Research Paper

Development and Validation of Stability Indicating High Performance Liquid Chromatography Method for Determination of Molnupiravir in Capsule Dosage Form

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A simple, rapid and novel reverse phase-high performance liquid chromatography method was developed for quantification of molnupiravir in its capsule dosage form which is recently approved for phase III clinical trials in moderate coronavirus disease patients in India. The chromatographic separation of Molnupiravir was achieved on reverse phase-high performance liquid chromatography using Eclipse Plus C18 (150×4.6 mm, 5 µ) column with buffer (pH 4.5) and methanol (70:30 v/v) as mobile phase. Method was validated in accordance with recommendations of International Council for Harmonisation Q2 (R1) guidelines. The linearity of the method was found to be excellent over the concentration range of 49.80-149.40 µg/ml. The mean of the coefficient of determinations (r^2, n=3) was found to be 0.9999. The precision values (percentage relative standard deviation) and overall percentage recovery was found to be acceptable. The proposed method effectively separated the drug from its degradation products. Hence, it can be used as a stability-indicating assay method for the routine analysis of molnupiravir in pharmaceutical formulations.

Key words: Severe acute respiratory syndrome coronavirus 2, molnupiravir, Reverse phase-high performance liquid chromatography, Coronavirus disease-19, antiviral, validation

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pathogen, which is a single-stranded Ribonucleic Acid (RNA) virus, causes Coronavirus Disease (COVID-19)[1]. COVID-19 is associated with a heavy disease burden and can severely impact several organ systems, including the lungs, kidneys, liver, muscles, and nervous system[2]. With the global spread of SARS-CoV-2 beginning in early 2020, significant efforts have been made in both vaccine research for the prevention of COVID-19, as well as in the identification and utilization of novel or repurposed therapeutics for the management of those with symptomatic COVID-19. To date, eleven agents have received emergency use authorization by the United States Food and Drug Administration (US FDA) for the management of patients with COVID, including one antiviral agent, the adenosine nucleoside analog remdesivir[3]. Other investigational agents are also being evaluated in clinical trials, including the ribonucleoside analog Molnupiravir (MLV), also known as EIDD-2801 or MK-4482[4,5].

MLV is chemically known as [(2R,3S,4R,5R)-3,4-Dihydroxy-5-[4-(hydroxyamino)-2-oxypyrimidin-1-yl]oxolan-2-yl]methyl 2-methylpropanoate (fig. 1)[6]. MLV has been authorized by Drug Controller General of India for the phase III clinical trials in moderate COVID patients[7]. MLV is the isopropyl ester pro-drug of N4-hydroxycytidine[8]. With improved oral bioavailability in non-human primates, it is hydrolysed *in vivo* and distributes into tissues where it becomes the active 5'-triphosphate form[9]. The active drug incorporates into the genome of RNA viruses, leading to an accumulation of mutations known as viral error catastrophe[10]. Recent studies have shown MLA inhibits replication of human and bat corona viruses including SARS-CoV-2, in mice and human airway epithelial cells[8].

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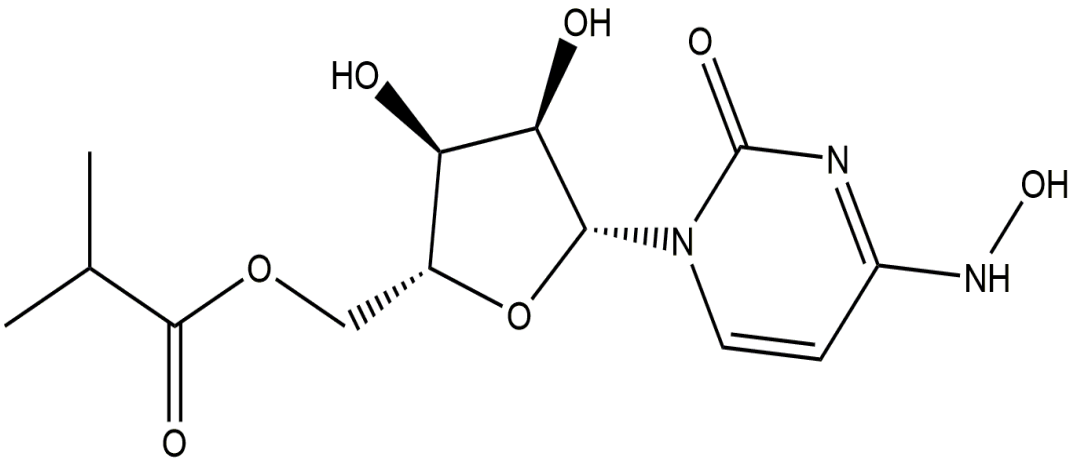

