Journal of Chromatography B, 879 (2011) 2443–2453

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Application of a validated ultra performance liquid chromatography–tandem mass spectrometry method for the quantification of darunavir in human plasma for a bioequivalence study in Indian subjects

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ARTICLE INFO

Article history:
Received 23 April 2011
Received in revised form 3 July 2011
Accepted 7 July 2011
Available online 18 July 2011

Keywords:
Darunavir
UPLC–MS/MS
Human plasma
Bioequivalence study
Incurred sample reanalysis

ABSTRACT

A simple, precise and rapid ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method has been developed and validated for the quantification of darunavir, a protease inhibitor, using darunavir-d9 as internal standard (IS). The method involved liquid–liquid extraction of darunavir and IS in methyl-*tert*-butyl ether from 50 μL human plasma. The chromatographic separation was achieved on an Acquity UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm particle size) analytical column under gradient conditions, in a run time of 1.6 min. The precursor → product ion transitions for darunavir (*m*/*z* 548.1 → 392.0) and IS (*m*/*z* 557.1 → 401.0) were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) and positive ion mode. The method was extensively validated for its selectivity, sensitivity, carryover check, linearity, precision and accuracy, reinjection reproducibility, recovery, matrix effect, ion suppression/enhancement, stability and dilution integrity. The linearity of the method was established in the concentration range of 1.0–5000 ng/mL. The mean relative recovery for darunavir (100.8%) and IS (89.8%) from spiked plasma samples was consistent and reproducible. The application of this method for routine measurement of plasma darunavir concentration was demonstrated by a bioequivalence study conducted in 40 healthy Indian subjects for a 600 mg tablet formulation along with 100 mg ritonavir as booster under fast and fed conditions. To demonstrate the reproducibility in the measurement of study data, an incurred sample reanalysis was done with 400 subject samples and the % change in concentration was within ±12%.

1. Introduction

Darunavir [DRV, Prezista (1R, 5S, 6R)-2,8-dioxabi cyclo[3.3.0]oct-6-yl]-N-[(2S,3R)-4-[(4-aminophenyl)sulfonyl-(2-methylpropyl)amino]-3-hydroxy-1-phenyl-butan-2-yl] carbamate, formerly known as TMC114], is a HIV peptidic protease inhibitor, with high levels of antiviral activity against wild-type virus and stains with phenotypic resistance to other protease inhibitors (PIs) [1,2]. DRV was licensed in June 2006 in the USA and subsequently in European Union in February 2007 [3]. It selectively inhibits the cleavage of HIV-encoded *gag-pol* polyproteins in virus-infected cells, thereby, preventing the formation of mature infectious virus particles [4]. DRV is a key component of many salvage therapies in multi-treated patients. The effectiveness of DRV against PI resistant strains was successfully demonstrated in patients who experienced a virological failure after several boosted PIs [5]. As with other PIs, the metabolism of DRV is cytochrome P450 (CYP) 3A4-dependent and is generally co-administered with low-dose of ritonavir. DRV is administered 600 mg twice a day along with 100 mg of ritonavir, which acts as a booster [6]. Ritonavir enhances the plasma concentration of DRV, primarily by inhibiting cytochrome P450 enzymes and transporters such as P-glycoprotein [7]. Like other PIs, DRV is highly protein bound (~95%), while a small unbound fraction (5%) is available to penetrate the central nervous system [8].

Sensitive and selective determination of anti-HIVs in plasma is essential for studying drug–drug interaction, pharmacokinetic/pharmacodynamic properties, and therapeutic drug monitoring [9]. Several methods have been published to quantify DRV in different biological matrices, alone [8,10,11] and in combination [4,12–22] with several other PIs, nucleoside and

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doi:10.1016/j.jchromb.2011.07.008

non-nucleoside reverse transcriptase inhibitors (NRTI and NNRTI), integrase inhibitor, raltegravir and an entry inhibitor, maraviroc. DRV as a single analyte has been determined in cerebrospinal fluid [8] and human plasma [8,10,11], with a limit of quantitation ≥5.0 ng/mL. Sekar et al. [12] determined DRV along with ritonavir in human plasma by LC–MS/MS with a sensitivity of 10 ng/mL. A novel LC–ESI-MS method has been described for the simultaneous determination of DRV, etravirine and ritonavir in human blood plasma [13]. The plasma samples (100 μL) were prepared by liquid–liquid extraction (LLE) in *tert*-butyl methyl ether and separated on a sub-2 μm particle column under gradient conditions within 7 min. The method was validated for dynamic linear range of 2–2000 ng/mL. Fayet et al. [14] determined three new antiretroviral agents raltegravir, maraviroc and etravirine along with DRV in plasma to study drug–drug interactions. All other methods deal with simultaneous determination of DRV along with six or more antiretrovirals in dried blood spots [15], human plasma [16–20] and peripheral blood mononuclear cells (intracellular concentration) [21,22] by LC–MS/MS. ter Heine et al. [15] developed a bioanalytical method to determine four PIs and two NNRTIs from dried blood spots by extracting in acetonitrile, methanol and zinc sulphate mixture. The other methods [16–20] developed in human plasma for simultaneous determination of DRV along with antiretrovirals had the lower limit of quantitation ≥15 ng/mL. ter Heine et al. [21] have quantified nine PIs, nelfinavir metabolite and two NNRTs in lysate of peripheral blood mononuclear cells (PBMC). Very recently, Avolio et al. [22] developed a highly sensitive HPLC–MS method for simultaneous quantification of 14 antiretrovirals including DRV in PBMC of HIV infected patients. The calibration curves for all the antiretroviral ranged from 0.1 to 32 ng/mL expect tipranavir. The chromatographic run time was 15 min, with retention of 10.2 min for DRV on an Atlantis T3 C-18 column.

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 [23,24]. After in-depth survey it was found that there were no reports for the determination of DRV in human plasma by UPLC–MS/MS. 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 DRV in subject samples. Also, evaluation of ion suppression effects is conducted for selective determination of DRV in presence of matrix components and 10 other antiretrovirals. The validated method presents excellent performance in terms of selectivity, ruggedness and efficiency (1.6 min per sample). The wide linear dynamic range ensures the estimation of DRV with desired accuracy and precision in human volunteers for bioequivalence study under fast and fed conditions.

2. Experimental

2.1. Chemicals and materials

Reference standard of darunavir (purity, 99.4%) and darunavir-d9 (IS, purity, 99.8%) were procured from Hetero Drugs Limited (Hyderabad, India) and Toronto Research Chemicals Inc. (Ontario, Canada) respectively. HPLC grade methanol, acetonitrile, methyl-*tert*-butyl ether and formic acid 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). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at −20 °C until use.

