Journal of Chromatographic Science 2012;50:625–635
doi:10.1093/chromsci/bms048 Advance Access publication May 4, 2012

Article

Simultaneous Quantitation of HIV-Protease Inhibitors Ritonavir, Lopinavir and Indinavir in Human Plasma by UPLC–ESI-MS-MS

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Received 26 July 2011; revised 17 September 2011

A selective, sensitive and high-throughput ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS-MS) method has been developed and validated for the quantification of HIV-protease inhibitors ritonavir (RTV), lopinavir (LPV) and indinavir (IDV) in human plasma. Sample clean-up involved protein precipitation of both drugs and fluconazole used as internal standard from 100 μL human plasma. All the analytes were chromatographically separated on a Waters Acquity UPLC BEH C18 (2.1 × 50 mm, 1.7 μm particle size) analytical column using 0.1% formic acid and methanol (40:60, *v*/*v*) as the mobile phase. The parent → product ion transitions for ritonavir (*m*/*z* 721.40→ 296.10), lopinavir (*m*/*z* 629.40→ 447.40) and indinavir (*m*/*z* 614.4→ 421.0) IS (*m*/*z* 307.10 → 220.10) were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring and positive ion mode. The method was validated over the concentration range of 30–15,000 ng/mL for LPV and IDV and 3–1500 ng/mL for RTV. The method was successfully applied to a pilot bioequivalence study in 36 healthy human subjects after oral administration of lopinavir 200 mg and ritonavir 50 mg tablet formulation under fasting conditions.

Introduction

The global AIDS epidemic has claimed the lives of more than 20 million people since 1981. Another 10 million are now living with HIV and most of these are likely to develop AIDS over the course of the next decade. In spite of the various treatment protocols available, including the mainstream highly active antiretroviral therapy (HAART), the number of people infected with HIV continues to rise. The most recent United Nations Programme on HIV/AIDS (UNAIDS)/World Health Organization estimates show that, in 2004 alone, 4.9 million people were newly infected with HIV (1). HIV is a virus that goes through many steps during its life cycle. Once HIV infects a human cell, the virus uses proteins and chemicals inside that cell to make more copies of it. Protease is a chemical, known as an enzyme that HIV needs to make new viruses (1–5). Protease inhibitors (PIs) block the protease enzyme. When protease is blocked, HIV makes copies of itself that cannot infect new cells (2, 3). Studies have shown that protease inhibitors can reduce the amount of virus in the blood and increase CD4 cell counts. In some cases, these drugs have improved CD4 cell counts, even when they were very low or zero (4–6).

The biggest news and the greatest benefits to people with HIV came when PIs were discovered and made into anti-HIV treatments. While using PIs in combination with other drugs, the number of people who became ill from opportunistic infections, or died from AIDS, dropped by approximately 70%. However, studies have also shown that these effects can wear off over time. This happens because HIV makes more of itself all the time (7). Each new HIV virus that gets made may be slightly different than the one it made before. The new protease that the virus has made may resist the drugs that worked for viruses with the older type of protease. This is what scientists call drug resistance. When this happens, other PIs usually become less effective as well.

The best way to avoid drug resistance is to stop or reduce HIV production in the body. The less HIV made in the body, the less chance of a virus created that is resistant to anti-HIV drugs. To keep HIV levels as low as possible, it is recommended that PIs be taken in combination with at least two other anti-HIV drugs. This is called HAART (2, 3). Studies have shown that when certain PIs are combined, the anti-HIV effect is increased, which can help prevent or overcome resistance. This is called protease boosting. Some doctors will order a drug resistance test to determine a patient's resistance profile to decide the best drug combination to use.

Seven United States Food and Drug Administration (FDA)-approved protease inhibitors are available so far: amprenavir (Agenerase), fosamprenavir (Lexiva), indinavir (IDV) (Crixivan), lopinavir (LPV)/ritonavir (RTV) (Kaletra), ritonavir (Norvir), saquinavir (Fortovase), and nelfinavir (Viracept).

Due to extensive use of these PIs in HAART, it has become essential to develop competent bioanalytical assays for their routine measurement in subject samples. Several analytical methods are developed and validated to determine LPV (8, 9) and RTV (10–13) separately in different biological matrices; namely, plasma, cerebral spinal fluid, serum, tissues and saliva. Other procedures present the determination of LPV with nevirapine (14), RTV with saquinavir (15, 16), indinavir and saquinavir (17), nelfinavir and saquinavir (18) and zidovudine and didanosine (19). Extensive literature is available (20–58) for their simultaneous quantitation with other protease, nucleoside and non-nucleoside reverse transcriptase inhibitors by capillary electrophoresis (20, 21), high-performance liquid chromatography (HPLC) (22–41), liquid chromatography–tandem mass spectrometry (LC–MS-MS) (42–51) and matrix-

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assisted laser desorption/ionization–tandem time-of-flight mass spectrometry (MALDI–TOF-)TOF (52).

In this paper, we describe a validated assay for the simultaneous determination of three protease inhibitors (ritonavir, lopinavir and indinavir) in human plasma sample using an optimized protein precipitation (PPT) procedure followed by an optimized ultra-performance liquid chromatography method with mass spectrometry detection (UPLC–MS-MS) with short chromatographic run time, which is beneficial for high throughput analysis of biological samples.

Experimental

Chemicals and materials

Reference standards of ritonavir (101.3%), lopinavir (98.5%) and indinavir (99.2%) and fluconazole (99.2%, internal standard; IS) were procured from Samex Overseas (Surat, India). HPLC-grade methanol and acetonitrile were obtained from Mallinckrodt Baker (S.A. de C.V., Mexico). Guaranteed reagent-grade formic acid was obtained from Merck Specialties, (Mumbai, India). Water used in the entire analysis was prepared from a Milli-Q water purification system procured from Millipore (Bangalore, India). Blank human plasma was procured from Supratech Micropath (Ahmedabad, India) and stored at −20°C until use.

Liquid chromatographic conditions

A Waters Acquity UPLC system (Milford, MA) consisting of binary solvent manager, sample manager and column manager was used for setting the reverse-phase liquid chromatographic conditions. The Waters Acquity UPLC BEH C18 [2.1 × 50 mm (length × inner diameter), with 1.7 μm particle size] was maintained at 40°C in column oven. The mobile phase consisted of 0.1% formic acid and acetonitrile in 40:60 (v/v) ratios. For isocratic elution, the flow rate of the mobile phase was kept at 0.200 mL/min; the total chromatographic run time was 2.7 min. The sample manager temperature was maintained at 5°C.

Mass spectrometric conditions

Ionization and detection of analyte and IS was carried out on a triple quadrupole mass spectrometer (MDS Sciex, API 4000 Canada), equipped with electrospray ionization (turbo Ion Spray) and operating in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor parent → product ion (m/z) transitions for ritonavir (m/z 721.40→ 296.10), Lopinavir (m/z 629.40→ 447.40) and Indinavir (m/z 614.40→ 421.00) and IS (m/z 307.10 → 220.10) respectively (Figure 1).

The source dependent parameters maintained for ritonavir, lopinavir and indinavir were: ion spray voltage (IS), 5.5kV; Curtain gas, 30; desolvation temperature, 400°C; nebulizer gas flow, 50; heater gas flow, 50. The optimum values for compound dependent parameters (MRM file parameters) like declustering potential and collision energy set were 80, 65 and 90 V and 21, 25 and 39 V for lopinavir, ritonavir and indinavir, respectively, and 39V for IS. The dwell time was set at 200 ms. Analyst software version 1.4.1 was used to control all parameters of UPLC and MS.