Fig. 1: Chemical structure of molnupiravir

Comprehensive literature survey reveals bio analytical method for the estimation of MLV in Human Plasma and Saliva[11]. MLV is not official in any pharmacopoeia, hence no official method is available for the estimation of MLV in their dosage forms. To the best of our knowledge, there is no stability indicating Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method reported for determination of MLV in its capsule dosage forms. Hence the objective of this work was to develop suitable stability indicating RP-HPLC method for drug product containing MLV.

MATERIAL AND METHODS

Chemicals and reagents:

A working standard of MLV was procured from JSK Chemicals, Ahmedabad, Gujarat, India (Potency: 99.83 % w/w on as is basis). MLV capsules were purchase form local pharmacy. The High Performance Liquid Chromatography (HPLC) grade solvents and Potassium dihydrogen orthophosphate, triethylamine, hydrochloric acid, ortho phosphoric acid, sodium hydroxide, methanol, acetonitrile, hydrogen peroxide, (Merck, Mumbai, India) were used in the analysis. HPLC grade water was prepared using Millipore purification system.

Instrumentation:

An HPLC instrument (Agilent 1260 Infinity II series) consisting Photodiode Array Detector (DAD) equipped with Open Lab CDS Software at wavelength 236 nm was used for this analysis. The chromatographic separations were performed on Eclipse Plus C18 (150×4.6 mm) column by keeping it on 25° using a flow rate of 1.0 ml/min with the run time of 12 min. Injection volume was set as 10 µl.

Mobile phase and diluent:

The mobile phase was a mixture of buffer (pH 4.5) and methanol (70:30 v/v), filtered through 0.22 µm nylon membrane filter and degassed prior to use. The buffer (pH 4.5) was prepared by dissolving about 0.68 g of potassium dihydrogen phosphate in 1000 ml of water and adjusting the pH to 4.5±0.05 with ortho phosphoric acid. Water and acetonitrile in the ratio of 30:70 was used as diluent.

Standard preparations:

Standard stock solution was prepared by dissolving 50 mg of MLV working standard in 50 ml of diluent. Further 5 ml of this solution is diluted up to 50 ml with diluent for final standard concentration of 100 ug/ml.

Sample preparations:

Transferred accurately weighed capsule filled powder equivalent to 200 mg of MLV into 100 ml volumetric flask. Added 50 ml of diluent and sonicate for 5 min. Made up the volume with diluent and mixed well. Centrifuged to get clear supernatant. Dilute 5 ml of this solution to 100 ml with diluent to get final concentration of 100 µg/ml

Validation:

The developed method has been validated for the assay of MLV in capsules, 200 mg capsule strength using following parameters as per International Conference on Harmonization Q2 (R1) guideline[12].

System suitability:

Five replicate injections of MLV standard preparation were injected into HPLC. The tailing factor for the MLV peak from the injection of the

standard preparation should be less than 2.0 and the column efficiency determined from MLV peak from the injections of the standard preparation should not be less than 1500 theoretical plates. The relative standard deviation for the mean area calculated for MLV peak from the five replicate injections of standard preparation should be less than 2.0 %.

Specificity:

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Specificity consisted of interference and forced degradation studies.

