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Determination of the new HIV-protease inhibitor atazanavir by liquid chromatography after solid-phase extraction

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Abstract

An HPLC method previously described for the simultaneous assay of amprenavir, ritonavir, indinavir, saquinavir, nelfinavir and efavirenz is proposed here for the simultaneous analysis of the new HIV protease inhibitor atazanavir (ATV) in human plasma, by off-line solid-phase extraction (SPE) followed by HPLC coupled with UV-diode array detection. After viral inactivation by heat (60 °C for 60 min), plasma (600 μl) with clozapine (internal standard) is diluted 1 + 1 with phosphate buffer pH 7 and subjected to a SPE on a C18 cartridge. Matrix components are eliminated with 2 × 500 μl of a solution of 0.1% H_3PO_4 neutralised with NaOH to pH 7. ATV is eluted with 3 × 500 μl MeOH. The resulting eluate is evaporated under nitrogen at room temperature and is reconstituted in 100 μl MeOH/H_2O 50/50. A 40 μl volume is injected onto a Nucleosil 100–5 μm C18 AB column. ATV is analysed by UV detection at 201 nm using a gradient elution program with solvents constituted of MeCN and phosphate buffer adjusted to pH 5.14. The mobile phase also contains 0.02% sodium heptanesulfonate, enabling an excellent separation of ATV from the other HIV protease inhibitors (PIs) amprenavir, indinavir, saquinavir, ritonavir, lopinavir, nelfinavir and the non-nucleoside reverse transcriptase inhibitors (NNRTIs) efavirenz and nevirapine. The calibration curves are linear up to 10 μg/ml, with a lower limit of quantification of 0.2 μg/ml. The mean absolute recovery of ATV is 96.4 ± 3.2%. The method is precise with mean inter-day CVs within 1.1–6.1%, and accurate (range of inter-day deviations +0.3 to +2.3%). The method has been validated and is currently applied to the monitoring of ATV in HIV patients.

Keywords: Atazanavir; Protease inhibitors; Non-nucleoside reverse transcriptase inhibitors

1. Introduction

The availability of highly active antiretroviral therapy has revolutionized the treatment of HIV infection, causing a dramatic reduction in HIV-related morbidity and mortality [1]. However, up to 50% of treatment-naïve patients do not have sustained antiviral response after one year of therapy [2]. Moreover, the extensive use of highly active antiretroviral therapy has led, to development of drug resistance and metabolic complications, which have clinical consequences for patients, and pose a problem for clinicians and for the community healthcare perspective. Atazanavir (ATV, BMS-232632, Fig. 1) is a novel and recently marketed azapeptide with potent inhibitory effect on the HIV protease [3] and with alternate resistance pattern [4]. Unlike other HIV protease inhibitors (PIs), ATV is reportedly not associated with significant dyslipidemia when used as a single PI in triple therapy regimen [4,5]. Furthermore, its good oral bioavailability and favorable pharmacokinetic profile may enable a once-daily dosing and a low pill burden [6].

Since increasing clinical evidences indicate that levels of systemic exposure to PIs and non-nucleoside reverse transcriptase inhibitors (NNRTIs) correlate with efficacy as well as with some of their adverse effects, adjusting PIs and

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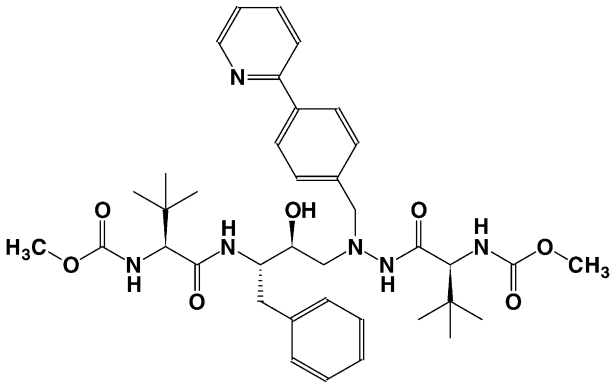

Fig. 1. Atazanavir chemical structure.

NNRTIs concentrations within a therapeutic window may be of benefit, especially in the light of the high inter-individual and relatively low intra-individual variability in plasma levels. Therefore, therapeutic drug monitoring (TDM) of PIs and NNRTIs is important for detecting sub-optimal drug exposure, contributing to the prevention of viral resistance development. TDM is also a practical tool for monitoring short-term adherence and in the detection, prevention and management of adverse drug reactions, drug-drug or drug-food interactions. Numerous high performance liquid chromatography methods have been developed to quantify simultaneously the HIV PIs (i.e. indinavir, amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir) and NNRTIs (efavirenz and nevirapine) ([7,8] and references cited therein). Two well detailed methods to quantify ATV in human plasma and in peripheral blood mononuclear cells (PBMCs) have been recently published, using liquid chromatography coupled to tandem mass spectrometry preceded by solid-phase extraction [9–11].

Since MS/MS facilities are not always available in standard hospital laboratories, we report here a simple method for the assay of ATV in human plasma, by offline solid-phase extraction followed by HPLC coupled with UV-diode array detection, using the sample processing previously proposed for the first five marketed PIs and efavirenz [7]. This method provides an excellent separation of ATV from the other HIV PIs (amprenavir, indinavir, saquinavir, ritonavir, lopinavir, nelfinavir) and the NNRTIs (efavirenz and nevirapine), enabling the accurate measurement of ATV in any concomitant antiretroviral treatment. The HPLC method for ATV has the required level of sensitivity and reproducibility for routine clinical application such as TDM.

