A Simple and Highly Sensitive HPLC Method with Fluorescent Detection for Determination of Pipecolic Acid in Mouse Brain Areas

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Pipecolic acid (PA), one of the imino acids, is the major metabolite of lysine in the mammalian brain.1) Previous studies have demonstrated high-affinity Na+-dependent uptake for PA in synaptosomes from mouse brain2) and Ca2+-dependent and K+-induced release of PA from brain slices.3) Other pharmacological and neurological studies suggested the involvement of PA as a neuromodulator or neurotransmitter with the γ-aminobutyric acid-ergic (GABAergic) transmission, which was shown to be region and site specific in the central nervous system.4–9) However, the pharmacological and neurological roles of PA are still unclear. Moreover, the levels of PA in blood, urine and cerebrospinal fluid are elevated in severe metabolic disorders of the nervous system such as infantile Refsum disease, Zellweger syndrome and neonatal adrenoleukodystrophy.10–12) To understand the roles of PA, it is important to estimate the regional distribution of PA in the brain. Therefore, a simple and sensitive method for determination of PA in brain areas is required.

For the determination of PA in brain, gas chromatography-mass spectrometry (GC-MS)3,14) and high-performance liquid chromatography (HPLC)15,16) have been reported. In these methods, while GC-MS methods are used for determination of PA in whole brain, HPLC methods that employ a pre-column derivatization technique using 1-dimethylaminonaphthalene-5-sulfonfyl chloride (Dansyl-Cl)15) or 2,4-dinitrofluorobenzene (DNFB)16) as a derivatization reagent were applied to determine the concentration of PA in rat or mouse brain areas. The HPLC method with electrophoretic detection using DNFB was employed following pre-treatment procedures: homogenization in trichloroacetic acid solution, washing of supernatant with ethyl acetate, separation with C18 cartridge column, derivatization with DNFB, solvent extraction to remove the excess reagent and two-dimensional thin-layer chromatography to separate the derivative of PA from other brain components before HPLC analysis. This HPLC method required laborious, time-consuming and complicated procedures because of the lack of specificity. In the HPLC method with fluorescent detection using Dansyl-Cl, the brain sample was treated as follows: homogenization in n-butanol, back extraction with HCl solution in the presence of n-heptane, separation with ion-exchange column and derivatization with Dansyl-Cl. Although the method with fluorescent detection was more specific, it was still tedious and time-consuming, and the derivative of Dansyl-Cl is light-sensitive. Furthermore, the detection limits of these HPLC methods were found to be around 3 pmol per injection. As the sensitivities of these HPLC methods were very poor, PA in brain areas was measured near the determination limit.

We previously established a highly sensitive and reliable HPLC method for the determination of PA in serum.17) In this method, PA is derivatized with 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride (DMS-Cl), which reacts quantitatively with amino acids to form stable and highly fluorescent sulfonamides.17,18) In the present study, we developed a simple and highly sensitive HPLC method for the determination of PA in mouse brain areas using the pre-column derivatization with DMS-Cl after the elimination of primary amino compounds coexisting with PA by the treatment with o-phthalaldehyde (OPA).

**Key words** pipecolic acid; brain; fluorescent detection; HPLC; 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride

**Materials and Methods**

**Chemicals and Solvents** All chemicals were of analytical-reagent grade, unless stated otherwise. DMS-Cl was prepared as described in a previous paper.18) PA and hydroxyproline were purchased from Sigma-Aldrich Fine Chemicals (Tokyo, Japan) and Kyowa Hakko (Tokyo, Japan), respectively. (2S,3S)-3-Methylpyrrolidine-2-carboxylic acid was obtained from ACROS ORGANICS (New Jersey, U.S.A.). OPA and HPLC-grade acetonitrile were obtained from Wako Pure Chemicals (Osaka, Japan). Deionized-distilled water was purified with the Milli-QII system (Yamato, Tokyo, Japan) prior to use.

