Chapter 5

STABILITY INDICATING HPLC METHOD DEVELOPMENT AND VALIDATION FOR DELSTRIGO COMBINATION

5.1 EXPERIMENTALS

5.1.1 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 in 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. . Veego VDA-8D Microprocessor Based Dissolution Test for dissolution testing. Photostability Test Chamber Sanwood SM-LHH-GSD-UV Series was utilised. Electronic analytical balance AUX-220 of the 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.

5.1.2 Materials and Reagents Utilised

The chemicals used working reference standard drugs Doravirine DOR, Lamivudine LAM, Tenofovir TEN drugs samples of 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.

5.1.3 Identification of Standard Drug Samples

5.1.3.1 Melting Point Determination

The working standard drugs Lamivudine LAM, Tenofovir TEN & Doravirine DOR 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 2.1.

Drug	Observed Melting Range	Standard Melting Range
DOR	283-285.5 ^{0}C	284.8 ^{0}C
TEN	114.5-115 ^{0}C	113-115 ^{0}C
LAM	161-162.5 ^{0}C	160-162-170 ^{0}C

Table 2.1: Melting Points of LAM, TEN & DOR

5.1.3.2 FTIR Spectral Determination for Identification of Standard drug samples LAM, TEN & DOR

The pure active pharmaceutical working standard drug substances LAM, TEN & DOR 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 2.1 for LAM, Fig 2.2 for TEN & Fig 2.3 for DOR. The principal IR peaks recorded and observed for the drugs are shown in Table 2.2, 2.3 & 2.4 for LAM, TEN & DOR respectively.

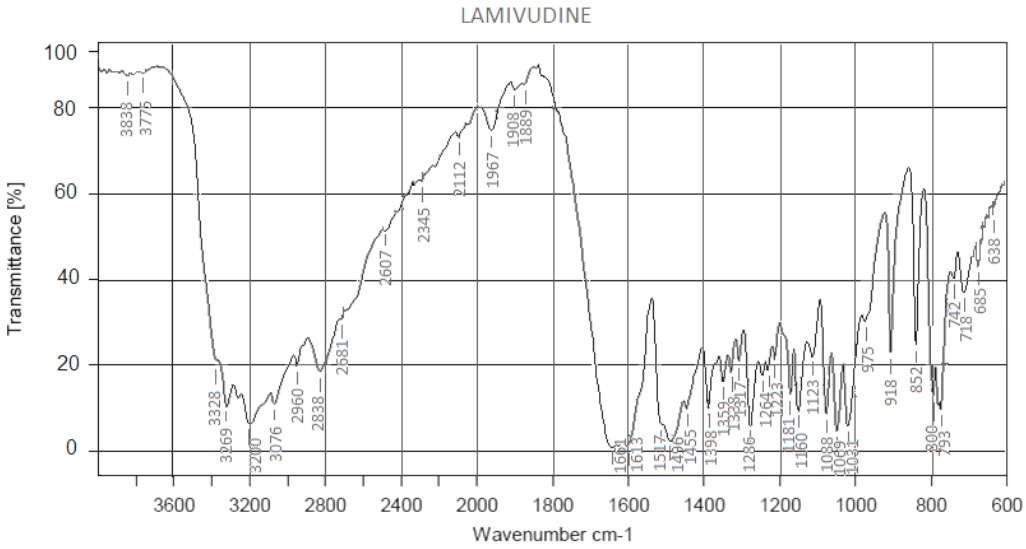


Figure 2.1: FTIR Spectra of Lamivudine LAM

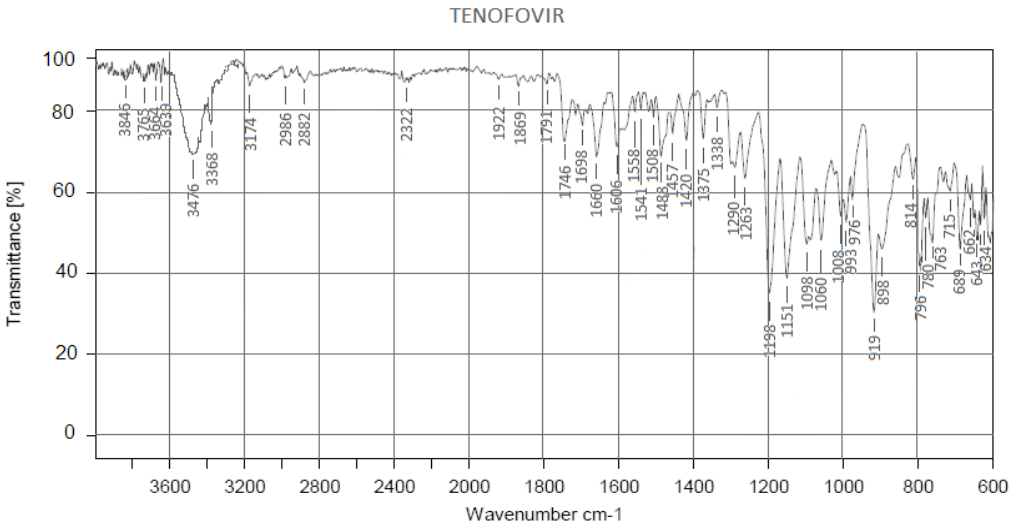


Figure 2.2: FTIR Spectra of Tenofovir TEN

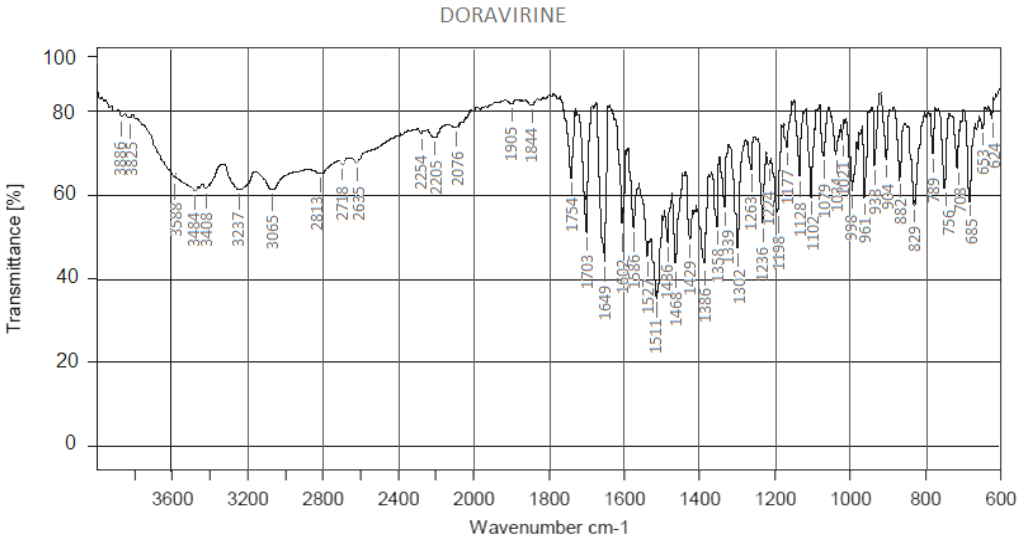


Figure 2.3: FTIR Spectra of Doravirine DOR

LAMIVUDINE					
Energy (Cm^{-1})	**Band Assignment**	**Peak Intensity**	**Energy (Cm^{-1})**	**Band Assignment**	**Peak Intensity**
1230-1270	C-N (S)	18.54 21.37	1340-1269	C-N Aromatic amine	19.68 17.36
690-687	C-S	43.29	1085-1055	OH Primary	8.77 4.91

1000-1305	C-O (ether)	5.44 4.76 18.85	2700-3200 3550-3200 3700-3585	O-H alcohol	12.69 5.48
1465-1300	C-H (S)	13.84 17.33	772-625	C-S-C cyclic	38.37 40.57
1650-1569	C=C Cyclic ene	4.66 3.83	1085-1150	C-O Cyclic ether	11.75 12.47
1500-1700	C=O	2.69	1690-1640	C=N imine	1.58
1200-1350	N-C (3^0 Amine)	22.46 17.17	1230-1270	C-N (S)	18.75 22.06

Table 2.2: FTIR Interpretation of Lamivudine LAM

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 2.3: FTIR Interpretation of Tenofovir TEN

DORAVIRINE					
Energy (Cm^{-1})	**Band Assignment**	**Peak Intensity**	**Energy (Cm^{-1})**	**Band Assignment**	**Peak Intensity**
857 - 867	C-Cl (S)	58.32 62.76	1342-1236	C-CN Aromatic	48.64 52.48
1183	C-O (S)	72.79	1275-1200	C-O Ether Aromatic	65.29 63.47
1647-1600	C=C (Aromatic)	54.69	1680- 1640-1630	C=O Amide	43.59
1749-1792	C-O (Ether)	62.17	2260-2222	C≡N nitrile	74.18
2350.21- 2360	N-H (S)	72.65	1690-1640	C=N imine	43.59 53.54
1180.0- 1281	$-CF_3$	57.48 65.36 68.19	3400- 3300-3200	N-H Sec amine	61.23 60.84
2200-2000	-CN	74.18 78.67	1250-1020	C-N amine	70.24 79.57 62.37

Table 2.4: FTIR Interpretation of Doravirine DOR

5.1.4 Preparation of Solutions

5.1.4.1 Preparation of standard solutions Doravirine DOR, Lamivudine LAM and Tenofovir TEN

The standard stock soln. of individual drugs prepared in 20:80 Methanol : Water solvent mixture. 30mg of LAM & 30mg of TEN were individually dissolved in solvent mixture and made upto 100ml with same solvent to give 300 µg/ml standard stock solutions of LAM & TEN were prepared and for DOR, 10mg DOR was dissolved in solvent mixture and made upto 100ml to give 100 µg/ml standard stock solution.

