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Short communication

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

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ABSTRACT

A high throughput and rugged ultra performance liquid chromatography tandem mass spectrometry (UPLC–ESI-MS/MS) method is developed and validated for the selective determination of protease inhibitors – lopinavir (LPV) and ritonavir (RTV) in human plasma. Plasma samples were prepared by solid phase extraction of the analytes and their deuterated analogs as internal standard (IS) using Waters Oasis HLB cartridges. The chromatographic separation was achieved in a run time of 1.2 min on Waters Acquity UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm) under isocratic conditions. The mobile phase consisted of 10 mM ammonium formate, pH 4.0 adjusted with formic acid and methanol (10:90, v/v). The protonated precursor → product ion transitions for lopinavir, ritonavir, d_8-lopinavir and d_6-ritonavir were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) and positive ion mode. A linear dynamic range of 2.9–1452 ng/mL and 29.6–14379 ng/mL was established for ritonavir and lopinavir respectively using 0.1 mL human plasma. The mean relative recovery of lopinavir (96.6%), ritonavir (97.5%), d_8-lopinavir (85.5%) and d_6-ritonavir (86.3%) from spiked plasma samples was consistent and reproducible. The method was successfully applied to a bioequivalence study of [200(lopinavir) + 50(ritonavir)] mg tablet formulation in 36 healthy human subjects under fasting conditions.

1. Introduction

Protease inhibitors are characterized pharmacologically by their ability to inhibit the viral protease enzyme and form an integral part of highly active antiretroviral therapy (HAART) regimens [1]. They prevent T-cells that have been infected from HIV, from producing new copies of the virus and in preventing maturation of the HIV virus [2,3]. Lopinavir (LPV) is a novel peptidomimetic HIV protease inhibitor developed from and structurally related to ritonavir (RTV). Both these drugs are currently marketed under the brand name Kaletra in a fixed-dose combination (133 mg LPV and 33 mg RTV) [4,5]. The low-dose of ritonavir significantly improves the pharmacokinetic properties and hence the activity of LPV against HIV-1 protease. RTV inhibits the cytochrome P450 CYP3A isoenzymes that inactivate LPV, thereby increasing its circulating levels. Co-formulated LPV/RTV-based regimens provide adequate and durable suppression of viral load and sustained improvements in CD4+ cell counts [6]. Due to their extensive binding to plasma proteins, essentially α1-acid glycoprotein and albumins (98–99%), they have limited distribution in the body. LPV and RTV are extensively metabolized by the liver and are eliminated in the urine and faces. Their half-life range varies from 3–6 h [7]. The simultaneous determination of these drugs in biological matrices, along with their pharmacokinetic study can assist in checking their effectiveness, treatment compliance, to prevent adverse events, and to formulate optimum dosages.

Due to extensive use of both these protease inhibitors 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 viz. plasma, cerebral spinal fluid, serum, tissues and saliva. Other procedures present determination of LPV with nevirapine [14]; RTV with saquinavir [15,16], indinavir and saquinavir [17], nelfinavir and saquinavir [18], zidovudine and didanosine [19]. An extensive literature is available for their simultaneous quantitation with other protease inhibitors by HPLC [20–27], LC–MS/MS

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[28–33] and MALDI–TOF/TOF [34]. So far only three LC–MS/MS methods [35–37] are reported for the simultaneous estimation of only LPV and RTV in biological samples. They were applied to monitor their concentration for bioequivalence study in subjects who received oral dose of Kaletra. Although the above methods are adequately sensitive, there has been no report of their simultaneous determination by UPLC–MS/MS. Due to ever increasing demands for assays with higher sensitivity and reduced overall analysis time, the use of UPLC has created a step-function improvement in chromatographic performance due to interlaced attributes of speed, sensitivity and resolution. UPLC coupled with MS/MS detection greatly improves the sensitivity and selectivity and causes a significant increase in sample throughput over traditional LC–MS/MS systems. Thus, the aim of the proposed work was to develop and validate a high throughput (overall analysis time), selective and rugged UPLC–MS/MS method for routine measurement of LPV and RTV in subject samples. The validated method presents excellent performance in terms of selectivity, ruggedness and efficiency (1.2 min per sample). Due to high inter-subject variability, a wide linear dynamic range was established, which ensures the estimation of both the inhibitors with desired accuracy and precision in healthy human volunteers for bioequivalence study.

