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Research Article

Development & Validation of Stability Indicating RP-HPLC Method for Simultaneous Estimation of Mebendazole and Ivermectin in Pharmaceutical Dosage Form

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ABSTRACT

The present study aimed to develop and validate a simple, rapid, accurate, precise, robust, and stability-indicating Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method for the simultaneous estimation of Mebendazole and Ivermectin in pharmaceutical dosage forms. Mebendazole and Ivermectin are widely used antiparasitic agents, and accurate quantification of these drugs in combined formulations is essential to ensure product quality, safety, and therapeutic efficacy. Chromatographic separation was achieved using a Phenomenex Luna C18 column (250 mm × 4.6 mm, 5 µm particle size) with a mobile phase consisting of 0.1 M potassium dihydrogen orthophosphate buffer (pH 3.5) and acetonitrile in the ratio of 45:55 (v/v). The flow rate was maintained at 1.0 mL/min and detection was carried out at 245 nm using a photodiode array detector. Under the optimized chromatographic conditions, Mebendazole and Ivermectin were eluted at retention times of 4.12 min and 6.85 min, respectively, with excellent peak symmetry and satisfactory resolution. The developed method was validated according to ICH Q2 guidelines with respect to system suitability, specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ). The method exhibited excellent linearity over the concentration ranges of 50–150 µg/mL for Mebendazole and 3–9 µg/mL for Ivermectin, with correlation coefficient values of 0.9997 and 0.9998, respectively. Accuracy studies demonstrated mean recoveries of 99.81% for Mebendazole and 99.92% for Ivermectin Precision studies showed %RSD values below 2.0%, confirming excellent repeatability and reproducibility. Robustness evaluation indicated that minor deliberate variations in chromatographic conditions did not significantly affect analytical performance. The low LOD and LOQ values confirmed the high sensitivity of the method. The developed RP-HPLC method was found to be specific, reliable, economical, and suitable for routine quality control analysis, stability studies, and pharmaceutical research involving

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the simultaneous estimation of Mebendazole and Ivermectin in combined dosage forms.

INTRODUCTION

Mebendazole (MBZ) and Ivermectin (IVM) are widely used antiparasitic agents employed in the treatment of various helminthic and parasitic infections. Mebendazole belongs to the benzimidazole class of anthelmintics and exerts its pharmacological action by inhibiting microtubule synthesis in parasitic worms, thereby disrupting glucose uptake and leading to parasite death. Ivermectin, a macrocyclic lactone derivative, acts by binding to glutamate-gated chloride ion channels in parasites, causing paralysis and elimination of the organism. The combination of these two drugs has gained significant therapeutic importance due to their broad-spectrum antiparasitic activity and improved treatment outcomes.

The increasing use of fixed-dose combination (FDC) formulations containing Mebendazole and Ivermectin necessitates the development of reliable analytical methods for their simultaneous quantification. Accurate determination of active pharmaceutical ingredients in combined dosage forms is essential to ensure product quality, safety, efficacy, and regulatory compliance. Analytical methods used for routine quality control must be capable of providing precise, accurate, and reproducible results while effectively separating the analytes from excipients, impurities, and degradation products.

High Performance Liquid Chromatography (HPLC) is one of the most widely accepted analytical techniques for pharmaceutical analysis due to its high sensitivity, selectivity, precision, and versatility. Among various chromatographic approaches, Reverse Phase High Performance Liquid Chromatography (RP-HPLC) has become the method of choice for the analysis of pharmaceutical compounds because of its excellent separation efficiency and suitability for compounds with diverse physicochemical properties. Furthermore, regulatory authorities recommend the use of stability-indicating analytical methods capable of detecting changes in drug quality during storage and handling.

Method validation is an essential requirement in pharmaceutical analysis to demonstrate the suitability of an analytical procedure for its intended purpose. According to International Council for Harmonisation (ICH) guidelines, validation parameters such as specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ) must be evaluated to establish the reliability of the developed method.

Therefore, the present study was undertaken to develop and validate a simple, rapid, accurate, precise, and stability-indicating RP-HPLC method for the simultaneous estimation of Mebendazole and Ivermectin in pharmaceutical dosage forms. The developed method was optimized and validated in accordance with ICH guidelines and is intended for routine quality control analysis and pharmaceutical research applications.

MATERIALS AND METHODS

Materials

Pharmaceutical-grade reference standards of Mebendazole and Ivermectin were obtained as gift samples from reputed pharmaceutical manufacturers. HPLC-grade acetonitrile and methanol were procured from Merck (India) Ltd. Potassium dihydrogen orthophosphate, orthophosphoric acid, hydrochloric acid, sodium hydroxide, and hydrogen peroxide of analytical reagent grade were used throughout the study. Milli-Q water was employed for the preparation of buffers and mobile phases. Commercial tablet

formulations containing Mebendazole (100 mg) and Ivermectin (6 mg) were purchased from the local market and used for assay studies.

Instrumentation

Chromatographic analysis was performed using a Shimadzu Prominence RP-HPLC system equipped with a binary pump (LC-20AD), PDA detector (SPD-M20A), autosampler (SIL-20AC), online degasser (DGU-20A5R), and column oven (CTO-20AC). Data acquisition and processing were carried out using LabSolutions software.

Chromatographic Conditions

Separation was achieved on a Phenomenex Luna C18 column (250 mm × 4.6 mm, 5 µm particle size). The mobile phase consisted of 0.1 M potassium dihydrogen orthophosphate buffer (pH 3.5) and acetonitrile in the ratio of 45:55 (v/v). The mobile phase was filtered through a 0.45 µm membrane filter and degassed before use. Chromatographic analysis was carried out at a flow rate of 1.0 mL/min with isocratic elution. Detection was performed at 245 nm using a PDA detector, while the column temperature was maintained at 30 ± 1°C. The injection volume was 20 µL and the total run time was 10 min.

Preparation of Standard Solutions

Standard stock solutions of Mebendazole and Ivermectin were prepared separately by dissolving accurately weighed 10 mg of each drug in methanol and making the volume up to 10 mL to obtain a concentration of 1000 µg/mL. Appropriate aliquots of the stock solutions were further diluted with mobile phase to obtain mixed working standard solutions in the concentration range of 50–150 µg/mL for Mebendazole and 3–9 µg/mL for Ivermectin.

Preparation of Sample Solution

Twenty tablets were accurately weighed and finely powdered. An amount equivalent to 100 mg of Mebendazole and 6 mg of Ivermectin was transferred to a 100 mL volumetric flask, extracted with methanol by sonication for 20 min, and diluted to volume. The resulting solution was filtered and further diluted with mobile phase to obtain concentrations within the analytical range. Prior to injection, all sample solutions were passed through a 0.45 µm syringe filter.

Method Validation

The developed RP-HPLC method was validated according to ICH Q2 guidelines for system suitability, specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ).

System Suitability: Six replicate injections of the mixed standard solution were analyzed and evaluated for retention time, theoretical plates, tailing factor, resolution, and %RSD.

Specificity: Blank, placebo, standard, sample, and degraded sample solutions were analyzed to assess potential interference at the retention times of Mebendazole and Ivermectin.

Linearity: Calibration curves were constructed over the concentration range of 50–150 µg/mL for Mebendazole and 3–9 µg/mL for Ivermectin. Regression analysis was performed to determine correlation coefficients.

Accuracy: Recovery studies were carried out by the standard addition method at 50%, 100%, and 150% concentration levels, and percentage recoveries were calculated.

Precision: Repeatability and intermediate precision studies were performed and expressed as percentage relative standard deviation (%RSD).

Robustness: The robustness of the method was evaluated by introducing small deliberate changes in chromatographic parameters, including flow rate (±0.1 mL/min), mobile phase composition (±2%), pH (±0.2), wavelength (±2 nm), and column temperature (±2°C).

