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Development and Validation of a Simple, Sensitive, Selective and Stability-Indicating RP-HPLC Method for the Determination of Darunavir and Cobicistat in pharmaceutical combined dosage forms

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Abstract: *The study describes development and subsequent validation of a stability indicating reverse-phase HPLC method for simultaneous estimation of the Darunavir and Cobicistat in combined Pharmaceutical dosage form using RP-HPLC. Separation was accomplished on BDS 250 x 4.6 mm, 5µm C_{18} column using 0.1% Perchloric acid buffer and acetonitrile (38:62 v/v) as mobile phase pumped through at a flow rate of 1 ml/min at 30°C. Optimized wavelength was 211 nm, retention time of Darunavir and Cobicistat were found to be 2.24 min and 2.63 min respectively. % RSD of the Darunavir and Cobicistat were found to be 0.3 and 0.5 respectively. Mean recovery were found to be 100.0% and 100.04% for Darunavir and Cobicistat respectively. The proposed method also proved to be suitable as a rapid and reliable quality control method.*

Keywords: *Darunavir, Cobicistat, RP-HPLC, Simultaneous determination, Degradation studies.*

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I. Introduction

Darunavir chemically described as [(1R,5S,6R)-2,8-dioxabicyclo[3.3.0]oct-6-yl] N-[(2S,3R)-4- [(4-aminophenyl)sulfonyl- (2-methylpropyl)amino]-3-hydroxy-1-phenyl- butan-2-yl] carbamate is a new HIV protease inhibitor that has demonstrated substantial antiretroviral activity against wild-type HIV-1 virus and multidrug-resistant strains [1]. Darunavir inhibits and is primarily metabolized by cytochrome P450 3A (CYP3A) isoenzymes resulting in increased plasma concentrations and allowing for a lower daily dose [2]. Studies conducted in HIV-negative healthy volunteers and in HIV-infected patients show that the potential for interactions is well characterized and the interactions are manageable. Darunavir co-administered with many other antiretroviral or non-antiretroviral medications commonly used in HIV-infected individuals [3-4].

Cobicistat chemically described as 1,3-thiazol-5-ylmethyl N-[(2R,5R)-5-[[(2S)-2-[[methyl-[(2-propan-2-yl-1,3-thiazol-4-yl)methyl]carbamoyl]amino]-4-morpholin-4-ylbutanoyl] amino]-1,6-diphenylhexan-2-yl] carbamate (Figure 2) a potent inhibitor of CYP3A and a pharmacoenhancer [5-7]. Cobicistat is used in the treatment of infection with human immunodeficiency virus (HIV) [8]. Cobicistat has no antiviral activity, does not induce CYP isozymes, and is more selective than ritonavir in terms of CYP3A inhibition. More specifically, cobicistat is indicated to increase systemic exposure of Darunavir (once daily dosing regimen) in combination with other antiretroviral agents in the treatment of HIV-1 infection [9-14]. Increasing systemic exposure of anti-retroviral (ARVs) without increasing dosage allows for better treatment outcomes and a decreased side effect profile.

The parent drug stability test guideline Q1A (R2) issued by the International Conference on Harmonization (ICH) suggests that stress testing is an essential part of development strategy and is carried out under more severe condition than accelerated conditions. These studies provide information to establish its inherent stability characteristics of the molecule such as the degradation pathways, leading to identification of degradation products and hence supporting the suitability of the proposed analytical procedures [15-16]. According to ICH guidelines stress testing should include the effect of temperature, light, oxidizing agents and susceptibility across a wide range of pH values and separation of drugs from degradation products [17]. It is also suggested that analysis of stability sample should be carried out using validated stability testing methods.

The reported methods available for the estimation for analysis of Darunavir and Cobicistat either alone or in combination with other drugs in pharmaceutical dosage forms or individually in biological fluids [18-19]. Stability indicating and RP-HPLC Simultaneous estimation of this combined dosage form has been reported in combination with other drugs in pharmaceutical dosage forms [20-22]. To our knowledge there has been no

Stability indicating RP-HPLC method development and validation reported for the simultaneous estimation of Darunavir and Cobicistat combination in which ICH recommended that stress conditions be applied. Therefore, the stability indicating method was also developed by applying different stress conditions like acidic, alkali, H_2O_2, thermal, and photo degradation. We have planned to develop a new, simple, precise, economic and accurate Stability indicating RP-HPLC method development and validation for the estimation of Darunavir and Cobicistat pharmaceutical dosage form according to ICH [23-25] Guidelines.

