The Validation of Plasma Darunavir Concentrations Determined by the HPLC Method for Protease Inhibitors

Masaaki TAKAHASHI, Yuichi KUDAKA, Naoya OKUMURA, Atsushi HIRANO, Kazuhide BANNO, and Tsuguhiro KANEDA

Department of Pharmacy, National Hospital Organization Nagoya Medical Center (Tokai Area Central Hospital for AIDS Treatment and Research); and Clinical Research Center, National Hospital Organization Nagoya Medical Center (Tokai Area Central Hospital for AIDS Treatment and Research); 4–1–1 Sannomaru, Naka-ku, Nagoya, Aichi 460–0001, Japan.

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Darunavir (DRV) is a new protease inhibitor (PI) used to treat human immunodeficiency virus (HIV) type-1. The aim of this study was to validate the determination of plasma DRV concentrations using HPLC method, a simple procedure for simultaneous determination of seven HIV protease inhibitors and efavirenz. The calibration curve was linear (range of 0.13 to 10.36 μg/ml). The average accuracy ranged from 100.7 to 105.6%. Both the interday and intraday coefficients of variation were less than 6.7%, which was similar to or much lower than previously reported values by the LC/MS/MS method. It is concluded that HPLC can be used to determine plasma DRV concentrations and routinely in the clinical setting; thus, this HPLC method enables further study of DRV pharmacokinetics in conventional hospital laboratories.

Key words: darunavir; human immunodeficiency virus type-1; HPLC; therapeutic drug monitoring

Darunavir (DRV), a new protease inhibitor (PI), is used to treat human immunodeficiency virus (HIV) type-1. According to in vitro experiments, DRV was active against HIV-1 with PI resistance mutations and against PI resistant clinical isolates. This drug is expected to be effective in antiretroviral treatment-experienced patients, such as those possessing HIV-1 strains which are resistant to more than one PI.

Bouche et al. recently determined plasma DRV concentrations using liquid chromatography-tandem mass spectrometry (LC/MS/MS). However, as LC/MS/MS equipment is very expensive and unavailable in conventional hospital laboratories, development of alternate methods is necessary.

We have already developed a simple HPLC method for simultaneous quantitative determination of seven HIV protease inhibitors and efavirenz. We expect DRV can be measured using this method because amprenavir, whose chemical structure is quite similar to DRV, was successfully measured.

In this study we aimed to validate the measurement of plasma DRV concentrations using the HPLC method. This is the first report where plasma DRV concentration has been measured using this HPLC method.

**MATERIALS AND METHODS**

**Standard Solutions and Chemicals**

DRV was supplied by Tibotec Pharmaceuticals Ltd. (Eastgate Village, Eastgate, Little Island, Co Cork, Ireland). The internal standard (IS), 6,7-dimethyl-2,3-di(2-pyridyl)-quinoxaline, was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Stock solutions of DRV and IS were prepared by dissolving accurately weighed amounts of each reference compound in water/ethanol (50 : 50, v/v) to yield concentrations of 259 μg/ml for DRV, and 588 μg/ml for IS. These stock solutions were stored at −80°C and thawed until the day of analysis. The stock solution was diluted in drug-free plasma to yield concentrations of 0.13, 1.30, 2.59, 5.18 and 10.36 μg/ml for DRV. All other chemicals and solvents were of analytical grade and have been described in our previous report.

**Chromatography**

The HPLC system consisted of a Waters pump (model 515), a 717 plus autosampler, and a 2487 dual λ absorbance detector coupled to the Empower™ software (Waters, Milford MA, U.S.A.). The analytical column was a Radial-Pak Nova-Pak C18 column (4 μm, 8×100 mm, Waters) protected by Guard-Pak Inserts Nova-Pak C18 precolumn. Absorbance was measured at 205 nm and separations were performed at 30°C. The mobile phase consisted of 39% 50 molar phosphate buffer (pH 5.9), 22% methanol and 39% acetonitrile. The assay run time was 30 min with a flow rate of 1.8 ml/min. Drugs were quantified by measuring the peak areas under the chromatograms. The other equipment and methodology used in this study have been described in our previous report.

