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Simultaneous quantification of Darunavir and Ritonavir in human plasma and pharmacokinetic study by LC MS/MS

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The combination of Darunavir (DRV) and Ritonavir (RTV) at a dose of 800/100mg has exhibit durable efficacy in both untreated and treated HIV positive patients with no observed DRV resistance-associated mutations (RAMs) and the RTV improves the pharmacokinetic profile of DRV by enhancing its bioavailability. Hence a sensitive high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method has been developed and validated for the simultaneous quantification of Darunavir (DRV) and Ritonavir (RTV) in human plasma. The chromatographic separation has been accomplished on Thermo Hypersil Gold (50×4.6mm, 3µ) analytical column using isocratic elution using 0.1% Formic Acid buffer solution/Acetonitrile (50:50, v/v) at a flow rate of 0.6 mL/min. The linearity of the method ranged 150.000 ng/mL to 15000.000 ng/mL & 10.000 ng/mL to 3000.00 ng/mL for DRV & RTV respectively, using 100 µL of plasma. The method was completely validated for its sensitivity, selectivity, linearity, accuracy and precision, recovery, matrix effect, stability, and dilution integrity. The absolute recovery for DRV ranged from 79.12 to 72.71 % while for RTV it ranged from 63.39% to 66.99 %. For DRV-D_9 and RTV-D_6 the recovery rates are 94.19 and 80.05% respectively. The method exhibit good intra-day and inter-day precision with low % CV of less than 5.0% at each quality control level for both the analytes. The developed method has been applied successfully for pharmacokinetic study in healthy humans by oral administration of Darunavir/Ritonavir tablets 400/50 mg (dose; 02x400/50 mg) in 77 healthy male volunteers under fed condition.

Keywords: Bioanalytical, Bioequivalence, Darunavir (DRV), Good Clinical Practice, LCMS/MS method Validation, Lower Limit of Quantification, Pharmacokinetic, Ritonavir (RTV)

There has been a remarkable transformation, in the treatment of HIV in recent years due to evolving potent antiretroviral (ART) combination therapies with better tolerability leading to increased life expectancy of HIV patients[1-3]. The most common antiviral dosing regimen recommended was a vital combination of two nucleotide reverse transcriptase inhibitors (NRTIs) with a third antiretroviral drug, preferably a protease inhibitor (PI) boosted with RTV. The HIV protease inhibitor DRV, boosted by RTV (DRV/RTV) is prescribed as a once-daily (q24h) ART at standard dosage regimens of 800/100 mg once daily in naive patients and 600/100 mg twice daily in treatment-experienced patients and with no DRV resistance-associated mutations (RAMs).

DRV a substrate and mild inhibitor of CYP3A4 is extensively metabolized by CYP3A4 in the liver and intestinal lumen[4]. RTV a strong CYP3A4 inhibitor prevents this fast pass metabolism of DRV and increases its bioavailability[5]. RTV further acts as a pharmacokinetic enhancer by strongly inhibiting the DRV transporter P-glycoprotein (P-gp) that result in 2.7-fold increase in intestinal permeability of DRV in mice and Caco-2 monolayers models. In a study of HIV-negative in healthy volunteers the absolute bioavailability of DRV (600 mg once daily) was observed to increase 82% in presence of RTV (100mg twice daily) in comparison to DRV was administered alone for which the observed increase was 37%.[6,7] However increasing the RTV dose at 200 mg twice daily with DRV 600mg once daily did not result in any relevant increase in plasma concentrations for DRV indicating 100 mg twice daily dose of RTV to be optimal for pharmacokinetic enhancement of DRV[6]. DRV in combination with RTV was evaluated in four different doses (600 or 400 mg twice daily, and 800 or 400 mg once daily with 100 mg RTV) for efficacy and safety in a treatment-experienced HIV-infected population of patients (pivotal POWER 1 and 2 clinical trials) in which the highest virological

response was obtained with twice daily 600/100 mg of DRV/RTV combination and was approved for treatment-experienced patients[8]. In another subgroup analysis for initial ART therapy similar responses was observed for the 800 mg once-daily and the 600 mg twice-daily doses leading to approval of the single daily 800 mg dose for naive patients[9].

The study of pharmacokinetic parameters is an important aspect for the reduction of Antiretroviral dose reduction that simplify the ART dosing regimens with reduced pill burden and less side-effects in HIV patients. Bioanalytical methods for measuring plasma drug concentration are indispensable to understand drug–drug interactions and pharmacokinetic/ pharmaco- dynamic properties[10]. Previously bioanalytical assays have been developed for the DRV and RTV in combination with other anti-retroviral or separately for each one of them[11], here we developed and validated a bioanalytical method for the simultaneous quantification of both DRV and RTV and an extensive validation studies had been performed to ensure the selectivity and sensitivity of the developed method. The developed model is simple, sensitive, selective, efficient, and validated and is reliable for the determination of DRV & RTV to ensure that the method is selective, specific, precise & accurate for the determination of DRV & RTV to support the bioequivalence and bioavailability study of DRV & RTV. The chemical structure of Darunavir (A) and Ritonavir (B) is given in Fig. 1.

Experimental Section

The reference standard samples of DRV (98.6%) and RTV (99.5%) were obtained from Mylan laboratories Ltd. and DRV-D_9 (99.25%) and RTV-D_6 (96.20%) were obtained from TLC pharmachem and TLC pharmaceuticalrespectively. Water used for the LC-MS/MS analysis was prepared by using Milli Q water purification system procured from Millipore (Bangalore, India). Formicacid of suprapure gradewas purchased from Merck. HPLC grade of acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, USA). The control human plasma sample was procured from Deccan's Pathological Lab's (Hyderabad, India).

