

## A VALIDATED RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF IVERMECTIN AND ALBENDAZOLE IN ITS BULK AND PHARMACEUTICAL DOSAGE FORMS

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### ABSTRACT

HPLC is the better analytical technique available for both the Qualitative and Quantitative analysis of natural, synthetic and semisynthetic compounds, drugs. As the most of Drugs are non-polar Normal phase mode of HPLC is not suitable for the analysis, for this Reverse phase mode HPLC was the best technique. In this present work RP-HPLC method was developed for the simultaneous estimation of Ivermectin and Albendazole in their bulk and dosage forms. Both of these drugs i.e., Ivermectin and Albendazole are used for treating parasitic infections. Now with this simultaneous study the quantities/amounts of these two drugs released can be well estimated.

**KEYWORDS:** RP-HPLC, Ivermectin, Albendazole.

### INTRODUCTION

High-Performance Liquid Chromatography also known as High-Pressure or High Price or High Speed Liquid Chromatography (HPLC) is a form of Column Chromatography used frequently in analytical chemistry and biochemistry to identify, separate, and quantify compounds. It is a powerful tool in analysis. It is basically an improved form of column chromatography which has been optimized to provide rapid high resolution separations. Early LC used gravity fed open tubular columns with particles 100s of microns in size; the human eye was used for a detector and separations often took hours or even days to develop. HPLC as compared with the classical technique is characterized by

- Small diameter (2-5 mm), reusable stainless steel columns without repacking and regeneration
- Column packings with very small (3, 5 and 10  $\mu$ m) particles and the continual development of new substances to be used as stationary phases
- Relatively high inlet pressures and controlled flow of the mobile phase

- Precise sample introduction without the need for large samples
- Special continuous flow detectors capable of handling small flow rates and detecting very small amounts
- Automated standardized instruments
- Rapid analysis
- Greater reproducibility due to close control of the parameters affecting the efficiency of separation
- Capable of handling macro molecule & viscous solutions
- Efficient analysis of labile natural products
- Reliable handling of inorganic or other ionic species

HPLC is probably the most universal type of analytical procedure. In addition HPLC also ranks as one of the most sensitive analytical procedures and is unique in that it easily copes with multi-component mixtures. Its application areas include quality control, process control, forensic analysis, environmental monitoring and clinical testing. It has achieved this position as a result of the constant evolution of the equipment used in LC to provide higher and higher efficiencies at faster and faster analysis times with a constant incorporation of new highly selective column packings.

**Reversed-phase chromatography** uses a non-polar stationary phase and a polar mobile phase. This is the most common type of HPLC separation in use today. A partition mechanism is typically used for separations by non-polar differences. For reversed phase, alkyl hydrocarbons are the preferred stationary phase; octadecyl (C18) is the most common stationary phase, but octyl (C8) and butyl (C4) are also used in some applications. In reversed phase chromatography, the most polar compounds elute first with the most non-polar compounds eluting last.

Albendazole is a benzimidazole derivative most commonly used for treating parasitic infestations like Neurocysticercosis which damages the nervous system, for this causative parasite is pork tapeworm. Albendazole can also be used for the treating the disease Cystic hydatid of Lungs, peritonium and liver, Tubulin modulator, destabilizer of the microtubules.

Ivermectin is the derivative of Avermectins, which is semisynthetically synthesized drug most widely used for the treatment of helmentic infections, Filariasis, whip, thread and round worm infestations and also to treat the disease River blindness.

Literature review reveals that there is no analytical method reported for the analysis of Ivermectin and Albendazole by simultaneous estimation by RP-HPLC. Spectrophotometer, HPLC and HPTLC are the reported analytical methods for compounds either individually or in combination with other dosage form. Hence, it was felt that, there is a need of new analytical method development for the simultaneous estimation of Ivermectin and Albendazole in pharmaceutical dosage form.

Present work is aimed to develop a new, simple, fast, rapid, accurate, efficient and reproducible RP-HPLC method for the simultaneous analysis of Ivermectin and Albendazole. The developed method will be validated according to ICH guidelines.

- The analytical method for the simultaneous estimation of Ivermectin and Albendazole will be developed by RP-HPLC method by optimizing the chromatographic conditions.

- The developed method is validated according to ICH guidelines for various parameters specified in ICH guidelines, Q2 (R1).

## MATERIALS AND METHODS

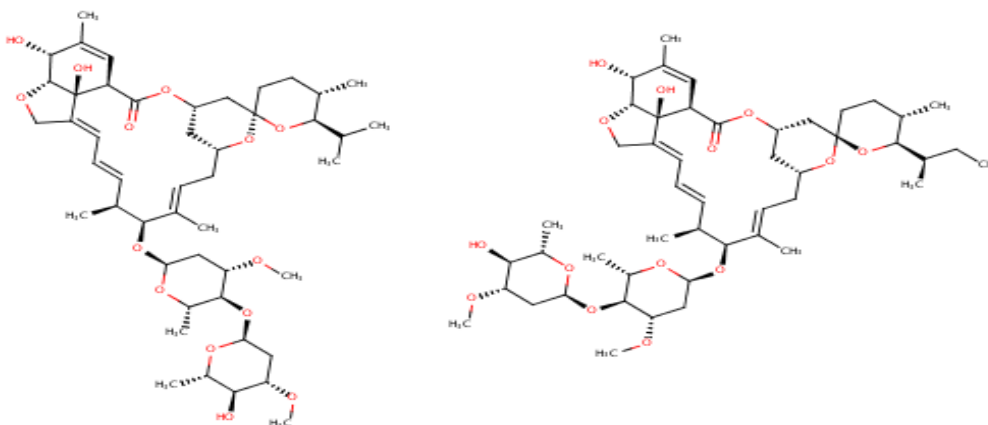
### Chemicals and standards used

Table.No.1: List of chemicals and standards used.

S. No.	Chemicals	Manufacturer	Grade
1.	Water	Merck	HPLC grade
2.	Methanol	Merck	HPLC grade
3.	Acetonitrile	Merck	HPLC grade
4.	Ortho phosphoric acid	Merck	G.R
5.	KH <sub>2</sub> PO <sub>4</sub>	Merck	G.R
6.	K <sub>2</sub> HPO <sub>4</sub>	Merck	G.R
7.	0.22µNylon filter	Advanced lab	HPLC grade
8.	0.45µfilter paper	Millipore	HPLC grade
9.	Tancodep-2	Torrent pharmaceuticals	Tablet form
10.	Ivermectin and Albendazole	In – House	In- House

## DRUG PROFILE

### Ivermectin



### Chemical Data

**IUPAC Name:** (1'R,2R,4'S,5S,6R,8'R,10'E,12'S,13'S,14'E,16'E,20'R,21'R,24'S)-21',24'-dihydroxy-12'-{[(2R,4S,5S,6S)-5-[(2S,4S,5S,6S)-5-hydroxy-4-methoxy-6-methyloxan-2-yl]oxy]-4-methoxy-6-methyloxan-2-yl]oxy}-5,11',13',22'-tetramethyl-6-(propan-2-yl)-3',7',19'-trioxaspiro[oxane-2,6'-tetracyclo[15.6.1.1<sup>4</sup>,8.0<sup>20,24</sup>]pentacosane]-10',14',16',22'-tetraen-2'-one; (1'R,2R,4'S,5S,6R,8'R,10'E,12'S,13'S,14'E,16'E,20'R,21'R,24'S)-6-[(2R)-butan-2-yl]-21',24'-dihydroxy-12'-{[(2R,4S,5S,6S)-5-[(2S,4S,5S,6S)-5-hydroxy-4-methoxy-6-methyloxan-2-yl]oxy]-4-methoxy-6-methyloxan-2-yl]oxy}-5,11',13',22'-tetramethyl-3',7',19'-trioxaspiro[oxane-2,6'-tetracyclo[15.6.1.1<sup>4</sup>,8.0<sup>20,24</sup>]pentacosane]-10',14',16',22'-tetraen-2'-one

**Chemical formula** : C<sub>95</sub>H<sub>146</sub>O<sub>28</sub>

**Molecular weight** : 1736.1589 g/mol

**CAS No** : 70288-86-7

**Physical Data**

**Description:** Ivermectin is a broad-spectrum anti-parasite medication. It was first marketed under the name Stromectol® and used against worms (except tapeworms), but, in 2012, it was approved for the topical treatment of head lice infestations in patients 6 months of age and older, and marketed under the name Sklice™ as well. Ivermectin is mainly used in humans in the treatment of onchocerciasis, but is also effective against other worm infestations (such as strongyloidiasis, ascariasis, trichuriasis and enterobiasis).

**Solubility:** Insoluble in water and soluble in methanol.

**Melting point:** 155 °C

**Category:** Insecticides, Antiparasitic Agents

**Mechanism of Action:** Ivermectin binds selectively and with high affinity to glutamate-gated chloride ion channels in invertebrate muscle and nerve cells of the microfilaria. This binding causes an increase in the permeability of the cell membrane to chloride ions and results in hyperpolarization of the cell, leading to paralysis and death of the parasite. Ivermectin also is believed to act as an agonist of the neurotransmitter gamma-aminobutyric acid (GABA), thereby disrupting GABA-mediated central nervous system (CNS) neurosynaptic transmission. Ivermectin may also impair normal intrauterine development of *O. volvulus* microfilariae and may inhibit their release from the uteri of gravid female worms.

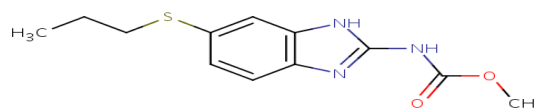
**Albendazole****Chemical Data**

**IUPAC Name:** methyl N-[6-(propylsulfanyl)-1H-1,3-benzodiazol-2-yl]carbamate

**Chemical formula:** C<sub>12</sub>H<sub>15</sub>N<sub>3</sub>O<sub>2</sub>S

**Molecular weight:** 265.331

**CAS No :** 54965-21-8

**Physical Data**

**Description:** Albendazole broad-spectrum anthelmintic structurally related to mebendazole that is effective against many diseases.

**Solubility:** Soluble in water.

**Melting point:** 208 °C

**Mechanism of Action:** 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.

**Instruments used****Table No. 2: List of instruments used.**

S. No.	Instrument	Model number	Software	Manufacturer
1	HPLC-auto sampler-UV detector	Separation module 2695, UV detector 2487	Empower-software version-2	Waters
2	U.V double beam spectrometer	UV 3000+	U.V win soft ware	Lab India
3	Digital weighing balance (sensitivity 5mg)	ER 200A	---	Ascotet
4	pH meter	AD 102U	---	ADWA
5	Sonicator	SE60US	---	Enertech

**Method development for the simultaneous estimation of Ivermectin and Albendazole by using RP-HPLC**

1. Selection of mobile phase
2. Selection of detection wavelength
3. Selection of column
4. Selection of solvent delivery system
5. Selection of flow rate
6. Selection of column temperature
7. Selection of diluent
8. Selection of test concentration and injection volume

**1. Selection of mobile phase**

- Phosphate buffer: Methanol (30: 70% v/v)
- Buffer pH should be between 2 to 8.
- Below 2: siloxane linkages are cleaved.
- Above 8: dissolution of silica.
- pH selected:  $3 \pm 0.05$
- pH controls the elution properties by controlling the ionization characteristics.
- Reasons: To decrease the retention and improve separation. Good Response, Area, Tailing factor, Resolution.