From the above stock solutions of individual drugs LAM, TEN & DOR each, 1ml from each was taken individually and diluted upto 10ml in individual volumetric

flasks to give LAM 30 µg/ml, TEN 30 µg/ml and DOR 10 µg/ml individual drug standard Final solutions.

5.1.4.2 Preparation of Sample Solutions

Sample solution from the tablet Delstrigo^{TM} containing combination of LAM 300 mg 300mg , TEN 300mg & DOR 100mg made. Accurately the avg. wt. of 10 tablets was done and crushed triturated, the tablet powder was taken weighing equivalent wt of LAM 300mg TEN 300mg & DOR 100mg was taken and dissolved in 50:50 Methanol : ACN 50ml solution in a volumetric flask and then sonicated, filtered and made upto 100ml to give stock solution A containing LAM 3000 µg/ml , TEN 3000 µg/ml & DOR 1000 µg/ml.

From this stock solution A, 1ml was taken aliquot and made upto 100ml in a volumetric flask to give final solution containing LAM 30 µg/ml , TEN 30 µg/ml & DOR 10 µg/ml .

5.1.4.3 Preparation of Optimized Mobile Phase

The mobile phase made by taking 80:20 ratio, 0.05M Phosphate buffer : ACN 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. The 1% OPA was prepared by taking (1.176ml) of 85%w/v orthophosphoric acid (MW 98) in 100ml HPLC grade water.

5.1.5 Selection of Wavelength for Detection

The Final standard solutions of LAM 30 µg/ml , TEN 30 µg/ml & DOR 10 µg/ml scanned in 200 - 400 nm in UV visible double beam spectrophotometer at a medium scanning speed. The overlain spectra shown in Fig. 2.4 of LAM 30 µg/ml , TEN 30 µg/ml & DOR 10 µg/ml were taken in 20:80 Methanol : Water and the 269nm wavelength was selected for estimation in the detection during the HPLC analysis.

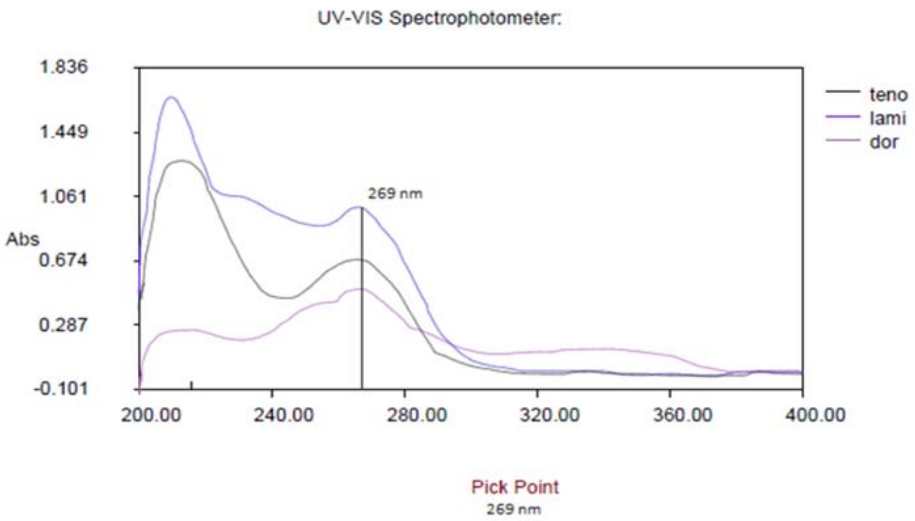


Figure 2.4: UV Spectra Overlay of LAM, TEN & DOR

5.1.6 Selection and Optimization of Mobile phase

For the detection analysis of the LAM, TEN & DOR 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 2.5

Sr No	Mobile Phase	pH	Ratio (v/v)	Retention Time (min)			REMARK
				LAM	TEN	DOR	
1	H_2O:MeOH	-	20:80	5.43	5.22	-	Merged peak and No peak of DOR detected
2	H_2O:MeOH	-	40:60	6.27	7.37	-	Merged peak and No peak of DOR detected
3	H_2O:MeOH	-	80:20	7.47	8.48	-	Merged peak and No peak of DOR detected
4	ACN: H_2O	-	40:60	6.31	6.74	-	Merged peak, Tailing, No peak of DOR detected

5	ACN: H_2O	-	80:20	8.09	8.91	-	Merged peak, Tailing, No peak of DOR detected
6	0.05 M - Phosphate - buffer : ACN	5	80:20	7.28	7.93	9.66	Not Good Resolution
7	0.05 M - Phosphate - buffer : ACN	5	70:30	6.91	7.51	9.17	Not Good Resolution, Peak tailing
8	0.05 M - Phosphate - buffer : ACN	4	80:20	6.21	7.11	8.81	Not Good Resolution
9	**0.05 M - Phosphate-buffer : ACN [Selected Mobile phase]**	**3.5**	**80:20**	**5.77**	**6.78**	**8.39**	**Good Separation & Resolution**

Table 2.5: Trials for Selection of Mobile Phase for LAM, TEN & DOR

5.1.7 Optimized Chromatographic Conditions

The optimized chromatographic conditions for the 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 80:20 pH - 3.5
Flow rate	1ml/min
Injection volume	20ul
Temp	Ambient Lab Temperature
Detection Wavelength	269.1nm
Retention Times (min)	LAM-5.77, TEN-6.78, DOR-8.39

Table 2.6: Optimized Chromatographic Conditions for LAM, TEN & DOR

5.2 STABILITY STUDIES BY FORCED DEGRADATIONS

The stability studies for the pure working standard drugs LAM, TEN & DOR as well as for the pharmaceutical marketed formulation Delstrigo™ 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.

5.2.1 Acid Degradation

For the acid degradation study, was performed in 0.1N HCl solution. The working standard drug solution of 1ml of LAM (300ug/ml) std stock soln, 1ml of TEN (300ug/ml) std stock soln, and 1ml of DOR (100ug/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 LAM 30ug/ml, TEN 30ug/ml and DOR 10ug/ml. And the analysed this sample by developed HPLC method. In the similar manner the combined drug sample of marketed Delstrigo™ formulation was prepared stock soln containing 300ug/ml LAM, 300ug/ml TEN and 100ug/ml DOR. 1ml from this stock soln 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 volu me with mobile phase to give LAM 30ug/ml, TEN 30ug/ml and DOR 10ug/ml. And the analysed this sample by developed HPLC method.

5.2.2 Base Degradation

The Base degradation study, performed in 0.1N NaOH solution. The working standard drug solution of 1ml of LAM (300ug/ml) std stock soln, 1ml of TEN (300ug/ml) std stock soln, and 1ml of DOR (100ug/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 LAM 30ug/ml, TEN 30ug/ml and DOR 10ug/ml. And the analysed this

sample by developed HPLC method. In the similar manner the combined drug sample of marketed Delstrigo^{TM} formulation was prepared stock soln containing 300ug/ml LAM, 300ug/ml TEN and 100ug/ml DOR. 1ml from this stock soln 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 to give LAM 30ug/ml, TEN 30ug/ml and DOR 10ug/ml. And the analysed this sample by developed HPLC method.

5.2.3 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 LAM (300ug/ml) std stock soln, 1ml of TEN (300ug/ml) std stock soln, and 1ml of DOR (100ug/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 LAM 30ug/ml, TEN 30ug/ml and DOR 10ug/ml. And the analysed this sample by developed HPLC method. In the similar manner the combined drug sample of marketed Delstrigo^{TM} formulation was prepared stock soln containing 300ug/ml LAM, 300ug/ml TEN and 100ug/ml DOR. 1ml from this stock soln 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 LAM 30ug/ml, TEN 30ug/ml and DOR 10ug/ml. And the analysed this sample by developed HPLC method.