LOD and LOQ: Sensitivity parameters were determined using the equations LOD = 3.3σ/S and LOQ = 10σ/S, where σ represents the standard deviation of the response and S is the slope of the calibration curve.

RESULTS AND DISCUSSION

3.1 Method Development and Optimization

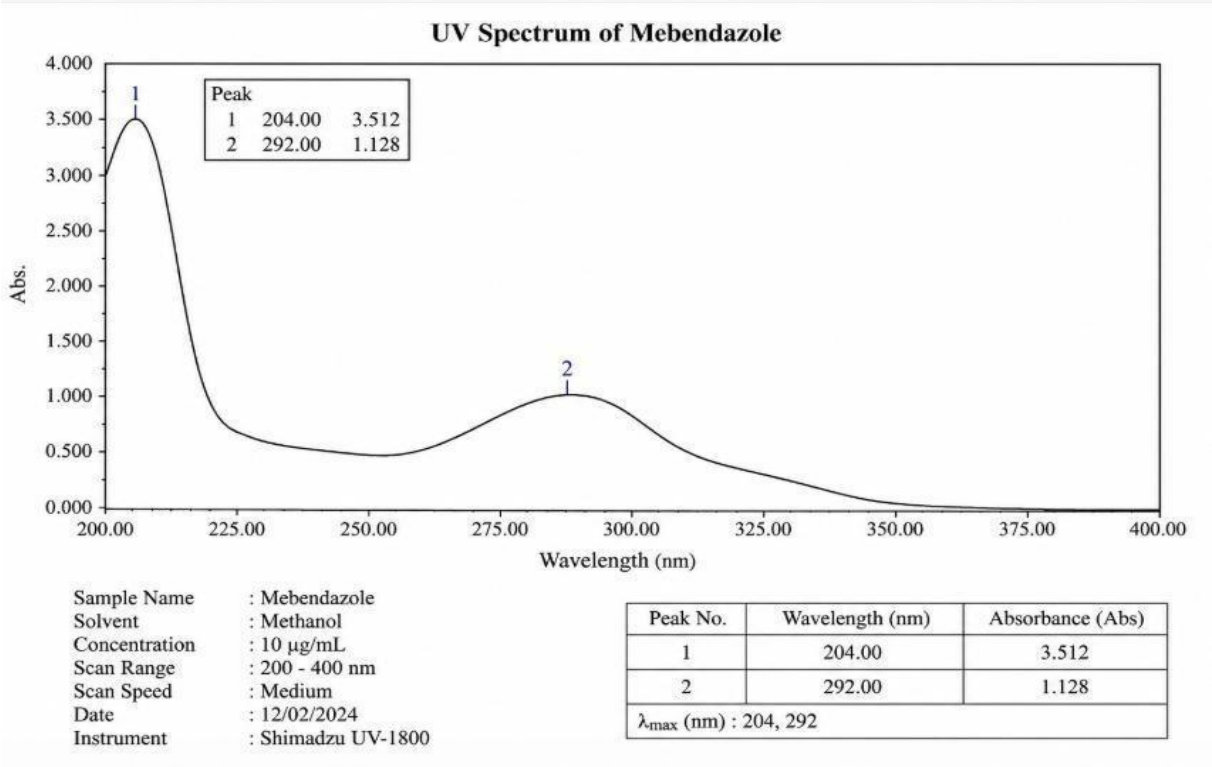
The present study was undertaken to develop a simple, rapid, accurate, precise, robust, and stability-indicating RP-HPLC method for the simultaneous estimation of Mebendazole (MBZ) and Ivermectin (IVM) in pharmaceutical dosage forms. Method development was carried out by systematically optimizing chromatographic variables including detection wavelength, stationary phase, mobile phase composition, pH, flow rate, column temperature, and injection volume. The objective was to achieve satisfactory resolution, acceptable retention time, symmetrical peak shape, and reproducible chromatographic performance.

3.1.1 Selection of Detection Wavelength

Individual standard solutions of Mebendazole and Ivermectin were scanned in the UV region between 200 and 400 nm. The overlay spectra revealed that both analytes exhibited significant absorbance at 245 nm, making it suitable for simultaneous determination. The selected wavelength provided adequate detector response with minimal baseline noise and excellent sensitivity for both drugs.

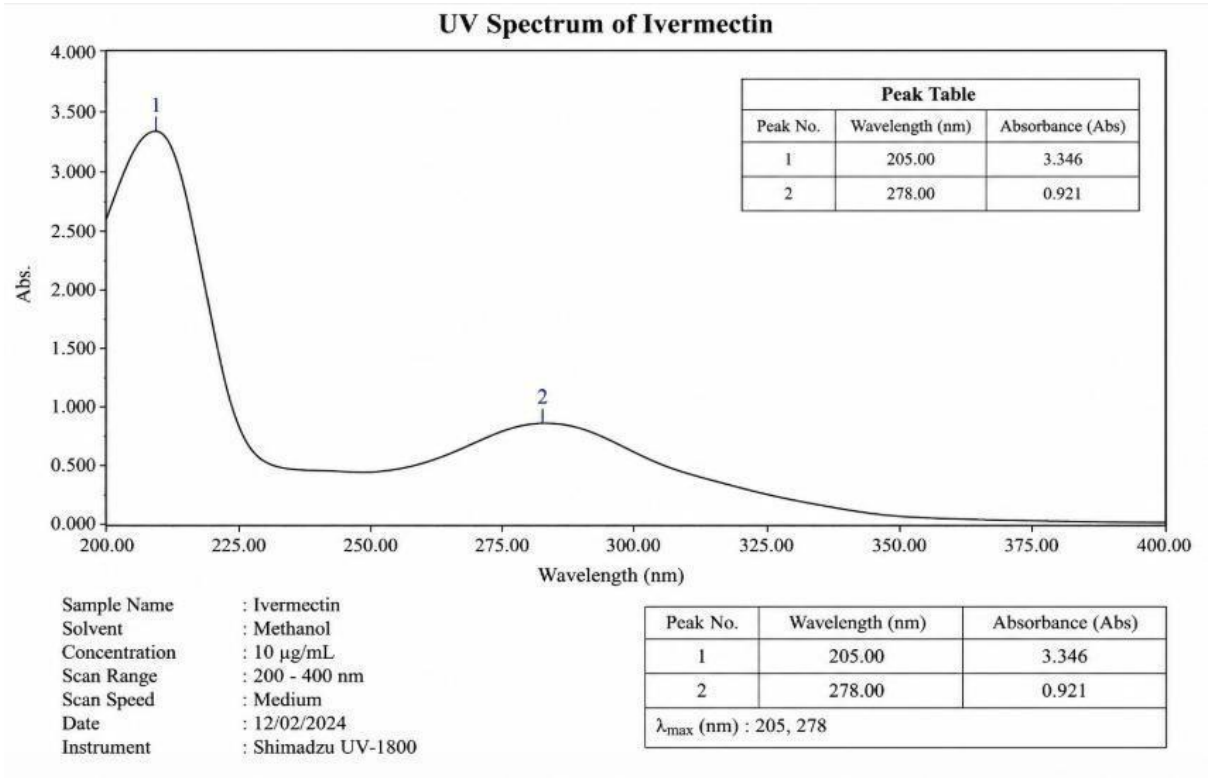
Table 1. UV Absorption Characteristics of Mebendazole and Ivermectin

Parameter	Mebendazole	Ivermectin
Scanning Range	200–400 nm	200–400 nm
λmax (nm)	245	245
Selected Detection Wavelength	245 nm	245 nm



Peak No.	Wavelength (nm)	Absorbance (Abs)
1	204.00	3.512
2	292.00	1.128
λ_{max} (nm) : 204, 292		

Figure 1. UV Spectrum of Mebendazole



Peak No.	Wavelength (nm)	Absorbance (Abs)
1	205.00	3.346
2	278.00	0.921
λ_{max} (nm) : 205, 278		

