A Simple HPLC Method for Simultaneous Determination of Lopinavir, Ritonavir and Efavirenz

Yoshiko USAMI,[a] Tsuyoshi OKI,[b] Masahiko NAKAI,[b] Masafumi SAGISAKA,[b] and Tsuguhiro KANEDA*,[a]

[a]*Clinical Research Center, Nagoya National Hospital (Tokai Area Central Hospital for AIDS Treatment and Research); and* [b]*Department of Pharmacy, Nagoya National Hospital (Tokai Area Central Hospital for AIDS Treatment and Research); 4–1–1 Sannomaru, Naka-ku, Nagoya, Aichi 460–0001, Japan.*

Received November 18, 2002; accepted March 8, 2003

We developed a simple HPLC method for the simultaneous determination of lopinavir (LPV), ritonavir (RTV) and efavirenz (EFV) to evaluate the efficiency of co-administration of LPV/RTV and EFV in Japanese patients enrolled in a clinical study. The monitoring of LPV plasma concentration is important because co-administration of LPV/RTV with EFV sometimes decreases plasma concentrations of LPV caused by EFV activation of cytochrome P-450 3A. A solution of acetonitrile, methanol and tetramethylammonium perchlorate (TMAP) in dilute aqueous trifluoroacetic acid (TFA) has been used as the mobile phase in a HPLC method to elute LPV and RTV. We found that a solvent ratio of 45 : 5 : 50 (v/v/v) of acetonitrile/methanol/0.02 M TMAP in 0.2% TFA optimized separation of LPV, RTV and EFV. A column temperature of 30 °C was necessary for the reproducibility of the analyses. Standard curves were linear in the range 0.060 to 24.06 μg/ml for LPV, 0.010 to 4.16 μg/ml for RTV, and 0.047 to 37.44 μg/ml for EFV. Coefficients of variation (CVs) of LPV, RTV and EFV in intraday and interday assays ranged from 1.5 to 4.0%, 2.5 to 16.8% and 1.0 to 7.7%, respectively. Accuracies ranged from 100 to 110%, 101 to 116% and 99 to 106% for LPV, RTV and EFV, respectively. The extraction recoveries were 77—87, 77—83 and 81—91% for LPV, RTV and EFV, respectively.

Key words human immunodeficiency virus (HIV)-1; HPLC; therapeutic drug monitoring; lopinavir; ritonavir; efavirenz

In recent years, the treatment of human immunodeficiency virus (HIV)-1 infection and AIDS 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 become nonresponsive to HAART even after experiencing a decrease in plasma HIV-RNA, because of the rapid development of drug-resistant variants of HIV-1. This undesirable outcome may result from a failure to achieve effective plasma concentrations of the anti-retroviral drugs. Therefore, monitoring plasma drug concentrations is important to ensure efficacious levels are achieved during HAART.

Combination therapy with the HIV protease inhibitors lopinavir (LPV)/ritonavir (RTV) (Kaletra®), and the non-nucleoside reverse transcriptase inhibitor efavirenz (EFV), has been shown to be effective against drug-resistant HIV-1.[1,2] These agents are metabolized by cytochrome P-450 (CYP) 3A in the liver.[3—5] When LPV is administered with RTV as Kaletra®, RTV inhibits the CYP3A-mediated metabolism of LPV, thereby providing increased plasma levels of LPV. In contrast to RTV, however, EFV enhances CYP3A. Therefore, co-administration of LPV/RTV and EFV can result in the decrease of LPV plasma concentrations.[6] To counteract this effect, the administration of an increased dose of LPV/RTV is required when used with EFV[6]; Clumeck *et al.*[1] also recommended use of an increased dose. Kaletra® was approved for use by the US Food and Drug Administration in September 2000 and became available for use in Japan in December 2000, but a Japanese clinical trial was not conducted due to a strong and urgent demand for the drug. However, an understanding of the pharmacokinetics of these drugs in Japanese patients remains essential.

A clinical study to evaluate the efficiency of co-administration of LPV/RTV and EFV in Japanese HIV-1-infected patients and determine the correlation between plasma concentrations of these drugs and their efficacy is planned. Prior to the onset of the clinical study, however, development of a simple and convenient method to monitor plasma concentrations of these drugs was necessary.