Interference study: This was demonstrated by preparing a placebo containing all excipients and injecting a placebo solution prepared as per sample preparations.

Force degradation study: This was demonstrated by carrying out forced degradation of the sample with 1 N HCl, 1 N NaOH, 30 % H_2O_2, heating in water bath at 60° for 30 mins and keeping under UV light for 24 h. The samples were prepared as per sample preparation and injected into HPLC system with a photodiode array detector.

Acid degradation: Transferred accurately weighed capsule filled powder equivalent to 200 mg of MLV into 100 ml volumetric flask. Added 50 ml of diluent and sonicate for 5 min. Add 0.5 ml of 1 N HCl and heated in a water bath at 60° for 30 min. Cooled and neutralized with 0.5 ml of 1 N NaOH. Made up the volume with diluent and mixed well. Centrifuged to get clear supernatant. Diluted 5 ml this solution upto 100 ml with diluent. Injected this solution in to the HPLC system.

Alkali degradation: Transferred accurately weighed capsule filled powder equivalent to 200 mg of MLV into 100 ml volumetric flask. Added 50 ml of diluent and sonicated for 5 min. Add 0.5 ml of 1 N NaOH at room temperature for 10 min and neutralized with 0.5 ml of 1 N HCl. Made up the volume with diluent and mixed well. Centrifuged to get clear supernatant. Dilute 5 ml of this solution to 100 ml with diluent. Inject this solution in to the HPLC system.

Peroxide degradation: Transferred accurately weighed capsule filled powder equivalent to 200 mg of MLV into 100 ml volumetric flask. Added 50 ml of diluent and sonicated for 5 min. Add 0.5 ml of 30 % H_2O_2 and heated in a water bath at 60° for 15 min. Cooled and made up the volume with diluent and mixed well. Dilute 5 ml of this solution to 100 ml with diluent. Inject this solution in to the HPLC system.

Thermal degradation: Transferred accurately weighed capsule filled powder equivalent to 200 mg of MLV into 100 ml volumetric flask. Added 50 ml of diluent and sonicate for 5 min, heated in a water bath at 60° for 30 min. Cooled and made up the volume with diluent and mixed well. Dilute 5 ml of this solution to 100 ml with diluent. Inject this solution in to the HPLC system.

UV degradation: Transferred accurately weighed capsule filled powder equivalent to 200 mg of MLV (Previously kept in UV light for 24 h) into 100 ml volumetric flask. Added 50 ml of diluent and sonicate for 5 min and made up the volume with diluent and mixed well centrifuged to get clear supernatant. Dilute 5 ml of this solution to 100 ml with diluent. Inject this solution in to the HPLC system. The peak purity of MLV peak should pass.

Linearity:

From the standard stock solution, a series of solutions were prepared at concentration levels ranging from 50 % to 150 % of standard concentration. The peak area responses of solutions at all levels in duplicate were measured. The peak response verses concentration data was treated by linear regression analysis and the linearity of response for MLV was determined by calculating correlation coefficient (acceptance criterion: correlation coefficient should not be less than 0.999).

Accuracy:

For accuracy study, known amount of placebo was taken separately into different volumetric flasks and spiked with known quantities of MLV active pharmaceutical ingredient at three different levels, in triplicate. Accuracy has been performed at about 50 % (Level 1), 100 % (Level 2) and 150 % (Level 3) of sample concentration. The samples were analysed by the proposed method and the amount of MLV recovered was calculated (acceptance criterion: percentage recovery shall be in the range of 98-102 %. Individual and overall % RSD of % recovery should not be more than 2.0).

Precision:

In precision study, system precision, method precision and intermediate precision have been carried out. The system precision was examined by analysing standard

solution in five replicates. % RSD of area counts of MLV peak was calculated. In method precision, six preparations of a single batch of MLV capsules, 200 mg capsule against MLV standard solution were examined.

Intermediate precision was repeated using different analysts, on different days, on different instruments and using column of different lot. Overall RSD for assay between the two sets of data was calculated.

