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Monitoring of lopinavir and ritonavir in peripheral blood mononuclear cells, plasma, and ultrafiltrate using a selective and highly sensitive LC/MS/MS assay

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Abstract

For the determination of the HIV protease inhibitors lopinavir and ritonavir in human plasma, plasma ultrafiltrate, and peripheral blood mononuclear cells (PBMCs) a highly sensitive and selective method has been developed, validated, and applied to samples of a healthy volunteer. BD Vacutainer® CPT™ and Amicon Centriplus® centrifugal filter devices were used for separation of PBMCs and for ultrafiltrate generation, respectively. After liquid/liquid-extraction extracts were chromatographed isocratically within 6 min on a Jupiter Proteo column. The drugs were quantified using $^{2}H_{5}$-saquinavir as internal standard and electrospray tandem mass spectrometry in the selected reaction monitoring mode. Limits of quantification for both analytes were 4.0 ng/mL in plasma, 0.2 ng/mL in ultrafiltrate, and 0.1 ng/cell pellet ($\sim 3 \times 10^{6}$ cells) in PBMCs. The calibration ranges were linear over more than three logs with an over-all accuracy varying between 98.7% and 111.5% and an over-all precision ranging from 6.2% to 14.0% (SD batch-to-batch). After a regular oral dose of Kaletra® (400 mg lopinavir, 100 mg ritonavir) analyte concentrations were detectable over a full dosing interval in plasma, ultrafiltrate, and PBMCs. The method is well suited for monitoring of free and total plasma, and intracellular lopinavir/ritonavir concentrations in samples from clinical trials.

Keywords: Kaletra®; Ritonavir; Lopinavir; PBMC; Protein binding; LC/MS/MS

1. Introduction

Lopinavir and ritonavir are antiretroviral drugs acting as inhibitors of human immunodeficiency virus (HIV) protease, a class of drugs that has markedly improved morbidity and mortality of HIV infected patients [1]. Within the highly active antiretroviral therapy (HAART) they are used in a fixed combination (33 mg ritonavir + 133 mg lopinavir) called Kaletra® with ritonavir added to boost lopinavir by inhibition of cytochrome P450 (CYP) CYP3A isozymes and active transport by P-glycoprotein [2,3]. Even though HAART has markedly improved the clinical outcome of HIV-infected patients, virological treatment failure often occurs already within the first year of therapy [4,5]. Evidence is growing that suboptimal drug concentrations at the site of virus replication (e.g. within $CD4^{+}$ cells) will confer selective pressure to the virus which ultimately results in the emergence of mutant viral isolates with reduced susceptibility to antiviral drugs [4,6]. Conversely high drug levels are linked to toxicity and increased rates of adverse events, which may limit treatment adherence.

Like ritonavir and lopinavir many of the protease inhibitors are substrates and inhibitors of CYP3A and P-glycoprotein and have the potential to mutually modify the pharmacokinetics. Therefore therapeutic drug monitoring (TDM) of protease inhibitor concentrations is a valuable tool to improve efficacy in children [7] and in treatment-naïve patients [8] as well as in treatment-experienced patients [9,10]. TDM is also well established in HIV positive patients to detect and consider factors limiting optimum therapeutic response like non-compliance [11] and large pharmacokinetic variability, often caused by drug interactions [5,12]. However, the value of (total) plasma concentration monitoring may be limited due to the large interindividual differences in protein binding leading to inter-patient variability of up to 48% in the free (active) fraction of the protease

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inhibitors. Moreover, TDM of total protease inhibitor concentrations has been increasing in the past few years; the pharmacological activity of protease inhibitors is dependent on unbound drug entering infected cells [13,14]. Because the leukocyte membrane is an actively regulated barrier between plasma and target site, plasma concentration monitoring may not closely reflect drug concentrations reaching the site of action [15]. Hence TDM of protease inhibitors within leukocytes or even their subfractions like $CD4^+$ cells might have distinct advantages over the determination of plasma concentration.

With increasing evidence for TDM of protease inhibitors methods depending on liquid chromatography (HPLC) were developed to quantify these drugs in plasma more and more precise. Whereas plasma concentrations are high enough to analyse the drugs using UV detection, determinations in other matrices (e.g. blood cells, cerebrospinal fluid, plasma ultrafiltrate, sanctuary sites) were mainly processed using HPLC coupled to mass spectrometry (LC/MS) or tandem mass spectrometry (LC/MS/MS) [15–19]. These methods are highly specific and sensitive and allow quantifications of HIV drugs below 1 ng/mL in fluids, respectively below 1 ng/cell pellet. Beside the LC/MS determination cell isolation procedures have to be developed mainly depending on ficoll density gradient centrifugation [15–19].

For the purpose of lopinavir and ritonavir drug monitoring in samples from clinical studies we established cell isolation procedures and developed analytical methods to quantify the protease inhibitors in plasma, plasma ultrafiltrate (determination of unbound drug concentration), and peripheral blood mononuclear cells (PBMCs). The in vivo application is demonstrated by the data of a single individual from a clinical study in healthy participants. We confirmed the feasibility and investigated the relationship between total plasma concentration, free plasma concentration, and intracellular concentration over a full dosing interval.

2. Participants, materials, and methods

2.1. Clinical study

The study protocol was approved by the Ethics Committee of the Medical Faculty of Heidelberg and was conducted at the Department of Internal Medicine VI, Clinical Pharmacology and Pharmacoepidemiology in accordance with good clinical practice guidelines, the Declaration of Helsinki, and German legal requirements.

After obtaining written informed consent from the healthy volunteer, three capsules of Kaletra® were orally administered (total dose 400 mg lopinavir/100 mg ritonavir) after a fasting period of 12 h.

2.2. Materials

Kaletra® capsules were purchased from Abbott (Mannheim, Germany). Lopinavir and ritonavir reference standards were kindly supplied by Abbott and the internal standard $^2H_5$-saquinavir mesylate (RO 31-8959/048) by Roche Products (Hertfordshire, UK). Vacutainer®CPT™ were obtained from Becton Dickinson (Heidelberg, Germany). Centriplus®Centrifugal Filter Devices YM-30 (cut off 30 kDa) were obtained from Amicon Bioseparations, Millipore Corporation (Bedford, USA). Phosphate buffered saline (PBS), Hanks' balanced salt solution (HBSS), 1 m Hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, and trypan blue were from Gibco (Auckland, New Zealand), and Ficoll-Paque™PLUS from Amersham Biosciences AB (Uppsala, Sweden). All other reagents and solvents used for chromatographic, spectroscopic, and sample preparation were of analytical or higher quality and were purchased from E. Merck (Darmstadt, Germany). Water was deionised and filtered by an HP 6UV/UF TKA system (TKA, Niederelbert, Germany).