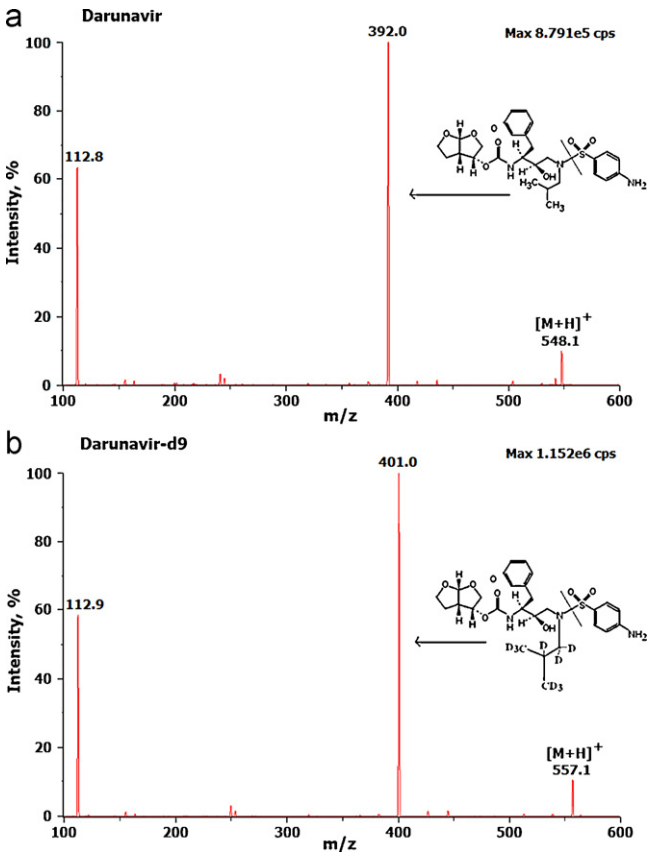

Fig. 1. Product ion mass spectra of (a) darunavir (*m/z* 548.1 → 392.0, scan range 100–600 amu) and (b) darunavir-d9 (IS, *m/z* 557.1 → 401.0, scan range 100–600 amu) in positive ion mode.

2.2. Liquid chromatographic conditions

A Waters Acquity UPLC (Massachusetts, USA) consisting of binary solvent manager, sample manager and column manager was used for setting the reverse-phase liquid chromatographic conditions. The separation of DRV and IS was performed on Waters Acquity UPLC BEH C18 (50 mm × 2.1 mm, length × inner diameter) column with 1.7 μm particle size and was maintained at 40 °C with an alarm band of ±6 °C in the column oven. For gradient elution, the mobile phase solvent consisted of (A) 0.5% formic acid in water and (B) 0.5% formic acid in acetonitrile: methanol (70:30, *v/v*) and the flow rate was maintained at 0.3 mL/min. Up to 0.4 min, the ratio of A and B was kept at 50:50 (*v/v*) and from 0.4 min to 1.2 min the ratio was changed to 30:70 (*v/v*), before returning to the starting conditions [A:B, 50:50 (*v/v*)] up to 1.6 min. The total chromatographic run time was 1.6 min. The sample manager temperature was maintained at 5 °C with an alarm band of ±3 °C and the average pressure of the system was 5500 psi.

2.3. Mass spectrometric conditions

Ionization and detection of analyte and IS was carried out on a Quattro Premier XE Mass spectrometer, Waters (Massachusetts, USA), equipped with ion spray interface and operating in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor precursor → product ion transitions for DRV *m/z* 548.1 → 392.0 and *m/z* 557.1 → 401.0 for IS (Fig. 1a and b). The source dependent parameters and analyzer

parameters are summarized in Table 1. Data collection, peak integration, and calculations were performed using Mass Lynx software version 4.1.

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

The standard stock solution of DRV (1000 μg/mL) was prepared by dissolving requisite amount in methanol. Calibration standards and quality control (QC) samples were prepared by spiking blank plasma (2% of total volume of blank plasma) with stock solutions. Calibration curve standards were made at 1.0, 2.0, 4.0, 10.0, 50.0, 100, 250, 500, 1000, 2500 and 5000 ng/mL concentrations while quality control samples were prepared at five concentration levels, 4000 ng/mL (HQC, high quality control), 2000 ng/mL (MQC-1, medium quality control-1), 120 ng/mL (MQC-2, medium quality control-2), 3.0 ng/mL (LQC, low quality control) and 1.0 ng/mL (LLOQ QC, lower limit of quantification quality control). Stock solution (500 μg/mL) of the IS was prepared by dissolving 2.5 mg of darunavir-d9 in 5.0 mL of methanol. An aliquot of 20 μL of this solution was further diluted to 10.0 mL in the same diluent to obtain a solution of 1.0 μg/mL. All the solutions (standard stock, calibration standards and quality control samples) were stored at 2–8 °C until use.

2.5. Sample extraction protocol

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 50 μL of spiked plasma sample, 50 μL of internal standard was added and vortexed for 20 s. Subsequently, 50 μL of 0.5% formic acid in water was added and vortexed for another 20 s. Additionally, 2.5 mL of methyl-*tert*-butyl ether was added and extracted on rotospin for 10 min at 32 × *g*. Samples were then centrifuged at 3204 × *g* for 5 min at 10 °C. After centrifugation, 2.0 mL of the supernatant organic layer was transferred to an evaporation tube. The supernatant was evaporated to dryness in a thermostatically controlled water-bath maintained at 40 °C under a stream of nitrogen. After drying, the residue was reconstituted in 100 μL of reconstitution solution [0.5% formic acid in water: acetonitrile and methanol (70:30) in 30:70, *v/v* ratio] and 10 μL was used for injection in the chromatographic system.

2.6. Bioanalytical method validation

A thorough and complete method validation of DRV in human plasma was done following the USFDA guidelines [25].

System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of DRV (2000 ng/mL) and internal standard (1.0 μg/mL) at the start of each batch during method validation. System performance was studied by injecting one extracted LLOQ sample with IS at the beginning of each analytical batch and before re-injecting any sample during method validation. Carry over effect of auto sampler was checked to verify any carryover of analyte at the start and at the end of each batch. The design of the experiment comprised of the following sequence of injections viz. extracted blank plasma → ULOQ sample → extracted blank plasma → LLOQ sample → extracted blank plasma.

The selectivity of the method towards endogenous plasma matrix components was assessed in ten different batches of plasma, of which, seven were normal K_3EDTA plasma and one each of lipidemic, haemolysed and heparinised plasma. The selectivity of the method towards commonly used medications in human volunteers was done for acetaminophene, cetirizine, domperidone,

Table 1
Optimized mass parameters for darunavir and darunavir-d9.

	Capillary/extractor voltage (kV/V)	RF lens (V)	Source temperature/desolvation temperature (°C)	Desolvation/cone gas flow (L/h)	LM 1/HM 1 resolution	Ion energy 1/ion energy 2	Entrance/exit	LM 2/HM 2 resolution	Cone voltage (V)	Collision energy (eV)	Dwell time (ms)
DRV and DRV-d9	4.0/3.0	0.0	100/400	900 ± 10/100 ± 10	15.0/15.0	0.2/1.0	−1/0.1	14/14	25.0	17.0	300

RF, radio frequency; LM, low mass; HM, high mass; DRV, darunavir; DRV-d9, darunavir-d9.

ranitidine, diclofenac and ibuprofen in six different batches of plasma having K_3EDTA as anticoagulant. The effect of potential concomitant antiretroviral drugs namely amprenavir, atazanavir, ritonavir, lopinavir, tipranavir, indinavir, saquanavir, nelfinavir, nevirapine and etravirine was studied for ionization (ion suppression/enhancement), analytical recovery (precision and accuracy) and chromatographic interference (interference with MRM of DRV and IS). Their stock solutions (1000 μg/mL) were prepared by dissolving requisite amount in methanol and water (95:5, *v/v*). Further, working solutions (200 ng/mL) of each drug were prepared in the same diluents, spiked in plasma and analyzed under the same conditions at LQC and HQC levels in triplicate. These sets were processed along with freshly processed calibration curve standards (CS) and two sets (8 samples) of qualifying QC samples (HQC, MQC-1, MQC-2 and LQC). As per the acceptance criteria, the % accuracy should be within 85–115%. The MRM transitions in the positive ionization mode for amprenavir (506.2/156.1), atazanavir (705.4/168.3), ritonavir (721.3/296.2), lopinavir (629.3/447.4), tipranavir (603.0/172.2), indinavir (614.1/421.0), saquanavir (671.2/225.1), nelfinavir (568.1/330.2), nevirapine (267.1/225.9) and etravirine (435.0/303.9) were studied.

