Chapter 14

HPLC METHOD DEVELOPMENT AND VALIDATION FOR MOLNUPIRAVIR

14.1 EXPERIMENTALS

14.1.1 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.

14.1.2 Materials and Reagents Utilised

The chemicals used working reference standard drug Molnupiravir MOLN samples of 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.

14.1.3 Identification of Standard Drug Sample

14.1.3.1 Melting Point Determination

The working standard drug Molnupiravir MOLN 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 11.1.

Drug	Observed Melting Range	Standard Melting Range
MOLN	158.49 ^{0}C	156-159 ^{0}C

Table 11.1: Melting Points of MOLN

14.1.3.2 FTIR Spectral Determination for Identification Standard drug samples MOLN

The pure active pharmaceutical working standard drug substances MOLN 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 11.1 for MOLN. The principal IR peaks recorded and observed for the drug are shown in Table 11.2, for MOLN.

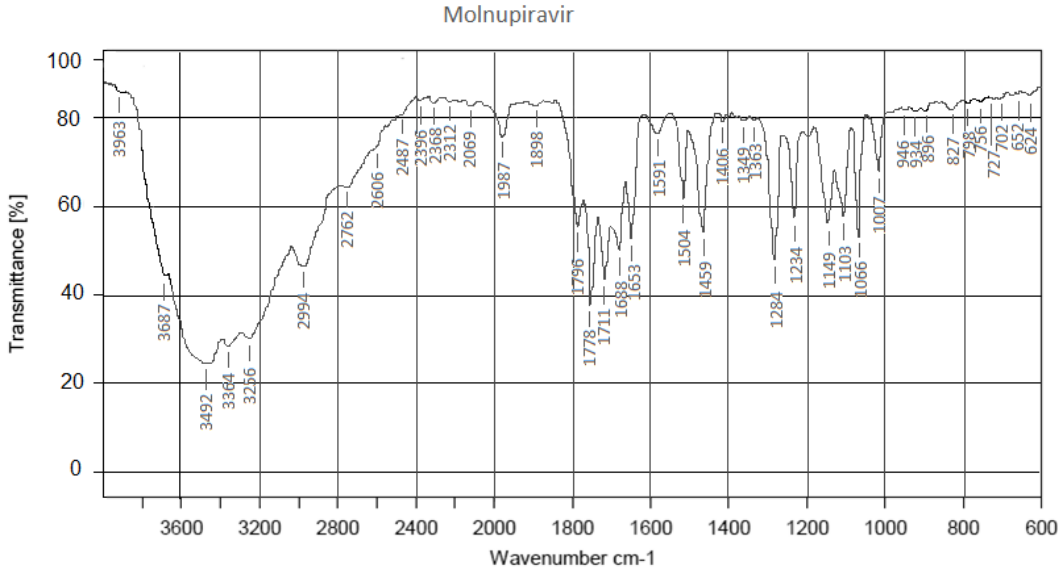


Figure 11.1: FTIR Spectra of Molnupiravir MOLN

MOLNUPIRAVIR					
Energy (Cm^{-1})	**Band Assignment**	**Peak Intensity**	**Energy (Cm^{-1})**	**Band Assignment**	**Peak Intensity**
1210-1136	C-O Ester	58.47	1647 1600-1553	C=C (Aromatic)	78.29
3300-3400	C-N	28.65	3000-2800 1450	C-H Methyl group	44.59
1749-1792 1275-1200	C-O (Ether)	57.38 38.46	1124-1087	C-O Alcohol	58.82
1690-1640	C=N	51.27 54.67	3350-3310	N-H 2^{0} Amine	30.46
1725-1705 1685-1666	C=O Ketone	43.29	1750-1735 1770-1780	C=O Ester	38.84
1500-1700	N-H	61.78	2700-3200 3550-3200 3700-3584	O-H Alcohol	31.07 43.78
2000-1650	C-H Aromatic	77.27	1650-1580	N-H	77.69

Table 11.2: FTIR Interpretation of Molnupiravir MOLN

14.1.4 Preparation of Solutions

14.1.4.1 Preparation of standard solutions of MOLN

The standard stock soln. drug prepared in Methanol solvent. 20mg of MOLN was dissolved in solvent mixture and made upto 100ml soln with same solvent to give 200 µg/ml standard stock solution of MOLN .From the above stock solutions of, 15ml from each was taken and diluted upto 100ml in to give MOLN 30 µg/ml drug standard Final solution.