2.3. Human blood samples

Before and 1, 2, 4, 6, 8, 10, and 12 h after dosing blood samples (4.5 mL) were drawn and immediately centrifuged ($3000 \times g$ for 15 min at 4 °C). The plasma was stored at −20 °C until analysis. Additional whole blood samples (4×8 mL for quadruplicate determination) were taken 2, 4, 6, and 12 h after administration in BD Vacutainer® CPT™ for intracellular drug determination and centrifuged within 20 min after drawing ($1700 \times g$, 20 min, ambient temperature). Plasma and PBMCs were decanted into a falkon tube and centrifuged for 5 min at $1700 \times g$ and 4 °C. Subsequently plasma was removed and PBMCs were re-suspended in ice cold HHBSS (HBSS supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) and centrifuged again (6 min, $700 \times g$, 4 °C). As suggested in earlier studies [15,16] this washing step was repeated twice to remove remaining and weakly adsorbed drug from the cells. During the last washing step 10 μL of each sample were taken and stained with trypan blue for cell counting. Washed cell pellets were stored at −20 °C until analysis.

For calibration and quality control (QC) samples, blank PBMCs were isolated from buffy coats obtained from the local blood bank and were diluted with PBS (1:1). Two parts of the mixture were carefully transferred onto one part of Ficoll-Paque™PLUS and centrifuged for 20 min ($700 \times g$) at ambient temperature. The supernatant was pipetted carefully into a fresh centrifuge tube, PBS was added, and the samples were centrifuged at $700 \times g$ for 5 min. After removing the supernatant, the pellet was re-suspended in PBS and centrifuged again ($700 \times g$, 5 min, ambient temperature) to wash off residual plasma and Ficoll-Paque™PLUS. After removal of the supernatant, cell density was adjusted to about 3×10^6 cells/mL by diluting with PBS and aliquotation into centrifuge tubes (about 3×10^6 cells each). After a last centrifugation step (5 min, $700 \times g$, 4 °C) and removal of the supernatant, cell pellets were stored at −20 °C until analysis.

Determination of unbound drug was performed by ultrafiltration. Amicon® Centriplus Centrifugal Filter Devices® were washed twice by addition of 3 mL of deionised water (about 35 °C) and centrifugation (15 min, $3000 \times g$, 30 °C) and subsequently dried by centrifugation (30 min, $3000 \times g$, 30 °C).

Plasma samples (1.5 mL) were pipetted into washed and dried filter devices and centrifuged (1 h, 3000 × *g*, 30 °C).

2.4. Standard solutions

The internal standard $^{2}H_5$-saquinavir-mesylate (RO 31-8959/048) was weighed (5.26 mg) into a volumetric flask (50 mL) and filled up with acetonitrile/water (1/1, v/v). From this stock solution an aliquot (183 μL) was diluted to a final volume of 20 mL (acetonitrile/water).

For calibration about 5 mg of drugs (4.58 mg lopinavir, 5.42 mg ritonavir) were weighed into 2 mL volumetric flasks and were filled up with acetonitrile/water. From these stock solutions the highest calibration solution was prepared by transferring 44 μL of lopinavir stock solution and 37 μL of ritonavir stock solution into a 10 mL volumetric flask and filled up with acetonitrile/water. All other calibration solutions were prepared by diluting this solution with acetonitrile/water.

For QC, standard stock solutions were prepared as described for calibration. Drug amounts weighed for standard solutions were 3.72 mg for lopinavir and 4.32 mg for ritonavir. From these solutions a QC stock solution was prepared in the same way as the highest calibration solution. QC solutions in three concentrations were prepared by diluting this QC stock solution.

2.4.1. Calibration samples

Blank plasma (25 μL) was spiked with 25 μL of respective calibration solutions, yielding plasma concentrations of 4.00, 40.0, 300, 925, 3500, 6750, and 10,000 ng/mL. Blank ultrafiltrate (500 μL) was also spiked with 25 μL of calibration solutions, yielding concentrations of 0.20, 2.00, 15.0, 46.3, 175, 338, and 500 ng/mL. For calibration of intracellular measurements, blank PBMCs were spiked with 25 μL of calibration solutions resulting in total drug amounts of 0.10, 1.00, 7.50, 23.1, 87.5, 169, and 250 ng/3 × 10^6 cells.

2.4.2. QC samples

QC samples were prepared as described for calibration samples. The concentrations represented in plasma were 13.0, 2843, and 5483 ng/mL for lopinavir and 12.8, 2790, and 5380 ng/mL for ritonavir. In ultrafiltrate the QC concentrations were 1.64, 114, and 222 ng/mL for ritonavir and 1.49, 104, and 201 ng/mL for lopinavir. Cell QC samples contained 0.32, 69.7, and 135 ng/3 × 10^6 cells of ritonavir and 0.33, 71.1, and 137 ng/3 × 10^6 cells of lopinavir.

2.5. Extraction procedures

Calibration and QC samples as well as study cell pellet (mean $9.9 \times 10^6 \pm 3.1 \times 10^6$ PBMCs), plasma (25 μL), and ultrafiltrate (500 μL) samples were spiked with internal standard solution (25 μL). Subsequently the samples were alkalinised (400 μL of 2 mM K_3PO_4), *tert*-butylmethylether (5 mL) was added, and the samples were shaken automatically (15 min) as previously described [20]. Cell pellets were additionally treated by ultrasonication (15 min) after alkalinisation to ensure complete cell lysis. After centrifugation (10 min, 2000 × *g*, 10 °C) the organic layers were separated and evaporated to dryness under a stream of nitrogen (40 °C). The extracts were reconstituted by adding LC mobile phase (200 μL) and ultrasonication (15 min). Forty microlitres were injected into the LC/MS/MS system.

2.6. Instrumental analysis parameters

The LC/MS/MS system (Thermo Electron, Dreieich, Germany) consisted of a quaternary LC pump (Model P4000), an autosampler (Gilson Abimed 232 Bio, Abimed GmbH, Langenfeld, Germany) and a triple stage quadrupole mass spectrometer (Model TSQ 7000 with API-2 ion source and performance kit). For isocratic chromatographic separation at 40 °C a Phenomenex® Jupiter™ Proteo column (C12, 90 A, 4 μm, 100 mm × 2 mm I.D., Phenomenex®, Aschaffenburg, Germany) with integrated guard column was used. The isocratic eluent (45% A/55% B) consisted of 0.1 vol.% aqueous acetic acid including 20 mM ammonium acetate (A) and acetonitrile (B). The flow rate was 0.35 mL/min and was introduced without splitting into the electrospray ion source (ESI) of the mass spectrometer. ESI interface parameters were as follows: middle position, spray voltage 4.5 kV, sheath gas (N_2) 90 psi, aux gas (N_2) 20 scales, capillary heater temperature 350 °C. The mass spectrometer was tuned automatically using a solution of myoglobin and Xcalibur 1.2 MS system software standard procedures. The voltages responsible for the ion beam focus (e.g. heated capillary, skimmer lens, etc.) were optimised during continuous delivery of standard solution into the LC eluent via a syringe pump and the intensity of base peak was monitored and adjusted to maximum. Selected reaction monitoring measurements were performed at 1.6 kV multiplier voltage. MS/MS transitions monitored in the positive ion mode were *m/z* 629.5 → *m/z* 447.2 at 20 V for lopinavir, *m/z* 721.4 → *m/z* 296.2 at 24 V for ritonavir and *m/z* 676.4 → *m/z* 575.3 at 36 V for $^{2}H_5$-saquinavir. The parameters influencing these transitions were optimised: the Ar pressure in the collision quadrupole was set to 2.2 mbar and the offset voltage was adjusted for each drug.

