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STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF RITONAVIR AND DARUNAVIR IN BULK AND ITS SYNTHETIC MIXTURE

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ABSTRACT

A simple, specific, precise and accurate Stability indicating RP-HPLC method for simultaneous estimation of Ritonavir (RTV) and Darunavir (DRV) In their Synthetic Mixture has been developed. A RP-HPLC method was developed for the simultaneous estimation of Ritonavir and Darunavir in their Combined Dosage Form has been developed. The separation was achieved by Waters 2695 (100 mm × 4.6 mm i.d, 5 µm) Hypersil ODS column and Phosphate buffer (pH 3.5): (Acetonitrile and Methanol 5:1) (30:70) as mobile phase, at a flow rate of 1 ml/min. Detection was carried out at 220 nm. Retention time of RTV and DRV were found to be 2.126 min and 2.813 min respectively. The method has been validated for linearity, accuracy, precision, robustness, LOD and LOQ. Linearity observed for RTV 20 – 100µg/ml and for DRV 120 - 600 µg/ml. Developed method was found to be accurate, precise and simple, specific for simultaneous estimation of RTV and DRV in their Combine Dosage Form. The drug was subjected to stress condition of hydrolysis, Oxidation, Photolysis and Thermal degradation. The proposed method was successfully applied for the simultaneous estimation of both the drugs in commercial combined dosage form.

INTRODUCTION:

Pharmaceutical products formulated with more than one drug, typically referred to as combination products. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods. The development and validation of analytical methods Spectrophotometric, High performance liquid chromatography (HPLC) and High performance thin layer chromatography (HPTLC) for drug products containing more than one active ingredient. The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug products. The number of drugs introduced into the

market is increasing every year. These drugs may be either new entities or partial structural modification of the existing ones. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs[3]. Darunavir[1] (DRV) is chemically (3R,3aS,6aR)-hexahydrofuro[2,3-b]furan-3-yl N-[(2S,3R)-3-hydroxy-4-[N-(2-methylpropyl)4-aminobenzenesulfonamido]-1-phenylbutan-2yl] carbamate Figure 1. It is a protease inhibitor used to treat HIV. It acts on the HIV aspartyl protease which the virus needs to cleave the HIV polyprotein into its functional fragments. Ritonavir[2] (RTV) is chemically 1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl})carbamoyl]amino}butanamido] 1,6-diphenylhexan-2-yl]carbamate Figure 2. It is an HIV protease inhibitor that interferes with the reproductive cycle of HIV. Although it was initially developed as an independent antiviral agent, it has been shown to possess advantageous properties in combination regimens with low-dose ritonavir and other protease inhibitors. There are few methods reported in the literature of Darunavir and Ritonavir alone or in combination with other drugs in the pure and pharmaceutical formulation by UV, HPLC and UPLC-MS [8-20]. In view of the need of suitable, cost effective RP HPLC method for routine analysis of simultaneous estimation of RTV and DRV in bulk and synthetic mixture (tablet dosage form), attempts we made do develop a simple, accurate, precise and cost effective analytical method for the estimation of RTV and DRV. The purpose of stability testing is to check the drug quality under the action of many environmental factors like temperature, acid, base and oxidative condition. This is necessary for establishment of re-test period for the drug products and for recommendation conditions for their storage. ICH guidelines therefore emphasize stability-indicating analytical methods[4]. Efforts were therefore made to develop a novel, fast and validated stability indicating HPLC procedure for determining simultaneously both the drugs in tablet dosage forms The proposed method will be validated as per ICH guidelines.

EXPERIMENTAL

Chemicals and Reagents: Working standards of DRV and RTV were from PharmaTrain Laboratories, Hyderabad, India. Acetonitrile, Methanol and water of HPLC grade, potassium dihydrogen phosphate, sodium hydroxide, ortho phosphoric acid and hydrochloric acid (analytical grade) were from FINER and MERCK chemical Ltd. Solutions were used for the study is filtered through nylon filter 0.45µm.

System conditions: The liquid chromatography utilized was of Waters. The system was equipped with Empower, 2695 separation module and UV detector. The detector was adjusted at 221 nm. The system was fitted with a auto sampler having a loop of 20 µL. Separations were achieved with Inertsil-ODS stationary phase (100 × 4.6 mm, 5µm). Mobile phase comprised of Phosphate Buffer pH 3.5 and acetonitrile + Methanol (500 + 100) in 30:70 %v/v. Solutions were degassed using ultrasonic

bath then filtered through 0.45µ filter under vacuum filtration before analysis. Mobile phase was delivered at 1.0 ml per min.

