RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF LEVAMISOLE AND ALBENDAZOLE IN PURE AND TABLET DOSAGE FORM

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ABSTRACT: A new, simple, precise, accurate and reproducible RP-HPLC method for Simultaneous estimation of Levamisole and Albendazole in bulk and pharmaceutical formulations. Separation of Levamisole and Albendazole was successfully achieved on a Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d. or equivalent in an isocratic mode utilizing Phosphate Buffer (0.2 M, pH=2): Acetonitrile in the ratio of 64:36% v/v at a flow rate of 1.0mL/min and eluates was monitored at 265nm, with a retention time of 2.131 and 2.816 minutes for Levamisole and Albendazole respectively. The method was validated and the response was found to be linear in the drug concentration range of 6µg/mL to 14µg/mL for Levamisole and 18µg/mL to 42µg/mL for Albendazole. The LOD and LOQ for Levamisole were found to be 0.4µg/mL and 0.12µg/mL respectively. The LOD and LOQ for Albendazole were found to be 0.07µg/mL and 0.21µg/mL respectively. This method was found to be good %recovery for Levamisole and Albendazole were found to be 100.415 and 100.264 respectively indicates that the proposed method is highly accurate. The specificity of the method shows good correlation between retention times of standard with the sample so, the method specifically determines the analytes in the sample without interference from excipients of tablet dosage forms. The method was extensively validated according to ICH guidelines for Linearity, Range, Accuracy, Precision, Specificity and Robustness.

Keywords: Levamisole and Albendazole, HPLC, Method Development, Validation.

I. INTRODUCTION

Levamisole is an antihelminthic drug that was commonly used for the treatment of parasitic, viral, and bacterial infections. It was manufactured by Janssen and first used in 1969 as an agent to treat worm infestations Levamisole[1] was approved by the FDA in 1990 as an adjuvant treatment for colon cancer. Prior to this, levamisole was used as an antirheumatic therapy in the 1970s and 1980s for patients with rheumatoid arthritis. Because of its immunomodulatory effects, this drug has been studied in the treatment of various immune-mediated diseases, with some studies showing positive results. This drug has also been used in combination with other drugs for the treatment of various cancers. Levamisole[2] was withdrawn from the American market in 2000 due to its ability to cause serious adverse effects, including agranulocytosis. Interestingly, levamisole has been found as an adulterant in cocaine and can lead to a variety of adverse effects in individuals using this drug. Levamisole is a synthetic imidazothiazole derivative that has been widely used in treatment of worm infestations in both humans and animals. As an anthelmintic, it probably works by targeting the nematode nicotinergic acetylcholine receptor. As an immunomodulator, it appears that Levamisole[3] is an immunostimulant which has been shown to increase NK cells and activated T-cells in patients receiving this adjuvantly along with 5FU for Stage III colon cancer. The IUPAC Name of Levamisole is (6S)-6-phenyl-2, 3, 5, 6-tetrahydroimidazo [2, 1-b] [1, 3] thiazole. The Chemical Structure of Levamisole is following