**2. Selection of wavelength** 10 mg of Ivermectin and Albendazole was dissolved in mobile phase. The solution was scanned from 200-400 nm the spectrum was obtained. The overlay spectrum was used for selection of wavelength for Ivermectin and Albendazole. The isobestic point was taken as detection wavelength. The overlay spectrums are shown in Fig.

**3. Selection of column**

- Heart of HPLC made of 316 grade stainless steel packed with stationary phase.

Silica based columns with different cross linking's in the increasing order of polarity are as follows:

←----- Non-polar-----moderately polar-----Polar-----→

$C_{18} < C_8 < C_6 < \text{Phenyl} < \text{Amino} < \text{Cyano} < \text{Silica}$

- In reverse phase chromatography, hydrophobic interaction between drug molecule and the alkyl chains on the column packing material.
- Column is selected based on solubility, polarity and chemical differences among analysts and Column selected: i.e. ACE C18 column (4.6 x150 mm) 5  $\mu$ .

- Reasons: Better separation, Good tailing factor.

#### 4. Selection of solvent delivery system

- Always preferable solvent delivery system.
- More chance of getting reproducible result on retention time of analytes.
- More economic than gradient technique.

#### 5. Selection of flow rate

Acceptable limit: - Not more than 2.5 ml/min

- Flow rate selected was 1ml/min
- Flow rate is selected based on
  1. Retention time
  2. Column back pressure
  3. Peak symmetry.
  4. Separation of impurities.

Reasons

- ❖ For earlier elution of analyte and elution of all impurities within 6.0 min.
- ❖ Information from the reference method in literature.

#### 6. Selection of diluent

- Selection of diluent is based on the solubility of the analyte
- Diluent selected: Phosphate buffer: Methanol (30: 70% v/v)

#### 7. Selection of column temperature

- Preferable temperature is ambient or room temperature.

Reasons

- ❖ To elute all impurities along with analyte with in 10.0 min of run time.
- ❖ Less retention time
- ❖ Good peak shape
- ❖ Higher theoretical plates.
- ❖ Good resolution.

#### 8. Selection of test concentration and injection volume

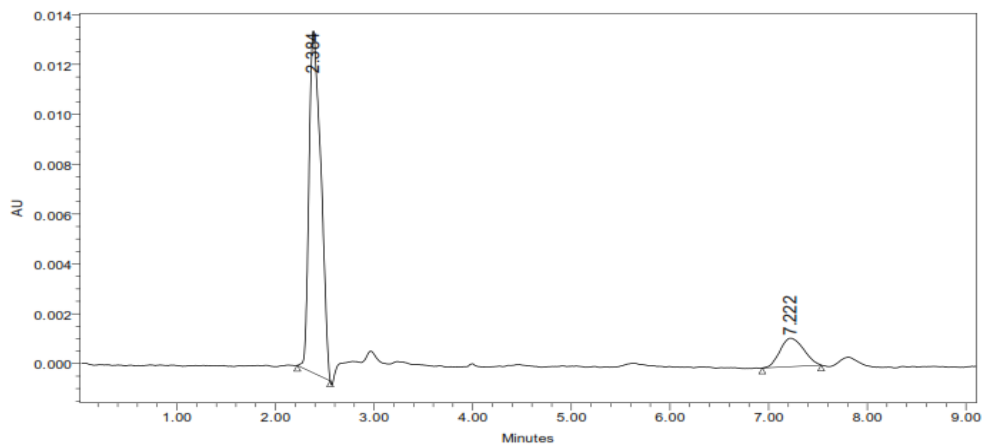
Test concentration is finalized after it is proved that API is completely extractable at the selected test concentration.

- Test concentration is fixed based upon the response of API peak at selected detector wavelength.
- And the test concentration selected is 10 ppm.
- Injection volume selected was 10 $\mu$ L.

Reason: good peak area, retention time, peak symmetry.

**9. Chromatographic trials for simultaneous estimation of Ivermectin and Albendazole by RP- HPLC.****Trial-1****Chromatographic conditions**

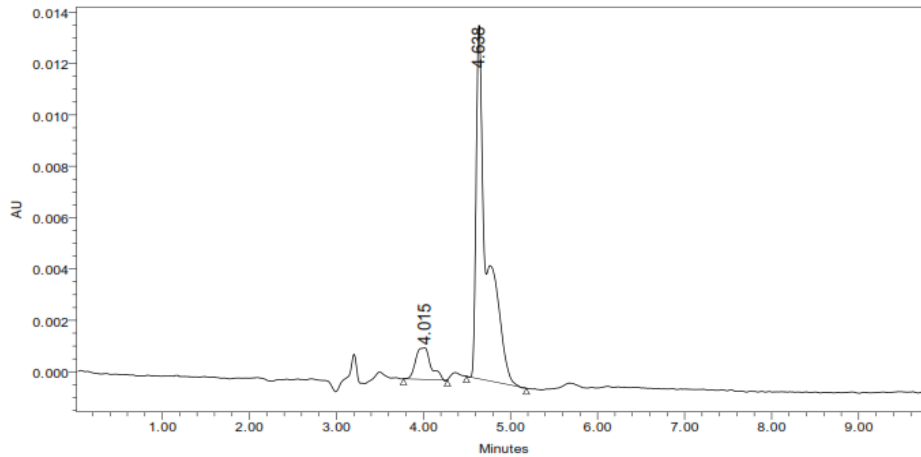
Column	: Thermosil C18 4.6x150mm, 5 $\mu$ m
Mobile phase ratio	: MeOH: H <sub>2</sub> O (60:40% v/v)
Detection wavelength	: 236nm
Flow rate	: 1ml/min
Injection volume	: 10 $\mu$ l
Column temperature	: Ambient
Auto sampler temperature	: Ambient
Run time	: 10min
Retention time	: 2.384 min & 7.222 min

**Fig.1: Chromatogram showing trial-1 injection.****Observation**

The trial shows no proper separation peaks in the chromatogram, so more trials were required for obtaining peaks.

**Trial - 2****Chromatographic conditions**

Column	: Symmetry C18 4.6x150mm 5 $\mu$ m
Mobile phase ratio	: ACN: Methanol (40:60% v/v)
Detection wavelength	: 254 nm
Flow rate	: 1ml/min
Injection volume	: 20 $\mu$ l
Column temperature	: Ambient
Auto sampler temperature	: Ambient
Run time	: 8.0 min
Retention time	: 4.015 min & 4.638 min.



**Fig.2: Chromatogram showing trial-2 injection.**

### Observation

In this trial two peaks were separated but don't have proper resolution. Still more trials were required for proper peaks.

### Trial-3

#### Chromatographic conditions

Column : Zodiacsil RPC8 4.5×150mm 5.0 μm

Mobile phase ratio : ACN: pH 4 buffer (70:30 % v/v)

Detection wavelength : 254 nm

Flow rate : 1.0ml/min

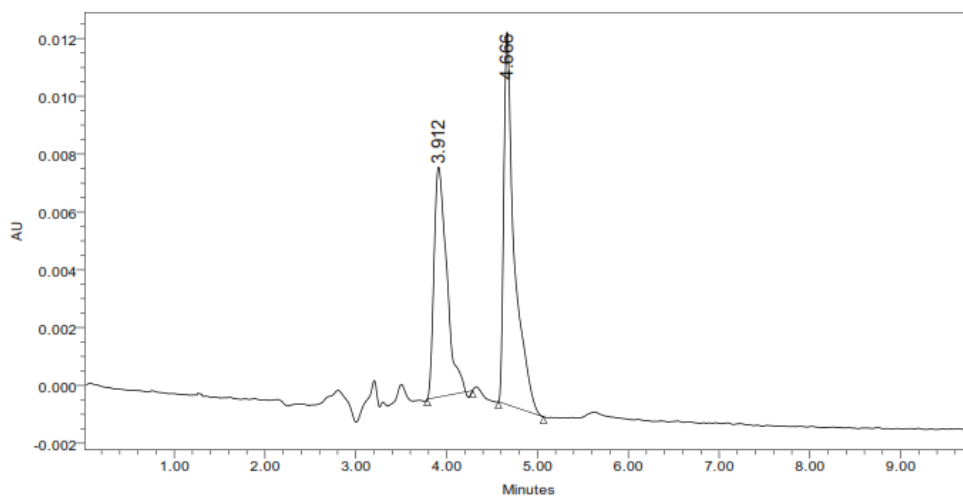
Injection volume : 20μl

Column temperature : Ambient

Auto sampler temperature : Ambient

Run time : 10.0 mins

Retention time : 3.912, 4.666 mins



**Fig. 3: Chromatogram showing trial-3 injection.**

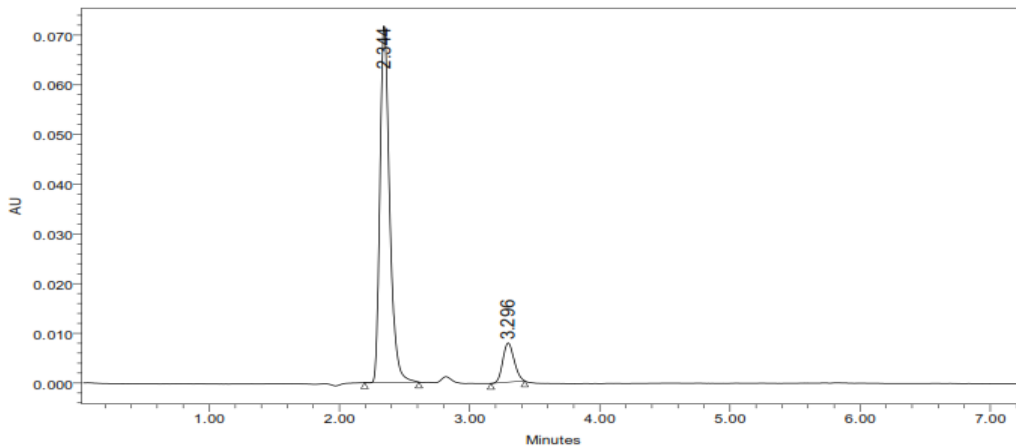


**Observation**

In this trial both Ivermectin and Albendazole were eluted but there is no proper resolution. Still more trials were required for better resolution in peaks.

