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Liquid chromatography-tandem mass spectrometric quantitative determination of the HIV protease inhibitor atazanavir (BMS-232632) in human peripheral blood mononuclear cells (PBMC): practical approaches to PBMC preparation and PBMC assay design for high-throughput analysis

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Abstract

A selective, accurate, and reproducible LC/MS/MS assay was developed and validated for the determination of the HIV protease inhibitor atazanavir (BMS-232632) in human peripheral blood mononuclear cells (PBMC) samples. In addition to the details of the validated LC/MS/MS method, a practical procedure is described in great detail for the preparation of large supplies of control (blank) PBMC from units of blood (each unit of blood is about 500 ml) for making the calibration standards and quality control (QC) samples. The PBMC assay design, intended for high-throughput sample analysis, is also described in some detail in regards to the composition and concentration expressions of the calibration standards and QC samples, the lysing procedure of the PBMC samples, and the final analysis/quantitation procedure. The method involved automated solid-phase extraction (SPE) of atazanavir and a stable isotope analog internal standard (I.S.) using 3M Empore® C2-SD 96-well plates. A portion of the reconstituted sample residue was injected onto a YMC Basic analytical column which was connected to a triple quad mass spectrometer for analyte determination by positive-ion electrospray in the selected reaction monitoring (SRM) mode. The standard curve, which ranged from 5 to 2500 fmol per one million cells (fmol/10^6 cells), was fitted to a quadratic regression model weighted by 1/concentration. The lower limit of quantitation (LLOQ) was 5 fmol/10^6 cells. The inter- and intra-run coefficients of variation (CV) for the assay were <9% and the accuracy was 94–104%. Atazanavir was stable in PBMC for at least 24 h at room temperature and for at least 129 days at −15 °C.

Keywords: Peripheral blood mononuclear cells; Atazanavir

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1. Introduction

Atazanavir (BMS-232632) is an azapeptide that is a highly potent human immunodeficiency virus type 1 (HIV-1) protease inhibitor with a half-life that allows for once-daily dosing [1,2]. Atazanavir can be used in combination with other available antiretroviral agents following multi-drug regimens [3].

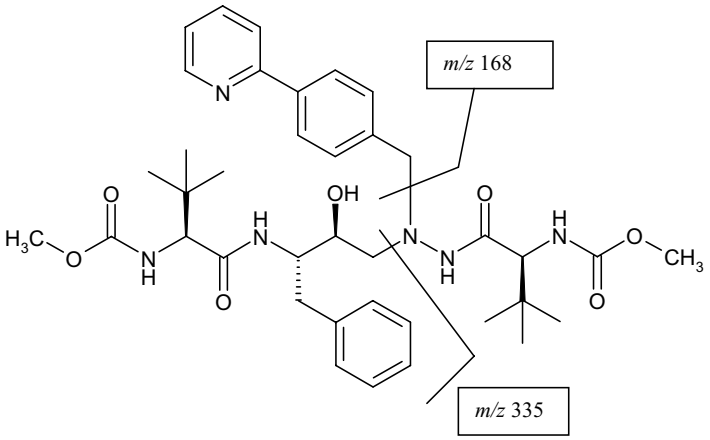
Lymphocytes, monocytes, and macrophages are considered to be primary targets for viral infection [4]. An effective antiviral agent should be able to penetrate into these cells where it can access the virus particle. Collectively, lymphocytes and monocytes in the blood are referred to as peripheral blood mononuclear cells (PBMC) [4,5]. The mononuclear cells may also be referred to as agranulocytes. PBMC makes up about one-third of the white blood cells (WBC), also known as leukocytes. The rest of the leukocytes consists of neutrophils, eosinophils and basophils, collectively known as polynuclear cells. The polynuclear cells may also be referred to as granulocytes. When anticoagulated blood is placed in a tube containing material (medium) of appropriate composition and density, such as Vacutainer® cell preparation tube (CPT), and then centrifuged, the cellular elements are divided into two main fractions [5,6]. The first fraction consists of the granulocytes and erythrocytes (red blood cells, RBC) which sediment to the bottom of the tube, and the second fraction consists of the mononuclear cells and platelets, which remain at the interface above the medium and below the plasma.

In anticipation of clinical studies designed to quantitate atazanavir in human PBMC samples obtained from blood specimens collected from human subjects following dosing with atazanavir, we were required to develop a sensitive PBMC assay based on liquid chromatography with tandem mass spectrometry (LC/MS/MS). The method, while similar in some aspects to the plasma method that we reported earlier [7], was designed with specific attentions to the handling, preparation, extraction and analysis of PBMC samples. The PBMC method has been designed for use in the large-scale (large-batch), high-throughput analysis of a large number of PBMC samples from clinical studies. The validation of this method was based on the recommendations published as a Conference Report of the Washington Conference on Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic studies [8].

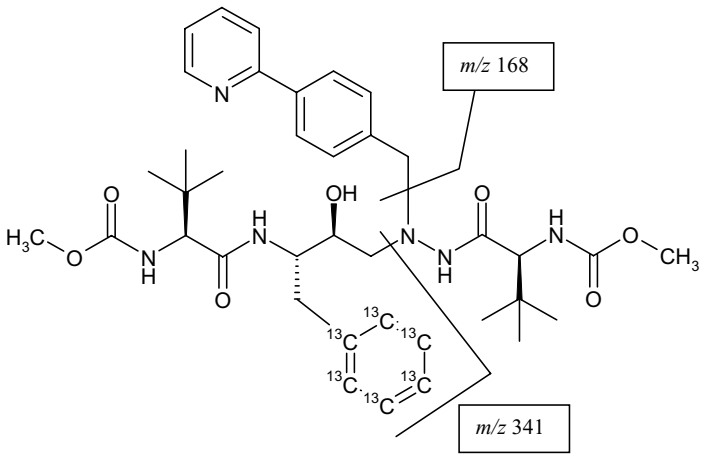
2. Experimental

2.1. Chemicals and reagents

Atazanavir (Fig. 1) was synthesized in large quantities at the Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ, USA) [9]. Likewise, the C^{13} stable label isotope analog used as the internal standard (Fig. 1, I.S.), was also obtained from the Bristol-Myers Squibb Pharmaceutical Research Institute. HPLC-grade methanol, acetonitrile, glacial acetic acid, and ammonium acetate, as well as formic acid, 88%, certified ACS, were obtained from Fisher Scientific (Fair Lawn, NJ, USA). The phosphate buffered saline (PBS) solution used in the preparation of PBMC matrix and the Accustain (a modified Wright's Stain) used in the counting of the PBMC cells were obtained from Sigma-Aldrich (St. Louis, MO, USA). The red-cell lysing solution used in the preparation of PBMC matrix was obtained from Gentra Systems (Minneapolis, MN, USA). Deionized water was produced in-house using a Barnstead Nanopure II Water Purification System (Barnstead/Thermolyne, Dubuque, IA, USA). A 60:40 methanol:water solution (lysing solution), used for lysing the PBMC cells, was prepared as needed by mixing methanol and water in the ratio 60:40 (v/v). A 0.1% acetic acid solution, prepared by mixing 0.50 ml of glacial acetic acid with 500 ml of water, was used to condition the solid-phase extraction (SPE) discs and as washing solution during sample extraction. The elution solvent was a 1:1 (v/v) mixture of methanol:acetonitrile. PBMC matrix was prepared from blood at Bioreclamation Inc. (Hicksville, NY, USA) where PBMC matrix can be commercially obtained. For the preparation, counting, storage, and recovery calculations for PBMC matrix, refer to Sections 2.1.1, 2.1.2, and 2.1.3. A control PBMC stock solution, prepared by adding an appropriate volume of lysing solution to PBMC pellets such that 200 μl of the resulting mixture contained 5×10^6 cells, was used to prepare the standard curve and quality control (QC) samples.



