Quantitative Analysis of Selegiline and Three Metabolites (N-Desmethylselegiline, Methamphetamine, and Amphetamine) in Human Plasma by High-Performance Liquid Chromatography–Atmospheric Pressure Chemical Ionization-Tandem Mass Spectrometry

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Abstract

This report describes a sensitive and specific high-performance liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry method for the detection of subnanogram concentrations of selegiline and its three principle metabolites, N-desmethylselegiline, methamphetamine, and amphetamine, in human plasma. The assay has a dynamic range of 0.1–20 ng/mL for selegiline and N-desmethylselegiline (norselegiline) and 0.2–20 ng/mL for methamphetamine and amphetamine. The inter- and intra-assay precision and accuracy varied by less than 11% for all analytes at 0.3, 2.5, and 15 ng/mL and less than 16% at the lower limit of quantitation (0.1 ng/mL for selegiline and norselegiline; and 0.2 ng/mL for methamphetamine and amphetamine). Selegiline and its metabolites showed no significant loss in quantitative accuracy after three freeze/thaw cycles or after up to 6 h at room temperature prior to extraction. Extracted plasma samples retained quantitative accuracy after storage for at least 7 days at -20°C or up to 70 h at room temperature. Methanolic stock solutions were stable for at least 6 h when kept at room temperature or at least 90 days when kept at -20°C.

Introduction

Selegiline (N-methyl-(1-phenyl-2-propyl)-N-propynylamine; deprenyl) is an irreversible inhibitor of monoamine oxidase B. It is used in the treatment of Parkinson's disease and other neurological disorders (1–3). It has recently been proposed to be a useful adjunct in the treatment of cocaine addiction (4). Selegiline is metabolized in the liver to N-desmethylselegiline and methamphetamine. Either of these metabolites can be further metabolized to amphetamine, which can be followed by hydroxylation and conjugation of the metabolites. The oxidative metabolism occurs primarily in the liver by cytochrome P450 (4,5). After chronic administration 10-mg daily dose of selegiline, average serum concentrations were 19 ng/mL for l-methamphetamine, 7.5 ng/mL for l-amphetamine, and 7.4 ng/mL for N-desmethylselegiline. Selegiline concentrations were below the limit of detection for the assay method used. A fatality involving selegiline overdose resulted in postmortem femoral blood concentrations 170 ng/mL of l-methamphetamine and 70 ng/mL of l-amphetamine (6). Recently selegiline has been proposed to be a useful adjunct in the treatment of cocaine addiction (7). In order to evaluate the efficacy of selegiline in the treatment of addiction it is necessary to understand the pharmacokinetics of selegiline in subjects who have been habituated to cocaine. Because orally administered selegiline undergoes significant first pass metabolism (8) and the doses of selegiline in addiction treatment are relatively small, it is important that sensitive and specific methods exist for the analysis of selegiline and its metabolites.

Several methods have been published for the analysis of selegiline and/or various combinations of its metabolites in urine (9–18), blood and/or plasma (13,17,19–23), hair (17), and other tissues (13,19,24). Analytical methods have used liquid chromatography (25–33), gas chromatography (12,13,15,23,34–37),
electrophoresis (9,10,38) or other indirect methods (21,22). Very few of these methods analyze for the parent compound (11,17), relying on the identification of metabolites to evaluate exposure/administration. Only recently have methods utilizing liquid chromatography coupled to mass spectrometry been utilized for the analysis of selegiline metabolites in urine (15,29).

This report describes a sensitive and specific method for the analysis of selegiline and its three principle metabolites N-desmethyleselegiline (norselegiline), methamphetamine, and amphetamine in human plasma using high-performance liquid chromatography (HPLC) atmospheric pressure chemical ionization (APCI) tandem mass spectrometry (MS-MS).

This method was fully validated to comply with good laboratory practices for bioanalytical methods (39). It has a lower limit of quantitation (LLOQ) for selegiline and norselegiline of 0.1 ng/mL and an LLOQ for methamphetamine and amphetamine of 0.2 ng/mL. The analytes were also demonstrated to be stable under a variety of storage and preparation conditions. This method is currently being used to support experiments determining the pharmacokinetics and pharmacodynamics of selegiline when used as a treatment drug for cocaine addiction.

