How to Cite:
Pandya, Y., & Patel, S. (2022). RP-HPLC stability method development & validation for anti-HIV drugs cabotegravir & rilpivirine in I.M. injection and in human plasma. *International Journal of Health Sciences*, *6*(S1), 9104–9117. https://doi.org/10.53730/ijhs.v6nS1.7056

RP-HPLC stability method development & validation for anti-HIV drugs cabotegravir & rilpivirine in I.M. injection and in human plasma

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***Abstract*---**In Pharmaceutical & Medical Health Sciences there are necessities for development of analysis methods for medicines in various dosage forms. Currently the use of RP-HPLC is primarily applied for testing of medicines in various dosage forms, and to study bio analysis in human plasma matrix. The present method is developed for analysis of anti HIV drugs cabotegravir CAB and rilpivirine RILP in pure api & Intramuscular Injection dosage forms, and also in human plasma. The HPLC method is optimized for analysis of these two drugs in combined forms for swift analysis with very less amount of drugs utilized for testing purposes. The concentration range used for the linearity studies is 2.5 to 15µg/ml for CAB cabotegravir & for Rilpivirine RILP it is 3.75 to 22.5µg/ml. Wavelength selected for estimation is 242.5nm and column used was Kinetex C-18 column (250mm x 4.6mm, 5 µm id). The Retention-time obtained were 2.14min for CAB & 3.12min for RILP. The R^2 was found to be 0.999 for both drugs. The method is applied for analysis of drugs in i.m. injections, individually & in combined forms. The Stability & forced-degradations studies were carried out in different stress conditions and the impurities & pure drugs, are efficiently detected by the developed HPLC method.

***Keywords*---**RP-HPLC, Stability, Antiviral, HIV, Cabotegravir, Rilpivirine, I.M. Injection, Human Plasma

Introduction

In pharmaceutical analysis for the medicinal agents & dosage forms new sophisticated chromatographic methods are utilized for the quality control purpose. There are currently new medicinal agents are updated for the treatment therapy for various diseases. Majorly in the therapy for viral infectious diseases Novel antiviral drugs and their combinations are repeatedly developed & synthesised for the therapy for viral diseases. Even currently the use of the antiviral agents has been extensively used for the management of newer diseases like AIDS, Hepatitis, COVID-19, and many other infectious diseases. (McPherson et al., 2018)

The newly developed antimicrobial agents like Cabotegravir CAB integrase inhibitor (Overton et al., 2020) & Rilpivirine RILP NNRIT Non Nucleoside Reverse Transcriptase inhibitor (De Clercq, 2012) are been widely applied in therapy for diseases like HIV infections (Durham & Chahine, 2021), (Fernandez & van Halsema, 2019) also for hepatitis. They are available in tablet oral dosage form as well as in i.m. injections. In pharmaceutical industries there are different individual methods of analysis for these drugs.

The literature reviews also suggests the individual and other combinational HPLC methods (Ramöller et al., 2022), (Vejendla et al., 2021), (Kovač et al., 2022) of these drugs, but the methods are for single drugs estimations as well as for the other drug combinations, and not available in combined i.m. injectable dosage form along with stability HPLC method. Hence there is a need for the rapid testing of these drugs by one single HPLC method, as other methods are for individual estimations as well as in combinations only. This HPLC method is developed in which drugs CAB & RILP are analysed and assayed in combined Injection form. The CAB is HIV Integrase Inhibitor class of drug , while RILP NNRTI Non Nucleoside Reverse Transcriptase Inhibitor. (Howe et al., 2021) They act by blocking the viral DNA replication in the hosts and are highly potent drugs of the antiviral class (Margolis et al., 2015), (Wang et al., 2019).