Method Development and Validation:

Wavelength selection: The UV spectrum of diluted solutions of 10µg/ml concentrations of RTV and DRV in diluents (water:methanol 80:20v/v) was recorded using UV spectrophotometer. The wavelength of the maximum absorbance was observed at 221nm. This wavelength was used for detection of ritonavir and darunavir.

Preparation of standard solution: Weigh accurately and transfer 20mg of RTV and 120mg of DRV standard into a 100ml clean dry volumetric flask, add about 70ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (stock solution). Further pipette 3.0ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of sample solution: Weigh and power 10 tablets. Find the average weight. Weight equivalent to 20mg of RTV and or 120mg of DRV standard into a 100ml clean dry volumetric flask, add about 70ml of diluent (mobile phase) and sonicate to dissolve it completely and make volume up to the mark with the same solvent (stock solution). Further pipette 3.0ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. Before analysis, the solution was filtered through nylon filter (0.45µm).

Method linearity: For the proposed method linear graphs were attained in the concentration range of 20-100 µg/ml (20, 40, 60, 80, 100 µg /ml) for RTV and 120-600 µg/ml (120, 240, 360,480, 600 µg/ml) for DRV. The data of peak area versus drug concentration were treated by linear least square regression analysis.

Method accuracy: The accuracy of the method was determined by calculating the recoveries of RTV and DRV by the standard addition method. Known amounts of standard solutions of RTV and DRV were added at (50%, 100% and 150%) concentration to pre-quantified sample solutions of RTV and DRV and the amount of drug recovered was estimated.

Method precision: Inter-day as well as intra-day precision methodology was utilized to demonstrate the repeatability of the proposed method. In intra-day methodology six standard solutions of RTV and DRV were injected into HPLC system in single day. In inter-day precision the same solutions were injected for three consecutive (un-interrupted) days. To express the precision, RSD % of the RTV and DRV peaks was calculated.

Stress induced studies (specificity): Specificity test was conducted according to different ICH stated stress situations[5]. ICH stress to include tests of samples under relevant stress conditions: light, heat, humidity, acid/base hydrolysis and oxidation.

Acid-stress studies: Acid stress studies were carried out at 60°C and relative humidity (RH) 75% in an environmental accelerated chamber using 0.1 N hydrochloric acid. For these studies 3 ml stock standard solution was transferred to 10 ml flask. 3 ml of 0.1 N hydrochloric acid then added into the flask and placed at 60° C/75% RH in the environmental accelerated chamber for half an hour. After completion of the stress studies, 0.1N sodium hydroxide was added to neutralize the solution. Volume was adjusted to mark using mobile phase.

Base-stress studies: Basic stress degradations studies were carried out using 0.1N sodium hydroxide at 60°C and 75% RH. In a volumetric flask of 10 ml, 3 ml stock standard solution and 3 ml of 0.1N sodium hydroxide were mixed and the flask placed at 60°C/75% RH for 10 min. After completion of the stress studies, about 0.1N

hydrochloric acid was added to neutralize the solution. The volume was adjusted to mark using mobile phase.

Oxidative-stress studies

These studies were conducted at room temperature. To 3 ml of stock standard solution was transferred to a 10 ml flask and mixed with 1 ml of 30% hydrogen peroxide. The flask was allowed to stand at room temperature for 15 min. After completion of the stress studies mobile phase was added to the mark. This solution was then injected in to the HPLC system.

Photolytic-stress studies

3 ml stock standard solution was transferred into10 ml flask. The flask was then placed in open sunlight for 24 hours. After that, mobile phase was added to the mark. This solution was then injected in to the HPLC system.

Thermal-stress studies

A 3 ml portion of the stock standard solution was transferred to 10 ml flask and placed over hot plate for 3 hours at 110°C. After 3 h, mobile phase was added to the mark. This solution was then injected in to the HPLC system.

Robustness

Deliberate changes were made in the experimental conditions to assess the robustness of the developed method. For this intention, slight changes were made in composition mobile phase ±10% as well as flow rate (0.9 to 1.1 ml per min) of the mobile phase. The effects of these changes on chromatographic parameters of theoretical plates, resolution and tailing factor were noted.

