

Chapter 6**HPLC METHOD DEVELOPMENT AND
VALIDATION FOR CABOTEGRAVIR AND
RILPIVIRINE****6.1 EXPERIMENTALS****6.1.1 Instruments Utilised**

The Shimadzu-HPLC system LC-20-AT-system with LC-Solution and Peak chrom software with both PDA & UV detector. Stationary phase column in reverse phase has been used C-18-Hypersil-BDS & -ODS-250 x 4.6 mm, 5 micron size has been selected.

Systronics UV-visible spectrophotometer was used along with other Shimadzu UV 1800 spectrophotometer & Systronics UV for the wavelength maxima estimation. FTIR Spectrometer Shimadzu 8400 series has been utilised for identification of drugs standard samples. Melting point apparatus Labtronics was used for melting point determinations.

Wist Temperature Chamber was used for drying the drug samples and thermal degradation study. Ultra-sonicator Lab Branson ultrasonic's corporation was utilised. Digital pH meter labtronics was utilised.

Photostability Test Chamber Sanwood SM-LHH-GSD-UV Series was utilised.

Electronic analytical balance AUX-220 Shimadzu has been used. Borosil glass-ware volumetric flasks measuring cylinder pipettes of analytical were used. 0.22 and 0.45 µm nylon Millipore filters caps were used.

6.1.2 Materials and Reagents Utilised

The chemicals used working reference standard drugs Cabotegravir CAB & Rilpivirine RILP drugs samples of solisom & upcare pharma has been utilised. Acetonitrile, Methanol, potassium dihydrogen ortho phosphate, orthophosphoric acid, used analytical HPLC Merck grade. H₂O₂, HCl, NaOH analytical grade of Rankem used. Milli-Q pure water is utilized.

6.1.3 Identification of Standard Drug Samples

6.1.3.1 Melting Point Determination

The working standard drugs Cabotegravir CAB & Rilpivirine RILP were identified by melting point determination. Melting point apparatus used was made of Labtronics™ Melting Point Apparatus. The melting points observed for the standard drug samples are shown in the Table 3.1.

Drug	Observed Melting Range	Standard Melting Range
CAB	241.42-244.5 °C	241-243, 248.49 °C
RILP	249.9-251.36 °C	248-251 °C

Table 3.1: Melting Points of CAB & RILP

6.1.3.2 FTIR Spectral Determination for Identification Standard drug samples CAB & RILP

The pure active pharmaceutical working standard drug substances CAB & RILP were scanned between 400-4000 cm^{-1} in FTIR Spectrometer Shimadzu 8400 series. The drug dry powder samples were made die pressed pellets with KBr and the FTIR spectra were determined shown in Fig 3.1 for CAB, Fig 3.2 for RILP. The principal IR peaks recorded and observed for the drugs are shown in Table 3.2, 3.3 for CAB & RILP respectively.