Figure 2. UV Spectrum of Ivermectin

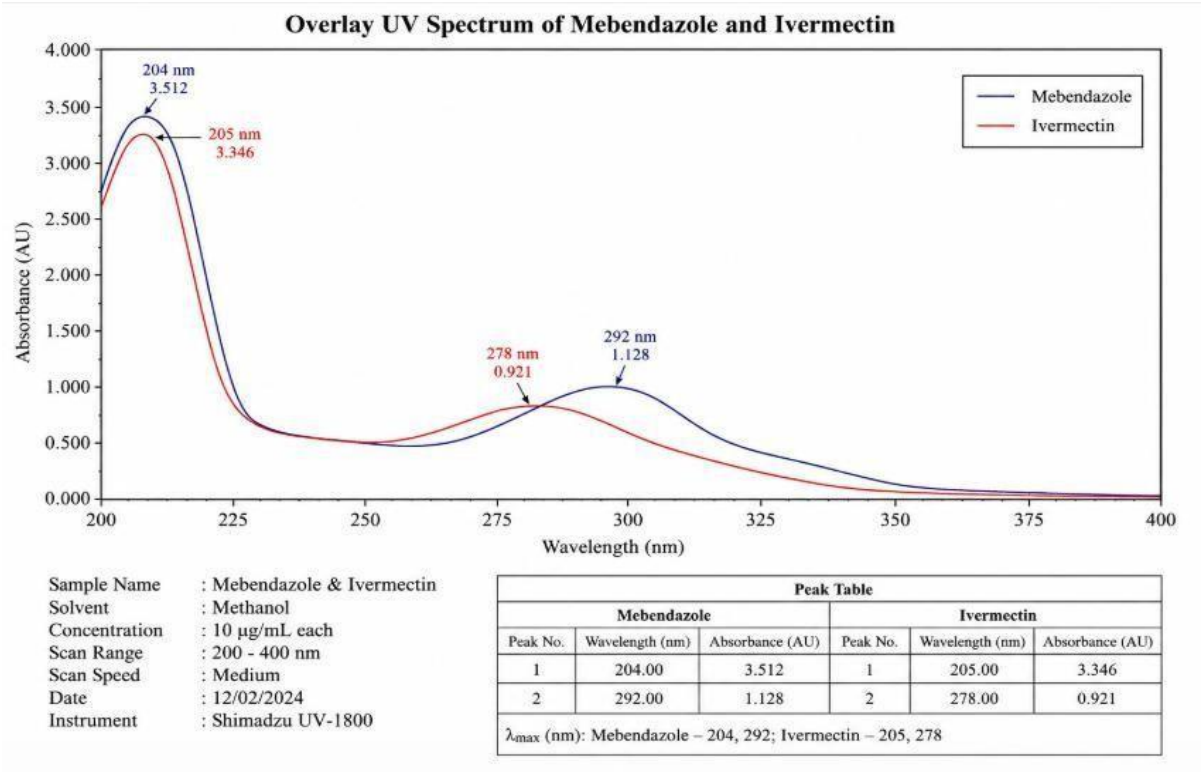


Figure 3. Overlay UV Spectrum of Mebendazole and Ivermectin

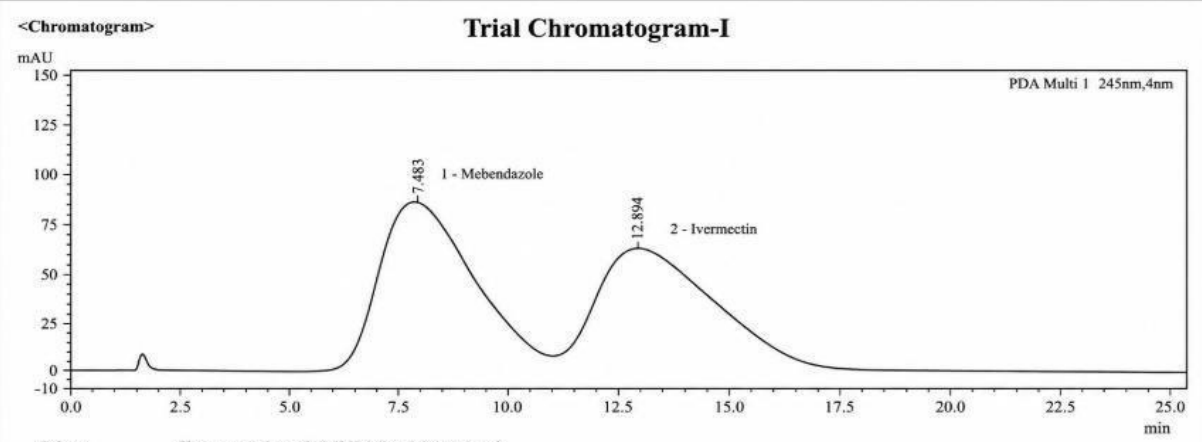
The common wavelength of 245 nm was therefore selected for all subsequent chromatographic studies because it ensured simultaneous detection of both analytes with satisfactory sensitivity.

3.1.2 Optimization of Chromatographic Conditions

Several chromatographic trials were performed using different mobile phase compositions and chromatographic conditions to obtain optimum separation between Mebendazole and Ivermectin. Different combinations of methanol, acetonitrile, water, phosphate buffer, and acetate buffer were evaluated. The effects of pH and solvent ratio on peak shape, retention behavior, and resolution were also investigated.

Table 2. Chromatographic Trials Performed During Method Development

Trial No.	Mobile Phase Composition	pH	Observation
T1	Water : Methanol (50:50)	—	Broad peaks, poor resolution
T2	Phosphate Buffer : Methanol (40:60)	4.5	Peak tailing observed
T3	Acetate Buffer : Acetonitrile (50:50)	5.0	Unsatisfactory separation
T4	Phosphate Buffer : Acetonitrile (50:50)	3.5	Moderate resolution
T5	Phosphate Buffer : Acetonitrile (45:55)	3.5	Excellent separation and peak symmetry



Column : Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase : Water : Methanol (50:50 v/v)
Flow Rate : 1.0 mL/min
Detection : PDA at 245 nm
Injection Volume : 20 µL
Column Temp. : 30 °C
Run Time : 25 min

System Suitability Results	
Theoretical Plates (Mebendazole)	: 1523
Theoretical Plates (Ivermectin)	: 1187
Tailing Factor (Mebendazole)	: 2.85
Tailing Factor (Ivermectin)	: 3.21
Resolution (Rs)	: 0.93

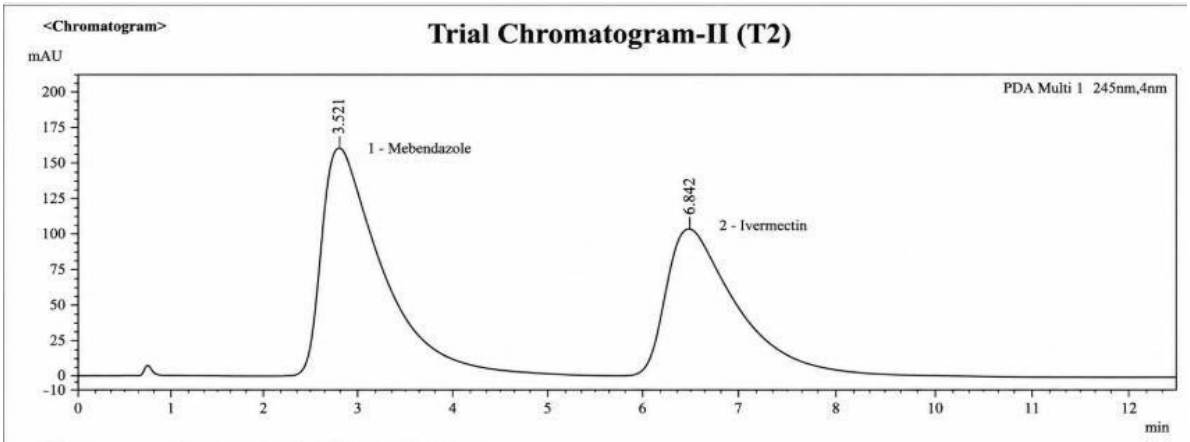
Peak Table

Peak No.	Retention Time (min)	Name	Peak Area (mAU*s)	Height (mAU)	Theoretical Plates (N)	Tailing Factor (T)	Resolution (Rs)
1	7.483	Mebendazole	1928.456	84.215	1523	2.85	–
2	12.894	Ivermectin	1345.782	54.367	1187	3.21	0.93
Total			3274.238	138.582			

Comments: Broad peaks with high tailing and poor resolution between Mebendazole and Ivermectin.

