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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR DETERMINATION OF LEVAMISOLE IN PURE AND DOSAGE FORM

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ABSTRACT: A reverse phase isocratic HPLC was developed and validated for the determination of levamisole in pure and tablet dosage forms. Method development was carried out on YMC C18 isocratic column, (250 mm × 4.6 mm i.d., particle size 5 µm, maintained at ambient temperature), Shimadzu LC–sol 2010 Prominence Liquid Chromatography. The mobile phase was a mixture of Acetonitrile: methanol 50:50 v/v and the flow rate was set at 1.3 ml/min and UV detection at 236 nm. Validation parameters were evaluated for the method according to the ICH guidelines. In the linearity study, linearity was observed from 2-10 µg/ml with a correlation coefficient of 0.9999 and regression coefficient of 0.999. The limit of detection and limit of quantitation for the method were 0.0209µg/ml and 0.069µg/ml, respectively. The statistical analysis shows that the method was found to be accurate, reliable, simple, and reproducible. The intra and interassay precisions were satisfactory; the values of relative standard deviations did not exceed 2%. The accuracy of the method was proved; the recovery of levamisole was within limits. The chromatographic retention time of the proposed method was 3.3 min, and the assay of content was found to be 98.9%-100.8%. The proposed method was successfully applied for the quantitative determination of levamisole in pure form and could be used for routine analysis with phenomenal accuracy and precisions.

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

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INTRODUCTION: Levamisole(LMS) is (S)-6-Phenyl-2,3,5,6 - tetrahydroimidazo[2,1 - b] [1,3] thiazole **Fig. 1**. LMS is a pharmaceutical with anthelminthic and immunomodulatory properties [1] 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 [1, 2, 3].

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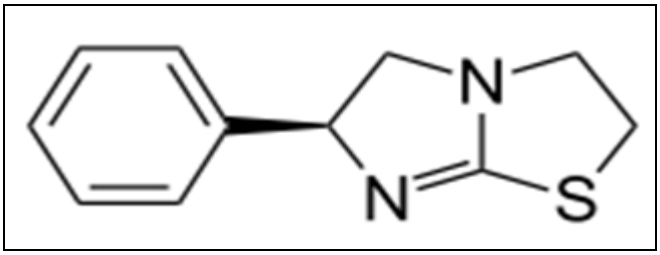

FIG. 1: STRUCTURE OF LEVAMISOLE

Literature survey reveals few analytical methods were reported for the determination of LMS in pure and pharmaceutical preparations and biological fluids by spectrophotometry [2], High-Performance Liquid Chromatography [3, 4, 5, 6]. However, most of the available methods have limitations such as long runtimes, low sensitivity, uneconomical, and have poor symmetry. Keeping given 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. The established method was validated concerning specificity, linearity, precision, accuracy, robustness, LOD, and LOQ according to ICH guidelines (ICH, 1997) [7, 8, 9, 10].

MATERIALS AND METHODS: Chemicals and Reagents an analytically pure sample of LMS standard was procured as a gift sample from Cipla Ltd., Mumbai, India. All the chemicals were analytical grade. HPLC grade acetonitrile and water used were of HPLC grade and purchased from Merck Specialties Private Ltd., Mumbai, India. Commercial tablets of LMS formulation was procured from a local pharmacy. LEVOMOL tablets containing LMS with labeled amount of50mg per pill are manufactured by Cipla Ltd., Mumbai, India.

Instruments and Chromatographic Conditions: The HPLC analysis was performed on Shimadzu LC-sol2010 Prominence Liquid Chromatography YMC C18 column (4.6 mm × 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 data N2000 software. The mobile phase consists of a mixture of acetonitrile and methanol in a ratio of 50:50% v/v. The mobile phase was set at a flow rate of 1.3 mL/min. Elute was monitored at 236 nm.

Preparation of Reagents and Standards:

Mobile Phase: The mobile phase was prepared by mixing of acetonitrile and methanol (all of HPLC grade) in the ratio of 50:50, v/v. It is filtered through a 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 sonicated to dissolve. The volume was made up to the mark with the mobile phase. This is standard stock solution of LMS with a concentration of 1000 µg/mL. Prepare five standard working solutions for calibration by adding defined volumes of the stock standard solution and diluting with the mobile phase. The concentrations of LMS are 2, 4, 6, 8 & 10 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 a 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 a 0.2 µm filter and degassed by sonication. The resulting solution is further diluted to give a concentration of approximately 20µg/mL.

Selection of Detection Wavelength: The UV spectrum of diluted solutions of various concentrations of LMS in the mobile phase was recorded using UV spectrophotometer. The wavelength of the maximum absorbance was observed at 236 nm. This wavelength was used for the 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 chromatogram of LMS calibration standard has been depicted in **Fig. 2**.

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 **Fig. 3.** The data of the analysis is presented in **Table 2**.

