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Development and Validation of an LC-MS/MS Method for the Quantification of Darunavir in Human Plasma

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A highly sensitive and reliable LC-MS/MS method was developed and validated for the quantification of Darunavir in human plasma using Verapamil as the internal standard. Chromatographic separation was achieved using an Agilent Zorbax XDB C18 column (2.1 × 50 mm, 5 µm) with a mobile phase comprising acetonitrile and 2 mM ammonium acetate with 0.1% formic acid in water (70:30 v/v) at a flow rate of 0.120 mL/min. The total run time was reduced to 3 minutes with a retention time of 1.35 min for Darunavir. The method demonstrated excellent linearity over the range of 10–2000 ng/mL, with a correlation coefficient exceeding 0.99. Intra- and interday precision and accuracy were within acceptable limits, with % CV values <3.5% and accuracy values ranging between 99.74% and 102.40%. Recovery studies showed consistent results across all QC levels, and no significant matrix effects were observed. Stability studies confirmed the analyte's stability under benchtop, stock solution, and long-term storage conditions. The method was validated according to regulatory guidelines, making it suitable for bioavailability, bioequivalence, and pharmacokinetic studies of Darunavir.

Keywords: Darunavir, LC-MS/MS, Method Development, Human Plasma, Validation, Protease Inhibitor, Stability Studies.

1. Introduction

Darunavir (Fig.1) is a synthetic antiretroviral drug belonging to the protease inhibitor (PI) class, used for the treatment of HIV-1 infection. It has a molecular formula of C27H37N3O7S and a molecular weight of 547.66 g/mol. Darunavir works by binding to the active site of HIV-1 protease, preventing the cleavage of viral polyproteins, which halts viral replication and results in the production of immature, non-infectious viral particles [1,2]. Though it is not directly derived from natural sources, its design is inspired by the structure-activity relationship of natural protease inhibitors, such as pepstatin. While Darunavir itself lacks phytochemical properties, plant-derived secondary metabolites like quercetin, baicalein, and curcumin have demonstrated protease inhibitory activity and continue to inspire the

development of HIV therapeutics. Darunavir has excellent bioavailability when boosted with ritonavir, exhibits high protein binding (~95%), and is metabolized primarily by CYP3A4 enzymes, with fecal excretion being the primary route of elimination [3].

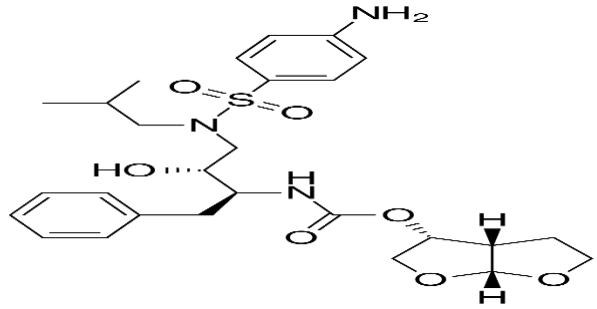

Figure 1: Chemical structure of Darunavir

2. 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.