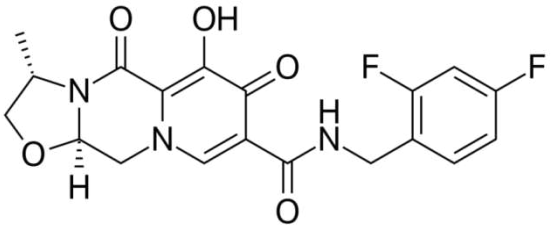

Figure 1. Structures of Cabotegravir

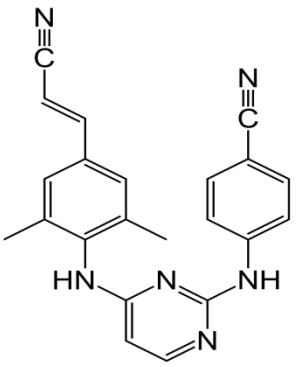

Figure 2. Structures of Rilpivirine

Materials and Methods

Materials

The Working Reference standards Cabotegravir CAB & Rilpivirine RILP have been obtained from Solesom & Bizotech pharma. The chemicals and reagents Methanol, Acetonitrile ACN, orthophosphoric acid, potassium-sodium-dihydrogen ortho phosphate, Azobisisobutyronitrile AIBN, has been used analytical grade Merck graded. HCl, NaOH, Trifluro-acetic acid, analytical grade of Rankem were used. Human plasma serum procured from red cross blood bank. Milli-Q pure water has been used for sample and mobile phase preparations

Instrumentation

Shimadzu HPLC system Class VP 2010 auto sampler & Agilent 1100 both systems has been used for the analysis equipped with both PDA & UV detector. Shimadzu UV 1800 spectrophotometer had been utilized for the wavelength maxima estimation. Wist Temperature Chamber was used for thermal degradation study. Photostability Test Chamber Sanwood SM-LHH-GSD-UV Series was utilised. Thermo scientific Heraeus refrigerated centrifuge was used.

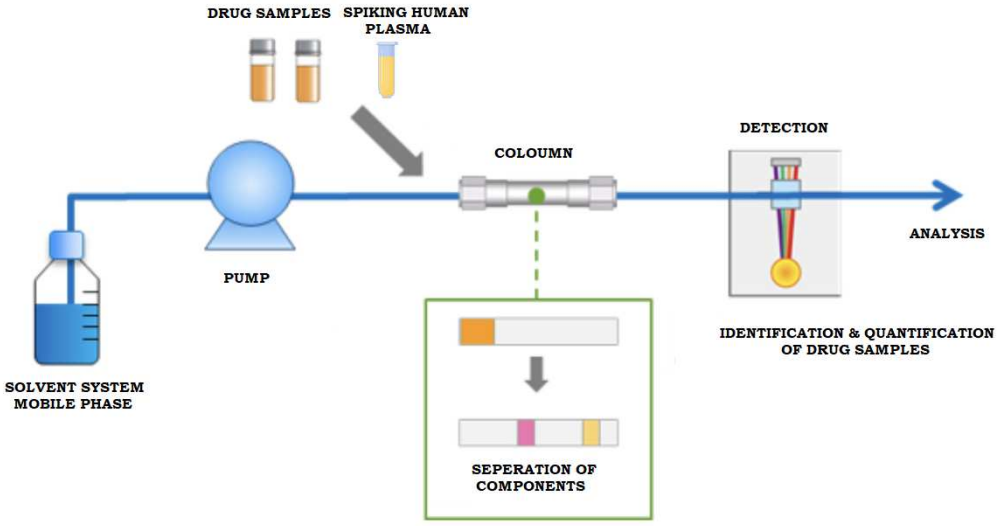

Figure 3. Chromatographic Analysis Flow Chart

Chromatographic conditions

The separation of the drugs Cabotegravir CAB & Rilpivirine RILP has been made by using two columns. Kinetex C-18 column & Hypersil ODS (250 mm x 4.6 mm, 5 µm id) for consistent results. The mobile phase is been utilised consists of ratio of % Percentage by volume of Acetonitrile ACN (35) : (65) Sodium Dihydrogen Phosphate buffer (0.05M) of pH 5.5. The flow rate adjusted 1ml/min and

detection wavelength was 242.5nm. The coloumn- temperature column oven kept at 25 ^{0}C.

