Pyridoxal in the cerebrospinal fluid may be a better indicator of vitamin B6-dependent epilepsy than pyridoxal 5′-phosphate

Supplementary material 2

Assays of 5-hydroxytryptophan

We modified the analysis method described in our previous study [1] to achieve a better separation of 5-hydroxytryptophan (5-HTP) from other co-eluting metabolites. In brief, we changed the mobile phase A to ammonium formate buffer (26.9 mmol of ammonium formate dissolved in 1 L of ultrapure water, adjusting the pH to ≈3.7 with 874.5 µL [23.1 mmol] of formic acid). The gradient setting is shown in Table 1. The column temperature was changed to 32°C. Injection volume was increased to 30 µL. Although this modification improved the separation of 5-HTP, the resolution of the peak of 5-hydroxyindoleacetic acid became worse in a few samples. Therefore, we used this modified method only to measure 5-HTP.

Table 1. Gradient settings for 5-HTP assay

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)	Gradient curve
Initial	1.0	100.0	0.0	0.0	
5.5	1.0	100.0	0.0	0.0	Linear
12.5	1.0	80.0	20.0	0.0	Linear
13.0	1.0	0.0	80.0	20.0	Step
17.0	1.0	100.0	0.0	0.0	Step
25.0	1.0	100.0	0.0	0.0	Linear

Assays of α-aminoadipic semialdehyde and pipecolic acid

Standard solutions

To prepare a standard solution of α-aminoadipic semialdehyde (AASA), we followed the method of Sadilkova et al. [2] to obtain 4 mmol/L AASA solution. Strictly speaking, this is a mixture of AASA and δ^1-piperideine-6-carboxylate, which are in equilibrium at a total

concentration of 4 mmol/L. We diluted this solution with ultrapure water to 10 μmol/L to be used as a calibrator. We also prepared a 10 μmol/L solution of pipecolic acid in ultrapure water as a calibrator. Additionally, we prepared quality control samples at 1 μmol/L by dilution with ultrapure water. These calibrators and quality controls of AASA and pipecolic acid were stored at –80°C. We made mixed amino acid solutions at 10 μmol/L, which were used to confirm that the peaks of AASA and pipecolic acid were clearly separated from those of other amino acids.

Sample preparation

Cerebrospinal fluid (CSF) samples (50 μL) with no dilution, serum samples diluted twofold by ultrapure water (100 μL), and standard solutions (50 μL) were added with an equivalent volume of acetonitrile for deproteinization. After centrifugation at 16,000 × *g* at 4°C for 10 min, 50 μL of the supernatant was transferred to another microtube.

For the AASA assay, 25 μL of 0.4 mol/L borate buffer (pH 9.0) was added. Subsequently, 25 μL of derivatization agent (20 mmol/L of 9-fluorenylmethyl chloroformate [FMOC-Cl] dissolved in acetonitrile) was added. After 5 min, the reaction was stopped by the addition of 50 μL of 100 mmol/L amantadine dissolved in a mixture of 0.02 mol/L HCl and acetonitrile [1:1, v/v]. After 3 min, the samples were filtered through 0.45 μm Millex LH filters (Merck Millipore, Billerica, MA, USA).

For the assay of pipecolic acid, primary amines were first derivatized by adding 25 μL of derivatization agent (75 mmol/L o-phthalaldehyde and 75 mmol/L dithiothreitol dissolved in a mixture of 0.4 mol/L borate buffer and acetonitrile [9:1, v/v]). After 3 min, the remaining secondary amines, including pipecolic acid, were derivatized by adding 25 μL of

derivatization agent (20 mmol/L FMOC-Cl dissolved in acetonitrile). After 3 min, the samples were filtered through 0.45 µm Millex LH filters.

Chromatographic setting

The chromatographic system consisted of a Waters Alliance 2695 module with a Waters 2475 multi λ fluorescence detector (Waters Japan, Tokyo, Japan).

For the AASA assay, the mobile phase A (pH ≈ 7.0) was generated by dissolving 5.082 g (35.8 mmol) of disodium hydrogen phosphate and 2.904 g (24.2 mmol) of sodium dihydrogen phosphate in 1 L of ultrapure water. The mobile phase B was 100% acetonitrile. The mobile phase C was ultrapure water. The gradient setting is shown in Table 2. The separation was performed through a CORTECS C18 column (2.7 µm, 3.0 mm × 150 mm, Waters) with a CORTECS C18 VanGuard cartridge (2.7 µm, 2.1 mm × 5 mm, Waters) at 35°C. The entire analysis time was 40 min.

