

Bioanalytical Method Development And Validation Of Darunavir In Biological Matrices Using Uplc-Ms-Ms

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DOI: 10.47750/pnr.2022.13.509.615

Abstract

Background: To develop a simple and accurate method for the determination of Darunavir in human plasma by liquid-liquid extraction method with Verapamil as an internal standard using UPLC-MS/MS. **Methods:** The separation was achieved on Agilent, Zorbax XDB C₁₈ (2.1 x 50 mm ID, 5 µm) column. The samples were chromatographed using the mobile phase consisting of acetonitrile and 2mM ammonium formate with 0.1% formic acid in water (70:30 v/v) at a flow rate of 0.12 ml/min in a mass spectrometer ESI chamber. The Darunavir and Verapamil were eluted at 1.35 and 1.13 min respectively with a total run time of 3 min. **Results:** A linear response was established at 10- 2,000 ng/ml with a correlation coefficient of 0.9919. The % accuracy was found to be 98% and 102.40%. A rapid method was developed for the determination of Darunavir in human plasma. The method was strictly validated according to the ICH guidelines.

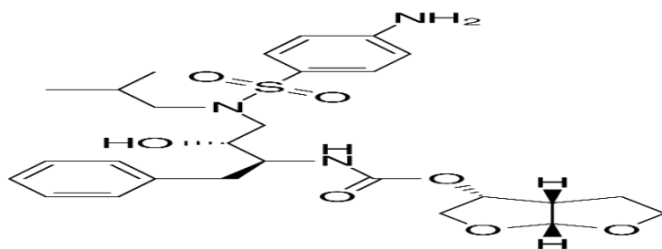
Keywords: Bioanalysis, Darunavir, HPLC-ESI-MS/MS, human plasma.

INTRODUCTION:

Darunavir is a protease inhibitor antiretroviral drug that has been used in treating the human immunodeficiency virus (HIV) in victims with a history of previous antiretroviral therapies.(1) It has been a second-generation protease inhibitor used with other drugs in the effective management of HIV infections. The chemical Nomenclature of Darunavir is written as [(1R,5S,6R)-2,8-dioxabicyclo [3.0.0]oct-6- yl]N-[2S,3R)-4-[(4-aminophenyl) sulphonyl-(2-methyl propyl)amino]-3-hydroxy-1-phenyl-butane- 2yl]carbamate and molecular formula C₂₇H₃₇N₃O₇S.(2)and molecular weight of 547.66g/mol. The point at which the drug melts is 74°C. It is an amorphous solid and white that dissolves easily in methanol, acetonitrile, and ethanol.(3) Darunavir is made up of a part called bis- tetrahydro-furanyl (bis-THF) and an isostere called sulfonamide. This drug is administered in its ethanolate salt.(4) The chemical structure was shown in (Figure 1)

Figure 1: Chemical Structure of Darunavir

In the literature, no bioanalytical methods were reported for the determination of Darunavir in human plasma. Therefore, it was thought desirable to develop a simple and accurate method using verapamil as the internal standard.



MATERIALS AND METHODS:

Chemical Resources

Darunavir and Verapamil were procured from Fisher chemicals in Mumbai, India. HPLC-grade acetonitrile and formic acid were purchased from Merck Mumbai, India. Human plasma (K2EDTA) was procured from the Doctors' pathological laboratory in Hyderabad. All experiments were conducted with Milli: Q system ultrapure water (Millipore, Bedford, MA, USA). Other chemicals and reagents were of analytical quality.

Instrument Resources

The processing and data collection were carried out by using a Waters Acquity - UPLC system, a Water Quattro - premier XE model mass spectrophotometer that was equipped with electrospray ionization interface (ESI) for the analysis, and Mass Lynx (4.1 SCN 805) software that helped in acquiring the data and results accurately.

Chromatographic conditions

The chromatographic separations were performed on Agilent, Zorbax, and XDB C₁₈ analytical columns with dimensions (2.1x50mm ID, 5µm) maintained at 30° C. The mobile phase consisted of acetonitrile and 2mM Ammonium formate with 0.1% formic acid in water (70:30 v/v) and was delivered at a flow rate of 0.120 ml/min. The two drugs: Darunavir and Verapamil were eluted at 1.35 min and 1.13 min respectively for a complete run span of 3.0 minutes per sample.

