RESEARCH PAPER

Stability Indicating Rp-Hplc Method Development And Impurity Profiling By Forced Degradation Study Of Antiretroviral Drugs

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Abstract

An error-free, accurate, precise and valid reverse-phase liquid chromatography method was developed for the quantitation of valganciclovir and darunavir in its bulk form as well as in dosage form by forced degradation studies. Chromatographic separation of these two drugs valganciclovir and darunavir, was achieved with an chemsil ods-c18 column (250 mm × 4.6 mm, 5 µm) reverse-phase analytical column with a 10 min analytical run time using a mixture of acetonitrile : ortho phosphate buffer of ph 4 (60:40% v/v) as mobile phase. The mobile phase was streamed at a flow rate of 1.0 ml min-1 with a column temperature of 250 °c, and detection wavelength was carried out at 257 nm. Darunavir and valganciclovir showed retention times of 4.1 minutes and 3.1 minutes, respectively. The limit of quantization (loq) for all degradation impurities was found to be 0.05%, matching the reporting threshold. The method demonstrated linearity and accuracy in the range, with accuracy values ranging between 99.2-99.4% and 97.82-99.82% respectively. Precision was confirmed by %rsd below 0.5%, and the method proved to be robust under minor variations. The proposed validation process is very simple as well as more accurate stability -indicating hplc process is developed to routine analysis to both the drugs includes valganciclovir as well as darunavir in bulk and tablet dosage forms. These compounds/substances are subjected to evaluation with forced degradation pertaining to several stress circumstances. This developed process separates both drugs and their products of degradation successfully also quantifies active contents at minute strength levels. This developed method is totally applied considering the rules and regulations given by ich to all parameters those are identified within acceptance criteria.

Keywords: Method Validation, Forced Degradation, Linearity, Accuracy, Robustness, Impurity Profiling.

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INTRODUCTION

Darunavir and Valganciclovir are two such antiviral agents with considerable clinical importance. Darunavir, a protease inhibitor, is primarily used in the treatment of HIV, often co-administered with other drugs to enhance bioavailability and therapeutic effect. Valganciclovir, a prodrug of ganciclovir, is widely used in the management of cytomegalovirus infections, particularly in immunocompromised patients. The combination of these drugs in a single dosage form could offer improved patient compliance, efficacy, and ease of therapy. However, such combination products pose analytical challenges, especially in impurity profiling and stability monitoring. [1-2]

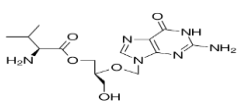

Figure No 1:Valganciclovir Structure

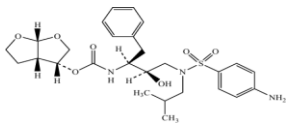

Figure No.2: Darunavir Structure

In RP-HPLC, separation efficiency can be optimized through either isocratic or gradient elution modes. Isocratic elution maintains a constant mobile phase composition throughout the run, while gradient elution varies the solvent strength over time to allow better resolution of late-eluting compounds.[3] The selection of

appropriate chromatographic conditions, including column type, mobile phase composition, pH, and flow rate, is vital for the successful development of a stability-indicating method that can effectively separate and quantify both drugs and their degradation products.[4] Stability studies are a regulatory requirement and a fundamental part of drug development. According to ICH and FDA guidelines, they assess how the quality of a drug substance or product varies with time under the influence of environmental factors like temperature, humidity, and light, these include long-term (real-time), accelerated, and intermediate studies. Complementing these are forced degradation (stress) studies, which subject the drug to extreme conditions (e.g., heat, light, oxidation, hydrolysis) to generate potential degradation products. These studies not only help in understanding the intrinsic stability and degradation pathways of active pharmaceutical ingredients (APIs) but also serve to validate the stability-indicating capability of the developed analytical method. [5,6] Forced degradation studies are critical for distinguishing between formulation-related and API-related degradation products and understanding the chemical behavior of the drug. They are instrumental in the development of stability-indicating methods, identification of degradation mechanisms (e.g., hydrolysis, oxidation), and optimization of formulations. Generally, a degradation level between 5–20% is considered suitable for chromatographic method validation. Conducted ideally during early stages of development, these studies help preempt stability issues and ensure robust formulation and manufacturing processes.[7,8] Impurity profiling further strengthens the analytical evaluation by identifying and quantifying both process-related and degradation impurities in bulk drugs and formulations. The presence of impurities, even in small quantities, can significantly affect the safety and efficacy of a drug product, especially when the impurities are pharmacologically active or toxic. Regulatory agencies like ICH mandate the thorough profiling of all potential impurities, especially in fixed-dose combination (FDC) products where drug - drug and drug- excipient interactions are more complex. [9,10]

