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Research Article

DEVELOPMENT AND VALIDATION OF LC-MS/MS METHOD FOR DETERMINATION OF DARUNAVIR IN HUMAN PLASMA FOR APPLICATION OF CLINICAL PHARMACOKINETICS

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ABSTRACT

A simple, rapid, sensitive, selective and high performance liquid chromatography method with MS/MS was developed and validated for determination of Darunavir (DRV) in human plasma. Extraction from the plasma was by solid phase extraction (SPE) extraction procedure. Carbamezepine was used as an internal standard (IS). The chromatographic separation was performed by Thermo Hypurity, advance column (50 X 4.6mm, 5µ) with mobile phase comprising of 5mM ammonium acetate: acetonitrile (15:85% v/v). Quantification was performed using multiple reactions monitoring (MRM) of the transition *m/z* 548.20 (parent ion) → *m/z* 392.30 (product ion); *m/z* 273.20 (parent ion) → *m/z* 194.10 (product ion) for DRV and IS respectively. The assay linearity ranged 50.14 to 2007.43 ng/mL and the lower limit of quantitation is 50.14 ng/mL. Frequently co-administered drugs did not interfere with the described methodology. The validated method is suitable to support a wide range of therapeutic drug monitoring and pharmacokinetic studies.

Keywords: Bionalysis, Darunavir, Pharmacokinetics, Human Plasma, LC-MS/MS.

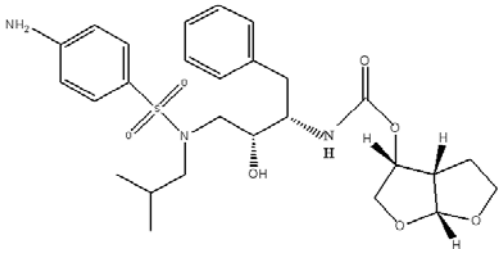
INTRODUCTION

Darunavir (DRV) is chemically [(1S,2R)-3-[[(4-aminophenyl) sulfonyl](2-methylpropyl)amino]-2-hydroxy-1-(phenylmethyl) propyl] -carbamic acid (3R,3aS,6aR)-hexahydrofuro[2,3-b]furan-3-yl ester monoethanolate [Fig.1.(I)]. It belongs to the protease inhibitors (PIs) class of medications. PIs are potent antiretroviral agents which in combination with other drugs have reduced morbidity and mortality of human immunodeficiency virus (HIV) infections[1]. However, virological failure continues to occur in a substantial proportion of HIV-infected individuals on highly active antiviral therapy (HAART). Cross-resistance is extensive within the PIs class and the efficacy of ritonavir-boosted PIs is greatly influenced by the extent of baseline protease resistance mutations. In general, the presence of ≥ 5 resistance mutations within the protease gene has been associated with a diminished response to all currently available PIs[2]. Therefore, the development of a new PI with a greater genetic barrier to resistance is crucial.

DRV was originally designed to be active against HIV strains resistant to other currently available PIs. The POWER trials have evaluated the safety and efficacy of DRV in highly treatment-experienced patients using other ritonavir-boosted PIs (as comparators, chosen as the most appropriate by the investigators). In all these studies, DRV has demonstrated significantly greater reductions in plasma HIV-RNA (ribonucleic acid) and increases in CD4+ (cluster of differentiation 4) counts over the active controls in patients with extensive PI resistance. Overall, 45% of patients on DRV had plasma HIV-RNA below 50copies/mL at week 48, more than twice seen in controls, with a similar profile of clinical and laboratory adverse events [3]. Information on DRV resistance is still scarce and mainly derived from clinical trials used for the registration of the drug. In the latest international acquired immune deficiency syndrome (AIDS) society-USA panel list[4], a total of 11 mutations were defined as specifically associated with DRV resistance. They were segregated as major (I50V, I54M, L76Vand I84V) or minor (V11I, V32I, L33F, I47V, I54L, G73S and L89V) resistance mutations[5].