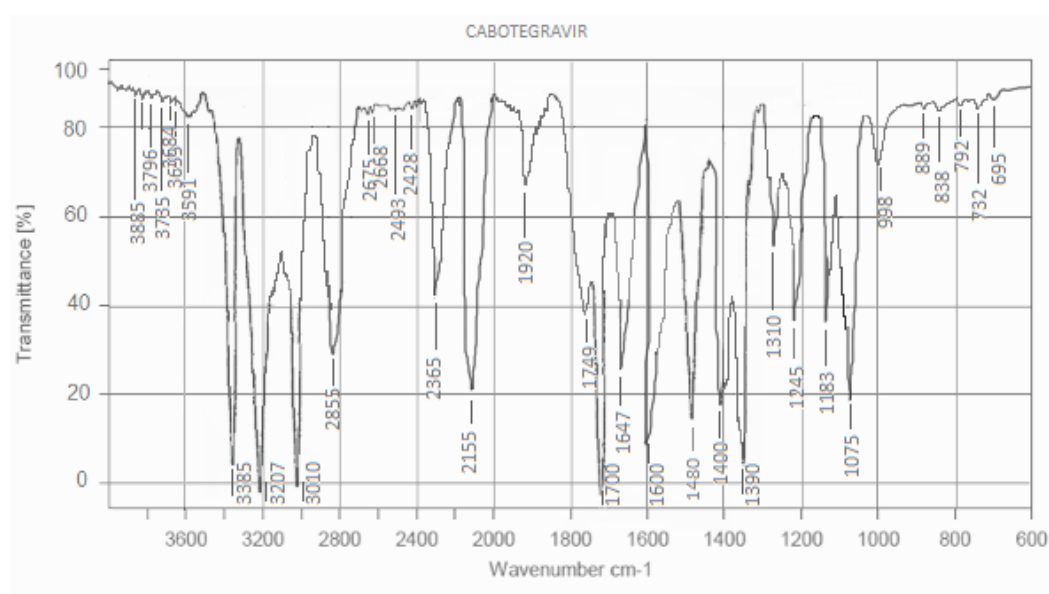


Figure 3.1: FTIR Spectra of Cabotegravir CAB

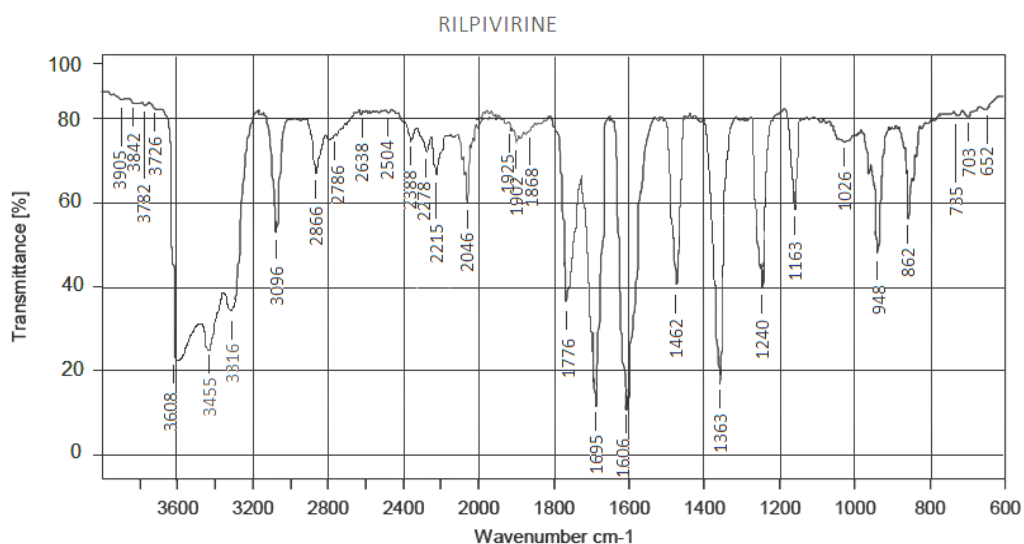


Figure 3.2: FTIR Spectra of Rilpivirine RILP

CABOTEGRAVIR					
Energy (Cm ⁻¹)	Band Assignment	Peak Intensity	Energy (Cm ⁻¹)	Band Assignment	Peak Intensity
1400-1390-1310	-OH phenolic	19.65 54.29	1680-1640-1630	C=O Amide	24.38
1183	C-O	38.25	2000-1650	C-H Aromatic	68.23
1600-1647-1700	C=C (Aromatic)	14.02	3000-2840	C-H Aliphatic	27.59
1749-1792 1275-1200	C-O (Ether)	39.26 38.74	1690-1640	C=N imine	14.02
2350.21-2360	N-H (S)	41.37	3400-3300	N-H Sec amine	4.67
1400-1000	-CF	19.65 7.47	1250-1020	C-N amine	19.57
1600-1700	C=C	9.63	1183	C-O	38.25

Table 3.2: FTIR Interpretation of Cabotegravir CAB

RILPIVIRINE					
Energy (Cm^{-1})	Band Assignment	Peak Intensity	Energy (Cm^{-1})	Band Assignment	Peak Intensity
1235-1268	C-N (S)	40.04	200-1650	C-H Aromatic	58.72
1675-1665	C=C Alkene	41.86	1250-1020	C-N Amine	76.48
1690-1640	C=N imine	16.56	2240-2200	-CN Aromatic Nitrile	66.25
1462	C-H Aromatic	41.23	3000-2800	C-H Methyl group	56.42
1500-1700	N-H (amine)	38.34	890-950	C=N Aromatic	48.67
3350-3310	N-H ₀ (2 Amine)	18.62	1675-1665	C=C Alkene	41.87

Table 3.3: FTIR Interpretation of Rilpivirine RILP

6.1.4 Preparation of Solutions

6.1.4.1 Preparation of standard solutions of CAB & RILP

The standard stock soln. individual drugs prepared in 50:50 Methanol : ACN solvent mixture. 10mg of CAB & 30mg of RILP were individually dissolved in solvent mixture and made upto 100ml soln with same solvent to give 100 $\mu\text{g/ml}$ standard stock solution of CAB were prepared and for RILP , 30mg RILP was dissolved in solvent mixture and made upto 200ml to give 150 $\mu\text{g/ml}$ RILP standard stock solution.

From the above stock solutions of individual drugs CAB & RILP each, 10ml from each was taken individually and diluted upto 100ml in individual volumetric flasks to give CAB 10 $\mu\text{g/ml}$, RILP 15 $\mu\text{g/ml}$, individual drug standard Final solutions.

6.1.4.2 Preparation of Sample Solutions

CABENUVA™ Each Vial contains CAB 400mg/2ml (200mg/ml) & RILP is 600mg/2ml (300mg/ml)

2ml from each vials, were taken containing CAB 400mg/2ml & RILP 600mg/2ml, was taken and Dissolved in 50ml Methanol : ACN (50:50), sonicated, filtered and make up to 100ml with Methanol : ACN (50:50) (Stock solution A) [4000:6000 ug/ml CAB:RILP]

From the Stock solution A, 2.5 ml was taken, diluted with mobile phase upto 100ml to give Solution B [100:150ug/ml CAB:RILP]

From the Solution B, 1ml was taken, diluted with mobile phase upto 10ml to give Final Solution C [10:15ug/ml CAB:RILP] used for analysis.

