Determination of ifenprodil by LC–MS/MS and its application to a pharmacokinetic study in healthy Chinese volunteers

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Abstract This paper reports the development and validation of an assay for ifenprodil based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) and its application to a pharmacokinetic study involving single and multiple intravenous infusions to healthy Chinese volunteers. After sample preparation of plasma by liquid–liquid extraction with ethyl acetate, the analyte and internal standard, urapidil, were separated by reversed phase chromatography in a run time of 4 min and detected by positive ion electrospray ionization followed by multiple reaction monitoring of the precursor-to-product ion transitions at m/z 326.2→308.1 for ifenprodil and m/z 388.4→205.3 for IS. The assay was linear in the concentration range 0.2–50.0 ng/mL with recovery ≥76.4%. In the pharmacokinetic study of single intravenous infusions of 5, 10 and 15 mg ifenprodil, peak plasma concentrations and areas under the plasma concentration–time curve were both linearly related to dose. In the pharmacokinetic study of multiple once daily intravenous infusions of 10 mg ifenprodil for 7 days, pharmacokinetic parameters were similar to those after the single dose showing that ifenprodil does not accumulate on repeated administration.

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1. Introduction

Ifenprodil [(1RS, 2SR)-4-[2-(4-benzylpiperidin-1-yl)-1-hydroxypropyl]phenol hemi-(2R,3R)-tartrate] (Fig. 1A) is a new class of N-methyl-D-aspartate (NMDA) receptor antagonist that selectively inhibits NMDA receptors containing the NR2B subunit\(^1\). The drug has been widely used in the treatment of cerebrovascular diseases and peripheral arterial obliterative disease\(^2\). However, pharmacokinetic studies of ifenprodil are limited to one involving a single dose administration\(^6\) and there is no literature reporting the pharmacokinetic studies of ifenprodil after multiple doses. The aim of the present study was to develop and validate an assay for ifenprodil based on liquid chromatography–tandem mass spectrometry (LC–MS/MS)\(^7\) and apply it to a pharmacokinetic study of ifenprodil involving single and multiple intravenous infusions to healthy Chinese volunteers.

2. Materials and methods

2.1. Reagents and materials

Ifenprodil tartrate for injection and ifenprodil reference standard (purity 98.7%) were obtained from Sihuan Kebao Pharmaceutical Co., Ltd. (Beijing, China). Urapidil for use as internal standard (IS) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). Water was deionized and purified using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). All other chemicals and reagents were of analytical grade and used as received. Drug-free human plasma from healthy volunteers was kindly provided by the Blood Center of Xijing Hospital (Shanxi, China) and was stored at \(-20^\circ C\) until use.

2.2. Instrumentation

LC–MS/MS was performed using an Agilent 1200 series HPLC equipped with a S Burril C18-EP column (150 mm x 2.1 mm i.d., 3 \(\mu\)m) maintained at 35 \(^\circ\)C and an Agilent 6410 Triple Quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent Technologies, USA) operating in the positive ionization mode. All data were acquired and processed using Agilent 6410 Quantitative Analysis processing software.

2.3. Preparation of the calibration standards and quality control (QC) samples

A stock solution of ifenprodil was prepared in methanol and serially diluted with methanol to give standard solutions with concentrations of 0.01, 0.1, 1.0, 10.0 and 100.0 \(\mu\)g/mL. In a similar manner, an IS working solution (203.2 ng/mL) was prepared by diluting a stock solution of urapidil (101.6 \(\mu\)g/mL). All solutions were kept at \(-20^\circ C\) until use. Calibration standards (0.02, 0.50, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 ng/mL) and QC samples (0.5, 4 and 40 ng/mL) were prepared by spiking 0.5 mL blank plasma samples with different volumes of working solutions.

2.4. Sample preparation

Frozen human plasma samples were thawed at ambient temperature and 0.5 mL aliquots mixed with 50 \(\mu\)L IS working solution. The mixture was subsequently subjected to liquid–liquid extraction (LLE) by vortex-mixing for 3 min with 4 mL ethyl acetate and centrifuging at 4000 rpm for 10 min. The organic layer was then removed and evaporated under a stream of nitrogen gas at 40 \(^\circ\)C. The residue was reconstituted in 120 \(\mu\)L mobile phase, vortex-mixed for 1 min, centrifuged at 16,000 rpm for 3 min and 5 \(\mu\)L of supernatant injected into the LC–MS/MS system.