Accurate measurement of PIs and non-nucleoside transcriptase inhibitors[8] (NNRTIs) plasma levels is crucial for pharmacokinetic analyses, drug-drug interaction studies, and therapeutic drug monitoring (TDM). The latter is currently considered a useful tool for the optimization of antiretroviral therapy in most international guidelines. Knowledge of pharmacokinetics of DRV in the clinical setting, namely pharmacokinetic properties and drug interaction profile, is still limited due to recent availability of this compound. Therefore, pharmacokinetic studies of DRV are requested to define the possible role of TDM of this drug in the clinical context. To date, several methods have been validated and published in combination with other drugs to determine the plasma DRV if present in high concentrations using liquid chromatography-tandem mass spectrometry[7,9-11] and HPLC[6,12]. However the reported methods had certain limitations viz., less sensitivity, long run time, facing difficulty in processing large samples and requires more plasma volume.

This paper describes a novel and selective approach, which enables the determination of darunavir with good accuracy at low drug concentrations in plasma using liquid chromatography coupled to tandem mass spectrometry (MS/MS) below the clinically relevant range of concentrations encountered in patients.



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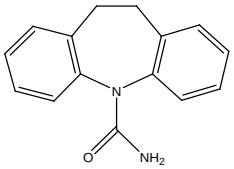


(II)

Fig. 1: Structure of Darunavir (I) and Carbamazepine (II)

MATERIAL AND METHODS

Chemicals and reagents

Reference standards of DRV and carbamazepine internal standard (IS) [Fig.1] were obtained from Hetero drugs Pvt. Ltd. (Hyderabad, India) and Neucon Pharma Pvt. Ltd, (Goa, India). Acetonitrile, methanol, hydrochloric acid obtained from J.T Baker (Phillipsburg,

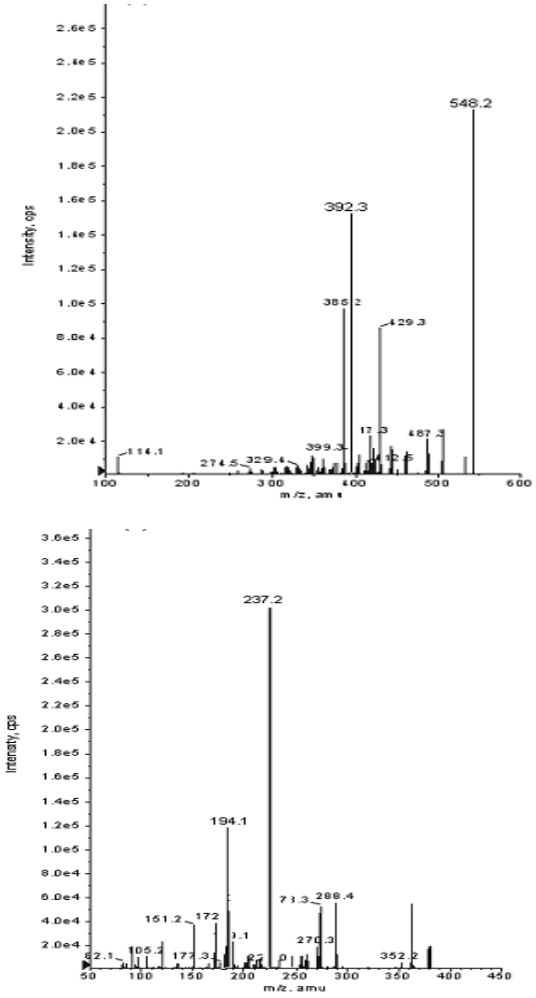
USA) and ammonium acetate were obtained from Worli (Mumbai, India). De-ionized water was prepared on MilliQ purification system from Millipore (Bangalore, India). Organic solvents and reagents used were of analytical grade. Drug-free (blank) human plasma was obtained from Cauveri blood bank (Hyderabad, India)

Instrumentation

The chromatographic system consists of Shimadzu HPLC connected with Thermo Hypurity advance column (Thermo, USA). Mass Spectrometric analysis were conducted using API 3000 triple quadrapole instrument (Applied Biosystem, Sciex, Canada), the fragmentation of ions was operated in positive ion mode. The whole system was controlled using Analyst software version 1.4.1. HLB 30mg 1cc solid phase extraction cartridge (Waters, Bangalore, India) was used for sample clean up solid phase extraction procedure.