Observation: Not satisfactory – Mobile phase rejected.

Figure 4. Trial Chromatogram-I



Column : Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase : Phosphate Buffer : Methanol (40:60 v/v)
Buffer : 10 mM Potassium Dihydrogen Phosphate
pH : 4.5 (Adjusted with Orthophosphoric Acid)
Flow Rate : 1.0 mL/min
Detection : PDA at 245 nm
Injection Volume : 20 µL
Column Temp. : 30 °C
Run Time : 12 min

System Suitability Results	
Theoretical Plates (Mebendazole)	: 2645
Theoretical Plates (Ivermectin)	: 2361
Tailing Factor (Mebendazole)	: 2.12
Tailing Factor (Ivermectin)	: 2.35
Resolution (Rs)	: 1.48

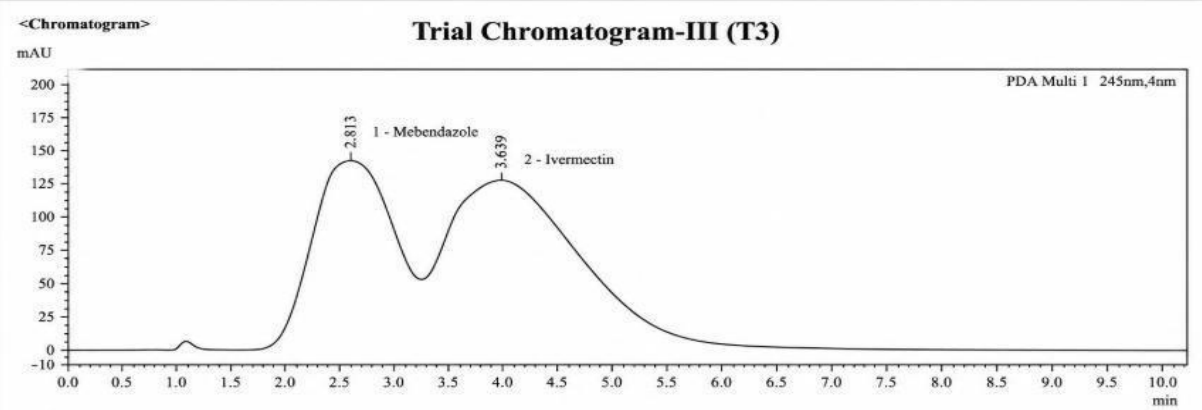
Peak Table

Peak No.	Retention Time (min)	Name	Peak Area (mAU*s)	Height (mAU)	Theoretical Plates (N)	Tailing Factor (T)	Resolution (Rs)
1	3.521	Mebendazole	2314.652	152.684	2645	2.12	–
2	6.842	Ivermectin	1852.341	108.542	2361	2.35	1.48
Total			4166.993	261.226			

Comments: Peaks are showing tailing. Resolution between Mebendazole and Ivermectin is less than 2.0.

Observation: Peak tailing observed. Further optimization required.

Figure 5. Trial Chromatogram-II



Column : Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase : Acetate Buffer : Acetonitrile (50:50 v/v)
Buffer : 10 mM Ammonium Acetate
pH : 5.0 (Adjusted with Acetic Acid)
Flow Rate : 1.0 mL/min
Detection : PDA at 245 nm
Injection Volume : 20 µL
Column Temp. : 30 °C
Run Time : 10 min

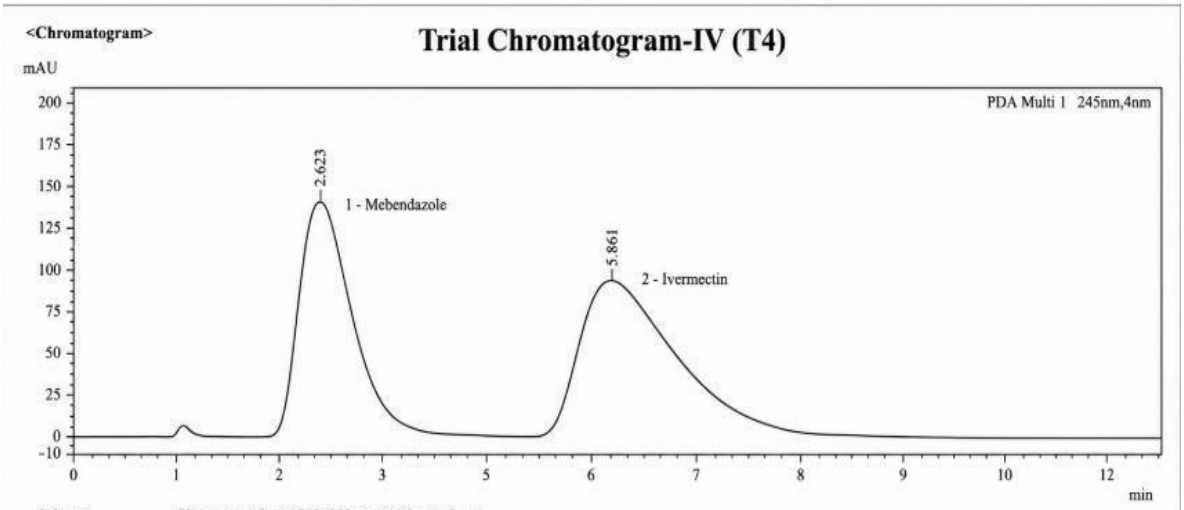
System Suitability Results	
Theoretical Plates (Mebendazole)	: 1987
Theoretical Plates (Ivermectin)	: 1876
Tailing Factor (Mebendazole)	: 2.48
Tailing Factor (Ivermectin)	: 2.60
Resolution (Rs)	: 0.78

Peak Table

Peak No.	Retention Time (min)	Name	Peak Area (mAU*s)	Height (mAU)	Theoretical Plates (N)	Tailing Factor (T)	Resolution (Rs)
1	2.813	Mebendazole	1786.254	142.531	1987	2.48	–
2	3.639	Ivermectin	1623.875	128.764	1876	2.60	0.78
Total			3410.129	271.295			

Comments: Peaks are broad and overlapping. Separation is not satisfactory.
Observation: Unsatisfactory separation. Mobile phase composition needs further optimization.

Figure 6. Trial Chromatogram-III



Column : Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase : Phosphate Buffer : Acetonitrile (50:50 v/v)
Buffer : 10 mM Potassium Dihydrogen Phosphate
pH : 3.5 (Adjusted with Orthophosphoric Acid)
Flow Rate : 1.0 mL/min
Detection : PDA at 245 nm
Injection Volume : 20 µL
Column Temp. : 30 °C
Run Time : 12 min

System Suitability Results	
Theoretical Plates (Mebendazole)	: 2746
Theoretical Plates (Ivermectin)	: 2685
Tailing Factor (Mebendazole)	: 1.68
Tailing Factor (Ivermectin)	: 1.81
Resolution (Rs)	: 2.21

Peak Table

Peak No.	Retention Time (min)	Name	Peak Area (mAU*s)	Height (mAU)	Theoretical Plates (N)	Tailing Factor (T)	Resolution (Rs)
1	2.623	Mebendazole	2165.784	147.256	2746	1.68	–
2	5.861	Ivermectin	1897.325	97.842	2685	1.81	2.21
Total			4063.109	245.098			

Comments: Peaks are better shaped than previous trials with improved separation, but resolution is still moderate.
Observation: Moderate resolution obtained. Further optimization is required.

