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Research Paper

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF COBICISTAT AND DARUNAVIR IN BULK AND PHARMACEUTICAL DOSAGE FORM

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A stability indicating RP-HPLC method has been developed and subsequently validated for the simultaneous estimation of cobicistat and darunavir in API and tablet dosage form. Optimized chromatographic condition were achieved by Eclipse XBD-C18(250mm x 4.6mm, 5 μ) column, mobile phase as a mixture of buffer (0.01M potassium dihydrogen phosphate, pH-3.0): acetonitril in the ratio of 40:60v/v with a flow rate of 1ml/min., UV detection was performed at 240nm and the sample temperature was maintained ambient which was injected manually. This developed method for simultaneous estimation of cobicistat and darunavir is linear over a range of 40 – 120 % and 40 -120 % respectively. The validation parameters as per ICH guide lines show good precision having 0.066 and 0.039 %RSD for cobicistat and darunavir which shows nice repeatability, limit of detection and limit of quantification for cobicistat and darunavir was found to be 0.02 and 0.06 and 0.02 and 0.06 % respectively. The developed method is easy to perform assay for indicating quality control determinations in bulk and formulated form with rapid, selective and stability indicating.

Keywords: RP-HPLC, Cobicistat, Darunavir, Prezcoibix, Validation

INTRODUCTION

Cobicistat

Cobicistat Systematic (IUPAC) name was given as 1,3-Thiazole-5-ylmethy [(2R,5R)-5-{{(2S)-2-[(methyl{[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl}carbamoyle)amino]-4-(morpholin-4-

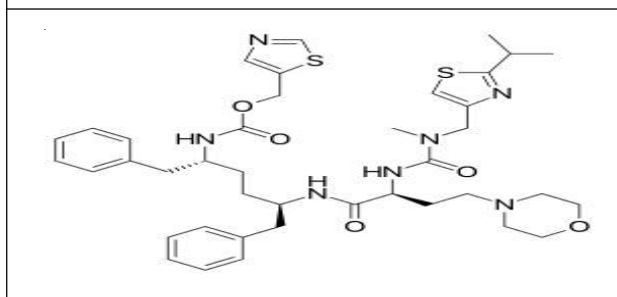
yl)butanoyl]amino}-1,6-diphenylhexane-2-yl]carbamate. Its molecular formula is C₄₀H₅₃N₇O₅S₂ and molecular mass is 776.023 g/mol¹. Cobicistat is adsorbed onto silicon dioxide. Cobicistat on silicon dioxide is a white to pale yellow solid with a solubility of 0.1 mg/ml in water

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at 20 °C drug shows reduced liability for drug interactions and may have potential improvements in tolerability over ritonavir. In addition it has high aqueous solubility and can be readily co formulated with other agents.

Figure 1: Molecular Structure of Cobicistat

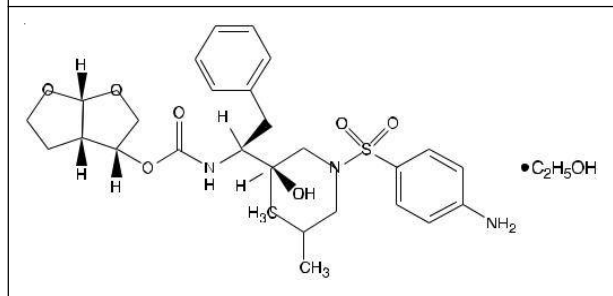


Cobicistat is an anti-retroviral drug from the protease inhibitor class used to treat HIV infection and AIDS. Cobicistat is frequently prescribed with highly active antiretroviral therapy, not for its antiretroviral action, but as it inhibits the same host enzyme that metabolizes other protease inhibitors. This inhibition leads to higher plasma concentrations of these latter drugs, allowing the clinician to lower their dose and frequency and improving their clinical efficacy. It also inhibits intestinal transport proteins, increasing the overall absorption of several HIV medications, including tenofovir and alafenamide. From the literature survey, it was found that Cobicistat was estimated by analytical methods such as spectrophotometric method, HPLC method and HPTLC method.

Darunavir

Darunavir³⁻⁵, in the form of darunavir ethanolate, has the following chemical name: [(1S,2R)-3-[[4-(4-aminophenyl)sulfonyl](2-methylpropyl)amino]-2-hydroxy-1-(phenylmethyl)propyl]-carbamic acid (3R,3aS,6aR)-hexahydrofuro[2,3-b]furan-3-yl ester monoethanolate. Its molecular formula is C₂₇H₃₇N₃O₇S • C₂H₅OH and its molecular weight is 593.73.

