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Stability Indicating Rp-Hplc Method For Impurity Profiling Of Darunavir Hydrate In Drug Product

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ABSTRACT

Drugs may degrade chemically or physically, changing their therapeutic usefulness or possibly having harmful side effects. Degradation products were discovered and degradation patterns were suggested using mass spectrometry. The optimized chromatographic setup used a CHEMSIL ODS-C18 column (250 mm × 4.6 mm, 5 µm). The mobile phase consisted of Phase A (Methanol: Acetonitrile: Water, 70:15:15). The flow rate was maintained at 1 mL/min, detection was carried out at 263 nm. The Limit of Quantization (LOQ) for all degradation impurities was found to be 0.05%, matching the reporting threshold. Therefore, the novel method showed to be linear from range 5-30 µg/ml, with high precision (CV < 2%) and accuracy (recuperation of 99.82%). It is simple and reliable, free of placebo interferences. Darunavir was more vulnerable to degradation when exposed to acid, alkali, and peroxide, as evidenced by the degradation products that were found. However, under photolytic and thermolytic conditions, no discernible deterioration effects were found. The HPLC method was able to quantify Darunavir and its degradation products. This study is novel because it identifies the variables that influence the stability of Darunavir hydrate in tablets, which is the first step in determining the degradation products. In low doses when linearity is present, the drug and the primary degradation products can be monitored using the new stability-indicating technique.

KEYWORDS: Darunavir, Method Validation, Forced degradation, Linearity, Accuracy, Robustness.

INTRODUCTION

Darunavir is effective against a broad panel of HIV-1 group M and group O primary isolates in cell culture, with EC50 values ranging from less than 0.1 to 4.3 Nm [1]. When human serum is present, darunavir's EC50 value rises by a median factor of 5.4. In acutely infected T-cell lines, human peripheral blood mononuclear cells, and human monocytes/macrophages, darunavir is effective against laboratory strains of HIV-1 and clinical isolates, as well as laboratory strains of HIV-2.When compared to the protease inhibitors amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, and ritonavir, darunavir did not exhibit antagonistic effects. [2-3].

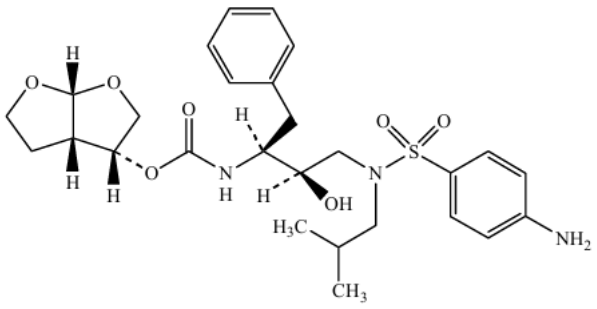

Figure 1: Structure of Darunavir

For the QC (Quality Control) of pharmaceutical formulations, it is strongly advised to construct stability indicating assays employing the stress testing methodology as established by the International Conference on Harmonization (ICH) recommendations. Darunavir is an antiviral medication, making it essential to examine the stability and degradation pathways of the drug through forced degradation studies. These studies typically occur under different stress factors, including temperature, humidity, light, and mechanical stress.[5-6] Forcible degradation studies are conducted to record how the quality of a drug substance or product evolves over time when exposed to various environmental conditions, and they assist in pinpointing possible degradation pathways and impurities that may arise during the manufacturing, storage, and transportation of different drug products.[7] When selecting the optimal formulation, packaging materials, and storage conditions to guarantee the stability and efficacy of pharmaceutical products throughout their shelf life, the results of these studies can be used as a guide [8]. Our research's primary objectives are to determine how forced degradation conditions such as heat, acid, base, and oxidizing agents affect Darunavir's stability and to create and improve a univariate spectrophotometric assay that can accurately predict stability for the simple quantification of Darunavir and its breakdown products.[9-10]

