How to Cite:
Manikanta, Y., & Jayakumari, S. (2022). LC-MS/MS method development and validation for the determination of antiviral drug in human plasma. *International Journal of Health Sciences*, *6*(S6), 7604–7612. https://doi.org/10.53730/ijhs.v6nS6.11412

LC-MS/MS method development and validation for the determination of antiviral drug in human plasma

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***Abstract*---**The validated protein precipitation method was applied to estimate Darunavir in human plasma with Verapamil as an internal standard. The analytical column was selected Agilent, Zorbax, and XDB C18 (2.1 x 50 mm ID, 5 µm). The column temperature was set at 30°C. The mobile phase composition was acetonitrile:2Mm Ammonium Formate with 0.1% formic acid in water (70:30 v/v). Source flow rate at 0.120 mL/min without a split. An injection volume of 10 µL. Darunavir and Verapamil were eluted at 1.35 &1.13 min, with a total run time of 3.0 min for each sample.

***Keywords*---**Bioanalysis, HPLC-ESI-MS/MS, Human plasma, Darunavir.

Introduction

Darunavir is an Antiretroviral drug from the protease inhibitor class used to treat HIV and AIDS. Nomenclature: - [(1S,2R)-3-[[(4- Aminophenyl)sulfonyl] (2-methyl propyl)amino]-2-hydroxy -1-(phenylmethyl) propyl] carbamic acid (3R,3aS,6aR)-hexahydrofuro[2,3-b]furan-3-yl ester. The molecular formula is $C_{27}H_{37}N_3O_7S$. [http://www.rxlist.com/ norvir-drug. htm]The molecular weight is 547.66. The melting point of the drug is 74⁰C.2 It is amorphous white, solid, freely soluble in methanol, acetonitrile, and soluble in ethanol. [Goldwirt L et al.] Darunavir contains a bis-tetrahydro-furanyl (bis-THF) moiety and sulfonamide isostere; the drug is administered as its ethanolate salt. [Shinde VR et al.].The chemical structure of Darunavir is (figure 1)

International Journal of Health Sciences ISSN 2550-6978 E-ISSN 2550-696X © 2022.
Manuscript submitted: 9 April 2022, Manuscript revised: 27 June 2022, Accepted for publication: 18 July 2022

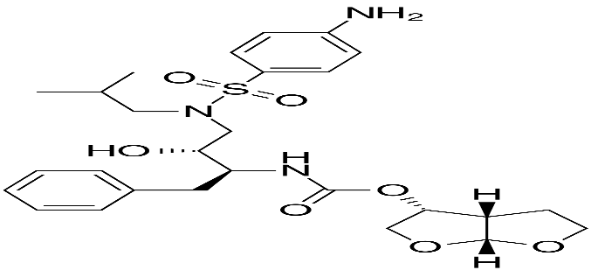

Figure 1. Chemical structure of Darunavir

Materials and Methods

Chemical Resources

Darunavir and Verapamil (Internal Standard) were procured from Fisher chemicals, Mumbai, India. Analytical Reagent grade formic acid and acetonitrile were procured from Merck (Mumbai, India). Human plasma (K2EDTA) was obtained from Doctors pathological Lab, Hyderabad. Ultrapure water from the Milli-Q system (Millipore, Bedford, MA, USA) was used throughout the study. All other chemicals in this study were of analytical grade.

Instrument Resources

An LC-MS/MS method was performed on a liquid chromatographic system consisting of a Waters AcquityUPLC system coupled with a Water Quattro Premier XE mass spectrometer with electrospray ionization (ESI) used for analysis, and Mass Lynx 4.1 SCN 805 software for processing and data collecting. Agilent, Zorbax, and XDB C18 (2.1 x 50 mm ID, 5 µm) are used as a stationary phase.

Chromatographic conditions

The analytical column was selected Agilent, Zorbax, and XDB C18 (2.1 x 50 mm ID, 5 µm). The column temperature was set at 30°C. Mobile phase composition was acetonitrile:2Mm Ammonium Formate with 0.1% formic acid in water (70:30 v/v). Source flow rate at 0.120 mL/min without a split. An injection volume of 10 µL. Darunavir and Verapamil were eluted at1.35 &1.13 min, with a total run time of 3.0 min for each sample.