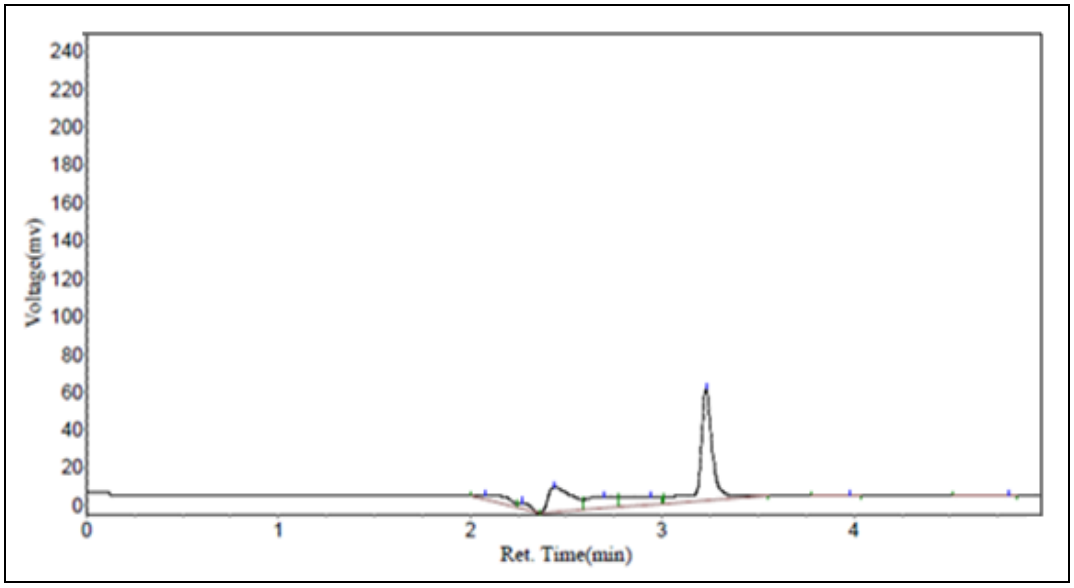

FIG. 2: 2µG CHROMATOGRAM OF LMS

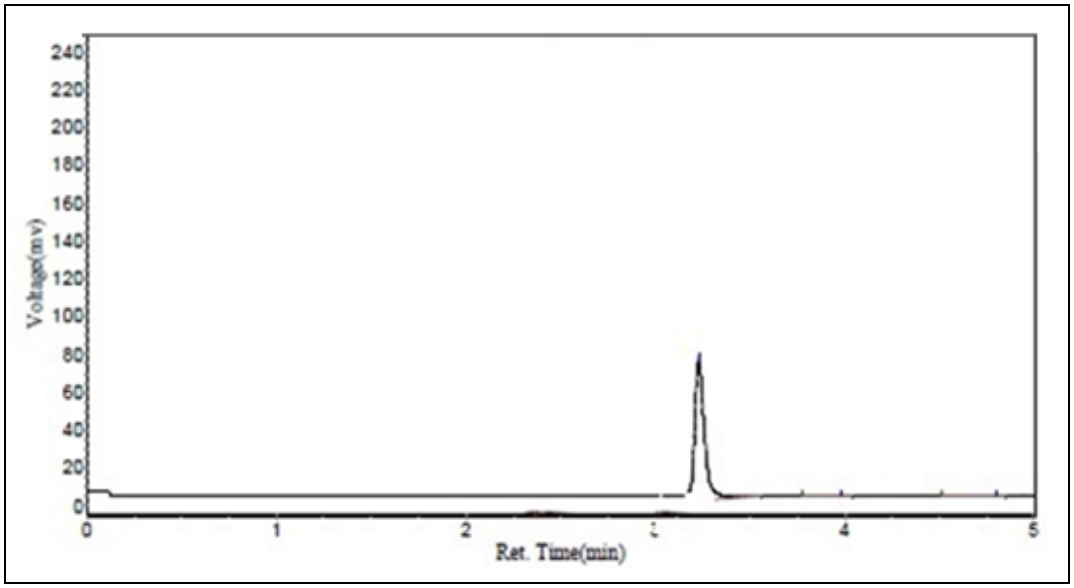

FIG. 3:4µG CHROMATOGRAM OF LMS

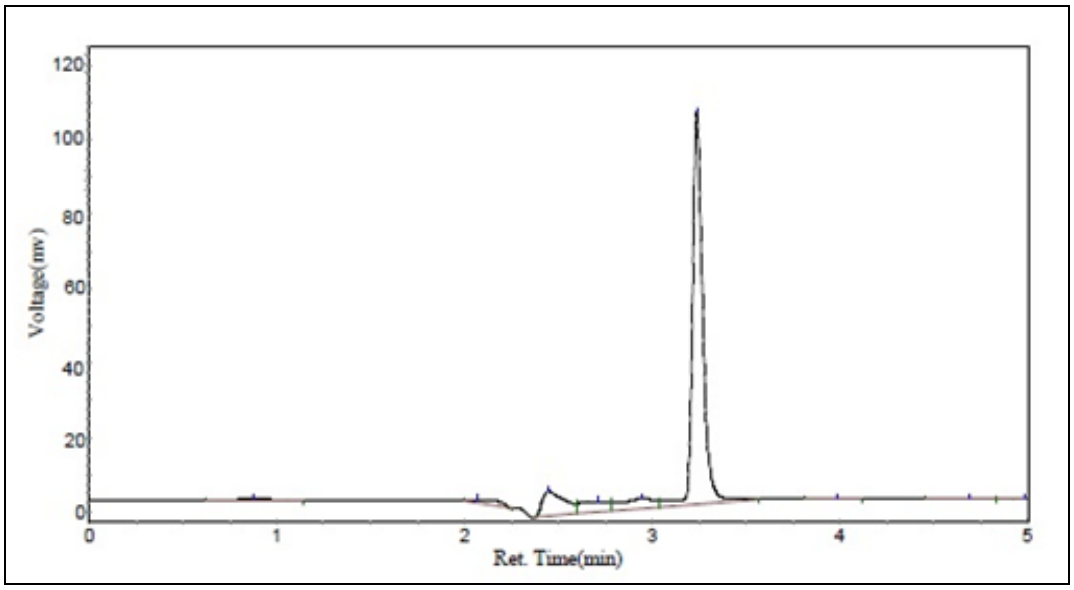

FIG. 4: 6µG CHROMATOGRAM OF LMS

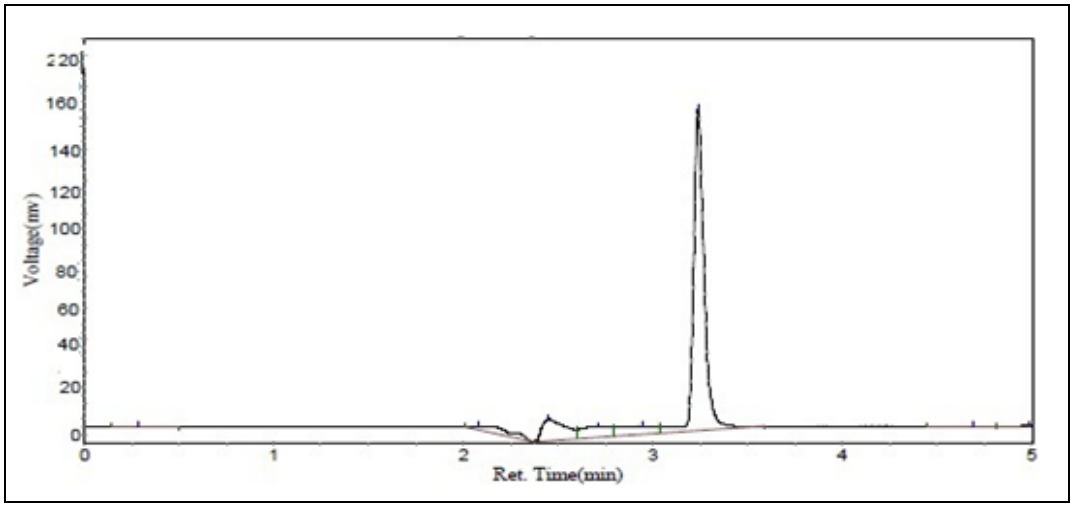

