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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPTLC METHOD FOR ESTIMATION OF DARUNAVIR

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Keywords:

High-performance thin-layer chromatography (HPTLC), Darunavir, method validation, ICH guidelines, stability-indicating method

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ABSTRACT: A simple, precise, and sensitive stability-indicating high-performance thin layer chromatographic (HPTLC) method has been developed and validated for the analysis of Darunavir in bulk and in the tablet dosage form. The separation was performed on pre-coated silica gel 60 GF_{254} plates using Toluene: Methanol: Triethylamine (8.5:1:0.5 v/v/v) as the mobile phase. The retention factor (R_f) was found to be 0.61 ± 0.89. The detection of a band was carried out at 267 nm. The drug was subjected to different stress conditions like acid, base hydrolysis, oxidation, thermal degradation, and photolysis. The method was successfully validated according to ICH Q_2 (R1) guidelines. The linear regression analysis data for the calibration plot showed a good linear relationship with R^2 = 0.992 in the range of 500-3000 ng $band^{-1}$. The method found to be accurate as results of the recovery studies are close to 100%. The developed method can be adopted for routine analysis of Darunavir in bulk and pharmaceutical dosage form.

INTRODUCTION: Darunavir is chemically [(3aS,4R,6aR)- 2,3,3a,4,5,6a-hexahydrofuro[2,3-b] furan-4-yl] N- [(2S,3R)-4- [(4-aminophenyl) sulfo-nyl-(2-methylpropyl)amino]- 3- hydroxy- 1- phenyl butan-2-yl] carbamate it is HIV protease inhibitor that is used in the treatment of AIDS and HIV infections. Darunavir is an antiretroviral protease inhibitor that is used in the therapy and prevention of human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS). Darunavir can cause transient and usually asymptomatic elevations in serum aminotransferase levels and has been linked to rare instances of clinically apparent, acute liver injury.

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In HBV or HCV co-infected patients, highly active antiretroviral therapy with darunavir may result of an exacerbation of the underlying chronic hepatitis B or C. Literature survey reveals that few analytical methods have been reported for the estimation of Darunavir including UV-Vis spectroscopy [1, 2], high-performance liquid chromatography (HPLC) [3, 4], high-performance thin-layer chromatography (HPTLC) [5, 6], Infrared Spectroscopy (IR) [7], Capillary Electrophoresis [8], LC-MS/MS [9, 10].

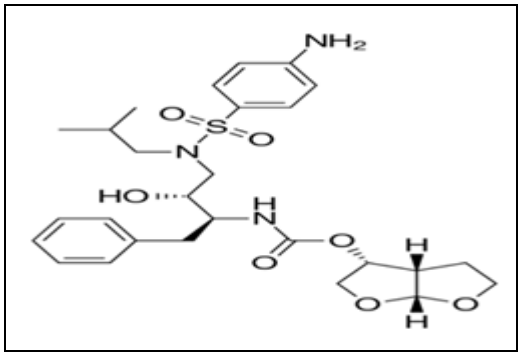

FIG. 1: STRUCTURE OF DARUNAVIR

MATERIALS AND METHODS:

Reagents and Chemicals: 20 tablets each containing 300 mg of Darunavir (Darunavir, Cipla Ltd.) was procured from the local market. Methanol (AR grade), Triethylamine (AR grade), toluene (AR grade) were purchased from S.D. Fine Chemical Laboratories, Mumbai. Hydrochloric acid (HCl), acetic acid (CH_3COOH), hydrogen peroxide (H_2O_2), and sodium hydroxide (NaOH); all AR grade were purchased from Loba Chemie Pvt. Ltd., Mumbai.

Chromatographic Conditions: Chromatographic separation of the drug was performed on aluminum plates precoated with silica gel 60 F_{254} (10 cm × 10 cm with 250 µm layer thickness). The sample was applied on the plate as a band of 5 mm width using Camag 100 µl sample syringe (Hamilton, Switzerland) with a linomat 5 applicator (Camag, Switzerland). The mobile phase was composed of Toluene: Methanol: Triehylamine (8.5:1:0.5 v/v/v). 10 cm × 10 cm Camagtwin trough glass chamber was used for linear ascending development of TLC plate under 15 min saturation conditions and 10 ml of mobile phase was used per run.

