Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 788 (2003) 377–386
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantitative determination of the HIV protease inhibitor atazanavir (BMS-232632) in human plasma by liquid chromatography–tandem mass spectrometry following automated solid-phase extraction

A. Schuster[a], S. Burzawa[b], M. Jemal[a], E. Loizillon[b], P. Couerbe[b], D. Whigan[a,*]

[a]*Drug Disposition and Bioanalytical Sciences, Bristol-Myers Squibb Pharmaceutical Research Institute, One Squibb Drive, New Brunswick, NJ 08903, USA*

[b]*Bioanalytical Department, Bristol-Myers Squibb Pharmaceutical Research Institute, F-44600 St-Nazaire, France*

Received 26 September 2002; received in revised form 23 December 2002; accepted 8 January 2003

Abstract

A selective, accurate, and reproducible LC–MS–MS assay was developed for the determination of the HIV protease inhibitor atazanavir (BMS-232632) in human plasma samples. The method involved automated solid-phase extraction of atazanavir and a stable isotope analog internal standard (I.S.) using Oasis HLB 10 mg 96-well SPE plates. A portion of the reconstituted sample residue was injected onto a C_{18} HDO analytical column which was configured with a triple quad mass spectrometer for analyte determination by positive ion electrospray. The assay was linear from 1.00 to 1000 ng/ml with a lower limit of quantitation of 1.00 ng/ml. The inter- and intra-day coefficients of variation (C.V.) for the assay were <4%, and the accuracy was 99–102%. Atazanavir was stable in human plasma for at least 109 h at room temperature and for at least 1 year at −20 °C.

Keywords: Atazanavir

1. Introduction

BMS-232632 (atazanavir) is an azapeptide human immunodeficiency virus (HIV) type 1 (HIV-1) protease inhibitor with a half-life that allows for once-daily dosing [1,2]. As a crucial part of the drug development process, a rapid, sensitive, and selective assay is required to measure drug concentrations in plasma from clinical studies. The use of LC–MS–MS in the bioanalysis of other anti-HIV agents has been reported in the literature [3,4], however there is no methodology reported to measure atazanavir in any biological matrix. We report an accurate and sensitive high-performance liquid chromatographic assay with tandem mass spectrometric detection (LC–MS–MS) for the determination of atazanavir in human plasma samples. The validation of this method was based on the recommendations published as a Conference Report of the Washington Conference on

*Corresponding author. Tel.: +1-732-519-1207; fax: +1-732-519-3979.
E-mail address: daisy.whigan@bms.com (D. Whigan).

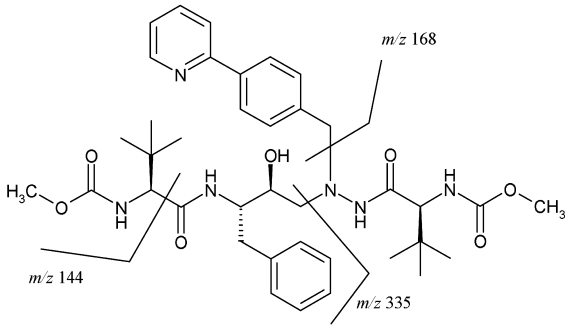
1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00063-1

Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic studies [5].

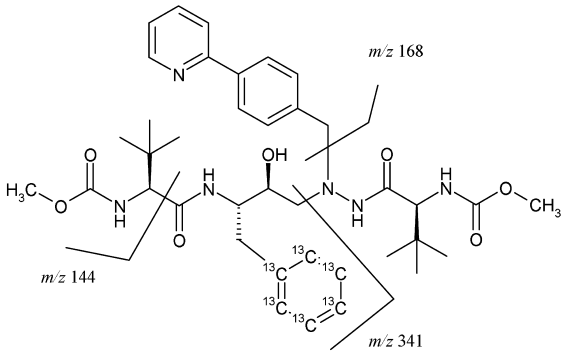
2. Experimental

2.1. Chemicals and reagents

Atazanavir and internal standard, I.S. ($^{13}C_6$-labeled atazanavir), were obtained from Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ, USA). The chemical structures of atazanavir and I.S. are provided in Fig. 1. The atazanavir reference material was the hydrogen sulfate salt which had a chemical purity of 99.8%.



