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Research Article

Analytical method development and validation for the simultaneous estimation of Darunavir and Ritonavir by RP-HPLC method

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ABSTRACT

A simple, Accurate, precise method was developed for the simultaneous estimation of the Darunavir and Ritonavir in tablet dosage form. Chromatogram was run through standard symmetry C18 (4.6 x 150 mm, 5µm). Mobile phase containing Buffer 0.01N KH_2PO_4: Acetonitrile taken in the ratio 45:55%v/v was pumped through column at a flow rate of 1ml/min. Optimized wavelength selected was 290 nm. Retention time of Darunavir and Ritonavir were found to be 2.131 min and 2.593 min. %RSD of the Darunavir and Ritonavir were and found to be 0.8 and 0.6 respectively. %Recovery was obtained as 99.59% and 99.94% for Darunavir and Ritonavir respectively. LOD, LOQ values obtained from regression equations of Darunavir and Ritonavir were 0.86, 2.60 and 0.09, 0.29 respectively. Regression equation of Darunavir is y = 12533x + 10387 and y = 9061x + 183.8 of Ritonavir. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

Key Words: Darunavir, Ritonavir, RP-HPLC

INTRODUCTION

Darunavir is a compound has a place with the class of natural mixes known as amino benzene sulfonamides. These are natural mixes containing a benzene sulfonamide moiety with an amine gathering joined to the benzene ring fig.no.1. Ritonavir, sold under the exchange name Norvir, is an antiretroviral prescription utilized alongside other medications to treat HIV/AIDS. This blend treatment is known as very dynamic antiretroviral treatment (HAART). Regularly a low portion is utilized with other protease inhibitors fig.no.2.

The present study was designed to develop a simple, precise, and rapid analytical RP-HPLC procedure, which can be used for the analytical method was developed for the simultaneous estimation of darunavir and ritonavir as there was only individual methods reported for both drugs. The combination of these two drugs is not official in any pharmacopoeia; hence no official method is available for the simultaneous estimation of these two drugs in their combined dosage forms. Literature survey of darunavir and ritonavir revealed several methods for detecting these drugs individually but there is only one method for their simultaneous estimation using RP-HPLC.

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Objective: Following are the objectives of the present work:
To develop a new stability indicating HPLC method for the simultaneous estimation of Darunavir and Ritonavir and to develop the validated method according to ICH guidelines. To apply the validated method for the simultaneous estimation of darunavir and ritonavir in pharmaceutical formulation.

EXPERIMENTAL

Chemicals and reagents: Darunavir and Ritonavir pure drugs (API) were from Rankem and combination of darunavir and ritonavir tablets (durart-r), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer and Ortho- phosphoric acid, were purchased from Rankem, Mumbai

Apparatus and chromatographic condition: Electronics Balance-Denver, P^H meter -BVK enterprises, India, Ultrasonicator-BVK enterprises, WATERS HPLC 2695 system equipped with binary pumps ,photo diode array detector and Auto sampler integrated with Empower 2 Software .Lab india UV double beam spectrophotometer with UV-win 5 software was used for measuring absorbances of Darunavir and ritonavir The mobile phase was prepared freshly, filtered, sonicated before use and delivered at a flow rate of 1ml/min and the detector wave length was set at 290nm injection volume was 10µL. Diluent used was acetonitrile and water taken in the ratio of 50:50

Preparation of standard and sample solutions
Standard solution: Accurately weighed 20mg of Darunavir, 2.5mg of Ritonavir and transferred to 50ml and 100ml individual volumetric flask and 3/4th of diluents was added to this flask and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. (400µg/ml of Darunavir and 50µg/ml of Ritonavir)

Standard working solution: 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (40µg/ml of Darunavir and 5µg/ml of Ritonavir)

Sample stock Solution: 20 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to one tablet was transferred into a 100ml volumetric flask. 25ml of diluent was added, sonicated for 50 min, further the volume made up with diluent and filtered. (4000µg/ml of Darunavir and 500µg/ml of ritonavir). It was centrifuged for 20 min. Then the supernatant was collected and filtered using 0.45 µm filters using (Millipore, Milford, PVDF). 2ml from sample stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (40µg/ml of Darunavir and 5µg/ml of Ritonavir)

Procedure: Inject 10µL of the standard and sample solution separately into the chromatographic system and measure the peak areas for Darunavir and ritonavir and calculate the % assay value.