6.1.4.3 Preparation of Optimized Mobile Phase

The mobile phase made by taking 65:35 ratio, 0.05M Phosphate buffer : ACN of pH 5.5. The phosphate buffer was prepared by accurately weighing 6.8gm KH_2PO_4 (MW. 136) in 1000ml HPLC grade milli-Q system purified water. The pH adjusted by 1% OPA Ortho-phosphoric acid. After filtration it was sonicated and the 1% OPA was prepared by taking (1.176ml) of 85%w/v orthophosphoric acid (MW 98) in 100ml HPLC grade water.

6.1.5 Selection of Wavelength for Detection

The Final standard solns of CAB 10 $\mu\text{g/ml}$, & RILP 15 $\mu\text{g/ml}$ scanned in 200 - 400 nm in UV-visible double beam spectrophotometer at a medium at scanning speed. The overlain spectra shown in Fig. 3.3 of CAB 30 $\mu\text{g/ml}$, & RILP 15 $\mu\text{g/ml}$ were taken in 50:50 Methanol : ACN and the 242.5nm wavelength was selected for estimation in the detection during the HPLC analysis.

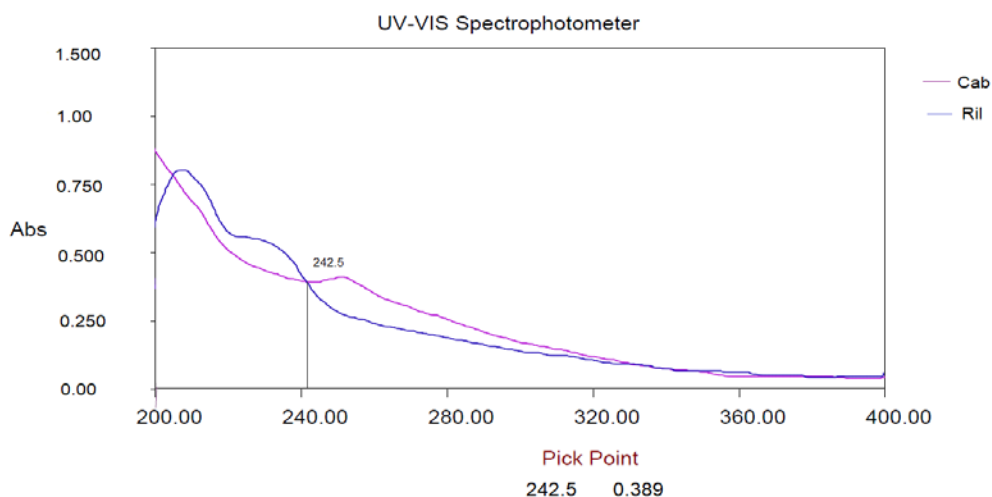


Figure 3.3: UV Spectra Overlay of CAB & RILP

6.1.6 Selection and Optimization of Mobile phase

For the detection analysis of the CAB & RILP drugs in the combined form in the working standard solutions by the HPLC method had been carried out in reverse phase by using polar solvents in mobile phase. The various trials with different mobile phase's has been carried out for the detection and separation of the drugs was carried out shown in Table 3.4

Sr No	Mobile Phase	pH	Ratio (v/v)	Retention Time (min)		REMARK
				CAB	RILP	
1	H ₂ O:MeOH	-	20:80	-	-	No peak detected
2	H ₂ O:MeOH	-	40:60	-	-	No peak detected
3	H ₂ O:MeOH	-	50 : 50	-	-	No peak detected
4	0.05 M Phosphate buffer : Methanol	7	80:20	11.71	11.20	Longer Run time, Peak Merged

5	0.05 M Phosphate buffer : Methanol	7	50:50	13.49	14.08	Longer Run time, Peak Merged and Tailing
6	0.05 M Phosphate buffer : Methanol	7.5	80:20	11.89	12.19	Longer Run time, Peak Merging and Tailing
7	0.05 M Phosphate buffer : Methanol	7	50:50	9.29	9.13	Peak Tailing
8	0.05 M Phosphate buffer : Methanol	8	80:20	8.64	10.37	Longer Run time, Tailing in peaks
9	0.05 M Phosphate buffer : Methanol	7.5	60:40	5.49	5.73	Peak tailing & merging
10	0.05 M Phosphate buffer : Methanol	6	60:40	4.24	4.57	Not good separation Peak merging
11	0.05 M Phosphate buffer : ACN [Selected Mobile phase]	5.5	65:35	2.14	3.21	Good Separation Less retention time

Table 3.4: Trials for Selection of Mobile Phase for CAB & RILP

6.1.7 Optimized Chromatographic Conditions

Optimized chromatographic conditions for developed HPLC analytical method are shown below-

Parameters	Conditions
Stationary Phase Coloumn	C18 Hypersil BDS 250 x 4.6mm , 5 micron
Mobile phase	Phosphate buffer : ACN 65:35 pH- 5.5
Flow rate	1ml/minl
Injection volume	20ul
Temp	Ambient Lab Temperature
Detection Wavelength	242.5nm
Retention Times (min)	CAB -2.14 & RILP -3.21