Atazanavir (BMS-232632)



$^{13}C_6$-Atazanavir (I.S.)

Fig. 1. Chemical structures of atazanavir and the $^{13}C_6$-labeled internal standard (I.S.).

All atazanavir concentrations are reported as the free base so the salt form was appropriately converted to the free base form. The I.S. had a chemical purity of 99.5% and an isotopic purity of 99%. HPLC-grade acetonitrile, HPLC-grade methanol, and glacial acetic acid (ref. 401422) were obtained from Carlo Erba Reagenti (Rodano, Mi, Italy). Deionized water (18 MΩ, TOC <100 ppb) was produced on-site. Ammonium acetate (ref. A-7272) was obtained from Sigma (St. Louis, MO, USA). A 10 m*M* ammonium acetate solution was prepared by dissolving 770 mg of ammonium acetate in one litre of deionized water. This solution was adjusted to pH 5.5 with glacial acetic acid and was used as part of the reconstitution solution. A 5 m*M* ammonium acetate solution, prepared by dissolving 385 mg of ammonium acetate in one litre of deionized water, was used as mobile phase A. A 0.1% acetic acid solution, prepared by mixing 0.50 ml of glacial acetic acid into 500 ml of deionized water, was used in the extraction of the samples. Control human plasma (with K_3EDTA as anticoagulant) was obtained from Biomedia (31100 Boussens, France).

2.2. Equipment

A Micromass Quattro Ultima mass spectrometer (Micromass UK, Altricam, UK) equipped with an atmospheric pressure ionization (API) electrospray interface and MassLynx v 3.5 system software was used for detection. The HPLC system consisted of the following components: mobile phase delivery pumps Models 422 and 422S (Kontron Instruments, Montigny le Bretonneux, France), mobile phase mixer Visco-Jet Micro-Mixer, 250 μl (The Lee, Westbrook, CT, USA), and a Perkin-Elmer Series 200 autosampler (Perkin-Elmer, Norwalk, CT, USA). The analytical column was an Uptisphere HDO C_{18}, 33×4.6 mm, 3 μm packing, supplied by Interchim (Montlucon, France). The analytical column can also be obtained from Analogix (Burlington, WI, USA). An Accurate Flow Splitter 1/20 (LC Packing, San Francisco, CA, USA) was used to split the effluent before introduction into the mass spectrometer. An IKA Model MS2 vortex mixer (IKA Works, Wilmington, NC, USA) was used for mixing. A Multi-Probe II extended (Packard Biosciences, Meriden, CT, USA) robotic liquid handling system was used

to automate the sample preparation. The 96-well SPE plates packed with Oasis HLB, 10 mg (Waters, Milford, MA, USA), were used for sample extraction; Costar 2-ml, 96-well collection plates (Corning, NY, USA) and Micromat TFE/SIL sealing mats to cover the plates (Varian, Les Ulis, France and Harbor City, CA, USA) were also used. A Micro-DS96, 96-well evaporator (Porvair Sciences, ATGC 77313 Marnes la Vallee, France) was used for drying the sample eluates.

2.3. Chromatographic and mass spectrometric conditions

HPLC separation was achieved with gradient elution using mobile phase A described in Section 2.1 and acetonitrile as mobile phase B following the sequence in Table 1. The flow-rate was 0.8 ml/min with a 1/20 flow split before the detector. The injection volume was 15 μl. The analytical column was kept at ambient conditions and the total run time was 4.5 min.

The mass spectrometer was operated in positive ion electrospray mode. The capillary sprayer voltage was 3.2 kV and the sample cone voltage was 80 V for both atazanavir and the I.S. The source temperature was 100 °C and the desolvation temperature was 350 °C. The nebulizing gas was nitrogen, the cone gas was nitrogen at 37 L/h, the desolvation gas was nitrogen at 500 L/h and the collision gas was argon at 2.6×10^{-3} mbar. All gases were ultra-high purity. The collision energy was set at 40 eV for both atazanavir and the I.S. Resolution was set at 0.7 mass units at half height for both the first and third quadrupoles (LM and HM at approximately 14 in each quadrupole).

