A Conventional LC-MS Method Developed for the Determination of Plasma Raltegravir Concentrations

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Raltegravir belongs to a new class of antiretrovirals acting for a human immunodeficiency virus (HIV)-1 integrase inhibition. Clinical trials of this drug have demonstrated potent antiviral activity in both therapy naïve and experienced patients. Thus, raltegravir has become an important component of combination treatment regimens used to treat patients with multidrug-resistant HIV-1. The quantification of raltegravir in human plasma is important to support clinical studies and determine pharmacokinetic parameters of raltegravir in HIV-1 infected patients. Recently, the LC-MS/MS superfine system was developed to determine plasma concentration of raltegravir; however, the system needs to be delicately set and the equipment is very expensive. Therefore, we developed a conventional LC-MS method to overcome these difficulties. Subsequently the method was validated by estimating the precision and accuracy for inter- and intraday analysis in the concentration range of 0.010—7.680 μg/ml. The calibration curve was linear in this range. Average accuracy ranged from 97.2 to 103.4%. Relative standard deviations of both inter- and intraday assays were less than 10.4%. Recovery of raltegravir was more than 80.6%. This novel LC-MS method is accurate and precise enough to determine raltegravir levels in human plasma samples.

Key words human immunodeficiency virus-1; LC-MS; therapeutic drug monitoring; raltegravir

The clinical treatment of patients with human immunodeficiency virus (HIV)-1 infection has been advanced by the success of highly active antiretroviral therapy (HAART). However, it became clear that the long-term administration of HAART was limited by toxicity associated with many of these treatments1,2 as well as by the development of resistance.3—6 Therefore, new antiretroviral drugs, which act on different action points from DNA elongation and protein processing in HIV-1 life cycle, are required to continue effective HAART for the treatment of HIV-1.

Raltegravir is one of a new class of antiretroviral agents that work by inhibiting the insertion of viral DNA into the cellular genome, resulting in virus replication prevention.7—10 Therefore, raltegravir is expected to treat therapy-experienced patients where protease inhibitor (PI) and/or reverse transcriptase inhibitor-resistant HIV-1 had developed.11—13 We have a routine system, by which all PI and efavirenz plasma concentrations are easily determined by HPLC,14 and therapeutic drug monitoring was performed as needed.15 In this study, we aimed to develop the determination method of plasma raltegravir.

Recently, a determination method using liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been reported.16 However, the MS-MS detector needs to be delicately set and LC-MS/MS equipment is very expensive. In addition, isotope labeled raltegravir as an internal standard (IS) is needed. To bypass these difficulties, we aimed to develop more conventional procedures for determining raltegravir using liquid chromatography coupled with mass spectrometry (LC-MS).

MATERIALS AND METHODS

Chemicals and Reagents Raltegravir was supplied by Merck Research Laboratories (Rahway, NJ, U.S.A.) 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-tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, was provided by Abbott Laboratories (Abbott Park, IL, U.S.A). Their chemical structures are shown in Fig. 1. Methanol, hexane, methylene chloride, and acetonitrile (Kanto Chemical, Tokyo, Japan) were 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 Corp., Bedford, MA, U.S.A.). All other chemicals and solvents were of analytical grade.

Fig. 1. Chemical Structures of Raltegravir and the Internal Standard A-86093

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Equipment A Waters Alliance 2695 HPLC and a Micromass ZQ-2000 MS (Waters Assoc., Milford, MA, U.S.A.), controlled with MassLynx version 4.0 software, were used for detection. The analytical column was a SunFire C\textsubscript{18} column (3.5 \(\mu\)m, 2.1\times50 mm, Waters), protected by a SunFire C\textsubscript{18} 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 (65 : 15 : 20) was used during the first 2 min of the run, followed by a linear gradient elution consisting of A–B–C (10 : 70 : 20) for the next 8 min. The final conditions were maintained for the final 5 min. The system was then reequilibrated for an additional 13 min using the initial conditions. The flow rate of the mobile phase was 0.2 ml/min, the column temperature was 40 °C, and the amount of injected sample was 5 \(\mu\)l.

The mass spectrometer was operated in positive ion electrospray mode. The capillary sprayer voltage was 3.5 kV and the sample cone voltage was 30 V for both raltegravir 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 is \(m/z\) 200—800 at 0.5 s per scan with a 0.1 s interscan delay. All mass spectra are acquired in centroid mode.

Quantitative analysis, carried out in Selected-ion recording (SIR) mode, detected raltegravir at \(m/z\) 445, and the internal standard (IS), A-86093, at \(m/z\) 748, all in the form of ions. The quantitation calculations were performed using analytical software, MassLynx version 4.0 (Waters).

Standard Solutions Stock solutions of raltegravir 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 384.0 \(\mu\)g/ml of raltegravir and 41.0 \(\mu\)g/ml of A-86093. These stock solutions were stored at \(-80^\circ\text{C}\) and thawed on the day of analysis. The stock solution was diluted in drug-free plasma to yield raltegravir concentrations of 0.010, 0.192, 1.920, 3.840 and 7.680 \(\mu\)g/ml.

Sample Preparation Two milliliters of methylene chloride/hexane (50 : 50, v/v) containing the IS (0.328 \(\mu\)g/ml) and 0.3 ml of 0.2 M ammonium acetate were added to a 500 \(\mu\)l plasma sample prepared from peripheral blood anticoagulated with heparin. The mixture was vortexed for 5 min and then centrifuged at 3500 \(\times\) g for 5 min. The upper layer was separated and evaporated dry. The dried material was then dissolved in 50 \(\mu\)l of a mobile phase solution. Lastly, 5 \(\mu\)l of the 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 five different concentrations of raltegravir five times on the same day and on three separate days to obtain the relative standard deviation (RSD). Accuracy was determined as the percentage of the nominal concentration. To assess the absolute recoveries of raltegravir extracted from plasma, the peak area ratios of the analytes to the internal standard were compared with those obtained from the mobile phase having the same concentration. The mean recoveries were determined in triplicate.

