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Effect of different sample pretreatment methods on the concentrations of excitatory amino acids in cerebrospinal fluid determined by high-performance liquid chromatography

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Abstract

Using high-performance liquid chromatography (HPLC), the effect of different sample pretreatment methods on the concentrations of excitatory amino acids (EAAs, glutamate and aspartate) measured in cerebrospinal fluid (CSF) was investigated. The results showed that the measured values of glutamate and aspartate were constant when the samples were stored at −80 °C and then methanol was used for CSF deproteinization before assay; the values of glutamate (Glu) increased when 0.3 *M* perchloric acid was used for CSF deproteinization with the CSF subsequently being stored at −20 °C; the values of Glu changed when the samples were stored at −20 °C over 8 weeks with methanol subsequently being used for CSF deproteinization before assay. This reference data suggested that the CSF sample would be better stored at −80 °C. If the sample is stored at −20 °C over 8 weeks, the Glu values change with the storage time. If strong acidic reagents are used for precipitation of protein in the CSF sample and then stored at −20 °C, Glu values are abnormally increased. From this study, an accurate and sensitive reversed-phase high-performance liquid chromatographic method has been developed for anti-excitotoxicity therapy and thorough study of EAAs in a clinical setting.

Keywords: Sample pretreatment; Glutamate; Aspartate

1. Introduction

Excitatory amino acids (EAAs) are necessary as neurotransmitters for normal function of the central nervous system, but superphysiological concentrations of EAAs in the extracellular fluid are known to be neurotoxic [1,2]. This notion is supported by some clinical evidence, such as the fact that EAAs in cerebrospinal fluid (CSF) are notably increased in traumatic brain injury, cerebral ischemia, anoxia of the brain and epilepsy etc. [1–4]; in traumatic brain injury, the more severe the trauma, the more obvious the excitotoxicity induced by EAAs; the more serious the secondary brain insult and the brain edema, the worse the outcome [5]. EAA receptor antagonists as treatment following severe head injury were suggested [6,7]. Therefore, it is very important to

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accurately determine the concentrations of EAAs in CSF of subjects suffering from brain injury. The determination of EAAs by HPLC is well established [8–11]. However, in previous literature reports, there is a wide deviation in measured values of Glu in CSF using HPLC when the samples were pretreated in different ways (including storage and deproteinization) [4,8–11]. The difference might be caused by the instability of Glu during storage or deproteinization. To develop an accurate and sensitive approach for determination of EAAs in a clinical setting, we determined the effect of three representative CSF sample pretreatment methods, reported in the literature [4,8,11], and evaluated the change in level of EAAs due to these methods using HPLC.

2. Experimental

2.1. Apparatus and reagents

A Perkin-Elmer HPLC system (Model 1022) was used with a gradient solvent-delivery system (Series-200), a fluorescence detector (Model 240), and a 50×4.6 mm guard column with 10 μm ODS stationary phase (Chromatographic Center, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences), and a 200×4 mm analytical column with 7 μm Nucleosil ODS stationary phase (National Chromatographic Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences). The excitation and emission wavelengths were 330 and 445 nm, respectively.

The *o*-phthalaldehyde (OPA; Fluka, Zurich, Switzerland) was of analytical reagent grade, the mercaptoethanol was of chemical reagent grade (No.4 Reagent Plant of Shanghai), and the methanol was HPLC-grade (Yu Chen Reagent Plant of Shandong). ''Ultra-pure'' water was prepared with an E-pure system (Barnstead Thermolyne). All other reagents were of analytical reagent grade and were obtained from commercial suppliers. The amino acid calibration solution was prepared by dissolving 2.46 μmol/l aspartic acid and 4.16 μmol/l Glu (No.3 Reagent Plant of Shanghai) in 0.5 mol/l HCl. Derivatization reagent was prepared by dissolving 50 mg of OPA in 2.5 ml of methanol and adding 50 μl of mercaptoethanol, the mixture was kept at 4 °C in the dark.

2.2. Chromatographic conditions

All HPLC solvents were filtered through 0.45 μm (pore size) filters and degassed. Mobile phase A was tetrahydrofuran–methanol–0.1 mol/l sodium acetate (pH 7; 5:95:900 v/v); mobile phase B was methanol. The gradient system was: 0 min, 0% mobile phase B, increased to 25% B at 5 min, to 35% B at 10 min, 55% B at 15 min and 95% B at 19 min, and held at 95% B until 22 min. The flow-rate was 1.0 ml/min.