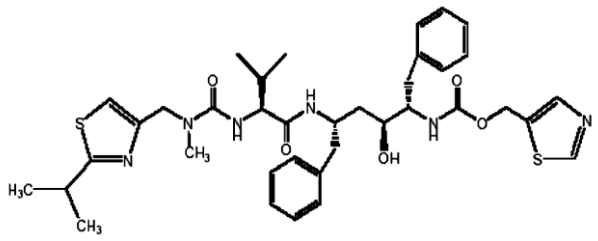

Fig. 1 — Chemical structure of (A) Darunavir (DRV) and (B) Ritonavir (RTV).

LC–MS system and conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a Thermo Hypersil Gold column (50×4.6mm, 3µ), a LC-20 AD Vp Pump (Shimadzu), an auto sampler (Shimadzu-SIL-HTc) and a solvent degasser (DGU-$20A_3$) were used for the study. The processed samples after reconstitution were injected (5µL)into the column maintained at a temperature of 40 ± 5 ºC. An isocratic mobile phase composition of acetonitrile and 0.1% formic acid (50:50) was used for the separation of the analytes transferred at 0.600 mL/min flow rate (with splitter of 50:50) into ionization chamber (electrospray mode) of the mass spectrometer. The detection and quantification of the analytes and the internal standards in MS-MS positive ionization mode was selected using MDS Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray™ interface at 450 ºC. The different source parameters such as the nebulizer gas (GS1-45 psi), curtain gas (CUR-20 psi), auxiliary gas (GS2-55 psi) and collision gas (CAD-7 psi), ion spray voltage (5500V) were optimized to obtain better detection response for the analytes and the internal standard within 5.5 min run time. The compound parameters were also optimized such as the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 70, 20, 10, 10 V for DRV and DRV d9 and 78, 30, 10, 10 V for RTV and RTV D_6 respectively. Multiple-reaction monitoring mode (MRM) was selected for detection of the ions, by selecting the transition pairs of *m/z* 548.300 precursor ion to the *m/z* 392.300 for DRV, *m/z* 557.400 precursor ion to the *m/z* 401.200 for DRV D_9 for the IS, and*m/z* 721.400 precursor ion to the *m/z* 296.100 for RTV, *m/z* 727.400 precursor ion to the *m/z* 302.100 for RTV d6 for the IS respectively. The quadrupoles Q1 and Q3 were set on unit resolution and the data analysis was performed using Analyst software™ (version 1.5.1).

Stock solutions

Stock solutions of DRV, RTV were prepared separately by dissolving in methanol at 2 mg/mL and 1mg/mL concentration respectively. RTV D_6 and DRV D_9 stock solutions were prepared by dissolving in methanol at 0.2 mg/mL and 0.4 mg/ml

concentration respectively. Different concentrations of the working solutions were prepared by stock dilution using diluent [methanol and water (60:40%, v/v)]. Intermediate solution containing both 200.000 µg/mL DRV and 100.000 µg/mL RTV were prepared by transferring both 1000µL of DRV stock solution (2000.000µg/mL) and RTV stock solution (1000.000µg/mL) using diluent in 10 mL volumetric flask. RTV-D_6 and DRV-D_9 working internal standard solution by transferring 1250 µL of RTV-D_6 and 3750 µL of DRV-D_9 stock solutions with diluent into a 50 mL of a volumetric flask.

Sample pretreatment

A 100 µL aliquot of human plasma sample was mixed with 25 µL of working internal standard (RTV-D_6+DRV-D_9 -5.000 µg/mL and 30.000 µg/mL). To this, 200µLof ExtractionAdditive (Milli-Q/HPLC grade Water) was added and vortexed for 10-20 seconds and keep aside for Solid phase extraction.

The conditioning and equilibration of cartridge (Oasis HLB, 30 mg, 1cc) were performed with 1mL of Methanol followed by 1mL of Milli-Q water. The spiked plasma samples of volume 325 µL (100 µL aliquot of human plasma + 25 µL of Working internal standard + 200µL of Extraction Additive) were loaded into cartridge and washed with 1mL of Milli-Q/HPLC grade water followed by washing with 1ml of 5% Methanol in water. Elution was performed by using methanol and evaporated the sample to complete dryness under a stream of nitrogen gas at ≤50°C. Reconstituted the residue with 1.000 mL of reconstitution solution (0.1% Formic Acid buffer solution with 250mL of Acetonitrile/Mobile phase). From these, a 5.0µL aliquot was injected into the chromatographic system. The developed method was completely validated by performing selectivity, sensitivity, linearity, precision and accuracy, recovery, matrix effect, stability, and dilution integrity experiments.

Calibration curves and Limit of quantitation

The assay was validated with a standard curve range of 150.074 to 15007.413 ng/mL & 10.007 to 3002.015 ng/mL for DRV &RTV respectively. The standard curve consisted of nine non-zero calibration standards, along with matrix blanks (with and without the addition of internal standard). The lowest standardconcentration (150.074 ng/mL & 10.007 ng/mL for DRV & RTV respectively) defined the lower limit of quantitation (LLOQ) for the assay, while the standard with the highest concentration (15007.413 ng/mL & 3002.015ng/mL for DRV & RTV respectively) defined the upper limit of the assay (ULOQ).

Linearity is defined as the square of the correlation coefficient (r) obtained from weighted linear regression of peak area ratio (analyte/internal standard) versus concentration. The criterion for acceptable linearity was ≥ 0.99. All validation standard curves used for accuracy and precision determinations surpassed this limit with r values of greater than 0.99. In any batch not less than 75% of all standards samples and 67% of all QCs (50% at each level) samples were required to have a percentage deviation within ± 15% except LLOQ where a percentage deviation within ± 20%.

