AN ANALYTICAL NEW RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ANTI-VIRAL DRUGS DARUNAVIR AND COBICISTAT IN BULK FORM AND MARKETED PHARMACEUTICAL TABLET DOSAGE FORM

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ABSTRACT: *A new analytical simple and precise, accurate and reproducible Reverse Phase - HPLC method for Simultaneous estimation of Darunavir and Cobicistat in bulk and pharmaceutical formulations. Separation of Darunavir and Cobicistat was successfully achieved on a Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d. or equivalent in an isocratic mode utilizing Phosphate Buffer (0.2 M, pH=2): Acetonitrile in the ratio of 64:36% v/v at a flow rate of 1.0mL/min and eluates was monitored at 265nm, with a retention time of 2.131 and 2.816 minutes for Darunavir and Cobicistat respectively. The method was validated and the response was found to be linear in the drug concentration range of 6µg/mL to 14µg/mL for Darunavir and 18µg/mL to 42µg/mL for Cobicistat. The LOD and LOQ for Darunavir were found to be 0.4µg/mL and 0.12µg/mL respectively. The LOD and LOQ for Cobicistat were found to be 0.07µg/mL and 0.21µg/mL respectively. This method was found to be good %recovery for Darunavir and Cobicistat were found to be 100.415 and 100.264 respectively indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard with the sample so, the method specifically determines the analytes in the sample without interference from excipients of tablet dosage forms. The method was extensively validated according to ICH guidelines for Linearity, Range, Accuracy, Precision, Specificity and Robustness.*

Keywords: *Darunavir and Cobicistat, HPLC, Method Development, Validation*

I. INTRODUCTION

Darunavir is an antiretroviral protease inhibitor that is used in the therapy and prevention of human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS). Darunavir[1] can cause transient and usually asymptomatic elevations in serum aminotransferase levels and has been linked to rare instances of clinically apparent, acute liver injury. In HBV or HCV coinfected patients, highly active antiretroviral therapy with Darunavir may result of an exacerbation of the underlying chronic hepatitis B or C. Darunavir[2] is a human immunodeficiency virus type 1 (HIV-1) protease nonpeptidic inhibitor, with activity against HIV. Upon oral administration, Darunavir selectively targets and binds to the active site of HIV-1 protease, and inhibits the dimerization and catalytic activity of HIV-1 protease. This inhibits the proteolytic cleavage of viral Gag and Gag-Pol polyproteins in HIV-infected cells. This inhibition leads to the production of immature, non-infectious viral proteins that are unable to form mature virions, and prevents HIV replication. The IUPAC Name of Darunavir[3] is [(3aS, 4R, 6aR)-2, 3, 3a, 4, 5, 6a-hexa hydro furo [2, 3-b] furan-4-yl] N-[(2S, 3R)-4-[(4-amino phenyl) sulfonyl-(2-methyl propyl) amino]-3-hydroxy-1-phenyl butan-2-yl] carbamate. The Chemical Structure of Darunavir is below

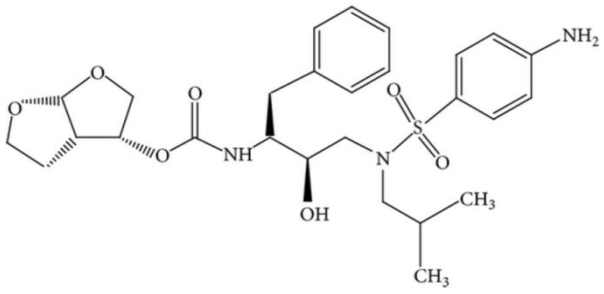

Fig.1. Chemical Structure of Darunavir

Cobicistat is a cytochrome P450 3A (CYP3A) inhibitor that can be used to enhance the pharmacokinetic profile of certain anti-HIV-1 agents. Upon administration, Cobicistat[4] inhibits the liver enzyme CYP3A4 and limits the breakdown of co-administered agents that are metabolized by CYP3A4, and increases the concentration, systemic

exposure and efficacy of the co-administered agent. Cobicistat[5] is a monocarboxylic acid amide obtained by formal condensation of the carboxy group of (2S)-2-({[(2-isopropyl-1, 3-thiazol-4-yl) methyl] (methyl) carbamoyl} amino)-4-(morpholin-4-yl) butanoic acid with the amino group of 1, 3-thiazol-5-yl methyl [(2R,5R)-5-amino-1,6-diphenyl hexan-2-yl] carbamate. Acts as a pharmacoenhancer in treatment of HIV-1 by inhibiting P450 enzymes that metabolise other medications. It has a role as a P450 inhibitor. It is a member of 1, 3-thiazoles, a member of morpholines, a member of ureas, a carbamate ester and a monocarboxylic acid amide. The IUPAC Name of Cobicistat is 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-yl butanoyl] amino]-1, 6-diphenyl hexan-2-yl] carbamate. The Chemical Structure of Cobicistat[6] is following