5.2.4 Thermal Degradation

It has carried out for the working standard drug powders LAM, TEN & DOR individually in Wist Temperature chamber oven at 105 ^{0}C for 12hrs. After thermal degradation, the drug powder LAM 30mg, TEN 30mg and DOR 10mg were taken in flask dissolved in 50ml of 20:80 Methanol : Water solvent, dissolved, sonicated , filtered and final volume made upto 100ml to give stock soln of 300ug/ml of LAM, 300ug/ml TEN & 100ug/ml DOR. From this stock soln, 1ml taken n diluted to 10ml with mobile phase to give final soln containing LAM 30ug/ml, TEN 30ug/ml and DOR 10ug/ml. This final solution was subjected to be analysed by developed HPLC method. In similar manner marketed formulation Delstrigo^{TM} tablet sample was powdered and kept in Wist Temperature chamber oven at 105 ^{0}C for 12hrs. After thermal degradation, the tablet powder weighing equivalent to LAM 30mg, TEN

30mg and DOR 10mg were taken in flask dissolved in 50ml of 20:80 Methanol : Water solvent, dissolved by heating on water-bath at 60 ^{0}C 20mins, shaken & sonicated , filtered and final volume made upto 100ml to give stock soln of 300ug/ml of LAM, 300ug/ml TEN & 100ug/ml DOR. From this stock soln, 1ml, taken and then it has been, diluted to 10ml with mobile phase to give final soln containing LAM 30ug/ml, TEN 30ug/ml and DOR 10ug/ml. This final solution was subjected to be analysed by the developed HPLC method.

5.2.5 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 LAM, TEN and DOR were kept into UV chamber for 24hrs. After photo degradation, the drug powder LAM 30mg, TEN 30mg and DOR 10mg were taken in flask dissolved in 50ml of 20:80 Methanol : Water solvent, dissolved, sonicated , filtered and final volume made upto 100ml to give stock soln of 300ug/ml of LAM, 300ug/ml TEN & 100ug/ml DOR. From this stock soln, 1ml taken and diluted to 10ml with mobile phase to give final soln containing LAM 30ug/ml, TEN 30ug/ml and DOR 10ug/ml. This final solution was subjected to be analysed by developed HPLC method. In similar manner marketed formulation Delstrigo^{TM} tablet sample was powdered and kept into UV chamber for 24hrs. After degradation, the tablet powder weighing equivalent to LAM 30mg, TEN 30mg and DOR 10mg were taken in flask dissolved in 50ml of 20:80 Methanol : Water solvent, dissolved by heating on water bath at 60 ^{0}C, 20mins, shaken & sonicated , filtered and final volume made upto 100ml to give stock soln of 300ug/ml of LAM, 300ug/ml TEN & 100ug/ml DOR. From this stock soln, 1ml taken and diluted to 10ml with mobile phase to give final soln containing LAM 30ug/ml, TEN 30ug/ml and DOR 10ug/ml. This final solution was subjected to be analysed by the developed HPLC - method .

5.3 METHOD VALIDATION

5.3.1 Linearity (Calibration Curve)

The working standard and sample solutions of Lamivudine LAM & Tenofovir TEN were 7.5, 15, 22.5, 30, 37.5, 45ug/ml, prepared in the serial dilutions for both drugs individually, while 2.5, 5. 7.5, 10, 12.5, 15ug/ml of Doravirine DOR, 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 .

5.3.2 Specificity and Selectivity

The selectivity and specificity parameters are utilised selective detection particular analyte which are in the matrix or along with other substances without any interventions. 30ug/ml of LAM & TEN and 10ug/ml of DOR 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.

5.3.3 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 LAM, TEN, DOR 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.

5.3.4 Precision

5.3.4.1 Repeatability (n=6)

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

5.3.4.2 Intraday Precision (n=3)

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

5.3.4.3 Interday Precision (n=3)

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

5.3.5 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

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

The developed analytical HPLC method is applied in the estimation-analysis of LAM, TEN, DOR in the Delstrigo^{TM} marketed tablet dosage form. Each tablet Delstrigo^{TM} contains LAM 300mg TEN 300mg and DOR 100mg.

The tablets were weighed and avg. wt, of 10 tablets was calculated, and the powder weighed equivalent to LAM 300mg, TEN 300mg and DOR 100mg was taken and dissolved in sufficient quantity of 50:50 Methanol:ACN, sonicated & filtered, and the final volume was made upto 100ml to give stock solution A containing LAM 3000ug/ml, TEN 3000ug/ml and DOR 1000ug/ml. From this stock solution A 1ml was diluted upto 100ml to give final solution containing LAM 30ug/ml, TEN 30ug/ml

and DOR 10ug/ml, and were prepared n=3 samples, analysed by the developed HPLC method. The working standard drugs LAM 30ug/ml, TEN 30ug/ml and DOR 10ug/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.

5.5 APPLICATION OF DEVELOPED METHOD IN DISSOLUTION STUDIES

The dissolution method has been developed & performed on the marketed formulation Delstrigo™ solid oral tablet dosage form, developed HPLC method has been utilised for qualitative, quantitative and % drug release, and % Cumulative drug release estimation.

5.5.1 Dissolution Medium

The dissolution medium was prepared by using 0.05M K_2HPO_4 phosphate buffer with 2% SLS Sodium lauryl sulphate pH adjusted to 6.8. [6.8gm KH_2PO_4 (MW 136) in 1000ml dist water with 20gm SLS (2%) And adjust pH 6.8 with 1% OPA, nearly the pH was found between range of 7-8.5, so it was adjusted to pH - 6.8 with with 1% OPA] [1 % OPA : (1.176ml) of orthophosphoric acid (85%w/v) (MW 98) in 100ml dist water]

5.5.2 Dissolution Instrument & Procedure

USP type-2 Paddle (Veego VDA-8D Dissolution Test Apparatus) was used.

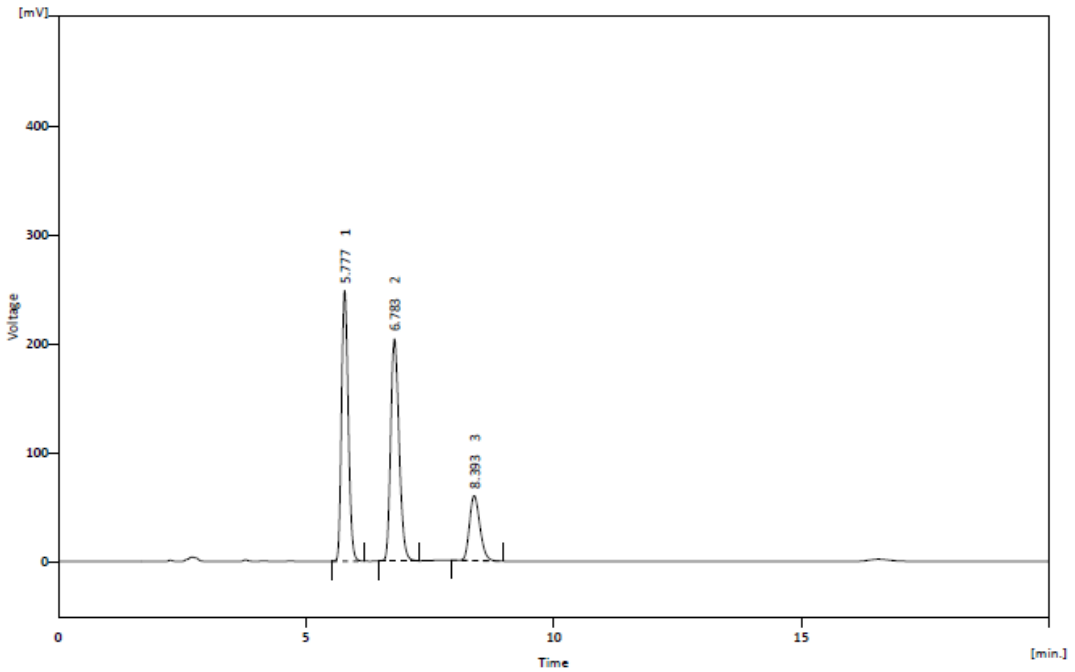
Bath Volume- 900ml, Bath Temp-$37^0C \pm 0.5$, Paddle RPM-50.