14.1.4.2 Preparation of Sample Solutions

MOVFOR ™ each capsule contains MOLN 200mg of Molnupiravir, powder equivalent to 600mg was taken and Dissolved in Methanol , sonicated, filtered and makeup to 100ml (Stock solutionA) [6000 ug/ml MOLN]

From the Stock solution A, 5ml was taken, diluted with mobile phase upto 100ml to give Solution B [300ug/ml MOLN]

From the Solution B, 1ml was taken, diluted with mobile phase upto 10ml to give Final Solution C [30ug/ml MOLN] used for analysis.

14.1.4.3 Preparation of Optimized Mobile Phase

The mobile phase made by taking 65:35 ratio, 0.05M Phosphate buffer : ACN of pH 3 with 1% TEA Triethyl amine. The phosphate buffer was prepared by accurately weighing 6.8gm KH_2PO_4 (MW. 136) with 10ml 1% TEA Triethyl amine 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.

14.1.5 Selection of Wavelength for Detection

The Final standard solns of MOLN 30 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. 11.2 of MOLN 30µg/ml , was taken in Methanol and the 236nm wavelength was selected for estimation in the detection during the HPLC analysis.

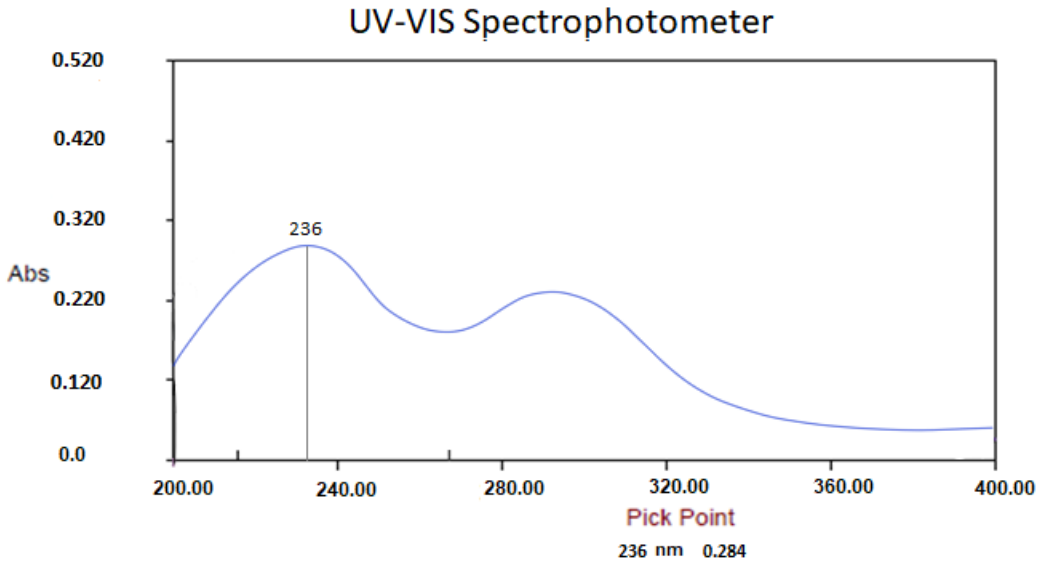


Figure 11.2: UV Spectra Overlay of MOLN

14.1.6 Selection and Optimization of Mobile phase

For the detection analysis of the MOLN 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 11.3