Materials and Methods

Chemicals and reagents

Selegiline, norselegiline, methamphetamine, and amphetamine and their respective deuterated isotopomers selegiline-d₆, norselegiline-d₁₁, methamphetamine-d₅, and amphetamine-d₅ were purchased from Cerilliant (Austin, TX). All had an established minimum purity of 99%. Concentrated hydrochloric acid was purchased from Mallinckrodt Specialty Chemicals Inc. (St. Louis, MO). Ammonium hydroxide was purchased from Fisher Co. (Fair Lawn, NJ). Concentrated (88%) formic acid was purchased from J.T. Baker (Phillipsburg, NJ). HPLC-grade solvents (n-butyl chloride, acetonitrile, methanol) were purchased from Burdick and Jackson (Muskegon, MI). Water used in the preparation of reagents and mobile phase was drawn from a Milli-Q filter apparatus (Millipore, Bedford, MA).

Figure 1. Product ion spectra for selegline (A), norselegline (B), methamphetamine (C), and amphetamine (D). Structures indicate where collision induced dissociation (CID) of the monitored product ion occurs. CID was achieved using argon as the collision gas at ~3mT. Offset voltage for all analytes was -19 V.
Calibrators and quality control (QC) samples

Stock solutions containing selegiline, norselegiline, methamphetamine, and amphetamine (100 ng/μL) used for the preparation of the calibration curves and QC samples were prepared in methanol and stored at -20°C. The stock solutions were used to prepare working solutions at 10, 1, 0.1, and 0.01 ng/μL of selegiline, norselegiline, methamphetamine, and amphetamine. The working solutions were used to prepare daily calibration curves and QC samples. Calibration curves were obtained by analyzing drug-free human plasma preserved with 1% NaF fortified with selegiline, norselegiline, methamphetamine, and amphetamine at 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2.5, 5, 10, and 20 ng/mL (n = 2 at each concentration). QC samples (0.1, 0.2, 0.3, 2.5, and 15 ng/mL) were prepared from stock solutions made from reference materials with different lot numbers from the reference materials used to prepare the calibrators.

Sample preparation and extraction

One milliliter each of calibrator, QC, or study sample plasma was pipetted into a labeled, silanized glass screw-cap tube. Internal standard (25 μL of a 0.1-ng/μL solution of selegiline-d₈, norselegiline-d₁₁, methamphetamine-d₅, and amphetamine-d₉) was added to each tube. Samples were vortex mixed and allowed to equilibrate for 20 min. One hundred microliters of ammonium hydroxide was added to each tube. Samples were vortex mixed again and allowed to equilibrate. Four milliliters of deuterated internal standards (25 μL of a 0.1-ng/μL solution of selegiline-d₈, norselegiline-d₁₁, methamphetamine-d₅, and amphetamine-d₉) was added to each tube. Samples were vortex mixed again and allowed to equilibrate for 20 min. One hundred microliters of 0.1% HCl in methanol was added to each tube, and the samples were mixed briefly. The organic solvent was evaporated to dryness under a stream of air at 30°C. The residues were reconstituted in 50 μL of methanol/0.1% formic acid in water (1:9) and transferred to 0.7-mL conical bottom autosampler vials. Twenty microliters of sample extract was injected into the HPLC-MS-MS.

HPLC–APCI-MS–MS analysis

HPLC–APCI-MS–MS analysis of sample extracts was performed using a ThermoFinnigan TSQ7000 (San Jose, CA) tandem MS interfaced with a Waters 626 HPLC pump and controller (Waters Corp., Milford, MA) equipped with a Leap A200S autosampler (Leap Technologies, Raleigh, NC). The HPLC mobile phase consisted of 73% water containing 0.1% formic acid and 27% methanol pumped isocratically at a rate of 0.25 mL/min at ambient temperature. Chromatographic separation of analytes was achieved using a MetaSil Basic 3 μ, 100- x 2-mm HPLC column (Metachem Technologies Inc., Lake Forest, CA).