Limit of Detection (LOD) and Limit of Quantification (LOQ):

A series of standard preparation of different concentrations of MLV standards were prepared by using linearity 50 % solution and injected in to HPLC till to get %RSD of area of three replicate injections below 10 was consider as a LOQ level and till to detect was consider as a LOD level.

Robustness:

Robustness of the method was investigated by varying the instrumental conditions such as wavelength of detection (±2 nm), column oven temperature (±5°), minor component in mobile phase (±5 % absolute), flow rate (±10 %) and pH of Buffer (±0.2 unit). System suitability of the standard solution was checked at each variable condition. The theoretical plates, tailing factor of MLV peak from standard solution and % RSD of area counts of standard solution for each set of data were calculated. Sample solution of MLV capsules, was prepared in triplicate and analysed under each condition and % label claim of MLV was calculated. Robustness of the method was indicated by the overall % RSD between the data of method precision and data at each variable condition (acceptance criterion: System suitability should pass and overall % RSD of % Assay shall not be more than 2.0).

Stability of analytical solution:

Freshly prepared Standard and sample solutions of MLV capsules were kept at 25°. Both the solutions were analysed initially and at different time intervals. Calculate percentage deviation form mean initial area count (acceptance criterion: % Deviation shall not be more than ±2.0 %).

RESULTS AND DISCUSSION

The chromatographic method was optimized by changing various parameters, such as the mobile phase composition and pH of the buffer used in the mobile phase. Retention time and separation of peak of MLV were dependent on the pH of the buffer and the percentage of methanol. Different mobile phases were tried, but satisfactory separation and good symmetrical peak were obtained with the mobile phase consisting of buffer (pH 4.5) and methanol in the ratio of 70:30 % v/v with Eclipse plus C18 (150×4.6mm), 5 µ column with run time of 12 min. The column selection and shorter run time depicts the cost effectiveness of the method. A typical chromatogram obtained by using the aforementioned mobile phase and 10 µl of the injected assay preparation is illustrated in fig. 2. Retention time for MLV was found to be 7.6 min. No blank peak and placebo interference at the retention time of MLV peak was obtained as shown in fig. 3.

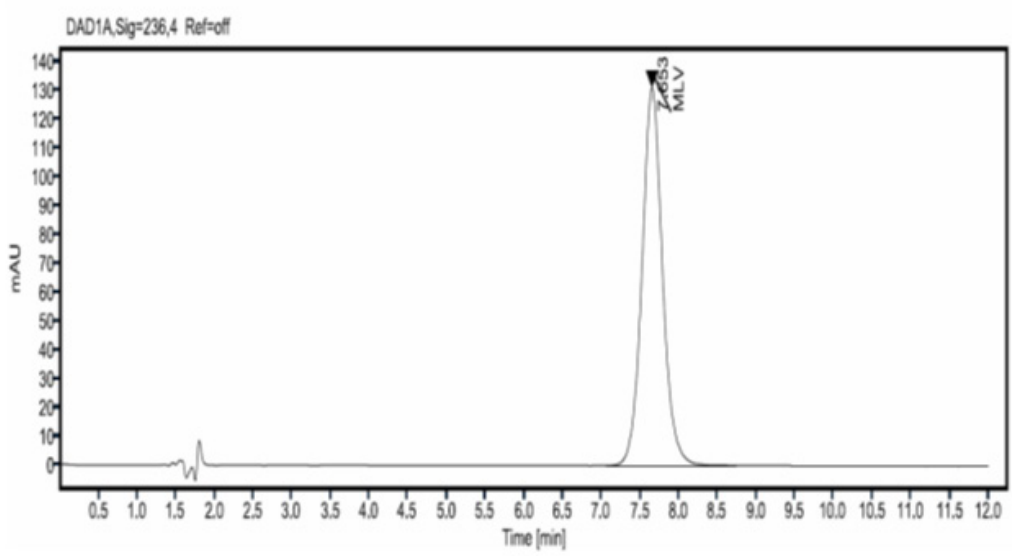

Fig. 2: Chromatogram of standard preparation"

