Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 113–118

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

An efficient HPLC method for the quantitative determination of atazanavir in human plasma suitable for bioequivalence and pharmacokinetic studies in healthy human subjects

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ARTICLE INFO

Article history:
Received 25 January 2010
Received in revised form 15 March 2010
Accepted 17 March 2010
Available online 27 March 2010

Keywords:
HPLC-UV
Atazanavir
Healthy human subjects
Plasma
Bioequivalence studies

ABSTRACT

An efficient HPLC method for the determination of atazanavir in human plasma has been developed and validated. A relatively simple mobile phase consisting of acetonitrile–ammonium formate buffer (pH 3; 10 mM) (45:55, v/v) was pumped at a low flow rate of 0.3 ml/min through a reverse phase Phenomenex® Luna C18 (2) (5 μm, 150 mm × 2.0 mm i.d.) column maintained at 30 °C. Diazepam was used as an internal standard and the eluent was monitored at 210 nm. The major advantage of this method over previously reported procedures is that the narrow-bore HPLC column used resulted in relatively short retention times for the internal standard (6.8 min) and atazanavir (8.3 min) with excellent peak resolution and associated reduction in solvent usage. Sample preparation involved liquid–liquid extraction using 400 μl plasma treated with sodium carbonate (2 M) and extracted with a mixed organic solvent consisting of ethyl acetate–n-hexane (50:50, v/v). The organic layer was removed and evaporated to dryness under nitrogen. Samples were reconstituted in mobile phase (100 μl) and 20 μl was injected onto the column. The procedures were validated according to international standards with good reproducibility and linear response with correlation coefficients (r) consistently ≥0.999. The intra- and inter-day accuracies were 97.1 ± 5.04 and 98.0 ± 11.3 respectively at the LLOQ and between 101 ± 4.48% and 104 ± 2.09% for the QC samples. The intra- and inter-day precision were ≤11.6% RSD at the LLOQ and ≤6.78% RSD across the entire QC concentration range. Mean recovery based on high, medium and low quality control standards ranged between 94.4 ± 1.07% and 100 ± 2.22%. Plasma samples were evaluated under short-term (ambient temperature for 6 h) and long-term (−10 ± 2 °C for 2 months) storage conditions and were found to be stable. The method described is efficient and has the necessary accuracy and precision for the rapid quantitative determination of atazanavir in human plasma and is thus highly suitable for use in pharmacokinetic/bioavailability/bioequivalence studies in healthy human subjects.

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1. Introduction

Atazanavir (ATV) belongs to the HIV protease inhibitor (PI) class of the antiretrovirals (ARVs), which have played an important role in lowering the morbidity and mortality of HIV/AIDS [1]. It is one of the newer PIs which has several advantages over the older ones, such as lopinavir, indinavir, ritonavir and saquinavir. The pharmacokinetics of ATV allows for once daily dosing [2], which may improve patient compliance. Furthermore, in patients who are ARV treatment-naïve, the co-administration of ritonavir as a booster may be avoided [3], thus reducing the PI-related adverse effects [4], including gastrointestinal problems [4], hyperlipidaemia [1,4] and insulin resistance [1].

Like other PIs, ATV may have an increased susceptibility to pharmacokinetic interactions with any drugs, food or other xenobiotics which have the ability to modulate the activities of CYP 450 enzymes and drug efflux transporters. Such interactions, which may compromise the safety and/or efficacy of ATV, can be determined by assessing measures of systemic exposure such as C_{max} and AUC. These values may be obtained from plasma concentration–time profiles of ATV in healthy human volunteers in the absence and presence of the potentially interacting drug, as demonstrated by Martin et al. [5] and Krishna et al. [6].

There are several methods described in the literature for the quantitative analysis of ATV in plasma, either alone [7–12] or in combination with other ARVs [13–24]. Some authors reported the use of mass spectrometry for detection [11,14,17,18,23],

Abbreviations: ATV, atazanavir; PI, protease inhibitor; ARVs, antiretrovirals; DIAZ, diazepam; IS, internal standard; QC, quality control; LLOQ, lower limit of quantification; % RSD, percentage relative standard deviation; % RE, percentage relative error.