II. Experimental

2.1 Materials and methods

Active pharmaceutical ingredients Darunavir and Cobicistat were obtained as a gift samples from Spectrum pharma research solutions, Hyderabad. The pharmaceutical dosage form Prezcobix was purchased from local pharmacy. The solvents used in this work were of HPLC grade and obtained from Ranchem Private Limited.

2.2 Instrumentation and chromatographic conditions

The analysis was performed on a high performance liquid chromatography system consisting of Waters 2695 with 2996 module Photo Diode Array detector equipped with a quaternary solvent delivery pump, automatic sample injector and column thermostat. The data acquisition and analysis was performed by using Empower2 software. The chromatographic separation was performed on BDS 250 x 4.6 mm, 5m C18 column using 0.1% Perchloric acid buffer and acetonitrile (38:62 v/v) as mobile phase at a flow rate of 1 ml/min, the column temperature was maintained at 30°C which gave acceptable retention time and good resolution between Darunavir and Cobicistat. The method was optimized at 211nm. The run time was taken as 6min.

2.3 Sample Processing:

Diluents: Based up on the solubility of the drug, diluents were selected, firstly dissolved in Methanol and made up with water: ACN (50:50).

2.3.1 Preparation of Standard stock solutions:

Accurately Weighed and transferred 40mg of Darunavir and 7.5mg of Cobicistat working Standards into a 25ml clean dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes and make up to the final volume with diluents. 1ml from the above two stock solutions was taken into a 10ml volumetric flask and made up to 10ml.

2.3.2 Preparation of Sample stock solutions:

Five tablets were weighed and their average weight was determined. The tablets were crushed to a homogenous powder and an amount equivalent to 1250 mg powder was weighted and then transferred into a 500mL volumetric flask, 300mL of diluent added and sonicated for 25 min, further the volume made up with diluent and filtered. From the filtered solution 1ml was pipeted out into a 10 ml volumetric flask and made upto 10ml with diluent.

2.3.3 Preparation of buffer

Buffer: (0.1%Perchoric acid)

1ML of Per choric acid solution in a 1000ml of Volumetric flask add about 100ml of milli-Q water and final volume make up to 1000 ml with milli-Q water.

2.3.4 Preparation of linearity solutions

Preparation of Standard stock solutions: Accurately Weighed and transferred 40 mg of Darunavir and 7.5 mg of Cobicistat working Standards into a 25 ml clean dry volumetric flask respectively, 3/4th of diluent was added, sonicated for 30 minutes and made up to the final volume with diluents. Flasks were made up with water and acetonitrile (50:50) and labelled as Standard stock solution 1, 2 and 3. From three stock solutions 0.25ml, 0.5ml, 0.75ml, 1.0ml, 1.25ml, 1.50ml into 10ml volumetric flask were pipette out to get 25%,50%, 75%, 100%, 125%, 150% of standard solutions

2.3.5 System suitability parameters

The system suitability parameters were determined by preparing standard solutions of Darunavir and Cobicistat the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined.

2.4 METHOD VALIDATION

The method was validated according to ICH guidelines. The different validation characteristics which were performed include: Linearity, Accuracy, Precision, Limit of Detection, Limit of Quantification, Robustness and the stability indicating capability.

2.4.1 Linearity

The linearity of the method was determined by preparing three individual series of solutions in the range of Darunavir (40-240µg/ml), and Cobicistat (7.5-45µg/ml). The obtained peak areas were plotted against concentration.

2.4.2 Accuracy

Accuracy was tested by the standard addition method at three different levels 50%, 100% and 150%. The percentage recoveries of Darunavir and Cobicistat present in the pharmaceutical dosage form were calculated.

