Chapter 9

STABILITY HPLC METHOD DEVELOPMENT AND VALIDATION FOR DOLUTEGRAVIR TENOFOVIR AND RILPIVIRINE

9.1 EXPERIMENTALS

9.1.1 Instruments Utilised

The Shimadzu-HPLC system LC-20-AT-system with LC-Solution and Peak chrom software with both PDA & UV detector. Stationary phase coloumn in reverse phase has been used C-18-Hypersil-BDS and Hypersil-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-wares volumetric flasks measuring cylinder pipettes of analytical were used. 0.22 and 0.45 µm nylon Millipore filters caps were used.

9.1.2 Materials and Reagents Utilised

The chemicals used working reference standard drugs Dolutegravir DOLU, Tenofovir TEN & Rilpivirine RILP drugs samples of sun-pharma, solisom & upcare pharma has been utilised. Acetonitrile, Methanol, potassium dihydrogen ortho phosphate, orthophosphoric acid, used analytical HPLC Merck grade. H_2O_2, HCl , NaOH analytical grade of Rankem used. Milli-Q pure water is utilized.

9.1.3 Identification of Standard Drug Samples

9.1.3.1 Melting Point Determination

The working standard drugs Dolutegravir DOLU, Tenofovir TEN & 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 6.1.

Drug	Observed Melting Range	Standard Melting Range
DOLU	192.32-195.04 ^{0}C	190-193 ^{0}C
TEN	112-115 ^{0}C	113-115 ^{0}C
RILP	249.9 – 251.36 ^{0}C	248-251 ^{0}C

Table 6.1: Melting Points of DOLU, TEN & RILP

9.1.3.2 FTIR Spectral Determination for Identification Standard drug samples DOLU, TEN & RILP

The pure active pharmaceutical working standard drug substances DOLU, TEN & RILP were scanned between 400-4000cm^{-1} in FTIR Spectrometer Shimadzu 8400 series. The drug dry powder samples were made die pressed pellets with KBr and the FRIR spectra were determined shown in Fig 6.1 for DOLU, Fig 6.2 for TEN & Fig 6.3 for RILP. The principal IR peaks recorded and observed for the drugs are shown in Table 6.2, 6.3 & 6.4 for DOLU, TEN & RILP respectively.

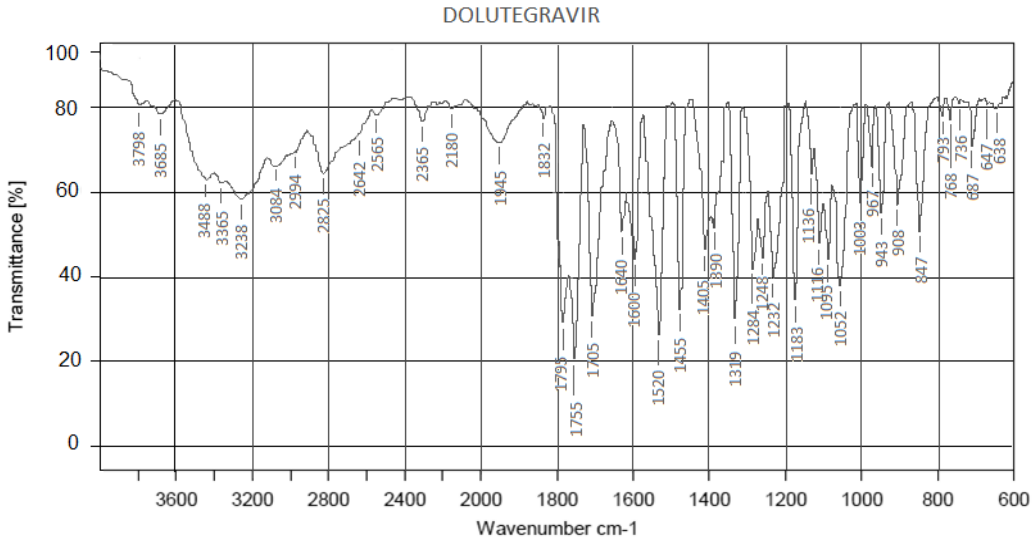

Figure 6.1: FTIR Spectra of Dolutegravir DOLU

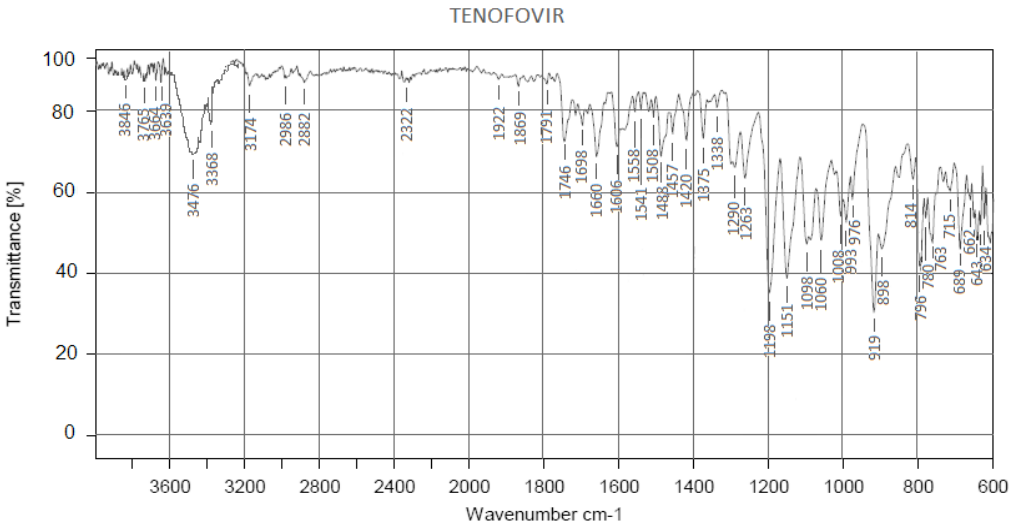

Figure 6.2: FTIR Spectra of Tenofovir TEN

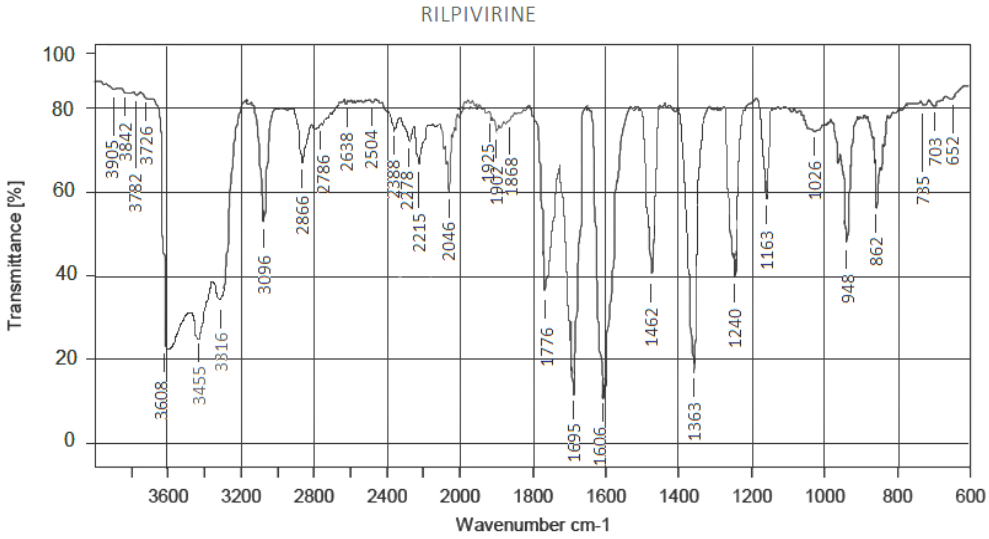

Figure 6.3: FTIR Spectra of Rilpivirine RILP

DOLUTEGRAVIR					
Energy (Cm^{-1})	**Band Assignment**	**Peak Intensity**	**Energy (Cm^{-1})**	**Band Assignment**	**Peak Intensity**
1647	C=C (Aromatic)	53.26	1462	C-H Aromatic	32.85
1400-1000	-CF	38.54 44.84 57.49	1500-1700	N-H (amine)	31.25 43.14
1800-1700	C=O ketone	20.65 31.25	3000-2800	C-H Methyl	64.29 71.03 68.46