Detection and quantitation limits

Signal/noise (S/N) ratio method was used to calculate the LOD and LOQ values. For this purpose, solution concentrations were prepared in descending order by way of spiking known concentrations of RTV and DRV. The solutions were made by the defined protocol and repeatedly analyzed to find S/N ratio. The average ratio at each level of concentration was used to determine the detection and quantitation limits. The lowest concentration of RTV and DRV which gives 10:1 signal to noise ratio where RTV and DRV can be quantitated with accuracy was represented as LOQ and the lowest level of concentration which gives 3:1 signal to noise ratio where RTV and DRV can be detected was expressed as detection limit.

RESULTS AND DISCUSSION

The purpose of this study was to develop a simple, fast and economic stability indicating high performance LC method for the instant and simultaneous determination of RTV and DRV in combination form. In order to accomplish this goal, chromatographic conditions were optimized. For optimization of conditions and to acquire symmetrical sharp peaks, different organic solvents and buffers with varied pH range in different proportions were tried in order to optimize the mobile phase composition. In addition, different stationary phases (Hypersil-100 C18, Hypersil-ODS, Symmetry ODS and Inertsil ODS-100) were initially chosen for optimization of stationary phase.

Optimizing mobile and stationary phases

Composition of mobile phase was studied systematically through a series of experiments to obtain symmetrical peaks of RTV and DRV. Different volume ratios (40:60, 20:80, 30:70, 50:50) of methanol and phosphate buffer and acetonitrile were tested and various columns (Hypersil-100 C18, Hypersil-ODS, Symmetry ODS and Inertsil ODS-100) used to obtain best resolution and separations but RTV and DRV showed peak broadening and asymmetry on all stationary phases. Inertsil ODS column provided almost symmetrical peak of RTV (tailing factor 1.25 with retention time 2.123) and asymmetrical peak of DRV retention time (tailing factor 1.08 and retention time

2.813). Highly sharp and symmetrical peaks of RTV and DRV were obtained with Phosphate buffer (pH 3.5): (Acetonitrile and Methanol 5:1) (30:70v/v) on Inertsil ODS column in contrast to the other stationary phases with better peak shape, theoretical plates and resolution Figure 3.

Analytical method validation

Validation of the proposed analytical method was conducted according to ICH guidelines[6,7]. Validation parameters included accuracy, precision, linearity, robustness, specificity, LOD and LOQ. For determination of linearity, different concentrations of RTV (20, 40, 60, 80, 100 µg /ml) and DRV (120, 240, 360, 480, 600 µg/ ml) were prepared and calibration curve was drawn using peak areas. Linear regression for RTV was Y = 13440x -11195 whereas for DRV it was Y = 15551x - 23939 with the value of correlation coefficient 0.9997 and 0.9999, respectively Figure 4 and 5. The LOD values calculated were 2.9 ng/ml for RTV and 3.1 ng/ ml for DRV (S/N ratio 3:1). The calculated LOQ was 9.90 ng/ ml for RTV and 10.10 ng/ ml for DRV (S/N ratio 10:1). To check the accuracy, known amounts of RTV and DRV were added into the sample solution containing known amounts of analytes. Results were then compared with the true values. True value was obtained by injecting the standard solutions of known concentration. Results of percentage recoveries are shown in the Table 1. The results revealed that method is accurate and appropriate for anticipated use. The results were very close to the true value and were within the range of theoretical expected value (98-102%). Both intraday precision and inter-day precision was performed to validate the proposed method. It was reported in term of RSD %. The results are presented in the Table 2 and 3. In both the cases % RSD is less than generally accepted theoretical value (< 2%).

During robustness study, the effects of changes in chromatographic conditions on the chromatographic parameters were negligible and show that method is robust for intended use. Results are shown in Table 4.

Specificity was evaluated by the application of various stress tests as acidic stress, basic stress, oxidative stress, thermal stress and photolytic stress to RTV and DRV in combination form. Under these circumstances both drugs not show any degradation and it was found to be stable. Typical chromatograms of basic, acidic, photolytic, oxidative and heat stresses are shown in the Figure 6,7,8,9 and10 respectively. Degradation results are reported in Table 5.

