VALIDATED REVERSE PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE SIMULTANEOUS DETERMINATION OF ALBENDAZOLE AND LEVAMISOLE IN API SAMPLES AND IN THEIR FIXED DOSE COMBINED TABLET DOSAGE FORM

Challa Kusuma Kumari [1]*, Dr.V.Anitha Kumari[1] , Dr.N.B.V Siva Ram[1], Ramya Sri.S[2]

[1]Department of Pharmaceutical Analysis, Nova College of Pharmacy ,Vegavaram ,West Godavari, Andhra Pradesh , India.

[2]Department of Pharmaceutics, University College of Technology,Osmania University, Hyderabad, Telangana, India

Corresponding Author:

Challa Kusuma Kumari,
Department of Pharmaceutical Analysis,
Nova College of Pharmacy,
Vegavaram (V), West Godavari(Dist.),
Andhra Pradesh , India.

ABSTRACT

Analytical Method Development and Validation for Albendazole and Levamisole in bulk and Combined Dosage Form by RPHPLC, New method was established for simultaneous estimation of Albendazole and Levamisole by RPHPLC method. The chromatographic co nditions were successfully developed for the separation of Albendazole and Levamisole b y using Symmetry ODS C18 (4.6mm×250mm, 5µm) particle size, flow rate was 1.0 ml/ min, mobile phase ratio was (30:70 v/v) Methanol: TEA buffer pH 3.8 (pH was adjusted with orthophosphoric acid), detection wavelength was 250nm. The instrument used was WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector. Th e retention times were found to be 2.246mins and 5.461mins. The % purity of Albendaz ole and Levamisole was found to be 101.27% and 99.76% respectively. The system suita bility parameters for Albendazole and Levamisole such as theoretical plates and tailing fa ctor were found to be 5387, 0.97 and 5398 and 1.26, the resolution was found to be 2. 97. The linearity study n Albendazole and Levamisole was found in concentration range of 30µg-70µg and 60µg-

140µg and correlation coefficient (r2) was found to be 0.999 and 0.999, % recovery was found to be 100.14% and 100.56%, %RSD for repeatability was 0.1 and 0.5, % RSD f or intermediate precision was 0.1 and 0.1 respectively. The precision study was precise, r obust, and repeatable.LOD value was 0.56 and 1.2, and LOQ value was 1.7 and 3.6 res pectively. Hence the suggested RP-
HPLC method can be used for routine analysis of Albendazole and Levamisole in API a nd Pharmaceutical dosage form.

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

INTRODUCTION

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY [1]

In the modern pharmaceutical industry, highperformance liquid chromatography (HPLC) is the major and integral analytical tool applied in all stages of drug discovery, developmen t and production. It is ideal for the analysis of many drugs in both dosage forms and bi ological fluids due to its simplicity, high specificity and good sensitivity.

High Performance Liquid Chromatography (HPLC) is a technique that has arisen from th e application to liquid chromatography the use of an instrumentation that was originally developed for gas chromatography. High Pressure Liquid Chromatography was developed in the mid-
1970 and was improved with the development of column packing material and the additi onal convenience of on-
line detectors. The various components of HPLC are pumps (solvent delivery system), mi xing unit, gradient controller and solvent degasser, injector (manual or au tomatic), guard column, analytical columns, detectors, recorders and/or int egrators. Recent models are equipped with computers and software for data acquis ition and processing. The mobile phase in HPLC refers to the solvent being continuously applied to the column or stationary phase at a flow rate of 1-
5 cm3/min. The mobile phase acts as a carrier for the sample solution. The chemical int

eractions of the mobile phase and sample with the column determine the degree of migr ation and separation of components contained in the sample. The mobile phase can be al tered in order to manipulate the interactions of the sample and the stationary phase.