MATERIAL AND METHOD

Materials

Darunavir Hydrate was purchased from Dr. SSK Labs Pvt. Ltd as active pharmaceutical ingredients (APIs). Analytical grade (AR) reagents including Potassium dihydrogen phosphate, Ortho phosphoric acid, Sodium hydroxide, and Trimethyl amine were procured from Sigma Aldrich.[11,12] High purity solvents such as Methanol, Acetonitrile, and HPLC grade Water were also obtained from Sigma Aldrich. Commercially available Daruvir 300 mg tablets, manufactured by Cipla, were purchased from the local market for analysis and method development.[13]

Instrumentation

The high-performance liquid chromatography (HPLC) system used for the study was of Thermo make, equipped with a P4000 Quaternary Gradient Pump, a Photo Diode Array (PDA) detector, and controlled via CHROMQUEST software.[14]

Selection of wavelength

To create 1.50 × 10–5 – 4.0 × 10–4 M Darunavir solutions, various aliquots of the Darunavir standard solution (5 × 10–3 M) were quantitatively diluted with deionized water.[15] Plotting the absorbance at 263 nm versus the concentration of Darunavir was done. In order to link the absorbance at 263 nm with the concentration of Darunavir, we calculated the regression equation.
.[16]

Chromatographic condition optimization

Preparation of standard Working solution

In order to create a stock solution of Darunavir Hydrate, 10 mg of the medication was precisely weighed, dissolved in 100 mL of mobile phase in a 100 mL volumetric flask, and then diluted to the appropriate concentration of 100 µg/mL. To reach a working concentration of 10 µg/mL, 1 mL of this stock was pipetted into a 10 mL volumetric flask and diluted to volume with mobile phase.[17] The resultant solution was filtered through the Whatman filter paper, and further dilutions were carried out as required.[18]

Preparation of Darunavir sample solution

10 Tablets of Darunavir were weighed and made powder in mortar and pestle. The powder equivalent to the amount of active ingredient present in 10 tablets was transferred into a 500 ml clean and dry volumetric

flask a total of 350 ml of diluent was introduced, and the mixture was agitated using a mechanical stirrer, followed by sonication for approximately 30 minutes, with shaking occurring at five-minute intervals. After that, the solution was further diluted to the necessary level and allowed to settle before an aliquot was taken out for further dilution. Before being injected into the HPLC system, 1 milliliter of the top clear solution was moved to a 10-milliliter volumetric flask, diluted with diluent until it reached the desired level, and then filtered through a 0.45 mcg/ml filter.[19]

Analytical Method Validation

Specificity

By assessing the separation of known impurities from both the peak of Darunavir Hydrate and from one another, the specificity was determined. Additional analysis of the drug peaks and blank impurity was also confirmed. In addition to visual separation, each known impurity and drug peak's peak purity was tracked.[19,20]

Forced degradation study

Forced acidic degradation

Pour 50 ml of the stock HCl solution (0.1 M) into a 150 ml glass stoppered flask, gently transfer 10 ml of the Darunavir stock standard solution (5 × 10–3 M), and reflux in a boiling water bath at 100 °C for five hours to expedite the degradation process. Use 50 milliliters of stock NaOH solution (0.1 M) to neutralize the mixture. Deionized water was used to quantitatively dilute the solution to 250 milliliters. In the 200–400 nm wavelength region, the absorbance spectra was measured against a 0.1 M NaCl solution. The degradation pattern was examined using mass spectrometry.[21]

Forced alkaline degradation

Fill a 150 ml glass stoppered flask with precisely 10 ml of the Darunavir stock standard solution (5 × 10–3 M). To expedite the degradation process, add 50 milliliters of the stock NaOH solution (0.1 M). Lastly, reflux for five hours at 100 °C in a water bath. Use 50 milliliters of stock HCl solution (0.1 M) to neutralize the mixture. Deionized water was used to quantitatively dilute the solution to 250 milliliters. In the 200–400 nm wavelength region, the absorbance spectra were measured against a 0.1 M NaCl solution. The degradation pattern was examined using mass spectrometry.[21,22]

Forced oxidative degradation

In a 150 ml glass flask with a stopper, carefully transfer 10 ml of Darunavir stock standard solution (5 × 10–3 M), then incorporate 50 ml of a 3% stock H2O2 solution to promote the degradation process. The mixture is refluxed in a boiling water bath at 100 °C for 5 hours, after which the flask is left open until the contents dry completely. The residue in the flask is then reconstituted with deionized water and diluted to a final volume of 250 ml with deionized water. The absorbance spectrum is recorded against deionized water over the wavelength range of 200–400 nm. Mass spectrometry was employed to analyze the degradation pattern.[22]

