchased from Pierce (Rockford, USA). Both sodium ^{13}C-cyanide (99% isotopic purity) and $^{2}H_2$-GABA (98% isotopic purity) were from MSD-isotopes (Montreal, Canada). $^{13}C_2$-bromoacetic acid (99% isotopic purity) was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Both 5% Pd/C and Amberlite IRA-68 ion-exchanger came from Fluka (Buchs, Switzerland). The CP-Sil 88 analytical GC-column was purchased from Chrompack (Middelburg, The Netherlands). All other chemicals and reagents were of analytical grade.

2.3. Synthesis of $^{13}C_3$-β-alanine

For the preparation of $^{13}C_3$-β-alanine,$^{13}C_2$-bromoacetic acid was treated with sodium ^{13}C-cyanide to provide $^{13}C_3$-cyanoacetic acid [7], followed by hydrogenation of the latter over 5% Pd/C at ambient pressure [8]. After purification over an Amberlite IRA-68 ion-exchange column, using water as eluent the product was obtained in a 60% overall yield as a white solid. The isotopic purity of $^{13}C_3$-β-alanine was assigned by GC–MS, under conditions described in paragraph 2.5, and found to be >99%.

2.4. Sample preparation

The sample preparation for the combined analysis of GABA and β-ALA is according to the procedure described by Kok et al. [6] with some modifications.

2.4.1. Free GABA and β-ALA sample preparation

To 1000 μl of CSF, 0.2 nmol $^{2}H_2$-GABA and 0.2 nmol $^{13}C_3$-β-ALA were added as internal standards. Eight hundred μl phosphate buffer (1 *M*, pH 11.5) and 50 μl methylchloroformate were then added to form *N*-methylcarbonyl derivatives. The mixture was allowed to react for 10 min at room temperature under continuous shaking. The mixture was acidified using 150 μl 6 *N* HCl and the formed derivatives were then extracted into 4 ml ethylacetate by manual shaking for 1 min. After 2 min of centrifugation the supernatant was transferred to another test tube and blown to dryness with nitrogen at 40°C. Pentafluorobenzyl (PFB) derivatives were formed by treating the residue with 10 μl tri-ethylamine and 100 μl 7% pentafluorobenzyl bromide in acetonitrile (v/v) at room temperature for 15 min. After adding 200 μl 0.5 *N* HCl the formed derivatives were extracted with 1 ml hexane. The hexane fraction was blown to dryness and redissolved in 50 μl hexane. From this final solution 1 μl was used for the GC–MS analysis.

2.4.2. Total GABA and β-ALA sample preparation

To an aliquot of 50 μl of CSF 0.2 nmol $^{2}H_2$-GABA and 0.2 nmol $^{13}C_3$-β-ALA were added as internal standards followed by an addition of 350 μl of water. The solution was acidified by adding 400 μl 6 *N* HCl and heated to 110°C for 4 h to accomplish hydrolysis of the GABA and β-ALA conjugates. After cooling to room temperature, the solution was neutralised by addition of 200 μl 12 *N* NaOH. 800 μl phosphate buffer (1 *M*, pH 11.5) and 50 μl methylchloroformate were then added to form *N*-methylcarbonyl derivatives. From this stage the procedure described for the free GABA and β-ALA sample preparation is followed. Calibration curves were established by carrying various amounts of GABA and β-ALA and constant amounts of internal standards through the entire procedure. The observed ratios of standard versus internal standard were used for linear regression analyses. The concentration in a

unknown sample was calculated by interpolation of the found ratio into the regression line.