Selectivity, matrix effect, recovery and carry over

The blank plasma samples 14 different lots (8 normal, 2 (0.5%) Haemolysed, 2 (1.0%) Haemolysed & 2 lipemic were evaluated for selectivity experiment during the validation experiment. The specificity run was performed using six extracted LLOQ standards. The analyte response in the blank plasma was compared to the LLOQ standard mean area of DRV, RTV and internal standards from the 14 different plasma lots.

To evaluate the matrix factor Six lots (4 Normal, 1 (1%) Haemolysed & 1 Lipemic) of interference free blank matrix from individual donor were taken, processed in triplicate from each lot and extracted according to the analytical method procedure. The post extracted LQC & HQC samples were obtained by spiking the analyte and internal standard into the extracted plasma blank samples. In the next step 12 aqueous (without matrix) LQC & HQC samples were prepared and compared with the post extracted LQC & HQC samples and calculation of the matrix factor was done for each matrix lot by calculating the ratio the mean peak area of unextracted samples to the peak area of post extracted samples. Additionally, Internal Standard Normalized Matrix Factor was also evaluated by calculating matrix factor of analytes by matrix factor of Internal Standards at each lot of matrix.

The extracted analyte peak area was compared with the non-extracted standard peak area to calculate recoveries of DRV and RTV. The recovery of DRV and RTV was calculated at a 450.0, 30.0 (LQC), 7500.0, 1500.0 (MQC-1) and 11300.0, 2300.0 (HQC) ng/mL, concentration levels respectively, and the IS was determined at concentration of (RTV-D6+DRV-

D9 -5.000 µg/mL and 30.000 µg/mL). In order to extend the upper concentration limit dilution integrity was performed with acceptable precision and accuracy. The dilution integrity experiment was performed by using 4 x ULOQ samples and dilution of the samples by using blank plasma was performed at six replicates of five times and ten times dilution (1 in 10 dilution) and freshly spiked calibration curve concentrations were determined against nominal comparison concentrations.

Accuracy and Precision

The intra batch accuracy and precision for DRV and RTV was determined by 4 sets of calibration curves and 4 sets of QC samples. The quality control samples (LLOQQC, LQC, MQC1, MQC2 and HQC) were taken in 6 replicates. The inter batch accuracy and precision was analyzed by performing four accuracy and precision batches using 1 sets of calibration curves and 6 replicates of QC samples (LLOQQC, LQC, MQC1, MQC2 and HQC). The batch acceptance criteria for the calibration curve standard and quality control samples were met if the accuracy of the samplesiswithin ±15% deviation (SD) from the nominal values, except for LLOQ QC, it is ±20%. The precision of ≤15% relative standard deviation (RSD), is accepted for the calibration curve standard and quality control samples except for LLOQ, where it should be ≤20%. The batch acceptance criteria were met if 67% of the total quality control samples and 50% at each quality control level is within the specified acceptance criteria.

Ruggedness

The ruggedness experiment was evaluated by processing one P&A by different analyst and reinjecting one P&A batch samples on different column with same specification. The run consisted of a calibration curve standard and 6 replicates at each of LLOQQC, low (LQC), medium (MQC1), medium (MQC2) and high (HQC) concentrations. The ruggedness experiment was also evaluated by reinjecting samples of one P&A on different column with same make and specification. Concentrations were calculated to determine precision and accuracy.

Dilution integrity

The dilution integrity experiment was performed using five times dilution (1 in 5 dilutions) and ten-time dilution (1 in 10 dilutions) of six replicates of approx. 4x ULOQ samples and the concentrations were calculated using the nominal concentrations of freshly spiked calibration curve samples.

Stability and re-Injection reproducibility

The bulk spiked samples were used for matrix stability experiments and the accuracy of these samples was determined previously to satisfy the acceptance criteria after spiking with the analyte. The bulk spiked samples of six replicates of LQC and HQC samples were determined for different stability conditions like the study sample analysis conditions. These stability experiments for the samples were performed in a single run where the samples were processed and analyzed with freshly processed calibration standards (CS) and six sets of QC samples.

The quality control samples of one P&A batch after analysis was reinjected to check the reproducibility bykeeping the samples in the auto sampler at 5°C and reinjected after more than 40 hrs. to check the acceptance limits of accuracy (±15% of their respective nominal concentration) and precision (%CV ≤15).

Pharmacokinetic study design

A randomized, open-label, balanced, two-treatment, two-period, two-sequence, single dose, crossover oral bioequivalence study was planned as per the ICH GCP guidelines. Bioequivalence study was conducted on 77 male subjects under Fedconditions.The study was conducted in groups due to relatively large number of subjects in the study. RTV half-life is 3-5 hours and the terminal elimination half-life of DRV was approximately 15 hours when co-administered with RTV. A washout period of 16 days was considered to be adequate. In this study, the pharmacokinetic profile of the test product (A) was characterized relative to that of the reference product (B) to assess bioequivalence for DRV/RTV tablets 400/50mg (Dose; 02 x 400/50 mg). Being a bioequivalence study with a crossover design, each subject act as his own control. Therefore, no control group was required for the study. The protocol was approved by the ethics committee and informed written consent was provided by the volunteers. After oral administration Blood samples were collected at pre-dose and 0.33, 0.67, 1, 1.33, 1.67, 2, 2.33, 2.67, 3, 3.33, 3.67, 4, 4.5, 5, 5.5, 6, 7, 8, 10, 12, 16, 24, 36, 48 and 72 hrs, in K3-EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The collected plasma samples were stored at –70 ± 15°C until use. Plasma samples were spiked with the IS and processed as per

the extraction procedure described earlier. The subject sample analysis was performed along with standard samples (calibration curve standards) and different level of QC samples (LQC, M1QC, M2QC and HQC) taken in triplicate.