Standard stock, calibration standards and quality control sample preparation

The standard stock solutions of 10 mg/mL of lopinavir and indinavir and 3 mg/mL for ritonavir were prepared by dissolving requisite amount in methanol. Calibration standards and quality control (QC) samples were prepared by spiking (2% of total volume of blank plasma) blank plasma with stock solution. Calibration curve standards were made at 30.9, 61.9, 103.1, 206.3, 412.5, 937.5, 1,875.1, 3,750.2, 7,500.3 and 15,000.0 ng/mL for lopinavir; 3.1, 6.2, 10.3, 20.6, 41.3, 93.8, 187.5, 375.0, 750.0 and 1,500.0 ng/mL for ritonavir; and 31.0, 62.0, 103.1, 206.3, 412.5, 937.5, 1,875.0, 3,750.0, 7,500.0 and 15,000.0 ng/mL for indinavir concentrations, respectively.QC samples were prepared at three levels: 13,800.6, 1,380.0 and 13,800 ng/mL for lopinavir, ritonavir and indinavir, respectively, for high quality control (HQC); 1,242, 124.2 and 1, 242 ng/mL for lopinavir, ritonavir and indinavir, respectively, for middle quality control (MQC); and 84.5, 8.5 and 84.5 ng/mL for lopinavir, ritonavir and indinavir, respectively, for low quality control (LQC). Stock solution (1 mg/mL) of the IS was prepared by dissolving 10 mg of fluconazole in 10 mL of methanol. An aliquot of 250 μL of this solution was further diluted to 25.0 mL in the same diluent to obtain a solution of 10.0 μg/mL. All solutions (standard stock, calibration standards and QC samples) were stored at 2–8°C until use.

Protocol for sample preparation

Before analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 100 μL of spiked plasma sample, 50 μL IS was added and vortexed for 20 s. Furthermore, 500 μL of acetonitrile was added and vortex mixed for 2 min. Centrifugation of the samples was done at 4,000 rpm for 5 min at 10°C. The supernatant (0.15 mL) was separated and 0.35 mL of 10 mM ammonium formate solution was added, then samples were vortexed for 20 s. Two microliters were used for injection in the chromatographic system.

Bio-analytical method validation

The method validation was performed as per the FDA and International Council on Harmonization (ICH) guidelines (59, 60). System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of lopinavir, ritonavir, indinavir and IS at the start of each batch during the method validation. The carryover effect of the autosampler was evaluated by injecting a sequence of injection solutions of aqueous standard (lopinavir, ritonavir, indinavir and IS), mobile phase, standard blank and extracted standard (lopinavir, ritonavir, indinavir and IS) equivalent to the highest standard in the calibration range.

The linearity of the method was determined by analysis of five linearity curves containing eight non-zero concentrations. The ratio of area response for analytes to IS was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression.

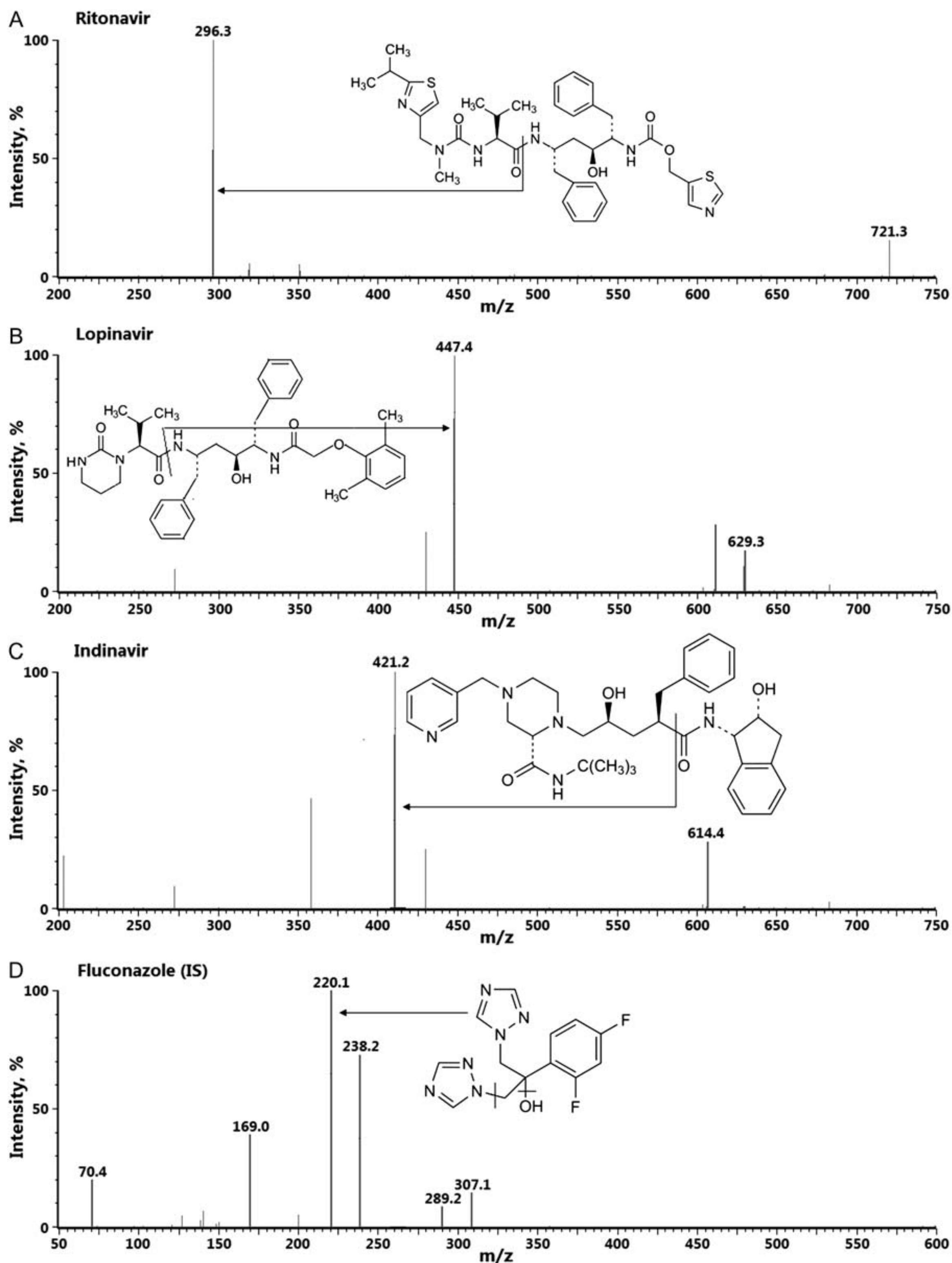

Figure 1. Product ion mass spectra in positive ionization mode: ritonavir (*m/z* 721.3 → 296.3, scan range 200–750 amu) (A); lopinavir (*m/z* 629.3 → 447.4, scan range 200–750 amu) (B); indinavir (*m/z* 614.4 → 421.2, scan range 200–750 amu) (C) and fluconazole (IS, *m/z* 307.1 → 220.1, scan range 50–600 amu) (D).

The lowest standard on the calibration curve was accepted as the lower limit of quantitation (LLOQ), if the analyte response was at least five times more than that of drug-free (blank) extracted plasma. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15.0%.

The selectivity of the method towards endogenous plasma matrix components was assessed in twelve batches (seven normal of K3 EDTA plasma, two haemolysed, two lipidemic and one heparinised) of blank human plasma. This was done to estimate the extent to which endogenous plasma components contribute toward the interference at the retention time of analytes and IS. The cross talk of MRM for analytes and IS was checked using highest standard on calibration curve and working solution of IS.

For determining the intra-day accuracy and precision, replicate analysis of plasma samples of lopinavir, ritonavir and indinavir was performed on the same day. The run consisted of a calibration curve and six replicates of LLOQ, LQC, MQC and HQC samples. The inter-day accuracy and precision were assessed by analysis of three precision and accuracy batches on three consecutive validation days. The precision of the method was determined by calculating the percent coefficient of variation (%CV) for each level. The deviation at each concentration level from the nominal concentration was expected to be within $\pm 15.0\%$, except LLOQ, for which it should be within $\pm 20.0\%$.

Ion suppression/enhancement effects on the MRM LC–MS-MS sensitivity were evaluated by the postcolumn analyte infusion experiment. A standard solution containing lopinavir, ritonavir and indinavir (at MQC level) and IS was infused postcolumn into the mobile phase at 10 μL/min employing the in-built infusion pump. Aliquots of 2 μL of extracted control plasma were then injected into the column by the autosampler and MRM LC–MS-MS chromatograms were acquired for the analytes and IS. Any dip in the baseline upon injection of double blank plasma (without IS) indicates ion suppression, while a peak at the retention time of analytes and IS indicates ion enhancement.