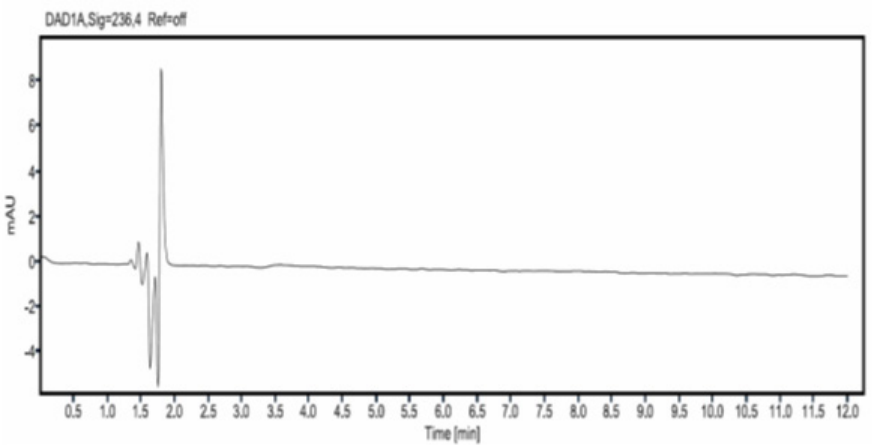

Fig. 3: Chromatogram of placebo preparation

Standard solution was injected on different days during the validation study. Open Lab CDS software, theoretical plates and tailing factor for MLV peak were calculated. Also, % RSD for five replicate injections was calculated. The tailing factor of MLV standard peak from the first injection of the standard preparation was 1.07, theoretical plates was 4161 and the % RSD calculated for MLV peak from the five replicate injections of standard preparations was 0.11. The above three system suitability parameters were met during the course of entire validation. System precision results are summarised in Table 1.

The result of interference study indicates that there are no coeluting peaks from placebo as well as no interference of MLV impurities with MLV peak as the peak purity indicates that the MLV peak is homogeneous. The peak purity of MLV peak was found above 998.88 in all the forced degradation samples fig. 4.

During forced degradation study, significant degradation was observed when MLV exposed to alkali hydrolysis and peroxide degradation but moderate degradation was observed in acid hydrolysis. MLV was found stable under thermal and photolytic condition. Results from peak purity testing confirmed MLV peak obtained by analysis of all the stress samples was homogenous and pure and unaffected by the presence of its degradation products, confirming the stability indicating nature of the method. The results from forced degradation studies are summarized in Table 2.

The method validated in the range of 49.80 to 149.40 µg/ml of standard concentration is significant. The regression equation from peak response *vs.* concentration data obtained Y=24.409X–1.8953 and correlation coefficient was 0.9999 indicating that the response is linear over the specified range. The result data is shown in Table 3 and the plot of Linearity shown in fig. 5.

The recovery of three sample preparation at each level was examined and ranged from 99.02 % to 100.73 %. As percentage recovery is found within the acceptance criteria, also individual and overall % RSD of % recovery is found 0.60 which proved the accuracy of the method. Results are summarized in Table 4.

TABLE 1: PRECISION DATA

System precision		Method precision		Intermediate precision	
Injection No.	Area Count (mAU)	Sample No.	Assay (% Label claim)	Sample No.	Assay (% Label claim)
1	2410.71	1	102.68	1	101.49
2	2416.59	2	100.20	2	100.67
3	2414.08	3	103.58	3	101.96
4	2417.27	4	100.88	4	100.96
5	2415.82	5	102.31	5	100.97
		6	101.25	6	100.63
Mean	2414.89	Mean	101.82	Mean	101.46
±SD	2.62	±SD	1.26	±SD	1.03
% RSD	0.11	% RSD	1.23	% RSD	1.01

