High-Performance Liquid Chromatographic-Mass Spectrometric Determination of Methamphetamine and Amphetamine Enantiomers, Desmethylselegiline and Selegiline, in Hair Samples of Long-Term Methamphetamine Abusers or Selegiline Users

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Abstract
We devised a highly sensitive method for simultaneously determining methamphetamine (MA) and amphetamine (AP) enantiomers, desmethylselegiline (DMSG) and selegiline (SG), in human hair using a derivatization technique and high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS). MA and AP enantiomers and DMSG were effectively converted to trifluoroacetic acid (TFA) derivatives, and the sensitivity of MA and DMSG increased five times over compared with that of free bases. The TFA derivatives of each compound were stable within one week in a stock solution of methanol or for 24 h in the HPLC mobile phase (mixture of methanol and ammonium formate buffer). Each compound was well separated, and calibration curves were linear in the concentration range 0.04–40 ng/mg for MA enantiomers, SG and DMSG, and 0.2–40 ng/mg for AP enantiomers. The accuracy and precision of the method were evaluated, and relative standard deviations were within 7%. Our method was successfully applied to hair samples obtained from long-term MA abusers and SG users. (+)-MA and (+)-AP were detected from three MA abusers at concentrations of 0.79–20.85 and 0.04–3.30 ng/mg, respectively. On the other hand, (-)-MA, (-)-AP, DMSG, and SG were detected in three SG users at concentrations of 2.48–9.05, 0.72–3.10, 0.12–0.59, and 0–0.04 ng/mg, respectively. Based on our obtained data, discrimination of MA abusers from SG users was considered to be possible by comparing optical isomers of MA and AP, the existence of DMSG and/or SG, and the concentration ratio of AP to MA in hair samples.

Introduction
Methamphetamine (MA) is a strong central nervous system stimulant, the abuse of which is a social problem in Japan. Selegiline (SG) [(-)-deprenyl] is a selective monoamine oxidase-type B inhibitor that has been used for the treatment of patients with Parkinson’s disease (1,2). Both drugs have a common characteristic in that MA and amphetamine (AP) are excreted in body fluids, such as urine, blood, etc. Main compounds excreted in urine are unchanged: MA and AP in MA abusers and desmethylselegiline (DMSG), MA, and AM in SG users. In SG users, the parent drug is not excreted into urine, and DMSG is excreted at only 1% of the given dose and rapidly disappears from urine. Two days from the intake of SG, MA and AP are the only compounds in urine (3). To distinguish medical SG users from illicit MA abusers, urinary analysis for the optical isomers of MA and AP has been carried out because MA abusers in Japan take mainly the (+)-enantiomer, which is more active than the (−)-enantiomer, whereas SG is metabolized in the body to (−)-enantiomer (3,4). Many reports have been published for the chiral analysis of MA and AP (5–10).

In forensic science, segmental hair analysis has been used to prove habitual abuse and/or to know the history of abuse (11,12). The general mechanism of drug incorporation into hair is one in which drugs (in the blood stream) enter hair in growing cells at the base of the hair follicle (13). Therefore, the existence of unchanged SG and DMSG in hair was expected, and DMSG was detected in hair samples (14–16). However, the amount of drugs in hair is only of a trace level (ng/mg hair order) even in addicts,
thus highly sensitive methods are required. We analyzed MA and AP as trifluoroacetic acid (TFA) derivatives and pentafluoropropionic acid (PFP) derivatives by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) with a semi-micro octadesyl silica (ODS) column. This acylation technique increased the sensitivity of MA about 5 times and was successfully applied to a MA abuser's hair (17).

We present here a method which can determine MA and AP enantiomers, DMSG and SG, simultaneously using a TFA derivatization technique and a semi-micro chiral column. Practical application of the method to hair samples of MA abusers and SG users of Parkinson's disease patients is also presented.

Experimental

Reagents
(+)-MA hydrochloride was purchased from Dainippon Pharmaceutical Company (Osaka, Japan), and (-)-MA hydrochloride, (+)-AP sulfate, and (-)-AP sulfate were received from the Ministry of Health, Labour, and Welfare. SG hydrochloride and DMSG hydrochloride were provided by Fujimoto Pharmaceuticals (Osaka, Japan). An internal standard (IS), N-n-butyylaniline, purchased from Wako Pure Chemical Industries (Osaka, Japan) was dissolved in 0.1M hydrochloric acid to give a concentration of 1 µg/mL. Trifluoroacetic anhydride (TFAA) was of gas chromatography grade (Nakarai Tesque, Kyoto, Japan), methanol was of high-performance liquid chromatography grade, and the other chemicals used were of analytical reagent grade. The Extrelut® NT3 column was purchased from Merck (Germany).

