RESEARCH PAPER

Development and Validation of Stability Indicating RP-HPLC Method for the Simultaneous Estimation of Antiretroviral Drugs by Forced Degradation Studies

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ABSTRACT

Valganciclovir and Darunavir are two antiretroviral drugs that have been approved for the treatment of HIV Infection. 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. The retention time was found to be 4.1 min for Valganciclovir and 3.1 min for Darunavir. The linearity limit of Valganciclovir and Darunavir was found to be in the range of 0.997 and 0.997. The method validation was carried out in terms of accuracy, linearity, precision, specificity, LOD, LOQ as per ICH Guidelines. The results obtained from the validation parameters show that the method developed can be useful in the quality control test of bulk and dosage forms of Valganciclovir and Darunavir. Valganciclovir and Darunavir were exposed to different stress conditions like acidic, basic, neutral, thermal and peroxide. Amongst all, the drug was found to be more degraded under acidic as well as basic degradation conditions.

Keywords: Valganciclovir, Darunavir, forced degradation, ICH Guidelines.

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INTRODUCTION

Intestinal and hepatic esterases quickly transform ganciclovir into valganciclovir, the L-valinyl ester of ganciclovirIt is an artificial counterpart of 2'-deoxyguanosine. It functions as both an antiviral medication and a prodrug[1,2,3,4]. It is an L-valyl ester and a purine. It is functionally related to a guanine and a ganciclovir. The U.S. Food and Drug Administration (FDA) has approved valganciclovir hydrochloride, an antiviral medication, to treat adults with AIDS who have cytomegalovirus retinitis (CMV retinitis)[5],[6,7,8]. The FDA has also approved valganciclovir hydrochloride for the prevention of CMV disease in organ transplant recipients who are susceptible to CMV infections[9,10,11,12]. Cytomegalovirus infections are treated with the antiviral drug valganciclovir hydrochloride (Valcyte, produced by Roche). As ganciclovir's L-valyl ester. In actuality, it is a prodrug for ganciclovir. Intestinal and hepatic esterases quickly transform it into ganciclovir after oral dosing[13,14,15]. Cytomegalovirus infections are treated with the antiviral drug valganciclovir. In cell culture, darunavir demonstrates antiviral activity against a broad panel of HIV-1 group M and group O main isolates[5]. Darunavir's EC50 value increases by a median factor of 5.4 in the presence of human serum.In acutely infected T-cell lines, human peripheral blood mononuclear cells, and human monocytes/macrophages, darunavir exhibits activity against laboratory strains and clinical isolates of HIV-1 and laboratory strains of HIV-2[16,17,18,19]. Darunavir showed no antagonistic effects in comparison to the protease inhibitors amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, and ritonavir[20,21].

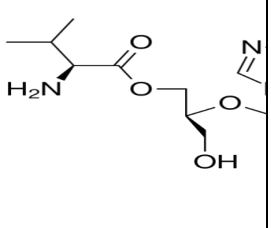

Figure No 1:Valganciclovir Structure

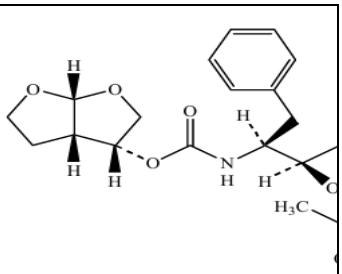

Figure No.2: Darunavir Structure

From the available literature, we found that some analytical methods were found to be less economical in terms of run

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times, mobile phase composition and column dimensions[22,23]. Hence, 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 and as well as in pharmaceutical dosage form[24,25].

MATERIALS AND METHODS

Instruments Used: HPLC Thermo, P4000 Quaternary pump, PDA Detector with CHROMQUEST software. UV/VIS spectrophotometer LABINDIA UV 3200 pH meter Thermo electron corporation Orion 2 star, Analytical balance Afcoset ER-200A.

Chemicals and Reagents: Potassium dihydrogen phosphate procured from finer chemical ltd AR grade, sodium hydroxide procured from Merck AR grade, hydrochloric acid procured from Merck GR grade, orthophosphoric acid procured from Merck GR grade, hydrogen peroxide procured from Merck, GR grade, acetonitrile procured from Merck HPLC grade, water Millipore HPLC grade.

Standard Preparation: Valganciclovir 45 mg and Darunavir 40 mg were weighed and transferred into a 100 mL volumetric flask. Then 7 mL of diluent was added and sonicated. The volume was made up to the mark with the same solvent. Then 3.0 mL of the above stock solution was transferred into a 10 mL volumetric flask and the volume was adjusted up to the mark with diluent.