Developed chromatographic method was also used for estimation of RTV and DRV in synthetic mixture. The results are presented in Table 6, which show high percent recovery and low % RSD.

CONCLUSION

A new method for the simultaneous estimation of RTV and DRV in bulk and synthetic mixture is reported. The described reversed phase HPLC method is very simple, specific, fast and isocratic. The proposed method is suitable and appropriate for routine quality control analysis and stability studies. The developed method is validated using ICH guidelines. The method is very fast and separation is achieved within 5 min. Sample preparation is very simple and is without the use of laborious and expensive solid phase extraction.

Acknowledgments:

I thank God for letting me complete my work successfully and I take privilege to sincerely thanks to my professor for his extreme support and guidance all throughout my work.

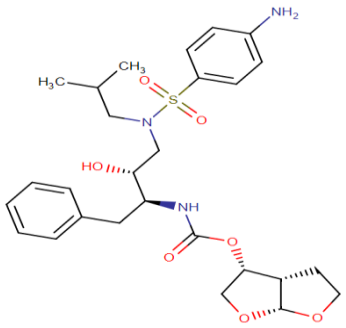

Figure – 1 Structure of Darunavir

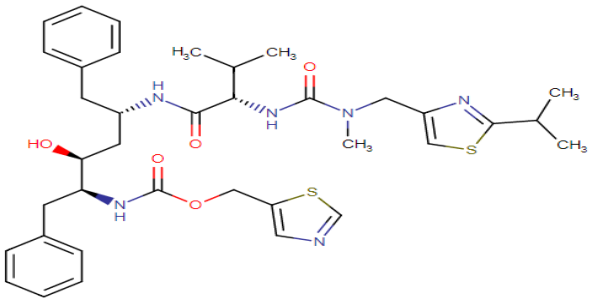

Figure – 2 Structure of Ritonavir

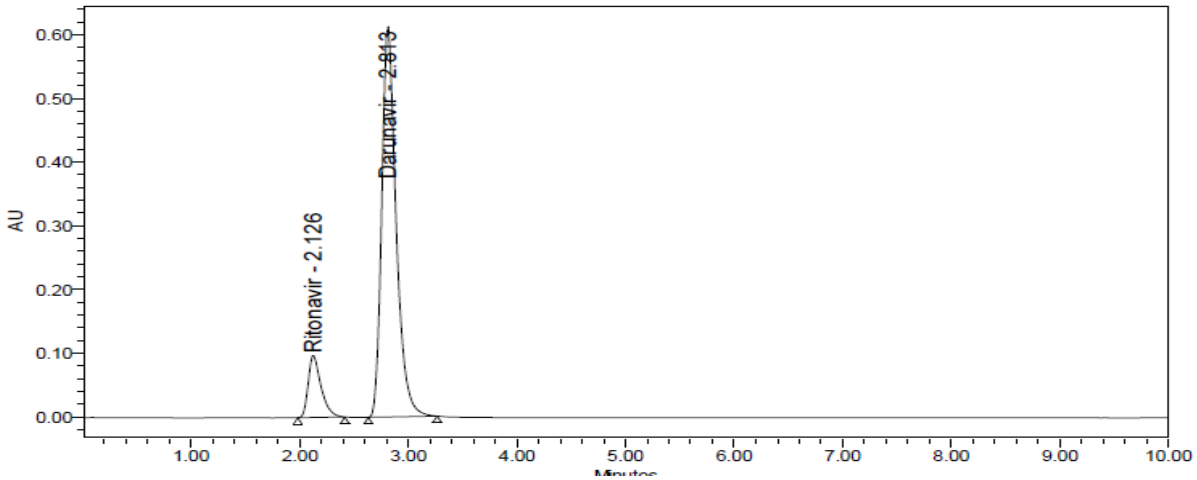

Figure – 3 Chromatogram of Ritonavir and Darunavir

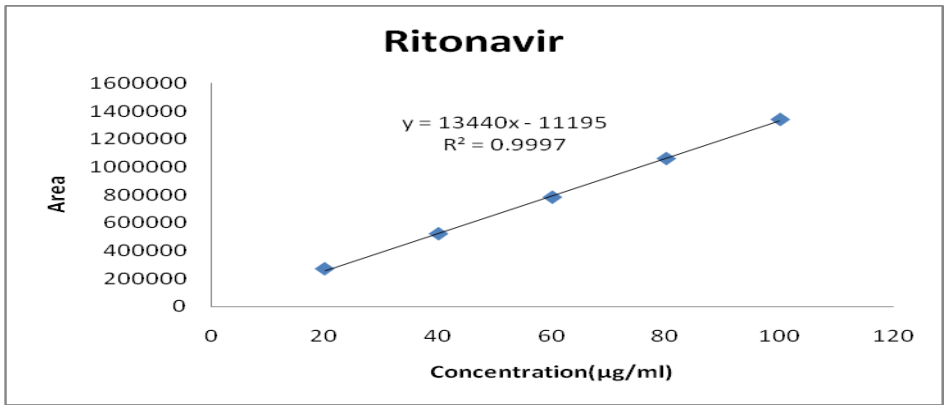

Figure – 4 Calibration curve of Ritonavir

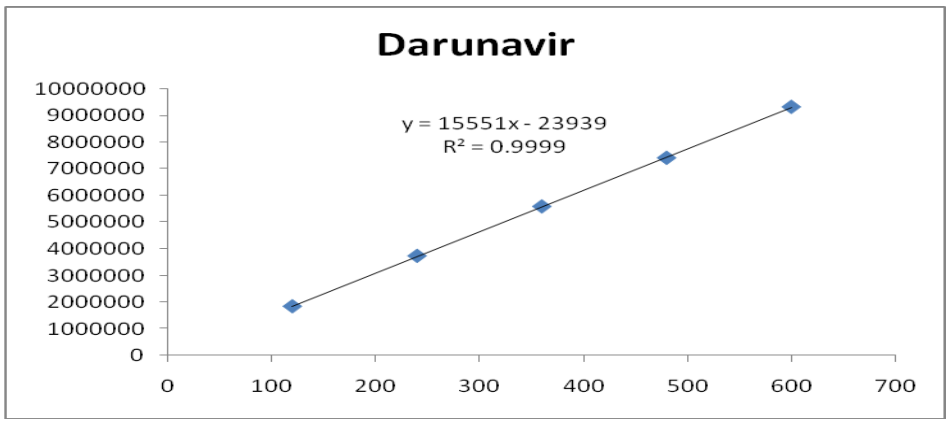

Figure – 5 Calibration curve of Darunavir

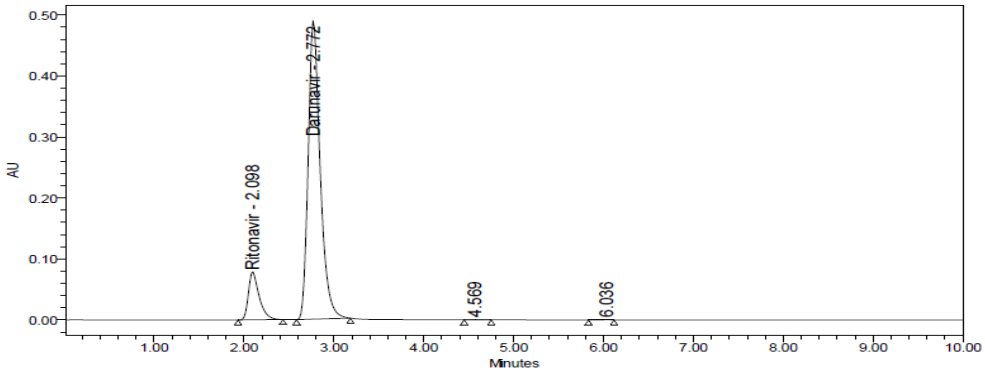

Figure - 6 Chromatogram of acidic stress induced degradation of Ritonavir and Darunavir