Liquid Chromatographic Conditions

The chromatography was performed on Shimadzu HPLC system with C18 Thermo Hypurity advanced column (50 x 4.6 mm, 5 µ), that contains packing of octadecylsilane chemically bonded to porous silica was used for chromatographic separation. The mobile phase was prepared with the combination of acetonitrile and 5 mM ammonium acetate (85:15 % v/v). The flow rate of 0.6 mL/min was used to carry out separation. The column temperature was set at 45˚C, the auto-sampler was conditioned at 10°C and the injection volume was 15 µL with a run time around 2.5 min.



(I) (II)

Fig. 2 Full-Scan Positive Ion Turbo Ionspray Product Ion Mass Spectra of (I) Darunavir, (II) Carbamazepine (I.S)

Mass Spectrometric Conditions and Data Processing

The Mass Spectrometry was operated in positive ion detection mode. Nitrogen was used as nebulizing turbo spry. The temperature of vaporizer was set at 500°C and the ESI needle voltage was 5500V. The declustering potential was set at 70 volts for DRV and 50 volts for IS. Collision energy for DRV and IS was 20 and 25V respectively. The mass spectrometer was operated at unit mass resolution with a dwell time of 200 milli seconds per transition. Quantification was performed using multiple reactions monitoring (MRM) of the transition *m/z* 548.20 (parent ion) → *m/z* 392.30 (product ion); *m/z* 273.20 (parent ion)↦ *m/z* 194.10 (product ion) for DRV and IS respectively [Fig.2]. The analytical data were processed by analyst 1.4.2.

Stock Solutions, Calibration Standards and Quality Control Samples

Stock solution (1 mg/mL) of reference compound was prepared by dissolving 10 mg of DRV in 10 mL of methanol. Spiking solution was prepared from stock solution by serial dilution method in methanol: water (1:1 % v/v). Ten levels of calibration curve standards were prepared by adding the spiking solution in plasma to achieve the concentration levels of 50.14, 100.29, 300.26, 1000.87, 2001.74, 4003.49, 8006.97, 12004.46, 16005.94 and 20007.43 ng/mL. Four levels of quality control samples were prepared by adding the spiking solutions in plasma to achieve the concentration levels of 150.07, 1250.57, 10208.76 and 17014.60 ng/mL.

Extraction Procedure

All the calibration standards (200µL) or QC samples (200µL) were taken in polypropylene tubes, 20µL of IS (10027.31 ng/mL of carbamazepine) was added and vortexed for 10 seconds. The samples were transferred to a 1cm ^{3}/30 mg Oasis HLB SPE column, which had been conditioned with 2.0mL of acetonitrile followed by 2.0mL of Milli Q water. After application of the samples, the SPE column was washed with 2.0mL Milli Q water followed by 2.0mL of washing solution (acetonitrile and Milli Q water, 10:90 %, v/v); was dried for 1.0min by applying positive pressure at maximum flow rate. The column was eluted with 1.5mL of mobile phase, vortexed for 10 seconds and an aliquot was transferred into 1mL LC vials for injection of 15µL into the LC system.