1400- 1390-1310	-OH phenolic	31.25 44.84	3350-3310	N-H (2^0 Amine)	62.06
1749-1792 1275-1200	C-O (Ether)	29.86 43.58	1235-1268	C-N amine	40.98 44.24
1680- 1640-1630	C=O Amide	52.63 45.42	2000-1650	C-H Aromatic	74.23 78.35 31.25
1250-1020	C-N Amine	46.52 37.94	1462	C-H Aromatic	32.85

Table 6.2: FTIR Interpretation of Dolutegravir DOLU

TENOFOVIR					
Energy (Cm^{-1})	**Band Assignment**	**Peak Intensity**	**Energy (Cm^{-1})**	**Band Assignment**	**Peak Intensity**
1200-1100	C-O (S)	44.53 36.26 39.41	1320-1440 909-1000	P=O P-O	80.52 72.37 31.21 59.17 56.18
1690-1640	C=N	77.54 68.56	1350-1250	N-C=N	81.46 63.34
1210- 1163-1300	C-O	65.72 64.51	1250-1020	C-N Amine	56.71 47.23 63.46
1100-1260	C=O	63.24 37.62	1200-1350	N-C (3^0 Amine)	36.67 80.13
3100- 2900-2840	C-H Alkyl groups	92.54 91.49	3500-3100	N-H (1^0 Amine)	67.71 78.51 85.84

Table 6.3: FTIR Interpretation of Tenofovir TEN

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 (2^{0} Amine)	18.62	1675-1665	C=C Alkene	41.86

Table 6.4: FTIR Interpretation of Rilpivirine RILP

9.1.4 Preparation of Solutions

9.1.4.1 Preparation of standard solutions of DOLU, TEN & RILP

The standard stock soln. individual drugs prepared in 50:50 Methanol : ACN solvent mixture. 10mg of DOLU, 30mg of TEN and 15mg of RILP were individually dissolved in solvent mixture and made upto 100ml with same solvent to give 100 µg/ml DOLU, 300 µg/ml TEN & 150 µg/ml RILP standard stock solution.

From the above stock solutions of individual drugs DOLU, TEN, RILP each, 10ml from each was taken individually and diluted upto 100ml in individual volumetric flasks to give DOLU 10 µg/ml , TEN 30 µg/ml , RILP 15 µg/ml individual drug standard Final solutions.

9.1.4.2 Preparation of Sample Solutions

Sample solution from tablets, DOLUVIR™ contains 50mg Dolutegravir, TENVIR™ contains 300mg Tenofovir, EDURANT™ contains 25mg of Rilpivirine. Accurately the avg. wt. of each 10 tablets individually was done and crushed triturated, the individually taken tablet powder was taken weighing equiv alent wt of DOLU 50mg dissolved in 50ml of 50:50 Methanol : ACN, & made upto 50ml to give (stock soln D1 1000ug/ml), and TEN 300mg in 100ml same solvent mixture to give (stock soln T1 3000ug/ml), and RILP 25mg in 10ml solvent mixture to give (stock soln R1 2500ug/ml). The combined solution B was made by mixing aliquots of 10ml of D1, 10ml of T1 & 6ml of R1 stock solns taken in a common single flask 100ml flask & made up to 100ml with solvent to give combined solution B (DOLU:TEN:RILP 100:300:150 ug/ml). From this combined solution B, 1ml was taken and diluted to 10ml to give final solution C (DOLU:TEN:RILP 10:30:15 ug/ml)

9.1.4.3 Preparation of Optimized Mobile Phase

The mobile phase made by taking 65:15:20 ratio, 0.05M Phosphate buffer : ACN : Methanol of pH -3.5. 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.

9.1.5 Selection of Wavelength for Detection

The Final standard solns of DOLU 10 µg/ml , TEN 30 µg/ml & RILP 15 µg/ml scanned in 200 - 400 nm in UV-visible double beam spectrophotometer at a medium scanning speed. The overlain spectra shown in Fig. 6.4 of DOLU 10 µg/ml , TEN 30 µg/ml & RILP 15 µg/ml were taken in 50:50 Methanol : ACN and the 229.6nm wavelength was selected for estimation in the detection during the HPLC analysis.

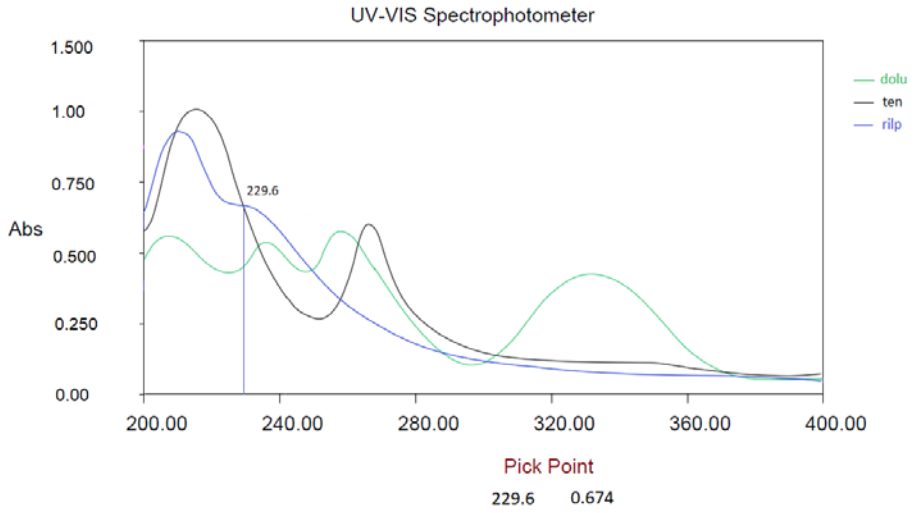

Figure 6.4: UV Spectra Overlay of DOLU, TEN & RILP

9.1.6 Selection and Optimization of Mobile phase

For the detection analysis of the DOLU, TEN & 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 seperation of the drugs was carried out shown in Table 6.5

Sr No	Mobile Phase	pH	Ratio (v/v)	Retention Time (min)			REMARK
				DOLU	TEN	RILP	
1	H_2O:MeOH	-	50:50	-	-	-	No peak detected
2	H_2O:MeOH	-	80:20	-	-	-	No peak detected
3	H_2O:MeOH	-	20 :80	-	-	-	No peak detected
4	ACN : Methanol	-	50:50	-	-	-	No peak detected

5	ACN : Methanol	-	80:20	-	-	-	No peak detected
6	ACN : Methanol	-	20:80	-	-	-	No peak detected
7	0.05 M Phosphate buffer:ACN	7	50:50	8.52	12.39	-	Peak Tailing No peak of RIL detected
8	0.05 M Phosphate buffer:ACN	7	20:80	14.49	-	-	Longer Run time, Tailing in peak No peak of TEN & RIL
9	0.05 M Phosphate buffer:ACN	6.5	80:20	8.15	8.76	8.89	Peak Merging & Tailing Not good separation
10	0.05 M Phosphate buffer:ACN	5	80:20	5.81	7.92	8.13	TEN+RIL peak merging
11	0.05 M Phosphate buffer:ACN	5	75:25	6.75	6.53	9.37	Peak Tailing Peak merging DOL+TEN
12	0.05 M Phosphate buffer:ACN	4	80:20	4.59	7.04	7.48	TEN+RIL peak merging Not good separation