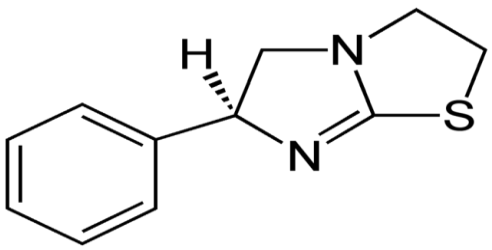

Fig-1: Chemical Structure of Levamisole

A benzimidazole broad-spectrum anthelmintic structurally related to mebendazole that is effective against many diseases. Albendazole[4] is a broad-spectrum, synthetic benzimidazole-derivative anthelmintic. Albendazole interferes with the reproduction and survival of helminths by inhibiting the formation of microtubules from tubulin. This leads to an impaired uptake of glucose, a depletion of glycogen stores, and results in the worm's death. Albendazole[5] is used in the treatment of dog and pork tapeworm-causing diseases, including hydatid disease and neurocysticercosis. Albendazole may also be used to treat a variety of other roundworm infections. Albendazole is a broad-spectrum anthelmintic. The principal mode of action for Albendazole is by its inhibitory effect on tubulin polymerization which results in the loss of cytoplasmic microtubules. Albendazole[6] causes degenerative alterations in the tegument and intestinal cells of the worm by diminishing its energy production, ultimately leading to immobilization and death of the parasite. It works by binding to the colchicine-sensitive site of tubulin, thus inhibiting its polymerization or assembly into microtubules. As cytoplasmic microtubules are critical in promoting glucose uptake in larval and adult stages of the susceptible parasites, the glycogen stores of the parasites are depleted. Degenerative changes in the endoplasmic reticulum, the mitochondria of the germinal layer, and the subsequent release of lysosomes result in decreased production of adenosine triphosphate (ATP), which is the energy required for the survival of the helminth. The IUPAC Name of Albendazole is methyl N-(6-propylsulfanyl-1H-benzimidazol-2-yl) carbamate. The Chemical Structure of Albendazole is as follows

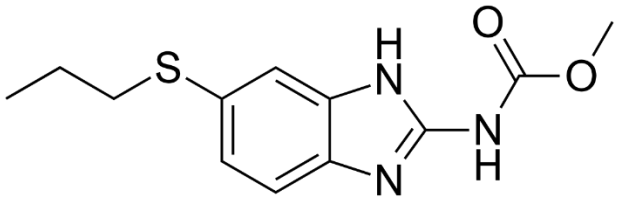

Fig-2: Chemical Structure of Albendazole

The literature revealed[32-35] that, no method was available for simultaneous determination of these two drugs in such bulk form and pharmaceutical preparations by HPLC. Therefore an HPLC method was developed for determination of Levamisole and Albendazole from bulk form and their combined dosage form. The method described is simple, fast, precise and accurate for simultaneous determination of Levamisole and Albendazole from bulk form and pharmaceutical preparations.

In the present research work, a reverse-phase HPLC method has been developed for simultaneous determination of Levamisole and Albendazole in bulk form and bulk form and pharmaceutical preparations.

II. MATERIALS AND METHODS

All the reagents used were of HPLC grade and analytical grade. Reference standard of Levamisole and Albendazole was supplied as gift sample from Endocard India Pvt Ltd. and Brutal Tablet (150mg/400mg) were procured from the local pharmacy in the market. A standard stock solution of Levamisole and Albendazole (1 mg/ml) was prepared by dissolving 10 mg of drug in 10 ml of mobile phase. Working standard solution (10µm/ml) was prepared from stock solution by proper dilution with mobile phase mixture. A HPLC with Empower2 Software with Isocratic with UV-Visible Detector (Waters). Develosil ODS HG-5 RP C18, 5µm, 15cmx4.6mm i.d. column and Empower2 Software were used. The mobile phase used was Phosphate Buffer (0.2 M, pH=2): Acetonitrile in the ratio of 64:36% v/v which was filtered through nylon 0.45 µm.

Preparation of Mobile Phase:
The mobile phase was prepared with the combination of Phosphate Buffer (0.2 M, pH=2) and Acetonitrile at the volume of 1000ml. 640ml of Phosphate Buffer and 360ml of Acetonitrile were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration.
Preparation of Standard Solutions:
10 mg of Levamisole & Albendazole was weighed accurately and transferred into 100 ml volumetric flask. About 10 ml mobile phase was added and sonicated to dissolve. The volume was made up to the mark with same solvent. The final solution contained about 10µg/ml and 10µg/ml of Levamisole & Albendazole respectively.

