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RESEARCH ARTICLE

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Development and Validation of New Analytical Methods for the Simultaneous Estimation of Levamisole and Albendazole in Bulk and Tablet Dosage Form by RP-HPLC

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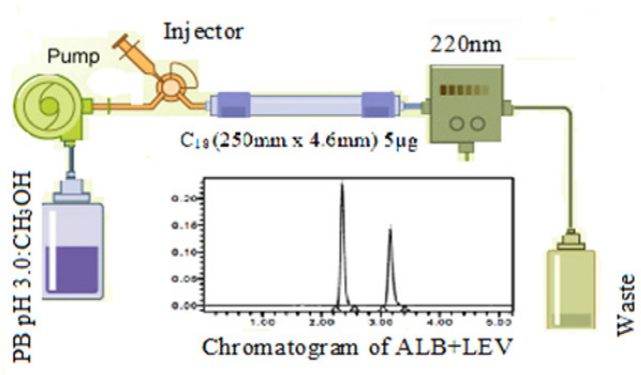
Abstract

A reverse-phase high-performance liquid chromatographic (RP-HPLC) method was developed to analyze levamisole and albendazole in tablet dosage forms. This method was simple, robust, and rapid reverse-phase high-performance liquid chromatography for the separation and quantitative determination of the Levamisole & Albendazole in combination forms. The victorious separation was achieved on Inertsil C18 (4.6 × 250mm, 5µm) Column, using Isocratic elution. The mobile phase contains 0.02M phosphate buffer (pH 3.0) and methanol in a ratio of (30:70v/v). The flow rate was monitored at 1.0 mL min^{-1} throughout the analysis and detection and quantitation of the main active pharmaceutical ingredients were achieved using a PDA detector at 220nm. The proposed method was validated according to the current ICH guidelines for specificity, linearity, accuracy, precision, ruggedness, and robustness. The proposed method was showed a linear response (R^2>0.999), accurate (recoveries 97-103%), precise (RS ≤ 1.0%), sensitive and specific. The anticipated reverse phase-liquid chromatography method was successfully helpful for the routine analysis of drugs in pharmaceutical dosage forms.

Keywords: HPLC; Levamisole; Albendazole; Method development; Validation

Abbreviations: HPLC: High-performance liquid chromatography; ICH: International conference on harmonization; ALB: Albendazole; LEV: Levamisole; LOD: Limit of detection; LOQ: Limit of quantification; Rt: Retention time; RSD: Relative Standard Deviation

Graphical Abstract



Introduction

Levamisole is an anti-helminthic medication [1] widely used in the treatment of infections caused by viral [2], bacterial [3], and also recently reported for covid-19 treatment [4]. It is chemically known as (6S)-6-phenyl-2H,3H,5H,6H-imidazo [2,1-b] [1,3] thiazole. It was manufactured by Janssen and used as an agent for treating worm infestations in 1969; Levamisole was approved as an adjuvant treatment for colon cancer by the FDA in 1990. Before this, levamisole was used as an antirheumatic therapy [5] for patients with rheumatoid arthritis. It shows immunomodulatory effects [6-9]; this drug was studied to treat various immune-mediated diseases; this drug was also used in combination with other drugs to treat various cancers. Levamisole was used in both humans and animals as an anthelmintic to treat worm infections. Levamisole acts as a nicotinic receptor agonist, which leads to paralysis of parasitic worms through continuous stimulation of their muscles. Levamisole has been used to treat many dermatological conditions, including skin infections, leprosy, warts, lichen planus, and aphthous ulcers. Levamisole drug is official in Indian Pharmacopoeia [10], European Pharmacopoeia [11], British Pharmacopoeia [12], and United States Pharmacopoeia [13].

Albendazole, also known as albendazolam, is a medication used to treat various parasitic worm infestations. It is used to treat giardiasis, trichuriasis, fil, neurocysticercosis, hydatid, pinworm and ascariasis.

It is chemically known asmethyl N-[6-(propylsulfanyl)-1H-1,3-benzodiazol-2-yl] carbamate.

Albendazole drug is official in British Pharmacopoeia [14], European Pharmacopoeia [15], and United States Pharmacopoeia [16].