13	0.05 M Phosphate buffer:ACN	3.5	80:20	3.16	5.52	6.06	TEN+RIL peak merging Not good separation
14	0.05 M Phosphate buffer : ACN : Methanol	7	80:10 :10	5.73	-	-	No peak of TEN, RILP detected
15	0.05 M Phosphate buffer : ACN : Methanol	6	50:25 :25	4.68	10.53	10.3 7	Tailing of peaks & Peak merging TEN+RIL
16	0.05 M Phosphate buffer : ACN : Methanol	5	60:20 :20	4.08	8.19	8.28	Peak merging TEN+RIL
17	0.05 M Phosphate buffer : ACN : Methanol	4.5	60:20 :20	3.24	6.83	7.14	Peak merging TEN+RIL Less Resolution
18	0.05 M Phosphate buffer : ACN : Methanol	4	60:20 :20	2.84	4.72	4.96	Peak merging TEN+RIL Less Resolution
19	**0.05 M Phosphate buffer : ACN: Methanol**	**3.5**	**65:15 :20**	**2.53**	**3.46**	**4.76**	**Good separation**

Table 6.5: Trials for Selection of Mobile Phase for DOLU, TEN & RILP

9.1.7 Optimized Chromatographic Conditions

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

Parameters	Conditions
Stationary Phase Coloumn	C18 Hypersil BDS 250 x 4.6mm , 5 micron
Mobile phase	Phosphate buffer :ACN:Methanol: 65:15:20- pH- 3.5
Flow rate	1ml/minl
Injection volume	20ul
Temp	Ambient Lab Temperature
Detection Wavelength	229.6nm
Retention Times (min)	DOLU-2.53, TEN-3.46, RILP-4.76

Table 6.6: Optimized Chromatographic Conditions for DOLU, TEN & RILP

9.2 STABILITY STUDIES BY FORCED DEGRADATIONS

The stability studies for the pure working standard drugs DOLU, TEN & RILP as well as for the pharmaceutical marketed formulation tablets, DOLUVIR™ contains 50mg Dolutegravir, TENVIR™ contains 300mg Tenofovir, EDURANT™ contain 25mg of Rilpivirine containing the triple combined drugs has been carried out by performing the forced-degradations stress testing method has been utilised in method. Developed- HPLC-analytical method is been applied in stability study as well as in the assay analysis and dissolution profile study. The stability study has been performed on the pure drug and marketed formulation samples under different types of stress conditions which helps in the forced degradations of the drug substances, under the conditions like thermal, acid, base-alkali, photo, & oxidative degradations were performed in accordance with the guideline ICH - guidelines and are effectively analysed by the developed HPLC method as well as validated.

9.2.1 Acid Degradation

For the acid degradation study, was performed in 0.1N HCl solution. The working standard drug solution of 1ml of DOLU (100ug/ml) std stock soln, 1ml of TEN (300ug/ml) std stock soln, and 1ml of RILP (150ug/ml) std stock were taken and 2ml of 0.1N HCl added and kept for 4hrs for degradation and then neutralized with

2ml of 0.1N NaOH soln, then it was made up soln to 10ml final volume with mobile phase solvent to give DOLU 10ug/ml, TEN 30ug/ml and RILP 15ug/ml. And the analysed this sample by developed HPLC method. In the similar manner the combined drug sample of marketed DOLUVIR™, TENVIR™ ,EDURANT™ formulation was prepared stock soln B containing 100ug/ml DOLU, 300ug/ml TEN and 150ug/ml RILP. 1ml from this stock soln B was taken and 2ml of 0.1N HCl was added and kept for 4hrs for degradation and then neutralized with 2ml 0.1N NaOH, and the made up soln to 10ml final volume with mobile phas e to give DOLU 10ug/ml, TEN 30ug/ml and RILP 15ug/ml. And the analysed this sample by developed HPLC method.

9.2.2 Base Degradation

The Base degradation study, performed in 0.1N NaOH solution. The working standard drug solution of 1ml of DOLU (100ug/ml) std stock soln, 1ml of TEN (300ug/ml) std stock soln, and 1ml of RILP (150ug/ml) std stock were taken and 2ml of 0.1N NaOH added and kept for 4hrs for degradation and then neutralized with 2ml of 0.1N HCl soln, was made up soln to 10ml final volume with mobile phase to give DOLU 10ug/ml, TEN 30ug/ml and RILP 15ug/ml. And the analysed this sample by developed HPLC method. In the similar manner the combined drug sample of marketed DOLUVIR™, TENVIR™ ,EDURANT™ formulation was prepared stock soln B containing 100ug/ml DOLU, 300ug/ml TEN and 150ug/ml RILP. 1ml from this stock soln B was taken and 2ml of 0.1N NaOH was added and it has been, kept for 4hrs for degradation and then neutralized with 2ml 0.1N HCl, and the made up soln to 10ml final made volume with mobile phase give DOLU 10ug/ml, TEN 30ug/ml and RILP 15ug/ml. And the analysed this sample by developed HPLC method.

9.2.3 Oxidative Degradation

The oxidative degradation study, was has been performed in 3% H_2O_2 solution as a oxidizing agent. The working standard drug solution of 1ml of DOLU (100ug/ml) std stock soln, 1ml of TEN (300ug/ml) std stock soln, and 1ml of RILP (150ug/ml) std stock were taken and 2ml of 3% H_2O_2 solution added and kept for 4hrs for degradation and then made up soln to 10ml final volume with mobile phase to give DOLU 10ug/ml, TEN 30ug/ml and RILP 15ug/ml. And the analysed this sample by developed HPLC method. In the similar manner the combined drug sample of marketed DOLUVIR™, TENVIR™ ,EDURANT™ formulation was

prepared stock soln B containing 100ug/ml DOLU, 300ug/ml TEN and 150ug/ml RILP. 1ml from this stock soln B was taken and 2ml of 3% H_2O_2 solution was added and kept for 4hrs for degradation and then made up soln to 10ml final volume with mobile phase to give DOLU 10ug/ml, TEN 30ug/ml and RILP 15ug/ml. And the analysed this sample by developed HPLC method.

9.2.4 Thermal Degradation

It has carried out for the working standard drug powders DOLU, TEN & RILP individually in Wist Temperature chamber oven at 60 ^{0}C for 24hrs. After thermal degradation, the drug powder DOLU 10mg, TEN 30mg and RILP 15mg were taken in flask dissolved in 50ml of 50:50 Methanol : ACN solvent, dissolved, sonicated , filtered and final volume made upto 10ml to give final soln of 10ug/ml of DOLU, 30ug/ml TEN & 15ug/ml RILP. This final solution was subjected to be analysed by developed HPLC method. In similar manner marketed formulation tablets sample was powdered and kept in Wist Temperature chamber oven at 60 ^{0}C for 24hrs. After thermal degradation, the tablet powders weighing equivalent to DOLU 50mg dissolved in 50ml of 50:50 Methanol : ACN, & made upto 50ml to give (stock soln D1 1000ug/ml), and TEN 300mg in 100ml same solvent m ixture to give (stock soln T1 3000ug/ml), and RILP 25mg in 10ml solvent mixture to give (stock soln R1 2500ug/ml). The combined solution B was made by mixing aliquots of 10ml of D1, 10ml of T1 & 6ml of R1 stock solns taken in a common single flask 100ml flask & made up to 100ml with solvent to give the a combined solution B (DOLU:TEN:RILP 100:300:150 ug/ml). From this combined solution B, 1ml was taken and diluted to 10ml to give final solution C (DOLU:TEN:RILP 10:30:15 ug/ml). This final solution was subjected to be analysed by the developed HPLC method.

9.2.5 Photo Degradation

The photo degradation has been carried out in UV chamber 1.2million-lux-hrs and 200-watt-hrs in a photo stability test chamber Sanwood SM-LHH-UV series. The standard drug powder of DOLU, TEN and RILP were kept into UV chamber for 24hrs.

