Journal of Chromatography B, 877 (2009) 575–580

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Short communication

Quantification of HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors in peripheral blood mononuclear cell lysate using liquid chromatography coupled with tandem mass spectrometry

R. ter Heine*, M. Davids, H. Rosing, E.C.M. van Gorp, J.W. Mulder, Y.T. van der Heide, J.H. Beijnen, A.D.R. Huitema

Slotervaart Hospital, Department of Pharmacy & Pharmacology, Louwesweg 6, 1066EC Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 25 September 2008
Accepted 7 January 2009
Available online 14 January 2009

Keywords:
Antiretroviral
HIV
Pharmacology
LC-MS
HPLC
TDM
Intracellular
LC-MS/MS

ABSTRACT

For pharmacokinetic monitoring, measurement of antiretroviral agents in plasma is the gold standard. However, human immunodeficiency virus protease inhibitors (PIs) or non-nucleoside reverse transcriptase inhibitors (NNRTIs) exert their action within the infected cell. Cell-associated concentrations may therefore more adequately reflect therapy outcome. Therefore, for the quantification of nine PIs (amprenavir, atazanavir, darunavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir and tipranavir), 1 active PI metabolite (nelfinavir M8) and 2 NNRTIs (efavirenz and nevirapine) in lysate of peripheral blood mononuclear cells (PBMCs) an assay was developed and validated, using liquid chromatography coupled with tandem mass spectrometry. Analytes were extracted from a PBMC pellet by means of a one-step extraction with 50% methanol containing the internal standards D6-indinavir, D5-saquinavir, 13C6-efavirenz and dibenzepine. Chromatographic separation was performed on a reversed phase C18 column (150 mm × 2.0 mm, particle size 5 μm) with a quick stepwise gradient using an acetate buffer (pH 5) and methanol, at a flow rate of 0.25 mL/min. The analytical run time was 10 min. The triple quadrupole mass spectrometer was operated in the positive ion-mode and multiple reaction monitoring was used for drug quantification. The method was validated over a range of 1–500 ng/mL in PBMC lysate for all analytes. The method was proven to be specific, accurate, precise and robust. The mean precision and accuracy was less than ±12% at all concentration levels. Using the developed assay and a previously developed assay for these analytes in plasma, the relationship between plasma and intracellular pharmacokinetics and their relationship with therapy outcome can now be determined.

1. Introduction

For therapeutic drug monitoring (TDM) and pharmacokinetic studies, antiretroviral drug concentrations are usually measured in plasma. The site of action of human immunodeficiency virus (HIV) protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), however, is not in plasma, but within the infected cell where the viral protease and reverse transcriptase catalyze virus replication. Previously, in vitro experiments have shown that cell-associated concentrations of PIs can be influenced by efflux transporter modulation and that genotypic resistance may arise when cellular accumulation of PIs is hampered [1–3]. Furthermore, the intracellular accumulation of PIs and NNRTIs most likely is a result of passive transport, active uptake and efflux from cells. The cellular accumulation of antiretroviral drugs may therefore vary from person to person and from drug to drug. Hence, measurement of cell-associated concentrations of PIs and NNRTIs may more adequately reflect treatment effectiveness than measurements of plasma concentrations.

Previously, only a few methods have been developed for the quantification of PIs and NNRTIs in peripheral blood mononuclear cell (PBMC) lysate using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [4]. Jemal et al. developed a sensitive method for the quantification of atazanavir, but were using a labour-intensive solid phase extraction [5]. Colombo et al. developed a method for the simultaneous quantification of nine antiretroviral drugs using a one-step extraction from PBMCs, while using two ionization methods and a 20 min chromatographic re-equilibration step [6].

We have previously developed a fast assay for the simultaneous determination of PIs and NNRTIs in plasma and dried blood spots, using LC-MS/MS with a total runtime of 10 min using only positive ionization [7,8]. We have adapted this method for the determination of PIs and NNRTIs in PBMC lysate and we are the first to report

* Corresponding author. Tel.: +31 20 5124737.
E-mail address: rob.terheine@slz.nl (R. ter Heine).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.01.011

Table 1
Monitored transitions, retention times, selected internal standards of all analytes.

