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Short communication

Simple determination of the HIV protease inhibitor atazanavir in human plasma by high-performance liquid chromatography with UV detection

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Abstract

A simple high-performance liquid chromatography method for the determination of the human immunodeficiency virus protease inhibitor atazanavir in human plasma samples was developed and validated. The method involved a rapid and simple solid-phase extraction of atazanavir using Oasis HLB 1 cc cartridges, an isocratic reversed-phase liquid chromatography on an XTerra RP18 (150 mm × 4.6 mm, 3.5 μm) column, and ultraviolet detection at 203 nm. The mobile phase consisted of phosphate buffer (pH 6, 52.5 mM) and acetonitrile (43:57, v/v). Up to 48 samples could be measured in one day since the run-time of one sample was 30 min. The assay was linear from 0.04 to 10 μg/ml with a lower limit of quantification of 0.04 μg/ml. The mean absolute recovery of ATV was 98.1%. The method was precise, with both intra-day and inter-day coefficients of variation ≤3.0%, and accurate (deviations ranged from −3.0% to 4.5% and from −3.6% to 4.7% for intra-day and inter-day analysis, respectively). There was no interference from 35 tested potentially co-administrated drugs. This method provides a simple, sensitive, precise and reproducible assay for the therapeutic drug monitoring of atazanavir in clinical routine of laboratories with standard equipment.

Keywords: Atazanavir; BMS-232632; HIV protease inhibitors; Solid-phase extraction; High-performance liquid chromatography; Therapeutic drug monitoring

1. Introduction

Atazanavir (ATV, formerly BMS-232632) is a novel and recently marketed azapeptide (Fig. 1) with potent inhibitory effect on the human immunodeficiency virus (HIV) protease [1]. ATV has good oral bioavailability and pharmacokinetic properties that allow a once-daily dosing and a low pill burden [1]. Moreover, unlike other protease inhibitors (PIs), ATV is not associated with significant dyslipidemia when used as a single PI in highly active antiretroviral therapy (HAART) [2]. The recommended non-boosted dosage of ATV is 400 mg once-daily. When co-administered with efavirenz or other anti-HIV interacting drugs, it is recommended that ATV (300 mg) should be boosted with ritonavir (100 mg) [3]. ATV trough plasma concentrations exhibit substantial inter-subject variability and range from 0.095 to 0.468 μg/ml for individuals on non-boosted regimens and from 0.646 to 0.902 μg/ml for those on boosted ATV [4].

Therapeutic drug monitoring (TDM) of antiretroviral drugs is increasingly used to determine the best dosage regimen adapted to each individual [5]. TDM may be particularly important with ATV, which is the first PI administered once-daily and is primarily metabolized by cytochrome P450 enzymes (CYP3A4) [1]. Indeed, since ATV is both a substrate and an inhibitor of CYP3A4, combination therapy is likely to be confronted with drug–drug interactions involving induction or inhibition of CYP3A4 metabolism and hence reduced or increased exposure to ATV [6]. Thus, a method sufficiently sensitive to measure trough plasma levels of ATV in different therapeutic regimens is needed.

Several analytical methods have been recently published for the determination of ATV in human plasma. Five methods using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) have been described [7–11], that have the sensitivity and selectivity required to measure plasma ATV concentration in clinical trials. However, since MS/MS facilities

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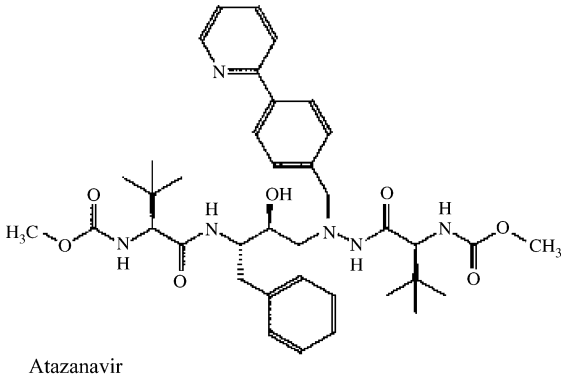

Atazanavir

logP= 4.25

Fig. 1. Atazanavir chemical structure. The log *P* value for ATV is also reported.

are not always available in standard hospital laboratories, analytical methods employing UV detection need to be developed. Some high-performance liquid chromatography (HPLC) methods using UV detection have been recently reported to determine ATV plasma level [12–18]. However, several of them require a multi-step, time-consuming liquid–liquid extraction [13–15] and gradient elution runs [12,13,16]. Although methods using simpler extraction procedures and isocratic elution have been developed [17,18], they show low drug recovery and high variability in the extraction step from plasma samples [18] and low sensitivity [17].