Sample Preparation: Valganciclovir 45 mg and Darunavir 40 mg were weighed and transferred into a 100 mL volumetric flask and about 7 mL of diluent was added and sonicated. The volume was adjusted up to the mark with the same solvent. It was kept aside for few minutes until the un dissolved excipient from the tablets gets settled at the bottom of the flask. Slowly the supernatant fluid was collected using a syringe and the solution was filtered with a 0.45 nylon membrane filter, which further removes any excipients present in the solution. Then 3.0 ml of the above stock solution was transferred into a 10 mL volumetric flask, and the volume was adjusted with diluent.

Preparation of OPA Buffer: 2.9 ml of o-phosphoric acid was pipette out into a 1000 ml volumetric flask. It was diluted with water and finally made up to 1000 ml. The pH of the buffer was adjusted to 4.0 with 0.5 M sodium hydroxide and finally filtered through 0.45µm nylon membrane filter.

Preparation of Mobile Phase: A mixture of Acetonitrile and 0.05 M ortho phosphate buffer (pH 4.0) in the ratio of 60:40 v/v was taken. Then the solution was filtered through 0.45µ nylon membrane filter, degassed and used as the mobile phase.

Diluent Preparation: The mobile phase was used as the diluent.

Method Development: Different chromatographic trials were performed with different columns and mobile phases. A number of trials were performed before choosing the chromatographic condition, with different solvents ratios, flow rate and temperatures to check the retention time (RT), peak shape, theoretical plates of the analyte and tailing factor (peak symmetry). The optimized chromatographic is shown in Fig. 3.

Table No. 1: Chromatographic Trials

Parameters	Trial 1	Trial 2	Trial 3	Trial 4
Column Used	symmetry, C18 4.6 × 150 mm, 5 µm	Zodiacsil C18 4.6 × 150 mm, 5 µm	Hypersil RPC8 4.5 × 150 mm, 5.0 µm	CHEMSIL ODS-C18 250 mm × 4.6 mm, 5 µm
Mobile phase	Methanol : Water (60:40%v/v)	Acetonitrile : Water (50:50%v/v)	Buffer ACN: pH 6.8 phosphate buffer (50:50)	Acetonitrile : Ortho Phosphate buffer of pH 4 (60:40%v/v)
Buffer				OPA
Flow rate	1 ml/min	1 ml/min	1 ml/min	1 ml/min
Wavelength	257 nm	257 nm	257 nm	257 nm
Temperature	30 °C	30°C	30 °C	30 °C
Injection Volume	20 µl	20 µl	20 µl	20 µl
Run time	5.0 min	5.0 min	8.0 min	10 min

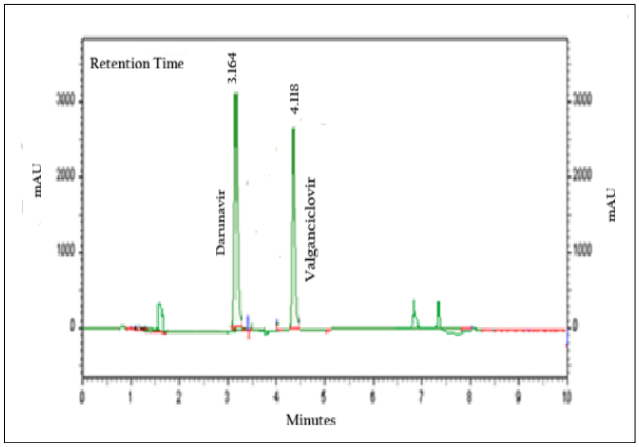

Figure No. 3: Optimized Chromatogram

Optimized Chromatographic Conditions: Temperature: Ambient (25 °C) Mode of separation: Isocratic mode Column : Buffer Mobile phase Flow rate Wavelength Injection volume Run time:10 min CHEMSIL ODS-C18 250 mm × 4.6 mm, 5 µm ,Acetonitrile : Ortho Phosphate buffer of pH 4 (60:40%v/v) 1.0 mL per min 257 nm 20 µl 10 min.

