Journal of Chromatography, 529 (1990) 417–423
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5323

Note

Determination of γ-aminobutyric acid in human cerebrospinal fluid by isocratic high-performance liquid chromatography

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(First received December 28th, 1989; revised manuscript received March 12th, 1990)

It is well known that γ-aminobutyric acid (GABA) is an important inhibitory neurotransmitter in the mammalian nervous system. GABA levels in cerebrospinal fluid (CSF) are considered to be an indicator of GABAergic neuronal functions in the central nervous system [1,2]. A decrease of the GABA level in CSF has been reported in several neurological disorders, as assayed by ion-exchange–fluorimetric and radioreceptor assay methods [3–6].

Automatic amino acid analysers have been used to investigate whether the amounts of amino acids in human CSF are altered in neurological and mental diseases. Owing to the low concentration of GABA in CSF, however, the increases of aspartic acid and glutamic acid, excitatory neurotransmitters, in the CSF from epileptic patients were reported [7,8].

In recent years, the fluorogenic reactions of amino acids with *o*-phthalaldehyde (OPA) and thiol have been exploited in reversed-phase high-perform-

0378-4347/90/$03.50

ance liquid chromatography (HPLC). Several HPLC systems have been used to measure concentrations of amino acids in CSF [9–16]. However, the concentration of GABA in CSF was reported in only some papers, because it is less than 10% of that of the other amino acids in CSF [11–13,15,16].

This paper describes a rapid isocratic HPLC method with fluorimetric detection for the determination of the OPA derivative of GABA in human CSF.

EXPERIMENTAL

Chemicals

4-Amino-*n*-butyric acid (GABA), L-2-amino-*n*-butyric acid, 2-aminoisobutyric acid, DL-3-aminoisobutyric acid, 5-amino-*n*-valeric acid (AVA), L-valine, DL-norvaline (2-amino-*n*-valeric acid) and acetonitrile (HPLC grade) were purchased from Nacalai Tesque (Kyoto, Japan). Citric acid monohydrate, perchloric acid and sodium hydroxide were from Wako (Osaka, Japan). *o*-Phthalaldehyde and 2-mercaptoethanol were from Sigma (St. Louis, MO, U.S.A.). Methanol (HPLC grade) was from Cica-Merck (Tokyo, Japan). Other chemicals were of analytical-reagent grade and were used without further purification.

Chromatographic equipment and procedures

The HPLC system consisted of a solvent-delivery pump (Model 635A, Hitachi, Tokyo, Japan), a manual injector (Model 635-0575, Hitachi) and a fluorescence spectrophotometer (Model 650-10LC, Hitachi). Elution was carried out at room temperature with a reversed-phase column (Wakosil 5C18, 250 mm × 4.6 mm I.D., Wako, Osaka, Japan) and a mobile phase of 0.1 *M* sodium citrate (pH 3.50)–acetonitrile–methanol (59:32:9, v/v) at a flow-rate of 1.5 ml/min. The fluorescence detector was set at 360 and 440 nm for excitation and emission wavelengths, respectively, and slit widths were 5 nm for both.

Sample preparation and precolumn derivatization

The CSF samples were obtained from neurologically normal patients by lumbar puncture. These samples were judged normal in terms of cell counts and total protein. CSF was also obtained from ten patients with spinocerebellar degeneration who were diagnosed on the basis of clinical signs as progressive cerebellar ataxia, extrapyramidal disorder and autonomic dysfunction and a computed-tomographic study. The CSF samples were kept frozen at −80°C until assayed for GABA concentration.

Prior to the HPLC analysis, the CSF samples (40 μl) were supplied with 10 μl of AVA (20 pmol) as an internal standard and were treated with 5 μl of 2 *M* perchloric acid. The mixture was centrifuged at 10 000 *g* for 10 min, and the supernatant was collected.