Detection

The pure drug solutions of Darunavir and Verapamil were prepared in ACN (10.00 ng/mL) and injected with a flow rate of 0.120 µL/ min into positive ion mode mass spectrometer for optimization of mass parameters like source temperature, IS, heater gas, nebulizer gas, curtain gas, CAD gas (all gas channels were purged with ultra-high pure nitrogen gas), EP, DP, CE, FP, and CXP were optimized. The analysis was performed using MRM positive ion mode with mass transitions of m/z (amu) 548.50 ®392 .10and 455.40 ® 165.00 for Darunavir and Verapamil, respectively. The mass fragmentation pattern of parent and product ions mass spectra is depicted in Figures 2 &3.

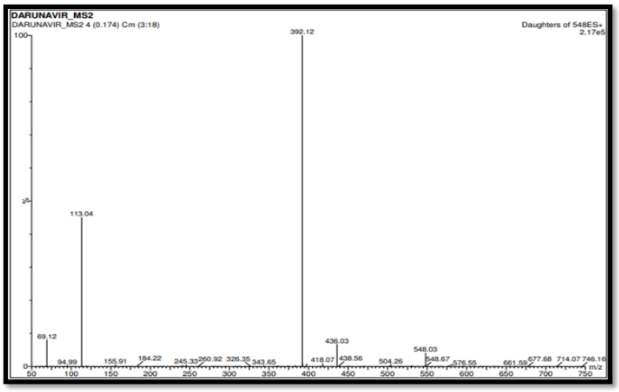

Figure 2. Mass fragmentation pattern of Darunavir

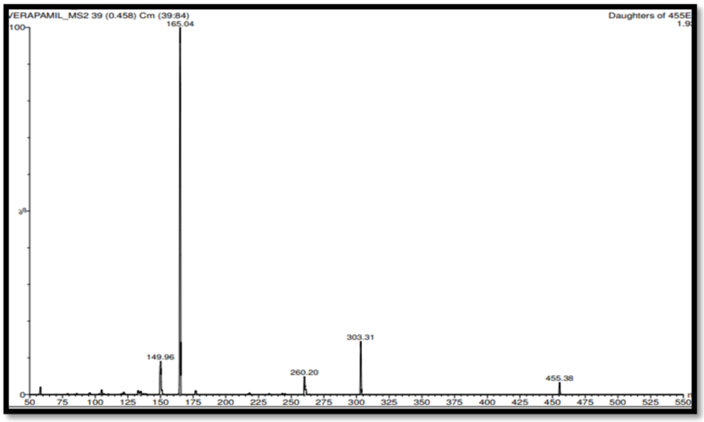

Figure 3. Mass fragmentation pattern of Verapamil

Standard calibration and quality control samples preparation

Stock solutions of Darunavir (1000.00 µg/mL) and verapamil (1000.00 µg/ml) were prepared in ACN. Stock solutions of Darunavir, Verapamil, and intermediate spiking solutions were stored in refrigerated conditions (2–8°C) until analysis. Calibration standards 10.00 to 2000.00 ng/mL, quality control samples of lower limit QC, low QC, mid-QC, and high QC (11.00, 25.00, 800.00, 1200.00 ng/mL) were used by spiking the appropriate amount of standard solution in the drug-free plasma and stored at –30°C till analysis.

Sample extraction

The Protein precipitation extraction procedure was used to isolate Darunavir from the plasma samples. Retrieved plasma blank, Quality control samples from Freezer and allowed them to reach room temperature. Vortexed the samples to ensure complete mixing of contents. Added 20 µL of 50% Methanol in water to a ria vial labeled as blank. Added 20 µL of ISTD (Mixed ISTD with approximately 2ug of Verapamil) to the pre-labeled ria vials (except blank), then transferred 100 µL sample to the vials from the specified samples and vortexed. Added 0.250 mL of acetonitrile vortexed and centrifuged at 4000 rpm, at 20°C and transferred the supernatant 150µL into auto-injector vials and Injected 10 µL onto LC-MS/MS system

Method validation

The developed method was validated over a linear concentration range of 10.00 to 2000.00 ng/mL. In addition, the validation parameters, selectivity and specificity, LLOQ, linearity, precision and accuracy, matrix effect, recovery, and stability (stock solution stability, autosampler, benchtop, short term) were evaluated under the validation section.

