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# ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF ALBENDAZOLE AND LEVAMISOLE IN ITS BULK AND PHARMACEUTICAL DOSAGE FORMS

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**ABSTRACT**

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Albendazole and Levamisole was done by RP-HPLC. The Phosphate buffer was $p^H$ 3.0 and the mobile phase was optimized with consists of Methanol: Phosphate buffer mixed in the ratio of 70:30 % v/ v. Inertsil $C_{18}$ column C18 (4.6 x 150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 260 nm. The solutions were chromatographed at a constant flow rate of 0.8 ml/min. the linearity range of Albendazole and Levamisole were found to be from 100-500 µg/ml of Albendazole and 1-5µg/ml of Levamisole. Linear regression coefficient was not more than 0.999. The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of Albendazole and levamisole. LOD and LOQ were found to be within limit.The results obtained on the validation parameters met ICH and USP requirements .it inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

**KEYWORDS:** Inertsil $C_{18,}$ Albendazole and Levamisole, RP-HPLC

**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.

## INTRODUCTION

### Albendazole

Albendazole is chemically known as Methyl N-[6-(propylsulfanyl)-1H-1,3-benzodiazol-2-yl] carbamate. It is used for the treatment of a variety of parasitic worm infection. Albendazole is an Antihelminthic medication with numerous indications such as cystic hydatid disease of the liver, lung, peritoneum resulting from the larval form of the dog tapeworm, Echinococcus granulosus.Albendazole causes degenerative alterations in the tegument and intestinal cells of the worm by binding to the colchicine-sensitive site of tubulin, thus inhibiting its polymerization or assembly into microtubules. The loss of the cytoplasmic microtubules leads to impaired uptake of glucose by the larval and adult stages of the susceptible parasites, and depletes their glycogen stores. The most common side-effects of Albendazole are biliary tract blockage, liver disease. Low blood counts like low white cell, platelet, or red cell counts. An unusual or allergic reaction to albendazole, other medicines, foods, dyes, or preservatives.

### Levamisole

Levamisole is chemically known as (6S)-6-phenyl- 2H, 3H, 5H, 6H-imidazo[2,1-b][1,3]thiazole**.** It is an antihelminthic drug that has been tried experimentally in rheumatic disorders where it apparently restores the immune response by increasing macrophage chemotaxis and T-lymphocyte function. Paradoxically, this immune enhancement appears to be beneficial in rheumatoid arthritis where dermatitis, leukopenia, and thrombocytopenia, and nausea and vomiting have been reported as side effects. Levamisole was withdrawn from the US and Canadian markets in 2000 and 2003, respectively, due to the risk of serious side effects and the availability of more effective replacement medications.

Levamisole is used as an antiparasitic agent that appears to be tied to its agnositic activity towards the L-subtype nicotinic acetylcholine receptors in nematode muscles.

This agonistic action reduces the capacity of the males to control their reproductive muscles and limits their ability to copulate.

And also levamisole is used as an anticancer drug in combination with fluorouracil is unknown. The effects of levamisole on the immune system are complex. The drug appears to restore depressed immune function rather than to stimulate response to above-normal levels. Levamisole can stimulate formation of antibodies to various antigens, enhance T-cell responses by stimulating T-cell activation and proliferation, potentiate monocyte and macrophage functions including phagocytosis and chemotaxis, and increase neutrophil mobility, adherence, and chemotaxis. The chemical structures of Levamisole and Albendazole were shown below in (Figure 1).

## MATERIALS AND METHODS

### Chemicals and reagents

All reagents used were analytical grade, and solvents used were HPLC grade; Lichrosolv Methanol and Water was purchased from MERCK Pharmaceuticals. $KH_2PO_4$ was procured from FINER chemical Ltd. Acetonitrile for HPLC was procured from MOLYCHEM. The orthophosphoric acid was procured from MERCK Pharmaceuticals. Albendazole was (100% pure) procured from MYLAN. Levamisole (100% pure) was procured from CIPLA.

### 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. UV/VIS spectrophotometer -Model LABINDIA UV $3000^{+.}$ The LC column is made of Inertsil C18 (4.6 x 150mm, 5mm). Weighing machine-Model Afcoset ER-200A, sonicator (make: LIFECARE), pH Meter (make: Adwa – AD 1020), Thermal oven (make: NEWTRONIC) were employed in this work.