2.7. Evaluation of the analytical methods

Analytical method validation for plasma, ultrafiltrate and PBMCs was performed in three analytical batches according to the recommendations published by the U.S. Food and Drug Administration (FDA) [21,22].

Accuracy was calculated as the ratio of the measurements averaged for individual batches divided by the nominal value and expressed in percent. Precision was defined as the ratio of standard deviation and mean calculated value in percent. These values are given within-batch and batch-to-batch. For this purpose validation batches ($n = 3$) each containing eight calibration samples and 18 quality control samples at three different concentrations were analysed. Accuracy and precision at the LOQ level was determined. From these values accuracy and precision of the method were calculated. Additional analytical batches ($n = 7$ plasma, $n = 3$ ultrafiltrate, $n = 11$ PBMCs) for quantification of plasma, ultrafiltrate, and PBMC samples from a pharmacoki-

Table 1
Summary of validation results for PBMCs

	Nominal analyte amount (ng/3 × 10^6 cells)	Lopinavir			Ritonavir		
		0.33	71.1	137	0.32	69.7	135
Within batch							
	Mean (ng/3 × 10^6)	0.37	80.5	146	0.36	81.5	142
1	Accuracy (%)	114.6	113.3	106.5	111.4	116.8	105.5
	Precision (%CV)	4.5	6.1	8.4	6.6	4.9	7.8
	Mean (ng/3 × 10^6)	0.33	80.3	144	0.32	81.1	149
2	Accuracy (%)	101.2	112.9	105.4	99.7	116.3	111.1
	Precision (%CV)	15.5	5.0	7.4	10	3.8	7.0
	Mean (ng/3 × 10^6)	0.34	67.3	135	0.30	67.8	140
3	Accuracy (%)	103.7	94.7	98.4	95.2	97.2	103.7
	Precision (%CV)	7.2	11.8	3.7	10.2	11.9	12.1
Batch to batch							
	Mean (ng/3 × 10^6)	0.35	75.7	142	0.33	75.5	143
	Accuracy (%)	106.1	106.6	103.4	102.5	108.2	106.5
	Precision (%CV)	11.1	11.3	7.3	10.7	11.8	9.2

netic study with Kaletra® included calibration samples, six quality control samples at three different concentrations, and different numbers of blank samples and provided further data on overall accuracy and precision. Extraction recovery rates for lopinavir, ritonavir and the internal standard were calculated for the matrices plasma, ultrafiltrate, and PBMCs within the validation procedure in double determination at two concentrations. Therefore resulting peak area of the respective compounds after extraction from the respective matrix was compared to the peak area of a pure solution containing the 100% amount. Selectivity for each matrix was measured according to the FDA Guideline [21] using blank matrix from six different individuals. These samples were processed according to the method described without addition of analytes and internal standard. No signals at the analytes retention times shall appear. Stability of the drugs was tested in three freeze-and-thaw cycles using ultrafiltrate matrix and the accuracies for both analytes were calculated. Influences of ion suppression onto the electrospray ionisation were monitored by analysing changes in the ion spray current. This parameter depends on the conductivity over the spray and can be influenced by charged or uncharged matrix compounds in both directions.

2.8. Calculations and statistical methods

Calibration curves were determined for both drugs using the respective calibration samples for each matrix. Peak area ratios of analytes and internal standards were calculated and weighted linear regressions ($1/x$) were performed for each analytical batch. For these procedures Thermo Finnigan software LCQuan 1.2 was used.

Data are expressed as mean values ± standard deviation (SD). Intracellular drug amounts were expressed as amount ng/3 × 10^6 cells. Calculation of intracellular drug concentrations for comparison to plasma concentrations a cell volume of 0.4 pL for a single cell was assumed [23] and drug concen-

Table 2
Summary of validation results for plasma

	Nominal analyte concentrations (ng/mL)	Lopinavir			Ritonavir		
		13.0	2843	5483	12.8	2790	5380
Within batch							
	Mean (ng/mL)	14.6	2995	4783	13.3	2897	4795
1	Accuracy (%)	112.2	105.4	87.2	104.6	103.8	89.1
	Precision (%CV)	5.4	11.8	9.3	6.7	9.3	7.0
	Mean (ng/mL)	13.4	3108	5711	13.0	3105	5742
2	Accuracy (%)	103.3	109.3	104.2	100	109.2	104.7
	Precision (%CV)	18.1	6.2	10.7	7.0	6.8	5.7
	Mean (ng/mL)	14.0	3318	5676	12.4	3370	6074
3	Accuracy (%)	107.7	116.7	103.5	95.1	118.5	110.8
	Precision (%CV)	10.0	2.2	10.0	11.8	3.1	9.0
Batch to batch							
	Mean (ng/mL)	14.1	3131	5410	12.9	3109	5506
	Accuracy (%)	108.3	110.1	98.7	101.1	111.5	102.3
	Precision (%CV)	10.7	8.6	12.4	8.8	9.1	12.2

Table 3
Summary of validation results for ultrafiltrate (free concentration)

	Nominal analyte concentrations (ng/mL)	Lopinavir			Ritonavir		
		1.49	104	201	1.64	115	222
Within batch							
	Mean (ng/mL)	1.66	115	223	1.60	121	229
1	Accuracy (%)	111.2	110.5	111.1	97.5	104.9	101.6
	Precision (%CV)	19.3	6.2	12.7	12.3	2.7	7.5
	Mean (ng/mL)	1.49	113	211	1.70	121	230
2	Accuracy (%)	100.3	108.8	104.8	103.7	105.0	103.5
	Precision (%CV)	10.1	5.0	10.4	7.1	6.0	8.2
	Mean (ng/mL)	1.56	110	212	1.62	117	217
3	Accuracy (%)	104.9	106.1	105.7	98.9	102.1	97.8
	Precision (%CV)	9.5	5.1	8.2	8.2	4.5	5.5
Batch to batch							
	Mean (ng/mL)	1.57	113	215	1.64	120	225
	Accuracy (%)	105.4	108.5	107.2	100.0	104.0	101.6
	Precision (%CV)	14.0	6.2	10.4	9.2	4.5	7.5

trations were calculated using the following equation: measured drug amount/(cell number × cell volume).