After photo degradation, the drug powder DOLU 10mg, TEN 30mg and RILP 15mg were taken in flask dissolved in 50ml of 50:50 Methanol : ACN solvent, dissolved, sonicated , filtered and final volume made upto 10ml to give final soln of 10ug/ml of

DOLU, 30ug/ml TEN & 15ug/ml RILP. This final solution was subjected to be analysed by developed HPLC method.

In similar manner marketed formulation tablets DOLUVIR™, TENVIR™ , EDURANT™ sample was powders and kept into UV chamber for 24hrs. After degradation, the tablet powders weighing equivalent to DOLU 50mg dissolved in 50ml of 50:50 Methanol : ACN, & made upto 50ml to give (stock soln D1 1000ug/ml), and TEN 300mg in 100ml same solvent m ixture to give (stock soln T1 3000ug/ml), and RILP 25mg in 10ml solvent mixture to give (stock soln R1 2500ug/ml). The combined solution B was made by mixing aliquots of 10ml of D1, 10ml of T1 & 6ml of R1 stock solns taken in a common single flask 100ml flask & made up to 100ml with solvent to give the a combined solution B (DOLU:TEN:RILP 100:300:150 ug/ml). From this combined solution B, 1ml was taken and diluted to 10ml to give final solution C (DOLU:TEN:RILP 10:30:15 ug/ml). This final solution was subjected to be analysed by the developed HPLC method.

9.3 METHOD VALIDATION

9.3.1 Linearity (Calibration Curve)

The working standard and sample solutions of DOLU was 2.5, 5, 7.5, 10, 12.5, 15 & TEN was 7.5, 15, 22.5, 30, 37.5, 45ug/ml, while 3.75, 7.5, 11.25, 15, 18.75, 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

9.3.2 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 DOLU & TEN 30ug/ml & 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.

9.3.3 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 DOLU, TEN, 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.

9.3.4 Precision

9.3.4.1 Repeatability (n=6)

The repeatability study has been performed by repeatedly n=6 sample standards injected 10ug/ml of DOLU & TEN 30ug/ml & RILP 15ug/ml and the area response of drugs was obtained and the %RSD had been calculated

9.3.4.2 Intraday Precision (n=3)

The intraday precision was performed by using the 2.5, 10, 15 ug/ml of DOLU & TEN was 7.5, 30, 45ug/ml, while 3.75, 15, 22.5 ug/ml for RILP was used, and the solutions were repeatedly injected analysed by HPLC three times on same day, obtained results calculated into the terms of %RSD.

9.3.4.3 Interday Precision (n=3)

The interday precision was performed by using the 2.5, 10, 15 ug/ml of DOLU & TEN was 7.5, 30, 45ug/ml, while 3.75, 15, 22.5 ug/ml for RILP was used, and the solutions were repeatedly injected analysed by HPLC three times in different days obtained results calculated into the terms of %RSD.

9.3.5 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 x 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 LOD = 10 x SD/slope

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

The developed analytical HPLC method is applied in the estimation-analysis of DOLU, TEN, RILP, in the tablets DOLUVIR^{TM} contains 50mg Dolutegravir, TENVIR^{TM} contains 300mg Tenofovir, EDURANT^{TM} contains 25mg of Rilpiviri ne. Accurately the avg. wt. of each 10 tablets individually was done and crushed triturated, the individually taken tablet powder was taken weighing equiv alent wt of DOLU 50mg dissolved in 50ml of 50:50 Methanol : ACN, & made upto 50ml to give (stock soln D1 1000ug/ml), and TEN 300mg in 100ml same solvent mixture to give (stock soln T1 3000ug/ml), and RILP 25mg in 10ml solvent mixture to give (stock soln R1 2500ug/ml). The combined solution B was made by mixing aliquots of 10ml of D1, 10ml of T1 & 6ml of R1 stock solns taken in a common single flask 100ml flask & made up to 100ml with solvent to give combined solution B (DOLU:TEN:RILP 100:300:150 ug/ml). From this combined solution B, 1ml was taken and diluted to 10ml to give final solution C (DOLU:TEN:RILP 10:30:15 ug/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.

9.5 RESULTS & DISCUSSIONS

9.5.1 Method Development

The developed analytical HPLC method found to be reliable, accurate.,- precise for analysis and quality control testing for DOLU, TEN and RILP in pure form, in marketed tablet dosage form's. The method is advantageous as the low cost solvents are used, good resolution and seperation 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:Methanol (65:15:20) pH 3.5, flow rate 1ml / min , detection wavelength at 229.6nm. 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 DOLU-2.53, TEN-3.46 and RILP-4.76 minutes. The chromatogram of the drugs are shown below.

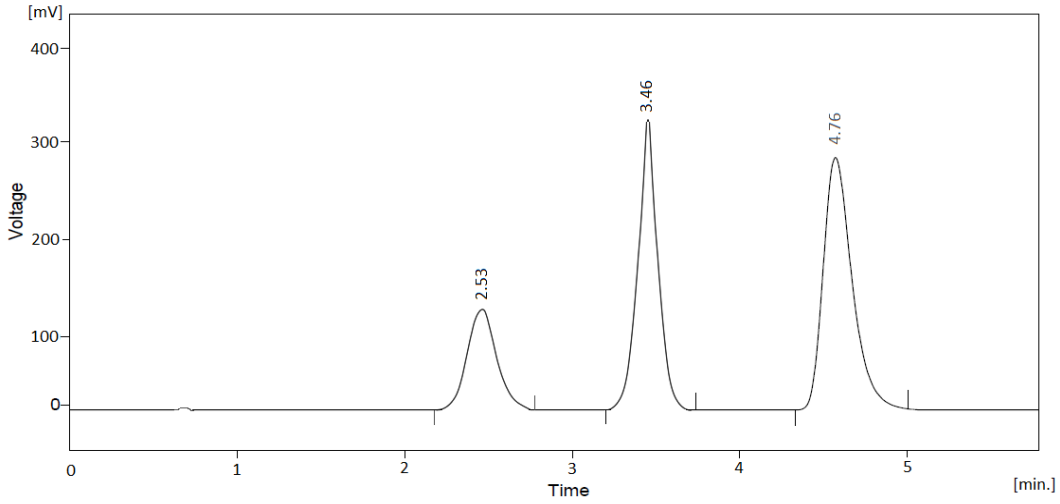

Figure 6.5: Chromatogram of Standard DOLU, TEN & RILP

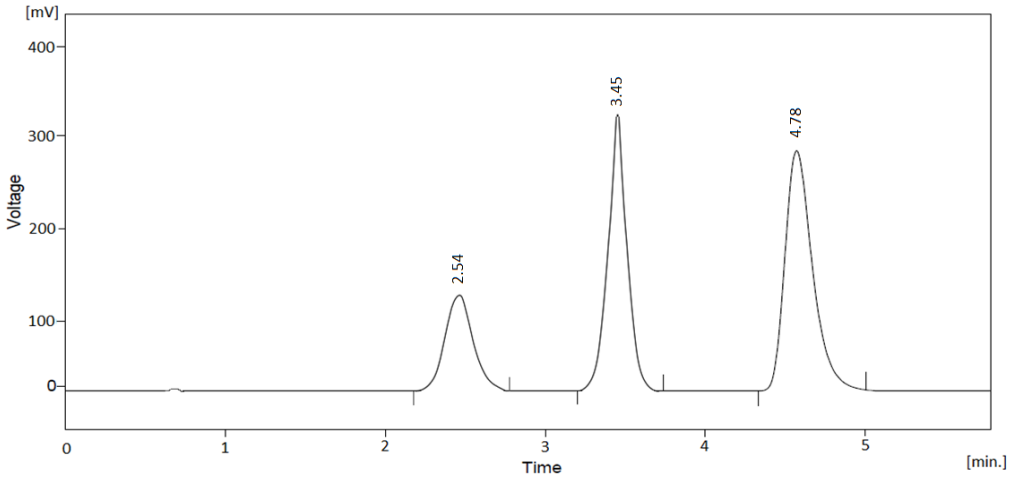

Figure 6.6: Chromatogram of Sample DOLU, TEN & RILP

9.5.2 Stability & Forced Degradation Studies

Stability studies of drug substances under forced degradation by acid, base, thermal, oxidative and photo degradation has been successively carried out for the working standard drugs DOLU, TEN & RILP and for the marketed formulation sample DOLUVIR™, TENVIR™ ,EDURANT™. Developed analytical HPLC method is competent to detect and quantify main peaks of the drugs, along with impurities, degraded products effectively without any interference or overlapping of other peaks. The chromatograms of drugs in different degradation conditions are shown below.