2. Experimental

2.1. Chemicals and materials

Reference standards of lopinavir (98.54%) and ritonavir (101.3%) were procured from Samex Overseas (Surat, India). Deuterated internal standards d_8-lopinavir (94.97%, IS for lopinavir) and d_6-ritonavir (98.0%, IS for ritonavir) were purchased from Toronto Research Chemicals Inc. (North York, Canada). HPLC grade methanol and acetonitrile were obtained from Mallinckrodt Baker, (S.A.de C.V. Mexico). Guaranteed reagent grade formic acid and ammonium formate were obtained from Merck Specialties Pvt. Ltd., (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system procured from Millipore (Bangalore, India). Oasis HLB extraction cartridges (30 mg/1 mL) were purchased from Waters (Bangalore, India). Blank human plasma was procured from Supratech Micropath (Ahmedabad, India) and was stored at −20 °C until use.

2.2. Liquid chromatographic conditions

A Waters Acquity UPLC system (Milford, MA, U.S.A.) consisting of binary solvent manager, sample manager and column manager was used for setting the reverse-phase liquid chromatographic conditions. The chromatography of LPV, RTV, d_8-LPV and d_6-RTV was performed on a Waters Acquity UPLC BEH C18, [50 mm × 2.1 mm (length × inner diameter), with 1.7 μm particle size] and was maintained at 40 °C in column oven. The mobile phase consisted of 10 mM ammonium formate, pH 4.0 adjusted with formic acid and methanol (10:90, v/v). For isocratic elution, the flow rate of the mobile phase was kept at 0.3 mL/min. The total chromatographic run time was 1.2 min. The sample manager temperature was maintained at 5 °C and the pressure of the system was 600 psi.

2.3. Mass spectrometric conditions

Ionization and detection of analytes and ISs was carried out on a Waters Quattro Premier XE (USA) triple quadrupole mass spectrometer, equipped with electro spray ionization and operating in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor protonated precursor → product ion transitions for LPV (m/z 629.3 → 447.4), d_8-LPV (m/z 637.4 → 447.4), RTV (m/z 721.3 → 296.3) and d_6-RTV (m/z 727.4 → 302.3).

The source dependent parameters maintained for LPV, RTV, d_8-LPV and d_6-RTV were cone gas flow: 100 ± 10 L/h; desolvation gas flow: 800 ± 10 L/h; ion spray voltage (ISV): 4000 V, source temperature: 100 °C; desolvation temperature: 400 °C; extractor volts: 4 V; collision activation dissociation gas (argon): 0.18 psig. The optimum values for compound dependent parameters like cone voltage and collision energy set were 25 and 15 V for LPV; 30 and 20 V for RTV; 25 and 15 V d_8-LPV; 25 and 20 V for d_6-RTV respectively. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 100 ms. MassLynx software version 4.1 was used to control all parameters of UPLC and MS.

2.4. Standard stock, calibration standards and quality control sample preparation

The standard stock solution of 10 mg/mL of LPV and 3 mg/mL of RTV were prepared by dissolving their requisite amount in methanol. Combined intermediate stock solution was prepared by mixing 1.5 mL of LPV, 0.5 mL of RTV and 8.0 mL of methanol. Calibration standards and quality control (QC) samples were prepared by spiking (2% of total plasma volume) blank plasma with combined intermediate stock solution. Calibration curve standards were made at 29.6, 59.3, 98.8, 198, 395, 899, 1797, 3595, 7189 and 14379 ng/mL concentrations for LPV and 2.9, 5.9, 9.9, 19.9, 39.9, 90.8, 182, 363, 726 and 1452 ng/mL for RTV, while quality control samples were prepared at four levels, viz. 1,3184 and 1333 ng/mL (HQC, high quality control), 1160 and 117 ng/mL (MQC, middle quality control), 84.5 and 8.5 ng/mL (LQC, low quality control) and 30.4 and 3.1 ng/mL (LLOQ QC, lower limit of quantification quality control) for LPV and RTV respectively. Separate stock solutions (1 mg/mL) of the internal standards were prepared in methanol. Their combined working solution (8 μg/mL of d_8-LPV and 1 μg/mL of d_6-RTV) was prepared in methanol: water (50:50, v/v). All the solutions were stored at 2–8 °C until use.