FIG. 5: 8µG CHROMATOGRAM OF LMS

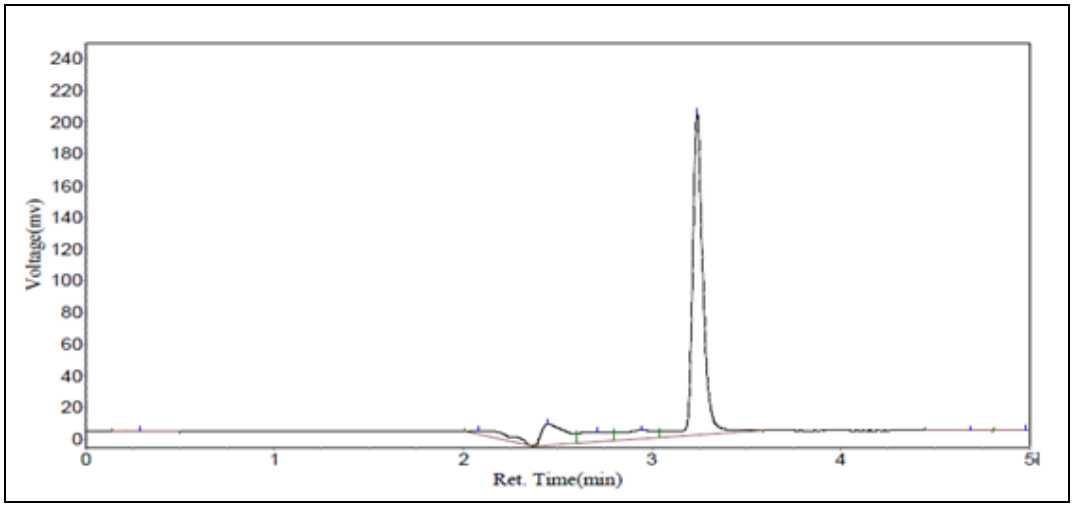

FIG. 6: 10 µG CHROMATOGRAM OF LMS

TABLE 1: LINEARITY

Concentration of levamisole(µg/ml)	Levamisole Peak Area (mV.sec)
2	721081
4	1446122
6	2181720
8	2884324
10	3624987