**Instrumental Conditions** An Agilent 1100 Series...
HPLC system (Agilent, California, U.S.A.), consisting of a binary pump, a micro-vacuum degasser, a micro-autosampler, a thermostatted column compartment, a programmable 3D fluorescence detector, a control module and a chemistation, was used. A TSK gel ODS-80Ts (150×4.6 mm, i.d., 5 μm, Tosoh, Tokyo, Japan) connected to a TSK guard-gel ODS-80Ts (15×3.2 mm, i.d., 5 μm, Tosoh) as a guard column was employed at 50 °C with a stepwise elution program using (A) acetic acid (30 mM)–(B) acetonitrile. The stepwise elution program consisted of isocratic elution of 28% B for 30 min, followed by a stepwise increase to 80% of B to wash the column for 10 min, and finally a stepwise decrease to 28% of B to re-equilibrate the column for 10 min. The flow rate was 1.2 ml/min. The fluorescence intensities were monitored at excitation and emission wavelengths of 316 nm and 403 nm, respectively.

Analytical Procedure The dissected brain sample (30—230 mg) was homogenized in 10 volumes (v/v) of methanol containing (2S,3S)-3-methylpyrrolidine-2-carboxylic acid as an internal standard (IS, 1 μm). The homogenate was centrifuged (3000 g, 10 min) and the supernatant (200 μl) was evaporated to dryness under a stream of nitrogen at 50 °C. To the residue, OPA (150 mM, in acetonitrile–borate buffer (0.2 m, pH 9.0)=(1 : 9), 50 μl) was added. After standing for more than 3 min at room temperature, the mixture was reacted with DMS-Cl (10 mM, in acetonitrile, 50 μl) at 70 °C for 15 min and then hydroxyproline (0.1 m, in borate buffer (0.2 m, pH 9.0), 50 μl) was added. After standing for more than 2 min at room temperature, the mixture was acidified with acetic acid (1 m, 50 μl) and centrifuged (3000 g, 10 min). The supernatant was filtered with a Cosmonice Filter W (0.45 μm, 4 mm, Nacalai Tesque, Kyoto, Japan) and then an aliquot of the filtrate (20 μl) was subjected to HPLC.

Mouse Brain Sample A male ddy mouse (8 weeks old) was anesthetized with ether and then decapitated. The brain was removed immediately under ice cooling and dissected into six regions. The dissected brain sample was stored at −20 °C until assay. The animal experiment was carried out according to the guidelines for animal experimentation, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University.

RESULTS AND DISCUSSION

Chromatographic Separation and Selection of IS The derivatives of PA and IS produced by the reaction with DMS-Cl were successfully separated on a reversed-phase column. Typical chromatograms obtained from a standard solution, a reagent blank and a mouse brain are shown in Fig. 1. The peaks due to IS and PA were eluted at 21.0 and 27.8 min, respectively, and were completely separated from the peaks of the reagent blank and other brain components under the described conditions. The separation of the peaks due to IS and PA was affected by the concentration of acetic acid in the mobile phase. The retention times of these peaks became longer with increasing concentration of acetic acid. Sufficient separation was obtained by employing 30 mM acetic acid. The peak due to PA in the mouse brain was identified by comparing the retention time with those of standard solution and co-chromatography of the standard solution and the mouse brain.

Some secondary amino acids were examined to select IS. Although the peaks due to isonipecotic acid, nipeptic acid and piperidin-2-yl-acetic acid were eluted at 16.8, 21.9 and 28.6 min, respectively, these peaks overlapped with peaks due to brain components. The peak due to (2S,3S)-3-methylpyrrolidine-2-carboxylic acid (21.0 min) was successfully separated from those of brain components.

Reaction Condition The derivatization reaction of PA with DMS-Cl was completed within 15 min at 70 °C in basic medium (pH 9) (derivatization yield: 100.8%).17 When the derivatization reaction of (2S,3S)-3-methylpyrrolidine-2-carboxylic acid as IS with DMS-Cl was examined, the reaction was completed under the same reaction conditions as PA. After the derivatization reaction of PA and IS, hydroxyproline was added to convert the excess of DMS-Cl to the hydroxyproline derivative because the excess of DMS-Cl was suspected of causing the guard column to degrade. The reaction of hydroxyproline with DMS-Cl took place at room temperature within 2 min and the derivative of hydroxyproline was eluted with the front peak of the blank at about 2 min. The reaction mixture was stable for at least 24 h at room temperature.

The reaction mixture was acidified with acetic acid before HPLC analysis because the peaks due to PA and IS were eluted as broad peaks without acidification.