Each tablet Delstrigo™ containing LAM 300mg TEN 300mg DOR 100mg was kept in dissolution medium n=6 and 5ml of the samples were withdrawn into soln. From bath, from dissolution bath at sampling time intervals of 10, 20, 30, 40, 50 & 60mins, and bath volume was maintained 900ml with dissolution medium. [0.33mg/ml or 333.33ug/ml of LAM & TEN and 0.11mg/ml or 111.11ug/ml DOR at proposed 100% Release]. The 5ml withdrawn sample filtered through nylon 0.20u filter cap and was made upto 10ml with (50:50 ACN:Methanol) to give Stock Soln [166.66ug/ml of LAM & TEN and 55.55ug/ml DOR at 100% release]. From above stock soln, 3ml was taken and made upto 50ml with mobile phase, the Final dilution *Sample Soln-B* used in analysis by HPLC. [10ug/ml of LAM & TEN and 3.33ug/ml DOR at proposed 100% Release]

5.6 RESULTS & DISCUSSIONS

5.6.1 Method Development

The developed analytical HPLC method found to be reliable, accurate.,- precise for analysis and quality control testing for LAM, TEN and DOR in pure form, in marketed tablet dosage form's., as well to perform the dissolution drug release profile studies from the dosage forms. 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 (80:20) pH - 3.5, flow rate 1ml / min , detection wavelength at 269nm. The 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 LAM-5.77, TEN-6.78 and DOR-8.39 minutes. The chromatogram of the drugs are shown below.



Result Table (Uncal - LAM-TEN-DOR Phosphate buffer ([pH 3.5)-ACN 80-20)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	W05 [min]
1	5.777	2336.756	247.635	41.599	0.15
2	6.783	2409.119	203.090	42.888	0.19
3	8.393	871.410	59.451	15.513	0.23
	Total	5617.285	510.176	100.000	

Figure 2.5: Chromatogram of Standard LAM, TEN & DOR

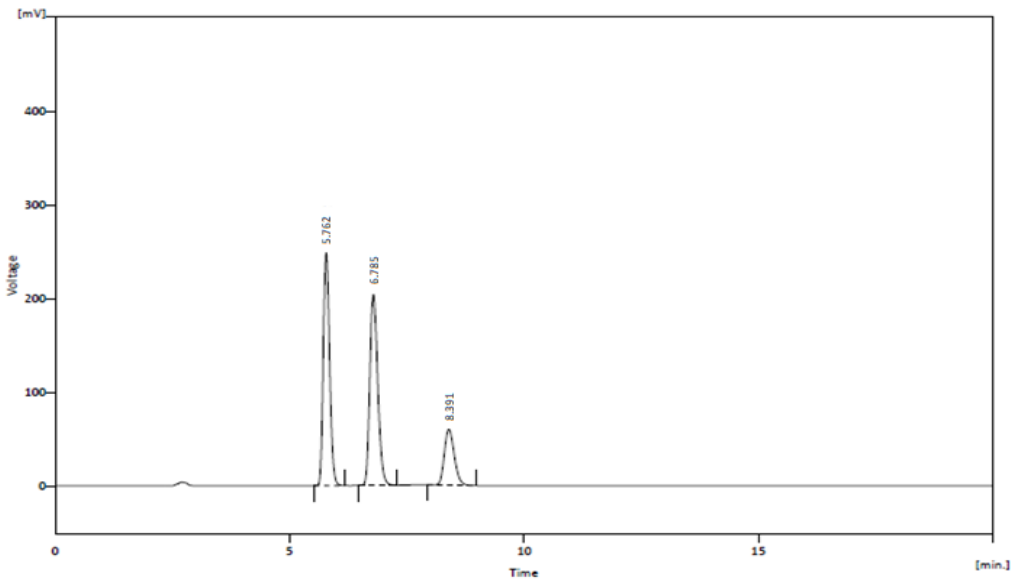


Figure 2.6: Chromatogram of Sample LAM, TEN & DOR

5.6.2 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 LAM, TEN & DOR and for the marketed formulation sample Delstrigo^{TM}. 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.