2.5. Protocol for sample preparation

To an aliquot of 100 μL of spiked plasma sample, 50 μL internal standard was added and vortexed for 10 s. Further, 100 μL of 10% formic acid was added and vortex mixed for another 10 s. The samples were loaded on Oasis HLB (1 cc, 30 mg) extraction cartridges which were preconditioned with 1 mL methanol followed by 1 mL of water. Subsequently, the cartridges were washed with 1 mL, 5% methanol in water and then dried for 2 min by applying 25 psi pressure at 2.4 L/min flow rate of nitrogen. Elution of analytes and ISs from the cartridges was carried out with 0.5 mL of 0.2% formic acid in methanol into pre-labeled tubes. The eluate was evaporated to dryness in a thermostatically controlled water-bath maintained at 40 °C under the stream of nitrogen for 5 min. After drying, the residue was reconstituted in 200 μL of reconstitution solution [10 mM ammonium formate: acetonitrile (20:80, v/v)] and 5 μL was used for injection in the chromatographic system.

2.6. Bio-analytical method validation procedures

A through and complete method validation was done following the USFDA guidelines [38]. System suitability tests and performance check was done before each batch to establish the overall compliance of the system. The acceptance criteria for these tests were based on change of ±2% in coefficient of variation (%CV) and a signal to noise ratio (S/N ≥10) respectively. The carryover effect of the autosampler was evaluated by sequentially injecting solutions

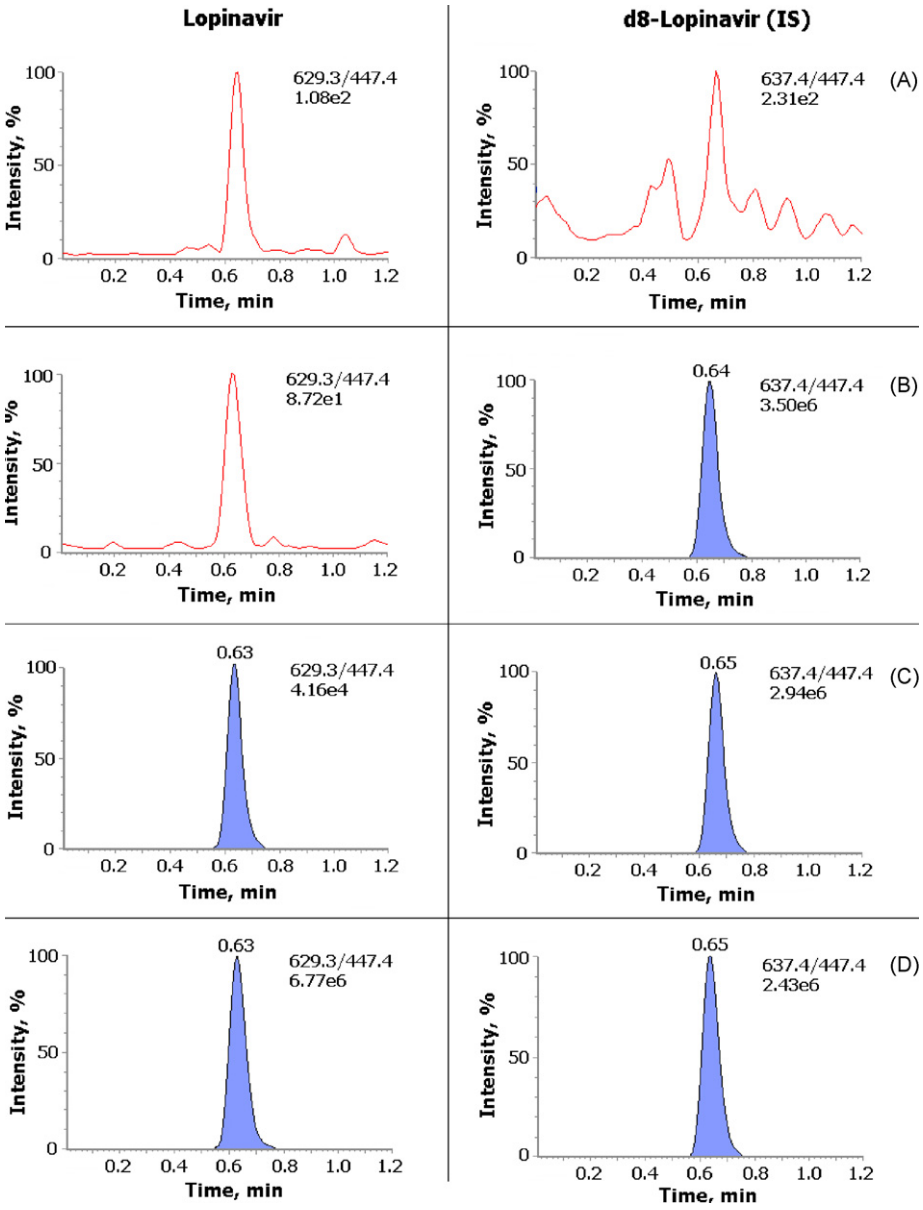

Fig. 1. MRM ion-chromatograms of lopinavir (m/z 629.3 → 447.4) and d_8-lopinavir (m/z 637.4 → 447.4) in (A) double blank plasma, (B) blank plasma with IS, (C) LLOQ and (D) real subject sample at 4 h.

of analytes (aqueous standard), reconstitution solution, standard blank and extracted standards of analytes, equivalent to highest standard in the calibration range.

