Chapter 12
HPLC METHOD DEVELOPMENT AND VALIDATION FOR MARIBAVIR

12.1 EXPERIMENTALS

12.1.1 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 & -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.

12.1.2 Materials and Reagents Utilised

The chemicals used working reference standard drug Maribavir MARI 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.

12.1.3 Identification of Standard Drug Sample

12.1.3.1 Melting Point Determination

The working standard drug Maribavir MARI was 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 9.1.

Drug	Observed Melting Range	Standard Melting Range
MARI	199.4 ^{0}C	198 ^{0}C

Table 9.1: Melting Points of MARI

12.1.3.2 FTIR Spectral Determination for Identification Standard drug samples MARI

The pure active pharmaceutical working standard drug substances MARI as 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 9.1 for MARI. The principal IR peaks recorded and observed for the drug are shown in Table 9.2, for MARI.

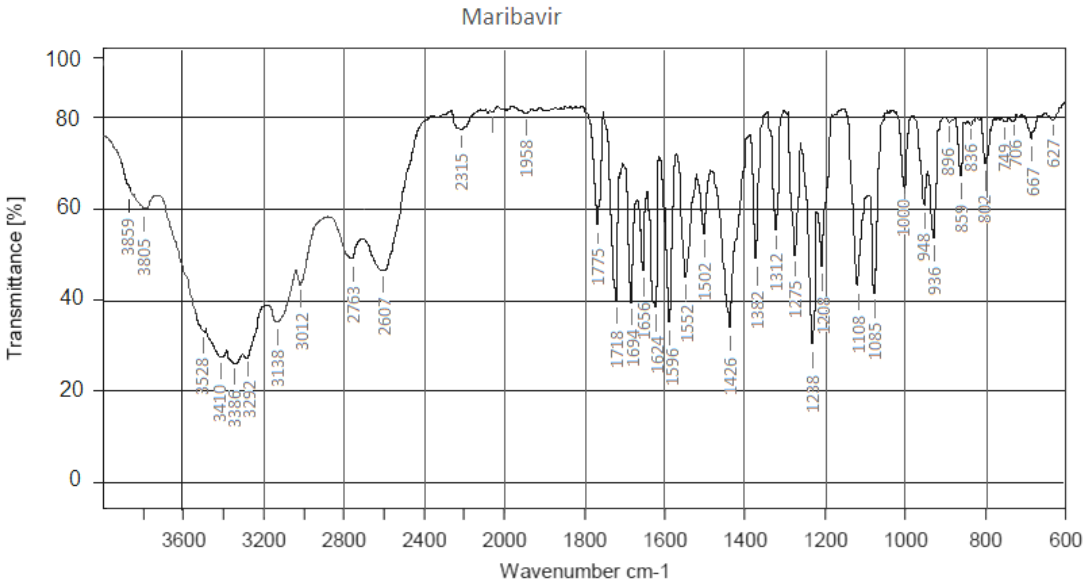


Figure 9.1: FTIR Spectra of Maribavir MARI

MARIBAVIR					
Energy (Cm^{-1})	**Band Assignment**	**Peak Intensity**	**Energy (Cm^{-1})**	**Band Assignment**	**Peak Intensity**
3350-3310	N-H 2^{0} Amine	27.25	2000-1650	C-H Aromatic	40.06 41.14
1124-1087	C-O Alcohol	41.83 43.28	850-550	C-Cl	70.04
1250-1020 3300-3400	C-N C-N	49.65 27.29	2700-3200 3550-3200 3700-3584	O-H Alcohol	48.83 33.42
1690-1640	C=N	40.06	1749-1792 1275-1200	C-O (Ether)	47.65
1647 1600-1553	C=C (Aromatic)	45.84	3000-2800 1450	C-H Methyl group	37.83
1500-1700 1650-1580	N-H	43.24 39.02 38.69	1124-1087	C-O	41.83 43.28

Table 9.2: FTIR Interpretation of Maribavir MARI

12.1.4 Preparation of Solutions

12.1.4.1 Preparation of standard solutions of MARI

The standard stock soln. drug prepared in 50:50 ACN : Methanol solvent mixture. 20mg of MARI was dissolved in solvent mixture and made upto 100ml soln with same solvent to give 200 µg/ml standard stock solution of MARI .From the above stock solutions of, 1ml from each was taken and diluted upto 10ml in to give MARI 20 µg/ml drug standard Final solution.

