International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.11, No.01, pp 28-34, 2018

Bioanalysis of Darunavir in Human plasma using Liquid Chromatography coupled with tandem mass spectrometry

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Abstract : This paper describes a simple and rapid LC-MS/MS method for the determination of Darunavir in human plasma using carbamazepine as internal standard (IS). Analyte and the IS was extracted from the 100 µL of human plasma *via* protein precipitation (PP). The chromatographic separation was achieved on a C_{18} column by using a mixture of 0.1% formic acid in acetonitrile – 5mM ammonium acetate buffer (75:25, v/v) as the mobile phase at a flow rate of 0.7 mL/min. Detection involved an API-4000 LC-MS/MS with electrospray ionization in the positive mode. The calibration curve obtained was linear ($r^2 \geq 0.99$) over the concentration range of 20.10–3501.23 ng/mL. Method validation was performed as per FDA guidelines and the results met the acceptance criteria. The proposed method can adopt for the regular bioequivalence study analysis and also can easily adoptable for clinical drug monitoring due to its simplicity and ruggedness.
Keywords : Darunavir in human plasma; Protein precipitation (PP); Liquid chromatography–tandem mass spectrometry; Method validation.

Introduction

Darunavir is an antiretroviral drug used to treat and prevent HIV/AIDS. In general, the drug was recommended to use with other antiretrovirals like ritonavir or cobicistat to increase darunavir levels. Darunavir belongs to a class of HIV drugs called protease inhibitors (PIs). PIs block an HIV enzyme called protease and thereby prevents the viral replication[1,2].

As per the literature, many LC-MS/MS methods have been reported for the simultaneous determination of darunavir along with the other anti-retroviral drugs[3,13]. But, those are having the limitation like 1) very complex and laborious extraction procedure, 2) longer run time, 3) lack of dynamic linearity range. But, the major concern in the world is treatment/control the progress of AIDS which demands the rapid turnover time in

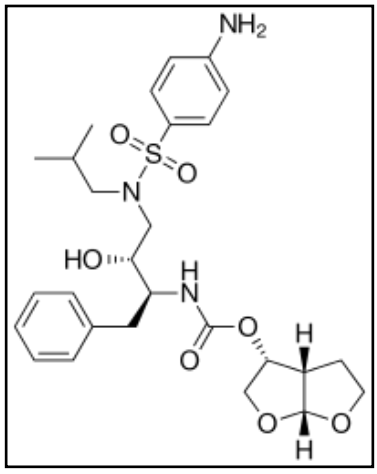
http://dx.doi.org/10.20902/IJPTR.2018.11104

research. Bioanalytical method should be gratify in terms of sensitivity, selectivity and rapid[14-16]. In the year 2011, Gupta *et al*., 2011[13] has been reported the UPLC-MS/MS method which exactly meets the requirement of the bioequivalence studies. But they have mainly focused on instrument time rather than sample preparation and able to get a minimal sample turnaround time. Moreover, they have used the UPLC which was not very common LC flat-form in many of the organizations/institutions. Hence, we adopted the simple and reliable approach for high throughput bioanalysis[17,18] of darunavir in human plasma. The developed method can compete with earlier reported UPLC-MS/MS method in order to make it use in almost all research institutes. Further, in the proposed methodology, the authors focused even on sample preparation to reduce the turnaround time further without affecting the quality.

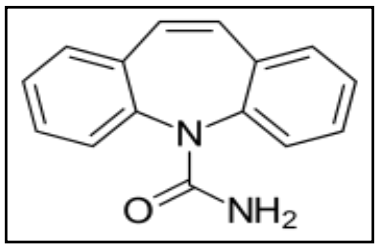
Experimental

Chemicals and reagents

Darunavir reference sample (99.7% pure) and carbamazepine (98.9% pure) was obtained from Vardha Biotech (Mumbai, India. Their structures are shown in Fig. 1. HPLC grade methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, USA). Analytical grade formic acid was purchased from Merck Ltd (Mumbai, India). Water used for the LC–MS/MS analysis was prepared by using Milli Q water purification system procured from Millipore (Bangalore, India). The control human plasma sample was procured from Deccan's Pathological Lab's (Hyderabad, India).