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.).

2.1.1. PBMC matrix preparation

Batches of PBMC matrix were prepared from citrated human blood using Vacutainer® CPT tubes (containing sodium citrate as the anticoagulant and a polyester gel layer and Ficoll solution which separate the mononuclear cells from other blood components) from Becton Dickinson Vacutainer® Systems (Franklin Lakes, NJ, USA) following the recommended procedure [5] with some modifications. The modifications were necessary since we used blood specimens drawn and collected in large volumes (units of blood, with one unit of blood amounting to approximately 500 ml), instead of small volumes of blood (approximately 8 ml) individually drawn into each Vacutainer® CPT tubes. Some of the modifications were done to ensure thorough washing of the PBMC samples with PBS solution and still obtain reasonable recovery of PBMC cells.

The procedure followed is described below in some detail.

The anticoagulant that is above the gel was discarded from each Vacutainer® CPT tube. A unit of citrated whole blood (about 500 ml) was aliquoted in 8–9 ml portions into CPT tubes after the anticoagulant has been decanted (needed 50–60 CPT tubes). The CPT tubes were capped and immediately centrifuged at 1500 × *g* for 25 min to separate the cells. The top layer containing the plasma and Ficoll reagent (part of the gel material in the CPT tube) was aspirated off and discarded, being careful not to disturb the cell layer. The 3–4 ml layer containing the mononuclear cells, platelets, and some Ficoll reagent was transferred with a disposable pipette into 15-ml conical centrifuge tubes. The CPT tubes were washed three times with PBS solution (2–3 ml) using a squeeze bottle and the washes were transferred to the corresponding 15-ml tube containing the mononuclear cells. The 15-ml tubes were filled to the mark with PBS solution, inverted gently several times to mix, then centrifuged at 300 × *g* for 20 min. As much of the wash solution on top as possible was aspirated off without disturbing the discernable 1–2 ml fraction containing the mononuclear cells at the bottom of the tube. The mononuclear cell fraction was washed a second time with PBS solution. After the second wash, the mononuclear cell fraction was transferred into a 50-ml conical centrifuge tube with a capillary pipet. The 15-ml tube was washed twice with PBS solution (2–3 ml) using a squeeze bottle and the washes were transferred to the 50-ml tube containing the mononuclear cells. This procedure was repeated using a second 15-ml tube transferring the mononuclear fraction to the same 50-ml tube being careful to insure complete transfer. This procedure was continued and the mononuclear cell fractions from about eight 15-ml tubes were transferred to the same 50-ml tube. The procedure was repeated by transferring from a second set of about eight 15-ml tubes to a second 50-ml tube. This was continued until finally all the mononuclear cell fractions from all 15-ml tubes have been transferred to about six to eight of the 50-ml tubes. After this, each 50-ml tube was filled to the mark with PBS solution, inverted several times to mix, then centrifuged at 300 × *g*. The mononuclear cell fractions were now present in the bottom 3 ml of the tubes. The PBS wash was carefully aspirated off, then the mononuclear cell fraction was washed a second time with PBS solution. The mixtures were centrifuged at 300 × *g* and the PBS wash was aspirated off. The mononuclear cell fractions thus obtained from six to eight 50-ml tubes were pooled into two new 50-ml conical centrifuge tubes. The two new tubes were brought to volume (50 ml) with PBS solution, gently inverted to mix, and then centrifuged at 300 × *g*. All but the last 4 ml in each tube was aspirated off. Red-cell lysing solution was added to each tube, mixed and allowed to stand for about 20 min at room temperature to lyse the red cells. PBS solution was added to the 50 ml mark, gently inverted to mix, and then centrifuged at 300 × *g*. The top layer with the reddish tint was aspirated off, being careful not to disturb the mononuclear cell fraction at the bottom of the tubes. The mononuclear cell fractions were washed twice with PBS solution. After carefully aspirating the PBS wash, the clean mononuclear cell pellet was about a 3-ml grayish-white jelly-like (viscous, but flowing) material at the bottom of each tube.

2.1.2. PBMC counting and storage

PBS solution was added to the mark of each of the two 50-ml tubes containing the pooled mononuclear cell fractions. The mixtures were mixed to get a homogeneous grayish-white suspension. A 20-μl aliquot of the suspension was taken for counting lymphocytes and monocytes using a Levy Double Neuburger Counting Chamber grid hemocytometer slide (Clay-Adams, Parsippany, NJ, USA) and Accustain. The mononuclear cells were thus stained for manual counting on the microscope slide. This procedure was used to calculate the total number of lymphocytes and monocytes (i.e. total number of mononuclear cells) contained in the pellet in each of the two 50-ml tubes. The number of cells was recorded on the label of each tube. After the 20-μl aliquots were taken for cell counting, the two 50-ml tubes were centrifuged at 1500 × *g* for 20 min and then all the PBS wash was aspirated off leaving the mononuclear cell compacted pellet. The tubes were immediately frozen in dry ice and then stored at −70 °C (to avoid cell lysing, it is recommended to flash freeze and then store the samples frozen).

2.1.3. Recovery (%) of PBMC cells

Before a blood specimen was processed to obtain PBMC matrix, a small portion of the blood

was processed through an automated three-part differential counter (model STKS coulter counter, Beckman-Coulter, Fullerton, CA, USA) to obtain the WBC (leukocytes) counts in the blood sample. It was then assumed that 31% of the WBC count (which has been reported and confirmed in our laboratories as well) was due to mononuclear cells. Thus, the total number of mononuclear cells in the unit of blood used for PBMC processing was obtained. Alternatively, the total number of mononuclear cells in a unit of blood can also be determined directly using a five-part differential counter, e.g. model Cell-Dyn 3200 (Abbott Labs, Abbott Park, IL, USA). Percent recovery of the mononuclear cells from blood was calculated by dividing the total count of the mononuclear cells in the two 50-ml tubes by the total number of mononuclear cells in the unit of blood and then multiplying by 100. Typically, the recovery obtained was 35–65%. This was lower than the recovery of about 70% claimed for Vacutainer® CPT tubes [5]. The relatively low recovery was mainly due to the fact that we processed the blood specimens after keeping them refrigerated for 24 h. This much time was needed to conduct the several tests required by the FDA before the blood could be used for PBMC harvesting. It has been reported [5], and confirmed in our laboratories, that the mononuclear cell recovery decreases by as much as $\geq$50% after the blood sample has been kept for 24 h before processing.

2.2. Equipment

A Sciex API 3000 TurboIonSpray mass spectrometer equipped with an atmospheric pressure ionization (API) electrospray interface (Applied Biosystems/MDS-Sciex, Foster City, CA, USA) was used for detection. The HPLC system consisted of the following components: Shimadzu LC-10AD pumps (Shimadzu Precision Instruments, Torrance, CA, USA), Perkin-Elmer Series 200 Autosampler (Perkin-Elmer, Norwalk, CT, USA). The analytical column was YMC Basic, 50 mm × 2 mm, 5 μm (Waters, Milford, MA, USA) and a similar guard column, YMC Basic, 10 mm × 2 mm, 5 μm, was also used. A Packard MultiPROBE 104 (Packard Instruments, Downers Grove, IL, USA) robotic liquid handling system equipped with a 3M Empore® 96-well vacuum manifold (3M, St. Paul, MN, USA) was used to automate the sample processing. The extraction plates used were 3M Empore® C2-SD 96-well plates also from 3M. The deep-well sample collection plates (2 ml) and mat lids for the collection plates were from VWR Scientific Products (Brisbane, CA, USA). A Fisher Scientific (Fair Lawn, NJ, USA) Model FS30 ultrasonic bath was used to sonicate the PBMC samples. Beckman TJ-6 and Beckman GPR (Beckman Coulter, Fullerton, CA, USA), Microfuge 12 (Beckman Instruments, Palo Alto, CA, USA), and Thermo IEC and IEC Model K (International Equipment, Needham Heights, MA, USA) centrifuges were used for centrifugation. A Maxi Mix II Vortexer (Barnstead/Thermolyne, Dubuque, IA, USA) and an S/P Multi Tube Vortexer (Baxter Healthcare, McGraw Park, IL, USA) were used for vortexing. A CEDRA Well-Evap® (CEDRA, Austin, TX, USA) was used for drying the sample eluents from SPE.

