Conventional HPLC Method Used for Simultaneous Determination of the Seven HIV Protease Inhibitors and Nonnucleoside Reverse Transcription Inhibitor Efavirenz in Human Plasma

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We developed a simple HPLC method for the simultaneous quantitative determination of seven HIV protease inhibitors: amprenavir (APV), atazanavir (ATV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), and a nonnucleoside reverse transcription inhibitor, efavirenz (EFV). This method involves a rapid liquid–liquid drug extraction from plasma, the use of an isocratic elution on a reversed-phase C18 column, and an ultraviolet detection at a single wavelength (205 nm). The mobile phase consisted of 39% 50 mM phosphate buffer (pH 5.9), 22% methanol and 39% acetonitrile. Forty-eight samples could be measured in one day since the runtime of one sample is 30 min. The assay has been validated over a concentration range of 0.05 to 12.20 μg/ml for APV, 0.09 to 12.05 μg/ml for ATV, 0.05 to 12.01 μg/ml for IDV, 0.12 to 12.36 μg/ml for LPV, 0.18 to 12.20 μg/ml for NFV, 0.12 to 12.33 μg/ml for RTV, 0.12 to 12.06 μg/ml for SQV, and 0.05 to 12.17 μg/ml for EFV. Calibration curves were linear in the described concentration ranges. The average accuracy ranged from 97.2 to 106.8%. Both the interday and intraday coefficients of variation for all drugs tested were less than 8.5%. This method provides a simple, accurate, and precise method for the therapeutic drug monitoring of the seven protease inhibitors and EFV in clinical routine use.

Key words HPLC; therapeutic drug monitoring; HIV protease inhibitor; human immunodeficiency virus (HIV)-1

The clinical treatment of patients with human immunodeficiency virus (HIV)-1 infection has been advanced by the development of highly active antiretroviral therapy (HAART). HAART reduces plasma HIV-RNA below detectable limits in most cases. However, some patients do not have a sustainable antiviral response, even after experiencing a decrease in plasma HIV-RNA, due to the development of drug resistance and metabolic complications. This undesirable outcome may result from a failure to achieve effective antiretroviral drug plasma concentrations. Therefore, monitoring plasma drug concentrations is essential to ensure optimal drug efficacy, to prevent viral resistance, to manage drug interactions, to avoid adverse effects, and to assess nonadherence.

In recent years several HPLC methods for simultaneous determination of antiretroviral drugs in plasma have been published. However, to popularize the simultaneous determination method, a simplified technique is necessary because the reported techniques require a solid-phase extraction, and/or use of a gradient elution, and/or an ultraviolet detection at multi wavelengths, all of which are not routinely available in conventional hospital laboratories. Therefore, we aimed to develop a simple procedure for simultaneous quantitative determination of seven protease inhibitors (PI): amprenavir (APV), atazanavir (ATV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), and the nonnucleoside reverse transcription inhibitor, efavirenz (EFV), in human plasma. Our technique involves rapid liquid–liquid drug extraction from plasma, the use of an isocratic elution, as well as an ultraviolet detection at a single wavelength. This assay is based on our previously published HPLC method.

MATERIALS AND METHODS

Chemicals and Reagents APV was kindly provided by Glaxosmithkline Research and Development, Ltd. (Park Road, Ware, U.K.). ATV was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ, U.S.A.). IDV and EFV were provided by Merck & Co., Inc. (Rahway, NJ, U.S.A.). LPV, RTV and the internal standard (IS), (S,S,S,S)-9-hydroxy-2-cyclopropyl-5-(1-methylthio)-1-[2-(1-methylthio)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, were generously provided by Abbott Laboratories (Abbott Park, IL, U.S.A.). NFV was provided by Agouron Pharmaceuticals (La Jolla, CA, U.S.A.) and SQV was provided by Roche Products (Welwyn Garden City, U.K.). 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.).