RESULTS AND DISCUSSIONS

All of the analytical validation parameters for this proposed method were determined according to ICH guidelines. Obtained validation parameters are presented in. Table.no1

Linearity: The calibration curve was constructed by plotting response factor against respective concentration of Darunavir and Ritonavir. The plots of peak area Vs respective concentration of Darunavir and Ritonavir were found to be linear in the range of 10-60 µg/mL and 1.25-7.5 µg/mL with coefficient of correlation (r^2) 0.999 for two drugs. The linearity of this method was evaluated by linear regression analysis. The slope and intercept calculated for Darunavir and ritonavir

Accuracy: Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 101.14% and 99.49% for Darunavir and Ritonavir respectively. The obtained results are presented in

Sensitivity: The limit of detection (LOD) was determined as lowest concentration giving response and limit of quantification (LOQ) was determined as the lowest concentration analyzed with accuracy of the proposed RP-HPLC method. The limit of detection (LOD) and limit of quantification (LOQ) were found to 0.86µg/ml and 2.60µg/ml for darunavir and 0.09µg/ml and 0.29µg/ml for ritonavir. The LOD and LOQ showed that the method is sensitive for Darunavir and ritonavir in table 4.

System suitability test: The specificity of this method was determined by complete separation of Darunavir and Ritonavir as shown in Fig. 3 with parameters like retention time, resolution and tailing factor. The tailing factor for peaks of Darunavir and Ritonavir was less than 2% and resolution was satisfactory. The average retention time for Darunavir and Ritonavir were 2.358 min and 3.099 min respectively for five replicates. The peaks obtained for Darunavir and Ritonavir were sharp and have clear baseline separation. Analysis was also performed for active Darunavir and Ritonavir, placebo sample (All the ingredients

except active Darunavir and Ritonavir) both at stressed and unstressed condition. After analysis it was found that there is no interference of peak in the placebo & active sample. Hence the developed method was specific for the analysis of this product**.**

Precision: From a single volumetric flask of working standard solution six injections were given. A study was carried out for intermediate precision with the same analyst on the different day for six sample preparations of marketed formulations. Robustness of the method was determined by small deliberate changes in flow rate, temperature and mobile phase ratio. The content of the drug was not adversely affected by these changes as evident from the low value of relative standard deviation indicating that the method was rugged and robust. The assay results of tablet dosage formulation by the proposed method are presented in Table 3.

Stability: In order to demonstrate the stability of both standard and sample solutions during analysis, both solutions were analyzed over a period of 24 hr at room temperature. The results show that for both solutions, the retention time and peak area of Darunavir and Ritonavir remained almost similar (% R.S.D. less than 2.0) and no significant degradation within the indicated period, thus indicated that both solutions were stable for at least 24 hr, which was sufficient to complete the whole analytical process. Further forced degradation studies were conducted indicating the stability of the method developed. The results of the degradation studies are presented in Table 7

Assay sample: The contents of darunavir and ritonavir (40 & 5 mcg each) were collected in 50 ml volumetric flask. Then 20ml acetonitrile was added, sonicated for 25 min and made up to mark to yield 1110&500µg/ml. It was centrifuged for 20 min. Then the supernatant was collected and filtered using 0.45 µm filters using (Millipore, Milford, PVDF). 2ml from sample stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (40µg/ml of Darunavir and 5µg/ml of Ritonavir) table.no.6

Acid degradation sample: To 1 ml of stock s solution Darunavir and Ritonavir, 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0c.The resultant solution was diluted to obtain 40µg/ml & 5µg/ml solution and 10µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample. The typical chromatogram of acid degradation was given in Fig. 7.

Base degradation sample: To 1 ml of stock solution Darunavir and Ritonavir, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60 ^{o}C. The resultant solution was diluted to obtain 40µg/ml & 5µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample. The typical chromatogram of acid degradation was given in Fig. 8.