Migration distance was 80 mm. Densitometric scanning was carried out using Camag TLC scanner at 267 nm, operated by win CATS software (version 1.4.3), slit dimensions were 4.00 × 0.45 mm and Deuterium lamp was used as a radiation source.

Preparation of Standard Stock Solution: The standard stock solution of Darunavir was prepared by dissolving 10 mg of drug in 10 ml of methanol to get a concentration of 1000 µg/ml. From the standard stock solution, the working standard solution was prepared to contain 100 µg/ml of Darunavir.

Selection of Analytical Wavelength: From the standard stock solution further dilutions were made using methanol and scanned over the range of 200 – 800 nm and the spectra were obtained. It was observed that the drug showed considerable absorbance at 267 nm **Fig. 2**.

Selection of Mobile Phase and Chromatographic Conditions: Chromatographic separation studies were carried out on the working standard solution of Darunavir 100 µg/ml. Initially, trials were carried out using various solvents in various proportions on normal TLC plates to obtain the desired R_f and shape for drug peak. After a few trials, Toluene: Methanol: Triethylamine (8.5:1:0.5 v/v/v) was chosen as the mobile phase, which gave acceptable peak parameters.

Preparation of Sample Solution: 20 tablets each containing 300 mg of Darunavir (Darunavir, Cipla Ltd.) was weighed and powdered. Powder equivalent to 10 mg of drug was transferred to 10 ml volumetric flask and volume was made up with acetonitrile to get concentration (1000 µg/ml) and was sonicated for 10 min. Solution was filtered, from this solution 1 ml of drug was taken in 10 ml volumetric flask and volume was made up with acetonitrile. Further dilution in acetonitrile was done to get concentration 10 µg/ml.

Densitogram and System Suitability Parameter of Drug: Solution of Darunavir (100 µg/ml) was prepared. 10 µl (1000 ng/band) of solution was applied on pre-activated TLC plate with the help of Hamilton syringe (100 µl), using Linomat 5 sample applicator. The development chamber was saturated with the mobile phase for 15 min. The spotted plate was placed in the saturated chamber and developed up to 80 mm distance. The plate was dried and was scanned over 90 mm distance at 267 nm. The retention factor was found to be: 0.61 ± 0.89 **Fig. 3**, **Table 1**.

Stress Degradation Studies of Bulk Drug: Stress degradation studies were carried under the condition of acid as well base hydrolysis, oxidation, and dry heat. Dry heat and photolytic degradation were carried out in solid-state.

Preparation of Standard Stock Solution: Accurately weighed 25 mg of Darunavir was transferred to the 5 ml pre-calibrated volumetric flask. Darunavir was dissolved in small quantity of water. Volume was made up to 5 ml with water to achieve a stock solution of 5000 µg/ml (Stock-1).

Preparation of Sample:

Degradation under Acid-Catalyzed Hydrolytic Condition: To 1 ml of 5000 µg/ml solution of Darunavir, 1 ml of 0.5 N HCl was added. The above solution was kept for 4 h at room temperature. After exposure the solution was neutralized with 0.5 N NaOH and volume was

made up to 10 ml with methanol 0.5 µl of the resultant solution was then applied at TLC plate and densitogram was developed. 19.77% Darunavir was recovered with no peak of degradant **Fig. 4**.

Degradation under Alkali Catalyzed Hydrolytic Condition: To 1 ml of 5000 µg/ml solution of Darunavir, 1 ml of 0.5 N NaOH was added. The above solution was kept for 4 h at room temperature. After exposure, the solution was neutralized with 0.5 N HCl and the volume was made up to 10 ml with methanol. 5 µl of the resultant solution was then applied at the TLC plate and densitogram was developed. Average 16.08% of Darunavir was recovered with no peak of degradation **Fig. 5**.

Degradation under Oxidative Condition: To 1 ml of 5000 µg/ml solution of Darunavir, 1 ml of 10 % H_2O_2 was added. The above solution was kept for 1 h at room temperature. The volume was made up to 10 ml with methanol. 5µl of the resultant solution was then applied at the TLC plate and densitogram was developed. Average 25.44% of Darunavir was recovered with no peak of degradant **Fig. 6**.