Table 1
Gradient elution used in the chromatographic system

Time (min)	% A	% B
0	50	50
0.5	50	50
0.6	40	60
2.3	40	60
2.4	50	50
4.5	50	50

The samples were analyzed via selected reaction monitoring (SRM) employing the transition of the $[M+H]^+$ precursor ions to product ions: m/z 705 to 168 for atazanavir and m/z 711 to 168 for the I.S. with an inter-channel delay of 0.1 s. The dwell time was 200 ms for each SRM transition. Data acquisition and integration of SRM chromatograms were performed using the Micromass Masslynx v 3.5/Quanlynx system software.

2.4. Standard solutions

A stock solution of atazanavir (500 μg/ml) was prepared in methanol. This solution was stable at −20 °C for at least 10 months. A stock solution of the I.S. (1000 μg/ml) was prepared in methanol. This solution was stored at −20 °C and was stable for at least 1 month. A working I.S. solution, prepared at 5.0 μg/ml by appropriately diluting the stock solution in deionized water, was used to spike the samples prior to extraction. This working I.S. solution was stored at +4 °C and used within 1 month of preparation.

2.5. Standard curve and quality control samples

The standard curve consisted of control human plasma samples (K_3EDTA) spiked at eight different concentrations of atazanavir over the range of 1.00 to 1000 ng/ml. These standard curve samples, prepared on the day of use, were extracted in duplicate for each analytical run.

Quality control (QC) samples were prepared by spiking control human plasma with atazanavir to obtain concentrations of 3.00, 600, 800, and 8000 ng/ml. The 8000 ng/ml QC, also known as dilution QC, was diluted 1:9 with control human plasma prior to analysis. This QC sample was spiked at a concentration higher than the upper limit of quantitation. These QC samples were used to determine the accuracy and precision of the method during validation and the acceptability of an analytical run during routine clinical sample analysis. Aliquots of these QC samples were stored appropriately to determine the stability of atazanavir in plasma at different stability conditions. During routine clinical sample

analysis, aliquots of these QC samples are stored together with the clinical samples at about −20 °C. When clinical samples have to be diluted prior to analysis, the dilution QC sample is also assayed in the same run.

2.6. Sample preparation

After the addition of 40 μl of the I.S. working solution and 0.3 ml of 0.1% acetic acid solution to 0.25 ml of plasma sample in a glass tube, the samples were manually mixed and placed on the robotic liquid handling system. The concentration of the I.S. was 800 ng/ml of plasma. The samples were loaded onto 96-well Oasis HLB 10 mg SPE plates previously activated with 1 ml of methanol followed by conditioning with 1 ml of 0.1% acetic acid solution. The loaded samples were first washed with 0.5 ml of 0.1% acetic acid solution and then with 0.5 ml of deionized water–methanol (80:20, v/v). The analyte and I.S. were eluted with 0.3 ml of methanol into a 96-well collection plate. The eluates were evaporated to dryness under a stream of nitrogen at about 60 °C. The dried extracts were reconstituted with 500 μl of the reconstitution solution in the 96-well collection plate. The reconstitution solution consisted of 40% 10 m*M*, pH 5.5, ammonium acetate solution (Section 2.1) and 60% of a methanol–acetonitrile mixture (50:50, v/v). A 15 μl portion of the reconstituted sample was injected onto the LC–MS–MS system.

2.7. Measurement and calculations

Chromatographic data acquisition and integration were automated using the MassLynx v 3.5/Quanlynx system software. The integrated peak areas of interest were imported into a Watson DMLIMS (Innaphase, Philadelphia, PA, USA) where subsequent calculations were performed. Peak area ratios (atazanavir peak area/I.S. peak area) versus concentrations of the standards were fitted to a linear regression equation, weighting each standard by the reciprocal of its concentration squared. The regression equation was used to calculate predicted concentrations of atazanavir in the samples.

2.8. Stability

The stability of atazanavir in plasma was studied at the following conditions: (1) heating at +60 °C, (2) room temperature, (3) freeze–thaw cycles, (4) long-term storage at −20 °C, (5) stability of processed samples. QC samples spiked at 3.00 and 800 ng/ml were assayed in six replicates for each stability determination. The deviations of the measured concentrations from the nominal concentrations were used to assess the stability of atazanavir.

