INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) treatment has made significant progress with the success of highly active antiretroviral therapy (HAART) using protease inhibitors and reverse transcriptase inhibitors. However, long-term use of HAART is currently limited by developed resistance (1-4) and toxicities (5, 6) associated with many of these treatments. New antiretroviral drugs with novel mechanisms of action and/or distinct HIV-1 resistance profiles are thus required to continue effective HAART for the treatment of HIV-1.

Maraviroc is one of a new class of antiretroviral compounds known as CCR5 antagonists. Maraviroc prevents HIV-1 from entering CCR5+ CD4+ cells by acting as an antagonist at the CCR5 coreceptor (7-10). This drug in combination with other antiretroviral drugs has been demonstrated to be useful for treating therapy-experienced and -naïve patients.
infected with CCR5 tropic HIV-1 (11, 12).

On the other hand, drug interaction studies show changes in maraviroc exposure when administered with other anti-HIV medications, including efavirenz and protease inhibitors (13, 14). These changes require alterations in the doses of maraviroc to use. Therefore, monitoring plasma maraviroc concentrations is essential.

Fayet et al. (15) and Martin et al. (16) have recently determined plasma maraviroc concentrations using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The LC-MS/MS assay is very sensitive and accurate, but these methods have several disadvantages in terms of cost performance and essential equipment. Notari et al. (17) also reported a determination method using HPLC-UV. However, HPLC method has some complicated procedures, such as the solid-phase drug extraction. In addition, according to our preliminary HPLC application, the sensitivity of LC-MS methods must at least be determined for clarification of measurements. To bypass these difficulties, we aimed to develop more conventional procedures for determining maraviroc using LC-MS. The principal advantages of our method are rapid liquid-liquid drug extraction from plasma and use of an available internal standard.

MATERIALS AND METHODS

Chemicals

Maraviroc was supplied by Pfizer (New York, NY, USA) and the internal standard (IS), A-86093 (5S,8S,10S,11S)-9-hydroxy-2-cyclopropyl-5-(1-methylethyl)-1-[(2-1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis (phenylmethyl)-2,4,7,12-tetraazatridecane-13-oic acid, 5-thiazolylmethyl ester, was provided by Abbott Laboratories (Abbott Park, IL, USA). Their chemical structures are shown in Figure 1. Methanol, hexane, methylene chloride and acetonitrile (Kanto Chemical, Tokyo, Japan) were of HPLC grade. Ammonium acetate, EDTA and acetic acid were purchased from Katayama Chemical (Osaka, Japan). Water was deionized and osmosed using a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals and solvents were of analytical grade.

Equipment

A Waters Alliance 2695 HPLC system and a Micromass ZQ-2000 MS system (Waters, Milford, MA, USA), controlled with MassLynx version 4.0 software (Waters), were used for detection. The analytical column was a SunFire C18 column (3.5 μm, 2.1×50 mm; Waters), protected by a SunFire C18 Guard column.

Chromatographic and Mass Spectrometric Conditions

The mobile phase was a mixture of 0.1 mM EDTA in 0.1% acetic acid (A), 100% acetonitrile (B) and 100% methanol (C). An isocratic mobile phase consisting of A-B-C (87 : 8 : 5) was used during the first 2 min of the run, followed by a linear gradient elution consisting of A-B-C (15 : 80 : 5) for the next 8 min. The final conditions (15 : 80 : 5) were maintained for the final 5 min. The system was then re-equilibrated for an additional 25 min using the initial conditions. The flow rate of the mobile phase was 0.2 ml/min, column temperature was 40°C, and the amount of injected sample was 5 μl.

The mass spectrometer was operated in positive ion electrospray mode. Capillary sprayer voltage was 3.5 kV and sample cone voltage was 30 V for both maraviroc and A-86093. The source temperature was 120°C and the desolvation temperature was 350°C. The desolvation and cone gas flow rates were set to 600 and 50 L/h, respectively. The acquisition mass range was m/z 200-800 at 0.5 s/scan with a 0.1-s interscan delay. All mass spectra were acquired in centroid mode.