The relative recovery, matrix effect and process efficiency were assessed. All three parameters were evaluated at HQC, MQC and LQC levels in six replicates. Relative recovery (RE) was calculated by comparing the mean area response of extracted samples (spiked before extraction) to that of unextracted samples (spiked after extraction) at each QC level. The recovery of IS was similarly estimated. Absolute matrix effect (ME) was assessed by comparing the mean area response of unextracted samples (spiked after extraction) with mean area of neat standard solutions. The overall process efficiency (%PE) was calculated as $(\mathrm{ME} \times \mathrm{RE})/100$. The assessment of relative ME was based on direct comparison of the MS-MS responses (peak areas) of the analytes spiked into extracts originating from different lots of plasma. The variability in these responses, expressed as %CV, was considered to be the measure of relative ME.

Stability experiments were carried out to examine the analyte stability in stock solutions and plasma samples under different conditions. Short-term stability at room temperature and long-term stability of spiked solution stored at −50°C were assessed by comparing the area response of stability sample of analyte and IS with the area response of sample prepared from fresh stock solutions. The solutions were considered stable if the deviation from nominal value was within $\pm 10\%$. Autosampler (wet extract) stability, bench top stability and freeze-thaw stability were performed at LQC and HQC, using six replicates at each level. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within $\pm 15\%$.

To authenticate the ruggedness of the proposed method, it was done on two precision and accuracy batches. The first batch was analyzed by two different analysts and the second batch was analyzed on a different lot of columns.

Application of the method in human subjects

The proposed validated method was successfully applied for the assay of LPV and RTV in healthy male volunteers in the age group of 18 to 45 years. Each volunteer was judged to be in good health through medical history, physical examination and routine laboratory tests. Written consent was taken from all the volunteers after informing them about the objectives and possible risks involved in the study. An independent ethics committee constituted as per Indian Council of Medical Research (ICMR) approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by ICH and FDA (59, 60).

Results and Discussion

Method development

Development and validation of an assay to simultaneously determine HIV PIs RTV, LPV and IDV by UPLC–MS-MS with accuracy, precision and specificity has been optimized and described. PI plasma concentrations demonstrate high interpatient variability; therefore, ranges of standard curves were chosen to encompass the broad spectrum of drug concentrations likely to be encountered in PK studies. It is important that the assay detects and quantifies below and above the defined minimum effective concentrations (MEC) and toxic levels of PIs, respectively. Therapeutic ranges of PIs have been reported in the literature (17, 18).

The RTV standard curve was validated over a lower concentration range because RTV is generally administered at low doses to boost concomitant PI exposure (8, 9); consequently, lower plasma concentrations of RTV result. The assay requires a small volume of plasma for analysis (100 μL). This is advantageous when measuring PI concentrations as part of clinical studies because they often necessitate hourly sampling to generate complete PK profiles; therefore, less blood can be drawn from the patient. This is identical to many assays measuring multiple PIs by LC–MS-MS (17, 23, 24, 27) however, two relatively recent methods required 250 μL and 1 mL, respectively (26, 27). The method combines a short run time of 2.7 min per sample with a quick and simple extraction procedure allowing a large number of samples to be processed quickly and efficiently. Similarly, it is beneficial that three PIs can be quantified within one assay, because combination PI therapy is common, particularly among antiretroviral-experienced patients.

During method development, it was observed that the pH of the mobile phase and selection of the right column is an important criterion. Thus, separation was tried using various combinations of methanol–acetonitrile, acidic buffers and additives like formic acid on four different reversed-phase columns with 5 mm particle size; namely, ACE Cyano (150 and 250 × 4.6 mm), Chromolith RP-18 (100 × 4.6 mm), Kromasil (50 and 100 × 4.6 mm) and Gemini C-18 (50 × 4.6 mm) to find the optimal column that produced the best sensitivity, efficiency

and peak shape. The analytes showed poor separation and reproducibility for the proposed linear range except for the Waters Acquity UPLC BEH C18, 2.1 × 50 mm, 1.7 µm that offered superior peak shape, efficient separation, and desired linearity and reproducibility. A 1.7-µm column instead of a 5-µm has shown better chromatography in terms of resolution, sharper peaks were observed due to modified column chemistry and the high surface area of column material compares to conventional columns. The mobile phase consisting of 0.1% formic acid in water and acetonitrile (40:60 v/v) ratio with a pH approximately 3.0 was found most suitable for eluting ritonavir, lopinavir, indinavir and IS at 1.54, 1.71, 0.63 and 0.69 min, respectively. Also, the reproducibility of retention times for the analytes, expressed as %CV, was ≤2% for 40 injections on the same column.

The inherent selectivity of MS-MS detection was also expected to be beneficial in developing a selective and sensitive method. The present study was conducted using electrospray ionization (ESI) as the ionization source because it gave high intensity for ritonavir, lopinavir, indinavir and IS, and a good linearity in regression curves. The intensity found was much higher in the positive mode for ritonavir, lopinavir and indinavir because they have similar sites for protonation. Also, the use of formic acid, pH 3.00, in the mobile phase further augmented the response of protonated precursor $[M + H]^+$ ions in the Q1 MS full scan spectra. A dwell time of 200 ms was adequate for the analytes and IS and no cross talk was observed between their MRMs.

The extraction of ritonavir, lopinavir, indinavir and IS was carried out via PPT with common solvents like acetonitrile, and after denaturing the plasma proteins, the cleaner supernatant was diluted with 10 mM ammonium formate (pH approximately 6.5) to minimize the ion saturation in mass spectroscopy. A general IS was used to minimize any analytical variation due to sample processing, integrity of the column and ionization efficiency of analytes. Fluconazole was used as an IS in the present study, which had similar chromatographic behavior and was quantitatively extracted with the proposed extraction procedure. Also, there was no effect of IS on analyte recovery, sensitivity or ion suppression.

In conclusion, the assay described is quick and easy, with a simple and rapid extraction procedure requiring only 100 µL of plasma.

System suitability and autosampler carryover

Throughout the method validation, the %CV of system suitability was observed below 4.0% at the retention times of ritonavir and the IS. Carry-over evaluation was performed in each analytical run to ensure that it does not affect the accuracy and the precision of the proposed method. Negligible carry-over (≤4%) was observed during the autosampler carryover experiment. No enhancement in the response was observed in double blank after subsequent injection of highest calibration standard (aqueous and extracted) at the retention times of analytes and IS.

Linearity and LLOQ

Due to high inter-subject variability observed in Indian volunteers, a wide linear dynamic range was essential to encompass different formulations of Kaletra. All five calibration curves analyzed during the course of validation were linear for the standards, ranging from 29.7–14,379 and 2.9–1,45 2ng/mL for LPV and RTV, respectively. A straight-line fit was made through the data points by least square regression analysis to give the linear equation $y = 0.0005x + 0.0005$ for LPV and $y = 0.0042x - 0.0020$ for RTV, where y is the peak area ratio of the analyte to the IS and x the concentration of the analyte. The standard deviation values for slope, intercept and correlation coefficient (r) observed during the course of validation were 1.0e-5, 1.64e-3 and 5.0e-5 for LPV and 3.0e-5, 1.6e-3 and 3.0e-5 for RTV respectively. The lowest concentration (LLOQ) in the standard curve that can be measured with acceptable accuracy and precision was found to be 29.7 and 2.9ng/mL for LPV and RTV in human plasma.

