High Performance Liquid Chromatography Using UV Detection for the Simultaneous Quantification of the New Non-nucleoside Reverse Transcriptase Inhibitor Etravirine (TMC-125), and 4 Protease Inhibitors in Human Plasma

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Etravirine (TMC-125, ETV) is a second-generation non-nucleoside reverse transcriptase inhibitor (NNRTI) that demonstrates potent activity against NNRTI-resistant strains of human immunodeficiency virus type-1 (HIV-1). Thus, ETV has been used in combination with ritonavir-boosted protease inhibitor (PI) and integrase inhibitor for therapy-experienced HIV-1-infected patients. On the other hand, as ETV is a substrate and inducer of cytochrome P450 3A4 (CYP3A4), ETV may induce metabolism of PI and alter the concentrations of co-administered PIs. In order to ensure optimal drug efficacy and prevention of resistance, it is essential to monitor plasma concentrations of ETV and PIs. Here we describe the application of HPLC with UV detection for the simultaneous assay of ETV and 4 PIs, darunavir (DRV), atazanavir (ATV), ritonavir (RTV) and lopinavir (LPV). In this study, the calibration curve of each drug was linear with the average accuracy ranging from 93.6 to 110.9%. Both intra- and interday coefficients of variation for each drug were less than 11.6%. The mean recovery of all drugs ranged from 88.0 to 97.5%. The limit of quantification was 0.04, 0.04, 0.04, 0.05 and 0.07 µg/ml for ETV, DRV, ATV, RTV and LPV, respectively. These results demonstrate that our HPLC-UV method can be used for routine determination of plasma concentrations of ETV and 4 PIs in clinical settings.

Key words: etravirine; HPLC; protease inhibitor; therapeutic drug monitoring

Etravirine (TMC-125, ETV) is a second-generation non-nucleoside reverse transcriptase inhibitor (NNRTI) that demonstrates potent activity against NNRTI-resistant strains of human immunodeficiency virus type-1 (HIV-1). According to the DUET studies (randomized, double-blind, placebo-controlled trials), overall, ETV was well tolerated in treatment-experienced patients infected with HIV-1, with its safety and tolerability profile generally comparable to placebo at week 24.1,2 Additionally, 48-week data pooled from these studies showed greater virologic and immunologic responses compared with placebo.3,4

In the latest HIV treatment, ETV has been used in combination with ritonavir-boosted protease inhibitor (PI) and integrase inhibitor for therapy-experienced HIV-1-infected patients. On the other hand, as ETV is a substrate and inducer of cytochrome P450 3A4 (CYP3A4), ETV may induce metabolism of PI and alter the concentrations of co-administered PIs.5 In order to ensure optimal drug efficacy and prevention of resistance, it is essential to monitor plasma concentrations of ETV and PIs.

Fayet et al.5 and Quaranta et al.6 succeeded in determining plasma concentrations of ETV and other drugs through the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS). Rezk et al.7 have also developed a method to measure plasma concentrations of ETV and PIs by LC-MS. LC-MS or LC-MS/MS assay is very sensitive and accurate. However, MS equipment is very expensive and unavailable in conventional hospital laboratories. Therefore, development of alternate methods is necessary.

Recently, D’Avolio et al.8,9 reported a new HPLC method that employs a photo diode array (HPLC-PDA) for quantification of ETV and other antiretroviral drugs. This method is simple, reliable, and sensitive, using cost-effective instrumentation when compared with others.5—7 However, this method requires a solid phase extraction. Furthermore, in general hospitals, a UV detector coupled with HPLC is more popular than a PDA detector. The HPLC-UV method is a user-friendly assay that is readily adaptable to standard laboratory equipment for routine therapeutic drug monitoring (TDM).

In this study, we propose the simultaneous quantitative assay of ETV and 4 PIs, darunavir (DRV), atazanavir (ATV), ritonavir (RTV) and lopinavir (LPV) in a simple procedure that is derived from a previously established HPLC-UV method.9 This method can be applied to pharmacokinetic studies of PIs and ETV, the newest NNRTI, and it is useful when evaluating the clinical significance of TDM for these drugs.