The aim of this study is to develop a simple and rapid HPLC method for the simultaneous determination of LPV, RTV and EFV.

Experimental

Chemicals LPV, RTV and the internal standard (IS), (5*S*,8*S*,10*S*,11*S*)-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, were generously provided by Abbott Laboratories (Abbott Park, IL, U.S.A.). EFV was kindly provided by Merck & Co., Inc. (Rahway, NJ, U.S.A.). Acetonitrile, methanol, ethyl acetate and *n*-hexane (Katayama Chemical, Osaka, Japan) were HPLC grade. L-Consera was purchased from Nissui Pharmaceutical Co., LTD. (Tokyo, Japan). Trifluoroacetic acid (TFA) and tetramethylammonium perchlorate (TMAP) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Sodium carbonate was purchased from Katayama Chemical (Osaka, Japan).

Chromatography The HPLC system consisted of a Waters pump model 6000A, 484 tunable absorbance detector, 741 data module, and WISP 710B auto sample processor. The analytical column was a Radial-Pak Nova-Pak C_{18} column (4 μm, 8×100 mm, Waters) protected by a Guard-Pak Inserts Nova-Pak C_{18} precolumn. Absorbance was measured at 205 nm. Drugs were quantified by measuring peak areas of chromatograms.

Standard Solutions A LPV/RTV stock solution was prepared at concentrations of 120.3/20.8 μg/ml in water/ethanol (50 : 50, v/v). An EFV stock solution of 93.6 μg/ml was prepared by dissolving the drug in water/ethanol (50 : 50, v/v). These stock solutions were stored at −80 °C and thawed on the day of analysis.

Each stock solution was diluted in drug-free serum to yield concentrations of 0.060, 0.120, 0.241, 0.602, 1.203, 2.406, 6.015, 12.03 and 24.06 μg/ml for LPV, 0.010, 0.021, 0.042, 0.104, 0.208, 0.416, 1.04, 2.08 and 4.16 μg/ml for RTV, and 0.047, 0.094, 0.187, 0.468, 0.936, 1.872, 4.68, 9.36, 18.72 and 37.44 μg/ml for EFV.

Sample Preparation Two milliliters of ethyl acetate/*n*-hexane (50 : 50, v/v) containing the IS (2.024 μg/ml) and 1 ml of 0.5 M sodium carbonate were added to 500 μl of serum sample. The mixture was vortexed and centrifuged at 2800×***g*** for 5 min. The organic layer was separated and evaporated to dryness. The dried material was dissolved in 100 μl of mobile phase

* To whom correspondence should be addressed. e-mail: kanedat@nagoya.hosp.go.jp

solution and centrifuged at 13000×***g*** for 5 min. Then 25 μl of the upper solution was injected into the HPLC.

Validation Intraday and interday precision values for the method were estimated by assaying control sera containing five different concentrations of LPV, RTV 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.

Recovery from serum was evaluated by analyzing triplicate samples with or without extraction.

Results

Selection of Mobile Phase When acetonitrile/methanol/0.01 M TMAP in 0.1% TFA (50 : 5 : 45, v/v/v) was used as the mobile phase solution at a flow rate of 1.5 ml/min, complete separation of LPV from RTV and the IS was achieved (Fig. 1a). HPLC performed with the same mobile phase solution using a standard solution containing LPV, RTV and EFV (see Experimental) resulted in incomplete separation; EFV eluted after LPV with a very similar retention time (Fig. 1b). As a first trial, the acetonitrile/methanol/0.01 M TMAP in 0.1% TFA ratio was changed to 45 : 5 : 50. EFV then eluted earlier than LPV, but separation was still incomplete (Fig. 1c). In a second trial, the concentration of TFA was increased to 0.2%, but the separation of LPV and EFV was not complete (Fig. 1d). When the concentration of TMAP was increased to 0.02 M in 0.1% TFA, separation of EFV from LPV was considerably improved; however, then the IS eluted close to EFV (Fig. 1e). Finally, we chose conditions of 0.02 M TMAP and 0.2% TFA, which resulted in separation of LPV, RTV, EFV and the IS (Fig. 1f).

