Analysis of Glutamate and GABA in brain homogenate by FA-3ODS column.

[HPLC Conditions]

HPLC-ECD HTEC-500 or 700 series
Separation column Eicompak FA-3ODS (3.0 mm.i.d. x 50 mm)
Pre-column(Guard column) CA-ODS (3 mm.i.d. x 4 mm)
Mobile phase(A) 0.1 mol/L Phosphate buffer (pH 6.0)-methanol-acetonitrile (80:7:13, v/v) containing 5 mg/L EDTA・2Na
Washout solution (B) 0.1 mol/L Phosphate buffer (pH 6.0)-acetonitrile (1:1, v/v) containing 5 mg/L EDTA・2Na
Flow rate 500 µL/min
Column temperature 40 degC
Working electrode WE-GC
Gasket GS-25 (25 µm)
Applied potential +600 mV vs. Ag/AgCl
Time constant 1.0 sec

Mobile phase switching By ELS-500
Switching Schedule Mobile phase (A) – (8.0 min) – Washout solution (B) – (3 min) – Mobile phase

[Flow Diagram]
Chromatogram obtained with standard solution

[Graph showing chromatogram obtained with standard solution]

1 μmol/L Standard solution

Chromatogram obtained with rat brain tissue homogenate

Rat Hippocampus homogenate (185 mg of Frozen sample).

One (1) μmol of homotaurine (IS) were added to the sample.

Final sample solution was diluted 100-fold.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>23.28 μmol/g frozen sample</td>
</tr>
<tr>
<td>GABA</td>
<td>9.45 μmol/g frozen sample</td>
</tr>
</tbody>
</table>
[Sample pretreatment]

Tissue

- Approx. 10-fold volume of methanol
- Appropriate amount of Homotaurine (IS) (e.g. 0.1 to 1 µmol in absolute amount)
- Homogenize by Polytron or Teflon homogenizer

Homogenate

- Leave in ice-water for 15 min
- Centrifuge, 20,000 x g for 15 min at 4 degC

Supernatant

- Filter 0.22 µm (Ultrafree-MC UFC30GV00, Millipore, 5,000 x g for 10 min at 4 degC)

Filtrate

- Dilute 50 to 500 times with 10 v/v% methanol

Diluted solution

- Delivatize with 4 mM OPA/2-ME solution (Sample : OPA = 4 : 1, v/v)

Delivatized mixture

- Apply onto HPLC

[OPA Derivatization]

0.5 M Carbonate buffer (pH 10.0)

6.9 g K$_2$CO$_3$ + 2.0 mL HCl (final pH 10.0) in a total of 100 mL water.

20 mM OPA solution

27 mg OPA + 1 mL methanol + 9 mL carbonate buffer.

Final OPA reagent

Add 20 µL 2-mercaptoethanol (2-ME) to 10 mL of 20 mM OPA solution. This gives a 20 mM OPA/2-ME solution. Dilute 20 mM OPA/2-ME solution to 4 mM with 0.5 M carbonate buffer (pH 10.0).

To 20 µL of samples, 5 µL of final OPA reagents are added (sample : OPA reagent = 4 : 1) and the mixtures are left at room temperature for 2.5 min.
[Preparation of mobile phase]

0.1 mol/L Phosphate buffer (pH 6.0)
Dissolve 13.45 g NaH$_2$PO$_4$·2H$_2$O and 4.94 g Na$_2$HPO$_4$·12H$_2$O in 1,000 mL water.

To 800 mL of 0.1 mol/L phosphaste buffer (pH 6.0), add 70 mL methanol, 130 mL acetonitrile and 5 mg EDTA·2Na.

[Reagents and standard chemicals]

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Manufacturer</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-Phtalaldehyde (OPA)</td>
<td>Wako Pure Chemical</td>
<td>161-09261</td>
</tr>
<tr>
<td>2-Mercaptoethanol (2-ME)</td>
<td>Wako Pure Chemical</td>
<td>137-06862</td>
</tr>
<tr>
<td>Potassium carbonate, anhydrous (K$_2$CO$_3$)</td>
<td>Wako Pure Chemical</td>
<td>162-03495</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>Wako Pure Chemical</td>
<td>083-02715</td>
</tr>
<tr>
<td>(2 mol/L Hydrochloric acid)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaH$_2$PO$_4$·2H$_2$O</td>
<td>Wako Pure Chemical</td>
<td>192-02815</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$·12H$_2$O</td>
<td>Wako Pure Chemical</td>
<td>196-02385</td>
</tr>
<tr>
<td>Methanol</td>
<td>Nacalai tesque</td>
<td>21929-23</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Nacalai tesque</td>
<td>00430-83</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Wako Pure Chemical</td>
<td>070-00502</td>
</tr>
<tr>
<td>GABA (γ-aminobutyric acid)</td>
<td>Wako Pure Chemical</td>
<td>010-02441</td>
</tr>
<tr>
<td>Homotaurine</td>
<td>Acros organics</td>
<td>104430010</td>
</tr>
<tr>
<td>(3-amino-1-propanesulfonic acid)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
[Reference data]

Chromatogram obtained with rat hippocampus homogenate without column washout (late eluting peaks)

[Detection limits]

Chromatogram obtained with $1 \times 10^{-7}$ mol/L standard solution

Detection limits (rough estimation)

- Glu: $1 \times 10^{-7}$ mol/L
- GABA: $1 \times 10^{-8}$ mol/L
Rat Hippocampus homogenate (185 mg of Freeze dried sample).
One (1) μmol of homotaurine (IS) were added to the sample.
Final sample solution was diluted 100-fold.

ASP and GLN are overlapped by reagent blank peaks.
[Application to brain microdialysate]

Brain microdialysis

Rat hippocampus (CA1, A -5.8 mm; L +5mm, V 3.5 mm from brain surface)

Probe: A-I-8-03

Perfusion medium: Ringer’s solution

Flow rate: 1 μL/min

Chromatogram obtained with rat brain microdialysate
[Memo for the method]

- Standard running pressure 10 to 11 MPa
- Don’t run pump when the column is cool to avoid excess pressure to the column.
- Seal the opening of mobile phase container with Parafilm. Acetonitrile evaporates day by day, and this results in the delay of retention time. Prepare fresh mobile phase daily if possible.
- Adjust the mobile phase switching timing depending on each system’s flow path volumes.
- Do not use PCA in homogenization of tissue. Glutamine is hydrolyzed to form glutamic acid under acidic condition.
- Glutamate and GABA contents in brain tissue are reported as below;
  
  Glutamate 10 μmol/g wet weight
  GABA 1-2.3 μmol/g wet weight

(Quantitative values obtained from frozen stock tissues used in this experiment are 2 to 5-fold higher than those previously reported. It might be partly because water was evaporated during storage period. Tissues looked dry in fact.)