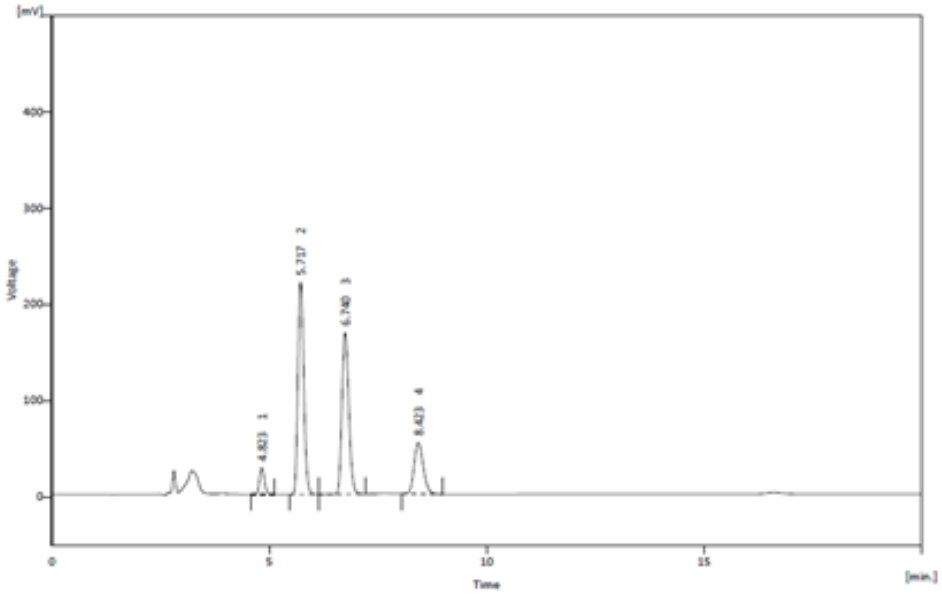


Figure 2.7 : Chromatogram of Acid Degradation Standard LAM, TEN & DOR

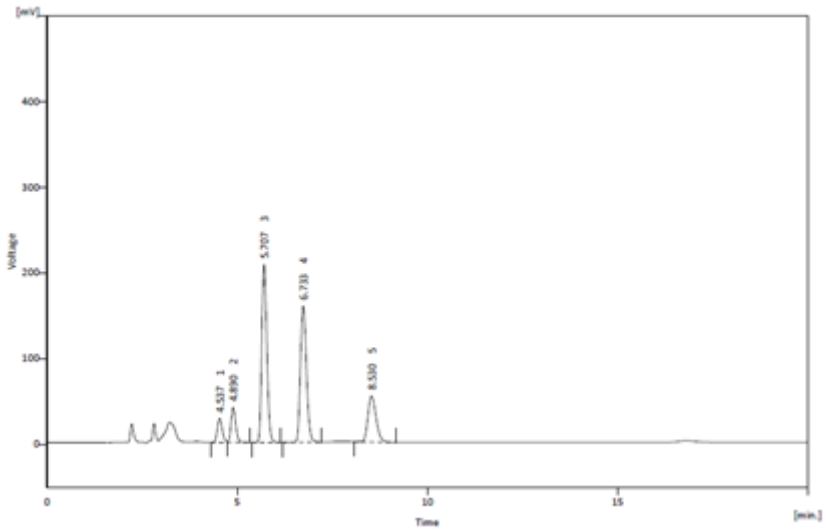


Figure 2.8: Chromatogram of Base Degradation Standard LAM, TEN & DOR

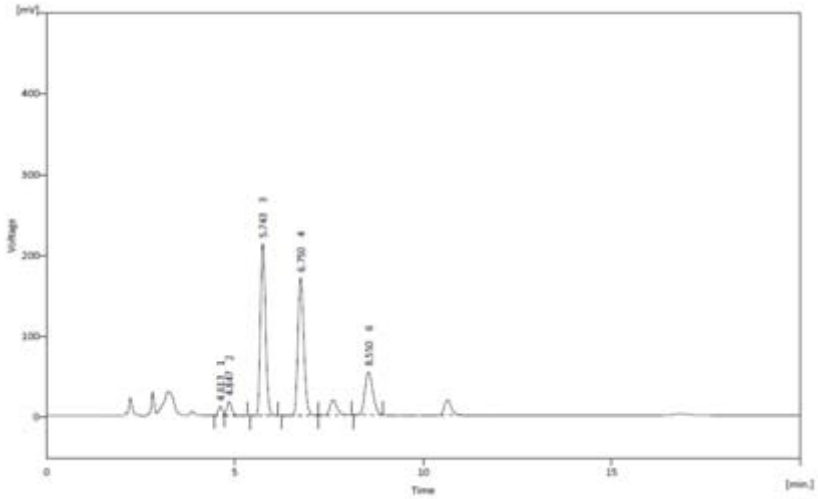


Figure 2.9: Chromatogram of Oxidative Degradation Standard LAM, TEN & DOR

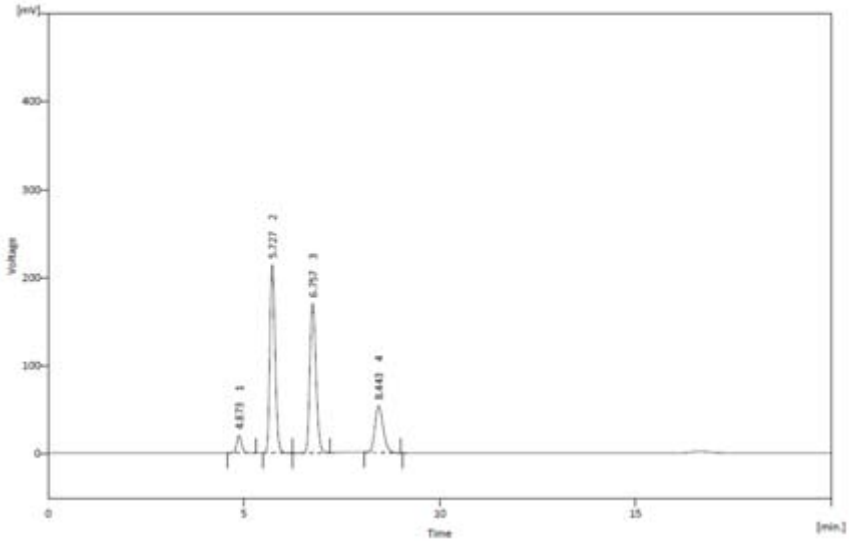


Figure 2.10: Chromatogram of Thermal Degradation Standard LAM, TEN & DOR

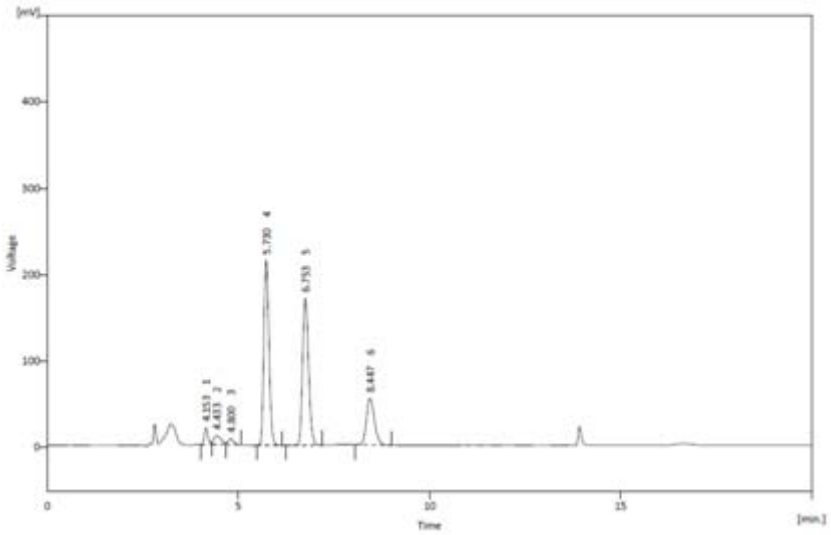


Figure 2.11: Chromatogram of Photo Degradation Standard LAM, TEN & DOR

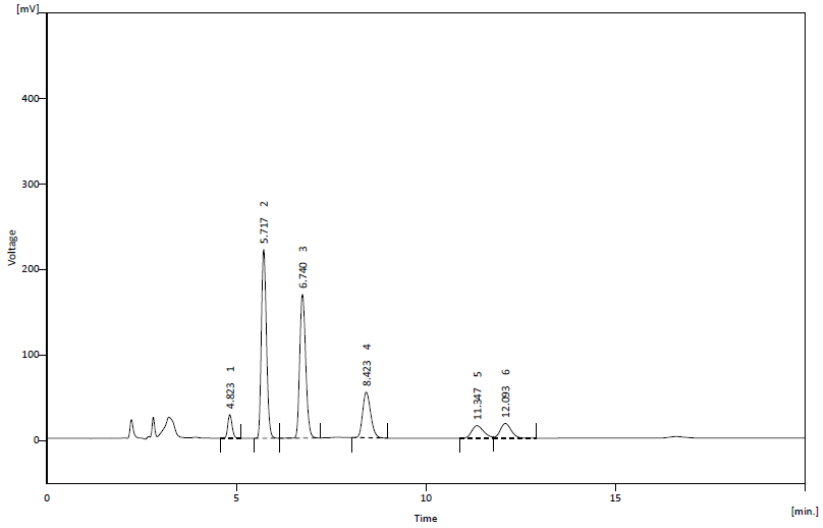


Figure 2.12: Chromatogram of Acid Degradation Sample LAM, TEN & DOR

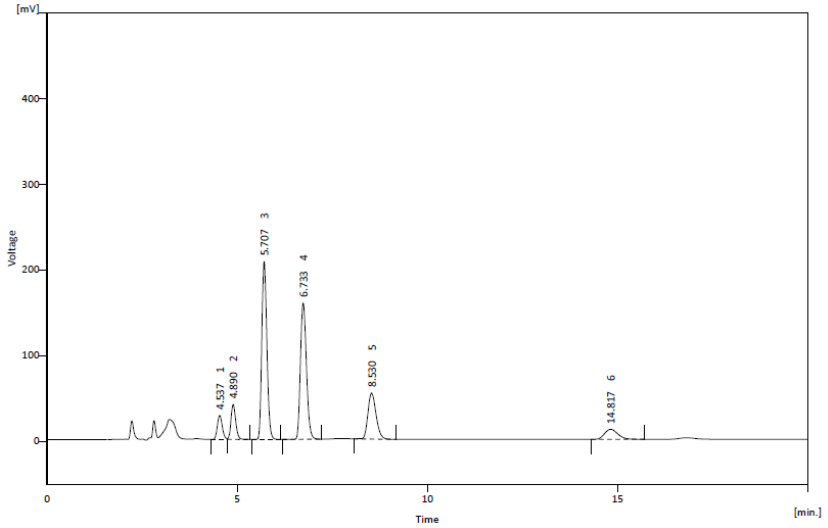


Figure 2.13: Chromatogram of Base Degradation Sample LAM, TEN & DOR

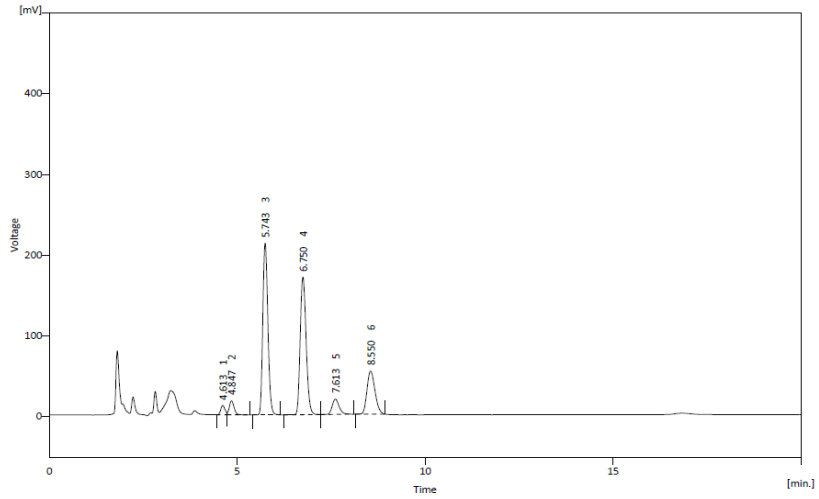


Figure 2.14: Chromatogram of Oxidative Degradation Sample LAM, TEN & DOR

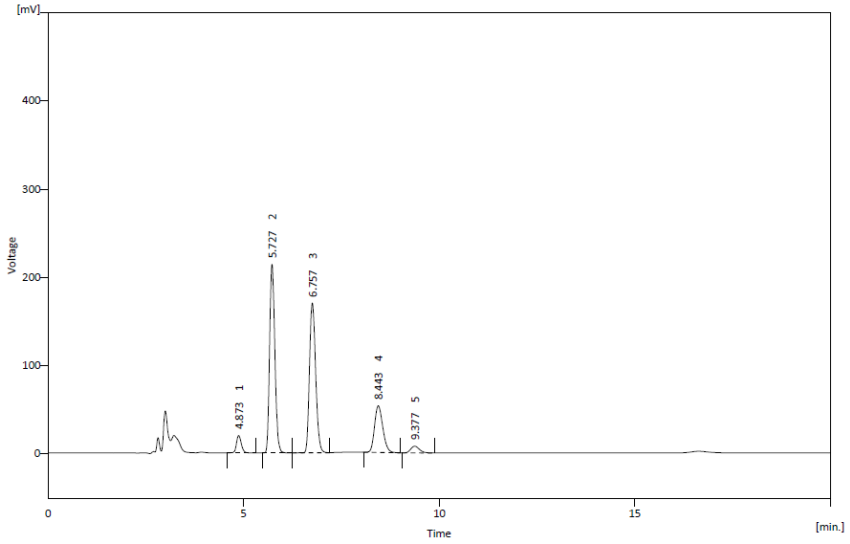


Figure 2.15: Chromatogram of Thermal Degradation Sample LAM, TEN & DOR

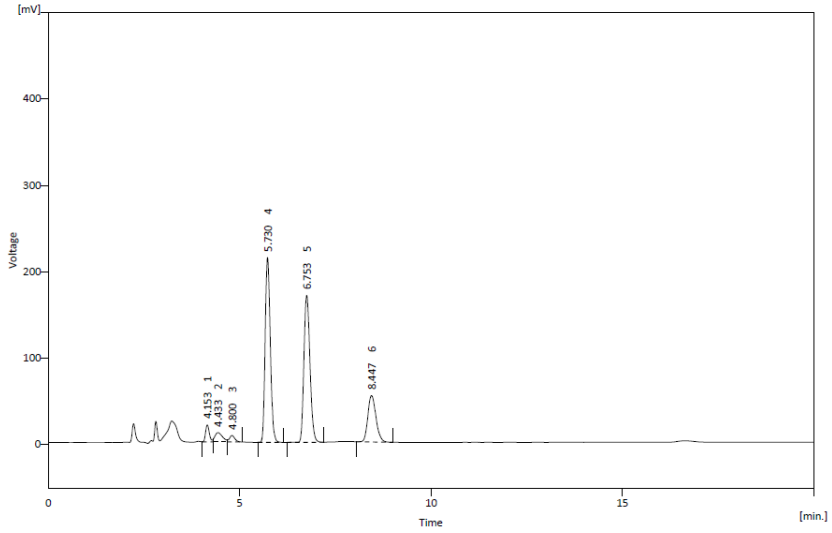


Figure 2.16: Chromatogram of Photo Degradation Sample LAM, TEN & DOR

Degradation Condition	Peak Area			% Drug Recovered			% Degraded		
	LAM	TEN	DOR	LAM	TEN	DOR	LAM	TEN	DOR