Table 3.5: Optimized Chromatographic Conditions for CAB & RILP

6.2 METHOD VALIDATION

6.2.1 Linearity (Calibration Curve)

The working standard and sample solutions of CAB 2.5, 5, 7.5, 10, 12.5, 15ug/ml, prepared in the serial dilutions for drugs individually, while 3.75, 7.5, 11.25, 15, 18.5, 22.5ug/ml of RILP , for conc. range, linearity, validation parameters and same con. ranges were used for the stability forced degradation studies. The calibration curves has been generated by plotting graph of peak area vs conc. for the drugs, and the regression equations, correlation coefficient R^2 value and the, Limit of Detection (LOD) & Limit of Quantification (LOQ) had been calculated .

6.2.2 Specificity and Selectivity

The selectivity and specificity parameters are utilised in selective detection particular analyte which are in the matrix or along with other substances without any interventions. 10ug/ml of CAB & RILP 15ug/ml were injected individually, and blank mobile phase as well as sample solutions from dosage form were compared to check the specificity & selectivity. Selectivity is a type of a qualitative determination of analytes, while the specificity is applied for both qualitative as well as quantitative estimations. The developed method must be selective and highly specific for the

analyte for which the method is intended to use, even in presence of impurities or any other degraded products, additives, excipients, reagents or other substances.

6.2.3 Accuracy (Recovery Studies)

Accuracy is one of the important validation parameter which describes the trueness-exactness of the test results in accordance with the true values. The accuracy studies has been performed by doing the drug recovery studies of deliberately added working standard drugs from the sample, n=3 samples taken for each drug CAB & RILP at 50%, 100% & 150% had performed at each level to the pre-analysed samples. The amount of drug-substance added and amount of drug-substance recovered were calculated from the sample peak area and total peak area and the % Recovery had been calculated.

6.2.4 Precision

6.2.4.1 Repeatability (n=6)

The repeatability study has been performed by repeatedly n=6 sample standards injected 10ug/ml of CAB & RILP 15ug/ml, and the area response of drugs was obtained and the %RSD had been calculated

6.2.4.2 Intraday Precision (n=3)

The intraday precision was performed by using the 2.5, 10, 15 ug/ml of CAB & RILP was 3.75, 15, 22.5 ug/ml was used, and the solutions were repeatedly injected analysed by HPLC three times on same day, obtained results calculated into the terms of %RSD.

6.2.4.3 Interday Precision (n=3)

The interday precision was performed by using the 2.5, 10, 15 ug/ml of CAB & RILP was 3.75, 15, 22.5 ug/ml was used, and the solutions were repeatedly injected analysed by HPLC three times in different days obtained results calculated into the terms of %RSD.

6.2.5 LOD and LOQ

The LOD Limit of Detection has been obtained from 5 set of the calibration curves performed in the linearity-range studies, the LOD is calculated as $LOD = 3.3 \times SD/Slope$

LOQ Limit of Quantitation has been obtained from the same 5 set of the calibration curves performed as per the linearity-range studies, the LOD is calculated as $LOQ = 10 \times SD/slope$

6.3 APPLICATION OF DEVELOPED ANALYTICAL METHOD AS A ASSAY METHOD FOR MARKETED FORMULATION

The developed analytical HPLC method is applied in the estimation-analysis of CABENUVA™ Each Vial contains CAB 400mg/2ml (200mg/ml) & RILP is 600mg/2ml (300mg/ml).

2ml from each vials, were taken containing CAB 400mg/2ml & RILP 600mg /2ml , was taken and Dissolved in 50ml Methanol : ACN (50:50) , sonicated, filtered and make up to 100ml with Methanol : ACN (50:50) (Stock solution A) [4000:6000 ug/ml CAB:RILP]

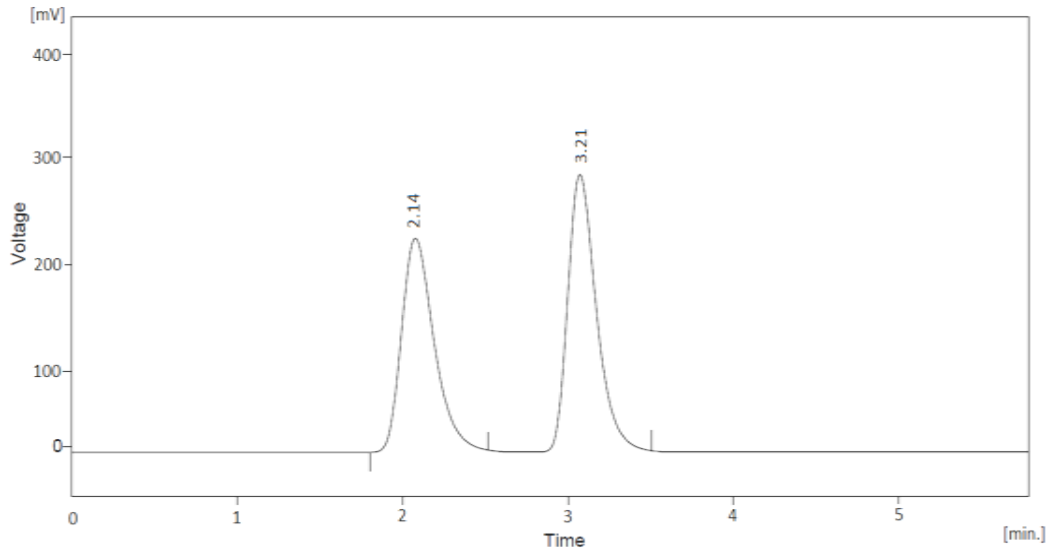
From the Stock solution A, 2.5 ml was taken, diluted with mobile phase upto 100ml to give Solution B [100:150ug/ml CAB:RILP]