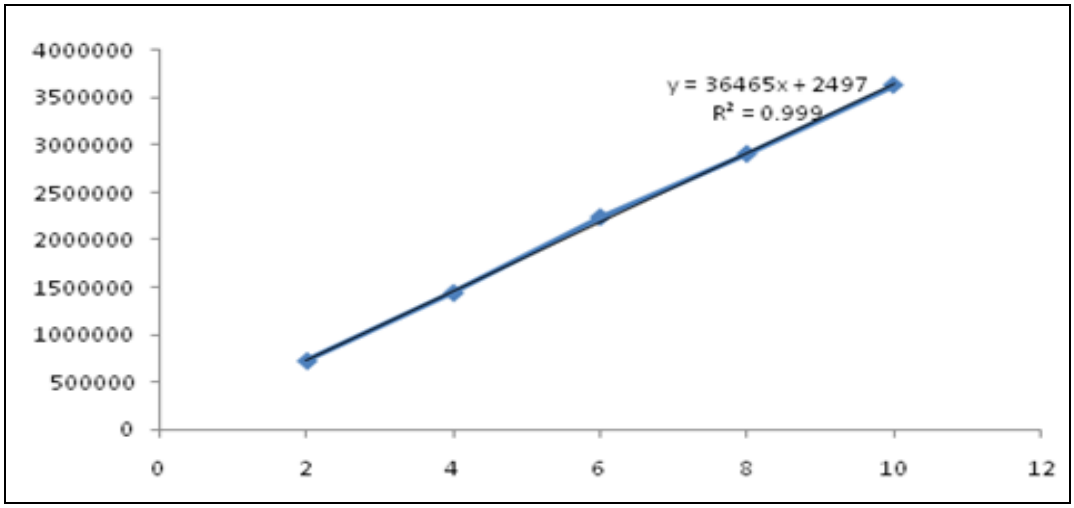

FIG. 7: LINEARITY GRAPH

System Precision:

TABLE 2: SYSTEM PRECISION

S. no.	Retention time	Peak area
1	3.313	723061
2	3.323	721081
3	3.357	727240
4	3.357	723262
5	3.348	713262
mean	3.3396	721581.2
Sd	0.0203	5159.84
%RSD	0.609	0.715