Acid	2390.46	2304.87	871.63	98.06	97.84	98.21	1.93	2.15	1.78
Base	2384.59	2279.65	878.04	97.81	96.77	98.93	2.18	3.22	1.06
Oxidative	2375.12	2286.14	869.13	97.43	97.04	97.93	2.56	2.95	2.06
Thermal	2374.07	2311.94	872.43	97.38	98.14	98.30	2.61	1.85	1.69
Photo	2409.67	2297.87	876.72	98.84	97.54	98.78	1.15	2.45	1.21

Table 2.7: % Drug Degraded & % Drug Recovered LAM, TEN & DOR

PEAK PURITY				
Drug	**Stress Type**	**Peak Purity Angle**	**Peak Purity Threshold**	**Peak Purity**
LAM	Untreated Sample	0.123	0.294	0.999
	Acid	0.156	0.311	0.998
	Base	0.148	0.397	0.999
	Oxidative	0.136	0.319	0.996
	Photo	0.178	0.413	0.997
	Thermal	0.122	0.356	0.995
TEN	Untreated Sample	0.113	0.246	0.999
	Acid	0.217	0.455	0.997
	Base	0.199	0.417	0.995
	Oxidative	0.269	0.497	0.996
	Photo	0.122	0.214	0.999
	Thermal	0.118	0.366	0.996
DOR	Untreated Sample	0.106	0.227	0.999
	Acid	0.211	0.345	0.996
	Base	0.133	0.339	0.997
	Oxidative	0.312	0.637	0.996
	Photo	0.233	0.412	0.997
	Thermal	0.132	0.377	0.995

Table 2.8: Peak Purity for LAM, TEN & DOR

5.6.3 Method Validation

5.6.3.1 Specificity

Method is found to be more - specific and selective as no other peaks of excipients- 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.