Darunavir



Carbamazepine

Figure 1. Chemical structures of darunavir and carbamazepine (IS).

LC–MS/MS instrument and conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a Hypurity advance column (50 mm ^{x} 4.6 mm, 5 µm; Thermo Scientific), a binary LC–20AD prominence pump, an auto sampler (SIL–HTc) and a solvent degasser (DGU–$20A_3$) was used for the study. Aliquot of 10 µL of the processed samples were injected into the Hypurity advance, 50*4.6mm, 5µ column, which was kept at 40 ºC. A mixture of 5mM ammonium acetate and 0.1% formic acid in acetonitrile (75:25, v/v) was used as mobile phase and pumped at flow rate of 0.7 mL/min. An API–4000 mass spectrometer (MDS Sciex, Foster City, CA, USA) operated in positive ion

mode was used for the quantification. The ion spray voltage was set at 5000 V and source temperature at 500 ºC. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 35, 40, 30, and 8 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 73, 19, 10, 10 V for darunavir and 50, 25, 10, 15 V for the IS. Detection of the ions was carried out in the multiple–reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 548.1 precursor ion to the *m/z* 392.0 for darunavir and *m/z* 237.1 precursor ion to the *m/z* 194.1 product ion for the IS. Quadrupoles Q1 and Q3 were set on unit resolution. The analysis data obtained were processed by Analyst Software™ (version 1.4.1).

Preparation of plasma standards and quality controls

The standard stock solution of darunavir and the IS (1 mg/mL) were prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in water–methanol (50:50, v/v; diluent). The IS working solution (1 µg/mL) was prepared by diluting its stock solution with diluent. The calibration standards in plasma were prepared by spiking 50 µL in 950 µL of plasma and the concentrations obtained were 20.10, 40.20, 80.30, 200.20, 405.52, 805.23, 1610.42, 2605.21 and 3501.23 ng/mL. Likewise, quality control (QC) samples were also prepared as a bulk based on an independent weighing of standard drug, at concentrations of 20.20 (LLOQ), 60.58 (low), 201.92 (middle 1), 1292.28 (middle 2) and 2907.64 ng/mL (high) as a single batch at each concentration. All the plasma samples prepared were stored in the freezer at –70 ± 10ºC until analyses.

Sample processing

A simple protein precipitation (PP) method was employed to extract the analytes from plasma. To an aliquot of 100 µL of plasma sample, 20 µL of internal standard dilution was added and vortex for 20 s. To this, 500 µL of 1% formic acid in acetonitrile was added. After vortex mixing for 2 min and centrifugation at 12000 rpm for 5 min, the supernatant (300 µL) was transferred to another clean test tube and diluted with 100 µL of 5mM ammonium acetate buffer and injected.

Bioanalytical method validation

The validation of the above method was carried out as per US FDA guidelines [19]. The parameters determined were selectivity, specificity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability.

Results and Discussion

Method development

The main goal of the present work was develop simple and rapid LC-MS/MS method for the determination of darunavir in plasma applicable to pharmacokinetic and bioequivalence studies. In this process we critically monitored mass spectrometry detection, chromatography and sample preparation conditions. During method development stage, mass parameters were tuned in both positive and negative ionization modes using electrospray ionization source. The highest response was obtained in positive ion mode than the negative ion mode. The most sensitive mass transition $[M+H]^+$ was observed from m/z 548.1 to 392.0 for darunavir and from m/z 237.1 to 194.1 for the IS. LC–MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. The dwell time for each transition was 200 ms.

The mobile phase, flow rate and column type was suitably evaluated to get better chromatographic conditions with short run time. Organic solvents like methanol and acetonitrile were tested in combination with volatile buffers like ammonium acetate and ammonium formate as well as acid additives like formic acid and acetic acid. The best chromatographic results were obtained with 0.1% formic acid in acetonitrile and 5mM ammonium acetate (75:25, v/v). The use of a Hypurity advance C_{18} column (50 mm x 4.6 mm, 5 µm) gave good peak shapes and response even at lowest concentration level for the analyte and IS. The mobile phase was pumped at a flow rate of 0.7 mL/min. The analyte and the IS was eluted at a retention time of 1.2 min, with a total run time of 2.2 min.