From the Solution B, 1ml was taken, diluted with mobile phase upto 10ml to give Final Solution C [10:15ug/ml CAB:RILP] were prepared, n=3 samples, analysed by the developed HPLC method. The working standard drugs CAB 10ug/ml and RILP 15ug/ml were prepared and analysed by HPLC and the % purity or % label claim was estimated by comparing the area & calculating from regression equation, for working standard drug and marketed formulation.

6.4 RESULTS & DISCUSSIONS

6.4.1 Method Development

The developed analytical HPLC method found to be reliable, accurate.,- precise for analysis and quality control testing for CAB & RILP in pure form, in marketed injectable dosage form's. The method is advantageous as the low cost solvents are used, good resolution and separation has been achieved, as well as the peak symmetry tailing factor are in greater acceptable limits. The isocratic mode adds the advantage of simplicity of the developed method. Method consists of the optimized mobile phase Phosphate buffer:ACN (65 : 35) pH - 5.5, flow rate 1ml / min , detection wavelength at 242.5nm. Excipients in the marketed formulation does not affect in the resolution, separations as well do not have any interfering peaks. The average retention times were found to be CAB -2.14 & RILP -3.21 minutes. The chromatogram of the drugs are shown below.



Result Table (Uncal - CAB- RIL- standard) Phosphate buffer pH - 5.5 : ACN 65 : 35

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Peak Purity
1	2.143	2250.337	237.906	47.862	0.998
2	3.215	2451.298	265.764	52.137	0.997
	Total	4701.635	503.670	100.000	

Column Performance Table (From - CAB- RIL- standard)

	Reten. Time	Asymmetry [-]	Efficiency [th.pl]	Resolution [-]
1	2.143	1.316	5412	-
2	3.215	1.248	7644	1.725

Figure 3.4: Chromatogram of Standard CAB & RILP

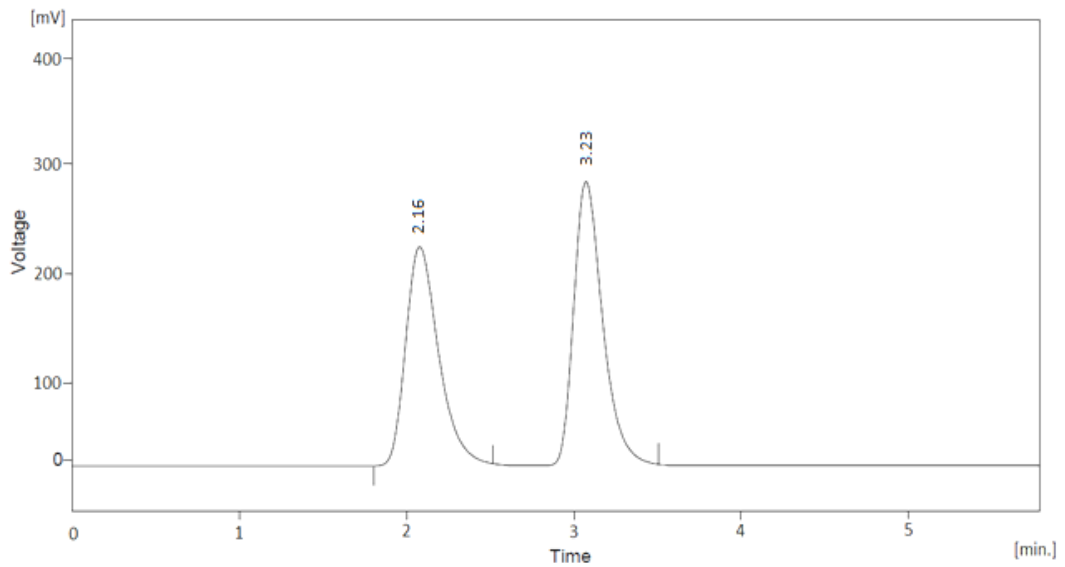


Figure 3.5: Chromatogram of Sample Standard CAB & RILP

6.4.2 Method Validation

6.4.2.1 Specificity

Developed method is specific and selective as the no other peaks of, mobile phase or any excipients impurities were interfering or overlapping in the chromatograms.