**Trial-4****Chromatographic conditions**

Column : Zodiac sil RPC18 4.6×250mm 5µm  
Mobile phase ratio : ACN: pH 3 buffer (65:35% v/v)  
Detection wavelength : 254 nm  
Flow rate : 1.0ml/min  
Injection volume : 20µl  
Column temperature : Ambient  
Auto sampler temperature : Ambient  
Run time : 7 min  
Retention time : 2.344 mins & 3.296 mins



**Fig. 4: Chromatogram showing trial-4 injections.**

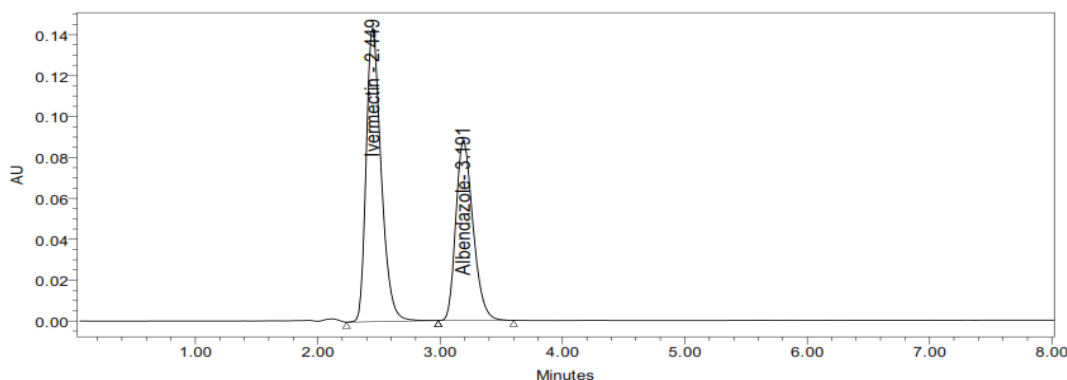
**Observation**

The separation was good; peak shape was good, still more trials were required to reduce the retention times of peaks.

**Trial -5 (optimised method)****Chromatographic conditions**

Column : ACE C18 (4.6×150 mm) 5.0µm  
Mobile phase ratio : Methanol: Phosphate buffer (70: 30 % v/v)  
Detection wavelength : 240 nm  
Flow rate : 1.2 ml/min  
Injection volume : 10µl  
Column temperature : Ambient  
Auto sampler temperature : Ambient  
Run time : 8min

Retention time : 2.449 & 3.191 mins



**Fig. 5: Chromatogram showing trial-5 injection.**

### Observation

The separation was good, peak shape was good, so we conclude that there is no required for reduce the retention times of peaks, so it is taken as final method.

### 10. Procedure

#### Preparation of phosphate buffer

2.58 grams of  $\text{KH}_2\text{PO}_4$  was weighed and taken into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water and pH was adjusted to 3 with orthophosphoric acid. The resulting solution was sonicated and filtered.

#### Preparation of mobile phase

Mix a mixture of above buffer 30 ml (30%) and 70 ml of Methanol (HPLC grade-70%) and degassed in ultrasonic water bath for 5 minutes. Filter through 0.22  $\mu$  filter under vacuum filtration.

#### Diluents preparation

Mobile phase was used as the diluent.

#### Preparation of the individual Ivermectin standard preparation

10 mg of Ivermectin working standard was accurately weighed and transferred into a 10 ml clean dry volumetric flask and add about 2 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette out 1.5 ml from the above stock solution into a 10 ml volumetric flask and was diluted up to the mark with diluent.

#### Preparation of the individual Albendazole standard preparation

10 mg of Albendazole working standard was accurately weighed and transferred into a 10 ml clean dry volumetric flask and add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette out 3 ml from the above stock solution into a 10 ml volumetric flask and was diluted up to the mark with diluent.

**Preparation of the Ivermectin and Albendazole standard and sample solution****Sample solution preparation**

10 mg of Ivermectin and 1 mg Albendazole tablet powder were accurately weighed and transferred into a 10 ml clean dry volumetric flask, add about 2ml of diluent and sonicate to dissolve it completely and making volume up to the mark with the same solvent(Stock solution). Further pipette 10ml of the above stock solution into a 100ml volumetric flask and was diluted up to the mark with diluent.

**Standard solution preparation**

10 mg Ivermectin and 1 mg Albendazole working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette out 1ml of the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

**Procedure**

10 $\mu$ L of the blank, standard and sample were injected into the chromatographic system and areas for the Ivermectin and Albendazole the peaks were used for calculating the % assay by using the formulae.

**System suitability**

- Tailing factor for the peaks due to Ivermectin and Albendazole in standard solution should not be more than 1.5.
- Theoretical plates for the Ivermectin and Albendazole peaks in standard solution should not be less than 2000.

**Assay calculation**

$$\text{Assay \%} = \frac{\text{sample area}}{\text{Standard area}} \times \frac{\text{dilution sample}}{\text{dilution of standard}} \times \frac{P}{100} \times \frac{\text{Avg.wt}}{Lc} \times 100$$

Where:

Avg.wt = average weight of tablets

P= Percentage purity of working standard

LC= Label Claim of Ivermectin mg/ml.

**ANALYTICAL METHOD VALIDATION****Validation parameters**

1. Specificity
2. Linearity
3. Range
4. Accuracy
5. Precision
6. Repeatability
7. Intermediate Precision
8. Detection Limit
9. Quantitation Limit
10. Robustness

### 1. Specificity

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The specificity was performed by injecting blank.

### 2. Linearity

#### Preparation of stock solution

10 mg of Ivermectin and 1 mg of Albendazole working standard were accurately weighed and were transferred into a 10ml clean dry volumetric flask, add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

#### Preparation of Level – I (50ppm of Ivermectin and 5 ppm of Albendazole)

0.5 ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

#### Preparation of Level – II (100ppm of Ivermectin and 10ppm of Albendazole)

1 ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

#### Preparation of Level – III (150ppm of Ivermectin and 15ppm of Albendazole)

1.5 ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

#### Preparation of Level – IV (200 ppm of Ivermectin and 20ppm of Albendazole)

2 ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

#### Preparation of Level – V (250 ppm of Ivermectin and 25ppm of Albendazole)

2.5 ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

### Procedure

Each level was injected into the chromatographic system and peak area was measured. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and the correlation coefficient was calculated.

### Acceptance criteria

- ❖ Correlation coefficient should be not less than 0.999.

### 3. Range

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 50 $\mu$ g/ml-250 $\mu$ g/ml and 5 $\mu$ g/ml-25 $\mu$ g/ml of Ivermectin and Albendazole respectively.

### 4. Accuracy

#### Preparation of standard stock solution

10mg of Ivermectin and 1mg of Albendazole working standard were accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette out 1 ml of the above stock solution into a 10 ml volumetric flask and was diluted up to the mark with diluent.

#### Preparation of sample solutions

##### For preparation of 50% solution (with respect to target assay concentration)

5mg of Ivermectin and 0.5 mg of Albendazole working standard were accurately weighed and transferred into a 10 ml clean dry volumetric flask add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the

mark with the same solvent (Stock Solution). Further pipette out 10 ml of the above stock solution into a 100ml volumetric flask and was diluted up to the mark with diluent.

**For preparation of 100% solution (with respect to target assay concentration)**

10 mg of Ivermectin and 1 mg of Albendazole working standards were accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette out 1ml of above stock solution into a 10 ml volumetric flask and was diluted up to the mark with diluent.

**For preparation of 150% solution (with respect to target assay concentration)**

15 mg of Ivermectin and 2 mg of Albendazole working standards into a 10ml clean dry volumetric flask add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette out 1ml of the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

**Procedure**

The standard solutions of accuracy 50%, 100% and 150% were injected into chromatographic system. Calculate the amount found and amount added for Ivermectin and Albendazole and calculate the individual % recovery and mean % recovery values.

**Acceptance criteria**

- ❖ The % recovery for each level should be between 98.0 to 102.0%

**5. Precision**

**5.1. Repeatability**

**Preparation of stock solution**

10 mg of Ivermectin and 1 mg of Albendazole working standard were accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette out 1ml of the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

**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 limits.

**Acceptance criteria**

- ❖ The % RSD for the area of five standard injections results should not be more than 2.

**5.2 Intermediate Precision/Ruggedness**

To evaluate the intermediate precision (also known as ruggedness) of the method, precision was performed on different days by using different make column of same dimensions.

**Preparation of stock solution**

10 mg of Ivermectin and 1mg of Albendazole working standard were accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2ml of diluent and sonic ate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette out 1ml of the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

**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 limits.

**Acceptance criteria**

❖ The % RSD for the area of five sample injections results should not be more than 2%.

**6. Limit of detection (LOD)**

LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

$$\text{LOD} = 3.3 \times \frac{\sigma}{S}$$

Where

$\sigma$  - Standard deviation (SD), S – Slope

**7. Limit of quantification**

LOQ's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula. Again, the standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

$$\text{LOD} = 10 \times \frac{\sigma}{S}$$

Where

$\sigma$  - Standard deviation. S - Slope

**8. Robustness**

As part of the robustness, deliberate change in the flow rate, mobile phase composition was made to evaluate the impact on the method.

- The flow rate was varied at 0.4ml/min to 0.6 ml/min. Standard solution 150 ppm of Ivermectin and 15 ppm of Albendazole was prepared and analysed using the varied flow rates along with method flow rate.
- The organic composition in the mobile phase was varied from 65% to 75 % standard solution 150 µg/ml of Ivermectin and 15 µg/ml of Albendazole were prepared and analysed using the varied mobile phase composition along with the actual mobile phase composition in the method.

### 9. System suitability

10 mg of Ivermectin and 1 mg of Albendazole working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette out 1ml of Ivermectin and Albendazole from the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

## RESULTS AND DISCUSSIONS

The present investigation reported in the thesis was aimed to develop a new method development and validation for the simultaneous estimation of Ivermectin and Albendazole by RP-HPLC method. Literature reveals that there are no analytical methods reported for the simultaneous estimation Ivermectin and Albendazole by RP-HPLC method. Hence, it was felt that, there is a need of new analytical method development for the simultaneous estimation of Ivermectin and Albendazole in pharmaceutical dosage form.

### Method Development

The detection wavelength was selected by dissolving the drug in mobile phase to get a concentration of 10 $\mu$ g/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400nm. The overlay spectrum of Ivermectin and Albendazole was obtained and the isobestic point of Ivermectin and Albendazole showed absorbance's maxima at 240 nm. The spectrums are shown in Fig.

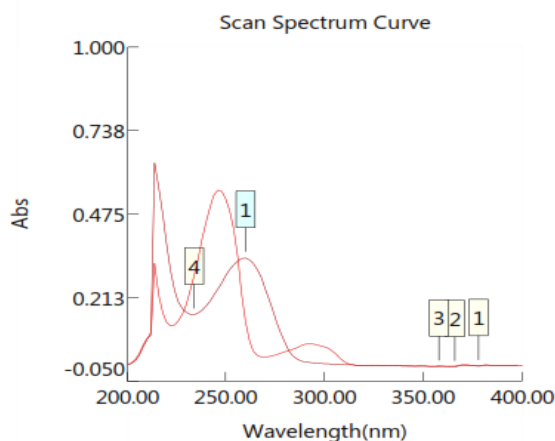


Fig. 6: Spectrum showing overlapping spectrum of IVERMECTIN and ALBENDAZOLE.