As a purpose to develop a simple and time cost extraction method, protein precipitation (PP) was tested. PP was carried out using ethanol, methanol and acetonitrile solvents. Of all the solvents tested, addition of 1% formic acid in acetonitrile to the plasma samples helped in obtaining consistent and reproducible response with good chromatography. For LC–MS/MS analysis, use of stable isotope–labeled drugs as internal standards proves to be helpful when a significant matrix effect is possible. Isotope–labeled analyte was not available to serve as IS, so, in the initial stages of this work, many compounds were investigated in order to find suitable IS, finally carbamazepine was found to be best for the present purpose.

Selectivity, chromatography and sensitivity

The selectivity of the method was assessed by analyzing blank human plasma samples obtained from different sources (Fig. 2A) and an extract spiked only with the IS (Fig. 2B). No significant direct interference in the blank plasma traces was found from endogenous substances in drug–free human plasma at the retention time of the analyte and IS (Fig. 2A) and also absence of direct interference from the IS to the MRM channel of the analyte (Fig . 2B). An LLOQ sample was presented in Fig. 2C.

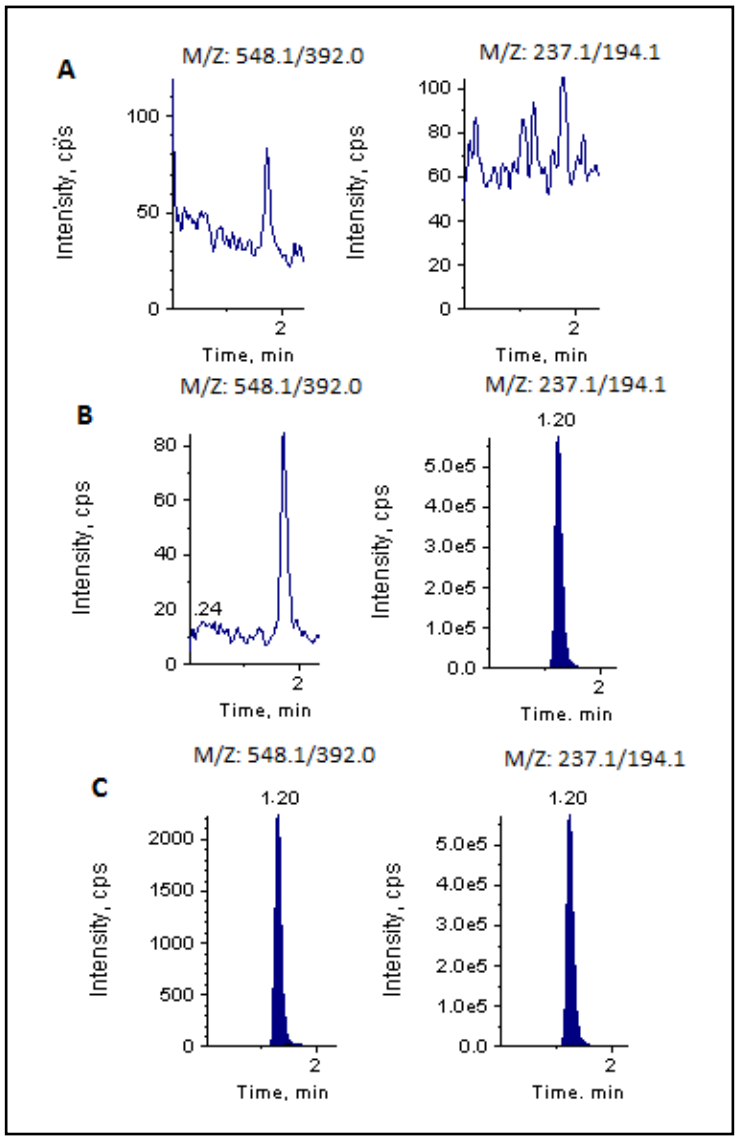


Figure 2. Typical MRM chromatograms of darunavir (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS (C).

The lowest limit of reliable quantification for the analyte was set at the concentration of the LLOQ (20.20 ng/mL). The precision and accuracy of analyte at LLOQ concentration was found to be 7.65 and 104%, respectively.