2. Materials and methods

2.1. Chemicals

Reyataz™ capsules (ATV sulfate, corresponding to ATV base 150 mg) were kindly provided by Bristol–Meyers Squibs (Baar, Switzerland). ATV stock solution (1 mg/ml) in MeOH was obtained by extraction of Reyataz™ capsules with MeOH in which ATV is reported to be freely soluble (>100 mg/ml) [6]. One Reyataz™ capsule (150 mg ATV base) was carefully opened to collect the solid content into a 150 ml-volumetric flask. The empty capsule shell was thoroughly rinsed with MeOH (ca. 5 ml) which was collected into the same flask. The resulting suspension was sonicated for 5 min and after allowing to equilibrate at room temperature (RT), was completed to the volume with MeOH, before being filtrated through a paper filter. Clozapine (Internal Standard, I.S.) stock solution (250 μg/ml) in MeOH was obtained by extraction with MeOH of Leponex® (Novartis, Basel, Switzerland) tablet. This solution was diluted down to 45 μg/ml before use. Acetonitrile (MeCN) for chromatography LiChrosolv®, methanol (MeOH) for chromatography LiChrosolv®, 100% acetic acid (AcOH) and 85% phosphoric acid (H_3PO_4) were from E. Merck (Darmstadt, Germany). Sodium hydroxide puriss. p.a. pellets and sodium heptanesulfonate p.a. were purchased from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade and used as received. Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore).

2.2. Chromatographic system

The chromatographic system consisted of a Hewlett-Packard 1090 Series II (Agilent, formerly Hewlett-Packard, Germany) pump equipped with a spectrophotometric UV-DAD detector set at 201 nm. The separation was performed at RT on a ChromCart® cartridge column (125 mm × 4 mm i.d.) filled with Nucleosil 100–5 μm C18 AB (Macherey-Nagel, Düren, Germany) and equipped with a guard column (8 mm × 4 mm i.d.) filled with the same packing material. The injection volume was 40 μl.

The mobile phase was delivered at 1 ml/min and the gradient program conditions are given in Table 1. Solvent A consisted of pure MeCN. Solution B was prepared by adding 11.8 ml H_3PO_4 8.5% and 0.2 g sodium heptanesulfonate to 988.2 ml H_2O with pH carefully adjusted to 5.13–5.15 with

Table 1
Gradient elution program

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
Atazanavir			
0	15	85	–
2	30	70	–
8	32	68	–
18	42	58	–
34	46	54	–
35	33	34	33
36	–	10	90
37	–	10	90
38	33	34	33
39	46	54	–
40	15	85	–
47	15	85	–

Solvent A: MeCN; solvent B: H_3PO_4 8.5% + 0.2 g/l sodium heptanosulfonate; pH 5.14; solvent C: MeCN + 0.3% AcOH. Temperature (°C): RT; flow: 1.0 ml/min; injection volume: 40 μl.

NaOH 10 N. The solvent C was 0.3% AcOH in MeCN. Solutions B and C were prepared prior to each series of analysis and stored in the dark at +4 °C prior to use. All solvents were degassed by sparging with helium.

The HP-ChemStation A.06.03 software loaded on a Compaq DeskPro EP 6/400c was used to pilot the HPLC instrument and to process the data (area integration, calculation and plotting of chromatograms) throughout the method validation. Baselines were visually inspected and were manually adjusted (in general, base line to base line) using peak start and end features of the HP-ChemStation software. In addition, the full-scan UV spectrum of atazanavir, recorded on-line by diode-array-detection, was carefully checked in all chromatograms from patients samples to verify that no co-eluting peak are present at ATV retention time.

2.3. Buffers solutions for samples processing

The buffer used for the dilution of plasma samples was prepared by mixing 413 ml of KH_2PO_4 1/15 M (9.97 g/l) and 587 ml of $Na_2HPO_4{\cdot}2H_2O$ 1/15 M (11.9 g/l) (buffer D). The solid-phase extraction (SPE) cartridge conditioning (see below) was performed with a solution of 0.1% H_3PO_4 neutralized with NaOH to pH 7 (buffer E).

2.4. Stock solution, working solution, plasma calibration and control samples

Stock solution of atazanavir at 1 mg/ml (calculated as base) in MeOH was further diluted with MeOH/H_2O 50:50 for the preparation of working solutions at concentrations of 2.5–100 μg/ml. Plasma calibration samples at 0.25, 1.0, 2.5, 5.0, 8.0, 10.0 μg/ml, together with plasma quality control samples at 0.75, 3.0, 9.0 μg/ml, were prepared by 1:10 dilution of the respective working solution by blank plasma from outdated transfusion bags (total added volume ≤10% of the biological sample volume), in accordance with the recommendations on bioanalytical method validation [12]. Of note, the total added volume of organic solvent in this preparation corresponds to 5% of biological sample. The stock solution may therefore be more appropriately diluted with MeOH/H_2O 20:80 for the preparation of working solutions without analytical consequences, albeit in accordance to new FDA guidelines [13] recommending that non-biological matrix (i.e. organic solvent) should correspond to only <2% of the volume of final biological samples.

The calibration standards and control samples were prepared in batches at the same occasion and were thermised at 60 °C for 60 min (see below), stored at −20 °C as 1.5 ml aliquots in 5 ml-polypropylene Eppendorf tubes, and thawed on the day of analysis.

2.5. Samples collection and thermisation (HIV inactivation)

Blood samples were taken from HIV patients during a regular visit, in case of unsatisfactory virological response or when patients developed adverse drug reactions, or if altered pharmacokinetics was suspected due to possible drug interactions, and finally to assess short term compliance.