Instrumentation [3-8]

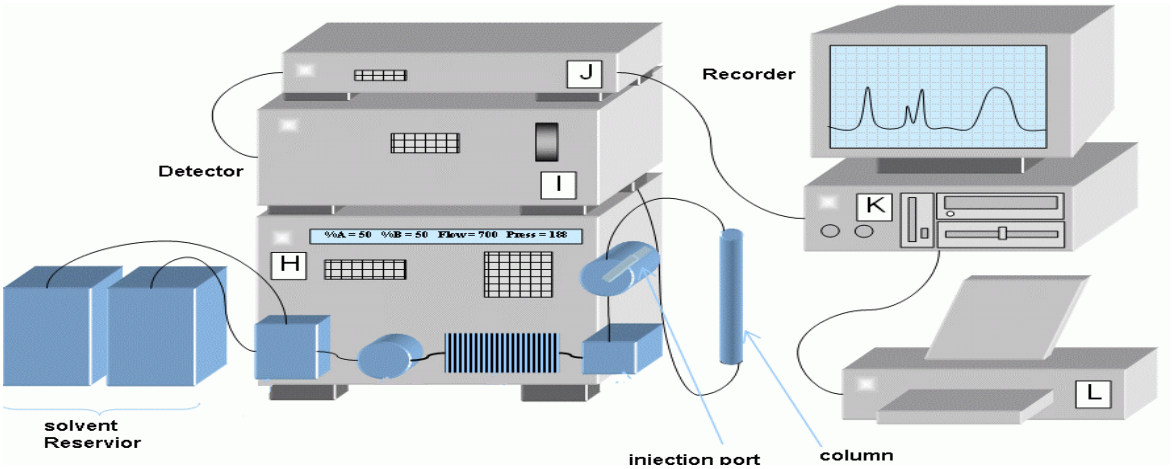

Figure-: Diagram of HPLC Instrument [3]

The basic components of HPLC are: [4-8]

1. Pumping System
2. Sample Introduction Device
3. Chromatographic Column
4. Detector
5. Data handling Device

Analytical Method Validation Requirement [10]

- Assuring quality
- Achieving acceptance of products by the international agencies.
- Mandatory requirement purposes for accreditation as per ISO 17025 guidelines.

- Validated methods are only acceptable for undertaking proficiency testing.

Importance of Method Validation:

Method validation has received considerable attention in the literature and from industrial com mittees and regulatory agencies. [34]

1. The U.S. FDA CGMP (1) request in section 211. 65 (e) methods to be validated: The accur acy, sensitivity, specificity, and reproducibility of test methods employed by the firm shall be established and documented.

2. The ICH has developed a text on the validation of analytical procedures. The doc ument includes definitions for eight validation characteristics.

Validation Parameters: [28,29-33]

1. Accuracy
2. Precision (Repeatability and Reproducibility)
3. Linearity
4. Range
5. Selectivity/ Specificity
6. Robustness/ Ruggedness
7. Limit of Detection (LOD)/ Limit of Quantification (LOQ)

MATERIALS

Albendazole, Levamisole, Water and Methanol for HPLC from LICHROSOLV (MERCK), Acetonitrile, Triethylamine from MERCK provided by **SURALABS** at **DILSUKNAGAR**.

METHODOLOGY

HPLC METHOD DEVELOPMENT:

TRAILS

Preparation of standard solution:

Accurately weigh and transfer 10 mg of Albendazole and Levamisole working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and r emoval of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.5ml of the Albendazole and 1ml of the Levamisole stock solutions into a 10 ml volumetric flask and dilute up to the mark with Methanol.

Preparation of Sample Solution:

Take average weight of one Tablet and crush in a mortar by using pestle and weight 10 mg e quivalent weight of Albendazole and Levamisole sample into a 10mL clean dry volumetric fla sk and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.5ml of the Albendazole and 1ml of the Levamisole stock solutions into a 10 ml volumetric flask and dilute up to the mark with Methanol

PREPARATION OF BUFFER AND MOBILE PHASE:

Preparation of Triethylamine (TEA) buffer (pH-4.2):

Dissolve 1.5ml of Ttiethyl amine in 250 ml HPLC water and adjust the pH 3.8. Filter and son icate the solution by vaccum filtration and ultra sonication.

Preparation of mobile phase:

Accurately measured 360 ml (36%) of Methanol and 640 ml of buffer (64%) a were m ixed and degassed in digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filt er under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Instrument used	:	Waters HPLC with auto sampler and PDA Detector 996 model.
Temperature	:	37ºC
Column	:	Symmetry ODS C18 (4.6mm×250mm, 5µm) particle size
Mobile phase	:	Methanol: TEA Buffer (pH-3.8) (30:70v/v)

Flow rate	:	1ml/min
Wavelength	:	250 nm
Injection volume	:	20 µl
Run time	:	10 min

METHOD VALIDATION PARAMETERS

SYSTEM SUITABILITY

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified li mits.