MATERIALS AND METHODS

Material

Valganciclovir Hydrochloride and Darunavir Hydrate were purchased from Cipla Pvt. Ltd., Mumbai, 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. High purity solvents such as Methanol, Acetonitrile, and HPLC grade Water were also obtained from Sigma Aldrich. Commercially available Valgan 450 mg tablets and Daruvir 300 mg tablets, both manufactured by Cipla, were purchased from the local market for analysis and method development. All reagents and solvents used in this study were of analytical or chromatographic grade, ensuring the accuracy and reliability of the analytical results.

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. This system was employed for method development and optimization studies related to the analysis of antiviral drug substances and products.

Selection of Wavelength:

The UV scan of the active ingredients Valganciclovir Hydrochloride and Darunavir Hydrate showed UV maxima (λmax) at 257 nm and 263 nm, respectively.

Optimization of Chromatographic Conditions:

Table No. 1: Chromatographic conditions for Valganciclovir and Darunavir

Particulars	Valganciclovir Hydrochloride	Darunavir Hydrate
Column:	CHEMSIL ODS-C18 (250 mm X 4.6 mm), 5µm column	CHEMSIL ODS-C18 (250 mm X 4.6 mm), 5µm column
Flow Rate:	1 ml/min	1 ml/min
Injection Volume:	20 µL	20 µL
Column Temperature:	Ambient	Ambient
Wavelength	257 nm	263 nm
Run time	10 min	10 min
Mobile phase	Acetonitrile : Ortho Phosphate buffer of pH 4 (60:40%v/v)	Methanol: Acetonitrile: Water 70: 15:15

The chromatographic conditions developed for the estimation and impurity profiling of Valganciclovir HCl and Darunavir Hydrate were based on reverse-phase high-performance liquid chromatography (RP-HPLC),

selected for its simplicity, robustness, and suitability for separating ionic or ionizable compounds.

RP-HPLC Method Development and Validation of Valganciclovir Hydrochloride and Darunavir Hydrate

Preparation of Standard Solution

A stock solution of Valganciclovir HCl was prepared by accurately weighing 10 mg of the drug and transferring it into a 100 mL clean, dry volumetric flask. About 70 mL of diluent was added, and the solution was sonicated to ensure complete dissolution. The volume was then made up to the mark with the same diluent. Similarly, a stock solution of Darunavir Hydrate was prepared by accurately weighing 10 mg of the drug, dissolving it in 100 mL of mobile phase in a 100 mL volumetric flask, and diluting it up to the mark to obtain a concentration of 100 µg/mL. From this stock, 1 mL was pipetted into a 10 mL volumetric flask and diluted to volume with mobile phase to achieve a working concentration of 10 µg/mL. The resultant solution was filtered through Whattman filter paper, and further dilutions were carried out in the same manner as required for method validation by RP-HPLC.

Preparation of Placebo Solution

An accurately weighed amount of tablet, equivalent to 50 mg of Valganciclovir and Darunavir , were transferred into a clean, dry 100 mL volumetric flask. About 80 mL of diluent was added, and the mixture was sonicated for 45 minutes with occasional shaking. After allowing the solution to cool to room temperature, the volume was made up to 100 mL with diluent and mixed well. Finally, the solution was filtered using a 0.20 µm Nylon (Millipore Millex-GN) filter, discarding the first 2 mL of the filtrate.

Preparation of Sample Solution

For the estimation of Valganciclovir HCl from its tablet formulation, 10 tablets were accurately weighed and powdered using a clean glass mortar and pestle. The powder equivalent to the total active content of 10 tablets was transferred into a 500 mL clean dry volumetric flask. About 350 mL of diluent was added, and the mixture was stirred mechanically and sonicated for 30 minutes, with intermittent shaking every five minutes to ensure complete extraction of the drug. The final volume was made up to the mark with the same diluent. The solution was allowed to stand until the insoluble residue settled. An aliquot of the clear supernatant (0.1 mL) was transferred into a 10 mL volumetric flask, diluted up to the mark with diluent, and filtered through a 0.45 µm membrane filter before injection into the HPLC system. For the estimation of Darunavir Hydrate from its tablet dosage form (Darunavir 300 mg, Cipla Pvt. Ltd.), 20 tablets were accurately weighed and powdered finely. Tablet powder equivalent to 10 mg of Darunavir was transferred into a 100 mL volumetric flask containing approximately 50 mL of mobile phase, followed by sonication for 30 minutes to dissolve the drug content. The solution was then filtered through Whattman filter paper, and the filter paper was rinsed with additional solvent. The combined filtrate was diluted to volume with the mobile phase. From this solution, 1 mL was pipetted into a 10 mL volumetric flask and diluted up to the mark to obtain a working concentration of 10 µg/mL. The final solution was again filtered through Whattman filter paper, and 20 µL aliquots were injected in triplicate into the HPLC system for analysis.