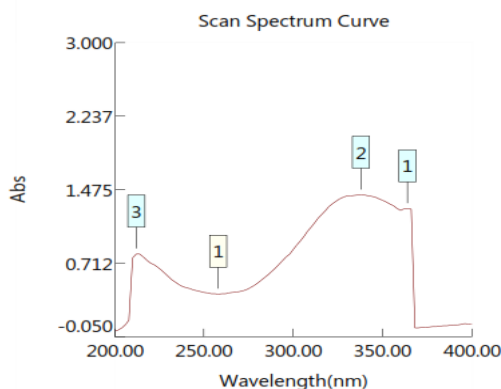
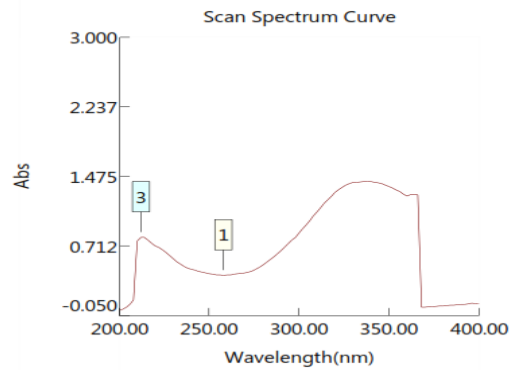


Fig.7: Spectrum showing wavelength of Ivermectin.

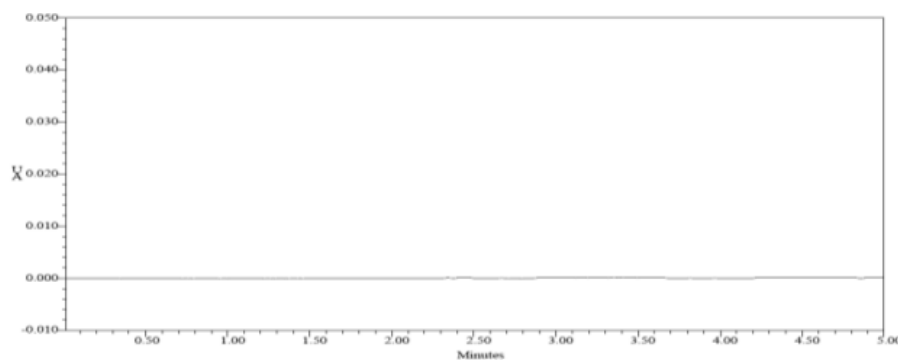


**Fig.8: Spectrum showing wavelength of Albendazole.**

The chromatographic method development for the simultaneous estimation of Ivermectin and Albendazole were optimized by several trials for various parameters as different column, flow rate and mobile phase, finally the following chromatographic method was selected for the separation and quantification of Ivermectin and Albendazole in API and pharmaceutical dosage form by RP-HPLC method.

#### **Optimized chromatographic conditions for simultaneous estimations of Ivermectin and Albendazole by RP-HPLC method**

Column	:	ACE C18 (4.6×150 mm) 5.0 μm
Column temperature	:	Ambient
Wavelength	:	240 nm
Mobile phase ratio	:	70:30
Methanol	:	Phosphate buffer
Flow rate	:	1.2 ml/min
Auto sampler temperature	:	Ambient
Injection volume	:	10μl
Run time	:	10.0 minutes



**Fig. 9: Chromatogram showing blank preparation (mobile phase).**

#### **Assay calculation for Ivermectin and Albendazole**

The assay study was performed for the Ivermectin and Albendazole. Each three injections of sample and standard were injected into chromatographic system. The chromatograms are shown in Fig.10-11 and results are tabulated in Table.No.3.



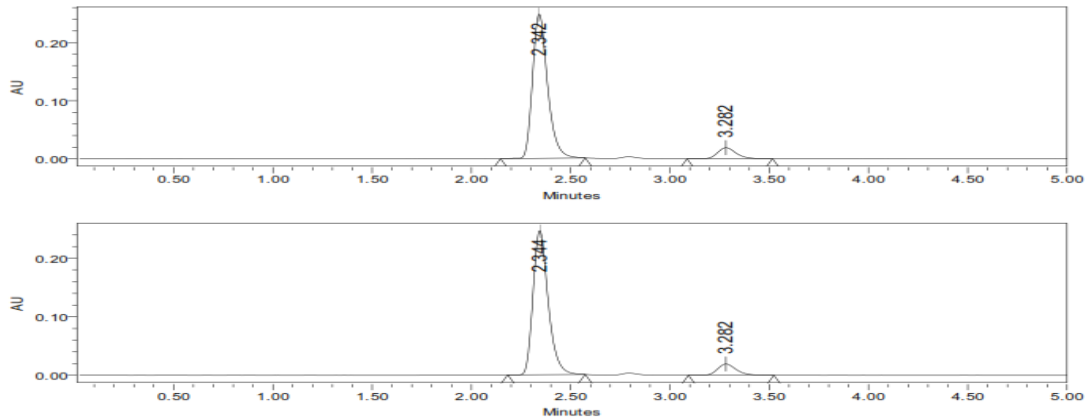


Fig. 10: Chromatogram showing assay of sample injection-1,2.

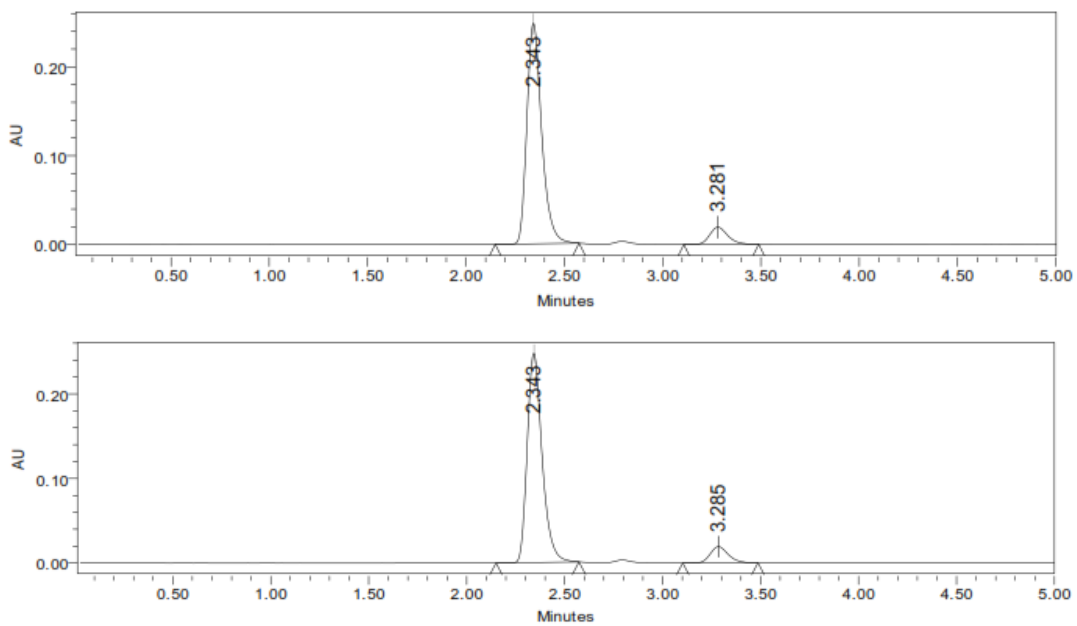


Fig.11: Chromatogram showing assay of sample injection -1,2,3.

Table 3: Showing assay results.

S. No.	Name of compound	Label claim	Amount taken	%purity
1.	Ivermectin	500	754.7	99.24
2.	Albendazole	2.5	735.6	101.04

The retention time of Ivermectin and Albendazole was found to be 2.344 mins and 3.284 mins respectively. The system suitability parameters for Ivermectin and Albendazole such as theoretical plates and tailing factor were found to be 4668, 1.3 and 6089, 1.2. Resolution was 6.0 The % purity Ivermectin and Albendazole in pharmaceutical dosage form was found to be 99.24 and 101.27% respectively.

**VALIDATION REPORT**

**Specificity**

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The study was performed by injecting blank. The chromatograms are shown in Fig.12-14.

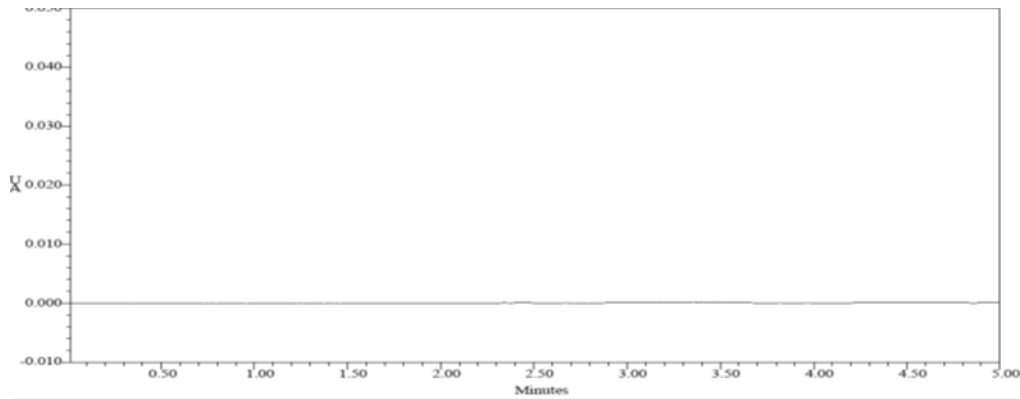


Fig. 12: Chromatogram showing blank (mobile phase preparation).

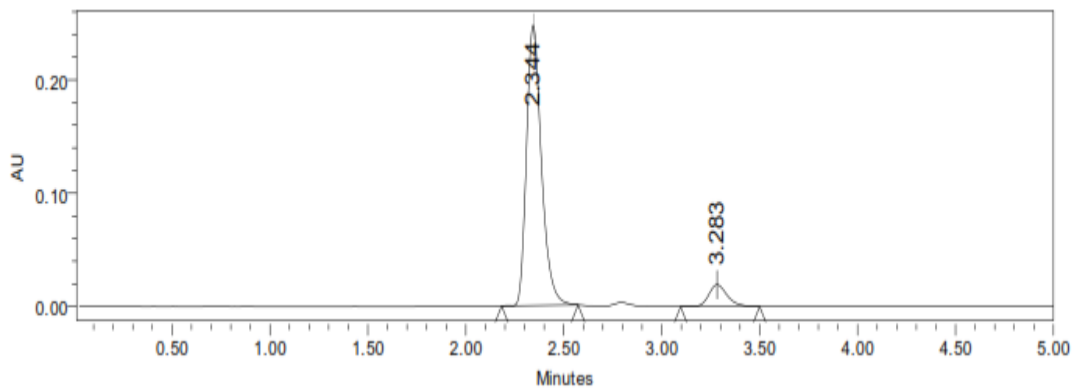


Fig.13: Chromatogram showing standard injection.

