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Reference values of neuroactive amino acids in the cerebrospinal fluid by high-performance liquid chromatography with electrochemical and fluorescence detection

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Abstract

Sampling and HPLC analysis procedures for CSF amino acid determinations were evaluated. In order to increase sensitivity, a precolumn derivatization of amino acids by o-phthalaldehyde–mercaptoethanol reagent was used. By using fluorimetric and electrochemical detection in series, positive peak identification can be obtained in a single chromatographic run. It is recommended to analyze freshly collected CSF. Amino acids are stable for short periods over a wide range of temperature, but storage at −80°C is recommended. The CSF samples for the calculation of the reference values were taken from 40 healthy subjects, hospitalized for lumbar disk herniation, placed on the same diet and kept drug-free for at least 1 week. The mean values (μmol/l) and the ranges (in parentheses) were: 0.27 (0.09–0.63), 0.62 (0.18–1.15), 5.32 (3.05–11.50), 6.16 (2.90–13.30), 0.16 (0.03–0.22) for aspartic acid, glutamic acid, glycine, taurine and γ-aminobutyric acid respectively.

Keywords: Amino acids; Neurotransmitters

1. Introduction

Free amino acids (AAs) are widely distributed within the central nervous system (CNS) and play an important role as neurotransmitters or neuromodulators. Since the content of free AAs in cerebrospinal fluid (CSF) has been demonstrated to correlate with brain AA content [1], reliable measurement of CSF AA levels is a potentially useful tool for investigating CNS function. It has been shown that abnormalities in AA metabolism are involved in the physiopathology of several neuropsychiatric disorders. Changes in levels of AAs with known excitatory function, e.g., aspartate (ASP) and glutamate (GLU), or putative inhibitory function, e.g., gamma-aminobutyric acid (GABA), glycine (GLY) and taurine (TAU), have been reported in cases of acute neuronal lesions, e.g., stroke [2], trauma [3] and hypoglycemia [4], viral diseases like AIDS [5] and also in chronic neurodegenerative disorders such as Alzheimer's dementia [6], Parkinson's [7] and Huntington's [8] diseases, amyotrophic lateral sclerosis [9] and olivopontocerebellar atrophy [10]. Such alterations have also been found in cases

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of epilepsy [11], schizophrenia [12], and migraine attacks [13].

Detection of CSF AA levels has been performed by several methods that use precolumn derivatization and high-performance liquid chromatography (HPLC). These methods are suitable for AA level determinations in the CSF, after deproteinization with acids or organic solvents, but results are discordant among various researchers. These discrepancies in the literature are probably related to different approaches in measuring CSF AA levels: for example, up to 100-fold differences have been reported for GLU levels [14].

Different analytical methods, lack of systematic evaluation of basic parameters (e.g., age, sex and severity of disease) or inappropriate CSF sample storage could also explain these conflicting results.

The aim of this work was to develop a highly sensitive chromatographic method for GABA, ASP, GLU, GLY and TAU determination in the CSF. Furthermore, we attempted to optimize separation parameters, reproducibility of measurements and handling and storage of CSF samples in order to obtain valid reference values.

2. Experimental

2.1. Chemicals and reagents

AAs were purchased from Sigma (St. Louis, MO, USA). All other reagents were the highest analytical grade available and obtained locally. *o*-Phthaldialdehyde reagent (OPA) was prepared and handled according to the method described by Donzanti and Yamamoto [15]. The borate buffer was prepared from 0.1 *M* boric acid solution and adjusted to pH 9.3 with 1 *M* sodium hydroxide.

2.2. Chromatography

The HPLC system consisted of a Bio-Rad 1350 pump equipped with a 7125 Rheodyne valve injector and a 10 μl loop for syringe injecting, coupled to a Coulochem 5100A electochemical detector (ESA Inc., Bedford, MA, USA) with a coulometric analytical cell (Model 5011) and to a Jasco FP-920 fluorescence detector (Jasco Int., Tokyo, Japan) with a 10-μl flow-through cell. A reversed-phase column packed with C_{18} Rosil HL, 150×4.6 mm I.D., 3 μm particle size (Bio-Rad Laboratories, Hercules, CA, USA) was used. The column temperature was set at 37°C. The flow rate was 1.0 ml/min. Electrochemical detection was performed with a potential of +0.15 V and +0.45 V for the first and the second electrode, respectively. The excitation and emission wavelengths for fluorescence detection were 330 and 450 nm, respectively. Peak height was used as a measure of the detector's response.