### Chromatographic conditions

The chromatographic separation was achieved by using the LC Inertsil C18 column (4.6 x 150mm, 5mm) with 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 0.8 mL.min-1 with a isocratic elution method. Detection and quantitation of the main active pharmaceutical ingredients were achieved using a PDA detector at 254 nm.

### Standard preparation

Accurately weigh and transfer 10 mg of Albendazole and Levamisole 10mg of working standard into a 10mL& 100ml clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 3ml& 0.3ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.The system suitability, results and evaluation were shown in table 1, and the standard chromatogram was illustrated in (Figure 2)

### Sample preparation

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 10 mg of Albendazole and Levamisole (marketed formulation) sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 3 ml of Albendazole e and Levamisole of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

## RESULTS AND DISCUSSION

### Method development strategy and optimization

To provide a suitable procedure for the routine quality control analysis of this multi-component drug mixture. 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 mobile phase, different types of mobile phase were tested entirely, such as ACN: water (30:70 v/v),water: Methanol pH 2.5 (30:70 v/v),ACN: Water (80:20 v/v),Phosphate buffer pH 4.5: Methanol (35:65 v/v),Phosphate buffer: Methanol pH 4.5 (65:35 v/v),Phosphate buffer: Methanol pH 4.5(20:80 v/v).The most desirable clear separation between the two primary compounds within a relatively short run time was obtained. Method Development Trails are done using the Inertsil C18 (4.6 x 150mm, 5mm) column; consequently, it became the Mobile phase of choice for this mixture. Other Mobile phase exhibited poor separation between the peaks of the target compounds. The excessive tailing for the peaks was another disadvantage of using the ACN: water (30:70 v/v), water: Methanol pH 2.5 (30:70 v/v), ACN: Water (80:20 v/v),Phosphate buffer pH 4.5: Methanol (35:65 v/v),Phosphate buffer: Methanol pH 4.5 (65:35 v/v) Mobile phases. 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 254nm. Further optimization was carried eluting peaks with optimal separation by varying the flow rates (0.8 mL/min and 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 phosphate buffer (pH 3.0) and methanol in a ratio of (30:70v/v). Isocratic elution at a 0.8mL/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.The Optimised Chromatographic Conditions are shown in table 2, Chromatogram was illustrated in (Figure 3).

**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.

**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 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.

**Intermediate precision**

- To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions.The % RSD for the area of five standard injections results should not be more than 2%.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.

LOD (mg/L) =3 × Noise/signal × Lowest concentration of the linearity samples
LOD (mg/L) =10 × Noise/signal × 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 4,5).

**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**

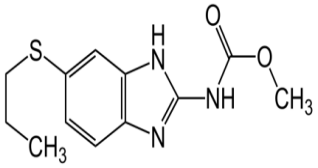
To demonstrate the robustness of the method, changes were made to the chromatographic conditions and system suitability parameters, such as tailing factor (<2.0), theoretical plate counts (>3000), and resolutions were between the nearest peaks (>2.0). Based on the results, the optimized method was proved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

**FIGURES**

**Albendazole**



**Levamisole**

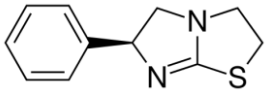



**Figure 1: Chemical Structures of Levamisole and Albendazole.**

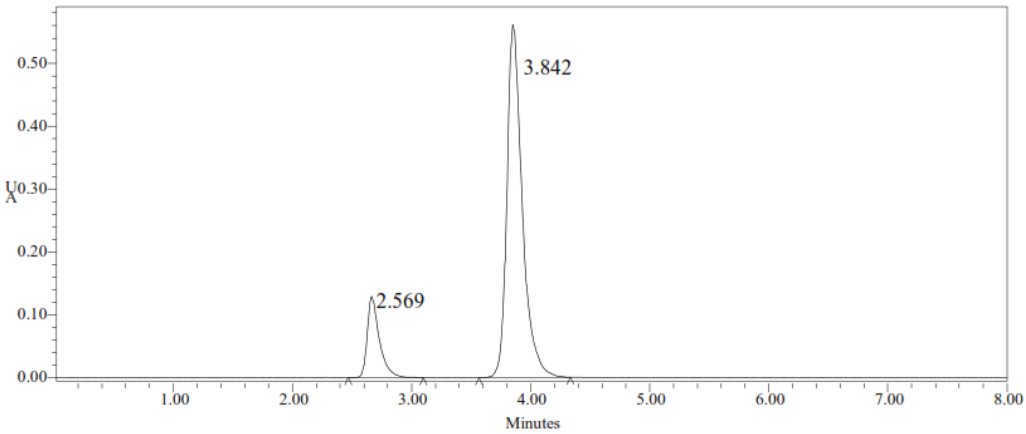



**Figure 2: Dilute Standard of Albendazole and Levamisole.**

**Chromatogram for Albendazole and Levamisole Standard Preparation**

Retention time of Albendazole – 2.569 min
Retention time of Levamisole - 3.842 min.