Figure 2: Molecular Structure of Darunavir



A few spectroscopic and liquid chromatographic procedures have been reported for the estimation of Darunavir and Cobicistat individually, but there is no method for simultaneous estimation by RP-HPLC. Therefore there is a need to develop rapid and reliable method and validated for simultaneous estimation of combined dosage form and API (Active Pharmaceutical Ingredient)⁶⁻¹¹.

EXPERIMENTAL METHODOLOGY

Instrumentation: Waters Alliance 2695 separation module (Waters Corporation, Milford, USA) equipped with 2998 UV detector with Empower-2 software was used for the analysis. The HPLC system was equipped with a column compartment with temperature control and an on-line degasser. Data acquisition, analysis, and reporting were performed by Empower-2 chromatography software. Eclipse XDB-C18 (250 mm x 4.6 mm I.D; 5 μm) was used as stationary phase. Solubility of the compound was enhanced by sonication. All the weights in the experiments were done with Essea model: AJ220 Digital Electronic Balance.

Chemicals and Reagents: The reference samples of cobicistat & darunavir were obtained from rainbow laboratories Ltd., Hyderabad. Purified water was prepared by using Milli-Q water

purification system. HPLC grade methanol (Merck, Mumbai), which was used for preparing dilutions and mobile phase. Analytical grade potassium di-hydrogen phosphate 0.01M, pH 3.0 was adjusted with dilute orthophosphoric acid (buffer) was obtained from Rankem Fine Chemicals Ltd., New-Delhi. Prezcofix, a formulation containing 150 mg of cobicistat and 800 mg darunavir was purchased from local market.

Preparation of standard solution: The standard solution was prepared by dissolving 150mg of cobicistat and 800mg of darunavir of working standard into a 100ml volumetric flask, dissolve and dilute to volume with diluent (water : acetonitril in ratio of 50:50). Pipette out 5ml into 50ml volumetric flask with and dissolve with diluents to get the working prepared standard solutions as 150 µg/ml and 800 µg/ml respectively. This prepared standard was injected and chromatogram was recorded at 240nm.

Preparation of sample solution: Twenty tablets were weighed and average weight was determined. Tablet powdered equivalent to cobicistat 150mg and darunavir 800mg (Avg. wt is 1100.5mg) of Prezcofix formulation transferred into a 100 ml standard volumetric flask, dissolve with diluent. Solution was ultrasonicated for 15min., filtered through Whatman filter paper No. 42. Filtrate was diluted upto the mark with diluent to obtain final concentration

pipette out 5 ml into 50 ml standard volumetric flask dissolve with diluent, this prepared sample (20 µl) was injected and chromatogram was recorded at 240nm. Content of drugs in sample solution was calculated by comparing mean peak area of sample with that of the standard. The typical chromatogram of cobicistat and darunavir in tablet dosage form.

METHOD DEVELOPMENT

Binary mixture of potassium di-hydrogen phosphate (0.001M) in water adjust pH 3.0 with dilute ortho phosphoric acid (solvent-A): acetonitrile (solvent –B) in 40:60 v/v proportions in isocratic mode of elution was used as mobile phase. The resultant solution was thoroughly mixed and filtered (poly-tetra-fluoro ethanol (PTFE) filter of 0.45 µm pore size) using vacuum pump and degassed by sonication to expel the dissolved gases in solvent system. The flow rate of mobile phase was adjusted at 1.0 mL/min and 20 µL solutions as injection volume were maintained. The eluted compounds were monitored at 240 nm by using UV detector. The column oven temperature was maintained at 30°C. Data acquisition, analysis, and reporting was performed by LC solution Software found to be an efficient system for elution of drug with good peak shape as well as retention time 2.979 min. and 4.260 min. for cobicistat and darunavir respectively with baseline stability.