Sensitivity of method by LOQ

Based on the maximum daily dose for each active pharmaceutical ingredient (API), a reporting threshold of 0.05% was established as the limit of quantification (LOQ). Therefore, a solution of each impurity at a concentration of 0.05% was injected to assess the Signal to Noise (S/N) ratio, which must be ≥10. The extremely high response of Darunavir was utilized to set the LOQ at a very low level of 0.005% and the LOD at 0.002%. Additionally, the solution containing all other impurities and Darunavir at the LOQ level was further diluted to 0.02% to create the LOD level solution, which was then injected to assess the S/N ratio, required to be above 3. The precision of the determined LOQ level was validated through six

replications of each impurity and the API, measuring % RSD for the obtained peak areas, which should remain below 10.0%.[22]

Linearity

The method's linearity was confirmed from the LOQ level up to 150% of the specified limit for every identified impurity. The results were validated by assessing the correlation coefficient through graphical analysis.

Precision

The precision study encompasses both system precision and method precision. For system precision, a standard solution was injected six times, and the results were assessed according to the established System Suitability (SST) criteria. The method precision was evaluated by analyzing six sets of tablet sample preparations, each spiked with all known impurities at their limit levels. The results were interpreted as the % RSD for each known impurity, a single maximum unknown impurity, and the total impurities.

Accuracy/Recovery

The accuracy was assessed by introducing each known impurity into the tablet sample solution at the levels of LOQ, 100%, and 150% in relation to the specification limit. The resulting percentage accuracy was determined by comparing the amount added to the amount detected for each impurity. A total of three replicate measurements were performed for each impurity and at each level, resulting in a total of nine determinations, from which the percentage RSD was calculated for the replicates at each level.[23]

Robustness

The robustness assessment was conducted by intentionally altering method parameters to observe their effect on the separation of impurities. The two primary parameters evaluated for system suitability and for distinguishing impurities from one another and from both API peaks were variations in flow rate and column oven temperature.

RESULTS

Method Validation study

Spectrophotometric assay

Darunavir shows a clear absorbance peak at 263 nm and the degradation products show no absorbance at that wavelength, the absorption spectra of Darunavir and its degradation products offered a simple way to distinguish Darunavir from its degradants. As a result, the medication can be analyzed using a direct spectrophotometric approach without requiring any previous separation or derivatization procedures. To get the regression equation using the direct approach, we create calibration curves that relate absorbance at 263 nm with Darunavir concentration. (as shown in Table 1). We followed the ICH validation guidelines, as outlined in Table 1. Within the concentration range of 1.50 × 10–5 to 4.0 × 10–4 M for Darunavir, the approach demonstrated satisfactory linearity, accuracy, and selectivity for Darunavir among its hydrolytic and oxidative degradants products. Linearity was evaluated by examining six distinct concentrations of Darunavir spanning from 1.50×10^{-5} to 4.0×10^{-4} M. The calibration process adhered to the practical linear range (Beer's law) to ensure accurate and precise results, as detailed in Table 1. By using the suggested approach to measure different Darunavir samples, accuracy was evaluated. The molar concentration of Darunavir was ascertained using the regression equation. Three distinct concentration levels of Darunavir were analyzed in order to assess within-day repeatability for precision testing. Similarly, the intermediate precision was assessed over three consecutive days, utilizing three concentration levels. The findings indicated a satisfactory level of precision, as shown in Table 1. Lab-prepared combinations with 10 to 90%

degradation products were analyzed to demonstrate the method's specificity at both extremes, as shown in Table 1.