The AA content was evaluated after comparison with an external AA standard solution. The phosphate buffer, prepared from 25 m*M* potassium phosphate (pH 5.9) and 15% acetonitrile, was applied as the mobile phase.

2.3. Subjects

Forty healthy subjects [23 men and 17 women, mean age 49.7±9.6 years (SD), age range 20–60 years], hospitalized for lumbar disk herniation were studied. All patients were placed on the same diet, and kept drug-free for at least 1 week. None of them had a history of severe head injury, drug abuse, or somatic or psychiatric diseases, and the routine CSF analysis was normal.

2.4. CSF collection

Four-ml aliquots of CSF were freshly collected by lumbar puncture in sterile tubes. The samples were centrifuged at 3000×*g* for 10 min at +4°C within 10 min after sampling. Then 0.2 ml of each CSF sample were immediately processed.

2.5. Sample preparation

The CSF samples were deproteinized with 10% trichloroacetic acid (TCA) (1:0.2, v/v). The resulting mixture was kept at 0°C for 15 min and then centifuged at 3000 g for 10 min at 4°C. 60 μl of the clear supernatant was adjusted to pH 9.3 with 30 μl of 0.2 *M* sodium bicarbonate and then 20 μl OPA were added to the supernatant. After a 1-min incuba-

tion at room temperature, 10 μl of this reaction mixture were injected into the chromatograph.

2.6. Sample stability

Samples from 10 different subjects were used to compare stability of the neuroactive AAs under various conditions of handling and storage. Each CSF sample was split into several aliquots which were processed in different ways and analyzed at different times. A first pair of aliquots of the freshly collected sample was processed and analyzed as follows:

(a) one was deproteinized and analyzed within 10 min after collection;
(b) one was incubated for 8 hours at room temperature, then deproteinized and analyzed.

A second pair of aliquots was immediately deproteinized and stored at −20°C and −80°C, respectively.

A third pair was immediately stored untreated at −20°C and −80°C, respectively.

2.7. Reproducibility

To verify the method's reproducibility, an AA standard mixture was analyzed 10 times in one day. Then, other series were performed on aliquots of a CSF sample pool and examined under two different conditions:

(a) 10 aliquots were analyzed consecutively (within-run).
(b) 10 aliquots were analyzed on different days (between-run). In this case, the CSF sample pool was stored at −80°C between runs.

2.8. Statistical analysis

Wilcoxon signed rank test was used for statistical evaluation of deproteinized and non-deproteinized samples. The significance of gender difference and linearity data were analyzed by Student's t-test. The results were expressed as means, standard deviations and ranges. Probability (p)<0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Chromatographic separation

Using a simple isocratic elution method, it was possible to separate the OPA derivatives of ASP, GLU, GLY, TAU and GABA in 55 min. Fig. 1 shows a chromatogram obtained by injection of a standard AA mixture and a CSF sample, respectively. Other identified compounds, such as glutamine (GLN) [retention time (RT)=11.00], asparagine (ASG) (RT=7.83), histidine (HIS) (RT=9.72), citrulline (CIT) (RT=14.63), threonine (THR) (RT=15.90) and arginine (ARG) (RT=18.52) were found not interfering. Severe criteria for choice of several parameters, e.g., mobile phase ionic strength, pH, organic solvent concentration and column temperature, must be used for a suitable chromatographic separation. By using a mobile phase at pH 5.9±0.02, the resolution and sensitivity for ASP and GLU are improved. At this pH value ASP and GLU were well separated from both solvent front and other interfering peaks. Furthermore, at this pH, the retention time of TAU was reduced and consequently the total running time became shorter.

The resolution of several AAs is strongly influenced by the PH value of the mobile phase. Fig. 2 shows a chromatogram obtained using a mobile phase at pH 5.4±0.02. Under this chromatographic condition, the GLU peak was entrapped between the ASG and the GLN peaks, and co-eluted with an unknown interfering peak.