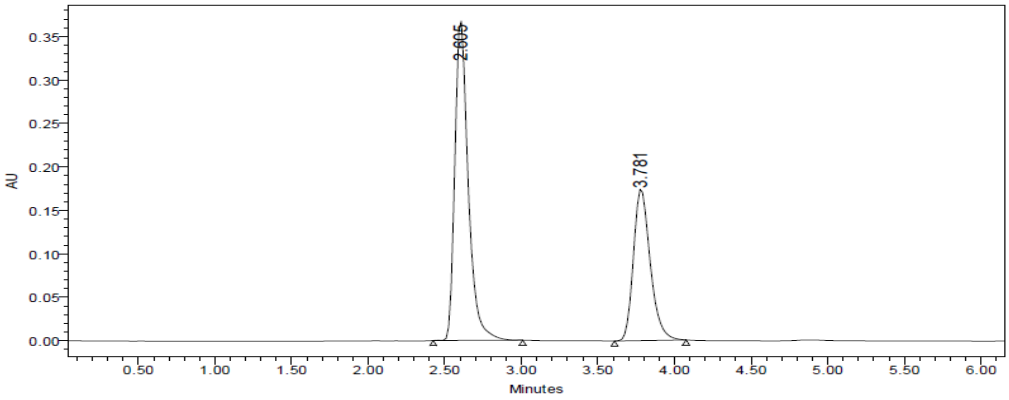



**Figure 3: Optimized Chromatogram.**

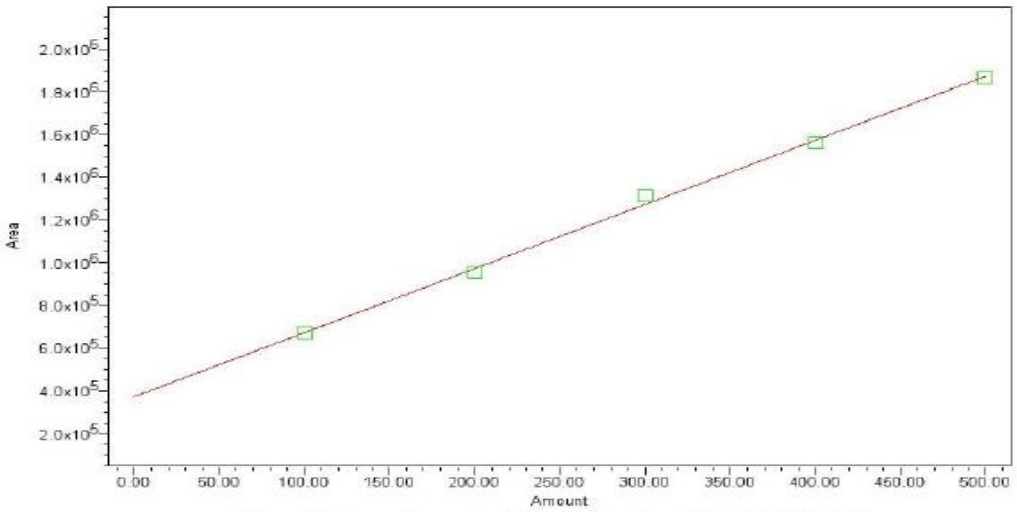



**Figure 4: Calibration graph for Albendazole at 254 nm.**

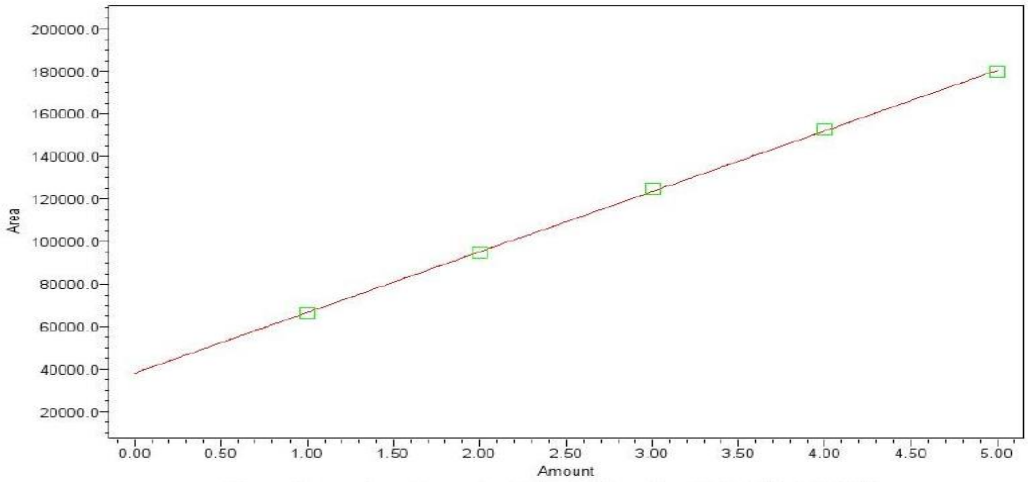



**Figure 5: Calibration graph for Levamisole at 254 nm.**

**TABLES**

**Table 1: System suitability evaluation.**

| S.No | Name | Retention time(min) | Area (µV sec) | Height (µV) | USP resolution | USP tailing | USP plate count |
|---|---|---|---|---|---|---|---|
| 1 | **Albendazole** | 2.5 | 124505 | 213642 | | 1.2 | 4673.4 |
| 2 | **Levamisole** | 3.9 | 1308495 | 154566 | 6 0 | 1.3 | 6090.3 |

**Table-2: Summary of Optimized Chromatographic Conditions.**

| Parameters | Description |
|---|---|
| Flow rate | 0.8ml $min^{-1}$ |
| Column | chromosil $C_{18}$ Column (250mm x 4.6mm)5µg. |
| Mobile Phase | Phosphate buffer:Methanol $P^H$ 4.5(20:80 v/v) |
| Buffer | Potassium dihydrogen orthophosphate PH 4.5 adjusted with Orthophosphoric acid |
| Detector | PDA |
| Column temperature | Ambient |
| Type of elution | Isocratic |
| Wavelength | 254 nm |
| Injection volume | 20µl |
| Run time | 10min |

**Table 3: Method validation results.**

| Parameters | Albendazole | Levamisole |
|---|---|---|
| **Linearity** | | |
| Range (µg $ml^{-1}$) | 100-500µg/ml | 5-25µg/ml |
| Slope | 66574 | 12529 |
| Intercept | 53592 | 50245 |
| Correlation Cofficient | 0.999 | 0.999 |
| **LOD (µg/mL-1)** S/N Ratio | 2.9 | 3 |