III. RESULTS AND DISCUSSION

Method Development

Selection of Wavelength:

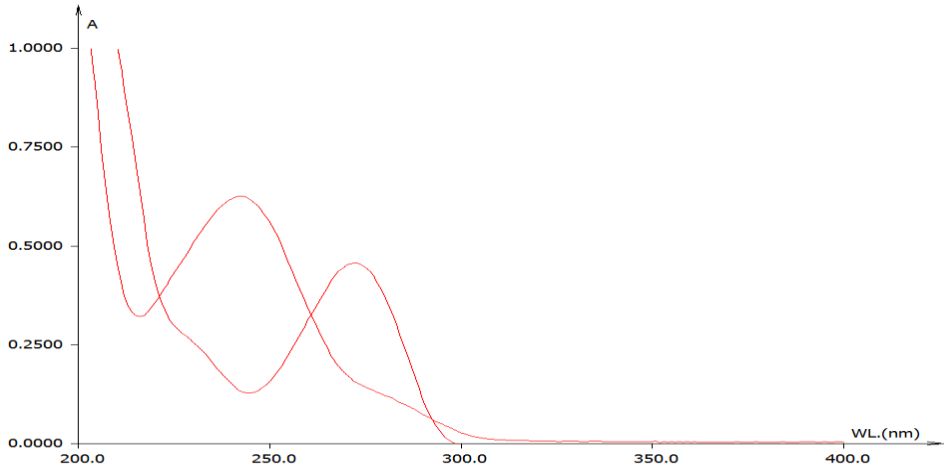

Fig-3: Isobestic point Levamisole & Albendazole

Observation: While scanning the Levamisole solution we observed the maxima at 275nm and for the Albendazole solution we observed the maxima at 248nm. The isobestic point for the drugs was found at 265nm. The UV spectrum[7] has been recorded on T60-LAB INDIA make UV – Vis spectrophotometer model UV-2450.

Trials for the Method Development

Table-1: Different Chromatographic used and their Optimizations

S.No.	Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
1	Symmetry C_{18}, 5µm, 25cmx4.6mm i.d.	ACN : Water = 70 : 30	0.8ml/min	265nm	Peaks did not separate	Method rejected
2	Waters C_{18}, 5µm, 25cmx4.6mm i.d.	Methanol: ACN = 40 :60	1.0 ml/min	265nm	Early elution of peak	Method rejected
3	Waters C_{18}, 5µm, 25cmx4.6mm i.d.	ACN: Phosphate buffer (0.02M) = 70:30	1.0 ml/min	265nm	Low resolution peak	Method rejected
4	Develosil ODS HG-5 RP C_{18}, 5µm,15cmx4.6mm i.d.	Phosphate buffer :Acetonitrile (0.01M) = 50:50	1.0 ml/ min	265nm	Resolution increases but Peak shapes not good	Method rejected
5	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.	Phosphate Buffer (0.2 M, pH=2) : Acetonitrile = 64:36	1.0 ml/min	265nm	Nice resolution & good peaks	Method Accepted

Summary of Optimized Chromatographic Conditions:

The Optimum conditions obtained from experiments can be summarized as below:

Table-2: Summary of Optimised Chromatographic Conditions

Mobile phase	Phosphate Buffer (0.2 M, pH=2): Acetonitrile = 64:36% v/v
Column	Develosil ODS HG-5 RP C_{18}, 5µm, 15cmx4.6mm i.d.
Column Temperature	Ambient
Detection Wavelength	265 nm
Flow rate	1.0 ml/ min.
Run time	10 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	10µl
Type of Elution	Isocratic