Mass Spectrometry operating conditions

Quantitation was achieved by Water Quattro - premier XE model mass spectrophotometer that was equipped with electrospray ionization interface (ESI) for the analysis, and Mass Lynx (4.1 SCN 805). The mass parameters such as the temperature of the source, internal standard, heating gas, nebulization gas, curtain gas and collision gas, and every gas channel were dismissed with ultra-pure nitrogen gas. Detection of ions was performed in the multiple reaction monitoring (MRM) mode, which was used to monitor Darunavir and Verapamil with the mass transition of m/z 548.50 @ 392.10 and 455.40 @ 165.00 respectively. The mass fragmentations for the parent and product ions were shown in Figure 2 and Figure 3 respectively.

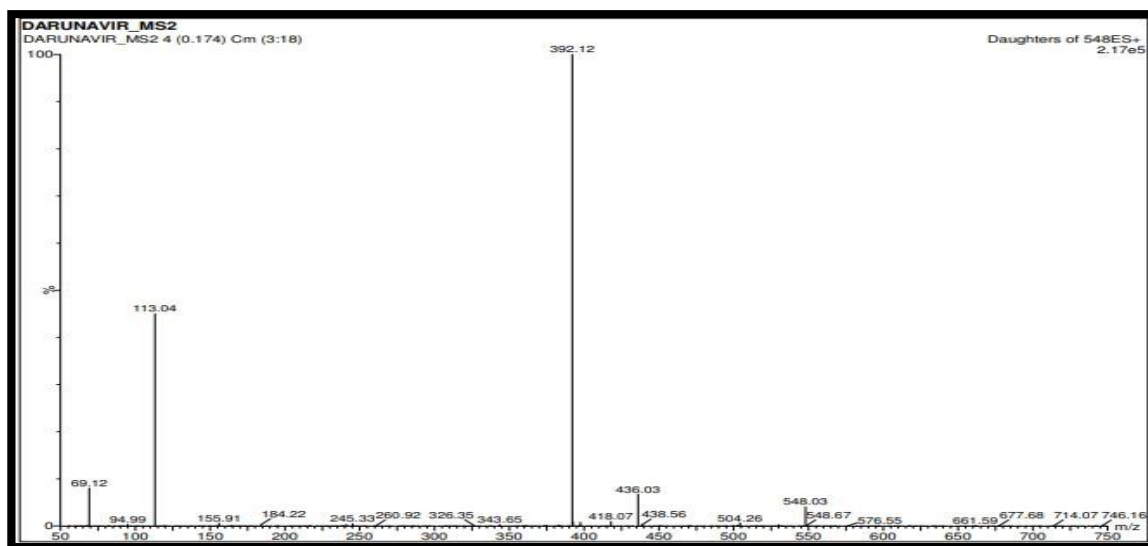
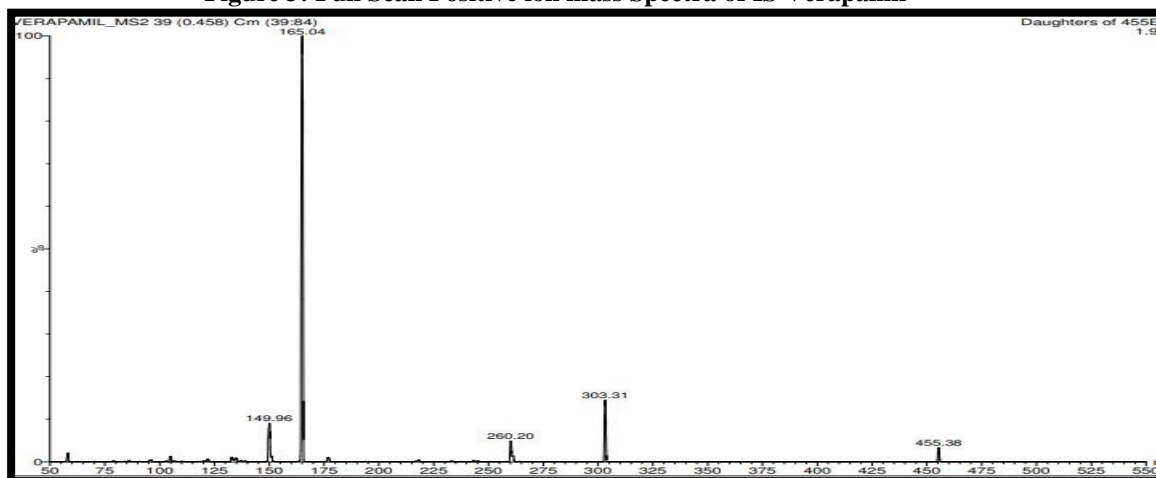