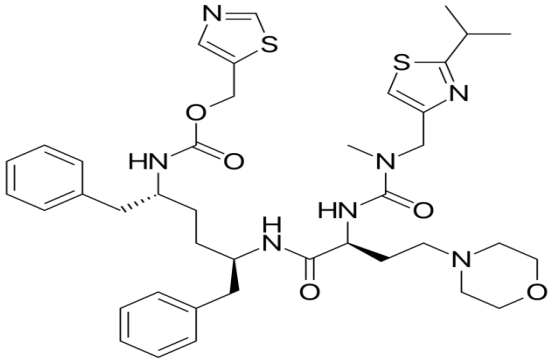

Fig.2. Chemical Structure of Cobicistat

II. MATERIALS AND METHODS

Table-1: List of Instrument used

S. No.	Instruments/Equipment/Apparatus
1.	HPLC with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	T60-LAB INDIA UV – Vis spectrophotometer
3.	Electronic Balance (SHIMADZU ATY224)
4.	Ultra Sonicator(Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry ODS RP C_{18},5µm, 15mm x 4.6mm i.d.
7.	pH Analyzer (ELICO)
8.	Vacuum filtration kit (BOROSIL)

Table-2: List of Chemicals used

S.N.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.

4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai

Method Development

Selection of Wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase[7] diluting with the same solvent. (After optimization of all conditions) for UV analysis. It is scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Darunavir & Cobicistat, so that the same wave number can be utilized in HPLC UV detector for estimating the Darunavir & Cobicistat. The scanned UV spectrum is attached in the following page.

Sample & Standard Preparation for the UV-Spectrophotometer Analysis

25 mg of Darunavir standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution[8] was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

25 mg of Cobicistat standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase. Further dilution was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

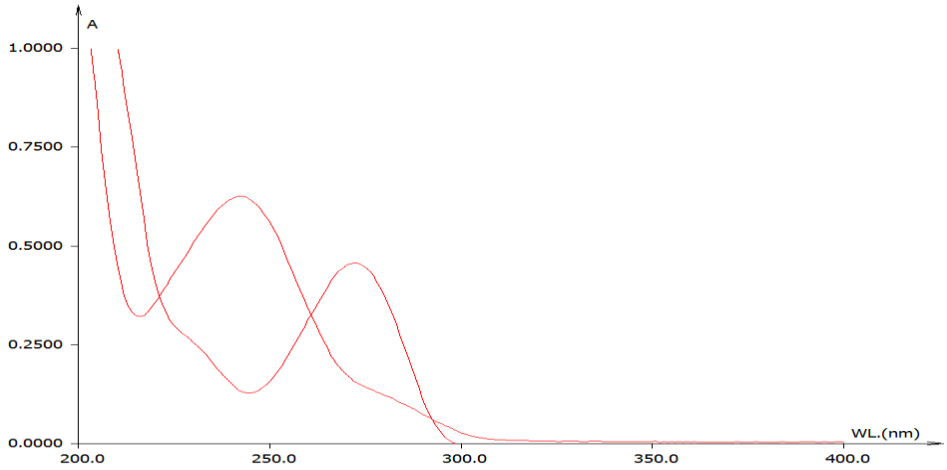

Fig.3. Isobestic point Darunavir & Cobicistat

Observation: While scanning the Darunavir solution we observed the maxima at 275nm and for the Cobicistat solution we observed the maxima at 248nm. The isobestic point for the drugs was found at 265nm. The UV spectrum[9] has been recorded on T60-LAB INDIA make UV – Vis spectrophotometer model UV-2450.