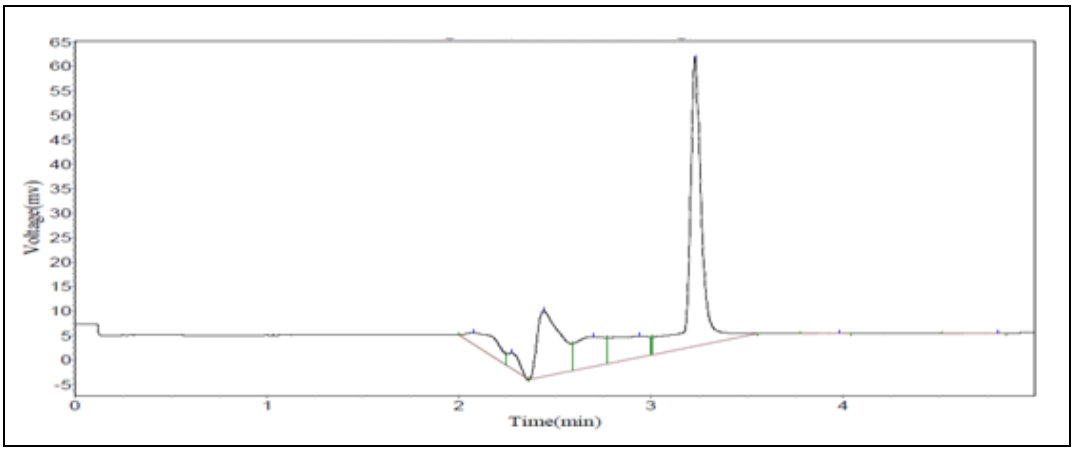

FIG. 8: CHROMATOGRAM 1 OF SYSTEM PRECISION OF LEVAMISOLE

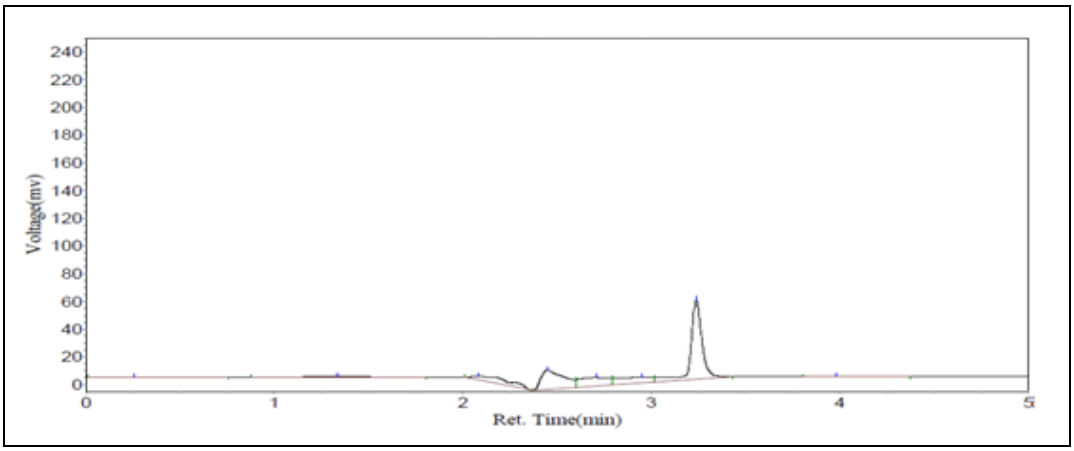

FIG. 9: CHROMATOGRAM 2 OF SYSTEM PRECISION OF LEVAMISOLE

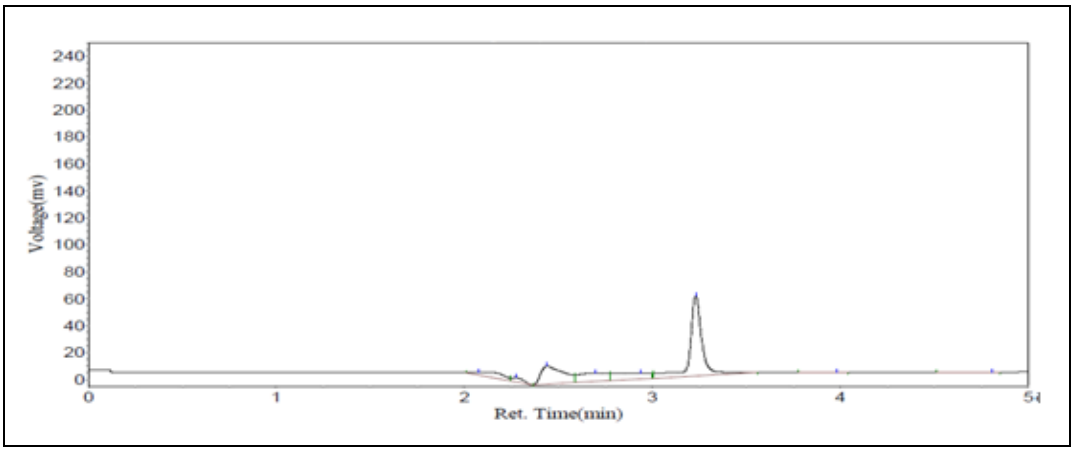

FIG. 10: CHROMATOGRAM 3 OF SYSTEM PRECISION OF LEVAMISOLE

Method Precision:

TABLE 3: METHOD PRECISION

S. no.	Levamisole	
	Area	% Labelled Claim
1	723061	100.20
2	721081	99.93
3	727240	100.784
4	723262	100.232
5	713262	98.847
Mean	721581.2	100.007
S.D	5159.85	0.71507
%RSD	0.71507	0.71507

