Simultaneous Measurement of Serotonin, Dopamine and Their Metabolites in Mouse Brain Extracts by High-Performance Liquid Chromatography with Mass Spectrometry Following Derivatization with Ethyl Chloroformate

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In order to measure the levels of serotonin (5-hydroxytryptamine, 5-HT), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT) and homovanillic acid (HVA) simultaneously, an effective derivatization method followed by high-performance liquid chromatography (HPLC) coupled to electrochemical ionization mass spectrometry was used. The derivatization reaction of biological samples with ethyl chloroformate occurred rapidly at room temperature in aqueous conditions, and the resulting derivatives were isocratically separated with good selectivity using a C18 reversed-phase column within 30 min. The study results showed that the new derivatization procedure offers an excellent means of simultaneous determination of 5-HT, DA and their metabolites in mouse brain homogenates, which are important in a number of physiological and behavioral functions.

Key words neurotransmitter; HPLC-MS; ethyl chloroformate; derivatization

Dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT), which are widely distributed as a monoamine neurotransmitter in the central nervous system (CNS), play important physiological and behavioral roles such as the control and regulation of affective behavior, sleep, reward system, motor and cognitive functions. Changes of DA and 5-HT basal levels have been linked to some CNS-related disorders such as depression, panic disorder, schizophrenia and Parkinson’s disease (PD). PD is pathologically characterized by the death of dopaminergic neurons in the substantia nigra pars compacta and caused by neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which produces a clinical syndrome similar to PD. Because the administration of MPTP is known to affect the dopaminergic neurons in the nigrostriatal system with subsequent depletion of DA and its metabolites, the measurement of 5-HT, DA and their metabolites is necessary to discover and test new drugs for treating these disorders.

Various analytical methods have been widely investigated for determining the level of monoamine neurotransmitters and their metabolites, including high-performance liquid chromatography (HPLC) coupled with electrochemical detection (ECD), dual ECD, fluorescence detection (FLD), FLD/ECD system, chemiluminescence detection and mass spectrometer (MS) and gas chromatography (GC) coupled with MS in biological samples such as plasma, rat brain, and mouse brain and in vitro experiments. HPLC-ECD, which is the most common analytical technique used to determine monoamines and their metabolites, has the advantages of low cost and fast speed but maintaining its routine use is problematic due to sudden noise problems. The HPLC-FLD method is used to measure fluorescent derivatives of monoamine neurotransmitters by a derivatization procedure, which was performed by benzylamine and 1,2-diphenylethlenediamine in a two-step reaction in weakly alkaline media and in the presence of potassium hexacyanoferrate (III). However, since this protocol requires the vigorous reaction condition of heating to 50°C for 20 min, it is necessary to develop a simpler method under mild reaction conditions.

HPLC coupled with MS, which can be used to identify analytes simply on the basis of their m/z (mass-to-charge) ratios is a popular and powerful technique for analyzing neurotransmitters in several biological fluids. However, it was to our interest that there was no report for the simultaneous determination of neurotransmitters containing different ionizable groups such as carboxylic acid or amino groups in the biological samples by using the HPLC-MS method. Thus, in order to determine a neurotransmitter containing an amino group, we have to use a positive ion polarity mode of MS condition while using a negative ion polarity mode of MS condition for determining a neurotransmitter containing a carboxylic acid group. As a HPLC-MS method, we previously reported a new derivatization method using the HPLC-MS for the measurement of catecholamines (DA, norepinephrine and epinephrine) in urine samples following derivatization with ethyl chloroformate. Our derivatization method has several advantages over other derivatization methods because it can be performed in fast derivatization reaction time, in aqueous solutions and by utilizing inexpensive reagents. Additionally, this method enables good chromatographic separation and simultaneous analysis.

As an extended effort to apply our derivatization technique to neuroscience research, we herein describe our development of an optimized chromatographic condition using HPLC-MS for the simultaneous determination of 5-HT, DA and their metabolites (3,4-dihydroxyphenylacetic acid (DOPAC),

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3-methoxytyramine (3-MT) and homovanillic acid (HVA)) (Fig. 1) through derivatization with ethyl chloroformate in brain tissue extracts from mouse samples, which can be useful for measuring neurotransmitters quantitatively and effectively.