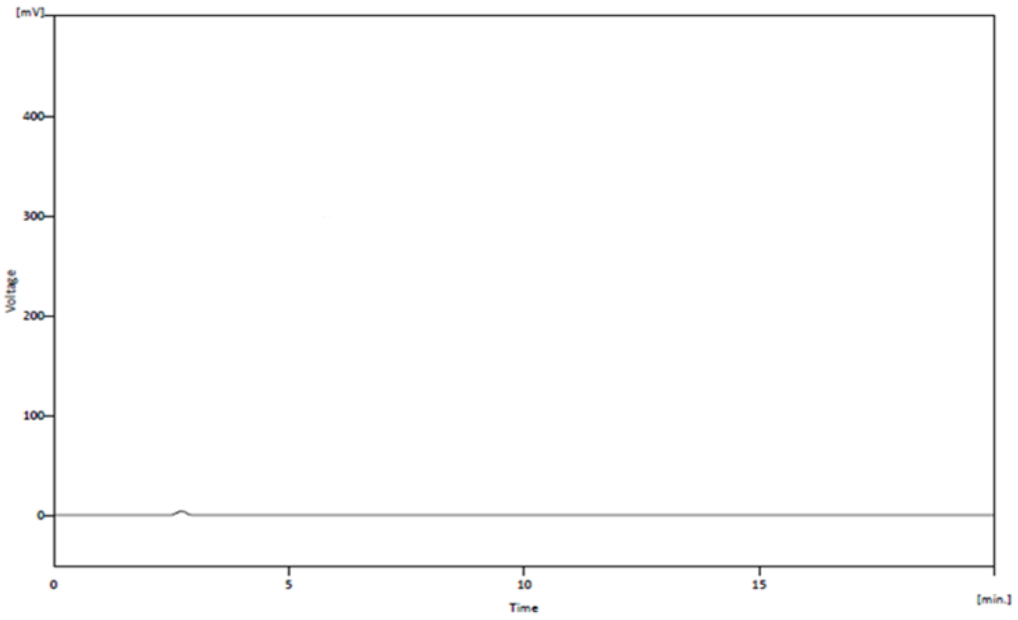


Figure 2.17: Blank Chromatogram

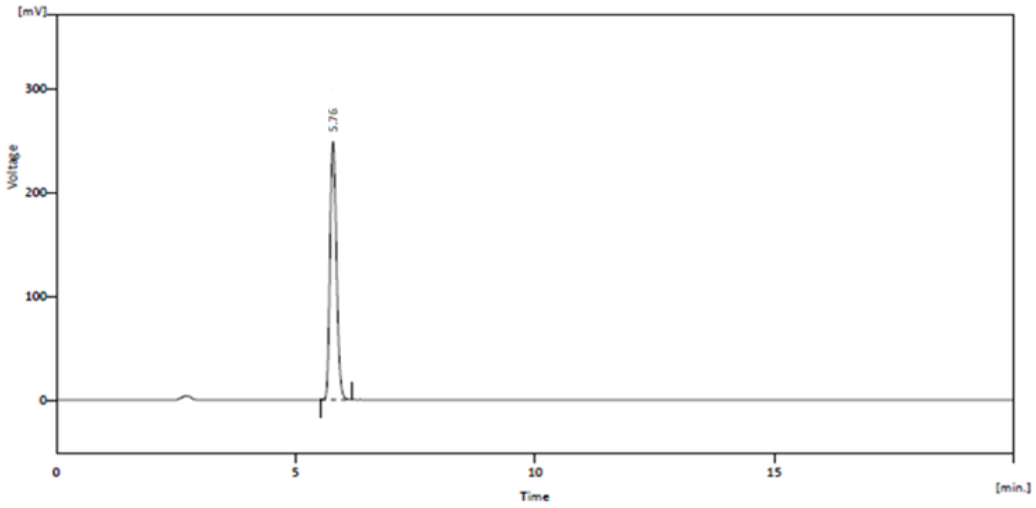


Figure 2.18: Chromatogram of LAM

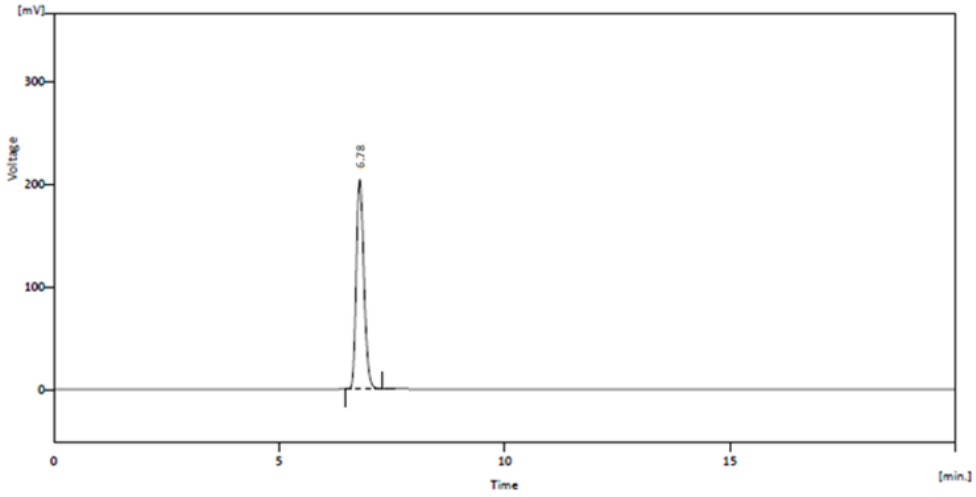


Figure 2.19: Chromatogram of TEN

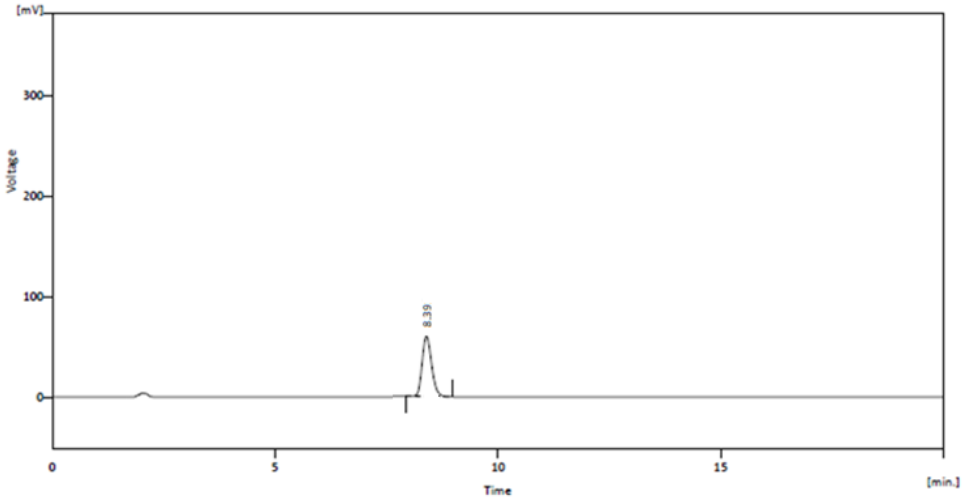


Figure 2.20: Chromatogram of DOR

5.6.3.2 Linearity and Range (n = 5)

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

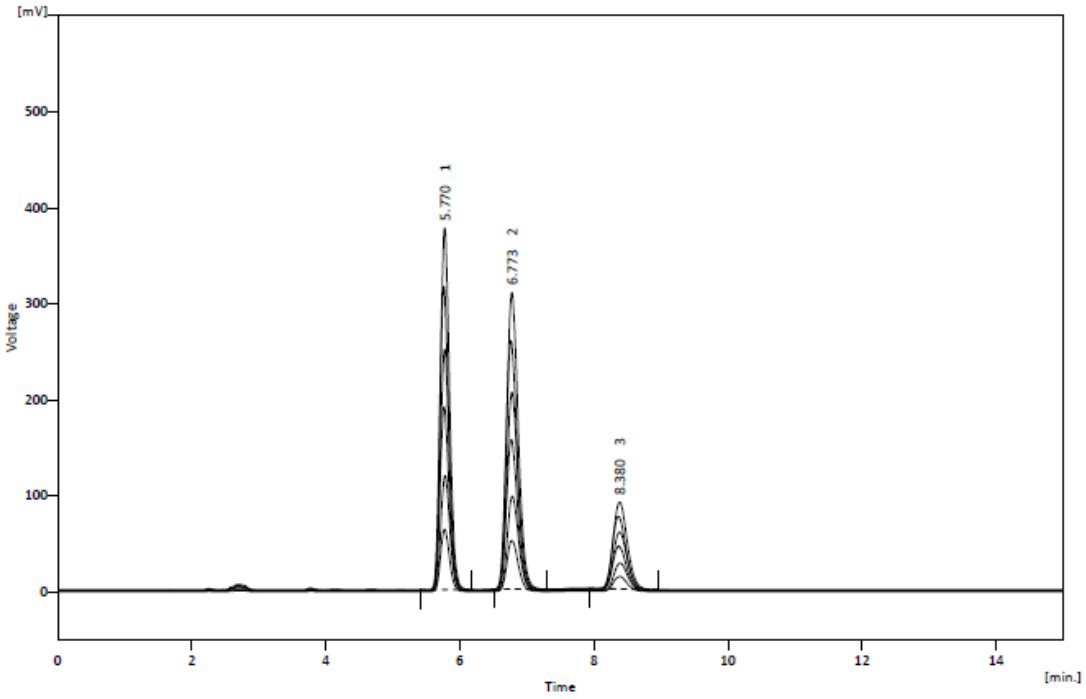


Figure 2.21: Overlain Chromatogram of Linearity for LAM, TEN & DOR

(x) Conc. µg/ml	(y) Area
7.5	628.03
15	1165.26
22.5	1858.71
30	2437.74
37.5	3051.53
45	3656.61
STD ERROR	36.96
Slope	81.45
LOD	1.49
LOQ	4.53

Table 2.9: Linearity data of LAM

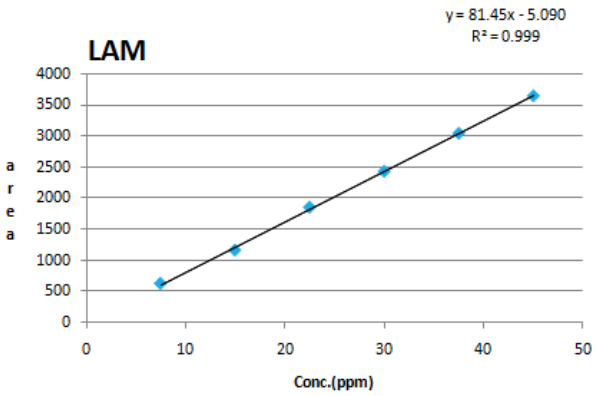


Figure 2.22: Calibration Curve for LAM

(x) Conc. µg/ml	(y) Area
7.5	612.56
15	1137.29
22.5	1811.76
30	2355.67
37.5	2988.56
45	3548.72
STD ERROR	38.57
Slope	79.15
LOD	1.60
LOQ	4.87

Table 2.10: Linearity data of TEN

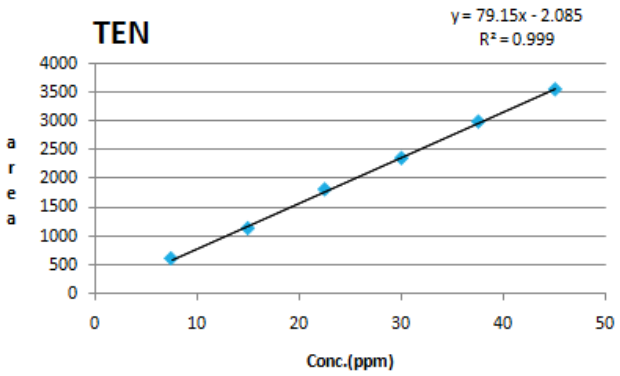


Figure 2.23: Calibration Curve for TEN

(x) Conc. µg/ml	(y) Area
2.5	242.71
5	429.19
7.5	677.48
10	887.49
12.5	1093.54
15	1329.74
STD ERROR	15.17
Slope	87.29
LOD	0.57
LOQ	1.73

Table 2.11: Linearity data of DOR

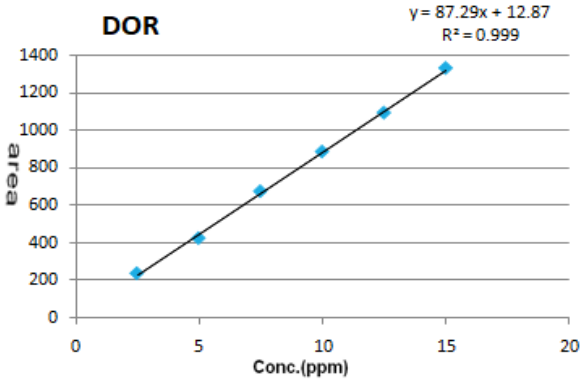


Figure 2.24: Calibration Curve for DOR

5.6.3.3 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 LAM, TEN and DOR. 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
LAM	15	50	7.5	7.44	99.30	99.43
	15	100	15	15.04	100.31	
	15	150	22.5	22.20	98.68	
TEN	15	50	7.5	7.45	99.46	99.38
	15	100	15	14.94	99.63	
	15	150	22.5	22.28	99.06	
DOR	5	50	2.5	2.51	100.66	100.24
	5	100	5	5.03	100.75	
	5	150	7.5	7.44	99.32	