Table 1: Method Validation Results of Darunavir

Sr. No.	Parameters	Results
1	Slope	6245.34
2	Intercept	y = 23.249x
3	Correlation coefficient	0.9973
4	Linearity range	5-30 µg/ml
5	Accuracy ± SD	98.78± 1.00
6	Precision %RSD	0.266%
7	Intermediate Repeatability	0.35%
8	LOD	3 ng/mL
9	LOQ	10.5 ng/mL
10	Specificity	99.27 ± 1.45

Forced Degradation (FD) Study

Darunavir was subjected to a variety of stress conditions, including thermal, acidic, alkaline, and oxidative degradation, in order to assess the drug's durability.

Thermal Degradation

After being refluxed in a boiling water bath at 100 °C for five hours, the medication remained stable despite thermal degradation. The drug was identical before and after the drying procedure, according to the mass spectrometry data and the UV absorption spectra.

Hydrolytic degradation

Darunavir was subjected to hydrolysis in both acidic and alkaline solutions by refluxing in a boiling water bath with 0.1 M HCl and 0.1 M NaOH for a duration of five hours. The resulting solutions were subsequently neutralized using 0.1 M NaOH and 0.1 M HCl, respectively. The occurrence of degradation was verified through a comparison of the absorption spectra, where the peak at 263 nm was completely eliminated; the proposed degradation pattern was validated by mass spectrometry, as illustrated in the figure.

Oxidative Degradation

The process of oxidative degradation involved dissolving the drug in deionized water and refluxing it in a boiling water bath with a 3% H_2O_2 solution. The reflux was conducted at 100 °C for a duration of 5 hours. After this, the flask was left uncovered until it dried completely. The degradation was verified by comparing the absorption spectra, where the peak at 263 nm was entirely absent, as illustrated in the figure. Mass spectrometry supported the suggested degradation pattern.