Figure 2: Full Scan Positive ion mass Spectra of Darunavir

Figure 3: Full Scan Positive ion mass Spectra of IS Verapamil



Preparation of Standard Calibration and Quality control solutions

Calibration solutions Darunavir and Verapamil were weighed accurately using an analytical microbalance. The stock solutions of Darunavir and Verapamil (1000 µg/ml) were prepared in acetonitrile. The stock solutions were stored at 2-8°C for Darunavir, Verapamil and intermediate spike solutions. Calibration standards samples (10 - 2000 ng/ml), and quality control samples consisting of the lower limit, low, middle and high-quality control limits [11, 25, 800 and 1200 ng/ml] were prepared and all the samples were stored at -30° C.

Sample extraction

Darunavir was discretely withdrawn from the plasma samples by applying the liquid-liquid extraction method after which the samples of plasma blank and quality control samples from the freezer storage were taken and thawed at room temperature. The samples were later subjected to a vortex (a whirling mass of air) to ensure the contents were mixed well. Then 20 µl of water with 50% methanol was added to a vial and labelled as "blank" after which a 20 µl of (ISTD mixed **with about 2g of Verapamil**) were added to the vials that were already labelled (except for the blank vial). Then 100µL of sample from the specified samples was transferred to the vials and mixed well. To this mixture, 0.250mL of acetonitrile was added and vortexed for 2 minutes, followed by centrifugation at 4000 rpm for 5 minutes at 20° C. Then, 150 µl of the supernatant liquid was collected and

transferred into auto-injector vials and 10 µl was infused into the LC-MS-MS system for analysis.

Method Validation

This method had been tested across a wide range of concentrations from 10 to 2000ng/ml. In addition to this, the validation process involved the evaluation of the parameters like selectivity, specificity, LLOQ, linearity, accuracy, precision, matrix effect, recovery and stability (stock solution stability, short-term, benchtop and auto sampler).

Selectivity and Specificity

After examining ten blank plasma samples, six lots devoid of interference were chosen to evaluate the assay's selectivity and specificity. For a blank sample to pass, the area of any endogenous or potentially interfering peaks must be below 20% of the LLOQ peak area for Darunavir retention time along with lower than 5 percent Verapamil retention time.

Linearity

Then the calibration standards with linearity ranges of 10, 20, 50, 100, 500, 1000, 1600, and 2000ng/ml were prepared and analyzed in five sections through five days. The ranges for the linearity standards were found to be as follows: 10, 20, 50, 100, 500, 1000, and 1600 and 2000ng/ml

Precision and Accuracy

A single batch of each of the calibration standards and the quality control standards were formulated in the analyzed plasma and examined in six clones on the same day (Intra-day) and on five consecutive days with four different concentrations of Low QC (11.00ng/ml), Low QC (25.00ng/ml), Mid QC (800.00ng/ml) and High QC (1200.00ng/ml).

Matrix Effect

Low QC (11.00ng/ml) was spiked into six withdrawn plasma samples and correlated with basic standards of the same quantity in three consecutive tests.

Recovery

To calculate the extraction recovery, six repetitions were run, each comparing the extracted and raw QC standards in three concentrations - A low concentration of 11,000ng/ml, a Medium concentration of 800,000ng/ml and a High concentration of (200,000ng/ml).

Stability Studies

Stability (Room Temperature Stability, 24 hours) Stability samples (Benchtop Stability Samples) had been preserved at room temperature for a day. These were contrasted to fresh samples of high and low concentrations (Comparative samples).