RESULTS AND DISCUSSION:

Validation: Precision: Precision is expressed in terms of the degree of agreement between replicate analyses of a homogenous sample, usually measured as the relative standard deviation (RSD). The precision was determined by taking 45 mg of Valganciclovir and 40 mg of Darunavir working standards. Both the working standards were transferred into a 100 mL clean, dry volumetric flask and about 7 mL of diluent was added and sonicated to dissolve it completely and the volume was adjusted up to the mark with the same solvent. (Stock solution) then 3.0 ml of the above stock solutions were transferred into a 10 mL volumetric flask and adjusted up to the mark with diluent. The results are summarized for Valganciclovir and Darunavir in Table 2 & 3.

Table No. 2: Summarized Precision Results for Valganciclovir And Darunavir

Injection	RT (Darunavir)	Area for Darunavir	RT (Valganciclovir)	Area for Valganciclovir
Injection-1	3.164	1228412	4.110	1610856
Injection-2	3.173	1223356	4.118	1609920
Injection-3	3.178	1213789	4.119	1619345
Injection-4	3.180	1201656	4.123	1608609
Injection-5	3.188	1228867	4.124	1610876
Injection-6	3.191	1220458	4.133	1618967
Average		1219423		1613095.5
Standard Deviation		10333.8		4768.3
%RSD		0.847		0.296

Specificity: The system suitability for specificity was carried out to determine whether there was any interference of any impurities in the retention time of the analytical peak. The study was performed by injecting blank and standard into the system. There was no interference of any peak in the blank with the retention time of the analytical peaks.

Accuracy: Accuracy is expressed as the nearness of agreement between the values found and values that are already available. Accuracy can be expressed in terms of closeness between the true value and precision.

It can be determined by using at least a minimum of 3 concentrations and 9 determinations over the specified range. 45 mg of Valganciclovir and 40 mg of Darunavir working standard were accurately weighed and transferred into a 100 mL volumetric flask, and about 7 mL of diluent was added and sonicated to dissolve it completely, and the volume was adjusted with the same solvent. (Stock solution) Further, 3.0 ml of the above stock solutions were transferred using a pipette into a 10 mL volumetric flask and diluted up to the mark with diluent. The accuracy results for Valganciclovir and Darunavir are shown in Tables 3 & 4.

Table No.3: Accuracy Results for Valganciclovir

% Concentration (at specification Level)	Area	% RSD	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	8095567	0.8	22.5	22.41	99.6	100.23
100%	1611745	0.2	45	45.18	100.4	
150%	2408434.6	0.3	67.5	67.99	100.7	

Table No.4: Accuracy Results for Darunavir

%Concentration (at specification Level)	Area	% RSD	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	617867.8	0.9	20	20.12	100.6	100.21
100%	1224245.3	0.1	40	39.91	99.77	
150%	1831567.7	0.3	60	60.17	100.28	

Linearity: Linearity may be defined as the capacity of an analytical method to produce outcomes that are directly related to the concentration of an analyte. Linearity was determined by taking 45 mg Valganciclovir, and 40 mg of Darunavir working standards both the standards were transferred into a 100 mL volumetric flask and 7 mL of diluent was added and sonicated for 10 min and the volume was adjusted with the same solvent. The linearity results for Valganciclovir& Darunavir are shown in Tables 5 & 6 and Fig. 4 & 5.

