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Analytical Method Development and Validation for the Simultaneous Estimation of Darunavir and Cobicistat by RP-HPLC Method in Bulk and Pharmaceutical Dosage Form

Sd. Asra Ayesha*, G. Devika Subramaniyan

Bomma Institute of Pharmacy, Allipuram, Khammam, Telangana, India

A B S T R A C T

A new, simple, precise, accurate and reproducible RP-HPLC method for simultaneous estimation of Darunavir and Cobicistat in bulk and pharmaceutical formulations was developed. Separation of Darunavir and Cobicistat was successfully achieved on C_{18} ODS(250mm x 4.6 mm), 5µm., column in an isocratic mode utilizing sodium acetate buffer pH 4.5:methanol (60:40v/v) at a flow rate of 1.0 ml/min and eluents were monitored at 253nm, with a retention time of 2.577and 4.967minutes for Darunavir and Cobicistat respectively. The method was validated and it was found to be linear. The values of the correlation coefficient were found to 0.992 for Darunavir and 0.9992 for Cobicistat respectively. The LOD for Darunavir and Cobicistat were found to be 19.07 and 0.28respectively. The LOD and LOQ for Darunavir and Cobicistat were found to be 57.78 and 2.47respectively. The percentage recoveries for Darunavir and Cobicistat were found to be within the limit indicates that the proposed method is highly accurate. The method was extensively validated according to ICH guidelines.

Keywords: Darunavir, Cobicistat, RP-HPLC

A R T I C L E I N F O

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***Corresponding Author**
Sd. Asra Ayesha
Bomma Institute of Pharmacy,
Khammam, Telangana, India
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1. Introduction

Analytical methods: 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 one. Often a time lag exists from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias[1]. 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[2]. Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International Conference on Harmonization (ICH) guidelines (Q2A and Q2B). [3,4]

Method development is a continuous process that progresses in parallel with the evolution of the drug product. The goal and purpose of the method should reflect the phase of drug development. During early drug development, the methods may focus on API behaviour[5]. They should be suitable to support preclinical safety evaluations, pre-formulation studies, and prototype product stability studies. As drug development progresses, the analytical methods are refined and expanded, based on increased API and drug product knowledge.

The methods should be robust and uncomplicated, while still meeting the appropriate regulatory guidelines. Scouting experiments are frequently performed during method development to establish the performance limits of the method, prior to formal validation experiments[6-9]. These may include forced degradation studies, which are an integral part of development of a stability-indicating method. API is typically subjected to degradation by acid, base, peroxide, heat, and light. This allows for a determination of the capability of the method to separate and quantify degradation products, while providing insight into the main mechanisms of degradation. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate potential degradation of the API in the presence of formulation excipients[10]

Darunavir[11] is a HIV protease inhibitor which prevents HIV replication by binding to the enzyme's active site, thereby preventing the dimerization and the catalytic activity of the HIV-1 protease. Darunavir selectively inhibits the cleavage of HIV encoded Gag-Pol polyproteins in virus-infected cells, which prevents the formation of mature infectious virus particles. Darunavir can also adapt to the changing shape of a protease enzyme because of its molecular flexibility. Darunavir is known to bind to two distinct sites on the enzyme: the active site cavity and the surface of one of the flexible flaps in the protease dimer

Cobicistat is a licensed drug for use in the treatment of infection with human immunodeficiency virus (HIV). Although it does not have any anti-HIV activity, cobicistat acts as a pharmacokinetic enhancer by inhibiting cytochrome P450 3A isoforms (CYP3A) and therefore increases the systemic exposure of coadministered agents that are metabolized by CYP3A enzymes. More specifically, cobicistat is indicated to increase systemic exposure of atazanavir or darunavir (once daily dosing regimen) in combination with other antiretroviral agents in the treatment of HIV-1 infection. Increasing systemic exposure of anti-retrovirals (ARVs) without increasing dosage allows for better treatment outcomes and a decreased side effect profile[12].

