

**DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR DETERMINATION OF LEVAMISOLE IN BULK AND DOSAGE FORM**P. RAVISANKAR<sup>1,2\*</sup>, G. DEVALA RAO<sup>3</sup>

<sup>1</sup>Department of Pharmaceutical Analysis and Quality Assurance, Vignan Pharmacy College, Vadlamudi, Guntur, A.P., India. <sup>2</sup>Faculty of Science, Sri Chandrasekharendra Saraswathi Viswa Maha Vidyalaya (SCSVMV University), Enathur, Kanchipuram, T.N., India. <sup>3</sup>Department of Pharmaceutical Analysis, KVSr Siddhartha College of Pharmaceutical Sciences, Vijayawada, A.P., India. E-mail: banuman35@gmail.com

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**ABSTRACT**

A reverse phase isocratic HPLC was developed and validated for the determination of levamisole in bulk and tablet dosage forms. Method development was carried out on Welchrom C<sub>18</sub> isocratic column, (250 mm × 4.6 mm i.d., particle size 5 μm, maintained at ambient temperature), Shimadzu LC-20AT Prominence Liquid Chromatograph. The mobile phase was a mixture of Methanol: Acetonitrile: Water 50:30:20 v/v, with apparent pH of 4.6 and the flow rate was set at 1.0 ml/min and UV detection at 225 nm. Validation parameters were evaluated for the method according to the ICH (Q2R1) guidelines. In the linearity study, linearity was observed from 2-10 μg/ml with correlation coefficient of 0.9999 and regression coefficient of 0.9998. The limit of detection and limit of quantitation for the method were 0.1219 μg/ml and 0.3695 μg/ml, respectively. The statistical analysis shows that the method was found to be accurate, reliable, simple and reproducible. The intra- and inter-assay precisions were satisfactory; the values of relative standard deviations did not exceed 2%. The accuracy of the method was proved; the mean recovery of levamisole was 99.36% to 100.56%. The chromatographic retention time of proposed method was 2.790 min and the mean assay of content was found to be 102.135 ± 0.933%. The proposed method was successfully applied for the quantitative determination of levamisole in bulk form and could be used for routine analysis with phenomenal accuracy and precisions.

**Keywords:** RP-HPLC, Levamisole, Validation, ICH guidelines.

**INTRODUCTION**

Levamisole (LMS) is (S)-6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-b][1,3]thiazole (Figure 1). LMS is a pharmaceutical with anthelmintic and immunomodulatory properties<sup>1</sup> that was previously used in both animals and humans to treat inflammatory conditions and cancer. LMS is the levorotatory isomer of tetramisole. Levamisole has been used in humans to treat parasitic worm infections, and has been studied in combination with other forms of chemotherapy for colon cancer, melanoma, and head and neck cancer.

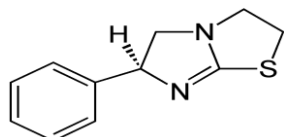


Figure 1: Structure of Levamisole

Literature survey reveals few analytical methods were reported for the determination of LMS in bulk and pharmaceutical preparations and in biological fluids by spectrophotometry<sup>2</sup>, High Performance Liquid Chromatography[3-9], LC-MS[10-11]. However most of the available methods have limitations such as a long runtimes, low sensitivity, uneconomical and have poor symmetry. Keeping in view of these, an attempt has been made to develop a simple, accurate, precise and reliable RP-HPLC method for the estimation of levamisole in pharmaceutical dosage forms. In fact the established method was validated with respect to specificity, linearity, precision, accuracy, robustness, LOD and LOQ according to ICH guidelines (ICH, 1997)[12].

**MATERIALS AND METHODS****Chemicals and Reagents**

An analytically pure sample of LMS standard was procured as gift sample from Cipla Ltd., Mumbai, India. All the chemicals were analytical grade. HPLC grade acetonitrile and triethylamine were procured from Merck Pharmaceuticals Private Ltd., Mumbai, India. Methanol and water used were of HPLC grade and purchased from

Merck Specialties Private Ltd., Mumbai, India. Commercial tablets of LMS formulation was procured from local pharmacy. LEVOMOL tablets containing LMS with labeled amount of 50mg per tablet are manufactured by Cipla Ltd., Mumbai, India.