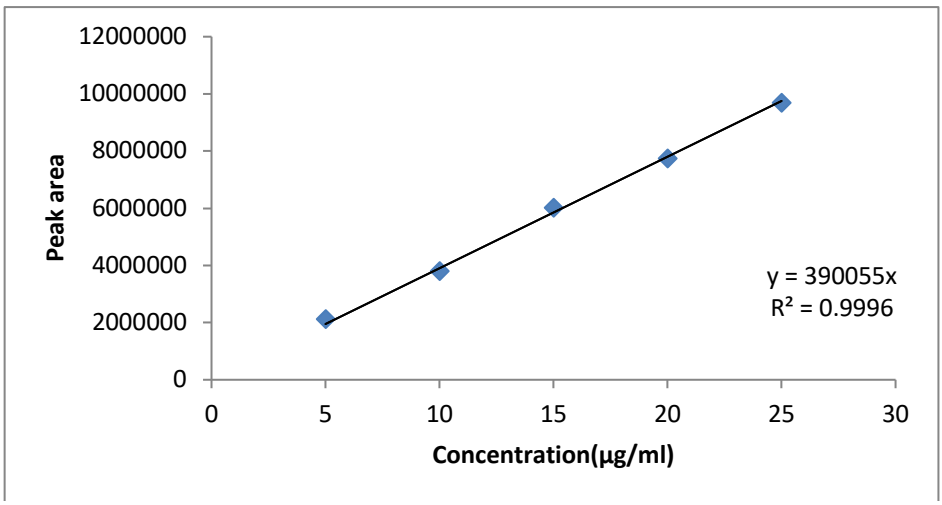

Figure No. 4: Linearity Graph of Valganciclovir

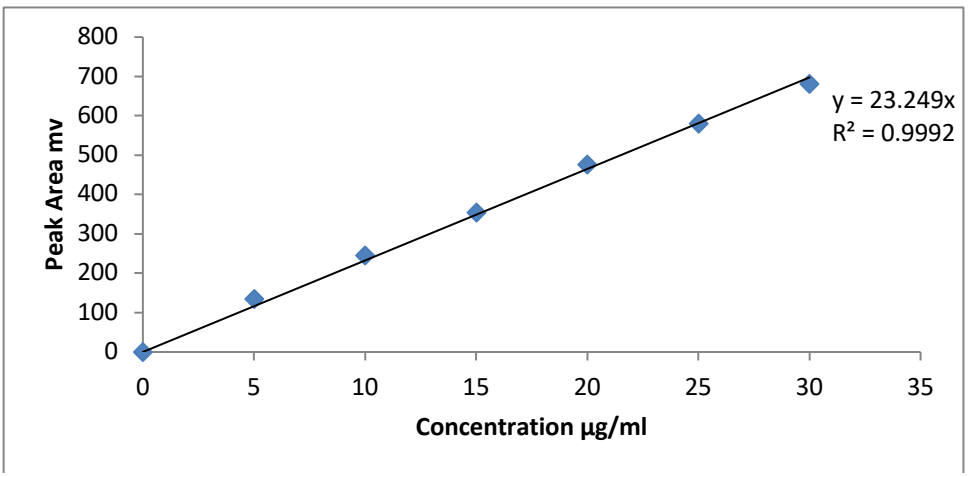

Figure No. 5: Linearity Graph of Darunavir

Table No. 5: Linearity Results of Valganciclovir

S. No	Linearity Level	Concentration	Area
1	I	05	2125970
2	II	10	3795876
3	III	15	6022163
4	IV	20	7757587
5	V	25	9690107
	Correlation Coefficient		0.997

Table No.6: Linearity Results of Darunavir

S. no	Linearity Level	Concentration	Area (mv)
1	I	05	135.245
2	II	10	245.789
3	III	15	354.132
4	IV	20	476.234
5	V	25	580.113
6	VI	30	680.567
	Correlation Coefficient		0.997

Robustness: As part of the robustness, deliberate change in the flow rate, mobile phase composition, temperature variation was made to evaluate the impact on the method. The robustness limit for mobile phase variation and flow rate variation is well within the limit; the % degradation results are in limits.

LOD & LOQ: LOD is expressed in terms of the lowest quantity of an analyte which can be detected by the chromatographical separation. A blank resolution is injected and the quantitative noise and peak-to-peak relation can be calculated from blank chromatograms. The limit of quantitation is the concentration level above which the concentration can be estimated with acceptable exactness and precision.

Degradation Studies: The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Valganciclovir and Darunavir using the proposed method.

Preparation of stock solution: Valganciclovir 45 mg and 40 mg of Darunavir working standards were accurately weighed and transferred into a 100 mL clean dry volumetric flask and about 7 mL of Diluent was added and sonicated to dissolve it completely and volume was adjusted up to the mark with the same solvent. (Stock solution)

Hydrolytic Degradation under Acidic Condition: A stock solution of 3 mL was pipetted out into a 10 mL volumetric flask and 3 mL of 0.1N HCl was added. Then, the volumetric flask was kept at 60 ºC for 24 h and then neutralized with 0.1 N NaOH and volume was made up to 10 ml with diluent. The solution was filtered with 0.44 microns syringe filters and placed in vials.