In this report, the development and validation of an user-friendly assay for routine quantification of ATV in human plasma is described. The proposed technique uses a simple and rapid solid-phase extraction (SPE) procedure, an isocratic elution on a reversed-phase column, and UV detection. The method is sufficiently sensitive to measure therapeutically relevant trough levels of ATV and is readily adaptable to standard laboratory equipment.

2. Experimental

2.1. Chemicals and reagents

ATV (hydrogen sulfate salt) was obtained from Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ, USA). Acetonitrile for chromatography (LiChrosolv) was from Merck (Darmstadt, Germany), while methanol for HPLC was from Carlo Erba (Milan, Italy). All other chemicals were of analytical grade and obtained from J.T. Baker (Deventer, The Netherlands). Ultrapure water was obtained from a Milli-Q apparatus (Millipore, Bedford, MA, USA). Control human plasma (with K_3EDTA as anticoagulant) was obtained from Roche (Milan, Italy; COBAS AmpliScreen kit).

2.2. Equipment and chromatographic conditions

The chromatographic system consisted of an Alliance 2695 Separations Module equipped with an online degasser and an automatic injector thermostated at 4 °C, and a 996 photodiode array detector coupled with the Empower data acquisition software (version 1.0) (Waters, Milford, MA, USA). Separations were performed on an XTerra RP18 (150 mm × 4.6 mm, particle size 3.5 μm; Waters) analytical column equipped with an XTerra RP18 (20 mm × 3.9 mm, particle size 5 μm; Waters) guard column. Both columns were maintained at 30 °C. The isocratic mobile phase consisted of a mixture of acetonitrile-phosphate buffer (pH 6, 52.5 mM) (43:57, v/v) and was filtered through a 0.2 μm nylon filter membrane (Millipore, Bedford, MA, USA) before use. The flow rate was 1.5 ml/min, and the assay runtime was 30 min. Absorbance was measured at 203 nm. An Extraction Manifold (Waters) liquid handling system was used to perform the sample preparation. A DRI-BLOCK DB-3 evaporator (Techne, Stone, United Kingdom) was used for drying the SPE eluates.

2.3. Preparation of stock solutions, working solutions, calibration standards and quality control (QC) samples

A stock solution of 878 μg/ml ATV (free base, corresponding to 1 mg/ml ATV sulfate) was prepared in methanol. The stock solution was diluted further with methanol to obtain working solutions with concentrations of 500, 375, 200, 100, 40, 20, 10, 4 and 2 μg/ml. The stock and working solutions were stored at −20 °C. Plasma calibration standards at 10.0, 7.5, 4.0, 2.0, 0.8, 0.4, 0.2, 0.08, 0.04 μg/ml were prepared by 1:50 dilution of the respective working solution in control human plasma. QC samples at the lower limit of quantification (LLOQ = 0.04 μg/ml), low (0.4 μg/ml), medium (2 μg/ml), high (8 μg/ml) and the upper limit of quantification (ULOQ = 10 μg/ml) concentration levels were prepared by diluting the working solutions in plasma. Of note, the total added volume of organic solvent in all samples corresponded to 2% of biological sample in accordance to FDA guidelines [19] recommending that non-biological matrix (i.e., organic solvent) should correspond to only ≤2% of the volume of final biological samples. The QC samples were prepared in batches at the same occasion, stored at −80 °C, and then thawed and thermized at 60 °C for 60 min on the day of analysis.