The method effectively analyses the drugs in pure form as well as in the marketed formulations with accuracy, and has reproducible results for individual drugs as well as for the combined formulation analysis.

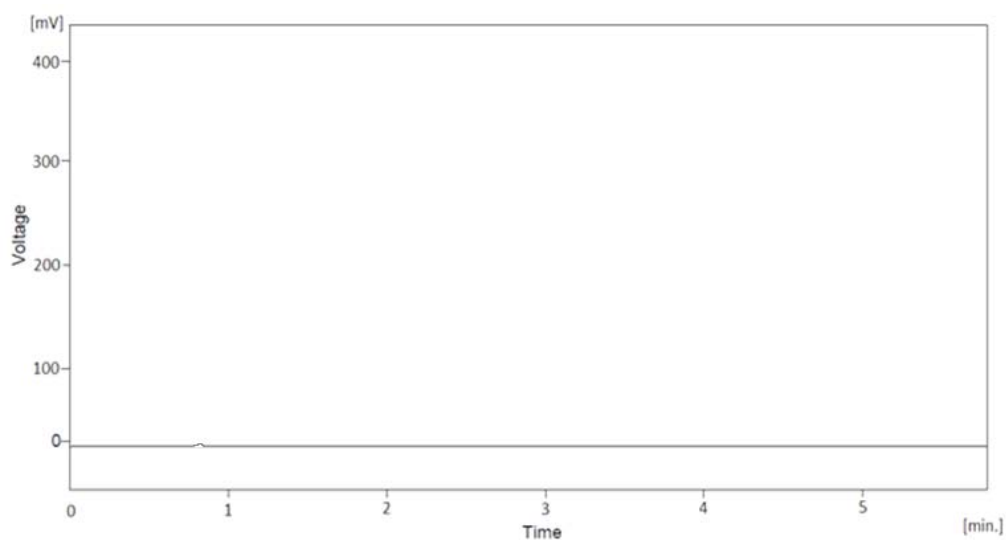


Figure 3.6: Blank Chromatogram

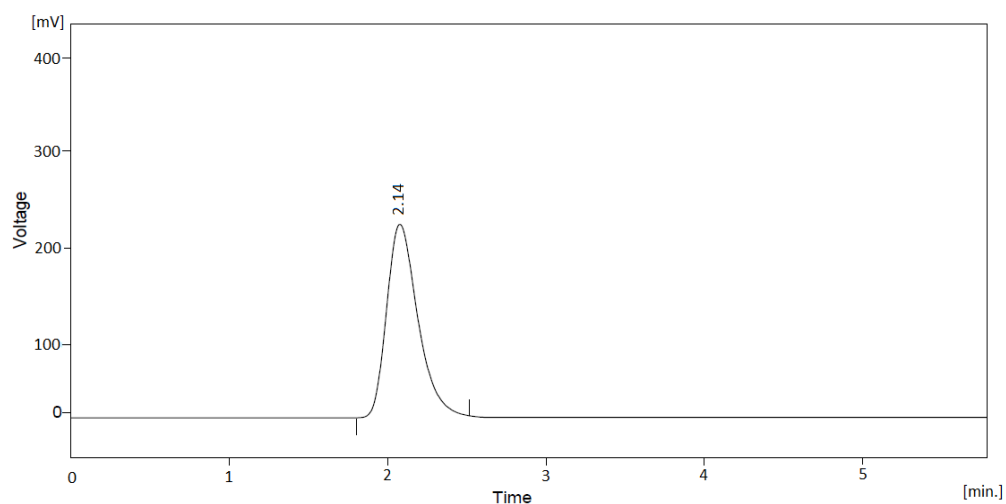


Figure 3.7: Chromatogram of CAB

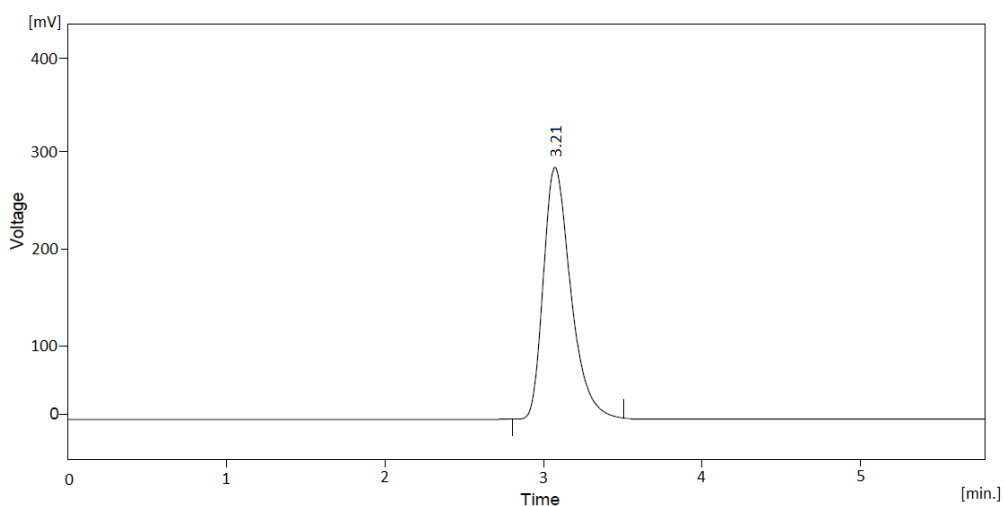


Figure 3.8: Chromatogram of RILP

6.4.2.2 Linearity and Range (n = 5)

Drugs CAB & RILP Linearity has been followed in a particular concentration ranges of 2.5-15ug/ml for CAB & RILP 3.75-22.5ug/ml. The linearity showing overlain chromatogram had been generated and the calibration curve been plotted of peak area vs conc. and straight line eqn. and correlation coefficient had been calculated.