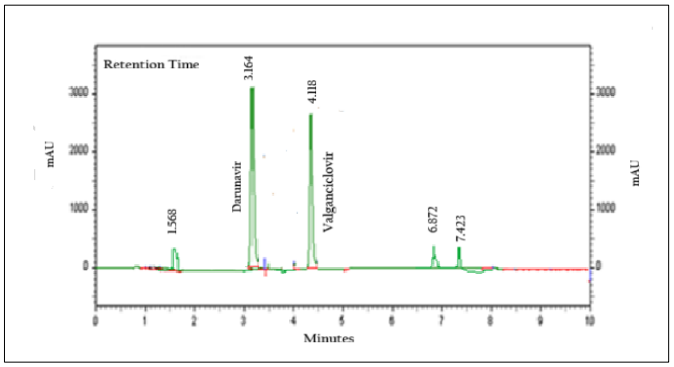

Figure No. 6: Acid Degradation Chromatogram

Table 7: Acid Degradation Results of Valganciclovir And Darunavir

S. No	Peak Name	RT	Area	Height	USP Resolution	USP Tailing	USP Plate Count
1		1.568	2570	680	1.07	3141.32	1.568
2	Darunavir	3.164	1207822	108152	3.52	1.40	3669.74
3	Valganciclovir	4.118	1583722	156664		1.42	2657.20
4		6.872	1243	182	10.47	1.01	9475.45
5		7.423	1417	161	1.06	0.87	2966.35

Hydrolytic Degradation Under Alkaline Condition: Stock solution of 3 mL was pipetted out into a 10 mL volumetric flask, and 3ml of 0.1N NaOH was added in 10 mL volumetric flask. The volumetric flask was kept at 60 ºC for 24 h and neutralized with 0.1N HCl and the volume was made up to 10 ml with diluent. The solution was filtered with 0.44 micron syringe filters and placed in vials.

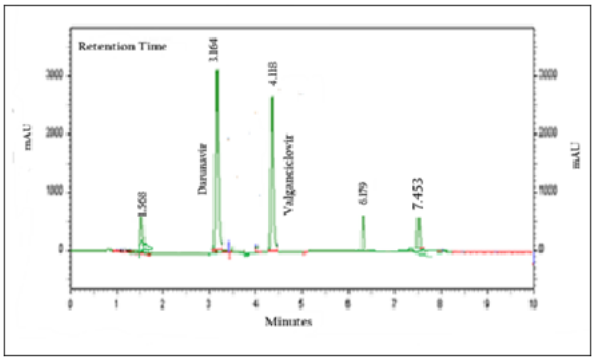

Figure No. 7: Base Degradation Chromatogram

Table No. 8: Alkaline Degradation Results of Valganciclovir and Darunavir

S.no	Peak Name	RT	Area	Height	USP Resolution	USP Tailing	USP Plate Count
1		1.568	2570	680		1.07	3141.32
2	Darunavir	3.164	11738332	1051108	3.52	1.40	3669.74
3	Valganciclovir	4.118	15283333	1511184	8.08	1.42	2657.20
4		6.179	1137	161	5.87	1.18	4220.48
5		7.453	1053	111	7.31	1.01	6559.29

Thermal Induced Degradation: Valganciclovir and Darunavir sample was taken in petridish and kept in Hot air oven at 1100 °C for 3 h. Then the sample was taken and diluted with diluents and injected into HPLC and analyzed.

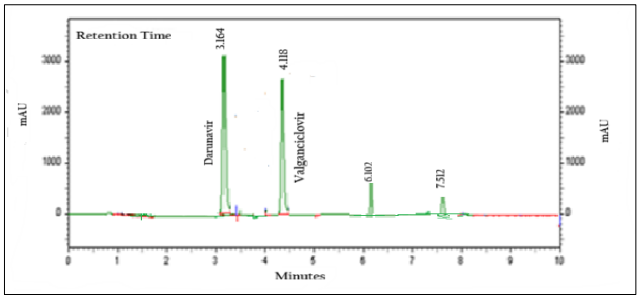

Figure No. 8: Thermal Degradation Chromatogram

Table No.9: Thermal Degradation Results Of Darunavir And Valganciclovir

S.no	Peak Name	RT	Area	Height	USP Resolution	USP Tailing	USP Plate Count
1	Darunavir	3.164	1196732	107159	3.25	1.40	3669.74
2	Valganciclovir	4.118	1492533	147643		1.42	2657.20
3		6.102	1460	190	5.60	0.91	8780.52
4		7.512	1819	215	8.13	0.73	7690.48

Oxidative Degradation: The stock solution of about 3 mL was pipetted into a 10 mL volumetric flask, and 1 mL of 30% w/v of hydrogen peroxide was added in 10 mL volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. The solution was filtered with 0.45 microns syringe filters and place in vials.