Degradation under Dry Heat: Dry heat studies were performed by keeping drug sample in the oven (80 °C) for a period of 4 h. The sample was withdrawn, dissolved in methanol and diluted to get 500 µg/ml. 5 µl of the resultant solution was then applied at TLC plate and densitogram was developed. Average 76.33% Darunavir was recovered with no peak of degradant **Fig. 7**.

Photo-Degradation Studies: The photo degradation stability study of the drug was studied by exposing the drug to UV light providing illumination of NLT 200 watt hr/m^2. After exposure accurately weighed 10 mg of drug was transferred to 10 ml of the volumetric flask; the volume was made up with methanol to obtain 1000 µg/ml solution. 5 ml of the resultant solution was then diluted with methanol to get a concentration of 500 µg/ml. 5µl of the resultant solution was then applied at the TLC plate, and densitogram was developed. Average 50.38% of Darunavir was recovered with no peak of degradant **Fig. 8**.

Validation of Analytical Method: [11] The method was validated as per ICH Q2 (R1) guidelines.

Specificity: The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than 0.998, indicating the non-interference of any other peak of degradation product or impurity.

Linearity: From the standard stock solution (1000µg/ml) of Darunavir, a solution was prepared to contain 100µg/ml. This solution was further used for spotting. Six replicates per concentration were spotted. The linearity (relationship between peak area and concentration) was determined by analyzing six concentrations over the concentration range of 500-3000 ng/band.

The results obtained are shown in **Table 2**, the peak areas were plotted against the corresponding concentrations to obtain the calibration curve as shown in **Fig. 9**.

Range: Darunavir = 500-3000 ng/band

Precision: The precision of the method was demonstrated by intra-day and inter-day variation studies. In the Intra-day studies, 3 replicates of 3 concentrations were analyzed on the same day, and percentage RSD was calculated. For the inter-day variation studies, 3 replicates of 3 concentrations were analyzed on 3 consecutive days and percentage RSD was calculated. For intraday precision and inter-day precision results obtained are shown in **Tables 3** and **4**.

Limit of Detection (LOD) and Limit of Quantification (LOQ):

LOD and LOQ are calculated from the formula: -

$$LOD = 3.3\ \sigma / S$$

$$LOQ = 10\ \sigma / S$$

Where, σ = standard deviation of Y intercept, S = slope of the calibration curve **Table 5**.

Assay: Darunavir tablet formulation analysis was carried out as mentioned under section preparation of sample solution. Procedure was repeated for six times. 2 µl volume of sample solution was applied and area was recorded.

Concentration and % recovery was determined from linear equation. The results obtained are shown in **Table 6**.

Accuracy: To check the accuracy of the method, recovery studies were carried by spiking the standard drug to the blend, at three different levels 50, 100 and 150%. The basic concentration of the sample chosen was 1000 ng/band.

% recovery was determined from the linearity equation. The results obtained are shown in **Table 7**.

Robustness: Robustness of the method was determined by carrying out the analysis under conditions during which detection wavelength, chamber saturation time were altered, time was also changed from spotting to development and development to scanning and the effects on the area were noted.

It was found that the method is robust. The results obtained are shown in **Table 8**.

Summary of Validation Study: The summary of validation parameters are summarized in **Table 9**.

RESULTS AND DISCUSSION:

Selection of Analytical Wavelength: Detection wavelength was found to be 267 nm.

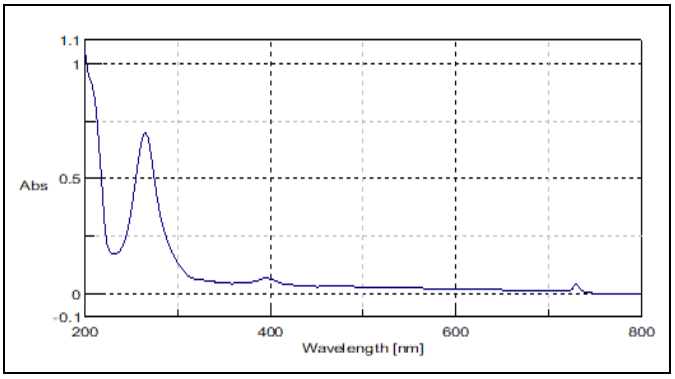

FIG. 2: UV SPECTRUM OF DARUNAVIR (10 µg/ml)