Summary of Optimized Chromatographic Conditions:

The Optimum conditions obtained from experiments can be summarized as below:

Table-3: Summary of Optimised Chromatographic Conditions

Mobile phase	Phosphate Buffer (0.2 M, pH=2): Acetonitrile = 64:36% v/v
Column	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.
Column Temperature	Ambient
Detection Wavelength	265 nm
Flow rate	1.0 ml/ min.
Run time	10 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	10µl
Type of Elution	Isocratic

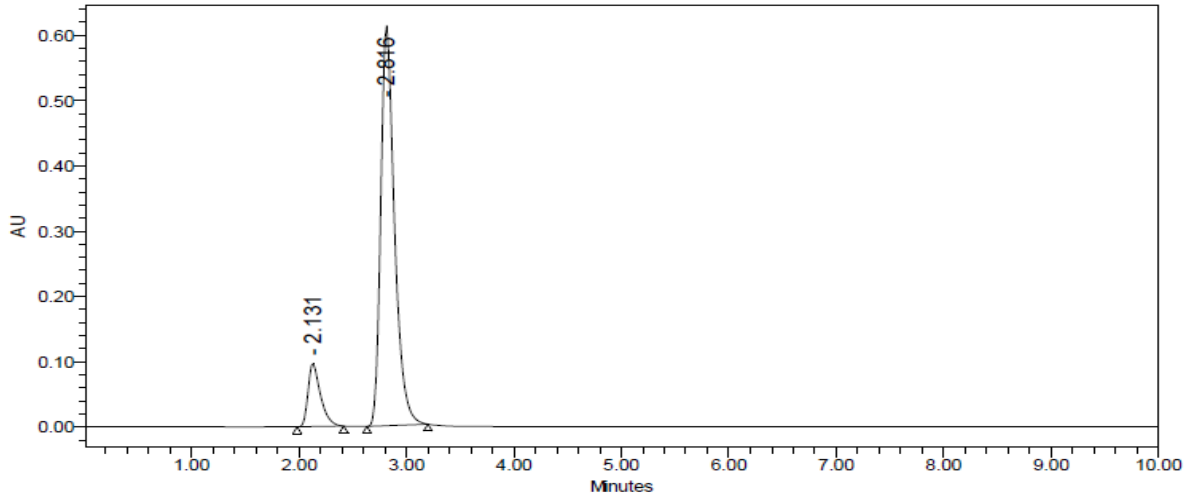

Fig.4. Chromatogram for Optimized Chromatographic Condition

Preparation of Mobile Phase:

The mobile phase was prepared with the combination of Phosphate Buffer (0.2 M, pH=2) and Acetonitrile at the volume of 1000ml. 640ml of Phosphate Buffer and 360ml of Acetonitrile were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration[10].

Preparation of Standard Solutions:

10 mg of Darunavir & Cobicistat was weighed accurately and transferred into 100 ml volumetric flask. About 10 ml mobile phase was added and sonicated to dissolve. The volume was made up to the mark with same solvent. The final solution contained about 10µg/ml and 10µg/ml of Darunavir & Cobicistat respectively.

Method Validation

1. Linearity and Range

To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 6-14µg/ml for Darunavir and concentration ranging from 12-28µg/ml for Cobicistat. The prepared solutions were filtered through Whatman filter paper (No.41). From these solutions, 10µl injections of each concentration were injected into the HPLC system[11] and chromatographed under the optimized conditions. Calibration curve was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