Results

LC-MS specification

During method development for the simultaneous quantification of DRV and RTV different detection and chromatography parameters were evaluated to optimize the detection of analytes. In positive ionization mode the mass parameters showed good response. The analyte response was optimized using the multiple reaction monitoring (MRM) modes. The monitoring of the most sensitive mass transition was performed by using the protonated parent ion in the Q1 spectrum form of each analyte and IS, $[M+H]^+$ion served as the precursor ion for Q3 product ion spectra. The mass spectrometry specification for DRV and RTV and the respective internal standards are mentioned in Table 1. The multiple-reactionmonitoring mode (MRM) was performed by monitoring the transition pairs of *m/z* 548.300 precursor ion to the *m/z* 392.300 for DRV, *m/z* 557.300 precursor ion to the *m/z* 401.200 for DRV-d9 for the IS, and *m/z* 721.400 precursor ion to the *m/z* 296.100 for RTV, *m/z* 727.400 precursor ion to the *m/z* 302.100 for RTV-d6 for the IS respectively.

Chromatography optimization

In order to achieve increased intensity and good resolution with shorter run time several trials were conducted to optimize the chromatographic conditions such as the composition of the mobile phase. Better response for both the analytes and the IS was observed using 0.1% Formic acid: Acetonitrile (50:50) while setting the MS detection in the positive ionization mode. The mobile phase delivered at a flow rate of 0.600 mL /min using the Thermo Hypersil Gold column (50×4.6mm, 3µ) provided better results such as good peak shape and response for both the analytes and IS. The retention time of DRV and RTV were 2.32 and 3.91 mins respectively and for the internal standard (DRV-D_9 and RTV-D_6) it was 2.30 and 3.86 mins respectively allowing a run time of 5.50 min.

Sample Pre-treatment Optimization

The low matrix effect and better recovery was obtained by using the solid phase extraction (SPE) technique that provided better extraction of the drug and IS from the matrix compared to the LLE technique. The auto sampler wash solution was optimized to avoid any carry over effect to 50% Acetonitrile/water. The intended sensitivity was obtained by adjusting the plasma sample volume to 100µL. The chromatographic conditions, extraction procedure and detection parameters were optimized to obtain accurate and precise detection for the analytes in human plasma.

Calibration curves and limit of quantitation

The assay was validated with a standard curve range of 150.074 to 15007.413 ng/mL & 10.007 to 3002.015 ng/mL for DRV &RTV respectively. The weighing factor selected was $1/x^2$. The measured volume of the working solutions (20 µL of DRV and 20 µL of RTV) for calibration curve (CC) standard samples were spiked in 960 µL of control human plasma with an, giving final concentrations of 150.0, 300.0, 800.0, 1200.0, 3000.0, 6000.0, 9000.0, 12000.0, and 15000.0 ng/mL for DRV, and 10.0, 20.0, 60.0, 200.0, 600.0, 1200.0, 1800.0, 2400.0, and 3000.0 ng/mL for RTV. In each batch the CC samples were analyzed along with the quality control (QC) samples. The QC samples were prepared at five different concentration levels of 150.0 (LLOQ), 450.0 (LQC), 3500.0 (M1QC), 7500.0 (M2QC) and 11300.0 (HQC) ng/mL for DRV and 10.0 (LLOQ), 30.0 (LQC), 300.0 (MQC1), 1500.0 (MQC2) and 2300.0 (HQC) ng/mL for RTV in blank plasma. The prepared samples were stored at −70 ±15°C. The criterion for acceptable linearity was $r \geq 0.99$. All validation standard curves used for accuracy and precision

Table 1 — Mass spectrometry specification for DRV and RTV and therespective internal standards.

Drug name	DRV	DRV-D9	RTV	RTV-D6
DP	70.00	70.00	78.00	78.00
EP	10.00	10.00	10.00	10.00
CE	20.00	20.00	30.00	30.00
CXP	10.00	10.00	10.00	10.00
Parent Mass	548.300	557.400	721.400	727.400
Product Mass	392.300	401.200	296.100	302.100
Dwell time (m sec)	200.00	200.00	200.00	200.00

determinations surpassed this limit with r values of greater than 0.99.

Selectivity, matrix effect, recovery and carry over

In all plasma lots no significant interferences were observed (normal, lipemic, hemolysed) in the selectivity experiments for DRV, RTV and their respective Internal Standard. Matrix effect is investigated to ensure that precision, selectivity and sensitivity are not compromised by the matrix. For matrix effect no significant interference was observed at the RT of both the analytes as the % CV of IS normalized matrix factor for DRV and RTV was below 4% for both HQC and LQC level that was within acceptance criteria(<15%).

The Meanrecovery values were approximately ≥75% for DRV and ≥65% for RTV respectively. The difference in %CV of recoveries across each QC level was within 15% for both the analytes and also for the respective IS. The results are presented in Table 2. There was no observed significant autosampler injector carry over for the analyte and IS.

Accuracy and precision

The acceptance criteria for the QC samples except LLOQ QC were satisfied if the mean % nominal value and %CV were in range of 85-115% and less than 15%, respectively for all analytes. The precision and accuracy results in plasma quality control samples for intra-day and inter-day are summarized in Table 3. The LQC, MQC1, MQC2 and HQC samples had precision deviation values all within 15% of the relative standard deviation (RSD) at the intra-day and inter day experiments and for LLOQ the relative standard deviation (RSD) is within 20%. Similarly, the accuracy deviation values for the LQC, MQC1, MQC2 and HQC samples for the intra-day and inter-day were all within 100 ± 15% of the actual values and for the LLOQ QCs level it is within 100± 20%. The obtained results satisfied the acceptance criteria in the precision and accuracy experiments. Thus, it indicates that method was accurate and precise over extended period and to cover actual study samples analysis time.