Albendazole and Levamisole combination was used to treat worm infections in humans. Albendazole works by preventing sugar (glucose) in consumed worms, so they lose their energy and die. Levamisole treats worm infections by decreasing the function of the muscles and causing the worms to become paralyzed. The chemical structures of Levamisole and Albendazole were shown below in (Figure 1).

Several analytical methods were reported to determine the ALB or LEV either individually or in other pharmaceuticals. Previous reported methods were described the use of GC-MS [17,18], LC-MS [19-24], UPLC [25,26], and HPLC [27-35].

Materials and Methods

Chemicals and reagents

All reagents used were analytical grade, and solvents used were HPLC grade; Methanol (JT beaker) with a certified purity of 99.9% was purchased from Avantor performance materials, LLC, Radnor, PA, USA. The orthophosphoric acid was procured from Macron chemicals, USA. High-quality In-House purity water was used for the experiments (TOC<500ppb, pH about 7.0, Conductivity <1.0µs/cm, finally exposed to UV radiation and followed filtered through 0.2µm filter). Albendazole was (100% pure) procured from MSN Laboratories Pvt Ltd. Hyderabad, India. Levamisole (100% pure) was procured from Shaanxi Hanjiang Pharmaceutical Group Co. China.

Instrumentation and software

Waters HPLC system Alliance e2695 separation module with an auto-injector, temperature controller for sample storage, and Empower 3 Software Build 3471 SPs were used to monitor the signal output. Feature Release 3 DB ID: 2639633283 has been installed. The LC column is made of Inertsil C18 (250mm × 4.6 mm) 5µm. It is manufactured by GL Sciences Inc. Analytical balance model CP225D (make: Sartorius), Top load balance model GP5202 (make: Sartorius) sonicator (make: LIFECARE), pH Meter (make: ORION 3 STAR), Thermal oven (make: NEWTRONIC) were employed in this work.

Chromatographic conditions

The chromatographic separation was achieved by using the LC Inertsil C18 column (250mm × 4.6mm 5µm) with 0.02 M phosphate buffer (pH 3.0) and methanol in the ratio of 30:70 (v/v) was used as mobile phase. The mobile phase was filtered through a 0.22µm filter, and the flow rate was 1.0mL.min^{-1} with a isocratic elution method. Detection and quantitation of the main active pharmaceutical ingredients were achieved using a PDA detector at 220 nm with an injection volume of 10µL.

Standard preparation

A standard stock solution, Levamisole & Albendazole (1000µg.mL^{-1}), was prepared by dissolving LEV, ALB (100mg), to make 100mL of solution in the mobile phase. Pipette out 10.0 mL of standard stock supernatant solution into 100mL volumetric flask and dilute to volume with diluent and mix well. The system suitability, results and evaluation were shown in table 1, and the standard chromatogram was illustrated in (Figure 2).

Sample preparation

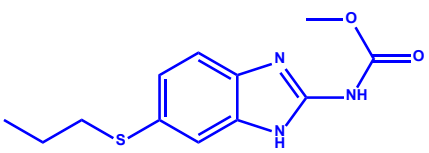
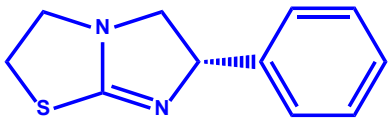
Accurately weighed ten tablets, crushed in a mortar and pestle, then equivalent to 100mg of Albendazole and Levamisole (marketed formulation) samples were transferred into a 100mL clean, dry volumetric flask, then 70mL of mobile phase was added and the mixture was sonicated for 20 min with occasional shaking. The mixture was then allowed to cool down to room temperature, diluted to volume with a mobile phase to get the concentration of 1000µg/mL and it was used for further analysis. 10mL of supernatant was withdrawn from the stock solution, transferred in 100mL volumetric flask, diluted to volume with a mobile phase mixed well.