Typical Densitogram of Darunavir:

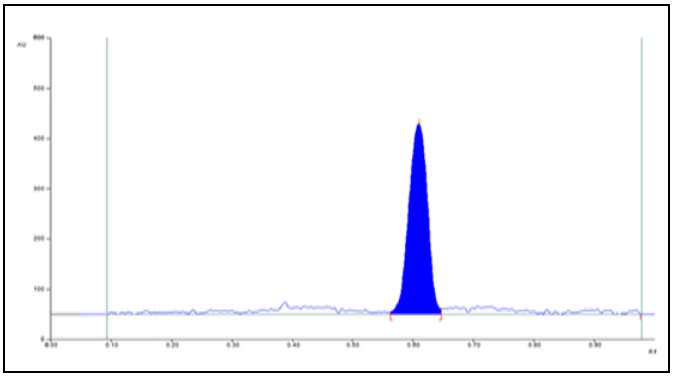

FIG. 3: A TYPICAL DENSITOGRAM OF STANDARD SOLUTION OF DARUNAVIR (1000 ng/band)

TABLE 1: SYSTEM SUITABILITY PARAMETERS FOR DARUNAVIR

Drug	Conc. (ng/band)	Rf	Area	Asymmetry
Darunavir	1000	0.61± 0.89	4267.6	1.13

Stress Degradation Studies of Bulk Drug:

Degradation under Acid-Catalyzed Hydrolytic Condition:

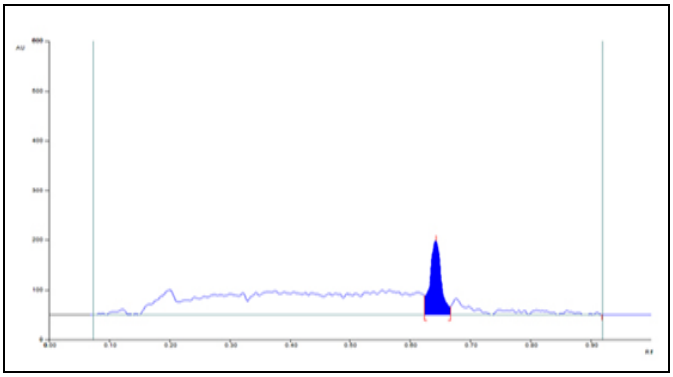

FIG. 4: REPRESENTATIVE DENSITOGRAM OF ACID INDUCED DEGRADATION OF DARUNAVIR (2500 ng/band)

Degradation under Alkali Catalyzed Hydrolytic Condition:

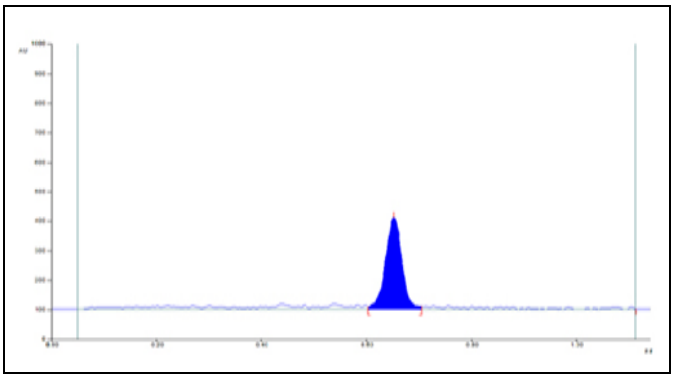

FIG. 5: REPRESENTATIVE DENSITOGRAM OF BASED INDUCED DEGRADATION OF DARUNAVIR (2500 ng/band)

Degradation under Oxidative Condition:

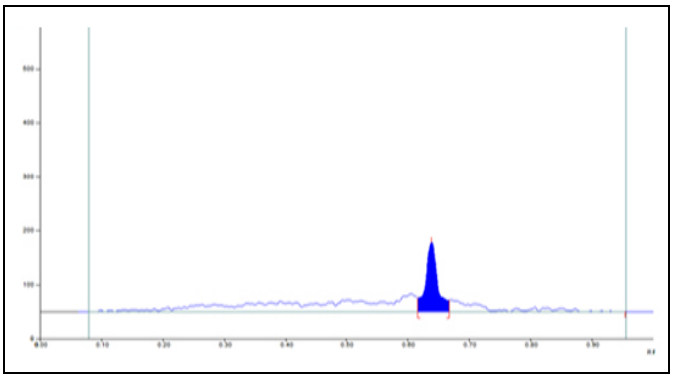

FIG. 6: REPRESENTATIVE DENSITOGRAM OF OXIDATIVE DEGRADATION OF DARUNAVIR (2500 ng/band)