Preparation of Solutions

Standard Solutions

The standard solutions of the three drugs working reference standards was prepared at concentrations range of 2.5 to 15 µg/ml for CAB cabotegravir & for Rilpivirine RILP it is 3.75 to 22.5 µg/ml

Sample Solution

The sample solutions were prepared from injections vials of the drugs CAB 400mg in 2ml inj vial & RILP 600mg in 2ml vial. And another second type vials contains CAB 600mg in 3ml inj vial & RILP 900mg in 3ml vial. (i.e. each vial type contains CAB 200mg/ml and RILP 300mg/ml). The contents of vials were dissolved in methanol 50 : acetonitrile 50 v/v ratio, sonicated, centrifuged & filtered off to give stock solutions of CAB & RILP. The final dilutions were prepared with mobile phase as 2.5 to 15 µg/ml that is 2.5, 5, 7.5, 10, 12.5, 15 µg/ml for CAB cabotegravir & for Rilpivirine RILP it is 3.75 to 22.5µg/ml that is 3.75, 7.5, 11.25, 15, 18.75, 22.5 µg/ml and used for linearity ranges.

The sample of standard concentration was also prepared by spiking with 100µl of human serum plasma in both standard and samples to give concentrations same as mentioned in here. The drug solutions dilutions as mentioned above were spiked with plasma were deproteinated by adding trifluoro-acetic acid solution and mixture vas vortexed, mixed and centrifuged at 2-4 ^{0}C at 10,000 rpm for 20mins. The supernatant solutions were filtered & used for the analysis purposes and injected in HPLC instrument. The final conc. of drug solutions were as per linearity. A separate blank solution of plasma serum were prepared similarly and utilised for analysis.

Method Validation

The analytical method is developed, for drugs Cabotegravir CAB & Rilpivirine RILP is been validated ICH guideline (ICH, 2005) in the terms of precision linearity accuracy LOD LOQ which are required to justify the purpose of the developed method. By employing the use of two columns Kinetex C-18 column & Hypersil ODS (250 mm x 4.6 mm, 5 µm id) for the separation and resolution of these drugs has been efficiently made. The developed analytical method proves to be more quick rapid and efficient on the basis of the validation as per the ICH guideline protocols. (I. C. H. Guideline, 2005)

System Suitability

The RP-HPLC method is an very accurate and precise method following the system suitability parameters as per the USP and ICH protocols. The system suitability has been justified from the data of the retention time is 2.14min for CAB & 3.12min for RILP of the drugs as well as on the basis of the tailing factors

of the chromatograms and from the values of the theoretical plates can be treated as accurate and efficient for the analysis purposes. System suitability parameters are summarised in results discussions Table 1 and Optimised chromatographic conditions are shown in Table 2.

Specificity

The chromatogram of the standard reference drugs it was found to be accurate for the individual drug analysis as well as in the combination, and no other impurity or other analyte found to be overlapped in the chromatogram. It was confirmed by individual drug injecting multiple times to confirm that method is highly specific for analysis. Also the placebo blank plasma serum spiked was injected individually for the sample analysis and it does not interfere in the chromatogram seen in Figure 5,7,8.

Linearity

The linearity of the drug response chromatogram Figure 6 shows that variable concentration has been found to be at the range of 2.5 to 15 µg/ml for CAB that is 2.5, 5, 7.5, 10, 12.5, 15 µg/ml and for RILP is 3.75 to 22.5µg/ml that is 3.75, 7.5, 11.25, 15, 18.75, 22.5 µg/ml mentioned in Table 3. The R^2 of both drugs was found to be 0.9999. The dugs CAB & RILP were individually & together optimized, so as to efficiently reduce the cost of the utilization of the working standard analyte drugs.