2.3. Preparation of standard stock and working solutions

A stock solution of atazanavir (1000 μg/ml) was prepared in methanol. An intermediate standard solution (2 μg/ml) was prepared by diluting 20 μl of the stock solution to 10 ml with methanol. Working standard solution A (35.2 pg/μl) was prepared by diluting 440 μl of the intermediate standard solution to 25 ml with methanol. Working standard solution B (0.704 pg/μl) was prepared by diluting 500 μl of working standard solution A to 25 ml with methanol. Working standard solutions A and B were used to prepare the standard curve samples. A second stock solution of atazanavir (1000 μg/ml) was used to prepare the QC samples. An intermediate quality control solution (100 μg/ml) was prepared by diluting 1 ml of the stock solution to 10 ml with methanol. Working quality control solution A (1760 ng/ml) was prepared by diluting 440 μl of the intermediate solution to 25 ml with methanol. Working quality control solution B (352 ng/ml) was prepared by diluting 88 μl of the intermediate solution to 25 ml with methanol. Working quality control solutions A and B were used to prepare the QC samples. A stock solution of the I.S. (1000 μg/ml) was prepared in methanol. A working I.S. solution, prepared at 200 ng/ml by diluting 20 μl of the stock solution to 100 ml with a 60:40 methanol: water solution, was used to spike the samples prior to

extraction. All stock, intermediate, and working solutions were stored at about +4 °C (refrigerator) and were stable for at least 1 month. If stored at −20 °C, the stock solutions were stable for at least 10 months [7].

2.4. Preparation of standard curve

2.4.1. Control PBMC stock solution

This was prepared by adding a specified volume of lysing solution (60:40 methanol:water) to PBMC matrix (pellet) so that a 200-μl aliquot of this stock solution contained 5×10^6 cells. Thus, if the PBMC pellet contained 200×10^6 cells, the required volume of the lysing solution was added to bring the final total volume to 8.0 ml. This was then mixed well to obtain a homogenous mixture.

2.4.2. Standard curve

A calibration standard was prepared by transferring the appropriate aliquot of a working solution of atazanavir (prepared in methanol) into a test tube, removing the methanol by evaporation, adding 200 μl of control PBMC stock solution, then vortexing to mix well. As an example, using working standard solution A having a concentration of 35.2 pg/μl (i.e. 50 fmol/μl, as the molecular weight of atazanavir base is 704.87), a 50 μl aliquot of the working solution would give 2500 fmol per tube, i.e. per 5×10^6 cells. Therefore, the concentration of this calibration standard would be 500 fmol/10^6 cells. Other calibration standards of different concentrations were prepared similarly. It should be noted that all calibration standards had the same matrix composition containing 5×10^6 cells. The standard curve range (Table 1) was 5–2500 fmol/10^6 cells and consisted of eight calibration standards, with a typical set consisting of 5, 10, 50, 100, 250, 500, 1000 and 2500 fmol/10^6 cells. Duplicate samples at each standard level were prepared for each standard curve.

2.5. Preparation of QC samples

2.5.1. Control PBMC stock solution

This was prepared as described in Section 2.4.1. Several control PBMC stock solutions were prepared and then combined to obtain a large supply of control PBMC stock solution for preparing large amounts of the QC samples. The control PBMC stock solution used to prepare the QC samples was different from that used to prepare the standard curves.

2.5.2. QC samples

The QC samples (Table 2) were prepared by spiking specified fmol of atazanavir into 20 ml of control PBMC stock solution prepared as described above. Four levels of QC samples were used, namely 15, 1000, 2000 and 10,000 fmol/10^6 cells. These QC samples were prepared such that, like the calibration standards, a 200-μl aliquot sample contained 5×10^6 cells

Table 1
Quadratic regression analysis of typical standard curves for atazanavir in PBMC

Spiked concentration (fmol/10^6 cells)	Predicted concentration (fmol/10^6 cells)			Mean (fmol/10^6 cells)	Deviation (%)	%CV
	Run 1	Run 2	Run 3			
5	5.311, 4.866	5.540, 4.838	4.943, 6.097	5.266	5.3	9.4
10	9.565, 9.403	9.542, 9.910	9.446, 9.819	9.614	−3.9	2.1
50	49.97, 52.37	49.10, 50.36	44.83, 44.15	48.46	−3.1	6.7
100	100.7, 101.4	95.48, 101.1	104.0, 102.6	100.9	0.9	2.9
250	254.5, 243.9	260.6, 247.2	256.3, 243.7	251.0	0.4	2.8
500	–[a], 511.6	503.2, 494.0	484.2, 533.8	505.4	1.1	3.7
1000	981.3, 999.9	1001, 997.4	1000, 982.9	993.8	−0.6	0.9
2500	2500, 2505	2475, 2526	2457, 2547	2502	0.1	1.3
Quadratic slope	−4.0121E−10	−3.5552E−09	−1.2456E−09			
Linear slope	3.5261E−04	3.5726E−04	3.4315E−04			
Intercept	−1.3860E−04	−3.1026E−04	−1.7918E−04			
r^2	0.9999	0.9998	0.9992			

[a] Rejected as an outlier.

Table 2
Assay accuracy and precision results ($n = 18$)

Nominal concentration (fmol/10^6 cells)	Mean predicted concentration (fmol/10^6 cells)	Deviation (%)	Intra-run precision (%CV)	Inter-run precision (%CV)
15 (low QC)	14.176	−5.5	8.6	0.0[a]
1000 (mid QC)	1035	3.5	2.9	0.0[a]
2000 (high QC)	2030	1.5	3.4	0.0[a]
10000 (dilution QC)[b]	10154	1.5	2.8	2.5

[a] No additional variation was observed as a result of analysis in different runs.
[b] Diluted 10-fold with control PBMC stock solution prior to analysis.

(75, 5000, 10,000, 50,000 fmol of atazanavir/200 μl of the sample). As an example, the 15 fmol/10^6 cells QC sample was prepared by transferring 15 μl of the 352 pg/μl (500 fmol/μl) working quality control solution B to a test tube, removing the methanol by evaporation and then adding 20 ml of control PBMC stock solution. Vortex well to ensure adequate mixing. The 10,000 fmol/10^6 cells QC sample was spiked at a concentration higher than the upper limit of quantitation (ULOQ) and is known as a dilution QC. It was diluted 10-fold with control PBMC stock solution prior to analysis. The QC samples were used to determine the accuracy and precision of the method during validation. The QC samples were sub-divided into 0.8 ml aliquots, which provided at least three 200-μl samples, and stored frozen.