Degradation under Dry Heat:

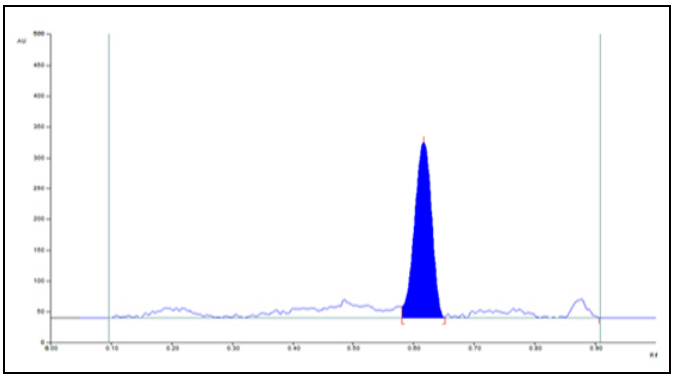

FIG. 7: REPRESENTATIVE DENSITOGRAM OF DRY HEAT DEGRADATION OF DARUNAVIR (2500ng/band)

Photo-Degradation Studies:

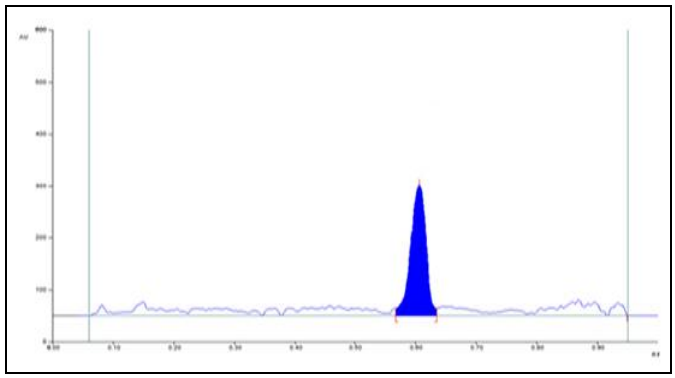

FIG. 8: REPRESENTATIVE DENSITOGRAM OF DARUNAVIR AFTER PHOTOLYSIS (2500 ng/band)

Validation of Analytical Method: Linearity:

TABLE 2: LINEARITY STUDY OF DARUNAVIR

Replicate	Concentrations of Darunavir (ng/band)					
	500	1000	1500	2000	2500	3000
	Peak Area					
1	1866.7	4267.6	6334	8025.5	9866.2	11066.3
2	1800.2	4298.7	6442.7	8154.3	9736.8	11375.6
3	1866.7	4305.3	6452.8	8035.7	9745.9	11543.2
4	1824.7	4267.6	6500	8035.4	9710	11587.3
5	1851.4	4267.3	6455.3	8025.9	9763.2	11563.7
6	1855.3	4355.7	6400.3	8305.7	9855.7	11480.3
Average	1844.16	4293.7	6430.85	8097.03	9779.63	11436.07
SD	26.47	34.82	57.12	31.62	65.37	96.45
% RSD	1.43	0.81	0.88	0.40	0.66	0.84

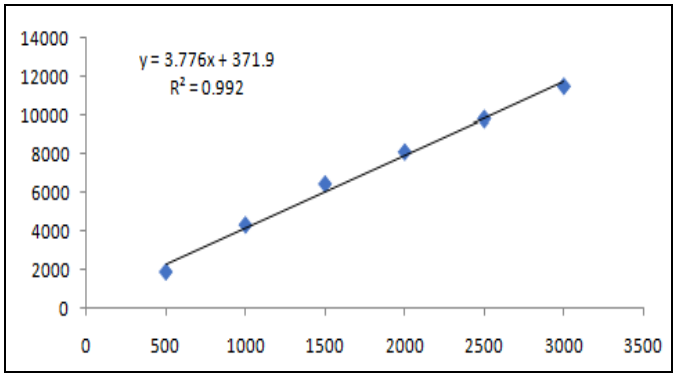

FIG. 9: CALIBRATION CURVE FOR DARUNAVIR

Range: Darunavir = 500-3000 ng/band

Precision:

TABLE 3: INTRA-DAY PRECISION OF DARUNAVIR

Conc. (ng/band)	Area	Mean area	SD	% RSD
1000	4587.9	4572	14.41	0.49
	4559.8			
	4568.3			
1500	6005.3	6017.3	15.63	0.39
	6012.3			
	6035.2			
2000	7455.9	7480.93	21.25	0.36
	7495.6			
	7488.9			

TABLE 4: INTER-DAY PRECISION OF DARUNAVIR

Conc. (ng/band)	Area	Mean area	SD	% RSD
1000	4556.3	4570.6	0.53	0.54
	4568.3			
	4587.3			
1500	6035.5	6089.3	0.18	0.17
	6210.3			
	6022.1			
2000	7485.9	7490.03	0.06	0.06
	7490.6			
	7493.6			

Limit of Detection (LOD) and Limit of Quantification (LOQ):

TABLE 5: LOD AND LOQ OF DARUNAVIR

Method	Avg. slope	S.D	LOD (ng/band)	LOQ (ng/band)
S.D of y-intercept	3.756	108.28	95.12	288.24

Assay:

TABLE 6: ASSAY OF MARKETED FORMULATION

S. no.	Peak area	Amount Recovered (ng/band)	% Recovery
1	4122.3	993.16	99.31
2	4123.9	993.59	99.35
3	4142.6	998.54	99.85
4	4152.3	1001.11	100.11
5	4147.3	999.78	99.97
6	4176.3	1007.46	100.74
Mean	4144.11	998.94	99.89
%RSD	0.48	0.53	0.55

Accuracy:

TABLE 7: RECOVERY STUDIES OF DARUNAVIR

Level (%)	Sample (ng/band)	Std. (ng/band)	Area	Recovered Conc. (ng/band)	% Recovery	± RSD
50	1000	50 0	6058.3	1505.85	100.39	0.26
			6065.9	1507.85	100.52	
			6087.3	1513.53	100.90	
100	1000	1000	7875.6	1987.10	99.35	0.18
			7852.6	1981.01	99.05	
			7877.5	1987.60	99.38	
150	1000	1500	9755.3	2484.7	99.39	0.17
			9765.9	2487.68	99.50	
			9787.3	2493.35	99.73	

Robustness:

TABLE 8: ROBUSTNESS STUDY

S. no.	Parameters	Variation	Concentration (ng/band)	% RSD
1	Time from application to development	(0, 30, 60, 90 min)	1000	0.48
			2000	0.76
			3000	0.82
2	Time from development to scanning	(0, 30, 60, 90 min)	1000	0.42
			2000	0.51
			3000	0.39
3	Scanning wavelength	267 ± 1 nm	1000	0.33
			2000	0.43
			3000	0.37

Summary of Validation Study:

TABLE 9: SUMMARY OF VALIDATION PARAMETERS

S. no.	Validation parameters	Darunavir
1	Linearity Equation	y = 3.7762x + 371.9
	(r^2) Range	r^2 = 0.992
		500- 3000ng/band
2	Precision (% RSD)	
	Intraday	0.25
	Interday	0.41
3	Assay ± RSD	99.89 ± 0.55
4	Accuracy ± RSD	% Recovery
	50	100.60 ± 0.26
	100	99.26 ± 0.18
	150	99.54 ± 0.17
5	Limit of Detection	95.12 ng/band
6	Limit of Quantitation	288.24 ng/band
7	Specificity	Specific
8	Robustness	Robust

CONCLUSION: An accurate, rapid, specific and stability-indicating HPTLC method was developed for the estimation of Darunavir Ethanoate in the bulk and pharmaceutical dosage form.

In the developed method, no interferences were observed from blank or excipients at Darunavir peak retention. The primary advantage of this method is that it is simple and accurate with a shorter run time.

Hence, the proposed method can be conveniently adopted for the routine quality control analysis in bulk and formulation. The developed method was validated according to ICH guidelines.

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CONFLICTS OF INTEREST: Nil

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