The resolution of GLY and CIT is a common problem in reversed-phase separation of AAs. GLY was resolved from CIT using a lower mobile phase ionic strength (25 mM) and operating the analytical column at 37°C. The increase of organic solvent concentration influences the elution of both GABA and TAU. Chromatographic separation of GABA, easily detectable in tissue samples and in animal brain dialysates collected with the microdyalisis technique, is rather difficult to obtain in CSF samples, given the greater complexity of the CSF matrix. We achieved the best result by increasing the percentage of acetonitrile in the mobile phase up to 20–25%, although an overlapping peak was found for GLY–THR under these conditions.

To avoid possible contamination or sample

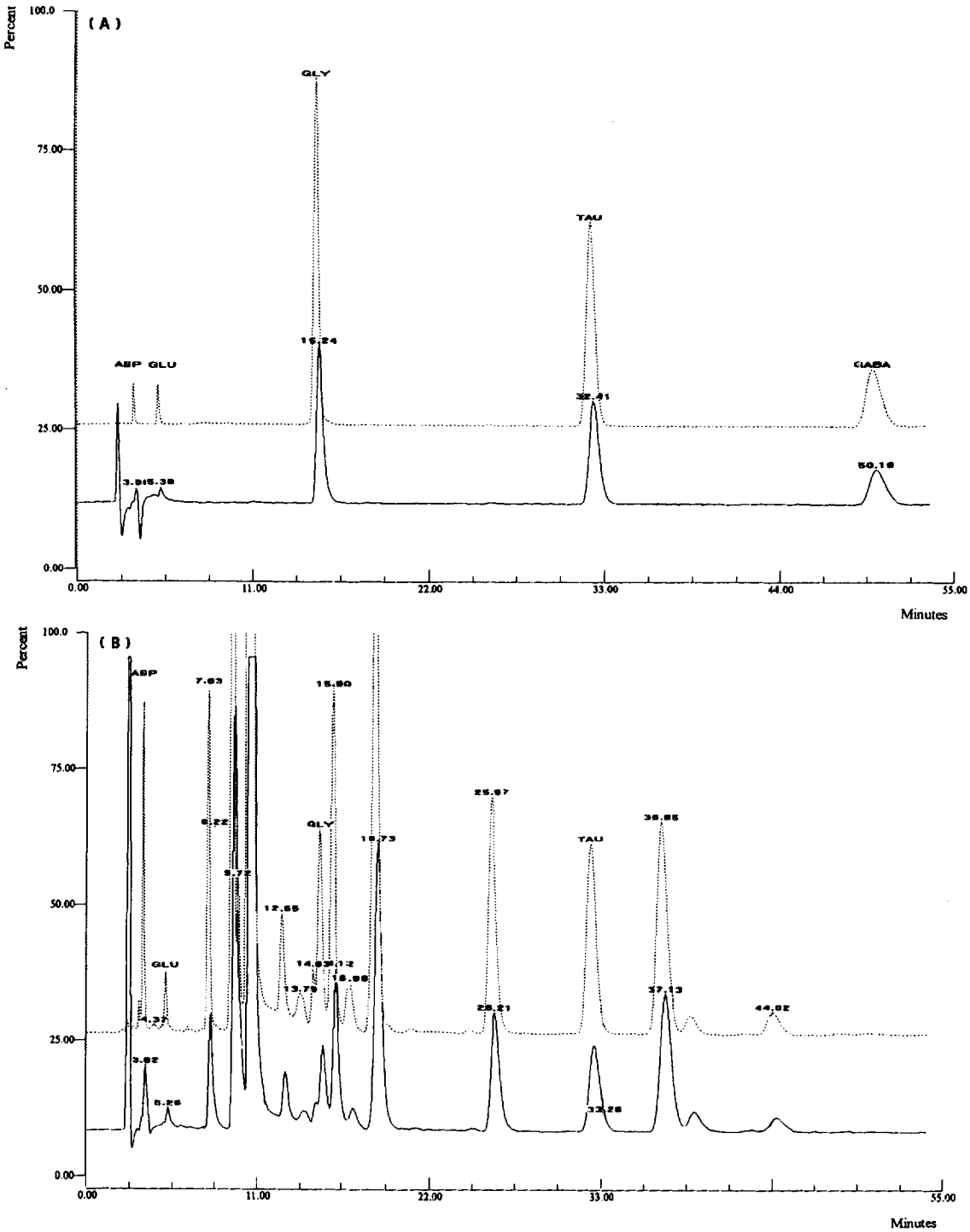

Fig. 1. Separation of ASP (RT=3′86″), GLU (RT=5′38″), GLY (RT=15′24″), TAU (RT=32′41″) and GABA (RT=50′19″) in standard mixture (A) and in CSF sample (B). Solid line=electrochemical response (250 nA), dashed line=fluorimetric response (100 mV×1). Separation was obtained on a RoSil C_{18}, 3 μm column, using a mobile phase at