Sr No	**Mobile Phase**	**pH**	**Ratio (v/v)**	**Retention Time (min)** **MOLN**	**REMARK**
1	0.05 M Phosphate buffer : ACN	6	50:50	12.164	Longer retention time, Asymmetric peak, tailing peak, tailing factor-2.95
2	0.05 M Phosphate buffer : ACN	5.5	50:50	10.322	Longer retention time, Asymmetric peak, tailing peak, tailing factor-2.87
3	0.05 M Phosphate buffer : ACN	5	40:60	7.547	Asymmetric peak, tailing peak, tailing factor-2.76
4	0.05 M Phosphate buffer : ACN	4.5	40:60	5.348	Asymmetric peak, tailing peak, tailing factor-2.64
5	0.05 M Phosphate buffer : ACN 1%TEA	4	40:60	4.426	Symmetric peak, tailing factor-2.18
6	0.05 M Phosphate buffer : ACN 1%TEA **Selected Mobile Phase**	3	35:65	2.537	Symmetric peak, tailing factor-1.21

Table 11.3: Trials for Selection of Mobile Phase for MOLN

14.1.7 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 1%TEA : ACN 65:35 pH- 3
Flow rate	1ml/minl
Injection volume	20ul
Temp	Ambient Lab Temperature
Detection Wavelength	236nm
Retention Times (min)	MOLN- 2.537

Table 11.4: Optimized Chromatographic Conditions for MOLN

14.2 METHOD VALIDATION

14.2.1 Linearity (Calibration Curve)

The working standard and sample solutions of MOLN 7.5, 15, 22.5, 30, 37.5, 45 ug/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 .

14.2.2 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. 30ug/ml of MOLN 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.

14.2.3 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 MOLN 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.

14.2.4 Precision

14.2.4.1 Repeatability (n=6)

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

14.2.4.2 Intraday Precision (n=3)

The intraday precision was performed by using the 7.5, 30, 45 ug/ml of MOLN 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.

14.2.4.3 Interday Precision (n=3)

The interday precision was performed by using the 7.5, 30, 45 ug/ml of MOLN was used, and the solutions were repeatedly injected analysed by HPLC three times in different days obtained results calculated into the terms of %RSD.

14.2.5 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

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

The developed analytical HPLC method is applied in the estimation-analysis of MOVFOR ™ each capsule contains MOLN 200mg of Molnupiravir, powder equivalent to 600mg was taken and Dissolved in Methanol , sonicated, filtered and makeup to 100ml (Stock solutionA) [6000 ug/ml MOLN].

From the Stock solution A, 5ml was taken, diluted with mobile phase upto 100ml to give Solution B [300ug/ml MOLN]. From the Solution B, 1ml was taken, diluted with mobile phase upto 10ml to give Final Solution C [30 ug/ml MOLN] used for analysis, were prepared, n=3 samples, analysed by the developed HPLC method.

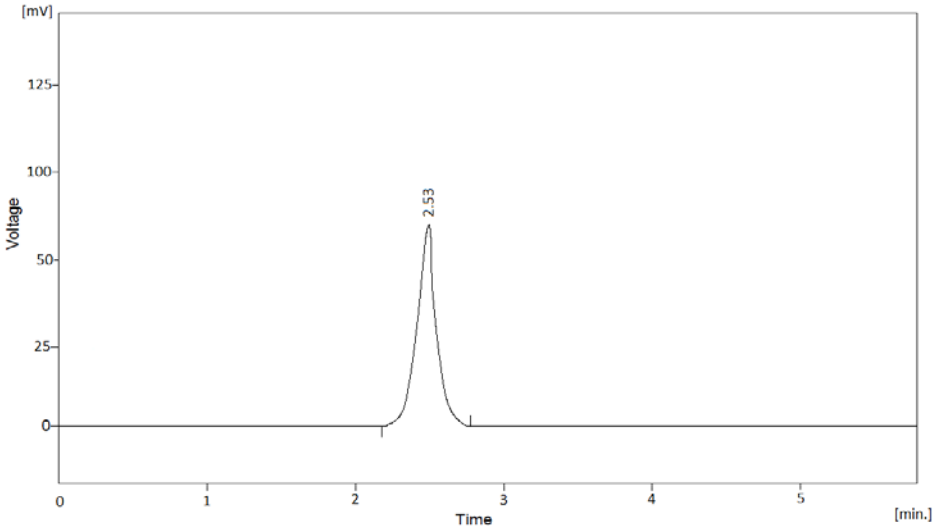
The standard stock soln. drug prepared in Methanol solvent. 20mg of MOLN was dissolved in solvent mixture and made upto 100ml soln with same solvent to give 200 µg/ml standard stock solution of MOLN .From the above stock solutions of, 15ml from each was taken and diluted upto 100ml in to give MOLN 30 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.