System Suitability

To confirm the reproducibility of the developed RP-HPLC method as per good chromatographic practices, system suitability parameters were evaluated for Valganciclovir HCl and Darunavir Hydrate prior to sample analysis. The parameters included USP plate count (N) not less than 3000, USP tailing factor (T) not more than 2.0, and % RSD for retention time and peak area from replicate injections (n=3) not more than 2.0%. All system suitability results were found within acceptable limits, indicating that the instrument, reagents, and column were suitable for performing the assay.

Force Degradation Study of Valganciclovir Hydrochloride and Darunavir

Control Sample: 10 Tablets of Valganciclovir Hydrochloride and 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, 350 ml of diluent was added in to it and was shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes each and was diluted up to the mark with diluent and allowed it to stand until the residue settles before taking an aliquot for further dilution. 1ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark and the solution was filtered through 0.45 mcg / ml filter before injecting into HPLC system.

Preparation of Aliquot of sample: Accurately weighed and transferred powder equivalent to 50 mg of

Valganciclovir hydrochloride and Darunavir into a 100 mL volumetric flask, containing 70 mL of methanol, and sonicated for 30 minutes.

Acid Degradation Sample: Acid decomposition was carried out in 1 M HCl and 0.1 M HCl at a concentration of 1.0 mg/mL Valganciclovir hydrochloride and Darunavir for RT and hrs at 35 ºC for 30 min and 1 hr. The stressed sample were cooled, neutralized and diluted with mobile phase to give a final concentration of 100 µg/mL and filtered before injection.

Base Degradation Sample: Stress studies in alkaline conditions were conducted using a concentration of 1.0 mg/mL in 0.1 M NaOH and 0.01 M NaOH for RT for 30 min and 15 min. After that the solution was neutralized and diluted with mobile phase to give a final concentration of 100 µg/mL and filtered before injection.

Oxidative Degradation: Solutions for oxidative stress studies were prepared using 3% H2O2 and 1% H2O2at a concentration of 1 mg/mL of Valganciclovir and Darunavir for RT and at 35 ºC for 30 min and 1 hr on the thermostat the sample solution were cooled and diluted with the mobile phase to give a final concentration of 100 µg/mL and filtered before injection.

Thermal Degradation Sample: For thermal stress testing, the drug solutions (1 mg/mL) were heated in thermostat at 60 ºC 70 ºC and 90 ºC for 5 days, and cooled and diluted with the mobile phase to give a final concentration of 100 µg/mL and filtered before injection.

Comparison of Degraded Impurities of Valganciclovir and Darunavir with Known Impurities for Retention Time

Preparation of Valganciclovir impurity spiked solution:

An accurately weighed quantity about 10.0mg of Valganciclovir standard was transferred to 100.0mL volumetric flask, about 50.0mL of diluent was added, sonicated to dissolve and cooled, from each impurity stock solution 3.0 mL was added and volume made up to the mark with diluent. (Concentration of Impurity 2.4µg/mL and Valganciclovir 100 µg/mL).

Preparation of Darunavir impurity spiked solution:

An accurately weighed quantity about 10.0mg of Darunavir standard was transferred to 100.0mL volumetric flask, about 50.0mL of diluent was added, sonicated to dissolve and cooled, from each impurity stock solution 3.0 mL was added and volume made up to the mark with diluent. (Concentration of Impurity 2.4µg/mL and Darunavir 100 µg/mL).

RESULTS AND DISCUSSIONS

Method Validation Study of Valganciclovir and Darunavir

Specificity

The chromatograms of standard and sample are identical with nearly same retention time. No interference due to placebo at the retention time of analyte which shows that the method was specific.