Pharmacokinetic parameters were calculated with WinNonlin Version 4.1 (Pharsight Corporation, Mountain View, CA, USA).

3. Results and discussion

3.1. Performance and validation of the laboratory methods

PBMC collection and isolation using BD Vacutainer®CPT™ is easy, fast, and with regard to the risk of infection a safe way compared to classical Ficoll isolation. Extracted cell pellets of the volunteer contained a mean of $8.3 \times 10^6 \pm 3.3 \times 10^6$ PBMCs/sample. Using this extraction procedure the number of PBMCs was sufficient to quantify drug concentrations for lopinavir and ritonavir during a full twelve hour dosing interval via the described LC/MS/MS assay.

All drug concentrations measured were within the calibration range (0.1–250 ng/3×10^6 cells, 4.0–10,000 ng/mL plasma, and 0.2–500 ng/ml ultrafiltrate) and could be analysed with appropriate precision and accuracy (Tables 1–3). Limit of quantification (LOQ) for both analytes in PBMCs was 0.1 ng/3×10^6 cells with an accuracy of 105% for lopinavir and 96% for ritonavir. Because a small blood volume of 8 mL yielded a sufficient number of PBMCs for analysis, this method allows repeated measurements even in children or within pharmacokinetic studies. The LOQ in plasma was 4.0 ng/mL (0.2 ng/mL in ultrafiltrate) for both analytes. Accuracies at the LOQ value were 105% (91%) for lopinavir and 96% (90%) for ritonavir.

Liquid–liquid extraction with *tert*-butylmethylether was chosen for a simple and fast sample preparation. Drug recovery rates were determined in double determinations for lopinavir (ritonavir) at concentration levels of QC B (middle concentration range) and QC C (high concentration range). In PBMCs the recoveries were 86.3% (93.2%) and 102.7% (100.8%), in

Table 4
Summary of QC results from running study batches

QC nominal amount (ng/3×10^6 cells)	Lopinavir			Ritonavir		
	0.42	92.1	178	0.36	79.5	153
PBMCs						
Mean ± SD (ng/3×10^6)	0.43 ± 0.04	91.9 ± 9.8	170 ± 20.1	0.37 ± 0.03	82.4 ± 5.4	152 ± 12
Accuracy (%)	102.6	99.9	95.7	102.8	103.7	99.0
Precision CV (%)	10.1	10.6	11.8	9.1	6.6	7.9
Plasma						
QC nominal concentration (ng/mL)	16.8	3682	7102	14.5	3179	6131
Mean ± SD (ng/mL)	17.8 ± 1.9	3739 ± 174	6871 ± 681	15.5 ± 1.6	3330 ± 221	6378 ± 603
Accuracy (%)	105.7	101.6	96.8	107.0	104.8	104.0
Precision CV (%)	10.8	4.6	9.9	10.4	6.7	9.5
Ultrafiltrate						
QC nominal concentration (ng/mL)	0.84	184	355	0.73	159	307
Mean ± SD (ng/mL)	0.85 ± 0.09	186 ± 25.3	322 ± 59.4	0.68 ± 0.22	160 ± 36.3	300 ± 41.9
Accuracy (%)	100.7	101.3	90.6	93.7	100.8	97.9
Precision CV (%)	10.5	13.6	18.5	32.1	22.7	14.0

plasma they were 88.0% (77.8%) and 74.1% (70.5%), and in ultrafiltrate they were 84.5% (81.6%) and 85.1% (85.0%). The recovery rate of the internal standard was 99.0% in PBMCs, 80.8% in plasma, and 53.8% in ultrafiltrate.

Three freeze and thaw cycles in ultrafiltrate at 104 ng/mL (lopinavir) and 115 ng/mL (ritonavir) were performed and no significant concentration decrease could be observed. The mean accuracy was 105.8 ± 7.8 ng/mL for lopinavir and 118.7 ± 7.2 ng/mL for ritonavir.

The LC parameters were optimised for a fast detection of ritonavir and lopinavir by tandem mass spectrometry. Therefore a narrow non-polar endcapped HPLC column was chosen and the solvent system was reduced to the specific requirements of the ESI source in order to introduce the eluate without splitting. The amount of acetonitrile in the eluent was adjusted to elute lopinavir and ritonavir within five minutes and to separate the compounds from the main sample matrix. Using specific tandem mass spectrometric detection selectivity of the method is given. No matrix interference or drug interference resulting in unexpected peaks were observed in blank plasma from six different individuals in the matrices plasma, ultrafiltrate, and PBMCs. In general, co-eluting sample matrix is able to influence chromatograms (baseline variation or unexpected peaks), the ionisation process (ion suppression) and to contaminate the ion source, resulting in increased variation, particularly at the LOQ level. This was not the case and no increase of the ESI spray current was measured, which can be observed when suppressing ions or matrix compounds coelute with the analytes. Representative spectra of lopinavir, ritonavir, the internal standard, and chromatograms of blanks, artificial samples, and study samples are shown in Figs. 1–4. The results of the analytical method validation for lopinavir and ritonavir in PBMCs, plasma, and ultrafiltrate are shown in Tables 1–3. The summary of quality control samples from running study batches is shown in Table 4. This data confirmed the validity of the results from clinical study samples. The validation results indicate that the method was accurate and precise in the calibration range for both drugs in all matrices.

Simple enrichment procedures (i.e. Vacutainer®CPT™, ultrafiltration, liquid/liquid-extraction), together with short chromatographic run times, and the excellent selectivity and sensitivity of current tandem MS systems are prerequisites for routine analysis of cell and plasma (total and free) concentrations within clinical trials and in individual patients.

3.2. Plasma and intracellular concentrations and intracellular/plasma-correlation

Although triple drug combination decreased plasma HIV-1 RNA levels below the limit of detection in most cases, some patients become non-responsive to HAART because of rapid development of drug resistant variants of HIV-1 likely as a result of inadequate drug levels [6,24–27]. Recently a possible link between intracellular drug levels and antiviral effect has been shown [26]. Because HIV replicates within $CD4^+$ cells, antiviral agents must reach the cytosol in concentrations sufficiently high to inhibit the reproductive cycle. For this purpose monitoring of intracellular concentrations will be a useful tool to ensure effective drug levels at the site of virus replication. Meanwhile different FDA conform validated methods have been established to analyse intracellular drug amounts [15–19] and they always depend on LC/MS or LC/MS/MS to meet the selectivity and sensitivity, which is a prerequisite to support respective studies in vivo. The differences between the methods can be found e.g. in