14.4 RESULTS & DISCUSSIONS

14.4.1 Method Development

The developed analytical HPLC method found to be reliable, accurate.,- precise for analysis and quality control testing for MOLN in pure form, in marketed capsule 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 with 1%TEA : ACN (65 : 35) pH- 3.

Flow rate 1ml / min , detection wavelength at 236.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 MOLN -2.537 minutes. The chromatogram of the drugs are shown below.



Result Table (Uncal MOLN- standard) ACN : phosphate buffer (1% TEA) pH- 3 35:65

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Peak Purity
1	2.537	732.251	68.719	100.000	0.999
	Total	732.251	68.719	100.000	

Column Performance Table (From -MOLN- standard)

	Reten. Time	Asymmetry [-]	Efficiency [th.pl]	Resolution [-]
1	2.537	1.218	3985	-

Figure 11.3: Chromatogram of Standard MOLN

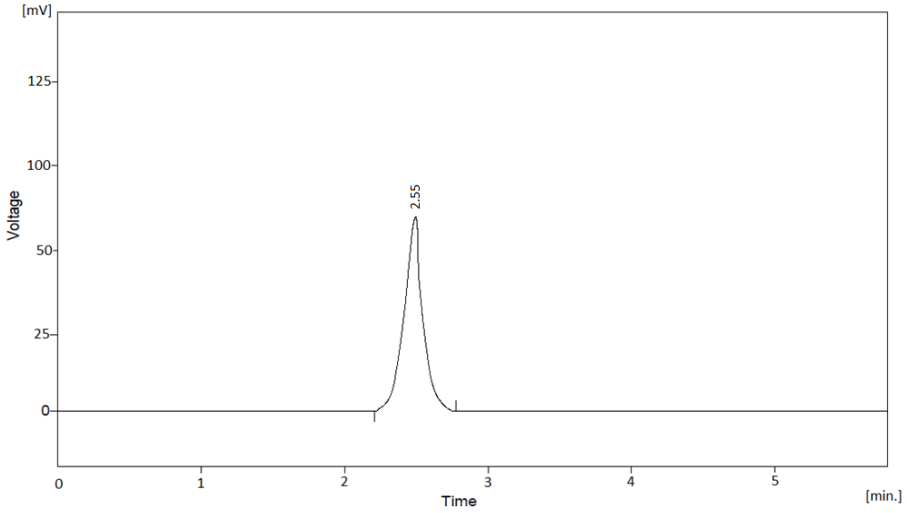


Figure 11.4: Chromatogram of Sample MOLN

14.4.2 Method Validation

14.4.2.1 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.