Ruggedness

The mean % nominal value and %CV were in range of 85-115% and less than 15%, respectively across all QC levels for all analytes for ruggedness experiment involving different analyst, column and instrument. The run consisted of a calibration curve

Table 2 — Matrix effect (factor)and recovery for DRV, RTV, DRV d_9 and RTV-d_6.

Analyte	Level	Mean/ %CV of IS-normalized matrix factor	Recovery(%)/CV(%)	Mean recovery
DRV	HQC	0.984/3.24	74.80/5.58	76.11
	M2QC	-	72.71/3.99	
	M1QC	-	79.12/4.81	
	LQC	0.994/1.13	77.81/4.90	
RTV	HQC	0.98/2.33	65.82/5.72	65.15
	M2QC	-	63.39/4.74	
	M1QC	-	66.99/8.83	
	LQC	0.999/1.88	64.41/9.98	
DRV d_9	-	3.53/0.989	94.19/4.83	-
RTV-d_6	-	0.990/2.00	80.05/7.42	-

Table 3 — Precision and accuracy results for DRV and RTV Precision and accuracy of DRV.

Nominal concentration in ng/mL	Intra-day (n=6)			Inter-day (n=24)		
	Mean	CV (%)	Accuracy (%)	Mean	CV (%)	Accuracy (%)
HQC (11321.688)	10709.917	3.53	94.60	10576.667	4.90	93.42
MQC1(7501.118)	7764.232	4.85	103.51	7733.750	4.42	103.10
MQC2(3500.522)	3687.759	2.34	105.35	3649.371	4.74	104.25
LQC (452.067)	441.445	2.79	97.65	452.190	4.95	100.03
LLOQQC (150.022)	152.072	4.96	101.37	153.156	4.33	102.09
			Precision and accuracy of RTV			
HQC (2302.918)	2330.272	1.23	101.19	2324.232	2.38	100.93
MQC1(1501.903)	1488.061	2.35	99.08	1511.947	3.25	100.67
MQC2(300.381)	307.362	1.95	102.32	307.849	3.91	102.49
LQC (30.038)	28.705	2.62	95.56	29.868	4.71	99.43
LLOQQC (10.013)	9.613	1.33	96.01	10.263	5.72	102.50

standard and 6 replicates at each of LLOQQC, low (LQC), medium (MQC1), medium (MQC2) and high (HQC) concentrations. The precision (%CV) and accuracy (%Nom) of DRV & RTV QC samples was within the range *viz.* the %CV is less than 15% and the mean % Nominal is between 85-115%. These results indicated that the method is rugged and reproducible even if sample processedby different analyst. The ruggedness experiment was also determined by calculating the precision and accuracy on different column with same make and specification by reinjecting the samples of one P&A batch and the results were found to be reproducible for both DRV & RTV. Ruggedness experiment was evaluated on different instrument of the same make with two P&A batches & Injector Carry Over. The correlation coefficient was found to be greater than or equal to 0.9994 & 0.9992 for DRV & RTV respectively.The results are presented in Table 4

Dilution integrity

The dilution integrity experiment was performed using five (1 in 5 dilution) and ten times diluted (1 in 10 dilution) samples at six replicates of approx. 4 x ULOQ samples and the concentrations were calculated and compared using calibration curve samples that are spiked freshly. The results were within the acceptance criteria. The results are presented in Table 5.

Reinjection reproducibility and stability

The QC samples of one P&A batch was used in partial reinjection reproducibility experiment by keeping them for approx. 44hr 21min in the auto sampler at 10°C and reinjected for analysis. The results confirmed that after reinjection in auto sampler at 5°C no significant variation was observed in the analyte concentration.

Similar to partial reinjection reproducibility samples of one P&A batch was reinjected to determine the whole batch reinjection reproducibility experiment. The results are reproducible after reinjection for both DRV & RTV that proved the stability of the samples for approx. 42hr 57 min post extraction in autosampler.

The stock (Prepared in Methanol) and working solutions of Darunavir, Ritonavir, Darunavir-D_9 and Ritonavir-D_6 (Prepared in Methanol: Water

Table 4 — Ruggedness experiment for DRV and RTV.

	LLOQ QC	LQC	M1QC	M2QC	HQC
			DRV (Different Analyst)		
Mean	153.755	441.691	3485.379	7436.600	9900.924
Accuracy	102.49	97.70	99.57	99.14	87.45
%CV	2.92	3.64	3.77	2.79	2.29
			DRV (Different column)		
Mean	145.067	452.164	3743.070	7555.645	10413.922
Accuracy	96.70	100.02	106.93	100.73	91.98
%CV	4.66	3.96	6.68	2.30	1.66
			RTV(Different Analyst)		
Mean	10.140	28.988	301.406	1471.470	2273.936
Accuracy	101.27	96.50	100.34	97.97	98.74
%CV	3.94	1.14	1.42	2.46	2.01
			RTV(Different column)		
Mean	10.464	29.800	305.266	1475.411	2269.805
Accuracy	104.50	99.21	101.63	98.24	98.56
%CV	3.70	1.74	1.95	1.44	1.49

Table 5 — Dilution integrity results for DRV and RTV.

Analyte	QC level	Dilution Factor: 5			Dilution Factor: 10		
		Mean	CV	% Bias	Mean	CV	% Bias
DRV	DQC*	59250.032	2.53	98.59	61312.329	2.95	102.03
RTV	DQC*	12115.098	0.76	100.79	11951.088	2.01	99.43

*For DRV the DQC concentration is 60094.728 ng/mL. For RTV the DQC concentration is 12019.998 ng/mL.