Table 1: Results of Assay from Tablet Dosage Form

S.No.	Drug	Label Claim (mg)	Amount Found (mg)	Recovery (%)
1.	Cobicistat	150	150.09	100.06
2.	Darunavir	800	780.08	97.60

METHOD VALIDATION

As per (ICH) International Conference on Harmonization guidelines, the method validation parameters such as specificity, linearity, precision, accuracy, LOD, LOQ and robustness were optimized^{13&14}.

a) Specificity: Specificity is the extension to which the procedure applies to analyte of interest and is checked by examining the formulation sample for any interfering peaks. The specificity of the method was evaluated with regard to interference due to presence of excipients. The excipients used in the formulation did not interfere with the drug peak and thus the method is specific. The HPLC chromatogram recorded for the drug matrix (mixture of the drug and excipients) showed almost no interfering peaks within retention time ranges. Figure 2 showed the representative chromatograms for standard

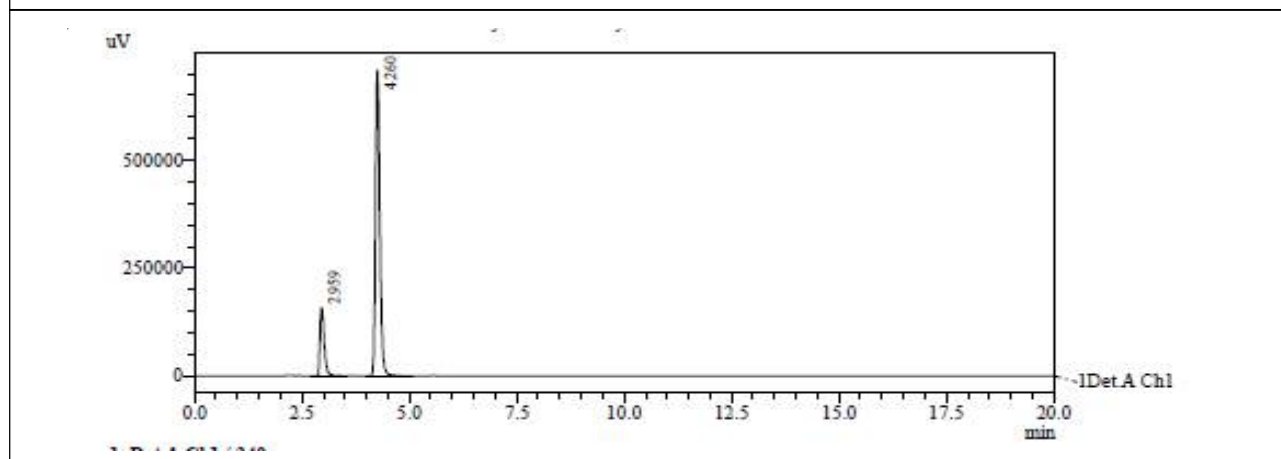
and the dosage form. The figure describes that the selected drug was clearly separated and the proposed HPLC method is selective.

b) Linearity: To establish linearity, the stock solutions were prepared as 1000 µg/ml using mobile phase, from the stock solution further dilutions were prepared in the concentration range of 600 - 100 µg/ml, elution's are made on HPLC by injecting 20 µg/ml of each concentration repeats it for two times. The coefficient of determination and regression coefficient (R^2) was obtained and shown in the table 2&3 and Figures 5 and 6.

Acceptance Criteria: Correlation coefficient (r^2) should be not less than 0.999.

c) Precision: The intraday and inter-day precision was determined by analyzing cobicistat (150 µg/ml) and darunavir (300 µg/ml) for six times on same day (intra-day) and repeated on the second day (inter-day) studies were given the

Figure 2: Chromatogram of Standard Solution of Cobicistat and Darunavir



Peak	Rt (min.)	Name	Area	% Area	Theoretical Plate	Tailing Factor	Resolution
1	2.959	Cobicistat	1080796	17.647	4011.857	1.659	0.000
2	4.260	Darunavir	5043880	82.353	7641.953	1.321	6.814
Total			6124676	100.00			

Table 2: Linearity Results for Cobicistat

Concentration of Drug (µg/mL)	Retention Time (min.)	Peak Area
40	2.993	453186
60	2.989	644079
80	2.982	853653
100	2.978	1092304
120	2.968	1331124

Figure 5: Calibration Curve of Cobicistat

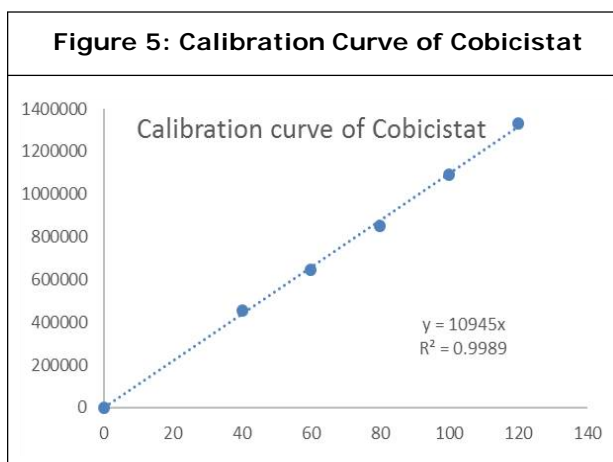


Table 3: Linearity Results for Darunavir

Concentration of Drug (µg/mL)	Retention Time (min.)	Peak Area
40	4.266	2251396
60	4.268	3155929
80	4.267	4062455
100	4.271	5086904
120	4.269	6089876