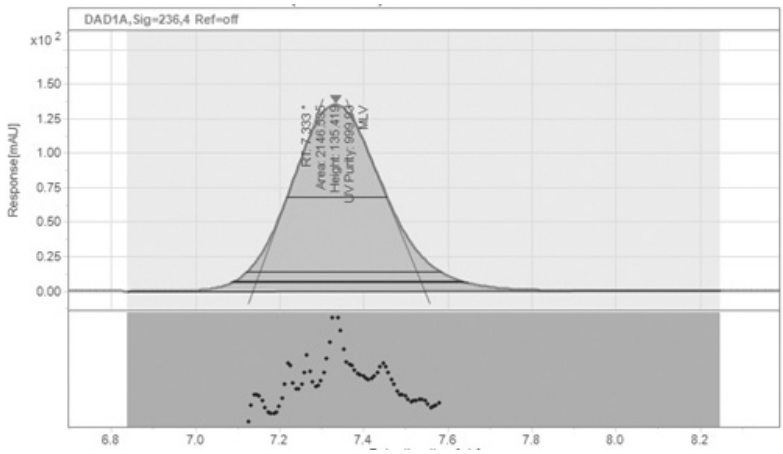

Fig. 4: Peak purity graph of MLV

TABLE 2: RESULTS OF FORCE DEGRADATION STUDY

Mode of degradation	Condition	Assay	% Degradation	UV Purity	Peak Purity
Sample as such	No treatment	101.78	-	999.93	Pass
Acid degradation	1 N HCl-0.5 ml/60°/30 min	97.43	4.35	999.92	Pass
Alkali degradation	1 N NaOH-0.5 ml/ RT/10 min	89.1	12.68	998.96	Pass
Peroxide degradation	30% H_2O_2-0.5 ml/60°/30 min	80.78	21	998.88	Pass
Thermal degradation	60° for 30 min	99.38	2.4	999.88	Pass
UV degradation	24 h at UV Chamber	101.82	0	999.93	Pass

TABLE 3: LINEARITY STUDY

Concentration (µg/ml)	Mean Area Counts
49.8	1223.05
79.68	1935.14
99.6	2412.99
119.52	2920.32
149.4	3652.91
Slope	24.41
Intercept	-1.90
Correlation Coefficient (r)	1.000

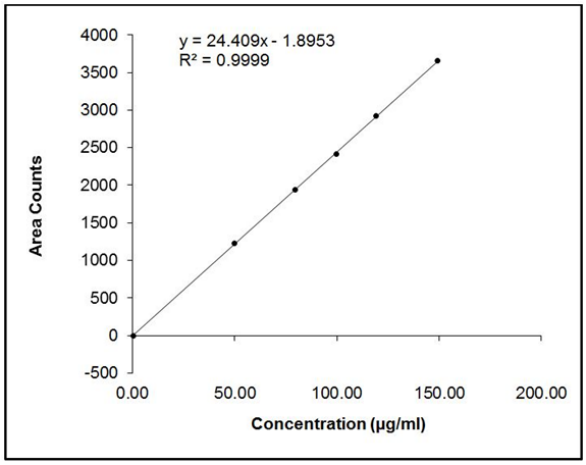

Fig. 5: Linearity of MLV

TABLE 4: ACCURACY RESULTS

Recovery levels	Amount added Conc. (µg/ml)	Amount recovered Conc. (µg/ml)	% Recovery
Level 1 (50 %)	50.8	50.4	99.21
	50.4	50.50	100.20
	51.2	50.70	99.02
Level 2 (100 %)	100.4	100.13	99.73
	100.8	99.98	99.19
	100.2	100.16	99.96
Level 3 (150%)	150.2	151.29	100.73
	150.8	151.47	100.44
	151.0	151.16	100.11
Mean			99.84
±SD			0.60
% RSD			0.60

The HPLC system has an acceptable level of precision as the acceptance criterion for individual and overall % RSD should not be more than 2.0 for precision, which was achieved successfully. The system is found to be precise as the %RSD of the area counts for five injections of the standard solution was 0.11 (Table 1). The method is also precise as the % RSD of amount present for MLV in the sample was 1.23. In intermediate precision, individual % RSD of amount present for MLV was found to be 1.23 for Set I (Method precision data), 1.01 for Set II and overall % RSD value is 1.12. Hence the method is found to be precised. Results are summarized in Table 1.