MATERIALS AND METHODS

Chemicals: ETV and DRV were supplied by Tibotec Pharmaceuticals Ltd. (Eastgate Village, Eastgate, Little Island, Co., Cork, Ireland). LPV and RTV were generously provided by Abbott Laboratories (Abbott Park, IL, U.S.A.). ATV was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ, U.S.A.). The internal standard (IS), 6,7-dimethyl-2,3-di(2-pyridyl)quinoxaline, was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Acetonitrile, methanol, ethyl acetate and n-hexane (Kanto
Chemical, Tokyo, Japan) were HPLC grade. Sodium carbonate was purchased from Katayama Chemical (Osaka, Japan). Water was deionized and osmosed using a Milli-Q® system (Millipore, Bedford, MA, U.S.A.). All other chemicals were of analytical grade and have been described in our previous report.9)

**Standard Solutions** Stock solutions of tested drugs and IS were prepared by accurately dissolving weighed amounts of each reference compound in water/ethanol (50:50, v/v) to yield concentrations of 191.0 μg/ml for ETV, 85.2 μg/ml for DRV, 502.0 μg/ml for ATV, 425.0 μg/ml for RTV, 95.1 μg/ml for LPV, and 588.0 μg/ml for IS. These stock solutions were stored at −80°C until the day of analysis. Each stock solution was diluted in drug-free plasma to yield concentrations of 0.08, 0.14, 0.42, 1.04 and 4.17 μg/ml for ETV, 0.07, 0.12, 0.37, 0.93 and 3.72 μg/ml for DRV, 0.09, 0.15, 0.44, 1.10 and 4.38 μg/ml for ATV, 0.07, 0.12, 0.37, 0.93 and 3.71 μg/ml for RTV, 0.08, 0.14, 0.42, 1.04 and 4.15 μg/ml for LPV.

**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, with separations performed at 30°C. The mobile phase consisted of 39% 50 mM phosphate buffer (pH 6.2), 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.

**Sample Preparation** A total of 2 ml of ethyl acetate/n-hexane (50:50, v/v) containing the IS (1.18 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 to dryness. 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 the National Hospital Organization Nagoya Medical Center approved this study. Plasma samples were prepared from patients after obtaining written informed consent.

**Validation** Intra- and interday precision values using this method were estimated by assaying control plasma containing five different concentrations of each drug 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. Plasma samples spiked with known amounts of both drugs and IS were extracted as usual. Blank plasma samples that contained only the IS were extracted and subsequently spiked with the same amount of analytes to give the 100% reference. The recovery was assessed by comparing the peak area ratio (analytes/IS) of extracts. The limit of quantification was defined as the lowest concentration for which both the CV% and the percent of deviation from the nominal concentration were less than 20%.

**RESULTS**

**Plasma Sample Chromatograms** Figure 1A is a chromatogram of a spiked plasma sample containing 3.77 μg/ml of DRV, 1.18 μg/ml of IS, 4.44 μg/ml of ATV, 3.76 μg/ml of RTV, 1.69 μg/ml of ETV and 4.21 μg/ml of LPV. Under the described chromatographic conditions, retention times were 3.4, 4.4, 8.3, 10.5, 11.7, 13.0 min for DRV, IS, ATV, RTV, ETV and LPV, respectively. At a detection wavelength of 205 nm, assays performed on drug-free human plasma demonstrated that there were no interfering peaks during the intervals of interest for the retention times (Fig. 1B).

Figure 2A is a chromatogram of a plasma sample from an HIV-1-infected patient treated with raltegravir, ETV, ATV, RTV and lamivudine. The patient was a Japanese male aged 37 years with a body weight of 72.6 kg. His CD4+ T cell count was 302/μl with a viral load of 7200 copies/ml. ETV and other antiretroviral agents were administered for 7 d. The plasma concentration at trough was 0.30, 0.48 and 0.24 μg/ml for ETV, ATV and RTV, respectively.