Compound	Mass transition (m/z)	Retention time (min)	Selected internal standard
Amprenavir	506 → 245	4.4	D6-indinavir
Atazanavir	705 → 168	5.4	D5-saquinavir
Darunavir	548 → 392	4.2	D5-saquinavir
Efavirenz	316 → 244	5.3	13C6-efavirenz
Indinavir	614 → 421	5.0	D6-indinavir
Lopinavir	629 → 447	6.0	D5-saquinavir
Nelfinavir	568 → 330	6.1	D5-saquinavir
M8	584 → 330	5.4	
Nevirapine	267 → 226	4.1	Dibenzepine
Ritonavir	721 → 296	5.2	D5-saquinavir
Saquinavir	671 → 570	5.5	D5-saquinavir
Tipranavir	603 → 411	5.9	D5-saquinavir

a validated method for the determination of tipranavir, the active nelfinavir metabolite M8 and darunavir in this matrix.

2. Experimental

All chemicals, reagents and equipment were as described previously [7,8]. All monitored mass transitions and the used internal standards are shown in Table 1 for completeness.

2.1. Preparations

The cell extraction solution was prepared by adding 17.5 μL of the D5-saquinavir and D6-indinavir stock solutions (0.4 mg/mL), 10 μL of the dibenzepine stock solution (1.5 mg/mL) and 65 μL of the 13C6-efavirenz stock solution (1 mg/mL) to 500 mL of 50% methanol in water (v/v).

Calibration standards and validation samples for the determination of all analytes in cell lysate were prepared in the cell extraction solution in a concentration range of 1–500 ng/mL.

Drug-free PBMCs were isolated from buffy coat as described before [9]. In summary, PBMCs from buffy coat were isolated using ficoll separation. After isolation the cells were washed twice with 10 mL of ice-cold PBS, after the last washing, the number of cells was counted and aliquots of 5×10^6 cells were centrifuged in eppendorf reaction vials for 10 min at $650 \times g$. The supernatant was aspirated and the cells were stored at −20 °C.

Clinical PBMC samples were obtained using BD vacutainer® CPT™ tubes, a single tube system for the collection of whole blood and the separation of mononuclear cells. Within 2 h of drawal, the tubes were centrifuged for 30 min at room temperature at $1500 \times g$. Thereafter, the cells were resuspended in plasma, transferred to a 15 mL volumetric tube and centrifuged for 10 min at 4 °C at $600 \times g$. The plasma was then aspirated off and the cell pellet was washed and centrifuged in ice-cold PBS, to prevent efflux of drugs from the cells [10], twice at 4 °C at $600 \times g$ before storage at −20 °C. Before the last washing step, the number of cells was counted.

2.2. Sample pre-treatment

For quantification, blank PBMC pellets were thawed and resuspended in 200 μL of the calibration standards or quality controls in the cell extraction solution. Clinical PBMC pellets were resuspended with 200 μL of the cell extraction solution only containing the internal standards. The resuspended pellets were subsequently sonicated for 10 min, centrifuged at $23{,}100 \times g$ and 180 μL of the supernatant was transferred to an autosampler vial with insert.

2.3. Analytical methods validation

2.3.1. Linearity

Calibration standards were prepared in duplicate for each run and analysed in three independent runs. Calibration curves (area ratio with the internal standard versus nominal concentration) were fitted by least-squares linear regression using $1/\text{concentration}^2$ as weighting factor. Deviations of the mean calculated concentrations over three runs should be within 85–115% of nominal concentrations for the non-zero calibration standards. At the lower limit of quantitation (LLQ) level a deviation of 20% was permitted.

2.3.2. Drug quantification accuracy and precision

Accuracy, intra- and inter-assay precision of the method were determined by assaying five replicates of each of the validation samples at the LLQ, low, mid and high concentration range in three separate runs. Accuracy was measured as the percentage of the concentration found as calculated with the calibration standards. The precision (relative standard deviation) should not exceed 15%, except for the LLQ, where 20% deviation was allowed.