**Instruments and Chromatographic conditions**

The HPLC analysis was performed on Shimadzu LC-20AT Prominence Liquid Chromatograph comprising a LC-20AT VP pump, Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom C<sub>18</sub> column (4.6 mm i.d. X 250 mm, 5 micron particle size). A manually operating Rheodyne injector with 20 μL sample loop was equipped with the HPLC system. The HPLC system was equipped with "Spinchrom" data acquisition software. The mobile phase consists of a mixture of methanol, acetonitrile and water (pH adjusted to 4.6 using o-phosphoric acid) in ratio of 50:30:20, v/v. Triethylamine was used as column modifier. The mobile phase was set at a flow rate of 1 mL/min. Eluate was monitored at 225 nm. In addition, an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), a sonicator (spectra lab, model UCB 40), UV-Visible Spectrophotometer (Systronics model 2203) were used in this study.

**Preparation of Reagents and Standards****Mobile phase**

The mobile phase was prepared by mixing of methanol, acetonitrile and water (all of HPLC grade) in the ratio of 50:30:20, v/v. Then pH is adjusted to 4.6 with 0.1N o-phosphoric acid and 0.5ml triethylamine is added as column modifier. It is filtered through 0.45 μm nylon membrane filter and then sonicated for degassing.

**Stock and Working Standard Solutions**

Accurately 10 mg of LMS was weighed and transferred to a 10 mL clean, dry volumetric flask and mobile phase was added and sonicate to dissolve. The volume was made up to the mark with mobile phase. This is stock standard solution of LMS with concentration of 1000 μg/mL. Prepare five working standard solutions for calibration by adding defined volumes of the stock standard solution and diluting

with mobile phase. The concentrations of LMS are 2.0, 4.0, 6.0, 8.0, 10.0 µg/mL, respectively.

#### Tablet Sample preparation

Weigh accurately not less than 20 tablets of LMS and determine average weight. Grind the tablets of LMS (LEVOMOL) into fine powder. Weigh accurately an amount of tablet powder equivalent to 50 mg of LMS and transfer into 50 mL volumetric flask. Add 40 mL mobile phase and place in an ultra sonication bath until dissolution is complete. Add mobile phase to bring up the volume to 50 mL. Pipette out 1.0 mL of the sample solution into a 10 mL volumetric flask and dilute with mobile phase up to the mark. Mix well. The resulting solution was filtered using 0.2 µm filter and degassed by sonication. The resulting solution is further diluted to give a concentration of approximately 10 µg/mL.

#### Selection of detection wavelength

The UV spectrum of diluted solutions of various concentrations of LMS in mobile phase was recorded using UV spectrophotometer. The wavelength of maximum absorbance was observed at 225nm. This wavelength was used for detection of LMS.

#### Calibration curve for Levamisole

20 µl of each calibration standard solutions (2, 4, 6, 8, 10 µg/mL) were injected into the HPLC system to get the chromatograms. The average peak area and retention time were recorded. Linearity curve was constructed by plotting concentration of LMS on X-axis and average peak areas of standard LMS on Y-axis and regression equations were computed for LMS. The linearity range was found to be 2-10 µg/mL. The results were presented in Table 1. The standard chromatograms of LMS calibration standards have been depicted in Figure 2 to Figure 6. Results show that a phenomenal correlation exists between peak area and concentration of drug within the linearity range. The regression graph for LMS is presented in Figure 7. The data of regression analysis is presented in Table 2.

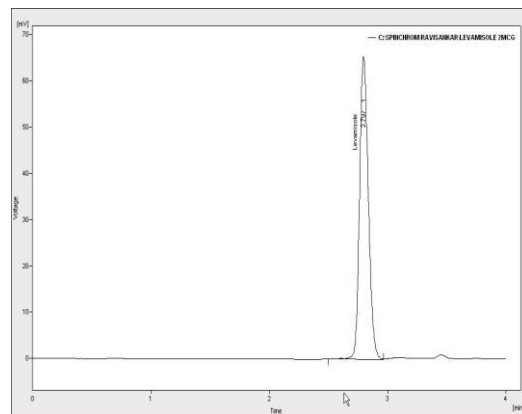
**Table 1: Calibration data of the proposed hplc method for estimation of levamisole**