12.1.4.2 Preparation of Sample Solutions

LIVTENCITY ^{TM} each tablet contains MARI 200mg of Maribavir was taken and Dissolved in 50ml ACN : Methanol (50:50) , sonicated, filtered and make up to 100ml (Stock solutionA) [2000 ug/ml MARI]

From the Stock solution A, 1ml was taken, diluted with mobile phase upto 10ml to give Solution B [200ug/ml MARI]

From the Solution B, 1ml was taken, diluted with mobile phase upto 10ml to give Final Solution C [20ug/ml MARI] used for analysis.

12.1.4.3 Preparation of Optimized Mobile Phase

The mobile phase made by taking 85:15 ratio, 0.05M Phosphate buffer : ACN of pH 4. 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.

12.1.5 Selection of Wavelength for Detection

The Final standard solns of MARI 20 µg/ml , scanned in 200 - 400 nm in UV-visible double beam spectrophotometer at a medium at scanning speed. The overlain spectra shown in Fig. 9.2 of MARI 20µg/ml , was taken in 50:50 Methanol : ACN and the 243nm wavelength was selected for estimation in the detection during the HPLC analysis.

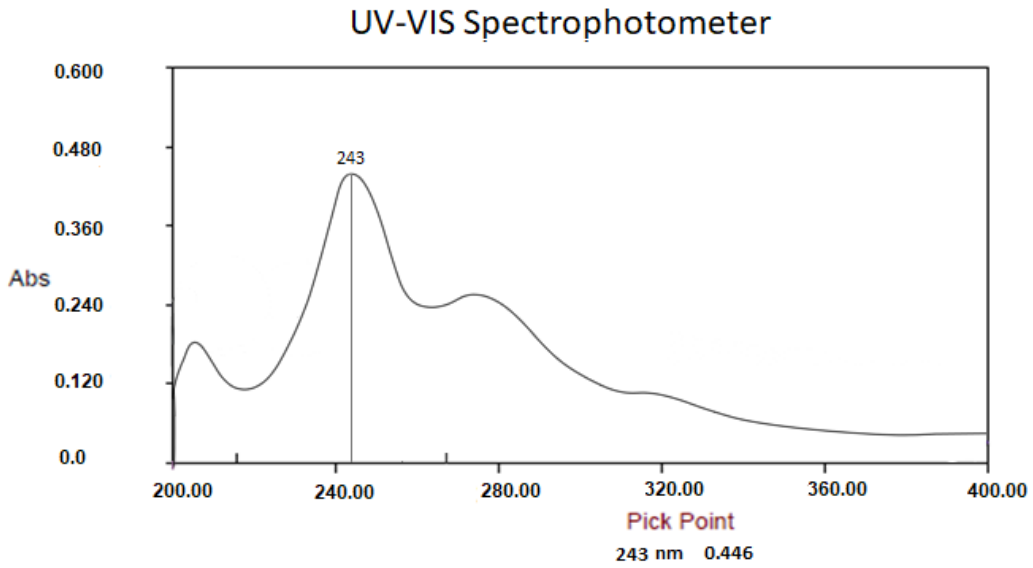


Figure 9.2: UV Spectra Overlay of MARI

12.1.6 Selection and Optimization of Mobile phase

For the detection analysis of the MARI drug in the 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 9.3

Sr No	Mobile Phase	pH	Ratio (v/v)	Retention Time (min) MARI	REMARK
1	ACN : Methanol	-	50:50	-	No peak detected
2	ACN : Methanol	-	80:20	-	No peak detected
3	ACN : Methanol	-	20:80	-	No peak detected
4	0.05 M Phosphate buffer : ACN	7	80:20	-	No peak detected
5	0.05 M Phosphate buffer : ACN	7	50:50	-	No peak detected
6	0.05 M Phosphate buffer : ACN	8	50:50	-	No peak detected
7	0.05 M Phosphate buffer : ACN	6.5	40:60	9.36	Tailing in peak, Asymmetry in peak - 3.21
8	0.05 M Phosphate buffer : ACN	6	30:70	8.51	Tailing in peak, Asymmetry in peak - 2.86
9	0.05 M Phosphate buffer : ACN	5	30:70	5.47	No tailing Good symmetry, Tailing factor- 2.65