Forced Degradation Studies

The HPLC method is moreover applicable in the stability study (I. H. T. Guideline, 2003) during forced- degradation stress studies (Singh & Bakshi, 2000) of the drug products and drug substances. It was carried out by ICH Q1 AR2 guidelines (I. H. T. Guideline, 2003) in which the degradation of these drug substances was been made out, - by different- stress conditions like Acid, Alkali, Hydrolytic, Thermal and Photo degradation for the drugs substances. (I. H. T. Guideline, 2003) In the forced degradation study the 0.1N HCl was utilised and the drug samples were subjected for degradation for at different time intervals of 60, 120 minutes and also on a thermostat heat bath at 60^0C temperature to accelerate the degradation process. Further Degradation was also carried out at 1N HCl, 2N HCl to analyse higher rate of degradations. Identical process was carried out in alkaline degradation by using 0.1N NaOH at 60, 120min time intervals and higher rate degraded by using 1N NaOH & 2N NaOH. The control and the samples were neutralized and then analyzed by the developed HPLC method,

The Thermal degradation was carried out in a controlled oven Wist Temperature Chamber at different ranges of 60, 80, 100 ^{0}C, and the samples were analysed at time intervals of 60mins and at longer durations 120mins, 180mins to 5hrs for analyzing higher degradation rates. Oxidative stress was applied by using 3% H_2O_2 and parallel with 0.01M Azobisisobutyronitrile AIBN for the degradation of the drug samples for 60,120 minutes has been done.

Photo UV stress has been applied by putting the drug samples in Photostability Test Chamber Sanwood SM-LHH-GSD-UV Series , Photolytic (1.2million lux hrs

and 200watt hrs)**,** the time interval was 6hrs, 12hrs, 24hrs and 48hrs to check the degradation pattern in longer duration of time. The samples and standard of different degradation methods were filtered through nylon membrane 0.45um and injected individually as well as in the combined forms. The developed HPLC method efficiently detects the drug samples in the chromatograms Figure 9,10,11,12 and can be compared with the reference standard chromatograms and % drug recovered in Table 5. The major peaks of individual drugs are unaffected by impurity peaks, and can be efficiently resolved with peak purity analysis in Figures 13,14 & Table 4 shows that justifies no interference, merging or overlapping of other peaks.

Precision

The developed method is validated and has been marked precise as per the validation parameter performed in this method. Different samples and standards in six sample and standards were injected multiple times and the SD and RSD was determined. The assay results were also performed and checked for each drug. Data shows the how the method is precise in Table 6

Accuracy

The accuracy studies- has been carried out as per the guideline at three different levels. The level of 50, 100 and 150 % has been made,- for justifies accuracy for developed method. For the api pure standard and the test samples the accuracy study had been done for showing accurate performance of the developed method shown in Table 7.

Assay of Marketed Formulations:

The foremost purpose of this method is to develop a quick & specific single assay,- method for the i.m. injections in vials. Each vial contains CAB 200mg/ml and RILP 300mg/ml. CABENUVA™ : (Cabotegravir 200 mg/1ml & Rilpivirine 300 mg/1ml) ER-Injection. CABENUVA 400-mg/600-mg Kit ER-Injection:- consists of single dose vial of 400 mg in 2ml (200 mg/1ml) cabotegravir & a single dose vial of 600 mg in 2 ml (300 mg/1ml) rilpivirine. CABENUVA 600-mg/900-mg Kit ER-Injection:- consists of single dose vial of 600 mg in 3ml (200 mg/1ml) cabotegravir & a single dose vial of 900 mg in 3ml (300 mg/1ml) Rilpivirine. The assay method is developed for both the Kits, individually & in combination of CAB 200mg + 300mg RILP. Assay results are Shown in the Table 8 & 9.

Results and Discussions

The New Rapid; Precise; Accurate, RP-HPLC method is been successfully developed for the Cabotegravir and Rilpivirine within a very short run time within 5 minutes & the drugs can be qualitatively and quantitatively analysed along with the stability studies.