**Sample Preparation**

Two milliliters of ethyl acetate/n-hexane (50:50, v/v) containing the IS (3.55 μg/ml) and 1 ml of 0.5 m sodium carbonate were added to a 500 μl plasma sample. The mixture was vortexed and then centrifuged at 3500×g for 5 min. The organic layer was separated and evaporated dry. The dried material was then dissolved in 100 μl of a mobile phase solution and centrifuged at 13000×g for 5 min. Lastly, 25 μl of the upper solution was injected into the HPLC column.

The institutional review board of National Hospital Organization Nagoya Medical Center approved this study and plasma samples were prepared from patients after obtaining written informed consent.

**Validation**

Intraday and interday precision values using this method were estimated by assaying control plasma containing five different concentrations of DRV five times on the same day and on three separate days to obtain the coefficient of variation (CV). Accuracy was determined as the percentage of the nominal concentration. Drug recovery from plasma was evaluated by analyzing triplicate samples with or without extraction.
RESULTS

Chromatograms of Plasma Sample  Figure 1A shows the chromatogram of a spiked plasma sample containing 5.18 μg/ml of DRV. Under the described chromatographic conditions, retention times were 3.8 min for DRV and 5.0 min for IS. Because the chemical structure of DRV is closely related to amprenavir (Fig. 1A), DRV retention time was similar to that of amprenavir in our previous report. At a detection wavelength of 205 nm, the assay performed on drug-free human plasma succeeded to show no interfering peaks during the interested retention time intervals (Fig. 1B).

Figure 2A shows a chromatogram of a plasma sample from an HIV-1-infected patient treated with atazanavir, ritonavir, abacavir and lamivudine. Figure 2B shows a chromatogram of a plasma sample from an HIV-1-infected patient treated with lopinavir, ritonavir, tenofovir and zidovudine. There are no interfering peaks affecting the quantification of DRV in these chromatograms.

Validation: Linearity, Precision, Accuracy and Recovery  Calibration curve of DRV appeared linear in the concentration range of 0.13 to 10.36 μg/ml with a correlation of 1.000.

Precision, accuracy and recovery for DRV are shown in Table 1. The selected concentration of DRV covers the expected plasma concentrations found in patients. The CVs calculated for DRV in the inter- and intraday assays ranged from

<table>
<thead>
<tr>
<th>Expected (μg/ml)</th>
<th>Intraday (n=5)</th>
<th>Interday (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured (μg/ml)</td>
<td>CV (%)</td>
</tr>
<tr>
<td>0.13</td>
<td>0.13±0.01</td>
<td>6.7</td>
</tr>
<tr>
<td>1.30</td>
<td>1.36±0.08</td>
<td>6.2</td>
</tr>
<tr>
<td>2.59</td>
<td>2.67±0.12</td>
<td>4.5</td>
</tr>
<tr>
<td>5.18</td>
<td>5.18±0.10</td>
<td>2.0</td>
</tr>
<tr>
<td>10.36</td>
<td>10.41±0.13</td>
<td>1.3</td>
</tr>
</tbody>
</table>

CV, coefficient of variation.

DRV, darunavir; APV, amprenavir; IS, internal standard.
1.3 to 6.7%, which were similar to or much lower than previously reported values.\textsuperscript{11,12} Accuracies ranged from 100.7 to 105.6%. Recoveries from plasma ranged from 88.0 to 100.9%. Mean extraction recovery of the IS was 80.4%.

**DISCUSSION**

In this study we demonstrated the determination of plasma DRV concentrations was possible using the HPLC method previously established in our laboratory for simultaneous detection of seven PIs. We validated the determination method using a concentration range between 0.13 and 10.36 mg/ml. The resulting HPLC method achieved reproducibility and accuracy for DRV detection.

Because the chemical structure of DRV is closely related to amprenavir, DRV retention time was similar to that of amprenavir in this study. DRV is expected to use in “salvage” therapy because this drug possesses low cross-resistance rates in patients who failed to respond to treatment with other PIs containing amprenavir. In another word, co-administration of DRV and amprenavir is not generally expected. Therefore, the similar retention times do not cause any problems in a practical use of the HPLC system.

The plasma DRV concentration was expected in the 2.8 to 5.8 µg/ml range when DRV was administered at the recommended dose with low-dose ritonavir.\textsuperscript{11} Our HPLC method successfully covered this region with both precision and accuracy.

In conclusion, our HPLC method can be routinely applied in the clinical setting and enables the study of DRV pharmacokinetics in conventional hospital laboratories.

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**REFERENCES**