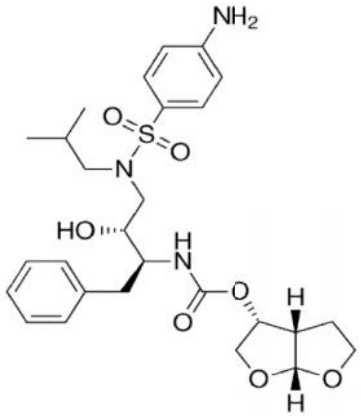

Figure 1: Darunavir

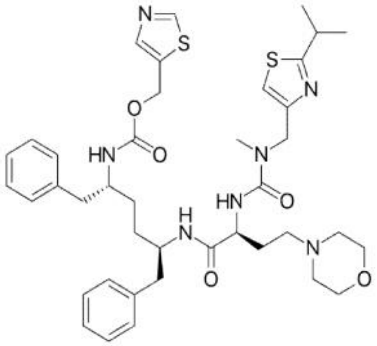

Figure 2: Cobicistat

2. Materials and Methods

Apparatus: The instrument used for the study was Shimadzu (LC20ATVP) HPLC, Separation module 2695, UV detector with Spin chrome software version 2.

Reagents and Materials

The solvents used were Methanol, Acetonitrile, Potassium dihydrogen ortho phosphate, Dipotassium hydrogen phosphate, Tri Ethyl Amine of HPLC Grade and HPLC Water.

Selection of detection wavelength:

The sensitivity of method that uses UV- Vis detector depends upon the proper selection of wavelength. An ideal wavelength is that gives maximum absorbance and good response for both the drugs to be detected. Standard solutions of Darunavir and Cobicistat were scanned in the

UV range (200-400nm) and the spectrums obtained were overlaid and the overlain spectrum was recorded. From the overlain spectrum, 253 nm was selected as the detection wavelength for the present study.

Selection of mobile phase

Initially the mobile phase tried was Methanol and water, Methanol, Buffer and water in various proportions. Finally, the mobile phase was optimized to Buffer: Methanol in proportion 60:40 v/v respectively at pH 4.5 adjusted with Orthophosphoric Acid

Chromatographic trials for Simultaneous Estimation of Darunavir and Cobicistat by RP- HPLC.

Trial-1Chromatographic conditions

Mobile phase : Phosphate buffer: ACN
pH : 4.0
Ratio : 37:63
Column : Inertsil ODS 3V (250×4.6× 5µ)
Wavelength : 253 nm
Flow rate : 1ml/min

Preparation of mixed standard solution

Weigh accurately 10 mg of Darunavir and Cobicistat in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of Darunavir and Cobicistat is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

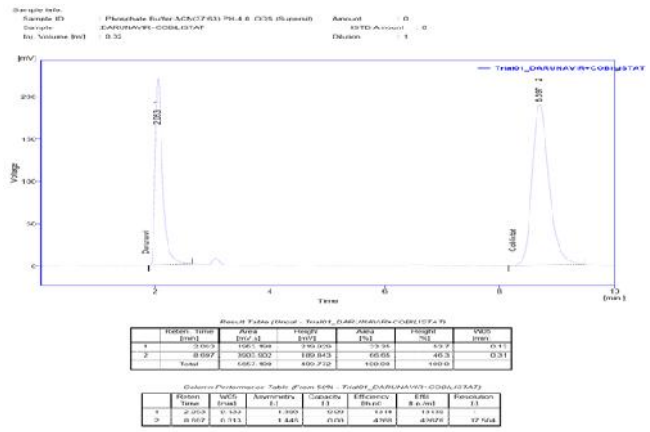

Figure 1: Chromatogram of Trail 1

Observation: Although the Efficiency was not satisfactory for Colibistat.The peak response of Darunavir was very less. Hence it was not taken for optimization.

Trial-2Chromatographic condition

Mobile phase : KH_2PO_4: MeOH
Ph : 6.0
Ratio : 55:45
Column : Inertsil ODS 3V (250×4.6 ×5µ)
Wavelength : 253nm
Flow rate : 1ml/min

Preparation of mixed standard solution

Weigh accurately 10 mg of Darunavir and Cobicistat in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of Darunavir and Cobicistat is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

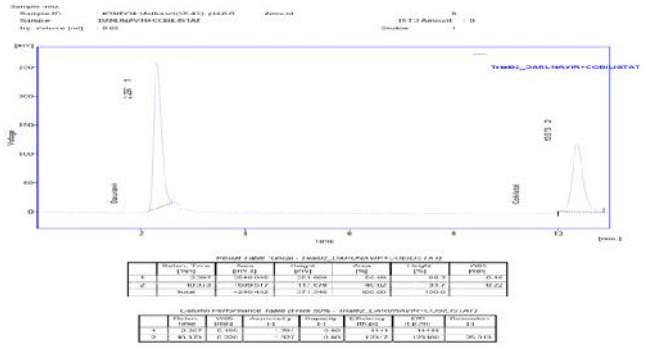

Figure 2: Chromatogram of Trail 2

Observation: Efficiency ofboth the drugs were good but the run time is very more and the peaks of darunavir and cobicistat showed tailing. Hence it was not taken for optimization.