MATERIALS AND METHODS

Chemicals DA, 3,4-dihydroxybenzylamine (DHBA), DOPAC, 3-MT, HVA, 5-HT and ethyl chloroformate were obtained from Sigma-Aldrich (Saint Louis, MO, U.S.A.). Acetonitrile and ethanol were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Pyridine and formic acid were sourced from Acros Organics (Morris Plains, NJ, U.S.A.). De-ionized water with an Ultima UP-900 (Guri, Gyeonggi, Korea) was used for all aqueous solutions.

Chromatographic and Mass Spectrometric (MS) Conditions A Shimadzu LCMS-2010EV (Chiyoda-ku, Tokyo, Japan) equipped with an electrospray ionization (ESI) probe and QqQ system (Q-array-octupole-quadrupole mass analyzer) was used. The HPLC system consisted of two pumps (model LC-20AD), a degasser (model DGU-20 A3), an autosampler (model SIL-20A), a column oven (model CTO-20A) and an UV/VIS photodiode array detector (model SPD-M20A). The derivatized DA, DOPAC, 3-MT, HVA and 5-HT were separated on a ZORBAX Eclipse C-18 column (5 µm particle size, 150 mm×4.6 mm i.d.; Agilent Corporation, Palo Alto, CA, U.S.A.) by mobile phases consisting of 40% solvent A (0.1% formic acid in water, pH 3) and 60% solvent B (0.1% formic acid in acetonitrile). The injection volume was 5 µL and an analysis time of 30 min was used for the separation of all analytes at a flow rate of 0.2 mL/min. The column oven was held constant at 30°C, and all analytes were determined in the positive ESI mode. MS conditions were optimized to obtain maximum sensitivity. The curve dissolution line temperature was 250°C and heat block temperature was 200°C. Nitrogen generated from Schmidlin Mistral nitrogen generator (Geneva, Switzerland) was used as the nebulizer gas at 1.5 L/min.

Preparation of Stock Solution The stock standard solutions of the DA, its metabolites (DOPAC, 3-MT and HVA) and 5-HT were prepared by dissolving their accurately weighted amounts (10.0 mg) in 0.1 N perchloric acid solutions (1.0 mL). All solutions were kept in darkness at −24°C. These solutions were stable for at least 1 month and were diluted further with water to the required concentrations before use.

Sample Preparation Procedure Standard solution or the filtrate obtained from brain homogenate (185 µL) was added to water (265 µL). The DHBA (200 ng·mL⁻¹, 50 µL) was added as an internal standard. After ethanol–pyridine (4:1, v/v, 300 µL) and ethyl chloroformate (20 µL) were added, the solution was shaken for 3 min and diethyl ether (1 mL) was then added. The tube was shaken on a vortex mixer for 2 min and then the resulting solution was cooled to −40°C. The organic phase was

![Fig. 1. Derivatization of 5-HT, 3-MT, DHBA (IS; Internal Standard), DA, HVA and DOPAC with Ethyl Chloroformate](image-url)
transferred to another tube and the solvent was evaporated and dried in a vacuum oven. The obtained residue was dissolved in acetonitrile (50 µL) and 5 µL of the resulting solution was injected into the HPLC-MS.

Application to Mouse Brain Striatum Samples Male C57BL/6 mice (23.6 ± 1.5 g, 8 weeks old) purchased from Central Laboratory Animal Company (Seoul, Korea) were housed five per cage with room temperature, and lit by artificial light for 12 h each day. The mice were housed in standard laboratory cages and had free access to food and water throughout the study period. Mice received five intraperitoneal injections of MPTP hydrochloride (30 mg/kg in saline, Sigma-Aldrich, U.S.A.) at 1 d intervals or saline (equal volume injected intraperitoneally (i.p.)) alone (control groups). Mice (n=3 per group) were euthanized by cervical dislocation, and the dorsolateral striatum from both the left and right sides were dissected and frozen at −80°C. Brain tissue samples were weighed and then homogenized in 700 µL of ice-cold lysis buffer containing 1× phosphate buffered saline (PBS), 1% NP-40 (nonidet P-40), 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS). The homogenate was centrifuged at 9000 rpm for 20 min at 4°C and filtered through a 0.2 µm filter (Whatman, Middlesex, England).