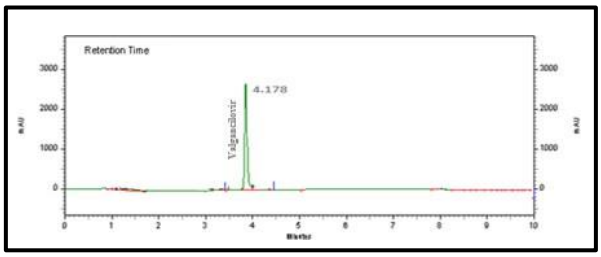

Figure No. 3 : Standard Chromatogram of Valganciclovir

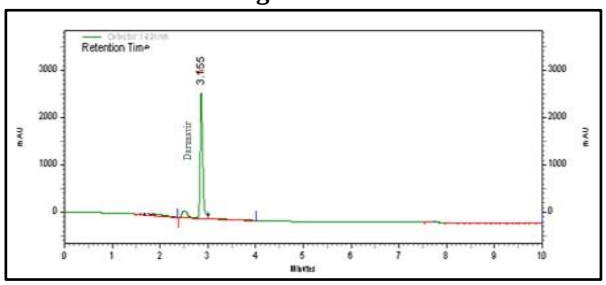

Figure No. 4 : Standard Chromatogram of Darunavir

Sensitivity of Method by LOD/LOQ

Valganciclovir HCl, the limit of detection was calculated as 4 ng/mL (S/N ratio ≥ 3), and the limit of quantification was 1.89 µg/mL. For Darunavir Hydrate, the LOD was found to be 1.86 µg/mL and LOQ was 6.78 µg/mL and 6.73 µg/mL respectively.

Accuracy/ Recovery

The accuracy study was performed over a range from LOQ to 150% of the specification level for both Valganciclovir hydrochloride and Darunavir. To meet the minimum requirement of nine determinations per impurity, triplicate sample preparations were analyzed at each level (LOQ, 50%, 100%, and 150%).

Robustness

The results of the robustness study indicated that while deliberate changes in flow rate and column oven temperature had an impact on peak retention times, system suitability criteria and impurity separation were not compromised. This confirmed that the analytical method remains robust under minor variations in chromatographic parameters.

Table No. 2: Characteristic parameters of Valganciclovir and Darunavir by the RP HPLC method

Sr. No.	Parameter	Valganciclovir Result	Darunavir Result
1	Calibration range (µg/ml)	5-25	5-30
2	Detection wavelength (nm)	257	263
3	Retention time	4.173 min	3.162 min
4	Regression equation (y*)	y = 5.58x + 2841.7	y = 23.249x
5	Slope (b)	33678	34568
6	Intercept (a)	72986	72589
7	Correlation coefficient(r2)	0.9979	0.9973
8	Limit of Detection (µg/ml)	1.89	1.86
9	Limit of Quantitation (µg/ml)	6.78	6.73
10	Precision	0.54	0.266
11	Accuracy	99.40	99.82
12	Specificity	99.98	99.97
13	Robustness	1.124	1.1245

Forced Degradation Study of Valganciclovir and Darunavir

Table No. 3: Results of Degradation study of Valganciclovir Hydrochloride

Stress test condition	Solvent	Temperature	Time	% of Valganciclovir HCl	% Degradation of Valganciclovir
Acidic	1 M HCl 1 M HCl 0.1 M HCl	Room temperature 35ºC 35ºC	30 min 30 min 1 h	74.6 43.1 64.4	25.4 56.9 35.6
Basic	1 M NaOH	Room temperature	30 min	14.2 48.4	85.8 51.6
	0.01 M NaOH	Room temperature	15 min		
Oxidative	3% H_2O_2 3% H_2O_2 1% H_2O_2	Room temperature 35ºC 35ºC	30 min 30 min 1 h	94.9 89.6 85.3	5.10 10.4 14.7
Photolytic UV light Visible light	Solid form Solid form	Room temperature Room temperature	5 days 5 days	97.5 99.3	2.5 0.7
Heat	Solid form	90ºC 70ºC 60ºC	5 days	99.7	0.3

