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SIMULTANEOUS DETERMINATION OF RILPIVIRINE AND CABOTEGRAVIR IN BULK AND PHARMACEUTICAL DOSAGE FORM: STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION

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ABSTRACT

A rapid stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous determination of Rilpivirine (RIL) and Cabotegravir (CAB) combination in API and pharmaceutical dosage forms. An octadecylsilane HPLC Column (Ascentis C18) with a five-micron particle size of 150 mm length and 4.6 mm internal diameter is used for analysis. A mixture of orthophosphoric acid buffer (0.1% OPA) and acetonitrile (ACN) solvent in the ratio of 3:2 is chosen as the mobile phase at a flow rate of 1.0 mL/min. A photodiode array detector was used at 257 nm for the detection. RIL and CAB solutions were analyzed in the range of 37.5 - 225 µg/mL and 25 -150 µg/mL respectively and the peak area response versus concentration curve obtained is rectilinear. The selectivity, specificity, linearity, robustness, accuracy, and precision were determined. The intended method was successful in the validation of the simultaneous determination of RIL and CAB in the pharmaceutical dosage form. The performance of the proposed method was found to be rapid and economical and is suitable for the QC and QA analysis.

Keywords: Reverse Phase - HPLC, Rilpivirine, Cabotegravir, Method Development, Validation, HIV Drugs.
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INTRODUCTION

Cabotegravir (CAB) and Rilpivirine (RIL) are suggested drugs in the treatment of human immunodeficiency virus type-1 infection (HIV-1) in adults.[1] Their chemical names are (3R,6S)-N-[(2,4-difluorophenyl)methyl]-10-hydroxy-6-methyl-8,11-dioxo-4-oxa-1,7-diazatricyclo [7.4.0.03,7]trideca-9,12-diene-12-carboxamide and 4-[[4-[4-[(E)-2-cyanoethenyl]-2,6-dimethylanilino]pyrimidin-2-yl]amino]benzonitrile respectively.[2,3] The chemical structures of the RIL and CAB are shown in the Fig.-1. The combination of these medicines is sold under the brand name Cabenuva. In this, RIL and CAB were packed together in two distinct vials. In these, CAB is an integrase strand transfer inhibitor that blocks the HIV's enzyme integrase, which prevents the integration of the HIV virus into the human cells' DNA. Hence, further spread of the virus is hampered as this is the necessary step for the virus to replication. RIL is a non-nucleoside reverse transcriptase inhibitor that binds to reverse transcriptase (NNRTIs), resulting in the prevention of activities related to RNA and DNA-dependent DNA polymerase.[4] Replication of viruses is one such activity. Molecular flexibility is another important asset for RIL to have multiple conformations, which makes it easy to bind with residues in the reverse transcriptase enzyme that has a reduced mutation rate.[5] Studies revealed that RIL and CAB are antiretroviral drugs that are suitable as long-acting injectable formulations.[6-9] In EU countries, this combination of drugs is suggested for the treatment of adults having less load of virus, generally, less than 50 copies/mL. United States Food and Drug Administration (FDA) has also approved this combination for medical use recently, in January 2021.[1] This combination is the first FDA-approved complete treatment for HIV-infected adults that is administered once a month in the form of injection.-The stability-indicating method is generally employed to study the stability of active pharmaceutical ingredients (API) under various conditions in which drugs undergo degradation.[10] In this procedure, degradation is detected and the change in concentration of various APIs in pharmaceutical products is determined.[11] Recently, reversed-phase HPLC has been the most used analytical technique because of its simplicity, versatility, and ability to analyze compounds of diverse polarity and molecular masses.[12] In this work, we successfully established an RP-HPLC method for the simultaneous determination

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of RIL, and CAB in pharmaceutical dosage form.

