Rapid and sensitive determination of donepezil in human plasma by liquid chromatography/tandem mass spectrometry: application to a pharmacokinetic study

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A liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was developed and validated for the determination of donepezil in human plasma samples. Diphenhydramine was used as the internal standard. The collision-induced transition \( m/z 380 \rightarrow 91 \) was used to analyze donepezil in selected reaction monitoring mode. The signal intensity of the \( m/z 380 \rightarrow 91 \) transition was found to relate linearly with donepezil concentrations in plasma from 0.1–20.0 ng/mL. The lower limit of quantification of the LC/MS/MS method was 0.1 ng/mL. The intra- and inter-day precisions were below 10.2% and the accuracy was between −2.3% and +2.8%. The validated LC/MS/MS method was applied to a pharmacokinetic study in which healthy Chinese volunteers each received a single oral dose of 5 mg donepezil hydrochloride. The non-compartmental pharmacokinetic model was used to fit the donepezil plasma concentration-time curve. Maximum plasma concentration was 12.3 ± 2.73 ng/mL which occurred at 3.50 ± 1.61 h post-dosing. The apparent elimination half-life and the area under the curve were, respectively, 60.86 ± 12.05 h and 609.3 ± 122.2 ng·h/mL. LC/MS/MS is a rapid, sensitive and specific method for determining donepezil in human plasma samples. Copyright © 2006 John Wiley & Sons, Ltd.

Alzheimer’s disease accounts for about 50% of clinically diagnosed dementia. Alzheimer’s dementia is a neurodegenerative disease characterized by progressive cognitive impairment. Mild to moderate Alzheimer’s dementia is usually treated with donepezil hydrochloride, \( \{2,3\text{-dihydro-5,6-dimethoxy-2-[1-(phenylmethyl)-4-piperidinyl]methyl}-1H\text{-inden-1-one}\} \) hydrochloride (Fig. 1), to slow down the degenerative progression of the disease and the patients’ cognitive impairment. Donepezil is an acetylcholinesterase inhibitor which enhances the acetylcholine level of the brain and its cholinergic function.

In humans, donepezil is metabolized mainly by the hepatic cytochrome P-450 2D6 and 3A4 isozymes. Elimination of donepezil from the blood is characterized by a dose-independent elimination half-life of about 70 h. Because plasma donepezil concentrations are related linearly to acetylcholinesterase inhibition, plasma donepezil concentration is a useful tool to predict donepezil efficacy.

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EXPERIMENTAL

Chemicals and reagents
Donepezil hydrochloride (purity ≥ 99.9%) and diphenhydramine hydrochloride (purity ≥ 99.8%) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol, acetonitrile and formic acid were purchased from Tedia Company Inc. (Beijing, China). All other reagents were of analytical grade. Blank human plasma was obtained from the Guangzhou Blood Donor Service (Guangzhou, China). Ultra-pure water was obtained from a Milli Q-plus system (Billerca, MA, USA).

Equipment
The LC/MS/MS system consisted of a Surveyor MS pump, a Surveyor autosampler (ThermoFinnigan, USA) and a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. Data acquisition was performed with Xcalibur 1.3 software (ThermoFinnigan, USA). Peak integration and calibration were performed using LCQuan software (ThermoFinnigan, USA).

Chromatographic conditions
Chromatographic separation was achieved using an Aquasil C18 column (150 mm × 2.1 mm i.d., 5 μm; Thermo Finnigan, USA) with a 4.0 mm × 2.0 mm i.d. Security Guard C18 (5 μm) guard column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of methanol/acetonitrile/1% formic acid (70:10:20, v/v/v), delivered at a flow rate of 0.3 mL/min. The column temperature was maintained at 25°C.

Mass spectrometric conditions
The mass spectrometer was operated in the positive mode. Quantification was performed using selected reaction monitoring (SRM) of the transitions of m/z 380 → 91 for donepezil and m/z 256 → 167 for diphenhydramine (IS), respectively, with a scan time of 0.3 s per transition. The tuning parameters were optimized for donepezil and IS by infusing a solution, containing 1 μg/mL of each analyte, at a flow rate of 10 μL/min into the mobile phase (0.3 mL/min) using a post-column T' connection. The optimal MS parameters obtained were as follows: the spray voltage was 3500 V with a source collision-induced dissociation (CID) voltage of 10 V, the heated capillary temperature was 350°C. Nitrogen was used as the sheath gas (50 psi) and auxiliary gas (12 psi). Argon was used as the collision gas at a pressure of approximately 1.2 mTorr (1 Torr = 133.3 Pa). The optimized collision energies chosen for donepezil and IS were 33 and 10 eV, respectively. Figure 2 shows the product ion mass spectra of the [M+H]+ ion of donepezil and IS.