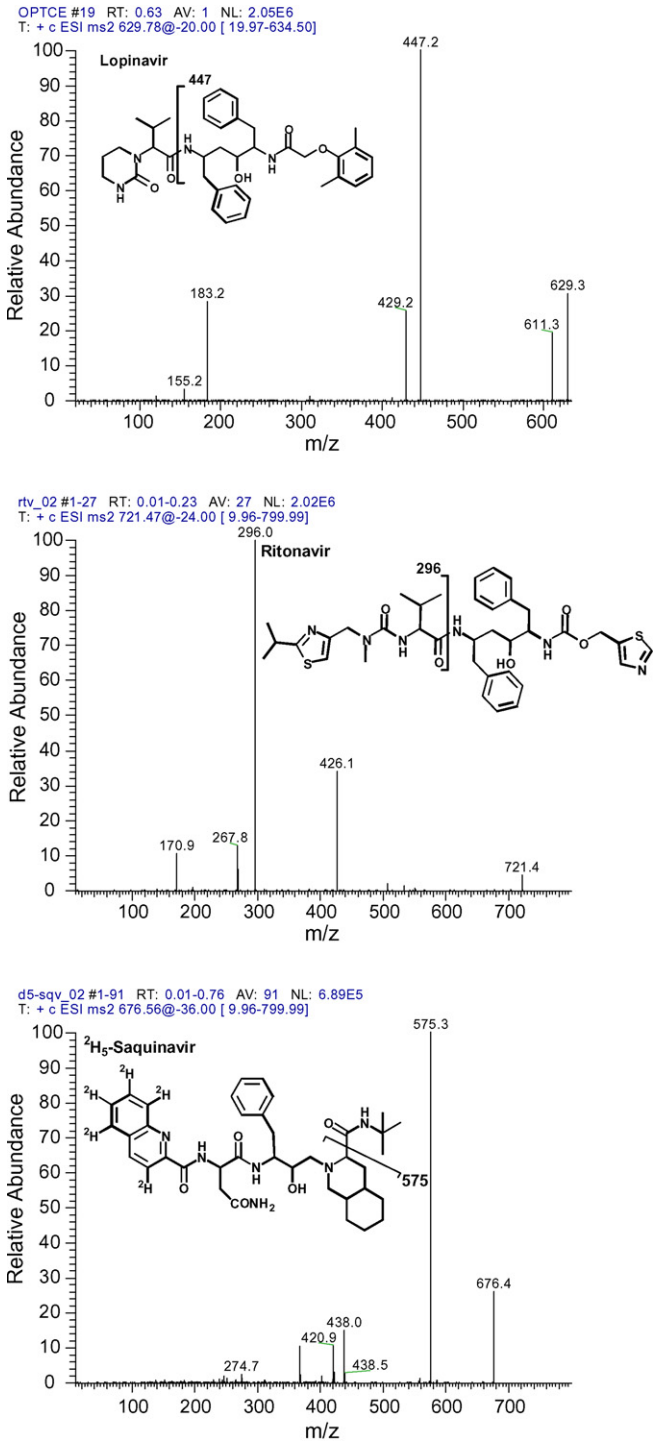

Fig. 1. ESI-tandem mass spectra (product ion scan) of lopinavir (precursor ion *m/z* 629), ritonavir (*m/z* 721), and $^2H_5$-saquinavir (*m/z* 676) and the respective fragmentations.

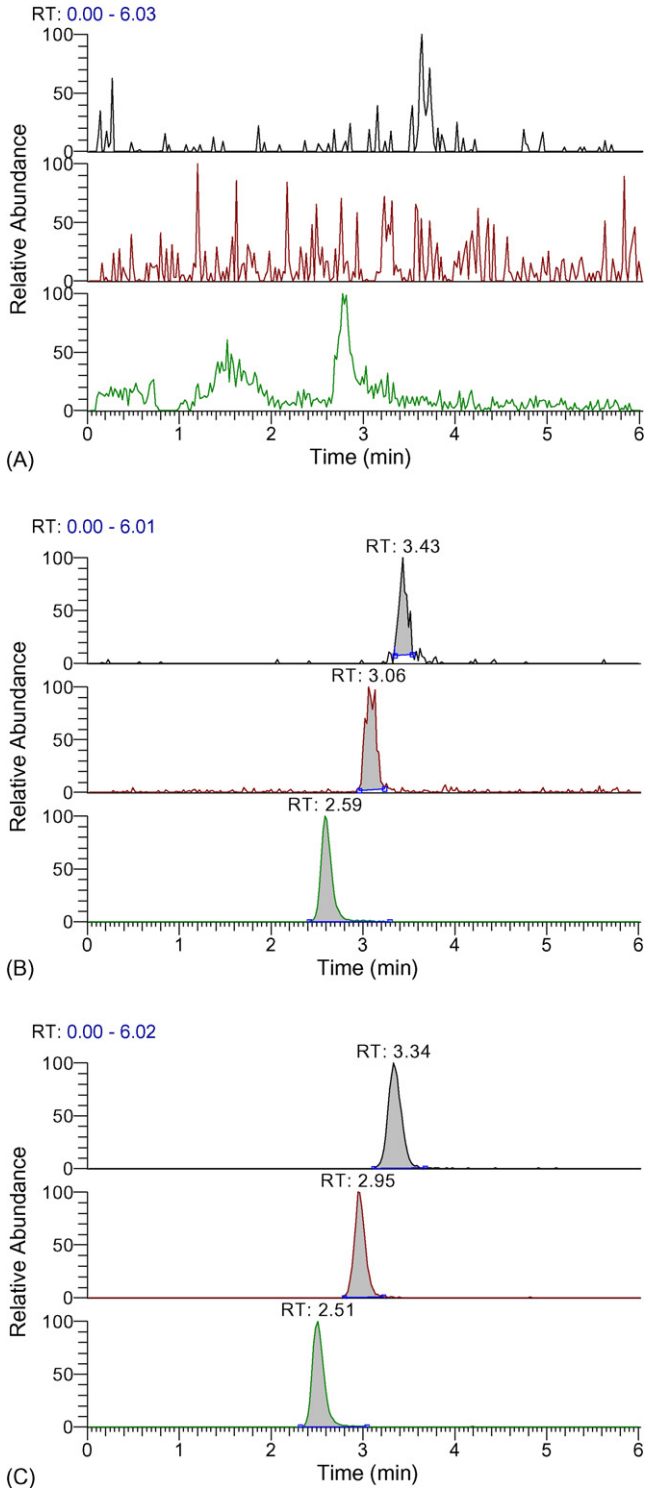

Fig. 2. LC/MS/MS chromatograms of extracted PBMC samples. Upper traces: lopinavir *m/z* 629.5 → *m/z* 447.2 at 20 V CE; middle traces: ritonavir *m/z* 721.4 → *m/z* 296.2 at 24 V collision energy; lower traces: $^{2}H_{5}$-saquinavir (internal standard) *m/z* 676.4 → *m/z* 575.3 at 36 V CE. (A) blank sample; (B) sample at LOQ; (C) sample of a volunteer 4 h after a single oral dose of 100 mg ritonavir/400 mg lopinavir (8.18 ng lopinavir and 2.04 ng ritonavir from 7.3×10^{6} PBMCs).