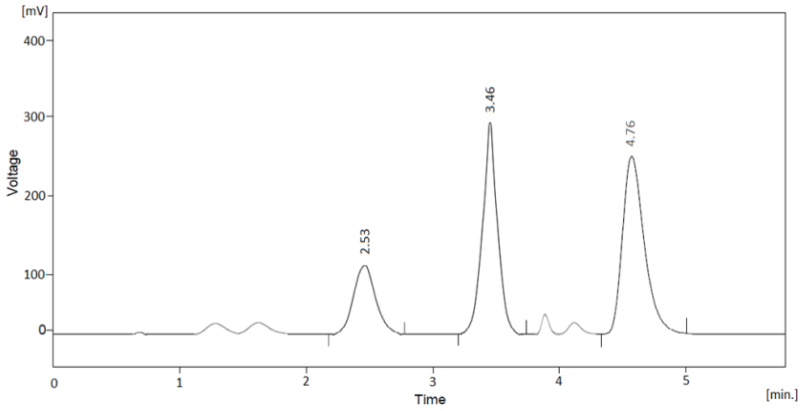

Figure 6.7: Chromatogram of Acid Degradation Standard DOLU, TEN & RILP

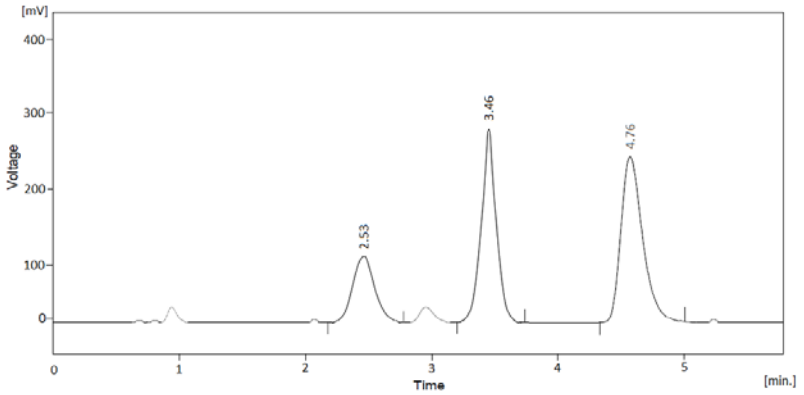

Figure 6.8: Chromatogram of Base Degradation Standard DOLU, TEN, RILP

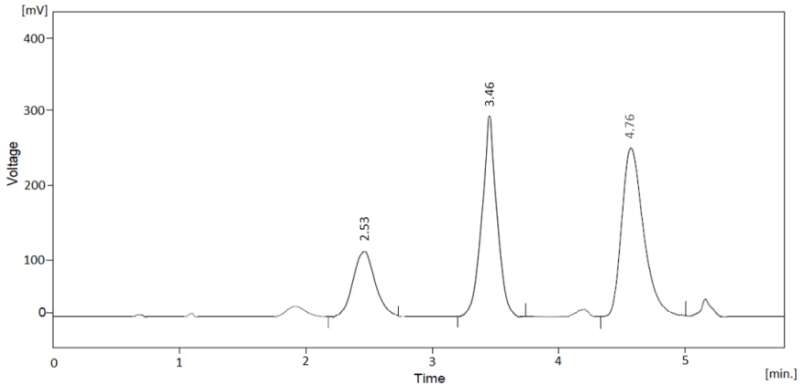

Figure 6.9: Chromatogram of Oxidative Degradation Standard DOLU, TEN, RILP

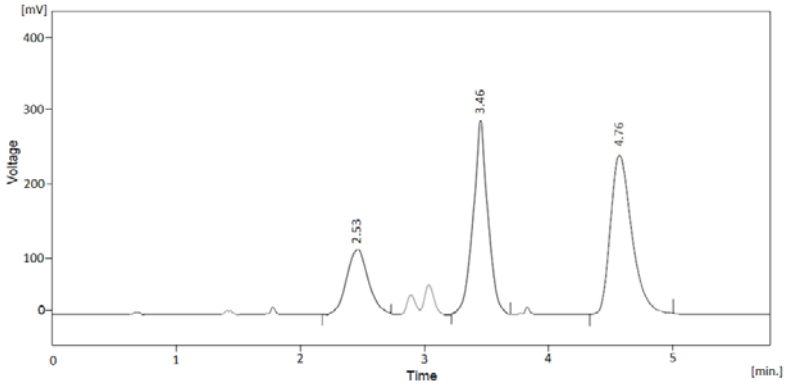

Figure 6.10: Chromatogram of Thermal Degradation Standard DOLU, TEN, RILP

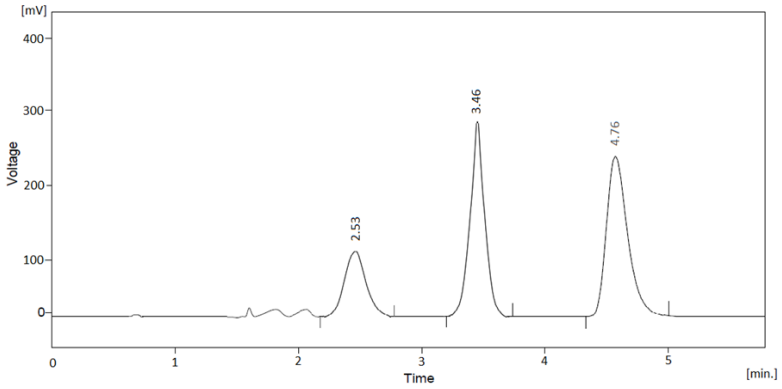

Figure 6.11: Chromatogram of Photo Degradation Standard DOLU, TEN, RILP

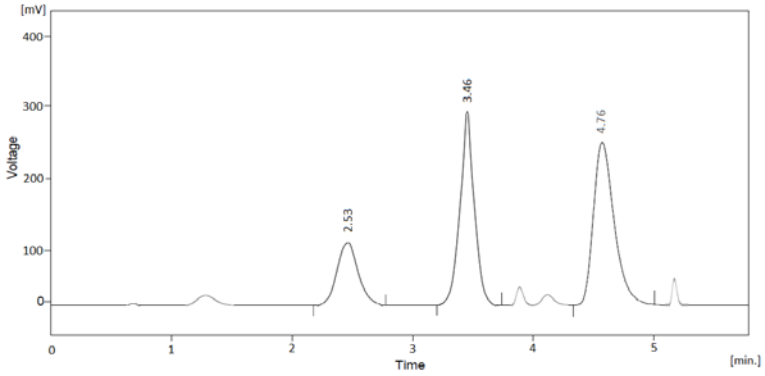

Figure 6.12: Chromatogram of Acid Degradation Sample DOLU, TEN, RILP

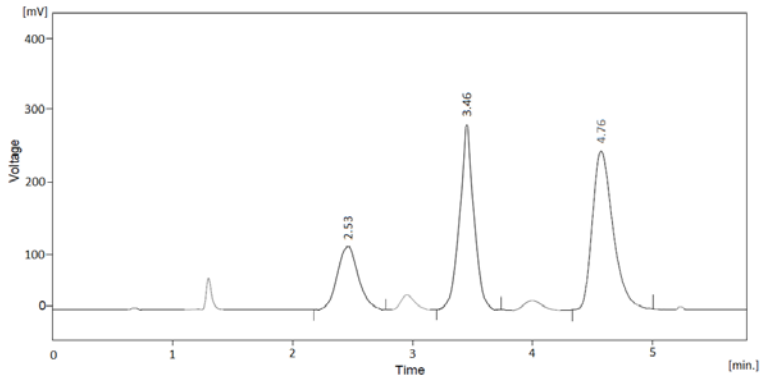

Figure 6.13: Chromatogram of Base Degradation Sample DOLU, TEN, RILP

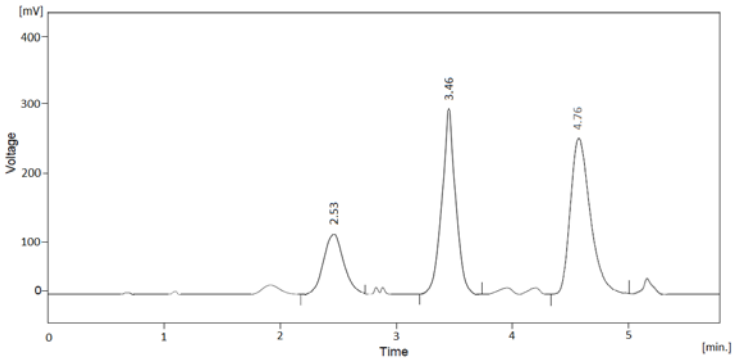

Figure 6.14: Chromatogram of Oxidative Degradation Sample DOLU, TEN, RILP

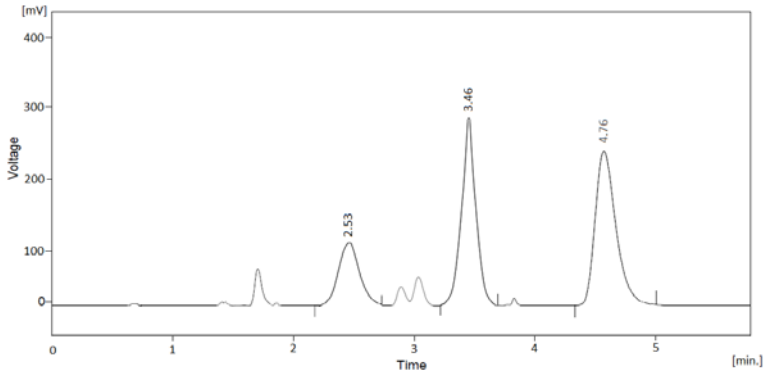

Figure 6.15: Chromatogram of Thermal Degradation Sample DOLU, TEN, RILP

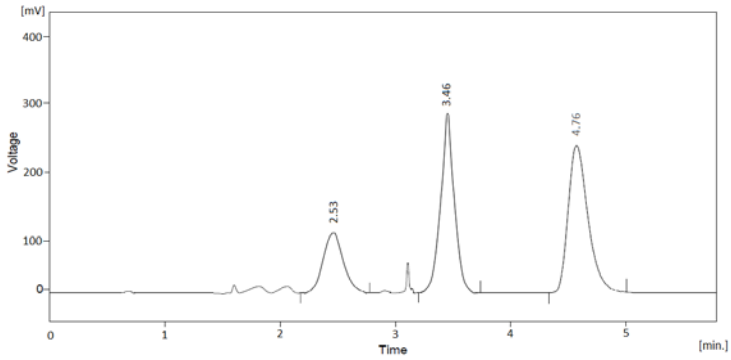

Figure 6.16: Chromatogram of Photo Degradation Sample DOLU, TEN, RILP

Degrading Condition	Peak Area			% Drug Recovered			% Degraded		