Validation

The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation[13]. The method was validated for selectivity, linearity, precision, accuracy, recovery, and stability. Selectivity was assessed, by comparing the chromatograms of six different batches of blank plasma obtained from six different sources (or donors) with those of corresponding standard plasma samples spiked with DRV and I.S. Sensitivity was determined by analyzing six replicates of blank human plasma and plasma spiked with lowest level of the calibration curve. All validation experiments were carried out at three quality control (QC) levels. For the determining of intraday accuracy and precision a replicates (n=6) analysis of plasma samples were performed on the same day. The inter-day accuracy and precision were assessed by analysis of three batches on different days. Recovery of DRV and I.S were determined by comparing the peak area of extracted analyte standard with the peak area of non-extracted standard. The matrix of plasma constituents over the ionization of analyte and IS was determined by comparing the responses of the post-extracted plasma standard QC samples (n = 6) with the response of analyte from neat samples at equivalent concentrations[14-16]. Matrix effect was determined at two levels (LQC and HQC) for DRV and for IS. Dilution integrity was performed to extend the upper concentration limit with acceptable precision and accuracy. The stability of the analytes and IS in human plasma under different temperature and time conditions, as well as their stability in the stock solutions, was assessed. QC samples were subjected to short-term room temperature conditions, to freeze-thaw stability studies, reinjection reproducibility and to long-term storage at -70^{0}C. All the stability studies were conducted at two concentration levels of 150.07 ng/mL (LQC) and 17014.60 ng/ml (HQC) for DRV with six replicates for each. All stability evaluations were based on back calculated

concentrations. Samples were considered to be stable if assay values were within the acceptable limits of accuracy and precision (i.e ± 15 %).

RESULTS AND DISCUSSION

During the method development mass spectrometric conditions, extraction procedure and chromatographic conditions were optimized. The ideal condition of MS/MS detection was expected to be advantageous in developing a selective and sensitive method. Optimum mass acquisition parameters were obtained by direct infusion of 500 ng/mL solution of both analyte and internal standard at a flow rate of 10 µL/min. The mass spectrometer was operated in the MRM condition under positive ion mode. The transition of ions were monitored m/z 548.2 (parent ion) - 392.3 (product ion) for DRV and m/z 273.2 (parent ion) - 194.1 (product ion) for internal standard (carbamazepine). An earlier report[9,10], had suggested the capillary temperature was 350°C and capillary voltage 4.0KV. However in the present, study capillary temperature 500°C and capillary voltage was 5.5KV which were optimized along with other tuning parameters for enhanced ionization, sensitivity and reproducibility.

Bouche MP et al[7], Antonio D'Avolio et al[9], Fayet A et al[10], Robter Heine et al[11] have reported a protein precipitation method to separate darunavir. As analyte is a polar drug, extraction was initially carried out via protein precipitation with common solvents like perchloric acid, acetonitrile, methanol, acetone, but reproducibility was very poor due to lot of matrix interference, which resulted in contamination of quadrapoles, leading to lesser sensitivity.