Selectivity, accuracy and precision

Figures 2, 3 and 4 demonstrate the selectivity experiments with the chromatograms of extracted blank plasma (A), blank plasma with IS (B), peak response of LPV, RTV and IDV at LLOQ (C) and a real sample for LPV and RTV at 4h after oral administration of [200 (LPV) + 50 (RTV)] mg tablet formulation (D). The combination of PPT and UPLC–MS-MS detection gave very good selectivity for the analytes and IS. No endogenous peaks were observed at the retention times of LPV (1.71 min), RTV (1.54 min), IDV (0.63 min) and IS (0.68 min) for any of the batches. The analysis time was short, which makes it an attractive method for routine analysis. None of the concomitant medications and antiretrovirals showed interfering signals at the retention times of LPV, RTV, IDV or IS. This demonstrates that the method is highly selective and free from interference due to matrix components and other prescribed medications. Precision (%CV) for intra-batch and inter-batch ranged from 0.8 to 4.7% for LPV, 2.7 to 7.5% for RTV and 3.2 to 5.4% for IDV, respectively. The accuracy results for intra-batch and inter-batch were within 93.0 to 111.3% for RTV, 94.4 to 100.6% for LPV and 94.4 to 99.1% for IDV, respectively, at all QC levels. The detailed results are presented in Table I.

Matrix effect, ion suppression, recovery

Matrix effect may be defined as a composite of some undesirable effects that originate from a biological matrix. These components may result in ion suppression/enhancement, decrease/increase in sensitivity of analytes over a period of time, increased baseline, imprecision of data, drift in retention time and distortion or tailing of a chromatographic output. The RE, absolute ME and PE data at LQC, MQC and HQC levels is presented in Table II. The recovery for IS in human plasma was 93.9%. Furthermore, the extent of matrix effect in different lots of plasma (spiked after extraction) was within the acceptable limit, as evident from the precision (%CV) values in Table III.

Stability

Stock solutions for short-term and long-term stability of the analytes and IS were stable at room temperature for minimum period of 7 h and between 2–8°C for seven days, respectively.

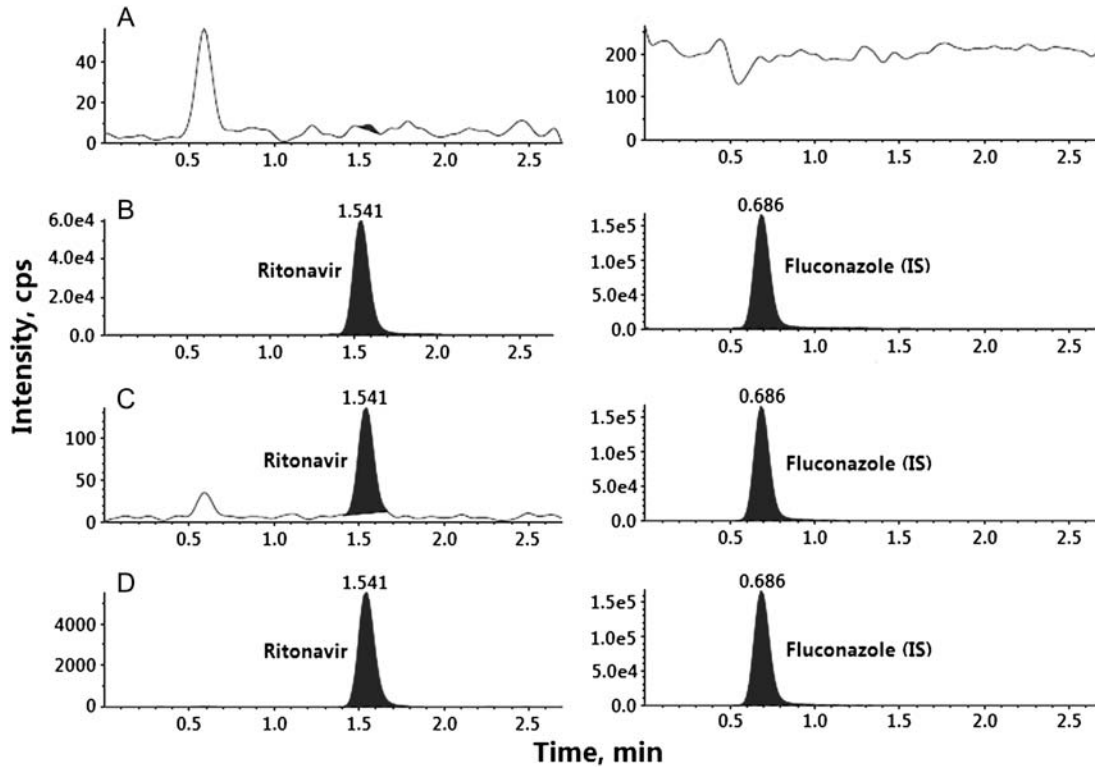

Figure 2. MRM ion-chromatograms of ritonavir (m/z 721.3 → 296.3) and IS (m/z 307.10 → 220.10) in double blank plasma (A); ULOQ with IS (B); LLOQ with IS (C); real subject sample at 4 h with IS (D).

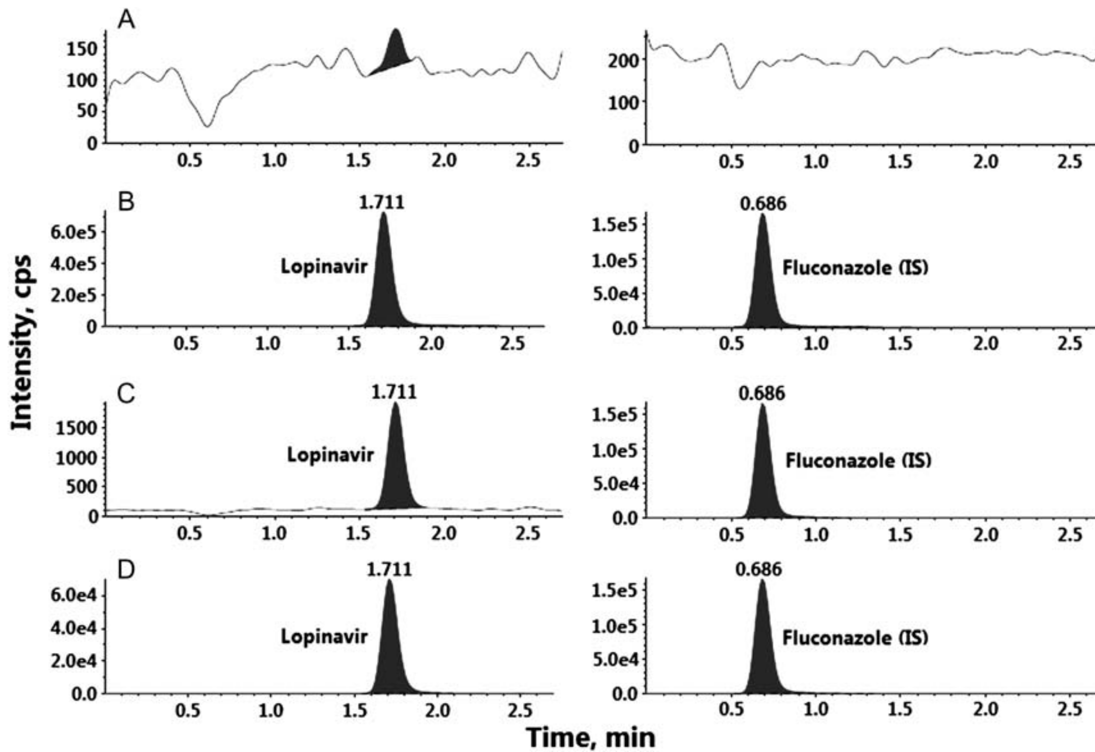

Figure 3. MRM ion-chromatograms of lopinavir (m/z 629.3 → 447.4) and fluconazole (m/z 307.10 → 220.10) in double blank plasma (A); ULOQ with IS (B); LLOQ with IS (C); real subject sample at 4 h with IS (D).