Chromatography The HPLC system consisted of a Waters pump (model 515), WISP 712 auto sample processor, and a 2487 dual absorbance detector coupled to the Millennium® version 3.21 software (Waters, Milford MA, U.S.A.). The analytical column was a Radial-Pak Nova-Pak C18 column (4.6 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 mM 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.
Standard Solutions  Stock solutions of all seven protease inhibitors and EFV were prepared by dissolving accurately weighed amounts of each reference compound in water/ethanol (50:50, v/v) to yield concentrations of 106.1 μg/ml for APV, 502.0 μg/ml for ATV, 100.1 μg/ml for IDV, 95.1 μg/ml for LPV, 305.0 μg/ml for NFV, 425.0 μg/ml for RTV, 67.0 μg/ml for SQV, and 93.6 μg/ml for EFV. These stock solutions were stored at −80 °C and thawed on the day of analysis. Each stock solution was diluted in drug-free plasma to yield concentrations of 0.05, 0.85, 2.12, 7.43, and 12.20 μg/ml for APV, 0.09, 0.88, 2.20, 6.02, and 12.05 μg/ml for ATV, 0.05, 0.80, 2.00, 6.01, and 12.01 μg/ml for IDV, 0.12, 0.95, 1.90, 6.18, and 12.36 μg/ml for LPV, 0.18, 0.92, 3.05, 6.10, and 12.20 μg/ml for NFV, 0.12, 0.86, 1.71, 6.16, and 12.33 μg/ml for RTV, 0.12, 0.92, 3.05, 6.10, and 12.06 μg/ml for SQV, and 0.05, 0.84, 1.87, 6.08, and 12.17 μg/ml for EFV.

Sample Preparation  Two milliliters of ethyl acetate/n-hexane (50:50, v/v) containing the IS (2.02 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 3500g 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 13000g for 5 min. Lastly, 25 μl of the upper solution was injected into the HPLC column. Before taking peripheral blood, written informed consent was obtained from all patients and a healthy volunteer.

Validation  Intraday and interday precision values using this method were estimated by assaying control plasma containing five different concentrations of APV, ATV, IDV, LPV, NFV, RTV, SQV, and EFV 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 plasma sample containing 2.12 μg/ml of APV, 2.20 μg/ml of ATV, 2.00 μg/ml of IDV, 1.90 μg/ml of LPV, 3.05 μg/ml of NFV, 1.71 μg/ml of RTV, 3.05 μg/ml of...
The selected concentration of each drug covers the expected plasma concentrations found in the patients.

The CVs calculated for APV in the intraday and interday assays ranged from 1.4 to 6.5% and 2.0 to 5.2%, respectively. In ATV CVs ranged from 1.2 to 5.6% and 2.0 to 5.0%, and in IDV, CVs ranged from 1.8 to 5.4% and 1.4 to 7.5%. For LPV, CVs ranged from 1.4 to 6.6% and 2.0 to 7.0%, NFV CVs ranged from 0.6 to 8.5% and 2.2 to 8.4%, RTV CVs ranged from 2.5 to 7.8% and 2.3 to 7.5%, SQV CVs ranged from 0.4 to 6.4% and 2.7 to 6.4%, while for EFV the CVs ranged from 2.0 to 3.8% and 2.6 to 6.5%. Accuracies ranged from 97.8 to 100.4%, 98.1 to 102.0%, 98.8 to 102.5%, 98.5 to 106.3%, 97.2 to 103.1%, 98.7 to 103.8%, 98.3 to 106.8% and 100.2 to 102.6% for APV, ATV, IDV, LPV, NFV, RTV, SQV and EFV, respectively. Drug recoveries from plasma ranged from 90.1 to 98.4%, 91.1 to 95.0%, 81.2 to 96.0%, 92.0 to 97.5%, 88.6 to 96.4%, 90.4 to 94.4%, 89.9 to 94.1% and 91.1 to 95.2% for APV, ATV, IDV, LPV, NFV, RTV, SQV and EFV, respectively. Extraction recovery of the IS was 100%.
Chromatograms of Patient Samples  Figure 2A shows a chromatogram of a plasma sample from an HIV-1-infected patient treated with ATV and RTV. Fig. 2B shows a chromatogram of a plasma sample from an HIV-1-infected patient treated with LPV and RTV. There is no significant drift from the baseline and no interfering peaks affecting quantification of ATV, LPV and RTV in this chromatogram. These results were validated by peak testing and library matching performed with Millenium32 software.