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doi:10.1016/j.jpba.2010.03.019

which is not routinely available in all laboratories. Other methods are somewhat more complex involving the use of gradient elution [9,13,15–18,22–24]. Pre-sample treatment has been performed mainly by solid-phase [7–11,14,15,18,22,24] or liquid–liquid [12,13,16,20,21] extraction. The former is expensive, especially considering that one cartridge is required per sample, whilst the liquid–liquid extraction methods described appeared to be uneconomical, primarily because of their use of relatively large plasma and/or solvent volumes per sample extracted. Furthermore, all the reported methods had run times exceeding 15 min, even those for the analysis of ATV alone. The aim of the present study was therefore to develop and validate a comparatively simple and rapid HPLC method for the quantitative determination of ATV in plasma for application to a pharmacokinetic interaction study between ATV and an African traditional medicine, *Sutherlandia frutescens*, in healthy human volunteers.

2. Experimental

2.1. Chemicals and reagents

ATV sulphate (100.9%) was donated by Aspen Pharmacare (Port Elizabeth, South Africa) and diazepam (DIAZ) used as an internal standard (IS) was obtained from the Biopharmaceutics Research Institute (Rhodes University, Grahamstown, South Africa). HPLC grade acetonitrile was purchased from Romil Ltd. (Cambridge, United Kingdom). Ammonium formate (98%) and formic acid (99.9%) were sourced from Riedel-de Haën (Seelze, Germany) and Associated Chemical Enterprises Pty Ltd. (Johannesburg, South Africa) respectively. Sodium carbonate (99.5%) and ethyl acetate (99–101%) were provided by BDH laboratory reagents (Poole, England). n-Hexane (≥98%) was purchased from Merck (Darmstadt, Germany) and water was purified by reverse osmosis and filtration through a Milli-Q purification system (Millipore, Milford, MA, USA). Human plasma with potassium edentate (K-EDTA) as an anticoagulant was obtained from the South African National Blood Services, Eastern Cape Headquarters (Port Elizabeth, South Africa) and was stored at −10 ± 2 °C.

2.2. Chromatographic conditions

The HPLC system consisted of an Alliance 2695 Separations module and a 2996 Waters photodiode array ultraviolet detector coupled to Empower data acquisition software (Waters, Milford, MA, USA). Chromatographic separation was achieved using a Luna C_{18} (2) (5 μm, 150 mm × 2.0 mm i.d.) column (Phenomenex®, USA) protected by a Luna C_{18} guard (4 mm × 2.0 mm i.d.) column (Phenomenex®, USA). Both columns were maintained at a temperature of 30 °C. The mobile phase consisted of acetonitrile–ammonium formate buffer (pH 3; 10 mM) (45:55, v/v), which was filtered under reduced pressure through a 0.45 μm (PVDF) membrane (Durapore®, Millipore, Bedford, MA, USA) and degassed using an Eyela Aspirator A-25 (Tokyo Rikakikai Co. Ltd., Tokyo, Japan) prior to use. The mobile phase was pumped at a flow rate of 0.3 ml/min and the eluent was monitored at 210 nm.

2.3. Preparation of calibration standards and quality control (QC) samples

A stock solution of ATV was prepared by dissolving an accurately weighed amount of ATV sulphate in mobile phase to provide the equivalent of 1.0 mg/ml ATV. A stock solution of the IS, DIAZ, was likewise prepared at a concentration of 10 μg/ml. All stock solutions were stored in a refrigerator at 4 ± 2 °C. A spiked plasma stock solution (20 μg/ml) was prepared by weight with blank plasma. The weight of blank plasma was converted to volume, using a plasma specific gravity of 1.025 g/ml [25]. Plasma calibration standards were prepared from the plasma stock solution by weight, using serial dilution with blank plasma to provide plasma concentrations in the range of 0.10–10 μg/ml. QC plasma samples at low (∼0.30 μg/ml), medium (∼1.5 μg/ml) and high (∼7.5 μg/ml) concentrations were prepared in the same manner, but from a separate plasma stock solution.