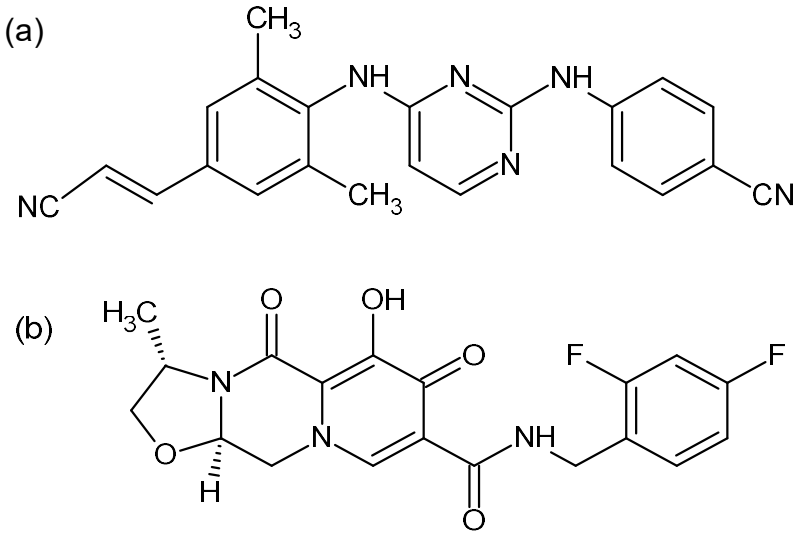

Fig.-1: Chemical Structures of (a) RIL and (b) CAB

EXPERIMENTAL

Chemicals and Reagents

Acetonitrile (Lichrosol), Merck Lifesciences Pvt. Ltd., and water, Thermo Fischer Scientific Pvt Ltd., of HPLC grade were used in this study. The working standards of Rilpivirine and Cabotegravir were provided by Hetero Pharma Ltd. Hyderabad, India as a generous gift. Cabenuva, is a co-packaged antiretroviral medication including Cabotegravir and Rilpivirine, which was acquired from a local marketplace in Hyderabad, India.

Instrumentation

HPLC waters acuity system provided with an in-built autosampler and photodiode array (PDA) detector was used for chromatographic separation and Empower-2 software was used for the analysis of eluents. Thermal degradation studies were carried out in a hot air oven. Photolytic degradation studies were carried out using a UV cross-linker in a UV chamber provided with a UV fluorescence lamp. Toshcon – Toshniwal made an ultrasonic bath, Adwa made an AD 1020 digital pH meter, and Lab India made a UV 3000 UV/VIS spectrophotometer for the analysis.

Operating Conditions of HPLC

Separation of analytes was achieved on Ascentis C18 HPLC Column (length of 150 mm and diameter of 4.6 mm), with octadecylsilane stationary phase, (5-micrometer particle size). Column temperature was maintained at 30 degrees Celsius for all determinations. A 10-microlitre sample was injected for the analysis. 0.1% OPA Buffer and ACN in the ratio of 3:2 is used as the mobile phase and a 1.0 mL/min flow rate is maintained while eluting the samples. Degassing was done for all the solvents, and solutions using ultra-sonication for 20 -25 min followed by filtered through 0.45 µm nylon (N66) 47mm membrane filter. Eluted components were monitored at 257 nm using a UV detector.

Preparation of Buffer, Mobile Phase, and Diluent

0.1% OPA buffer was prepared by taking one milliliter of o-phosphoric acid buffer solution in a 1000 mL standard flask and was diluted to 1000 mL using Milli-Q water. 0.1% OPA Buffer and Acetonitrile were mixed in the proportion of 3:2 for the mobile phase solution. All solutions used were filtered and degassed. The solution used for dilution was prepared by mixing Acetonitrile and water in a proportion of 1:1.

Preparation of Standard Solutions

75 mg of RIL working standard weighed with accuracy and the same is transferred into a 50 mL volumetric flask and 10 mL of diluent added to it. To get the homogeneous solution, it is subjected to sonication for

about 10-15 min and made to 50 mL by adding a diluent. The resultant solution concentration is 1500 µg/mL. Similarly, a standard solution of 1000 µg/mL CAB was prepared. From these solutions, 150 µg/mL and 100 µg/mL solutions of RIL and CAB were prepared in two separate volumetric flasks by diluting 1 mL of the stock solution to 10 mL.

Preparation of Sample Solution

1 mL of RIL injected into a 100-millilitre volumetric flask and approximately 50 mL of diluent solution was added and then sonicated for 20 - 25 minutes. A 100-millilitre solution was made by adding with diluent solution. Thus, 3000 µg/mL concentrated solution of RIL was prepared. Similarly, by injecting 1 mL of CAB, a 2000 µg/mL concentration of CAB solution was prepared. From these stock solutions, 0.5 mL was taken in a 10 mL volumetric flask and diluted to prepare 150 µg/mL RIL and 100 µg/mL of CAB.

Method Validation

The method was validated according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines.[13]

System Suitability Test

As per the USP general chapter on chromatography <621>, HPLC system suitability was optimized.[14] Standard solutions of RIL and CAB were analyzed in six replicate injections of 10 µL each using the chromatographic system. All the parameters were calculated to determine the system's suitability for the intended method.