Figure 6: Calibration Curve of Darunavir

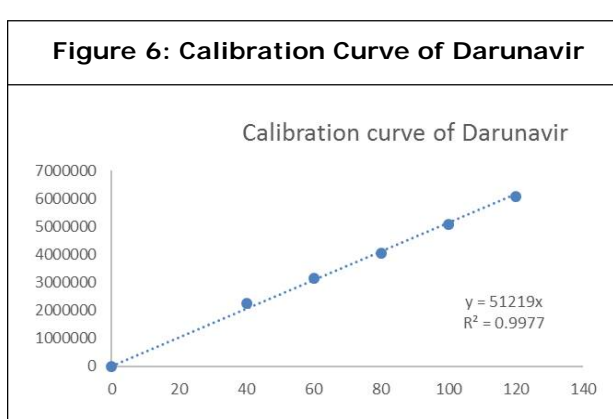


Table 4: Inter-day Precision for Cobicistat

Inj	Retention Time (min)	Peak Area
1	2.967	1067096
2	2.965	1069882
3	2.966	1068209
4	2.967	1067920
5	2.966	1068921
6	2.971	1068140
Mean	2.967	1068361
Std.Dev	0.002	948
%RSD	0.066	0.089

Table 5: Intra-day Precision for Cobicistat

Inj	Retention Time (min)	Peak Area
1	2.965	1075615
2	2.966	1068499
3	2.964	1070863
4	2.966	1069530
5	2.967	1044376
6	2.967	1069655
Mean	2.966	1066423
Std.Dev	0.001	11087
%RSD	0.039	1.040

Tables 4 and 5 for Cobicistat and 6 and 7 for Darunavir.

d) Accuracy: The accuracy of the method shall

be demonstrated through determination on samples in three concentrations from 120% , 100%, and 80% three replicates of each of

Table 6: Inter-day Precision for Darunavir

Inj	Retention Time (min)	Peak Area
1	4.258	5073355
2	4.259	5073719
3	4.261	5086606
4	4.265	5093012
5	4.264	5100941
6	4.271	5106060
Mean	4.263	5088949
Std.Dev	0.005	13666
%RSD	0.108	0.269

the theoretical concentrations employed as per the usual procedure and the results are summarized in Tables 8 and 9.

Acceptance Criteria: The mean % recovery at each level should be NLT 98.0% & NMT 102.0%.

e) Limit of detection (LOD) and limit of quantification (LOQ): A series of 11 replicate concentrations were analyzed and quantified. Set up the described chromatographic conditions and allow the system to equilibrate.

Table 7: Intra-day Precision for Darunavir

Inj	Retention Time (min)	Peak Area
1	4.265	5094551
2	4.267	5123695
3	4.265	5098612
4	4.266	5102669
5	4.266	5090259
6	4.265	5112281
Mean	4.266	5103678
Std.Dev	0.001	12367
%RSD	0.017	0.242

Starting with concentration 20%, 10%, 5%, 2%, 1%, 0.5%, 0.2%, 0.1%, 0.05%, 0.02% and 0.01% peak area values were given in the Table 10 for Cobicistat and darunavir.

e) **Robustness:** The robustness of the method was determined as per USP guidelines, under different conditions including change in flow rate, different column, pH of buffer, and buffer concentration. The results obtained by deliberately variation in method parameters and data are summarized in Table 11.

Table 8: Accuracy Results for Cobicistat

S.No.	Recovery at 120 µg/ml (80%) Dilution Level Peak Areas		Recovery at 150 µg/ml (100%) Dilution Level Peak Areas		Recovery at 180 µg/ml (120%) Dilution Level Peak Areas	
	Standard	Spiked (10%)	Standard	Spiked (10%)	Standard	Spiked (10%)
1	844435	971235	1087081	1197395	1322505	1444310
2	849927	971232	1091089	1199754	1323229	1442036
3	849915	969436	1090560	1197458	1322153	1441607
Mean	848092	970634	1089577	1198202	1322629	1442651
Std.Dev	3167	1037.79	2177	1344	549	1452.66
%RSD	0.373	0.107	0.200	0.112	0.041	0.101
% Recovery	116.20		100.6		109.10	
Average % Recovery -108.63						