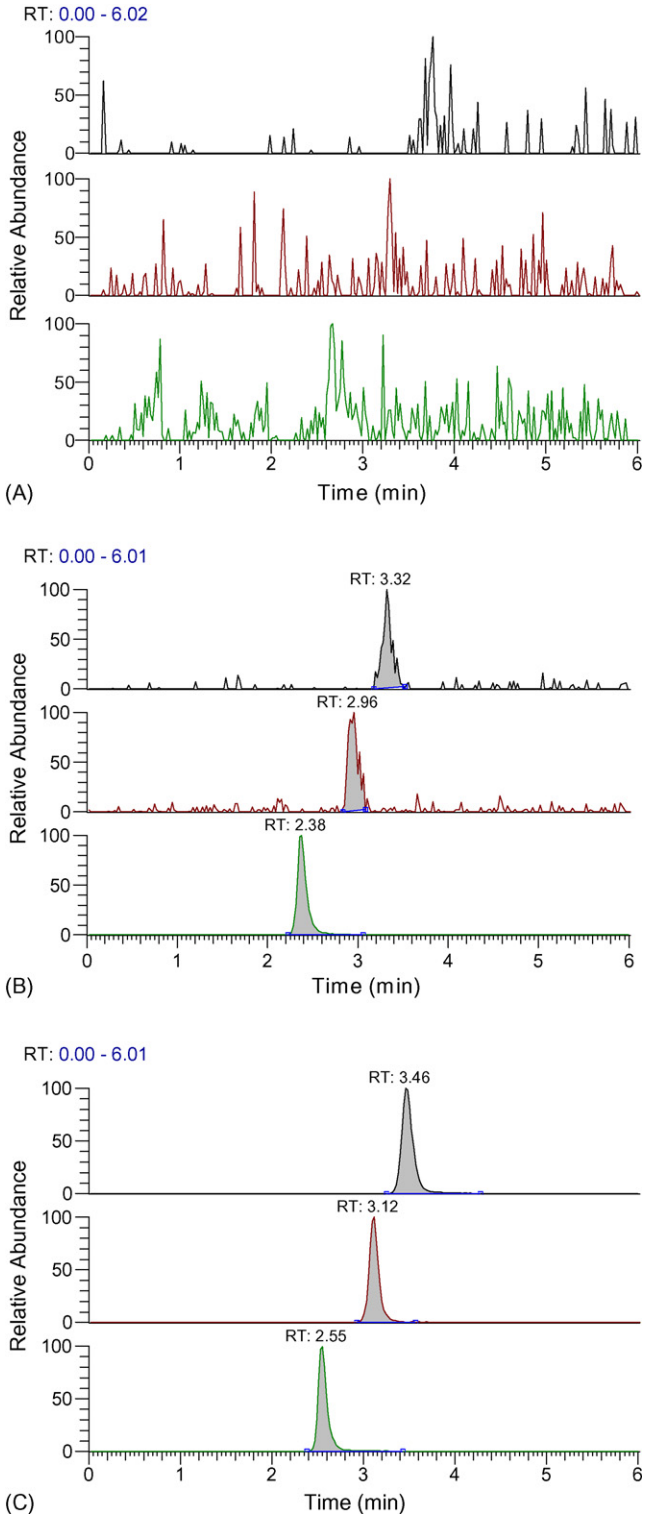

Fig. 3. LC/MS/MS chromatograms of extracted plasma samples. Upper traces: lopinavir *m/z* 629.5 → *m/z* 447.2 at 20 V CE; middle traces: ritonavir *m/z* 721.4 → *m/z* 296.2 at 24 V collision energy; lower traces: $^{2}H_{5}$-saquinavir (internal standard) *m/z* 676.4 → *m/z* 575.3 at 36 V CE. (A) blank sample; (B) sample at LOQ; (C) sample of a volunteer 4 h after a single oral dose of 100 mg ritonavir/400 mg lopinavir (7577 ng/mL lopinavir and 601 ng/mL ritonavir).