Table 6 — Stability study results for DRV and RTV.

Storage period and storage condition	QC level	DRV			RTV		
		Mean	CV (%)	Accuracy (%)	Mean	CV (%)	Accuracy (%)
Whole Blood Stability (RT)	HQC	1.4103	2.50	101.42	1.6591	1.36	102.53
(02 Hr 09 min)	LQC	0.0618	3.56	100.82	0.0227	3.99	99.56
Bench top stability	HQC	10211.957	3.16	90.20	2173.432	1.34	94.38
(26 hrs 58 min)	LQC	430.835	4.46	95.30	28.298	3.24	94.21
Auto sampler stability at 5±3°C	HQC	10704.221	2.45	94.55	2279.130	2.99	98.97
(73 Hrs 15 min)	LQC	473.420	1.97	104.72	28.974	3.96	96.46
freeze thaw cycles	HQC	10484.914	3.43	92.61	2217.322	2.18	96.28
(-20±5^{0}C) for 6 cycles	LQC	440.797	4.87	97.51	27.247	3.19	90.71
freeze thaw cycles	HQC	10565.812	2.70	93.32	2197.791	3.29	95.44
(-70±15^{0}C) for 6 cycles	LQC	459.399	3.13	101.62	27.118	2.77	90.28
Dry Extract Stability (RT)	HQC	10014.516	2.58	88.45	2120.150	4.42	92.06
26 hrs 12 min	LQC	418.835	3.10	92.65	27.896	2.96	92.87
Post Extracted Stability (RF)	HQC	10073.584	3.22	88.98	2232.473	1.66	96.94
25 hrs 42 min	LQC	431.825	3.01	95.52	28.068	1.84	93.44
Coolant Stability	HQC	9765.589	3.80	86.26	2109.550	2.82	91.60
93 Hrs 56 min	LQC	414.419	4.34	91.67	27.221	2.74	90.62

{60:40})was found to be stable for 16 days in the refrigerator at 0-10ºC. The bench top stability was evaluated by retrieving 6 LQC and HQC samples from deep freezer condition and kept for 26 hr 58 min at room temperature after which they were processed and compared with freshly prepared comparison LQC and HQC samples and calibration standards samples. The mean % changes in concentrations were calculated to determine the stability period that was 26 hrs 58 min at room temperature for both DRV & RTV.The results are presented in Table 6.

The stability of the analytes in the biological matrix kept in dry ice during transportation was evaluated by keeping six HQC and LQC samples in dry ice for approximately 93 hr 56 min.

The stability samples (HQC and LQC) after the desired period were processed and analysed along with freshly processed comparison samples (LQC and HQC samples) and calibration curve standards samples. The results prove the stability of both DRV &RTV during sample shipment in dry ice to be stable for approx. 93 hr. 56 min.The results are presented in Table 6.

The post extracted refrigerator stability in matrix was evaluated by processing six LQC and HQC samples in biological matrix kept for desired time duration. The six LQC and HQC samples after retrieval from deep freezer conditions were processed, reconstituted and stored for approx. 25 hr 42 min in refrigerator at 0-10ºC. The stability of the samples was compared with freshly prepared calibration standards and comparison samples (freshly processed LQC and HQC) after the desired period of time. The results indicate that post extracted samples kept at refrigerator conditions (0-10ºC) both DRV & RTV were stable for approx. 25 hr 42 min. The results are presented in Table 6.

The freeze and thaw stability of the samples was evaluated at set temperatureof -70ºC &-20ºC by taking six replicates of LQC and HQC levels that were subjected to six freeze and thaw cycles (stability samples). After six freeze and thaw cycles these samples were processed and compared with freshly processed LQC and HQC samples (comparison samples) and calibration standard samples. The mean % change in concentrations were calculated to determine the stability of DRV& RTV that showed both the analytes are stable after six freeze thaw cycles.The results are presented in Table 6.

The autosampler stability for DRV and RTV was assessed by taking 3 sets of LQC and HQC

(6 samples at each level). The initial samples were analysed at 0.00 hr. followed by second batch of processed samples that were kept at 5ºC and analysed after 73 Hrs 15 min. The comparison of the second batch samples were done against the initial set samples along with freshly prepared calibration standard samples. The mean % change in concentrations during stability period were calculated to determine the stability of DRV and RTV. The results indicated both DRV and RTV were stable at 5°C for approx. 73 Hrs. 15 min.The results are presented in Table 6.

The whole blood stability was performed by spiking six replicates of LQC and HQC samples of whole blood (Stability samples) and kept at room temperature. The comparison samples consisted of six aliquots of whole blood at HQC and LQC levels, which were aliquoted separately and compared with the stability samples after a period of 02 hr 09 min. The separation of the plasmawas done by centrifuging both the stability & comparison samples with a speed of 3000 RPM at 4°C for about 10 min. The processing of the plasma samples was done as per the specified method. The results are presented in Table 6.

The dry extract stability of DRV & RTV was performed by storing the dry extract (after extraction and sample preparation and no reconstitution solution) in the refrigerator (0-10ºC) and compared with the freshly prepared extracted replicates of LQC and HQC concentrations. The LQC and HQC concentrations for DRV & RTV in six replicates after extraction and in dry extract conditions were kept for approx. 26 hr 12 min in a refrigerator at 0-10ºC prior to sample analysis. The dry extract samples were compared with freshly prepared comparison (LQC & HQC) samples and calibration curve standards. The results are presented in Table 6. The results indicate both DRV & RTV were stable in dry extract for at least 26 hr 12 min during storage in the refrigerator (0-10ºC).