The selectivity of the method towards endogenous plasma matrix components and concomitant medications was assessed in 12 batches (six normal of K3 EDTA, two haemolysed two lipemic and two heparinised) of blank human plasma. Cross talk of MRM channels for analytes and internal standards were checked using highest concentration from linear calibration curve and the working solution of internal standard. The effect of 10 antiretroviral drugs (nucleoside reverse transcriptase inhibitors – zidovudine, didanosine, stavudine, lamivudine, emtricitabine, abacavir; non-nucleoside reverse transcriptase inhibitors – efavirenz and nevirapine; protease inhibitors – indinavir, nelfinavir) was studied under the same conditions. Check for interference due to commonly used medications in human volunteers was done for acetaminophen, chlorpheniramine maleate, caffeine, acetylsalicylic acid and ibuprofen. Their stock solutions (1000 μg/mL) were prepared by dissolving requisite amount in methanol: water (50:50, v/v). Further, working solutions (1.0 μg/mL) were prepared in the mobile phase to check any possible interference.

The linearity of the method was determined by analysis of five standard plots containing ten non-zero concentrations. Peak area ratios of analyte/IS were utilized for the construction of calibration curves, using weighted ($1/x^2$) linear least squares regression of the plasma concentrations and the measured peak area ratios.

For determining the intra-day accuracy and precision, replicate analysis of plasma samples was performed on the same day. The inter-day accuracy and precision were assessed by analysis of three precision and accuracy batches on three consecutive validation days.

Ion suppression/enhancement effects on the MRM UPLC–MS/MS sensitivity were evaluated by the post column analyte infusion experiment. A standard solution containing all the analytes (at MQC level in methanol) was infused post column via a 'T' connector into the mobile phase at 10 μL/min employing in-built infusion pump. Aliquots of 5 μL of reconstitution solution and extracted control plasma were then injected into the column.