Oxidation degradation sample: To 1 ml of stock solution of Darunavir and Ritonavir, 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60^0c. For HPLC study, the resultant solution was diluted to obtain 40µg/ml and 5µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample. The typical chromatogram of oxidative degradation was given in Fig. 9.

Neutral Degradation Studies: Stress testing under neutral conditions was studied by refluxing the drug in water for 1hours at a temperature of 60^o. For HPLC study, the resultant solution was diluted to 40µg/ml and 5µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample. The typical chromatogram of oxidative degradation was given in Fig. 10.

Photo Stability studies: The photochemical stability of the drug was also studied by exposing the 250µg/ml & 625µg/ml solution to UV Light by keeping the beaker in UV Chamber for 1days or 200-Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 40µg/ml and 5µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample. The typical chromatogram of thermal degradation was given in Fig. 11.

CONCLUSION

A suitable chromatographic method was developed through optimization by changing various parameters such as the mobile phase, injection volume, flow rate etc. In the present method a standard symmetry C_{18} (4.6 x 150mm, 5µm) column has been used For Darunavir and Ritonavir respectively. Mobile phase used was Buffer 0.01N KH_2PO_4: Acetonitrile(45:55%v/v) for Darunavir and Ritonavir respectively, Retention of Darunavir and Ritonavir has more dependence on the mobile phase. The separation of the two peaks was also dependent on the buffer and the percentage of mobile phases. Darunavr and Ritonavir were eluted at acceptable retention times and got good resolution. Several assay methods has been

developed for the determination of Darunavir and Ritonavir in pharmaceutical dosage forms and in biological fluids but this method is most economic and accurate so this method is very useful for the determination of Darunavir and Ritonavir in bulk and pharmaceutical dosage forms. This method was validated as per ICH-Q2 (R1) guidelines and met the regulatory requirements for selectivity, accuracy and stability. Considering the obtained data, it was possible to affirm that the proposed method was fast, simple and suitable for the accurate determination of Darunavir and Ritonavir.