2.3.3. Cell count precision

Intracellular concentrations were calculated using a previously determined volume of 0.4 pL for each PBMC [10]. As the number of cells is therefore in the denominator of the concentration fraction, the cell count should be accurate and precise. A cell suspension containing approximately 20×10^6 cells/mL PBS was prepared and stored at 4 °C. At time 0, 2 and 5 h a cell count was performed in sixfold. Precision was considered to be acceptable when it was found to be less than 5%, expressed as relative standard deviation. Relative accuracy (compared to the cell count at time 0 h) should not change more than ±5% over time.

2.3.4. Matrix effect

The number of cells in clinical PBMC samples vary from sample to sample due to natural variation in the amount of systemic circulating PBMCs. We therefore investigated the matrix effect of PBMC extract on analytical results in triplicate at three concentration levels in three different amounts of cells. Cell pellets containing either 3×10^6, 15×10^6 or 30×10^6 cells were prepared, a range covering the number of cells that can be recovered from a clinical sample. When at all concentration levels in every cell pellet the amount of recovered analyte was within 85–115% of nominal concentrations, the number of cells in the pellets was supposed not to influence the analytical results in the determined range.

2.3.5. Selectivity, specificity and carry-over

To ensure selectivity and specificity of the developed assay, six different blank batches of PBMCs, as well as six different blank PBMC batches spiked at the LLQ level were tested. Areas of peaks co-eluting with the analytes should not exceed 20% of the area at the LLQ level. The deviation of the nominal concentration for the LLQ samples should be within ±20%. Carry-over was assessed by the analysis of a blank sample after the analysis of a sample at the upper limit of quantitation (500 ng/mL).

Ion-suppression was assessed using a previously described post-column infusion experiment [7]. During post-column infusion of the analytes (at a concentration of 275 ng/mL) and internal standards at a concentration of 150 ng/mL of D5-saquinavir, D6-indinavir, 250 ng/mL dibenzepine and 1500 ng/mL 13C6-efavirenz, either a blank PBMC extract or eluent was injected into the column. In the elution window of the analyte no difference in signal should be observed between the chromatograms of the blank PBMC extract injection and the eluent injection, indicating no interference