2.6. Sample extraction

To 200 μl of each calibration standard and QC sample in a culture tube, each containing 5×10^6 cells, 50 μl of the working I.S. solution (I.S. concentration is 2820 fmol/10^6 cells) were added. For the dilution QC sample, 20 μl of sample was used and then diluted to 200 μl with control PBMC stock solution. Each sample was vortexed thoroughly. The mixture was sonicated for 10 min and then centrifuged at $2600 \times g$ for 10 min to obtain a supernatant layer. The supernatant layer was transferred into a clean 12 cm × 75 mm culture tube and then evaporated to dryness. The dried extract was reconstituted with 50 μl of methanol and then 200 μl of water and 250 μl of 0.1% acetic acid were added. The mixture was mixed well. Sample processing was then continued on a MultiPROBE with 3M Empore® C2-SD 96-well plates. Using the MultiPROBE, each well was activated with 250 μl of methanol, followed by 500 μl of 0.1% acetic acid. The wells were not allowed to dry. The sample was manually loaded onto a well. The entire sample was allowed to pass through the well with vacuum for about 2 min. Using the MultiPROBE, each well was washed with 500 μl of 0.1% acetic acid. The wells were dried by applying vacuum for about 2 min. The waste reservoir was then replaced with a deep-well collection plate. Using the MultiPROBE, each well was eluted twice with 200 μl of elution solvent pulling the vacuum for a minute to dryness between elutions. The collection plate was transferred to the Well-Evap® and the collected eluents were evaporated at about 60 °C for approximately 40 min under nitrogen. Each dried extract was reconstituted with 200 μl of mobile phase by vortexing. The collection plate was capped and placed in the autosampler for analysis.

2.7. Chromatographic and mass spectrometric conditions

The isocratic mobile phase was prepared by adding 300 ml of acetonitrile into a 1-l graduated cylinder, adding methanol to the 600 ml mark, adding water to the 1000 ml mark and then adding 250 μl of 88% formic acid to the mixture. The entire mixture was mixed well, then placed in an ultrasonic bath for 20 min to degas. The analytical column was YMC Basic, 50 mm × 2 mm, 5 μm (Waters, Milford, MA, USA, Part No. BA995050502WTA) and the guard column was also YMC Basic, 10 mm × 2 mm, 5 μm (Part No. BA995050102WDA). Both columns were kept at ambient conditions. The flow rate was 0.25 ml/min, the injection volume was 20 μl, and the cycle time was set at 4.0 min.

The mass spectrometer was operated in the positive TurboIonSpray mode at 400 °C. The samples were

analyzed via selected reaction monitoring (SRM) employing the transition of the $[M + H]^+$ precursor ions to product ions: *m*/*z* 705→335 for atazanavir and *m*/*z* 711→341 for the I.S. The IonSpray voltage was set at 4600 V and the declustering potential was 56 V. The entrance and focusing potentials were −10 and 220 V, respectively. The nebulizer, curtain, and collision gas were ultra high purity nitrogen and the settings were 9, 9, and 5, respectively. The TurboIon gas (nitrogen) flow rate was 8.0 l/min. The collision energy was set at 42 V and the collision cell exit potential was set at 24 V. Both the first and third quadrupoles were set at unit resolution. The dwell time was 500 ms for both atazanavir and I.S. and the pause time was 5 ms.

2.8. Measurement and calculations

Chromatographic data acquisition was automated using the system Mass Chrom 1.1 software (Applied Biosystems/MDS-Sciex, Foster City, CA, USA) and peak integration was done using TurboQuan software. The integrated peak areas of interest were imported into a Watson DMLIMS (Innaphase Inc., Philadelphia, PA, USA) where subsequent calculations were performed. Peak area ratios (atazanavir peak area/I.S. peak area) versus concentrations of the standards, expressed as fmol/10^6 cells, were fitted to a quadratic regression equation, weighting each standard by the reciprocal of its concentration ($1/x$). The concentration of a QC sample (or unknown sample) is obtained by multiplying the concentration obtained from regression equation by the dilution factor (df), where df = 5 million cell count divided by the cell count (million) of the portion of the QC sample (or unknown sample) used for analysis.

2.9. Stability

The stability of atazanavir in PBMC was studied at the following conditions: (1) room temperature, (2) freeze-thaw cycles, (3) storage at −15 °C, (4) stability of processed samples. For each stability determination, QC samples spiked at 15, 1000, and 2000 fmol/10^6 cells were assayed in triplicate. The deviations of the mean predicted concentrations from the nominal concentrations as well as the percentage of coefficients of variation (%CV) of the predicted concentrations were used to assess stability.

2.10. Recovery

The extraction recoveries of both atazanavir and I.S. were evaluated by comparing peak areas of pre-extraction spiked samples (analyte spiked into PBMC before extraction) with peak areas of corresponding post-extraction spiked samples (analyte spiked into the SPE extract of blank PBMC). Recoveries for atazanavir were evaluated at 15 and 2000 fmol/10^6 cells and for the I.S. at 2820 fmol/10^6 cells, each in six replicates.

2.11. Application to clinical samples

2.11.1. Sample collection

The following sample collection procedure is recommended for a clinical study. At the designated time-point draw about 8 ml of blood into a Vacutainer® CPT tube (with sodium citrate). CPT tubes should be stored upright at room temperature according to the Package Insert. Since the CPT tubes contain chemical additives, it is important to prevent backflow from the tube to the subject. Following blood collection, gently invert (do not shake) the tube 3–4 times to ensure mixing the blood with the anticoagulant. After mixing, store the CPT tube upright at room temperature until centrifugation. Centrifuge within 1 h of collection for maximum cell recovery. Prior to centrifugation, remix the blood by gently inverting the CPT tube 6–8 times. Centrifuge at 1500 × *g* for 25 min, maintaining the centrifuge temperature at 18–25 °C. After centrifugation, aspirate off and discard the top layer containing the plasma and Ficoll reagent, being careful not to disturb the cell layer (see package insert). With a disposable pipette, transfer the 3–4 ml layer containing the mononuclear cells, platelets, and some Ficoll reagent into a clean 15-ml centrifuge tube. Wash the CPT tube with about 3–5 ml of PBS solution using a squeeze bottle and add the wash to the sample in the clean 15-ml centrifuge tube. Fill to the mark of the 15-ml tube with PBS solution, invert gently several times to mix, then immediately centrifuge at 300 × *g* for about 20 min at 18–25 °C. Aspirate off and discard as much of the wash solution on top as possible without disturbing the discernible 0.5-ml fraction containing the mononculear cells at the bottom of the tube. Using a capillary pipet, transfer as much of the PBMC layer as possible into a micro-tube (Fisher 1.5 ml microcentrifuge tube with secure

lock, natural color, #05-669-32, Fisher Scientific, Fair Lawn, NJ, USA). Wash the 15-ml tube with a small volume of PBS solution and add the washings to the microtube. Centrifuge at 18,200 × *g* for 6 min, then aspirate off the PBS wash. Add 1.0 ml of PBS solution, invert the microtube twice, then gently vortex for 20 s to obtain a homogeneous mixture. Take a 20-μl aliquot of the mixture for counting under the hemocytometer/microscope. Calculate and record the number of PBMC cells remaining in the microtube. There is no need to correct for the 20-μl aliquot used for counting. After taking the 20 μl for cell counting, immediately centrifuge at 18,200 × *g* (18–25 °C) for 20 min. Aspirate off all the liquid, then invert the microtube on a blotting paper to drain off any remaining liquid. The PBMC pellet forms a thin layer at the bottom of the microtube and the volume can be considered to be insignificant compared to the amount of lysing solution to be added in the bioanalytical laboratory. The tube is then immediately capped and frozen at −70 °C until analysis.