2.5. Gas chromatography and mass spectrometry conditions

GC–MS analyses were performed using a Finnigan MAT GCQ gas chromatograph connected to a Finnigan Automass II quadrupole mass spectrometer (San Jose, California, USA). Chromatographic separation was achieved on CP-Sil 88 (25 m×0.32 mm I.D., film thickness 0.25 μm) capillary column coated with a polar phase. Samples were injected splitless at a temperature of 240°C. The initial oven temperature was held for 1 min at 80°C, followed by a ramp of 30°C/min to 240°C. The temperature of the transfer line to the mass spectrometer was set at 240°C. The GC-column was inserted directly into the ion source, which was set at 200°C. Ammonia was used as reagent gas at an optimised gas pressure. The mass spectrometer was operated under electron capture negative chemical ionisation in the single ion monitoring mode. The negative ions measured for GABA were: m/z 160.2 (endogeneous GABA) and m/z 162.2 (internal standard). The negative ions measured for β-ALA were: m/z 146.2 (endogeneous β-ALA) and m/z 149.2 (internal standard).

3. Results

3.1. Negative chemical ionisation mass spectra of GABA and β-ALA

The use of PFB-derivatives under NCI-conditions results in the release of the PFB-group yielding a single $[M\text{-}PFB]^-$ fragment. As can be seen in Fig. 1, the mass spectrum of β-ALA only shows the pseudo molecular $[M\text{-}PFB]^-$ ion; m/z 146.2. In the lower part, the mass spectrum of the labelled internal standard is shown.

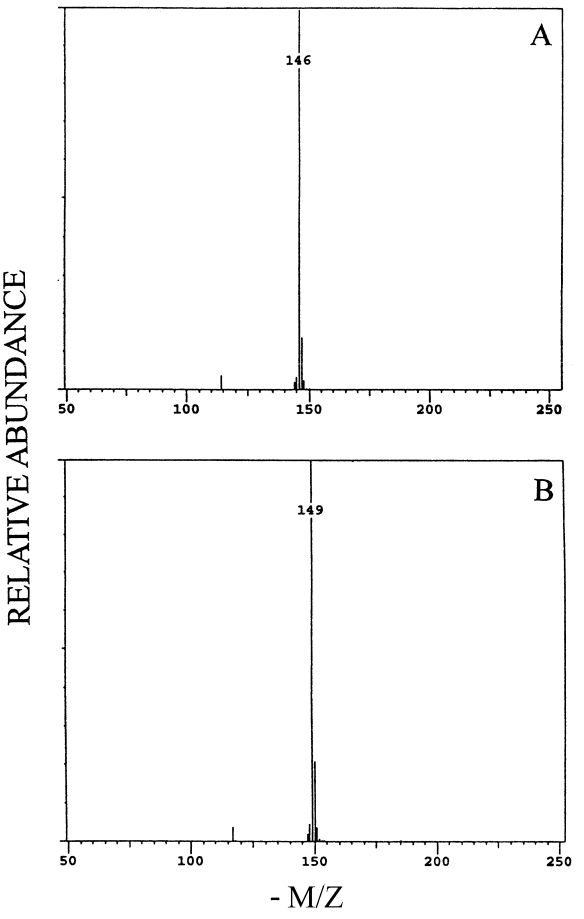

Fig. 1. Negative chemical ionisation mass spectra of the *N*-methylcarbonyl-PFB derivatives of β-ALA (A) and $^{13}C_3$-β-ALA (B).

3.2. Standard curves of free and total GABA and β-ALA

For the free GABA and β-ALA assay, the linearity was determined over a range of 0.02 to 0.2 nmol absolute amount of added standards. The equation of the linear regression analysis of the obtained data for GABA was: $y=(5.30\pm0.02)x+(0.044\pm0.002)$ and for free β-ALA: $y=(5.52\pm0.014)x+(0.079\pm0.001)$. The regression coefficients (r) for free GABA and free β-ALA were in both cases 0.9999. For the total GABA and β-ALA assay the linearity was determined over a range of 0.1 to 1.0 nmol absolute amount of added standards, resulting in $y=(4.06\pm0.20)x+(0.22\pm0.10)$, $r=0.993$, and $y=(4.59\pm0.13)x+(0.18\pm0.06)$, $r=0.998$ respectively. A typical mass fragmentogram of a CSF sample is shown in Fig. 2.