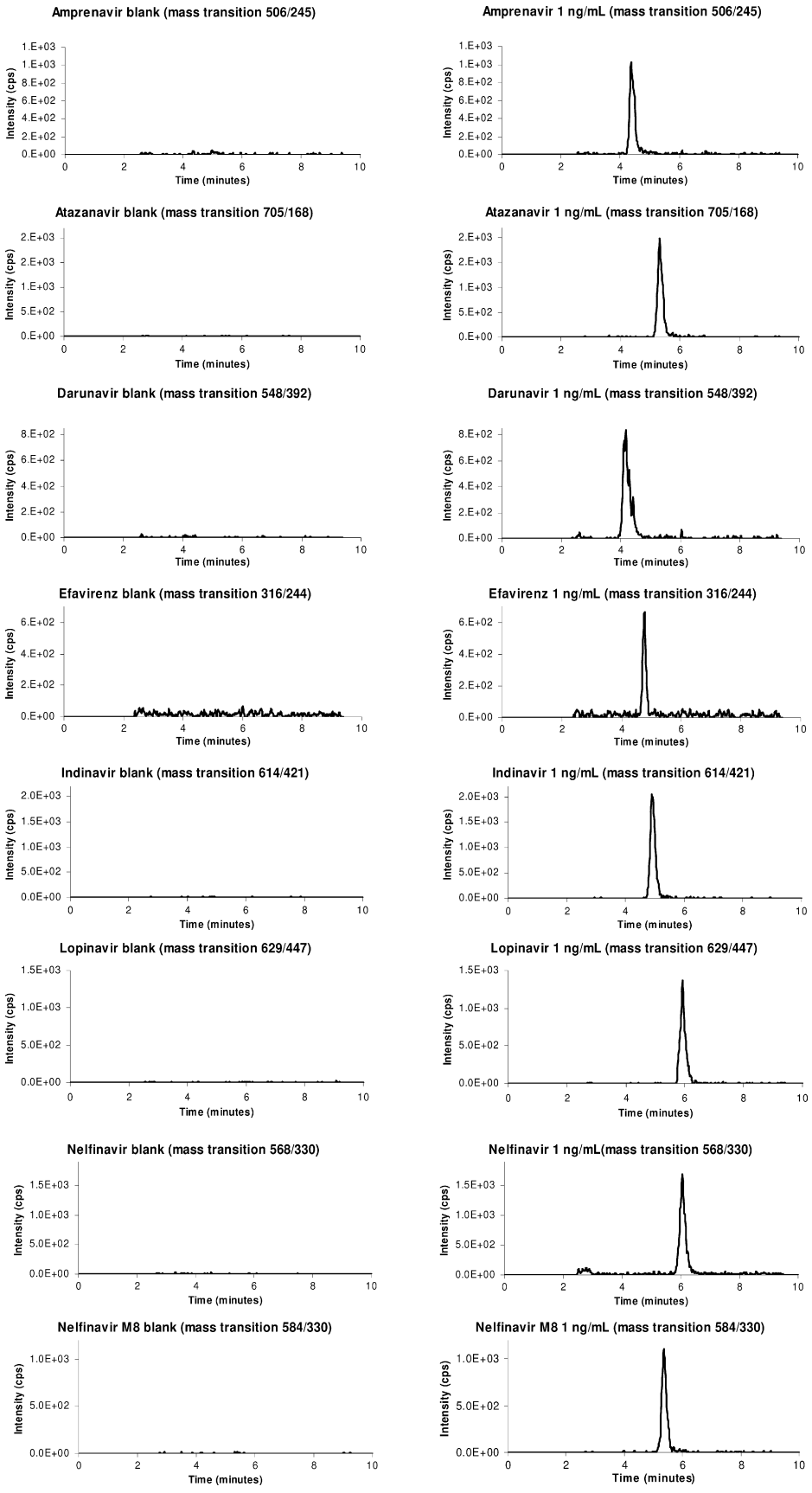

Fig. 1. Chromatograms of all analytes at the LLQ level and their respective blanks.