pH 5.9±0.02. Fluorescence intensity was monitored at an excitation wavelength of 330 nm and an emission wavelength of 450 nm. Electrochemical detector conditions were: detector 1 at +0.15 V, detector 2 at +0.35 V. The standard mixture contained 8.00 μmol/l each of ASP and GLU, 13.00 μmol/l of GLY, 7.30 μmol/l of TAU and 9.7 μmol/l of GABA. CSF sample contained the following concentrations: ASP: 6.11 μmol/l, GLU: 0.81 μmol/l, GLY: 6.02 μmol/l, TAU: 6.18 μmol/l, GABA not detectable.

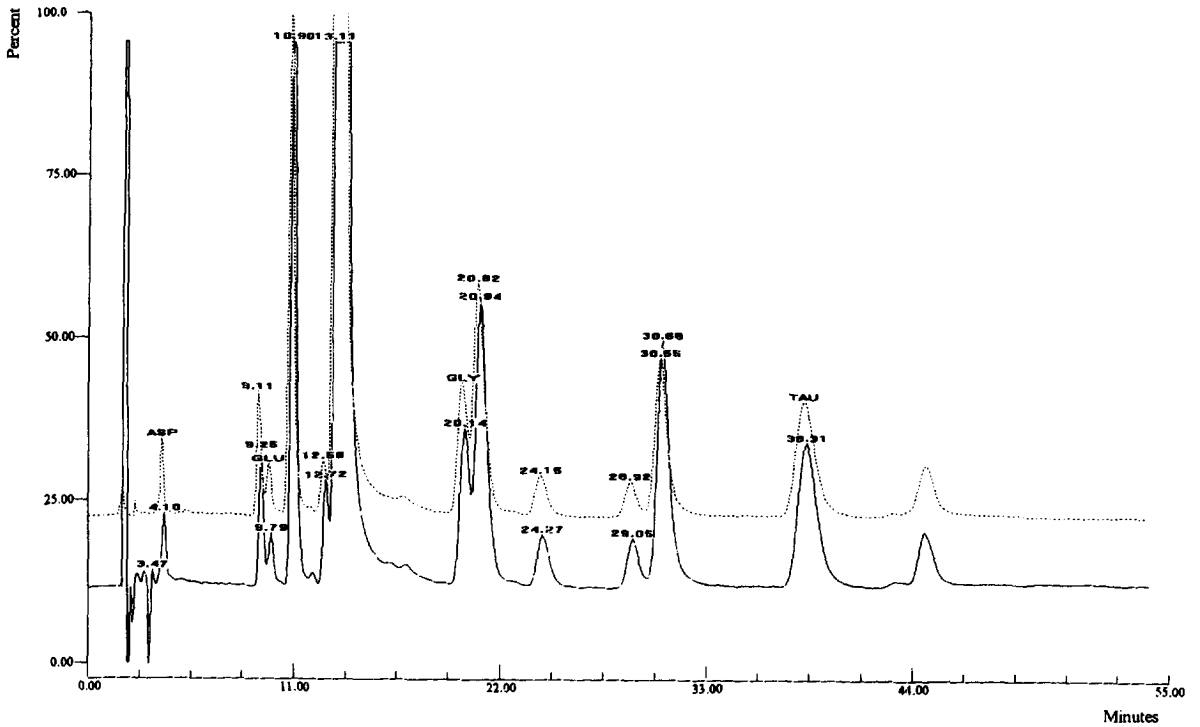

Fig. 2. Chromatograms of a CSF sample. Solid line=electrochemical response (250 nA), dashed line=fluorimetric response (100 mV×1). Separation of ASP (RT=4′10″), GLU (RT=9′79″), GLY(RT=20′14″), TAU (RT=30′31″), GABA (not detectable), has been obtained by using a mobile phase at pH 5.4±0.02. Column as in Fig. 1.

carryover between injections, great care was taken in cleaning the injection loop with methanol and deionized water (1:1, v/v). Following the above mentioned procedures, an adequate resolution was obtained for the peaks of interest and no problems were encountered with late eluting peaks.