2.5. LC–MS/MS conditions

The mobile phase was methanol–10 mM ammonium acetate solution (90:10, \(v/v\)) delivered at a flow rate of 0.2 mL/min. Detection was by multiple reaction monitoring (MRM) of the precursor-to-product ion transitions at \(m/z\) 326.2 \(\rightarrow\) 308.1 for ifenprodil and \(m/z\) 388.4 \(\rightarrow\) 205.3 for IS. Optimized MS parameters were as follows: nebulizer pressure 40 psi; drying gas temperature 350 \(^\circ\)C; dry gas flow rate 10 L/min; dwell time per transition 200 ms; EMV 200 V; fragmentor voltage 140 V (ifenprodil) and 150 V (IS); collision energies 19 eV (ifenprodil) and 45 eV (IS).

2.6. Assay validation

Assay validation was carried out according to the USFDA guidelines\(^8\). Specificity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Linearity was determined by weighted \((1/C^2)\) least-squares linear regression of calibration curves based on eight calibration standards prepared from three batches of plasma. The acceptance criteria for the validation were a correlation coefficient \((r) \geq 0.99\) and that each back-calculated concentration was within 15\% of the nominal value. The lower limit of quantitation (LLOQ) was defined as the lowest concentration on the calibration curve at which the precision (as relative standard deviation, RSD) was 20\% and accuracy (as relative error, RE) was \(\pm 20\%). Precision and accuracy of the method were determined by assay of 5 replicates of QC samples on three validation days. Intra- and inter-batch precisions (as RSD calculated by one-way analysis of variance) were required to be \(< 15\%\) with accuracy (as RE) of \(\pm 15\%). Recovery of ifenprodil was determined by comparing peak areas for ifenprodil in 5 replicates of QC samples with those of blank plasma extracts to which ifenprodil was added after extraction at corresponding levels.

Figure 1 Chemical structures of (A) ifenprodil and (B) urapidil.
concentrations. Matrix effects were evaluated by comparing the peak areas of ifenprodil in 5 replicates of QC samples with those for the analyte dissolved in mobile phase. If the peak area ratio is <85% or >115%, a matrix effect is implied. Stability of ifenprodil was evaluated in 5 replicates of QC samples stored under the following conditions: short-term stability at room temperature for 24 h; long-term stability at −20 °C for 30 days; freeze-thaw stability after 3 freeze-thaw cycles. Post-preparative stability of processed samples was also assessed after storage at room temperature for 20 h.

2.7. Pharmacokinetic study

The pharmacokinetics of ifenprodil were determined after intravenous infusions of ifenprodil tartrate for injection to healthy Chinese volunteers in accordance with the Declaration of Helsinki (2008) for biomedical research involving human subjects and Good Clinical Practice. The protocol and associated informed consent statements were reviewed and approved by the Committee on Human Rights Related to Human Experimentation at Xijing Hospital. All volunteers signed the informed consent statements.

Thirty healthy Chinese healthy subjects (15 males, 15 females aged 29–40, body weight 56–68 kg) were included in the study. They had no history of cardiovascular, hepatic, renal, gastrointestinal, hematologic, nervous, or any acute or chronic diseases or drug allergy, and were not allowed to take drugs during the 2 weeks before the study. Physical examination and laboratory tests showed no abnormal findings. For the single dose study, subjects were randomly divided into three groups A, B and C (5 males, 5 females per group) and after an overnight fast were administered over 30 min single intravenous infusions containing 5 mg, 10 mg and 15 mg ifenprodil tartrate for injection diluted in 100 mL water for injection, respectively. Water intake was allowed 2 h post-dose and standard meals were provided at 4 h and 10 h post-dose. The subjects were required to refrain from smoking, alcohol, caffeine and strenuous exercise during the study and were under direct medical supervision at the study site. Blood samples (4 mL) were collected at 0 h (pre-dose) and 5, 10, 20, 30 min, 1, 1.5, 2, 2.5, 3.5, 4.5, 6.5, 8.5 and 12.5 h post-dose into heparinized tubes. Plasma was collected after centrifugation at 4000 rpm for 10 min and stored at −20 °C until analysis. In the multiple dose study, Group B received single daily doses of 10 mg ifenprodil tartrate for injection at 8:00 am on 7 consecutive days. On days 4, 5 and 6, a venous blood sample (4 mL) was drawn prior to the dose to determine trough plasma concentrations. On day 7, the pharmacokinetics was determined as described for the single dose study.