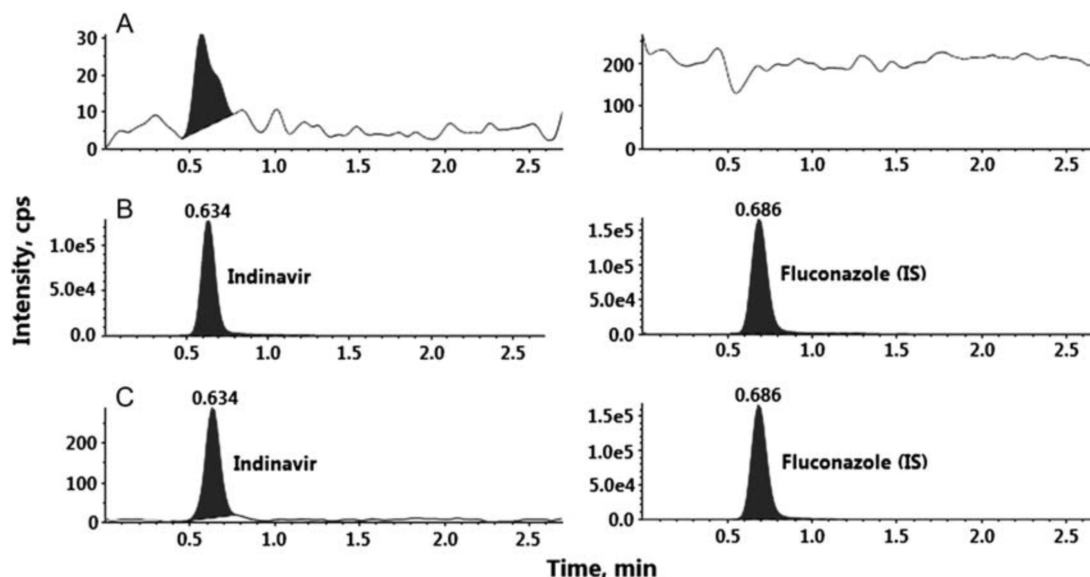

Figure 4. MRM ion-chromatograms of indinavir (m/z 614.4 → 421.2) and fluconazole (m/z 307.10 → 220.10) in double blank plasma (A); ULOQ with IS (B); LLOQ (C).

RTV, LPV and IDV in control human plasma (bench top) at room temperature were stable for at least for 6 h at 25 ± 3°C and for minimum of three freeze and thaw cycles. Spiked plasma samples stored at −20°C and −70°C for the long-term stability experiment were stable for a minimum of 60 days. Autosampler stability of the spiked QC samples maintained at 5°C was also determined up to 23 h. Different stability experiments in plasma and the values for the precision and percent change are shown in Tables IV, V and VI.

Ruggedness and dilution integrity

The results of the ruggedness study for RTV, LPV and IDV were well within the acceptance limit of 15% in precision and 85.0 to 115.0% in mean accuracy. The precision and accuracy values for both experiments at LLOQ, LQC, MQC and HQC levels for RTV ranged from 2.6 to 7.9% and 99.1 to 104.4%. For LPV, the precision and accuracy values observed were within 2.5 to 8.6% and 97.7 to 102.9%; and for IDV, the precision and accuracy values observed were within 3.5 to 7.6% and 92.7 to 108.9%, respectively, for all QC levels.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentration above the upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis. The precision and accuracy values for 1/5th and 1/10th dilution ranged from 5.0–5.6% and 102.1–105.1% for RTV, LPV and IDV.

Table I
Comparison of Intra-Batch and Intra-Batch Precision and Accuracy for Ritonavir, Lopinavir and Indinavir*

QC ID	Nominal concentration (ng/mL)	Intra-batch				Inter-batch			
		N	Mean concentration observed (ng/mL)†	% CV	% Accuracy	N	Mean concentration observed (ng/mL)‡	% CV	% Accuracy
Ritonavir									
HQC	1,338	6	1,489	4.8	111.3	15	1,338	3.8	108.6
MQC	120	6	122	2.7	101.2	15	120	3.9	99.5
LQC	8.48	6	8.30	4.7	97.9	15	8.48	3.9	100.2
LLOQ QC	3.05	6	2.84	7.5	93.0	15	3.05	9.3	101.5
Lopinavir									
HQC	13,303	6	12,564	1.6	94.4	15	13,303	2.2	92.7
MQC	1,197	6	1,205	1.6	100.6	15	1,197	1.9	99.7
LQC	84.3	6	82.2	0.8	97.5	15	84.3	0.9	97.2
LLOQ QC	30.3	6	28.9	4.7	95.2	15	30.3	4.1	93.6
Indinavir									
HQC	13,656	6	13,218	3.8	94.9	15	13,656	6.5	93.4
MQC	1,229	6	1,225	3.2	97.8	15	1,229	5.1	100.7
LQC	83.6	6	82.7	4.7	97.0	15	83.6	6.3	100.3
LLOQ QC	31.8	6	32.1	5.4	99.1	15	31.8	8.3	101.9

**N*: total number of observations.
†Mean of six replicates at each concentration.
‡Mean of 15 replicates at each concentration.

Table II
Absolute Matrix Effect, Relative Recovery and Process Efficiency for Ritonavir, Lopinavir and Indinavir

Analyte	A* (%CV)	B† (%CV)	C‡ (%CV)	Absolute matrix effect(% ME)§	Relative recovery(% RE)**	Process efficiency (% PE)††
LQC						
Ritonavir	3,642 (3.01)	3,940 (7.80)	4,175 (2.71)	108.2	106.0	114.6
Lopinavir	43,024 (2.10)	47,484 (1.84)	47,297 (2.82)	110.4	99.6	109.9
Indinavir	5,923 (3.6)	6,067 (4.8)	5,754 (3.2)	102.4	94.8	97.1
MQC						
Ritonavir	49,583 (4.85)	50,996 (4.88)	48,473 (1.91)	102.8	95.1	97.8
Lopinavir	582,941 (1.66)	613,358 (1.42)	650,713 (1.85)	105.2	106.1	111.6
Indinavir	81,595 (1.87)	76,818 (4.26)	79,742 (2.9)	94.1	103.8	97.7
HQC						
Ritonavir	559,102 (1.70)	596,244 (3.37)	571,788 (1.26)	106.6	95.9	102.3
Lopinavir	5,652,098 (1.83)	5,980,575 (1.63)	5,879,164 (1.40)	105.8	98.3	104.0
Indinavir	784,991 (3.13)	757,591 (3.32)	741,977 (3.7)	96.5	97.9	94.5

*Mean area response of six replicate samples prepared in mobile phase (neat samples).
†Mean area response of six replicate samples prepared by spiking in extracted blank plasma.
‡Mean area response of six replicate samples prepared by spiking before extraction.
§B/A × 100.
**C/B × 100.
††C/A × 100 = (ME × RE)/100.

Application of the method in healthy human subjects

The proposed validated method was successfully applied for the assay of LPV and RTV in healthy male volunteers in the age group of 18 to 45 years. Figure 5 shows the mean plasma concentration-time profile following oral administration of [200 (LPV+ 50 (RTV)] mg tablet test formulation and Kaletra (lopinavir and ritonavir) tablets, 200/50 mg (Abbott Laboratories, Chicago, IL) reference to 36 healthy Indian male subjects under fasting condition. In all, approximately 3,000 samples, including the calibration and QC samples with volunteer samples, were run and analyzed during a period of 10 days and the precision and accuracy for calibration and QC samples

Table III
Relative Matrix Effect in Six Different Lots of Human Plasma (Spiked After Extraction) for Ritonavir, Lopinavir and Indinavir at LLOQ Level ($n = 6$)

Ritonavir			Lopinavir		Indinavir	
Lot No.	Mean area response	%CV	Mean area response	%CV	Mean area response	%CV
1	1,213	3.1	20,489	4.3	1,508	1.9
2	1,233	2.9	20,752	3.9	1,490	2.7
3	1,271	3.5	21,085	2.5	1,798	1.1
4	1,191	3.9	19,251	4.9	1,565	3.5
5	1,209	1.2	20,062	3.2	1,589	1.5
6	1,144	1.7	20,512	2.7	1,503	1.8

Table IV
Stability of Ritonavir Under Various Conditions ($n = 3$)

Storage conditions	Ritonavir		
	Nominal concentration (ng/mL)	Mean calculated concentration (ng/mL) ± SD	% Mean accuracy
Bench top stability, 6 h, Ambient			
HQC	1,338	1,511 ± 69.6	113.0
LQC	8.48	8.49 ± 0.6	100.1
Freeze and thaw stability, 3 cycles			
HQC	1,338	1,480 ± 16.0	110.6
LQC	8.48	8.54 ± 0.7	100.7
Wet extract stability, 23 h			
HQC	1,338	1,478 ± 44.6	110.5
LQC	8.48	8.04 ± 0.40	94.8
Long-term stability in plasma at −78°C for 60 days			
HQC	1,338	1,418 ± 22.7	106.3
LQC	8.48	8.86 ± 0.90	104.5