2.9. Recovery

The overall recovery of atazanavir from human plasma was determined at 3.00, 200, and 400 ng/ml by comparing the peak area response obtained from extracted human plasma (six replicates) with the peak area response from neat solutions (six replicates) prepared at the same concentration levels. The overall recovery of the internal standard was determined similarly at 800 ng/ml from six replicates.

3. Results and discussion

3.1. Method development

Both atazanavir and I.S. were extracted from plasma samples by solid-phase extraction. Originally, Empore C2-SD disks (3M Filter Products, St. Paul, MN, USA) were used but plugging problems occurred in the robotic sampling system. In addition, the extraction recovery with the C2 disks was lower than that obtained with the OASIS HLB 10 mg 96-well extraction plates. Using the C2 disks, washing with just 0.1% acetic acid was adequate to obtain clean extracts. But with the OASIS HLB extraction plates, it was necessary to wash the plasma samples further with a water–methanol (80:20, v/v) mixture. Mixtures containing varying percentages of methanol were tried. The 80:20 mixture gave optimum results in terms of recovery and selectivity. Efforts to elute the analytes with the reconstitution solution were not successful because of poor recovery. The recovery was improved by eluting with pure methanol, however the extracted analytes exhibited poor autosampler stability in this medium. It was therefore neces-

sary to evaporate the eluate and reconstitute the extracts in the reconstitution solution. The addition of the pH 5.5 buffer solution to the reconstitution solution resulted in good analyte stability prior to injection onto the LC–MS–MS system. The use of an Uptisphere HDO C_{18} column resulted in excellent analyte peak shapes. The use of a 4.6 mm internal column diameter minimized the risk of column plugging and improved the robustness of the chromatographic system. The 1/20 split of the eluate before entering the mass spectrometer resulted in good linearity over the standard curve range and good reproducibility without compromising the sensitivity. Additionally, the split minimized the need to clean the ion source. Typically, with the split, the mass spectrometer was able to handle about 1000 injections before a slight preventive source cleaning was performed (cone and baffle).

Prior to automating the sample preparation, a similar manual extraction procedure using OASIS HLB 30 mg cartridges (Waters, Milford, MA, USA) was validated to assay atazanavir in human plasma. In the absence of a robotic sampling system, manual extraction can be employed.

A method using a structural analog as internal standard was previously validated for the quantitative analysis of atazanavir in human plasma. The use of a stable isotope analog instead of the structural analog as internal standard improved the performance parameters of the method. For precision, within-run C.V. values were reduced to 3.9, 2.2, and 3.5% instead of 4.9, 4.4 and 4.2% at 3.00, 600 and 800 ng/ml, respectively, while between-run C.V. values were 2.3, 2.4 and 1.9% instead of 7.4, 5.6 and 6.5% for the same concentration levels. For accuracy, the improvement was less significant, as shown by the percent deviation recorded at the three levels, respectively: 0.3, 1.8 and −0.1% using the stable isotope analog, instead of 0.0, 3.4 and 3.6% using the structural analog.

Positive-ion electrospray mass spectra (Q1) and the MS–MS product-ion spectra (Q3) of atazanavir and the I.S. are shown in Figs. 2 and 3, respectively. For each compound, the $[M+H]^+$ was the predominant ion in the Q1 spectrum, which was used as the precursor ion for obtaining the MS–MS product-ion spectrum. The proposed fragments for the three major product ions are shown in Fig. 1.

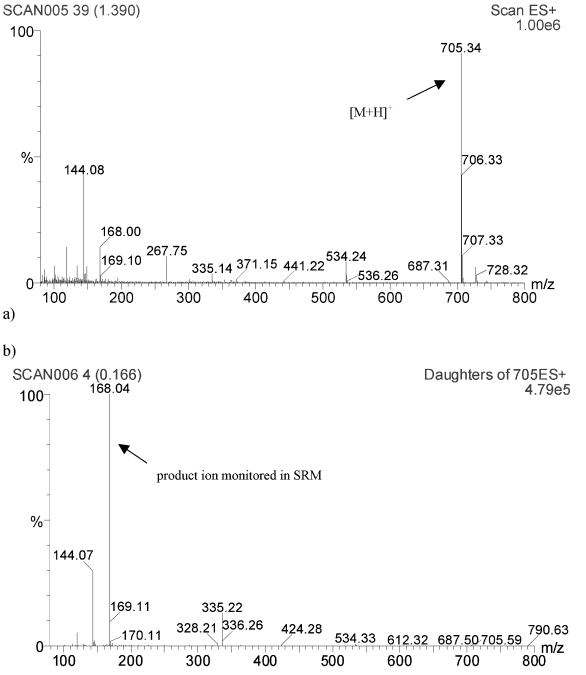


Fig. 2. (a) Positive-ion electrospray mass spectrum (Q1) for atazanavir. (b) MS–MS product-ion spectrum (Q3) for atazanavir, with m/z 705 as the precursor ion.