10	0.05 M Phosphate buffer : ACN	5	20:80	4.82	No tailing Good symmetry, Tailing factor- 2.49
11	**0.05 M Phosphate buffer : ACN** **Selected Mobile Phase**	4	15:85	3.13	Good symmetry, Tailing factor- 1.27

Table 9.3: Trials for Selection of Mobile Phase for MARI

12.1.7 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 85:15 pH- 4
Flow rate	1ml/minl
Injection volume	20ul
Temp	Ambient Lab Temperature
Detection Wavelength	243nm
Retention Times (min)	MARI- 3.13

Table 9.4: Optimized Chromatographic Conditions for MARI

12.2 METHOD VALIDATION

12.2.1 Linearity (Calibration Curve)

The working standard and sample solutions of MARI 5, 10, 15, 20, 25, 30ug/ml, prepared in the serial dilutions for drug, 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 .

12.2.2 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. 20ug/ml of MARI was 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.

12.2.3 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 drug MARI 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.

12.2.4 Precision

12.2.4.1 Repeatability (n=6)

The repeatability study has been performed by repeatedly n=6 sample standards injected 20ug/ml of MARI, and the area response of drugs was obtained and the %RSD had been calculated

12.2.4.2 Intraday Precision (n=3)

The intraday precision was performed by using the 5, 20, 30 ug/ml of MARI was was used, and the solutions were repeatedly injected analysed by HPLC three times on same day, obtained results calculated into the terms of %RSD.

12.2.4.3 Interday Precision (n=3)

The interday precision was performed by using the 5, 20, 30 ug/ml of MARI was used, and the solutions were repeatedly injected analysed by HPLC three times in different days obtained results calculated into the terms of %RSD.

12.2.5 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 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

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

The developed analytical HPLC method is applied in the estimation-analysis of LIVTENCITY ^{TM} each tablet contains MARI 200mg of Maribavir was taken and Dissolved in 50ml ACN : Methanol (50:50) , sonicated, filtered and make up to 100ml (Stock solutionA), [2000 ug/ml MARI].

From the Stock solution A, 1ml was taken, diluted with mobile phase upto 10ml to give Solution B [200ug/ml MARI].

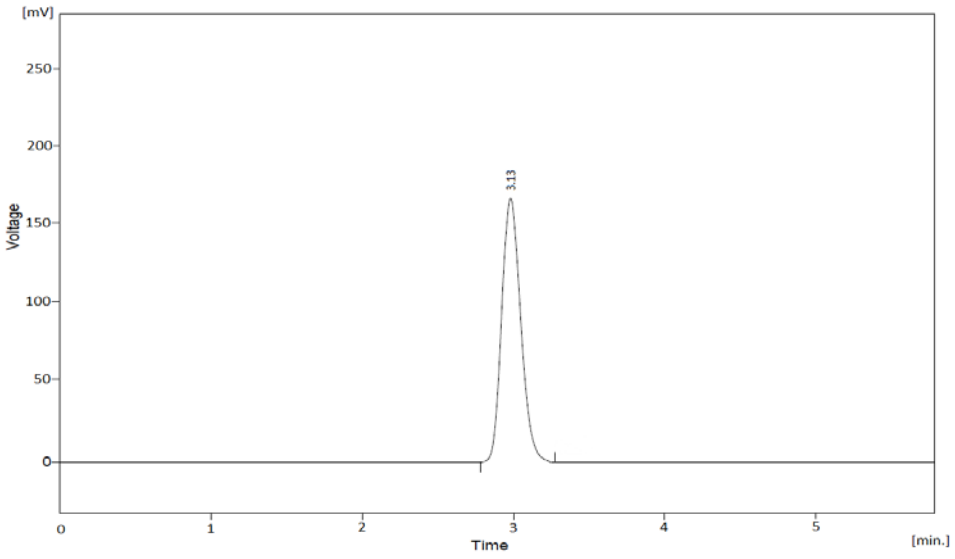
From the Solution B, 1ml was taken, diluted with mobile phase upto 10ml to give final Solution C [20ug/ml MARI] used for analysis. were prepared, n=3 samples, analysed by the developed HPLC method.

The standard stock soln. drug prepared in 50:50 ACN : Methanol solvent mixture. 20mg of MARI was dissolved in solvent mixture and made upto 100ml soln with same solvent to give 200 µg/ml standard stock solution of MARI . From the above stock solutions of, 1ml from was taken and diluted upto 10ml in to give MARI 20 µg/ml drug standard Final solution, was 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.