	DOLU	TEN	RILP	DOLU	TEN	RILP	DOLU	TEN	RILP
Acid	1164.29	2318.69	2416.71	96.42	97.43	98.57	3.57	2.57	1.42
Base	1171.07	2326.24	2421.65	96.98	97.74	98.77	3.01	2.25	1.23
Oxidative	1161.95	2291.46	2394.32	96.22	96.28	97.65	3.77	3.71	2.34
Thermal	1174.38	2297.17	2405.93	97.25	96.52	98.13	2.74	3.47	1.86
Photo	1187.21	2337.38	2413.54	98.32	98.21	98.44	1.67	1.78	1.55

Table 6.7: % Drug Degraded & % Drug Recovered DOLU, TEN, RILP

PEAK PURITY				
Drug	**Stress Type**	**Peak Purity Angle**	**Peak Purity Threshold**	**Peak Purity**
DOLU	Untreated Sample	0.102	0.387	0.999
	Acid	0.121	0.306	0.998
	Base	0.118	0.247	0.999
	Oxidative	0.124	0.284	0.997
	Photo	0.132	0.316	0.998
	Thermal	0.119	0.251	0.998
TEN	Untreated Sample	0.113	0.349	0.998
	Acid	0.127	0.288	0.999
	Base	0.226	0.325	0.998
	Oxidative	0.134	0.279	0.998
	Photo	0.128	0.254	0.997
	Thermal	0.125	0.286	0.999
RILP	Untreated Sample	0.103	0.397	0.999
	Acid	0.239	0.324	0.998
	Base	0.124	0.273	0.998
	Oxidative	0.217	0.310	0.997
	Photo	0.226	0.367	0.998
	Thermal	0.119	0.286	0.999

Table 6.8: Peak Purity for DOLU, TEN, RILP

9.5.3 Method Validation

9.5.3.1 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.