Experimentally LOQ was found 0.5 µg/ml where % RSD of three replicate injection was found 9.83 and LOD was found 0.25 µg/ml so we can say that method is sensitive and can detect and quantify very small amount of Concentration. Results are summarized in Table 5.

In all the deliberate varied chromatographic conditions (wavelength of detection, column oven temperature, composition of mobile phase, flow rate and pH of buffer) the results obtained were well within the limit. Results were summarized in Table 6.

In standard solution, % deviation form mean initial area counts up to 24 h at room temperature condition is 0.8 %. In sample solution, % deviation form mean initial area counts up to be 24 h at room temperature condition is 1.1 %. As the % deviation form mean initial area counts of standard solution and sample solution of MLV, within the acceptable limits ± 2.0 %, it reveals that standard and sample are stable in analytical solution for at least 24 h at room temperature. Results are summarized in Table 7.

TABLE 5: LOD and LOQ STUDY

Injection	Area count						
	5.0 (µg/ml)	2.5 (µg/ml)	1.25 (µg/ml)	0.75 (µg/ml)	0.5 (µg/ml)	0.25 (µg/ml)	0.05 (µg/ml)
1	123.41	62.32	31.44	14.24	11.17	5.12	ND
2	121.73	60.26	30.29	15.23	13.61	8.27	ND
3	121.99	61.45	30.00	15.86	12.59	6.41	ND
Mean	122.38	61.34	30.57	15.11	12.45	6.60	-
±SD	0.90	1.03	0.76	0.82	1.22	1.58	-
% RSD	0.73	1.68	2.49	5.4	9.83	23.99	-

Note: ND: Not detected

TABLE 6: ROBUSTNESS DATA

Method parameters	Standard Solution			Assay of MLV (% Claim)
	Theoretical plate	Tailing factor	% RSD	
As such (Method precision)	4161	1.07	0.11	101.82
Variation in wavelength (234 nm)	4216	1.08	0.12	102.68
Variation in wavelength (238 nm)	4158	1.07	0.15	100.20
Variation in column oven temperature (20°)	4072	1.05	0.15	100.88
Variation in column oven temperature (30°)	4198	1.02	0.13	102.31
Variation in minor component in mobile phase (- 5 % of methanol)	3892	1.14	0.21	101.25
Variation in minor component in mobile phase (+ 5 % of methanol)	4254	1.05	0.16	100.96
Variation in flow rate (0.8 ml/min)	4098	1.16	0.14	100.97
Variation in flow rate (1.2 ml/min)	3941	1.04	0.13	101.49
Variation in pH of Buffer solution (pH 4.3)	4028	1.05	0.14	101.96
Variation in pH of Buffer solution (pH 4.7)	4121	1.02	0.15	100.63

TABLE 7: SOLUTION STABILITY

Time (h)	Standard solution		Sample solution	
	Area count	% Deviation	Area count	% Deviation
Initial	2410.71	0.0	2489.05	0.0
4	2425.78	0.6	2492.90	0.1
10	2423.56	0.5	2498.92	0.4
15	2431.25	0.8	2505.86	0.6
24	2426.51	0.6	2517.03	1.1

In conclusion, the present HPLC method for the determination of assay of MLV in capsule dosage form is simple, rapid, economical, precise, accurate and rouged. The method has been validated and satisfactory results were observed for all the tested validation parameters. The developed method can be conveniently used for determination of assay of MLV in marketed formulation and stability samples. Moreover, the lower solvent consumption along with the short analytical run time of 12 min leads to cost effective chromatographic method.

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Conflict of interests:

The authors declared no conflict of interests.

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