Stock Solution Stability

The standard stock solutions of the drugs Darunavir and Verapamil (Stock stability samples) were accommodated on a bench for up to 9.5 hours and compared with freshly made stock solutions to initiate the determination of the stability of stock solutions at room temperature (These are Comparison samples).

Long-term stability (86°F, 64 days)

Freshly generated low and high concentrations were correlated to low and high concentrations that were stored for 64 days at 86°F (or -30 degrees Celsius) (LT stability samples) (comparison samples).

RESULTS AND DISCUSSION

Method Development

Mass Spectrometry

Full-scan mass spectra of Darunavir and IS were obtained in MRM positive ion mode with the introduction of standard solutions directly into the ESI source. The product ion mass spectra of Darunavir and IS Verapamil were shown in Figures 2 and 3. The mass fragmentation for the parent and product ions showed a better response over the other transitions, hence it was used for the quantitative purpose.(5)

Liquid Chromatography

The optimized chromatographic conditions, especially the composition of the mobile phase were selected through several trials to reach good resolution and a symmetric peak of Darunavir and Verapamil, as well as a short run time. The mobile phase comprising acetonitrile and 2Mm ammonium formate with 0.1% formic acid in water (70:30 v/v) using Zorbax C₁₈ column (2.1 x 50mm 5 μm) at a flow rate of 0.12ml/min produced sharp peaks and a short run time of 3 minutes. The liquid extraction techniques help to separate the analytes from the plasma matrix. As a result, both drugs had favorable peak profiles.(6,7)

Method Validation

Selectivity, Specificity and Lower Limit of Quantification (LLOQ)

Representative chromatograms of blank plasma and LLOQ samples exhibited no significant interference in the retention times of Darunavir and Verapamil IS. The lowest limit of reliable quantification was set at 10.0ng/ml.(8) A typical chromatogram was shown in Figure.4.

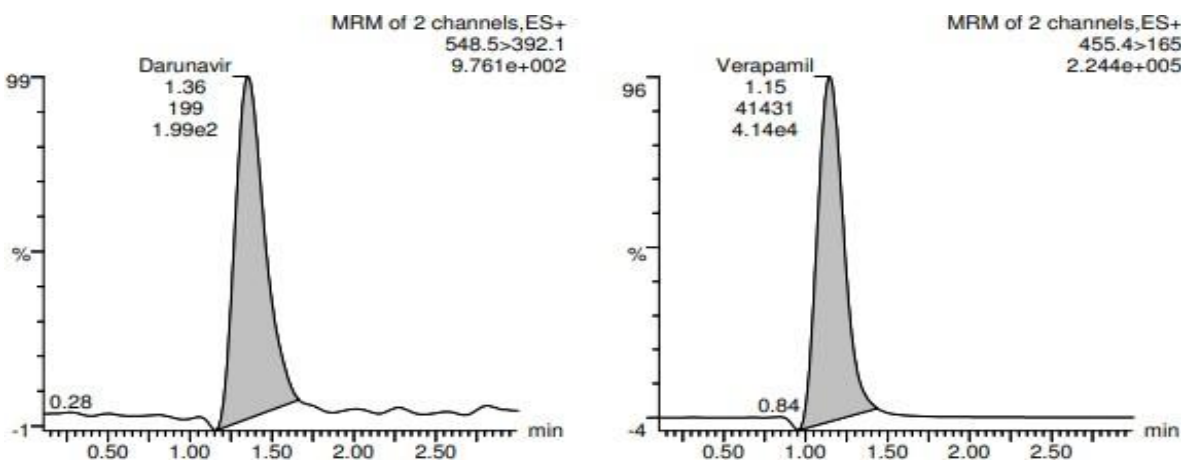


Figure 4: Chromatogram of LLOQ sample (Darunavir with Verapamil).

Linearity

A regression equation with a weighting factor of $1/(\text{concentration ratio})^2$ of analyte and IS concentration was judged to produce the best fit for the concentration detector response relationship for Darunavir in human plasma. The correlation coefficient r^2 of the calibration curves was >0.991 in the concentration range of 10 to 2,000ng/ml and was persistently well-aimed and errorless. The standard curve was linear in the calibration range. The observed mean back-calculated concentration with an accuracy of 98 and 102.4 % and precision of less than 15%.(9) The linear graph was shown in Figure.5 and the results are tabulated in Table 1.