The linearity of the method was determined by analysis of five calibration curves containing eleven non-zero concentrations. The area ratio response for analyte/IS obtained from multiple reaction monitoring was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression which was finalized during pre-method validation. A correlation coefficient (r^2) value >0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was accepted as the LLOQ, if the analyte response was at least ten times more than that of drug free (blank) extracted plasma. In addition, the analyte peak of LLOQ sample should be identifiable, discrete, and reproducible with a precision (%CV) not greater than 20% and accuracy within 80–120%. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15%.

For determining the intra-batch accuracy and precision, replicate analysis of plasma samples of DRV was performed on the same day. The run consisted of a calibration curve and six replicates of LLOQ QC, LQC, MQC-2, MQC-1 and HQC samples. The inter-batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive validation days. The deviation (%CV) at each concentration level from the nominal concentration was expected to be within ±15% except LLOQ, for which it should be within ±20%. Similarly, the mean accuracy should not deviate by ±15% except for the LLOQ where it can be ±20% of the nominal concentration.

Ion suppression/enhancement effects on the MRM LC–MS/MS sensitivity were evaluated by the post column analyte infusion experiment. A standard solution containing DRV (400 ng/mL) was infused post column via a 'T' connector into the mobile phase at 10 μL/min employing in-built infusion pump. Aliquots of 10 μL of extracted control plasma (six samples) were then injected into the column by the auto-sampler and MRM LC–MS/MS chromatogram was acquired for DRV. Any dip in the baseline upon injection of double blank plasma (without IS) would indicate ion suppression, while a peak at the retention time of DRV indicates ion enhancement.

The relative recovery, matrix effect and process efficiency were assessed as recommended by Matuszewski et al. [26]. All three parameters were evaluated at HQC, MQC-1, MQC-2 and LQC level. 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. As per the acceptance criteria, recovery should be consistent, precise and reproducible. 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 (ME × RE)/100. Further, the effect of plasma matrix (relative matrix effect) on analyte quantification was also checked in ten different batches of plasma. From each batch, six samples at LLOQ level were prepared (spiked after extraction) and checked for the % accuracy and precision (%CV). The deviation of the standards should not be more than ±20% and at least 90% of the lots at LLOQ level should be within the aforementioned criteria.

All stability results were evaluated by measuring the area response (DRV/IS) of stability samples against freshly prepared comparison standards at LQC and HQC levels. Stock solutions of DRV and IS were checked for short term stability at room temperature and long term stability at 5 °C. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Autosampler stability (wet extract), bench top (at room temperature), dry extract and freeze–thaw stability were performed at LQC and HQC using six replicates at each level. Freeze–thaw stability was evaluated by successive cycles of freezing (at −20 °C and −70 °C) and thawing (without warming) at room temperature. Long term stability of spiked plasma samples stored at −20 °C and −70 °C was also studied at both these levels. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ±15.0%.

To authenticate the ruggedness of the proposed method, it was performed on two precision and accuracy batches. The first batch was analyzed by different analyst while the second batch was studied on two different columns. Dilution integrity experiment was evaluated by diluting the stock solution prepared as spiked standard at 8000 ng/mL concentration in the screened plasma. The precision and accuracy for dilution integrity standards at 1/2 (4000 ng/mL) and 1/10th (800 ng/mL) dilution were determined by analyzing the samples against calibration curve standards.

2.7. Bioequivalence study design and incurred sample reanalysis

The design of the study comprised of "An open label, balanced, randomized, two treatment, two period, two sequence, single dose, crossover bioequivalence study of test (600 mg tablets from an Indian Company) and a reference (PREZISTA®, 600 mg darunavir tablets, manufactured by JOLLC, Gurabo, Puerto Rico for Tibotec Inc., NJ, USA) formulation of darunavir in 40 healthy, adult (18–45 years) Indian subjects under fast and fed conditions". Each subject was judged to be in good health through medical history, physical examination and routine laboratory tests. Written consent was taken from all the subjects 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 International Conference on Harmonization and USFDA [27]. Initially, for period 1, the drug under investigation (600 mg of DRV, test/reference) was co-administered with Norvir® (100 mg ritonavir tablets from Abbott Laboratories, North Chicago, USA) and subsequently ritonavir was given twice a day at an interval of 12 h for three consecutive days (total seven tablets, 7 × 100 mg) with 240 mL water. After the wash out period of 7 days, for period 2, 600 mg of DRV (test/reference) was again co-administered with 100 mg ritonavir, and subsequently ritonavir was given twice a day at an interval of 12 h for three consecutive days (total seven tablets, 7 × 100 mg) with 240 mL water. The subjects for both the studies were fasted 10 h before administration of the drug formulation. Further, under fed conditions the subjects were given high fat and high calorie breakfast (consisting of 250 mL milk with 5 g sugar, 35 g walnuts, two slices of bread with cheese and two cheese cutlets, total 969 calories) 30 min prior to giving the drug under investigation.

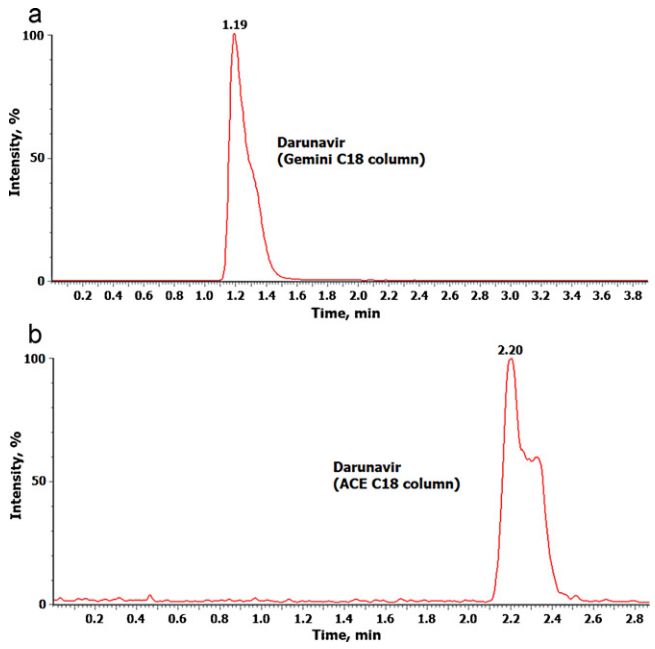

Fig. 2. Chromatograms of darunavir (*m/z* 548.1 → 392.0) obtained on Phenomenex Gemini C18 (50 mm × 4.6 mm, 5 μm) and ACE C18 (100 mm × 4.6 mm, 5 μm) analytical columns.