Preparation of stock and working solutions
The stock solutions of donepezil (400 μg/mL) and diphenhydramine (400 μg/mL) were prepared in methanol and serially diluted to produce a 4 μg/mL stock solution in methanol/water (50:50, v/v). The donepezil stock solution was then diluted to give working solutions of 1, 2, 8, 20, 50, 100 and 200 ng/mL in methanol/water (50:50, v/v). Diphenhydramine working solution (200 ng/mL) was also prepared by diluting the 400 μg/mL stock solution of diphenhydramine with methanol/water (50:50, v/v). All stock solutions and working solutions were stored at 4°C.

Preparation of calibration standards and quality control (QC) samples
Calibration curves were prepared by spiking 50 μL of the appropriate working solution to 500 μL blank human plasma. Effective donepezil concentrations in plasma samples were 0.1, 0.2, 0.8, 2.0, 5.0, 10.0, and 20.0 ng/mL. The QC samples used in the validation and during the pharmacokinetic (PK) study were prepared in the same way as the calibration standards. The nominal donepezil plasma concentrations of QC samples were 0.2, 2.0 and 20.0 ng/mL. The spiked plasma samples (standards and QC samples) were extracted in each analytical batch along with the unknown samples.

Sample preparation
To a 500-μL aliquot of plasma sample, 50 μL of IS (200 ng/mL diphenhydramine), 50 μL of methanol/water (50:50, v/v) and 0.5 mL of 10 mmol/L phosphate buffer (pH 14) were

Figure 1. Structures of donepezil (I) and diphenhydramine (II), the internal standard.

Figure 2. Product ion mass spectra of [M+H]+ ions of (A) donepezil and (B) diphenhydramine (internal standard).
added. The sample was briefly mixed and ethyl acetate (3 mL) was added. The mixture was vortex-mixed for approximately 1 min, then shaken on a mechanical shaker for 15 min. After centrifugation at 3000 rpm for 10 min, the upper organic layer was removed and evaporated to dryness at 40°C under a gentle stream of nitrogen. The dry residue was reconstituted in 200 μL of the mobile phase, then vortex-mixed. A 10-μL aliquot of the resulting solution was injected onto the LC/MS/MS system.

**Method validation**

The method was validated by linearity, LLOQ, accuracy and precision. To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on three separate days. Accuracy and precision were also assessed by determination of QC samples using six replicate preparations of plasma samples at three concentration levels (Table 1) for donepezil on three validation days. Accuracy was expressed by relative error (RE) and precision by relative standard deviation (RSD).

The LLOQs, defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, were evaluated by analyzing samples which were prepared in six replicates as follows: spiking 50 μL of the standard solution, containing 1 ng/mL donepezil, to 500 μL of blank human plasma.

For the determination of extraction recovery, blank human plasma was processed according to the sample preparation procedure as described above. The organic layer was evaporated to dryness, and dry extracts were reconstituted in the mobile phase adding appropriate standards at concentrations corresponding to the final concentration of the extracted plasma samples. These spike-after-extraction samples represented 100% recovery. The extraction recoveries of donepezil were determined by comparing the mean peak areas of six extracted low, medium and high QC samples to mean peak areas of six spike-after-extract samples at the same concentrations. Recovery of IS was also evaluated by comparing the mean peak areas of six extracted medium QC samples to mean peak areas of six reference solutions spiked in extracted plasma samples of the same concentrations.

Stability of processing (three freeze/thaw cycles, benchtop for 4 h), chromatography (re-injection) and sample storage (−20°C for 45 days) was assessed by analyzing triplicates (n = 3) of QC samples (at the concentrations of 0.2, 2.0 and 20.0 μg/mL for donepezil), which were exposed to different time and temperature conditions. The results were compared with those QC samples freshly prepared, and the percentage concentration deviation was calculated.