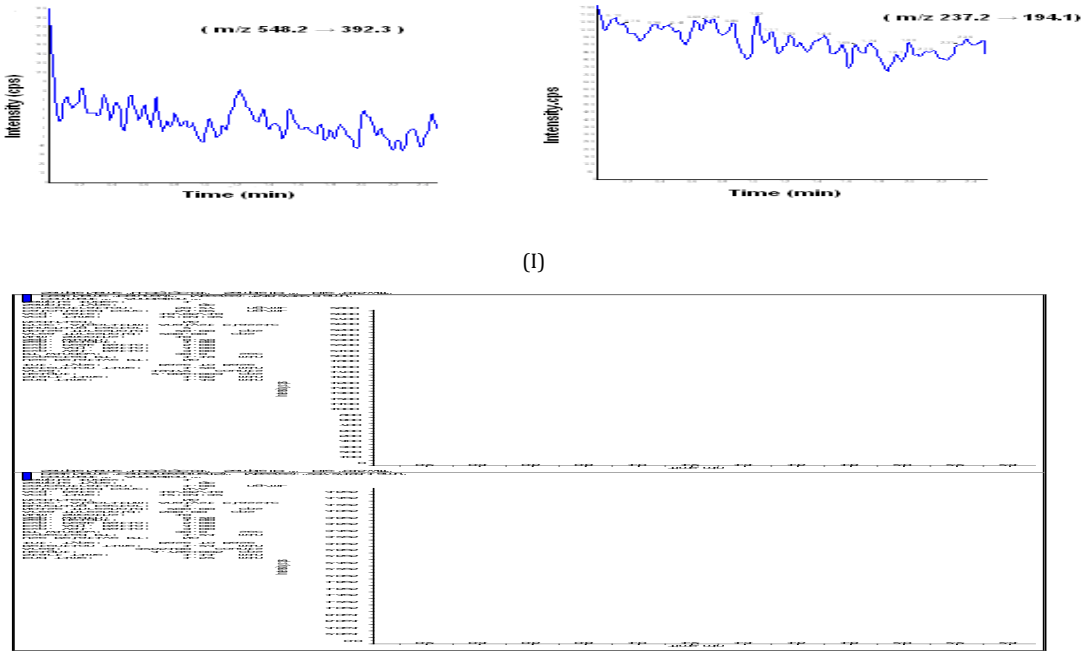
Masaaki T et al[6] has reported a liquid-liquid extraction method to extract DRV from human plasma. Analyte being a polar drug, moderate polar solvents like methyl *tert* butyl ether, isopropyl alcohol and ethyl acetate in acidic and basic conditions were used to extract DRV and IS. None of the above extractions produced desired sensitivity and reproducibility. Based on earlier report[12], in present study evaluated the extraction method in solid phase extraction using Water Oasis MCX, Waters Oasis HLB, Waters Oasis WAX, Bond Elut C18 and Phenomenex Strata cartridges. Waters Oasis HLB produced better repeatability and reproducibility, when compared with other cartridges. Hence Waters Oasis HLB has been chosen for the extraction of DRV and IS.

It was essential to have a chromatographic separation of the drugs and to minimize any interference during quantitation. Chromatographic analysis of darunavir and IS was initiated under isocratic conditions to obtain adequate response, sharp peak shape and a shorter run time. The separation was attempted using various combinations of methanol/acetonitrile, acidic buffers and additives like formic acid, ammonium acetate, ammonium formate on different reversed-phase columns [ACE C18 (50×4.6 mm, 5 µm), Gemini C18 (50×4.6 mm, 5µm), Kromasil C18 (50×4.6 mm, 5 µm), and Thermo Hypurity advance C18 (50×4.6 mm, 5 µm)]. Best results in terms of reproducibility, complete separation and peak shape without any interference were obtained with Thermo Hypurity advance C18 (50×4.6 mm, 5 µm) column compared to others and hence was selected for further study. Earlier reports[9,10] used gradient mobile phase condition, which results slow equilibration of the column and shifting of base line. In our method a mobile phase consisting of acetonitrile-ammonium acetate (5 mM) (85:15 % v/v) was found most suitable for eluting DRV and IS at around 2.5 min. A flow-rate of 0.6 mL/min produced good peak shape and sensitivity. Earlier publications[6,7, 9-12] have reported longer run times (5 to 30 min) when compared with this chromatographic condition. By virtue of its similarity in chromatographic behaviour and ionization pattern carbamazepine was selected as IS.

Method Validation

Selectivity

Possible interferences at the retention times of DRV and IS from endogenous compounds were checked during the validation by testing six different batches of K_2EDTA human plasma, one lipemic blank plasma and one lot of haemolysed blank in order to check the absence of signals for the retention times of each compound. Selectivity was carried out by analyzing six blank plasma samples spiked with DRV (LLOQ level) and IS. Representative chromatograms of extracted blank plasma are presented in Fig.3. (A) Extracted blank plasma sample; (B) Extracted lower limit of quantification plasma sample.