Table 9: Accuracy Results for Darunavir

S.No.	Recovery at 80% Dilution Level Peak areas		Recovery at 100% Dilution Level Peak areas		Recovery at 120% Dilution Level Peak areas	
	Standard	Spiked (10%)	Standard	Spiked (10%)	Standard	Spiked (10%)
1	4061180	4580420	5084776	5575802	6062373	6572822
2	4055456	4580106	5076211	5578826	6061037	6564472
3	4056214	4576846	5085511	5584140	6053013	6564525
Mean	4057617	4579124	5082166	5579589	6058807.7	6567273
Std.Dev	3109	1979	5170	4221	5063	4806
%RSD	0.077	0.043	0.102	0.076	0.084	0.073
% Recovery	102.80	97.60	100.30			
Average % Recovery -100.23						

Table 10: LOD and LOQ Values for Prezcoibix

S.No.	% Concentration	Peak Area	
		Cobicistat	Darunavir
1	20	222098	1161716
2	10	113777	597142
3	05	56285	310533
4	02	23274	125347
5	01	11490	62394
6	0.5	6109	31271
7	0.2	2565	12288
8	0.1	1451	6178
9	0.05	718	3264
10	0.02	298	1508
11	0.01	ND	ND
Limit of Detection (LOD):		0.02%	0.02%
Limit of Quantification (LOQ):		0.06%	0.06%
Note: ND* - Not Detected.			

Acceptance Criteria: There should be no significant effect on the result by doing small

deliberate changes in the system as well as in method parameters.

g) System suitability: For system suitability, six replicates of the working standard samples were injected and the parameters like – plate number (N), retention time (Rt), and peak asymmetry of samples were calculated for Prezcofix and given in Table 12.

Acceptance Criteria: The % RSD for the retention times of principal peak from 5 replicate injections of each standard solution should be not more than 2.0%.

RESULTS AND DISCUSSION

The aim of the present study was to develop a

simple, sensitive, precise, and accurate RP-HPLC method for the analysis of Cobicistat & Darunavir in bulk and tablet formulation. To optimize the mobile phase, various combinations of buffer and acetonitrile solvents were studied, on a column Eclipse XBD-C18 (250 mm x 4.6 mm, 5 μ). Finally by using mixture of 0.01M potassium di-hydrogen phosphate pH 3.0 (adjusted with ortho phosphoric acid) as buffer and acetonitrile in the ratio of (40:60 v/v) found to be an efficient system for elution of drug with good peak shape as well as retention time 2.959 and 4.260 min., for Cobicistat and Darunavir respectively, flow rate 1.0 mL/min. at UV

Table 11a: Robustness Results for Cobicistat

Parameter	Peak Areas for Flow Rate		Peak Areas for Variable Column	
	Flow Rate 1.2 ml	Flow Rate 0.8 ml	Zobrax Eclipse XBD-C18	Inertsil ODS - C18
Injection-1	1321967	1329137	1089359	1099621
Injection-2	1319868	1327695	1093130	1092293
Injection-3	1327966	1327069	1089006	1091219
Mean	1323267	1327967	1090498	1094378
Std. dev	4203	1060.49	2285.91	4572.5
% RSD	0.318	0.080	0.210	0.418