3.2. Linearity and reproducibility

Correlation between peak height and AA concentration was examined over the range 0.1–6 μmol/l. Linear regression analysis of the peak height versus concentration of each AA gave a coefficient of regression greater than 0.999 in all cases.

The analytical performance was evaluated by performing ten consecutive injections of a five-component AA standard solution (1 μmol/l) which showed a coefficient of variation (CV) between 1.8% and 2.5% for the different AAs. The reproducibility was evaluated with a pooled CSF sample containing 0.87 μmol/l of ASP, 0.62 μmol/l of GLU, 13.00 μmol/l of GLY, 4.27 μmol/l of TAU and 0.38 μmol/l of GABA. The CSF sample was examined under two different conditions:1) one sample was split into 10 portions which were analyzed consecutively, with a mean CV of 2.06% (within-run).2) one sample was split into 10 portions which were analyzed consecutively on different days and with different standards, with a CV of 3.57% (between-run).

Complete (97–100%) and linear recoveries were obtained for all AAs in the range from 10 to 5000 ng/ml of standard mixture added.

Absolute detection limits (amount giving a signal twice the peak-to-peak noise level) was 60, 74, 76, 178 and 210 ftmol for GABA, ASP, GLU, GLY and TAU, respectively.

3.3. Accuracy

Specificity of the isocratic separation was evaluated using fluorimetric and electrochemical detection in series. The choice of the voltage used for the working electrodes was based on the "current/voltage plot" relative to ASP and GLU, two AAs at lower concentrations in CSF.

The excitation wavelength was varied in steps from 320 to 360 nm. Within this range the fluorescence responses of the OPA derivatives of individual AAs varied less than 10%. Thus 330 nm was considered satisfactory.

Chromatograms in Fig. 1 shows that each peak on the fluorescence plot, joins a peak on the electrochemical plot, if setting the working electrode at +0.35 V. At this lower potential, only the isoindolic OPA derivatives were active, in contrast to the indoles and phenols which required a potential of +0.5–0.6 V.

Since the fluorimetric and electrochemical peak heights do not co-vary across different AAs, the ratio of fluorescent to electrochemical signal, at a given potential, can be used as a tool for a positive identification of AAs in a single chromatographic run. Table 1 shows the good correlation of the ratio obtained for standard AAs and for the AAs in the CSF sample.

3.4. Deproteinization

CSF samples can be injected directly into the column without any clean-up procedures if they are diluted enough prior to derivatization. However, the dilution step itself may introduce contaminants which interfere at subpicomole sensitivity levels and may produce incomplete derivatization. Moreover, after a few runs, strongly adsorbed compounds accumulated on the top of the column, affecting precision and accuracy of chromatographic performance. To minimize these drawbacks, in previous studies ultrafiltration [16], hydrolysis procedures [17], sulphosalicylic acid [18], perchloric acid [19], TCA [20], or organic solvents such as ethanol [21] and acetonitrile [22] were used to precipitate proteins. However, there is no agreement among the data obtained after deproteinization by different procedures. We evaluated the effects of several precipitating agents on the levels of free AAs in CSF. The use of acid agents results in an overestimate of free GLU and TAU (up to 5 and 2 times, respectively), while no differences were observed on GABA, ASP and GLY concentrations. GABA, GLY and TAU were stable in organic solvents, despite the high degradation of ASP and GLU (more than 20% in a few hours).

From the above results, TCA was chosen as the most suitable sample protein remover (more than 99%). Stable AAs were obtained in a protein-free supernatant using low amounts of TCA.

Table 1
Ratios of fluorescent (Fl) to electrochemical (Ec) signal for neuro active AAs in a standard mixture and in CSF

Amino acid	*Fl/Ec ratio	
	Standard	CSF
Aspartate	5.00	5.00
Glutamate	3.00	3.10
Glycine	2.04	2.06
Taurine	1.70	1.72
GABA	0.50	0.50

*Fl/Ec ratio is the ratio of peak height on the fluorimetric detector at 100 mV to that on the electrochemical detector at +0.35 V, f.s.d.=250 nA.

3.5. Sample stability

As illustrated in Table 2, no significant changes were found in AA levels of CSF after 8 hours storage at room temperature compared to "$t=0$", except for ASP showing a significant increase ($p<0.05$ Wilcoxon's test).