**Application to PK study**

The method was applied to determine the plasma concentrations of donepezil from a clinical trial study in which 20 healthy male volunteers each received orally a tablet containing 5 mg donepezil hydrochloride. Venous blood samples (5 mL) were withdrawn from each volunteer and placed into heparinized tubes according to the following time schedule: 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 48.0, 96.0, 144.0 and 192.0 h post-dosing. Blood samples were centrifuged immediately at 3500 rpm for 10 min to obtain the plasma. The plasma samples were labeled and kept frozen at −20°C until analysis. The volunteers were all adult Chinese who were selected after completing a thorough medical, biochemical and physical examination. Informed consent was obtained from all subjects after explaining the aims and risks of the study. The study protocol was approved by the Human Investigation Ethical Committee at the School of Pharmaceutical Sciences at Central South University, Changsha, China.

The plasma concentration-time profiles obtained from the experimental subjects were analyzed by non-compartmental analysis using the TopFit 2.0 software package (Thomae GmbH, Germany). Maximum plasma concentration (C max) and the time-to-maximum concentration (t max) were estimated by visual inspection of semi-logarithmic plots of the concentration-time curves. The area under the curve (AUC 0→∞) was calculated using the linear-trapezoidal rule, with extrapolation to infinity (AUC 0→∞) from the last detectable concentration using the terminal elimination rate constant (k e) calculated by linear regression of the final log-linear part of the drug concentration-time curve. Apparent elimination half-life (t 1/2) was calculated as t 1/2 = 0.693/k e.

**RESULTS AND DISCUSSION**

**Mass spectrometry**

Electrospray ionization (ESI) was used in the present study because ESI mass spectra of donepezil are more sensitive than atmospheric pressure chemical ionization (APCI) spectra in detecting donepezil (data not shown). Following the selection of the ESI source, donepezil and diphenhydramine, respectively, were found to form protonated molecules [M+H] + at m/z 380 and 256 that could be used as precursor ions for the analysis of donepezil. Figure 2 shows the product ion mass spectra of the [M+H] + ion of donepezil and diphenhydramine; fragment ions at m/z 91 and 167 were observed for donepezil and diphenhydramine, respectively. Based on the results of these studies, the transitions of m/z 380 →91 for donepezil and m/z 256 →167 for diphenhydramine were used to analyze the human plasma donepezil samples in selected reaction monitoring (SRM) mode. Several LC/MS/MS parameters were optimized to obtain the maximum sensitivity for the m/z 380 →91 transition. The collision energy in this study was set at 30 eV because the collision behavior of the [M+H] + ion of donepezil was found to depend strongly on this parameter. In contrast, the capillary temperature and the spray voltage

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**Table 1. Accuracy and precision for donepezil at the plasma concentrations of the LLOQ**

<table>
<thead>
<tr>
<th>Nominal plasma conc. (ng/mL)</th>
<th>Mean measured conc. (ng/mL)</th>
<th>RE (%)</th>
<th>Intra-day RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>0.09</td>
<td>2.05</td>
<td>6.90</td>
</tr>
<tr>
<td>0.10</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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were left unchanged at the recommended values of 350°C and 3.5 kV, respectively, because they were not found to affect the detection sensitivity of the analytes significantly. Other LC/MS/MS parameters including the source CID and the collision pressure also were maintained at the auto-tuned values since they did not significantly affect the spectral behavior of the analytes.

HPLC mobile phase

The retention times of donepezil and diphenhydramine in the HPLC chromatogram were 2.0 and 2.2 min, respectively. The HPLC mobile phase consisted of a solution of methanol, acetonitrile and 1% formic acid because: (1) the composition of the solvents could reduce the run time of each sample to about 2.8 min which was much shorter than most methods reported for donepezil analysis;19 (2) the use of 70% methanol provided a low background chromatogram and a rapid separation of donepezil and diphenhydramine; (3) the addition of a small amount of formic acid in the mobile phase improved analyte detection and sensitivity; and (4) a symmetrical peak was achieved only after adding 10% acetonitrile to the mobile phase.