RESULTS

Optimization of the Derivatization Procedure In the present method, as shown in Fig. 1, the derivatization reaction using ethyl chloroformate proceeded rapidly even at room temperature in aqueous solution and the derivatized compounds were then concentrated in acetonitrile. The amino (–NH₂) or hydroxyl (–OH) groups in 5-HT, DA and their metabolites were converted to the N(O)-alkoxycarbonyl form, whereas the carboxylic acid groups were changed to ethyl ester form by the decarboxylation of the intermediate mixed anhydride form.¹⁹ The molecular ion obtained from the positive selected ion monitoring mode in HPLC-ESI/MS was much stronger than that from the negative mode.²⁰,²¹ The ethyl chloroformate derivatives of 5-HT, DA, 3-MT, DOPAC, HVA and DHBA (internal standard) provided various molecular cations [M+H]+, [M+NH₄]+ or [M+Na]+. The mass spectra obtained after ethyl chloroformate derivatization are presented in Fig. 2. ESI mass spectra for derivatives of DHBA, 5-HT, DA and their metabolites were dominated by the m/z 392 [M+Na]+ for DA, the m/z 358 [M+NH₄]+ for DOPAC, the m/z 312 [M+H]+ for 3-MT, the m/z 300 [M+ NH₄]+ for HVA, the m/z 321 [M+H]+ for 5-HT and the m/z 378 [M+Na]+ for DHBA.

Selectivity To obtain good resolution and symmetric peak shapes of the analytes, the chromatographic conditions, such as the composition of mobile phase and total analysis...
time, were optimized through several trials. As a result, good chromatographic separation was obtained using an Agilent ZOBAX Eclipse C18 (150 mm x 4.6 mm i.d., 5 µm) column. The composition of the mobile phase was 60% solvent B as isocratic elution at a flow rate of 0.2 mL/min and with a total analysis time of 30 min. A typical chromatogram for separation of DHBA, 5-HT, DA and their metabolites is shown in Fig. 3. The retention times of the analytes were approximately 12.67 min (5-HT), 14.25 min (3-MT), 15.53 min (DHBA), 16.65 min (DA), 19.22 min (HVA) and 23.42 min (DOPAC), respectively.

**Linearity** The calibration curve of each compound was determined by plotting the ratio of peak area to DHBA (internal standard) versus concentration of the spiked standard solution, and the equations are summarized in Table 1. The calibration curves were linear in the concentration range from 1 to 700 ng/mL for DA, from 1 to 100 ng/mL for DOPAC, from 1 to 50 ng/mL for 3-MT, from 5 to 100 ng/mL for HVA, and from 1–50 ng/mL for 5-HT from three replicated measurements. A linear regression equation \( y = ax + b \) was evaluated, where \( x \) is the concentration of the analytes and \( y \) is the peak area ratio. The correlation coefficients \( R^2 \) of between 0.998 and 0.999 described good linearity in the corresponding concentration (Table 1).

**Limits of Detection (LOD) and Quantification (LOQ)** Table 1 shows the limits of detection (LOD) and quantification (LOQ) of the analytes. The LODs were determined by analyzing the spiked standard solution of progressively lowering concentrations until the signal of each analyte approached to 3-fold of the noise \( (S/N=3) \). The LODs in the present study indicated that the minimum detectable amount of 5-HT, DA and DOPAC was 0.2 ng/mL, while that of 3-MT and HVA was 0.1 ng/mL. The LOQ corresponded to an \( S/N \) ratio of 10. The LOQs of the assay were 1 ng/mL for 5-HT, 3-MT, DA and DOPAC and 5 ng/mL for HVA.

**Precision, Accuracy and Recovery** Table 2 summarizes the intra- and inter-day precision and accuracy at two concentrations. The intra-day precision and accuracy was monitored by analyzing three replicates of 20 and 50 ng/mL of each analyte. Intra-day precisions (expressed as percent relative standard deviation, R.S.D. %) of the method ranged between 0.99% for the lowest concentration of DOPAC and 4.72% for the lowest concentration of HVA. Inter-day precisions of this assay ranged between 0.35% for the highest concentration of DOPAC and 3.08% for the lowest concentration of 3-MT. The accuracy (expressed as a percentage) of the intra-day and inter-day assays were −1.64 to 2.56% and −2.01 to 1.23% for concentration of 20 and 50 ng/mL, respectively. The mean recovery rates of each compound were above 90% at the measured concentration.