3.2. Selectivity

The sample extraction and chromatographic analysis were developed to produce a selective assay for the analytes. At least six different lots of commercial K_3EDTA control human plasma were carefully evaluated for interference in the assay. The degree of interference was assessed by inspection of SRM chromatograms. Typical chromatograms of blank plasma (Figs. 4 and 5) show that no significant interfering peaks from human plasma were found at the retention time and in the ion channel of either atazanavir or the I.S.

3.3. Calibration curve

An eight-point calibration standard curve in human plasma, ranging from 1.00 to 1000 ng/ml for atazanavir, was prepared in duplicate for each run. The standard curve is satisfactorily described by linear regression weighted by $1/x^2$, as the back-calculated values were all within ±15% of the

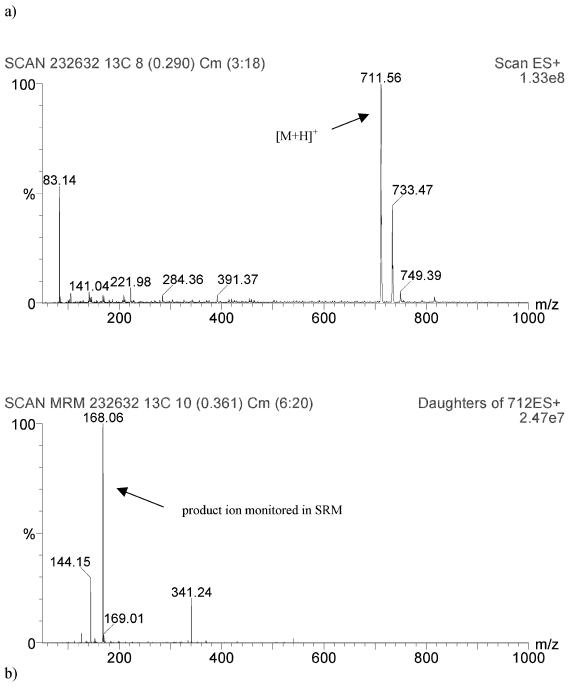


Fig. 3. (a) Positive-ion electrospray mass spectrum (Q1) for the I.S. (b) MS–MS product-ion spectrum (Q3) for the I.S., with m/z 711 as the precursor ion.

nominal concentrations (±20% at the LLOQ) in the three validation runs. A summary of the standard curve regression parameters obtained during the three validation runs is presented in Table 2.

3.4. Accuracy and precision

The accuracy and precision of the assay were determined by assaying QC samples in six replicates on each of three different days. One-way analysis of variance (ANOVA) of the results established good accuracy and precision of the method. For the three runs, the mean accuracy ranged from 99 to 102%. Within- and between-run precision were <4% C.V. for all QC samples (Table 3).

In the analysis of patient samples from one clinical study, the slopes of the calibration curves were reproducible with values exhibiting 3.3% C.V. over 11 runs, spanning 21 days, further demonstrating good precision of the method.

3.5. Lower and upper limits of quantitation

The lower limit of quantitation (LLOQ) is defined as the lowest concentration in the standard curve that back-calculates with adequate precision and accuracy. A plasma sample spiked at the LLOQ was assayed in six replicates on each of three different days. A one-way analysis of variance (ANOVA) of the results over the three runs showed a mean accuracy of 107% and within- and between-run precision values of <6% C.V. at the LLOQ of 1.00 ng/ml (Table 3).

In addition, the six different lots of commercial control human plasma that were evaluated for specificity were spiked at 1.00 ng/ml of atazanavir to obtain six LLOQ samples. These LLOQ samples were assayed and the results, presented in Table 4, show good accuracy. The LLOQ was established at 1.00 ng/ml. A typical chromatogram of an LLOQ sample is shown in Fig. 4.