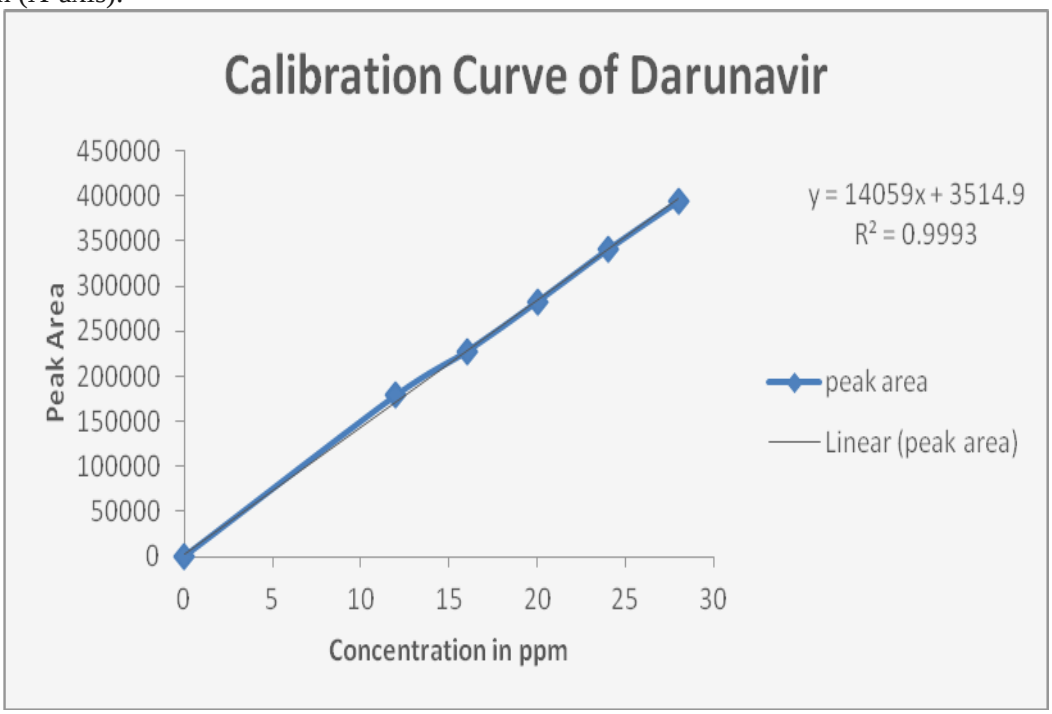

Fig.5. Standard curve for Darunavir

Table-4: Linearity Results for Darunavir

CONC. (µg/ml)	AUC (n=6)
0	0
6	119571
8	167873
10	211264
12	255428
14	299987

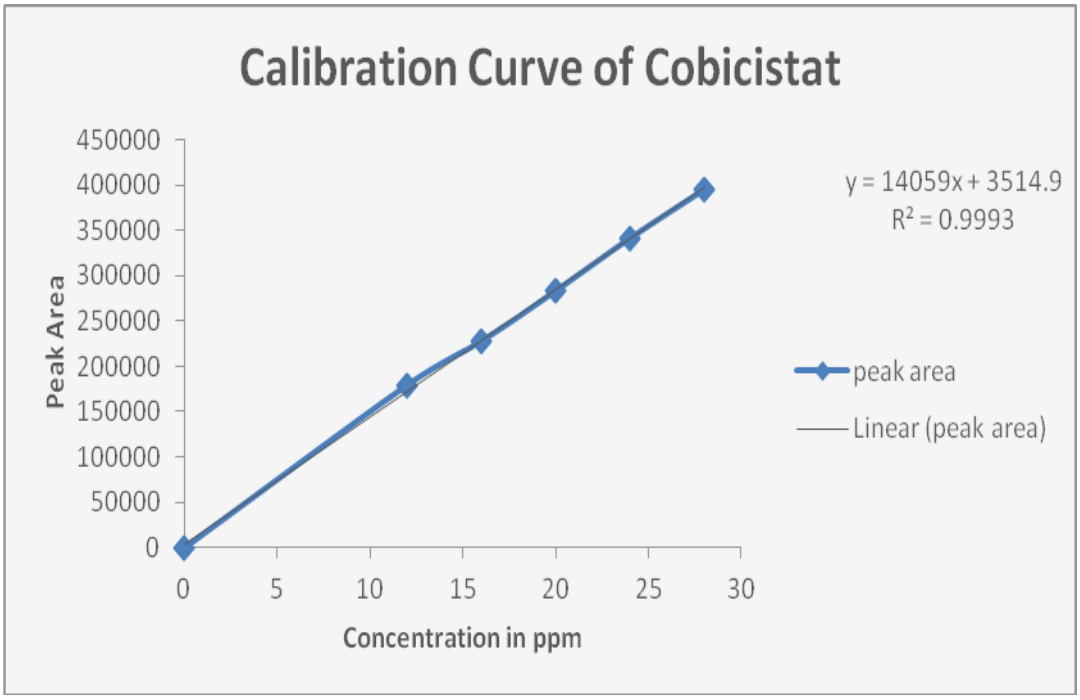

Fig.6. Standard curve for Cobicistat

Table-5: Linearity Results for Cobicistat

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
12	179371
16	227893
20	283264
24	341428
28	394987

Results & Discussion: The linearity range was found to be 6-14 µg/ml for Darunavir. The correlation coefficient was found to be 0.999, the slope was found to be 14059 and the intercept was found to be 3514 for Darunavir. Linearity[12] range was found to be 12-28 µg/ml for Cobicistat. The correlation coefficient was found to be 0.999, the slope was found to be 14059 and the intercept was found to be 3514 for Cobicistat.

2. Accuracy:

Recovery study: For Darunavir

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Darunavir were taken and added to the pre-analyzed formulation of concentration of 10µg/ml. From that percentage recovery[13] values were calculated. The results were shown in table-6.