Optimization Chromatographic trial

Chromatographic conditions

Mobile phase : Mixed phosphate buffer: Methanol
pH : 4.5
Ratio : 60:40
Column : Inertsil ODS 3V column,C18(250x4.6 ID) 5µm
Wavelength : 253 nm
Flow rate : 1.0ml/min

Preparation of mixed standard solution

Weigh accurately 60 mg of Darunavir and 40 mg of Cobicistat in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 60 µg/ml of Darunavir and 40 µg/ml of Cobicistat is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

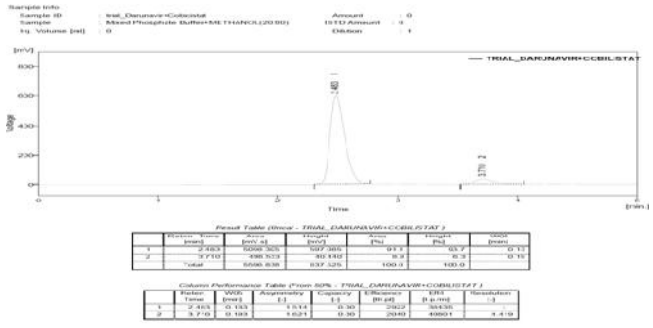

Figure 3: Optimized Chromatogram

Observation: All the system suitability requirements were met. The peak Asymmetry factor was less than 2 for both Cobicistat and Darunavir. The efficiency was more than 2000 for Cobicistat and Darunavir.Resolution between two peaks >1.5 and hence this method was selected as optimized method.

Procedure

Preparation of Mixed Phosphate Buffer:

1.652 gm of potassium di hydrogen phosphate (KH_2PO_4) and 0.3 gm of Dipotassium Hydrogen phosphate was weighed and dissolved in 550ml of water. The buffer was filtered through 0.45µ filters to remove all fine particles.

Preparation of samples for Assay

Preparation of mixed standard solution

Weigh accurately 10 mg of Darunavir and Cobicistat in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of Darunavir and Cobicistatis prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Tablet sample

10 tablets are weighed accurately each tablet contains Cobicistat 160 mg and Darunavir 320 mg were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of Darunavir and Cobicistat were prepared by dissolving weight equivalent to 10 mg of Darunavir and Cobicistat and dissolved in sufficient mobile phase. After that filter the solution using 0.45 micron syringe filter and sonicate for 5 min and dilute to 50ml with mobile phase. Further dilutions are prepared in 5 replicates of 10µg/ml of Darunavir and Cobicistat was made by adding 1 ml of stock solution to 10 ml of mobile phase.

Assay

Preparation of samples for Assay

Standard solution

Weigh accurately 8 mg of Darunavir and 1.5 mg of Cobicistat in 25 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. This solution contains 320 µg/ml of Darunavir and 60 µg/ml of Cobicistat. This solution is used for recording chromatogram.

Tablet sample

10 tablets (each tablet contains Darunavir800 mg and Cobicistat 150 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solution of Darunavirand Cobicistat (µg/ml) was prepared by dissolving weight equivalent to 800 mg of Darunavir and 150mg of Cobicistatdissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 25 ml with mobile phase. This solution contains 320 µg/mL of Darunavir and 60 µg/mLof Cobicistat. This solution is used for recording chromatogram.

Calculation

The amount of Ipratropium Bromide and Albuterol present in the formulation by using the formula given below, and results shown in above table:

$$\%\ \text{Assay} = \frac{\text{AT}}{\text{AS}} \times \frac{\text{WS}}{\text{DS}} \times \frac{\text{DT}}{\text{WT}} \times \frac{\text{P}}{100} \times \frac{\text{AW}}{\text{LC}} \times 100$$

Where,

AS: Average peak area due to standard preparation

AT: Peak area due to assay preparation

WS: Weight of Ipratropium Bromide /Albuterol in mg

WT: Weight of sample in assay preparation

DT: Dilution of assay preparation

3. Results and discussions

Method Validation Parameters

1. Specificity: The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The specificity was performed by Injecting blank and sample.