Blood samples (5 ml) were collected in Monovettes® (Sarstedt, Nümbrecht, Germany), with K-EDTA or Li heparinate as anticoagulant. Samples were immediately sent to the laboratory and were processed under a protection hood wearing nitrile gloves and long-sleeve lab coats. The Monovettes were centrifuged at 1850 × *g* (3000 rpm) for 10 min at +4 °C (Beckmann Centrifuge, Model J6B) and the plasma was separated and transferred into 5 ml-polypropylene test tubes before being heated at 60 °C for 60 min in a thermostated water bath (Memmert® WB 7, Schwabach, Germany). This treatment has been shown to effectively inactivate HIV particles present in the samples [14–17]. The stability of atazanavir under these conditions is reported in the method validation (see below). The processed plasma samples were stored at −20 °C until analysis.

2.6. Sample preparation

On the day of analysis, calibration, quality control and patient samples were thawed and allowed to equilibrate at room temperature.

The sample preparation procedure is identical to that previously described for the former PIs (IDV, APV, RTV, SQV, NFV, LPV) or nevirapine and efavirenz [7,8].

Briefly, 600 μl plasma samples (calibration, control, patients) were diluted with 500 μl of solution D and 100 μl of I.S. solution in an Eppendorf microvial. The resulting solution was vortexed and centrifuged for exactly 5.0 min on a benchtop centrifuge at 20,000 × *g* (14,000 rpm) at 4 °C (Hettich® Benchtop Universal 16R centrifuge, Bäch, Switzerland).

The clean up procedure of diluted plasma samples was performed by solid-phase extraction (SPE) using a 24 tubes vacuum manifold Macherey–Nagel (Düren, Germany). The C18 cartridges Supelclean LC-18 from Supelco (Sigma–Aldrich, Buchs, Switzerland) were 1 ml-cartridges containing 100 mg of octadecyl silica gel packing with a diameter of 40–45 μm and a porosity of 60 Angströms. The C18 cartridges were conditioned with 2 × 1 ml MeOH followed by 2 × 1 ml buffer E. An aliquot (1000 μl) of the diluted plasma sample was applied onto the cartridge and drawn through completely under vacuum (typically 1–2 mm Hg).

The cartridge was washed twice with 500 μl of buffer E and light vacuum (6–7 mm Hg) applied for 5 min. ATV (and any PIs/NNRTIs present in the samples) were subsequently desorbed with three times 500 μl of MeOH, with a final drying step (6–7 mm Hg vacuum) after the third elution. The eluted solutions were evaporated under nitrogen steam at room temperature for approximately 30 min and the residue reconstituted in 100 μl of MeOH/H_2O 50:50. The resulting solutions were carefully vortexed and centrifuged at 20,000 × *g* for 5 min at 4 °C. The supernatants were introduced into 200 μl HPLC microvials (Agilent,

Germany) and a volume of 40 μl was used for HPLC analysis.

2.7. Calibration curves

Quantitative analysis ATV was performed using the internal standard (I.S. = clozapine) method.

The calibration curves were fitted by least-squares linear regression using 1/concentration ($1/x$) as weighting factor of the peak-area ratio of ATV to I.S. versus the ratio of the injected amount of the respective ATV to I.S., in each standard samples. The calibration was established over the range 0.25–10.0 μg/ml for ATV.

2.8. Analytical method validation

The validation of the method was based on the guidelines published on-line by the FDA [13] as well as on the recommendations of the Conference Report of the Washington Conference on "analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies" [12] and of the Arlington Workshop "bioanalytical methods validation—a revisit with a decade of progress" [18].

Each level of the calibration curve was measured with two sets of calibrators: one at the beginning and the second at the end of the run. Throughout patient sample analysis, control samples at three concentrations levels (low, medium and high: i.e. 0.75, 3.0 and 9.0 μg/ml) were assayed every five samples.

Replicate analysis ($n = 6$) of quality control samples were used for the precision and accuracy determination, the three concentrations were chosen to encompass the range of the calibration curve corresponding to the ATV levels expected to occur in patient samples. Precision being calculated as the coefficient of variation (C.V., %) within a single run (intra-assay) and between different assays (inter-assays), and the accuracy as the percentage of deviation between nominal and measured concentration.

Both experimental lower limit of quantification (LLOQ) and limit of detection (LOD) were determined by diluting the calibration samples. The LLOQ for APV in plasma was experimentally chosen as the minimal concentration in plasma samples which could be confidently determined in accordance with the Conference Report on analytical method validation [12,18] and the FDA [13] recommending that the deviation between measured and nominal concentration at LLOQ should not deviate more than ±20%. The limit of detection (LOD) was considered as the concentration of ATV that provides a signal corresponding to three times the HPLC background signal.

2.9. Stability of ATV

The stability of ATV in plasma under the thermisation process (60 °C for 60 min) was assessed as follows: two series of calibration samples at the six concentrations reported above (0.25–10.0 μg/ml) were analysed in parallel. One was heated at 60 °C for 60 min, while the thermisation procedure was omitted in the second. Aliquots (600 μl) of both series were submitted simultaneously to solid-phase extraction according to the procedure described above. The slope was compared between the resulting calibration curves.

Further stability studies included:

(a) The long term stability of plasma samples kept frozen at −20 °C: six series of calibration and quality control plasma samples spiked with ATV were prepared. Three series were either immediately analysed (i.e. without being frozen) while the three remaining series were stored during 30 days at −20 °C. The ATV levels in samples were compared as well as the slope of the calibrations curves.