To an OPA solution (10 mg per 200 μl of methanol), 800 μl of 0.4 *M* borate

buffer (pH 10.5) and 20 μl of 2-mercaptoethanol were added. The OPA reagent was prepared before each series of experiments because of the instability [17]. Exactly 2 min before the injection on the column, equal volumes (25–50 μl in a standard procedure) of the OPA reagent and the sample (authentic GABA and AVA solutions or CSF samples after their treatment) were mixed and incubated at 25 °C. Quantitation was based on the measurement of the ratio of the fluorescence intensity of the GABA peak to that of AVA, the internal standard.

Analysis of data

Results were expressed as mean ± S.D. and were statistically evaluated using the analysis of variance and Student's *t*-test: $P < 0.05$ was considered to be significant.

RESULTS

As shown in Fig. 1, structurally related amino acids (C_{4-5}) were separated from GABA, although not all of these amino acids are present in human CSF. The retention times of GABA and AVA were 10 and 13 min, respectively. When solutions of pH above or below 3.50 were used as the mobile phase, the peak of 3-aminoisobutyric acid overlapped with that of GABA. This amino acid is reported to exist in mammalian tissues. We also examined Leu, Ile, Phe, Tyr, Trp, Thr, Ser, Asp, Asn, Glu, His, Arg, Lys, Met, Cys and taurine, but they did not interfere with the determination of GABA.

The reproducibility of the method was studied by injecting 22 samples of 20 pmol each of GABA and AVA. The coefficients of variation (C.V.) were 3.0%. This reproducibility covers both the precolumn derivatization procedures with

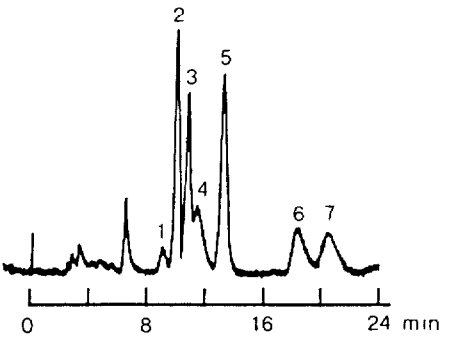

Fig. 1. Separation of GABA. Samples containing GABA and C_{4-5} amino acids (20 pmol each) in 25 μl of 0.2 *M* perchloric acid were derivatized with 25 μl of OPA reagent and injected onto the reversed-phase column. Peaks: 1 = 2-aminoisobutyric acid; 2 = GABA; 3 = 3-aminoisobutyric acid; 4 = 2-amino-*n*-butyric acid; 5 = AVA; 6 = valine; 7 = norvaline.

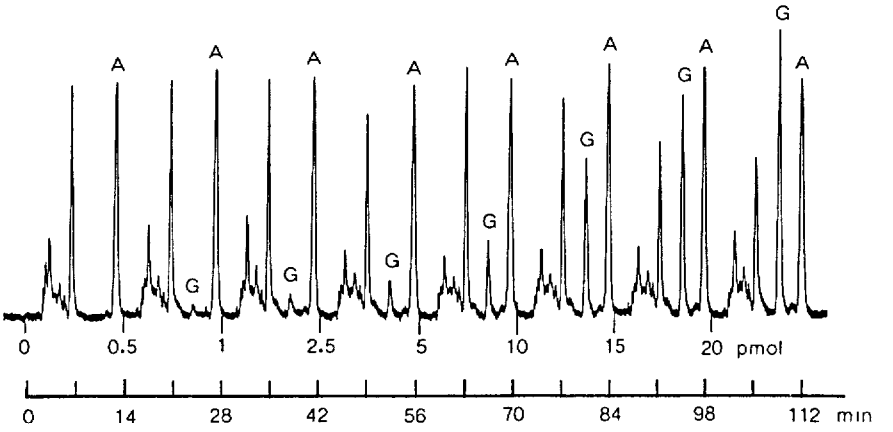

Fig. 2. Chromatogram of standard GABA and AVA. Standard samples containing 0–20 pmol of GABA and 20 pmol of AVA were derivatized with OPA reagent. Exactly 2 min after the reaction, the mixture was injected at 14-min intervals. GABA and AVA were eluted at 10 and 13 min, respectively. Peaks: G = GABA; A = AVA.