S.No	Concentration, µg/mL.	Retention time, (t <sub>r</sub> )min.	Peak area, mV.s.
1.	0	-	0
2.	2	2.797	297.924
3.	4	2.790	584.628
4.	6	2.787	881.176
5.	8	2.790	1162.192
6.	10	2.790	1475.255
Slope		146.651814	
Intercept		0.270095	
Correlation Coefficient [CC] (r)		0.999925	
Squared CC (R <sup>2</sup> )		0.999851	
Residual sum of squares		224.256590	

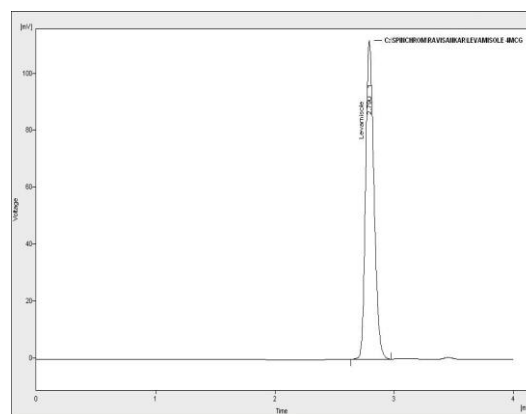
**Table 2: Linear regression data of the proposed hplc method of levamisole**

Parameter	Method
Detection wavelength( λ <sub>max</sub> )	UV at 225 nm
Linearity range (µg/mL)	2-10 µg/mL
Regression equation (Y = a + bX)	Y = 0.2701 + 146.65X
Slope(b)	146.65
Intercept(a)	0.2701
Standard error of slope (S <sub>b</sub> )	0.894939
Standard error of intercept (S <sub>a</sub> )	5.419126
Standard error of estimation (S <sub>e</sub> )	7.487599
Regression coefficient (R <sup>2</sup> )	0.9998
% Relative standard deviation* i.e., Coefficient of variation(CV)	0.9315
Percentage range of errors* (Confidence limits)	
0.005significance level	0.827986
0.001 significance level	1.298756

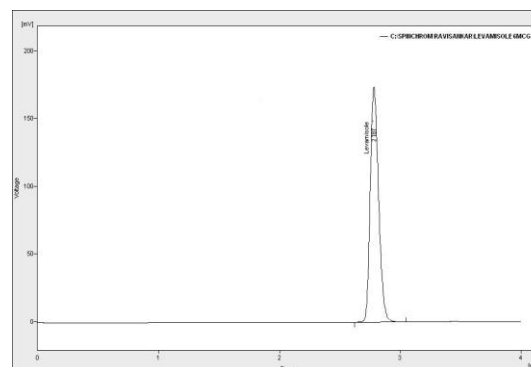
\*Average of 6 determinations; Acceptance criteria < 2.0.



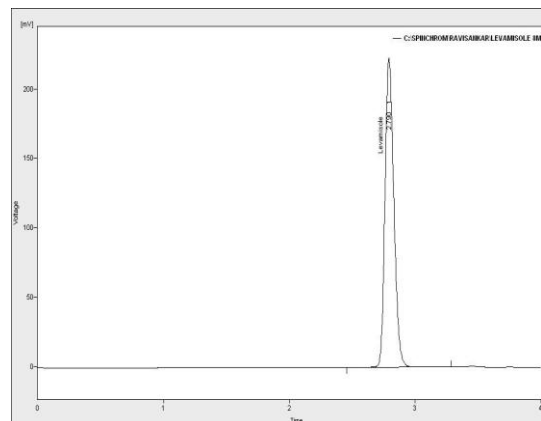
**Figure 2: Standard chromatogram of Levamisole (2 µg/ml)**



**Figure 3: Standard chromatogram of Levamisole (4 µg/ml)**



**Figure 4: Standard chromatogram of Levamisole (6 µg/ml)**



**Figure 5: Standard chromatogram of Levamisole (8 µg/ml)**

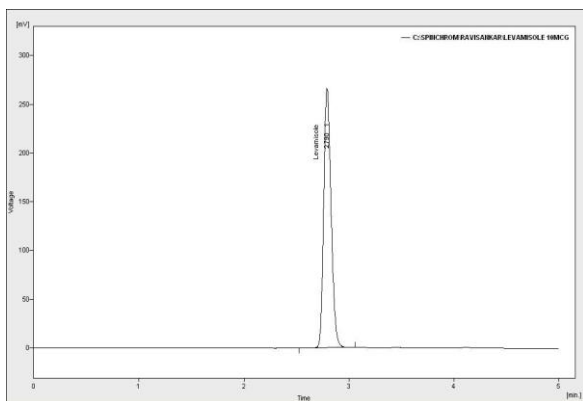


Figure 6: Standard chromatogram of Levamisole (10 µg/ml)