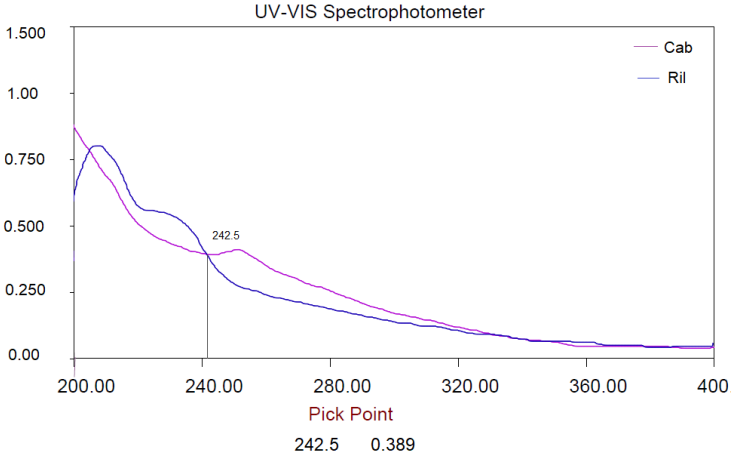

Figure 4. UV overlay Spectra of CAB & RILP 242.5nm wavelength for estimation

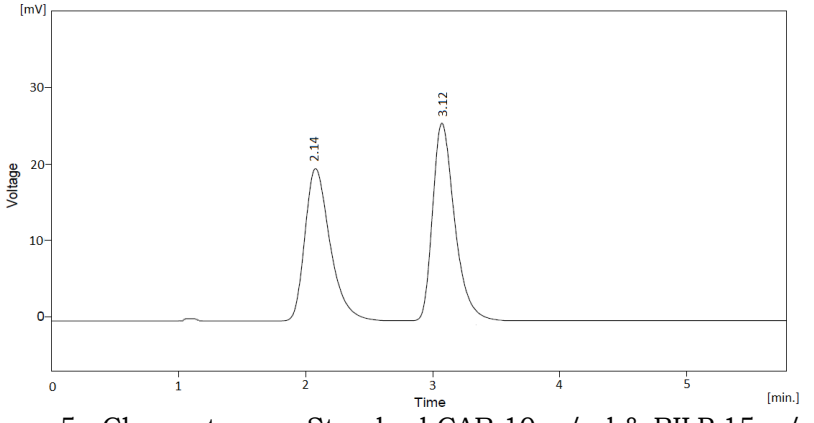

Figure 5. Chromatogram Standard CAB 10µg/ml & RILP 15µg/ml

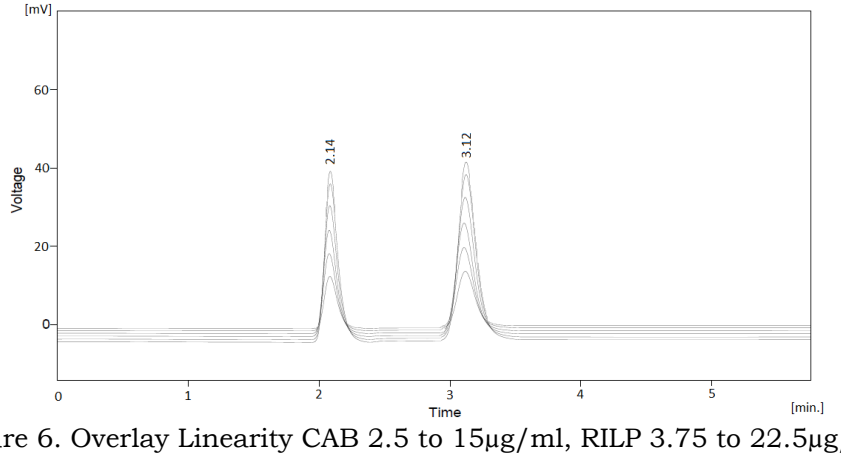

Figure 6. Overlay Linearity CAB 2.5 to 15µg/ml, RILP 3.75 to 22.5µg/ml

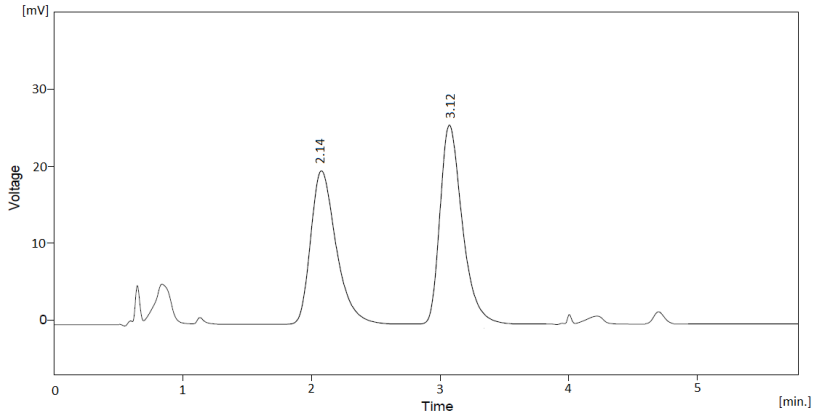

Figure 7. Chromatogram Spiked with plasma CAB 10µg/ml & RILP 15µg/ml

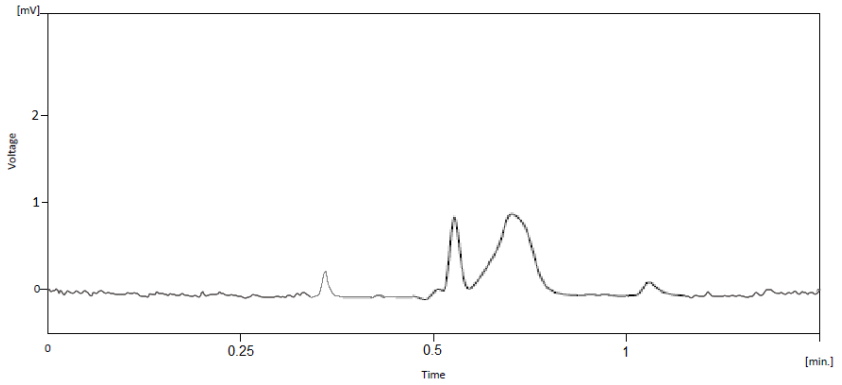

Figure 8. Chromatogram of Blank Spiked with plasma

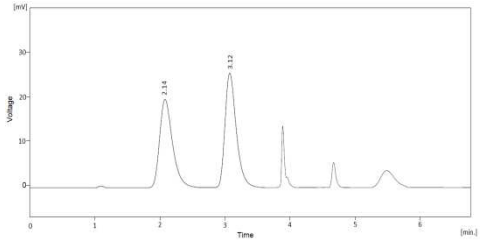

Figure 9. Acid Degradation

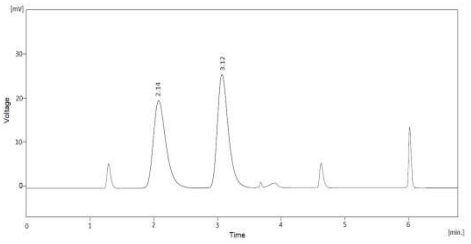

Figure 10. Base Degradation

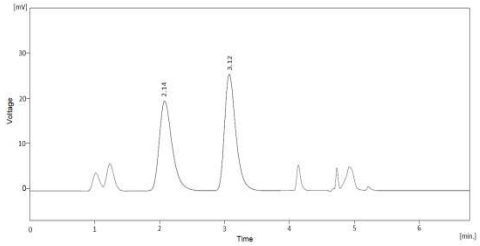

Figure 11. Base Degradation

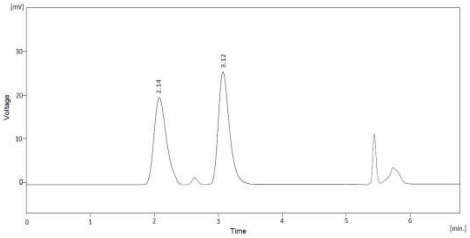

Figure 12. Oxidative Degradation

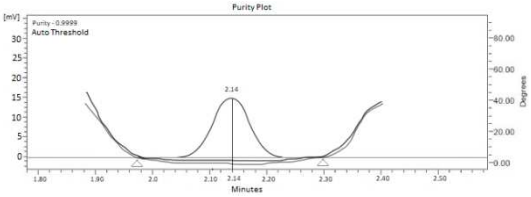

Figure 13. Peak Purity of CAB

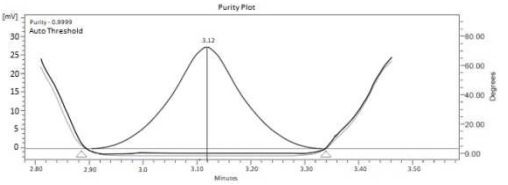

Figure 14. Peak Purity of RILP

Table 1
