High-performance Liquid Chromatographic Ultraviolet Detection of Nilotinib in Human Plasma from Patients with Chronic Myelogenous Leukemia, and Comparison with Liquid Chromatography Tandem Mass Spectrometry

Ryosuke Nakahara,* Yuhki Satho, and Hiroki Itoh

Department of Clinical Pharmacy, Oita University Hospital, Yufu City, Japan

Background: A method for determining nilotinib concentration in human plasma is proposed using high-performance liquid chromatography and ultraviolet detection. **Materials & Methods:** Nilotinib and the internal standard dasatinib were separated using a mobile phase of 0.5% $\text{Na}_2\text{PO}_4\text{H}_2\text{O}$ (pH 2.5)-acetonitrile-methanol (55:25:20, v/v/v) on a Capcell Pak C18 MG II column (250 × 4.6 mm) at a flow rate of 1.0 ml/min, and ultraviolet measurement at 250 nm. **Results:** The calibration curve exhibited linearity over the nilotinib concentration range of 50–2,500 ng/ml at 250 nm, with relative standard deviations ($n = 5$) of 7.1%, 2.5%, and 2.9% for 250, 1,500, and 2,500 ng/ml,

respectively. The detection limit for nilotinib was 5 ng/ml due to three blank determinations ($\rho = 3$). **Conclusion:** This method was successfully applied to assaying nilotinib in human plasma samples from patients with chronic myelogenous leukemia. In addition, we compared the results with those measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) at BML, Inc. (a commercial laboratory). A strong correlation was observed between the nilotinib concentrations measured by our high-performance liquid chromatographic method and those obtained by LC-MS-MS ($r^2 = 0.988$, $P < 0.01$). J. Clin. Lab. Anal. 30:1028–1030, 2016. © 2016 Wiley Periodicals, Inc.

Key words: assay; high-performance liquid chromatography; liquid chromatography tandem mass spectrometry; nilotinib

Nilotinib (nilotinib mesylate, 4-methyl-*N*-[3-(4-methyl-1*H*-imidazol-1-yl)-5-(trifluoromethyl)phenyl]-3-[(4-pyridin-3-ylpyrimidin-2-yl) amino]benzamide) is a second-generation transduction inhibitor that targets Bcr-Abl, c-kit, and PDGF, for the potential treatment of chronic myelogenous leukemia (CML) (1). Nilotinib inhibits the tyrosine kinase activity of the Bcr-Abl protein. Nilotinib binds the ATP-binding site of the Bcr-Abl protein with higher affinity than imatinib, over-riding resistance caused by mutations (2). With the advent of Bcr-Abl tyrosine kinase inhibitors (TKIs), the prognosis of chronic myeloid leukemia (CML) has improved dramatically. However, the inter-individual variability in adverse events and clinical efficacy, as well as high drug cost remain major issues, and have become a major obstacle to treatment. Cytogenetic and molecular responses to imatinib correlate with the trough plasma concentration of imatinib in patients with CML (3, 4). Therefore, therapeutic drug monitoring (TDM) of TKIs is an important tool for the management of CML patients (5).

Some high-performance liquid chromatographic (HPLC) methods for quantifying nilotinib in human plasma have been described. Methods reported previously employed HPLC coupled with UV-diode array detection (6,7).

The objective of this study was to develop an HPLC-UV method for determining trough nilotinib concentration in human plasma, which is simple, sensitive, rapid, and inexpensive, and requires only equipment widely available in most hospital laboratories. We validated our method by comparing with liquid chromatography tandem mass spectrometry (LC-MS/MS) as described by Yin et al. (8), which is highly sensitive

*Correspondence to: Ryosuke Nakahara, Department of Clinical Pharmacy, Oita University Hospital, 1-1 Hasama, Yufu City 879-5593, Japan. E-mail: ryosuke-n@oita-u.ac.jp

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and accurate but requires sophisticated high-cost equipment available only in a few specialized hospitals.

Nilotinib and dasatinib were purchased from Funakoshi Co. (Tokyo, Japan). Oasis hydrophilic lipophilic balance (HLB) extraction cartridge (1 ml, 30 mg) was purchased from Waters (Milford, MA). All other reagents were purchased from Wako Pure Chemical Industries (Tokyo, Japan). All solvents were HPLC grade. Stock solutions for generating standard curves of nilotinib and dasatinib were prepared by dissolving the dry reagents in methanol to yield a concentration of 1.0 mg/ml. Working standard solutions of nilotinib (100, 500, 1,000, 3,000, and 5,000 ng/ml) and dasatinib (100, 500, 1,000, 3,000, and 5,000 ng/ml) were prepared by serial dilution with methanol.