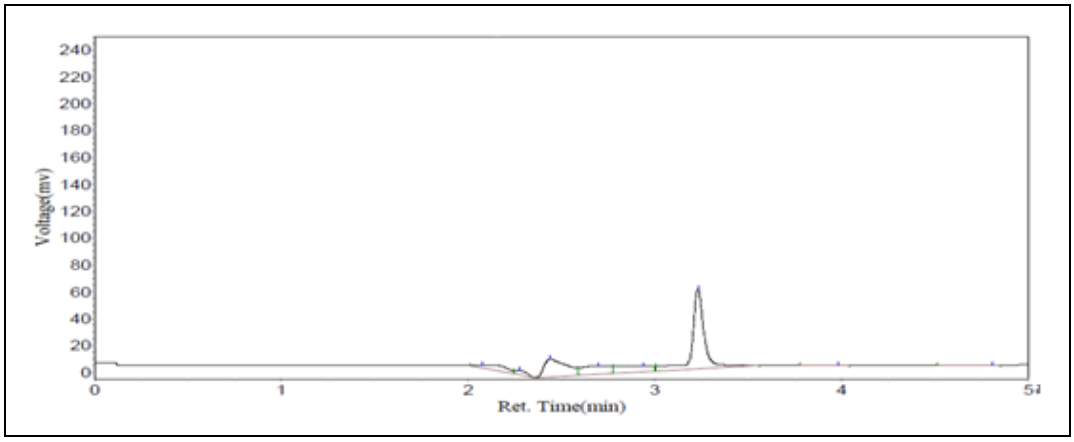

FIG. 11: CHROMATOGRAM 1 OF METHOD PRECISION OF LEVAMISOLE

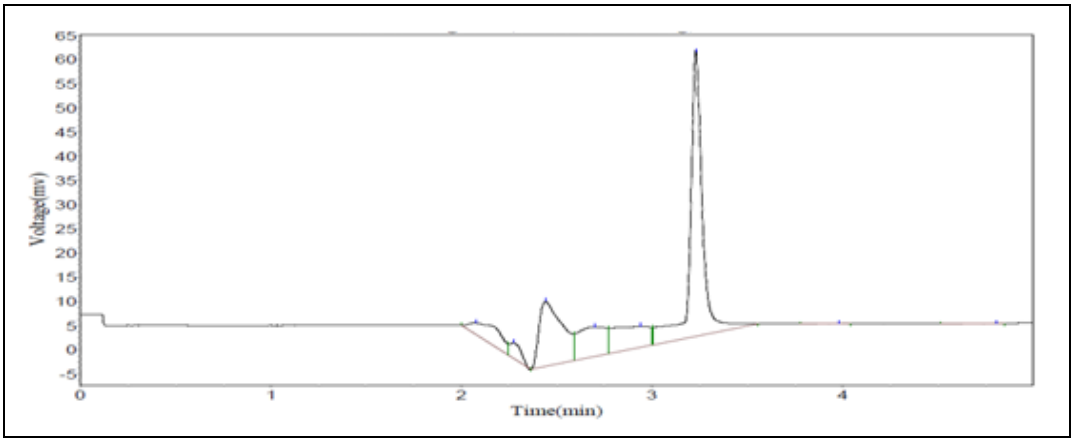

FIG. 12: CHROMATOGRAM 2 OF METHOD PRECISION OF LEVAMISOLE

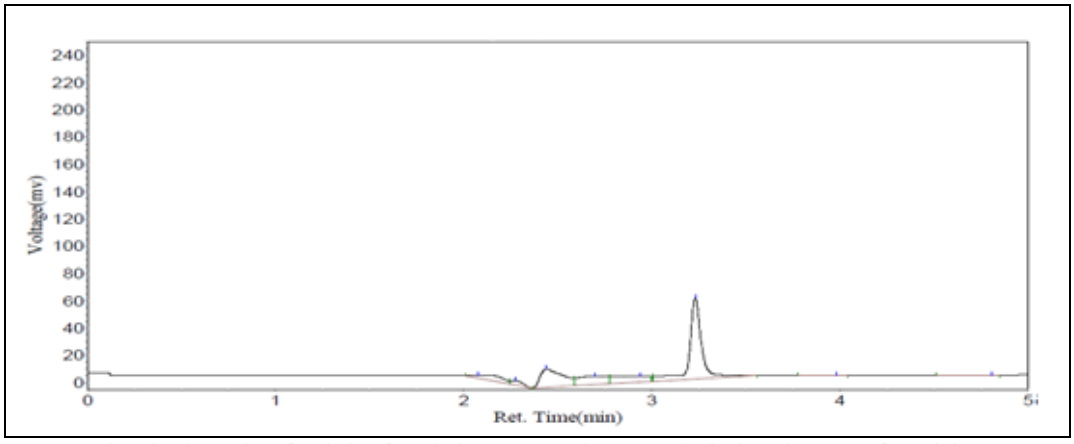

FIG. 13: CHROMATOGRAM 3 OF METHOD PRECISION OF LEVAMISOLE

Intermediate Precision:

TABLE 4: INTERMEDIATE PRECESSION

	S. no.	ANALYST – 1		ANALYST – 2	
		Area (Mv.sec)	Retention time	Area (Mv.sec)	Retention time
Levamisole	1	723061	3.309	723061	3.313
	2	721081	3.323	721081	3.323
	3	727240	3.317	727240	3.357
	4	723262	3.327	723262	3.357
	5	717262	3.338	717262	3.348
	MEAN	721581.2	3.3228	721581.2	3.3396
	S.D	5159.8498	0.010872	5159.8498	0.0203
	% RSD	0.71507543	0.327193	0.71507543	0.6098