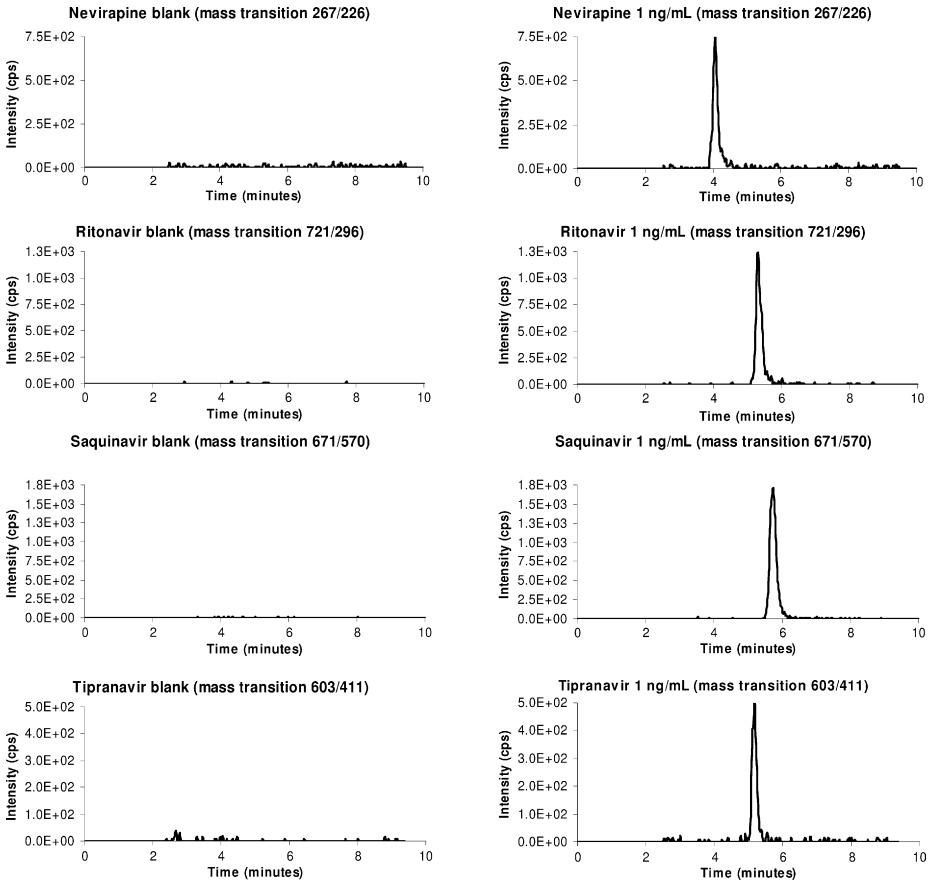

Fig. 1. (*Continued*).

of endogenous compounds from the PBMC lysate on the quantification.

2.3.6. Stability and re-injection reproducibility

Stability of the final extract and re-injection reproducibility were investigated at three concentration levels in five-fold after storage at 4 °C for 4 days. Stability was assured when 85–115% of the nominal concentration was found in the stored stability samples when compared with a freshly prepared calibration curve. The re-injection was considered to be reproducible when 85–115% of the nominal concentration was recovered.

3. Results and discussion

Previously, we have determined lopinavir in clinical PBMC samples by lysing the cell pellet with chloroform, subsequent evaporation of the chloroform and dilution of the lysate in 100 μL of human plasma. The sample pre-treatment then consisted of protein precipitation and subsequent dilution of the supernatant, resulting in a six-fold dilution of the sample. For quantification, 10 μL of the plasma extract was then injected into the column [11]. We have increased the sensitivity of the method almost a ten-fold by performing a direct extraction of the cell pellets using 200 μL of extraction solution and by increasing the injection volume to 30 μL. However, using the new sample pre-treatment and the increased injection volume we observed a gradual increase in pressure on the chromatographic system. This was traced back to clogging of the pre-column, probably because of precipitation of proteins left in the PBMC-lysate, due to a sudden increase in the amount of modifier in the eluent after injection. This phenomenon was not previously observed during analysis of plasma or dried blood spot extracts and the clogging could not be removed by rinsing the pre-column with methanol or tetrahydrofuran. This finding stresses the importance to use a pre-column and to replace it when necessary. Our upper chromatographic pressure limit was 250 bar and we replaced the pre-column approximately every 100 injections.

3.1. Chromatograms

Chromatograms of all analytes at the LLQ level and their respective blanks are depicted in Fig. 1. As observed, the used chromatographic setup allows simultaneous determination of a wide range of different analytes and all analytes show sufficient response at a concentration of 1 ng/mL in PBMC lysate (of 5×10^6 cells per 200 μL) for quantification.

3.2. Validation results

3.2.1. Linearity

The assay was linear over the tested concentration range of 1–500 ng/mL for all analytes.

3.2.2. Accuracy and precision

Assay performance data for all analytes are summarised in Table 2. Intra- and inter-assay accuracies are defined in terms of deviation from nominal concentration with a precision in terms of relative standard deviation, all determined at four concentration levels. The intra-assay accuracy was within all predefined limits at all concentration levels in all matrices. As observed, the mean precision and accuracy was less than ±12% at all concentration levels.