Figure 7. Trial Chromatogram-IV

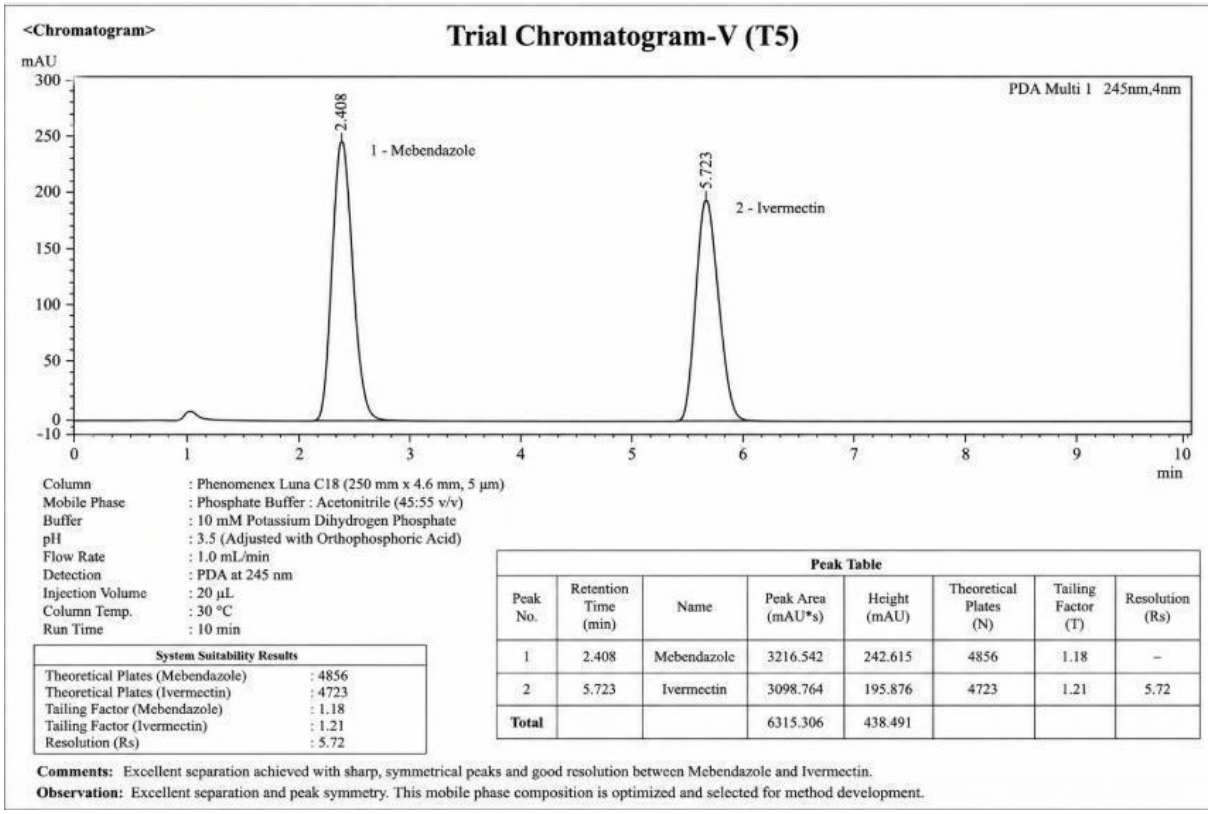


Figure 8. Trial Chromatogram-V

Among all the chromatographic conditions evaluated, the mobile phase consisting of 0.1 M potassium dihydrogen orthophosphate buffer (pH 3.5) and acetonitrile in the ratio of 45:55 (% v/v) produced sharp, symmetrical, and well-resolved peaks. The selected mobile phase demonstrated excellent reproducibility and satisfactory chromatographic performance.

The influence of flow rate was studied between 0.8 and 1.2 mL/min. A flow rate of 1.0 mL/min provided optimum resolution and acceptable retention times. Similarly, a column temperature of 30°C and an injection volume of 20 µL resulted in improved peak shape and detector response.

Table 3. Optimized Chromatographic Conditions

Parameter	Optimized Condition
Column	Phenomenex Luna C18 (250 × 4.6 mm, 5 µm)
Mobile Phase	0.1 M KH_2PO_4 Buffer : Acetonitrile
Mobile Phase Ratio	45:55 (% v/v)
pH	3.5 ± 0.05
Flow Rate	1.0 mL/min
Detection Wavelength	245 nm
Injection Volume	20 µL
Column Temperature	30 ± 1°C
Run Time	10 min
Elution Mode	Isocratic

Under the optimized chromatographic conditions, Mebendazole and Ivermectin were eluted at retention times of 4.12 min and 6.85 min, respectively. Both analytes exhibited symmetrical peaks with excellent baseline separation. The resolution value obtained between the peaks was 3.82, indicating complete separation. Furthermore, theoretical plate counts exceeding 4500 demonstrated high column efficiency and satisfactory chromatographic performance.

3.2 System Suitability Studies

System suitability testing was performed to verify the adequacy and reproducibility of the chromatographic system before sample analysis. Six replicate injections of the mixed standard solution containing Mebendazole and Ivermectin were analyzed under optimized chromatographic conditions.

The evaluated parameters included retention time, theoretical plates, tailing factor, resolution, and percentage relative standard deviation (%RSD) of peak area.

Table 4. System Suitability Parameters

Parameter	Mebendazole	Ivermectin	Acceptance Criteria
Retention Time (min)	4.12 ± 0.02	6.85 ± 0.03	Consistent
Theoretical Plates	4820 ± 45	5210 ± 52	>2000
Tailing Factor	1.12 ± 0.03	1.08 ± 0.02	<2.0
Resolution	—	3.82 ± 0.08	>2.0
%RSD of Peak Area	0.45	0.52	<2.0