The drug Valganciclovir hydrochloride was found to undergo moderate degradation in basic hydrolysis and oxidation. It was observed that 9Valganciclovir hydrochloride was found to degrade in basic hydrolysis and oxidation with 3% H_2O_2. In acidic conditions, it was observed that the drug was found to show 25.4% degradation at room temperature with 1 M Hydrochloric acid, the drug was refluxed with 1 M Hydrochloric acid for 30 min at 35˚C. About 56.9% of degradation was observed with two additional peaks at the retention time of 2.102 and 3.0 min respectively. Valganciclovir hydrochloride was found to be highly susceptible to basic hydrolysis. Almost 85.8% was observed when the drug was incubated with 1 M sodium hydroxide at room temperature for 30 min. To minimize the effect of basic hydrolysis, the drug was kept with 0.1M sodium hydroxide at room temperature. Almost 51% degradation was observed. The drug was more stable to Oxidative degradation. When an aqueous solution was subjected to stress conditions i.e. reflux for 1 hr at 35˚C, only 14.7 % degradation was observed. In oxidative degradation, it was observed that the drug was found to show 5% degradation at room temperature with 3% hydrogen

peroxide and 10% degradation when refluxed at 35˚ C for 30 min. Oxidation degradation was observed with two additional peaks at 2.231 and 3.113 min. Valganciclovir hydrochloride was not susceptible to photolysis as the degradation was less than 10% in solution as well as in solid state.

The results of the Forced degradation study of Darunavir hydrate

Table No. 4 : Results of Degradation study of Darunavir Hydrate

Stress test condition	Solvent	Temperature	Time	% of Darunavir hydrate	% Degradation of Darunavir
Acidic	1 M HCl 1 M HCl 0.1 M HCl	Room temperature 35ºC 35ºC	30 min 30 min 1 h	81.1 24.5 66.4	18.9 75.5 33.6
Basic	1 M NaOH 0.01 M NaOH	Room temperature Room temperature	30 min 15 min	20.4 44.1	79.6 55.9
Oxidative	3% H_2O_2 3% H_2O_2 1% H_2O_2	Room temperature 35ºC 35ºC	30 min 30 min 1 h	93.8 28.9 81.1	6.2 71.1 18.9
Photolytic UV light Visible light	Solid form Solid form	Room temperature Room temperature	5 days 5 days	99.7 99.9	0.3 0.2
Heat	Solid form	90ºC 70ºC 60ºC	5 days	99.8	0.2

The results of acidic hydrolysis showed degradation peak at 1.947 min along with the drug peak. The peak area showed that 75.5 % of degradation of the drug occurred when the drug was kept in 1 M HCl. Darunavir hydrate upon alkaline degradation in 1 M NaOH up to 30 min underwent percentage degradation was 79.6 % in the above condition. Darunavir hydrate did not degrade after it was kept under direct sunlight. No peak other than the drug peak was found in the chromatogram of that sample. Darunavir hydrate was not degraded after it was kept in the UV chamber. In the oxidative degradation study, Darunavir hydrate showed percentage degradation 71.1. In acidic condition drug was found degraded up to 76% . Darunavir hydrate was found to be more labile. The drug was degraded about 80% after 30 min exposure to 1 M NaOH at room temperature. The degradation was slower under exposure to 0.01 M NaOH and 55.9% degradation was observed after 15 min at room temperature. Darunavir hydrate was found to degrade in 1% H_2O_2 to an extent of 18.9% after 1h. More degradation ie. 71.1 %was observed by using 3% H_2O_2 at room temperature. Darunavir hydrate bulk powder was stable under exposure to heat, UV light, and visible light and no significant degradation was observed.

Comparison of Degraded Impurities of Valganciclovir with Known Impurities

Table no. 5: comparison of degraded impurities of Valganciclovir with known impurities

Conditions	Retention time(minute)						
	Valganciclovir HCl	Unknown Impurity		Known Impurity			
		Imp I	Imp II	Imp V1	Imp V2	ImpV3	ImpV4
Degradation obtained by Acid	4.182	2.980	6.102	2.245		6.102	7.371

Degradation obtained by Base	4.178	2.143	3.124	2.245	3.145	-	-
Degradation obtained by Oxidation	4.182	-	-	-	-	-	-
Degradation obtained by using Thermal study	4.178	-	-	-	-	-	-
Degradation obtained by Photolytic activity	4.178	-	-	-	-	-	-