DISCUSSION

There have been some recent reports of HPLC methods for simultaneous determination of antiretroviral drugs. However, these methods have several disadvantages in terms of cost performance, time consumption and necessary equipment; for example, the use of expensive disposable cartridges at the solid-phase drug extraction, gradient elution control by a gradient HPLC pump system, and the ultraviolet detection at multiple wavelengths.

We describe the development, validation, and application of a simple HPLC method for simultaneous quantitative determination of seven PIs currently on the market as well as EFV. The principal advantages of our method are a rapid liquid-liquid drug extraction from plasma, an isocratic elution on a reversed-phase C18 column, and ultraviolet detection at a single wavelength (205 nm).

Generally, the retention time of antiretroviral drugs are dependent on pH of the mobile phase buffer. In fact, some previously reported assays used the mobile phase buffer at a variety of pH values. We sought the optimum pH of the mobile phase buffer by changing pH every 0.5 from pH 2 to pH 11. Finally, we determined pH 5.9 phosphate buffer for the mobile phase to separate each drug. A mobile phase with a pH 9.4 phosphate buffer also enabled excellent drug separation. The overlapping profile of some peaks was not excluded in the pH condition except about 6 and 9.5. However, we recommend pH 5.9 phosphate buffer, because alkaline buffer rapidly degrades the C18 reversed-phase column and causes a baseline drift.
In this study, calibration curves of all the drugs used were linear in the described concentration ranges and the average accuracy ranged from 97.2 to 106.8%. Both interday and intraday CVs for all drugs were less than 8.5%, which is similar to or much lower than previously reported values.\(^1\)\(^{-15}\) Mean extraction recoveries varied from 81.2% (IDV) to 98.4% (APV). These results indicate that the method developed here achieves a high degree of reproducibility and accuracy.

When antiretroviral drugs are administered at the recommended dose, plasma concentrations are expected in the 0.3 to 8.2 \(\mu g/ml\) range for APV,\(^{12}\) the 0.2 to 10 \(\mu g/ml\) range for ATV,\(^{17}\) the 0.1 to 7.7 \(\mu g/ml\) range for IDV,\(^{18,19}\) the 5.5 to 9.6 \(\mu g/ml\) range for LPV,\(^{12}\) the 0.1 to 4.0 \(\mu g/ml\) range for NFV,\(^{18,20}\) the 0.1 to 11.2 \(\mu g/ml\) range for RTV,\(^{18,20}\) the 0 to 0.2 \(\mu g/ml\) range for SQV,\(^{16}\) and the 1 to 4 \(\mu g/ml\) range for EFV.\(^{21}\) Our method successfully covers these regions with good precision and accuracy.

Figure 1 illustrates the chromatogram of a plasma sample containing IS, APV, ATV, IDV, LPV, NFV, RTV, SQV, and EFV. Our isocratic elution yields sharp peaks for all the drugs tested and gives an excellent separation for each. The chromatogram of the blank plasma sample shows that there was no significant drift from the baseline and no interfering peaks affecting the quantification of all the drugs. Furthermore, it turns out that no analytical interference was encountered from endogenous substances or other co-administered drugs at the retention time for each drug, even in the chromatogram of a plasma sample from an HIV-1-infected patient treated with either ATV/RTV or LPV/RTV. Therefore, our method can be made available for optimal follow-up of HIV-infected patients through therapeutic drug monitoring.

In conclusion, this simple HPLC method can be conveniently used as a routine clinical application and enables study of the drug pharmacokinetics in conventional hospital laboratories. This method can also offer continuous measurement of 48 samples in one day.

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