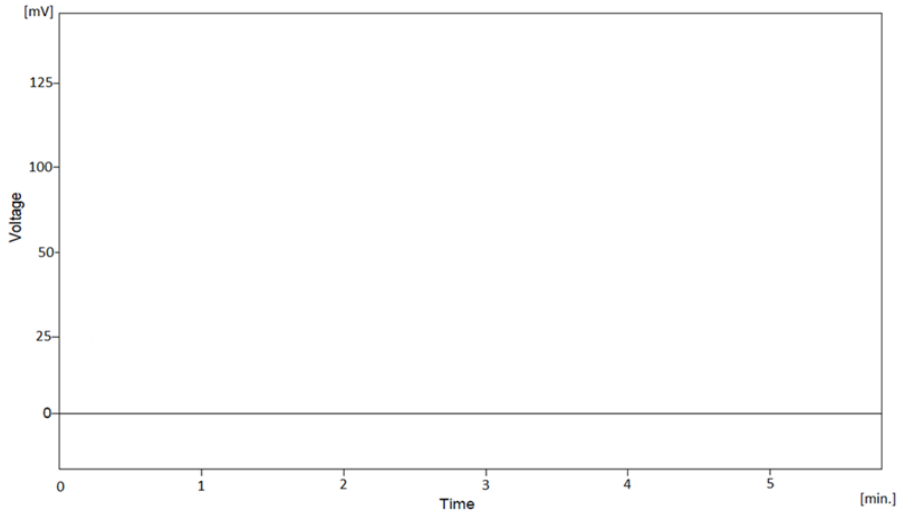


Figure 11.5: Blank Chromatogram

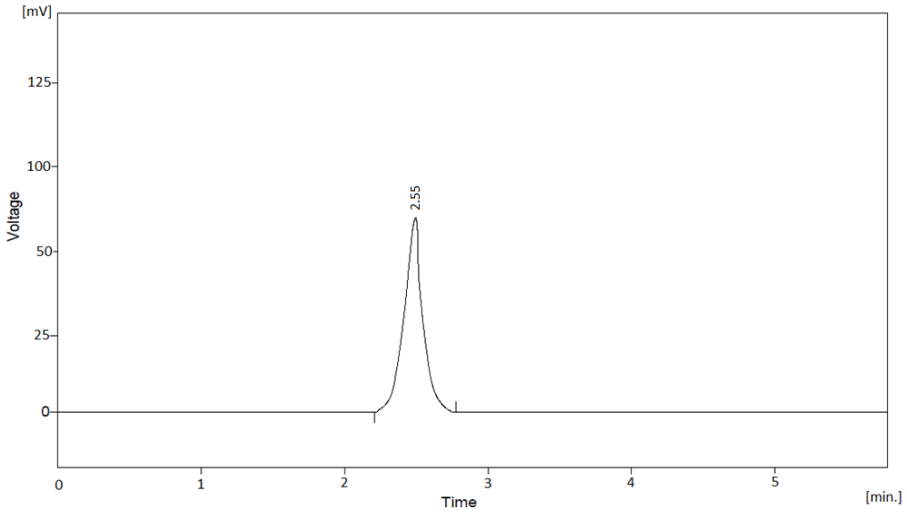


Figure 11.6: Chromatogram of Sample MOLN

14.4.2.2 Linearity and Range (n = 5)

Drugs MOLN Linearity has been followed in a particular concentration ranges of 7.5, 15, 22.5, 30, 37.5, 45ug/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.

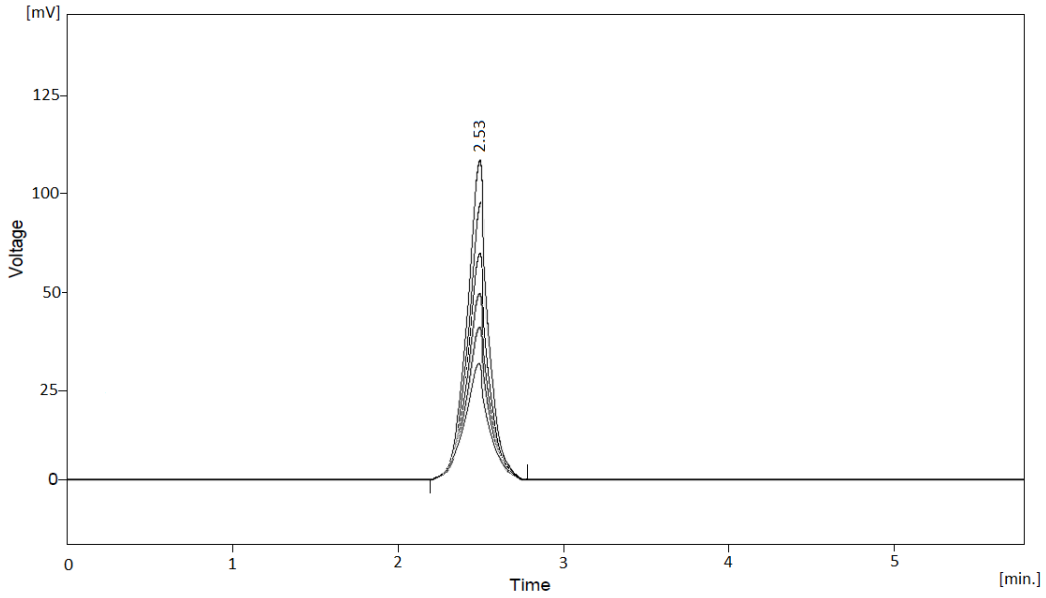


Figure 11.7: Overlain Chromatogram of Linearity for MOLN

(x) Conc. µg/ml	(y) Area
7.5	190.24
15	359.45
22.5	552.49
30	732.25
37.5	918.45
45	1120.24
STD ERROR	8.87
Slope	24.78
LOD	1.181
LOQ	3.581