12.4 RESULTS & DISCUSSIONS

12.4.1 Method Development

The developed analytical HPLC method found to be reliable, accurate.,- precise for analysis and quality control testing for MARI 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 (85 : 15) pH - 4, flow rate 1ml / min , detection wavelength at 243.nm. Excipients in the marketed formulation does not affect in the resolution, separations as well do not have any interfering peaks. The average retention time was found to be MARI -3.13 minutes. The chromatogram of the drugs are shown below.



Result Table (Uncal - MARI- standard) ACN : phosphate buffer pH - 4.0 15 : 85

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Peak Purity
1	3.135	1722.324	163.241	100.000	0.999
	Total	1722.324	163.241	100.000	

Column Performance Table (From - MARI- standard)

	Reten. Time	Asymmetry [-]	Efficiency [th.pl]	Resolution [-]
1	3.135	1.276	3641	-

Figure 9.3: Chromatogram of Standard MARI

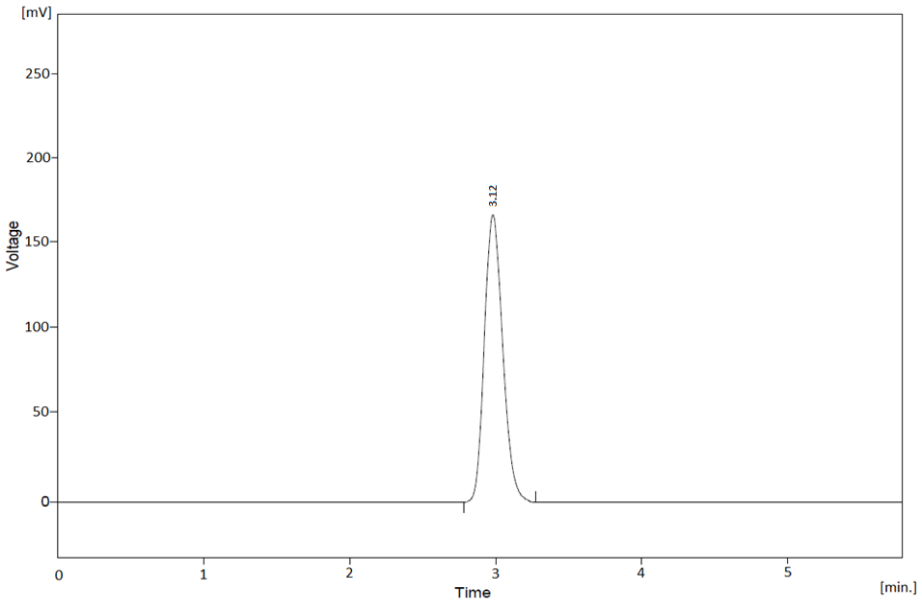


Figure 9.4: Chromatogram of Sample MARI

12.4.2 Method Validation

12.4.2.1 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 drug 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.