Specificity

Standard solutions of RIL and CAB, blank, and placebo were analyzed in the specificity test to check out for any interference in the parameters such as retention time of RIL and CAB peaks with any other impurities.

Linearity

A series of the solutions of RIL and CAB with different concentrations were made by diluting the standard stock solutions. 225, 187.5, 150, 112.5, 75 and 37.5 µg/mL of RIL and 150, 125, 100, 75, 50, and 25 µg/mL of CAB solutions were prepared. Peak area vs concentration of RIL and CAB plots were plotted, and linearity was determined from the regression analysis.

Precision

The precision of the method was analyzed using a known concentrated solution of RIL and CAB by injecting 10 µL into the HPLC column repeatedly about six times a day. Also, the same samples were analyzed on dissimilar days.

Accuracy

To test the accuracy, a standard addition method was used. A known fixed volume of the standard RIL and CAB solutions was added to the blank solution at discrete levels of 50, 100, and 150%.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and limit of quantification (LOQ) were estimated using the Calibration curve method. A graph is plotted between the average area under the peak and the concentration. The slope method is used to calculate LOD and LOQ.

Robustness

The robustness of the analytical method was evaluated by modifying HPLC conditions slightly. Flow rate, Column temperature, and the mobile phase composition are the important parameters to check the robustness of the method.

Forced Degradation Study

Alkaline, acidic, oxidative stress, photostability, dry heat degradation, and neutral degradation analysis were carried out.

Alkali Hydrolysis
One milliliter of two normal sodium hydroxides was added to one milliliter of stock RIL and CAB solutions and heated to reflux for 0.5 hours at 60°C.

Acid Hydrolysis
One milliliter of two normal hydrochloric acids is added to one milliliter of stock RIL and CAB solutions and heated to reflux for 0.5 hours at 60°C.

Oxidative Stress
One milliliter of 20% hydrogen peroxide (H_2O_2) was added to 1 mL stock RIL and CAB solutions and heated to reflux for 0.5 hours at 60°C.

Photo Stability studies
A fixed volume of stock solutions of RIL and CAB stock solutions are subjected to UV Light in a UV Chamber for one week.

Dry Heat Degradation Studies
The standard drug solution was placed in an oven at 105°C for six hours and then analyzed.

Neutral Degradation Studies
one milliliter of HPLC grade water was added to one milliliter of stock RIL and CAB solutions and heated to reflux for 0.5 hours at 60°C. All these resultant solutions obtained in the degradation studies were diluted to get 150 µg/mL of RIL, and 100 µg/mL of CAB. 10 microlitres of these diluted solutions were introduced into the HPLC system and chromatograms were recorded.

RESULTS AND DISCUSSION

Optimization of the Method

The method development includes providing suitable conditions for the separation of RIL and CAB using an octadecyl silane column at an ambient temperature. Detection of the eluents was done at 257 nm using photodiode. The chromatogram recorded under the optimized conditions showed the appropriate and reliable retention times, good peak shape, and good resolution for RIL and CAB. The mobile phase was optimized first by taking different compositions of OPA buffer and Acetonitrile mixture. The adjusted mobile phase has of 3:2 v/v mixture of phosphate buffer (pH adjusted to 3.0) and Acetonitrile. Based on lower pKa values of RIL and CAB compounds, the mobile phase was selected. Later, the flow rate was optimized as 1.0 mL/min. Retention time observed was 2.345 min for RIL and 3.277 min for CAB (Figure 2). The sufficient retention of RIL and CAB is the basis for the selection of columns.

Validation of Optimized Method
ICH guidelines were followed to validate the proposed method[13]. Under optimized conditions, the retention times of RIL and CAB were obtained as 2.345 min and 3.277 min respectively. Peak symmetry was obtained at less than 1.5 for each peak and the theoretical plate numbers were observed greater than 2000, resolution is greater than 4, and the percentage of RSD calculated between areas obtained for six standard samples was lower than 2. Observations tabulated in Table-1 are in good agreement with the USP general chapter on chromatography <621>.[14] Interference of excipients in the pharmaceutical dosage form was evaluated from a prepared solution of placebo from which we found the method specifically determines the concentrations of RIL and CAB. Optimized chromatograms of RIL and CAB are shown in Fig.-2. The chromatogram indicates the potential of the proposed method to appraise the RIL and CAB in the pharmaceutical dosage form, i.e., in the presence of excipients. Near the retention times corresponding to the RIL and CAB, there were no peaks observed in blank and placebo samples. The chromatograms of blank and placebo are shown in Fig.-3. Linearity was evaluated for both RIL and CAB in the concentration varies from 37.5- 225 µg/mL and 25-150 µg/mL respectively. Solutions of 25, 50, 75, 100, 125, and 150% of 150 µg/mL of RIL component and 100 µg/mL of CAB component were prepared and analyzed. Table-2 comprises the peak area data corresponding to the concentrations of RIL and CAB. A graph was plotted by taking the peak area on the y-axis and the concentration of corresponding RIL or CAB on the x-axis. A