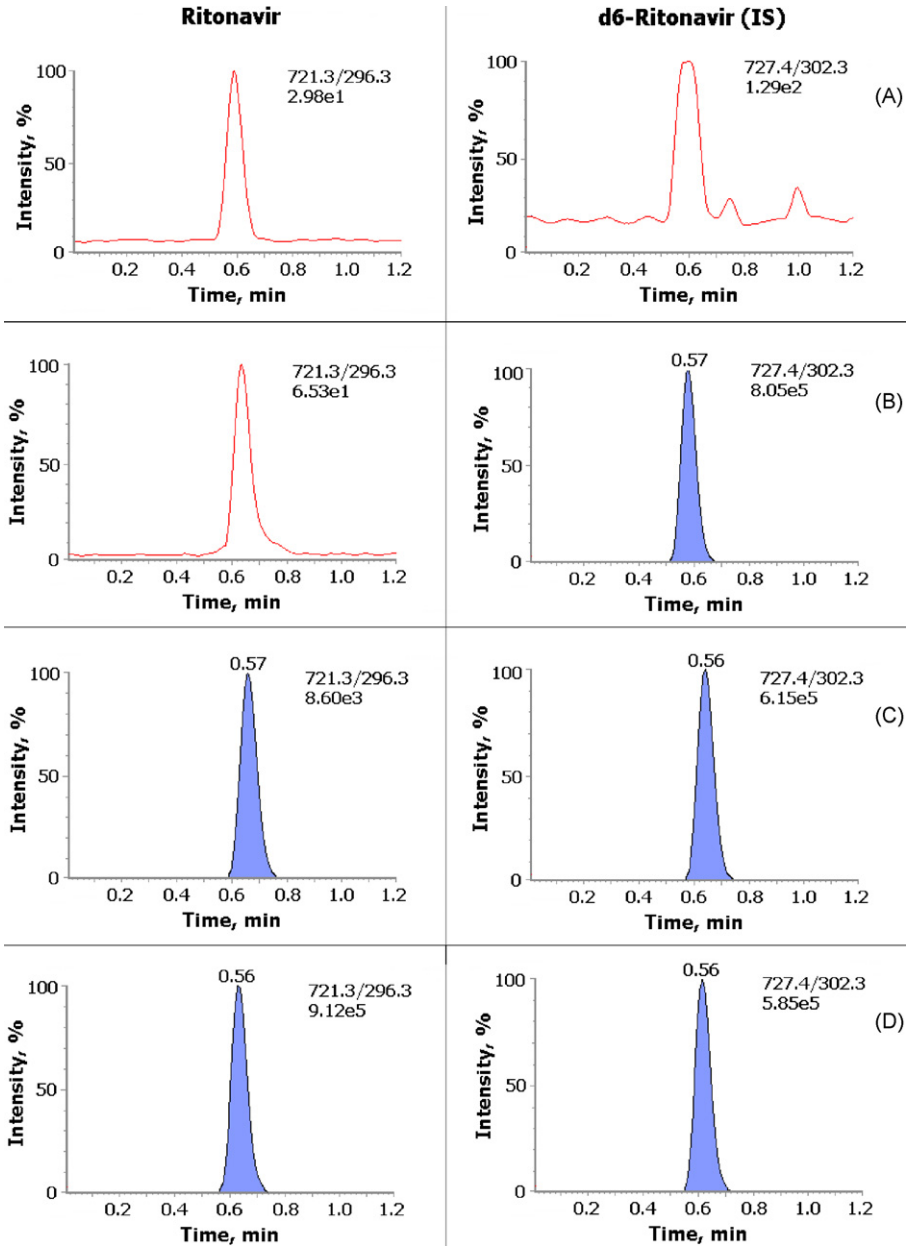

Fig. 2. MRM ion-chromatograms of ritonavir (*m/z* 721.3 → 296.3) and d_6-ritonavir (*m/z* 727.4 → 302.3) in (A) double blank plasma, (B) blank plasma with IS, (C) LLOQ and (D) real subject sample at 4 h.

The relative recovery, absolute matrix effect and process efficiency were assessed at HQC, MQC and LQC levels as recommended by Matuszewski et al. [39]. Further, relative matrix on analyte quantification was checked in seven different batches/lots of plasma. From each batch, six samples at HQC and LQC levels were prepared (spiked after extraction) and checked for precision (%CV).

Short term and long term stock solution stability at room temperature was assessed by comparing the area response of stability sample of analytes and ISs with the area response of sample prepared from fresh stock solutions. Autosampler stability (wet extract), bench top stability, dry extract stability and freeze-thaw stability were performed at LQC and HQC using three replicates at each level. Long term stability of analytes in human plasma was determined at −20 and −70 °C.

To authenticate the ruggedness of the proposed method, it was done on three precision and accuracy batches. The first batch was analyzed by different analysts, second batch was analyzed on two different columns and third batch was analyzed on two different equipments. Dilution integrity experiment was performed by diluting the stock solution of 2,9955 and 3025 ng/mL in the screened plasma for LPV and RTV respectively. The precision and accuracy for dilution integrity standards at 1/5th (5991and 605 ng/mL for LPV and RTV respectively) and 1/10th (2995.5 and 302.5 ng/mL for LPV and RTV respectively) dilution were determined by analyzing the samples against calibration curve standards.

3. Results and discussion

3.1. Bio analytical method development

The present study was conducted using positive electrospray ionization as it gave higher response under MRM with a signal to noise ratio >1700 (with validated/finalized integration parameters) and a good linearity in regression curves. The most stable

Table 1
Comparison of intra-batch and inter-batch precision and accuracy for lopinavir and ritonavir.

QC ID	Nominal concentration (ng/mL)	Intra-batch				Inter-batch			
		n	Mean concentration observed (ng/mL)[a]	%CV	%Accuracy	*n*	Mean concentration observed (ng/mL)[b]	%CV	%Accuracy
Lopinavir									
HQC	13184	5	13172	1.2	99.9	15	13094	1.3	99.3
MQC	1160	5	1118	2.0	96.3	15	1112	1.9	95.9
LQC	84.5	5	80.6	1.8	95.4	15	81.4	2.0	96.3
LLOQ QC	30.4	5	29.4	1.9	96.8	15	29.6	2.3	97.4
Ritonavir									
HQC	1333	5	1381	1.8	103.6	15	1384	1.8	103.8
MQC	117	5	118	2.1	100.7	15	117	1.7	100.1
LQC	8.5	5	8.5	4.4	99.5	15	8.4	3.6	98.4
LLOQ QC	3.1	5	3.0	1.4	98.8	15	3.0	4.7	96.5

n, total number of observation; CV, coefficient of variance.
[a] Mean of six replicates at each concentration level.
[b] Mean of 15 replicates at each concentration level.

and consistent product ions for LPV, RTV, d_8-LPV and d_6-RTV were observed at *m*/*z* 447.4, 296.3, 447.4 and 302.3 respectively. A dwell time of 100 ms for both the drugs was adequate and no cross talk was observed between their MRMs.