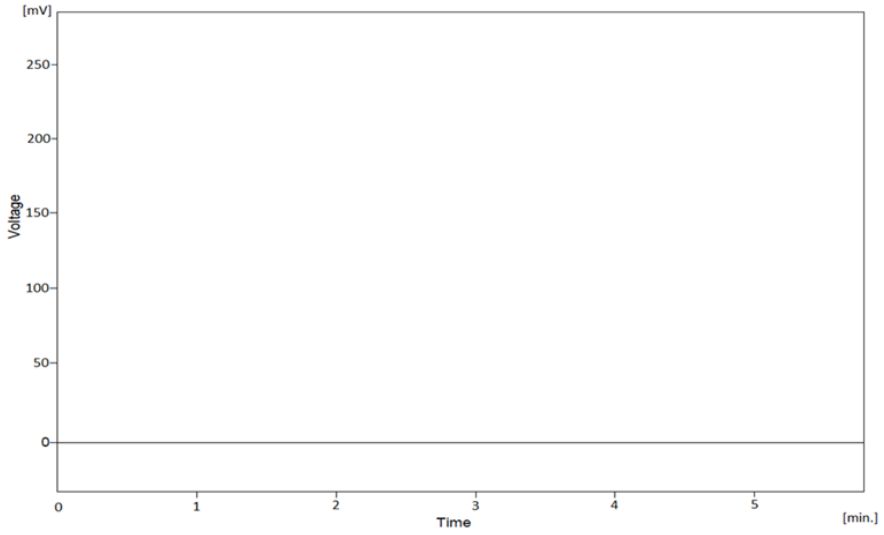


Figure 9.5: Blank Chromatogram

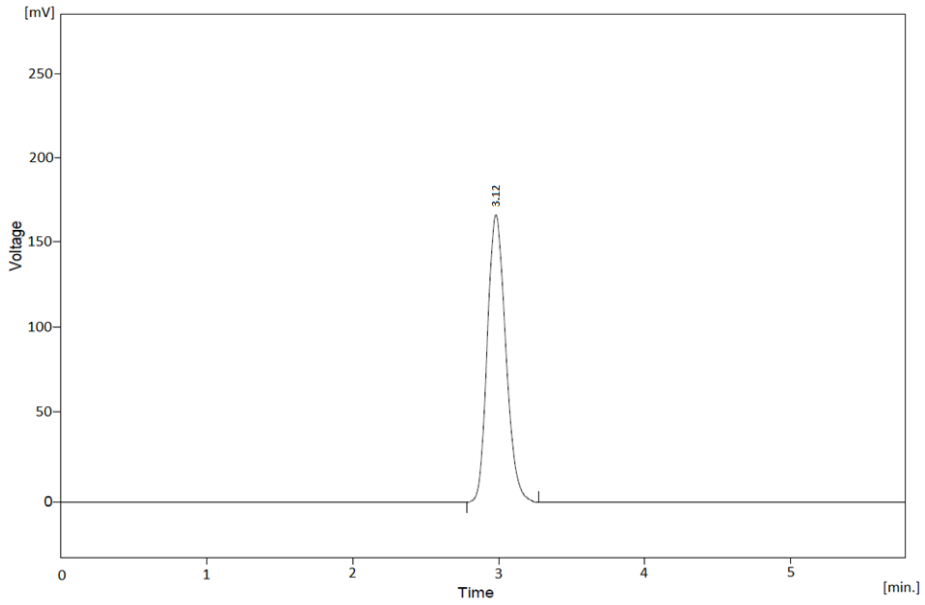


Figure 9.6: Chromatogram of Sample MARI

12.4.2.2 Linearity and Range (n = 5)

Drugs MARI Linearity has been followed in a particular concentration ranges of 5, 10, 15, 20, 25, 30ug/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.

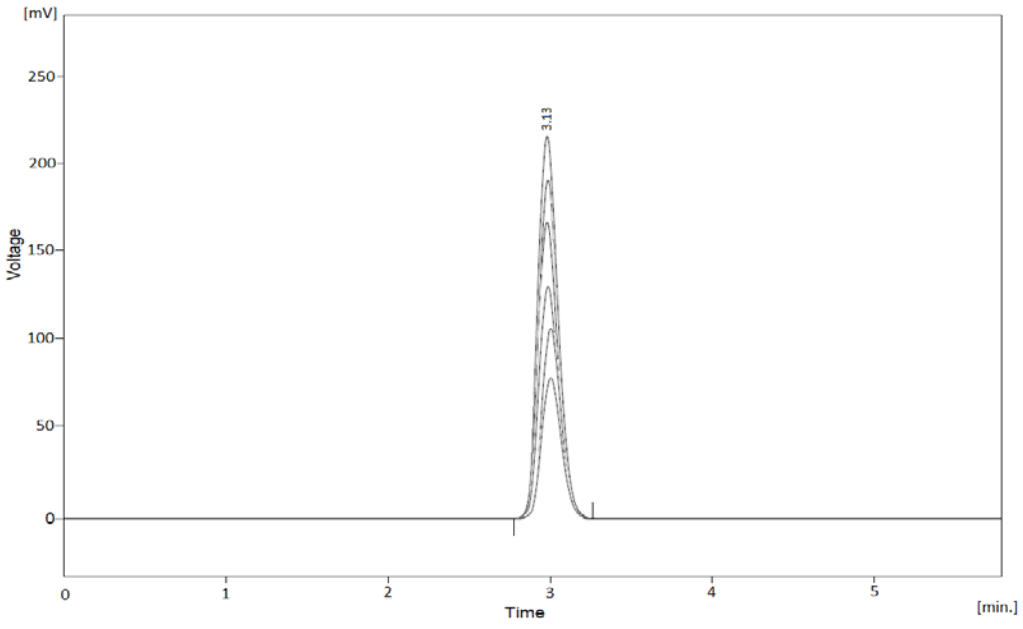


Figure 9.7: Overlain Chromatogram of Linearity for MARI

(x) Conc. µg/ml	(y) Area
5	438.64
10	865.76
15	1280.45
20	1722.32
25	2160.46
30	2581.25
STD ERROR	7.97
Slope	85.93
LOD	0.306
LOQ	0.927