2.8. Data analysis

Plasma concentration versus time profiles were prepared for each subject and peak plasma concentration (C_{max}) and time to C_{max} (T_{max}) values obtained by inspection of the data. Other pharmacokinetic parameters were calculated by non-compartmental analysis using WinNonlin software (version 6.2). The elimination rate constant (k) was calculated as the slope of log plasma concentration versus time data for measurable points in the elimination phase. Apparent elimination half-life (t_{1/2}) was calculated as ln 2/k. Area under the plasma concentration–time curve from time zero (pre-dose) to the last measurable concentration (AUC_{0–t}) was calculated using the linear trapezoidal rule. The AUC from time zero to infinity (AUC_{0–∞}) was calculated as AUC_{0–t}+C_{t}/k where C_{t} is the last measurable drug concentration. The apparent volume of distribution (V_{d}) was calculated as Dose/C_{0}, where log C_{0} was calculated as the intercept of the log plasma concentration versus time data for the last four measurable points. Clearance rate (CL)

Figure 2 Full-scan positive product ion mass spectra of (A) precursor ion of ifenprodil; (B) product ion of ifenprodil; (C) precursor of IS and (D) product ion of IS.
was calculated as \( k \times V_d \). The minimum steady state plasma concentration \( (C_{ss\text{min}}) \), average steady state plasma concentration \( (C_{av}) \) and area under the plasma concentration–time curve for steady-state conditions \( (\text{AUC}_{ss}) \) were also calculated.

3. Results and discussion

3.1. Method development

In the present work, sample preparation by LLE was evaluated using diethyl ether, trichloromethane and ethyl acetate. The latter produced clean chromatograms for blank plasma and provided a recovery \( >74.6\% \). As regards chromatography, a mobile phase containing acetonitrile gave higher background noise than methanol. A mixture of methanol–10 mM ammonium acetate solution \((90:10, v/v)\) was found to provide symmetric peak shapes of analyte and IS in a short run time. In selecting the IS, several analogs of ifenprodil were evaluated on the basis that uncertainty about matrix effects would thereby be largely eliminated. Urapidil (Fig. 1B) was finally chosen since it gave similar recovery and chromatography to those of the analyte. In relation to mass spectrometry, the analytes and IS yielded major \([M+H]^+\) ions at \( m/z \) 326.2 and 388.4 respectively. The product ion mass spectra (Fig. 2) show that the most sensitive transitions were at \( m/z \) 326.2→308.1 for ifenprodil and \( m/z \) 388.4→205.3 for IS.

3.2. Assay validation

Fig. 3 shows typical MRM chromatograms of blank plasma, blank plasma spiked with ifenprodil and the IS, and a plasma sample from a volunteer after a single intravenous infusion. The retention times of ifenprodil and IS were 2.3 and 2.9 min, respectively, and the assay was free of interference at these times in all plasma samples.

The LLOQ was established at 0.2 ng/mL at which concentration precision and accuracy were within the required limits \((\text{RSD} 6.9\%, \text{RE} -3\%, n=6)\). The assay was linear in the range 0.2–50 ng/mL \((r=0.997)\) with a typical regression equation of \( Y=1.1759C+0.0731 \).

Precision and accuracy data are summarized in Table 1. The data demonstrate that the precision and accuracy values were within acceptable ranges. Recoveries of ifenprodil in low, medium and high QC samples were 74.6±7.3%, 78.4±7.6% and 76.1±6.6%, respectively. Considering matrix effects, concentrations of low, medium and high QC samples were found to be 95.3±4.2%, 95.8±6.9% and 103.6±6.6%, respectively, of nominal values, indicating matrix effects are not an issue.

Results of stability studies summarized in Table 2 show that no significant degradation of ifenprodil was observed in plasma or in post-preparative samples under the storage conditions tested.