Table 2. Gradient settings for AASA assay

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)	Gradient curve
Initial	0.5	76.0	24.0	0.0	
14.0	0.5	76.0	24.0	0.0	Linear
14.1	0.5	0.0	60.0	40.0	Step
15.0	0.5	0.0	80.0	20.0	Step
16.0	0.5	0.0	24.0	76.0	Step
17.0	0.5	76.0	24.0	0.0	Step
40.0	0.5	76.0	24.0	0.0	Linear

For the pipecolic acid assay, the mobile phase A (pH ≈ 7.2) was generated by dissolving 6.019 g (42.4 mmol) of disodium hydrogen phosphate and 2.112 g (17.6 mmol) of sodium dihydrogen phosphate in 1 L of ultrapure water. The mobile phase B was 100% acetonitrile.

The mobile phase C was ultrapure water. The gradient setting is shown in Table 3. The separation was performed through an XBridge BEH C18 XP column (2.5 μm, 3.0 mm × 50 mm, Waters) with an XBridge BEH C18 XP VanGuard cartridge (2.5 μm, 2.1 mm × 5 mm, Waters) at 32°C. The entire analysis time was 15 min.

Table 3. Gradient settings for pipecolic acid assay

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)	Gradient curve
Initial	0.5	74.0	26.0	0.0	
7.0	0.5	74.0	26.0	0.0	Linear
7.1	0.8	0.0	60.0	40.0	Step
10.0	0.8	0.0	26.0	74.0	Step
11.0	0.8	74.0	26.0	0.0	Step
15.0	0.8	74.0	26.0	0.0	Linear

For both assays, fluorescence detection was performed with excitation at 254 nm and emission at 313 nm. Concentration was determined by the heights of the peaks. The samples were cooled to 4°C in the autosampler, and the injection volume was 5 μL. At the beginning of each analysis run, the concentration of a quality control sample was measured and was required to have a relative error (RE) within 15% (0.85 to 1.15 μmol/L).

Validation of the assays

The linearity of the calibration curve was assessed using Pearson's correlation coefficient. The limit of detection (LOD) was determined based on a signal-to-noise (S/N) ratio ≥ 3. The limit of quantification (LOQ) was determined based on an S/N ratio ≥ 10 and RE within 20%. Recovery was evaluated by spiking five serum samples by 1 μmol/L and 5 μmol/L.

Results of validation

The retention time of FMOC-AASA was 15.0 min. We did not observe other extra peaks

compared with blank samples with the same derivatization process. We checked the identity of the FMOC-AASA peak by obtaining the fraction of this peak and flow-injection mass spectrometry (negative mode, *m/z* 366.13). The LOD was below 0.01 µmol/L and the LOQ was 0.05 µmol/L (RE –6.0% to +14.0%, n = 5). The calibration curve was linear (r > 0.999) in the range of 0.01 to 10 µmol/L, and the intercept was not significantly different from zero. Therefore, we chose to use a single-point calibrator at 10 µmol/L. The peak of FMOC-AASA was clearly separated from those of other amino acids, and its height did not show a reduction after 24 h. The recoveries calculated from five serum samples were 84.6 to 98.8% (mean, 93.1%) for samples spiked by 1 µmol/L, and 88.6 to 99.7% (mean, 93.5%) for samples spiked by 5 µmol/L. The intra-day coefficient of variation (CV) for the quality control samples was 0.7% (n = 5), and the inter-day CV was 1.6% (n = 5). Example chromatograms are presented in Figure 1.

The retention time of pipecolic acid was 6 min. The LOD was 0.02 µmol/L and the LOQ was 0.05 µmol/L. The calibration curve was linear (r > 0.999) in the range of 0.01 to 10 µmol/L, and the intercept was not significantly different from zero. Therefore, we chose to use a single-point calibrator at 10 µmol/L. The peak of pipecolic acid was clearly separated from those of other amino acids, and its height did not show a reduction after 24 h. The recoveries calculated from five serum samples were 92.8 to 126.6% (mean, 111.4%) for samples spiked by 1 µmol/L, and 88.5 to 106.0% (mean, 99.8%) for samples spiked by 5 µmol/L. Intra-day CV for the quality control samples was 1.2% (n = 5) and inter-day CV was 2.7% (n = 5). Example chromatograms are demonstrated in Figure 1.