RESULTS

LC-MS Chromatograms Figures 2A and B show selected-ion recording chromatograms obtained from a spiked plasma sample containing 0.192 \(\mu\)g/ml of raltegravir and 0.328 \(\mu\)g/ml of A-86093 (IS). Under the described chromatographic conditions, retention times were 8.2 min for raltegravir and 12.9 min for A-86093. Figures 2C and D show chromatograms obtained from a blank plasma sample. Assays performed on drug-free human plasma succeeded to show no interfering peaks during the interested intervals of the retention times. Figure 2D is the expanded figure of the

![Fig. 2. Selected-ion Recording Chromatograms for Raltegravir and A-86093](image-url)
baseline part of Fig. 2B. These peaks did not affect the quantification of IS. Figures 2E and F show chromatograms of a plasma sample from an HIV-1-infected patient treated with raltegravir. There were no interfering peaks affecting quantification of raltegravir in this chromatogram. Anticoagulants of heparin and EDTA did not hinder the selected-ion record-
ification of raltegravir in this chromatogram. Anticoagulants
of heparin and EDTA did not hinder the selected-ion recording chromatograms for raltegravir and A-86093.

**Validation: Linearity, Precision, Accuracy and Recovery** Calibration curves of raltegravir appeared linear in the concentration range of 0.010 to 7.680 \( \mu g/ml \) with a correlation of 1.000.

Precision, accuracy, and recovery of our LC-MS method are shown in Table 1. The selected concentration of raltegra-
vir covers the expected plasma concentrations found in the patients. The RSDs calculated for raltegravir in the inter-
and intraday assays ranged from 1.3 to 10.4%, which are similar to previously reported values.\(^\text{14}\) Accuracies ranged from 97.2 to 103.4%. Recoveries from plasma ranged from 80.6 to 87.4%. Mean extraction recovery of the IS was 87.9%. These results indicate that this method achieves a high degree of reproducibility and accuracy.

**Raltegravir Concentrations in Plasma** Plasma raltegra-
vir concentrations in an HIV-1-infected patient are shown in Table 2. The patient received oral administration of 400 mg raltegravir twice daily. The samples were collected on day 8 after the start of HAART. When raltegravir is admin-
istered by a single 400 mg dose, plasma concentrations are expected in the 0.01 to 4.71 \( \mu g/ml \) range.\(^\text{16–18}\) In this study, raltegravir concentrations at steady state following multiple-dose administration ranged from 1.2 to 5.2 \( \mu g/ml \).

**DISCUSSION**

Clinical trials of raltegravir have demonstrated potent antiviral responses in both therapy naïve and experienced patients.\(^\text{11,12,20}\) Moreover, raltegravir has demonstrated a clean safety profile in these studies and may not have the tox-

icity and tolerability issues as the current anti-HIV drugs.

Thus, raltegravir has become an important component of combination treatment regimens and its use has been initi-
ated for the treatment of heavily pretreated patients with a multidrug-resistant virus.

We first wanted to judge whether therapeutic drug moni-
toring of raltegravir is necessary. To achieve this, the de-
velopment of determination method for raltegravir is essential. Until now there has been a methodological report for the
determination of raltegravir using LC-MS/MS.\(^\text{16}\) However,
this method has several disadvantages in terms of cost per-
formance and essential equipment; for example, the authors used isotoped labeled raltegravir and/or the setting of the LC-
MS-MS equipment.

To avoid such disadvantages we decided to use an LC-MS method using an available IS (A-86093) for determining plasma protease inhibitor concentrations. The reason we chose ritonavir analogue A-86093 is the stability and better elution point of the compound on the HPLC as were reported previously.\(^\text{14}\) Validation showed our method was successful in measuring plasma raltegravir with high precision and satisfactory RSD values. The raltegravir calibration curve was linear at the concentration range of 0.010 to 7.680 \( \mu g/ml \), and the average accuracy ranged from 97.2 to 103.4%. Both inter- and intraday RSDs for raltegravir were less than 10.4%, which is similar to previously reported values.\(^\text{10}\) Recovery of raltegravir was more than 80.6%. These results indicate our newly developed method achieves the same level of reproducibility and accuracy as the LC-MS/MS method. As plasma concentrations of raltegravir are expected in the 0.01 to 4.71 \( \mu g/ml \) range when raltegravir is administered at single dose of 400 mg,\(^\text{16–18}\) our method successfully covers this region with good precision and accuracy. Actually, the raltegravir concentration change was clearly demonstrated; it rose from 1.2 (0 h) to 5.2 \( \mu g/ml \) (1 h), then decreased to 1.5 \( \mu g/ml \) (6 h) when raltegravir was orally administered 400 mg twice daily in an HIV-1-infected patient.

Recently, Poirier *et al.* reported the HPLC method for
determining plasma raltegravir concentration with fluores-
cence detection.\(^\text{21}\) Our’s and Poirier’s methods can specifically determine the raltegravir concentration and the sensitivities seem almost equivalent. Therefore, an alternative use is possible according to the availability of the equipments. This conventional LC-MS method can provide a routine clinical application, and permits management of drug inter-
actions and toxicity.

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REFERENCES