3.3. Pharmacokinetic study

There were no adverse events observed during the study and the drug was well tolerated by all volunteers. Mean plasma concentration–time profiles are presented in Figs. 4 and 5 and corresponding non-compartmental pharmacokinetic parameters are listed in Table 3. The \( t_{1/2}, C_{\text{max}} \) and AUC after the single intravenous administration are consistent with previously reported values\(^8\). The results show that \( C_{\text{max}} \) and AUC \( 0-12 \) are both linearly related to dose over the dose range studied. The additional steady state parameters were as follows: \( C_{\text{ssmin}} 0.53±0.18 \text{ ng/mL; } C_{av} 1.78 \pm 0.29 \text{ ng/mL; } \text{DF} 11.76 \pm 0.65; \text{AUC}_{ss} 42.72 \pm 6.90 \text{ ng·h/mL.} \) The \( t_{1/2} \) and AUC for the last dose are similar to those after the single-dose \((P>0.05)\), indicating that ifenprodil does not accumulate on repeated administration.

4. Conclusions

A rapid and sensitive assay for ifenprodil in human plasma based on LC–MS/MS has been developed, validated and successfully applied to a pharmacokinetic study in healthy volunteers. The results show ifenprodil does not accumulate on repeated administration.
venous infusion containing 10 mg ifenprodil. Data are means
Figure 5
Mean plasma concentration
Figure 4
Mean plasma concentration–time profiles of ifenprodil in
Chinese healthy volunteers following a single intravenous infusion containing 5, 10 and 15 mg ifenprodil. Data are means ± SD, n = 10.

Table 1 Intra- and inter-batch precision and accuracy for determination of ifenprodil in human plasma.

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Intra-batch (n = 5)</th>
<th>Inter-batch (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured (ng/mL)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>40.00</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 2 Short-term, post-preparative, freeze-thaw and long-term stability of ifenprodil in human plasma.

<table>
<thead>
<tr>
<th>Stability</th>
<th>Mean percentage remaining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>Short-term</td>
<td>86.6</td>
</tr>
<tr>
<td>Post-preparative</td>
<td>94.1</td>
</tr>
<tr>
<td>Freeze-thaw</td>
<td>101.3</td>
</tr>
<tr>
<td>Long-term</td>
<td>95.8</td>
</tr>
</tbody>
</table>

Table 3 Main pharmacokinetic parameters of ifenprodil following single intravenous infusions of 5, 10 and 15 mg ifenprodil tartrate for injection and following single daily intravenous infusions of 10 mg ifenprodil tartrate for injection for 7 days to groups of healthy Chinese volunteers.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>5 mg</th>
<th>10 mg</th>
<th>15 mg</th>
<th>10 mg (ss)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (ng/mL)</td>
<td>10.13 ± 4.02</td>
<td>19.1 ± 5.9</td>
<td>28.9 ± 6.2</td>
<td>20.4 ± 5.1</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>0.45 ± 0.08</td>
<td>0.50 ± 0.00</td>
<td>0.45 ± 0.08</td>
<td>0.42 ± 0.09</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>3.87 ± 0.93</td>
<td>3.61 ± 0.97</td>
<td>4.07 ± 0.69</td>
<td>4.25 ± 1.26</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>3.44 ± 0.34</td>
<td>3.72 ± 0.27</td>
<td>3.54 ± 0.16</td>
<td>3.87 ± 0.35</td>
</tr>
<tr>
<td>AUCinf (ng·h/mL)</td>
<td>19.9 ± 4.8</td>
<td>37.7 ± 8.5</td>
<td>57.1 ± 13.0</td>
<td>41.5 ± 5.0</td>
</tr>
<tr>
<td>AUC0–inf (ng·h/mL)</td>
<td>21.7 ± 4.8</td>
<td>41.3 ± 9.3</td>
<td>63.1 ± 13.6</td>
<td>47.0 ± 8.1</td>
</tr>
<tr>
<td>CL (L/h)</td>
<td>241 ± 53</td>
<td>254 ± 58</td>
<td>249 ± 61</td>
<td>218 ± 34</td>
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<tr>
<td>Vd (L)</td>
<td>1373 ± 566</td>
<td>1306 ± 425</td>
<td>1500 ± 635</td>
<td>1292 ± 241</td>
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</table>

References