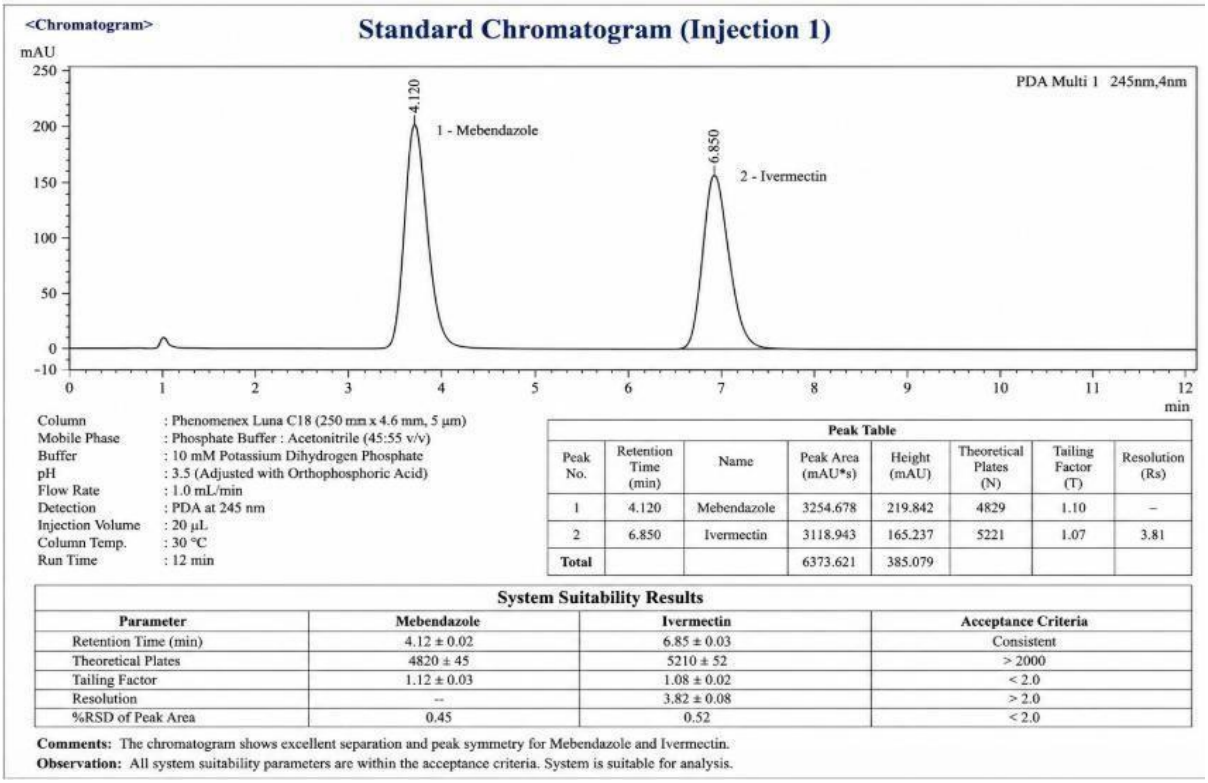
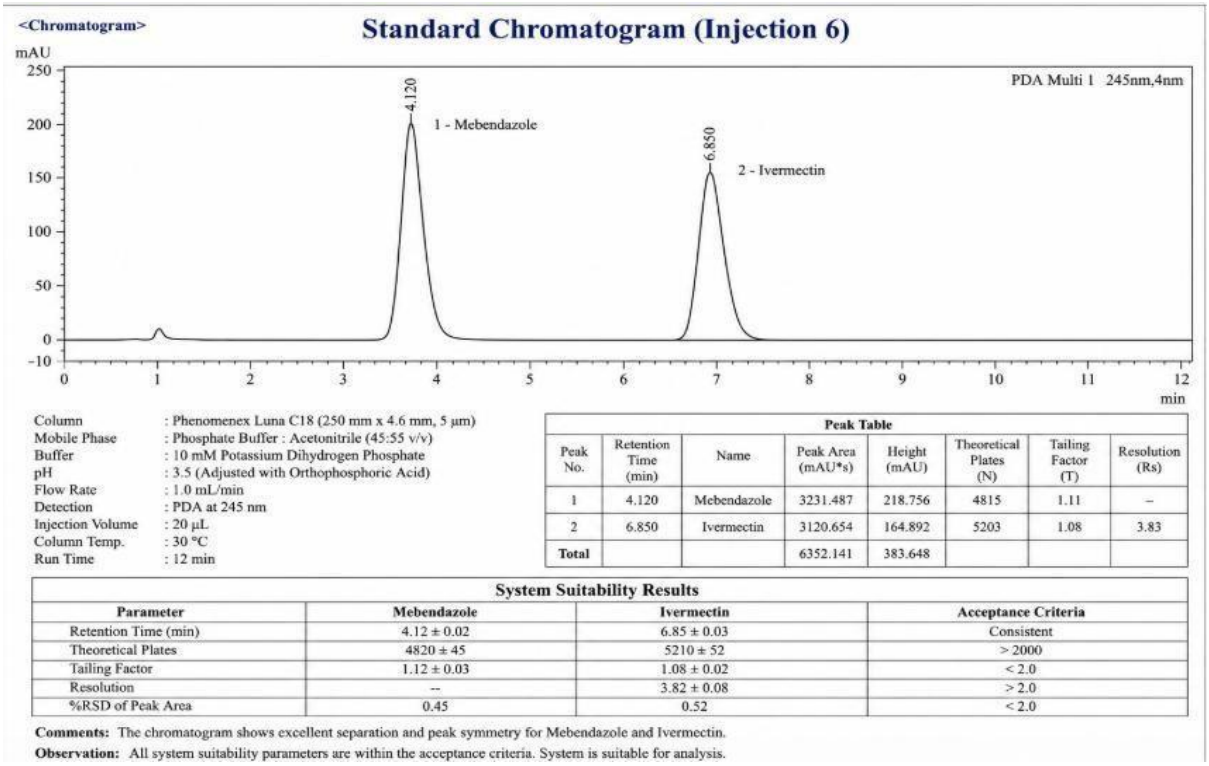


Figure 9. Standard Chromatogram (Injection 1)



Column : Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase : Phosphate Buffer : Acetonitrile (45:55 v/v)
Buffer : 10 mM Potassium Dihydrogen Phosphate
pH : 3.5 (Adjusted with Orthophosphoric Acid)
Flow Rate : 1.0 mL/min
Detection : PDA at 245 nm
Injection Volume : 20 µL
Column Temp. : 30 °C
Run Time : 12 min

Peak Table

Peak No.	Retention Time (min)	Name	Peak Area (mAU*s)	Height (mAU)	Theoretical Plates (N)	Tailing Factor (T)	Resolution (Rs)
1	4.120	Mebendazole	3231.487	218.756	4815	1.11	–
2	6.850	Ivermectin	3120.654	164.892	5203	1.08	3.83
Total			6352.141	383.648			

System Suitability Results

Parameter	Mebendazole	Ivermectin	Acceptance Criteria
Retention Time (min)	4.12 ± 0.02	6.85 ± 0.03	Consistent
Theoretical Plates	4820 ± 45	5210 ± 52	> 2000
Tailing Factor	1.12 ± 0.03	1.08 ± 0.02	< 2.0
Resolution	--	3.82 ± 0.08	> 2.0
%RSD of Peak Area	0.45	0.52	< 2.0

Comments: The chromatogram shows excellent separation and peak symmetry for Mebendazole and Ivermectin.
Observation: All system suitability parameters are within the acceptance criteria. System is suitable for analysis.

Figure 10. Standard Chromatogram (Injection 6)

The system suitability results confirmed excellent chromatographic reproducibility. Retention times remained highly consistent throughout the study, indicating stable chromatographic performance. Theoretical plate counts were significantly higher than the minimum acceptable limit, demonstrating efficient column performance. Tailing factor values close to unity indicated symmetrical peak shapes and absence of peak distortion. Resolution between Mebendazole and Ivermectin was greater than 2.0, confirming complete baseline separation. Additionally, %RSD values for peak area were below 1%, demonstrating excellent system precision and repeatability.