Blood samples were collected at 0.0 (pre-dose), 0.33, 0.67, 1.0, 1.33, 1.67, 2.0, 2.33, 2.67, 3.0, 3.33, 3.67, 4.0, 4.33, 4.67, 5.0, 6.0, 8.0, 10.0, 12.0, 16.0, 24.0, 36.0, 48.0 and 72.0 h after oral administration of test and reference formulation in labeled K_3EDTA-vacutainers. The maximum volume of blood withdrawn during the entire study was 294 mL, which included (other than for measurement) up to 10 mL for screening, about 10 mL for post study safety assessment (hematology and biochemical tests) while a total of 24 mL of heparinised blood was discarded prior to sampling through venous cannula for each subject in both the periods. Plasma was separated by centrifugation and kept frozen at −20 °C till the completion of period and then below −70 °C until analysis. During study, subjects had a standard diet while water intake was unmonitored. The samples were processed based on the proposed extraction protocol for quantification of DRV. An incurred sample re-analysis (ISR) was also conducted by computerized random selection of subject samples, 10% of total samples analyzed. The selection criteria included samples which were near the C_{max} and the elimination phase in the pharmacokinetic profile of the drug. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the value should not be more than ±20% [28].

2.8. Statistical analysis

The pharmacokinetic parameters of DRV were estimated by non-compartmental model using WinNonlin software version 5.2.1 (Pharsight Corporation, Sunnyvale, CA, USA). The C_{max} values and the time to reach maximum plasma concentration (T_{max}) were estimated directly from the observed plasma concentration vs. time data. The area under the plasma concentration–time curve from time 0 to 72 h (AUC_{0-72}) was calculated using the linear trapezoidal rule. The AUC_{0-inf} was calculated as: $AUC_{0-inf} = AUC_{0-72} + C_t/K_{el}$, where C_t is the last plasma concentration measured and K_{el} is the elimination rate constant; K_{el} was determined using linear regression analysis of the logarithm linear part of the plasma concentration–time curve. The $t_{1/2}$ of DRV was calculated as: $t_{1/2} = \ln 2/K_{el}$. To determine whether the test and reference formulations were pharmacokinetically equivalent, C_{max}, AUC_{0-72}, and AUC_{0-inf} and their ratios (test/reference) using log transformed data were assessed; their means and 90% CIs were analyzed by using SAS® software version 9.1.3 (SAS Institute Inc., Cary, NC, USA). The drugs were considered pharmacokinetically equivalent if the difference between the compared parameters was statistically non-significant ($P \geq 0.05$) and the 90% confidence intervals (CI) for these parameters fell within 80–125%.

3. Results and discussion

3.1. Method development

To develop a rapid, rugged and precise method it was important to optimize the chromatographic and mass spectrometric conditions, as well as to have an efficient and simple extraction procedure for DRV. The inherent selectivity of MS/MS detection was also expected to be beneficial in developing a selective and sensitive method. Initially, the precursor and product ions were optimized by infusing 200 ng/mL solutions in the mass spectrometer between *m/z* 100 and 600 range. The present study was conducted using electrospray ionization (ESI) in the positive mode as the analyte and IS have a primary and secondary amino groups. Also, the use of 0.1% formic acid in the mobile phase further augmented the response of protonated precursor $[M+H]^+$ ions at *m/z* 548.1 and 557.1 for DRV and IS respectively in the MS1 scan spectra. Stable and consistent product ions at *m/z* 392.0 and 401.0 were found due to elimination of *p*-aminophenyl sulfonyl group by applying 17 eV collision energy for DRV and IS. The MRM state file parameters like cone gas, desolvation gas, capillary and extractor voltage, source and desolvation temperature were suitably optimized to obtain a consistent and adequate response for the analyte. A dwell time of 300 ms for DRV and IS was adequate and no cross talk was observed between their MRMs.

Reported procedures have employed either protein precipitation [14,16–18,20], liquid–liquid extraction [10,13,19] or solid phase extraction [11] for DRV sample preparation from human plasma. Initially, protein precipitation was tried with solvents like acetonitrile and methanol in acidic conditions. However, selectivity and peak shapes were significantly affected with frequent clogging of the column. SPE under acidic condition gave good peak shapes but the response was not adequate. Goldwirt et al. [11] reported a mean recovery of 75.7% for DRV by solid phase extraction on C18 Bond Elut column. Thus, liquid–liquid extraction was tested to isolate the drug from plasma using diethyl ether, *n*-hexane, methyl-*tert*-butyl ether (MTBE) and ethyl acetate as extracting solvents which have been used in previous studies [10,13,19] with quantitative recoveries. Reproducibility and recovery data supported LLE with MTBE under acidic conditions (0.5% formic acid) as the best solvent for the extraction of DRV from human plasma. The plasma volume employed for sample preparation was only 50 μL, which is less compared to reported procedures [10–14,16,18–20] involving 100 μL and is same as reported by Avolio et al. [17].

To have an efficient chromatography, prime consideration was given to achieve a short run time in order to ensure high throughput, high sensitivity and minimizing the matrix effects as well as maintaining good peak shapes. Initially, to evaluate the analytical potential of different columns for fast chromatographic separation, four columns having different dimensions and particle sizes were evaluated namely, Gemini C18 (50 mm × 4.6 mm, 5 μm), ACE C18 (100 mm × 4.6 mm, 5 μm), Luna CN (50 mm × 2.0 mm, 3 μm), Chromolith RP-18 (100 mm × 4.6 mm). Separation was tried using various combinations of methanol/acetonitrile in acidic buffer (ammonium formate) and additives like formic acid (0.01–0.1%) on these columns to find the optimal mobile phase that pro-