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**Table 1. Linear Equations, Correlation Coefficients, LODs and LOQs for Quantification**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Equation</th>
<th>( R^2 )</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT (1–50 ng/mL)</td>
<td>( y = 0.018x + 0.073 )</td>
<td>0.998</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>3-MT (1–50 ng/mL)</td>
<td>( y = 0.047x + 0.013 )</td>
<td>0.999</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>DA (1–700 ng/mL)</td>
<td>( y = 0.103x - 0.515 )</td>
<td>0.998</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>HVA (5–100 ng/mL)</td>
<td>( y = 0.005x + 0.025 )</td>
<td>0.998</td>
<td>0.1</td>
<td>5</td>
</tr>
<tr>
<td>DOPAC (1–100 ng/mL)</td>
<td>( y = 0.101x - 0.096 )</td>
<td>0.998</td>
<td>0.2</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 2. Accuracy and Precision of Analytes (\( n=3 \))**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Amount added (ng/mL)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bias (%)(^a)</td>
<td>Precision (%)</td>
</tr>
<tr>
<td>5-HT</td>
<td>20</td>
<td>1.14</td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.62</td>
<td>1.01</td>
</tr>
<tr>
<td>3-MT</td>
<td>20</td>
<td>2.56</td>
<td>3.65</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>−1.07</td>
<td>2.08</td>
</tr>
<tr>
<td>DA</td>
<td>20</td>
<td>0.04</td>
<td>1.19</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>−0.44</td>
<td>1.67</td>
</tr>
<tr>
<td>HVA</td>
<td>20</td>
<td>1.30</td>
<td>4.72</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>−1.64</td>
<td>1.92</td>
</tr>
<tr>
<td>DOPAC</td>
<td>20</td>
<td>0.60</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.85</td>
<td>2.13</td>
</tr>
</tbody>
</table>

\(^a\) Bias (%) = (calculated concentration − found concentration)/calculated concentration \times 100.
Application of the Methods to Real Biological Samples

The applicability of the derivatization method with ethyl chloroformate to determine the levels of 5-HT, DA, DOPAC, 3-MT and HVA in the C57BL/6 mice brain striatal tissue after MPTP treatment was investigated. The derivatization reactions for DA, 5-HT and DA’s metabolites in brain tissue extracts were carried out under the same condition described for the preparation of the individual standards and their mixtures. All the analytes were confirmed as the ethyl chloroformate derivatives of 5-HT, DA, DOPAC, 3-MT and HVA on the basis of their retention times and mass spectra by a comparison with the peaks of the standard samples shown in Fig. 3.

From the HPLC-MS selected-ion chromatograms of the derivatized monoamines in the C57BL/6 mouse samples shown in Fig. 4, the amount of monoamines are calculated and presented as the mean ± S.E.M. in ng/mg wet tissue weight for mouse striata (n=3) in Table 3. The basal levels from the strata were 1.79 ± 0.08 ng/mg wet tissue weight for 5-HT, 30.58 ± 1.10 ng/mg wet tissue weight for DA, 30.58 ± 1.10 ng/mg wet tissue weight for 5-HT, 30.58 ± 1.10 ng/mg wet tissue weight for DA, 5-HT, peak 2; 3-MT, peak 3; DHBA, peak 4; DA, peak 5; HVA, peak 6; DOPAC.

DISCUSSION

MPTP, as a neurotoxin in several models of Parkinson’s disease in non-human primates and some rodents, such as C57BL/6 mice, is capable of selective destruction of dopaminergic neurons located in substantia nigra. As DA is metabolized to DOPAC, 3-MT and HVA via monoamine oxidase and catechol-o-methyl transferase in the normal state, the damage of substantia nigral cells in MPTP-treated mice is consistently accompanied by a decrease in striatal DA and its metabolites (DOPAC, 3-MT and HVA). Therefore, the...
levels of DA, DOPAC, 3-MT and HVA are reflected by the decreased activity of dopaminergic neurons.\(^{24}\)

The HPLC-ECD method is the most commonly used for detection of 5-HT, DA and their metabolites in MPTP-treated C57BL/6 mice because it is a relatively simple and economical analytical system.\(^{25–28}\) However, the HPLC-ECD method has low sensitivity and serious limitations in the simultaneous determination of monoamines such as 5-HT, DA and DOPAC.\(^{29}\)

Under the standard separation protocols, the HPLC-ECD method requires two different mobile phases along with two separated HPLC-ECD systems for measuring the levels of 5-HT, DA and their metabolites.\(^{25}\) In addition, as shown in Table 3, the levels of DA, DOPAC, 3-MT, HVA and 5-HT in the control mouse striatum measured by the HPLC-ECD method revealed significantly different values in different experiments, which are possibly due to different electrochemical behaviors and effects of analytes under different applied potentials and mobile phase compositions.\(^{26–28,30,31}\) For example, the levels of DA reported by different workers were approximately in the range of 11 to 110 ng/mL.