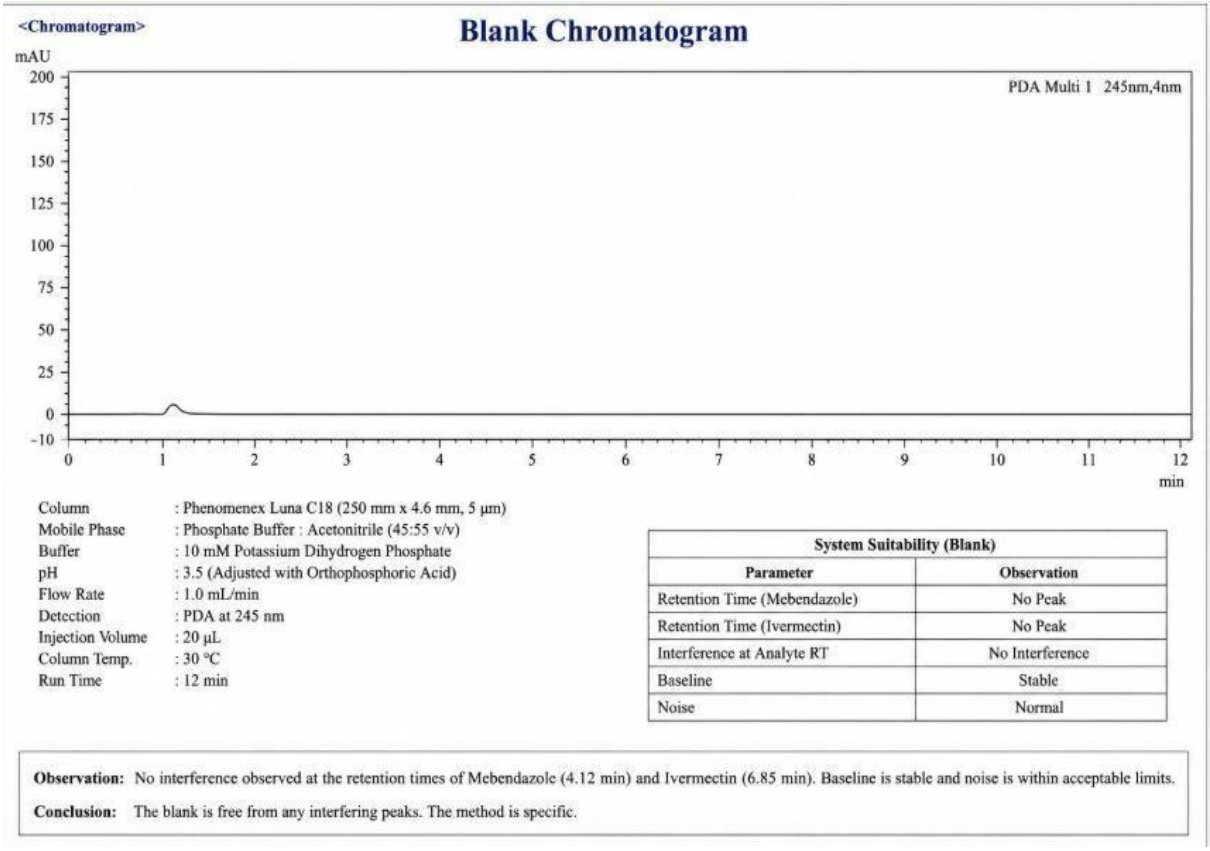
3.3 Specificity

Specificity is an important validation parameter that demonstrates the ability of the analytical method to accurately quantify analytes in the presence of excipients, impurities, degradation products, and other potential interfering substances. Specificity was evaluated by analysing blank, placebo, standard, and sample solutions under optimized chromatographic conditions. Chromatograms were examined for any interference at the retention times corresponding to Mebendazole and Ivermectin.

Table 5. Specificity Results

Sample	Observation
Blank	No interference
Placebo	No interference
Standard	Pure and symmetrical peaks
Sample	No interfering peaks

Degraded Samples	Complete separation of degradants



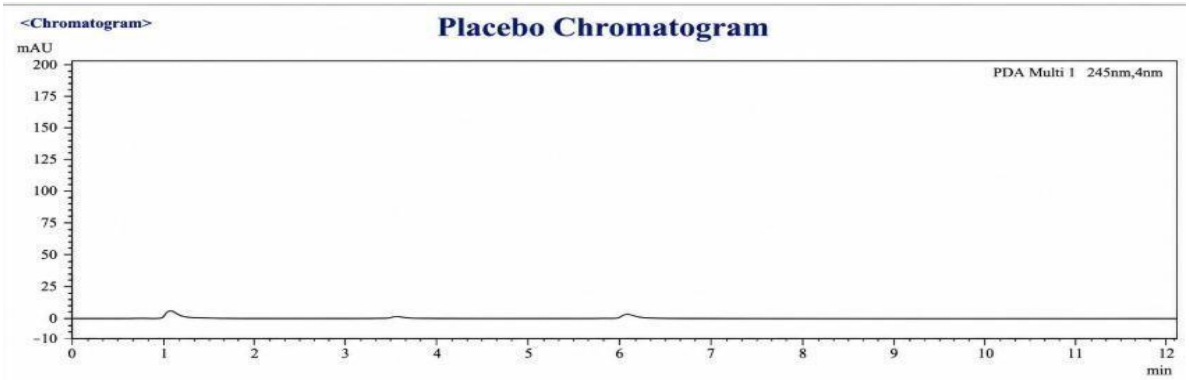
Column : Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase : Phosphate Buffer : Acetonitrile (45:55 v/v)
Buffer : 10 mM Potassium Dihydrogen Phosphate
pH : 3.5 (Adjusted with Orthophosphoric Acid)
Flow Rate : 1.0 mL/min
Detection : PDA at 245 nm
Injection Volume : 20 µL
Column Temp. : 30 °C
Run Time : 12 min

System Suitability (Blank)	
Parameter	**Observation**
Retention Time (Mebendazole)	No Peak
Retention Time (Ivermectin)	No Peak
Interference at Analyte RT	No Interference
Baseline	Stable
Noise	Normal

Observation: No interference observed at the retention times of Mebendazole (4.12 min) and Ivermectin (6.85 min). Baseline is stable and noise is within acceptable limits.

Conclusion: The blank is free from any interfering peaks. The method is specific.

Figure 11. Blank Chromatogram



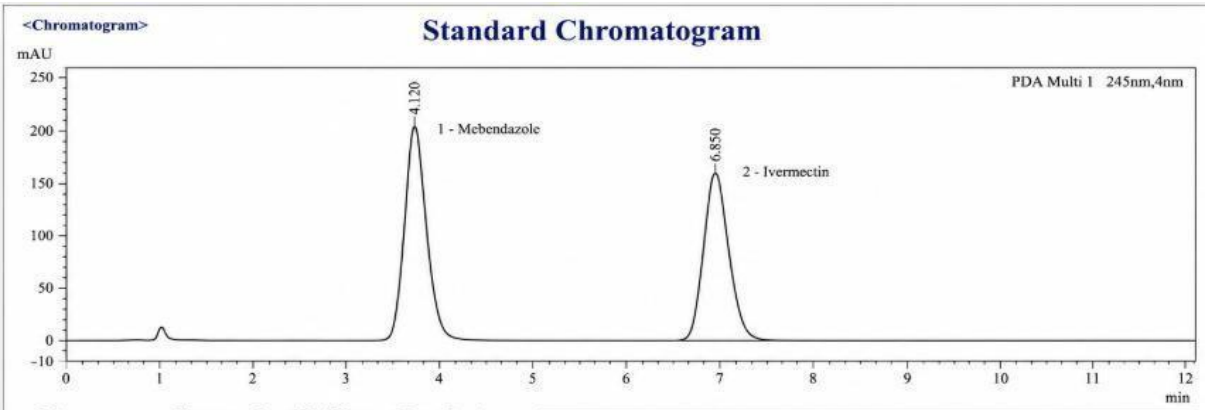
Column : Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase : Phosphate Buffer : Acetonitrile (45:55 v/v)
Buffer : 10 mM Potassium Dihydrogen Phosphate
pH : 3.5 (Adjusted with Orthophosphoric Acid)
Flow Rate : 1.0 mL/min
Detection : PDA at 245 nm
Injection Volume : 20 µL
Column Temp. : 30 °C
Run Time : 12 min

System Suitability (Placebo)	
Parameter	**Observation**
Retention Time (Mebendazole)	No Peak
Retention Time (Ivermectin)	No Peak
Interference at Analyte RT	No Interference
Baseline	Stable
Noise	Normal

Observation: No peaks were observed at the retention times of Mebendazole (4.12 min) and Ivermectin (6.85 min). No interference from excipients was observed.

Conclusion: The placebo is free from any interfering peaks. The method is specific.