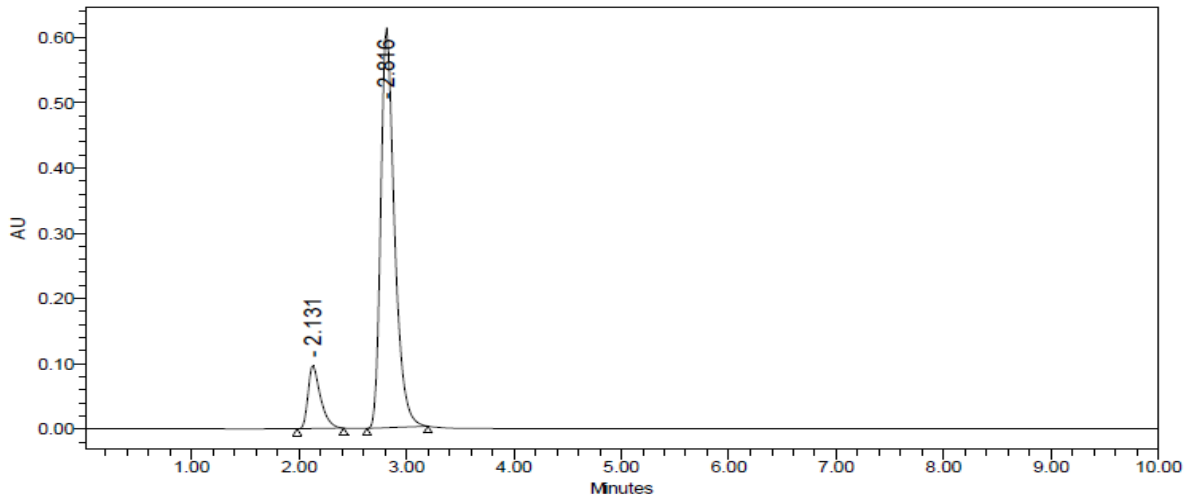

Fig-4: Chromatogram for Optimized Chromatographic Condition

Method Validation

1. Linearity and Range

Method: To evaluate the linearity, serial dilution[8] of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 6-14µg/ml for Levamisole and concentration ranging from 12-28µg/ml for Albendazole. The prepared solutions were filtered through Whatman filter paper (No.41). From these solutions, 10µl injections of each concentration were injected into the HPLC system and chromatographed under the optimized conditions. Calibration curve[9] was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