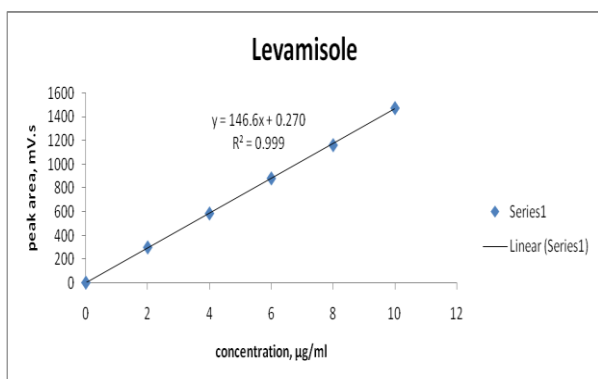


Figure 7: Calibration plot of Levamisole

#### Assay of Levamisole tablets:

The developed method was applied to the assay of LMS tablets. The drug content was calculated as an average of six determinations and assay results were shown in Table 3. The results were very close to the labeled value of commercial tablets. The representative sample chromatogram of LMS is shown in Figure 8.

Table 3: Assay Results Of Levamisole Formulation

S.No	Formulations	Labelled amount	Amount found*	% Assay ±SD*
1	LEVOMOL tablets (Cipla Ltd., Mumbai, India)	50 mg/tablet	51.06 mg/tablet	102.13 ±0.933%

\*Average of 6 determinations; SD is standard deviation.

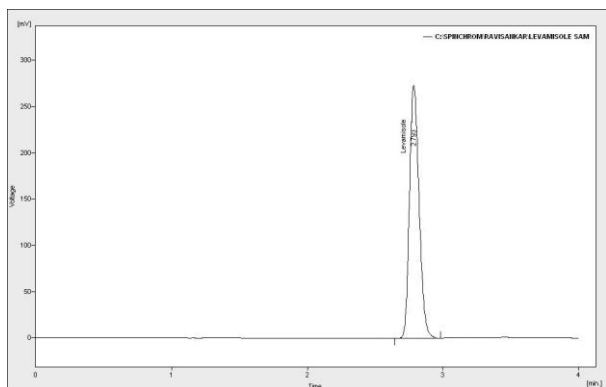


Figure 8: Chromatogram of market formulation (LEVOMOL 10 mg tablets) of Levamisole

#### VALIDATION OF THE PROPOSED METHOD

The developed method of analysis was validated as per the ICH for the parameters like system suitability, specificity, linearity, precision, accuracy, robustness and system suitability, limit of detection (LOD) and limit of quantitation (LOQ).

**System suitability:** The chromatographic systems used for analysis must pass the system suitability limits before sample analysis can commence. Set up the chromatographic system, allow the HPLC system to stabilize for 40 min. Inject blank preparation (single injection) and standard preparation (six replicates) and record the chromatograms to evaluate the system suitability parameters like resolution (NLT 2.0), tailing factor (NMT 1.5), theoretical plate count (NLT 3000) and % RSD for peak area of six replicate injections of LMS standard (%RSD NMT 2.0). The system suitability data is reported in Table 4.

**Specificity:** The specificity of the method was determined by observing interference of any encountered ingredients present in the formulations. The test results obtained were compared with the results of those obtained for standard drug. It was shown that those ingredients were not interfering with the developed method. Furthermore the well-shaped peaks also indicate the specificity of the method. The results for specificity are tabulated in Table 5. The chromatogram for placebo indicating the specificity of developed method is presented in Figure 9.