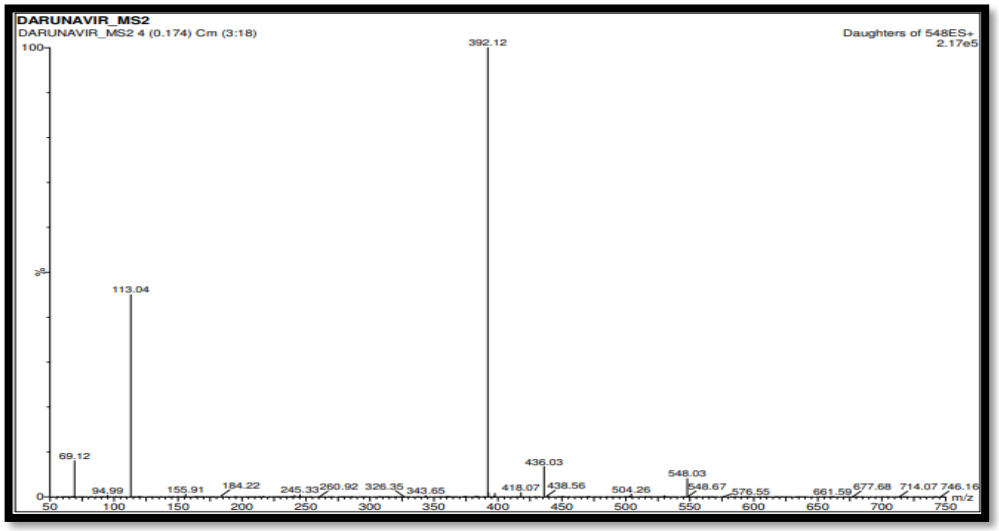

Figure 2: Mass fragmentation pattern of Darunavir

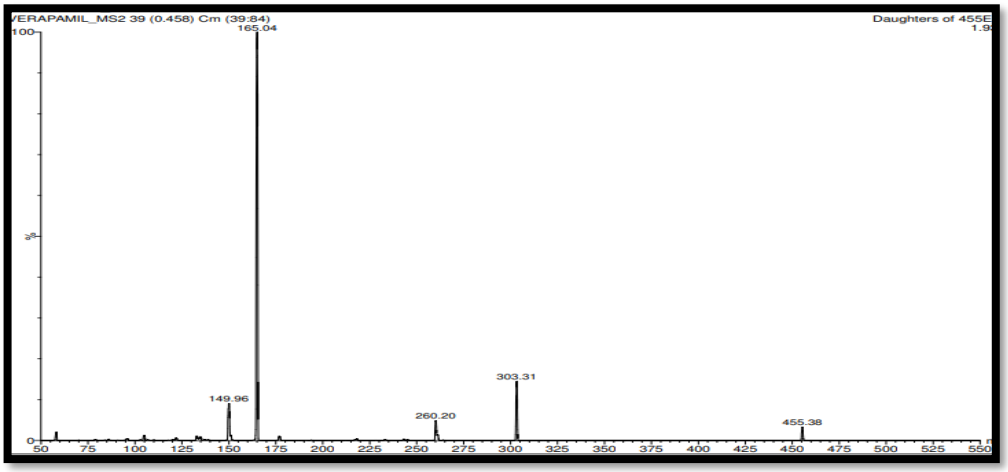

Figure 3:Mass fragmentation pattern of Verapamil

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 20ºC 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[4-12].

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:
The linearity of the method was assessed by preparing calibration standards across a concentration range of 10, 20, 50, 100, 500, 1000, 1600, and 2000 ng/mL. Each concentration was analyzed in five replicates over five consecutive days to evaluate the consistency and reliability of the calibration curve. The data demonstrated a strong linear relationship between the spiked plasma concentrations and the measured values, ensuring the method's suitability for quantitative analysis over this range. The precision and reproducibility of the calibration points were consistent across the five days, confirming the robustness of the method for routine analysis.

Precision and Accuracy

To evaluate precision and accuracy, one set of calibration standards and one set of quality control (QC) samples at four concentrations—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)—were analyzed. Intraday precision and accuracy were determined by analyzing six replicates of each QC concentration on the same day, while interday precision and accuracy were evaluated over five consecutive days. The results demonstrated high reproducibility, with % CV values within acceptable limits and accuracy values close to the nominal concentrations, confirming the reliability of the method for both short-term and long-term analysis.

Matrix Effect

The matrix effect was evaluated to assess any potential interference from plasma components that could affect ionization efficiency during LC-MS analysis. Six blank plasma samples were extracted in three replicates and spiked with the un-extracted concentration of low QC (11.00

ng/mL). These samples were then compared to un-extracted standard solutions of the same concentration. The results showed no significant difference between the extracted and un-extracted samples, indicating that the plasma matrix had minimal impact on the analyte's ionization. This confirms the method's robustness and ability to provide accurate measurements in biological matrices.

Recovery:
Sample recovery was determined using a liquid-liquid extraction (LLE) method, which ensures clean extraction of the analyte from the plasma matrix. The extraction recovery was evaluated in six replicates at three different QC levels—low (11.00 ng/mL), medium (800.00 ng/mL), and high (2000.00 ng/mL)—by comparing the concentrations of extracted QC standards to un-extracted QC standards. The recovery results were consistent and reproducible across all concentration levels, demonstrating efficient and reliable extraction of the analyte. The high recovery percentage confirmed the suitability of the LLE method for extracting Darunavir and Verapamil without significant loss.

Stability Studies:

Benchtop Stability (Room Temperature Stability, 24 hours)

To evaluate the benchtop stability, spiked plasma samples at low and high concentrations were left at ambient room temperature for 24 hours. After this period, the samples were processed and compared to freshly prepared samples at the same concentrations. The results showed no significant degradation of the analytes, indicating that both Darunavir and Verapamil remained stable at room temperature for up to 24 hours.