2.4. Sample pretreatment and preparation

Blood samples (5 ml) were collected in tubes with K_3EDTA as anticoagulant and transported to the laboratory on ice. The tubes were centrifuged at 3000 rpm (705 × *g*) for 10 min at 4 °C (Sigma Centrifuge, Model 2K15) and the plasma was collected and stored at −80 °C until analysis. On the day of analysis, plasma samples were thawed and heated at 60 °C for 60 min. This treatment has been shown to effectively inactivate HIV particles present in the samples [20]. The analysis of stability of ATV under these conditions is reported in the method validation (see below). Aliquotes (0.5 ml) of heat-inactivated plasma samples were loaded onto Oasis HLB 1 cc cartridges (Waters, Milford, MA, USA) previously activated with 1 ml of methanol followed by conditioning with 1 ml of Milli-Q water. The cartridges were then washed with 1 ml of 5% methanol (v/v). The analytes were successively eluted with

0.5 ml of methanol. The eluates were evaporated to dryness under a stream of nitrogen at 40 °C. The dried extracts were reconstituted with 125 μl of mobile phase. The reconstituted sample was centrifuged at 13,000 rpm for 5 min at room temperature and 20 μl of the supernatant was injected onto the HPLC system.

2.5. Stability

The stability of ATV in plasma under the thermization process (60 °C for 60 min) was assessed as follows: two series of samples at the LLOQ, low-, medium-, high-QC, and ULOQ level were prepared. One was heated with the procedure described above, while the thermization was omitted in the second. The two sample series were then subjected simultaneously to SPE and analyzed. Additionally, reconstituted samples were kept for 24 and 48 h at room temperature and for 48 and 96 h in the autosampler thermostated at 4 °C to investigate the stability of ATV under these conditions.

2.6. Recovery

The overall recovery of ATV from human plasma was determined at the LLOQ, low-, medium-, high-QC, and ULOQ level by comparing the peak area response obtained from extracted human plasma (six samples for each concentration level) with the peak area response from neat solutions of ATV prepared in mobile phase at the same concentration levels (six samples for each concentration level). The extraction recovery was calculated using the ratio of the response and the concentration factor of the assay (500:125) and was expressed as a percentage of the response of the calculated amount of ATV diluted in mobile phase and directly injected onto the HPLC, which corresponds to 100% recovery.

2.7. Analytical method validation

The validation of the assay was based on the FDA guidelines for Bioanalytical Method Validation [19]. Assay validation involved linearity, specificity, accuracy, precision, limit of detection (LOD) and lower limit of quantification (LLOQ) determination. Intra-day and inter-day precision values were estimated by assaying plasma samples containing five different concentrations of ATV five times on the same day and on four separate days to obtain the coefficient of variation (CV). Accuracy was determined as the percentage of deviation between nominal and measured concentration (% bias). To validate the accuracy and precision of the analysis of samples originally above the upper limit of quantification, a sample two times higher than the upper limit of the calibration curve was diluted five-fold with control human plasma and processed and analyzed according to the described method. Analytic interferences from endogenous substances were investigated by testing at least ten different lots of blank human plasma. The method specificity was also investigated by analyzing both plasma spiked with anti-HIV drugs and possible co-administered drugs, and patient samples.

3. Results and discussion

3.1. Method development

To develop an efficient and reproducible method for ATV extraction from plasma, several extraction solvents were initially tested (i.e., ethylacetate, ether, chloroform, dichloroethane and dichloromethane), as the use of liquid–liquid extraction has been shown to be a good choice in the development of LC-UV assays for other PIs [21]. However, liquid–liquid extraction methods showed to be time-consuming and gave low recovery and/or low reproducibility (data not shown). In contrast, the clean-up procedure by SPE provided a fast and efficient sample preparation, with high recovery and reproducibility (see below). Among the SPE cartridges and procedures proposed for PI extraction (e.g. [7]) that we tested, the Waters Oasis HLB cartridge proved to be the most adequate in terms of efficiency and reliability when SPE was performed with the standard procedure recommended by the producer.

The mobile phase that gave optimal separation from plasma endogenous peaks and from other antiretroviral drugs was a