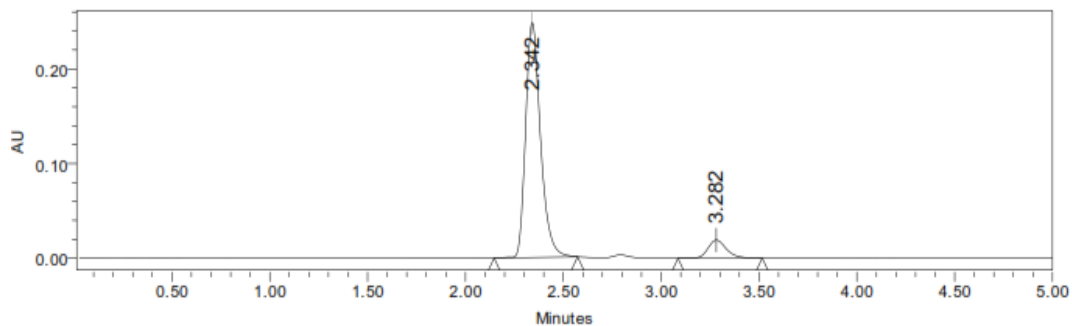


Fig. 14: Chromatogram showing sample injection.

The specificity test was performed for Ivermectin and Albendazole. It was found that there was no interference of impurities in retention time of analytical peak.

#### Linearity

The linearity study was performed for the concentration of 50 ppm to 250 ppm and 5ppm to 25 ppm level. Each level was injected into chromatographic system. The area of each level was used for calculation of correlation coefficient. The chromatograms are shown in Fig.15-17 and results are tabulated in Table. No.4-5. Calibration graph for MET and LINA are shown in Fig.No.41-42.

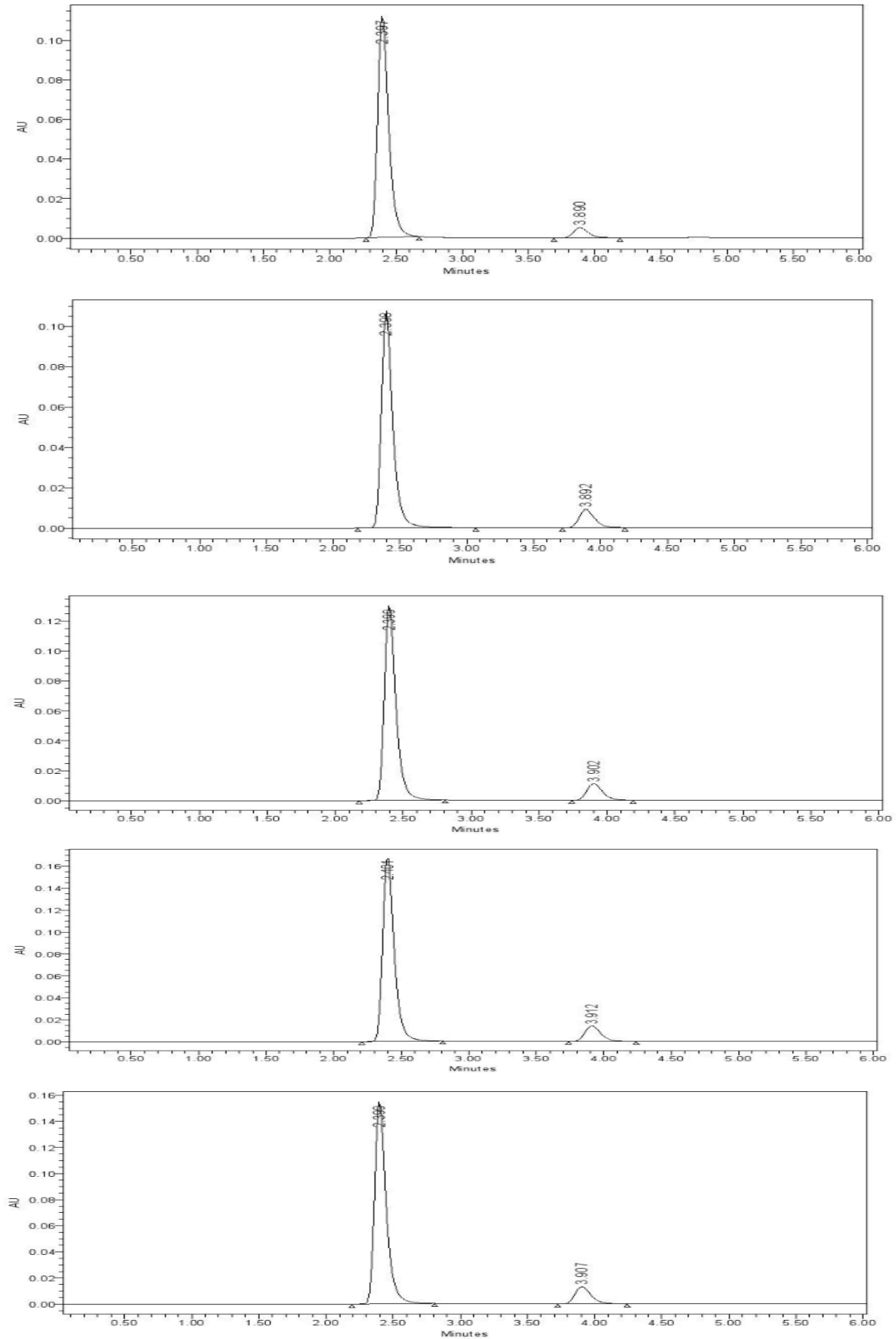
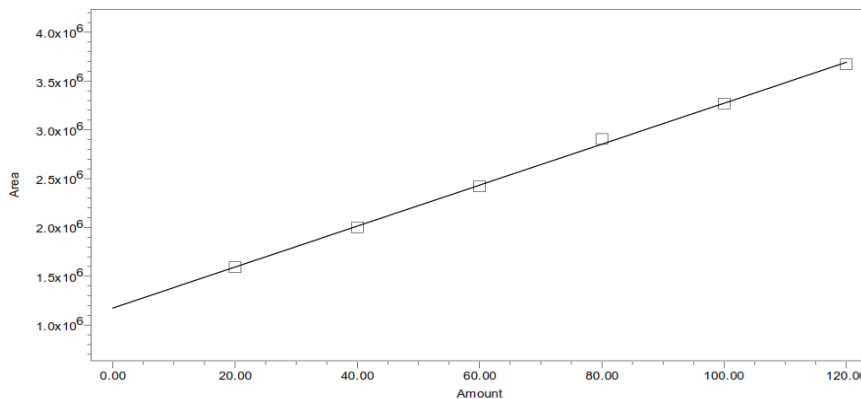


Fig. 15: Chromatograms showing linearity level-1 to level-5 (50ppm-250 ppm of IVERMECTIN and 5ppm - 50ppm of ALBENDAZOLE) injections.

**Linearity Results for Ivermectin**

**Table No. 4.**

S. No.	Linearity Level	Concentration	Area
1	I	50 ppm	471543
2	II	100 ppm	656277
3	III	150 ppm	794999
4	IV	200 ppm	946124
5	V	250 ppm	1002139
Correlation Coefficient			0.999



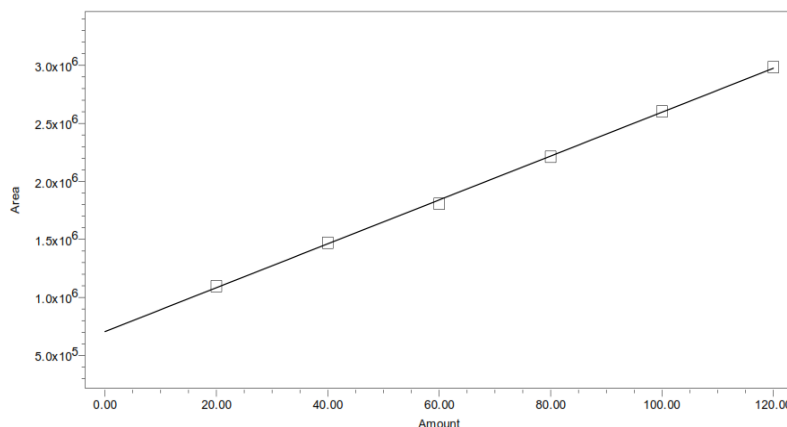
Ivermectin  $r^2 = 0.999$

**Fig. 16: Showing calibration graph for Ivermectin.**

**Linearity Results for Albendazole**

**Table No. 5.**

S. No.	Linearity Level	Concentration	Area
1	I	5ppm	56472
2	II	10 ppm	73841
3	III	15ppm	92655
4	IV	20ppm	111541
5	V	25ppm	130567
Correlation Coefficient			0.999



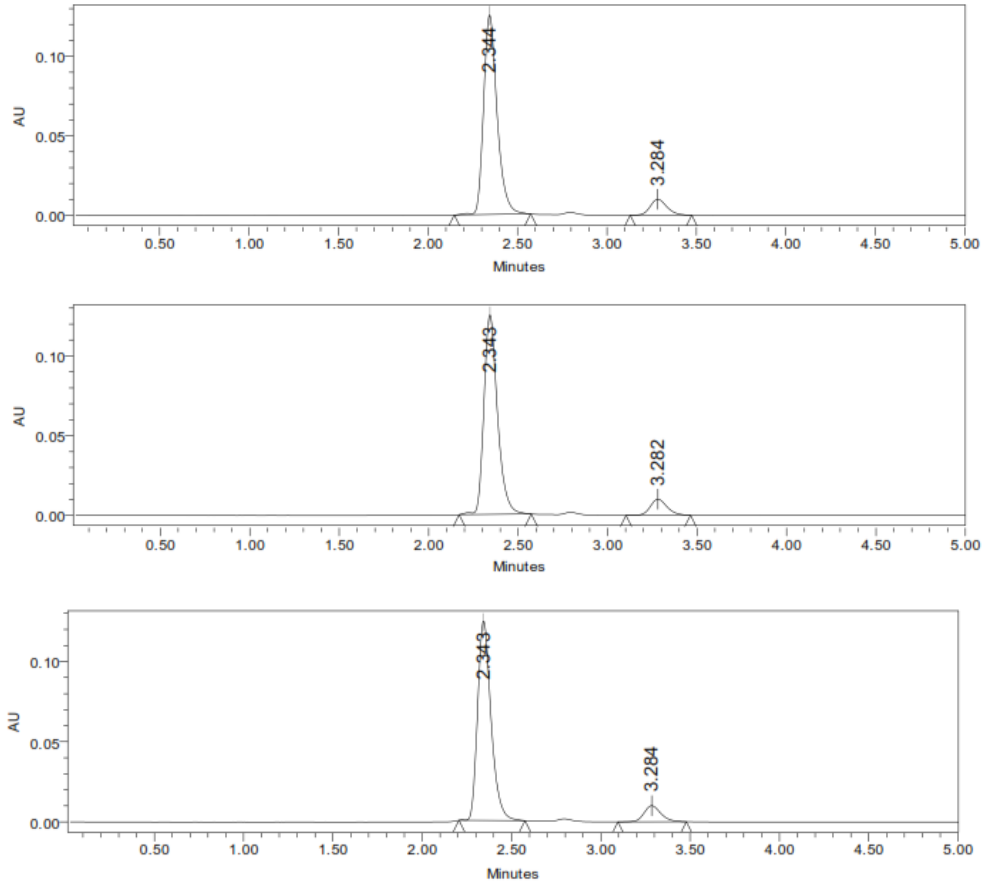
Albendazole  $r^2 = 0.999$

**Fig. 17: Showing calibration graph for Albendazole.**

The linearity study was performed for concentration range of 50.µg-250µg and 5µg-50µg of Ivermectin and Albendazole and the correlation coefficient was found to be 0.999 and 0.999. (NLT 0.999).