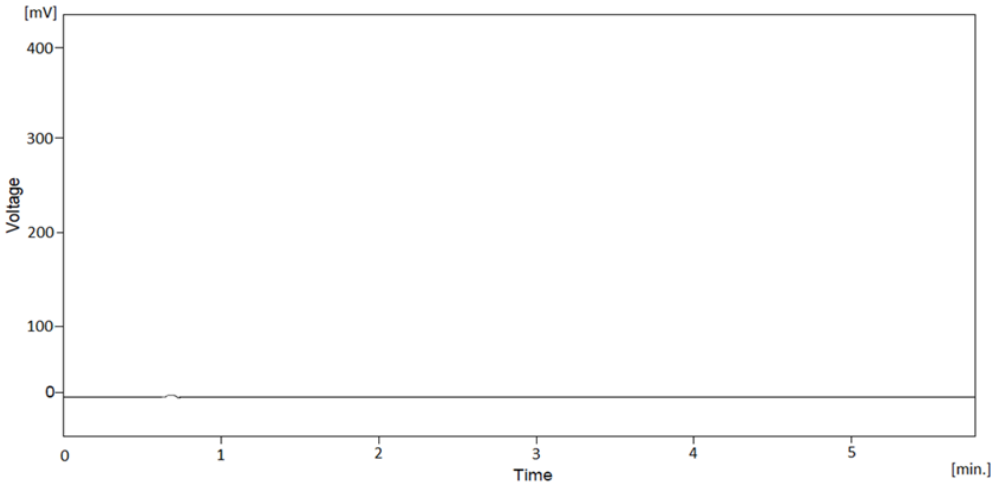

Figure 6.17: Blank Chromatogram

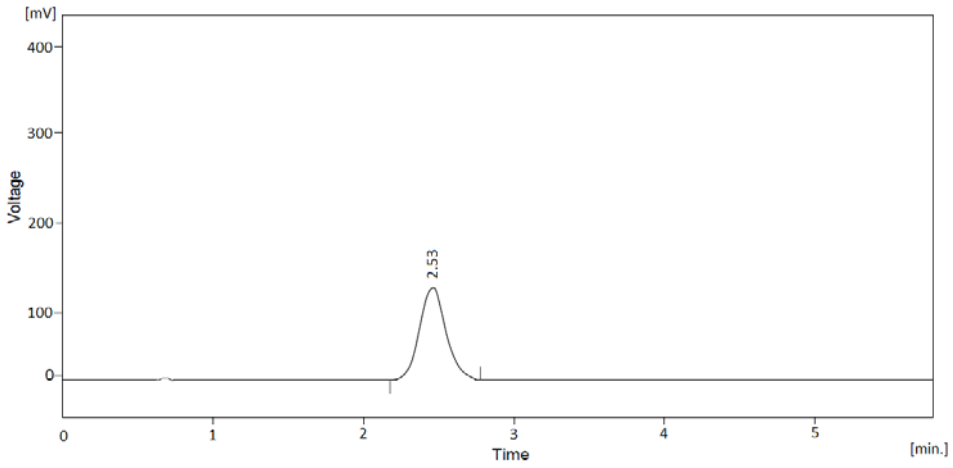

Figure 6.18: Chromatogram of DOLU

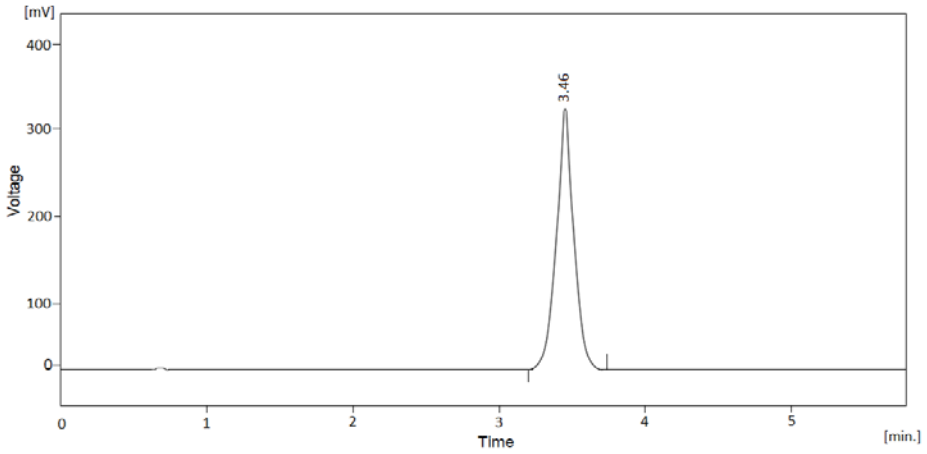

Figure 6.19: Chromatogram of TEN

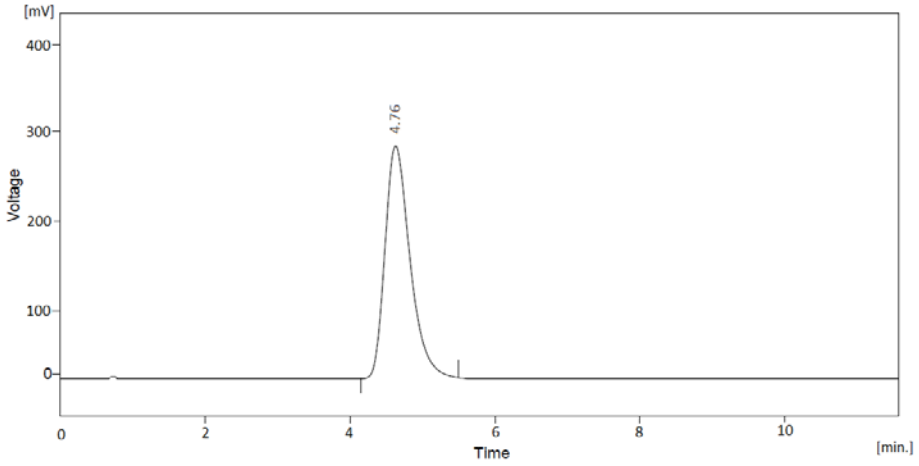

Figure 6.20: Chromatogram of RILP

9.5.3.2 Linearity and Range (n = 5)

Drugs LAM, TEN and DOR Linearity has been followed in a particular concentration ranges of 2.5-15ug/ml for DOLU, 7.5-45ug/ml for TEN and 3.75 - 22.5ug/ml for RILP. 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.

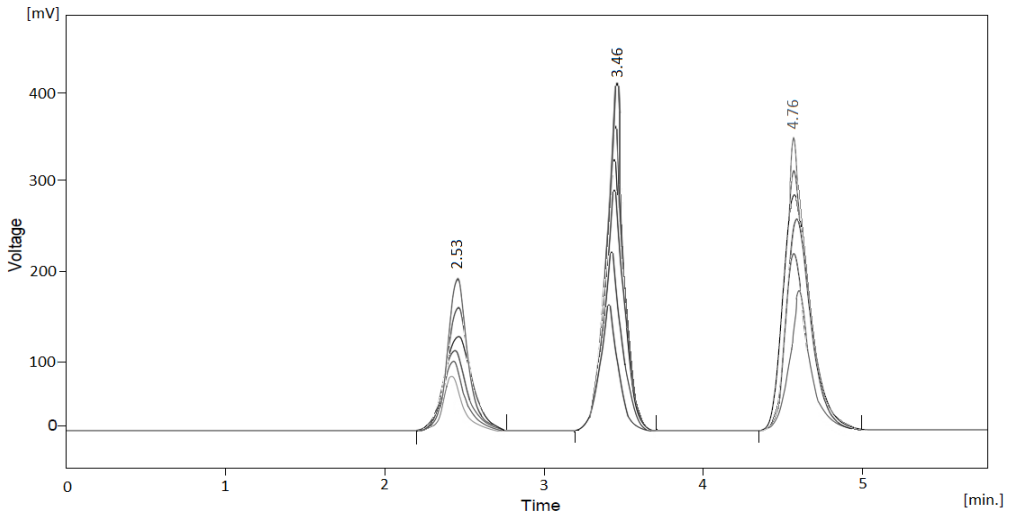

Figure 6.21: Overlain Chromatogram of Linearity for DOLU, TEN & RILP

(x) Conc. µg/ml	(y) Area
2.5	288.16
5	608.27
7.5	900.37
10	1210.59
12.5	1490.09
15	1822.94
STD ERROR	11.75
Slope	121.4
LOD	0.31
LOQ	0.96

Table 6.9: Linearity data of DOLU

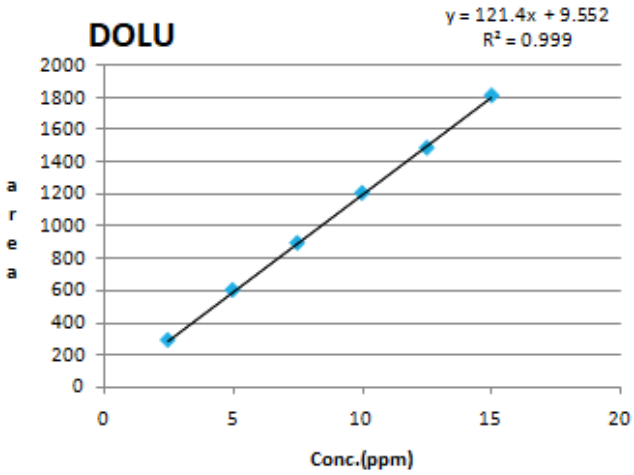

Figure 6.22: Calibration Curve for DOLU

(x) Conc. µg/ml	(y) Area
7.5	615.66
15	1142.27
22.5	1795.17
30	2375.85
37.5	2988.46
45	3561.38
STD ERROR	31.14
Slope	79.42
LOD	1.29
LOQ	3.92