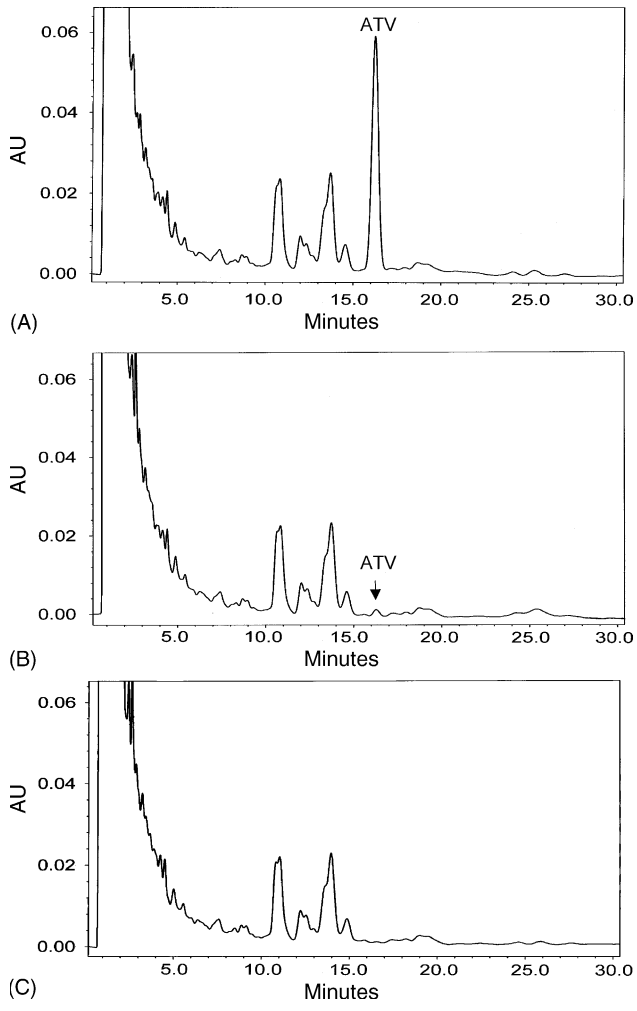

Fig. 2. Representative chromatograms obtained after extraction of (A) spiked plasma sample containing ATV 10 μg/ml (ULOQ), (B) spiked plasma sample containing ATV 0.04 μg/ml (LLOQ), and (C) blank plasma sample.

simple mixture of acetonitrile and phosphate buffer (43:57, v/v). A higher percentage of acetonitrile led to chromatographic interference with endogenous substances. Generally, the retention time of PIs is dependent on pH of the mobile phase buffer [22]. In fact, some previously reported assays used the mobile phase buffer at a variety of pH values. The optimum pH of the mobile phase buffer was determined by changing pH every 0.5 from pH 2 to 11, since the XTerra columns can be used in a wide pH range. Finally, pH 6.0 phosphate buffer for the mobile phase appeared to be optimal to separate ATV. Isocratic delivery of the mobile phase was preferred, as gradient elution requires control by a gradient HPLC pump system, long re-equilibration time, perfect solvent mixing, etc. Analyses were performed at 30 °C, as less reproducibility in ATV retention time was observed at lower temperatures. Using these chromatographic conditions, retention time of ATV was 16.2 min. Representative chromatograms of a control human plasma sample and spiked samples at the LLOQ and ULOQ level are shown in Fig. 2. At the selected detection wavelength, i.e., 203 nm, assays performed on drug-free human plasma (Fig. 2C) did not show the presence of any interfering peak at the retention time of interest.

3.2. Calibration curve and linearity

A nine-point calibration standard curve of ATV in plasma, ranging from 0.04 to 10 μg/ml for ATV, was prepared in triplicate for each run. The calibration curves were linear over the tested concentration range, with correlations of 0.997–0.999.

3.3. Lower and upper limits of quantification

The LLOQ, defined as the lowest concentration in the standard curve that back-calculates with good accuracy and precision (accuracy = −3.6/4.5%, CV = ≤3.0%, $n = 20$), was 0.040 μg/ml. A typical chromatogram of an LLOQ sample is shown in Fig. 2B. The LLOQ was lower compared with all the existing LC-UV assays for ATV [12–18]. This LLOQ is below the lower limit of ATV trough plasma concentrations recently reported (0.095 μg/ml [4]) and thus provides sufficient sensitivity for routine analysis of human plasma samples both for individuals on boosted ATV and for those on non-boosted regimens. The LOD, defined as the concentration giving a signal-to-noise ratio of 3, was 0.020 μg/ml. The ULOQ, defined as the highest concentration in the standard curve that was analyzed using the chromatographic and detection parameters, was 10 μg/ml. Samples originally above the ULOQ could be quantified with good accuracy and precision after a five-fold dilution with control human plasma. When performing this procedure, two high-QC samples were diluted in duplicate by the same dilution factor to allow validation of the dilution process.