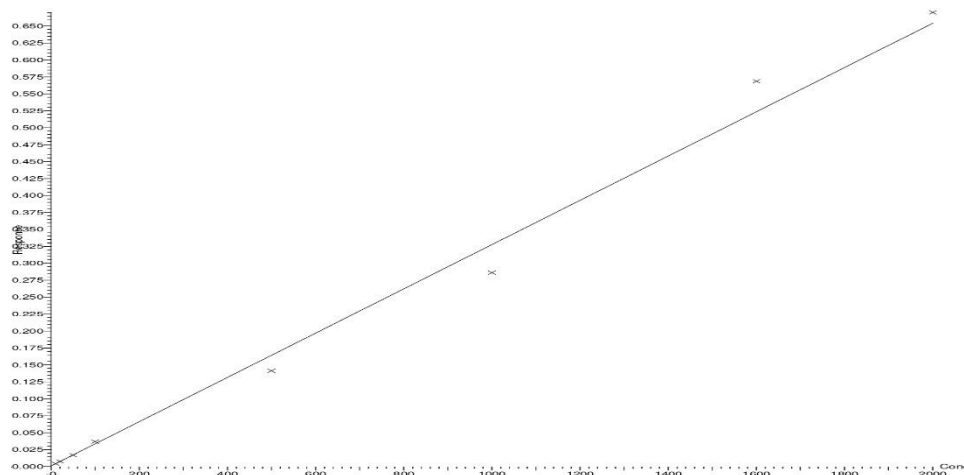


Figure 5: Linearity Graph of Darunavir

Table 1 – Calibration curve features of Darunavir

The spiked concentration of Plasma (ng/ml)	Concentration measured (ng/ml) (Mean±S.D)	% CV (n=6)	% Accuracy
10.00	10.07±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±4.10	0.41	99.77
1600.00	1601.47±6.77	0.42	100.09
2000.00	2001±6.48	0.32	100.06

Matrix Effect

No Significant matrix effect was observed in six batches of plasma for Darunavir at low and High QC concentrations. The %CV was found to be 1.71%.

Precision and Accuracy

The intra and inter-batch % accuracy range in the case of darunavir was determined to be 99.79% to 100.24% and 99.74% to 100.61% respectively. The intra and inter-batch %CV ranges from 0.5% to 3.5% and 0.9% to 3.3% respectively. The outcome observations are recorded in Tables 2 and 3.

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

The spiked concentration of Plasma (ng/ml)	Concentration measured (n=6ng/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
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Table 3: Precision and accuracy (Interday samples at three different concentrations)

The spiked concentration of Plasma (ng/ml)	Concentration measured (n=6ng/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 recovery comparison samples of Darunavir were compared against extracted samples of LQC, MQC and HQC of precision and accuracy batch. The overall mean % recovery was found to be 98.12%, 99.76%, and 100.6%, respectively. Darunavir had a mean percent recovery of 99.12% and a percent CV of 2.12% across all QC levels. The mean recovery and coefficient of variation for Verapamil i.e., the internal standards are 99% and 3% respectively. The recovery of Darunavir and IS was good and reliable, accurate and reproducible. Consequently, the method has proved to be robust for high-throughput bioanalysis.(10)

Stability studies

In stability Studies for short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a time exceeding that expected to be encountered during routine sample preparation (around 24 h). Samples were extracted and analyzed as described above and the results indicated reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of quality control samples kept in the auto sampler for 28 h was also assessed. The results indicated that solutions of the analytes and the IS can remain in the auto sampler for at least 28 h without showing a significant loss in the quantified values, indicating that samples should be processed within this period. Refrigerated stock solution stability of Darunavir and IS was carried out by injecting six replicates of stock dilutions. The stability data of the analytes in plasma over these cycles indicate that the analytes are stable in human plasma when stored at below -50 C and thawed to room temperature(11–13) and are represented in Table 4.