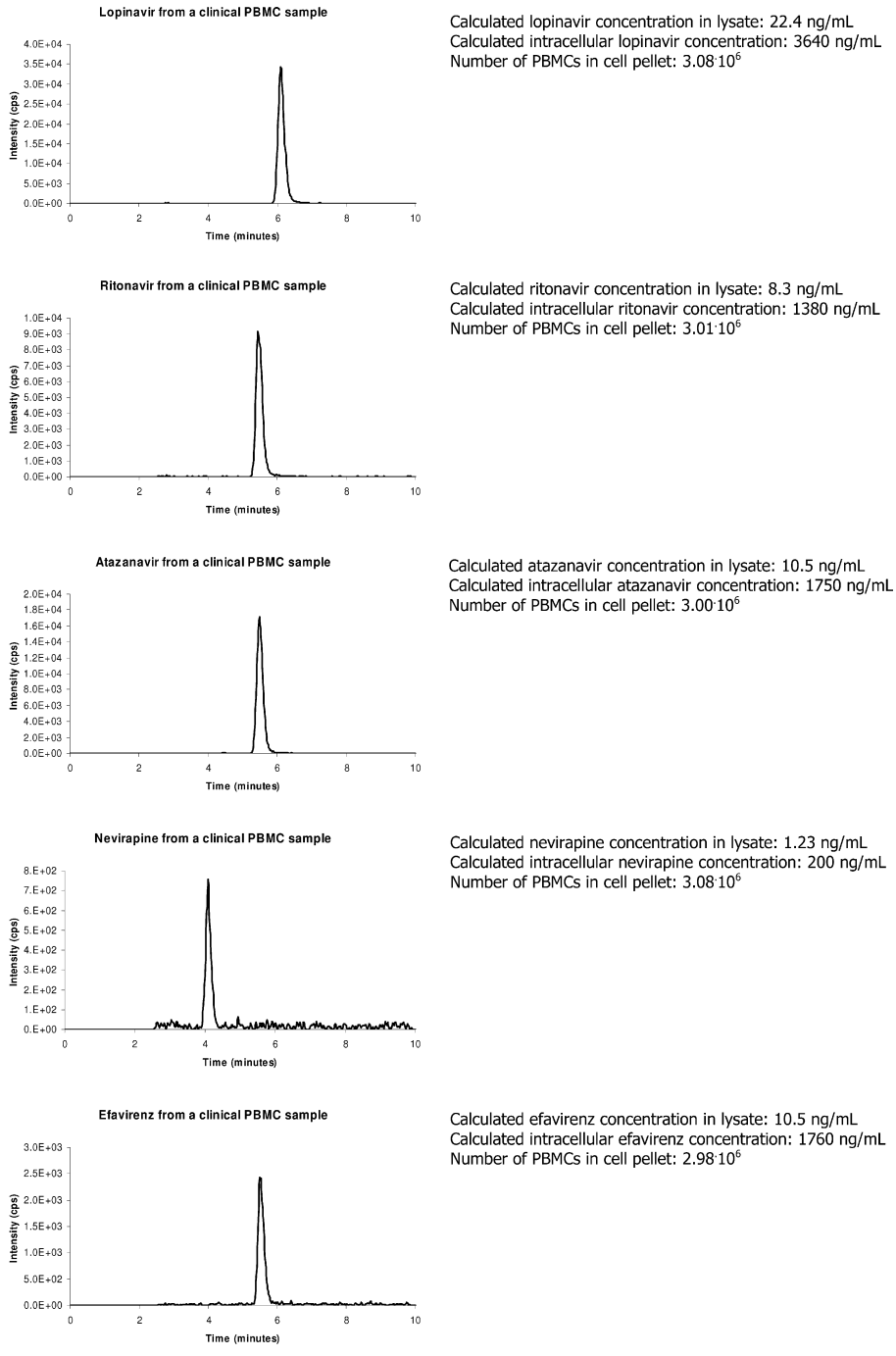

Fig. 2. Chromatograms of clinical samples. (Panel 1) Calculated lopinavir concentration in lysate: 22.4 ng/mL. Calculated intracellular lopinavir concentration: 3640 ng/mL. Number of PBMCs in cell pellet: 3.08×10^6. (Panel 2) Calculated ritonavir concentration in lysate: 8.3 ng/mL. Calculated intracellular ritonavir concentration: 1380 ng/mL. Number of PBMCs in cell pellet: 3.01×10^6. (Panel 3) Calculated atazanavir concentration in lysate: 10.5 ng/mL. Calculated intracellular atazanavir concentration: 1750 ng/mL. Number of PBMCs in cell pellet: 3.00×10^6. (Panel 4) Calculated nevirapine concentration in lysate: 1.23 ng/mL. Calculated intracellular nevirapine concentration: 200 ng/mL. Number of PBMCs in cell pellet: 3.08×10^6. (Panel 5) Calculated efavirenz concentration in lysate: 10.5 ng/mL. Calculated intracellular efavirenz concentration: 1760 ng/mL. Number of PBMCs in cell pellet: 2.98×10^6.