2.4. Sample preparation

Plasma samples (400 μl) spiked with ATV were transferred to 2 ml microcentrifuge tubes (Optima Scientific, Cape Town, South Africa). IS stock solution (25 μl), 2 M sodium carbonate (400 μl) and ethyl acetate–n-hexane (50:50, v/v) (800 μl) were added. The resultant mixture was then vortexed at 1600 rpm (Mixmate®, Eppendorf AG, Hamburg, Germany) for 10 min and centrifuged at 10,000 rpm (Ministar Plus®, Hangzhou Allsheng Instruments Co. Ltd., Hangzhou City, China) for 5 min. The aqueous layer was frozen in liquid nitrogen before the organic layer was transferred to 1.5 ml microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) for evaporation under a gentle stream of nitrogen gas (N-EVAP® Analytical Evaporator, Organomation Associates Inc., MA, USA). The residues were reconstituted in mobile phase (100 μl), vortexed at 2000 rpm (Mixmate®, Eppendorf AG, Hamburg, Germany) for 5 min and centrifuged at 10,000 rpm (Ministar Plus®, Hangzhou Allsheng Instruments Co. Ltd., Hangzhou City, China) for 5 min. The supernatants were transferred to micro-inserts (Separation Scientific SA Pty Ltd., Johannesburg, South Africa) and 20 μl aliquots were injected onto the column.

2.5. Method validation

2.5.1. Calibration curve

Nine point calibration curves were constructed in the range of 0.1–10 μg/ml ATV, using least squares linear regression analyses with a weighting factor of $1/y^2$. Each plasma calibration standard was extracted in triplicate and the mean peak area ratio, ATV/IS was plotted versus concentration of ATV.

2.6. Lower limit of quantification (LLOQ)

The LLOQ was determined based on a signal to noise ratio of 5:1 [26], from the determination of 5 replicate spiked plasma samples.

2.7. Accuracy and precision

Accuracy was defined by the regressed (measured) concentration represented as a percentage of the target (nominal) concentration. Intra-assay accuracy was determined from the analysis of five replicate samples at each of the low, medium and high QC concentrations. After intra-assay accuracy was assessed thrice, inter-assay accuracy was calculated from the 15 samples at each of the high, medium and low QC concentrations. The limits for the accuracy values were set as the range, 85–115%, except at the LLOQ where values between 80% and 120% were accepted [26]. The percent relative standard deviation (% RSD) of the regressed (measured) concentrations was used to report precision. Intra-assay and inter-assay precision were assessed on the same samples used to determine intra- and inter-assay accuracy. Precision for all concentrations was accepted if the % RSD fell within ±15%, except at the LLOQ, where the limit was extended to ±20% [26].

2.8. Selectivity

Blank plasma samples and spiked LLOQ samples prepared from six different batches of plasma were extracted and checked for interference of ATV and the IS by endogenous compounds. A plasma sample from a healthy volunteer following 5 days oral administration of a twice daily regimen of Sutherlandia SU1™ tablets (Phyto Nova Natural Medicines, Cape Town, South Africa) each containing 300 mg *S. frutescens* and who had been administered a single oral dose of 400 mg ATV (2 × 200 mg Reyataz™ capsules, Bristol Myers Squibb, Bedfordview, South Africa) was collected 2.5 h post-ATV dose. This was to determine whether the metabolites of ATV and the components and/or metabolites of the Southern African medicinal plant, *S. frutescens* would interfere with the assay of ATV.

2.9. Recovery

Extraction efficiency was determined from the ATV peak area of the QC samples in the low, medium and high QC concentrations and expressed as a percentage of ATV peak area of equivalent unextracted standards prepared in mobile phase.