Figure 2B shows a chromatogram of a plasma sample from an HIV-1-infected patient treated with raltegravir, ETV, DRV, RTV and lamivudine. The patient was a Brazilian male aged 49 years with a body weight of 83.0 kg. His CD4+ T cell count was 157/μl with a viral load of 44 copies/ml. ETV...
and other antiretroviral agents were administered for 3 weeks. The plasma concentration at trough was 0.33, 3.30, and 0.29 μg/ml for ETV, DRV and RTV, respectively.

In chromatograms of Figs. 2A and B, the peaks of raltegravir and lamivudine were not detected.

**Precision, Accuracy, Recovery, Linearity and Limit of Quantification** The precision and accuracy for all tested drugs are shown in Table 1. The analyses show satisfactory precision with intra- and interassay coefficients of variation less than 11.6%. Accuracy ranged from 93.6 to 110.9%. The mean recovery of all drugs ranged from 88.0 to 97.5%. The regression coefficients of determination (R²) values of the calibration curves for each drug were 0.99 or greater. The limit of quantification was 0.04, 0.04, 0.04, 0.05 and 0.07 μg/ml for ETV, DRV, ATV, RTV and LPV, respectively.

**DISCUSSION**

ETV has activity in vitro against viral strains with mutations that confer resistance to efavirenz and nevirapine. The EC₅₀ of ETV was <100 nm (43.5 ng/ml) against clinically derived recombinant viruses resistant to at least one of the currently marketed NNRTIs. However, clinical investigations have yet to determine the therapeutic range of ETV concentrations that are associated with the desired therapeutic response. In addition, there is the potential problem for complex drug interactions due to the fact that ETV is a substrate and inducer of CYP3A4, as well as a substrate and inhibitor of 2C9 and 2C19. In clinical treatment, ETV is co-administered with other antiretroviral agents including RTV-boosted PI. Therefore, too-low or too-high plasma concentrations of these drugs may decrease treatment efficacy or increase the risk of adverse effects. To solve these problems, a simple drug monitoring system for these agents is needed. Here we describe the application of HPLC with UV detection for simultaneously assaying ETV and 4 PIs. HPLC-UV equipment is frequently used in conventional hospital laboratories.

In this study, the calibration curve of each drug was linear with the average accuracy ranging from 93.6 to 110.9%. Both intra- and interday coefficients of variation for each drug were less than 11.6%. These results demonstrate that our HPLC-UV method has advantages in both reproducibility and accuracy in measuring plasma concentration of ETV and 4 PIs in a single run.

In our clinical cases, the ETV plasma concentrations, measured at trough, were 0.30 or 0.33 μg/ml for the HIV-1-infected patients. These values were similar to the previously reported findings in DUET studies. In each case, the trough concentration of DRV or ATV was more than the suggested minimum target trough value in the guideline. The viral load has been decreasing in these patients and treatment success is expected in the future. We, thus, proposed maintaining the current daily dose of these drugs. Conversely, the peaks of co-administered raltegravir and lamivudine were not

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**Table 1. Intra- and Interday Precision and Accuracy for ETV, ATV, RTV, LPV and DRV**

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<th>Drug</th>
<th>Expected (μg/ml)</th>
<th>Measured (μg/ml)</th>
<th>CV (%)</th>
<th>Measured (μg/ml)</th>
<th>CV (%)</th>
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ETV, etravirine; ATV, atazanavir; RTV, ritonavir; LPV, lopinavir; DRV, darunavir; CV, coefficient of variation.
detected because these drugs were not extracted from plasma by our liquid–liquid extraction technique. As these drugs are not metabolized by cytochrome P450, there are no drug interactions with ETV.

In conclusion, we have successfully constructed a protocol for the simultaneous quantification of ETV and 4 PIs by HPLC-UV. We believe our method enables accurate monitoring of ETV and co-administered PIs and may guide optimized administration of these drugs and prevent potential drug interactions and toxicity in treatment.

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REFERENCES AND NOTES