Pharmacokinetic parameters

The mean and SD of pharmacokinetic parameters estimated for test product (A) and reference product (B) were listed in Table 7. The geometric least squares mean, ratio of test product (A) and reference product (B),(A /B), 90% confidence intervals, Intra Subject Variability (CV in%) and power (in %) for the untransformed pharmacokinetic parameters Cmax, AUC0-t and AUC0-inf for Darunavir and Ritonavir were summarizedin Table 8

The ratio of geometric least squares mean for the C_{max} of test product (A) and reference product (B) treatments of log-transformed pharmacokinetic parameter C_{max} was 114.19%. The two one-sided 90% confidence interval for the ratio of the geometric least squares mean was found 110.39-118.13 % with power

Table 7 — Pharmacokinetic parameters for DRV in test and reference product under fed conditions.

Parameters (Units)	Un-transformed Data (Mean ± SD)	
	Test Product(A) n=77	Reference Product (B) n=77
*T_{max} (hr)	4.00(1.33-5.00)	4.00(1.33-5.00)
C_{max} (ng/mL)	9152.511±2074.3019	7999.547±1733.2324
AUC_{0-t} (ng.hr/mL)	96420.313±31248.6686	82021.719±27910.2291
AUC_{0-inf} (ng.hr/mL)	102385.648±33664.6866	87324.115±28510.3070
K_{el} (1/hr)	0.101±0.0276	0.100±0.0289
$t_{½}$ (hr)	7.368±1.9736	7.471±2.1343
(AUC_{0-t}/AUC_{0-inf})*100	5.572±5.6187	6.278±5.7754

*Median, Minimum and Maximum values reported for $T_{max.}$

Table 8 — Pharmacokinetic parameters for RTV in test and reference product under fed conditions.

Parameters (Units)	Un-transformed Data (Mean ± SD)	
	Test Product(A) n=77	Reference Product (B) n=77
*T_{max} (hr)	4.50(2.33-6.00)	4.50(3.00-6.00)
C_{max} (ng/mL)	933.797±341.1617	830.664±308.1748
AUC_{0-t} (ng.hr/mL)	6279.856±2527.0849	5564.254±2451.4327
AUC_{0-inf} (ng.hr/mL)	6491.992±2558.0749	5759.936±2493.6955
K_{el} (1/hr)	0.125±0.0362	0.121±0.0328
$t_{½}$ (hr)	6.048±1.8252	6.161±1.7626
(AUC_{0-t}/AUC_{0-inf}) *100	3.468±1.9416	3.652±1.7437

*Median, Minimum and Maximum values reported for $T_{max.}$

100.0%. The ratio of geometric least squares mean of test product (A) and reference product (B) treatments of log-transformed pharmacokinetic parameter AUC_{0-t} was 118.11%. For AUC_{0-t} the two one-sided 90% confidence interval for the ratio of the geometric least squares mean was found 112.58-123.92 % with power 100.0 %. For the log-transformed pharmacokinetic parameter AUC0-inf the ratio of geometric least squares mean of test product (A) and reference product (B) treatments was 117.17 % and the two one-sided 90% confidence interval for the ratio of geometric least squares mean was found 111.97-122.61 % with power 100.0%. In DRV for all the pharmacokinetic parameters the 90% confidence interval is within the acceptance limits of 80.00 – 125.00% (Table 9).

For RTV the ratio of geometric least squares mean of test product (A) and reference product (B) treatments of log-transformed pharmacokinetic parameter Cmax and AUC0-t was 112.37% and 114.98% respectively. The two one-sided 90% confidence interval for the ratio of the geometric least squares mean for the Cmax and AUC0-t was found 106.02-119.11 % and 109.84-120.36% with power 100.0% respectively. In case of log-transformed pharmacokinetic parameter AUC0-inf the ratio of geometric least squares mean of test product (A) and reference product (B) treatments of was 114.76%. The two one-sided 90% confidence interval for the ratio of geometric least squares mean for AUC0-inf was found 109.92-119.81 % with power 100.0 %. For RTV also all the pharmacokinetic parameters are within the acceptance limits of 80.00-125.00% at 90% confidence interval. The Group*Formulation effect was found statistically insignificant for the pharmacokinetic parameters Cmax, AUC0-t and AUC0-∞ on log-transformed data for both DRV and RTV. The linear and semi log plot of mean plasma concentration versus time curves of Darunavir and Ritonavir after administration of test product (A) and reference product (B) under fed conditions are represented in Fig. 2 and Fig. 3 that indicates the test product (A) compared to the Reference product (B), met the bioequivalence criteria under fed conditions.

Discussion

The current study reports the development, validation, and clinical application of a novel bioanalytical method of DRV and RTV using electrospray ionization (ESI) in the positive ionization mode with DRV-D_9, and RTV-D_6 as the respective internal standards. This study exclusively reports the simultaneous quantification of DRV and RTV. The chromatographic conditions were optimized to have adequate response, good peak shapes and shorter run time, under isocratic conditions on an HPLC system connected with mass spectrometry using Thermo Hypersil Gold column (50×4.6mm, 3µ). All mass parameters were suitably optimized to obtain a stable and adequate response for the analytes. The difference

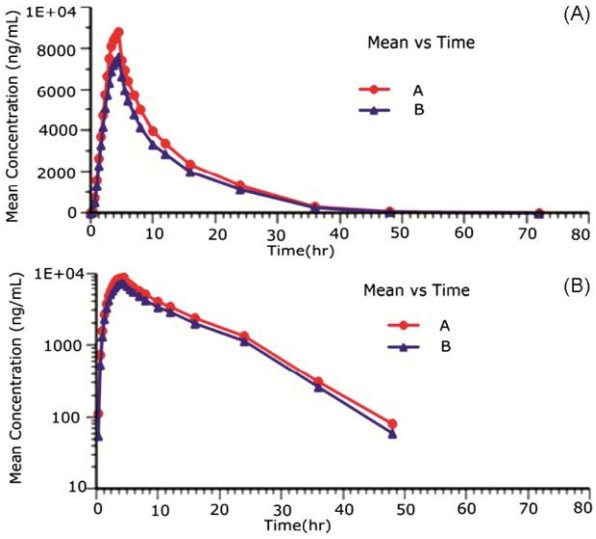

Fig. 2 — (A)Linear and (B) semi log plot of mean plasma concentration versus time curves of DRV after administration of test product (T) and reference product (R) under fed conditions.