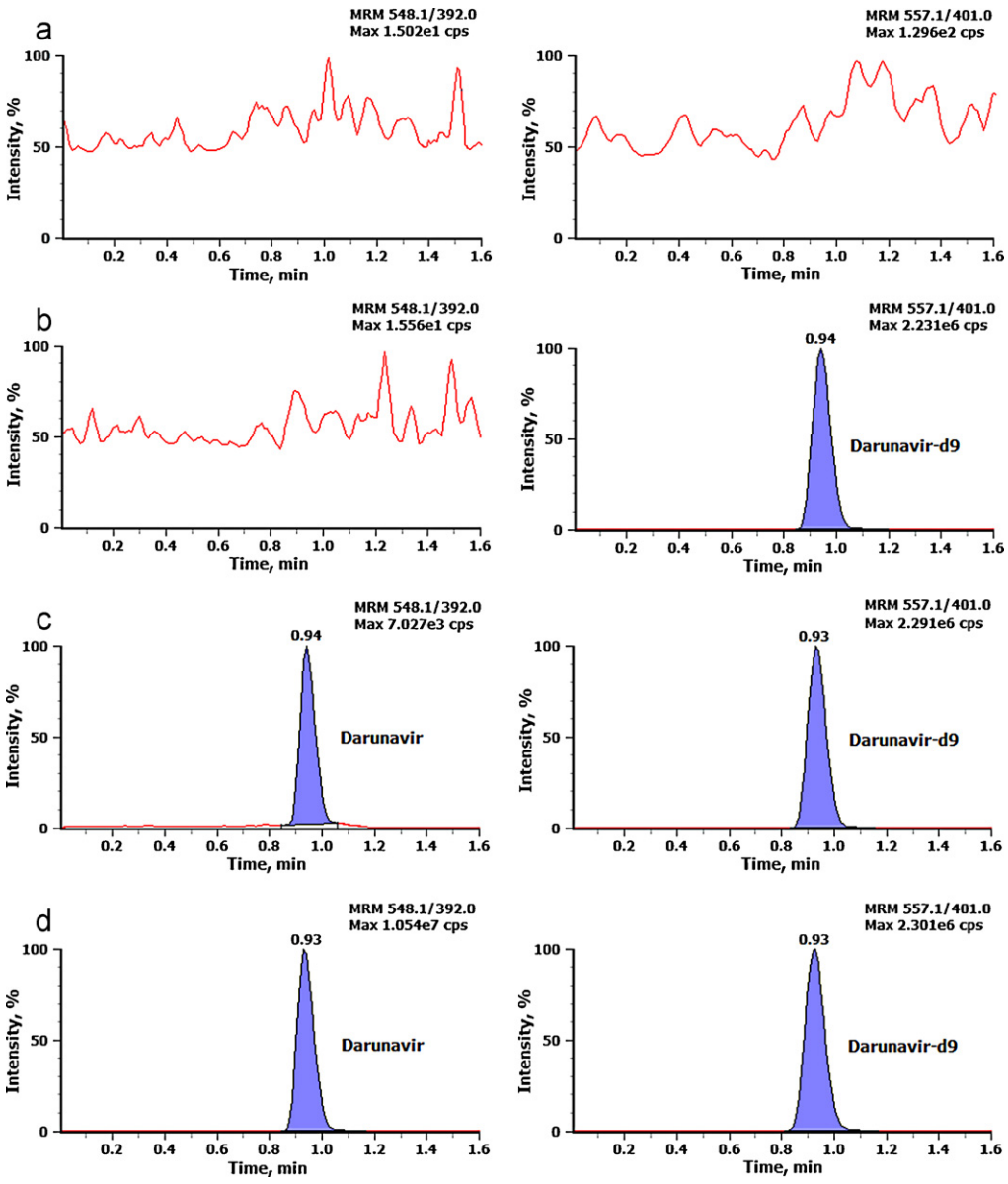

Fig. 3. MRM ion-chromatograms of darunavir (*m/z* 548.1 → 392.0) and darunavir-d9 (IS, *m/z* 557.1 → 401.0) in (a) double blank plasma (without analyte and IS), (b) blank plasma with IS, (c) darunavir at LLOQ and IS (d) real subject sample at 1 h after administration of 600 mg dose of darunavir.

duced the best sensitivity, efficiency and peak shape. The response obtained was inadequate, with poor chromatography on Gemini C18 and ACE C18 columns as shown in Fig. 2a and b respectively. Thus, both these columns were not considered for further study. Luna CN column offered superior peak shape and good response for DRV and IS compared to the previous two columns, however, the drug was not adequately retained on the column and eluted at 0.5 min. Further, Chromolith revered phase C18 column was investigated as their selectivity is comparable with conventional silica C18 columns and the separation efficiency is better than 5 μm particle columns and equivalent to 3.5 μm columns [29]. This column showed good response, retention and analysis time but the peak shapes were not satisfactory, especially at the LLOQ level. In one of the previous studies [13], Agilent Zorbax XDB C8 column (50 mm × 3.0 mm, 1.8 μm) was used to separate DRV, ritonavir and etranavir with good peak shapes and short run time. Nevertheless, in the present work, the best chromatographic conditions as a function of analyte peak intensity, peak shape, adequate retention and analysis run time was achieved with Acquity UPLC BEH C18 (50 mm × 2.1 mm, 1.7 μm) using the mobile phase A and B under gradient conditions. The total chromatographic run time was 1.6 min with a retention time of 0.94 min for DRV, was the shortest compared to previous assays [8,10–24]. Moreover, the use of deuterated internal standard DRV-d9 helped in maintaining the integrity of the column and ionization efficiency of the analyte. Additionally, it has negligible memory effects and is not present in healthy subjects or patients. Further, the reproducibility of retention time for DRV, expressed as %CV was ≤1% for 100 injections on the same column. The sensitivity achieved for DRV in the present work was 1.0 ng/mL, which is greater compared to other methods reported in human plasma [8,10–14,16–20]. Only one method [22] has higher sensitivity (0.1 ng/mL) than the work presented here, which involves simultaneous quantification of 14 antiretroviral agents in peripheral blood mononuclear cell of HIV infected patients. It was possible to further lower the LLOQ by ten folds (S/N ≥ 100), however, it was not required based on DRV concentration observed in subject samples.

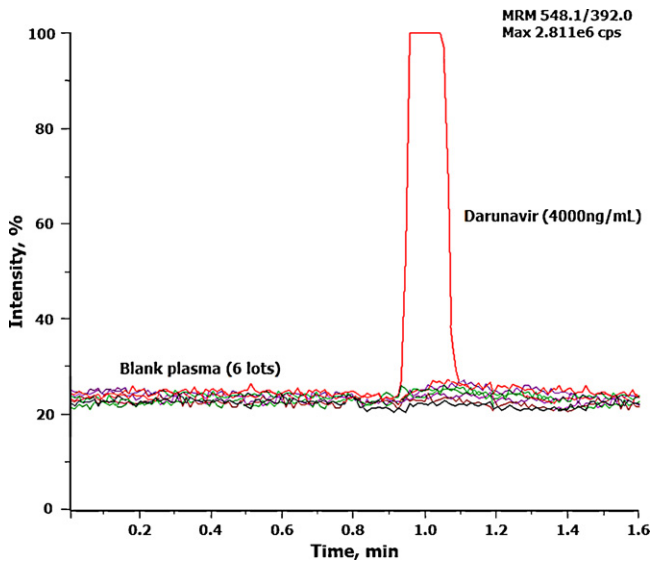

Fig. 4. MRM LC–MS/MS chromatograms of six blank plasma extracts with post column infusion of darunavir at 400 ng/mL concentration. Also shown is the chromatogram of darunavir at the HQC level.

Representative MRM ion chromatograms in Fig. 3a–d of extracted blank human plasma (without IS and analyte), blank plasma fortified with IS (*m/z* 557.1 → 401.0), DRV at LLOQ (*m/z* 548.1 → 392.0) and an actual subject sample (after 1.0 h) at peak plasma concentration demonstrates the selectivity of the method. The extraction procedure together with mass detection gave very good selectivity for the analysis of DRV and IS in the blank plasma. The chromatograms showed excellent peak shape for both the drug and its deuterated analog. None of the concomitant antiretroviral drugs studied or the commonly used medications by human volunteers interfered in the determination of DRV. Except nevirapine which eluted at 0.81 min and etravirine at 1.2 min, the retention time for all other antiretroviral drugs was close to that of DRV in the range of 0.91–1.08 min. However, due to their different MRM transitions there was no interference in the quantification of DRV. The % accuracy results were within 94.2–103.5% at both the QC levels. The result of post-column infusion experiment with six extracted blank plasma samples in Fig. 4 indicates no ion suppression and enhancement at the retention time of DRV, as evident from the flat base-line.