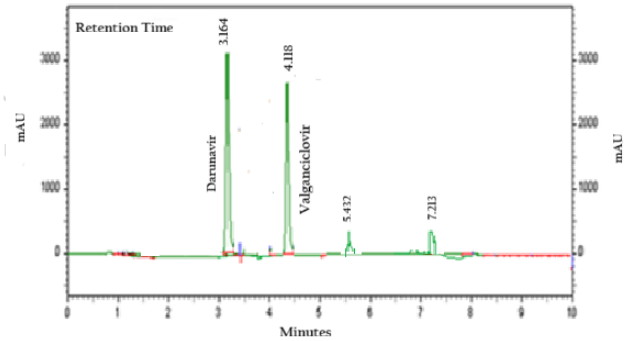

Figure No. 9: Oxidative Degradation Chromatogram

Table 10: Oxidative Degradation Results of Valganciclovir And Darunavir

S.no	Peak Name	RT	Area	Height	USP Resolution	USP Tailing	USP Plate Count
1	Darunavir	3.164	1146223	102636	3.52	1.40	3669.74
2	Valganciclovir	4.118	1558673	154186	3.23	1.42	2657.20
3		5.432	1113	171	8.35	0.96	8299.87
4		7.213	1216	182	13.38	0.49	7297.85

Photo Degradation: Photo Degradation: About 3 ml of the stock solution was pipetted out into a 10 mL volumetric flask and exposed to sunlight for 24 h and the volume was made up to the mark with diluent. The solution was filtered with 0.45 microns syringe filters and place in vials.

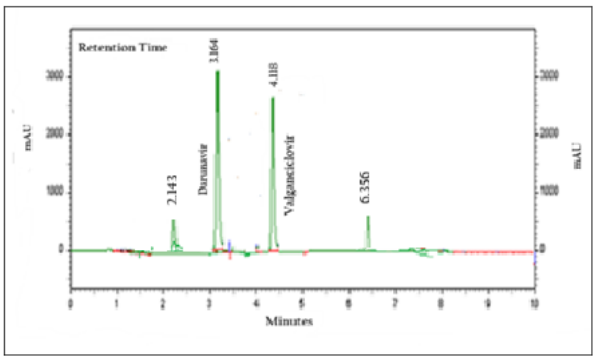

Figure No. 10: Photo degradation Chromatogram

Table No.11: Photo degradation Results of Valganciclovir and Darunavir

S.no	Peak Name	RT	Area	Height	USP Resolution	USP Tailing	USP Plate Count
1		2.143	1286	170		0.86	7322.54
2	Darunavir	3.164	11278897	100995	3.52	1.40	3669.74
3	Valganciclovir	4.118	15093356	149307	5.05	1.42	2657.20
4		6.356	1167	162	11.40	0.79	4664.85

Table No. 12: Degradation Results Of Valganciclovir and Darunavir

Valganciclovir			Darunavir	
Sample Name	Area	% Degraded	Area	% Degraded
Standard	1602702		1224118	
Acid	1583722	5.18	1207822	6.33
Base	1528333	4.64	1173832	4.11
Peroxide	1558673	2.75	1146223	2.36
Thermal	1492533	2.87	1196732	2.24
Photo	1509356	1.82	1127897	1.86

DISCUSSION: A simple, precise and selective RP-HPLC method was developed for the determination of Valganciclovir and Darunavir. Chromatographic separation was achieved by using mobile phase consisting of a mixture of Acetonitrile : Ortho Phosphate buffer of pH 4 (60:40%v/v) on CHEMSIL ODS-C18 column (250 mm × 4.6 mm, 5 µm) column, with a detection limit of 257 nm. Linearity was observed in the range 5-25 µg /ml for Valganciclovir and 5-30 µg/ml for Darunavir for the amount of drugs estimated by the proposed methods were in good agreement with the label claim. The proposed method was validated. The accuracy of the methods was assessed by recovery studies at three different levels. Recovery experiments indicated the absence of interference from commonly encountered pharmaceutical additives or excipients. The method was found to be precise as indicated by the repeatability analysis, showing % RSD less than 2. All statistical data prove the validity of the methods and can be used for routine analysis of pharmaceutical dosage forms.

CONCLUSION: The proposed HPLC method was found to be rapid, simple, specific, precise, accurate, and economical for the simultaneous estimation of Valganciclovir and Darunavir in the pharmaceutical dosage form. From the above experimental results and parameters, it was concluded that a new method was established for simultaneous estimation of Valganciclovir and Darunavir by the RP-HPLC method. Precision and recovery studies were also found to be within the range. The drug gets more degraded under acidic degradation in Valganciclovir and basic degradation in Darunavir degradation studies. There was a decrease in retention times and so the run time also decreased, so the method developed was simple and economical that can be adopted in regular quality analysis tests in Industries.

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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