(b) The stability of plasma samples after multiple freeze-thaw cycles: aliquots of plasma spiked with ATV at 0.75, 3.0 and 9.0 μg/ml, underwent three freeze-thaw cycles: frozen samples were allowed to thaw at ambient temperature for 2 h and were subsequently refrozen. The ATV levels in samples collected at each step of the freeze-thaw cycles were analysed in the same series, to eliminate the inter-assay variability. The variations of ATV concentrations were expressed in percentage of the levels of samples not subjected to the freeze-thaw cycles.

(c) The stability of plasma extracts into HPLC vials: processed samples (i.e. reconstituted in MeOH 50:50) containing ATV at low, medium and high concentration were analysed in duplicate either immediately after preparation, or after being left 120 h at room temperature in the auto-sampler rack. The results were compared.

(d) The short-term temperature stability at RT: triplicate aliquots of plasma spiked with ATV at 0.25, 1.0, 2.5, 5.0, 8.0, 10.0 μg/ml were thawed and the volume was divided into two tubes (A and B). Tubes A were kept at RT for 24 h, which corresponds to the maximal duration that samples may be left in this condition. Tubes B were stored for 24 h at 4 °C. The slopes of ATV calibration curves in both groups were compared (Student' *t*-test).

2.10. Recovery

The efficiency of the solid phase extraction was determined with quality control samples at three levels (0.75, 3.0 and 9.0 μg/ml of ATV) with $n = 3$ for each level. The absolute recovery of ATV from plasma was obtained as the peak-area response of the processed samples, expressed as a percentage of the response of the calculated amount of ATV prepared in MeOH/H_2O 50/50 (contained into the 40 μl injection volume) directly injected onto the HPLC, which corresponds to the 100% recovery.

2.11. Selectivity

The selectivity of the proposed analytical method has been previously ascertained [7,8] for the following drugs:

stavudine, zidovudine/lamivudine, didanosine, nevirapine, M8 (pharmacologically active metabolite of nelfinavir), calcium folinate, atorvastatine, pancreatine, sulfadiazine loperamide, trimethoprim/sulfamethoxazole, pyrimethamine, clindamycin. Further drugs likely to be prescribed to HIV patients and/or employed in the treatment/prophylaxis of opportunistic infections have also been tested and include: tenofovir disoproxil fumarate (tenofovir DF), azithromycine, abacavir, flucytosine, itraconazole, cefepim, imipenem, amoxicillin/clavunalic acid, clarithromycine, metronidazole, rifampicin, ceftriaxone, amikacin, ciprofloxacin, levofloxacin, piperacilin/tazobactam, meropenem, teicoplanin, gentamicin, fluconazole, ceftazidime, vancomycine, amphotericin, voriconazole, caspofungin and ciclosporin.

2.12. Applications

This method is currently used as a part of our routine TDM of PIs and NNRTIs for HIV patients, in addition to the previously reported assay for the other PIs/NNRTI [7,8].

3. Results and discussion

3.1. Chromatograms

The proposed HPLC method enables the measurement of ATV in plasma with UV detection at 201 nm. With the gradient program used (Table 1), the retention times for clozapine and ATV are 8.9 and 24.4 min, respectively (Fig. 2). Interestingly, this gradient program, previously described for the simultaneous assay of amprenavir, ritonavir, indinavir, saquinavir, nelfinavir and efavirenz in plasma, gives an excellent separation for ATV and is eluted between SQV and RTV (retention time: 22.3 and 27.3 min, respectively) (Fig. 3). This gradient elution program yields sharp peaks for all PIs, including ATV without producing any significant drift of the baseline, even at such a low wavelength.

Fig. 4 shows the chromatogram of a blank plasma, using the gradient program reported in Table 1. Fig. 5 shows the chromatographic profile of a plasma obtained from a patient receiving a regimen of atazanavir 400 mg QD, didanosine 400 mg QD and abacavir 300 mg BID. The level of ATV measured 3 h 45 min after the Reyataz® intake is 2.6 μg/ml.

3.2. HPLC solvents composition

A careful control of the elution conditions, including pH of solution B (at 5.13–5.15), mobile phase composition and gradient program, is important for consistent peak shape and retention time of ATV, and for satisfactory separation from matrix peaks. Solvents are prepared regularly (e.g. every 3 days) and stored in the dark at +4 °C prior to use.

Solvent was found stable at room temperature throughout the series of analyses that may last up to 48 h: the pH remained identical (pH ±0.01) during this period of time and there were no signs of microbiological contamination. The retention time of ATV was stable (24.4 ± 0.4 min (mean of 10 series of analysis). In fact, pH adjustment at 5.14 was especially important for the other PI nelfinavir whose retention time at 33 min (Fig. 3) was found more sensible to pH variation—so that nelfinavir will not interfere with other antiretrovirals, notably ATV.

For the ATV analysis at 201 nm, it is recommended, as for the others PIs, to introduce at the end of the elution a rinsing step with a 10/90 mixture of solvent B and the additional solvent C, followed by a re-equilibration with 15/85 solvent A/solvent B, for avoiding perturbation of the next analysis by late eluting peaks.

3.3. Selectivity

No significant interfering peaks were observed at the retention time of clozapine and ATV at 8.9 and 24.4 min, respectively. A small peak at 23.9 min arising from an endogenous matrix component, was observed just prior to ATV in some blank plasma used for the preparation of the calibration samples and in some patients. However, this signal was comparatively small producing only some drift of the baseline, which did not preclude an acceptable determination of the clinically relevant range of ATV concentrations (see below).