2.11.2. Addition of lysing solution and calculation during analysis

Upon thawing of the sample, make sure that the sample pellet is dry. Remove any traces of liquid droplets by room-temperature evaporation under nitrogen. Using the number of cells in the sample, add the appropriate amount of lysing solution such that 40 μl of the resulting sample mixture contain 10^6 cells. As an example, the lysing solution volumes added to samples A, B, and C, containing 5×10^6, 20×10^6, and 4×10^6 cells, respectively, would be 200, 800 and 160 μl, respectively. For the initial analysis, the aliquots used to analyze samples A, B, and C would be 200, 200, and 160 μl, respectively. To sample C, 40 μl of control PBMC stock solution (Section 2.4.1) will be added before extraction in order to bring the cell count to 5×10^6 cells. The df (Section 2.8) for samples A, B, and C would be 1.0, 1.0, and 1.25. The df of 1.25 for sample C is obtained by dividing 5 million cells specified by the method for each analysis by the 4 million cells in the sample actually used for analysis. If a repeat analysis were to be carried out on sample B using only 50 μl of the lysed sample (because the initial analysis showed a drug concentration that was above the upper limit of the standard curve), the 50 μl would be brought to 200 μl with control PBMC stock solution before extraction in order to bring the cell count to 5×10^6 cells. The df for the re-analysis would be 4.0 (5 million cells specified by the method for each analysis divided by 1.25 million cells in the sample actually used for analysis). Record the amount of lysing solution added to the sample, together with the amount taken out for analysis and immediately re-freeze any remaining lysed sample.

3. Results and discussion

3.1. Method development

Since the analysis of drugs in PBMC samples is still a rarity, experiences in handling PBMC samples is limited. Our expression of the concentrations of the calibration standards and QC samples as fmol/10^6 cells was based on similar units of measurement reported in the literature when analyzing PBMC samples [10,11]. This requires accurate counting of the number of cells in unknown sample pellets.

The method described here is based on using a PBMC sample amount of 5×10^6 cells per analysis. The rationale for choosing this sample amount is as follows. For the anticipated clinical studies, the PBMC samples are to be obtained from 8 ml blood drawn into Vacutainer® CPT tubes. This amount of blood from the different subjects is expected to provide a mean count of 13×10^6 cells, with a range ($n = 10$) of 7×10^6 to 21×10^6 cells [5]. Our choice of 5×10^6 cells for sample amount per analysis would allow the maximum sample amount assayed to obtain the lowest possible limit of quantitation, and at the same time provide some sample for re-extraction and then re-analysis should the need arise. The remaining sample, already in lysing solution, will be kept frozen until re-analysis time.

The lysing solution is expected to lyse the cells and then extract atazanavir contained within the cells. The use of 60% methanol in water as lysing solution has been reported in the literature [12,13]. To the best of our knowledge, there is no discussion as to why this particular medium is chosen. We chose this as the lysing solution because atazanavir has been shown to be very soluble and stable in methanol. Methanol is also known to denature proteins and while doing so it will release atazanavir from the mononuclear cells. In

the absence of in vitro or in vivo study PBMC samples (i.e. incurred samples) known to contain atazanavir within the cells, it is impossible to gauge the true extraction recovery obtained using this particular lysing solution. The extraction recovery (Sections 2.10 and 3.6) obtained using spiked samples is not necessarily an accurate indicator of the true recovery. No other lysing solutions were tried during the development of this method.

Cognizant of the fact that the amount of PBMC cells recovered from the 8-ml blood draw during the clinical studies would vary from sample to sample (due to variability in recovery from tube to tube and/or due to variability in the PBMC contents from subject to subject), our method design is based on using different volumes of the lysing solution for different PBMC sample pellets. The volume is adjusted in proportion to the cell count in the sample pellet. This keeps the number of cells per volume of lysing solution the same for all samples. Thus, the lysing efficiency is expected to stay the same, as indicated by recovery, cleanliness of solution, and ease of centrifuging from sample to sample. In addition, the LC/MS/MS matrix effect will stay the same from sample to sample, assuming there is no batch to batch difference.

We chose a lysing solution volume of 40 μl per 10^6 cells. This volume, which is about twice that used in the literature [12,13], is expected to provide good lysing and extraction efficiency. Thus, in accordance with the design of our method, the volume of the lysing solution used for PBMC pellets obtained from 8-ml blood draws into CPT tubes will range from 280 to 840 μl for the expected cell count range of 7×10^6 to 21×10^6 cells. This statement presupposes that the volume of the pellet sample is insignificant compared to the volume of the lysing solution added. It is therefore important to remove all traces of liquid in the sample before adding the lysing solution.

It should be noted that the I.S. addition is not part of the lysing solution addition. While the lysing solution is added to the entire unknown sample received, the I.S. solution is added to the aliquot of the sample used for analysis. If the I.S. was contained in the lysing solution that is added to the entire unknown sample received, the repeat analysis of sample B described in Section 2.11.2 would not have been possible since the analyte/I.S. ratio, on which the calculation from the regression model is based, would have been the same irrespective of the aliquot of the lysed sample used for analysis.

The method described is based on receiving the unknown samples from clinical sites as pellets. However, the method is equally applicable if the samples were to be received after adding the lysing solution (without internal standard) at the clinical site. The advantage of this latter approach is that the patient samples would likely have been disinfected prior to arrival at the analytical laboratory, thus providing for better laboratory personnel safety. A disadvantage would be the potential for losing part of the sample due to spillage during transit. This would cause a problem since the analytical laboratory would be relying on the original absolute total cell count. The samples that come from the clinic suspended in the lysing solution would be ‘dried’ by evaporation before analysis. Then the prescribed volume (as discussed above) of lysing solution would be added to the dried residue.

As described above, the method presented here is based on adding varying volumes of the lysing solution in direct proportion of the cell counts in the samples and then using the same aliquot volume of the lysed solution for all samples. An equally viable approach would be to use the same volume of lysing solution, e.g. 200 μl, for all samples, irrespective of the cell counts, and then using for analysis an aliquot of the lysed sample whose volume is varied in inverse proportion to the original sample cell counts. Each aliquot used for analysis will contain 5×10^6 cells (except for those samples whose total cell count is lower than 5×10^6). Before performing the steps of adding the internal standard and follow-up extraction, the aliquots will be brought to 200 μl with additional lysing solution. The regression scheme and concentration calculations for unknown samples, including the dilution factors, will be the same as described above for the method.

After adding the internal standard, lysing the sample, and centrifugation, the supernatant containing the analytes was transferred and evaporated to dryness so that the extract could be taken up in a matrix suitable for solid-phase extraction. At this stage, solubility was an issue. The extract had to be reconstituted in pure methanol first to dissolve the analytes then diluted nine-fold with water/0.1% acetic acid before extraction using C2-SD plates on the MultiPROBE. A

single wash with 500 μl of 0.1% acetic acid was sufficient to obtain clean extracts. Elution with a 50/50 mixture of methanol and acetonitrile gave good recovery of the atazanavir and I.S. Using a 1:1:2 mixture of methanol:acetonitrile:10 mM ammonium acetate solution as the eluting solvent gave very low recoveries (about 25%) for atazanavir and I.S. The elution solvent was then evaporated off so that the residue could be reconstituted with mobile phase where the analytes are stable.

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 MS/MS product-ion spectrum. The proposed fragments for the two major product ions are shown in Fig. 1. The relative intensities of the product ions are different from those observed for the plasma assay [7] because of the different mass spectrometers and settings used in each assay. The settings on the mass spectrometer were optimized for the production of the ions that are monitored.

3.2. Selectivity

The sample extraction and chromatographic analysis were developed to produce a selective assay for the analytes. The product ions monitored for this assay are not the most predominant. They were chosen to maximize selectivity of the assay.