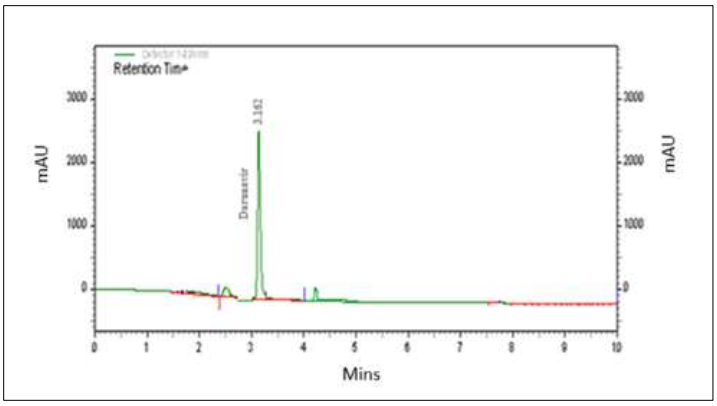

Figure 2: Standard Chromatogram of Darunavir Hydrate

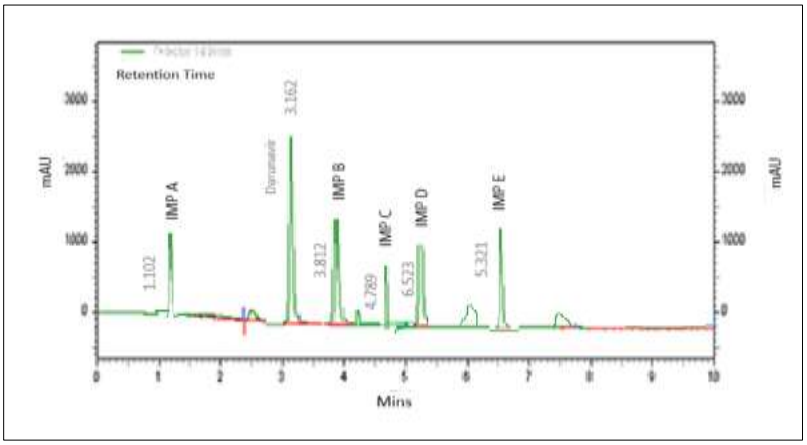

Figure 3: Sample chromatogram prepared using drug product Darunavir tablet

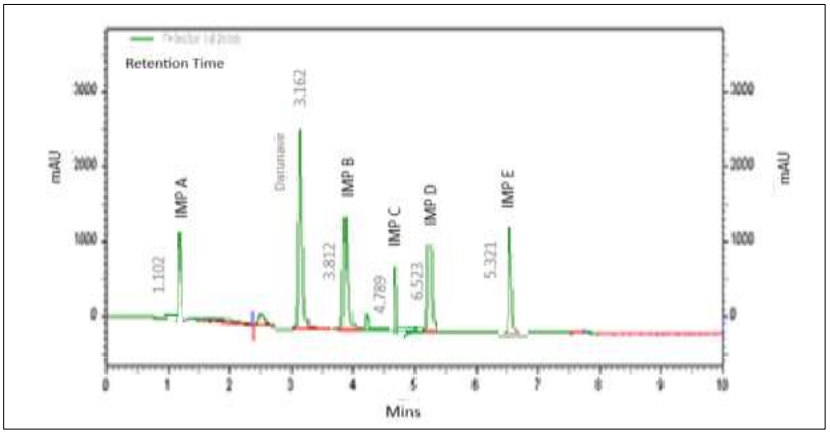

Figure 4: Selectivity study chromatogram of tablet sample solution spiked with known impurities.

Table 2: Specificity and Forced degradation study outcome

Sr. No	Stress type	%Impurity / %Degradation						
		Imp A	Imp B	Imp C	Imp D	Imp E	Any Unspecific Impurity	Total Impurities/ Degradation
1	Untreated sample	0.06	1.45	0.21	0.05	0.04	0.04	0.56
2	Acid degradation	0.55	1.75	0.19	0.15	0.06	0.17	2.31
3	Base degradation	0.04	15.31	0.20	0.06	0.03	0.04	16.98
4	Peroxide degradation	0.09	0.29	0.24	0.27	0.05	0.05	0.65
5	Thermal degradation	ND	ND	ND	ND	ND	ND	ND
6	Humidity degradation	ND	ND	ND	ND	ND	ND	ND
7	Photolytic degradation	ND	ND	ND	ND	ND	ND	ND
8	Retention time	1.102	3.812	4.789	6.523	5.321	-	-

ND= Not Detected, Imp- Impurity

Table 3 : Peak Purity data

Sr. No	Stress type	Purity Angle/Purity Threshold					
		Impurity A	Impurity B	Impurity C	Impurity D	Impurity E	Conclusion
1	Untreated	24.512 / 57.100	20.134 / 53.456	18.765 / 52.301	22.345 / 55.234	10.121/56.321	Pass
2	Acid degradation	8.743 / 51.236	9.238 / 52.789	6.987 / 50.123	22.345 / 55.234	5.432/54.587	Pass
3	Base degradation	4.512 / 53.678	5.213 / 54.321	3.798 / 51.452	22.345 / 55.234	4.324/45.234	Pass
4	Peroxide degradation	21.987 / 56.789	19.543 / 52.345	17.321 / 50.000	22.345 / 55.234	10.876 / 49.321	Pass

5	Thermal degradation	NA	NA	NA	NA	NA	Pass
6	Humidity degradation	NA	NA	NA	NA	NA	Pass
7	Photolytic degradation	NA	NA	NA	NA	NA	Pass

Interpretation: Peak purity passes if purity Angle is less than purity threshold. N.A. = Not Applicable.

Sensitivity of method by LOD/LOQ

Each impurity and the Darunavir active pharmaceutical ingredient (API) peak were confirmed to have a limit of detection (LOD) of 0.02% and a limit of quantification (LOQ) of 0.05% (depending on the test concentration). The results were recorded as a signal-to-noise (S/N) ratio, which shouldn't be less than 10. The LOD and LOQ for darunavir were determined to be 0.002% and 0.005%, respectively. The peak area derived from six replicate injections of each impurity and both APIs at the LOQ level was also measured to determine the percent relative standard deviation (% RSD), which shouldn't be higher than 10.0%.