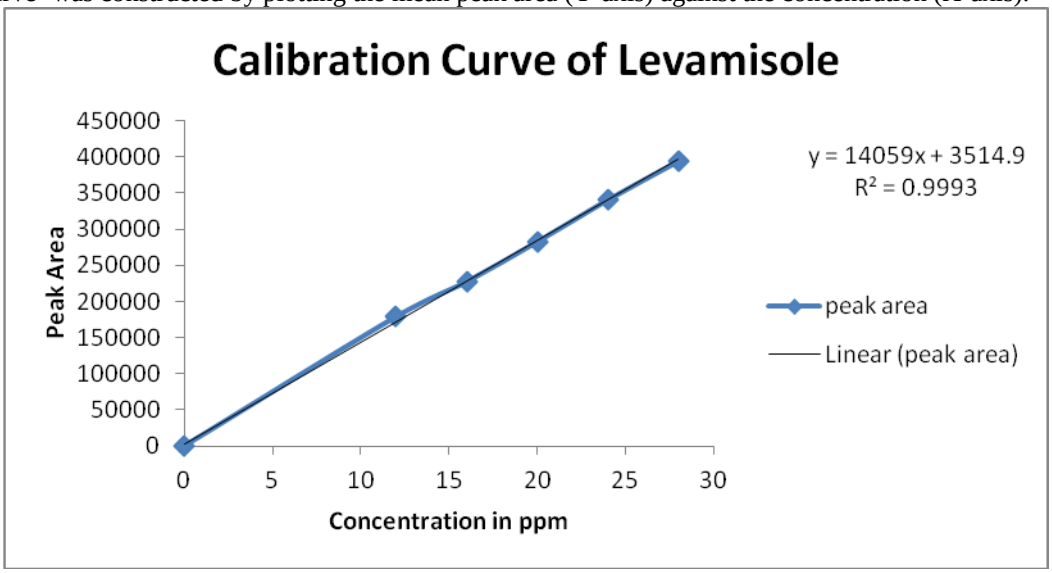

Fig-5: Standard curve for Levamisole

Table-3: Linearity Results for Levamisole

CONC. (µg/ml)	AUC (n=6)
0	0
6	119571
8	167873
10	211264

12	255428
14	299987

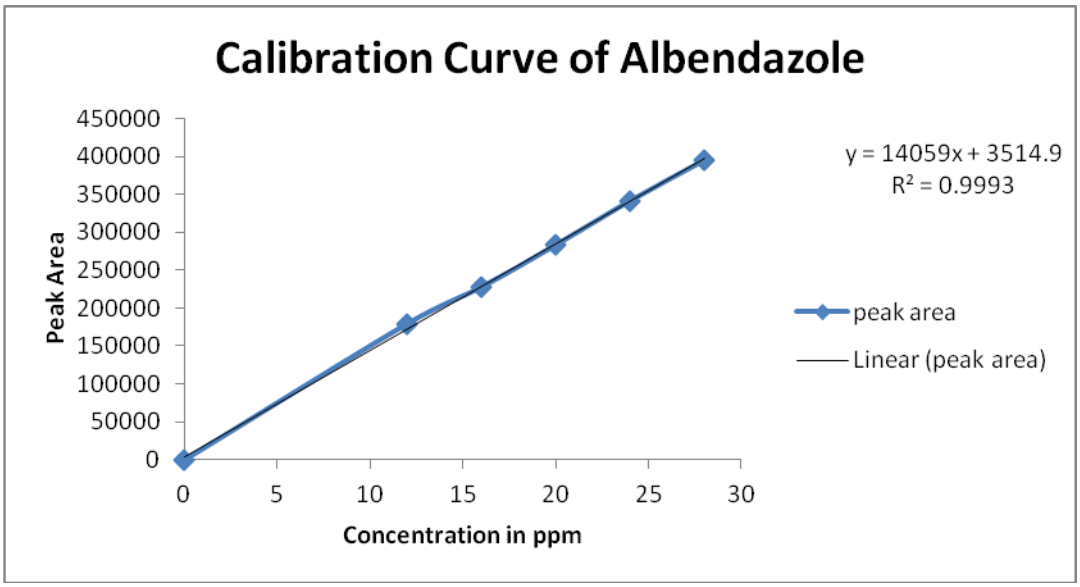

Fig-6: Standard curve for Albendazole

Table-4: Linearity Results for Albendazole

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
12	179371
16	227893
20	283264
24	341428
28	394987

Results & Discussion:

Linearity range was found to be 6-14 µg/ml for Levamisole. The correlation coefficient was found to be 0.999, the slope was found to be 14059 and intercept[10] was found to be 3514 for Levamisole.

Linearity range was found to be 12-28 µg/ml for Albendazole. The correlation coefficient was found to be 0.999, the slope was found to be 14059 and intercept was found to be 3514 for Albendazole.

2. Accuracy:

Recovery study: For Levamisole

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Levamisole were taken and added to the pre-analyzed formulation of concentration 10µg/ml. From that percentage recovery[11] values were calculated. The results were shown in table-5.

Table-5: Accuracy Readings for Levamisole

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	8	7.997368	115949	99.9671	Mean= 100.7003% S.D. = 0.6884036 % R.S.D.= 0.683616%
S_2 : 80 %	8	8.106622	117485	101.3328	
S_3 : 80 %	8	8.064087	116887	100.8011	
S_4 : 100 %	10	9.904901	142767	99.04901	Mean= 100.36157% S.D. = 1.346221 R.S.D.= 1.3413706%
S_5 : 100 %	10	10.02966	144521	100.2966	
S_6 : 100 %	10	10.17391	146549	101.7391	
S_7 : 120 %	12	12.01807	172476	100.1506	Mean= 100.183756% S.D. = 1.19411 % R.S.D. = 1.19191%
S_8 : 120 %	12	11.88079	170546	99.00657	
S_9 : 120 %	12	12.16729	174574	101.3941	

Observation : From the Accuracy Method, we observed that the mean %Recovery[12] of the drug are 100.7003%, 100.36157% and 100.183756% which is within the range of 98-102% and %RSD is within the range <2 i.e. 0.683616%, 1.3413706% and 1.19191% respectively.