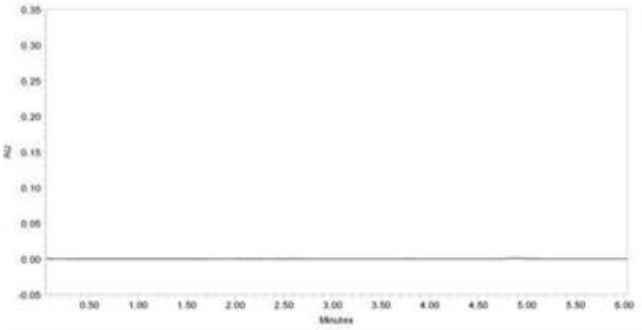

Figure 4: Chromatogram of Blank

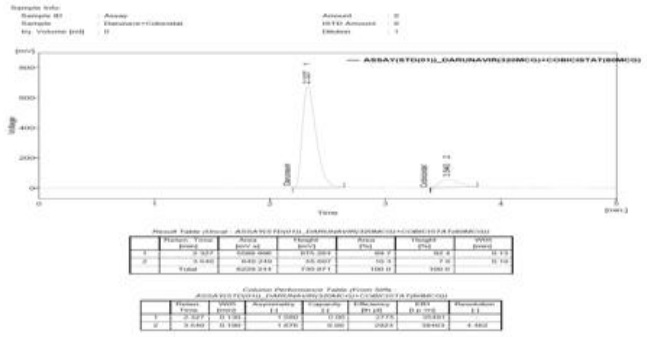

Figure 5: Chromatogram of Sample

2. Linearity

The linearity of an analytical method is it stability to elicit test results that aredirectly, by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.Weigh accurately 16mg of Darunavir and 3 mg of Cobicistat in 50 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. This solution contains 160-480 µg/ml of Darunavir and 30-90 µg/ml of Cobicistat

Acceptance criteria: Correlation coefficient should be not less than 0.999.

3. Range

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 160-480 ppm and 30-90 ppm for Darunavir and Cobicistat respectively

4. Accuracy

Accuracy of the method was determined by recovery experiments. There are mainly 2types of recovery studies are there.

a) Standard addition method: To the formulation, the reference standard of the respective drug of known concentration was added, analyzed by HPLC and compared with the standard drug concentration.

b) Percentage method: For these assay method samples are prepared in three concentrations of 80%, 100%, and 120% respectively.

Acceptance criteria:

The mean % recovery of the Darunavir and Cobicistat at each level should be not less than 95.0% and not more than 105.0%.

Assay procedure

10µL of the standard and sample solutions of Darunavir and Cobicistat were injected into the HPLC system and the chromatograms were recorded. Amount of drug present in the Tablets were calculated using the peak areas.

5. Precision

Method precision also called as repeatability/Intra-day precision indicates whether a method gives consistent results for a single batch. Method precision was demonstrated by preparing six test solutions at 100% concentration as per the test procedure & recording the chromatograms of six test solutions. The % RSD of peak areas of six samples was calculated. The method precision was performed on Darunavir and Cobicistat formulation.

Acceptance criteria:

The % RSD for the area of sample injections results should not be more than 2.

Selection of solvent

Solutions of Darunavir and Cobicistat were prepared by dissolving in mobile phase and UV spectrum of each was recorded by scanning between 200-400 nm.

Validation of the Method

Linearity

Darunavir and Cobicistat:

Serial dilutions of Darunavir and Cobicistat (160-480 ppm and 30-90 ppm) were injected into the column and detected at a wavelength set at 253 nm. The calibration curve was obtained by plotting the concentration vs. peak area and the correlation coefficient was found to be 0.992 and 0.992 respectively.