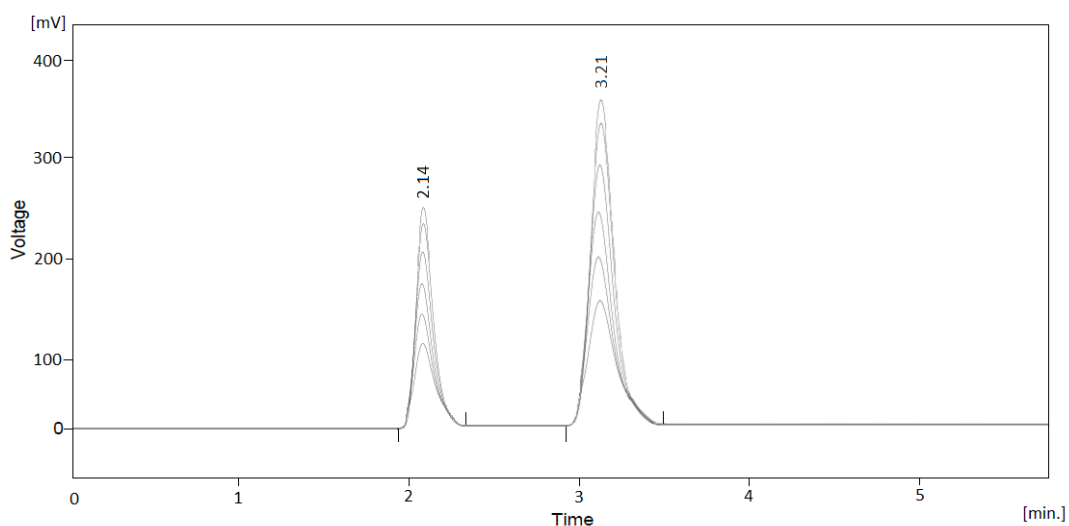


Figure 3.9: Overlain Chromatogram of Linearity for CAB & RILP

(x) Conc. µg/ml	(y) Area
2.5	561.47
5	1126.34
7.5	1722.36
10	2250.33
12.5	2812.21
15	3385.31
STD ERROR	17.98
Slope	225.2
LOD	0.26
LOQ	0.79

Table 3.6: Linearity data of CAB

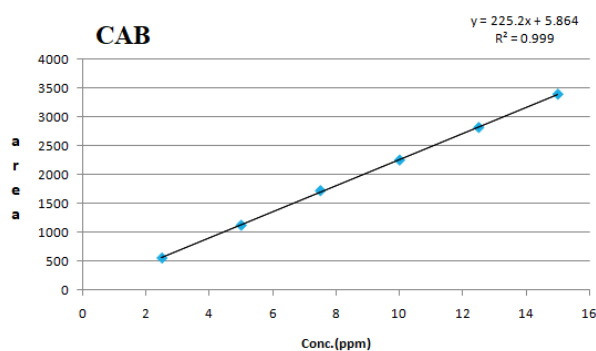


Figure 3.10: Calibration Curve for CAB

(x) Conc. µg/ml	(y) Area
3.75	612.75
7.5	1235.87
11.25	1840.25
15	2451.29
18.75	3089.19
22.5	3675.46
STD ERROR	10.03
Slope	163.6
LOD	0.20
LOQ	0.61

Table 3.7: Linearity data of RILP

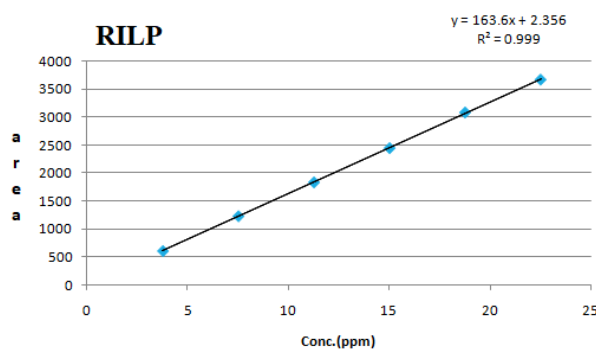


Figure 3.11: Calibration Curve for RILP

6.4.2.3 Accuracy (Recovery Studies) (n = 3)

The accuracy has been done by performing the recovery studies of the working standard drug from the pre-analysed sample of the drugs CAB & RILP. The

recovered drug from the samples has been calculated as % Recovery is been reported in the table below.