Recovery study: Albendazole

To determine the accuracy[13] of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Albendazole were taken and added to the pre-analysed formulation of concentration 50µg/ml. From that percentage recovery values were calculated. The results were shown in table-6.

Table-6: Accuracy Results for Albendazole

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	16	16.08685	229679	100.5428	Mean= 100.54488% S.D. = 0.97847% R.S.D.= 0.9731%
S_2 : 80 %	16	15.93079	227485	99.56745	
S_3 : 80 %	16	16.2439	231887	101.5244	
S_4 : 100 %	20	20.07632	285767	100.3816	Mean= 99.97095% S.D. = 0.395406 % R.S.D.= 0.39552%
S_5 : 100 %	20	19.98769	284521	99.93847	
S_6 : 100 %	20	19.91856	283549	99.59279	
S_7 : 120 %	24	23.75432	337476	98.97634	Mean= 100.27718% S.D. = 1.21262 % R.S.D. = 1.20927%
S_8 : 120 %	24	24.11494	342546	100.4789	
S_9 : 120 %	24	24.33032	345574	101.3763	

Observation : From the Accuracy Method, we observed that the mean %Recovery of the drug are 100.54488%, 99.97095% and 100.27718% which is within the range of 98-102% and %RSD is within the range <2 i.e. 0.9731%, 0.39552% and 1.20927% respectively.

3. Precision:

Repeatability: The precision[14] of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug Levamisole & Albendazole (API). The percent relative standard deviation[15] was calculated for Levamisole & Albendazole are presented in the table-7.

Table-7: Data showing repeatability analysis for Levamisole & Albendazole

HPLC Injection Replicates	AUC for Levamisole	AUC for Albendazole
Replicate – 1	113568	241022
Replicate – 2	113241	240137
Replicate – 3	115408	242911
Replicate – 4	117412	245245
Replicate – 5	112541	241941
Replicate – 6	112546	240444
Average	**114119.3333**	**241356.6667**
Standard Deviation	**1925.83838**	**1416.95812**
% RSD	**1.68756**	**0.58708**

Result & Discussion: The repeatability study[16] which was conducted on the solution having the concentration of about 10µg/ml for Levamisole and 20µg/ml for Albendazole (n =6) showed a RSD of 1.68756% for Levamisole and 0.58708% for Albendazole. It was concluded that the analytical technique showed good repeatability.

Intermediate precision

The Intermediate Precision[17] consists of two methods:-

Intra Day: In Intra Day process, the 80%, 100% and 120% concentration are injected at different intervals of time in same day.

Inter Day: In Inter Day process, the 80%, 100% and 120% concentration are injected at same intervals of time in different days.

Table-8: Data for Levamisole analysis

Conc. of Levamisole (API) (µg/ml)	Observed Conc. of Levamisole (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
8	8.17	0.35	8.28	0.48
10	10.19	0.56	10.66	0.65
12	12.26	0.76	12.56	0.46

Table-9: Data for Albendazole analysis

Conc. of Albendazole (API) (µg/ml)	Observed Conc. of Albendazole (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
16	16.33	0.24	16.56	0.33
20	20.56	0.48	20.76	0.67
24	24.23	0.63	24.63	0.43

Observations: The intra & inter day variation[18] of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Levamisole and Albendazole revealed that the proposed method is precise.