Table 4: Optimized chromatographic conditions and system suitability parameters of proposed rp-hplc method for levamisole

Parameter	Chromatographic conditions
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph
Column	WELCHROM C <sub>18</sub> Column (4.6 mm i.d. X 250 mm, 5 µm particle size)
Detector	SHIMADZU SPD-20A prominence UV-Vis detector
Diluents	Methanol: Acetonitrile: Water (50:30:20, v/v, pH-4.6 using o-phosphoric acid)
Mobile phase	Methanol: Acetonitrile: Water (50:30:20, v/v, pH-4.6 using o-phosphoric acid)
Column modifier	Triethylamine (0.5 mL)
Flow rate	1 mL/min.
Detection wave length	UV at 225 nm.
Run time	5 minutes
Column back pressure	156 kgf
Temperature	Ambient temperature (25°C)
Volume of injection loop	20 µL
Retention time (t <sub>R</sub> )	2.790 min
Theoretical plates [th.pl] (Efficiency)	6,738
Theoretical plates per meter [t.p/m]	134,762
Tailing factor (asymmetry)	1.079

Table 5: Specificity study for levamisole

Name of the solution	Retention time, (t <sub>R</sub> ) min.
Mobile phase	No peaks
Placebo	No peaks
Levamisole, 10 µg/mL	2.790 min.

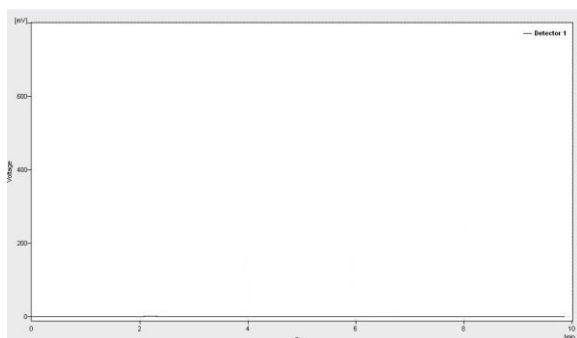


Fig 9: Chromatogram of placebo

### Precision

Intra-day precision was investigated by replicate applications and measurements of peak area for LMS for six times on the same day under similar conditions. Inter-day precision was obtained from % RSD values obtained by repeating the assay six times on two different days. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0. The intra-day and inter-day precision results were shown in Table 6 and Table 7 respectively.

Table 6: Results of precision study (intra-day) for levamisole

Sample	Concentration (µg/mL)	Injection no.	Peak area (mV.s)	%RSD#
Levamisole	10	1	1475.255	0.3934
		2	1478.118	
		3	1468.942	
		4	1472.647	
		5	1462.746	
		6	1466.068	

# Acceptance criteria &lt; 2.0.

Table 7: Results of precision study (inter-day) for levamisole

Sample	Concentration (µg/mL)	Injection no.	Peak area (mV.s)	%RSD#
Levamisole	10	1	1478.884	0.5119
		2	1462.462	
		3	1470.642	
		4	1458.873	
		5	1474.546	
		6	1466.362	

# Acceptance criteria &lt; 2.0.

### Accuracy/Recovery

Accuracy is the degree of agreement between a measured value and the accepted reference value. The accuracy of the method was tested by triplicate samples at 3 different concentrations equivalent to 80%, 100% and 120% of the active ingredient, by adding a known amount of LMS standard to a sample with pre-determined amount of LMS. The recovered amount of LMS, %RSD of recovery, % recovery of each concentration is calculated to determine the accuracy. The recovery results for accuracy study of LMS are presented in Table 8.

### Robustness

Robustness is the ability to provide accurate and precise results under a variety of conditions. In order to measure the extent of method robustness, the most critical parameters were interchanged while keeping the other parameters unchanged and in parallel, the chromatographic profile was observed and recorded. The studied parameters were: the composition of mobile phase, flow rate, detection wavelength. The results for robustness study in Table 9 indicated that the small change in the conditions did not significantly affect the determination of LMS

Table 8: Recovery data of for levamisole

Recovery level	Amount added (mg)	Total amount (mg)	Amount found (mg)	Amount recovered (mg)	% recovery	Mean % Recovery ± SD	%RSD#
80%	39.76	90.82	90.72	39.66	99.74	99.55 ± 0.188	0.1893
	39.94	91.00	90.82	39.76	99.54		
	39.79	90.85	90.60	39.54	99.37		
100%	49.86	100.92	101.20	50.14	100.56	100.32 ± 0.240	0.2398
	49.79	100.85	100.89	49.83	100.08		
	49.92	100.98	101.14	50.08	100.32		
120%	59.87	110.93	110.77	59.71	99.73	99.93 ± 0.219	0.2193
	59.78	110.84	110.94	59.88	100.16		
	59.97	111.03	110.97	59.91	99.89		

#acceptance criteria &lt; 2.0.