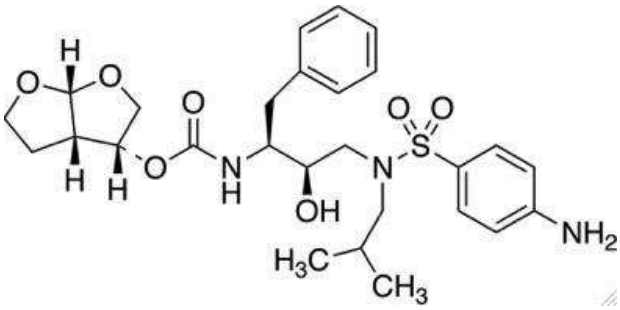

Figure-1: Chemical Structure of Darunavir

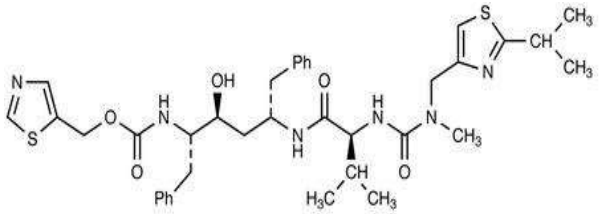

Figure-2: Chemical Structure of Ritonavir

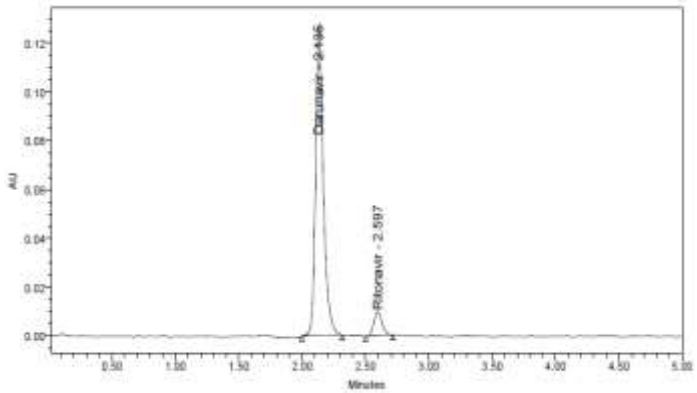

Fig -3 Optimized Chromatogram of Darunavir and Ritonavir

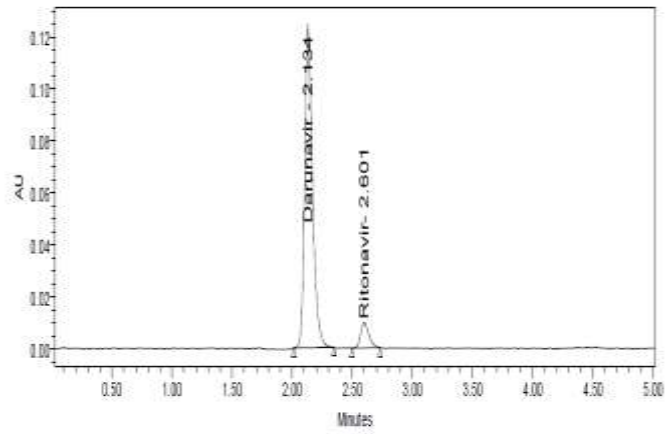

Fig No. 4 Chromatogram of working sample solution

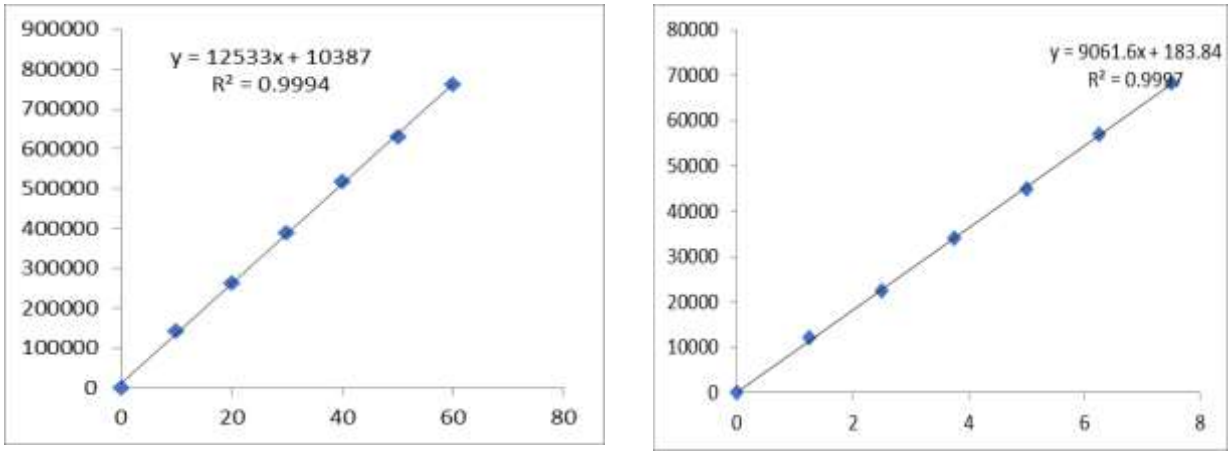

Fig. 5 and 6: Calibration curve of Darunavir and Ritonavir

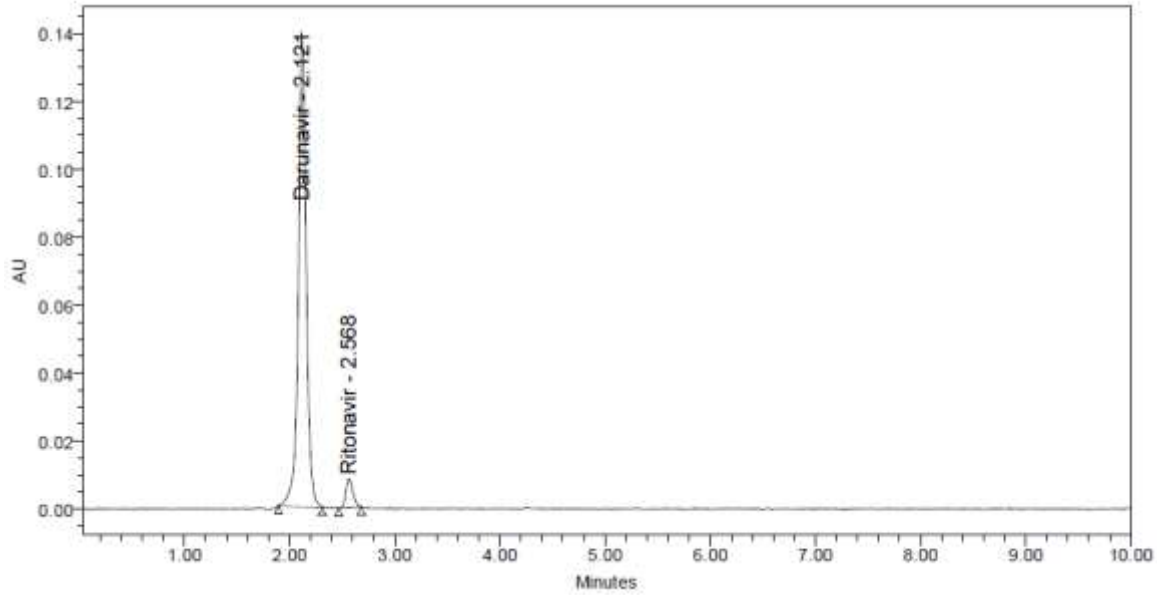

Fig 7: Acid Chromatogram of Darunavir and Ritonavir

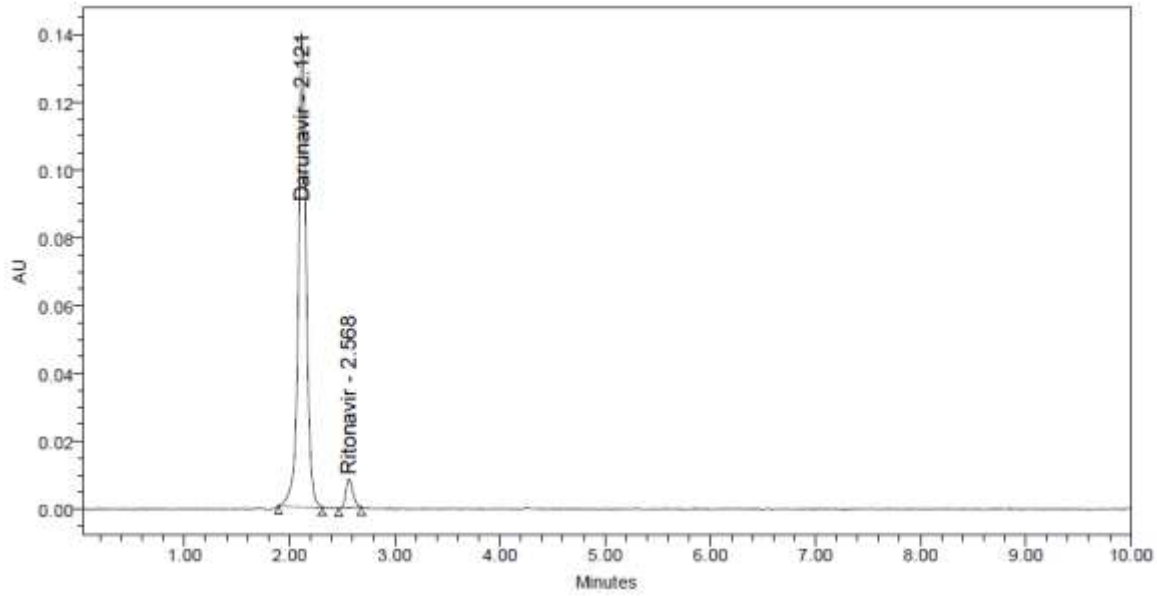

Fig No. 8 Base Chromatogram of working sample solution