Fig. 1. Chemical structures of maraviroc and the internal standard A-86093.
Quantitative analysis, performed in selected-ion recording (SIR) mode, detected maraviroc at $m/z$ 514.7, and the IS (A-86093) at $m/z$ 748.0, all in the form of ions. Quantitation calculations were performed using MassLynx version 4.0 analytical software.

**Standard Solutions**

Stock solutions of maraviroc and A-86093 were prepared by dissolving accurately weighed amounts of each reference compound in water/ethanol (50:50, v/v) to yield concentrations of 87.5 μg/ml maraviroc and 41.0 μg/ml A-86093. These stock solutions were stored at -80°C and thawed on the day of analysis. The stock solution was diluted in drug-free plasma to yield maraviroc concentrations of 0.011, 0.109, 0.438, 1.094 and 2.188 μg/ml.

**Sample Preparation**

A 2-ml aliquot of methylene chloride/hexane (50:50, v/v) containing the IS (0.164 μg/ml) and 0.3 ml of 0.2 M ammonium acetate were added to 500 μl plasma sample prepared from peripheral blood anticoagulated with heparin. The mixture was vortexed for 5 min and then centrifuged at 3500 × g for 5 min. The upper layer was separated and evaporated dry. This dried material was then dissolved in 50 μl mobile phase solution. Lastly, 5 μl upper solution was injected into the LC-MS system. The institutional review board of National Hospital Organization Nagoya Medical Center approved this study and each subject provided written informed consent.

**Validation**

Inter- and intraday precision values using this method were estimated by assaying control plasma containing 5 different concentrations of maraviroc 5 times on the same day and on 3 separate days to obtain the relative standard deviation (RSD). Accuracy was determined as a percentage of the nominal concentration. To assess absolute recovery of maraviroc extracted from plasma, peak area ratios of analytes to IS were compared with those obtained from the mobile phase with the same concentration. Mean recoveries were determined in triplicate.

**RESULTS**

**LC-MS Chromatograms**

Figures 2A and 2B show selected-ion recording chromatograms obtained from a spiked plasma sample containing 0.875 μg/ml maraviroc and 0.164 μg/ml A-86093.

(A) $m/z$ 514.7

(B) $m/z$ 748.0

(C) $m/z$ 514.7

(D) $m/z$ 748.0

Fig. 2. Selected-ion recording chromatograms for maraviroc and A-86093.

(A,B) Spiked plasma containing 0.875 μg/ml of maraviroc monitored with $m/z$ 514.7 (A) and 0.164 μg/ml of A-86093 (IS) monitored with $m/z$ 748.0 (B).

(C,D) A blank plasma sample monitored with $m/z$ 514.7 (C) and $m/z$ 748.0 (D).

(C) and (D) are the expanded figures of the baselines in (A) and (B), respectively.
ml A-86093 (IS). Under the described chromatographic conditions, retention times were 8.00 min for maraviroc and 13.6 min for A-86093. Figures 2C and 2D show chromatograms obtained from a blank plasma sample. Assays performed on drug-free human plasma successfully showed no interfering peaks during the examined intervals of retention times. Figure 2D represents an expanded figure of the baseline part of Figure 2B. These peaks did not affect the quantification of IS. Anticoagulants heparin and EDTA did not hinder the selected-ion recording chromatograms for maraviroc and IS.

Validation: Linearity, Precision, Accuracy and Recovery

Calibration curves of maraviroc appeared linear in the concentration range of 0.011-2.188 μg/ml, with a correlation of 1.000.

The precision, accuracy, and recovery of our LC-MS method are shown in Table 1. The selected concentration of maraviroc covers the expected plasma concentrations found in patients. RSDs calculated for maraviroc in inter- and intraday assays ranged from 3.2% to 7.1%, accuracy from 92.7% to 99.7% and recovery from plasma from 86.7% to 89.0%. Mean extraction recovery of IS was 87.9%. These results indicate that this method achieves a high degree of reproducibility and accuracy.