The results in Table 3 show the stability of CSF AAs after one month under different conditions of storage and handling. GLY and ASP are significantly increased in acid-treated and in untreated samples, respectively, when stored at −20°C for 30 days ($p<0.05$ Wilcoxon's test). Due to the tendency for increase amino acid values at higher temperature, a storage at −80°C is suggested.

3.6. Reference values of AAs in CSF

Reference values for each AA are depicted in Table 4. No significant age-related changes in CSF levels of AAs were found, whereas females showed

Table 2
Stability of CSF neuroactive AAs stored at room temperature

Amino acid	Storage time $t=0$		Storage time $t=8$ h	
	Mean±S.D.	Range	Mean±S.D.	Range
Aspartate	0.08±0.01	0.06–0.12	0.12±0.02*	0.06–0.18
Glutamate	0.58±0.24	0.20–0.45	0.49±0.26	0.24–0.78
Glycine	5.00±1.12	3.59–6.70	4.87±1.21	3.70–7.30
Taurine	6.18±2.05	3.59–9.49	6.04±1.84	3.57–8.50
GABA	0.20±0.02	0.08–0.49	0.19±0.01	0.08–0.28

*$p<0.05$ vs $t=0$. Values are expressed in μmol/l. Number of samples=10.

Table 3
Stability of CSF neuroactive AAs under various conditions of storage

Amino acid	Native $t=0$	Untreated		Deproteinized	
		30 days at −20°C	30 days at−80°C	30 days at −20°C	30 days at −80°C
Aspartate	0. 11±0.06	0.14±0.05*	0.11±0.05	0.13±0.04	0.13±0.04
	(0.07–0.28)	(0.08–0.30)	(0.13–0.16)	(0.08–0.23)	(0.11–0.30)
Glutamate	0.59±0.28	0.62±0.30	0.61±0.32	0.65±0.30	0.62±0.25
	(0.20–1.15)	(0.23–1.19)	(0.22–1.08)	(0.26–1.18)	(0.23–0.98)
Glycine	5.60±1.17	5.62±1.53	6.00±1.62	6.23±1.30*	6.04±1.12
	(3.59–6.99)	(3.99–7.99)	(3.95–9.15)	(3.95–7.88)	(4.31–7.36)
Taurine	6.5±1.78	6.54±1.50	6.46±2.17	6.21±1.87	6.07±1.46
	(5.21–9.48)	(4.92–8.42)	(3.65–9.47)	(3.94–9.90)	(4.62–8.13)
GABA	0.17±0.05	0.15±0.06	0.17±0.06	0.21±0.08	0.18±0.05
	(0.05–0.21)	(0.09–0.33)	(0.10–0.35)	(0.10–0.24)	(0.10–0.35)

*$p<0.05$ vs $t=0$. Values are expressed as means, μmol/l±standard deviations. Ranges, μmol/l, are in parentheses. Number of samples=10.

a significantly higher level of ASP ($p<0.05$ Student's t-test) when compared with males.

4. Conclusion

We have reported a chromatographic characterization of the AA profile in CSF. We have also documented methodological parameters which can influence HPLC analysis of AAs after precolumn derivatization with OPA. Particularly, we suggested that the choice of the analytical methodology is the primarily responsible for the discrepancies among the published data. The procedures described here provide a specific, reproducible, sensitive and linear method for AA analysis of CSF. In addition, electrochemical detection coupled with fluorimetric detection of OPA derivatives offers improved versatility and specificity.

The concentration of the AAs in CSF were

Table 4
Reference values of CSF neuroactive AAs

Amino acid	Females		Males	
	Mean±S.D.	Range	Mean±S.D.	Range
Aspartate	0.37±0.16*	0.09–0.63	0.26±0.14	0.09–0.61
Glutamate	0.72±0.23	0.29–1.08	0.65±0.23	0.18–1.15
Glycine	6.34±2.13	3.13–10.4	6.52±2.26	3.05–11.50
Taurine	6.70±1.98	4.81–13.3	5.82±1.66	2.90–9.26
GABA	0.11±0.03	0.08–0.12	0.15±0.06	0.03–0.22

*$p<0.05$ vs males. Values are expressed in μmol/l. Number of females=17, males=23.

evaluated using native samples at the time of collection. The initial sample handling and the analysis should be made at room temperature. If storage is necessary, a temperature of −80°C is recommended.

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