Stock Solution Stability

Stock solution stability was assessed by storing standard stock solutions of Darunavir and Verapamil at room temperature for 9.5 hours. These solutions were compared with freshly prepared stock solutions to determine any potential degradation. The findings demonstrated that the stock solutions remained stable, with no significant change in concentration, ensuring their reliability for routine use within the specified time frame.

Long-Term Stability (-30°C, 64 Days)

Long-term stability was evaluated by storing spiked plasma samples at -30°C for 64 days. After the stability period, six replicates of low and high concentrations were processed and compared to freshly prepared samples. The results confirmed that the analytes exhibited excellent stability under long-term storage conditions, with no significant loss of concentration or degradation over the 64-day period. This ensures the suitability of the method for the long-term analysis of stored plasma samples.

3. Results and Discussion

Method development

In the present study, an optimized mobile phase consisting of acetonitrile and 2 mM ammonium acetate with 0.1% formic acid in water (70:30 v/v) was employed for the analysis of Darunavir and Verapamil. This combination provided the best signal intensity and a

significant improvement in peak shape for both analytes. The use of the Agilent Zorbax XDB C18 column (2.1 × 50 mm ID, 5 µm) further enhanced chromatographic performance by delivering sharp, well-resolved peaks. The column's specific dimensions and particle size facilitated efficient separation and improved analyte retention, which are critical for achieving high-quality chromatographic results.

To optimize throughput and minimize run time, a flow rate of 0.120 mL/min was applied without the use of a flow splitter. This adjustment reduced the total run time to just 3.0 minutes, improving analytical efficiency without compromising separation quality. The column oven temperature was maintained at a stable 30°C, which contributed to consistent analyte retention and reproducible results. An injection volume of 10 µL was carefully selected to ensure effective ionization and enhance chromatographic performance, particularly for low-concentration analytes.

It is important to note that prior to loading the sample onto the LC system, co-extracted proteins must be removed from the prepared solution. Protein removal is a critical step as it prevents interference during analysis, improves column longevity, and enhances method reliability. Proper sample preparation ensures cleaner chromatograms, better sensitivity, and accurate quantification of Darunavir and Verapamil. This optimized method demonstrates significant improvements in efficiency, precision, and overall chromatographic quality, making it ideal for the rapid and reliable analysis of these drugs.[13-14].

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

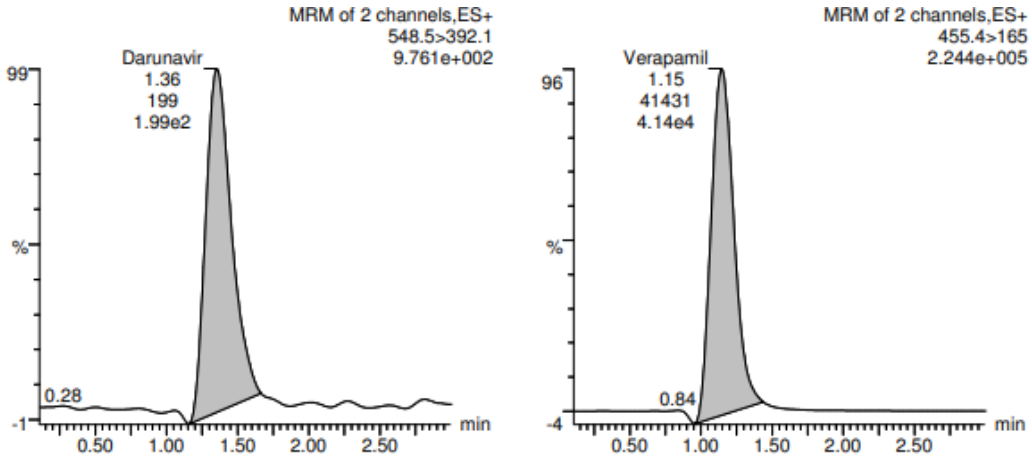

Figure 4: Chromatogram of LLOQ sample (Darunavir with Verapamil).

Linearity

Table 1 summarizes the calibration curve details of Darunavir across a range of spiked plasma concentrations (10.00 ng/mL to 2000.00 ng/mL). At the lowest concentration (10.00 ng/mL), the mean measured concentration was 10.17 ± 0.08 ng/mL with a % CV of 0.78% and % accuracy of 102.35%, indicating excellent precision and accuracy. For 20.00 ng/mL, the concentration measured was 20.49 ± 0.44 ng/mL, with a % CV of 2.14% and % accuracy of 102.40%. At 50.00 ng/mL, the mean measured was 49.25 ± 1.58 ng/mL, with a % CV of 3.22% and % accuracy of 98.33%. For mid to high concentrations (100.00 ng/mL to 2000.00 ng/mL), the % CV values ranged from 0.32% to 1.43%, and % accuracy values remained within 99.67% to 100.66%, reflecting high precision and reliability of the method. The overall data demonstrate the calibration curve's robustness, with excellent linearity, accuracy, and precision across the entire tested concentration range.