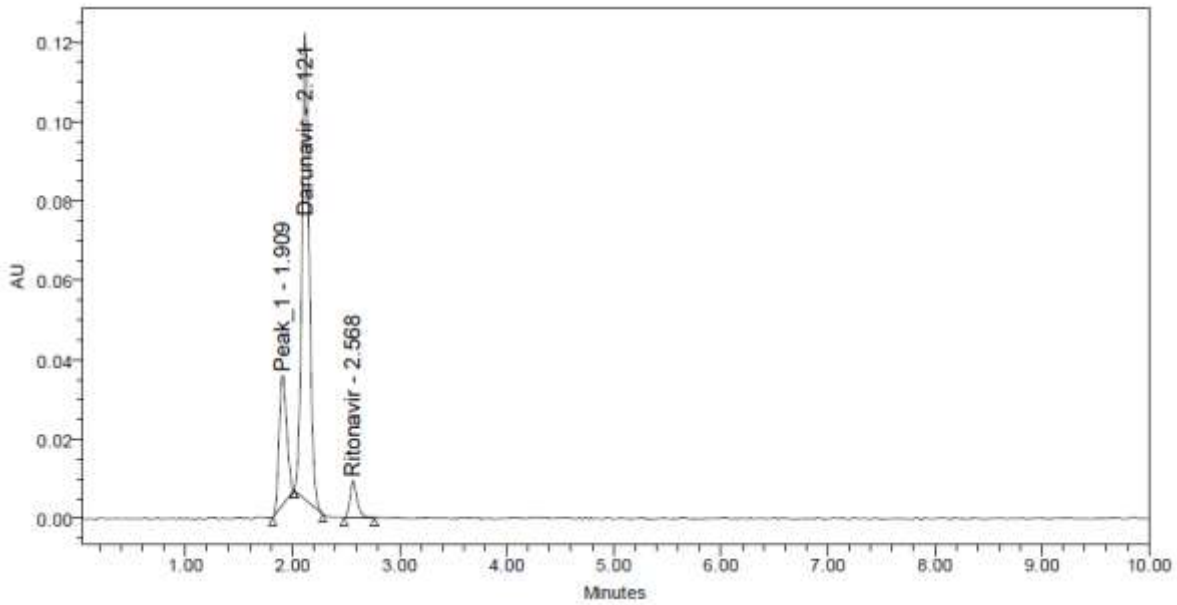

Fig. no:9 Peroxide chromatogram of Darunavir and ritonavir

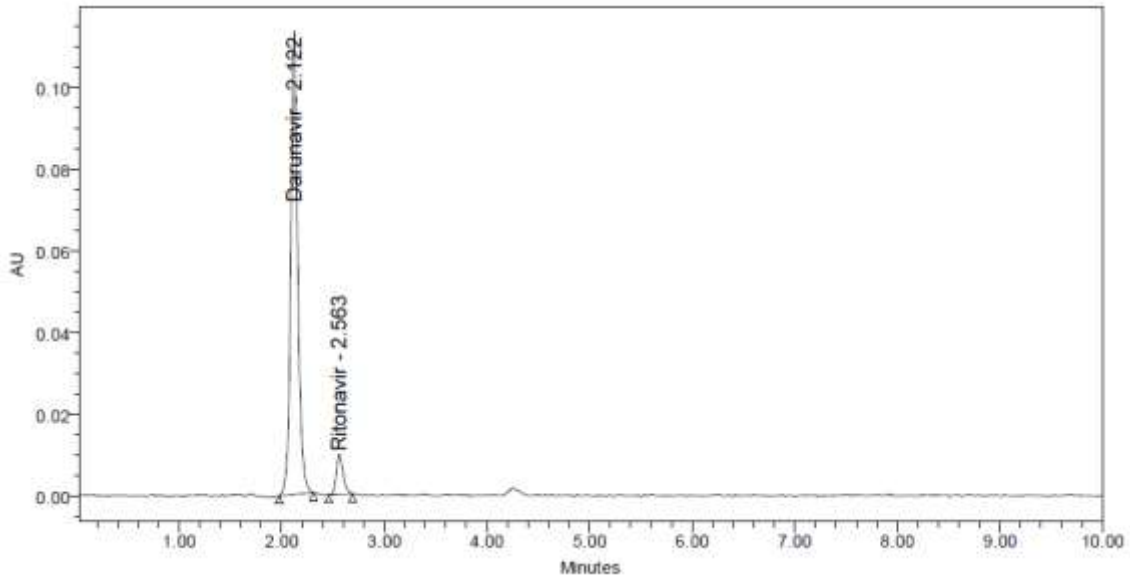

Fig. no:10 Thermal chromatogram of Darunavir and Ritonavir

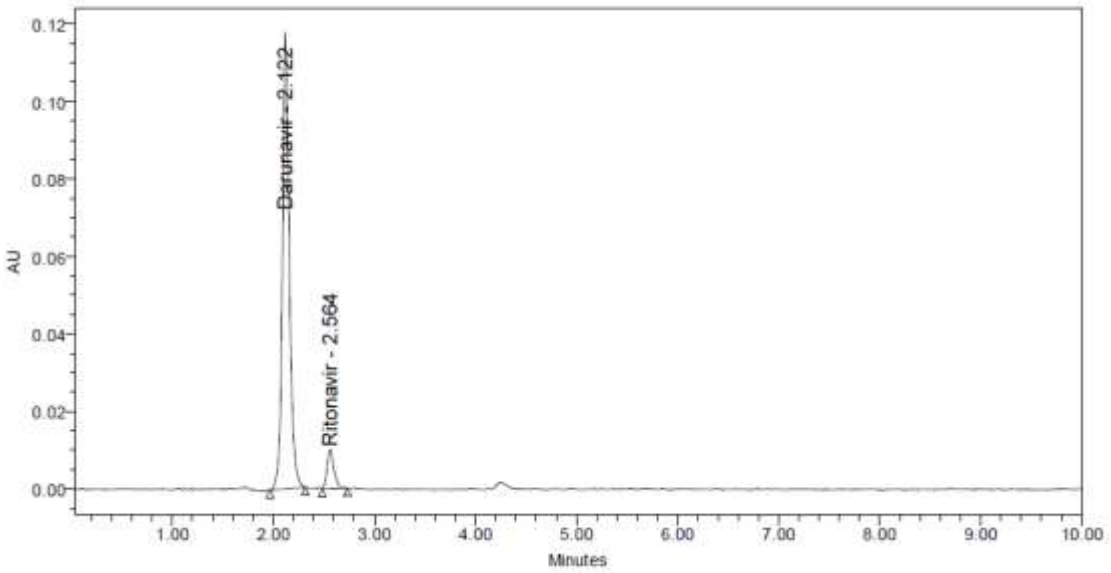

Fig.no: 11 UV degradation chromatogram of Darunavir and Ritonavir

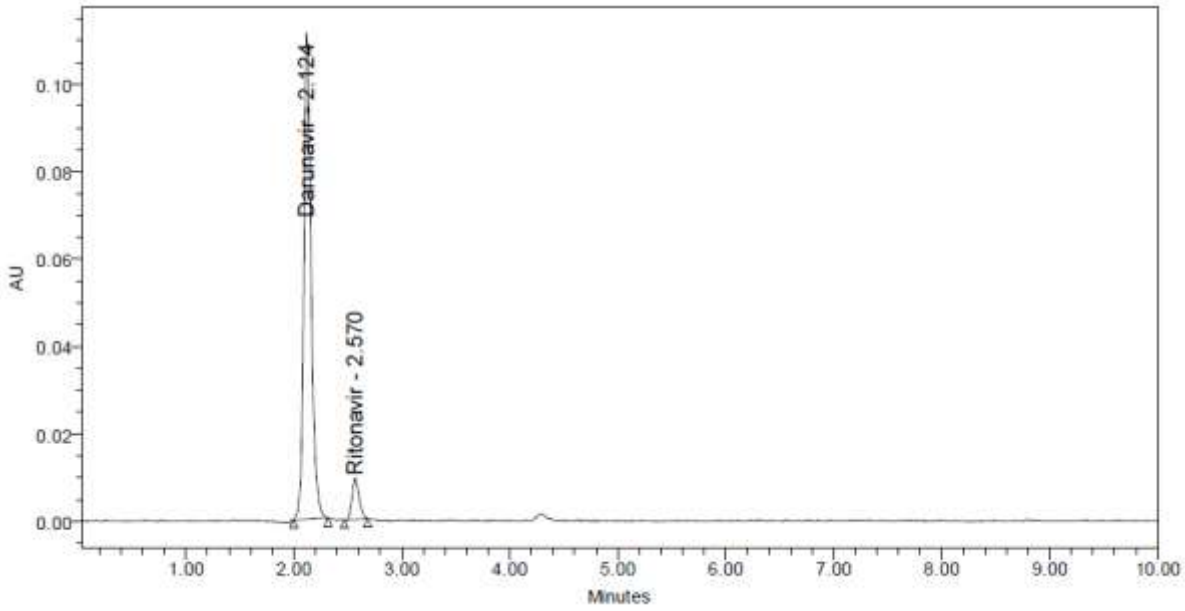

Fig.no:12 Water degradation chromatogram of Darunavir and Ritonavir

Table 1: Analytical validation parameters (System suitability and Linearity)

Parameter	**Darunavir**	**Ritonavir**
Linearity (µg/ml)	10-60 µg/ml	12.25-7.5µg/ml
Slope(m)	12533	9061
Intercept(c)	10387	183.8
Regression equation (Y=mx+c)	y = 12533x + 10387	y = 9061x + 183.8