Temperature can affect peak separation in samples containing ions. To obtain better separation of LPV and EFV, the effect of temperature was investigated. Complete separation of LPV and EFV was achieved when the column temperature was increased from room temperature to 30 °C (Fig. 1g).

Validation: Linearity, Precision, Accuracy, and Recovery Standard curves of LPV, RTV and EFV showed linearity in the concentration range of 0.060 to 24.06 μg/ml for LPV, 0.010 to 4.16 μg/ml for RTV, and 0.047 to 37.44 μg/ml for EFV, with correlation coefficients of 1.000, 0.9998, and 0.9994, respectively.

Precision, accuracy, and extraction recovery of the method are shown in Table 1. The selected concentrations of each drug cover the expected plasma concentrations found in the patients.

The CVs calculated for LPV in the intraday and interday assays ranged from 1.5 to 3.1% and 2.3 to 4.0%, respectively, which are similar to those reported by Marzolini *et al.*,[7] Poirier *et al.*,[8] Titier *et al.*,[9] and Tribut *et al.*[10] For RTV, CVs in the intraday and interday assays ranged from 2.5 to 12.5% and 2.8 to 16.8%, respectively, which are comparable to values reported by others.[8—10] A slightly high CV value of greater than 10% was obtained at the RTV concentrations of 0.021 and 0.208 μg/ml. Intraday and interday CVs of EFV ranged from 1.0 to 4.2% and 3.4 to 7.7%, respectively, which are similar to or much lower than previously reported values.[8—10]

Accuracies ranged from 100 to 110% and 99 to 106% for LPV and EFV, respectively. For RTV, accuracy values at concentrations of 0.208 μg/ml and higher ranged from 101 to 116%, but the value at the low concentration of 0.021 μg/ml was somewhat high at 146.7%.