TABLE 9: ROBUSTNESS RESULTS OF LEVAMISOLE

S. no	Parameter <sup>a</sup>	Optimized	Used	Retention time (t <sub>R</sub> ), min	Plate count <sup>§</sup>	Peak asymmetry <sup>#</sup>	Remark
1.	Flow rate (±0.2 mL/min)	1.0 mL/min	0.8 mL/min	3.308	7094	1.094	*Robust
			1.2 mL/min	2.548	6467	1.109	*Robust
2.	Detection wavelength (±5 nm)	225 nm	230 nm	2.797	6775	1.093	Robust
			240 nm	2.793	6742	1.102	Robust
3.	Mobile phase composition (methanol content ±5 %)	50:50, v/v	55:45, v/v	3.148	6329	1.112	*Robust
			45:55, v/v	2.582	7175	1.105	*Robust

Acceptance criteria (Limits):#Peak Asymmetry &lt; 1.5, §Plate count &gt; 3000, \* Significant change in Retention time

**LOD and LOQ** Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of Detection and Limit

of Quantitation were calculated using following formula LOD= 3.3σ/S and LOQ= 10σ/S, where SD=standard deviation of response (peak area) and S= slope of the calibration curve. The LOD and LOQ values are presented in Table 10. The results of LOD and LOQ supported the sensitivity of the developed method.

**Table 10: LOD and LOQ**

Limit of Detection(LOD)	0.1219 µg/mL
Limit of Quantitation(LOQ)	0.3695 µg/mL

**RESULTS AND DISCUSSION**

The present study was aimed at developing a precise, sensitive, rapid and accurate HPLC method for the analysis of LMS in bulk drug and in pharmaceutical dosage forms. In order to achieve phenomenal retention time and peak asymmetry, a C<sub>18</sub> stationary phase column (250mm X 4.6mm i.d, 5 µm particle size) and mobile phase composed of methanol, acetonitrile and HPLC grade water in a ratio of 50:30:20, v/v, with pH adjusted to 4.6 using o-phosphoric acid and triethylamine as column modifier at a flow rate of 1mL/min was selected. The retention time for LMS was found to be 2.790 min. UV spectra of LMS showed that the drug absorbed maximum at 225 nm, so this wavelength was selected as the detection wavelength. The correlation coefficient (0.9999) of regression was found almost equal to 1 in the range of 2-10 µg/mL which states that the method was linear to the concentration versus peak area responses. On slight variation in the mobile phase ratio of up to ±5 %, the change in the peak asymmetry, plate count and retention time are within the limits which indicated that the method is robust and also indicating lack of influence on the test results by operational variable for the proposed method. This shows that the method is having phenomenal system suitability parameters under given conditions. The comparison of chromatograms of placebo, standard and sample, there was no interference observed from the peaks of placebo, standard and sample. It shows that the method is specific. The precision studies were performed and the % RSD of the determinations was found to be 0.3934 for intra-day precision and 0.5119 for inter-day precision which are within the limits. Hence the proposed method was found to be precise. The accuracy of the method was found to be good with the overall % RSD for recovery at 80%, 100% and 120% levels were all within the limits. This indicates that the proposed method was found to be accurate. Method validation following ICH guidelines indicated that the developed method had high sensitivity with LOD of 0.1219 µg/mL and LOQ of 0.3695 µg/mL. The assay results of tablets by applying the HPLC method was found to be within the pharmacopoeial limits and the assay values were found to be 102.13±0.933%.

**CONCLUSION**

The developed RP-HPLC method for the quantification of LMS has various advantages like less retention time, good peak symmetry and phenomenal linearity, highly sensitive, simple, precise, accurate and robust. The mobile phase can be easily prepared and diluent is economical and readily available and it does not need sample preparation with sophisticated techniques or instruments. The drug solutions employed in the study were stable up to 48 hours. These attribute the high quality of the method. The proposed method can be used for the routine analysis of LMS in bulk preparations of the drug and in pharmaceutical dosage forms for routine application in quality control laboratories without interference of excipients.

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