Table 4 – Stability studies of Darunavir

	Benchtop Stability		Stock solution stability		Long term stability	
	24hrs		9.5hrs		64 days	
Spiked Plasma concentration (ng/ml)	Concentration measured(n=6ng/ml, Mean±S.D)	%CV (n=6)	Concentration measured(n=6ng/ml, Mean±S.D)	%CV (n=6)	Concentration measured(n=6ng/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:

A simple, specific and accurate analytical method for the determination of Darunavir and IS was developed and validated. The plasma concentrations extending from 10 to 2,000 ng/ml were used for testing and validating the procedure presented in this article. The precision (percent CV) was less than 15% within and between the batches, and the accuracy was 99.64 % - 100.614 %. The technique used in this research study is not only simple and fast, but it is also accurate, precise, selective and dependable. Consequently, the method's ease of use and fast liquid extraction time

of fewer than three minutes per sample should improve highly throughout Darunavir bio analytics.

REFERENCES:

1. Busse KHS, Penzak SR. Darunavir: A second-generation protease inhibitor. Vol. 64, American Journal of Health-System Pharmacy. 2007.
2. Mastrolorenzo A, Rusconi S, Scozzafava A, Barbaro G, Supuran C. Inhibitors of HIV-1 Protease: Current State of the Art 10 Years After their Introduction. From Antiretroviral Drugs to Antifungal, Antibacterial and Antitumor Agents Based on Aspartic Protease Inhibitors. *Curr Med Chem.* 2007;14(26).
3. Goldwirt L, Chhun S, Rey E, Launay O, Viard JP, Pons G, et al. Quantification of darunavir (TMC114) in human plasma by high-performance liquid chromatography with ultra-violet detection. *J Chromatogr B Anal Technol Biomed Life Sci.* 2007;857(2).
4. Maddiboyina B, Jhawat V, Desu PK, Gandhi S, Nakkala RK, Singh S. Formulation and Evaluation of Thermosensitive Flurbiprofen In Situ Nano Gel for the Ocular Delivery. *J Biomater Sci Polym Ed.* 2021 May;1–12.
5. Kanneti R, Jaswanth KI, Neeraja KR, Bhatt PA. Development and validation of LC-MS/MS method for determination of Darunavir in human plasma for application of clinical pharmacokinetics. *Int J Pharm Pharm Sci.* 2011;3(SUPPL. 5).
6. Patel BN, Suhagia BN, Patel CN. RP-HPLC method development and validation for estimation of Darunavir ethanolate in tablet dosage form. *Int J Pharm Pharm Sci.* 2012;4(SUPPL.3).
7. Patel BN, Suhagia BN. Simultaneous determination and validation of darunavir ethanolate and ritonavir in binary mixture by liquid chromatography. *Int J PharmTech Res.* 2012;4(4).
8. Nagaraju P, Ravi Chandra S, Pavithra B, Akhil S, Altaf S. DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF DARUNAVIR AND COBICISTAT IN COMBINED TABLET DOSAGE FORMS. *Int J Res Pharm Chem.* 2020;10(2).
9. Ramesh B, Ramakrishna S, Reddy RKK, Babu KH, Sarma VUM, Devi PS. Hptlc method for determination of darunavir in rat plasma and its application in pharmacokinetic studies. *J Liq Chromatogr Relat Technol.* 2013;36(2).
10. Sindu Priya D, Gowri Sankar D, Jaya Chandrika D. Stability indicating RP-HPLC method for the simultaneous estimation of darunavir ethanolate and cobicistat in bulk and tablet dosage form. *Der Pharm Lett.* 2016;8(14).
11. Ghante MR, Shelar RS, Sawant SD, Kadam MM. DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC METHOD FOR ESTIMATION OF DARUNAVIR ETHANOLATE IN BULK AND TABLET DOSAGE FORM. *Int J Pharm Pharm Sci.* 2014;6(7):240–242.
12. Patel BN, Suhagia BN, Patel CN, Panchal HJ. A simple and sensitive HPTLC method for quantitative analysis of darunavir ethanolate tablets. *J Planar Chromatogr - Mod TLC.* 2011;24(3).
13. Rizwan SH, Girija Sastry V, Gazi S, Imad Q, Bhameshan KM. A new and validated stability indicating RP-HPLC analysis of darunavir and cobicistat in bulk drug and tablet dosage form. *Int J Pharm Sci Rev Res.* 2016;36(1).