Linearity

The linearity assessment was conducted from the Limit of Quantitation (LOQ) up to 150% of the specification levels for each impurity, along with the Darunavir Active Pharmaceutical Ingredient (API). The concentration ranges selected for Darunavir (0.20-12.0 µg/mL), Impurity A (0.25-2.25 µg/mL), Impurity B (0.25-19.5 µg/mL), Impurity C (0.25-2.25 µg/mL), Impurity D (0.25-2.25 µg/mL), and Impurity E (0.25-6.07 µg/mL) were taken into account. The linearity results were assessed by creating a calibration curve that plots average area (X axis) against concentration for each component (Y axis). The final interpretation was done through a statistical analysis evaluating the correlation coefficient, slope, and Y-intercept for each impurity and the API. It was confirmed that the linearity outcomes for correlation coefficients met or exceeded the threshold of ≥0.995. The correlation coefficient results for the linearity of each impurity were recorded.

Precision

System precision

By evaluating the system appropriateness criteria in accordance with the technique, system precision was ascertained. Six replicate injections of the standard preparation for the Darunavir peak are used to calculate the percentage RSD of peak area, which should be less than 10.0%. The USP plate count for Darunavir was noted to be greater than 30000. Furthermore, the tailing factor for both component peaks was found to be within the range of 0.8 to 1.5. The overall % RSD result for the Darunavir peak was documented.

Table 4: System precision and Method precision study outcome

SST Solution/ Test solution (Impurity Spiked)	Darunavir peak area	Impurity A	Impurity B	Impurity C	Impurity D	Impurity E	Single maximum unknown impurity	Total impurities
TES-1	30543	0.15	0.18	0.11	0.21	0.09	0.12	0.65
TES-2	40625	0.16		0.12	0.34		0.11	0.66

			0.17			0.10		
TES-3	40476	0.14	0.16	0.10	0.41	0.08	0.13	0.61
TES-4	50765	0.15	0.17	0.11	0.56	0.09	0.12	0.64
TES-5	56543	0.15	0.18	0.10	0.34	0.10	0.11	0.64
TES-6	61234	0.16	0.17	0.11	0.54	0.09	0.12	0.65
Average	55514	0.152	0.171	0.108	0.51	0.091	0.118	0.642
SD(±)	458.93	0.007	0.007	0.008	0.45	0.007	0.007	0.017
% RSD	0.43%	4.61%	4.09%	7.41%	0.47	7.69	5.93%	2.65%

(SST Solution=System suitability solution.)

Method precision

A drug product sample solution that had been spiked with every known impurity at the specification level was used for the technique precision investigation. The percentage RSD was calculated using statistical analysis of the results from the six sample preparations for each known impurity, maximum single unknown impurity, and overall impurities. The evaluation of the six replicate analyses for the % impurity result was performed to confirm that the % RSD remained below 10.0%.

Accuracy/Recovery

The accuracy study was conducted across a range from the limit of quantification (LOQ) to the 150% level by introducing known impurities during sample preparation, except for the LOQ level where all known impurities were added to a placebo solution. Taking into account the minimum requirement of nine determinations for each impurity, triplicate preparations at each level were analyzed, and the results were calculated using the formula: % Recovery = (% impurity found / % impurity added). The general criteria consistent with industry standards, which states that recovery should not be less than 80.0% and not exceed 120.0%, was adhered to for the interpretation of data. The compiled results of the % recovery for each specific impurity were documented.

Table 5: Accuracy/Recovery study outcome.

Impurity/ Level	Impurity A		Impurity B		Impurity C		Impurity D		Impurity E	
	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD	% Recovery	% RSD
LOQ	98.6	4.2	97.9	3.8	99.2	3.5	96.8	4.1	97.3	3.9
50%	99.1	2.7	98.3	2.9	98.8	2.5	97.5	3.0	98.1	2.6
100%	100.3	1.8	99.7	1.5	100.1	1.9	99.2	2.1	99.6	1.8
150%	101.2	1.5	100.8	1.3	101.0	1.6	100.5	1.7	100.9	1.4

Robustness

Two important deliberate changes—flow rate and column oven temperature—were the focus of the robustness research. To ascertain the impact of these intentional modifications, the results of system suitability parameters and Retention Time (RT) for Darunavir and all known impurities were compared to method precision data. Each condition's comparison data findings were documented. The robustness study's conclusions show that intentional adjustments to flow rate and column oven temperature have an impact on peak retention, but they had no effect on system appropriateness or impurity separation.