Clinical application

Figures 3 and 4 show chromatograms of a plasma sample obtained from an HIV-1 infected patient on

Table 1. Intra- and interday precision and accuracy for maraviroc

<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>RSD (%)</td>
</tr>
<tr>
<td>0.011</td>
<td>0.010±0.001</td>
<td>7.1</td>
</tr>
<tr>
<td>0.109</td>
<td>0.106±0.007</td>
<td>6.4</td>
</tr>
<tr>
<td>0.438</td>
<td>0.437±0.024</td>
<td>5.5</td>
</tr>
<tr>
<td>1.094</td>
<td>1.096±0.048</td>
<td>4.4</td>
</tr>
<tr>
<td>2.188</td>
<td>2.164±0.070</td>
<td>3.2</td>
</tr>
</tbody>
</table>

RSD, relative standard deviation. Values indicate mean±SD.

Fig. 3. Selected-ion recording chromatograms of a plasma sample obtained from patient 1.

Fig. 4. Selected-ion recording chromatograms of a plasma sample obtained from patient 2.
maraviroc.

The patient 1 was a Japanese man aged 43 years with a body weight of 38.0 kg. He had been treated with maraviroc, lamivudine, etravirine, darunavir and ritonavir for one month. Maraviroc was administered at 150 mg once daily, because a strong CYP3A4 inhibitor, darunavir, was coadministered with maraviroc. However, his CD4+ count was still 20/μl with a viral load of 100,000 copies/ml. Successful virological treatment was not achieved. The maraviroc plasma concentration, measured at trough, was 0.024 μg/ml. In the DHHS guidelines (18), the suggested minimum target trough maraviroc concentration is more than 0.050 μg/ml. This treatment failure may have been due to a low maraviroc trough level and thus to elevate the trough level, we proposed increasing the maraviroc dose to 300 mg twice daily.

The patient 2 was a Japanese man aged 34 with a body weight of 75.4 kg. He had been treated with maraviroc, lamivudine, efavirenz, enfuvirtide, tipranavir and ritonavir for two weeks. Maraviroc was administered at 600 mg twice daily, because a strong CYP3A4 inducer, efavirenz, was coadministered with maraviroc. At the start of this regimen, his CD4+ count was 23/μl with a viral load of 220,000 copies/ml. After two weeks, his CD4+ count was 69/μl with a viral load of 3,500 copies/ml. The maraviroc trough plasma concentration was 0.177 and 0.133 μg/ml. In this case, the maraviroc trough levels were more than 0.050 μg/ml. The viral load has been decreasing and treatment success is expected in the future. We thus proposed maintaining the current daily dose of maraviroc.

DISCUSSION

Clinical trials of maraviroc have demonstrated potent antiviral responses in patients infected with CCR5 tropic HIV-1 who had previously received triple classes of antiretroviral drugs and/or were identified as triple-class resistant (19). Moreover, maraviroc has demonstrated a clean safety profile in these studies and may not have the toxicities and tolerability issues seen with current anti-HIV drugs. Maraviroc has thus become an important component of combination treatment regimens for therapy-experienced patients infected with CCR5 tropic HIV-1.

Maraviroc is a substrate of CYP3A4 and Pgp, and its pharmacokinetics is likely to be modulated by inhibitors and inducers of these enzymes/transporters (13, 14). Therefore, a dose adjustment is required when maraviroc is coadministered with those drugs. To manage these drug interactions and ensure optimal drug efficacy, monitoring plasma maraviroc concentrations is essential. For this purpose, we developed a method for determining plasma maraviroc concentrations using LC-MS. The maraviroc calibration curve was linear within the concentration range of 0.011 to 2.188 μg/ml, and average accuracy ranged from 92.7% to 99.7%. Both inter- and intraday RSDs for maraviroc were less than 7.1%. Recovery of maraviroc ranged from 86.7% to 89.0%. Thus, our newly developed method achieved a high degree of reproducibility and accuracy. In addition, as plasma concentrations of maraviroc are reportedly within 0.03-0.89 μg/ml when maraviroc is administered at 300 mg twice daily (13), our method successfully covers this region with good precision and accuracy.

Our LC-MS method provides a conventional, accurate and precise way to determine the maraviroc concentration in human plasma. This method enables a dose adjustment based on monitoring plasma maraviroc concentrations for HIV-1-infected patients and permits management of drug interactions and toxicity.

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REFERENCES