The average matrix factor value calculated as the response of post spiked sample/response of neat solution in mobile phase at the LLOQ levels was 0.99, which indicates a minor suppression of 1%.

3.2. Assay performance and validation

Right through the method validation, the precision (%CV) of system suitability test was observed in the range of 0.01–1.48% for the retention time and 0.42–0.87% for the area ratio response of DRV/IS. The signal to noise ratio for system performance was ≥100 for both the analytes and IS. Carry-over evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. There was negligible carry over (≤0.04%) observed during auto-sampler carryover experiment. No enhancement in the response was observed in extracted blank plasma (without IS and analytes) after subsequent injection of highest calibration standard at the retention time of DRV and IS as evident from Fig. 5.

All five calibration curves were linear over the concentration range of 1.0–5000 ng/mL for DRV. A straight-line fit was made through the data points by least square regression analysis to give the mean linear equation $y = (0.0022 \pm 0.0008)x - (0.000086 \pm 0.000089)$, where y is the peak area ratio of the DRV/IS and x the concentration of the DRV. The mean and standard deviation value for correlation coefficient (r^2) observed were 0.9992 and 0.00031 respectively. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 96.2 to 102.2% and 0.50 to 3.60% respectively for DRV as shown in Table 2. The lowest concentration (LLOQ) in the standard curve that can be measured with acceptable accuracy and precision was found to be 1.0 ng/mL in plasma at a signal-to-noise ratio (S/N) of ≥100. The intra-batch and inter-batch precision and accuracy were established from validation runs performed at HQC, MQC-1, MQC-2, LQC and LLOQ QC levels (Table 3). The intra-batch precision (%CV) ranged from 1.6 to 5.7 and the accuracy was within 101.5 to 105.3% for DRV. Similarly, for the inter-batch experiments, the precision varied from 3.7 to 4.6 and the accuracy was within 96.8 to 102.2%. The relative recovery, absolute matrix effect and process efficiency data for DRV at LQC, MQC and HQC levels is presented in Table 4. The process efficiency/absolute recovery obtained for DRV was greater than 94% at all QC levels. The mean recovery for IS in human plasma was 89.8% at these QC levels. Further, the relative matrix effect, which compares the precision (%CV) values between different lots (sources) of plasma (spiked after extraction) samples varied from 2.2 to 3.6 for DRV at LLOQ level. The accuracy results were between 96.2 and 103.6% at the LLOQ level.

The stability of DRV and IS in human plasma and stock solutions was examined under different storage conditions. Samples for short-term stability remained stable up to 12 h, while the stock solutions for long term stability of DRV and IS were stable for minimum of 17 days at refrigerated temperature of 5 °C. DRV in control human plasma (bench top) at room temperature was stable at least for 14 h at 25 °C and for minimum of five freeze and thaw cycles at −20 °C and −70 °C. Dry extract stability of the spiked quality control samples stored at −20 °C was determined up to 30 h. Autosampler stability (wet extract) of the spiked quality control samples maintained at 5 °C was determined up to 30 h without significant loss of the analytes. Spiked plasma samples stored at −20 °C and −70 °C for long term stability experiment were found stable for a minimum period of 42 days. The percentage change for different stability experiments in plasma at two QC levels is shown in Table 5.

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 for dilution integrity of 1/2 and 1/10th dilution were 1.2 and 5.6%, while the accuracy results were 94.7 and 96.6% respectively which is within the acceptance limit of 15% for precision (%CV) and 85.0–115.0% for accuracy. Method ruggedness was evaluated using re-injection of analyzed samples, two different columns of the same make and also with different analysts. The precision (%CV) and accuracy values for two different columns ranged from 1.0% to 2.9% and 94.8% to 100.1% respectively at all five quality control levels. For the experiment with different analysts, the results for precision and accuracy were within 1.1–6.3% and 95.4–101.0% respectively at these levels.

3.3. Application to a bioequivalence study and incurred sample reanalysis assessment

The validated method has been successfully used to quantify DRV concentration in the human plasma samples after co-administration of a single 600 mg oral dose of DRV and 100 mg ritonavir, and subsequently twice a day for three consecutive

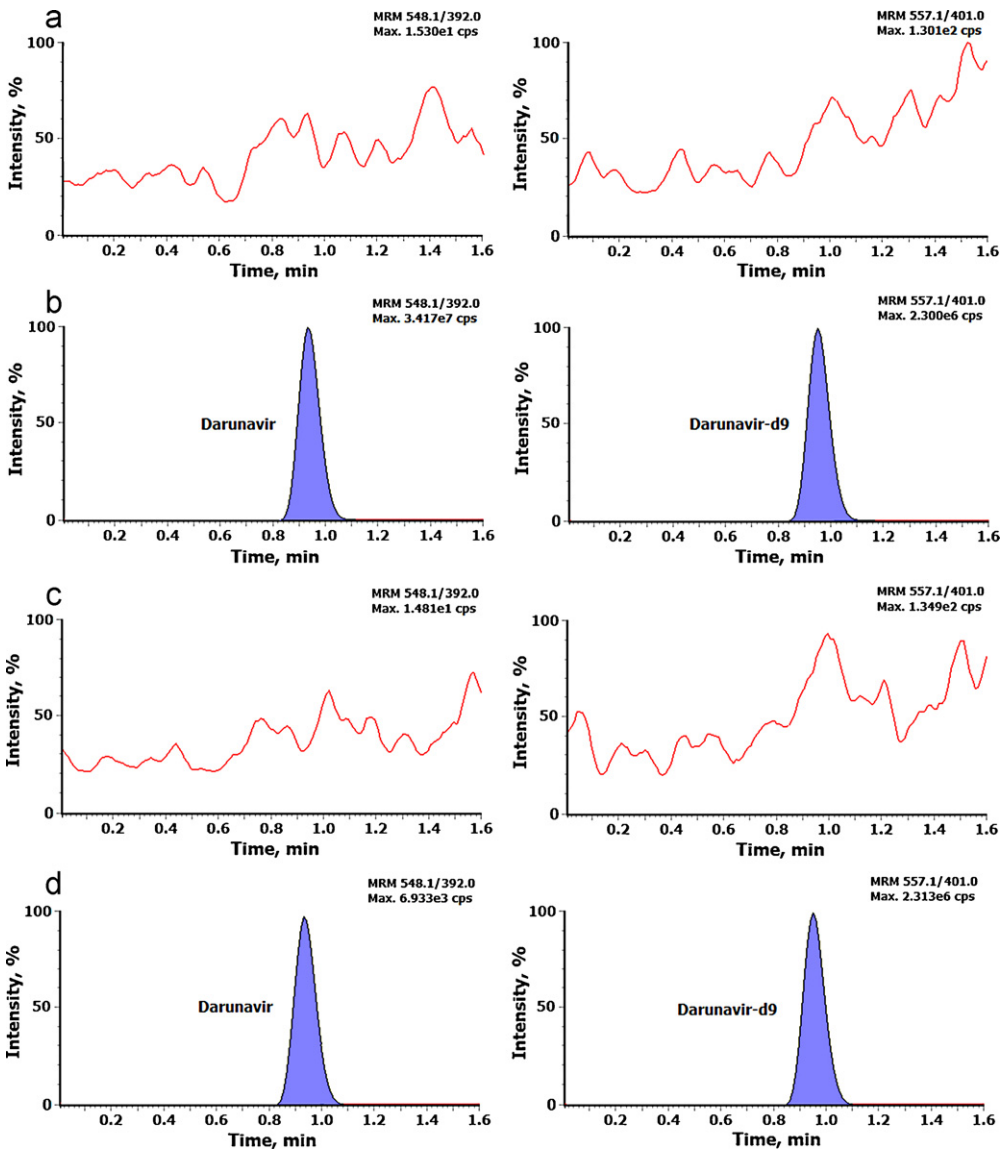

Fig. 5. MRM ion-chromatograms for carry over test of darunavir (*m/z* 548.1 → 392.0) and darunavir-d9 (IS, *m/z* 557.1 → 401.0). (a) double blank plasma (without analyte and IS), (b) darunavir at ULOQ and IS (c) double blank plasma (without analyte and IS) and (d) darunavir at LLOQ and IS.