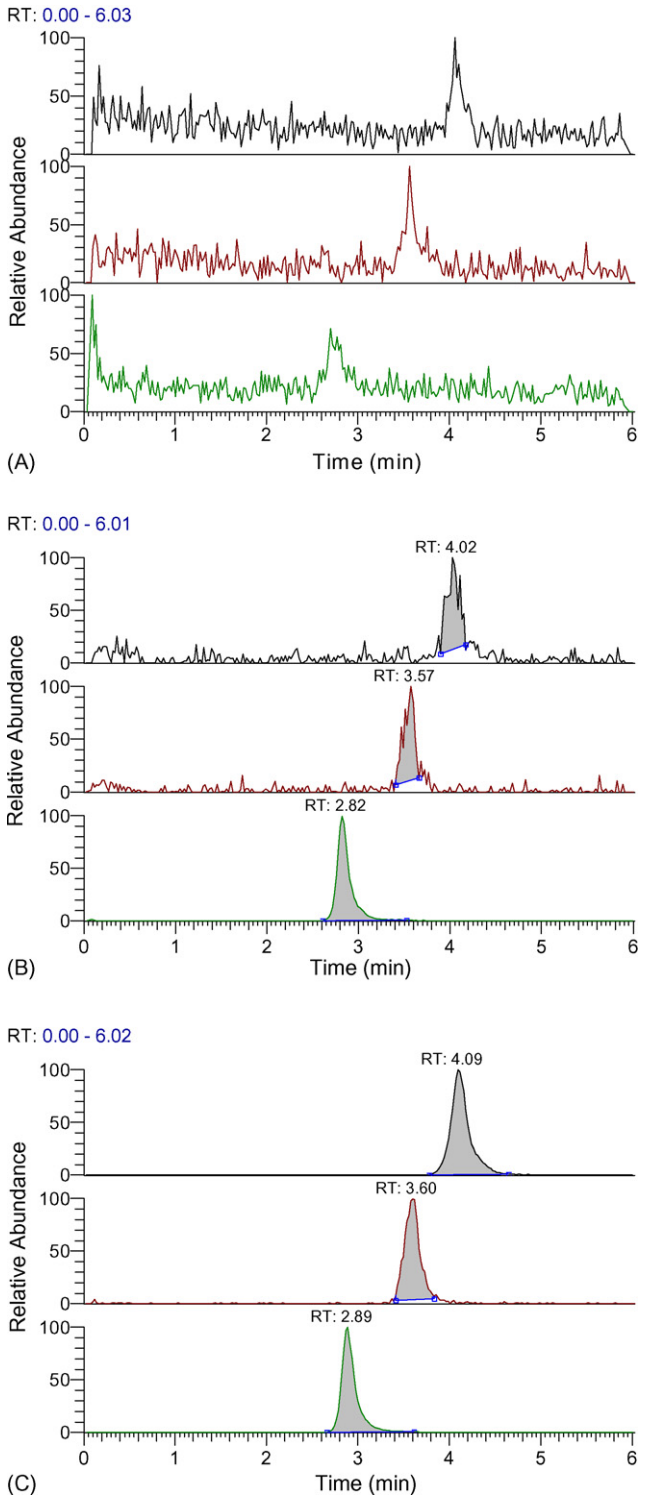

Fig. 4. LC/MS/MS chromatograms of extracted ultrafiltrate samples. Upper traces: lopinavir m/z 629.5 → m/z 447.2 at 20 V CE; middle traces: ritonavir m/z 721.4 → m/z 296.2 at 24 V collision energy; lower traces: $^{2}H_{5}$-saquinavir (internal standard) m/z 676.4 → m/z 575.3 at 36 V CE. (A) blank sample; (B) sample at LOQ; (C) sample of a volunteer 4 h after a single oral dose of 100 mg ritonavir/400 mg lopinavir (28.9 ng/mL lopinavir and 1.26 ng/mL ritonavir).

the application purpose, choice of protease inhibitors, run times, limits of quantification, cell isolation procedures, and number of different biological matrices. Therefore the work of Pelerin et al. [19] for TDM comprises all registered protease inhibitors and analyses in PBMCs and plasma using tandem mass spectrometry. Runtimes are about 20 min with LOQs of 0.25 ng/PBMC pellet and 50 ng/ml plasma. Rouzes et al. [18] analysed four protease inhibitors and efavirenz especially in PBMCs using single mass spectroscopy resulting 15 min run time and LOQs between 1 and 2 ng/3 × 10^6 PBMC cells.

We established and validated an analytical assay for the monitoring of only lopinavir and ritonavir, two protease inhibitors often used in HAART and in clinical studies. The assay should meet the specific demands of Kaletra® (concentration ranges in three different matrices) and had to include the biological matrix PBMC and additionally plasma and ultrafiltrate, because total and free concentrations are often of interest comparing the intracellular concentration and to achieve data concerning active transport over the leucocytes cell membrane. We used tandem mass spectrometry according to achieve maximum selectivity and sensitivity, resulting in short chromatograms (6 min) and low LOQs (0.1 ng/3 × 10^6 PBMCs, 4.0 ng/mL plasma, and 0.2 ng/mL ultrafiltrate). The application of this method will be clinical studies with patients and healthy individuals. Concentration time profiles for lopinavir and ritonavir in plasma and PBMCs of a healthy individual are shown in Fig. 5. Due to the excellent sensitivity of the assay we were able to use small blood samples and measured the pharmacokinetic profiles of both protease inhibitors over a full dosing interval after a regular single oral dose of Kaletra®. Calculated plasma AUCs were 51.3 h × mg/L for lopinavir and 3.0 h × mg/L for ritonavir. Intracellular AUCs were 30.2 h × mg/L for lopinavir and 6.8 h × mg/L for ritonavir within a selected healthy study participant. The clinical data of the whole study will be published elsewhere.

While intracellular lopinavir concentrations were below concurrent plasma concentrations (intracellular/plasma-ratio: 0.59), intracellular concentrations of ritonavir exceeded plasma concentrations (intracellular/plasma-ratio: 2.27) suggesting distinct mechanisms (e.g. active transport) controlling the access of lopinavir and ritonavir to PBMCs which is in accordance with published data [28]. Indeed for many protease inhibitors interactions with ATP-dependent influx [29] and efflux transporters in the cell membrane of $CD4^+$ cells have been reported [30–32]. The assay described here was developed to study these effects in vivo and to quantify the pharmacokinetics of lopinavir/ritonavir in the different body compartments of interest.

We also evaluated the correlation between total and/or free plasma concentration and the concentration inside PBMCs. The free plasma concentrations ranged between 14.1 and 54.1 ng/mL for lopinavir and between 0.24 and 2.44 ng/mL for ritonavir, resulting in 0.52% free fraction for lopinavir and 0.34% for ritonavir, which is in accordance with published data [13]. Free plasma time profiles are shown in Fig. 5. The calculated AUCs were 269 h × ng/mL for lopinavir and 10.0 h × ng/mL for ritonavir in this individual. Concentrations in PBMCs were

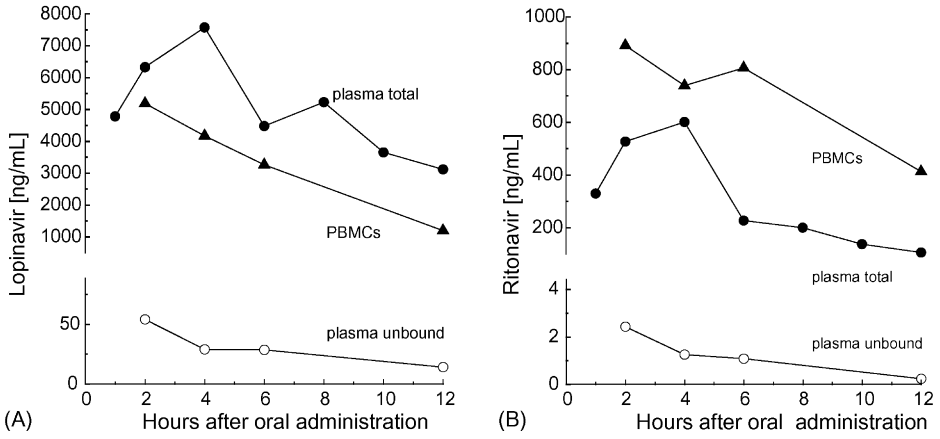

Fig. 5. Concentration time curves of lopinavir (A) and ritonavir (B) in plasma (unbound and total concentrations) and PBMCs after a single oral dose of Kaletra® (100 mg ritonavir/400 mg lopinavir) in a healthy individual.

related to plasma concentrations and there is a linear increase even more closely to free plasma than to total plasma concentrations giving evidence for the possibility of calculating intracellular concentrations from free plasma concentrations. Correlation coefficients (r^2) were below 0.5 but for significance calculations higher numbers of individuals have to be included.

3.3. Limitations

Although we have developed a valid, selective, and highly sensitive assay there are some limitations for its use in research as well as in clinical routine. One critical step is the PBMC washing procedure. On the one hand, PBMCs must be washed thoroughly to remove extracellular medium (i.e. plasma), on the other hand, drug loss due to efflux out of PBMCs must be prevented. To minimise such effects all washing procedures were performed at 4 °C and within 60 min as recommended in earlier studies [15,16] which will limit its usability in clinical routine.