3.2.3. Cell count precision

The cell count precision was always less than 3% and the number of cells did not change more than 1% within a 5 h time interval. Therefore, an accurate cell count could be performed up to 5 h after isolation of PBMCs.

3.2.4. Matrix effect

All recovered concentrations were within 85–115% of nominal concentrations. Therefore, in the range of 3×10^6–30×10^6 cells, all clinical samples, calibration standards and quality control samples can be treated with an equal amount of cell extraction solution.

3.2.5. Selectivity, specificity, carry-over and ion-suppression

No interfering peaks co-eluting with the analytes were observed in the six different batches of blank PBMCs, neither did the recovered concentrations of the samples spiked at the LLQ level in the different batches of PBMCs exceed the 80–120% accuracy limits. The carry over was less than 20% for all analytes.

Table 2
Assay performance ($n = 5$ in three separate runs).

Nominal concentration (ng/mL)	Accuracy (%)	Mean intra-assay precision (%)	Inter-assay precision (%)
Amprenavir			
1	−7.3	6.6	11.3
3	−11.1	5.8	8.0
275	−7.2	2.3	4.8
400	−2.5	3.4	3.9
Atazanavir			
1	−8.2	4.3	5.5
3	−6.9	6.9	7.9
275	−7.8	2.9	3.7
400	−5.5	3.0	4.4
Darunavir			
1	−8.2	9.4	10.1
3	−3.4	4.7	3.4
275	−1.2	3.0	2.4
400	−0.9	3.7	2.5
Efavirenz			
1	−8.0	9.9	9.4
3	−10.1	7.1	7.7
275	−8.6	2.7	3.0
400	−10.6	3.0	3.9
Indinavir			
1	−1.5	4.1	4.9
3	−3.2	3.4	3.2
275	−1.7	1.9	1.8
400	−1.1	2.0	2.3
Lopinavir			
1	−4.8	5.1	7.2
3	−0.9	6.7	9.5
275	−0.4	3.6	3.8
400	−0.1	1.5	4.7
Nelfinavir			
1	−3.3	5.1	5.8
3	2.0	5.1	7.2
275	−1.3	2.3	3.1
400	−1.7	2.4	3.4
Nelfinavir M8			
1	4.3	7.6	12
3	7.5	6.3	6.3
275	2.1	3.3	5.4
400	−1.0	2.5	4.0
Nevirapine			
1	−6.0	9.4	9.6
3	−6.9	2.8	4.2
275	−5.5	2.0	3.0
400	−8.5	1.5	5.2
Ritonavir			
1	−3.3	6.9	9.2
3	−3.7	4.9	5.7
275	−5.9	2.6	2.8
400	−2.4	2.5	4.3
Saquinavir			
1	1.9	4.5	4.7
3	−1.6	2.1	2.8
275	−0.4	2.2	2.1
400	−0.1	1.8	2.3
Tipranavir			
1	−6.3	6.9	11.0
3	−4.1	11.2	12.3
275	−3.2	6.5	6.9
400	1.8	3.3	4.9

Ion-suppression or ion enhancement was typically observed 2–3 min after injection, but never at the time of elution of the analyte or internal standard. Therefore, ion-suppression or enhancement was not supposed to influence the analysis.

3.2.6. Stability and re-injection reproducibility

Re-injection reproducibility and stability of the final was assured for 4 days, when samples were stored at 4 °C.

3.3. Chromatograms of clinical samples

Fig. 2 shows chromatograms, the measured concentration in the lysate and the calculated cell-associated concentration of clinical samples of aliquots of approximately 3×10^6 PBMCs containing lopinavir, ritonavir, atazanavir, nevirapine and efavirenz, showing the applicability of the method and the adequacy of the chosen calibration range for these analytes. Clinical samples containing other analytes were not available at the time of validation.