At least six different batches of PBMC matrix were analyzed and checked for interference in the assay. Inspection of SRM chromatograms showed minimum interference from PBMC matrix at the retention time and in the ion channel of either atazanavir or the I.S. Fig. 4 shows a typical SRM chromatogram. The accurate quantitation of atazanavir in diluted samples (Table 2) and in the lower limit of quantitation (LLOQ) samples (Table 3) indicates that matrix effect from PBMC, if present, does not adversely affect accuracy and precision of the method. The use of a stable isotope analog for I.S. is expected to help in this regard.

3.3. Calibration curve

An 8-point calibration standard curve in PBMC, ranging from 5 to 2500 fmol/10^6 cells for atazanavir, was prepared in duplicate for each run. The standard curve is satisfactorily described by quadratic regression weighted by $1/x$, as the back-calculated values were within ±15% of the nominal concentrations (±20% at the LLOQ) in the three validation runs (Table 1). This range was chosen to include the anticipated concentrations of atazanavir in PBMC samples collected post-dose. The regression equation is as follows:

$$\text{peak area ratio} = (\text{quadratic slope})\,(\text{concentration})^2 + (\text{linear slope})\,(\text{concentration}) + \text{intercept}$$

Table 3
Lower limit of quantitation (LLOQ) results

Predicted concentration (fmol/10^6 cells)	Deviation (%)	Mean predicted concentration (fmol/10^6 cells)	Mean deviation (%)
5.124	2.5	5.066	1.3
4.763	−4.7		
4.928	−1.4		
4.996	−0.1		
5.203	4.1		
5.382	7.6		

Nominal concentration = 5 fmol/10^6 cells.

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 ANOVA of the results established good accuracy and precision of the method. For the three runs, the mean accuracy ranged from 94 to 104%. Estimates of the within- and between-run precision were <9% CV for all QC samples (Table 2).

3.5. Lower and upper limits of quantitation

The LLOQ is defined as the lowest concentration in the standard curve that back-calculates with adequate precision and accuracy. For the standard curves in the three accuracy and precision runs, the mean predicted concentration at the LLOQ was within 6% of the nominal value with a CV of 9.4%. These accuracy and precision values are excellent.

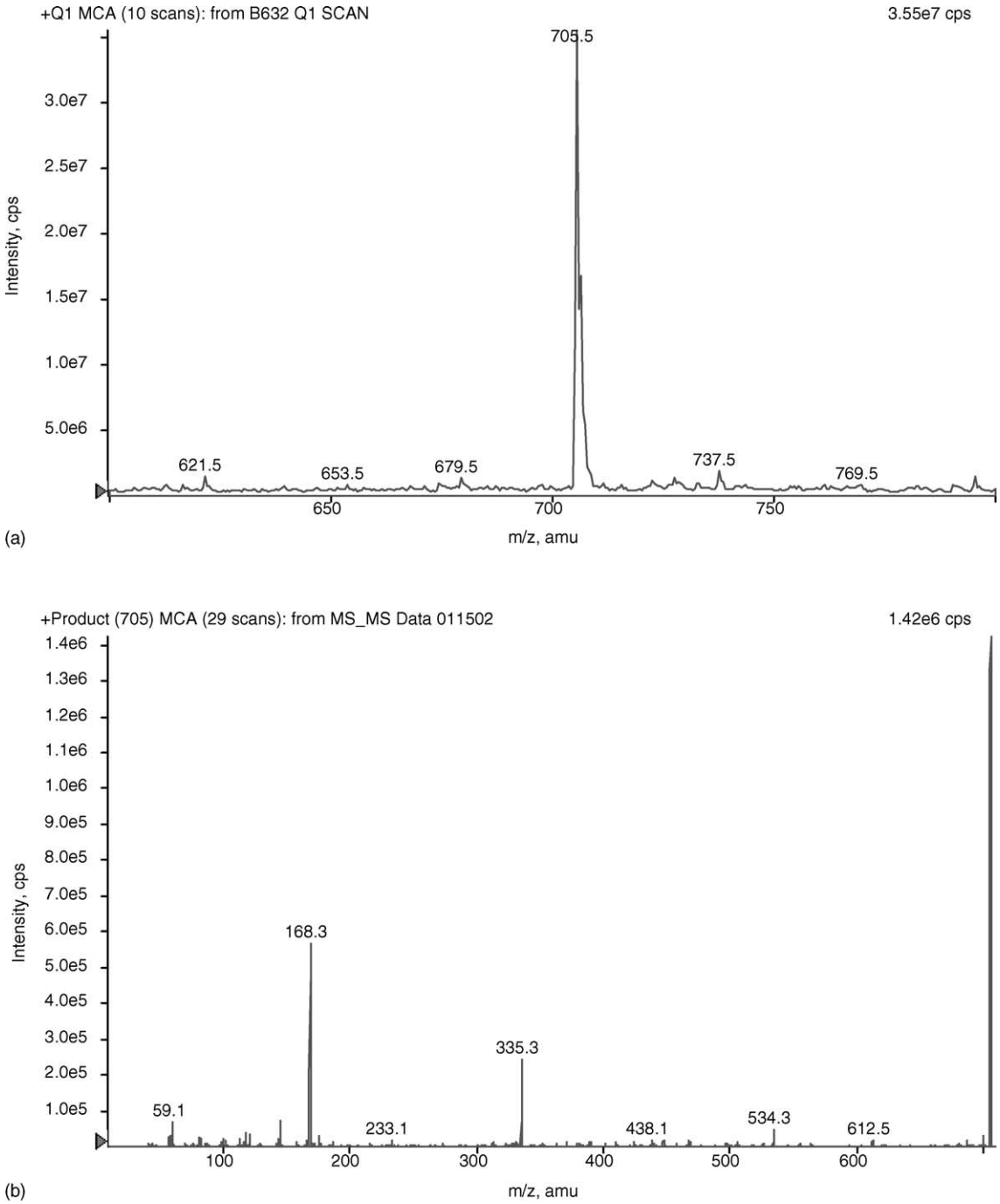


Fig. 2. (a) Positive-ion electrospray mass spectrum (Q1) for atazanavir, $[M + H]^+ = 705$. (b) MS/MS product-ion spectrum (Q3) for atazanavir with *m*/*z* 705 as the precursor ion; *m*/*z* 335 was monitored.