2.10. Stability

Five replicate plasma samples at each of the low and high QC concentrations were analyzed for stability. Bench-top stability was assessed by storing unextracted plasma samples for 6 h at ambient temperature (22 ± 3 °C), before extraction and analysis. Long-term stability was determined for 1 week, 1 month and 2 months after storage in a freezer at −10 ± 2 °C. After three 24 h freeze–thaw cycles, plasma samples were also extracted and analyzed. Extracted plasma samples were tested for post-preparative stability, which included autosampler stability and re-injection analysis. For the former, samples were analyzed after being stored in the autosampler at 22 ± 3 °C for 24 h. The latter involved the re-assay of previously injected samples 24 h after initial injection. All stored plasma samples were considered stable if the 90% confidence interval of the regressed stored plasma sample concentrations calculated as a percentage of the regressed fresh plasma sample concentrations fell within the range 85–115% [27]. Stock solutions of ATV in mobile phase were assessed for stability at 22 ± 3 °C for 6 h and following storage in a refrigerator at 5 ± 3 °C for 1 week. All stored stock solutions were considered stable if the 90% confidence interval of the peak area of ATV from the stored sample calculated as a percentage of the peak area of ATV from a freshly prepared sample fell within 93–107% [28].

2.11. Application of the analytical method

The feasibility of the clinical application of this analytical method was assessed by determining the plasma concentration–time profile over 24 h following the administration of a single oral dose of 400 mg ATV (2 × 200 mg Reyataz™ capsules, Bristol Myers Squibb, Bedfordview, South Africa) under fed conditions to a healthy human subject.

3. Results and discussion

3.1. Method development

Preliminary experiments were conducted to compare the performance of two Luna C_{18} columns which differed only in internal diameter (i.d.). The column with an i.d. of 4.6 mm required a higher flow rate than the column with an i.d. of 2 mm to obtain the same retention time of ATV and IS, thereby increasing the volume of mobile phase solvents used for the analysis of samples. Moreover, the sensitivity of the method was lower when the column of 4.6 mm i.d. was used.

A simple binary mobile phase of acetonitrile–water (50:50, v/v) was initially chosen as the mobile phase for the chromatographic separation of unextracted and extracted standard solutions of ATV and IS dissolved in mobile phase. However, for the analysis of plasma samples, unacceptable peak tailing was observed (data not shown). Using buffers with pH's below the basic pK_a's of ATV and DIAZ, which were 4.65 ± 0.25 [29] and 3.4 ± 0.1 [30] respectively, instead of water as the aqueous component of the mobile phase, improved the symmetry of the peaks. A formate buffer was selected rather than phosphate buffers used by other authors [9,10,15,19–21], since the possibility of precipitation in the HPLC system is less likely. This will help to protect and ensure the longevity of the HPLC pump and column. Fig. 1(a) depicts a representative chromatogram of an extract from plasma spiked with IS and ATV to give a final concentration of 2.5 and 10 μg/ml respectively. As shown in this chromatogram, IS and ATV eluted at retention times of 6.8 and 8.3 min respectively, allowing the run time to be set at 10 min. This is an improvement on all previously reported isocratic HPLC-UV methods for the determination of ATV in plasma [7,8,10,19–21]. Moreover, despite the shorter run time, the selection of a column with a small i.d. of 2 mm made it possible to use a lower flow rate than those used in previously published methods, thereby decreasing the volume of mobile phase required for each run. The injection volume of 20 μl was the same as used by Loregian et al. [10] and lower than all other HPLC-UV [7–9,12,13,15,16,19–21] methods.

Using the above chromatographic conditions resulted in the development of an efficient and reproducible method for the quantitative determination of ATV in plasma. The use of a simple protein precipitation extraction procedure was investigated but proved to be unacceptable due to interferences and low sensitivity. Liquid–liquid extraction was then investigated according to the procedures described by Takahashi et al. [19], using sodium carbonate (0.5 M) and ethyl acetate–n-hexane (50:50, v/v). In an effort to achieve concomitant multi-sample preparation, the method was modified such that the volume of a 2 ml microcentrifuge tube was adequate for the extraction of each sample. A plasma volume of 400 μl instead of 500 μl was used and the ratio of plasma:sodium carbonate:ethyl acetate–n-hexane (50:50, v/v) was reduced from 1:2:4 to 1:1:2, giving a total extraction volume of 1.6 ml, instead of 3.5 ml. This initially resulted in both poor recovery and poor reproducibility. However, when the concentration of sodium carbonate was increased from 0.5 to 2 M, the extraction efficiency was much improved from a recovery of 83.3 ± 4.94% to 94.4 ± 1.07%.