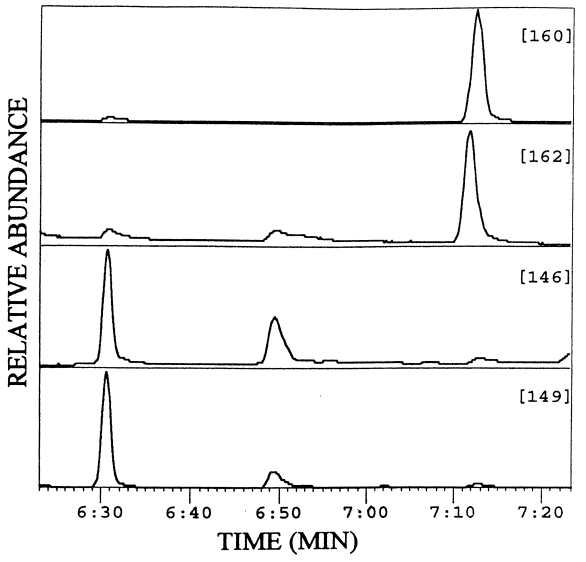

Fig. 2. Typical mass fragmentogram of a CSF sample, T_r β-ALA: 6.3 min, T_r GABA: 7.1 min.

3.3. Inter- and intra-assay analyses of the method

The intra-assay variability was established by the serial analysis of five identical samples prepared at the same time. The inter-assay variability was established by processing and measuring one sample in five independent sample preparations on five different days. The results of the inter- and intra-assay determinations are listed in Table 1.

3.4. Limit of detection and limit of quantification

The lower limit of detection (LOD) ($S/N=5$) of both GABA and β-ALA in the described sample volume is 0.005 μmol/l, whereas the lower limit of quantification (LOQ) ($S/N=10$) is 0.01 μmol/l.

Table 1
Inter- and intra-assay variability of free and total GABA and β-ALA determinations; concentration in μmol/l, relative standard deviation between brackets

	GABA		β-ALA	
	Free	Total	Free	Total
Intra-assay $n=5$	0.22±0.003 (1.5%)	5.7±0.12 (2.1%)	0.12±0.001 (1.1%)	1.6±0.050 (3.4%)
Inter-assay $n=5$	0.22±0.015 (6.6%)	5.9±0.39 (6.5%)	0.11±0.010 (9.0%)	1.7±0.13 (7.9%)

Table 2
Control values of free and total GABA and β-ALA in CSF (μmol/l)

	GABA		β-ALA	
	Free	Total	Free	Total
age <2 years	0.017–0.067 ($n=9$)	4.2–13.4 ($n=10$)	0.049–0.11 ($n=9$)	2.1–4.6 ($n=9$)
age >2 years	0.032–0.17 ($n=13$)	3.3–12.2 ($n=14$)	0.021–0.058 ($n=14$)	0.91–3.5 ($n=13$)

3.5. Control values of GABA and β-ALA

Control values for both GABA and β-ALA are age dependent. Therefore we used two categories; the first group were children aged below two years and the second group were children older than two years. Control values of free GABA and β-ALA are approximately 50 to 100 fold lower than the total GABA and β-ALA values (Table 2).

4. Discussion

The combined method for the measurement of GABA and β-ALA in CSF presented here, has been validated and proved to be successful for diagnostic use [9]. A labelled internal standard, $^{13}C_3$-β-ALA was synthesised to accomplish absolute quantification of free and total β-ALA in CSF. The levels of free β-ALA tend to decrease with an increase of age of the patient, whereas the levels of free GABA tend to increase with an increase of the patient's age. During our research we were faced with problems related to the hydrolysis of total β-ALA resulting in non-reproducable results. The hydrolysis step performed with 20% sulphosalicylic acid for 24 h at 110°C, used for the total GABA determination was replaced by hydrolysis with 6 *N* HCl for 4 h at 110°C. Using this procedure complete hydrolysis was accomplished for both total GABA and total β-ALA, which was beneficial with regards to the time needed for the sample preparation. For more then two years the combined method has been used successfully in our laboratory with reproducable

outcomes for control CSF samples included in our series of patients samples.

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