Summarized Results for The Developed HPLC Method

Serial No	Parameters	Criteria of Acceptance	Results
1	System Suitability	Theoretical Plates NLT 2000	CAB- 15412 RILP- 17644
		Tailing Factor NMT 2.0	CAB- 1.12 RILP- 1.14
		Retention Time Min	LAM- 2.14 RILP- 3.12
2	Precision	% RSD NMT 2.0	CAB- 0.74 RILP- 0.58
3	Linearity	Correlation Coefficient NLT 0.999	LAM- 0.9999 RILP- 0.9999
4	Accuracy	% Recovery 98-102%	CAB- 99.44 RILP- 100.23
5	LOD	1:3 (Conc. in µg/ml)	CAB- 0.263 RILP- 0.202
6	LOQ	1:10 (Conc. in µg/ml)	CAB- 0.798 RILP- 0.613
7	Assay	% Label Claim	CAB- 99.80 RILP- 99.95

Table 2
Optimised Chromatographic Conditions

Parameters	Chromatographic Conditions	Parameters	Chromatographic Conditions
Mobile Phase Ratio	Acetonitrile ACN (35) : (65) Sodium Dihydrogen Phosphate buffer (0.05M) of pH 4.5	Coloumn Temp	25 ^{0}C
Coloumn	Kinetex C-18 column & Hypersil ODS (250 mm x 4.6 mm, 5 µm id)	Wavelength	242.5nm
Detector	PDA & UV	Flow Rate	1ml/min

Table 3
Linearity Data of CAB & RILP

Linearity	CAB		RILP	