Standard stock solutions (1 µg/mL each) of MA enantiomers and AP enantiomers, SG and DMSG, were prepared by dissolving each drug in 0.1M hydrochloric acid to give a concentration of 1 µg/mL. Trifluoroacetic anhydride (TFAA) was of gas chromatography grade (Nakarai Tesque, Kyoto, Japan), methanol was of high-performance liquid chromatography grade, and the other chemicals used were of analytical reagent grade. The Extrelut® NT3 column was purchased from Merck (Germany).

Hair samples and sample preparation
Hair samples were voluntarily obtained with informed consent under the ethical guidelines prepared by the Ministry of Health, Labor, and Welfare in Japan. The samples came from: one man and two women, MA abusers arrested by Oita Prefecture Police; and one man and two women, SG users who had been treated with anti-Parkinson therapy in the Asaki Hospital. Hair samples were collected directly from scalps for MA users, and deciduous hair was used for SG users. Hair was cut

![](Image)

Figure 1. Mass spectra obtained from (+)- and (-)-MA-TFA (A), (+)- and (-)-AP-TFA (B), SG (C), DMSG-TFA (D), and IS-TFA (E) by directly injected ESI-MS.

![](Image)

Figure 2. Total and extracted ion chromatograms obtained from control hair (A) and spiked hair containing 10 ng/mg each drug (B). Peaks: 1, (-)-AP-TFA; 2, (+)-AP-TFA; 3, (-)-MA-TFA; 4, (+)-MA-TFA; 5, IS-TFA; 6, DMSG-TFA; and 7, SG.
into 1 cm long segments from the root. All strands were washed by sonication five times each with 1% sodium lauryl-sulphate (SDS) and methanol, respectively, and dried at room temperature. Five mg of dried hair were accurately weighed and transferred into a test-tube. To ensure decontamination, the final methanol washes were evaporated by rotary evaporator, and the residue was injected onto the LC–ESI–MS following TFAA treatment as described later.

To the test tube, 1 mL of 2.5M sodium hydroxide and 0.1 mL of diethyl ether were added, and the mixture was left to stand for 3 to 4 h at room temperature. After complete dissolution of the hair, 1 mL of 2M hydrochloric acid was added for pH adjustment, and 25 µL of IS solution (N-n-butylaniline) was added to the dissolved solution. The resultant mixture was applied to an Extrelut® NT3 column. After standing for 5 min, drugs were eluted with 15 mL of ethyl acetate. The eluent was concentrated by rotary evaporator to approximately 50 µL and transferred into a micro-sample-tube.

50 µL of TFAA was added to the tube and heated at 60~ for 30 min, and the reaction mixture was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 µL of methanol, and a 5-µL aliquot was injected onto the LC–ESI–MS.

Table I. Calibration Curves and Detection Limits Obtained by the Present Method

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration Range (ng/mg)</th>
<th>Regression Equation</th>
<th>Correlation Coefficient</th>
<th>Detection Limit (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-MA</td>
<td>0.04–40</td>
<td>(y = 0.1301x - 0.4266)</td>
<td>(r^2 = 0.998)</td>
<td>0.01</td>
</tr>
<tr>
<td>(-)-MA</td>
<td>0.04–40</td>
<td>(y = 0.1194x - 0.1782)</td>
<td>(r^2 = 0.999)</td>
<td>0.01</td>
</tr>
<tr>
<td>(+)-AP</td>
<td>0.2–40</td>
<td>(y = 0.0618x - 0.0838)</td>
<td>(r^2 = 0.999)</td>
<td>0.05</td>
</tr>
<tr>
<td>(-)-AP</td>
<td>0.2–40</td>
<td>(y = 0.0651x - 0.2096)</td>
<td>(r^2 = 0.998)</td>
<td>0.05</td>
</tr>
<tr>
<td>SG</td>
<td>0.04–40</td>
<td>(y = 0.4062x + 1.387)</td>
<td>(r^2 = 0.999)</td>
<td>0.01</td>
</tr>
<tr>
<td>DMSG</td>
<td>0.04–40</td>
<td>(y = 0.0856x + 0.0243)</td>
<td>(r^2 = 0.995)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table II. Within- and Between-Day Reproducibilities of Determination of MA and AP Enantiomers, SG and DMSG