linear relation is observed through which an equation is extracted for RIL and CAB "y = 33881x + 63156" and "y = 30734x + 42936" respectively. The coefficient of regression (R^2) was 0.999 for both RIL and CAB which is on par with ICH guidelines.[13] To evaluate method precision, 150 µg/mL of RIL and 100 µg/mL of CAB were investigated by six replicate injections using the HPLC system in a day, and on different days. The results are tabulated in Table-3. %RSD data was found to be in the range of 0.2 to 0.7 which is within the limits (<2). The accuracy of the method was determined by analyzing the series of samples prepared by adding a fixed amount of the standard RIL and CAB to the blank sample at various levels such as 50, 100, and 150%. The mean recovery percentage of RIL and CAB was found to be between 98.73 - 100.64% and 98.51 - 100.80% respectively. The findings were tabulated in Table-4 indicating the good accuracy for the proposed method. The LOD values were obtained as 1.55 µg/mL and 0.46 µg/mL and the LOQ values obtained were 4.71 µg/mL and 1.39 µg/mL respectively for RIL and CAB. The LOD and LOQ were calculated using the different concentration solutions obtained by successive dilutions of RIL and CAB standard stock solutions and a signal-to-noise ratio of 3:1 for LOD and 10:1 for LOQ was observed. By modifying the conditions slightly from the optimized method, various samples of RIL and CAB were analyzed. The observations were tabulated in Tables-5. The results indicate the robustness of the method as we did not observe many significant changes with all variant conditions. Hence, the parameters in the proposed method were suitable and the method proposed is robust. Forced degradation studies are required to demonstrate to specificity of the method in identifying, separating, and quantifying the drug from its impurities formed under different conditions of stress. RIL and CAB were exposed to various stress conditions and the resultant solutions were analyzed. The results obtained after Acid hydrolysis, Alkali hydrolysis, oxidative degradation, thermal degradation Neutral degradation, and photolytic degradation studies were tabulated in Table-6 and the corresponding chromatograms were shown in supporting information. It is observed that the purity angle is always less than the purity threshold, which indicates the validity of the method.

Table-1: System Suitability Results

Parameter	RIL	CAB
Peak area	5065785	3072860
Theoretical plates	3012	3751
Retention time	2.345	3.258
Tailing factor	1.38	1.49
USP Resolution	4.6	

Table-2: Linearity data

Conc. RIL (µg/mL)	Peak area	Conc. CAB (µg/mL)	Peak area
37.5	1337225	25	811372
75	2717736	50	1669919
112.5	3897515	75	2346087
150	5051666	100	3056948
187.5	6442395	125	3905363
225	7676995	150	4646301
R^2	0.999	0.999	

Table-3: Method Precision Data

S. No.	RIL (150 µg/mL)		CAB (100 µg/mL)	
	Peak area	% Assay	Peak area	% Assay
1	5031658	99.23	3082219	100.74
2	5044110	99.47	3067177	99.02
3	4999386	98.59	3072860	100.30
4	5033201	99.26	3078351	100.18
5	5105629	100.69	3082563	99.86
6	5023281	99.06	3078351	100.27
Avg	5055433	99.38	3076920	100.06
SD	14816.75	0.70	5923.29	0.58
%RSD	0.3	0.7	0.2	0.6

Table-4: Method Validation Recovery Results of RIL and CAB

Name	RIL		CAB	
Sample name	% Recovery	Statistical Analysis	% Recovery	Statistical Analysis
50%	98.8	Mean =99.29	99.40	Mean =99.7
50%	99.7	SD =0.434	99.53	SD =0.48
50%	99.4	%RSD =0.44	100.29	%RSD =0.48
100%	98.7	Mean =98.22	100.61	Mean =99.88
100%	99.4	SD =1.4867	99.13	SD =0.74
100%	96.6	%RSD =1.51	99.89	%RSD =0.74
150%	100.1	Mean =99.90	99.98	Mean =99.95
150%	99.1	SD =0.6740	99.99	SD =0.05
150%	100.4	%RSD =0.67	99.90	%RSD =0.05