| **LOQ (µg/mL-1)** S/N Ratio | 10.03 | 10.1 |
| **Accuracy(a)(% of Recovery)** | | |
| 50% | 100.7 | 100.8 |
| 100% | 100.0 | 100.01 |
| 150% | 98.78 | 99.68 |
| **Precision(b)(%RSD)** | 0.2 | 0.6 |
| **Intermediate precision** | 0.2 | 0.1 |

*All the parameters $R^2$, S/N Ratio, %RSD should be within limits.

**Table 4: Robustness evaluation.**

| Parameter | Albendazole | | Levamesole | |
|---|---|---|---|---|
| | USP Tailing | USP Plate Count | USP Tailing | USP Plate Count |
| Normal (0.8 ml/min) | 4673.4 | 1.3 | 6090.3 | 1.2 |
| Less Flow (0.6 ml/min) | 5339.9 | 1.4 | 7063.3 | 1.3 |
| More Flow (1.0 ml/min) | 5216.0 | 1.4 | 6998.0 | 1.3 |
| *Actual Organic Composition | 4673.4 | 1.4 | 6090.3 | 1.2 |
| High Organic (10%) Methanol) | 4318.1 | 1.3 | 6232.5 | 1.2 |
| Low Organic (10% Methanol) | 4508.4 | 1.3 | 6387.7 | 1.2 |

## CONCLUSION

A sensitive & selective stability indicting RP-HPLC method has been developed & validated for the analysis of Levamisole & Albendazole in bulk and pharmaceutical dosage form. Based on peak purity results, obtained from the analysis of samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Levamisole & Albendazole indicated that the developed method is specific for the simultaneous estimation of Levamisole & Albendazole in the bulk and pharmaceutical dosage forms. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

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