Table V
Stability of Lopinavir Under Various Conditions ($n = 3$)

Storage conditions	Lopinavir		
	Nominal concentration (ng/mL)	Mean calculated concentration (ng/mL) ± SD	% Mean accuracy
Bench top stability, 6 h, Ambient			
HQC	13,303	13,462 ± 148	101.2
LQC	84.3	82.4 ± 0.85	97.7
Freeze and thaw stability, 3 cycles			
HQC	13,303	14,008 ± 102	105.3
LQC	84.3	86.6 ± 0.73	102.7
Wet extract stability, 23 h			
HQC	13,303	14,340 ± 157	107.8
LQC	84.3	86.5 ± 0.65	102.6
Long-term stability in plasma at −78°C for 60 days			
HQC	13,303	14,606 ± 186	109.8
LQC	84.3	85.9 ± 0.48	101.9

Table VI
Stability of Indinavir Under Various Conditions ($n = 3$)

Storage conditions	Indinavir		
	Nominal concentration (ng/mL)	Mean calculated concentration (ng/mL) ± SD	% Mean accuracy
Bench top stability, 6 h, Ambient			
HQC	13,656	12,876 ± 637	92.4
LQC	83.6	77.8 ± 3.6	91.3
Freeze and thaw stability, 3 cycles			
HQC	13,656	13,107 ± 962	94.1
LQC	83.6	86.0 ± 3.5	92.5
Wet extract stability, 23 h			
HQC	13,656	11,824 ± 369	86.6
LQC	83.6	88.8 ± 3.2	106.2
Long-term stability in plasma at −78°C for 60 days			
HQC	13,656	14,420 ± 283	105.6
LQC	83.6	84.6 ± 2.6	101.2

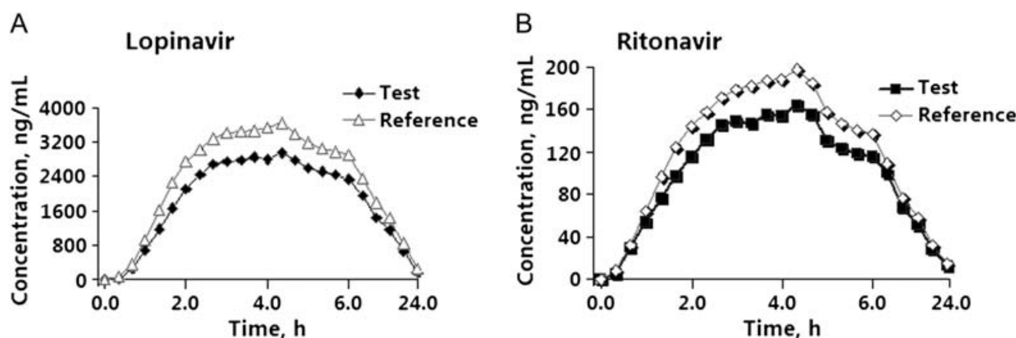

Figure 5. Mean plasma concentration-time profile after oral administration of [200 (LPV) +50 (RTV)] mg of test and reference tablet formulation to 36 healthy volunteers under fasting conditions: lopinavir (A); ritonavir (B).

Table VII
Mean Pharmacokinetic Parameters of Lopinavir and Ritonavir After Oral Dose of 200/50 mg of Lopinavir/Ritonavir Tablet Formulation to 36 Healthy Human Subjects Under Fasting Condition

Parameter	Lopinavir		Ritonavir	
	Test Mean ± SD	Reference* Mean ± SD	Test Mean ± SD	Reference* Mean ± SD
C_{max} (ng/mL)	3,303 ± 1,175	3,656 ± 1,484	202 ± 105	203 ± 111
T_{max} (h)	4.0 ± 0.7	3.3 ± 1.2	3.9 ± 0.9	3.2 ± 1.2
$t_{1/2}$ (h)	4.4 ± 1.5	4.5 ± 1.7	5.4 ± 1.2	5.3 ± 1.0
AUC_{0-24h} (h.ng/mL)	32,060 ± 13,531	34,997 ± 17,859	1,685 ± 869	1,633 ± 857
AUC_{0-inf} (h.ng/mL)	34,077 ± 15,295	37,023 ± 19,416	1,805 ± 949	1,747 ± 924

*Kaletra (lopinavir and ritonavir) tablets, 200/50 mg (Abbott Laboratories, Chicago, IL).

were well within the acceptable limits. The mean pharmacokinetic parameters; that is, C_{max}, AUC_{0-t}, AUC_{0-inf}, T_{max}, K_{el} and $t_{1/2}$, calculated for test and reference formulation are presented in Table VII. C_{max} values for LPV in more than 50% of subject samples were higher than the ULOQ reported in previous studies (53, 55). Similarly, for RTV, the C_{max} values were much higher than ULOQ value (55) for approximately 40% subject samples. This is evident from large variation in the standard deviation values in Table VII. The 90% confidence interval of individual ratio geometric mean for test/reference was within 80–125% for AUC_{0-t}, AUC_{0-inf} and C_{max}. These observations confirm the bioequivalence of the test sample with the reference product in terms of rate and extent of absorption. Furthermore, no adverse event occurred during the course of the study.

Conclusions

The UPLC–MS-MS methodology presented for simultaneous estimation of RTV, LPV and IDV in human plasma is highly selective and rugged for routine measurement of these drugs in combination therapy. The wide linear dynamic range ensures measurement of the drugs in all currently available formulations of different strengths. The method involved a simple, efficient and specific sample preparation with shorter chromatographic time. The small plasma requirement for processing is beneficial, especially for patients infected with HIV. The overall analysis time is promising compared to other reported procedures (53–55) for their simultaneous determination.

Acknowledgments

The authors are thankful to scientists and management of Veeda Clinical Research for providing technical support and infrastructure facility respectively to carrying out this work.

References

1. Gallant, J.E.; Initial therapy of HIV infection; *Journal of Clinical Virology*, (2002); 25:317–333.
2. Clevenbergh, P., Mouly, S., Sellier, P., Badsi, E., Cervoni, J., Vincent, V., *et al.*; Improving HIV infection management using antiretroviral plasma drug levels monitoring: a clinician's point of view; *Current HIV Research*, (2004); 2:309–321.
3. Boffito, M., Acosta, E., Burger, D., Fletcher, C.V., Flexner, C., Garaffo, R., *et al.*; Current status and future prospects of therapeutic drug monitoring and applied clinical pharmacology in antiretroviral therapy; *Antiviral Therapy*, (2005);10:375–392.
4. von Hentig, N.; Decrease of atazanavir and lopinavir plasma concentrations in a boosted double human immunodeficiency virus protease inhibitor salvage regimen; *Drugs Today (Barc.)*, (2007); 43:221–247.
5. Martin, T.M., Morse, G.D., Kurpewski, J., Difrancesco, R., Caliendo, A.M., Flanigan, T.P., *et al.*; Plasma and cerebrospinal pharmacokinetics and pharmacodynamics in subjects taking lopinavir/ritonavir; *AIDS*, (2006); 20:1085–1087.
6. Porche, D.J. Lopinavir/Ritonavir; *Journal of the Association of Nurses in AIDS Care*, (2001); 12:101–104.
7. Cvetkovic, R.S., Goa, K.L.; Lopinavir/ritonavir: a review of its use in the management of HIV infection; *Drugs*, (2003); 63: 769–802.
8. Azoulay, S., Nevers, M.C., Creminon, C., Heripret, L., Garraffo, R., Durant, J., *et al.*; Sensitive enzyme immunoassay for measuring