Recoveries from serum ranged from 77 to 87%, 77 to 83%

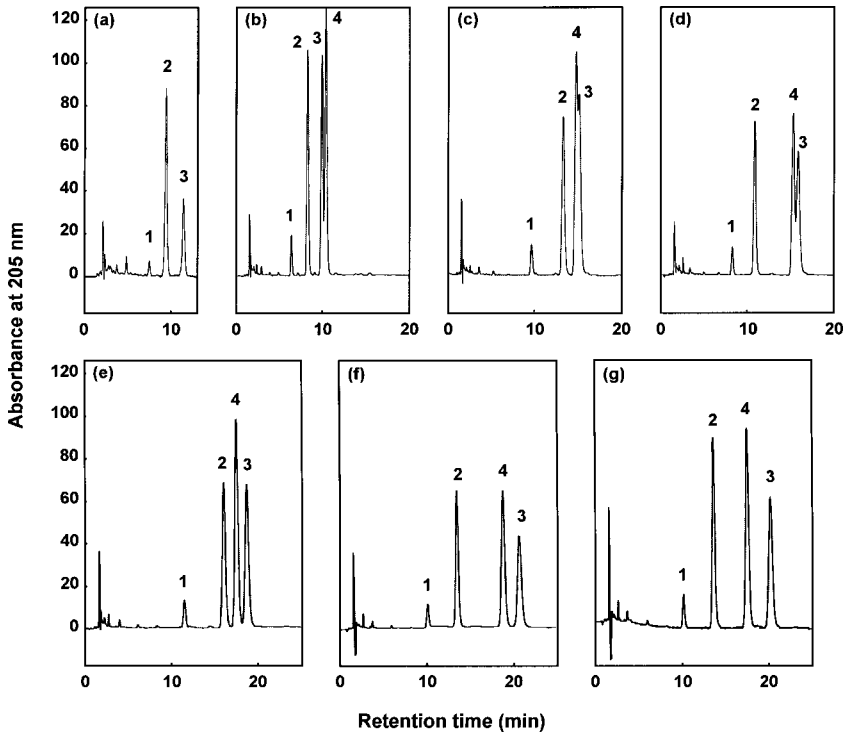

Fig. 1. Chromatograms under Different Mobile Phase Solution Conditions

Peak 1, RTV; 2, IS; 3, EFV; 4, LPV. (a) LPV, RTV and the IS were separated with a mobile phase of 50 : 5 : 45 acetonitrile/methanol/0.01 M TMAP in 0.1% TFA. LPV, RTV, EFV and the IS were separated with mobile phase solutions of (b) 50 : 5 : 45 acetonitrile/methanol/0.01 M TMAP in 0.1% TFA, (c) 45 : 5 : 50 acetonitrile/methanol/0.01 M TMAP in 0.1% TFA, (d) 45 : 5 : 50 acetonitrile/methanol/0.01 M TMAP in 0.2% TFA, (e) 45 : 5 : 50 acetonitrile/methanol/0.02 M TMAP in 0.1% TFA, and (f) 45 : 5 : 50 acetonitrile/methanol/0.02 M TMAP in 0.2% TFA at room temperature. (g) LPV, RTV, EFV and the IS were separated with the same mobile phase solution as (f) at 30 °C.

Table 1. Intraday and Interday Precision and Accuracy for LPV, RTV and EFV

	Expected (μg/ml)	Intraday (n=5)		Interday (n=15)		Accuracy (%)	Recovery (%)
		Measured (μg/ml)	CV (%)	Measured (μg/ml)	CV (%)		
LPV	0.060	0.064±0.002	2.8	0.066±0.002	3.2	109.4±3.5	77.6±1.9
	0.120	0.129±0.002	1.3	0.128±0.004	3.0	106.0±3.1	86.4±0.7
	1.203	1.240±0.038	3.1	1.258±0.029	2.3	104.6±2.4	78.8±0.5
	6.015	6.068±0.138	2.3	6.314±0.250	4.0	105.0±4.2	81.2±2.1
	24.060	23.331±0.351	1.5	24.288±0.837	3.4	100.9±3.5	84.5±3.3
RTV	0.021	0.034±0.004	11.7	0.031±0.005	16.8	146.7±24.7	83.0±9.6
	0.208	0.253±0.032	12.5	0.240±0.024	10.0	115.4±11.6	77.8±2.5
	1.040	1.050±0.037	3.5	1.058±0.033	3.1	101.8±3.2	77.2±2.3
	4.160	4.136±0.102	2.5	4.218±0.119	2.8	101.4±2.6	82.8±0.6
EFV	0.047	0.048±0.002	4.0	0.049±0.004	7.7	105.2±8.1	86.3±3.9
	0.094	0.095±0.004	4.2	0.097±0.006	5.9	103.7±6.1	90.8±1.0
	0.936	0.966±0.036	3.7	0.991±0.033	3.4	105.9±3.6	87.3±2.2
	4.680	4.669±0.115	2.5	4.951±0.265	5.4	105.8±5.7	81.6±2.3
	18.720	17.389±0.167	1.0	18.595±0.917	4.9	99.3±4.9	84.6±2.6

and 81 to 91% for LPV, RTV and EFV, respectively. Extraction recovery of the IS was 100%.

These results indicate that the method developed in this study achieves a high degree of reproducibility and accuracy.

Chromatograms of Serum or Plasma Samples Figure 2 shows chromatograms obtained after extraction of control sera containing (a) 6.015 μg/ml of LPV, 1.04 μg/ml of RTV and 4.68 μg/ml of EFV and (b) plasma from an HIV-1-infected patient treated with Kaletra® and EFV. There was no significant drift of the baseline and no interfering peaks affected quantification of LPV, RTV and EFV. In the case of the HIV-1-infected patient (b), RTV could not be determined because RTV plasma concentration was below the limits of detection.

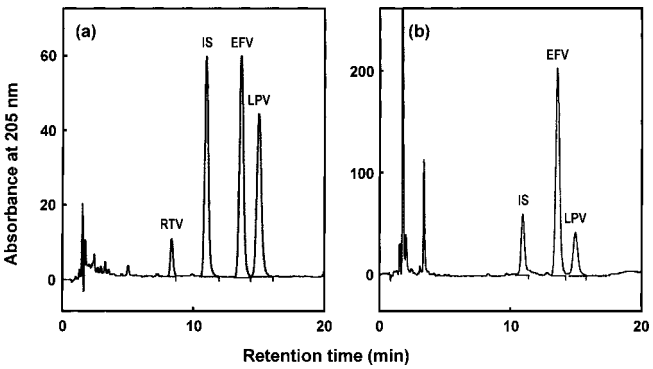

Fig. 2. Chromatograms Obtained after Extraction of (a) Control Serum Sample Containing 6.015 μg/ml of LPV, 1.04 μg/ml of RTV and 4.68 μg/ml of EFV and (b) Plasma Sample from an HIV-1-Infected Patient Treated with Kaletra® and EFV