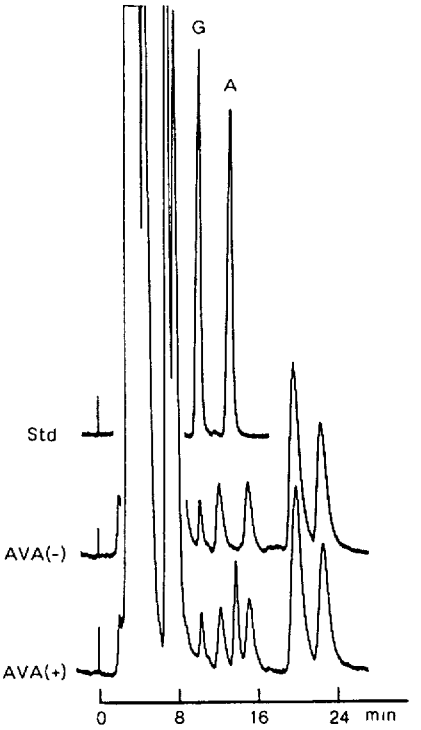

Fig. 3. Chromatograms of standard sample (Std) and human CSF with or without AVA [AVA(+) or AVA(−)]. Deproteinized human CSF (50 μl), after the addition of 0 or 15 pmol of AVA, was derivatized with the OPA reagent (50 μl) and injected onto the HPLC column. A standard sample containing authentic GABA (40 pmol) and AVA (40 pmol) was also analysed. The concentration of GABA was calculated to be 104 pmol/ml. Peaks: G = GABA; A = AVA.

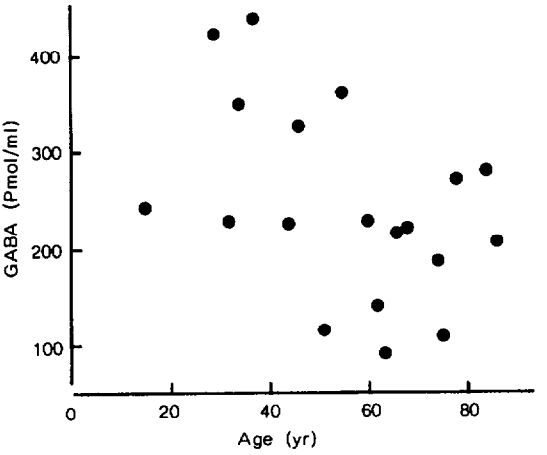

Fig. 4. Concentrations of GABA in human CSF. The CSF samples were obtained from neurologically normal patients and analysed as described in Experimental. GABA levels (y) in CSF samples were plotted as a function of the age (x). The linear regression equation is $y=-2.2x+368$ (correlation coefficient $=-0.440$; $P>0.05$).

OPA and the separation on the HPLC column because of the instability of OPA derivatives.

Fig. 2 shows a chromatogram of authentic GABA (0–20 pmol) and AVA (20 pmol). The peak height of GABA seemed to correspond to the injected amount of GABA. However, when the peak heights of GABA (y) were plotted as a function of the injected amounts (x) without correction, the linear regression equation and correlation coefficient between them were $y=1.780x+0.540$ and 0.9988, respectively. This correlation was improved when the peak heights of GABA were corrected with the corresponding peak heights of AVA. The linear regression equation and correlation coefficient between the corrected peak heights (y) and the amounts (x) of GABA were $y=1.820x+0.279$ and 0.9995, respectively. These results indicate that AVA is a suitable internal standard for the determination of GABA content.