Table 9 — Statistical results for DRV and RTV test and reference products under fed condition.

Parameters (Units)	Ratio of Geometric Least Squares Means			Intrasubject variability	Power	90% Confidence Limits
	Test product (A)	Reference product (B)	(A/B) %			(A vs. B)
DRV						
C_{max} (ng/mL)	8929.135	7819.385	114.19	12.7	100	110.39-118.13
AUC_{0-t} (ng.hr/mL)	91693.847	77633.630	118.11	18.0	100	112.58-123.92
AUC_{0-inf} (ng.hr/mL)	97293.655	83035.569	117.17	17.0	100	111.97-122.61
RTV						
C_{max}(ng/mL)	876.584	780.061	112.37	21.9	100	106.02-119.11
AUC_{0-t} (ng.hr/mL)	5825.403	5066.554	114.98	17.1	100	109.84-120.36
AUC_{0-inf} (ng.hr/mL)	6036.644	5260.172	114.76	16.1	125.00	109.92-119.81

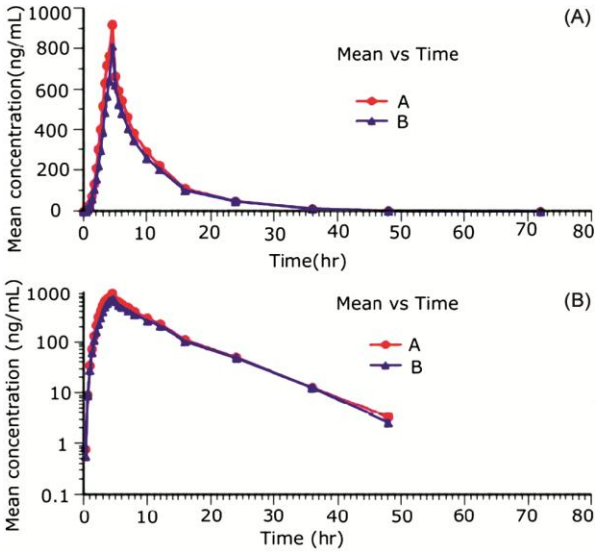

Fig. 3 — (A)Linear and (B) semi log plot of mean plasma concentration versus time curves of RTV after administration of test product (T) and reference product (R) under fed conditions.

in retention time of DRV and RTV were 2.32 and 3.91 mins respectively and for the Internal standard (DRV-D_9 and RTV-D_6) 2.30 and 3.86 mins respectively, allowing a good separation for both the analytes. Further, use of deuterated internal standards helped to compensate variability during extraction and HPLC-MS/MS analysis.

The validation was carried out as per US FDA guidelines. The parameters determined were selectivity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, stability and dilution integrity. No Matrix effect was observed for six different lots of K_3-EDTA plasma and the blank plasma samples were also analyzed to confirm the absence of direct interferences. The results of the P&A batches confirm the reproducibility of the method with an excellent ruggedness for different analyst and column. This optimized and validated LC–MS/MSmethod was applied to quantify plasma DRV and RTV concentration for a bioequivalence study in 77 healthy subjects(dosed in 3 groups)after oral administration of test DRV/RTV tablets 400/50mg (Dose; 02 x 400/50 mg) with reference PREZISTA® (DRV) tablets 800 mg of Janssen Ortho LLC Gurabo, PR 00778 and NORVIR® (RTV) Tablets 100 mg of AbbVie Inc, North Chicago, IL 60064, U.S.A under fed conditions.

Conclusion

The current study describes a LC-MS/MS assay method for the rapid, simple and sensitive quantification of both DRV and RTV in human plasma. The developed method was validated by following the US FDA guidelines. The study is unique and reports the simultaneous assay and sensitive determination of both DRV and RTV in human plasma that was further applied for pharmacokinetic studies in humans. The developed method can be further used in routine therapeutic drug monitoring to quantify both DRV and RTV or in bioequivalence (BA/BE) study with desired precision and accuracy.

Abbreviations

DRV: Darunavir; RTV: Ritonavir; FDA: Food and Drug Administration; Cmax: Maximum plasma concentration; ICH: International Conference on Harmonization; HPLC: High Performance Liquid Chromatography; USA: United State on America; LCMS: Liquid Chromatography; Mass Spectrometry; K_3EDTA: Tripotassium Ethylene Diamine Tetra Acetate; BE: Bioequivalence; LLOQ: Lower Limit of Quantification; MS: Mass Spectrometry; GS: Gas; DP: Clustering Potential; EP: Entrance Potential; CE: Collision Energy; CXP: Cell Exit Potential; QC: Quality Control, CS: Calibration Standard; WIS: Working Internal Standard; HLB: Hydrophilic Lipophilic Balance; EMA: European Medicines Agency; LQC: Low QC; MQC: Med QC; HQC: High QC; DQC: Diluted QC; ULOQ: Upper Limit of Quantification; PB: Plasma Blank; GCP: Good Clinical Practice, CV: Co-efficient of Variation; SD: Standard Deviation

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