2.3. Derivatization

CSF sample or calibration solution (50 μl) and 50 μl ethanolamine (32.74 μmol/l, dissolved in methanol, internal standard) were added into a polyethylene microfuge tube; 50 μl of derivatization reagent (OPA) and 450 μl borate buffer (0.4 mol/l, pH 9.25) were added. The mixture was shaken for 10 s and was centrifuged at 15,000 *g* for 1 min at room temperature; 20 μl of the reaction mixture were injected into the HPLC system 2 min later. Fig. 1 shows chromatograms of Glu and Asp.

2.4. Collection of CSF sample

The research project was approved by the Ethics Committee of The First Affiliated Hospital Lanzhou Medical College. Six adults (four men and two women, average age 48 years, age range 17–58) with acute head injury were included in this study. About 20 ml of CSF were collected freshly via draining from every subject. Because there are some red blood cells and other cells in CSF via draining, these cells might rupture and the amino acids in them would alter the EAA concentration, so the fresh samples were centrifuged within 30 min at 15,000 *g* for 2 min. After CSF was centrifuged, no cell remained in the supernatant.

2.5. Sample pretreatment

An aliquot of the CSF supernatant of every subject was assayed immediately and then the CSF was divided into nine tubes, 1 ml in each tube. According

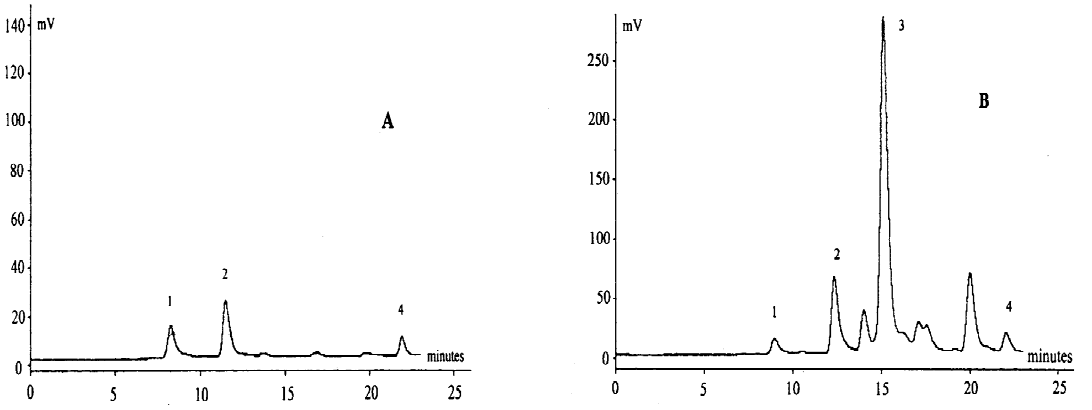

Fig. 1. Chromatogram of Asp and Glu. (A) Calibration solution (2.46 μmol/l Asp and 4.16 μmol/l Glu. See Experimental section for derivatization process); (B) CSF sample (50 μl CSF sample and ethanolamine 32.74 μmol/l). Mobile phase A was tetrahydrofuran–methanol–0.1 mol/l sodium acetate (pH 7; 5:95:900, v/v); mobile phase B was methanol. The gradient system was: 0 min, 0% mobile phase B, gradually increased to 25% B at 5 min, to 35% B at 10 min, 55% B at 15 min and 95% B at 19 min, and held at 95% B until 22 min; injection volume, 20 μl; flow-rate, 1 ml/min; detection, the excitation wavelength 330 nm and emission wavelength 445 nm. Peaks: 1, Asp; 2, Glu; 3, glutamine; 4, ethanolamine (internal standard).

to the literature, three CSF pretreatment procedures are as follows.