2.4.3 Precision

a) Method precision (repeatability)

The method precision/ repeatability can be determined by injecting six working standard solutions and six sample injections. The areas of all the injections were taken and standard deviation, %Relative standard deviation, %assay were calculated.

b) Intermediate precision

The intermediate precision can be determined by injecting six working standard solutions and six sample injections on different days by different operators or by different instruments. The areas of all the injections were taken and standard deviation, %Relative standard deviation, %assay were calculated. The results obtained were within the acceptance criteria.

2.4.4 Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) of Darunavir and Cobicistat were determined by calibration curve method. Solutions of Darunavir and Cobicistat were prepared in linearity range and injected in triplicate. Average peak area of three analyses were plotted against concentration

2.4.5 Method robustness

The robustness can be determined by varying the following parameters:

Robustness of the developed method was determined by making small deliberate changes in flow rate (±0.1ml/min), column temperature (±5%), organic mobile phase ratio (±10%), along with the optimized method.

2.5 DEGRADATION PROCEDURE

2.5.1 Oxidation:

To 1 ml of stock solution of Cobicistat, Darunavir, 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 30µg/ml&160µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

2.5.2 Acid Degradation Studies:

To 1ml of stock s solution Cobicistat, Darunavir, 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0c .The resultant solution was diluted to obtain 30µg/ml & 160µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

2.5.3 Alkali Degradation Studies:

To 1 ml of stock solution Cobicistat, Darunavir, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 30µg/ml & 160µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

2.5.4 Dry Heat Degradation Studies:

The standard drug solution was placed in oven at 105 °C for 6 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 30µg/ml & 160µg/ml solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

2.5.5 Photo Stability studies:

The photochemical stability of the drug was also studied by exposing the 300µg/ml&1600µg/ml solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 30µg/ml & 160µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

2.5.6 Neutral Degradation Studies:

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60º. For HPLC study, the resultant solution was diluted to 30µg/ml & 160µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample..

III. Results And Discussions

The present work was focused on development of a stability indicating RP-HPLC method for the simultaneous estimation of Darunavir and Cobicistat in pharmaceutical dosage form. The solubility of the active pharmaceutical ingredient was checked in different solvents like methanol, water, acetonitrile and in different ratios but finally the standard was first dissolved in methanol and made up with water:ACN (50:50). So it was chosen as a diluent. Different mobile phases like methanol and water, acetonitrile and 0.01N potassium dihydrogen ortho phosphate buffer and acetonitrile and sodium dihydrogen phosphate buffer were used in compositions at a different flow rates but the peak resolution, retention time and tailing factor were not satisfactory, so at last acetonitrile and perchloric acid was selected as a buffer at flow rate of 1ml/min. The chromatographic separation was performed on BDS (250mm x 4.6mm x 5µ) kept at 30°C with a run time of 6 minutes. Finally the method was optimized by altering the mobile phase composition / ratio and the optimized wavelength of two drugs Darunavir and Cobicistat were found to be at 211nm.

3.1 System suitability parameters

The system suitability tests were conducted before performing the validation and the parameters were within the acceptance criteria like retention times were 2.24 min and 2.63 min for Darunavir and Cobicistat, plate count was >2000, peak tailing was <2 and the %RSD of peak areas of six injections were ≤ 2% (Table 1). Hence the proposed method can be successfully applied to routine analysis. Chromatograms are shown in Fig 3(a), 3(b), 3(c).

3.2 Linearity range

The linearity range was in the interval of Darunavir (40-240 µg/ml), and Cobicistat (7.5-45µg/ml) respectively. These were represented by a linear regression equation as follows: Darunavir is y = 17079x + 32976 (r^2 = 0.999 and Cobicistat is y = 24142x + 16018 (r^2 = 0.999). Regression line was established by least squares method and correlation coefficient (r^2) for Darunavir and Cobicistat were found to be greater than 0.999. Hence the curves established were linear (Table 2, Graph 1). Chromatograms are shown in Fig 4.

3.3 Precision

Six replicates injections at the same concentration were analyzed on same day and two different days for verifying the variation in the precision and the % RSD for Darunavir and Cobicistat were within acceptable limit of ≤2. Hence the method is reproducible on different days with different analyst and column. This indicates that the method is precise (Table 3).