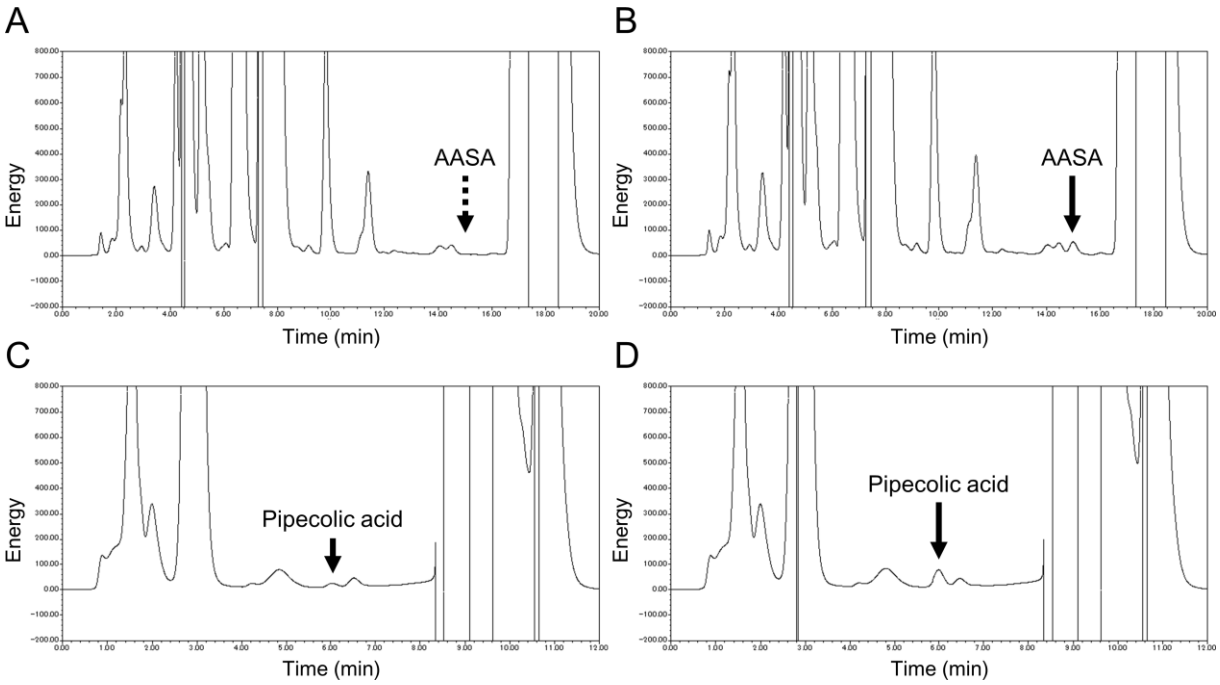
Reference values are demonstrated in Table 4. They are comparable to previous reports [3,4].

Table 4. Reference values for AASA and pipecolic acid

Age	Serum (μmol/L)		CSF (μmol/L)	
	AASA	Pipecolic acid	AASA	Pipecolic acid
0–12 months	<0.3	0.4–5.4	<0.1	<0.4
	n = 60	n = 75	n = 91	n = 139
≥ 1 year	<0.3	0.4–2.8	<0.1	<0.1
	n = 30	n = 26	n = 38	n = 30

Figure 1. Example chromatograms of the assays for AASA and pipecolic acid

Panel A: AASA assay for a serum sample from a heathy adult. AASA was not detected. Panel B: AASA assay for the same serum sample spiked with 5 μmol/L AASA. AASA was detected at 15.0 min. Panel C: Pipecolic acid assay for the same serum sample with no spiking. Pipecolic acid was detected at 6.0 min. Panel D: Pipecolic acid assay for the same serum sample spiked with 5 μmol/L pipecolic acid. Elevation of the peak height was observed.



References

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