In order to determine whether a weighting factor would be required to fit the data using linear regression analysis, the percentage relative error (% RE), which expresses the deviation of the regressed concentration obtained for each weighting factor from the nominal concentration, was determined. Weighting factors of 1 (unweighted), $1/x^{1/2}$, $1/x$, $1/x^2$, $1/y^{1/2}$, $1/y$, $1/y^2$ were tested [31] and the one which produced the lowest sum of absolute % RE across the whole concentration range of three sets of calibration standards prepared and assayed on three different days was selected [31].

3.2. Method validation

3.2.1. Linearity

All calibration curves were linear, with a correlation coefficient (r) consistently ≥0.999. The average slope of the calibration curves was 0.657 ± 0.0437.

3.2.2. Lower limit of quantification

The LLOQ was found to be 0.1 μg/ml. This was similar to that of Takahashi et al. [19], who obtained an LLOQ of 0.09 μg/ml but was

Table 1
Accuracy and precision.

QC concentrations (μg/ml)	Intra-assay (n = 5)		Inter-assay (n = 15)	
	Accuracy[a] (mean % ± SD)	Precision[b] (% RSD)	Accuracy[a] (mean % ± SD)	Precision[b] (% RSD)
LLOQ (0.1)	97.1 ± 5.04	5.18	98.0 ± 11.3	11.6
Low (0.3)	104 ± 2.09	1.88	101 ± 4.10	6.78
Medium (1.5)	101 ± 2.81	3.05	102 ± 4.11	4.10
High (7.5)	102 ± 1.47	1.44	101 ± 4.48	3.86

[a] Accuracy is calculated as the mean and standard deviation of the [regressed concentration/nominal concentration] × 100.
[b] Precision is calculated as % RSD of regressed concentration.

an improvement on the LLOQ of 0.156 μg/ml reported by Cateau et al. [7].

3.2.3. Accuracy and precision

The intra- and inter-assay accuracies and precision of the LLOQ and QC sample concentrations, shown in Table 1, were all within the acceptance criteria. The intra- and inter-assay accuracies at the LLOQ were found to be 97.1 ± 5.04% and 98.0 ± 11.3% respectively. The accuracies for the low, medium and high QC sample concentrations fell within the range of 101 ± 4.48–104 ± 2.09%. The intra- and inter-day precision were ≤11.6% RSD at the LLOQ and ≤6.78% RSD for all the QC sample concentrations. The accuracy and precision of the method at all QC concentrations and the LLOQ was generally better than most previously reported methods [7,8,10,12–19,21].

3.2.4. Selectivity

Fig. 1 depicts chromatograms showing (a) spiked plasma extract containing ATZ (10 μg/ml) and the IS; (b) blank plasma extract; (c) spiked plasma extract containing ATZ at the LLOQ and IS; (d) plasma extract of a healthy human volunteer 2.5 h after a single oral dose of 400 mg ATV (2 × 200 mg Reyataz™ capsules, Bristol Myers Squibb, Bedfordview, South Africa), where a twice daily regimen of 300 mg Sutherlandia SU1™ tablets (Phyto Nova Natural Medicines, Cape Town, South Africa) had been administered for 5 days. There were