(I)

(II)

Fig. 3: Representative Chromatogram of Darunavir (I) Extracted Blank Plasma Sample (II) Extracted LLOQ + IS Plasma Sample

Linearity

Linearity of the method was evaluated using bulk spiked plasma samples in the concentration range as mentioned above using the method of least squares. Five such linearity curves were analyzed. Each calibration curve consisted of a blank sample, a zero sample (blank + IS) and ten concentrations. The standard curves were linear over the concentration range of 50.14 - 20007.43 ng/mL. The mean correlation coefficient was 0.9991. Samples were quantified using the ratio of peak area of analyte to that of IS. A weighting factor linear regression ($1/x^2$) was performed with the nominal concentrations of calibration levels. Peak area ratios were plotted against plasma concentrations. The limit of quantitation was found to be 50.14 ng/mL.

Recovery

The extraction efficiency of DRV from human plasma at the concentrations of LQC, MQC and HQC was found to be 70.44, 66.93 and 66.06% respectively. The mean recovery for the internal standard was 77.20% [Table 1].

Precision and Accuracy

The precision for the six plasma samples spiked with DRV at LLOQ concentration was 1.53% with a mean accuracy of 100.95% [Table 2]. The inter-batch assay accuracy ranged between 100.15 - 101.51%, whereas intra-batch accuracy ranged between 101.44 – 105.76%. The inter-batch precision ranged between 3.80 - 5.3% and intra batch precision ranged between 2.59 - 4.02%. The results are presented in Table 3. All the results were found within the acceptable limit of precision was not more than 15.0% and accuracy was between 85.0 and 115.0%.

Matrix Factor

The matrix effect intended method was assessed using chromatographically screened human plasma. The precision (%CV) at HQC and LQC levels was within 1.4 - 4.0% [Table 4].

The matrix effect was not observed at analyte and IS retention times. All the results were found within the acceptable limit of precision not more than 15.0%.

Dilution Integrity

The precision for dilution integrity standards at 1:2 and 1:5 were 0.7% and 0.6% respectively. The mean accuracy for dilution integrity of 1:2 and 1:5 were 100.2% and 100.8% [Table 5].

All the results were found within the acceptance criteria of not more than 15.0% for precision and 85.0-115.0% for accuracy.

Table 1: Percentage Recovery of DRV and IS

Nominal concentrations (ng/mL)	% Recovery	
	DRV	IS
150.07	70.44	78.75
10208.76	66.93	75.28
17014.60	66.06	77.57
Mean Recovery	67.81	77.20

Table 2: Precision and Accuracy Data of Back-Calculated Concentration of Calibration Samples for DRV in Human Plasma

Conc. added (ng/mL)	Conc. determined (mean ± S.D) (ng/mL)	Precision (%)	Accuracy (%)
50.14	50.62±0.774	1.53	100.95
100.29	98.99±3.366	3.40	98.70
300.26	291.91±13.632	4.67	97.21
1000.87	1020.01±13.464	1.32	101.91
2001.74	2026.04±73.950	3.65	101.21
4003.49	4005.46±152.608	3.81	100.04
8006.97	8296.18±141.035	1.70	103.61
12004.46	11749.21±363.051	3.09	97.87
16005.94	15684.57±425.052	2.71	97.99
20007.43	20095.35±729.461	3.63	100.43

Table 3: Precision and Accuracy of the Method for Determining DRV Concentration in Plasma Samples

Conc. added (ng/mL)	Intra-day precision (n=6)			Inter-day precision (n=30)		
	Conc. determined (mean ± S.D) (ng/mL)	Precision (%)	Accuracy (%)	Conc. determined (mean ± S.D) (ng/mL)	Precision (%)	Accuracy (%)
150.07	154.35±6.205	4.02	102.61	150.30±5.711	3.80	100.15
10208.76	10355.18±358.289	3.46	101.44	10229.10±545.211	5.33	100.19
17014.60	17996.91±466.120	2.59	105.76	17272.011±775.513	4.49	101.51