4. Limit of detection and limit of quantification

The LOD was found to be 0.04µg/ml and LOQ[19] was found to be 0.12µg/ml for Levamisole respectively which represents that sensitivity of the method is high.

The LOD[20] was found to be 0.07µg/ml and LOQ was found to be 0.21µg/ml for Albendazole respectively which represents that sensitivity of the method is high.

5. Method Robustness:

Influence of small changes in chromatographic conditions[21] such as change in flow rate (± 0.1ml/min), Wavelength of detection (±2nm) & organic phase content in mobile phase (±2%) studied to determine the robustness[22] of the method are also in favour of (Table-10, % RSD < 2%) the developed RP-HPLC method for the analysis of Levamisole (API).

Table-10: Result of Method Robustness Test for Levamisole

Change in parameter	% RSD
Flow (0.8 ml/min)	0.23
Flow (1.2 ml/min)	0.39
More Organic	0.83
Less Organic	0.76
Wavelength of Detection (277 nm)	0.56
Wavelength of detection (273 nm)	0.43

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Wavelength of detection (±2nm) & organic phase content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-11, % RSD < 2%) the developed RP-HPLC method[23] for the analysis of Albendazole (API).

Table-11: Result of Method Robustness Test for Albendazole

Change in parameter	% RSD
Flow (0.8 ml/min)	0.37
Flow (1.2 ml/min)	0.57
More Organic	0.76
Less Organic	0.53
Wavelength of Detection (250 nm)	1.21
Wavelength of detection (246 nm)	0.39

6. System Suitability Parameter

System suitableness[24] testing is associate degree integral a part of several analytical procedures. The tests are supported the idea that the instrumentality, physics, associate degree analytical operations and samples to be analyzed represent an integral system which will be evaluated intrinsically. Following system suitableness take a look at parameters were established. The information is shown in Table-12.

Table-12: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs> 2	2.57
2	Asymmetry	T ≤ 2	Levamisole = 0.46 Albendazole = 0.77
3	Theoretical plate	N > 2000	Levamisole = 2946 Albendazole = 3076

7. Estimation of Levamisole and in Pharmaceutical Dosage Form

Twenty Tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder[25] equivalent to 25 mg of drugs were transferred to 25 ml volumetric flask, make and solution was sonicated for 15 minutes, there after volume was made up to 25 ml with same solvent. Then 10 ml of the above solution was diluted to 100 ml with mobile phase. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas[26]. The solution prepared was injected in five replicates into the HPLC system and the observations were recorded.

A duplicate injection of the standard solution[27] was also injected into the HPLC system and the peak areas were recorded. The data are shown in Table-13.

ASSAY:

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:

AT = Peak Area of drug obtained with test preparation
AS = Peak Area of drug obtained with standard preparation
WS = Weight of working standard taken in mg
WT = Weight of sample taken in mg
DS = Dilution of Standard solution
DT = Dilution of sample solution
P = Percentage purity of working standard

Table-13: Recovery Data for estimation Levamisole and Albendazole

Brand name of Levamisole and Albendazole	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Brutal	150/400	149.856 (±0.422) / 399.578 (± 0.372)	99.5 (±0.576) / 99.4 ± 0.822)

Result & Discussion: The assay of Brutal Tablets containing 150mg of Levamisole & 400mg of Albendazole was found to be 99.5% and 99.4% respectively.

Forced Degradation Studies

The results of the forced degradation studies[28-30] indicated the specificity of the developed method that has been developed. Levamisole and Albendazole were stable only in acidic, basic and thermal stress conditions and photolytic stress conditions[31]. The results of stability studies are given in the following Table-14.

Table-14: Results of Force Degradation Studies of Levamisole and Albendazole API.

Stress Condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	95.62	4.38	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	97.13	2.87	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	96.24	3.76	100.00
UV (254nm)	24Hrs.	95.43	4.57	100.00
3% Hydrogen peroxide	24Hrs.	96.16	3.84	100.00

IV. 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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