Due to high protein binding of these drugs, protein precipitation was tried initially with acetonitrile and methanol, but the response for RTV was inconsistent with significant matrix effect. Liquid–liquid extraction (LLE) has been reported for the simultaneous determination of these drugs in earlier reports [35–37]. Thus, LLE was tried with ethyl acetate, *tert*-butyl methyl ether, hexane and their combinations, however, the results for RTV especially at the LLOQ levels had very low precision (%CV), greater than 20%. Solid phase extraction of the analytes and ISs was tried on Waters Oasis HLB, Waters Oasis MCX and Phenomenex Strata cartridges for quantitative recoveries. Addition of formic acid during sample preparation helped in breaking drug–plasma binding and maintaining the analytes in the ionized form. Superior results with better retention was obtained on Waters Oasis HLB as compared to other cartridges in terms of precision, selectivity, reproducibility and recovery for calibration standards, quality control and subject (real) samples. Washing of cartridges was optimized through trials with water/methanol in combination with different concentrations of formic acid (v/v), however, 5% (v/v) methanol in water was the most acceptable choice in the present study. Further, elution of the analytes and ISs was realized with 2% formic acid in methanol after many attempts with different volume combinations of methanol/acetonitrile with formic acid. During sample preparation, different reconstitution solutions (10 mM ammonium formate, formic acid and methanol in different volume ratios), were tested to get clear solutions for the extracted drugs in the dried extract. Use of methanol along with 10 mM ammonium formate, pH 4.0 gave improved response with good peak shapes and negligible matrix effect. However, the solutions were slightly turbid, which was overcome by replacing methanol with acetonitrile in 80:20 (v/v) ratio. Quantitative and precise recoveries were obtained at all quality control levels with minimum matrix interference.

Chromatographic analysis of the analytes and ISs was initiated under isocratic conditions to obtain adequate response, sharp peak shape and a short run time. Previous reports have used long columns for their separation viz. Waters Symmetry C18 (150 mm × 3.9 mm, 5 μm) [35], Phenomenex Jupiter Proteo C12 (100 mm × 2 mm, 4 μm) [36], LiChrocart C18 (125 mm × 4 mm, 5 μm) [37]. Though, efficient separation was achieved for both the drugs, the run time was ≥4 in all the cases. Thus, a short column with smaller particle size, Waters Acquity UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm) was used for higher resolution and fast throughput. Mobile phase pH was studied in the range of 2–5

Table 2
Absolute matrix effect, relative recovery and process efficiency for lopinavir and ritonavir.

Analyte	A[a] (%CV)[b]	B[c] (%CV)[b]	C[d] (%CV)[b]	Absolute matrix effect (%ME)[e]	Relative recovery (%RE)[f]	Process efficiency (%PE)[g]
LQC						
Lopinavir	0.048 (0.00)	0.046 (1.55)	0.044 (0.01)	95.8 (98.6)[h]	95.7 (84.7)[h]	91.7 (83.5)[h]
Ritonavir	0.052 (2.96)	0.048 (1.49)	0.047 (0.01)	92.3 (98.9)[i]	97.9 (85.8)[i]	90.4 (84.9)[i]
MQC						
Lopinavir	0.658 (0.97)	0.625 (0.11)	0.583 (0.12)	95.0 (99.1)[h]	93.3 (86.1)[h]	88.6 (85.3)[h]
Ritonavir	0.606 (0.35)	0.545 (0.520)	0.527 (0.27)	90.0 (98.7)[i]	96.7 (86.7)[i]	87.0 (85.6)[i]
HQC						
Lopinavir	7.556 (0.44)	6.593 (1.15)	6.648 (0.34)	87.3 (99.3)[h]	100.8 (85.3)[h]	88.0 (84.7)[h]
Ritonavir	6.741 (0.76)	5.907 (1.97)	5.780 (1.02)	87.6 (99.2)[i]	97.9 (86.2)[i]	85.8 (85.5)[i]