3.4 Accuracy

The percentage recoveries for Darunavir and Cobicistat were found to be 100.00% and 100.04% respectively (Table 4, 5). The results of the recovery studies undoubtedly demonstrate accuracy of the proposed method.

3.5 Limit of detection (LOD) and limit of quantification (LOQ)

The determined values of LOD and LOQ were calculated by using slope and Y-intercept. The LOD and LOQ values for Darunavir were found to be 0.13 and 0.39 µg/ml, Cobicistat were found to be 0.39 and 1.20 µg/ml respectively (Table 6).

3.6 Robustness

Robustness of the proposed method demonstrated a non-significant alteration through analysis of the sample and standard Darunavir and Cobicistat (Table 7, 8). After this the results obtained were compared with that of optimized method. It was confirmed that by the deliberate changes in the parameters there was no any significant changes in standard deviation, relative standard deviation, theoretical plates, retention time and USP tailing factor.

3.7 Assay

The Content of Darunavir and Cobicistat in the pharmaceutical dosage form were found by using the developed method. The percentage purity of Darunavir and Cobicistat were found to be 100.1% and 100.75% and %RSD values for Darunavir and Cobicistat were within limit of ≤2.

3.8 Forced degradation studies

The forced degradation studies were conducted and all the parameters for Darunavir and Cobicistat were within the limits (Table 9). Darunavir and Cobicistat have shown significant sensitivity towards the treatment of HCl, NaOH and peroxide solutions [26]. The drugs gradually undergone degradation with time and prominent degradation was observed. Darunavir and Cobicistat were stable under forced thermal degradation, photolytic and neutral degradations [27-28]. From the degradation studies, Peak purity test results derived from PDA detector, confirmed that the Darunavir and Cobicistat peaks were homogeneous and pure in all the analyzed stress samples. Chromatograms are shown in Fig 6.

IV. Conclusion

A new, simple, rapid and precise stability indicating reversed-phase high performance liquid chromatographic method was developed for the simultaneous estimation of Darunavir and Cobicistat in bulk and combined pharmaceutical dosage forms. The method is simple, accurate, linear, sensitive and reproducible as well as economical for the effective quantitative analysis of Darunavir and Cobicistat in bulk and combined dosage forms. The method was validated, and all the method validation parameters were tested and shown to produce satisfactory results. The method is free from interactions of the other ingredients and excipients used in the formulations. Finally, concluded that the method is suitable for use in the routine quality control of Darunavir and Cobicistat in active pharmaceutical ingredients and in pharmaceutical dosage forms.

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Table 1:

S no	Darunavir			Cobicistat		
Inj	RT(min)	N	Tailing	RT(min)	N	Tailing
1	2.24	4673	1.09	2.623	4703	1.07
2	2.24	4634	1.09	2.625	4552	1.05
3	2.24	4496	1.08	2.626	4650	1.05
4	2.25	4648	1.08	2.635	4603	1.05
5	2.25	4380	1.08	2.635	4779	1.05
6	2.25	4771	1.08	2.637	4880	1.04

System suitability parameters for Darunavir and Cobicistat

Table 2: Linearity and Statistical analysis data for Darunavir and Cobicistat

S.No	Pipetted from stock (mL)	Volume of flask (mL)	Concentration in ppm (Darunavir)	Concentration in ppm (Cobicistat)	%Linearity Level
1	0.25	10	40	7.5	25
2	0.5	10	80	15	50
3	0.75	10	120	22.5	75
4	1	10	160	30	100
5	1.25	10	200	37.5	125
6	1.5	10	240	45	150

Darunavir			Cobicistat		
Conc. (□g/mL)	Peak area	Correlation Coefficient	Conc. (□g/mL)	Peak area	Correlation Coefficient
25	713120	0.999	25	214333	0.999
50	1496396		50	387051.3	
75	2046002		75	554898	
100	2730724		100	738383.3	
125	3453109		125	912216	
150	4138231		150	1107545	

Table 3: Determination of repeatability and intermediate precision

Drug Name	Repeatability			Intermediate		
	Peak Area	Std Dev	%RSD	Peak Area	Std Dev	%RSD
Darunavir	2780193	16000	0.6	2751575	12491	0.5
Cobicistat	741876	4631	0.62	731022	7302.8	1.0