3.4. Recovery

The average recovery (±S.D.) obtained for ATV during the extraction from plasma ranged from 96.5 ± 4.2% to 99.3 ± 3.7% in the tested concentration range (0.04–10 μg/ml). These results

Table 1
Stability of ATV in plasma samples heated for 60 min at 60 °C and in reconstituted extracts under different storage condition

Nominal concentration (μg/ml)	ATV-spiked plasma samples				Reconstituted extracts							
	60 min at 60 °C				48 h at room temperature				96 h at 4 °C			
	Mean measured concentration at $t = 0$ (μg/ml)	Mean concentration recovered (μg/ml)	Deviation (%)	CV (%)	Mean measured concentration at $t = 0$ (μg/ml)	Mean concentration recovered (μg/ml)	Deviation (%)	CV (%)	Mean concentration recovered (μg/ml)	Deviation (%)	CV (%)	
0.04 (LLOQ)	0.041	0.039	−4.9	3.3	0.038	0.036	−5.3	2.9	0.040	5.2	3.6	
0.4 (low QC)	0.394	0.383	−2.8	2.8	0.389	0.394	1.3	0.9	0.372	−4.4	0.6	
2.0 (medium QC)	1.945	1.975	1.5	0.6	2.043	1.998	−2.2	1.8	1.899	−7.0	1.4	
8.0 (high QC)	8.179	8.007	−2.1	1.1	7.856	7.775	−1.1	3.1	7.942	1.1	2.6	
10.0 (ULOQ)	9.523	9.847	3.4	0.9	9.789	9.657	−1.3	2.4	9.849	0.6	0.7	

indicate that the extraction method developed here achieves a high degree of efficiency and reproducibility.

3.5. Stability

Good stability of ATV in methanol at −20 °C and in human plasma under various conditions (i.e., 24–48 h at room temperature, 4 °C for 9 days, after 1–3 freeze-thaw cycles, and long-term storage at −20 °C) has been conclusively demonstrated by several authors [7–9,12,13,18]. In contrast, some debate still exists about ATV stability during heating HIV-inactivation, as Schuster et al. [7] and Sparidans et al. [18] reported a 15–46% decrease in ATV concentration in plasma samples treated at 60 °C for 30–60 min, while no degradation of ATV in plasma after heating at 56–60 °C for 30–60 min was observed in other studies [9,10,12]. These discrepancies put the stability of ATV under heating in question, and demand further investigation, as heating HIV-inactivation of plasma samples is recommended for safety issues. While several reasons could be adduced for the thermal instability of ATV observed previously (e.g. the extraction procedure, or the extremely low ATV plasma concentration), our stability studies, performed with plasma samples at 0.04–10 μg/ml ATV concentration range, show no major effect of the thermization procedure (60 °C for 60 min) on ATV levels (Table 1).

In addition, the stability of the processed samples in mobile phase was assessed. Two sets of extracted and reconstituted LLOQ, ULOQ and QC samples in mobile phase were analyzed immediately after extraction and after 24 and 48 h of storage at room temperature and 48 and 96 h in the autosampler thermostated at 4 °C. These samples were analyzed simultaneously with a freshly prepared calibration standard curve. After 48 h at room temperature or 4 days of storage in the autosampler at 4 °C, extracted samples were in the range 93–105% of their nominal concentrations (Table 1), indicating that ATV is stable in the reconstituted extracts under the tested conditions.

3.6. Accuracy and precision

The accuracy and precision of the assay were determined by assaying LLOQ, ULOQ and QC samples in five replicates on each of four different days. The accuracy (expressed as % bias) and precision (expressed as % CV) data are summarized in Table 2. The intra-assay deviation (% bias) from the nominal concentrations was between −3.0% and 4.5% and the range of inter-day deviations always ≤4.7%. The CVs calculated for ATV in the intra-day and inter-day assays ranged from 0.6% to 3.0% and from 1.1% to 2.9%, respectively. Both accuracy values and inter-day and intra-day CVs are similar to or much lower than previously reported values for LC-UV assays measuring ATV concentration in human plasma [12–18]. These results indicate that the method developed here achieves a high degree of reproducibility and accuracy.