[a] Mean area ratio (analyte/IS) response of six replicate samples prepared in mobile phase (neat samples).
[b] Coefficient of variation.
[c] Mean area ratio (analyte/IS) response of six replicate samples prepared by spiking in extracted blank plasma.
[d] Mean area ratio (analyte/IS) response of six replicate samples prepared by spiking before extraction.
[e] B/A × 100.
[f] C/B × 100.
[g] C/A × 100 = (ME × RE)/100;
[h] Values for internal standard (IS) d_8-LPV.
[i] Values for internal standard (IS) d_6-RTV.

using different combinations of ammonium acetate/acetic acid and ammonium formate/formic acid with methanol and acetonitrile. However, the best peak shape and adequate response was obtained at pH 4.0 with ammonium formate/formic acid and methanol (10:90, v/v). The total run time for both the drugs and their internal standards was 1.2 min with retention time of 0.63, 0.57 0.65 and 0.56 min for LPV, RTV, d_8-LPV and d_6-RTV respectively. Initially, use of adefovir and lamivudine as internal standards was associated with significant matrix effect. All possible combinations of extraction buffers of different pH, strength and ionization sources (ESI and APCI) tested, were inadequate to minimize or eliminate matrix effect on the analytes. Thus, deuterated standards of both the drugs (d_8-LPV and d_6-RTV) were selected as internal standards to achieve best results in the present study.

3.2. System suitability, performance check and auto sampler carry-over test

During method validation, the %CV of system suitability test was observed in the range of 0.01–0.72 for the retention time of analytes and the ISs, while 0.76–1.29% for the response of drug and its internal standard. The mean signal to noise ratio observed for LPV and RTV was 1782 and 1886 respectively. There was no carry-over observed during autosampler carryover experiment. Also, no enhancement in the response was observed in double blank after subsequent injection of highest calibration standard (aqueous and extracted) at the retention time of LPV, RTV, d_8-LPV and d_6-RTV respectively.

3.3. Linearity and lower limit of quantification (LLOQ)

All five calibration curves were linear for the standards ranging from 29.7 to 1,4379 ng/mL and 2.9 to 1452 ng/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 was 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.

3.4. Selectivity of the method, accuracy and precision

Figs. 1 and 2 demonstrates the selectivity experiments with the chromatograms of extracted (A) blank plasma, (B) blank plasma with IS, (C) peak response of LPV and RTV at LLOQ and (D) a real sample at 4 h after oral administration of [200(LPV) + 50(RTV)] mg tablet formulation. None of the concomitant medications and antiretrovirals considered showed interfering signals at the retention time of LPV, RTV, d_8-LPV or d_6-RTV. The intra-batch and inter-batch precision (%CV) and accuracy (%) results at all the QC levels for LPV and RTV are presented in Table 1.

3.5. Ion-suppression, recovery and matrix effect

Results of post-column analyte infusion experiment indicated no ion suppression or enhancement at the retention time of LPV, RTV, d_8-LPV or d_6-RTV. Minor ion suppression was observed around 0.1 min, which did not affect the quantification of the analytes. The relative recovery, absolute matrix effect and process efficiency data at LQC, MQC and HQC levels is presented in Table 2. Further, the precision values (%CV) for relative matrix affect studied in different lots of plasma (spiked after extraction) was ≤2.35% for both the analytes.

Table 3
Stability of lopinavir and ritonavir under various conditions ($n = 3$).

Lopinavir				Ritonavir		
Storage conditions	Nominal concentration (ng/mL) ± SD	Mean calculated concentration (ng/mL) ± SD	%Mean accuracy	Nominal concentration (ng/mL) ± SD	Mean calculated concentration (ng/mL) ± SD	%Mean accuracy
Bench top stability, at 5 °C, 45 h						
HQC	1,3184	1,3077 ± 98.6	−0.8	1333	1342 ± 12.9	0.7
LQC	84.5	82.6 ± 1.2	−2.2	8.5	8.4 ± 0.2	−1.2
Freeze & thaw stability, at −20 °C, 3 cycles						
HQC	1,3184	1,2424 ± 125	−5.8	1333	1291 ± 11.4	−3.1
LQC	84.5	78.0 ± 1.0	−7.6	8.5	8.1 ± 0.0	−5.5
Dry extract stability, at 5 °C, 45 h						
HQC	1,3184	1,3114 ± 64.9	−0.5	1333	1328 ± 38.0	−0.4
LQC	84.5	84.9 ± 2.4	0.6	8.5	8.8 ± 0.2	2.5
Wet extract stability, at 5 °C, 45 h						
HQC	1,3184	1,2963 ± 199	−1.7	1333	1328 ± 12.1	−0.3
LQC	84.5	80.9 ± 1.1	−4.2	8.5	8.4 ± 0.2	−5.9
Long term stability in plasma at −20 °C, 63 days						
HQC	1,3184	1,3340 ± 217	1.2	1333	1417 ± 22.7	6.3
LQC	84.5	82.5 ± 0.9	−2.3	8.5	8.9 ± 0.2	4.7
Long term stability in plasma at −70 °C, 63 days						
HQC	1,3184	1,3100 ± 191	−0.6	1333	1408 ± 28.5	5.7
LQC	84.5	67.5 ± 2.1	−4.0	8.5	8.9 ± 0.2	4.8