4. Conclusion

We have successfully validated a quantitative assay for the determination of PIs and NNRTIs in PBMC lysate, showing the applicability of the same LC-MS/MS method across three matrices (plasma, dried blood spots and PBMC lysate). Using a rapid extraction procedure and a runtime of only 10 min, a total of 11 antiretroviral agents and one active metabolite could be quantified from PBMC lysate. Previously, some work has been performed establishing the relationship between plasma and intracellular concentrations for several antiretroviral drugs [11–15]. Using our method, the relationship between plasma and cellular pharmacokinetics for all other PIs and NNRTIs drugs can now be established.

References

[1] B. Chandler, M. Detsika, A. Owen, S. Evans, R.C. Hartkoorn, P.A. Cane, D.J. Back, S.H. Khoo, Antivir. Ther. 12 (2007) 831.
[2] O. Janneh, A. Owen, B. Chandler, R.C. Hartkoorn, C.A. Hart, P.G. Bray, S.A. Ward, D.J. Back, S.H. Khoo, AIDS 19 (2005) 2097.
[3] O. Janneh, E. Jones, B. Chandler, A. Owen, S.H. Khoo, J. Antimicrob. Chemother. 60 (2007) 987.
[4] K.M. Crommentuyn, A.D. Huitema, J.H. Beijnen, J. Pharm. Biomed. Anal. 38 (2005) 139.
[5] M. Jemal, S. Rao, M. Gatz, D. Whigan, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 795 (2003) 273.
[6] S. Colombo, A. Beguin, A. Telenti, J. Biollaz, T. Buclin, B. Rochat, L.A. Decosterd, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 819 (2005) 259.
[7] R. ter Heine, C.G. Alderden-Los, H. Rosing, M.J. Hillebrand, E.C. van Gorp, A.D. Huitema, J.H. Beijnen, Rapid Commun. Mass Spectrom. 21 (2007) 2505.
[8] R. ter Heine, H. Rosing, E.C. van Gorp, J.W. Mulder, W.A. van der Steeg, J.H. Beijnen, A.D. Huitema, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 867 (2008) 205.
[9] S.A. Veltkamp, M.J. Hillebrand, H. Rosing, R.S. Jansen, E.R. Wickremsinhe, E.J. Perkins, J.H. Schellens, J.H. Beijnen, J. Mass Spectrom. 41 (2006) 1633.
[10] S.H. Khoo, P.G. Hoggard, I. Williams, E.R. Meaden, P. Newton, E.G. Wilkins, A. Smith, J.F. Tjia, J. Lloyd, K. Jones, N. Beeching, P. Carey, B. Peters, D.J. Back, Antimicrob. Agents Chemother. 46 (2002) 3228.
[11] K.M. Crommentuyn, J.W. Mulder, A.T. Mairuhu, E.C. van Gorp, P.L. Meenhorst, A.D. Huitema, J.H. Beijnen, Antivir. Ther. 9 (2004) 779.
[12] S. Colombo, A. Telenti, T. Buclin, H. Furrer, B.L. Lee, J. Biollaz, L.A. Decosterd, Ther. Drug Monit. 28 (2006) 332.
[13] J. Ford, M. Boffito, A. Wildfire, A. Hill, D. Back, S. Khoo, M. Nelson, G. Moyle, B. Gazzard, A. Pozniak, Antimicrob. Agents Chemother. 48 (2004) 2388.
[14] J. Ford, D. Cornforth, P.G. Hoggard, Z. Cuthbertson, E.R. Meaden, I. Williams, M. Johnson, E. Daniels, P. Hsyu, D.J. Back, S.H. Khoo, Antivir. Ther. 9 (2004) 77.
[15] J. Ford, M. Boffito, D. Maitland, A. Hill, D. Back, S. Khoo, M. Nelson, G. Moyle, B. Gazzard, A. Pozniak, J. Antimicrob. Chemother. 58 (2006) 1009.