Pretreatment and OPA Treatment Before HPLC analysis, the brain sample was treated as follows: homogenization in methanol, dryness of supernatant with a stream of nitrogen, OPA treatment and derivatization with DMS-Cl. As OPA treatment is effective to eliminate primary amino compounds coexisting with secondary amino compounds,17,18 OPA treatment of brain was employed prior to the derivatization reaction of PA with DMS-Cl. As a result, a simple chro-
matogram (Fig. 1c) was obtained and the determination of PA was achieved.

**Linearity, Recovery, Precision and Detection Limit**

Linearity was examined using methanol or the supernatant of homogenate (200 mg) spiked with various amounts of standard PA (25, 50, 100, 200, 400 pmol). The relationship between the peak-area ratio of PA to IS and the amounts of PA was linear (Table 1).

The recovery test and precision were examined using whole brain samples mashed homogeneously with agate mortar on ice. When standard PA (2 nmol) was added to the mashed brain samples (200 mg) before homogenization in methanol, the recoveries were 106.0—108.0% (mean ± S.D.: 106.7±1.2% (n = 3), Table 2). The precision was obtained from six replicate assays in 1 d using a mashed brain sample (200 mg). The concentration (mean ± S.D., n = 6) was 4.7±0.15 nmol/g and the relative standard deviation was 3.2%.

The detection limit (signal-to-noise ratio = 3) of PA was 13 fmol per injection. This indicates that the proposed method is extremely sensitive compared with previously reported methods (detection limit: around 3 pmol per injection).  

**Determination of PA in Mouse Brain Areas**

The regional distribution of PA in brain from male ddY mice (8 weeks old) was determined by the present method. The regional distribution of PA in mouse brains dissected into six regions is given in Table 3. The mean values of PA in thalamus and cerebral cortex were similar to, but those in cerebellum, pons-medulla oblongata, midbrain and hippocampus were 20—30% lower than the values reported previously.  

**CONCLUSION**

We have established a simple and highly sensitive HPLC method for the determination of PA in mouse brain areas using DMS-Cl as a pre-column fluorescent derivatization reagent. The proposed method has achieved the determination of low concentrations of PA in a small amount of mouse brain area by a simple pretreatment compared with the previous methods.  

**Acknowledgements**

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**REFERENCES**


**Table 1. Linearity of PA**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Regression equation$^{(a)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>$y = 3.10x + 0.008$ (r = 0.999)</td>
</tr>
<tr>
<td>Supernatant</td>
<td>$y = 2.99x + 0.764$ (r = 0.998)</td>
</tr>
<tr>
<td>Sample 1</td>
<td>$y = 3.26x + 0.286$ (r = 0.996)</td>
</tr>
<tr>
<td>Sample 2</td>
<td>$y = 3.27x + 0.271$ (r = 0.999)</td>
</tr>
</tbody>
</table>

(a) Various amounts of PA were added to methanol or the supernatant of homogenate (200 μl). x, amount of PA (pmol/test tube, range: 25—400); y, peak-area ratio of PA to IS.

**Table 2. Recovery of PA from Mashed Brain Sample**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Content (nmol/g)</th>
<th>Expected (nmol/g)</th>
<th>Found (nmol/g)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain 1</td>
<td>5.7</td>
<td>15.7</td>
<td>16.5</td>
<td>108.0</td>
</tr>
<tr>
<td>Brain 2</td>
<td>5.6</td>
<td>15.6</td>
<td>16.2</td>
<td>106.0</td>
</tr>
<tr>
<td>Brain 3</td>
<td>7.5</td>
<td>17.5</td>
<td>18.1</td>
<td>106.0</td>
</tr>
</tbody>
</table>

(a) PA (2 nmol) was added to the mashed brain sample (200 mg) before homogenization in methanol.

**Table 3. Regional Distribution of PA in Mouse Brain**

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Weight (mg)</th>
<th>Content (nmol/g wet tissue, n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebellum</td>
<td>62.7—79.3</td>
<td>5.90±2.25</td>
</tr>
<tr>
<td>Pons-medulla oblongata</td>
<td>52.3—63.6</td>
<td>5.02±1.98</td>
</tr>
<tr>
<td>Midbrain</td>
<td>46.1—82.8</td>
<td>4.44±1.57</td>
</tr>
<tr>
<td>Thalamus</td>
<td>66.6—97.9</td>
<td>4.13±1.65</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>33.6—57.5</td>
<td>3.35±1.14</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>153.8—228.7</td>
<td>3.35±1.18</td>
</tr>
</tbody>
</table>