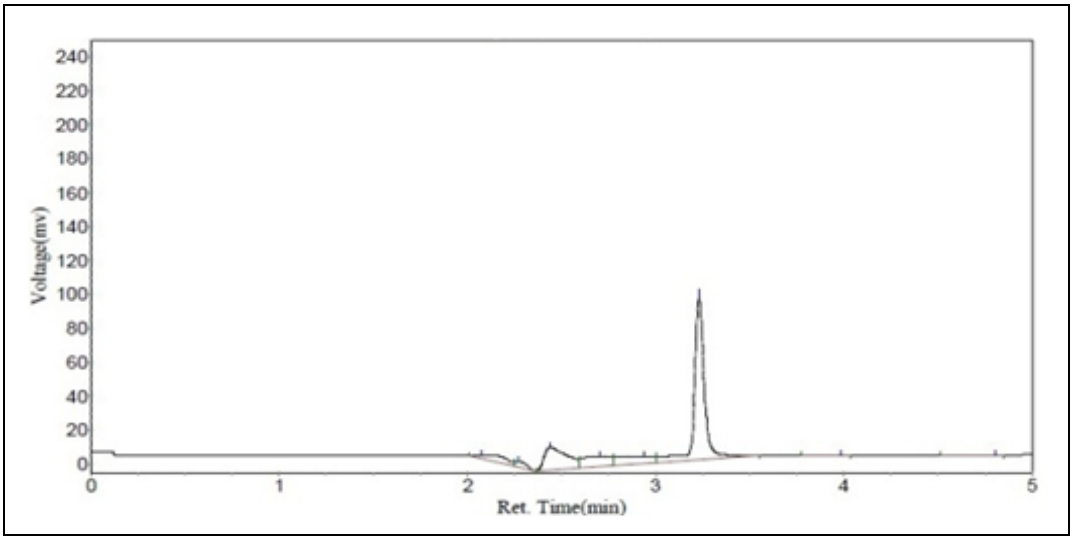

FIG. 14: CHROMATOGRAM 1 OF INTERMEDIATE PRECESSION OF LEVAMISOLE-ANALYST 1

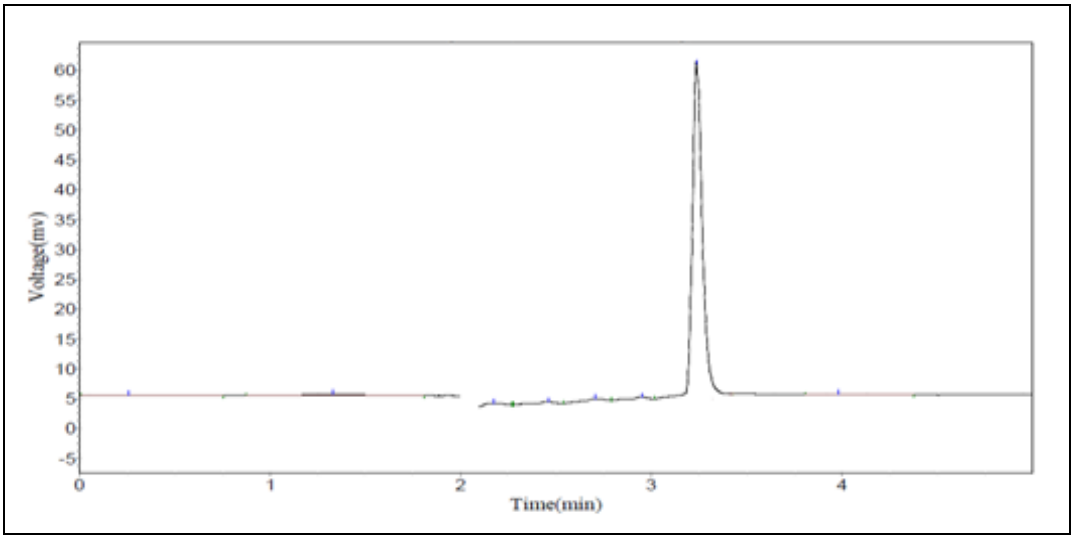

FIG. 15: CHROMATOGRAM 2 OF INTERMEDIATE PRECISION OF LEVAMISOLE-ANALYST 2

Accuracy:

Results for Recovery Studies:

TABLE 5: LEVEL 2(50%) OF ACCURACY OF LEVAMISOLE.

Standard	Level	Amt added (mg)	Total amt recovery	Recovered	% Recovered
4	2	2	6.02	2.02	101
4	2	2	5.95	1.95	97.5
4	2	2	5.92	1.92	96
4	2	2	5.9	1.9	95
4	2	2	6.01	2.01	100.5

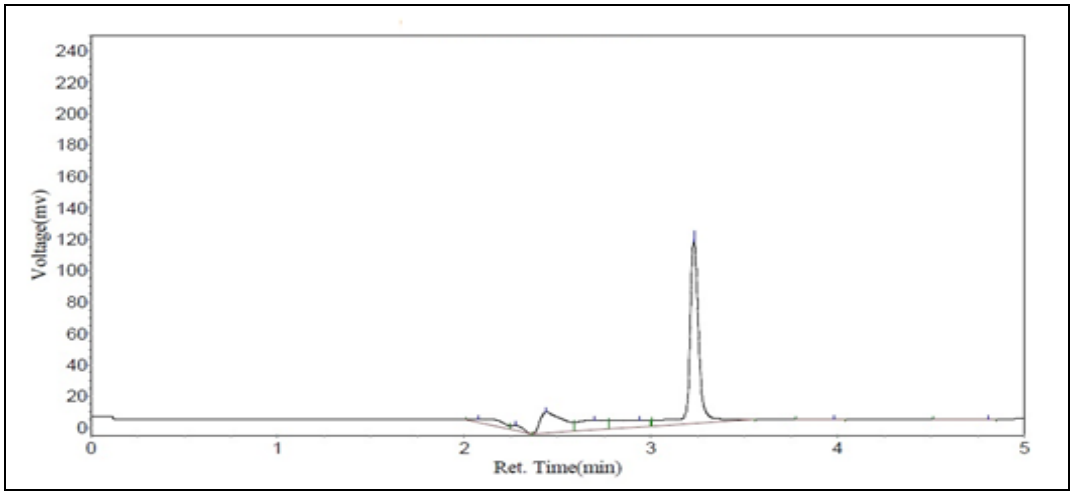

FIG. 16*:* CHROMATOGRAM OF ACCURACY OF LEVAMISOLE STANDARD (4µg/ml) + LEVAMISOLETEST (2µg/ml)

TABLE 6: LEVEL 4(100%) OF ACCURACY OF LEVAMISOLE

Standard	Level	Amt added (mg)	Total amt recovery (mg)	Recovered (mg)	% Recovered
4	4	4	8.05	4.02	100.5
4	4	4	7.92	3.92	98
4	4	4	8.19	4.19	104.75
4	4	4	7.85	3.85	96.25
4	4	4	7.96	3.96	99