Discussion

Using HPLC, we now can measure plasma concentrations of protease inhibitors routinely in HIV-1-infected patients treated with HAART. Measurement of plasma concentrations of LPV/RTV in an HIV-1-infected patient treated with Kaletra® and EFV by HPLC revealed that EFV eluted very closely to LPV. Combination therapy with Kaletra® and EFV is effective, and its use is expected to increase. A pharmacokinetic clinical study on the combination therapy in Japanese patients necessitated the development of a method for simultaneous quantification of the concentrations of LPV, RTV and EFV. The method needed to be convenient and time-efficient because of the need for frequent measurement of the drugs in patient plasma samples. The method described here requires only 90 min for one sample measurement, including the time for drug extraction. The isocratic condition of our method contributes to the convenience of serial runs.

When EFV is administered at the recommended dose of 600 mg once daily postprandial, plasma concentrations are expected in the 1 to 5 μg/ml range.[11] When LPV/RTV is administered at a dose of 400/100 mg twice daily postprandial, plasma concentrations are expected in the 1 to 10 or 0.5 to 1 μg/ml range, respectively.[6] Our method successfully covers these regions with good precision and accuracy.

This simple and timesaving HPLC method can be performed using conventional instruments and will be useful for monitoring combination therapy of LPV/RTV and EFV in HIV-1-infected patients to prevent treatment failure.

Acknowledgements This study was supported in part by a Health Science Research Grant for Research on HIV/AIDS from the Ministry of Health, Labor, and Welfare of Japan (No. H13-AIDS-001 to TK).

YU is a research resident of the Japanese Foundation for AIDS Prevention.

References

1) Clumeck N., Brun S., Sylte J., Isaacson J., Chen S., Lazzarin A., Girard P. M., Rockstroh J., Becker S., Telenti A., Bergmann F., Danner S., Ho D., Tubiana R., Carosi G., Bertz R., Hsu A., King M., Kempf D., Sun E., 8th Conference on Retroviruses and Opportunistic Infections, Chicago, on 4—8 February, 2001.
2) Corbett A. H., Lim M. L., Kashuba A. D., *Ann. Pharmacother.*, **36**, 1193—1203 (2002).
3) Kumar G. N., Rodrigues A. D., Buko A. M., Denissen J. F., *J. Pharmacol. Exp. Ther.*, **277**, 423—431 (1996).
4) Kumar G. N., Jayanti V., Lee R. D., Whittern D. N., Uchic J., Thomas S., Johnson P., Grabowski B., Sham H., Betebenner D., Kempf D. J., Denissen J. F., *Drug Metab. Dispos.*, **27**, 86—91 (1999).
5) Adkins J. C., Noble S., *Drugs*, **56**, 1055—1064 (1998).
6) Product information, Kaletra® (lopinavir/ritonavir), Abbott Park, IL: Abbott Laboratories, 2000.
7) Marzolini C., Béguin A., Telenti A., Schreyer A., Buclin T., Biollaz J., Decosterd L. A., *J. Chromatgr. B*, **774**, 127—140 (2002).

8) Poirier J.-M., Robidou P., Jaillon P., *Ther. Drug Monit.*, **24**, 302—309 (2002).
9) Titier K., Lagrange F., Péhourcq F., Edno-Mcheik L., Moore N., Molimard M., *Ther. Drug Monit.*, **24**, 417—424 (2002).
10) Tribut O., Arvieux C., Michelet C., Chapplain L.-M., Allain H., Bentué-Ferrer D., *Ther. Drug Monit.*, **24**, 554—562 (2002).
11) Product information, Sustiva (efavirenz), Wilmington, DE: DuPont Pharmaceuticals Company, 2001.