Table 2.12: Accuracy Study of LAM, TEN & DOR (n = 3)

5.6.3.4 Precision

5.6.3.4.1 Repeatability (n = 6)

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

Conc. of LAM (µg/ml)	Area	Conc. of TEN (µg/ml)	Area	Conc. of DOR (µg/ml)	Area
30	2432.41	30	2358.46	10	882.82
	2446.68		2342.26		895.82
	2438.13		2359.54		886.45
	2441.69		2362.89		889.26
	2448.25		2359.10		884.32
	2411.97		2363.12		879.35
Mean	2436.52	Mean	2357.56	Mean	886.34
SD	13.33	SD	7.75	SD	5.72
% RSD	0.54	% RSD	0.32	% RSD	0.64

Table 2.13: Repeatability Study of LAM, TEN & DOR (n = 6)

5.6.3.4.2 Intraday Precision (n = 3)

The Intraday precision for the LAM, TEN & DOR 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.

LAM			TEN			DOR		
Conc. (µg/ml)	**Mean area ± SD**	**% RSD**	**Conc. (µg/ml)**	**Mean area ± SD**	**% RSD**	**Conc. (µg/ml)**	**Mean area ± SD**	**% RSD**
7.5	628.5 ± 4.0	0.64	7.5	619.7 ± 8.0	1.29	2.5	242.51 ± 2.5	1.06
30	2432.3 ± 5.7	0.23	30	2345.4 ± 8.7	0.37	10	882.7 ± 4.2	0.47
45	3668.4 ± 15.8	0.43	45	3549.5 ± 17.5	0.49	15	1322.7 ± 13.9	1.05

Table 2.14: Intraday Precision of LAM, TEN & DOR (n = 3)

5.6.3.4.3 Interday Precision (n = 3)

The Interday precision for the LAM, TEN & DOR 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.

LAM			TEN			DOR		
Conc. (µg/ml)	**Mean area ± SD**	**% RSD**	**Conc. (µg/ml)**	**Mean area ± SD**	**% RSD**	**Conc. (µg/ml)**	**Mean area ± SD**	**% RSD**
7.5	626.3 ± 6.0	1.04	7.5	622.4 ± 7.07	1.13	2.5	239.6 ± 3.3	1.40
30	2438.9 ± 3.5	0.14	30	2344.9 ± 10.2	0.43	10	879.5 ± 8.5	0.97
45	3598.9 ± 51.7	1.43	45	3547.6 ± 4.14	0.11	15	1334.3 ± 6.5	0.49

Table 2.15: Interday Precision of LAM, TEN & DOR (n = 3)

5.6.3.5 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 1.49, 1.60, and 0.57 ug respectively for LAM, TEN and DOR, and the LOQ values are found to be 4.53, 4.87 and 1.73 ug respectively for LAM, TEN and DOR.

5.6.4 Application of the Developed Analytical Method to Formulation

The proposed analytical method been tested in assay analysis % Assay of the Label claim on the Delstrigo^{TM} tablet dosage form containing LAM 300mg, TEN 300mg & DOR 100mg. 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
TEN	1	300	289.61	96.54	96.03	0.50	0.52
	2	300	288.07	96.02			
	3	300	286.63	95.54			
LAM	1	300	290.41	96.80	96.25	0.53	0.55
	2	300	288.67	96.22			
	3	300	287.21	95.74			
DOR	1	100	96.25	96.25	97.47	0.64	0.66
	2	100	97.37	97.37			
	3	100	96.88	96.88			

Table 2.16: Assay of Formulation Delstrigo^{TM} (n = 3)

5.6.5 Dissolution Studies

The dissolution profile method is developed of the drugs LAM, TEN & DOR has been performed from the tablet dosage form n=6 and it shows % Drug release and % Cumulative drug release Dissolution. The analytical developed method is applied successfully in the dissolution profile studies. The results of dissolution studies are shown below.

Time Min	Area of Sample	Drug Release Conc. ug/ml	Drug Release in mg as per Label claim	% DR Drug Release	% CDR Cumulative Drug Release
LAM					
			Label Claim LAM 300mg		
10	426.72	5.20	156.05	52.01	52.00
20	627.13	7.66	229.92	76.64	76.92
30	714.29	8.73	262.04	87.34	87.764
40	807.41	9.87	296.36	98.78	99.26
50	816.53	9.99	299.72	99.90	100.44
60	821.67	10.05	301.61	100.53	101.08
TEN					
			Label Claim TEN 300mg		
10	297.48	3.68	110.41	36.80	36.79
20	412.63	5.13	154.01	51.33	51.50
30	685.27	8.574	257.23	85.74	85.98
40	786.54	9.85	295.57	98.52	98.97
50	798.36	10.00	300.04	100.01	100.54
60	803.49	10.06	301.99	100.66	101.15
DOR					
			Label Claim DOR 100mg		
10	239.24	1.14	34.25	34.25	34.22
20	423.38	1.99	59.85	59.85	60.03
30	673.10	2.78	83.62	83.62	83.94
40	882.74	3.26	98.09	98.09	98.53
50	1094.70	3.32	99.70	99.70	100.23
60	1324.11	3.36	100.86	100.86	101.38

Table 2.17: Dissolution Study of Formulation Delstrigo™

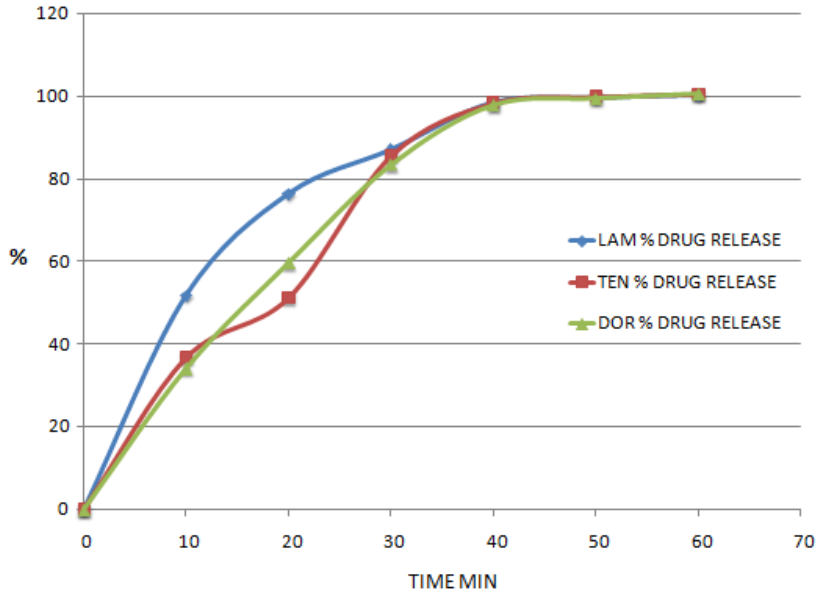


Figure 2.25: % Drug Release LAM, TEN & DOR

5.6.6 Summary of Results

Sr No	Parameters	Results		
		LAM	TEN	DOR
1	System Suitability: Theoretical plates- Tailing Factor- Retention time min-	 4532 1.09 5.76	 6283 1.21 6.79	 7193 1.19 8.38
2	Precision (%RSD)	0.54	0.32	0.64
3	Linearity (R^2)	0.9996	0.9995	0.9998
4	Accuracy (% Recovery)	99.43	99.38	100.24
5	LOD (ug/ml)	1.49	1.60	0.57
6	LOQ (ug/ml)	4.53	4.87	1.73
7	% Assay	96.25	96.03	97.47
8	Dissolution % Drug Release at 40min	99.26	98.97	98.53

5.7 CONCLUSIONS

The Stability Indicating HPLC method for LAM, TEN & DOR combinational drugs has been successfully developed and validated. The analytical method is optimized for the testing even in degraded conditions and analysis for LAM, TEN, DOR 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 dissolution method for these combinational drugs have been developed for solid dosage form. The HPLC analytical method is applied in the estimation for dissolution profile studies of the combined tablet dosage form. The accurate precise method can be used for analysis of LAM, TEN & DOR combination as well as individual in as Assay method and dissolution testing procedures in academics, research, analytical laboratories and in pharmaceutical industries.