Table-5: Robustness Results

	Operating conditions			% Assay of	
	Flow rate	mobile phase	column temp	RIL	CAB
Low	0.8	60:40	30	99.01	100.52
	1.0	60:40	28	99.21	100.59
	1.0	62:38	30	99.08	100.37
As such	1.0	60:40	30	99.21	100.46
High	1.2	60:40	30	99.25	100.28
	1.0	60:40	32	99.11	100.53
	1.0	58:42	30	99.27	100.54

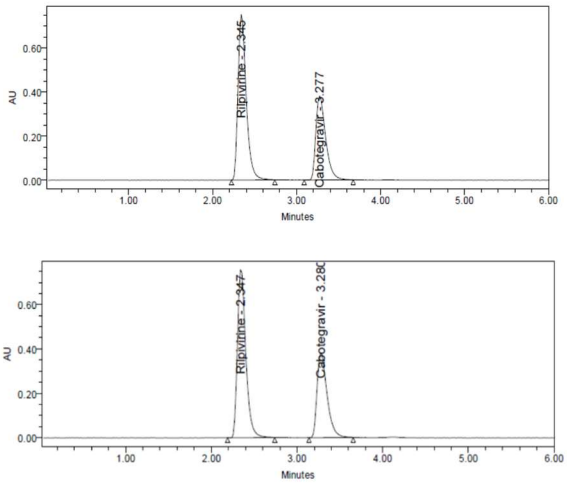

Fig.-2: Typical Chromatograms of (a) Standard RIL (150 µg/mL) and Standard CAB (100 µg/mL) (b) Typical Chromatograms of (b) Pharmaceutical Dosage RIL (150 µg/mL) and CAB (100 µg/mL)

Table-6: Forced Degradation Studies

Name	RIL			CAB		
Sample Name	(%)	Purity Angle	Purity Threshold	(%)	Purity Angle	Purity Threshold
Unstressed sample	-	0.243	0.428	-	0.358	0.584
Acid Degradation	95.18	0.284	0.419	94.95	0.382	0.540
Alkali Degradation	94.52	0.450	0.458	94.34	0.396	0.637
Peroxide Degradation	95.97	0.231	0.425	95.89	0.353	0.548
Thermal Stress	97.35	0.245	0.417	97.96	0.422	0.625
Photo Stress Sample	98.52	0.261	0.422	98.61	0.395	0.626
Water Stress	99.29	0.255	0.433	99.33	0.386	0.618

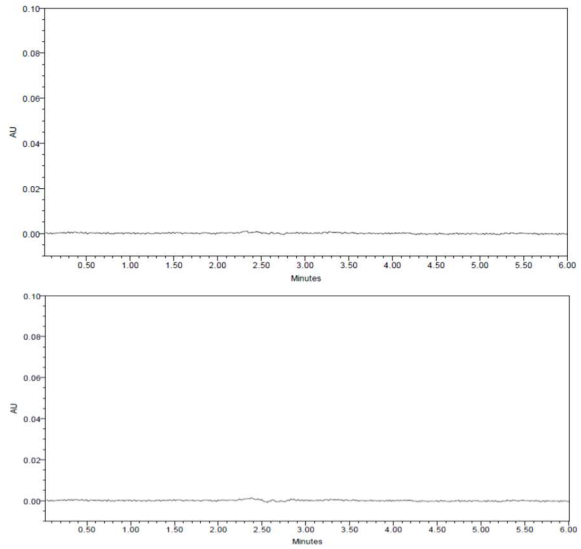

Fig.-3: Typical Blank Chromatograms of (a) Blank and (b) Placebo

CONCLUSION

RIL and CAB were analyzed with reliable and reproducible retention times, good peak shape, and good resolution. Linearity was proven in the range of 37.5-225 µg/mL for RIL and 25-150 µg/mL for CAB with R^2=0.999. The % recovery of RIL and CAB was observed in the range of 98.0-102.0%, which was inside the limits of the criteria of acceptance. The percentage RSD was in the acceptable range, less than 2%, proving that the method is precise. Hence, the proposed Reverse Phase HPLC method is found specific and robust for the simultaneous determination of RIL and CAB in both combined dosage forms.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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