Table 4: Recovery data for the proposed RP-HPLC method for Darunavir

Concentration level	Amount added	Amount found	%Recovery	Mean %Recovery

(%)	(□g/mL)	(□g/mL)		
50	15	15.00	100.04	100.25
		15.09	100.61	
		15.01	100.11	
100	30	30.10	100.07	99.81
		29.84	99.46	
		29.96	99.89	
150	45	45.38	100.86	100.07
		45.04	100.09	
		44.66	99.25	

Table 5: Recovery data for the proposed RP-HPLC method for Benzhexol

Concentration level (%)	Amount added (□g/mL)	Amount found (□g/mL)	%Recovery	Mean %Recovery
50	10	9.87	98.80	99.18
		9.92	99.24	
		9.94	99.50	
100	20	20.25	101.28	100.46
		20.11	100.60	
		19.89	99.49	
150	30	30.37	101.26	100.2
		29.91	99.73	
		29.88	99.61	

Table 6: Sensitivity table of Darunavir and Cobicistat

Drug Name	LOD(µg/ml)	LOQ(µg/ml)
Darunavir	0.13	0.39
Cobicistat	0.39	1.19

Table 7: Robustness results of the proposed RP-HPLC method for Darunavir

S.no	Parameters			Peak area	RT *	% RSD
	Optimized		*Used*			
1	Flow rate (ml/min)	1.0	1.1	2664386	2.13	0.5
			0.9	3062803	2.45	0.2
4	Mobile phase	38:62	43:67	2822427	2.26	0.5
			33:57	2822447	2.27	0.5
6	Temperature	30^0C	35^0C	2780073	2.23	0.1
			25^0C	2847502	2.22	0.4

***RT=Retention Time**

Table 8: Robustness results of the proposed RP-HPLC method for Cobicistat

S.no	Parameters			Peak area	RT *	% RSD
	Optimized		*Used*			
1	Flow rate (ml/min)	1.0	1.1	696132	2.51	0.6
			0.9	802720	2.88	0.2
4	Mobile phase	45:55	43:67	686283	2.71	0.4
			33:57	686283	2.71	0.4
6	Temperature	30^0C	35^0C	724102	2.62	0.2
			25^0C	683463	2.61	0.4

***RT=Retention**

Table 9: Forced Degradation results of proposed RP-HPLC method

Degradation Condition	Darunavir			Cobicistat		
	%Drug Degraded	Purity of		% Drug Degraded	Purity of	
		Angle	Threshold		Angle	Threshold
Control Sample	-	-	-	-	-	-
Acid	3.23	0.170	0.332	3.75	0.625	0.644
Alkali	2.02	0.138	0.331	2.93	0.478	0.643
Oxidation	1.29	0.168	0.340	2.00	0.614	0.620
Thermal	0.86	0.152	0.330	0.68	0.521	0.610
UV	0.64	0.155	0.334	0.51	0.528	0.530
Water	0.09	0.155	0.327	0.15	0.502	0.507