Selectivity and Specificity

Ten blank plasma samples were analyzed, out of which six lots free from interference were selected for assessing the selectivity and specificity. The endogenous/potential interfering peak areas for blank samples must be less than 20% of the LLoQ peak area of Darunavir retention time and less than 5% for Verapamil retention time.

Linearity

Calibration standards were prepared to obtain a linearity range of 10, 20, 50, 100, 500, 1000, 1600, & 2000 ng/ ml and assayed in five replicates on five days.

Precision and Accuracy

One set of calibration standards and one set contains four different concentrations of quality control standards of lower limit QC (11.00 ng/mL), low QC (25.00 ng/mL), mid-QC (800.00 ng/mL), and high QC (1200.00 ng/mL) concentrations were prepared in screened plasma and analyzed each quality control (QC) standards in six replicates on the same day (Intra-day) and five different days (Inter-day).

Matrix Effect

Six extracted blank plasma samples in three replicates were spiked with the un-extracted concentration of low -QC (11.00 ng/mL) and compared with un-extracted standards of the same concentration.

Recovery

The recovery of samples was performed by the Liquid-liquid extraction method. The extraction recovery was determined in six replicates by comparing the extracted QC standards with un-extracted QC standards at three different concentrations of low 11.00 ng/mL), medium (800.00 ng/mL), and high (2000.00 ng/mL).

Stability studies

Benchtop Stability (Room Temperature Stability, 24 hours) Six replicates of spiked low and high concentrations (Benchtop stability samples) were set aside at ambient temperature for up to 24 hours. Samples were processed and compared with newly prepared low and high concentrations (comparison samples).

Stock solution stability

Room temperature stock solution stability was determined using standard stock solutions of Darunavir and verapamil (ST stability samples) were set aside on the bench up to 9.5 h and compared with newly prepared stock solutions (comparison samples).

Long-term stability (-30°C, 64 Days)

After completion of the stability period stored at -30 °C (64 days), six replicates of low and high concentrations (LT stability samples) were compared with newly prepared low and high concentrations (comparison samples).

Results and Discussion

Method development [Ana Carolina Kogawa et al . – kiado et al.]

In a Mobile phase containing acetonitrile and 2Mm Ammonium acetate with 0.1% formic acid in water (70:30 v/v), the best signal and a marked improvement in the peak shape were observed for Darunavir and Verapamil. The best signal and good peak shape were obtained using the Agilent, Zorbax, and XDB C18 (2.1 x 50 mm ID, 5 µm) column. It gave good peak shapes for both Darunavir and Verapamil. A flow rate of 0.120mL/min without a splitter was used, reducing the run time to 3.0 min. The column oven temperature was maintained at a constant temperature of about 30°C. An injection volume of a 10µL sample is adjusted for better ionization and chromatography. Before loading the sample for LC injection, the co-extracted proteins should be removed from the prepared solution.

Method validation

Selectivity and Specificity, Lower Limit of Quantification (LLOQ)

No significant response was observed at Darunavir and verapamil retention times in blank plasma compared to LLOQ and blank with IS samples. The limit of quantification for this method was proven as the lowest concentration of the calibration curve, which was proven to be 10.0 ng/mL. Representative chromatograms are shown in Figure 4

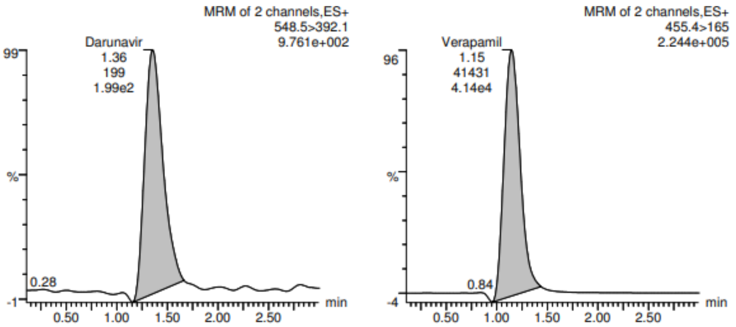

Figure 4. Chromatogram of LLOQ sample (Darunavir with Verapamil)

Linearity

Linearity was plotted as a peak area ratio (Darunavir peak area/ verapamil peak area) on the y-axis against Darunavir concentration (ng/mL) on the x-axis. Calibration curves were consistently accurate and precise for Darunavir over a linearity range of 10.00 to 2000.00 ng/mL. The correlation coefficient was greater than 0.9919 for Darunavir. The %CV was less than 15%, and the mean %accuracy ranged between 98.33-102.40%. The results are presented in Table 1.