The intracellular free drug concentration is crucial for the inhibition of virus replication. However, in this assay we measured total drug concentrations inside PBMCs. Assuming an intracellular protein binding comparable to plasma protein binding (about 99%) a blood volume of at least 80 mL per sample would be needed for reliable quantification of free intracellular drug concentrations. This high blood volume will not allow repeated measurements for pharmacokinetic analyses and will preclude repeated sampling in patients.

4. Conclusion

With the presented cell isolation and liquid/liquid–extraction procedure in combination with LC/MS/MS we were able to monitor intracellular, free, and total plasma lopinavir and ritonavir concentrations through a full dosing interval. The high sensitivity of the assay and thus low sample volume even for intracellular determinations allows repeated sampling even in conditions, when large blood volumes should be avoided.

Acknowledgement

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References

[1] F.J. Palella, K.M. Delaney, A.C. Moorman, M.O. Loveless, J. Fuhrer, G.A. Satten, D.J. Aschman, S.D. Holmberg, N. Engl. J. Med. 338 (1998) 853.
[2] C.S. Ernest, S.D. Hall, D.R. Jones, J. Pharmacol. Exp. Ther. 312 (2005) 583.
[3] C.G. Lee, M.M. Gottesman, C.O. Cardarelli, M. Ramachandra, K.T. Jeang, S.V. Ambudkar, I. Pastan, S. Dey, Biochemistry 37 (1998) 3594.
[4] R.E. Aarnoutse, J.M. Schapiro, C.A.B. Boucher, Y.A. Hekster, D.M. Burger, Drugs 63 (2003) 741.
[5] R.P.G. van Heeswijk, Ther. Drug Monit. 24 (2002) 323.
[6] N.A. Roberts, J.C. Craig, J. Sheldon, AIDS 12 (1998) 453.
[7] P.L.A. Fraaij, N. Rakhmanina, D.M. Burger, R. de Groot, Ther. Drug Monit. 26 (2004) 122.
[8] D. Burger, P. Hugen, P. Reiss, I. Gyssens, M. Schneider, F. Kroon, G. Schreij, K. Brinkman, C. Richter, J. Prins, R. Aarnoutse, J. Lange, AIDS 17 (2003) 1157.
[9] D.J. Back, S.H. Khoo, Br. J. Clin. Pharmacol. 55 (2003) 473.
[10] M. Boffito, E. Acosta, D. Burger, C.V. Fletcher, C. Flexner, R. Garaffo, G. Gatti, M. Kurowski, C.F. Perno, G. Peytavin, M. Regazzi, D. Back, Antivir. Ther. 10 (2005) 469.
[11] P.W.H. Hugen, D.M. Burger, R.E. Aarnoutse, P.A. Baede, P.T. Nieuwkerk, P.P. Koopmans, Y.A. Hekster, Ther. Drug Monit. 24 (2002) 579.
[12] J.E. Ray, D. Marriott, M.T. Bloch, A.J. McLachlan, Br. J. Clin. Pharmacol. 60 (2005) 291.
[13] M. Boffito, P.G. Hoggard, W.E. Lindup, S. Bonora, A. Sinicco, S.H. Khoo, G.D. Perri, D.J. Back, Ther. Drug Monit. 26 (2004) 35.
[14] K.A. Koeplinger, T.J. Raub, G.E. Padbury, Z. Zhao, J. Pharm. Biomed. Anal. 19 (1999) 399.
[15] E.R. Meaden, P.G. Hoggard, P. Newton, J.F. Tjia, D. Adam, D. Cornforth, J. Lloyd, I. Williams, D.J. Back, S.H. Khoo, Antimicrob. Agents Chemother. 50 (2002) 583.
[16] S. Colombo, A. Beguin, A. Telenti, J. Biollaz, T. Buclin, B. Rochat, L.A. Decosterd, J. Chromatogr. B 819 (2005) 259.
[17] Y. Huang, E. Zurlinden, E. Lin, X. Li, J. Tokumoto, J. Golden, A. Murr, J. Engstrom, J. Conte, J. Chromatogr. B 799 (2004) 51.
[18] A. Rouzes, K. Berthoin, F. Xuereb, S. Djabarouti, I. Pellegrin, J.L. Pellegrin, A.C. Coupet, S. Augagneur, H. Budzinski, M.C. Saux, D. Breilh, J. Chromatogr. B 813 (2004) 209.

[19] H. Pelerin, S. Compain, X. Duval, F. Gimenez, H. Benech, A. Mabondzo, J. Chromatogr. B 819 (2005) 47.
[20] J. Burhenne, K.D. Riedel, M. Martin-Facklam, G. Mikus, W.E. Haefeli, J. Chromatogr. B 784 (2003) 233.
[21] Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, May 2001. Available from: URL: http://www.fda.gov/cder/guidance/4252fnl.htm (accessed August 31, 2006).
[22] V.P. Shah, K.K. Midha, J.W. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551.
[23] P.A. Furman, J.A. Fyfe, M.H. St Clair, K. Weinhold, J.L. Rideout, G.A. Freeman, S.N. Lehrman, D.P. Bolognesi, S. Broder, H. Mitsuya, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 8333.
[24] J.H. Condra, W.A. Schleif, O.M. Blahy, L.J. Gabryelski, D.J. Graham, J.C. Quintero, A. Rhodes, H.L. Robbins, E. Roth, M. Shivaprakash, D. Titus, T. Yang, H. Teppler, K.E. Squires, P.J. Deutsch, E.A. Emini, Nature 374 (1995) 569.
[25] P.G. Hoggard, A. Owen, J. Antimicrob. Chemother. 51 (2003) 493.
[26] D. Breilh, I. Pellegrin, A. Rouzes, K. Berthoin, F. Xuereb, M. Munck, H. Budzinski, H. Fleuery, M.C. Saux, J.L. Pellegrin, AIDS 18 (2004) 1305.
[27] M. Hennessy, S. Clarke, J.P. Spiers, F. Mulcahy, D. Kelleher, E. Meadon, B. Maher, C. Bergin, S. Khoo, J. Tjia, P. Hoggard, D. Back, M. Barry, Antivir. Ther. 8 (2003) 191.
[28] K.M.L. Crommentuyn, J.W. Mulder, A.T.A. Mairuhu, E.C. van Gorp, P.L. Meenhorst, A.D.R. Huitema, J.H. Beijnen, Antivir. Ther. 9 (2004) 779.
[29] Y. Su, X. Zhang, P.J. Sinko, Mol. Pharm. 1 (2004) 49.
[30] J.W. Polli, S.A. Wring, J.E. Humphreys, L. Huang, J.B. Morgan, L.O. Webster, C.S. Singh, J. Pharmacol. Exp. Ther. 299 (2001) 620.
[31] B. Chandler, L. Almond, J. Ford, P. Hoggard, S. Khoo, D. Back, JAIDS 33 (2003) 551.
[32] D. Vishnuvardhan, L. Moltke, C. Richter, D.J. Greenblatt, AIDS 17 (2003) 1092.