Fig. 3 shows typical chromatograms for the determination of GABA in human CSF samples containing 0 or 15 pmol of AVA as an internal standard [AVA(−) or AVA(+)]. The peak heights of GABA in the CSF sample were the same, regardless of the addition of AVA. In the CSF sample without AVA [AVA(−)], no peak was observed at 13 min, where AVA is eluted. The analyses of human CSF samples can be repeated at 30-min intervals, because of the late-eluting hydrophobic amino acids. The lower limit of detection was found to be 0.5 pmol, allowing the detection of 10 pmol/ml GABA in human CSF. The overall recovery of added AVA was $96.6\pm5.6\%$ (mean ± S.D., $n=30$), including the reproducibility of precolumn derivatizations. Both the within-

day and the day-to-day C.V. of GABA in separate determinations on pooled CSF were less than 3% ($n=4$ and 5, respectively).

Concentrations of GABA in human CSF from neurologically normal patients ($n=19$) ranged from 92 to 443 pmol/ml. The mean and S.D. were 246 ± 96 pmol/ml. As shown in Fig. 4, although the CSF levels of GABA had a tendency to decrease with age, this was not statistically significant ($P>0.05$). There was also no significant difference between GABA concentrations in CSF from female (213 ± 85 pmol/ml, $n=8$) and male (269 ± 101 pmol/ml, $n=11$) subjects. The GABA levels in CSF obtained from patients with spinocerebellar degeneration were 132 ± 43 pmol/ml ($n=10$), which is significantly different ($P<0.05$) from those of normal controls matched by age and sex (186 ± 54 pmol/ml, $n=10$).

DISCUSSION

Although several systems have been used to determine amino acid concentrations in CSF [3,9–16], the content of GABA was not always detected. These systems aimed to separate all amino acids on a reversed-phase or ion-exchange column with a gradient of organic solvent (methanol or acetonitrile). It appears to be difficult to measure the GABA levels in CSF with these systems because of the low concentration of GABA and the presence of substances with similar properties to GABA (Fig. 1). In the chromatograms obtained with these systems for the determination of amino acid levels in CSF, the GABA peak was asymmetric or overlapped the other peaks, owing to incomplete separation from unknown substances.

On the other hand, HPLC with fluorescence detection was reported to measure the GABA content in rat brain tissue [18]. This method was validated by comparison with capillary gas chromatography and mass spectrometry. However, the amount of GABA in rat brain is 227.2 ± 11.1 $\mu g/g$ wet weight, which is 10^4 times higher than that in human CSF. Although we tried to adopt this method to measure the GABA content in human CSF, the GABA peak could not be separated from the peak of 3-aminoisobutyric acid.

We developed a highly sensitive, rapid and simple HPLC method for determination of GABA in human CSF by an isocratic mobile phase with fluorimetric detection of the corresponding OPA derivative. In order to measure the GABA level in human CSF, it is necessary to separate it completely from the structurally related substances, because the GABA level in CSF is less than 10% of that of the other amino acids [11–13,15,16]. Although only GABA can be measured by this method, the other amino acid levels in CSF are detectable by any automatic amino acid analysers [7,8].

The normal level of GABA in human CSF was found to be 246 ± 96 pmol/ml. This value was in good agreement with previous data: 225 ± 39 pmol/ml by the radioreceptor assay [2] and 239 ± 76 pmol/ml by the ion-exchange–fluor-

imetric method [4]. The GABA level was significantly reduced to 132 ± 43 pmol/ml in CSF from patients with spinocerebellar degeneration. In this disease, the degeneration of Purkinje cells in the cerebellum is suggested by pathological examinations. Since Purkinje cells contain GABA as an inhibitory neurotransmitter, the reduced content of GABA in CSF may reflect the decreased function of those cells. Therefore, the rapid HPLC method for determination of GABA in CSF may be a diagnostic procedure for some neurological disorders.

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