1. CSF samples were stored at −20 °C, and immediately before assay proteins were precipitated with methanol [8]. The methanol volume added to the CSF sample was two times that of the CSF. To avoid the CSF sample being too dilute, as stated above we prepared the derivatization reagent solution and internal standard with methanol. Fifty μl of derivatization reagent and 50 μl of ethanolamine (internal standard) were added to 50 μl CSF sample.
2. CSF samples were stored at −80 °C, with deproteinization using methanol immediately before assay [11]. The methanol volume added to the CSF sample was two times that of the CSF, as described in (1) above.
3. CSF samples (1 ml) were precipitated with 250 μl of 0.3 *M* perchloric acid and the mixture was stored at −20 °C [4].

The concentrations of Glu and Asp were assayed in samples with the different pretreatment procedures when the CSF samples were stored for 0, 4, 8, and 12 weeks.

2.6. Statistical analysis

Data were compared by Student's *t*-test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Method validation

To evaluate the between-run variation of the method, the same CSF sample was measured five times during 2 weeks. The relative standard deviations (RSD) for Asp and Glu were 7.3% (Asp 0.92 μmol/l) and 5.3% (Glu 6.95 μmol/l), respectively. For within-assay reproducibility studies, we assayed the same CSF sample five times in one analytical run. The RSD for Asp and Glu were 5.8% (Asp 0.92 μmol/l) and 5.5% (Glu 6.95 μmol/l), respectively. The mean analytical recovery was 97.9% (RSD 4.8%, $n=3$ for Asp) and 98.7% (RSD 4.3%, $n=3$ for Glu), as estimated by adding known quantities of amino acids to the CSF samples (adding Asp at 3.08 μmol/l and Glu at 5.20 μmol/l). The calibration curve for Asp was linear at 0.32 to 10.29 μmol/l ($y_{\mathrm{Asp}} = 0.0791 + 0.051x$, $r = 0.9998$, where x is the concentration and y is the ratio of peak areas between Asp and internal standard), while for Glu, it

Table 1
The concentrations of aspartate and glutamate in CSF stored at −20 °C with methanol deproteinization before assay (μmol/l)[a]

Subject	0 weeks		4 weeks		8 weeks		12 weeks	
	Asp^0	Glu^0	Asp^4	Glu^4	Asp^8	Glu^8	Asp^{12}	Glu^{12}
1	2.35	13.91	2.19	13.55	2.24	12.60	2.29	11.86
2	1.21	10.98	1.12	10.11	1.20	9.15	1.24	8.87
3	2.60	15.70	2.65	15.67	2.41	15.39	2.58	14.70
4	2.42	14.54	2.37	14.34	2.31	13.10	2.27	12.86
5	1.57	12.56	1.60	12.45	1.53	11.93	1.49	11.20
6	1.11	8.12	0.99	8.02	1.13	7.13	1.12	7.01
Mean	1.88	12.64	1.82	12.36	1.80	11.55	1.83	11.08
SD	±0.66	±2.75	±0.69	±2.84	±0.58	±2.96	±0.62	±2.77
P			$P>0.05$ vs. Asp^0	$P>0.05$ vs. Glu^0	$P>0.05$ vs. Asp^0	$P<0.05$ vs. Glu^0	$P>0.05$ vs. Asp^0	$P<0.05$ vs. Glu^0

[a] Average of three determinations for Asp and Glu.

was linear at 0.63–20.26 μmol/l ($y_{Glu} = 0.0654 + 0.0631x$, $r = 0.9997$).

3.2. Effect of sample pretreatment

The concentrations of Glu and Asp in CSF with different treatment methods and storage time are shown in Tables 1–3.

In Tables 1–3, Asp concentrations stored for 4, 8, and 12 weeks were compared with concentrations for 0 weeks. The results showed that Asp concentrations were not statistically significantly changed ($P>0.05$), namely, the Asp in CSF is stable independent of the three pretreatment methods, when stored for 4, 8, and 12 weeks. The results showed that Glu concentrations were significantly decreased after storage for 8 weeks and 12 weeks at −20 °C when deproteinized with methanol ($P<0.05$). However, there was no statistically significant change after storage within 4 weeks at −20 °C ($P>0.05$). Thus, Glu in CSF is stable at −20 °C for 4 weeks (Table 1).

For CSF samples stored for 4, 8 and 12 weeks at −80 °C with methanol precipitation, Glu concentrations were not significantly different ($P>0.05$). That means Glu in CSF is stable at −80 °C. The results are shown in Table 2.