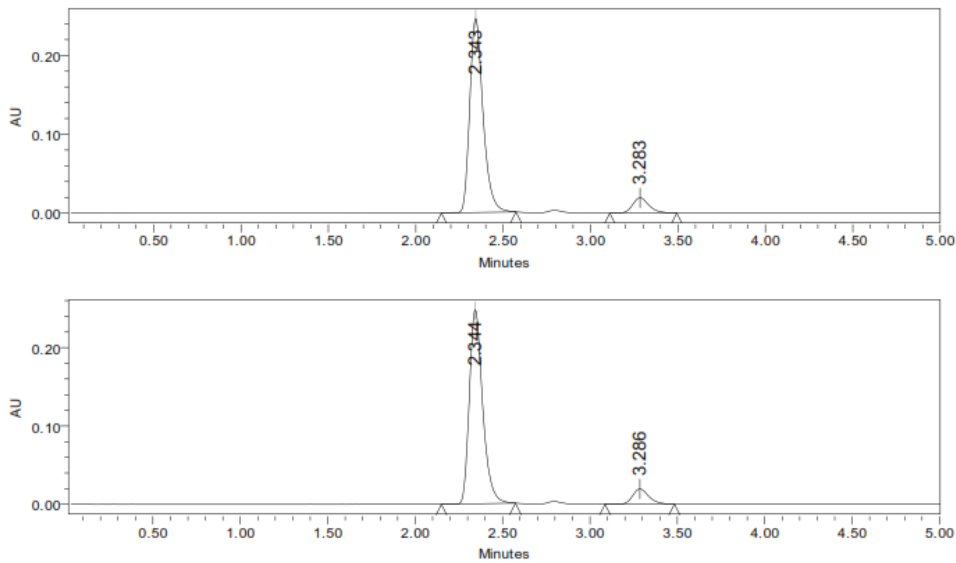
**Accuracy**

The accuracy study was performed for 50%, 100% and 150 % for Ivermectin and Albendazole. Each level was injected in triplicate into chromatographic system. The area of each level was used for calculation of % recovery. Chromatograms are shown in Fig.18-20 and results are tabulated in Table.No.6-7.



**Fig.18: Chromatograms showing Accuracy-50% injection-1,2,3.**

**Accuracy -100%**



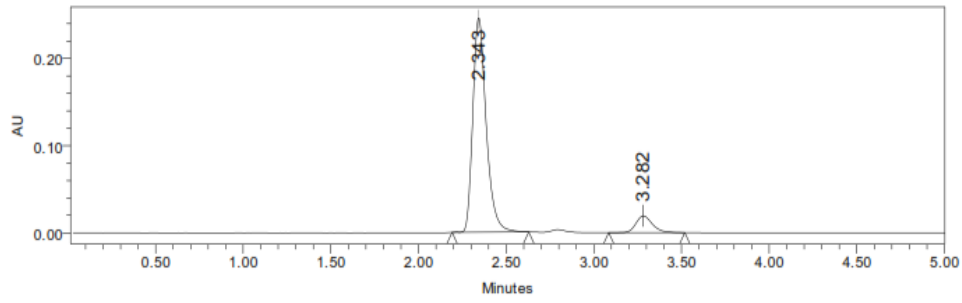


Fig. 19: Chromatogram showing accuracy -100% injection-1,2,3.

Accuracy 150%

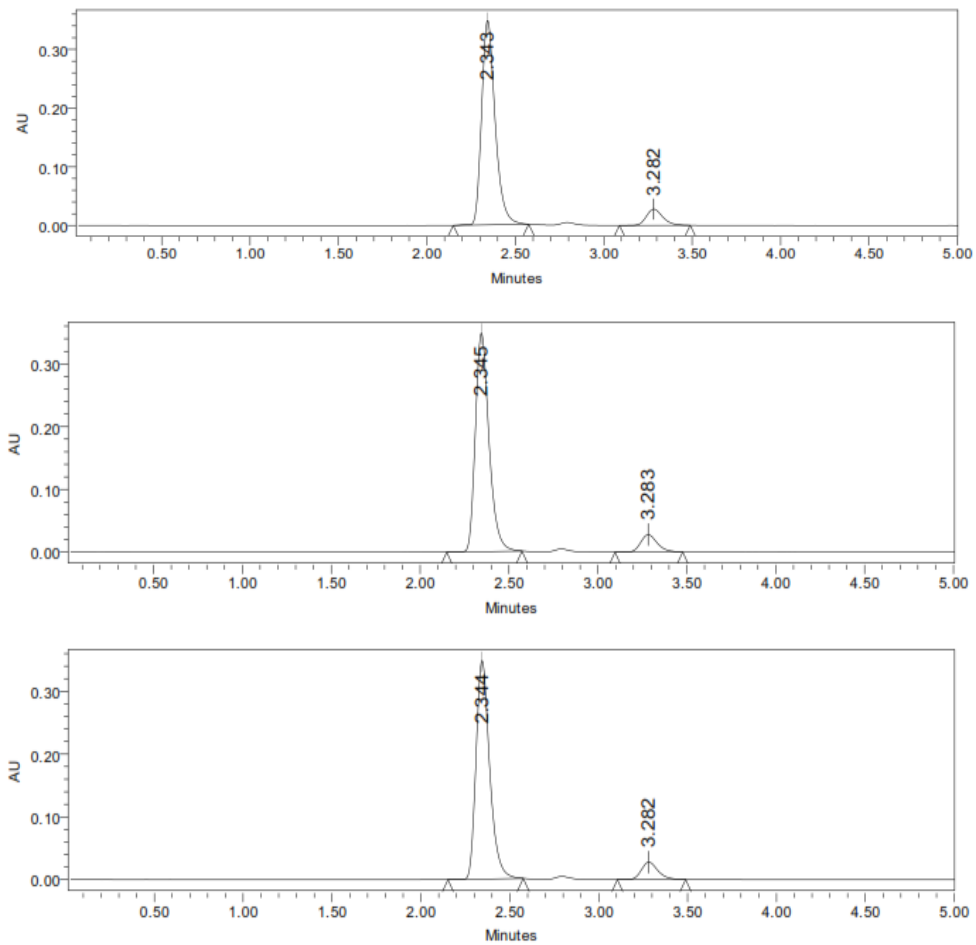


Fig. 20: Chromatogram showing accuracy -150 % injection-1,2,3.

Table No.6: Showing accuracy results for Ivermectin.

% Concentration (at specification level)	Average area	Amount added (mg)	Amount found (mg)	% Recovery	Mean recovery
50%	656659	5	4.96	99.91%	99.56%
100%	1304258	10	9.98	99.18%	
150%	1854608	15	15.02	99.60%	

**Table No. 7: Showing accuracy results for Albendazole.**

%Concentration (at specification level)	Average Area	Amount added(mg)	Amount found (mg)	% Recovery	Mean recovery
50%	5312	0.5	0.99	99.53%	99.47%
100%	24509	1.0	0.05	99.38%	
150%	78517	1.5	0.495	99.52%	

The accuracy study was performed for % recovery of Ivermectin and Albendazole. The % recovery was found to be 99.56% and 99.47% respectively (NLT 98% and NMT 102%).

### Precision

- ❖ Repeatability
- ❖ Intermediate Precision

### Repeatability

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 limits.

### Intermediate precision/Ruggedness

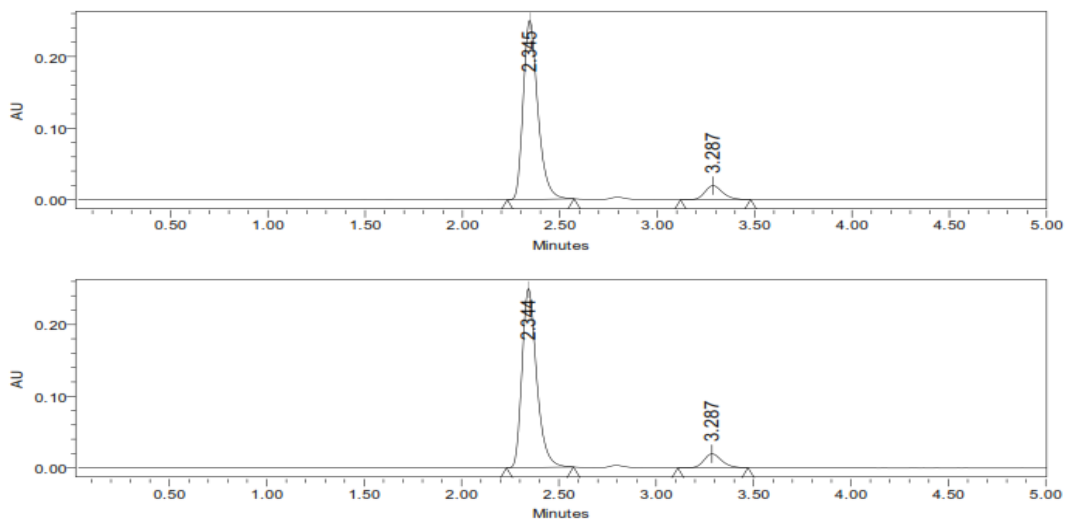
The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

### Repeatability

The precision study was performed for five injections of Ivermectin and Albendazole standard injection was injected into chromatographic system.