Table 6: Robustness Study Outcome

Parameter	Condition	Darunavir			RT of Imp A	RT of Imp B	RT of Imp C	RT of Imp D	RT of Imp E
		RT	N	T					
Method Precision	Temp=55 Flow=0.22	3.167	12848	0.87	1.102	3.812	4.789	6.523	5.321
Change in Column Oven Temp. (ºC)	+5=60	3.023	18090	0.89	1.153	3.233	4.502	6.871	5.324
	-5=50	4.012	31240	0.98	1.785	4.761	5.785	7.365	4.812
Chang in Flow rate (mL/min)	+10%=0.24	3.154	34380	0.92	1.891	3.146	4.644	6.116	4.474
	-10%=0.20	4.562	31356	0.86	1.125	4.675	6.123	8.192	4.231

Temp=Temperature, RT=Retention time, N=Theoretical plates, T=Tailing factor, Imp=Impurity.

DISCUSSION

The literature and pharmacopeia monographs now in publication offer techniques for examining the composition and contaminants of specific pharmaceutical items. In order to monitor and manage all possible degrading impurities in a fixed-dose medication combination product, this study set out to create a single chromatographic technique.

In order to effectively elute and separate both known and unknown impurities of Darunavir, traditional HPLC methods often necessitate long run times. The goal is to achieve the desired separation while also developing a method that is shorter and more cost-effective. The newly developed method incorporates volatile salts in the mobile phase, making it suitable for LC-MS analysis if identification of unknown impurities is needed. This method has been found to be selective for all anticipated known degradation impurities, and the results from the forced degradation study validate its stability-indicating capability for the intended application. The process used for manufacturing the drug product involves hot melt extrusion, during which Darunavir is subjected to elevated temperatures in the presence of excipients, resulting in the formation of Darunavir impurity C as a principal degradation product, along with impurity E. This impurity is also subject to evaluation using the HPLC method. The method has been validated according to ICH guidelines for all necessary parameters to ensure its suitability. The results for all validation parameters were compiled and documented as a summary of the method validation discussion.

Table 7: Discussion Summary of Method Validation

Components Name	Selectivity by Retention time	S/N Ratio/ RSD for LOQ Precision	Linearity Correlation Coefficient Range: LOQ to 150%	Method Precision % RSD	Accuracy / Recovery (Min and Max) Range: LOQ to 150%
Darunavir	3.242	47/8.6%	0.9996	N.A.	N.A.
Impurity A	1.102	35/2.1%	0.9998	0.45	Min=92.1, Max=101.2
Impurity B	3.812	46/3.8%	0.9993	1.42	Min= 96.5, Max=103.5
Impurity C	4.789	26/6.4%	0.9994	4.67	Min= 97.2, Max=106.8
Impurity D	6.523	27/8.5%	0.9999	N.A.	N.A.
Impurity E	5.321	34/2.3%	0.9992	2.12	Min=90.89,

					Max=97.03

Note: Considered 0.05% as LOQ level for all impurities and both active ingredient. N.A. =Not applicable, Min=Minimum, Max=Maximum

CONCLUSION

A method utilizing HPLC has been established and validated for measuring all potential degradation impurities in the drug product. The method demonstrated sensitivity and the ability to track all possible impurities created during the manufacturing process as well as during the stability assessment of the drug product. The validation results for linearity, accuracy, precision, and robustness indicate the method's reliability and its appropriateness for the intended purpose. The forced degradation study verifies the method's specificity for all potential degradation impurities stemming from both the active ingredients and the manufacturing process, as well as potential interactions with excipients. The methodology employs an HPLC instrument, which allows for a brief run time of just 10 minutes and a minimal flow rate of 0.22 mL/min, leading to significantly reduced solvent consumption; therefore, the method is considered economical. Additionally, the mobile phase comprises all volatile salts and solvents, allowing the same technique to be directly utilized for LC-MS applications to determine the mass of unknown impurities. Thus, the established and validated HPLC method with a PDA detector can be used for impurity profiling of the Darunavir drug combination dosage form, and this method may also be employed for routine quality control and stability testing of the drug product.

CONFLICT OF INTEREST

None of the authors have any conflicts of interest to declare.

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