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration Added (ng/mg)</th>
<th>Within-day (n = 5)</th>
<th>Between-day (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Found (ng/mg)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>(+)-MA</td>
<td>1</td>
<td>1.03</td>
<td>2.39</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.91</td>
<td>1.55</td>
</tr>
<tr>
<td>(-)-MA</td>
<td>1</td>
<td>1.01</td>
<td>3.65</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.70</td>
<td>1.69</td>
</tr>
<tr>
<td>(+)-AP</td>
<td>1</td>
<td>0.96</td>
<td>3.76</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.08</td>
<td>1.74</td>
</tr>
<tr>
<td>(-)-AP</td>
<td>1</td>
<td>0.98</td>
<td>3.30</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.80</td>
<td>2.27</td>
</tr>
<tr>
<td>SG</td>
<td>1</td>
<td>0.99</td>
<td>4.06</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.59</td>
<td>1.41</td>
</tr>
<tr>
<td>DMSG</td>
<td>1</td>
<td>1.00</td>
<td>3.13</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.88</td>
<td>2.99</td>
</tr>
</tbody>
</table>

* Relative standard deviation.

Validation

To know the stability of TFA derivatives in the mobile phase solutions, spiked hair samples containing 50 ng of each drug were treated using the present method, and the derivatized extract was dissolved in 250 µL of the mobile phase (40% solvent A and 60% solvent B) instead of methanol. An aliquot (5 µL) of the mixture was injected into LC–ESI–MS at intervals of 2 h, and peak area of each compound was measured for 24 h.

Calibration curves were obtained by plotting the peak-area ratio of each drug to IS versus the amount of each drug added to control hair. Within- and between-day reproducibilities of the proposed method were evaluated using control hair samples containing 1 or 10 ng/mg each drug. These samples were analyzed as previously described over a period of 5 days.

Results

To know the most suitable condition in ESI–MS, standard samples (50 ng each) treated by TFAA were dissolved in the mobile phase (40% solvent A and 60% solvent B) and were directly injected into the ESI–MS. As shown in Figure 1, strong protonated molecular ions ([M+H]+) were obtained in (+)- and (-)-MA-TFA, DMSG–TFA, SG, and IS–TFA at \(m/z\) 246, 234, 188, and 270, respectively.
270, 188, and 246 with 30-, 20-, and 30-V of cone voltages, respectively. However, (+)- and (−)-AP-TFA showed weak protonated ions compared with ammonium adduct ion ([M+NH₄]⁺) or fragment ion ([C₆H₅CH₂CHCH₃]⁺, m/z = 119) in the cone voltage range of 10–40 V. Therefore, ammonium adduct ion of m/z 249 was selected for the measurement of (+)- and (−)-AP-TFA with 15 V of cone voltage, which increased the sensitivity more than 10 times in SIM mode.

The strongest ions for all analytes were obtained with 2 to 3 mM of ammonium formate buffer among 1–10 mM in the mobile phase, and the pH of the buffer did not affect the sensitivity and separation of each compound within the examined pH 3–7. Therefore, 2 mM and pH 5 of the ammonium formate buffer was chosen for the best sensitivity and protection of the column, respectively. TFA derivatives of each compound were stable for 24 h in the mobile phase solution (40% solvent A and 60% solvent B).

Figure 2 illustrates typical total and extracted ion chromatograms of control hair sample and spiked hair sample containing 10 ng/mg each drug. Although DMSG-TFA and IS-TFA gave the same retention time in total ion chromatogram, both compounds were separated by the monitoring ions of m/z 270 for DMSG-TFA and m/z 246 for IS-TFA (Figure 2B). The other analytes showed complete separation even in total ion chromatograms. There were no interfering peaks in any control hair samples examined. The abuse of metylenedioxymethamphetamine (MDMA) has been increasing recently in Japan. So MDMA was examined by our method. TFA derivatives of optical isomers of MDMA showed a strong [M+H]⁺ at m/z 290 with 20 V of cone voltages. The peaks of (−)-enantiomer and (+)-enantiomer were observed at 56.4 and 60.0 min, respectively. Therefore, the peaks of MDMA-TFA enantiomers were discriminated from those of the target analytes.

Table I summarizes the parameters of calibration curves and detection limits at a signal-to-noise ratio of 3. Calibration curve of each compound was linear over the wide range, which can cover the practical concentrations in hair samples. The detection limits were between 0.01 and 0.05 ng/mg in the SIM mode. Sensitivity of MA enantiomers in this method is about two times higher than those of column switching LC–MS in determination for free base (20), and MA enantiomers and DMSG are five times more sensitive than a result of determination for free base in our laboratory using the same apparatus. However, sensitivity of AP enantiomers is not increased compared with that of column switching LC–MS and determination results of free bases in our laboratory. The minimum amounts of com-

Figure 3. Total and extracted ion chromatograms obtained from the final methanol wash (A) and hair sample for MA abuser (abuser A) (B). Peaks: 2, (+)-AP-TFA; 4, (+)-MA-TFA; and 5, IS-TFA.