Table 9.5: Linearity data of MARI

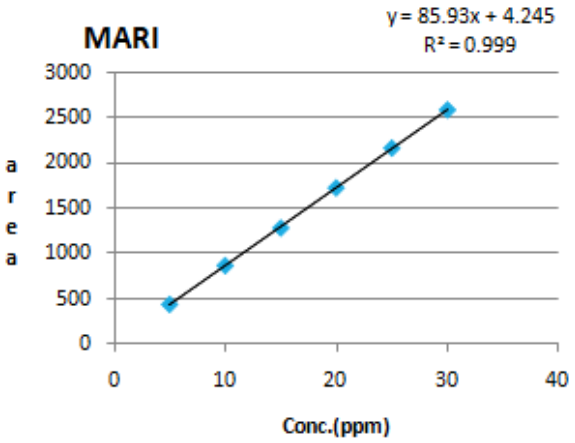


Figure 9.8: Calibration Curve for MARI

12.4.2.3 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 drug MARI. 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
MARI	20	50	10	9.90	99.01	99.79
	20	100	20	19.99	99.99	
	20	150	30	30.10	100.36	

Table 9.6: Accuracy Study of MARI (n = 3)

12.4.2.4 Precision

12.4.2.4.1 Repeatability (n = 6)

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

Conc. of MARI (µg/ml)	**Area**
20	1732.71
	1728.47
	1724.57
	1735.82
	1718.98
	1734.63
Mean	1729.19
SD	6.51
% RSD	0.37

Table 9.7: Repeatability Study of MARI (n = 6)

12.4.2.4.2 Intraday Precision (n = 3)

The Intraday precision for the MARI 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.

MARI		
Conc. (µg/ml)	**Mean area ± SD**	**% RSD**
5	436.2 ± 2.9	0.68
20	1721.4 ± 9.9	0.57
30	2580.0 ± 3.1	0.12

Table 9.8: Intraday Precision of MARI (n = 3)

12.4.2.4.3 Interday Precision (n = 3)

The Interday precision for the MARI 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.

MARI		
Conc. (µg/ml)	**Mean area ± SD**	**% RSD**
5	439.7 ± 5.9	1.35
20	1722.9 ± 8.3	0.48
30	2583.5 ± 4.4	0.17

Table 9.9: Interday Precision of MARI (n = 3)

12.4.2.5 LOD and LOQ

It has been calculated from the n=5 samples from the calibration curve slope and standard deviation. The LOD & LOQ value are found to be 0.306 & 0.927 ug respectively for MARI.

12.4.3 Application of the Developed Analytical Method to Formulation

The proposed analytical method been tested in assay analysis % Assay of the Label claim on the LIVTENCITY™ Each tablet contains MARI Maribavir 200mg. 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
MARI	1	200	194.66	97.33	97.73	0.39	0.40
	2	200	195.49	97.75			
	3	200	196.22	98.11			

Table 9.10: Assay of Formulation LIVTENCITY™ (n = 3)

12.4.4 Summary of Results

Sr No	Parameters	Results MARI
1	System Suitability: Theoretical plates- Tailing Factor- Retention time min-	 3641 1.27 3.13
2	Precision (%RSD)	0.48
3	Linearity (R^2)	0.999
4	Accuracy (% Recovery)	99.79
5	LOD (ug/ml)	0.306
6	LOQ (ug/ml)	0.927
7	% Assay	97.73

12.5 CONCLUSIONS

The Analytical HPLC method for MARI drug has been successfully developed and validated. The analytical method is optimized in testing, analysis of the drug in individual as well in the in formulation 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 MARI drug as well as individual in as Assay method and dissolution testing procedures in academics, research, analytical laboratories.