In acidic conditions, it was observed that the drug was found to show 25% degradation at room temperature with 0.1 M Hydrochloric acid, the drug was refluxed with 0. 1 M Hydrochloric acid for 24 hrs at 60˚C. About 56% of degradation was observed with two additional peaks at the retention time of 2.980 and 6.102 min respectively. Valganciclovir hydrochloride was found to be highly susceptible to basic hydrolysis. It was observed that the drug was found to show 65% degradation. Degradation was observed when the drug was incubated with 0.1 M sodium hydroxide at room temperature for 2 hours. Degradation was observed with two additional peaks at the retention time of 2.143 and 3.124 min respectively. When the Retention time of unknown impurities compare with known impurities it was observed that the Retention time of impurity found in acidic degradation was 2.980 and 6.102 min respectively which is similar to the retention time of known impurity V1 and V3 respectively.Similarly retention time of impurity found in basic degradation was 2.143 and 3.124 min respectively which is matches with Retention time of known impurity V1 and V2 respectively. In oxidative degradation, it was observed that the drug was found negligible degradation at room temperature with 3% hydrogen so no significant degradation was found. Valganciclovir Hydrochloride bulk powder was stable under exposure to heat, UV light, and visible light and no significant degradation was observed.

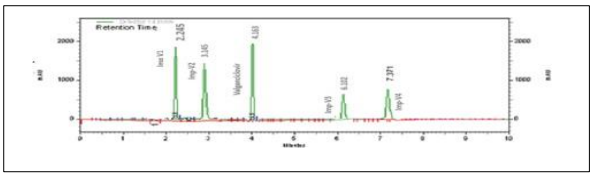

Figure No. 5: Selectivity study chromatogram of sample solution spiked with known impurities.

Comparison of Degraded Impurities of Darunavir with Known Impurities

Table No. 6: comparison of degraded impurities of Darunavir with known impurities

Conditions	**Retention time(minute)**							
	Darunavir Hydrate	**Unknown Impurity**		**Known Impurity**				
		ImpI	**ImpII**	**ImpA**	**ImpB**	**ImpC**	**ImpD**	**ImpE**
Degradation obtained by Acid	3.137	1.947	7.371	1.824	4.536	-	6.324	7.458
Degradation obtained by Base	3.288	-	7.523	-	-	-	-	7.458
Degradation obtained by Oxidation	3.140	-	5.271	-	-	5.312	-	-

Degradation obtained by using Thermal study	3.155	-	-	-	-	-	-	-
Degradation obtained by Photolytic activity	3.157	-	-	-	-	-	-	-

In the present investigation, Darunavir hydrate HCl was subjected to its stability studies under different conditions as per the ICH guidelines. The results of acidic hydrolysis showed a degradation peak at RT 1.947 min along with the drug peak. The peak area showed that 72
% of degradation of the drug occurred when the drug was kept in 1 M HCl. Darunavir hydrate upon alkaline degradation showed a degradation peak at RT 7.523 min in 1 M NaOH up to 30 min underwent percentage degradation was 46 % in the above condition. Darunavir hydrate was found to degrade in 1% H_2O_2 to an extent of 18% after 1h it showed degradation peak at RT 5.271 min.

When the Retention time of unknown impurities compare with known impurities it was observed that the Retention time of impurity found in acidic degradation was 1.947 and 7.371 min respectively which is similar to the retention time of known impurity A and E respectively. Similarly retention time of impurity found in basic degradation was 7.523 min which is matches with Retention time of known impurity E. In oxidative degradation, it was observed that the retention time of unknown impurity was 5.271 which was matches to known impurity C. Darunavir hydrate bulk powder was stable under exposure to heat, UV light, and visible light and no significant degradation was observed.

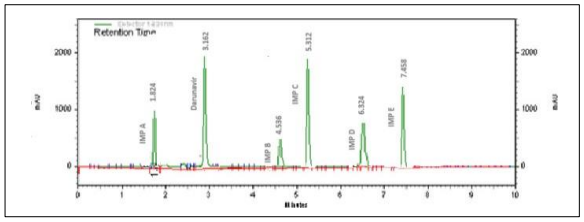

Figure No. 6: Selectivity study chromatogram of sample solution spiked with known impurities

CONCLUSION

From all above studies, concluded that the proposed validation process is very simple as well as more accurate stability -indicating HPLC process is developed to routine analysis to both the drugs includes Valganciclovir as well as Darunavir in bulk and tablet dosage forms. These compounds/substances are subjected to evaluation with forced degradation pertaining to several stress circumstances. This developed process separates both drugs and their products of degradation successfully also quantifies active contents at minute strength levels. This developed method is totally applied considering the rules and regulations given by ICH to all parameters those are identified within acceptance criteria. Finally, described process may be recommended to regular analysis as well as to check quality during stability studies for these drugs.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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