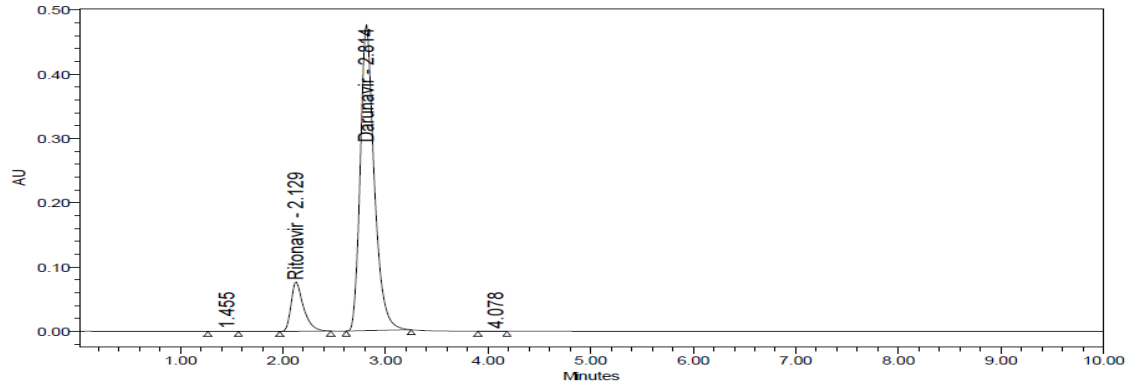

Figure - 7 Chromatogram of basic stress induced degradation of Ritonavir and Darunavir

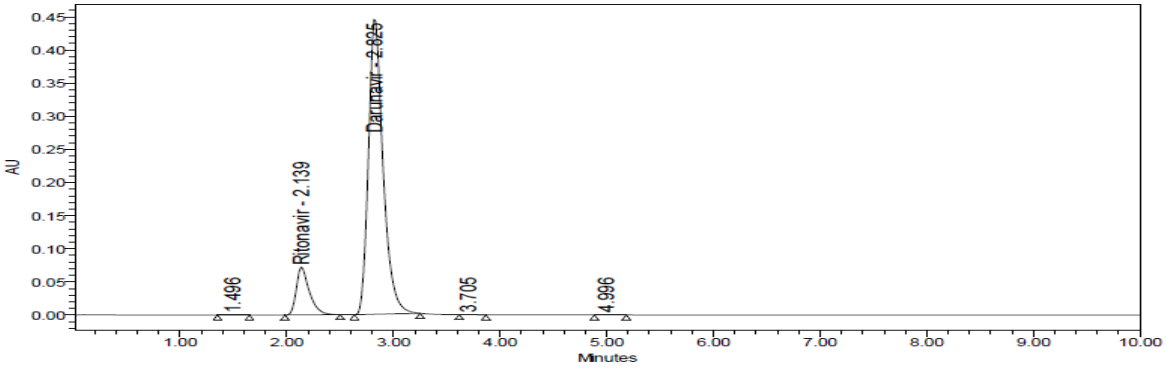

Figure - 8 Chromatogram of oxidative stress induced degradation of Ritonavir and Darunavir

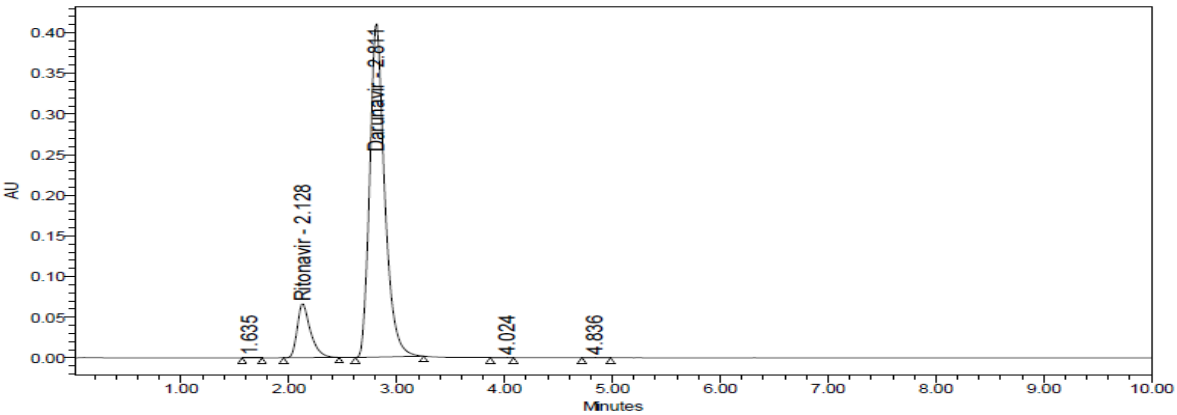

Figure - 9 Chromatogram of photolytic stress induced degradation of Ritonavir and Darunavir