The upper limit of quantitation (ULOQ) is the highest concentration in the standard curve that was analyzed using the chromatographic and mass spectrometer parameters. Samples that contain analyte concentrations higher than the ULOQ can be assayed after dilution with control human plasma. When this occurs, a dilution QC sample is assayed together with the study (post-dose) samples.

3.6. Recovery

The mean overall recovery for atazanavir was 68%. The overall recovery for the I.S. at the concentration used in the assay was 72%. The sample extraction procedure using OASIS HLB 10 mg 96-well extraction plates results in clean extracts and adequate recovery to obtain the required sensitivity for the assay. Since the recovery was calculated against neat standards, this demonstrated that ion suppression related to the matrix effect was not significant.

3.7. Stability

3.7.1. Heating at 60 °C for 30 min

Heated QC samples were assayed against a non-heated calibration curve. The low QC samples showed a decrease of about 40% while the high QC

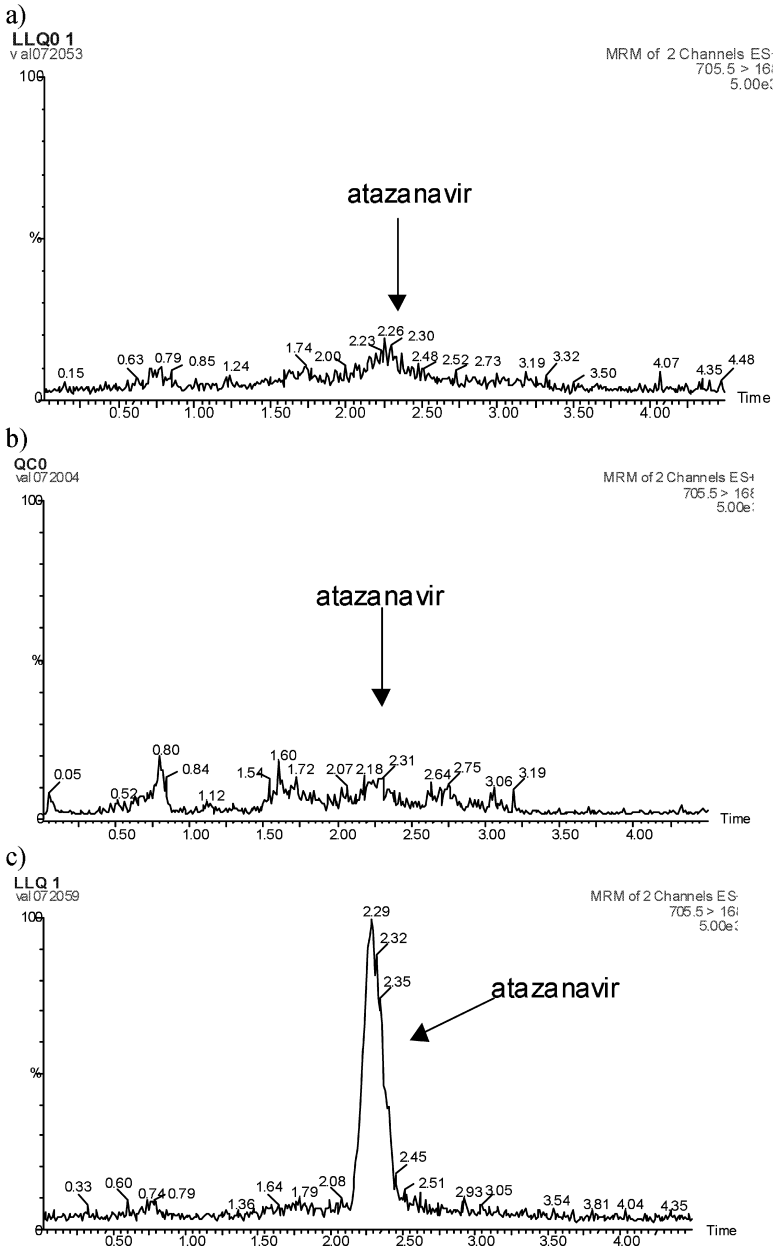


Fig. 4. SRM chromatograms for atazanavir obtained from (a) control human plasma, (b) human plasma containing I.S. only, and (c) LLOQ plasma sample (1.00 ng atazanavir/ml plasma).

sample showed a decrease of about 15% after heating the samples at 60 °C for 30 min. The results indicate that atazanavir is not stable when heated at 60 °C for 30 min.