The APCI source was operated with a vaporizer temperature of 350°C, a corona discharge current of 4.5 μA, and 20 psi of sheath gas (high purity N₂). The heated capillary was maintained at 150°C. Positive ion precursors for selegiline (m/z 188→91, RT: 4.16, NL: 1.40E3), norselegiline (m/z 196→99, RT: 4.13, NL: 5.26E3), methamphetamine (m/z 155→92, RT: 4.44, NL: 3.07E4), and amphetamine (m/z 136→91, RT: 3.99, NL: 3.09E4) were detected. The residues were reconstituted in 50 μL of methanol/0.1% formic acid in water (1:9) and transferred to 0.7-mL conical bottom autosampler vials. Twenty microliters of sample extract was injected into the HPLC-MS-MS.
Quantitative analysis

Quantitative concentrations of selegiline and its metabolites in plasma were determined by calculating peak-area ratios for the product ions of each analyte and its respective deuterated isotopomer internal standard. Linear curve fits with 1/Y weighting were used to ensure accurate quantitation across the dynamic range of the assay (0.1–20 ng/mL for selegiline and norselegiline; 0.2–20 ng/mL for methamphetamine and amphetamine). Linear curve fits with 1/Y weighting were used to ensure accurate quantitation across the dynamic range of the assay (0.1–20 ng/mL for selegiline and norselegiline; 0.2–20 ng/mL for methamphetamine and amphetamine).

Product ions monitored in the third quadrupole were m/z 91 and 92 (selegiline and selegiline-ds, respectively), m/z 91 and 98 (norselegiline and norselegiline-d11, respectively), m/z 91 and 92 (methamphetamine and methamphetamine-ds, respectively), and m/z 91 and 93 (amphetamine and amphetamine-ds, respectively). The scan time was 0.2 s/scan.

Precision and accuracy experiments

Intra-assay precision and accuracy was evaluated by analyzing QC samples at each concentration (n = 5 at each concentration) and determining the mean as a percentage of the target concentration (%target) and the percent coefficient of variation (%CV) for each analyte at each concentration. Interassay precision and accuracy were determined from five analytical runs. The mean QC values for each concentration, in each run, were used to determine %target and %CV over the five analytical runs.

Stability experiments

QC samples used for determining analyte stability were fortified prior to experimentation. For freeze-thaw stability experiments, QC samples at 0.3 and 15 ng/mL were stored at −20°C for at least 12 h. Samples were removed and allowed to thaw unassisted at room temperature. This cycle was repeated two more times prior to the day of extraction and analysis. Samples were analyzed and quantitated using a freshly prepared calibration curve and compared to quantitative values from freshly extracted QC samples to determine any loss of quantitative accuracy and precision in the freeze-thaw-cycled samples.

QC samples used for determining bench-top stability at room temperature were prepared at 6, 4, or 0 h prior to extraction. Quantitative results for the 4- and 6-h time points were compared to quantitative results for the 0-h time point to determine any loss of quantitative precision and accuracy. QC samples at 0.3 and 15 ng/mL were used for processed sample stability. Prepared extracts were analyzed initially to determine concentrations. The analyzed extracts were then stored at either −20°C or on the autosampler at room temperature to determine frozen and autosampler storage limits for prepared extracts, respectively. Samples were re-injected with a freshly prepared calibration curve and quantitated using both the fresh and stored curves to evaluate any loss of quantitative accuracy and precision in the stored samples.

To determine stock solutions' stability, stock solutions prepared at the beginning of method development were compared with stock solutions prepared at the conclusion of validation experiments. In addition, aliquots from the new stock solution were evaluated for room temperature stability at 4 and 6 h. The methanolic stock solutions were appropriately diluted and analyzed directly by HPLC-APCI-MS-MS without extraction.

Recovery experiments

Analyte recovery was determined at 0.3, 2.5, and 15 ng/mL. Internal standard recovery was determined at 2.5 ng/mL. One set of drug-free plasma (n = 5 at each concentration) was fortified with analyte and processed through the extraction procedure. A second set (n = 5 at each concentration) was only fortified with analyte (i.e., no matrix). This second set was not extracted, but the methanolic solutions were evaporated and reconstituted in 0.1% formic acid in water/methanol (9:1). Percent recovery was determined at each concentration by di-

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<th>Table I. Intra-assay Precision and Accuracy*</th>
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<td>QC Target Concentration (ng/mL) % Target ± % CV Selegiline Norselegiline Amphetamine Methamphetamine</td>
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<tr>
<td>LLOQ 0.1/0.2 100.0 ± 13.0 108.0 ± 12.0 116.5 ± 3.6 118.5 ± 3.8</td>
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<tr>
<td>QC1 0.3 102.3 ± 5.9 112.7 ± 1.5 112.0 ± 2.7 105.7 ± 7.9</td>
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<tr>
<td>QC2 2.5 103.2 ± 5.0 105.6 ± 4.5 110.0 ± 2.3 106.8 ± 3.4</td>
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<tr>
<td>QC3 15 102.0 ± 5.2 104.7 ± 2.5 110.0 ± 3.6 99.3 ± 5.4</td>
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* N = 5 at each concentration. Mean values at each concentration were used to calculate the % target and the %CV. LLOQ for selegiline and norselegiline = 0.1 ng/mL, and methamphetamine and amphetamine = 0.2 ng/mL.