days. As with other protease inhibitors, the metabolism of DRV is cytochrome P450 (CYP) 3A4-dependent. In clinical practice, administration of DRV with low dose ritonavir acts as a booster by inhibiting CYP3A4 isozyme and increasing its plasma concentration. The presence of ritonavir reduces the metabolism of DRV with consequent increase in the systemic exposure to DRV [4]. It has been observed that HIV-infected patients receiving ritonavir boosted DRV had statistically significantly higher virological and immunological responses than those receiving DRV alone. Moreover, the absolute oral bioavailability of a single 600 mg dose of

Table 2
Summary of calibration curves with back calculated concentration for darunavir.

Linearity	CS-1	CS-2	CS-3	CS-4	CS-5	CS-6	CS-7	C -8	CS-9	CS-10	CS-11	Regression Parameters		
	Nominal concentration (ng/mL)											Slope	Intercept	r^2
	5000	2500	1000	500	250	100	50	10	4	2	1			
1	4762.8	2504.7	1029.9	507.3	256.2	102.1	50.7	9.7	3.8	2.1	1.0	0.00192	−0.00003	0.99895
2	5070.3	2491.9	1038.7	479.2	237.5	99.0	50.6	10.3	3.9	2.0	1.0	0.00297	0.00014	0.99920
3	5070.0	2470.0	1020.0	500.0	243.0	102.0	50.5	10.0	3.8	2.0	1.0	0.00270	0.00015	0.99948
4	4965.5	2547.7	1019.9	506.6	246.4	99.7	50.5	10.2	3.9	2.0	1.0	0.00237	0.00001	0.99965
5	5038.6	2386.0	999.6	529.8	255.9	98.7	50.0	10.1	3.8	2.0	1.0	0.00105	0.00016	0.99895
Mean	4981.5	2480.0	1021.6	504.6	247.8	100.3	50.5	10.1	3.8	2.0	1.0	0.0022	0.000086	0.9992
SD	129.5	59.7	14.6	18.1	8.2	1.6	0.3	0.2	0.1	0.0	0.0	0.0008	0.000089	0.00031
%CV	2.6	2.4	1.4	3.6	3.3	1.6	0.5	2.3	1.8	1.3	1.3			
% Nominal	99.6	99.2	102.2	100.9	99.1	100.3	100.9	100.5	96.2	101.1	100.6			

CS, calibration standard; SD, standard deviation; CV, coefficient of variation; r^2, correlation coefficient.

Table 3
Intra-batch and inter-batch precision and accuracy for darunavir.

QC ID	Nominal conc. (ng/mL)	Intra-batch				Inter-batch			
		n	Mean conc. observed (ng/mL)[a]	% CV	% Accuracy	*n*	Mean conc. observed (ng/mL)[b]	% CV	% Accuracy
HQC	4000	6	4213	2.2	105.3	30	4089	3.7	102.2
MQC-1	2000	6	2066	1.6	103.3	30	1990	4.0	99.5
MQC-2	120	6	122	1.9	101.5	30	116	4.6	96.8
LQC	3.0	6	3.1	3.3	104.2	30	2.9	4.3	97.2
LLOQ QC	1.0	6	1.0	5.7	102.5	30	1.0	3.7	99.5

CV, coefficient of variance; *n*, total number of observations.
[a] Mean of 6 replicates at each concentration.
[b] Mean of 6 replicates for five precision and accuracy batches.

Table 4
Absolute matrix effect, relative recovery and process efficiency for darunavir.

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					
0.0069 (5.2)	0.0066 (5.6)	0.0066 (5.9)	95.6	100.1	95.7
MQC-2					
0.28 (1.9)	0.26 (3.2)	0.27 (2.7)	92.8	103.8	96.3
MQC-1					
4.59 (0.5)	4.42 (1.4)	4.34 (0.6)	96.3	98.2	94.6
HQC					
9.50 (0.8)	9.23 (1.8)	9.34 (0.6)	97.2	101.2	98.4

[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] $B/A \times 100$.
[f] $C/B \times 100$.
[g] $C/A \times 100 = (\text{ME} \times \text{RE})/100$.

DRV increases from 37% to 82% after co-administration with 100 mg ritonavir twice daily [30].

As the presence of food increases exposure to DRV [3], a comparative study was conducted under fed as well as fasting conditions. High fat, high calorie diet was given to subjects to assess the effect of food on bioequivalence study. Food can change the bioavailability of a drug and can influence the bioequivalence between test and reference products. Meals that are high in total calories and fat content are more likely to affect the gastrointestinal physiology and thereby result in a larger effect on the bioavailability of a drug product. Fig. 6 shows the plasma concentration vs. time profile of DRV in healthy human subjects under fast and fed conditions. The method was sensitive enough to monitor the plasma concentration up to 72 h. In all approximately 7800 samples including the calibration, QC and volunteer samples were run and analyzed during a period of 18 days and the precision and accuracy were well within the acceptable limits. The mean pharmacokinetic parameters obtained for the test and reference formulation are presented in Table 6. The 90% confi-

Table 5
Stability of darunavir under different conditions ($n = 6$).

Storage condition	Nominal conc. (ng/mL)	Calculated conc. (ng/mL)	
		Mean, stability samples ± SD	% Change[a]
Bench top stability; 14 h			
HQC	4000	3765 ± 33.5	−5.87
LQC	3.0	2.84 ± 0.2	−5.33
Wet extract stability; 30 h			
HQC	4000	3925 ± 30.6	−1.87
LQC	3.0	2.87 ± 0.1	−4.33
Dry extract stability; 30 h			
HQC	4000	3915 ± 37.9	−2.13
LQC	3.0	2.82 ± 0.1	−6.00
Freeze and thaw stability; 5 Cycles, −20 °C			
HQC	4000	3765 ± 106.9	−5.87
LQC	3.0	2.77 ± 0.1	−7.66
Freeze and thaw stability; 5 Cycles, −70 °C			
HQC	4000	3795 ± 105.2	−5.13
LQC	3.0	2.75 ± 0.1	−8.33
Long term matrix stability; 42 days, −20 °C			
HQC	4000	4040 ± 67.6	1.00
LQC	3.0	3.02 ± 0.1	0.67
Long term matrix stability; 42 days, −70 °C			
HQC	4000	4065 ± 134.2	1.63
LQC	3.0	2.96 ± 0.1	−1.33

[a] $\%\text{Change} = \frac{\text{Mean stability samples} - \text{Mean comparison samples}}{\text{Mean comparison samples}} \times 100$.