Sample preparation

Attempts were made to prepare the plasma samples using either protein precipitation or liquid-liquid extraction (LLE). Protein precipitants such as methanol, acetonitrile, ethanol and acetone produced sample extracts that reduced the sensitivity of the mass spectral detector (data not shown). Different LLE extraction conditions also were evaluated in order to reduce extraction time and improve donepezil recovery. These included the use of different organic solvents and aqueous pH modifiers such as NH₃ buffer (10 mmol/L, pH 8), 0.5 mol/L Na₂CO₃, 1.0 mol/L NaOH, and 10 mmol/L phosphate buffer (pH 14) in the extraction. Phosphate buffer (pH 14) was found to be the best aqueous pH modifier for the LC/MS/MS method; plasma analyte and IS in phosphate buffer could be extracted by ethyl acetate with a high recovery (≥65% for donepezil and IS) and a small standard deviation (SD ≤7.4%).

Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma with the chromatograms from the same plasma samples but that had been spiked with 0.1 ng/mL of donepezil (LLOQ concentration). Figure 3 shows a typical chromatogram of the plasma blank, the spiked plasma sample, and the 96 h plasma sample from a healthy volunteer after receiving donepezil. As shown in Fig. 3, no endogenous chemical peak was found to interfere with the analysis of the analytes.

Matrix effects

Although matrix-matched calibration standards were used in the analytical procedure, matrix effects due to co-eluted endogenous substances might reduce the selectivity, sensitivity, accuracy or precision of the assay. Therefore, an assessment of matrix effects (MEs) was conducted according to the procedure of Matuszewski et al.24 the absolute MEs of five different batches of blank plasma were evaluated by comparing the peak areas of donepezil (0.2, 2.0, and 20 ng/mL) and the IS (20 ng/mL) in five QC samples spiked post-extraction with standard solutions that had been prepared in the same way as the QC samples except that water was substituted for drug-free plasma. For donepezil, the absolute MEs were found to be between −96.8% and +103.8%. For the IS, the absolute ME was 3.7%. These observations indicated that no endogenous substances significantly affect the ionization of these analytes. The relative standard deviations (RSDs) of absolute MEs at different concentrations of donepezil in the five batches of human plasma were below 8.2%. For the IS, the RSD of absolute ME was below 6.2%. These data also indicate that the relative MEs for donepezil and IS are small in this assay.

Linearity of calibration curve and LLOQ

Peak area ratio versus donepezil concentration plots were analyzed by the least-squares linear regression with a

![Figure 3. Representative SRM chromatograms for (A) drug-free plasma; (B) plasma (500 μL) spiked with 0.1 ng/mL of donepezil and 20 ng/mL of internal standard (IS); and (C) a volunteer’s 96 h plasma sample after an oral dose of 5 mg donepezil hydrochloride. I: donepezil; II: diphenhydramine (IS).](image-url)
weighting of \(1/x^2\). The straight line could be described by the equation, \(y = 1.187 \times 10^{-3} + 4.749 \times 10^{-2}x\) with \(r^2 = 0.9937\), where \(y\) represents the ratios of the donepezil peak area to that of diphenhydramine and \(x\) represents the plasma concentrations of donepezil. Based on the results of this analysis, peak area ratio is found to be linearly related to plasma donepezil concentrations ranging from 0.1 to 20.0 ng/mL.

The LLOQ was established at 0.1 ng/mL for donepezil, which was sufficient for clinical PK studies in humans following oral administration of donepezil. The precision and accuracy values corresponding to the LLOQ are shown in Table 1.

**Table 1.** Accuracy and precision results for donepezil in human plasma (3 days, 6 replicates per day)

<table>
<thead>
<tr>
<th>Nominal plasma conc. (ng/mL)</th>
<th>Mean measured conc. (ng/mL)</th>
<th>Intra-day RE (%)</th>
<th>Inter-day RE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>0.20</td>
<td>-2.25</td>
<td>8.58</td>
</tr>
<tr>
<td>2.00</td>
<td>2.02</td>
<td>0.83</td>
<td>3.55</td>
</tr>
<tr>
<td>20.00</td>
<td>20.56</td>
<td>2.79</td>
<td>3.84</td>
</tr>
</tbody>
</table>

The intra-day and inter-day precisions were less than 10.2% for each QC level of donepezil. The accuracy, determined from QC samples, was within \(\pm 10.2\%\) for each QC level of donepezil. The results are summarized in Table 2.