Figures 1, 2: Structures of Darunavir and Cobicistat

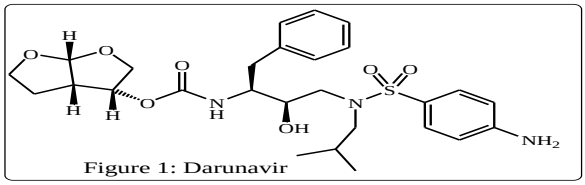


Figure 1: Darunavir

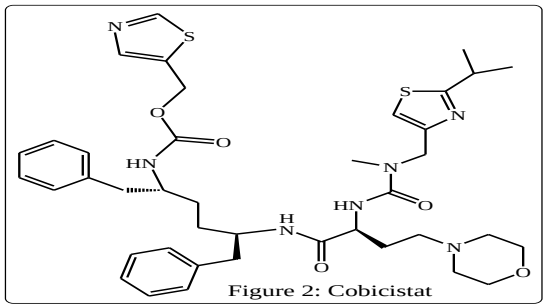


Figure 2: Cobicistat

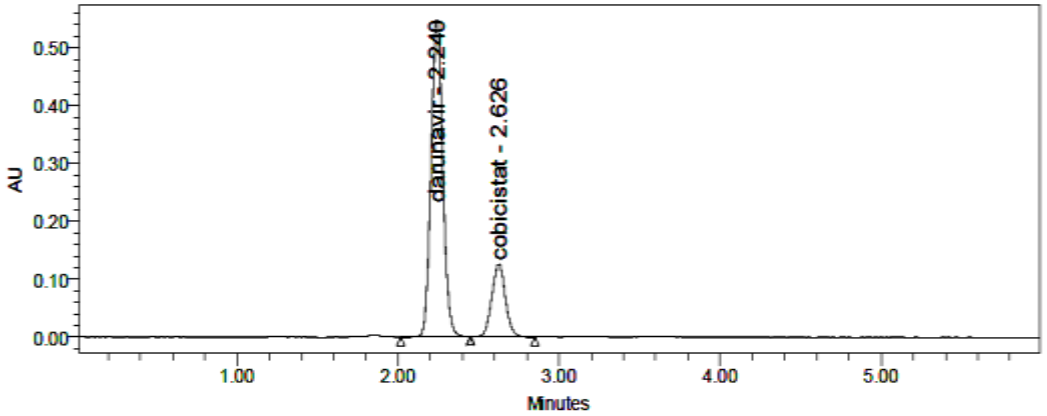


Figure 3 (a): RP-HPLC Chromatogram of Darunavir and Cobicistat