3.7.2. Room temperature

QC samples were left at room temperature for 24 h prior to analysis against a freshly prepared standard curve. The mean deviations were −12 and −4% for

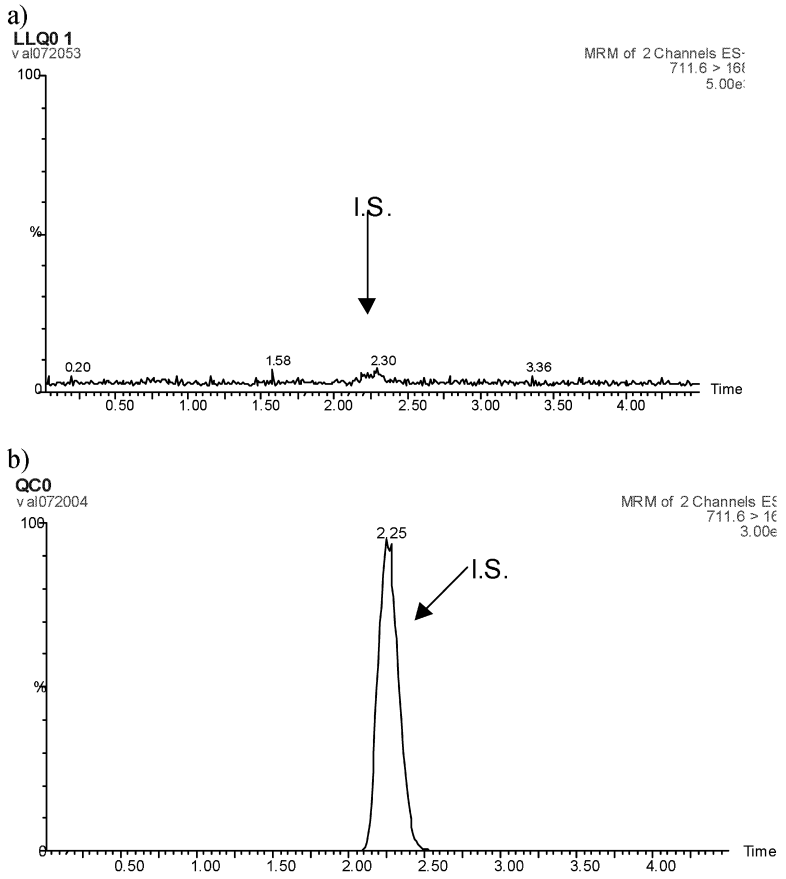


Fig. 5. SRM chromatograms for the I.S. obtained from (a) control human plasma, and (b) human plasma containing the I.S. only at 800 ng/ml.

the low and high QC samples, respectively, indicating that atazanavir is stable for at least 24 h at room temperature. Results in other laboratories indicate that atazanavir is stable in human plasma for at least 109 h at room temperature [6].

Table 2
Standard curve regression analysis results

Run No.	Linear slope	Intercept	r^2
1	0.00153635	0.00006307	0.9981
2	0.00163472	0.00020145	0.9981
3	0.00158724	0.00052996	0.9981
Mean	0.0015861	0.00026483	0.9981
SD	4.9192E−05	0.00023981	0
% C.V.	3.1	90.6	

3.7.3. Freeze–thaw cycles

QC samples were subjected to three complete freeze–thaw cycles. A freeze–thaw cycle consisted of thawing for less than 1 h and freezing for more than 14 h. After the third cycle, the samples were analyzed against a freshly prepared standard curve. The mean deviations were −3 and −10% for the low and high QC samples, respectively, indicating that atazanavir is stable through at least three freeze–thaw cycles. Results in other laboratories indicate that atazanavir is stable through at least six freeze–thaw cycles [6].