**Table 2.** Accuracy and precision results for donepezil in human plasma (3 days, 6 replicates per day)

<table>
<thead>
<tr>
<th>Nominal plasma conc. (ng/mL)</th>
<th>Mean measured conc. (ng/mL)</th>
<th>Intra-day RSD (%)</th>
<th>Inter-day RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>0.20</td>
<td>8.58</td>
<td>10.18</td>
</tr>
<tr>
<td>2.00</td>
<td>2.02</td>
<td>3.55</td>
<td>7.51</td>
</tr>
<tr>
<td>20.00</td>
<td>20.56</td>
<td>3.84</td>
<td>4.33</td>
</tr>
</tbody>
</table>

**Precision and accuracy**

Precision and accuracy of the assay were determined by performing the complete analytical runs of replicate QC samples \((n = 6)\) at three concentrations on the same day and on the three consecutive days. The data from these QC samples were examined by a one-way analysis of variance (ANOVA). The intra-day and inter-day precisions were less than 10.2% for each QC level of donepezil. The accuracy, determined from QC samples, was within \(\pm 2.8\%\) for each QC level. The results are summarized in Table 2.

**Extraction recovery and stability**

Extraction recoveries for donepezil at 0.2, 2.0 and 20.0 ng/mL were 66.9 \(\pm 7.4\%\), 65.5 \(\pm 3.1\%\) and 66.7 \(\pm 2.5\%\) \((n = 3)\), respectively. The RSDs of extraction recoveries were better than 7.4% for the entire standard concentration ranges, indicating consistent recoveries of donepezil from plasma samples. Extraction recovery for the IS (20 ng/mL) was 72.0 \(\pm 2.6\%\) \((n = 3)\).

The stability of donepezil in human plasma was also investigated under a variety of storage and process conditions. The analyte was found to be stable \((RE < 8.6\%)\) in human plasma after three freeze \((-20^\circ C)/thaw\) (room temperature) cycles. The analyte also was stable after being left on the laboratory bench for 4 h at room temperature \((RE < 8.8\%)\) and in reconstitution solutions at room temperature for 24 h \((RE < 7.3\%)\). Therefore, frozen plasma samples could be kept at \(-20^\circ C\) for at least 45 days \((RE < 5.8\%)\) without significant donepezil degradation.

**Application of the method to a PK study in healthy volunteers**

The validated LC/MS/MS method was used to obtain the plasma kinetic profile of donepezil in humans after receiving an oral dose of 5 mg donepezil hydrochloride. Figure 4 shows the mean donepezil plasma concentration versus time curve. Table 3 lists the pharmacokinetic parameters of donepezil.

**Table 3.** Pharmacokinetic parameters of donepezil in humans after receiving a single oral dose of 5 mg donepezil hydrochloride

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Oral of 5 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C_{max}) (ng/mL)</td>
<td>12.3 (\pm 2.73)</td>
</tr>
<tr>
<td>(T_{max}) (h)</td>
<td>3.50 (\pm 1.61)</td>
</tr>
<tr>
<td>(t_{1/2}) (h)</td>
<td>6.08 (\pm 1.20)</td>
</tr>
<tr>
<td>(k_e) (L/h)</td>
<td>0.11 (\pm 0.002)</td>
</tr>
<tr>
<td>(AUC_{0-\infty}) (ng·h/mL)</td>
<td>809.3 (\pm 122.2)</td>
</tr>
<tr>
<td>(AUC_{0-12}) (ng·h/mL)</td>
<td>989.6 (\pm 233.7)</td>
</tr>
</tbody>
</table>

**CONCLUSIONS**

An LC/MS/MS method with an ESI interface was developed and validated for determining donepezil in human plasma. The method had several advantages over the previously reported methods; it was rapid, sensitive, robust and highly selective. Also, this method has a short run time (about 2.8 min) and requires only a simple sample preparation procedure. In our hands, more than 150 samples were analyzed in one day. The method was successfully applied to determine plasma donepezil concentrations in a pharmacokinetic study involving healthy Chinese volunteers. The pharmacokinetic profile in this study was similar to those reported in previous studies.

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