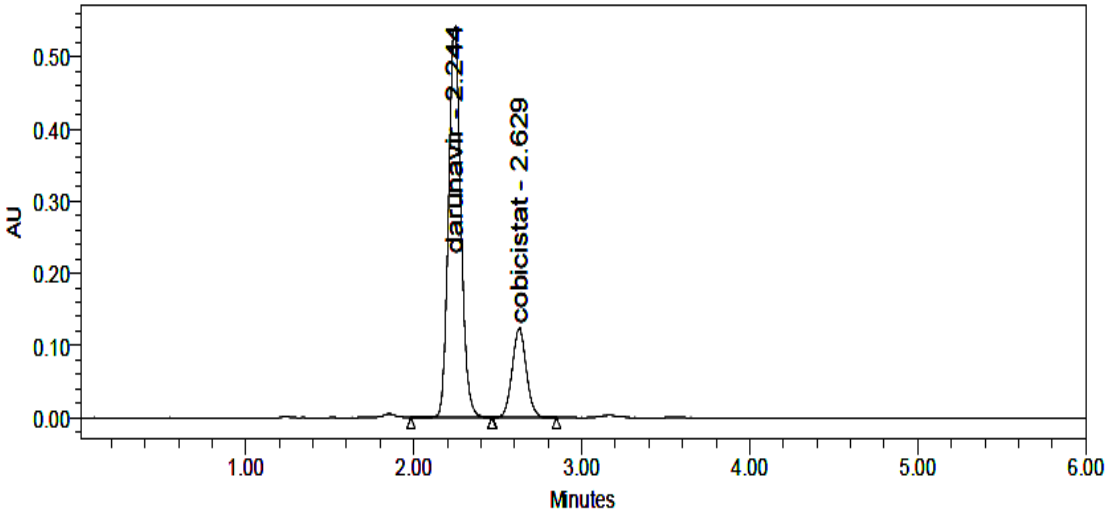


Figure 3 (b) RP-HPLC Chromatogram of Darunavir and Cobicistat Formulation

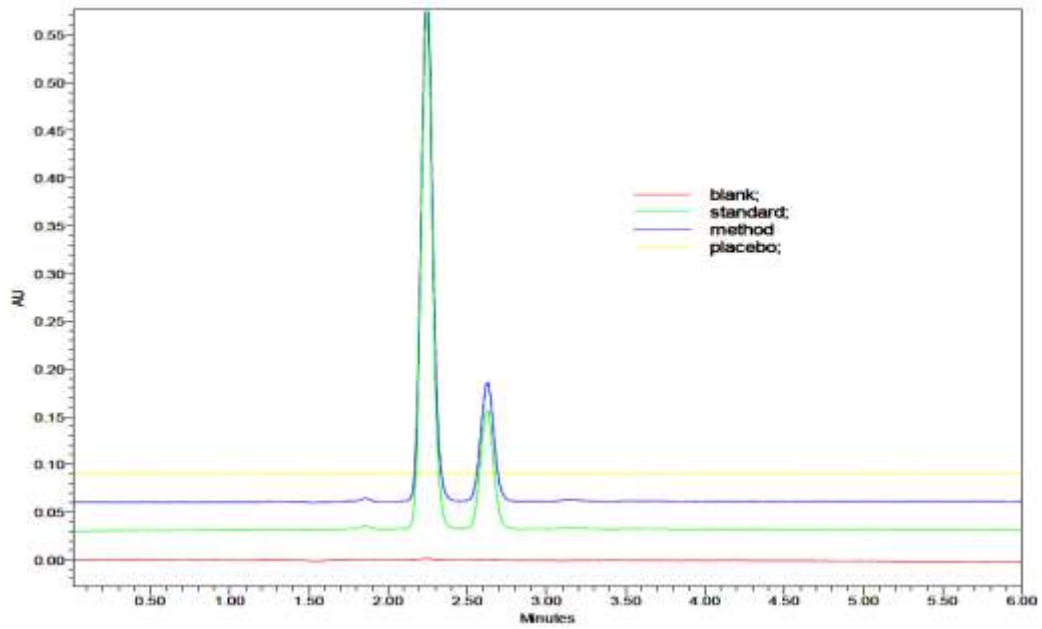


Figure 3 (c): Specificity overlay chromatogram of blank, standard, placebo and marketed sample of Darunavir and Cobicistat