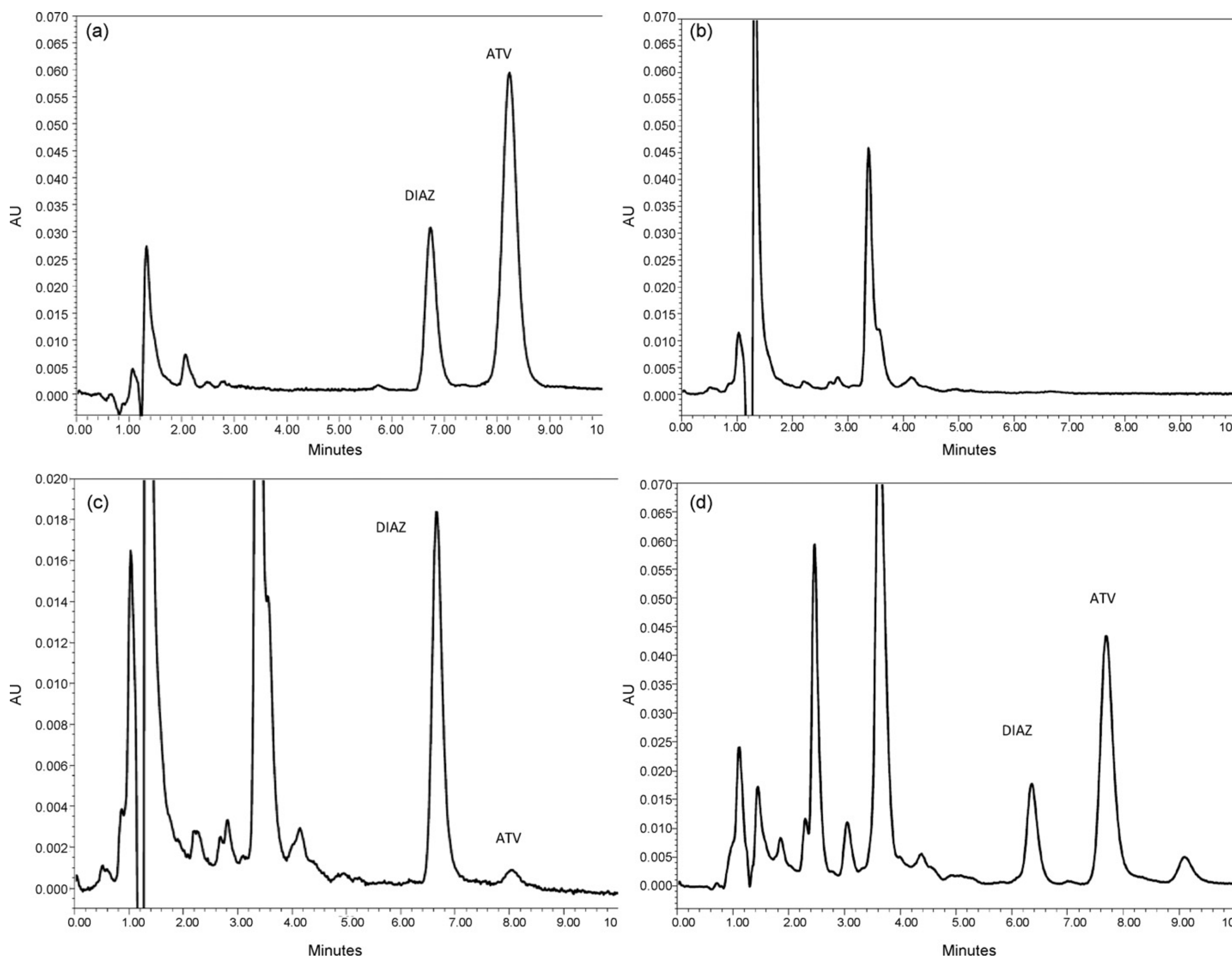

Fig. 1. Chromatograms showing selectivity. (a) Spiked plasma extract containing ATZ (10 μg/ml) and DIAZ; (b) blank plasma extract; (c) spiked plasma extract containing ATZ at the LLOQ and DIAZ; (d) plasma extract of a healthy human volunteer 2.5 h after single oral dose of 400 mg ATV (2 × 200 mg Reyataz™ capsules) and a twice daily regimen of 300 mg Sutherlandia SU1™ tablets.

Table 2
Stability of ATV.

Storage conditions	QC concentration (μg/ml)	90% Confidence interval[a] (%)
Unextracted plasma		
6 h at 22 ± 3 °C	0.3	89.4–110
	7.5	96.2–102
Three freeze–thaw cycles	0.3	90.6–102
	7.5	94.9–102
1 week at −10 ± 2 °C	0.3	90.0–97.2
	7.5	101–106
1 month at −10 ± 2 °C	0.3	91.6–107
	7.5	94.2–108
2 months at −10 ± 2 °C	0.3	86.9–103
	7.5	85.6–91.8
Final extract		
24 h at 22 ± 3 °C	0.3	85.6–103
	7.5	95.6–107
Re-injection analysis	0.3	84.5–101
	7.5	97.0–108
Stock solution		
6 h at 22 ± 3 °C		99.5–101
1 week at 5 ± 3 °C		96.8–99.1

[a] 90% confidence interval of the regressed stored plasma sample concentrations calculated as a percentage of the regressed fresh plasma sample concentrations.

no co-eluting interferents in any of the 6 different batches of human plasma nor in the plasma sample drawn from a healthy human volunteer.