The method selectivity was confirmed by analysing all the currently prescribed anti HIV drugs and/or drugs possibly employed in the treatment/prophylaxis of opportunistic infections in the HIV+ patients cohort (listed in Material in Methods). All drugs are eluted at different times and do not interfere with ATV analysis. As standard substances of drug metabolites are difficult to obtain (with the notable exception of M8, an active metabolite of nelfinavir), their potential interference can not be fully excluded. Thus, it is recommend to always inspect the full scan UV absorbance spectrum of ATV provided by the diode-array detector, to circumvent any incorrect peak assignment due to the recognized limited selectivity of a single wavelength UV detection at 201 nm. Of note, direct injection of tenofovir disoproxil fumarate (obtained by extraction with H_2O of Viread® tablet (TRB Chemedica International SA, Genève, Switzerland) produces a peak at 8.9 min, at the same retention time as clozapine. However, since tenofovir disoproxil fumarate is a pro-drug cleaved in vivo by enzymatic hydrolysis, the pro-drug is unlikely to be detectable in patients plasma. Indeed, none of the chromatographic profiles of plasma from patients on Viread® had a detectable signal at this retention time. Moreover, when tenofovir disoproxil fumarate is added into plasma, no signal is observed at 8.9 min, suggesting that the hydrolysis already takes place in vitro in presence of plasma proteins.

3.4. Calibration samples preparation

The proposed method for the ATV assay is using the same methodology previously published for the simultaneous analyses of other PIs and efavirenz [7,8]. Thus, it

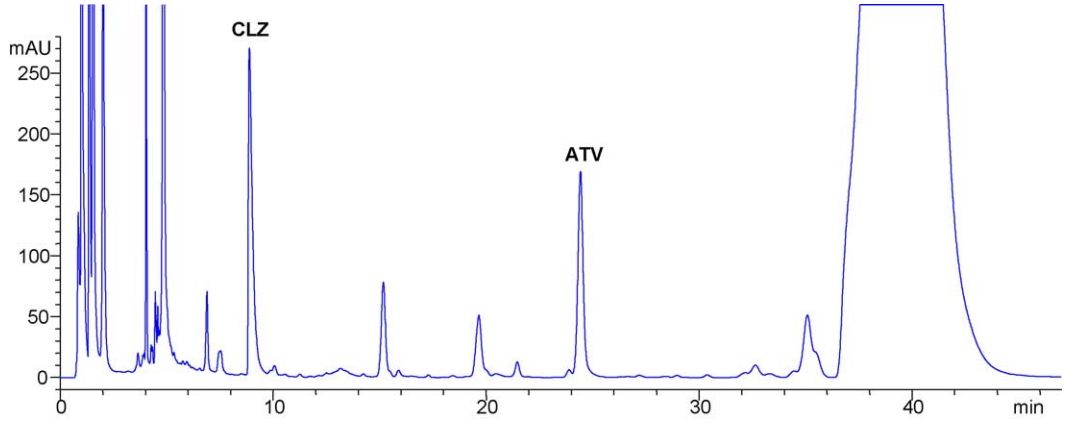

Fig. 2. Chromatographic profile of a plasma calibration sample of ATV (8000 ng/ml) spiked with I.S. (CLZ).

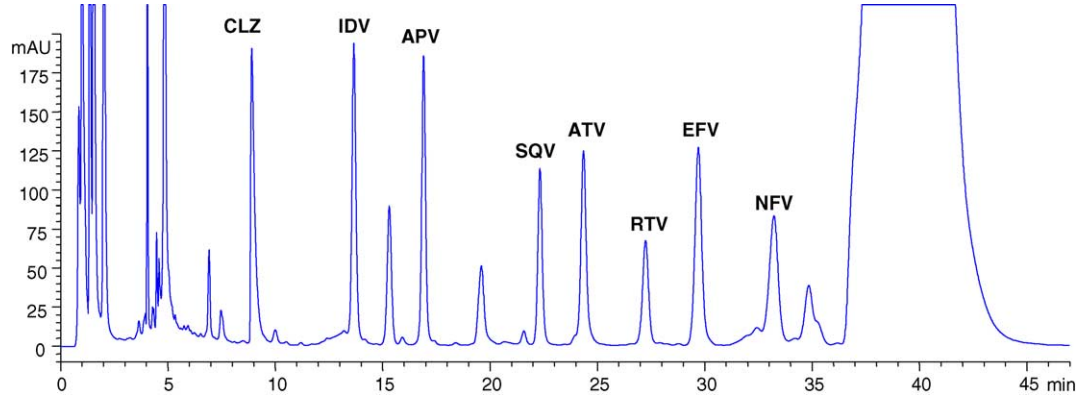

Fig. 3. Chromatographic profile of a plasma calibration sample of ATV with PIs/NNRTIs (8000 ng/ml) spiked with I.S. (CLZ).

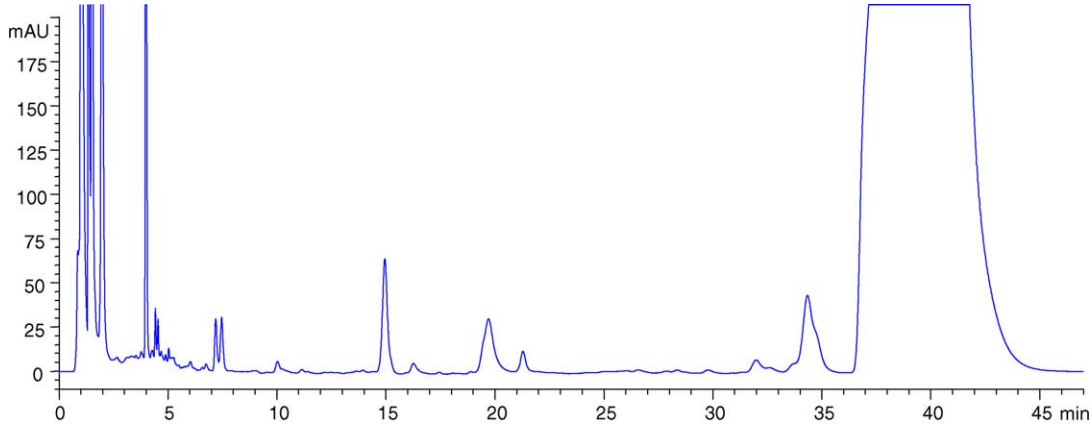

Fig. 4. Chromatographic profile of a blank plasma.