3.7. Selectivity and specificity

At least ten different lots of commercial control human plasma were evaluated for interference in the assay. No significant interfering peaks from human plasma were found at the retention time of ATV (see Fig. 2C). In addition, the following

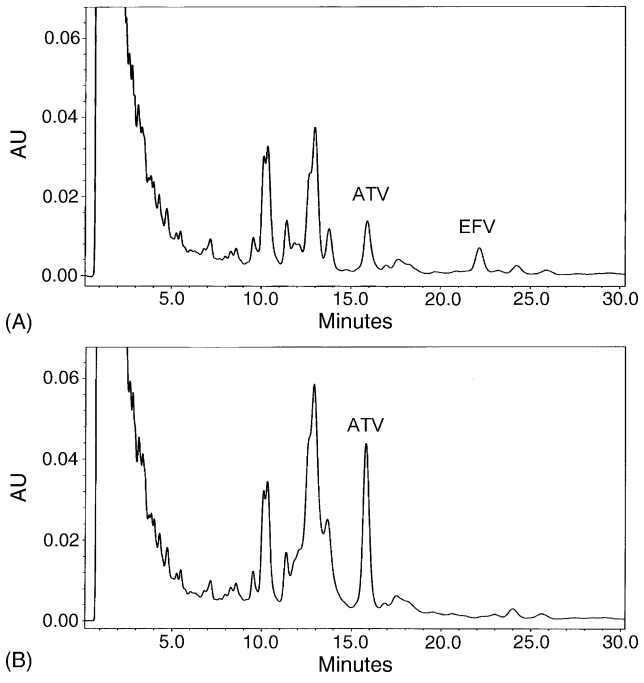

Fig. 3. Chromatograms of plasma samples from HIV-infected patients treated with ATV in combination with (A) ritonavir, efavirenz (EFV), and tenofovir, or (B) abacavir and lamivudine. The measured concentration of ATV was 1.93 μg/ml in sample (A) and 5.87 μg/ml in sample (B). Efavirenz (whose peak is marked), ritonavir, tenofovir, abacavir, and lamivudine did not interfere or were not retained on the column.

Table 2
Intra-day and inter-day accuracy and precision results

Nominal concentration (μg/ml)	Intra-day ($n=5$)			Inter-day ($n=4$)		
	Mean measured concentration (μg/ml)	Accuracy[a] (% bias)	Precision (% CV)	Mean measured concentration (μg/ml)	Accuracy (% bias)	Precision (% CV)
0.04 (LLOQ)	0.0418	4.5	3.0	0.0385	−3.6	2.9
0.4 (low QC)	0.409	2.3	0.6	0.419	4.7	2.8
2.0 (medium QC)	1.94	−3.0	0.9	2.05	2.6	1.5
8.0 (high QC)	8.25	3.1	2.2	7.80	−2.5	1.1
10.0 (ULOQ)	9.71	−2.9	1.9	10.22	2.2	2.6

[a] Accuracy = [(measured concentration − nominal concentration)/nominal concentration] × 100.

antiretroviral and non-antiretroviral potentially co-administered drugs were analyzed with the current procedure: abacavir, acetaminophen, acyclovir, amprenavir, didanosine, efavirenz, enfuvirtide, fluconazole, ganciclovir, gentamicin, ibuprofen, indinavir, itraconazole, lamivudine, lopinavir, methadone, nelfinavir, nelfinavir metabolite (M8), nevirapine, oxazepam, pyrazinamide, pyrimethamine, ribavirin, rifampicin, ritonavir, saquinavir, stavudine, sulphamethoxazole, teicoplanin, tenofovir, trimethoprim, vancomycine, voriconazole, zalcitabine, zidovudine. All drugs eluted at different times or were not retained on the column (Fig. 3 and data not shown). Therefore, none of these drugs was found to interfere with the assay. The absence of analytic interference was also confirmed by the use of the peak purity testing system and the library matching of the Empower software.

3.8. Analysis of patient samples

This analytical method is used daily to measure ATV plasma concentrations of samples received by our TDM Service and has proved to be reliable and sensitive enough for monitoring concentrations of ATV in plasma from HIV-infected individuals. Furthermore, the method is being applied in ongoing research studies. Fig. 3 shows representative chromatograms of TDM samples from patients receiving ATV.

4. Conclusion

A simple, precise, selective, and sensitive isocratic HPLC assay with UV detection for the quantification of ATV in plasma has been developed and validated. The applicability of the method and the appropriateness of the validated concentrations ranges have been demonstrated in the analysis of plasma samples of HIV-infected subjects. This simple HPLC method can enable measurement of up to 48 samples in one day and can be conveniently used for routine TDM of ATV in conventional hospital laboratories wherein LC–MS/MS is not available.

Acknowledgments

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