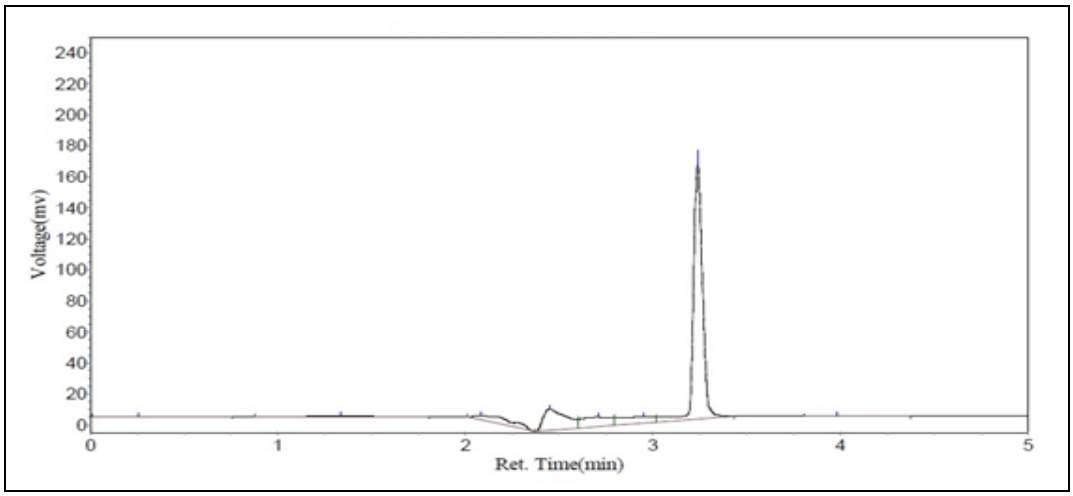

FIG. 17: CHROMATOGRAM OF ACCURACY OF LEVAMISOLESTANDARD (4µg/ml) + LEVAMISOLE TEST (4µg/ml)

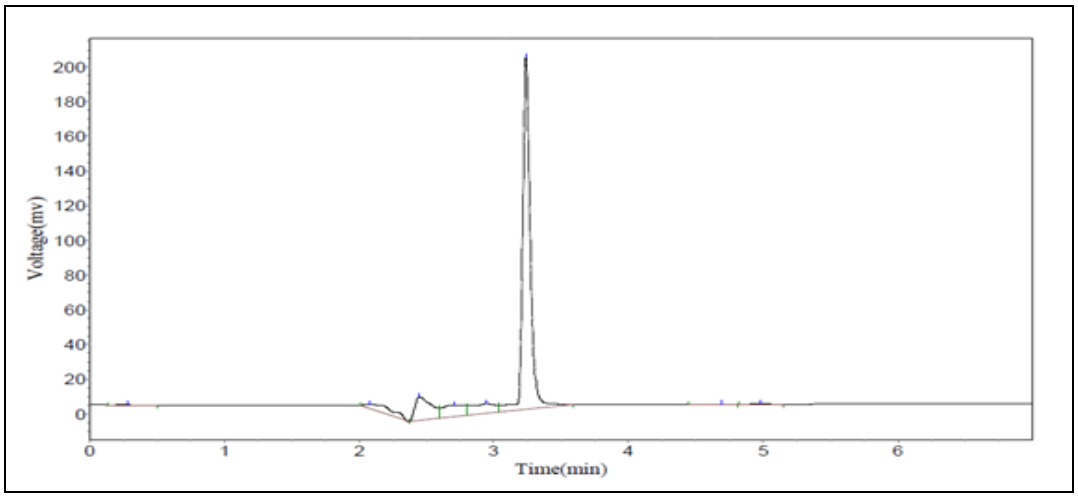

FIG. 18: CHROMATOGRAM OF ACCURACY OF LEVAMISOLESTANDARD (4MG/ML) +LEVAMISOLE TEST (6µg/ml)

TABLE 7: LEVEL 6(150%) OF ACCURACY OF LEVAMISOLE

Standard	Level	Amt added (mg)	Total amt recovery (mg)	Recovered (mg)	% Recovered
4	6	6	10.09	6.02	100.5
4	6	6	9.92	5.92	98.66
4	6	6	9.89	5.89	98.16
4	6	6	10.12	6.12	102
4	6	6	9.95	5.95	99.16

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 within limits. The results were very close to the labeled value of commercial tablets.

TABLE 8: DATA OF ANALYSIS

Parameters		Levamisole
Linearity range		2-10 (µg\ml)
Regression equation		y = 364654x + 2497
slope		364654
Intercept		2497
Correlation coefficient		0.999
System precision (% RSD)		0.609
Method precision (% RSD)		0.715
Intermediate precision	Analyst-I	0.327
	Analyst-II	0.609
% Recovery	Leve-2(50%)	95-101
	Level-4(100%)	96.25-104.75
	Level-6(150%)	98.16-102
LOD		0.0209 µg/ml
LOQ		0.069 µg/ml
% Assay		98.9%-100.8%.

RESULTS AND DISCUSSION: The present study was aimed at developing a precise, sensitive, rapid, and accurate HPLC method for the analysis of LMS in pure drug and pharmaceutical dosage forms. To achieve phenomenal retention time and peak asymmetry, a C18 stationary phase column (250mm × 4.6mm, 5 µm particle size) and mobile phase composed of acetonitrile and methanol in a ratio of 50:50 v/v, at a flow rate of 1.3mL/min, was selected. The retention time for LMS was found to be 6.5 min. UV spectra of LMS showed that the drug absorbed maximum at 236 nm, so this wavelength was selected as the detection wavelength. The correlation coefficient (0.9999) of regression was found almost equal to1 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 has 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. The accuracy of the method was found to be good with the overall % RSD for recovery at 50%, 100% and 150% levels were all within 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 0.0209 µg/mL and LOQ of 0.069 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 98.9%-100.8%.

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. These attribute the high quality of the method. The proposed method can be used for the routine analysis of LMS in pure preparations of the drug and in pharmaceutical dosage forms for routine application in quality control laboratories without the interference of excipients.

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CONFLICT OF INTEREST: Nil

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