was initially attempted to prepare calibration samples containing atazanavir together with the former PIs (indinavir, amprenavir, saquinavir, ritonavir, nelfinavir) and efavirenz. By doing so however, a definite loss of linearity of the established calibration curves was observed at high levels (10,000 ng/ml) with plasma becoming viscous. This phenomenon arises most probably because of solubility problems due to an excessive number of analytes solubilised in plasma with MeOH (i.e. total added MeOH volume $\leq$10% of the biological sample volume [7,8]). Thus, it is recommended to prepare a separate series of atazanavir plasma calibration samples. The HPLC analysis of the previous protease inhibitors has been previously reported and is not discussed here [7,8].

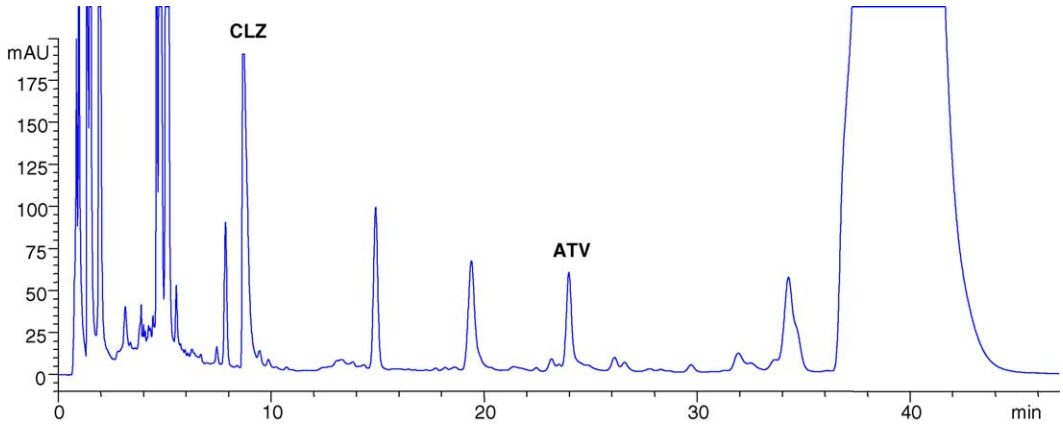

Fig. 5. Plasma chromatogram of one HIV-patient receiving abacavir, atazanavir and didanosine (details in the text).

3.5. Calibration curves

The calibrations curves have been calculated and fitted by least-squares linear regression either unweighted, or using 1/concentration ($1/x$) and 1/concentration^2 ($1/x^2$) as weighting factor. In order to establish the best weighting factor, back calculated concentrations were determined. The model with the lowest total bias and the most constant bias across the concentration range was considered to be the best fit. Visual inspection of the plot of residuals (Fig. 6) of the $1/x$ weighted regression indicates that there are no trend in variability throughout the delineated range of concentrations. Moreover, the homogeneity of variances have been statistically verified according to Levene's test [19] yielding Pr values $>F = 0.161$, verifying the hypothesis of homoscedasticity, and indicating that the chosen $1/x$ model is indeed adequate.

The slope of the calibration curves obtained throughout method validation and initial analysis of patients samples was stable, with values averaging 0.74 (±0.02%) (n = 9). Over the concentration range 0.25–10.0 μg/ml for ATV, the regression coefficient r^2 of the calibration curves remained excellent, always greater than 0.999.

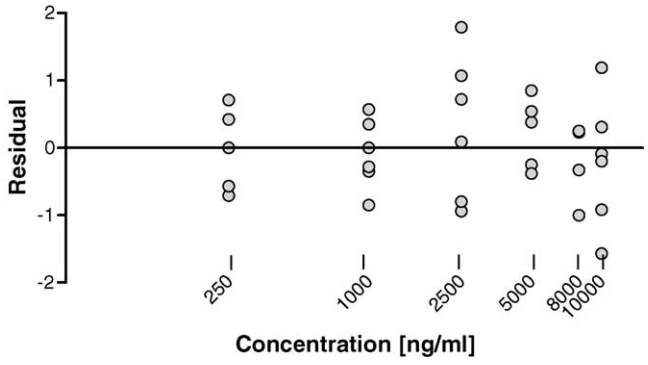

Fig. 6. Residual plot of ATV plasma calibration samples back-calculated with a $1/x$ weighted regression (n = 6).

3.6. Validation of the HPLC method: precision, accuracy and LLOQ/LOD

Precision and accuracy of the control samples are given in Table 2. The concentration levels of control samples of ATV (750, 3000 and 9000 ng/ml) were selected to encompass the range of concentrations expected in plasma samples.

Throughout these concentration ranges, the mean intra-assay precision was similar, always lower than 5.5%. Overall, the mean inter-day precision for ATV was good with mean CVs within 1.1–6.1%. The intra-assay deviation (bias) from the nominal concentrations of ATV was between +0.1 and +4.1% and the range of inter-day deviation was always <2.3%.