plasma and intracellular nevirapine levels in human immunodeficiency virus-infected patients; *Journal of Immunological Methods*, (2004); 295:37–48.
9. DiFrancesco, R., DiCenzo, R., Vicente, G., Donnelly, J., Martin, T.M., Colon, L.A., *et al.*; Determination of lopinavir cerebral spinal fluid concentrations by liquid chromatography coupled to tandem mass spectrometry; *Journal of Pharmaceutical and Biomedical Analysis*, (2007); 44:1139–1146.
10. Marsh, K.C., Eiden, E., McDonald, E.; Determination of ritonavir, a new HIV protease inhibitor, in biological samples using reversed-phase high-performance liquid chromatography; *Journal of Chromatography B*, (1997); 704: 307–313.
11. Granda, B.W., Giancarlo, G.M., von Moltke, L.L., Greenblatt, D.J.; Analysis of ritonavir in plasma/serum and tissues by high-performance liquid chromatography; *Journal of Pharmacological and Toxicological Methods*, (1998); 40: 235–239.
12. Hoetelmans, R.M.W., vanEssenberg, M., Profijt, M., Meenhorst, P.L., Mulder, J.W., Beijnen, J.H.; High-performance liquid chromatography of ritonavir in human plasma, saliva and cerebrospinal fluid; *Journal of Chromatography B*, (1998); 705: 119–126.
13. Akeb, F., Ferrua, B., Creminon, C., Roptin, C., Grassi, J., Nevers, M.C., *et al.*; Quantification of plasma and intracellular levels of the HIV protease inhibitor ritonavir by competitive ELISA; *Journal of Immunological Methods*, (2002); 263: 1–9.
14. Marzolini, C., Begiun, A., Telenti, A., Schreyer, A., Buclin, T., Biollaz, J., *et al.*; Transplacental passage of protease inhibitors at delivery; *Journal of Chromatography B*, (2002); 774: 127–140.
15. Frappier, S., Breilh, D., Diarte, E., Ba, B., Ducint, D., Pellegrin, J.L., *et al.*; Simultaneous determination of ritonavir and saquinavir, two human immunodeficiency virus protease inhibitors, in human serum by high-performance liquid chromatography; *Journal of Chromatography B*, (1998); 714: 384–389.
16. Albert, V., Modamio, P., Lastra, C.F., Marino, E.L.; Determination of saquinavir and ritonavir in human plasma by reversed-phase high-performance liquid chromatography and the analytical error function; *Journal of Pharmaceutical and Biomedical Analysis*, (2004); 36: 835–840.
17. Langmann, P., Klinker, H., Schirmer, D., Zilly, M., Bienert, A., Richter, E.; High-performance liquid chromatographic method for the simultaneous determination of HIV-1 protease inhibitors indinavir, saquinavir and ritonavir in plasma of patients during highly active antiretroviral therapy; *Journal of Chromatography B*, (1999); 735: 41–50.
18. Janoly, A., Bleyzac, N., Favetta, P., Gagneu, M.C., Bourhis, Y., Coudray, S., *et al.*; Simple and rapid high-performance liquid chromatographic method for nelfinavir, M8 nelfinavir metabolite, ritonavir and saquinavir assay in plasma; *Journal of Chromatography B*, (2002); 780: 155–160.
19. Fin, B., Stewart, J.T.; Determination of zidovudine/lamivudine/nevirapine in human plasma using ion-pair HPLC; *Journal of Pharmaceutical and Biomedical Analysis*, (2002); 30: 955–960.
20. Gutleben, W., Tuan, N.D., Stoiber, H., Dietrich, M.P., Sarcletti, M., Zemann, A.; Capillary electrophoretic separation of protease inhibitors used in human-immunodeficiency-virus therapy; *Journal of Chromatography A*, (2001); 922: 313–320.
21. Pereira, E.A., Micke, G.A., Tavares, M.F.M.; Determination of antiretroviral agents in human serum by capillary electrophoresis; *Journal of Chromatography A*, (2005); 1091: 169–176.
22. Ray, J., Pang, E., Carey, D.; Simultaneous determination of indinavir, ritonavir and lopinavir (ABT 378) in human plasma by high-performance liquid chromatography; *Journal of Chromatography B*, (2002); 775:225–230.
23. Usami, Y., Oki, T., Nakai, M., Sagisaka, M., Kaneda, T.; A simple HPLC method for simultaneous determination of lopinavir, ritonavir and efavirenz; *Chemical and Pharmaceutical Bulletin*, (2003); 51: 715–718.
24. van Heeswijk, R.P.G., Hoetelmans, R.M.W., Harms, R., Meenhorst, P.L., Mulder, J.W., Lange, J.M.A., *et al.*; Simultaneous quantitative determination of the HIV protease inhibitors amprenavir, indinavir, nelfinavir, ritonavir and saquinavir in human plasma by ion-pair high-performance liquid chromatography with ultraviolet detection; *Journal of Chromatography B*, (1998); 719: 159–168.
25. Hugen, P.W.H., Verweij-van Wissen, C.P.W.G.M., Burger, D.M., Wuis, E.W., Koopmans, P.P., Hekster, Y.A.; Simultaneous determination of the HIV-protease inhibitors indinavir, nelfinavir, saquinavir and ritonavir in human plasma by reversed-phase high-performance liquid chromatography; *Journal of Chromatography B*, (1999); 727: 139–149.
26. Marzolini, C., Telenti, A., Buclin, T., Biollaz, J., Decosterd, L.A.; Simultaneous determination of the HIV protease inhibitors indinavir, amprenavir, saquinavir, ritonavir, nelfinavir and the nonnucleoside reverse transcriptase inhibitor efavirenz by high-performance liquid chromatography after solid-phase extraction; *Journal of Chromatography B*, (2000); 740: 43–58.
27. Proust, V., Toth, K., Hulin, A., Taburet, A.M., Gimenez, F., Singlas, E.; Simultaneous high-performance liquid chromatographic determination of the antiretroviral agents amprenavir, nelfinavir, ritonavir, saquinavir, delavirdine and efavirenz in human plasma; *Journal of Chromatography B*, (2000); 742: 453–458.
28. Aymard, G., Legrand, M., Trichereau, N., Diquet, B.; Determination of twelve antiretroviral agents in human plasma sample using reversed-phase high-performance liquid chromatography; *Journal of Chromatography B*, (2000); 744: 227–240.
29. Simon, V.A., Thiam, M.D., Lipford, L.C.; Determination of serum levels of thirteen human immunodeficiency virus-suppressing drugs by high-performance liquid chromatography; *Journal of Chromatography A*, (2001); 913: 447–453.
30. Yamada, H., Kotaki, H., Nakamura, T., Iwamoto, A.; Simultaneous determination of the HIV protease inhibitors indinavir, amprenavir, saquinavir, ritonavir and nelfinavir in human plasma by high-performance liquid chromatography; *Journal of Chromatography B*, (2001); 755: 85–89.
31. Dailly, E., Thomas, L., Kergueris, M.F., Jolliet, P., Bourin, M.; High-performance liquid chromatographic assay to determine the plasma levels of HIV-protease inhibitors (amprenavir, indinavir, nelfinavir, ritonavir and saquinavir) and the non-nucleoside reverse transcriptase inhibitor (nevirapine) after liquid-liquid extraction; *Journal of Chromatography B*, (2001); 758: 129–135.
32. Sarasa-Nacenta, M., Lopez-Pua, Y., Mallolas, J., Blanco, J.L., Gatell, J.M., Carne, X.; Simultaneous determination of the HIV-protease inhibitors indinavir, amprenavir, ritonavir, saquinavir and nelfinavir in human plasma by reversed-phase high-performance liquid chromatography; *Journal of Chromatography B*, (2001); 757: 325–332.
33. Justesen, U.S., Pedersen, C., Klitgaard, N.A.; Simultaneous quantitative determination of the HIV protease inhibitors indinavir, amprenavir, ritonavir, lopinavir, saquinavir, nelfinavir and the nelfinavir active metabolite M8 in plasma by liquid chromatography; *Journal of Chromatography B*, (2003); 783: 491–500.
34. Turner, M.L., Reed-Walker, K., King, J.R., Acosta, E.P.; Simultaneous quantitation of protease inhibitors and non-nucleoside reverse transcriptase inhibitors in human plasma using a single high-performance liquid chromatography methodology; *Journal of Chromatography B*, (2003); 784: 331–341.
35. Rezk, N.L., Tidwell, R.R., Kashuba, A.D.M.; High-performance liquid chromatography assay for the quantification of HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors in human plasma; *Journal of Chromatography B*, (2004); 805: 241–247.
36. Notari, S., Bocedi, A., Ippolito, G., Narciso, P., Paolo, L.P., Tossini, G., *et al.*; Simultaneous determination of 16 anti-HIV drugs in human plasma by high-performance liquid chromatography; *Journal of Chromatography B*, (2006); 831: 258–266.
37. Colombo, S., Beguin, A., Marzolini, C., Telenti, A., Biollaz, J., Decosterd, L.A.; Determination of the novel non-peptidic HIV-protease inhibitor tipranavir by HPLC–UV after solid-phase extraction; *Journal of Chromatography B*, (2006); 832: 138–143.
38. Hirabayashi, Y., Tsuchiya, K., Kimura, S., Oka, S.; Simultaneous determination of six HIV protease inhibitors (amprenavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir), the active metabolite of nelfinavir (M8) and non-nucleoside reverse transcriptase inhibitor