SPECIFICITY STUDY OF DRUG:

Procedure:

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

PREPARATION OF DRUG SOLUTIONS FOR LINEARITY:

Accurately weigh and transfer 10 mg of Albendazole and 10mg of Levamisole working standa rd into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to disso lve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (30 ppm of Albendazole & 60ppm of Levamisole):

Pipette out 0.3ml of Albendazole and 0.6ml of Levamisole stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (40 ppm of Albendazole & 80ppm of Levamisole):

Pipette out 0.4ml of Albendazole and 0.8ml of Levamisole stock solutions was take in a 10m l of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (50 ppm of Albendazole & 100ppm of Levamisole):

Pipette out 0.5ml of Albendazole and 1ml of Levamisole stock solutions was take in a 10ml o f volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (60 ppm of Albendazole & 120ppm of Levamisole):

Pipette out 0.6ml of Albendazole and 1.2ml of Levamisole stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (70 ppm of Albendazole & 140ppm of Levamisole):

Pipette out 0.7ml of Albendazole and 1.4ml of Levamisole stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Procedure:

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

PRECISION

REPEATABILITY

Procedure: The standard solution was injected for five times and measured the area for all five in jections in HPLC. The %RSD for the area of five replicate injections was found to be within the s pecified limits.

INTERMEDIATE PRECISION:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:

DAY 1:

The standard solution was injected for six times and measured the area for all six injections in HP LC. The %RSD for the area of six replicate injections was found to be within the specified limits.

DAY 2:

The standard solution was injected for six times and measured the area for all six injections in HP LC. The %RSD for the area of six replicate injections was found to be within the specified limits

ACCURACY:

For preparation of 50% Standard stock solution:

Further pipette 0.25ml of the Albendazole and 0.5ml of the Levamisole stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 100% Standard stock solution:

Further pipette 0.5ml of the Albendazole and 1ml of the Levamisole stock solutions into a 10 ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 150% Standard stock solution:

Further pipette 0.75ml of the Albendazole and 1.5ml of the Levamisole stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were ma de under the optimized conditions. Recorded the chromatograms and measured the peak respo nses. Calculate the Amount found and Amount added for Albendazole and Levamisole and cal culate the individual recovery and mean recovery values.

ROBUSTNESS:

The analysis was performed in different conditions to find the variability of test results. The f ollowing conditions are checked for variation of results.

Effect of Variation of flow conditions:

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditi ons are same. 20µl of the above sample was injected and chromatograms were recorded

Effect of Variation of mobile phase organic composition:

The sample was analyzed by variation of mobile phase i.e. Methanol: TEA Buffer was taken i n the ratio and 35:65, 25:75 instead (30:70), remaining conditions are same. 20µl of the above sample was injected and chromatograms were recorded.

RESULTS AND DISCUSSION

Optimized Chromatogram (Standard) and (Sample)

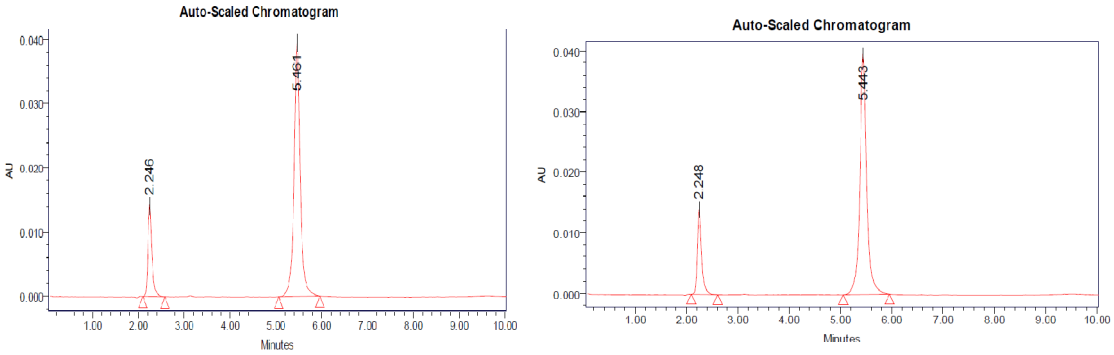

Fig-: Optimized ChromatogramFigure-: Optimized Chromatogram (Sample)

Observation: From the above chromatogram it was observed that the Albendazole and Levamisole pe aks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

It was found from above data that all the system suitability parameters for developed method were wi thin the limit.