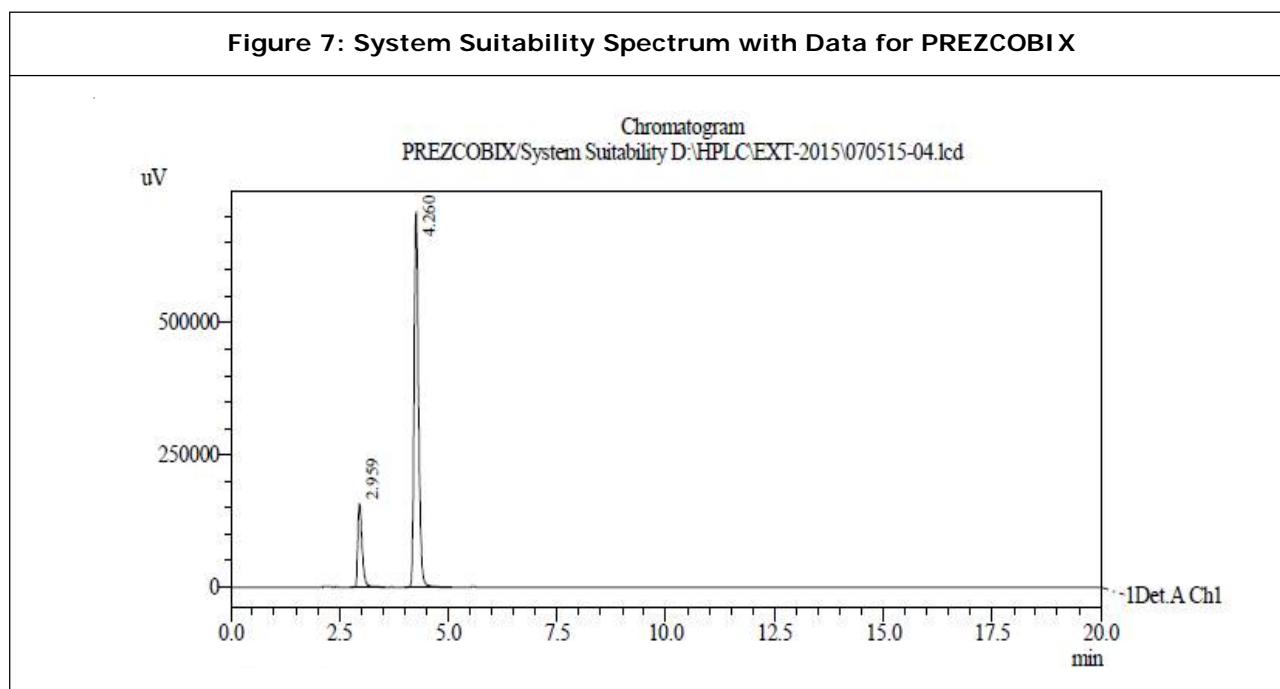
Table 11b: Robustness Results for Darunavir

Parameter	Peak Areas for Flow Rate		Peak Areas for Variable Column	
	Flow Rate 1.2 ml	Flow Rate 0.8 ml	Zobrax Eclipse XBD-C18	Inertsil ODS - C18
Injection-1	6087482	6097145	5061257	5045599
Injection-2	6093124	6097369	5060817	5038877
Injection-3	6108172	6091574	5064823	5045098
Mean	6096259	6095363	5062299	5043191
Std. dev	10695	3283	2197	3745
% RSD	0.175	0.054	0.043	0.074

Table 12: System Suitability Parameters

Parameter	Results of the Proposed HPLC Method	
	Cobicistat	Darunavir
Retention time (min)	2.959	4.260
Theoretical plates (n)	4011.872	7641.953
Peak Area	1080796	5043880
Peak asymmetry (Tailing)	1.659	1.321
Linearity range (%)	40-120	40-120
Regression coefficient (R2)	0.9989	0.9977
Limit of Detection (%)	0.02	0.02
Limit of Quantification (%)	0.06	0.06

Figure 7: System Suitability Spectrum with Data for PREZCOBIX



Detector A Ch1 240 nm

Peak Table

Peak	Retention Time (min)	Name	Peak Area	% Peak Area	Theoretical Plate	Tailing Factor	Resolution
1	2.959	COBICISTAT	1080796	17.647	4011.872	1.659	0.000
2	4.260	DARUNAVIR	5043880	82.353	7641.953	1.321	6.814
Total			6124676	100.00			

wavelength of 240 nm. Quantitative linearity was obeyed in the percentage range of 40 to 120% and 40 to 120% the regression equations of concentration over their peak areas were found to be $Y = 10945.X$, $r^2 = 0.9989$, and $Y = 51219.X$, $r^2 = 0.9977$ for cobicistat and darunavir respectively where Y is the peak area and X is the concentration of drug. The number of theoretical plates obtained was 4011.872 for cobicistat and 7641.953 for darunavir which indicate the efficient performance of the column. The Limit of detection was 0.02% and 0.06% and limit of quantification was 0.02% and 0.06% for cobicistat and darunavir, which indicates the sensitivity of the method the high percentage recovery, indicates that the proposed method is highly accurate. No interfering peaks were found in the chromatogram indicating that excipients used in tablet formulation did not interfere with the estimation of the drug by the proposed RP-HPLC method.

CONCLUSION

In conclusion a new isocratic RP-HPLC method was developed and validated for the estimation of Cobicistat & Darunavir in bulk and combined tablet dosage form. The developed method is simple, precise and accurate and satisfactory results were obtained through the method validation data. The present method can be easily applicable for routine drug analysis in laboratories and pharmaceutical industry.

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