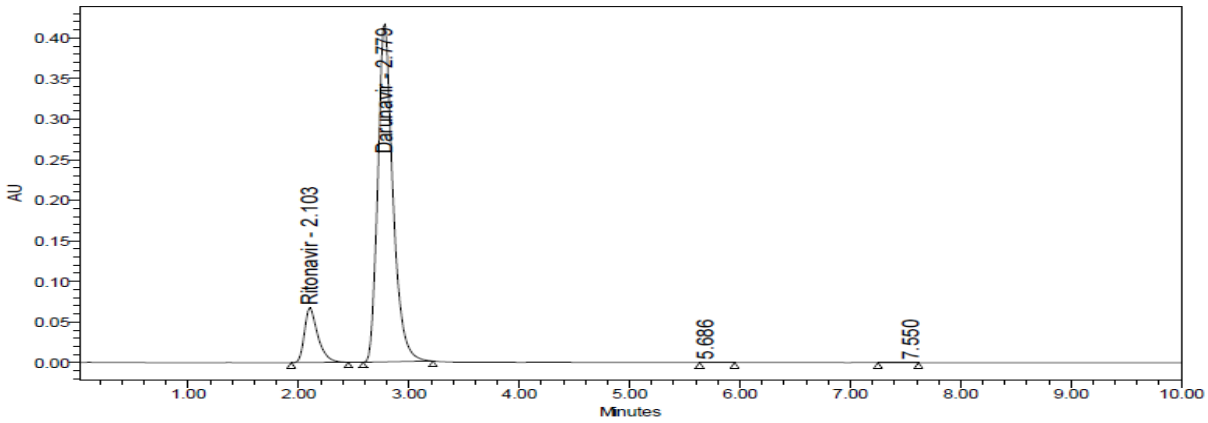

Figure - 10 Chromatogram of thermal stress induced degradation of Ritonavir and Darunavir

Sample	%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
Darunavir	50%	396812	10	10.08	100.80	100.06
	100%	787039	20	20.00	100.00	
	150%	1173386.0	30	29.82	99.40	
Ritonavir	50%	2754176	60	59.84	99.73	99.86
	100%	5551291.7	120	120.61	100.51	
	150%	8229366.3	180	178.79	99.33	

Table – 1 Recovery (Accuracy) data for Ritonavir and Darunavir

Injection	Area for Ritonavir	Area for Darunavir
I	789316	5523508
II	785334	5528488
III	780020	5591669
IV	786180	5523942
V	781227	5539053
VI	782839	5567550
Average	784152.7	5545701.7
Standard Deviation	3450.5	27917.4
%RSD	0.4	0.5

Table – 2 Precision data for Ritonavir and Darunavir

Injection	Area for Ritonavir	Area for Darunavir
I	784589	5550899
II	787669	5526967
III	788979	5549869
IV	783607	5543117
V	786196	5540984
VI	781921	5582718
Average	785493.5	5549092.3
Standard Deviation	2627.3	18577.0
%RSD	0.3	0.3

Table – 3 Intermediate Precision data for Ritonavir and Darunavir

Chromatographic Conditions	Resolution	Ritonavir		Darunavir	
		USP Tailing	USP Plate Count	USP Tailing	USP Plate Count
Flow rate (0.9ml/min)	2.93	1.42	3497.58	1.30	6032.68
Flow rate (1.0ml/min)	3.04	1.45	3568.55	1.35	5239.73
Flow rate (1.1ml/min)	2.50	1.25	3191.13	1.15	5428.68
Mobile phase composition (10% less)	2.59	1.10	3631.56	1.50	5528.47
Mobile phase composition (Actual)	3.94	1.25	6037.41	1.08	7679.28
Mobile phase composition (10% more)	3.08	1.54	3662.04	1.47	5509.65

Table – 4 Robustness data for Ritonavir and Darunavir

Nature of the Stress	Ritonavir		Darunavir	
	Area	% Degraded	Area	% Degraded
Standard	785386		5512235	
Acid	763563	2.78	5312622	3.62
Base	757893	3.50	5286737	4.09
Peroxide	759376	3.31	5297856	3.89
Thermal	735422	6.36	5215762	5.38
Photo	745353	5.10	5257689	4.62

Table – 5 Stress testing data for Ritonavir and Darunavir

Ingredients	Label (mg)	% Recovery ±%RSD
Ritonavir	20	99.58% ±0.35
Darunavir	120	100.21% ±0.40

Table – 6 Assay Results of Ritonavir and Darunavir in synthetic Mixture

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