Table-6: Accuracy Readings for Darunavir

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	8	7.997368	115949	99.9671	Mean= 100.7003% S.D. = 0.6884036 % R.S.D.= 0.683616%
S_2 : 80 %	8	8.106622	117485	101.3328	
S_3 : 80 %	8	8.064087	116887	100.8011	
S_4 : 100 %	10	9.904901	142767	99.04901	Mean= 100.36157% S.D. = 1.346221 R.S.D.= 1.3413706%
S_5 : 100 %	10	10.02966	144521	100.2966	
S_6 : 100 %	10	10.17391	146549	101.7391	
S_7 : 120 %	12	12.01807	172476	100.1506	Mean= 100.183756% S.D. = 1.19411 % R.S.D. = 1.19191%
S_8 : 120 %	12	11.88079	170546	99.00657	
S_9 : 120 %	12	12.16729	174574	101.3941	

Observation : From the Accuracy Method, we observed that the mean %Recovery of the drug are 100.7003%, 100.36157% and 100.183756% which is within the range of 98-102% and %RSD is within the range <2 i.e. 0.683616%, 1.3413706% and 1.19191% respectively.

Recovery study: Cobicistat

To determine the accuracy[14] of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Cobicistat were taken and added to the pre-analysed formulation of concentration 50µg/ml. From that percentage recovery values were calculated. The results were shown in table-7.

Table-7: Accuracy Results for Cobicistat

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	16	16.08685	229679	100.5428	Mean= 100.54488% S.D. = 0.97847% R.S.D.= 0.9731%
S_2 : 80 %	16	15.93079	227485	99.56745	
S_3 : 80 %	16	16.2439	231887	101.5244	
S_4 : 100 %	20	20.07632	285767	100.3816	Mean= 99.97095% S.D. = 0.395406 % R.S.D.= 0.39552%
S_5 : 100 %	20	19.98769	284521	99.93847	
S_6 : 100 %	20	19.91856	283549	99.59279	
S_7 : 120 %	24	23.75432	337476	98.97634	Mean= 100.27718% S.D. = 1.21262 % R.S.D. = 1.20927%
S_8 : 120 %	24	24.11494	342546	100.4789	
S_9 : 120 %	24	24.33032	345574	101.3763	

Observation : From the Accuracy Method, we observed that the mean %Recovery of the drug are 100.54488%, 99.97095% and 100.27718% which is within the range of 98-102% and %RSD is within the range <2 i.e. 0.9731%, 0.39552% and 1.20927% respectively.

3. Precision:

Repeatability: The precision[15] of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug Darunavir & Cobicistat (API). The percent relative standard deviation[16] was calculated for Darunavir & Cobicistat are presented in the table-8.

Table-8: Data showing repeatability analysis for Darunavir & Cobicistat

HPLC Injection Replicates	AUC for Darunavir	AUC for Cobicistat
Replicate – 1	113568	241022
Replicate – 2	113241	240137
Replicate – 3	115408	242911
Replicate – 4	117412	245245
Replicate – 5	112541	241941

Replicate – 6	112546	240444
Average	**114119.3333**	**241356.6667**
Standard Deviation	**1925.83838**	**1416.95812**
% RSD	**1.68756**	**0.58708**

Result & Discussion: The repeatability study[17] which was conducted on the solution having the concentration of about 10µg/ml for Darunavir and 20µg/ml for Cobicistat (n =6) showed a RSD of 1.68756% for Darunavir and 0.58708% for Cobicistat. It was concluded that the analytical technique showed good repeatability.

Intermediate precision

The Intermediate Precision[18] consists of two methods:-

Intra Day: In Intra Day process, the 80%, 100% and 120% concentration are injected at different intervals of time in same day.

Inter Day: In Inter Day process, the 80%, 100% and 120% concentration are injected at same intervals of time in different days.

Table-9: Data for Darunavir analysis

Conc. of Darunavir (API) (µg/ml)	Observed Conc. of Darunavir (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
8	8.17	0.35	8.28	0.48
10	10.19	0.56	10.66	0.65
12	12.26	0.76	12.56	0.46

Table-10: Data for Cobicistat analysis

Conc. of Cobicistat (API) (µg/ml)	Observed Conc. of Cobicistat (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
16	16.33	0.24	16.56	0.33
20	20.56	0.48	20.76	0.67
24	24.23	0.63	24.63	0.43

Observations: The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Darunavir and Cobicistat revealed that the proposed method is precise.