	Conc. µg/ml	Area	Conc. µg/ml	Area
1	2.5	561.47	3.75	612.75
2	5	1126.34	7.5	1235.87
3	7.5	1722.36	11.25	1840.25
4	10	2250.33	15	2451.298
5	12.5	2812.21	18.75	3089.19
6	15	3385.31	22.5	3675.46

Table 4
Stress Degradation Peak Purity Data

Conditions	Peak Purity Angle		Peak Purity Threshold		Peak Purity	
	CAB	RILP	CAB	RILP	CAB	RILP
Acid	0.113	0.123	0.295	0.298	0.999	0.999
Base	0.134	0.134	0.316	0.314	0.997	0.998
Oxidative	0.139	0.133	0.346	0.366	0.998	0.999
Thermal	0.126	0.136	0.311	0.297	0.999	0.998
Photolytic	0.134	0.129	0.321	0.388	0.999	0.998
Hydrolytic	0.137	0.211	0.346	0.347	0.998	0.999

Table 5
Stress Degradation Study Summarized Data

Degradation Condition	Peak Area		% Drug Recovered		% Degraded	
	CAB	RILP	CAB	RILP	CAB	RILP
Acid	2248.32	2249.36	99.90	99.92	0.08	0.07
Base	2238.23	2438.32	99.46	99.47	0.53	0.52
Oxidative	2233.02	2428.23	99.23	99.05	0.76	0.94
Thermal	2232.12	2437.66	99.19	99.44	0.80	0.55
Photolytic	2229.32	2433.25	99.06	99.26	0.93	0.73
Hydrolytic	2248.22	2430.21	99.09	99.13	0.09	0.86