By analysing plasma from outdated transfusion bags spiked with decreasing concentrations of ATV (0.2–0.0125 μg/ml), the lower limit of quantification was experimentally found to be 0.2 μg/ml for ATV. The limit of detection of ATV with this HPLC-UV assay was 0.05 μg/ml.

3.7. Recovery

The mean absolute recovery of ATV measured with the high, medium and low QC controls were 96.4 ± 3.2%. (Table 3). The clean-up procedure by SPE was found to be a reliable way of eliminating interfering material from plasma, with low recovery variability.

3.8. Samples stability

3.8.1. Stability during thermisation (HIV inactivation)

The slope of the calibration curves of ATV established in samples submitted to the thermisation procedure (60 °C for 60 min) was slightly lower (−4.6 ± 0.1) than that obtained with non-heated samples, as shown in Table 4. In addition, the Table 5 shows the back-calculated values of both thermised and non thermised QCs samples using calibration curves established with samples subjected or not to the same

Table 2
Precision and accuracy of the assay for ATV in plasma (750, 3000 and 9000 ng/ml)

Nominal concentration (ng/ml)	Concentration found (ng/ml)	S.D.	Precision (C.V., %)	Accuracy bias (%)
(A) Intra-assay (n = 6)				
750	781	±43	5.5	4.1
3000	3020	±27	0.9	0.7
9000	9008	±134	1.5	0.1
(B) Inter-assay (n = 5)				
750	762	±47	6.1	1.6
3000	3069	±102	3.3	2.3
9000	9026	±95	1.1	0.3

(Found − Nominal)/Nominal × 100.

Table 3
Absolute recovery of ATV after SPE from plasma

Nominal concentration (ng/ml)	750			3000			9000		
Area (w/SPE)	386	377	377	1335	1304	1305	3821	3950	3586
Area (w/o SPE)	396	397	423	1333	1315	1301	3887	4022	3987
Absolute recovery (%)		93.8			99.9			95.5	

Mean absolute recovery (%) = 96.4 ± 3.2.

Table 4
Parameters of the calibration curves for ATV before and after plasma thermisation at 60 °C for 60 min (n = 2)

Sample treatment (n = 2)	m	r^2	b	Variation (%)
(1) Thermisation 60 min at 60 °C	0.7589	0.9999	−2.01E-02	
No thermisation	0.7949	0.9999	−1.27E-02	−4.5
(2) Thermisation 60 min at 60 °C	0.7578	0.9996	−2.30E-03	
No thermisation	0.7950	0.9995	−1.20E-02	−4.7
Mean ± S.T.D.				−4.6 ± 0.1

m: slope, r^2: coefficient of determination, b = y-axis intercept.

Table 5
Back calculated values of both thermised and non thermised QCs samples using calibration curves established with samples subjected or not to the same treatment

Nominal concentration (ng/ml)	T vs. T (ng/ml)	Accuracy (%)	T vs. NT (ng/ml)	Accuracy (%)	NT vs. T (ng/ml)	Accuracy (%)	NT vs. NT (ng/ml)	Accuracy (%)
750	805	7.3	860	14.7	760	1.3	780	4.0
	790	5.3	860	14.7	762	1.6	760	1.3
3000	2900	−3.3	2760	−8.0	2945	−1.8	2950	−1.7
	2870	−4.3	2835	−5.5	2985	−0.5	2955	−1.5
9000	9100	1.1	9015	0.2	9340	3.8	9120	1.3
	8990	−0.1	8685	−3.5	9085	0.9	8980	−0.2

T vs. T: thermised QC samples back-calculated with the calibration established with thermised samples; T vs. NT: thermised QC samples back-calculated with the calibration established with non-thermised samples; NT vs. T: non-thermised QC samples back-calculated with the calibration established with thermised samples; NT vs. NT: non-thermised QC samples back-calculated with the calibration established with non-thermised samples.

treatment. Considering the experimental variability (Table 2) and by comparison with stability studies performed with previous PIs submitted to the same procedure [7,8], these results indicate that such a procedure does not affect, or only slightly, ATV concentrations, within the considered concentrations range. These data contrast with a previously published study on atazanavir stability reporting a 15 and 40% decrease in the concentration of QC at 800 and 3 ng/ml, respectively [10]. The latter stability study was performed by LC–MS–MS at a much lower and narrow concentrations range (3–800 ng/ml), suggesting that at low levels (<1 μg/ml), atazanavir may be more affected by heat treatment. Our stability study, performed with plasma samples at the clinically relevant range of concentration (250–10,000 ng/ml) usually encountered in therapeutic drug monitoring, shows no major effect of the thermisation procedure on ATV levels. Nevertheless, and for the sake of standardisation, all calibration and control samples were treated similarly and heated at 60 °C for 60 min.

3.8.2. Stability of plasma samples at room temperature

The mean slope of calibration curves (n = 3) established with samples let 24 h at RT (m = 0.69) was not different than the slope of calibration curves calculated with samples stored during the same time at 4 °C (m = 0.71; n = 3), (P =

Table 6
Stability after one, two and three freeze-thaw cycles for the QC plasma samples G, H and I, at nominal concentration of 9000, 3000, 750 ng/ml, respectively

Number of thaw-freeze cycles	Nominal Concentration 9000 ng/ml			Mean	± S.D.	Percentage of initial concentration	±
	G1	G2	G3				
0	9240	9120	9290	9217	87		
1	9305	9215	9220	9247	51	100.3	0.5
2	9170	9270	9250	9230	53	100.1	0.6
3	9105	9090	9135	9110	23	98.8	0.2
Number of thaw-freeze cycles	Nominal Concentration 3000 ng/ml			Mean	±S.D.	Percentage of initial concentration	±
	H1	H2	H3				
0	3100	3090	3125	3105	18		
1	3075	3080	3060	3072	10	98.9	0.3
2	3100	3125	3080	3102	23	99.9	0.7
3	3040	3025	3130	3065	57	98.7	1.8
Number of thaw-freeze cycles	Nominal Concentration 750 ng/ml			Mean	±S.D.	Percentage of initial concentration	±
	I1	I2	I3				
0	755	765	720	747	24		
1	755	770	765	763	8	102.2	1.0
2	750	730	760	747	15	100.0	2.0
3	720	785	750	752	33	100.7	4.4

Concentration expressed in % of the initial concentration. 0: initial concentration.