Table 11.5: Linearity data of MOLN

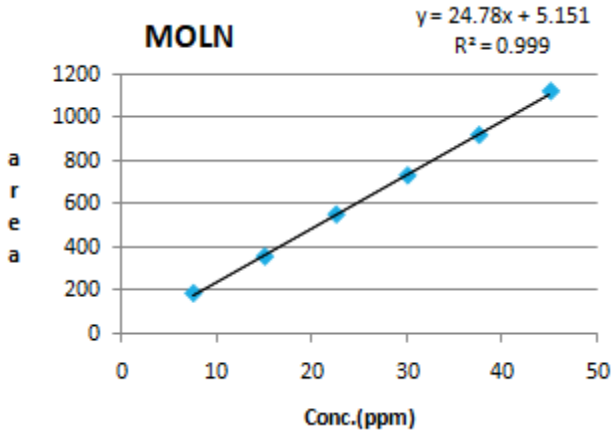


Figure 11.8: Calibration Curve for MOLN

14.4.2.3 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 MOLN. 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
MOLN	30	50	15	14.75	98.33	99.45
	30	100	30	29.93	99.79	
	30	150	45	45.07	100.16	

Table 11.6: Accuracy Study of MOLN (n = 3)

14.4.2.4 Precision

14.4.2.4.1 Repeatability (n = 6)

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

Conc. of MOLN (µg/ml)	Area
30	733.64
	734.59
	729.66
	724.51
	739.87
	735.16
Mean	732.90
SD	5.25
% RSD	0.71

Table 11.7: Repeatability Study of MOLN (n = 6)

14.4.2.4.2 Intraday Precision (n = 3)

The Intraday precision for the MOLN 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.

MOLN		
Conc. (µg/ml)	**Mean area ± SD**	**% RSD**
7.5	192.1 ± 1.7	0.92
30	731.9 ± 1.5	0.21
45	1124.0 ± 3.8	0.34

Table 11.8: Intraday Precision of MOLN (n = 3)

14.4.2.4.3 Interday Precision (n = 3)

The Interday precision for the MOLN 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.

MOLN		
Conc. (µg/ml)	**Mean area ± SD**	**% RSD**
7.5	192.6 ± 2.7	1.40
30	733.4 ± 1.4	0.19
45	1122.6 ± 3.0	0.26

Table 11.9: Interday Precision of MOLN (n = 3)

14.4.2.5 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 1.181 & 3.581 ug respectively for MOLN.

14.4.3 Application of the Developed Analytical Method to Formulation

The proposed analytical method been tested in assay analysis % Assay of the Label claim on the MOVFOR™ Each capsule contains MOLN Molnupiravir 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
MOLN	1	200	197.81	98.90	98.20	0.90	0.92
	2	200	194.36	97.18			
	3	200	197.04	98.52			

Table 11.10: Assay of Formulation MOVFOR™ (n = 3)

14.4.4 Summary of Results

Sr No	**Parameters**	**Results MOLN**
1	System Suitability: Theoretical plates- Tailing Factor- Retention time min-	 3985 1.21 2.53
2	Precision (%RSD)	0.21
3	Linearity (R^2)	0.999
4	Accuracy (% Recovery)	99.45
5	LOD (ug/ml)	1.181
6	LOQ (ug/ml)	3.581
7	% Assay	98.20

14.5 CONCLUSIONS

The Analytical HPLC method for MOLN 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 MOLN drug as well as individual in as Assay method and dissolution testing procedures in academics, research, analytical laboratories.