Table 6
Precision Repeatability Data

Number of Injections	CAB Area 10 µg/ml	RILP Area 15 µg/ml
1	2250.33	2451.29
2	2252.46	2452.12
3	2255.31	2455.32
4	2253.88	2459.67
5	2255.98	2454.41
6	2253.44	2449.22
AVG	2253.56	2453.67
SD	2.03	3.66
% RSD	0.09	0.14

Table 7
Accuracy Data at Three Different Levels

Drug	Sample Amount µg/ml	% Conc. Level	Total Area	Net Area	Amount STD Added µg/ml	Amount STD Recovered µg/ml	% Recovery	Mean Recovery
CAB	10	50	3350.66	1100.32	5	4.88	97.79	99.44
		100	4513.45	2263.11	10	10.05	100.56	
		150	5624.98	3374.64	15	14.99	99.97	
RILP	15	50	3679.21	1227.91	7.5	7.51	100.18	100.23
		100	4910.24	2458.94	15	15.04	100.31	
		150	6135.65	3684.35	22.5	22.54	100.20	

Table 8
Assay of Drugs in Cabenuva 400-mg/600-mg Kit ER – Injection
Contains of vial of 400mg CAB in 2ml (200 mg/1ml) & 600mg RILP in 2 ml (300 mg/1ml)

	Sample No	Label Claim (mg)	Result (mg)	% Label Claim	% Avg Assay	SD	% RSD
CAB	1	400	399.59	99.89			
	2	400	399.15	99.78	99.80	0.08	0.08
	3	400	398.89	99.72			
RILP	1	600	599.70	99.95			
	2	600	598.99	99.83	99.95	0.12	0.12
	3	600	600.44	100.07			

Table 9
Assay of Drugs in Cabenuva 600-mg/900-mg Kit ER – Injection
Contains of vial of 900mg CAB in 3ml (200 mg/1ml) & 900mg RILP in 3 ml (300 mg/1ml)

	Sample No	Label Claim (mg)	Result (mg)	% Label Claim	% Avg Assay	SD	% RSD
CAB	1	600	598.33	99.72			
	2	600	599.74	99.95	99.64	0.35	0.35
	3	600	595.57	99.26			
RILP	1	900	894.23	99.35			
	2	900	898.72	99.85	99.52	0.28	0.28
	3	900	894.23	99.36			

The method is helpful in the assay analysis for the drugs CAB & RILP, having % Assay results CAB 99.64-99.80% & RILP 99.52-99.95% as compared with the label claimed. The method has very accurate working response within very lower range of concentration of 2.5 to 15µg/ml for CAB and 3.75 to 22.5µg/ml for RILP in individual as well as in the dosage forms. And in the spiked plasma matrix also shows that method is sensitive, no interference of plasma matrix and can be quantified in plasma serum samples. This helps in the detection of the analyte drugs by using less amount of the working standards making the method very cost effective.

Conclusion

The developed analytical method is able to detect the drugs Cabotegravir CAB, Rilpivirine RILP in individual as well as in the samples vials and also in the combined dosage forms with the accuracy and precision parameters as per the ICH guideline. The Stability method i.e. forced degradation study helps to understand the different impurities, degraded products, generated in stress conditions and the method effectively detects the drug analytes pure peaks, without any interference of other peaks. Also the analytical method is successfully validated as per the ICH guidelines and is useful for the assay, recovery study, from the marketed formulations and in in-vitro serum plasma matrix studies. The HLPC run time is 5mins which is beneficial and time saving for the quick analysis of the drugs in different individual dosage-forms as well as in the combinations.

Ethical approval
No ethical approval or permissions are required for this research work

Acknowledgments

The authors favorably thank the contributors and co-workers in this research work, and under the guidance of Dr Samixa Patel without their support and acquaintance it would be a difficult task. The authors are also thankful to the industrial support from Bizotech life and Solisom pharma care for providing materials, chemicals, equipments and instrumentation facilities for the completion of the research.

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