Table 4: Matrix Effect of DRV

HQC			LQC			Internal Standard		
Processed (A)	Aqueous (B)	Matrix Factor(A/B)	Processed (A)	Aqueous (B)	Matrix Factor (A/B)	Processed (A)	Aqueous (B)	Matrix Factor (A/B)
5067487	5387913	0.941	44696	47787	0.935	3114128	3678412	0.847
4970289	5456234	0.911	42256	48228	0.876	3298321	3775214	0.874
5098654	5490705	0.929	41267	47997	0.860	3023672	3726507	0.811
5124569	5478514	0.935	42373	48126	0.880	3300421	3698023	0.892
4962487	5445203	0.911	40605	48205	0.842	3231710	3702340	0.873
4978403	5455179	0.913	44213	48078	0.920	3211672	3745207	0.858
Mean	5452291.3	0.92		48070.17	0.89		3720951	0.86
SD		0.013			0.035			0.028
% CV		1.4			4.0			3.3

Table 5: Dilution Integrity for DRV

Concentration of the Dilution Integrity Sample 34030.285ng/mL				
Description	**Concentration Obtained for 1/2 Dilutions (ng/mL)**		**Concentration Obtained for 1/5 Dilutions (ng/mL)**	
	Sample concentration	**With dilution factor**	**Sample concentration**	**With dilution factor**
Aliquot 1	17177.357	34354.713	6840.461	34202.303
Aliquot 2	17147.701	34295.402	6837.596	34187.978
Aliquot 3	17000.062	34000.123	6870.925	34354.627
Aliquot 4	16905.647	33811.293	6817.942	34089.711
Aliquot 5	16911.664	33823.328	6922.427	34612.136
Aliquot 6	17166.305	34332.609	6891.255	34456.274
Mean	17051.456	34102.911	6863.434	34317.172
SD	127.865	255.731	38.924	194.618
% CV	0.7		0.6	
% Mean Accuracy	100.2		100.8	

Stability Study

The bench top stability at room temperature was determined for 6 h by comparing the accuracy of the mean concentrations for the low and high QCs which were found to be 103.5 and 102.9 % respectively. Bench top stability, the results allowed us to conclude that the analytes are stable 6 h at room temperature. The freeze-thaw stability was determined at -20°C for the low and high QCs, which underwent four freeze thaw cycles. In each freeze thaw cycle, the frozen plasma samples were thawed at room temperature for 2-3 h and refrozen for 12-24 h. The accuracy of the mean concentrations for the low and high QCs were found to be 100.1 and 101.0 %. Auto sampler stability of the plasma samples were over 53h established at 10°C. Freeze and thaw stability results indicated that the repeated freeze and thaw (four cycles) did not affect the stability of analytes. All the stability results were within the acceptable limit of precision not more than 15.0% and accuracy between 85.0 and 115.0 % [Table 6].

Table 6: Stability Results for DRV

Stability	**Spiked conc. (ng/mL)**	**Mean calculated comparison sample conc. (ng/mL)**	**Mean calculated stability sample conc. (ng/mL)**	**Precision %**	**Accuracy %**
Process	150.07	150.10	146.35	2.2	97.5
	17014.60	17019.26	17172.43	1.6	100.9
Bench top	150.07	150.14	155.39	2.7	103.5
	17014.60	17006.80	17500.00	1.3	102.9
Freeze and thaw	150.07	150.08	150.23	1.6	100.1
	17014.60	17025.22	17195.473	1.9	101.0

CONCLUSION

A simple, specific, rapid and sensitive LC-MS/MS method has been developed for the determination of darunavir in human plasma and fully validated according to FDA guidelines. The developed assay method was validated and showed good precision (inter batch 3.80-5.3 %; intra batch 2.59-4.02 %) and accuracy (inter batch 100.51-101.51 % ; intra batch 101.44-105.76 %) over a wide concentration range (50.14-20007.43 ng/mL) with no interference by endogenous compounds. This method will be helpful in processing of large number of samples with minimal run time (2.5 min) for clinical pharmacokinetic and therapeutic drug monitoring studies. Our method suitably answers the demands of clinicians for monitoring novel drugs, often as salvage therapy, to heavily pre-treated patients, in whom exposure, tolerance and adherence assessments are critical issues. Further studies will determine its contribution to risk minimization and to therapy optimization.

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