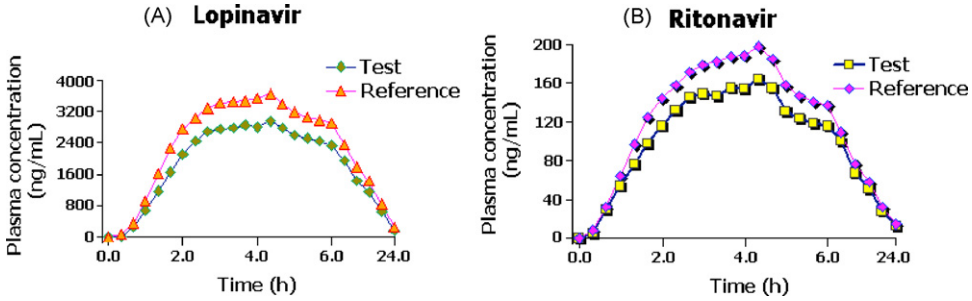

Fig. 3. Mean plasma concentration-time profile of (A) lopinavir and (B) ritonavir after oral administration of [200(lopinavir) + 50(ritonavir)] mg of test and reference tablet formulation to 36 healthy volunteers under fasting condition.

3.6. Stability, method ruggedness and dilution integrity

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 and 8 °C for 7 days respectively. Different stability experiments in plasma and the values for precision and percent change are shown in Table 3.

For ruggedness experiments, the precision and accuracy at LLOQ, low, middle and high quality control samples ranged from 0.9% to 3.1% and 95.3% to 105.1% for LPV and RTV.

The precision for dilution integrity of 1/5th and 1/10th dilution were 1.0% and 1.1%; 1.0% and 1.5% for LPV and RTV respectively, while the accuracy results were 99.0% and 98.5% for LPV; 106.4% and 105.4% for RTV respectively.

3.7. Application of the method in healthy human subjects

The design of the study comprised of "An open label, balanced, randomized, single-dose, two-treatment, two-sequence, two period crossover bioequivalence study of 200/50 mg LPV and RTV test (Indian Pharmaceutical Company) and reference (Kaletra, Abbott Laboratories, Chicago, IL60064, USA) tablet formulations in human subjects under fasting conditions." The work was approved and subject to review by an Institutional Ethics Committee. The procedures followed while dealing with human subjects were based on International Conference on Harmonization, E6 Good Clinical Practice (ICH, E6 GCP) guidelines [40]. Fig. 3 shows the mean plasma concentration-time profile following oral administration of [200(LPV + 50(RTV)] mg tablet formulation to 36 healthy Indian male subjects under fasting condition. The mean pharmacokinetic parameters viz. C_{max}, $AUC_{0-24\,h}$, AUC_{0-inf}, T_{max} and $t_{1/2}$, calculated for test/reference formulation were $3303 \pm 1175/3656 \pm 1484$, $32060 \pm 13531/34997 \pm 17859$, $34077 \pm 15295/37023 \pm 19416$, $4.0 \pm 0.7/3.3 \pm 1.2$ and $4.4 \pm 1.5/4.5 \pm 1.7$ for LPV and $202 \pm 105/203 \pm 111$, $1685 \pm 869/1633 \pm 857$, $1805 \pm 949/1747 \pm 924$, $3.9 \pm 0.9/3.2 \pm 1.2$ and $5.4 \pm 1.2/5.3 \pm 1.0$ for RTV respectively. It was observed that C_{max} values for LPV in more than 50% of subject samples was higher than the upper limit of quantification reported in previous studies [35,37]. Similarly, for RTV the C_{max} values were much higher than ULOQ value [37] for about 40% subject samples, which is evident from large variation in the standard deviation values.

4. Conclusion

The UPLC–MS/MS methodology presented for simultaneous estimation of LPV and RTV 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 Kaletra of different strengths. The method involved an efficient and specific sample preparation by solid phase extraction followed by isocratic chromatographic separation in 1.2 min. 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 [35–37] for their simultaneous determination.

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