Results and Discussion

Method development strategy and optimization

The previous literature was assessed, concludes the report, and discussed in table 2. To provide a suitable procedure for the routine quality control analysis of this multi-component drug mixture. The liquid chromatographic method coupled with diode array detection. The developed process was carefully designed and optimized to separate the cited compounds. The most critical aspect in LC method development is the achievement of sufficient resolution of the analytes with good peak symmetry in a reasonable analysis time. Many experiments were carried out to optimize both the stationary and mobile phases for better results. In these trials, evaluation was based on efficient resolution between the two analytes peaks. For optimization of the stationary phase, different types of columns were tested entirely, such as Thermo Hypersil BDS C8 (4.6 × 150mm), Durashell-C18 (4.6 × 250mm), Venusil XBP CN (4.6 × 250mm), and Inertsil C18 (250mm × 4.6mm) 5µm. The most desirable clear separation between the two primary compounds within a relatively short run time was obtained

Figure 1: Chemical Structures of Levamisole and Albendazole.

S.No	Name	Chemical Formula	IUPAC Name	M.Wt	Structure
1	Albendazole	$C_{12}H_{15}N_3O_2S$	methyl N-[6-(propylsulfanyl)-1H-1,3-benzodiazol-2-yl] carbamate	265.331	
2	Levamisole	$C_{11}H_{12}N_2S$	(6S)-6-phenyl-2H,3H,5H,6H-imidazo[2,1-b][1,3] thiazole	204.291	

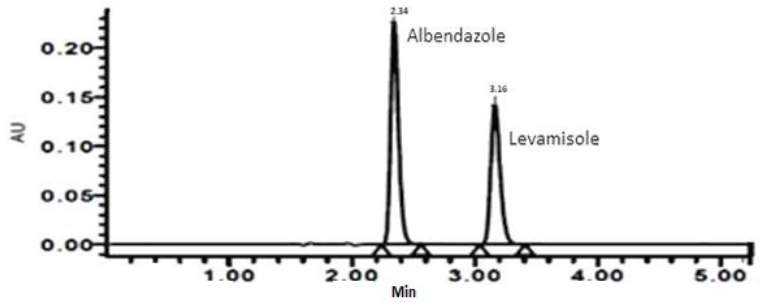


Figure 2: Dilute Standard of Albendazole and Levamisole.

Table 1: Comparison of published LC method conditions.

Publication Title & Reference	Chromatographic Conditions	Inference
Development of RP-HPLC Method for the Simultaneous Quantitation of Levamisole and Albendazole: Application to Assay Validation [34]	Mobile phase: Buffer of pH 3.5 and Acetonitrile (70:30 v/v) Column: Inertsil ODS C18 (4.6 × 150 mm, 5µm) Flow rate: 1.0 mL/min Detector: 224nm Injection volume: 20µL Column temperature: 25°C	1. The concentration of the sample is deficient and challenging to extract the subject sample at 30µg mL and 80µg mL of LEV+ALB 2. Responses of Levamisole and albendazole is too low 3. Broad peak shape of active compounds
Stability indicating method development and validation for simultaneous estimation of Levamisole HCl and albendazole in tablet dosage form [37]	Mobile phase: Phosphate Buffer pH 5.0: Acetonitrile (30:70 % v/v) Column: Shiseido C18 (250 mm × 4.6 mm, 5µm) Flow rate: 1.0mL/min Detector: 217nm Injection volume: 20µL Column temperature: 25°C	1. The concentration of the sample is deficient, and difficult to extract the subject sample at 7.5µg mL and 20µg mL of LEV+ALB 2. Responses of Levamisole and albendazole is too low
Current Proposed Method A rapid, simple, specific, reproducible, and robust reversed-phase HPLC method was developed and validated per ICH guidelines for the determination of Albendazole and Levamisole in bulk and tablet dosage form. Both albendazole and levamisole were detected within 5 minutes of run time using the Inertsil C_{18} (4.6 × 250mm, 5µm) column. With simple isocratic mobile phase contains 0.02M phosphate buffer (pH 3.0) and methanol in a ratio of 30:70(v/v). The flow rate is 1.0 mL min^{-1}, and the injection volume is 10µl. Detection was at 220nm.		

Table 2: System suitability evaluation.