Regression coefficient	0.999	0.999
System precision %RSD	0.7	0.8
Method precision %RSD	0.8	0.6
LOD	0.86	0.09
LOQ	2.60	0.29
Retention Time (min)	2.131	2.593

Table 2: Accuracy table of Darunavir and Ritonavir

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean % Recover y	% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	20	19.88	99.42	99.59%	50%	2.5	2.48	99.15	99.94%
	20	19.85	99.24			2.5	2.50	99.94	
	20	19.92	99.62			2.5	2.50	99.92	
100%	40	40.26	100.64		100%	5	5.04	100.86	
	40	39.84	99.59			5	5.04	100.73	
	40	39.90	99.75			5	5.04	100.74	
150%	60	59.73	99.54		150%	7.5	7.45	99.40	
	60	59.50	99.16			7.5	7.45	99.38	
	60	59.59	99.32			7.5	7.45	99.36	

Table 3: Precision of Darunavir and Ritonavir

Drug	**Sample Weight (mg)**	**Inter-day precision**		**System precision**		**Repeatability**	
		SD	**% RSD**	**SD**	**%RSD**	**SD**	**%RSD**
Darunavir	20	2916.0	0.7	3564.3	0.7	4195.2	0.8
Ritonavir	2.5	2916.0	0.7	285.0	0.7	244.3	0.6

Table 4: Sensitivity table of Darunavir and Ritonavir

Molecule	**LOD**	**LOQ**
Darunavir	0.86	2.60
Ritonavir	0.09	0.29

Table 5: Robustness data for Darunavir and Ritonavir.

S.no	**Condition**	**%RSD of Darunavir**	**%RSD of Ritonavir**
1	Flow rate (-) 0.9ml/min	0.8	0.7
2	Flow rate (+) 1.1ml/min	0.7	0.8
3	Mobile phase (-) 44:56 solvent	0.8	1.0
4	Mobile phase (+) 46:54 solvent	0.9	1.0

Table 6: Assay result of pharmaceutical dosage formulation

Drug	**Label strength (mg)**	**% Assay**
Darunavir	400	99.96%
Ritonavir	50	100.45%

Table 7: Degradation studies of Darunavir and Ritonavir

Type of degradation	**Darunavir**		
	%DEGRADED	PURITY ANGLE	PURITY THRESHOLD
Acid	3.19	0.210	0.402
Base	4.49	0.110	0.402
Peroxide	2.66	0.210	0.402
Thermal	2.00	0.126	0.421
Uv	2.28	0.208	0.419
Water	6.27	0.218	0.423

Type of degradation	Ritonavir		
	%DEGRADED	PURITY ANGLE	PURITY THRESHOLD
Acid	4.23	1.267	1.661
Base	2.27	1.662	2.136
Peroxide	2.88	1.501	1.862
Thermal	4.68	1.244	1.657
Uv	3.27	1.326	1.639
Water	3.24	1.465	1.742

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