Figure 4. Total and extracted ion chromatograms obtained from the final methanol wash (A) and hair sample for SG user (patient C) (B). Peaks: 1, (−)-AP-TFA; 3, (−)-MA-TFA; 5, IS-TFA; 6, DMSG-TFA; and 7, SG.
Abuser A had been taking 0.04 g MA once or twice a week, and with relative standard deviations of under 7% for all compounds. Abuser B had taken 0.02 g MA six times in a month. Abuser C didn’t remember the exact time of abuse and the amount of compounds were obtained.

Hair samples obtained from MA abusers (abuser A–C) and SG users (patient A–C) were analyzed using the present method. Abuser A had been taking 0.04 g MA once or twice a week, and abuser B had taken 0.02 g MA six times in a month. Abuser C didn’t remember the exact time of abuse and the amount of MA. The patients A–C had been taking SG orally for more than one year. Patient A took 2 or 4 tablets (containing 2.5 mg SG hydrochloride) a day, and patient B and C took 2 tablets a day. Total and extracted ion chromatograms obtained from the final methanol wash and hair samples of abuser A or patient C are shown in Figures 3 and 4, respectively, and quantification data are summarized in Table III. The final methanol washes of hair samples of abuser A or patient C showed the existence of DMSG in SG users, which was not detected in the hair of patient B.

Discussion

Both PFP and TFA derivatives were successfully applied for analysis of MA in our previous study using an ODS column (17). However, PFP derivatives of (+)-MA and (-)-MA were not separated even by the chiral column used in this study; thus, TFA derivatives were used in this study. TFA derivatives of optical isomers of MA and AP were completely separated by using a mixture of 4 mM ammonium formate and methanol (50:50, v/v) as mobile phase at a flow rate of 0.1 ml/min. But SG was not eluted with this mobile phase within 120 min. Complete separation of optical isomers and elution of all analytes from a column within 80 min was carried out using gradient program. [M+H]+ ions for MA, AP, and DMSG were shifted to a higher mass range from m/z 150, 136, and 174 to m/z 246, 232, and 270 by TFA derivatization, respectively. As many background peaks derived from the mobile phase appeared around the mass range of m/z 150, this shift contributed to an increased sensitivity of each drug.

External contamination on the surface of the hair should be removed before analysis. Nakahara (12) reported that external MA contamination on the surface of the hair could be removed thoroughly by washing hairs with 0.1% SDS two or three times. Therefore, we washed hair samples by sonication five times each with SDS and methanol after cutting them into 1-cm segments. From the results obtained by the analysis of the final methanol wash, we knew that the external contamination on the surface of the hair could be removed by this method. However, it is also reported that trace amount of MA and AP would be detected when control hair was soaked in more than 20 μg/mL or 0.5 μmol/dm3 of MA or AP solution for 24 h or overnight (12,18). It was presumed that part of the adsorbed drugs permeated into the hair shaft rather than being adsorbed on the surface. Therefore, the external contamination should not be underestimated. When a hair sample is analyzed, it is important not only to detect the metabolite of the drug but also to investigate the past behavior of a suspect.

As concentrations of MA in hair samples varied significantly because different amounts of MA were taken by abusers because of drug tolerance, the data obtained in this study were evaluated based on the concentration ratio of each compound. The concentration ratios of AP to MA (AP/MA) in the hair samples of MA abusers and SG users were 0.05–0.16 and 0.24–0.34, respectively. Nakahara (19) reported that the ratio in MA abusers’ hair did not exceed 0.2. The ratio in our experiment (0.05–0.16) was also below 0.2 and was approximately the same value that had been reported in the other papers (20–23). The ratios in SG users’ hair (0.24–0.34) in our cases were almost same as those of Cronstrand et al. (0.39), who studied long-term SG users (16), but were lower than those of Kikura and Nakahara (0.32–0.54), who reported on short-time SG users (15 mg a day during five days) (14).

In this experiment, we proved that TFA derivatives usually used for gas chromatography are also useful for LC–MS analysis, and we established a highly sensitive method that can determine MA enantiomers and AP enantiomers, DMSG and SG, simultaneously in hair samples of MA and SG users. Hair analyses showed differences in optical isomers of MA and AP and in the values of AP/MA between two groups, and it also showed the existence of DMSG in SG users, which was not al-
ways detected in urine samples. Thus, the present method should be useful to distinguish therapeutic SG users from MA abusers in forensic toxicological analysis.

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References