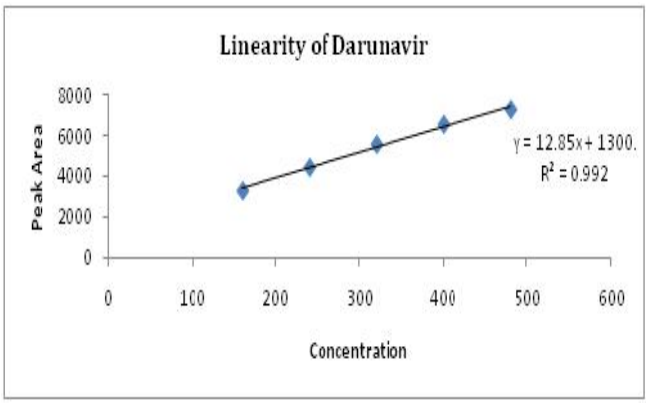

Figure 6: Calibration graph of Darunavir

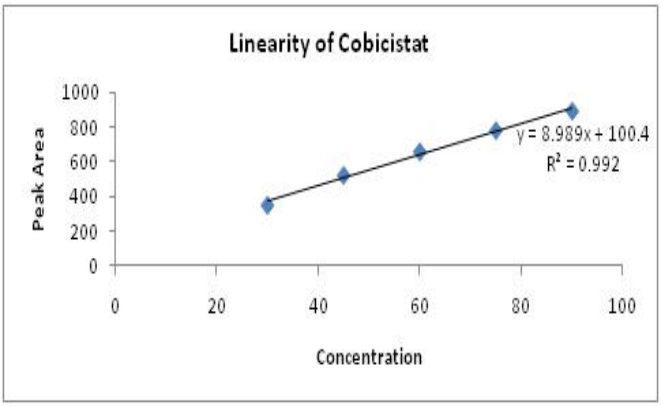

Figure 7: Calibration graph of Cobicistat

Table 1: Calibration data of Cobicistat

S.No.	Conc.(µg/ml)	Area
1	30	347.912
2	45	520.885
3	60	657.488
4	75	780.529
5	90	892.314

Table 2: Calibration data of Darunavir

S.No.	Conc.(µg/ml)	Area
1	160	3236.788
2	240	4409.861
3	320	5560.106
4	400	6560.326
5	480	7303.508

Table 3: Showing accuracy results for Darunavir

Recovery level	Accuracy Darunavir					Average % Recovery
	Amount taken(µg/ml)	Area	Average area	Amount recovered (mcg/ml)	% Recovery	
80%	240	4662.113	**4671.027**	254.21	99.30	100.023
	240	4682.749				
	240	4668.220				
100%	320	5595.271	**5589.477**	321.69	100.53	
	320	5588.966				
	320	5584.193				
120%	400	6296.468	**6313.257**	384.94	100.24	

	400	6324.060				
	400	6319.242				

Table 4: Showing accuracy results for Cobicistat

Recovery level	Accuracy Cobicistat					Average % Recovery
	Amount taken (µg//ml)	Area	Average area	Amount recovered (mcg/ml)	% Recovery	
80%	45	558.057	**560.812**	48.45	100.94	100.61
	45	563.349				
	45	561.030				
100%	60	657.465	**657.722**	60.02	100.04	
	60	659.972				
	60	655.729				
120%	75	**751.964**	**755.928**	72.64	100.88	
	75	**755.857**				
	75	**759.963**				

Table 5: Result of Robustness study

Parameter	Darunavir		Cobicistat	
	Retention time(min)	Tailing factor	Retention time(min)	Tailing factor
Flow Rate				
1.0 ml/min	2.713	1.731	4.147	1.605
1.4 ml/min	2.083	3.140	3.140	1.668
Wavelength				
251nm	2.323	1.669	3.523	1.637
255nm	2.323	1.669	3.520	1.568

Table 6: Results for Method precision of Darunavir and Cobicistat

Darunavir		
S.No.	Rt	Area
1	2.32	5599.365
2	2.327	5626.643
3	2.323	5600.893
4	2.327	5606.288
5	2.317	5593.455
6	2.327	5593.358
avg	2.3235	5603.334
stdev	0.0043	12.417
%RSD	**0.18**	**0.22**

Cobicistat		
S.No.	Rt	Area
1	3.523	707.341
2	3.520	692.14
3	3.537	687.02
4	3.530	696.712
5	3.520	700.091
6	3.530	695.011
avg	3.527	696.386
stdev	0.007	6.950
%RSD	**0.19**	**1.00**

Table 7: Results for Ruggedness

Darunavir	%Assay	Cobicistat	%Assay
Analyst 01	100.51	**Analyst 01**	100.00
Anaylst 02	99.35	**Anaylst 02**	95.30

Table 8: Results for **LOD & LOQ**

Drug name	LOD (µg)	LOQ (µg)
Darunavir	19.07	57.78
Cobicistat	0.28	2.47

4. Conclusion

A new method was established for simultaneous estimation of Darunavir and cobicistat by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Darunavir and cobicistat by using C18 column (4.6×250mm)5µ, flow rate was 1ml/min, mobile phase ratio was (40:60 v/v) methanol: Buffer, detection

wavelength was 253nm. Precision and recovery studies were also found to be with the range.The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of Darunavir and cobicistat in pharmaceutical dosage form. The developed method was validated in terms of accuracy, precision, linearity, robustness and ruggedness, and results will be validated statistically according to ICH guidelines. The Sample recoveries in all formulations were in good agreement with their respective label claims. Hence the suggested RP-HPLC method can be used for routine analysis of Darunavir and cobicistat in API and Pharmaceutical dosage form

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