Table 6.10: Linearity data of TEN

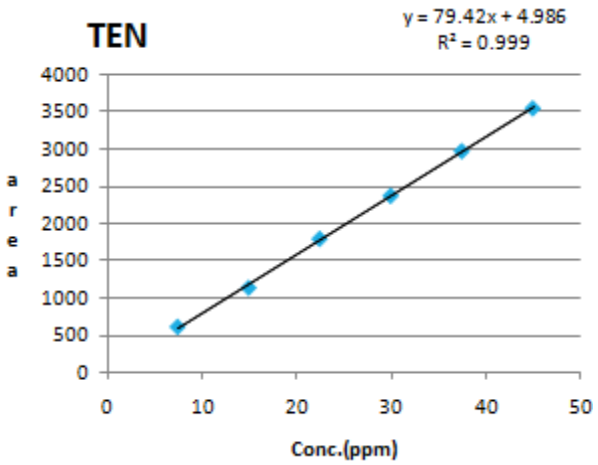

Figure 6.23: Calibration Curve for TEN

(x) Conc. µg/ml	(y) Area
3.75	615.53
7.5	1224.75
11.25	1849.21
15	2455.54
18.75	3086.17
22.5	3679.87
STD ERROR	6.67
Slope	163.3
LOD	0.13
LOQ	0.40

Table 6.11: Linearity data of RILP

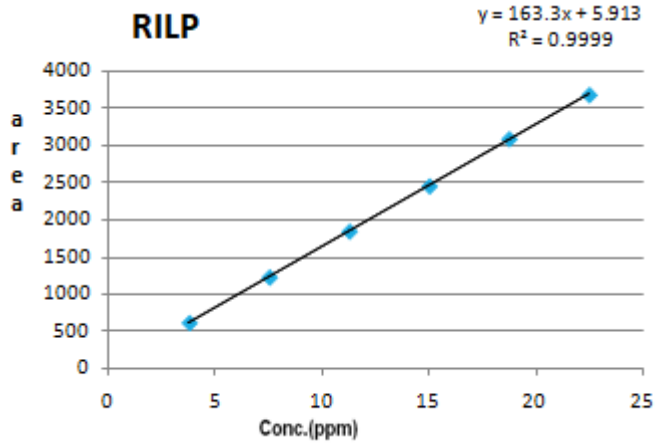

Figure 6.24: Calibration Curve for RILP

9.5.3.3 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 DOLU, TEN and 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
DOLU	10	50	5	4.91	98.30	98.78
	10	100	10	9.84	98.45	
	10	150	15	14.93	99.58	
TEN	30	50	15	14.88	99.20	99.29
	30	100	30	29.83	99.44	
	30	150	45	44.66	99.24	
RILP	15	50	7.5	7.52	100.29	100.28
	15	100	15	15.03	100.25	
	15	150	22.5	22.56	100.30	

Table 6.12: Accuracy Study of DOLU, TEN & RILP (n = 3)

9.5.3.4 Precision

9.5.3.4.1 Repeatability (n = 6)

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

Conc. of DOLU (µg/ml)	Area	Conc. of TEN (µg/ml)	Area	Conc. of RILP (µg/ml)	Area
10	1211.46	30	2381.25	15	2381.25
	1210.13		2385.94		2385.94
	1218.98		2378.63		2378.63
	1209.93		2375.19		2375.19
	1211.76		2380.07		2380.07
	1214.76		2375.86		2375.86
Mean	1212.83	Mean	2379.49	Mean	2379.49
SD	3.47	SD	3.93	SD	3.93
% RSD	0.28	% RSD	0.16	% RSD	0.16

Table 6.13: Repeatability Study of DOLU, TEN & RILP (n = 6)

9.5.3.4.2 Intraday Precision (n = 3)

The Intraday precision for the DOLU, TEN & 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.

DOLU			TEN			RILP		
Conc. (µg/ml)	Mean area ± SD	% RSD	Conc. (µg/ml)	Mean area ± SD	% RSD	Conc. (µg/ml)	Mean area ± SD	% RSD
2.5	287.0 ± 4.0	1.39	7.5	612.7 ± 4.8	0.78	3.75	618.1 ± 2.8	0.45
10	1218.3 ± 5.1	0.42	30	2378.5 ± 5.5	0.23	15	2455.1 ± 3.4	0.14
15	1820.3 ± 7.3	0.40	45	3559.1 ± 4.8	0.13	22.5	3677.27 ± 5.4	0.14

Table 6.14: Intraday Precision of DOLU, TEN & RILP (n = 3)

9.5.3.4.3 Interday Precision (n = 3)

The Interday precision for the DOLU, TEN & 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.

DOLU			TEN			RILP		
Conc. (µg/ml)	**Mean area ± SD**	**% RSD**	**Conc. (µg/ml)**	**Mean area ± SD**	**% RSD**	**Conc. (µg/ml)**	**Mean area ± SD**	**% RSD**
2.5	287.4 ± 5.3	1.80	7.5	617.9 ± 2.1	0.34	3.75	617.8 ± 5.5	0.90
10	1213.6 ± 4.6	0.38	30	2371.8 ± 2.4	0.10	15	2460.5 ± 5.5	0.22
15	1820.4 ± 3.6	0.24	45	3559.1 ± 6.4	0.18	22.5	3680.2 ± 6.8	0.18

Table 6.15: Interday Precision of DOLU, TEN & RILP (n = 3)

9.5.3.5 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 0.31, 1.29, and 0.13 ug respectively for DOLU, TEN & RILP, and the LOQ values are found to be 0.96, 4.92 and 0.40 ug respectively for DOLU, TEN & RILP.

9.5.4 Application of the Developed Analytical Method to Formulation

The proposed analytical method been tested in assay analysis % Assay of the Label claim on the DOLUVIR™ contains 50mg Dolutegravir, TENVIR™ contains 300mg Tenofovir, EDURANT™ contains 25mg of Rilpivirine.

Analytical method successfully applied to the estimation of drugs in marketed product 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
DOLU	1	50	49.04	98.08	98.03	0.27	0.28
	2	50	48.87	97.74			
	3	50	49.14	98.28			
TEN	1	300	296.95	98.98	99.01	0.17	0.17
	2	300	296.49	98.83			
	3	300	297.53	99.18			
RILP	1	25	24.75	99.0	98.25	0.14	0.14
	2	25	24.52	98.09			
	3	25	24.59	98.36			

Table 6.16: Assay of Formulations (n = 3)

9.5.5 Summary of Results

Sr No	**Parameters**	**Results**		
		DOLU	**TEN**	**RILP**
1	System Suitability:			
	Theoretical plates-	5537	6772	7781
	Tailing Factor-	1.41	1.21	1.32
	Retention time min-	2.53	3.46	4.76
2	Precision (%RSD)	0.42	0.23	0.14
3	Linearity (R^2)	0.9999	0.9999	0.9998
4	Accuracy (% Recovery)	99.78	99.29	100.28
5	LOD (ug/ml)	0.31	1.29	0.13
6	LOQ (ug/ml)	0.96	3.92	0.40
7	% Assay	98.03	99.01	98.25

9.6 CONCLUSIONS

The Stability Indicating HPLC method for DOLU, TEN & RILP combinational drugs has been successfully developed and validated. The analytical method is optimized for the testing even in degraded conditions and analysis for DOLU, TEN & RILP in individual as well in combined forms and all the validation parameters are performed in the acceptance criteria as per ICH regulatory guideline. Developed method is accurate., & precise to detect the main drug peaks without any interference or overlap of degraded impurities & products produced during forced degradation stress conditions.

The accurate precise method can be used for analysis of DOLU, TEN & RILP combination as well as individual in as Assay method and dissolution testing procedures in academics, research, analytical laboratories and in pharmaceutical industries.