WaterSTM 515 HPLC Pump equipped with a WaterSTM 486 tunable absorbance detector was used. The HPLC column was a Capcell Pak C18 MG II (250 × 4.6 mm i.d.; Shiseido, Tokyo, Japan). This column is supplied with packing material made of totally porous spherical silica coated with a silicone polymer monolayer containing octadecyl (C18) groups. The mobile phase was composed of 0.5% Na₂PO₄H₂O (pH 2.5), acetonitrile, and methanol (55:25:20, v/v/v), which was degassed in an ultrasonic bath prior to use. Before mixing with acetonitrile, the pH of 0.5% Na₂PO₄H₂O was adjusted with 50% phosphoric acid. The flow rate was 1.0 ml/min at ambient temperature and sample detection was carried out at 250 nm.

Fifty microlitres of dasatinib (50 ng) was added as internal standard to 100 µl of plasma sample and then the plasma sample was diluted with 900 µl of water and vortexed for 60 sec. This mixture was applied to an Oasis HLB extraction cartridge that had been

activated previously with methanol and water (1.0 ml each). The cartridge was then washed with 1.0 ml water and 1.0 ml 60% methanol in water, and eluted with 1.0 ml 100% methanol. Eluates were evaporated to dryness in a vacuum at 40°C using a rotary evaporator (Iwaki, Tokyo, Japan). The residues were dissolved in 50 µl of methanol (vortex mixed for 60 sec) and then 50 µl of the mobile phase was added to each sample (vortex mixed for another 60 sec). An aliquot of 50 µl of each sample was then applied to the HPLC.

This study was approved by the Ethics Committee of Oita University. Plasma samples were obtained from 11 patients with CML receiving nilotinib treatment. Sampling was performed after a steady state was reached. Blood samples were collected by venipuncture 24 h after oral administration of nilotinib. Plasma was separated by centrifugation at 1,900 × g for 15 min and stored at -40°C until analysis. Plasma samples (100 µl) were then extracted as described above. The same 11 samples were also sent to a commercial laboratory (BML, Inc.; Tokyo, Japan) for assaying nilotinib concentration by LC equipped with tandem mass spectrometric detectors (8).

The total analytical time of the chromatogram was 30 min. Nilotinib peak was detected at the retention time of 25.0 min. The chromatograms are those from 100 µl plasma samples spiked with nilotinib (50, 250, 500, 1,500, and 2,500 ng) and dasatinib (50 ng) as internal standard. There was no interference by plasma components or nilotinib metabolites.

A calibration curve for nilotinib was constructed by the peak height method. A good linear relationship and wide dynamic range were observed over 50–2,500 ng/ml

Fig. 1. Comparison of nilotinib trough plasma concentrations in 11 patients with CML. Concentrations were determined with the newly developed method and by an LC-MS/MS method at BML, Inc.

of nilotinib. The typical calibration curve (obtained using the least-squares method) was expressed as $y = 0.002x - 0.155$ ($r^2 = 0.9991$), where y is the peak height ratio and x is the concentration in ng/ml. The limit of detection, defined as $(3.3 \times \text{S.D. of blank})/(\text{slope of analytical calibration})$, was 5 ng/ml. The correlation coefficient was 0.9991. The relative standard deviations (RSD) for intraday precision were 7.1%, 2.5%, and 2.9% for 250, 1,500, and 2,500 ng/ml, respectively, of nilotinib. The RSD for intra-day and inter-day precision were less than 7.1%. The proposed method is more sensitive and reproducible than previous methods.

The proposed method was used for the determination of recovery in human plasma. Recovery of nilotinib was determined by adding three known nilotinib concentrations (250, 1,500, and 2,500 ng/ml) to drug-free plasma. The extraction recovery rates for nilotinib ranged from 98.2% to 107.7%.

The plasma concentrations of nilotinib obtained by the present method were almost the same as those assayed by LC-MS/MS at BML, Inc. (Fig. 1). Thus, this proposed HPLC method can be considered a simple and low-cost alternative to LC-MS/MS for TDM of nilotinib.

In conclusion, we have described a simple ultraviolet detection method for the determination of nilotinib, which has high sensitivity and large dynamic range. This study demonstrates the reliability of a simple, robust, rapid, and inexpensive HPLC-UV method for nilotinib TDM in patients with CML. The results can be expected to extend the availability of nilotinib

TDM, because our HPLC-UV method requires only standard equipment available in most hospital laboratories. We are conducting a prospective clinical trial to assess the usefulness of nilotinib TDM performed using our assay method.

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