The area of each standard injection was used for calculation of %RSD

The chromatograms are shown in Fig.21. and results are tabulated in Table.No.8-9



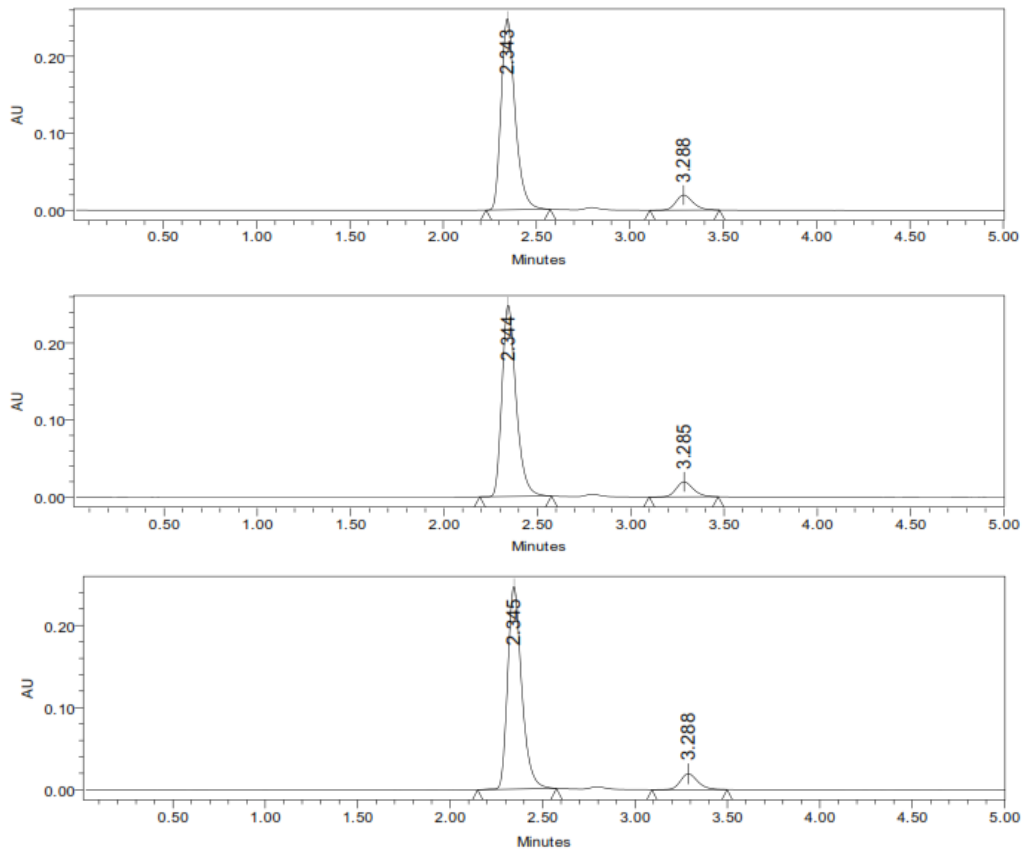


Fig.21: Chromatograms showing precision injections -1 to 5.

Table.No.8. Showing % RSD results for Ivermectin

**Peak Name: Ivermectin**

	Peak Name	RT	Area (μV*sec)	Height (μV)
1	Ivermectin	2.343	1302729	248455
2	Ivermectin	2.344	1309759	248699
3	Ivermectin	2.344	1302947	249526
4	Ivermectin	2.345	1303977	246695
5	Ivermectin	2.345	1303236	250012
Mean			1304529.8	
Std. Dev.			2961.1	
% RSD			0.2	

Table.No.9. Showing %RSD results for Albendazole

**Peak Name: Albendazole**

	Peak Name	RT	Area (μV*sec)	Height (μV)
1	Albendazole	3.285	124263	19458
2	Albendazole	3.287	124487	19634
3	Albendazole	3.287	124175	19600
4	Albendazole	3.288	124894	19327
5	Albendazole	3.288	124495	19540
Mean			124462.7	
Std. Dev.			278.6	
% RSD			0.2	



The Method precision study was performed for the %RSD of Ivermectin and Albendazole was found to be 0.2 and 0.2 (NMT 2).

#### Intermediate precision/Ruggedness

The intermediate precision study was performed for five injections of Ivermectin and Albendazole. Each standard injection was injected into chromatographic system. The area of each standard injection was used for calculation of % RSD. The chromatograms are shown in Fig.22 and results are tabulated in Table 10-11.

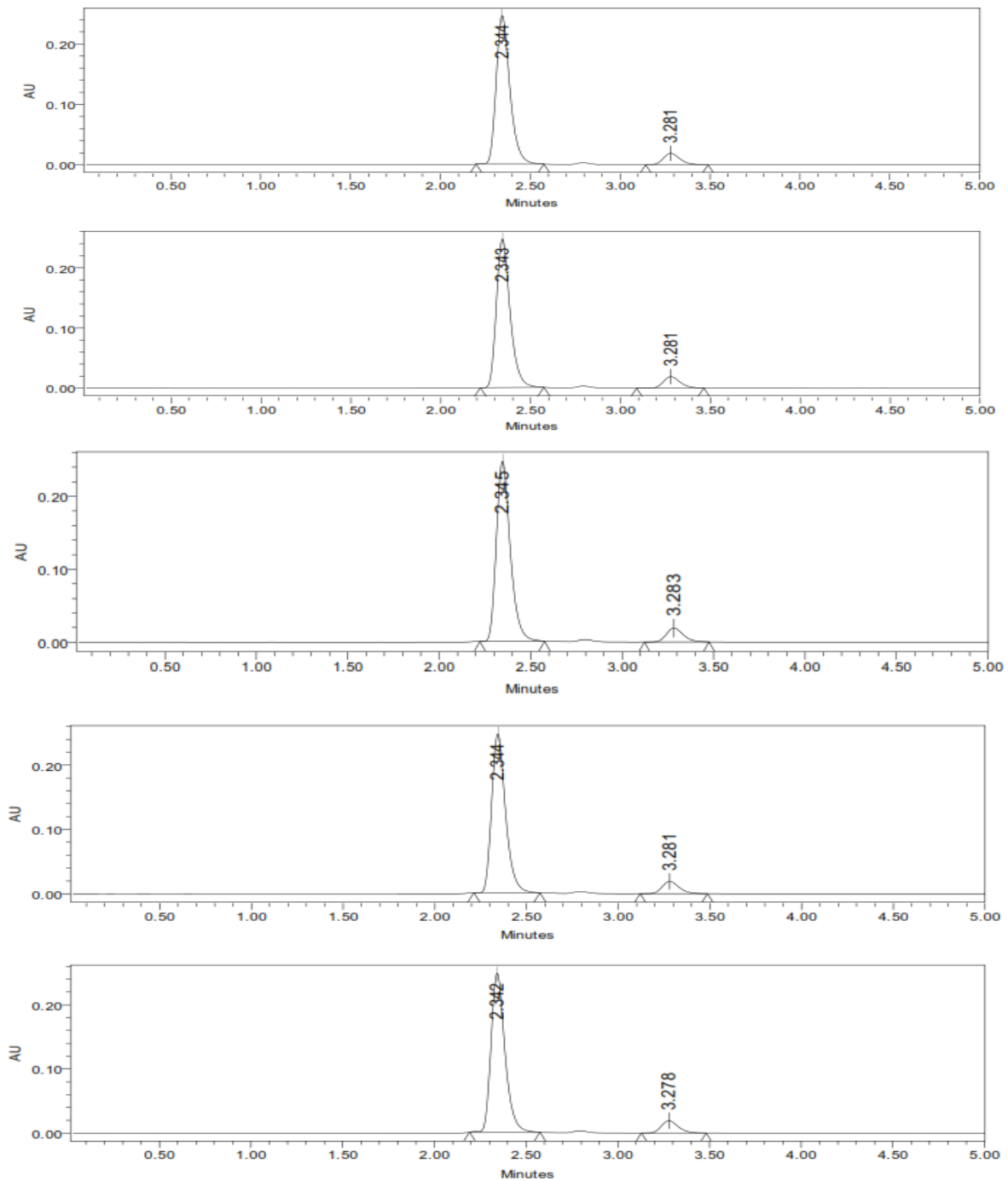


Fig.22: Chromatograms showing intermediate precision injections -1to 5.

Table No.10: Showing results for intermediate precision of Ivermectin.

**Peak Name: Ivermectin**

	Peak Name	RT	Area ( $\mu\text{V}\cdot\text{sec}$ )	Height ( $\mu\text{V}$ )
1	Ivermectin	2.342	1305937	247870
2	Ivermectin	2.343	1306476	246764
3	Ivermectin	2.344	1304520	245696
4	Ivermectin	2.344	1300148	247140
5	Ivermectin	2.345	1308271	247280
Mean			1305070.2	
Std. Dev.			3061.8	
% RSD			0.2	

Table No.11: Showing results for intermediate precision of Albendazole.

**Peak Name: Albendazole**

	Peak Name	RT	Area ( $\mu\text{V}\cdot\text{sec}$ )	Height ( $\mu\text{V}$ )
1	Albendazole	3.278	122962	19165
2	Albendazole	3.281	122487	19115
3	Albendazole	3.281	122632	19073
4	Albendazole	3.281	122626	19003
5	Albendazole	3.283	122702	19123
Mean			122681.8	
Std. Dev.			174.8	
% RSD			0.1	

The intermediate precision was performed for %RSD of Ivermectin and Albendazole was found to be 0.2 and 0.1 respectively (NMT 2).

#### Detection limit

LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

$$\text{LOD} = 3.3 \times \frac{\sigma}{S}$$

Where

$\sigma$  - Standard deviation (SD), S - Slope

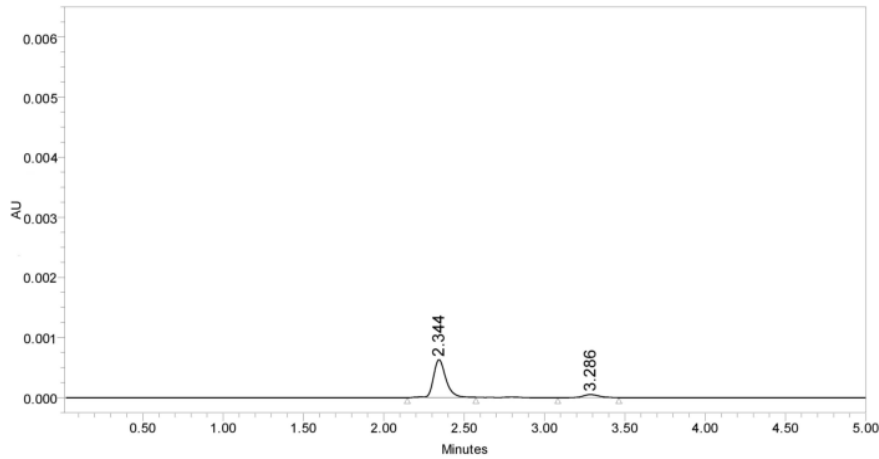


Fig.23: Showing results for Limit of Detection.

Table No 12: Showing results for Limit of Detection.

Drug name	Standard deviation( $\sigma$ )	Slope(s)	LOD( $\mu\text{g}$ )
Ivermectin	382625.50	572175863	3.17
Albendazole	5862.40	467579210	0.0172

The LOD was performed for Ivermectin and Albendazole was found to be 3.17 and 0.0172 respectively.