Compound	USP Tailing	RT	USP Plate Count	Resolution	% RSD
Albendazole	1.13	2.34	5643	NA	0.8
Levamisole	1.22	3.16	7564	3.5	0.5

RT-Retention time; RSD-Relative standard deviation

using the Inertsil C18 (250mm × 4.6mm) 5µm column; consequently, it became the column of choice for this mixture. Other columns exhibited poor separation between the peaks of the target compounds. Some other trials revealed insufficient resolution between ALB and LEV. The excessive tailing for the peaks was another disadvantage of using the Thermo Hypersil BDS, Durashell-C18, and Venusil XBP CN columns.

The multi-wavelength ranges were evaluated to measure each analyte at its maximum wavelength to verify the sensitivity. ALB and LEV show stronger UV absorption with prominent peaks at 220nm.

Further optimization was carried eluting peaks with optimal separation by varying the flow rates (0.5 mL/min to 1.0 mL/min) and column temperature (range from 25°C to 40°C).

Estimation of Levamisole & Albendazole in different mobile phases, solvent-buffer ratios were tried to proposed final chromatographic conditions. The shape of the peaks, the symmetry, and resolution of Levamisole & Albendazole were good with mobile phase contains 0.02M phosphate buffer (pH 3.0) and methanol in a ratio of (30:70v/v). Isocratic elution at a 1.0mL/min flow rate, sample, and column temperature was maintained at 25°C. The developed method was successfully helpful to estimate the amount of Levamisole & Albendazole in bulk and tablet dosage form.

Method validation

Analytical method validation is essential to ensure that the analytical procedure employed for a specific test is appropriate for its intended. After method development, analytical techniques were validated

before the duration of routine use. The parameters evaluated during contemporary method development include specificity, linearity, range, accuracy, robustness, & precision.

The proposed method was validated based on the International Conference on Harmonization (ICH) Q2 (R1) guidelines [36,37].

Specificity

An essential obligatory ICH guideline for method validation is specificity or selectivity. In other words, specificity is the capability to evaluate the purity of the analyte in the being there of the co-eluting or co-migrating impurity. The method specificity was illustrated by demonstrating that no excipients interfere with the retention time of both drugs in the assay sample chromatogram.

Method precision

In method precision, a homogenous test of a single batch was analyzed six times. The results determine whether a method produces consistent results for a single batch. Calculate the average results (X), standard deviation (SD), and the percent relative standard deviation (%RSD). The proposed method was found to be precise since the RSD values method precision was below 1.0. The summary results were shown in table 3.

LOD (Limits of Detection) and LOQ (Limits of Quantification)

The LOQ and LOD are calculated using signal-to-noise ratios at analytical responses of 3×10 times the background noise. The method validation results were shown in table 3.

$$\text{LOD (mg/L)} = 3 \times \frac{\text{Noise}}{\text{signal}} \times \text{Lowest concentration of the linearity samples}$$

$$\text{LOD (mg/L)} = 10 \times \frac{\text{Noise}}{\text{signal}} \times \text{Lowest concentration of the linearity samples}$$

Linearity

An assay can obtain test results directly proportional to the concentration of an analyte in the sample. The determination of this parameter will define the range of the analytical assay. The linearity of the method was determined by drawing the calibration curves. Standard solutions of Levamisole & Albendazole of different concentrations levels (10%-150%) prepared by serial dilution of standard stock solution) were used for this purpose. The summary results were presented in table 3, and linearity curve was shown in (Figure 3).

Accuracy

The accuracy of an analytical method is the closeness of the test results obtained by the process to the actual value. Accuracy may often be expressed as a percent of recovery by testing known added amounts of analyte. Accuracy was the measurement of the exactness of the analytical method.

In this HPLC method, the recovery of the samples was verified with three concentration levels (50%, 100% & 150%). The recovery was performed by API + placebo and injected into the HPLC (triplicate). The summary results were presented in table 3.

Robustness

To demonstrate the robustness of the method, changes were made to the chromatographic conditions and system suitability parameters, such as tailing factor (<2.0), theoretical plate counts (>3000), and resolutions were between the nearest peaks (>2.0). Based on the results, the optimized method was proved robust, even under changed conditions. The summary results were presented in table 4.