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<th>Table II. Inter-assay Precision and Accuracy*</th>
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<td>QC Target Concentration (ng/mL) % Target ± % CV Selegiline Norselegiline Amphetamine Methamphetamine</td>
</tr>
<tr>
<td>LLOQ 0.1/0.2 107.0 ± 8.4 110.0 ± 6.4 111.5 ± 5.8 116.0 ± 2.2</td>
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* Determined from five analyses on five different days. The mean QC values for each concentration, in each run, were used to determine %target and %CV over the five runs. LLOQ for selegiline and norselegiline = 0.1 ng/mL, methamphetamine and amphetamine = 0.2 ng/mL.
viding the average peak area at each concentration from the extracted samples by the average peak area for each concentration from the unextracted samples and multiplying by 100.

Specificity experiments
Specificity of the method was evaluated by analyzing blank human plasma collected from six volunteers who had not been administered selegiline or other structurally similar drugs. Each individual plasma sample (n = 3 aliquots per individual) was spiked with internal standard; an additional aliquot from each individual containing no analyte or internal standard was also prepared. These samples were extracted and analyzed by the described method. Any signal observed at the retention times for each analyte was compared to the signal at the LLOQ for each analyte.

Results and Discussion

Analytical method
The product ion spectra for selegiline, norsesegiline, methamphetamine, and amphetamine are shown in Figures 1A–1D. Although a common fragment ion corresponding to the benzylic moiety is the predominant product ion after CID, the fact that each is derived from a unique precursor ion ensures that each ion current collected is unique to each analyte. Figure 2 shows an extracted ion chromatogram from an extracted blank sample demonstrating that no discernable interference is attributable to the plasma matrix. Figure 3 shows an extracted ion chromatogram from a QC sample fortified at 0.2 ng/mL. All traces show gaussian peak shape and good signal-to-noise ratios. Although the ion current peaks for methamphetamine and amphetamine are not completely resolved chromatographically, they do have unique retention times and are derived from unique MS–MS transitions. A MetaSil Basic column was selected because it was able to more efficiently resolve all four analytes within a reasonable time frame and with minimal tailing compared to traditional C18 bonded phases. During method development, conventional ionization (ESI) was compared with APCI. It was determined that APCI resulted in a much better signal-to-noise ratio for methamphetamine and amphetamine and was therefore chosen over ESI for this method. ESI also exhibited a noticeable ion suppression effect that made it less favorable to APCI.

Method validation experiments
Precision and accuracy. Intra-assay precision and accuracy are summarized in Table I. All analytes were within 18.5% of the target at the LLOQ and within 13% at the higher QC concentrations. %CVs were less than 14% for the LLOQ and less than 8% for the higher QC concentrations. Interassay precision and accuracy were also acceptable, as shown in Table II. All analytes were within 16% of the target at the LLOQ and within 11% at the higher QC concentrations. The %CVs were less than 9% at all concentrations tested.

Specificity and recovery. Specificity experiments demonstrated that no substantial interferences were observed that could be attributable to the matrix at the retention times observed for each analyte. When ion currents at the retention time of the analytes were quantitated and the resulting peak-area ratios compared to that of the LLOQ, no signal greater than 8.6% of the LLOQ was found for any analyte. Figure 2 shows a representative extracted blank chromatogram fortified with internal standards only.

Recoveries of selegiline and its metabolites from human subjects ranged from 85% to 109% and are summarized in Table III. The fact that amphetamine was recovered in excess of 100% at 0.3 ng/mL is likely a technical artifact of the technique employed.