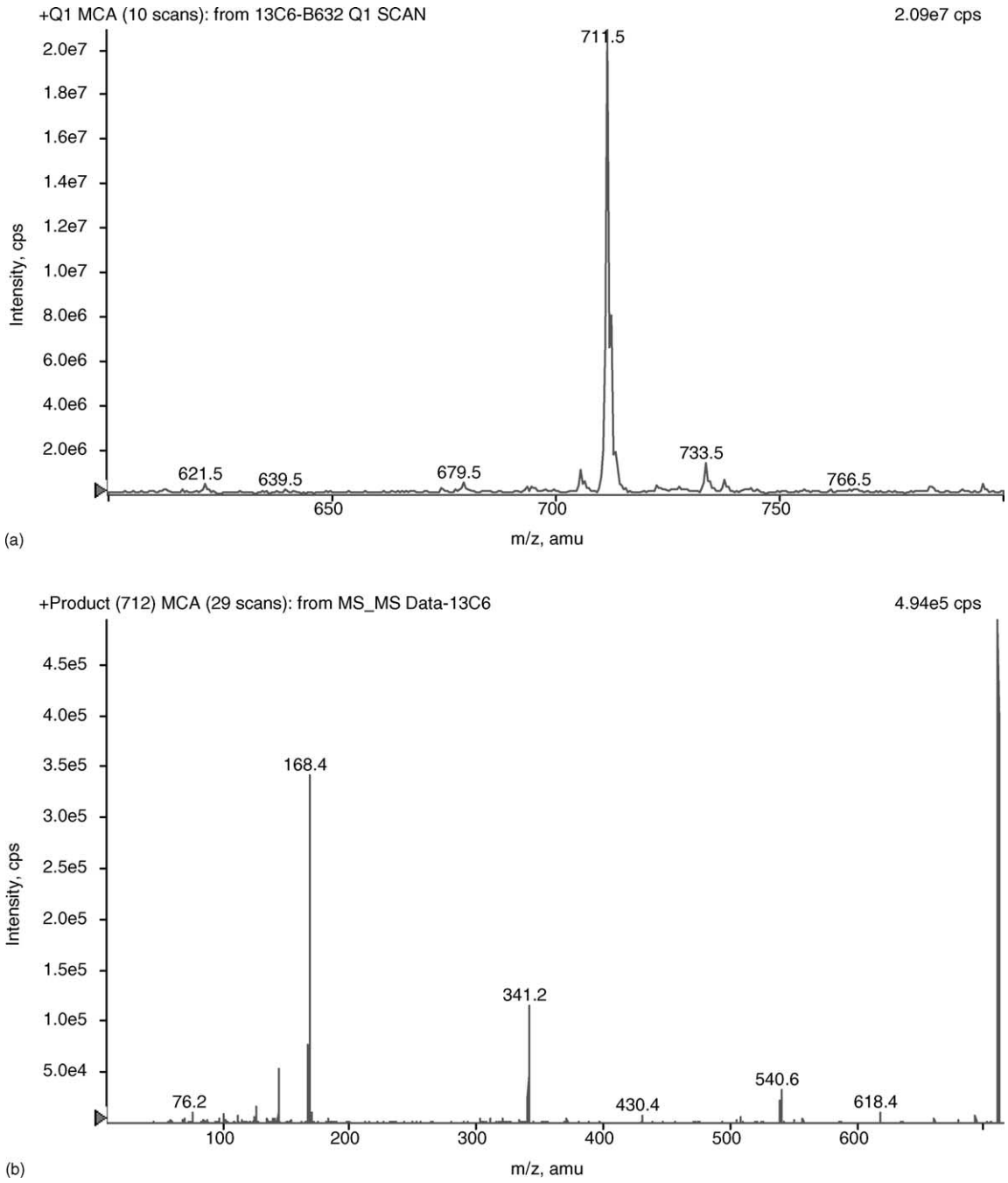


Fig. 3. (a) Positive-ion electrospray mass spectrum (Q1) for the I.S., $[M + H]^+ = 711$. (b) MS/MS product-ion spectrum (Q3) for the I.S. with *m*/*z* 711 as the precursor ion; *m*/*z* 341 was monitored.

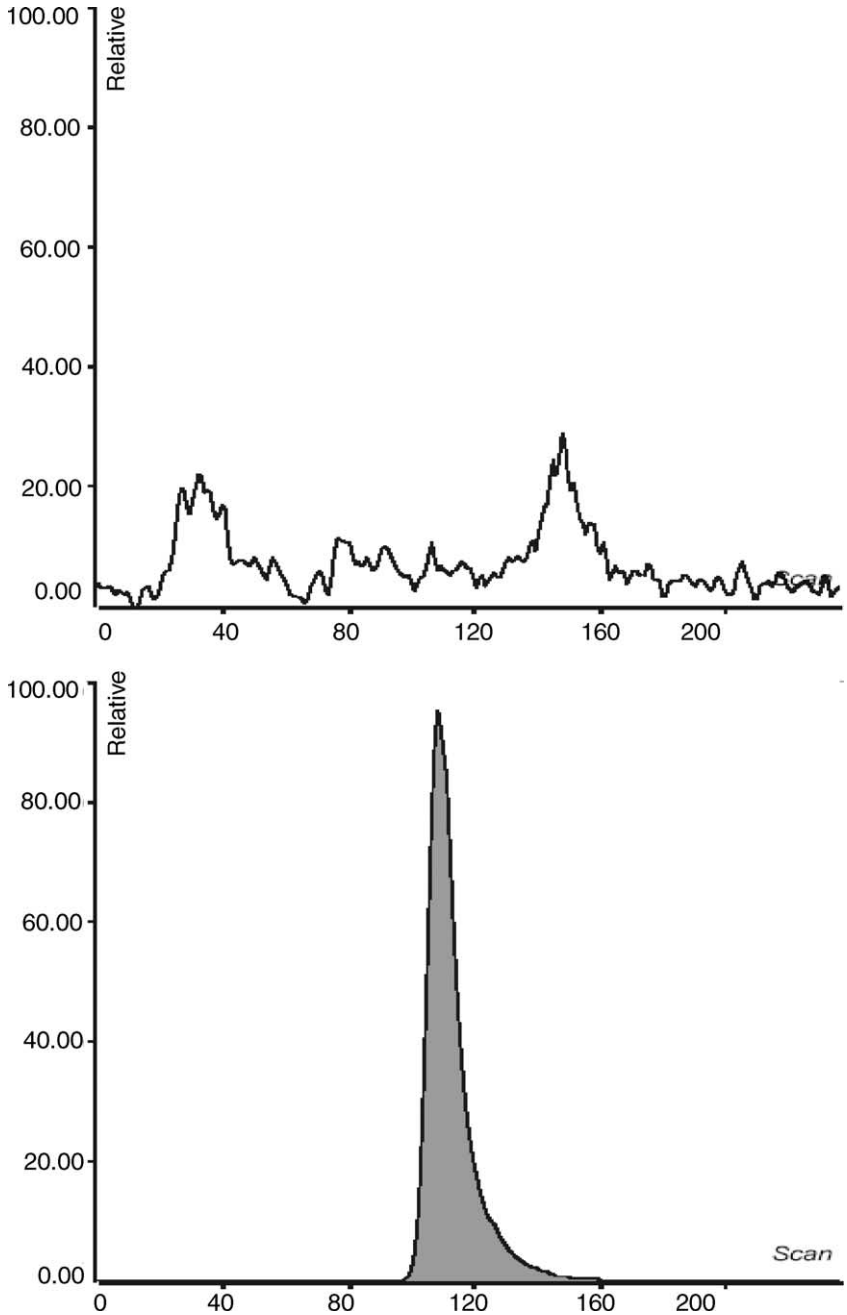


Fig. 4. SRM chromatograms obtained from PBMC matrix containing I.S. only at 2820 fmol/10^6 cells: monitored in the atazanavir channel, *m*/*z* 705→335 (top panel); monitored in the I.S. channel, *m*/*z* 711→341 (bottom panel).