4. Limit of detection and limit of quantification

The LOD[19] was found to be 0.04µg/ml and LOQ was found to be 0.12µg/ml for Darunavir respectively which represents that sensitivity of the method is high.

The LOD was found to be 0.07µg/ml and LOQ[20] was found to be 0.21µg/ml for Cobicistat respectively which represents that sensitivity of the method is high.

5. Method Robustness:

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Wavelength of detection (±2nm) & organic phase content in mobile phase (±2%) studied to determine the robustness[21] of the method are also in favour of (Table-11, % RSD < 2%) the developed RP-HPLC method for the analysis of Darunavir (API).

Table-11: Result of Method Robustness Test for Darunavir

Change in parameter	% RSD
Flow (0.8 ml/min)	0.23
Flow (1.2 ml/min)	0.39
More Organic	0.83
Less Organic	0.76
Wavelength of Detection (277 nm)	0.56
Wavelength of detection (273 nm)	0.43

Influence of small changes in chromatographic conditions[22] such as change in flow rate (± 0.1ml/min),

Wavelength of detection (±2nm) & organic phase content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-12, % RSD < 2%) the developed RP-HPLC method for the analysis of Cobicistat (API).

Table-12: Result of Method Robustness Test for Cobicistat

Change in parameter	% RSD
Flow (0.8 ml/min)	0.37
Flow (1.2 ml/min)	0.57
More Organic	0.76
Less Organic	0.53
Wavelength of Detection (250 nm)	1.21
Wavelength of detection (246 nm)	0.39

6. System Suitability Parameter

System suitableness testing is associate degree integral a part of several analytical procedures. The tests are supported the idea that the instrumentality, physics, associate degree analytical operations and samples to be analyzed represent an integral system which will be evaluated intrinsically. Following system suitableness take a look at parameters[23] were established. The information is shown in Table-13.

Table-13: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs> 2	2.57
2	Asymmetry	T ≤ 2	Darunavir = 0.46 Cobicistat = 0.77
3	Theoretical plate	N > 2000	Darunavir = 2946 Cobicistat = 3076

7. Estimation of Darunavir and in Pharmaceutical Dosage Form

Twenty Tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 25 mg of drugs were transferred to 25 ml volumetric flask, make and solution was sonicated for 15 minutes, there after volume was made up to 25 ml with same solvent. Then 10 ml of the above solution was diluted to 100 ml with mobile phase. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. The solution prepared was injected in five replicates into the HPLC system and the observations were recorded.

A duplicate injection of the standard solution was also injected into the HPLC system and the peak areas were recorded (Assay[24]). The data are shown in Table-14.

ASSAY:

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:

AT = Peak Area of drug obtained with test preparation

AS = Peak Area of drug obtained with standard preparation

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

Table-14: Recovery Data for estimation Darunavir and Cobicistat

Brand name of Darunavir and Cobicistat	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Prezcobix	800/150	799.856 (±0.422) / 149.578 (± 0.372)	99.5 (±0.576) / 99.4 ± 0.822)

Result & Discussion: The assay of Prezcobix Tablets containing 800mg of Darunavir & 150mg of Cobicistat was found to be 99.5% and 99.4% respectively.

Forced Degradation Studies

The different types of forced degradation pathways/studies[25] are studied here are acid hydrolysis, basic hydrolysis, thermal degradation and oxidative degradation.

Table-15: Results of Force Degradation Studies of Darunavir and Cobicistat

Stress Condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	95.62	4.38	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	97.13	2.87	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	96.24	3.76	100.00
UV (254nm)	24Hrs.	95.43	4.57	100.00
3% Hydrogen peroxide	24Hrs.	96.16	3.84	100.00

III. RESULTS AND DISCUSSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Darunavir and Cobicistat, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d. column was preferred because using this column peak shape, resolution and absorbance were good.

Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, Acetonitrile, water, 0.1N NaOH, 0.1NHCl). Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Darunavir and Cobicistat it is evident that most of the HPLC work can be accomplished in the wavelength range of 200-300 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 10µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay which can help in the analysis Darunavir and Cobicistat in different formulations

IV. CONCLUSION

A sensitive & selective stability indicting RP-HPLC method has been developed & validated for the analysis of

Darunavir & Cobicistat in bulk and pharmaceutical dosage form. Based on peak purity results, obtained from the analysis of samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Darunavir & Cobicistat indicated that the developed method is specific for the simultaneous estimation of Darunavir & Cobicistat in the bulk and pharmaceutical dosage forms. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

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