VALIDATION PARAMETERS

LINEARITY

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY:

Albendazole:

Concentration µg/ml	Average Peak Area
30	51476
40	67598
50	84897
60	101114
70	119554

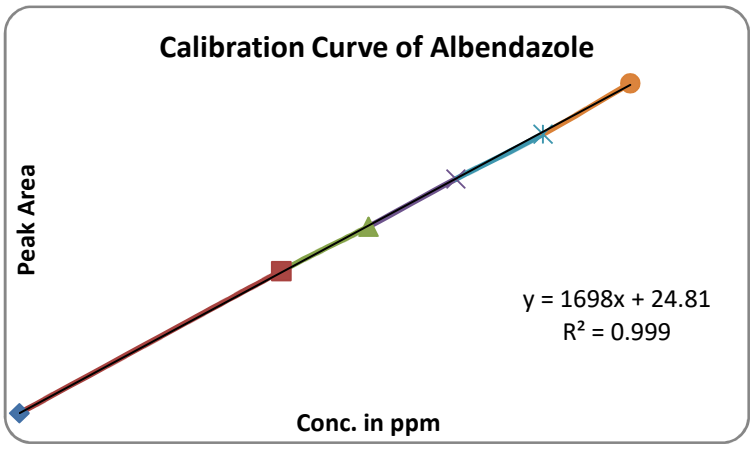

Figure : Calibration graph for Albendazole

Levamisole

Concentration µg/ml	Average Peak Area
60	2286598
80	3086587
100	3867579
120	4758517
140	5604874

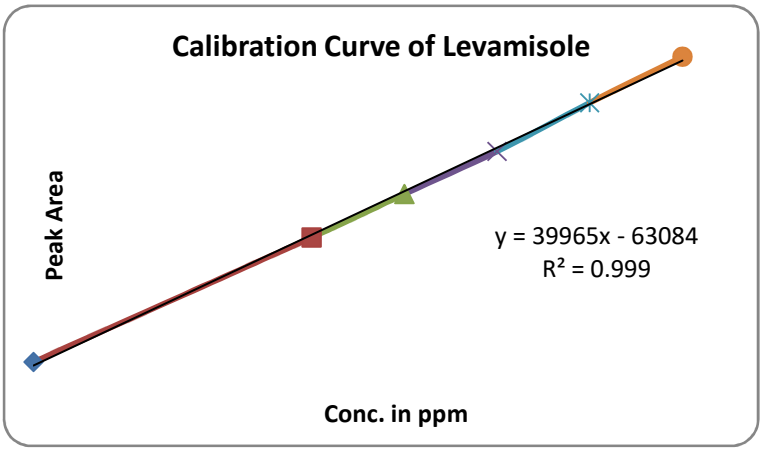

Figure : Calibration Graph for Levamisole

SPECIFICITY

Table-: Peak results for assay standard

S.No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Albendazole	2.256	759868	71255		1.7	5689	1
2	Levamisole	5.427	2458754	215654	2.04	1.6	5362	1
3	Albendazole	2.249	759458	72541		1.7	5748	2

4	Levamisole	5.430	2465885	226565	2.00	1.6	5452	2
5	Albendazole	2.248	759245	72584		1.7	5584	3
6	Levamisole	5.443	2489578	221542	2.04	1.6	5456	3

Table-: Peak results for Assay sample

S.No.	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Albendazole	2.247	756985	68958		0.98	7253	1
2	Levamisole	5.452	2569856	198564	2.06	1.23	8836	1
3	Albendazole	2.246	758745	69857		1.05	6530	2
4	Levamisole	5.461	2598654	195682	2.04	0.99	7270	2
5	Albendazole	2.243	756848	69588		1.7	7586	3
6	Levamisole	5.466	2587454	192541	2.04	1.6	8371	3

$$\%\text{ASSAY} = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Albendazole and Levamisole in pharmaceutical dosage form was found to be 99.76 %.