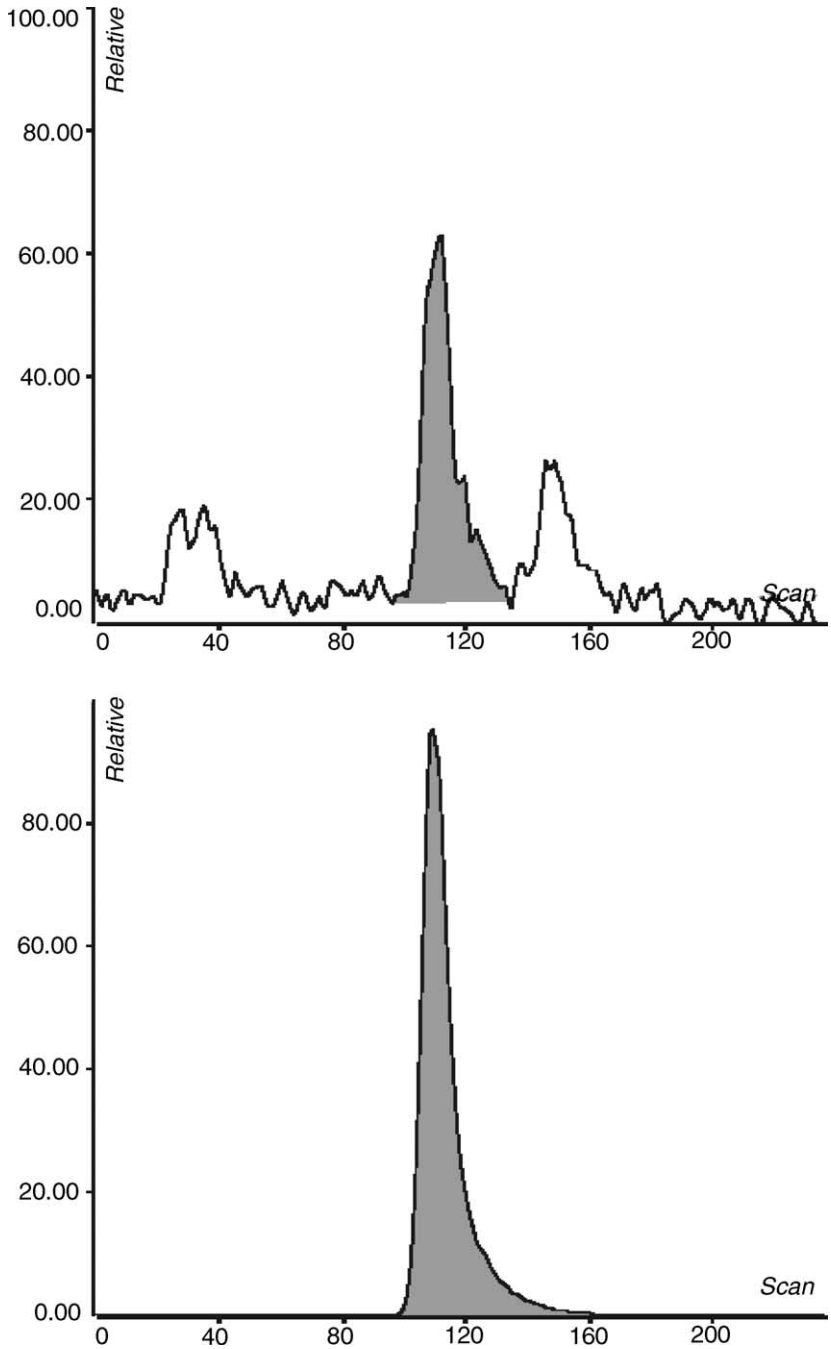


Fig. 5. SRM chromatograms obtained from LLOQ sample (5 fmol/10^6 cells) containing I.S. at 2820 fmol/10^6 cells: atazanavir SRM channel (top panel); I.S. SRM channel (bottom panel).

In addition, the six different batches of PBMC matrix that were evaluated for specificity were spiked at 5 fmol/10^6 cells to obtain six LLOQ samples. These LLOQ samples were assayed and the results presented in Table 3 show good accuracy. The LLOQ was thus established at 5 fmol/10^6 cells. A typical chromatogram of an LLOQ sample is shown in Fig. 5.

The ULOQ of 2500 fmol/10^6 cells 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 PBMC stock solution. When this occurs, a dilution QC sample is assayed together with the study (post-dose) samples.

3.6. Recovery

The extraction recovery for atazanavir was about 100%. Likewise, the extraction recovery for the I.S. at the concentration used in the assay was about 100%. The sample extraction procedure results in clean extracts and good recovery to obtain the required sensitivity for the assay. Good precision and accuracy of the results for the dilution QC sample (Table 2) which was diluted with different batches of control PBMC stock solution on different analysis days prior to analysis suggest insignificant adverse effect due to ion suppression, if any, from the matrix. Furthermore, the results in the evaluation of the LLOQ using different batches of PBMC matrix point to the same conclusion.

3.7. Stability

It should be noted that atazanavir was found to be extremely stable in methanolic solution and in human plasma [7].

3.7.1. Room temperature

QC samples were left standing at ambient conditions for 24 h prior to analysis against a freshly prepared standard curve. The results in Table 4 indicate that atazanavir is stable in PBMC for at least 24 h at room temperature.

3.7.2. Freeze-thaw cycles

QC samples were subjected to three freeze-thaw cycles. Each freeze-thaw cycle consisted of keeping the samples frozen for a minimum of 12 h. After the third freeze-thaw cycle, the samples were analyzed against a freshly prepared standard curve. The results in Table 4 indicate that atazanavir is stable in PBMC through at least three freeze-thaw cycles.

3.7.3. Long-term storage at −15 °C

QC samples were stored at −15 °C. After 129 days, the samples were analyzed against a freshly prepared standard curve. The results in Table 4 indicate that atazanavir is stable in PBMC for at least 129 days at −15 °C.

3.7.4. Autosampler stability

Autosampler stability was investigated as part of a regular validation run. Freshly spiked standards and QCs were extracted and injected for precision and ac-

Table 4
Stability of BMS-232632 in human PBMC samples

Storage conditions	Nominal concentration (fmol/10^6 cells)					
	15		1000		2000	
	Mean predicted concentration	Deviation (%) (%CV)	Mean predicted concentration	Deviation (%) (%CV)	Mean predicted concentration	Deviation (%) (%CV)
Room temperature (24 h)	14.82	−1.2 (6.6)	941.3	−5.8 (1.3)	2054	2.7 (5.5)
Freeze-thaw (three cycles)	14.50	−3.3 (7.5)	1075	7.5 (9.3)	1747	−12.6 (5.5)
Long-term storage[a]	14.83	−1.1 (4.4)	1026	2.6 (1.5)	2049	2.4 (0.8)
Autosampler stability[b]	14.44	−3.7 (5.6)	1071	7.1 (0.6)	2114	5.7 (2.1)

[a] For 129 days at −15 °C.
[b] At room temperature for 17 h.

curacy. Also, an extra set of QC samples was extracted as part of the run, but not injected. These extra QC samples were left in the autosampler at room temperature for 17 h and then injected. These were analyzed against the original curve. The results in Table 4 indicate that atazanavir is stable in the reconstituted PBMC extracts for at least 17 h at room temperature.

4. Conclusions

A sensitive and robust method for determining atazanavir in PBMC samples has been developed and validated. It was important to understand the preparation of PBMC matrix from whole blood. PBMC matrix was routinely prepared as a pellet by obtaining units of citrated whole blood and then proportioning the blood into Vacutainer® CPT tubes where the PBMC samples were harvested from the blood.

The experiences with the plasma assay were helpful in the development of this assay in PBMC samples. Atazanavir showed excellent stability in PBMC samples as it did in plasma samples. The preparation of the PBMC sample and the counting of the cells is very important in the analysis. As discussed in Section 2.1.3, the recovery of PBMC depends partly on the time elapsed between blood collection and PBMC processing. When feasible, it is recommended to process the blood collected to obtain the PBMC sample as soon as possible. Since the results are expressed as femtomoles per million cells, it is important to get an accurate cell count in the samples. Automation of the extraction procedure is desirable both for productivity and safety reasons. The use of a mass spectrometer as the detector coupled with solid-phase extraction makes this method quite selective. The use of a stable isotope analog as internal standard contributes to the overall robustness of the method.

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