Filter validation and solution stability

Two different types of 0.45µm filters (Nylon and PVDF) were used to determine the filter's effect on the sample. Concentrations of both

Table 3: Method validation results.

Parameters	Albendazole	Levamisole
Linearity		
Range (µg mL^{-1})	10-150	10-150
Slope	10956.22	9380.96
Intercept	6173.49	4452.59
Correlation Coefficient	0.999	0.999
STYX SD	9679.18	6666.72
LOD (µg/mL^{-1})	4.2	3.8
LOQ (µg/mL^{-1})	14.3	16.5
Accuracy[a](% of Recovery)		
50% Mean ± SD	100.3 ± 0.38	99.5 ± 1.15
100% Mean ± SD	100.4 ± 0.15	100.1 ± 0.21
150% Mean ± SD	101.0 ± 0.61	99.8 ± 0.67
Precision[b](%RSD)		
Repeatability	0.9	0.5
Intermediate precision	1.1	1
95% Confidence interval	99.825, 100.775	100.540, 101.460

[a]Average of three determinations of each concentration level.
[b]% RSD of six determinations of each component.

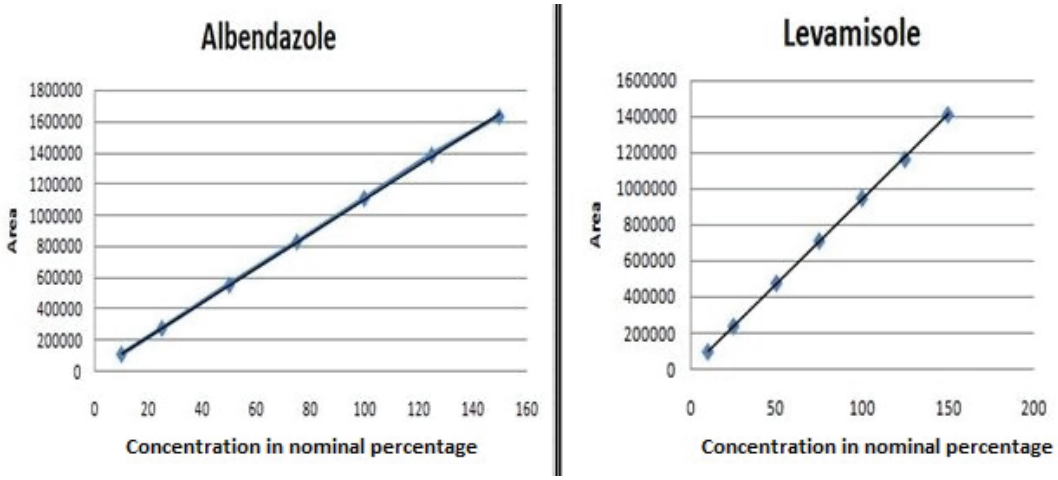


Figure 3: Dilute Standard of Albendazole and Levamisole.

Table 4: Robustness evaluation.

Parameter	Albendazole			Levamesole			Resolution b/w ALB & LEV
	Retention Time	USP Tailing	USP Plate Count	Retention Time	USP Tailing	USP Plate Count	
Normal	2.3	1.2	5512	3.2	1.1	7423	3.4
Low Flow (0.8mL/min)	2.6	1.1	5342	3.5	1.2	7564	3.6
High Flow (1.2 mL/min)	2.05	1	5453	2.95	1.3	7012	2.9
High Temperature (30ºC)	2.2	1.2	5544	3.1	1.2	7715	3.1
High Organic (105%) Methanol)	1.98	1.1	5098	2.89	1.1	7432	2.8
Low Organic (95% Methanol)	2.7	1	5231	3.7	1	7654	3.7

types of filtered samples were calculated and compared against the centrifuged sample and showed no difference in results. The sample solution was stable for up to 24h on the bench.

Conclusion

A rapid, simple, reproducible, and robust reversed-phase HPLC method was developed and validated as per ICH guidelines for the determination of Albendazole and Levamisole in bulk and tablet dosage form. The method provides selective quantification of ALB+LEV mixture without interference of the blank and placebo. The proposed method will be readily adapted to the routine quality control analysis.

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