Precision:

Table-: Results of Intermediate precisionDay 1 for Albendazole

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Albendazole	2.248	758955	68986	5785	1.6
2	Albendazole	2.245	759869	68957	5698	1.4
3	Albendazole	2.242	758985	68545	5689	1.6
4	Albendazole	2.239	756894	68952	5781	1.9
5	Albendazole	2.243	759854	68595	5785	1.7
6	Albendazole	2.246	756985	68952	5693	1.6
Mean			758590.3			
Std. Dev			1339.793			
% RSD			0.176616			

Table: Results of Intermediate precisionDay 1 for Levamisole

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Levamisole	5.284	2659852	190025	5485	1.5	2.04
2	Levamisole	5.293	2648574	190048	5421	1.6	2.03
3	Levamisole	5.306	2659865	190054	5468	1.6	2.01
4	Levamisole	5.319	2658547	190078	5487	1.6	2.05
5	Levamisole	5.346	2648981	190016	5492	1.6	2.02
6	Levamisole	5.352	2654652	190057	5463	1.6	2.03
Mean			2655079				

Std. Dev			5242.086				
% RSD			0.197436				

Table-: Results of Intermediate precision Day 2 for Albendazole

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Albendazole	2.255	766895	69858	5586	1.5
2	Albendazole	2.260	765988	69854	5636	1.6
3	Albendazole	2.242	766532	69824	5432	1.6
4	Albendazole	2.245	766214	69875	5468	1.6
5	Albendazole	2.260	765897	69854	5546	1.9
6	Albendazole	2.255	765245	69848	5507	1.7
Mean			766128.5			
Std. Dev			567.7234			
% RSD			0.074103			

Table-: Results of Intermediate precision for Levamisole

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Levamisole	5.266	2653254	190110	5428	1.6	7.98
2	Levamisole	5.265	2648985	190058	5452	1.6	6.4
3	Levamisole	5.306	2658213	190142	5498	1.6	8.9
4	Levamisole	5.293	2653652	190031	5442	1.5	8.3

5	Levamisole	5.265	2648978	190058	5489	1.5	7.5
6	Levamisole	5.266	2658985	190047	5463	1.6	5.3
Mean			2653678				
Std. Dev			4313.355				
% RSD			0.162543				

Acceptance criteria:

- %RSD of Six different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is rugged.

ACCURACY:

Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was c alculated.

Table-: The accuracy results for Albendazole

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	% Mean Recovery
50%	42594.67	25	25.070	100.280%	100.14%
100%	84867	50	49.965	99.930%	
150%	127654	75	75.164	100.218%	

Table-: The accuracy results for Levamisole

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	% Mean Recovery
50%	2079124	50	50.445	100.890%	100.56%

100%	4082412	100	100.571	100.571%	
150%	6070195	150	150.309	100.206%	

Acceptance Criteria:

The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurat e

LIMIT OF DETECTIONLIMIT OF QUANTITATION

LOD= 3.3 × σ / sLOQ=10×σ/S

Result:Result:

Albendazole:0.56µg/ml**Albendazole:**1.2µg/ml

Levamisole:1.7µg/ml**Levamisole:**3.6µg/ml

Robustness

Variation in flow

Albendazole**:**

Table-: Results for Robustness

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plat es	Tailing factor
Actual Flow rate of 1.0 mL/min	765789	2.246	5387.0	0.97
Less Flow rate of 0.9 mL/min	758698	2.505	5458	0.96
More Flow rate of 1.1 mL/min	7689584	2.046	5696	0.94
Less organic phase	758412	2.505	5586	0.92
More organic phase	769852	2.046	5355	0.95

Levamisole:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	2532158	5.461	5398	1.26
Less Flow rate of 0.9 mL/min	2458692	5.599	5329	1.25
More Flow rate of 1.1 mL/min	2658642	4.576	5256	1.24
Less organic phase	2452148	5.599	5214	1.23
More organic phase	2653894	4.576	5524	1.22

Acceptance criteria:

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RPHPLC method was developed for the quantitative estimation of Albendazole and Levamisole in bulk drug and pharmaceutical dosage forms.

Albendazole was found to be soluble in dimethyl sulfoxide, strong acids, and strong bases. It is slightly soluble in methanol, chloroform, ethyl acetate, and Acetonitrile. Albendazole is practically insoluble in water. Levamisole was found to be freely soluble in water; soluble in ethanol (~750 g/L), soluble in methanol and propylene glycol, slightly soluble in chloroform.

Methanol: TEA Buffer (pH-3.8) (30:70v/v) was chosen as the mobile phase. The solvent system used in this method was economical.

The %RSD values were within 2 and the method was found to be precise.

The results expressed in Tables for RP-HPLC method was promising. The RP-

HPLC method is more sensitive, accurate and precise compared to the Spectrophotome tric methods.

This method can be used for the routine determination of Albendazole and Levamisole in bulk drug and in Pharmaceutical dosage forms.

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