3.2.5. Recovery

Extraction efficiencies of ATV from plasma were 100 ± 2.22%, 95.0 ± 0.777% and 94.4 ± 1.07% at the low, medium and high QC sample concentrations respectively.

3.3. Stability

The stability of stored stock solutions as well as stored plasma samples at low and high QC concentrations, as shown in Table 2, were all acceptable according to the acceptance criteria. The 90% confidence interval for ATV after storing for 1 week in the refrigerator at 5 ± 3 °C was 96.8–99.1%, and the 90% confidence interval of unextracted and extracted plasma samples under all the storage conditions tested was within the range of 85.0–110%.

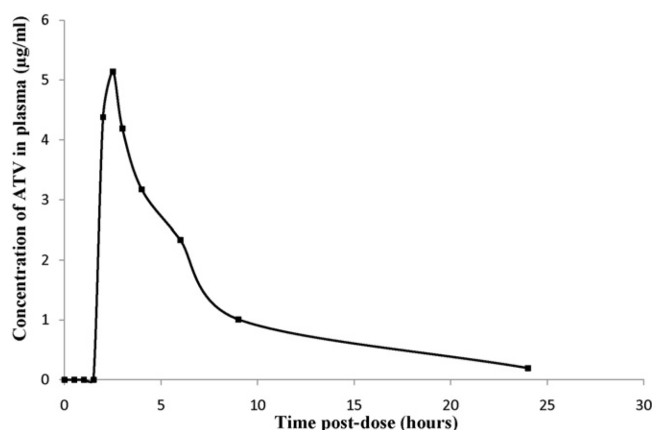

Fig. 2. ATV plasma concentration–time profile after a single oral dose of 400 mg ATV (2 × 200 mg Reyataz™ capsules) to a healthy human volunteer, in the fed state.

3.4. Application of the clinical method

The plasma concentration–time profile of ATV from a healthy human volunteer is depicted in Fig. 2 and shows a C_{max} of 5.14 μg/ml occurring at a t_{max} of 2.5 h. These single dose values are similar to steady state values reported following the chronic administration of a daily dose of 400 mg ATZ to 214 HIV negative volunteers in the fed state [32]. Martin et al. [5] also reported a similar steady state C_{max} value and a t_{max} of 2 h, in 24 healthy volunteers.

4. Conclusions

A relatively simple, accurate, precise and rapid method suitable for use in pharmacokinetic/bioavailability/bioequivalence studies in human subjects to determine ATV in plasma has been developed and validated. This method has several advantages compared to previously reported methods such as the use of a narrow-bore HPLC column which allowed for the reduction in flow rate and consequently decreased the volume of organic solvent used in the mobile phase. In addition, the use of organic solvents in the extraction procedure was also minimised, demonstrated by a low total extraction volume of 1.6 ml, containing only 800 μl of ethyl acetate–n-hexane (50/50, v/v) per sample. The method was comparatively simple, due to the use of HPLC-UV with isocratic elution and provided accuracy and precision which was better than most previously reported methods. Also, despite the use of a low flow rate of 0.3 ml/min, relatively short retention times for the IS (6.8 min) and ATV (8.3 min) resulted. Hence a short run time of 10 min was achieved compared to previously reported run times of at least 15 min using isocratic elution and those involving gradient elution were associated with run times of 28 min and even longer. The current relatively short run time of 10 min has the advantage of facilitating and enhancing the efficiency of processing large numbers of plasma samples obtained from pharmacokinetic/bioavailability/bioequivalence studies in healthy human subjects.

Acknowledgements

Financial support from the Rhodes University Joint Research Committee (IK), the Medical Research Council of South Council of South Africa (IK), the National Research Foundation of South Africa (ACM) and the Rhodes University Atlantic Philanthropies Scholarship Fund (ACM) are acknowledged.

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