0.3, Student's *t*-test), indicating the good stability of ATV in plasma at RT.

3.8.3. Stability of plasma samples kept frozen at −20 °C

No evidence of ATV decomposition was found during plasma samples storage in the freezer at −20 °C for at least 3 months. This confirms the good atazanavir stability at −20 °C previously reported in detail in another studies [11]. Calibration and quality control samples wee prepared in batches, and distributed in 5 ml-polypropylene tubes for use up to one month in our routine PIs/NNRTI monitoring facility. The concentrations of the control samples remained stable at least up to one month, and the slope of the calibrations curves constant, as previously stated. Plasma collected from patients samples were stored in the same type of tubes at −20 °C prior to the analysis that usually takes place within one week.

3.8.4. Stability of plasma samples after one, two and three freeze-thaw cycles

The variations of ATV concentrations when submitting control plasma to successive three freeze-thaw cycles are reported in Table 6. Taking into account the analytical variability, this indicates that no significant loss of ATV is to be expected after up to three freeze-thaw cycles.

3.8.5. Stability of extracts samples into HPLC vials (i.e. ready for HPLC analysis) at room temperature

The stability of plasma extracts (i.e. reconstituted in MeOH/H_2O 50/50) submitted to HPLC analysis was checked at RT for 5 days (120 h) and is reported in Table 7. The variations over time of ATV, expressed in percentage of the starting levels (i.e. after immediate analysis), were less then −4.2 ± 0.4% in samples left at room temperature for 120 h. These results indicate that, considering the analytical variability, the processed samples stability is acceptable throughout the HPLC run performed over one day.

3.9. Clinical applications

This HPLC assay is currently used for the analysis of samples collected as part of the TDM in HIV-positive patients, contributing to patient management, in particular for assessing compliance and evaluating drug-drug interactions. Since ATV is both a substrate and an inhibitor of CYP3A4, combination therapy is likely to be confronted with interactions involving induction and/or inhibition of CYP3A4 metabolism. Drug/drug interaction has been notably documented for ATV whose AUC, Cmax and Cmin are dramatically reduced (by 74, 59 and 93%, respectively) by the co-administration of EFV, a potent CYP3A4 inducer [20,21]. To

Table 7
Stability of ATV in extract samples left at room temperature (RT) for 5 days

750 ng/ml	3000 ng/ml	9000 ng/ml
Stability of atazanavir in extracts samples left at room temperature (RT) for 120 h (% change of the initial concentration ± S.D.; *n* = 2)		
−4.2 ± 0.4	−3.2 ± 1.0	−2.0 ± 1.1

overcome the CYP3A4 induction due to efavirenz, atazanavir has been associated with 100 mg of ritonavir once daily. In the presence of ritonavir as pharmacokinetic enhancer, atazanavir and efavirenz may hence be administered in association [22]. Finally, the isoenzyme CYP3A4 displays a large inter-individual variability in its expression and activity and is the major isoenzyme responsible for the metabolism of ATV.

More generally, the monitoring of new antiretroviral drugs is important for detecting interactions modifying their systemic disposition likely to impair the virologic response. Such pharmacokinetic interactions have been notably observed when the NRTI tenofovir DF is co-administered with un-boosted dose (i.e. without ritonavir added as pharmacokinetic enhancer) of 400 mg of ATV once daily. The atazanavir AUC and Cmin were decreased by approximately 25 and 40% respectively, via a mechanism not yet elucidated [23]. This advocates again for the monitoring of novel drugs administered in combination to heavily pretreated patients having experienced treatment failure, and in whom exposure, tolerance and adherence assessments are critical issues.

4. Conclusions

This HPLC method provides a robust procedure for determining the new PI atazanavir in plasma by using samples processing, column type, solvent mixture and gradient program previously proposed for the first PIs generation, and run routinely in our laboratory for more than three years [7,8]. This is a clear advantage for a laboratory where large numbers of samples containing multiple analytes are to be analysed on a routine basis.

To the best of our knowledge, this is the first report describing an assay of atazanavir by HPLC-UV after solid phase extraction from plasma. When using UV detection, the retention time of potentially co-administered drugs must be carefully ascertained. Though the selectivity issue is certainly more easily solved by the LC–MS/MS technology, the latter is not available in all hospital laboratories.

Our method, though relatively time consuming, uses instruments available in conventional hospital laboratories and enables the analysis of the seven protease inhibitors marketed to date (atazanavir, saquinavir, ritonavir, indinavir, nelfinavir, amprenavir and lopinavir) and two NNRTIs (nevirapine and efavirenz). It can be used conveniently on a routine basis, with minor adjustments, with one single sample processing procedure followed by a chromatographic step on an identical column type, using the same solvent mixture, changing only the elution gradient program for nevirapine and lopinavir [8]. Our approach represents therefore a useful and convenient tool contributing to the optimal follow-up of HIV patients through TDM.

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