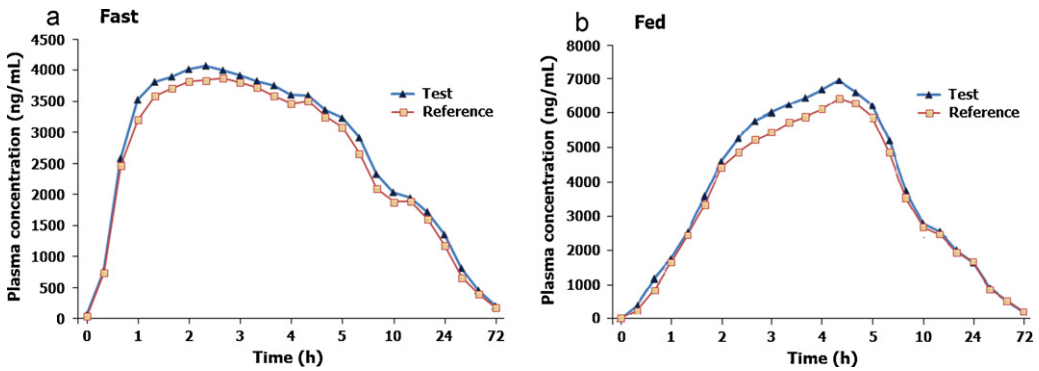

Fig. 6. Mean plasma concentration–time profile of darunavir after oral administration of test (600 mg darunavir tablets of an Indian Company) and a reference (PREZISTA®, 600 mg darunavir tablets from Tibotec Inc., NJ, USA) formulation along with 100 mg ritonavir (Norvir® from Abbott Laboratories, North Chicago, USA, twice a day) tablets to 40 healthy Indian subjects under (a) fast and (b) fed conditions.

Table 6
Mean pharmacokinetic parameters following oral administration of 600 mg tablet formulation (test and reference) of darunavir along with 100 mg ritonavir (7 × 100 mg) in 40 healthy Indian subjects under fast and fed condition.

Parameter	Fast		Fed	
	Test Mean ± SD	Reference Mean ± SD	Test Mean ± SD	Reference Mean ± SD
C_{max} (ng/mL)	4678 ± 1604	4250 ± 1224	7456 ± 1888	6918 ± 1993
T_{max} (h)	2.13 ± 1.38	2.31 ± 1.03	3.96 ± 0.99	4.02 ± 1.30
$t_{1/2}$ (h)	12.77 ± 4.88	12.40 ± 3.62	11.62 ± 5.38	12.16 ± 6.67
K_{el} (1/h)	0.061 ± 0.021	0.061 ± 0.019	0.068 ± 0.025	0.067 ± 0.029
$AUC_{0-72\,h}$ (h ng/mL)	77,612 ± 48,434	71,690 ± 32,416	10,2108 ± 37,568	98,633 ± 39,854
AUC_{0-inf} (h ng/mL)	81,887 ± 54,717	74,769 ± 33,559	106,508 ± 41,466	103,626 ± 45,926

C_{max}, maximum plasma concentration; T_{max}, time point of maximum plasma concentration; $t_{1/2}$, half life of drug elimination during the terminal phase; AUC_{0-t}, area under the plasma concentration–time curve from 0 h to 72 h; AUC_{0-inf}, area under the plasma concentration–time curve from 0 h to infinity.

Table 7
Log(ln) transformed pharmacokinetic parameters of darunavir in 40 healthy Indian subjects under fast and fed condition.

Parameter	Ratio (test/reference), %		90% CI (Lower–upper), %		Power (%)		Intra subject variation, %CV	
	Fast	Fed	Fast	Fed	Fast	Fed	Fast	Fed
Ln C_{max} (ng/mL)	107.4	108.1	98.9–116.6	102.6–113.8	99.7	100.0	22.33	14.72
Ln AUC_{0-72} (h ng/mL)	104.4	103.7	96.3–113.3	98.2–109.5	99.7	100.0	22.17	15.54
Ln AUC_{0-inf} (h ng/mL)	105.0	103.5	96.7–114.0	98.1–109.2	99.7	100.0	22.37	15.23

dence interval of individual ratio geometric mean for test/reference was within 80–125% for AUC_{0-t}, AUC_{0-inf} and C_{max} for fast and fed conditions as shown in Table 7. The results indicate 1.3–1.8 times increase in C_{max}, T_{max} and AUC values for fed conditions. However, the $t_{1/2}$ values were not significantly different in both the studies. Additionally, the intra-subject variability expressed as %CV was much higher under fasting compared to fed conditions. It has been shown previously that exposure to food and type of food increases DRV concentration [31]. Two previous studies have investigated DRV/ritonavir (400/100 mg) pharmacokinetics in HIV healthy volunteers under fast [4] and fed [3] conditions. Though the present work was done with DRV/ritonavir (600/100 mg) formulation, due to lack of dose proportionality in DRV pharmacokinetics, significant overlap in plasma concentrations for 400/100 mg and 600/100 mg doses can be imagined [3]. The $t_{1/2}$ and C_{max} values obtained in the present study for fasting were comparable with those reported by Vermeir et al. [4], however, T_{max} and AUC values were much higher in our work. Similarly, for fed study C_{max}, T_{max} and AUC values were higher compared to the previous report [3]. This variation is some parameters can be ascribed to genetic difference, gender type (body size and muscle mass), type of food, etc. Further, there was no adverse event during the course of the study.

Incurred sample reanalysis (ISR) study has now become an integral part of the bioanalytical process to assess the quality of bioanalytical assays. ISR study data reaffirms the reproducibility and reliability of a validated bioanalytical method. This was done by random selection of subject samples (10% of total samples analyzed). Out of 400 incurred samples studied, 90% samples showed % change for assay reproducibility within ±7% and the remaining 10% samples were within ±12%. This authenticates the reproducibility of the proposed method.

4. Conclusion

To summarize, the UPLC–MS/MS method for the quantitation of DRV in human plasma was developed and fully validated as per USFDA guidelines. The method offers significant advantages over those previously reported, in terms of lower sample requirements, simplicity of extraction procedure and overall analysis time. The efficiency of liquid–liquid extraction and a chromatographic run time of 1.6 min per sample make it an attractive procedure in high-throughput bioanalysis of DRV. Also, the on-column loading of sample at LLOQ level (5 pg per injection volume) was much lower compared to other reported procedures. A wide linear dynamic range ensures application of the method for higher dose strength with acceptable precision and accuracy. With dilution integrity up to 10-fold, it is possible to extended the upper limit of quantification to 8000 ng/mL. Incurred sample reanalysis with 400 samples demonstrates the reproducibility in the measurement of subject samples. The current method has shown acceptable precision and

highest sensitivity for the quantification of DRV in human plasma in a clinical study.

Acknowledgements

The authors are thankful to scientists, Mr. Pradeep Sharma, Mr. Trupesh Pethani, the chief operating officer, Mr. E. Venu Madhav and directors, Mr. Apurva Shah and Mr. Binoy Gardi of Veeda Clinical Research Pvt. Ltd. (India) for providing infrastructure facility to carrying out this work.

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