Drug	Amt of Sample Taken (µg)	% Amt of Std Added	Spiked Std Drug Amount (µg)	Spiked Amt Recovered Mean (µg)	% Recovery	% Mean Recovery
CAB	10	50	5	4.88	97.79	99.44
	10	100	10	10.05	100.56	
	10	150	15	14.99	99.97	
RILP	15	50	7.5	7.51	100.18	100.23
	15	100	15	15.04	100.31	
	15	150	22.5	22.54	100.20	

Table 3.8: Accuracy Study of CAB & RILP (n = 3)

6.4.2.4 Precision

6.4.2.4.1 Repeatability (n = 6)

The repeatability study of CAB & RILP have been performed by multiple injections of the samples of the drugs (n = 6). The repeatability data for the CAB & RILP is shown in the table below.

Conc. of CAB (µg/ml)	Area	Conc. of RILP (µg/ml)	Area
10	2250.76	15	2451.29
	2263.59		2452.12
	2247.28		2462.79
	2260.85		2459.67
	2249.97		2454.41
	2256.62		2449.22
Mean	2254.84	Mean	2454.92
SD	6.53	SD	5.25
% RSD	0.28	% RSD	0.21

Table 3.9: Repeatability Study of CAB & RILP (n = 6)

6.4.2.4.2 Intraday Precision (n = 3)

The Intraday precision for the CAB & RILP has been performed by taking multiple injections (n = 3) in a same day at different 25, 100, 150 % Levels. The data for the intraday precision is shown in table below.

CAB			RILP		
Conc. (µg/ml)	Mean area ± SD	% RSD	Conc. (µg/ml)	Mean area ± SD	% RSD
2.5	565.8 ± 4.2	0.74	3.75	615.0 ± 3.6	0.58
10	2253.3 ± 2.9	0.12	15	2448.9 ± 2.4	0.10
15	3390.9 ± 6.6	0.19	22.5	3671.0 ± 5.1	0.13

Table 3.10: Intraday Precision of CAB & RILP (n = 3)

6.4.2.4.3 Interday Precision (n = 3)

The Interday precision for the CAB & RILP has been performed by taking multiple injections (n = 3) in different day at different 25, 100, 150 % Levels. The data for the intraday precision is shown in table below.

CAB			RILP		
Conc. (µg/ml)	Mean area ± SD	% RSD	Conc. (µg/ml)	Mean area ± SD	% RSD
2.5	565.5 ± 4.8	0.85	3.75	617.5 ± 5.6	0.92
10	2254.4 ± 7.9	0.35	15	2457.8 ± 7.6	0.31
15	3379.6 ± 9.0	0.26	22.5	3674.5 ± 12.5	0.34

Table 3.11: Interday Precision of CAB & RILP (n = 3)

6.4.2.5 LOD and LOQ

It has been calculated from the n=5 samples from the calibration curve slope and standard deviation. The LOD value are found to be 0.26 & 0.20 ug respectively for CAB & RILP, and the LOQ values are found to be 0.79 & 0.61ug respectively for CAB & RILP.

6.4.3 Application of the Developed Analytical Method to Formulation

The proposed analytical method been tested in assay analysis % Assay of the Label claim on the CABENUVA™ Each Vial contains CAB 400mg/2ml that is (200mg/ml) & RILP is 600mg/2ml that is (300mg/ml). Analytical method successfully applied to the estimation of drugs in marketed pro-duct by comparing with the standard and the sample formulation. The assay result are shown in the table below.

	Serial No	Label claim (mg)	Result (mg)	% Label Claim	Avg % Assay	SD	% RSD
CAB	1	400	399.59	99.89	99.80	0.08	0.08
	2	400	399.15	99.78			
	3	400	398.89	99.82			
RILP	1	600	599.70	99.95	99.95	0.12	0.12
	2	600	598.99	99.83			
	3	600	600.44	100.07			

Table 3.12: Assay of Formulation CABENUVA™ (n = 3)

6.4.4 Summary of Results

Sr No	Parameters	Results	
		CAB	RILP
1	System Suitability:		
	Theoretical plates-	5412	7644
	Tailing Factor-	1.31	1.24
	Retention time min-	2.14	3.21
2	Precision (%RSD)	0.12	0.10
3	Linearity (R ²)	0.9999	0.9999
4	Accuracy (% Recovery)	99.44	100.23
5	LOD (ug/ml)	0.26	0.20
6	LOQ (ug/ml)	0.79	0.61
7	% Assay	99.80	99.95

6.5 CONCLUSIONS

The Analytical HPLC method for CAB & RILP combinational drugs has been successfully developed and validated. The analytical method is optimized in testing, analysis of these selected drugs in individual as well in the combined forms and all validation parameters., are performed in the acceptance criteria as per ICH regulatory guideline. Method that has been developed., is been, optimized to analyse minimum conc. of drugs in-pure form and, in testing-analysing marketed formulation. Accurate precise method developed., can be used for analysis of CAB & RILP combinational drugs injectable forms, combination as well as individual in as assay method and quality control testing procedures in academics, research, analytical laboratories and pharmaceutical industries.