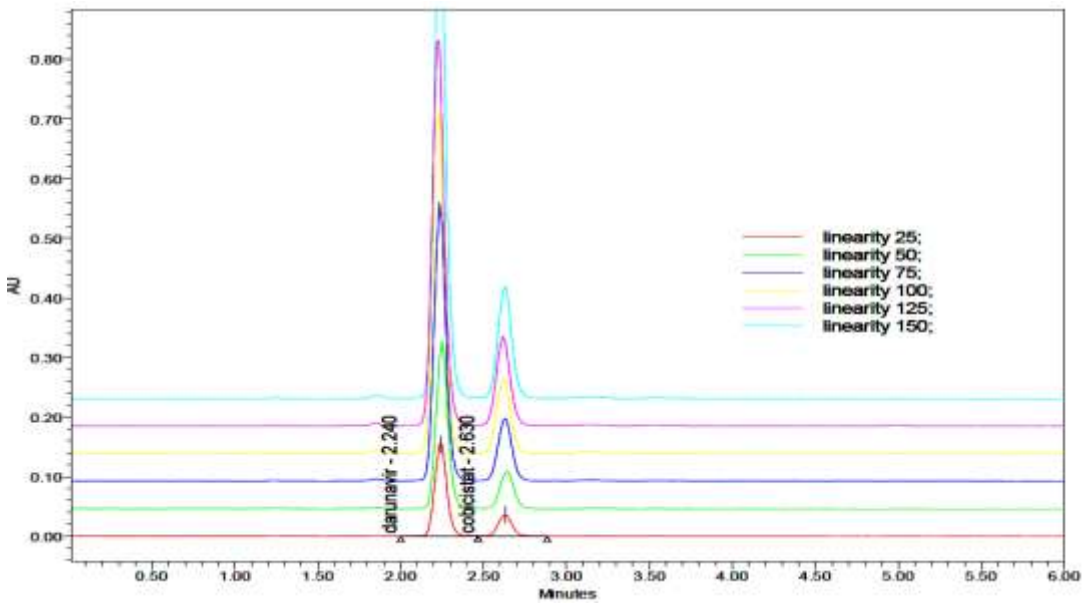


Figure 4: Linearity overlay chromatograms of Darunavir and Cobicistat

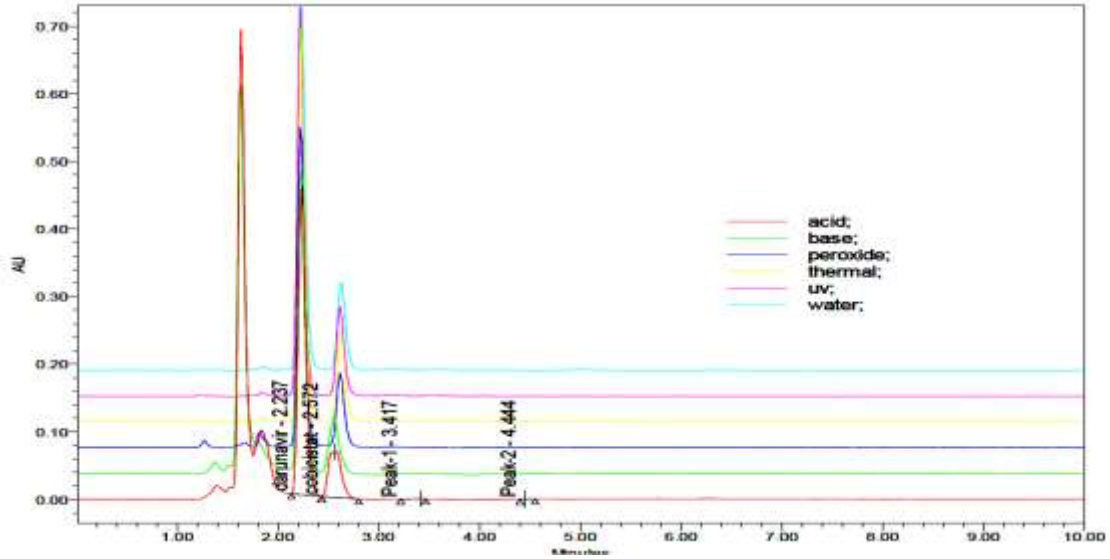
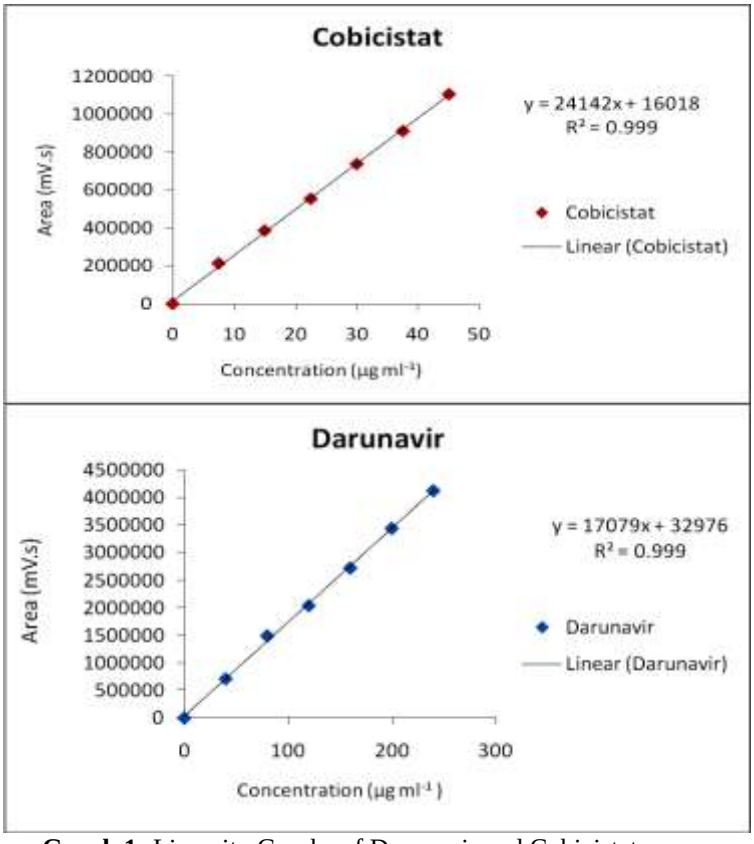


Figure 5 : Forced degradation overlay Darunavir and Cobicistat



Graph 1: Linearity Graphs of Darunavir and Cobicistat

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Mathews Bommella. "Development and Validation of a Simple, Sensitive, Selective and Stability-Indicating RP-HPLC Method for the Determination of Darunavir and Cobicistat in pharmaceutical combined dosage forms." IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) 12.4 (2017): 69-78.