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

Table 2 presents the precision and accuracy of intraday samples at three different spiked plasma concentrations (25 ng/mL, 800 ng/mL, and 1200 ng/mL). At the low concentration (25 ng/mL), the mean measured concentration was 25.07 ± 0.9 ng/mL, with a % CV of 3.5% and % accuracy of 100.02%, indicating good precision and accuracy. For the medium concentration (800 ng/mL), the measured mean was 798.3 ± 4.66 ng/mL, showing excellent reproducibility with a % CV of 0.5% and % accuracy of 99.79%. At the high concentration (1200 ng/mL), the mean concentration was 1203 ± 12.01 ng/mL, with a % CV of 0.9% and % accuracy of 100.24%, confirming the method's high precision and reliability within the same day. Overall, these results highlight the method's robustness and accuracy for intraday analysis across all tested concentration levels.

Table 3 presents the precision and accuracy results for interday samples at three different spiked plasma concentrations (25 ng/mL, 800 ng/mL, and 1200 ng/mL). At the low concentration (25 ng/mL), the mean measured concentration was 25.07 ± 0.84 ng/mL with a % CV of 3.3% and % accuracy of 100.25%, demonstrating excellent reproducibility and accuracy. For the medium concentration (800 ng/mL), the measured mean was 804 ± 10.48 ng/mL with a % CV of 1.3% and % accuracy of 100.61%, indicating high precision and

reliability. At the high concentration (1200 ng/mL), the mean concentration was 1196 ± 10.80 ng/mL with a % CV of 0.9% and % accuracy of 99.74%, further confirming the method's accuracy and precision. These results demonstrate that the method is highly precise and accurate across all tested concentration levels over multiple days.

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)

The table 4 summarizes the stability evaluation of spiked plasma samples at two concentration levels (25 ng/mL and 1200 ng/mL) under three conditions: benchtop stability (24 hours), stock solution stability (9.5 hours), and long-term stability (64 days). For the low concentration (25 ng/mL), the measured mean values remained consistent across all conditions with minimal variation, as indicated by % CV values of 3.2%, 1.2%, and 3.58%, respectively. Similarly, at the high concentration (1200 ng/mL), the mean concentrations showed acceptable stability under benchtop (1203 ± 0.77 ng/mL, % CV 1.58%) and stock solution conditions (1195.95 ± 13.02 ng/mL, % CV 4.05%). However, long-term stability was slightly increased (1298.23 ± 0.25 ng/mL, % CV 6.32%), suggesting some variability over extended storage. Overall, the results demonstrate good stability across the tested conditions with low variability, ensuring the method's reliability for analyzing spiked plasma samples.

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

4. Conclusion

The present study successfully developed and validated a robust LC-MS/MS method for the simultaneous quantification of Darunavir in human plasma using Verapamil as an internal standard. The optimized chromatographic conditions, including the use of a mobile phase comprising acetonitrile and 2 mM ammonium acetate with 0.1% formic acid in water (70:30 v/v) and the Agilent Zorbax XDB C18 column, ensured excellent resolution, sharp peak shapes, and high signal intensity within a short run time of 3 minutes. The method demonstrated strong linearity across the concentration range of 10.00 to 2000.00 ng/mL with consistent precision, accuracy, and reproducibility, as evidenced by intra-day and inter-day validation results. Recovery studies confirmed efficient extraction using the protein precipitation method, while matrix effect analysis highlighted minimal interference from plasma components. Stability evaluations, including benchtop, stock solution, and long-term stability, demonstrated the reliability of the method over extended periods, further supporting its applicability in routine bioanalytical studies.The high sensitivity, accuracy, and precision of this method make it ideal for pharmacokinetic and bioequivalence studies of Darunavir in clinical settings. Additionally, the use of Verapamil as an internal standard enhanced method reliability and ensured minimal variability in results. Overall, this validated LC-MS/MS method provides a reliable, fast, and efficient analytical tool for the quantification of Darunavir, addressing the critical requirements for therapeutic drug monitoring and clinical pharmacokinetic evaluations.

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