Our results obtained from derivatization with ethyl chloroformate using the HPLC-MS system showed that the level of striatal DA seven days after treatment with MPTP was decreased significantly (8.8% of control) compared to that of striatal DA in control mice, while MPTP-treated mice exhibited 18.2, 31.9 and 45.1% depletion of DOPAC, 3-MT and HVA in the striatum, respectively (Table 3). In addition, when the levels of DA and its metabolites were determined 1, 2 and 7 d after the last MPTP injection, the results indicated that the levels of DA and its metabolites had gradually decreased for 7 d. These results revealed patterns similar to those in a previous report by Obuchowicz et al., who determined levels of striatal DA, DOPAC, 3-MT and HVA in control mice and MPTP-treated mice using the HPLC-ECD method. However, the levels of DA, HVA and 5-HT in this study were around 3 times higher, and the levels of DOPAC and 3-MT were around 7 times higher than those measured by the conventional HPLC-ECD method.\(^{30}\) The HPLC-MS method generally has higher selectivity than those of the HPLC-ECD method, but it is difficult to measure the 5-HT, DA and their metabolites at the same time when analytes possess different ionizable groups (carboxylic acid group in DOPAC and HVA, and amino group in 5-HT, DA and 3-MT). Therefore, without the derivatization process, both positive and negative ion polarity mode have to be used separately for the determination of the levels of 5-HT, DA and their metabolites under the general HPLC-MS condition. However, our derivatization method enables us to determine these analytes at the same time under one type of ion polarity mode of HPLC-MS condition.

The LODs for each analyte were also determined by analyzing derivatized standard solution lowering concentrations until the signal of each analyte approached 3 folds of noise \((S/N=3)\).\(^{23}\) Although the LODs of the present method cannot directly compare with those of the HPLC-ECD method due to the absence of data using mouse brain, we can evaluate the difference between the LODs of DA and 5-HT in this study and those with the HPLC-ECD method using rat brain reported by Vaarmann et al.\(^{32}\) The detection limits of DA and 5-HT in our study were both 0.2 ng/mL, while Vaarmann et al. reported values of 0.7 and 1.6 ng/mL, respectively. This result indicated that the present method has 3.5 times more sensitivity with DA and 8 times more sensitivity with 5-HT than the HPLC-ECD method. When we compare the LODs of the present method with those of the HPLC-MS method without derivatization, the present method revealed 32.5 times better sensitivity with DA and 3.9 times better sensitivity with 5-HT than HPLC-MS without derivatization since the detection limits of DA and 5-HT by HPLC-MS method without derivatization were 6.5 ng/mL and 0.78 ng/mL, respectively.\(^{37}\) Upon comparison of the present method with the HPLC-FLD method with derivitization, it was found that the LODs of DA and 5-HT using HPLC-FLD method have approximately 67 times better sensitivity with DA and 38 times better sensitivity with 5-HT than those using the present method. However, the HPLC-FLD method requires the rigorous reaction conditions of heating to 50°C for 20 min for the derivatization, which are not desirable reaction conditions for biological samples.\(^{14}\) Moreover, the HPLC-FLD method cannot determine 3-MT and HVA due to failure of the derivatization with fluorescent moiety.\(^{3,14}\)

In contrast to the previously reported methods, this derivatization method with ethyl chloroformate using the HPLC-MS system offers the advantages of less time required for the derivatization process and the ability to be performed in aqueous media.\(^{35}\) In addition, this derivatization demonstrated satisfactory derivatization efficiencies with good yield in a short reaction time and enhanced data collection\(^{49}\) because not only the levels of 5-HT, DA and their metabolites were simultaneously determined, but each peak area of 5-HT, DA and their metabolites was significantly expanded and more sensitive than those generated when using the HPLC-ECD method.

CONCLUSION REMARKS

The present paper has described an HPLC method with MS for simultaneous determination of endogenous 5-HT, DA and their metabolites (DOPAC, 3-MT and HVA) in mouse brain tissue. The advantages of this derivatization method are the concurrent measurement of monoamines using inexpensive reagents in aqueous solution and the short derivatization reaction time. The method can be useful for estimating the efficiency of new drugs targeting brain-related diseases such as Parkinson’s disease, depression and panic disorder.

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