Stability. The stability of selegiline and its metabolites in human plasma was evaluated under a number of conditions. The conditions were chosen to mimic real-life handling of study samples under conditions that would likely be encountered under normal laboratory operating situations. Currently no other studies are available which assess the stability of selegiline under these conditions. At room

| Table III. Recovery of Selegiline and Metabolites Extracted from Human Plasma* |
|----------------------------------|------------------|------------------|------------------|
| % Recovery Non-deuterated Analytes | % Recovery Internal Standard |
| 0.3 ng/mL | 2.5 ng/mL | 15 ng/mL | 2.5 ng/mL |
| Selegiline | 94.5 | 89.0 | 91.9 | 72.7 |
| Norselegiline | 86.0 | 91.5 | 96.7 | 70.8 |
| Amphetamine | 109.1 | 96.0 | 99.1 | 71.2 |
| Methamphetamine | 88.4 | 85.1 | 94.0 | 66.5 |

* N = 5 at each concentration. Values calculated by dividing the mean peak area at each concentration for extracted samples by the mean peak area for each concentration for unextracted samples and multiplying by 100.

| Table IV Stability of Selegiline and Metabolites in Plasma Stored at Room Temperature and following Three Freeze-Thaw Cycles* |
|----------------------------------|------------------|------------------|------------------|
| Analyte | Target Concentration (ng/mL) | Hours at Room Temperature (% Target) | Three Freeze-Thaw Cycles (% Target) |
| 0 | 4 | 6 |
| Selegiline | 0.3 | 95.3 | 95.7 | 91.7 | 106.7 |
| | 15 | 90.7 | 97.3 | 102.0 | 99.3 |
| Norselegiline | 0.3 | 89.0 | 101.3 | 101.0 | 102.7 |
| | 15 | 86.0 | 93.3 | 98.0 | 98.0 |
| Amphetamine | 0.3 | 107.3 | 98.0 | 99.3 | 105.3 |
| | 15 | 104.0 | 110.7 | 112.0 |
| Methamphetamine | 0.3 | 108.7 | 112.3 | 111.7 | 111.0 |
| | 15 | 96.0 | 103.3 | 106.0 | 101.3 |

* N = 3 at each concentration. Values represent the % target of the mean.
temperature, QC samples at 0.3 and 15 ng/mL ($n = 3$ at each concentration and time point) were allowed to sit for the time it took to prepare the calibration curves (time zero) plus an additional 4 or 6 h. The stability of selegiline and metabolites after three freezing-thawing cycles were also evaluated in QC samples at 0.3 and 15 ng/mL ($n = 3$ at each concentration). Under these conditions 94 of 96 quantitative results were within ±15% of the target concentration (data not shown), and there were no time-related changes relative to the 0-h control (Table IV). These results demonstrate that selegiline and its metabolites are stable under these laboratory-handling conditions.

Processed sample stability was determined by comparing the quantitative results from an initial analysis to the quantitative results obtained when the sample extracts were reanalyzed after a storage period of either 7 days at −20°C or 70 h at room temperature on the instrument autosampler. The reanalyzed batch data was also quantitated using a freshly prepared and analyzed calibration curve. Results indicated that selegiline and metabolites, on average, accurately quantitated to within 20% of the target concentration at the LLOQ and within 15% of the target concentration for the higher QC concentrations with both the reanalyzed calibration curve and the freshly prepared calibration curve after either storage scenario (Table V). These results indicate that extracts of selegiline and metabolites are stable under the storage conditions and time-frames described.

Stock solution stability was determined by comparing the peak-area ratios of selegiline and metabolites to their respective internal standard obtained from methanolic stock solutions that had either been stored at −20°C for 90 days or at room temperature for 6 h. These stored solutions responses were compared with freshly prepared methanolic stock solutions using freshly prepared internal standard solutions to determine peak-area ratios. Internal standard was added just prior to analysis and was not subjected to the storage interval. Results indicated (Table VI) that no significant loss in response was observed for selegiline or its metabolites when methanolic solutions are stored for extended periods of time in a freezer (i.e., 90 days at −20°C) or when left out on a benchtop at room temperature for at least 6 h.

## Conclusions

This report describes a sensitive and specific method for the analysis of selegiline and three of its principle metabolites (norselegiline,
methamphetamine, and amphetamine) in plasma using HPLC–APCI-MS–MS. The assay has a LLOQ of 0.1 ng/mL for selegiline and norselegiline and 0.2 ng/mL for methamphetamine and amphetamine. The assay was shown to have good quantitative precision and accuracy under a number of storage and processing conditions intended to mimic normal sample handling situations. This method has been validated and accepted for use in the analysis of clinical samples collected in the process of evaluating selegiline as an effective therapeutic adjunct in the treatment of cocaine addiction.

Acknowledgment

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References