(efavirenz) in human plasma by high-performance liquid chromatography; *Biomedical Chromatography*, (2006); 20: 28–36.
39. Weller, D.R., Brundage, R.C., Balfour, H.H., Jr., Vezina, H.E.; An isocratic liquid chromatography method for determining HIV non-nucleoside reverse transcriptase inhibitor and protease inhibitor concentrations in human plasma; *Journal of Chromatography B*, (2007); 848: 369–373.
40. Verbesselt, R., Wijngaerden, E.V., de Hoon, J.; Simultaneous determination of 8 HIV protease inhibitors in human plasma by isocratic high-performance liquid chromatography with combined use of UV and fluorescence detection: Amprenavir, indinavir, atazanavir, ritonavir, lopinavir, saquinavir, nelfinavir and M8-nelfinavir metabolite; *Journal of Chromatography B*, (2007); 845: 51–60.
41. Choi, S.O., Rezk, N.L., Kashuba, A.D.M.; High-performance liquid chromatography assay for the determination of the HIV-protease inhibitor tipranavir in human plasma in combination with nine other antiretroviral medications; *Journal of Pharmaceutical and Biomedical Analysis*, (2007); 43: 1562–1567.
42. Naidong, W., Bu, H., Chen, Y.-L., Shou, W.Z., Jiang, X., Halls, T.D.J.; Simultaneous development of six LC-MS-MS methods for the determination of multiple analytes in human plasma; *Journal of Pharmaceutical and Biomedical Analysis*, (2002); 28: 1115–1126.
43. Chi, J., Jayewardene, A.L., Stone, J.A., Motoya, T., Aweeka, F.T.; Simultaneous determination of five HIV protease inhibitors nelfinavir, indinavir, ritonavir, saquinavir and amprenavir in human plasma by LC/MS/MS; *Journal of Pharmaceutical and Biomedical Analysis*, (2002); 30: 675–684.
44. Frerichs, V.A., DiFrancesco, R., Morse, G.D.; Determination of protease inhibitors using liquid chromatography-tandem mass spectrometry; *Journal of Chromatography B*, (2003); 787: 393–403.
45. Rentsch, K.M.; Sensitive and specific determination of eight antiretroviral agents in plasma by high-performance liquid chromatography-mass spectrometry; *Journal of Chromatography B*, (2003); 788: 339–350.
46. Rouzes, A., Berthoin, K., Xuereb, F., Djabarouti, S., Pellegrin, I., Pellegrin, J.L., *et al.*; Simultaneous determination of the antiretroviral agents: amprenavir, lopinavir, ritonavir, saquinavir and efavirenz in human peripheral blood mononuclear cells by high-performance liquid chromatography–mass spectrometry; *Journal of Chromatography B*, (2004); 813: 209–216.
47. Dickinson, L., Robinson, L., Tjia, J., Khoo, S., Back, D.; Simultaneous determination of HIV protease inhibitors amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir in human plasma by high-performance liquid chromatography-tandem mass spectrometry; *Journal of Chromatography B*, (2005); 829: 82–90.
48. Colombo, S., Beguin, A., Telenti, A., Biollaz, J., Buclin, T., Rochat, B., *et al.*; Intracellular measurements of anti-HIV drugs indinavir, amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir, atazanavir, efavirenz and nevirapine in peripheral blood mononuclear cells by liquid chromatography coupled to tandem mass spectrometry; *Journal of Chromatography B*, (2005); 819: 259–276.
49. Gehring, A.-K., Mikus, G., Haefeli, W.E., Burhnne, J.; Electrospray tandem mass spectroscopic characterisation of 18 antiretroviral drugs and simultaneous quantification of 12 antiretrovirals in plasma; *Rapid Communications in Mass Spectrometry*, (2007); 21: 2704–2716.
50. Avolio, A.D., Siccardi, M., Sciandra, M., Lorena, B., Bonora, S., Trentini, L., *et al.*; HPLC-MS method for the simultaneous quantification of the new HIV protease inhibitor darunavir, and 11 other antiretroviral agents in plasma of HIV-infected patients; *Journal of Chromatography B*, (2007); 859: 234–240.
51. Ter Heine, R., Rosing, H., van Gorp, E.C.M., Mulder, J.W., van der Steeg, W.A., Beijnen, J.H., *et al.*; Quantification of protease inhibitors and non-nucleoside reverse transcriptase inhibitors in dried blood spots by liquid chromatography–triple quadrupole mass spectrometry; *Journal of Chromatography B*, (2008); 867: 205–212.
52. Notari, S., Mancone, C., Tripodi, M., Narciso, P., Fasano, M., Ascenzi, P. Determination of anti-HIV drug concentration in human plasma by MALDI-TOF/TOF; *Journal of Chromatography B*, (2006); 833:109–116.
53. Wang, P.C., Wei, J.S., Kim, G., Chang, M., El-Shourbagy, T.; Validation and application of a high-performance liquid chromatography-tandem mass spectrometric method for simultaneous quantification of lopinavir and ritonavir in human plasma using semi-automated 96-well liquid–liquid extraction; *Journal of Chromatography A*, (2006); 1130: 302–307.
54. Ehrhardt, M., Mock, M., Haefeli, W.E., Mikus, G., Burhenne, J.; Monitoring of lopinavir and ritonavir in peripheral blood mononuclear cells, plasma, and ultrafiltrate using a selective and highly sensitive LC/MS/MS assay; *Journal of Chromatography B*, (2007); 850: 249–258.
55. Estrela, R.C., Ribeiro, F.S., Seixes, B.V., Suarez-Kurtz, G.; Determination of lopinavir and ritonavir in blood plasma, seminal plasma, saliva and plasma ultra-filtrate by liquid chromatography/tandem mass spectrometry detection; *Rapid Communications in Mass Spectrometry*, (2008); 22: 657–664.
56. Dickinson, L., Robinson, L., Tjia, J., Khoo, S., Back, D.; Simultaneous determination of HIV protease inhibitors amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir in human plasma by high-performance liquid chromatography-tandem mass spectrometry; *Journal of Chromatography B*, (2005); 829: 82–90.
57. Crommentuyn, K.M., Rosing, H., Nan-Offeringa, L.G., Hillebrand, M.J., Huitema, A.D., Beijnen, J.H.; Rapid quantification of HIV protease inhibitors in human plasma by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry; *Journal of Mass Spectrometry*, (2003); 38: 157–166.
58. Yadav, M., Rao, R., Kurani, H., Singhal, S., Goswami, S., Shrivastav, P.S.; Application of a rapid and selective method for the simultaneous determination of protease inhibitors, lopinavir and ritonavir in human plasma by UPLC-ESI-MS/MS for bioequivalence study in Indian subjects; *Journal of Pharmaceutical and Biomedical Analysis*, (2009); 49: 1115-1122.
59. Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), (2001). http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf (assessed September 17, 2011).
60. Guidance for Industry: ICH E6Good Clinical Practice, U.S. Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Centre for Biologics Evaluation and Research (CBER), (1996). http://www.fda.gov/downloads/regulatoryinformation/guidances/ucm129515.pdf (assessed September 17, 2011).