Matrix effect and recovery

No significant matrix effect was observed in all the six batches of human plasma for the analyte at low and high quality control concentrations. The precision at LQC and HQC concentrations were found to be 3.15% and 4.85%. Consistent and reproducible recoveries were obtained with simple PP technique. The mean overall recovery of darunavir was 78.36±2.59% with the precision range of 0.21–0.57%.and the recovery of IS was 80.25% with the precision range of 1.68–5.80%.

Linearity and precision and accuracy

The nine–point calibration curve was found to be linear over the concentration range of 20–3501 ng/mL for darunavir. A weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration–detector response relationship. The mean correlation coefficient was ≥ 0.99. The results for intra–day and inter–day precision and accuracy in plasma quality control samples are summarized in Table 1. The results revealed good precision and accuracy.

Table 1 Precision and accuracy data for darunavir.

Quality control	Run	Concentration found Mean±SD (ng/mL)	Precision (%)	Accuracy (%)
Intra–day variations (*n*=12 at each concentration)				
LLOQ		20.02 ± 1.60	7.99	99.10
LQC		64.89 ± 3.23	4.97	107.11
MQC1		211.35 ± 9.44	4.47	104.67
MQC2		1347.48 ± 88.24	6.55	104.27
HQC		3064.30 ± 146.68	4.79	105.39
Inter–day variations (*n*=30 at each concentration)				
LLOQ		22.10 ± 2.15	9.77	109.35
LQC		65.30 ± 4.19	6.41	107.78
MQC1		208.60 ± 12.41	5.95	103.30
MQC2		1326.55 ± 74.46	5.61	102.65
HQC		2957.40 ± 204.35	6.91	101.71
Nominal concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 20.20, 60.58, 201.92, 1292.28 and 2907.64 ng/mL, respectively.				

Dilution integrity

The upper concentration limit of darunavir can be extended to 5252 ng/mL for by 1/2 and 1/4 dilutions with screened human blank plasma. The mean back–calculated concentrations for 1/2 and 1/4 dilution samples were within 90–110% of their nominal value. The coefficients of variation (%CV) for 1/2 and 1/4 dilution samples were less than 2%.

Stability studies

In all the stability experiments like bench top stability (8 h), repeated freeze–thaw cycles (4 cycles), and long term stability at –70 °C for 60 days and processed samples stability autosampler stability (48 h), reinjection stability (30 h), wet extract stability (38 h at 2–8 °C), the mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels (Table 2).

Table 2 Stability data for darunavir in plasma (*n*=6).

Stability test	QC (spiked concentration, ng/mL)	Mean±SD (ng/mL)	Accuracy/ Stability (%)	Precision (%)
Aautosampler stability (at 10°C for 48 h)	60.58 2907.64	56.14 ± 2.93 2834.57 ± 102.78	92.67 97.49	5.22 3.63
Wet extract stability (at 2–8 °C for 38 h)	60.58 2907.64	63.25 ± 1.17 2886.52 ± 54.93	104.41 99.27	1.85 1.90
Bench top stability (8 h at room temperature)	60.58 2907.64	63.43 ± 1.08 2876.61 ± 66.62	104.71 98.93	1.71 2.32
Freeze–thaw stability (four cycles)	60.58 2907.64	65.12 ± 2.34 2855.42 ± 296.07	107.49 98.20	3.59 10.37
Reinjection stability (30 h)	60.58 2907.64	63.96 ± 1.63 2777.22 ± 53,73	105.58 95.51	2.55 1.93
Long–term Stability (at –70°C for 60 days)	60.58 2907.64	62.19 ± 4.04 2808.55 ± 34.72	102.66 96.59	4.04 1.24

Conclusions

The HPLC-MS/MS assay method described is rapid, simple and specific for quantification of darunavir in human plasma and validated as per the FDA guidelines. This method employs simple and one step PP technique for the sample preparation. Moreover, the total analysis time (extraction and chromatography) is the shortest compared to all these methods. Thus, the advantage of this method is that a relatively more number of samples can be analyzed in short time, thus increasing the output. The validated method can successfully apply to assay human plasma samples from the clinical study of darunavir.

Acknowledgements

The authors gratefully acknowledge PCR Laboratories, India for providing necessary facilities to carry out this work.

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