In Table 3, Glu concentrations in CSF stored for 4, 8, and 12 weeks after perchloric acid precipitation were compared with concentrations for 0 weeks. They were significantly increased ($P<0.05$), with mean Glu concentrations after storage for 4, 8 and 12

Table 2
The concentrations of aspartate and glutamate in CSF stored at −80 °C and deproteinization with methanol before assay (μmol/l)[a]

Subject	0 weeks		4 weeks		8 weeks		12 weeks	
	Asp^0	Glu^0	Asp^4	Glu^4	Asp^8	Glu^8	Asp^{12}	Glu^{12}
1	2.35	13.91	2.34	13.68	2.43	14.09	2.36	14.04
2	1.21	10.98	1.19	11.02	1.25	10.54	1.26	10.21
3	2.60	15.70	2.62	15.90	2.57	15.86	2.65	16.00
4	2.42	14.54	2.40	14.25	2.43	14.45	2.39	14.69
5	1.57	12.56	1.55	12.10	1.62	11.87	1.64	12.76
6	1.11	8.12	1.00	7.81	1.15	8.23	1.00	8.34
Mean	1.88	12.64	1.85	12.46	1.90	12.51	1.88	12.67
SD	±0.66	±2.75	±0.69	±2.84	±0.64	±2.83	±0.68	±2.89
P			$P>0.05$ vs. Asp^0	$P>0.05$ vs. Glu^0	$P>0.05$ vs. Asp^0	$P>0.05$ vs. Glu^0	$P>0.05$ vs. Asp^0	$P>0.05$ vs. Glu^0

[a] Average of three determinations for Asp and Glu.

Table 3
The concentrations of aspartate and glutamate in CSF sample deproteinized with 0.3 *M* perchloric acid and then stored at −20 °C (μmol/l)[a]

Subject	0 weeks		4 weeks		8 weeks		12 weeks	
	Asp^0	Glu^0	Asp^4	Glu^4	Asp^8	Glu^8	Asp^{12}	Glu^{12}
1	2.35	13.91	2.36	28.72	2.47	41.91	2.53	58.90
2	1.21	10.98	1.36	23.12	1.10	33.51	1.27	44.19
3	2.60	15.70	2.73	31.10	2.68	42.19	2.51	59.09
4	2.42	14.54	2.45	31.94	2.37	45.33	2.59	60.32
5	1.57	12.56	1.41	26.37	1.46	37.56	1.65	49.46
6	1.11	8.12	1.10	19.93	0.95	31.86	1.26	43.85
Mean	1.88	12.64	1.90	26.86	1.84	38.73	1.97	52.64
SD	±0.66	±2.75	±0.69	±4.68	±0.76	±5.32	±0.65	±7.73
P			$P>0.05$ vs. Asp^0	$P<0.05$ vs. Glu^0	$P>0.05$ vs. Asp^0	$P<0.05$ vs. Glu^0	$P>0.05$ vs. Asp^0	$P<0.05$ vs. Glu^0

[a] Average of three determinations for Asp and Glu.

weeks increasing to 212.5%, 306.4%, 416.5% of the time zero values, respectively.

4. Discussion and conclusion

The finding that Glu in CSF is stable at −80 °C is in agreement with a previous report [11]; in that work, Glu levels were not changed even if the samples were stored for 9 months at −80 °C.

The concentrations of Glu in CSF stored at −20 °C for 8 and 12 weeks were decreased. This may be due to some enzymes in the CSF sample that still have a little activity at −20 °C.

It was found that concentrations of the Glu in CSF stored at −20 °C in 0.3 *M* perchloric acid were significantly increased as storage time goes on. The strong acid can lead to falsely high measured values for Glu. The explanation for this increase probably is that glutamine in CSF can be hydrolyzed into Glu and ammonia (or amine) under acidic conditions [8,11]. Previous observations also indicate that Glu concentrations would increase if trichloroacetic acid or sulfosalicylic acid were used for CSF deproteinization [9].

The following conclusions can be drawn: (1) it would be better to store the CSF sample at −80 °C when EAAs in CSF are determined. (2) If the CSF sample is left at −20 °C, the storage time should not exceed 4 weeks. (3) Strong acids are not suitable as deproteinizing reagents in the measurement of EAAs in CSF.

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