**Quantitation limit**

LOQ's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula. Again, the standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

$$LOQ = 10 \times \frac{\sigma}{S}$$

Where

$\sigma$  - Standard deviation, S - Slope

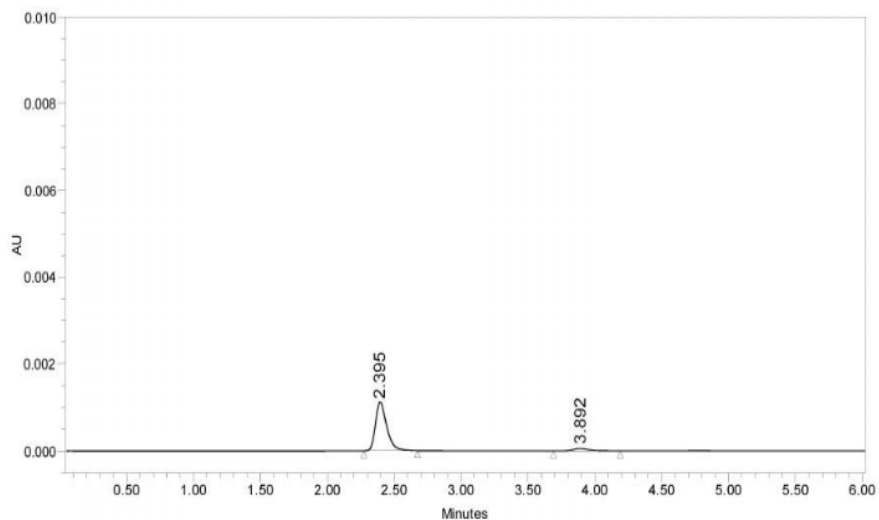


Fig.24: Showing results for Limit of Quantitation.

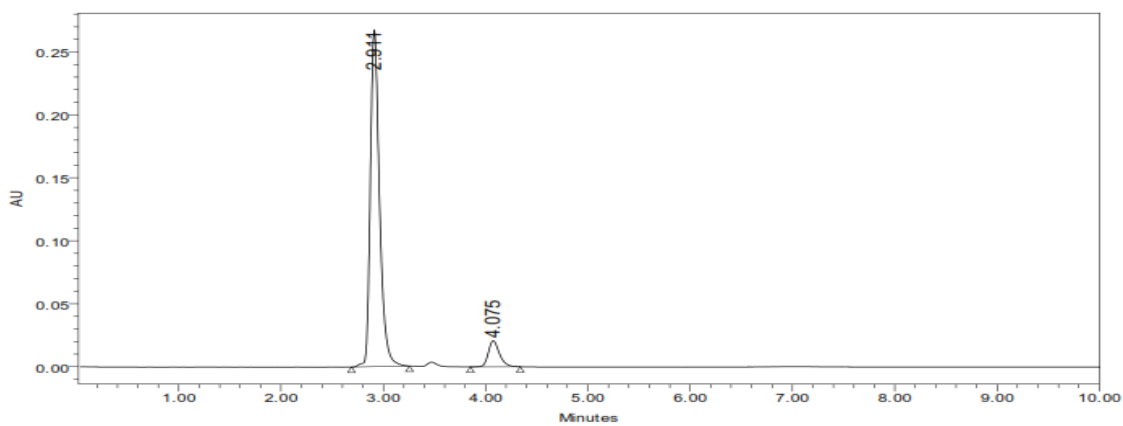
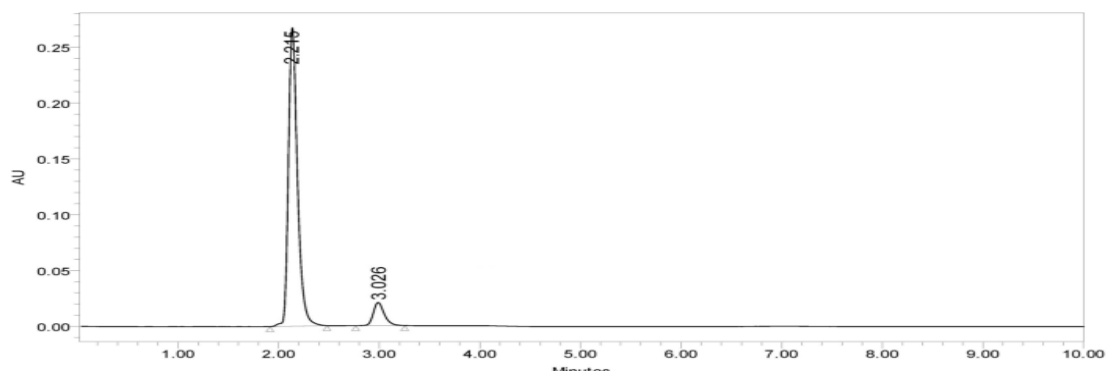
**Table No.13: Showing results for Limit of Quantitation.**

Drug name	Standard deviation( $\sigma$ )	Slope(s)	LOQ( $\mu\text{g}$ )
Ivermectin	381727.80	583265980	5.80
Albendazole	5681.30	469828490	0.212

The LOQ was performed for Ivermectin and Albendazole was found to be 5.80 and 0.212 respectively.

### Robustness

The robustness was performed for the flow rate variations from 0.4ml/min to 0.6ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Ivermectin and Albendazole. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase  $\pm 5\%$ . The chromatograms are shown in Fig.25-26 and results are tabulated in Table.No.14-15.

**Fig. 25: Chromatogram showing less flow rate 0.8ml/min.****Fig. 26: Chromatogram showing less flow rate 1.2 ml/min.**

The results are summarized on evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate  $\pm 0.2\text{ml/min}$ . The method is robust only in less flow condition.

**Table No.14: Showing system suitability results for Ivermectin.**

S. No.	Flow rate (ml/min)	System suitability results	
		USP Plate Count	USP Tailing
1	0.8	5339	1.4
2	1	4668	1.3
3	1.2	5216	1.4

Table No.15: Showing system suitability results for Albendazole.

S. No.	Flow rate (ml/min)	System suitability results	
		USP Plate Count	USP Tailing
1	0.8	7036	1.3
2	1	<b>6089</b>	<b>1.2</b>
3	1.2	6998	1.3

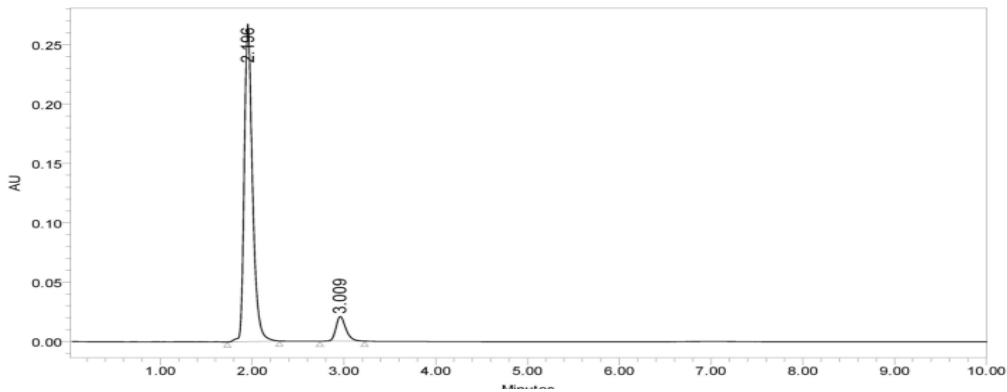


Fig. 27: Chromatogram showing more organic phase ratio.

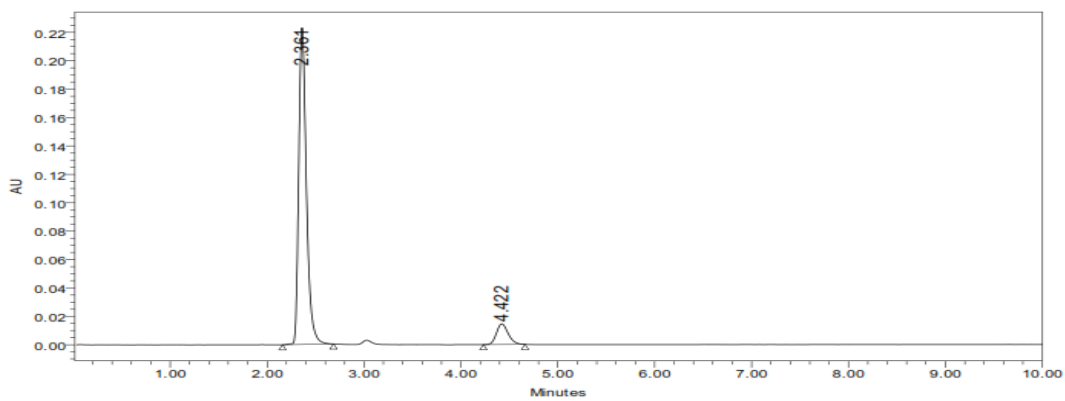


Fig. 28: Chromatogram showing less organic phase ratio.

On evaluation of the above results, it can be concluded that the variation in  $\pm 5\%$ . Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the mobile phase  $\pm 5\%$ .

Table No.16: Showing system suitability results for Ivermectin.

S. No.	Change in organic composition in the mobile phase	System suitability results	
		USP Plate Count	USP Tailing
1	5 % less	6232	1.4
2	<b>*Actual</b>	<b>4668</b>	<b>1.3</b>
3	5 % more	6387	1.4

Table No. 17: Showing system suitability results for Albendazole.

S. No.	Change in organic composition in the mobile phase	System suitability results	
		USP Plate Count	USP Tailing
1	5 % less	5437	1.3
2	<b>*Actual</b>	<b>6089</b>	<b>1.2</b>
3	5 % more	4817	1.2

## CONCLUSION

A new method was established for simultaneous estimation of Ivermectin and Albendazole by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Ivermectin and Albendazole by using ACE C18 column (4.6×150mm) 5 $\mu$ , flow rate was 1.2 ml/min, mobile phase ratio was (70:30 v/v) Methanol:Phosphate buffer pH 3 (pH was adjusted with orthophosphoric acid), detection wavelength was 240nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2690, photo diode array detector 996, Empower-software version-2. The retention times were found to be 2.344 mins and 3.284 mins. The % purity of Ivermectin and Albendazole was found to be 101.27% and 99.97% respectively. The system suitability parameters for Ivermectin and Albendazole such as theoretical plates and tailing factor were found to be 4668, 1.3 and 6089 and 1.2, the resolution was found to be 6.0. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study for Ivermectin and Albendazole was found in concentration range of 50 $\mu$ g-250 $\mu$ g and 5 $\mu$ g-50 $\mu$ g and correlation coefficient ( $r^2$ ) was found to be 0.999 and 0.999, % recovery was found to be 99.56% and 99.48%, %RSD for repeatability was 0.2 and 0.2, % RSD for intermediate precision was 0.2 and 0.1 respectively. The precision study was precise, robust, and repeatable. LOD value was 3.17 and 5.68, and LOQ value was 0.0172 and 0.2125 respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Ivermectin and Albendazole in API and Pharmaceutical dosage form.

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