3.7.4. Long-term storage stability at −20 °C

QC samples were stored at −20 °C. After 3 months, the samples were analyzed against a freshly

Table 3
Assay accuracy and precision results ($n=18$)

Nominal conc. (ng/ml)	Mean observed conc. (ng/ml)	Deviation (%)	Between-run precision (% C.V.)	Within-run precision (% C.V.)
(LLOQ)				
1.00	1.07	7.0	1.3	5.2
(Low QC)				
3.00	3.01	0.3	2.3	3.9
(Mid QC)				
600	610.52	1.8	2.4	2.2
(High QC)				
800	799.39	−0.1	1.9	3.5
Dilution QC[a]				
8000	7936.21	−0.8	2.6	1.9

[a] Diluted 10-fold with control human plasma prior to analysis.

prepared standard curve. The mean deviations were −5 and −7% for the low and high QC samples, respectively, confirming that atazanavir is stable in human plasma for at least 3 months at −20 °C. Results in other laboratories indicate that atazanavir is stable in human plasma for at least 1 year at −20 °C [6].

3.7.5. Autosampler stability

Reconstituted plasma extracts of test QC samples were analyzed initially against a standard curve that was extracted together with the test QC samples. The same test QC sample extracts were left in the autosampler at room temperature. After 24 and 48 h, these test QC sample extracts were analyzed against a freshly prepared standard curve. After 48 h, the mean deviations were 1 and −4% for the low and high QC samples, respectively, indicating that atazanavir is stable in the reconstituted plasma extracts for at least 48 h at room temperature.

Table 4
Lower limit of quantitation (LLOQ) results; nominal concentration 1.00 ng/ml

Observed conc. (ng/ml)	Deviation (%)	Mean observed conc. (ng/ml)	Mean % deviation
1.16	16.0	1.13	13.0
1.14	14.0		
1.18	18.0		
1.08	8.0		
1.14	14.0		
1.06	6.0		

3.8. Matrix substitution

Fresh QC samples at low, middle and high concentration levels were prepared in K_3EDTA and in heparin/sodium plasma. These were assayed against a fresh standard curve prepared in K_3EDTA plasma. The mean deviations were 8, −3 and −1% for the low, middle and high QC samples in K_3EDTA, and 1, 2 and −1% in heparin/sodium, respectively. The results indicate that clinical samples can be assayed accurately according to the method described here irrespective of the anti-coagulant used.

4. Conclusions

A sensitive and robust method for determining atazanavir concentrations in human plasma has been developed and validated. The automated version of the sample extraction procedure has been described. Although a similar manual extraction procedure using Oasis cartridges can be used, automation of the extraction procedure is desirable because it minimizes manual intervention in the sample preparation, particularly when infected samples are assayed. The use of a stable isotope analog instead of a structural analog as internal standard improved the precision and accuracy of the method.

This reported method has been used to assay plasma samples collected from Phase I, II, and III studies. The method is applicable to the analysis of plasma using either heparin or K_3EDTA as the anti-coagulant. However, it is recommended that the standard and QC samples should be prepared in the same matrix as the study samples. Because of the selectivity of the mass spectrometer as a detector, several co-administered drugs and their major metabolites have been shown not to interfere in the assay. These include ritonavir, rifabutin, efavirenz, ddI, d4T, ketoconazole, ethinyl estradiol, norethindrone, atenolol, clarithromycin, and diltiazem.

Acknowledgements

The authors wish to acknowledge MDS Pharma Services, Sunnyvale, CA, USA for their extended stability studies of atazanavir in human plasma at −20 °C, room temperature, and through several freeze–thaw cycles.

References

[1] Y.-F. Gong, B.S. Robinson, R.E. Rose, C. Deminie, T.P. Spicer, D. Stock, R.J. Colonno, P.-F. Lin, Antimicrob. Agents Chemother. 44 (2000) 2319.
[2] G.L. Drusano, J.A. Bilello, S.L. Preston, E. O'Mara, S. Kaul, S. Schnittman, R. Echols, J. Infect. Dis. 183 (2001) 1126.
[3] N.G. Knebel, S.R. Sharp, M.J. Madigan, J. Mass Spectrom. 30 (1995) 1149.
[4] M.E. Fitzsimmons, J.M. Collins, Drug Metab. Dispos. 25 (1997) 256.
[5] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelley, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Eur. J. Drug Metab. Pharmacokinet. 16 (1991) 249.
[6] Personal communication.