Table 1
Calibration curve details of Darunavir

Spiked Plasma Concentration (ng/mL)	Concentration measured (ng/ml) (Mean ± S.D	% CV (n = 6)	% Accuracy
10.00	10.17±0.08	0.78	102.35
20.00	20.49±0.44	2.14	102.40
50.00	49.25±1.58	3.22	98.33
100.00	100.66±1.44	1.43	100.66
500.00	498±3.6	0.72	99.67
1000.00	997.81±4.1036	0.41	99.77
1600.00	1601.47±6.77	0.42	100.09
2000.00	2001±6.48	0.32	100.06

Precision and Accuracy

Intra and inter-batch % accuracy for Darunavir ranged between 99.79 to 100.24 and 99.74 to 100.61. % CV is 0.5 to 3.5 and 0.9-3.3. Results are presented in Tables 2 & 3

Table 2
Precision and accuracy (Intraday samples at three different concentrations)

Spiked Plasma Concentration (ng/mL	Concentration measured (n = 6;ng/mL;mean ± S.D	% CV (n = 6	% Accuracy
25	25.07±0.9	3.5	100.02

800	798.3±4.66	0.5	99.79
1200	1203±12.01	0.9	100.24

Table 3
Precision and accuracy (Interday samples at three different concentrations)

Spiked Plasma Concentration (ng/mL	Concentration measured (n = 6n ng/mL;mean ± S.D	% CV (n = 6	% Accuracy
25	25.07±0.84	3.3	100.25
800	804±10.48	1.3	100.61
1200	1196±10.80	0.9	99.74

Recovery

The mean % recovery for LQC, MQC, and HQC samples of Darunavir were 98.12%, 99.76%, and 100.06%, respectively. The overall mean %recovery and %CV of Darunavir across QC levels is 99.12% and 2.12%. For the Verapamil (internal standard), the mean % recovery and % CV is 100.68% and 3.09%, respectively.

Matrix Effect

No significant matrix effect was found in different sources of human plasma tested for Darunavir verapamil. The % CV was found to be 1.71.

Stability (benchtop, Stock solution stability, long-term stability)

Quantification of the Darunavir in plasma subjected to room temperature (Benchtop), long-term stability details as shown in Table 4.

Table 4
Stability studies of Darunavir

	Benchtop stability		Stock solution stability		Long term stability	
	24 Hr		9.5 h		64 days	
Spiked Plasma Concentration (ng/mL)	*Concentration measured (n = 6n ng/mL;mean ± S.D*	*% CV (n = 6*	*Concentration measured (n = 6n ng/mL;mean ± S.D*	*% CV (n = 6*	*Concentration measured (n = 6n ng/mL;mean ± S.D*	*% CV (n = 6*
25	24.89 ±0.98	3.2	25.78±1.48	1.2	25.98 ±0.56	3.58
1200	1203±0.77	1.58	1195.95±13.02	4.05	1298.23±0.25	6.32

Conclusion

The method described in this manuscript has been developed and validated over the concentration range of 10.00 –2000.00 ng/ml in human plasma. The intra and inter-batch precision (% CV) was less than 15.0%, and % the accuracy ranged from 99.64%–100.61%. The overall % recovery for Darunavir, and Verapamil, was greater than 95%. This method's selectivity, sensitivity, precision, and accuracy make it suitable for the present study. In conclusion, the present study's method is easy and fast to perform; it is also characterized by adequate accuracy, precision, selectivity, and stability. Furthermore, the method's simplicity and rapid liquid extraction with less run time of 3.0 min per sample make it an attractive procedure for high-throughput Darunavir bioanalysis.

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