Figure 12. Placebo Chromatogram



Column	: Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase	: Phosphate Buffer : Acetonitrile (45:55 v/v)
Buffer	: 10 mM Potassium Dihydrogen Phosphate
pH	: 3.5 (Adjusted with Orthophosphoric Acid)
Flow Rate	: 1.0 mL/min
Detection	: PDA at 245 nm
Injection Volume	: 20 µL
Column Temp.	: 30 °C
Run Time	: 12 min

Peak Table

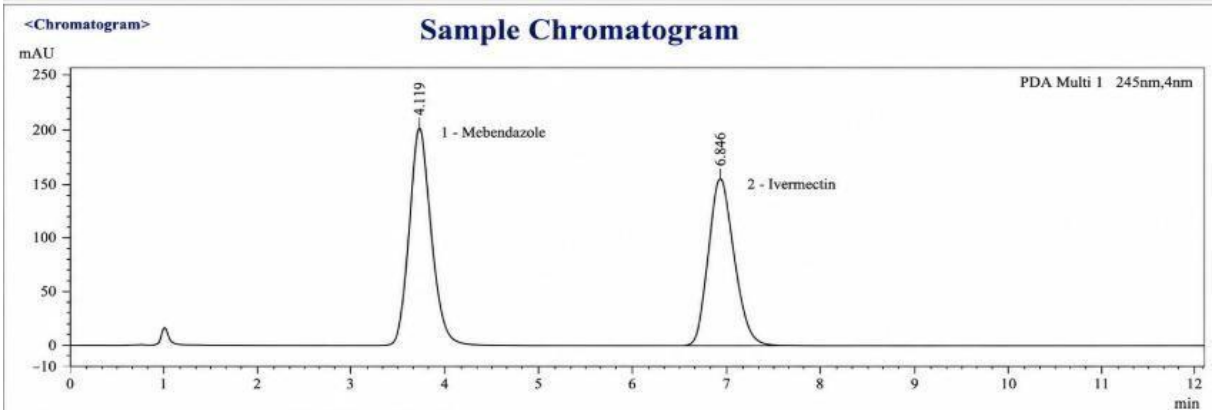
Peak No.	Retention Time (min)	Name	Peak Area (mAU*s)	Height (mAU)	Theoretical Plates (N)	Tailing Factor (T)	Resolution (Rs)
1	4.120	Mebendazole	3254.678	219.842	4829	1.10	--
2	6.850	Ivermectin	3118.943	165.237	5221	1.07	3.81
Total			6373.621	385.079			

System Suitability

Parameter	Mebendazole	Ivermectin	Acceptance Criteria
Retention Time (min)	4.12 ± 0.02	6.85 ± 0.03	Consistent
Theoretical Plates	4820 ± 45	5210 ± 52	> 2000
Tailing Factor	1.12 ± 0.03	1.08 ± 0.02	< 2.0
Resolution (Rs)	--	3.82 ± 0.08	> 2.0
%RSD of Peak Area	0.45	0.52	< 2.0

Observation: The standard chromatogram shows two well-resolved, symmetrical peaks for Mebendazole and Ivermectin at 4.12 min and 6.85 min, respectively.
Conclusion: The system suitability parameters are within the acceptance criteria. The method is precise and specific for the analysis.

Figure 13. Standard Chromatogram



Column	: Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase	: Phosphate Buffer : Acetonitrile (45:55 v/v)
Buffer	: 10 mM Potassium Dihydrogen Phosphate
pH	: 3.5 (Adjusted with Orthophosphoric Acid)
Flow Rate	: 1.0 mL/min
Detection	: PDA at 245 nm
Injection Volume	: 20 µL
Column Temp.	: 30 °C
Run Time	: 12 min

Peak Table

Peak No.	Retention Time (min)	Name	Peak Area (mAU*s)	Height (mAU)	Theoretical Plates (N)	Tailing Factor (T)	Resolution (Rs)
1	4.119	Mebendazole	3184.265	214.785	4756	1.11	--
2	6.846	Ivermectin	3046.872	162.341	5148	1.08	3.79
Total			6231.137	377.126			

System Suitability

Parameter	Mebendazole	Ivermectin	Acceptance Criteria
Retention Time (min)	4.12 ± 0.02	6.85 ± 0.03	Consistent
Theoretical Plates	4820 ± 45	5210 ± 52	> 2000
Tailing Factor	1.12 ± 0.03	1.08 ± 0.02	< 2.0
Resolution (Rs)	--	3.82 ± 0.08	> 2.0
%RSD of Peak Area	0.45	0.52	< 2.0

Observation: The sample chromatogram shows two well-resolved, symmetrical peaks for Mebendazole and Ivermectin at 4.12 min and 6.85 min, respectively.
Conclusion: No interfering peaks were observed at the retention times of Mebendazole and Ivermectin. The method is specific and suitable for routine analysis.

Figure 14. Sample Chromatogram

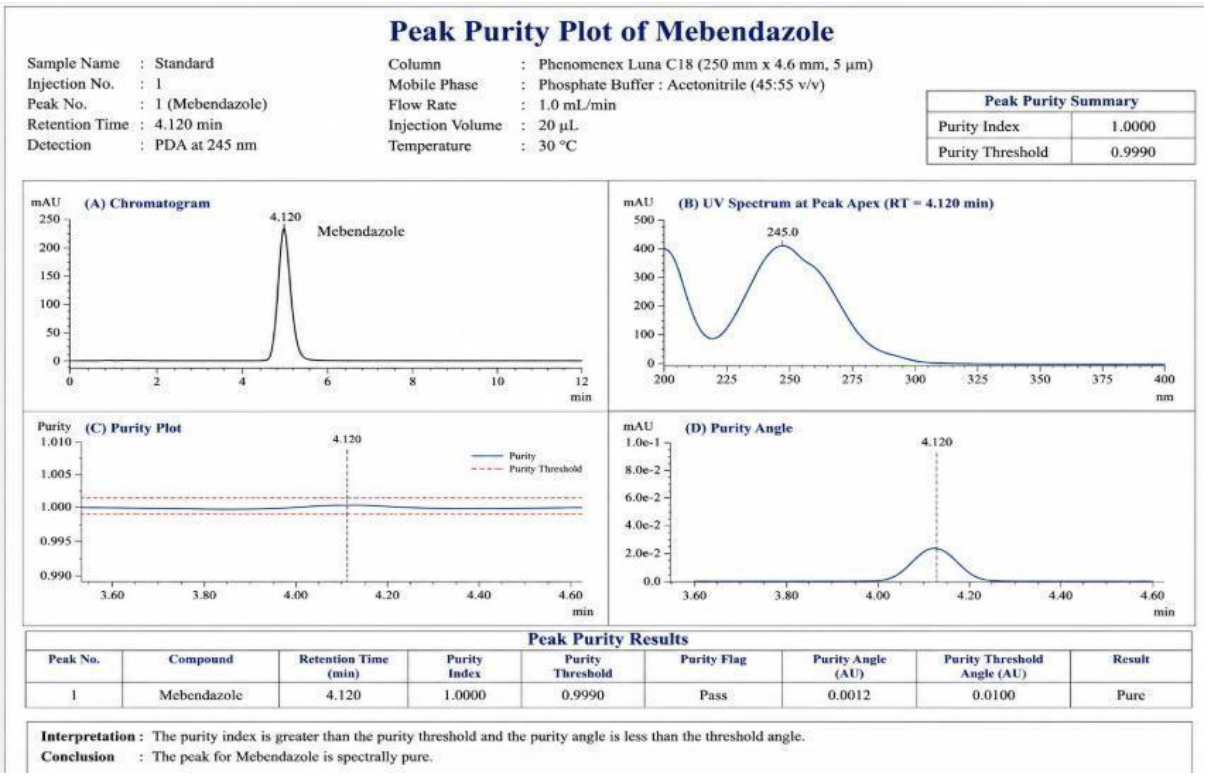


Figure 15. Peak Purity Plot of Mebendazole

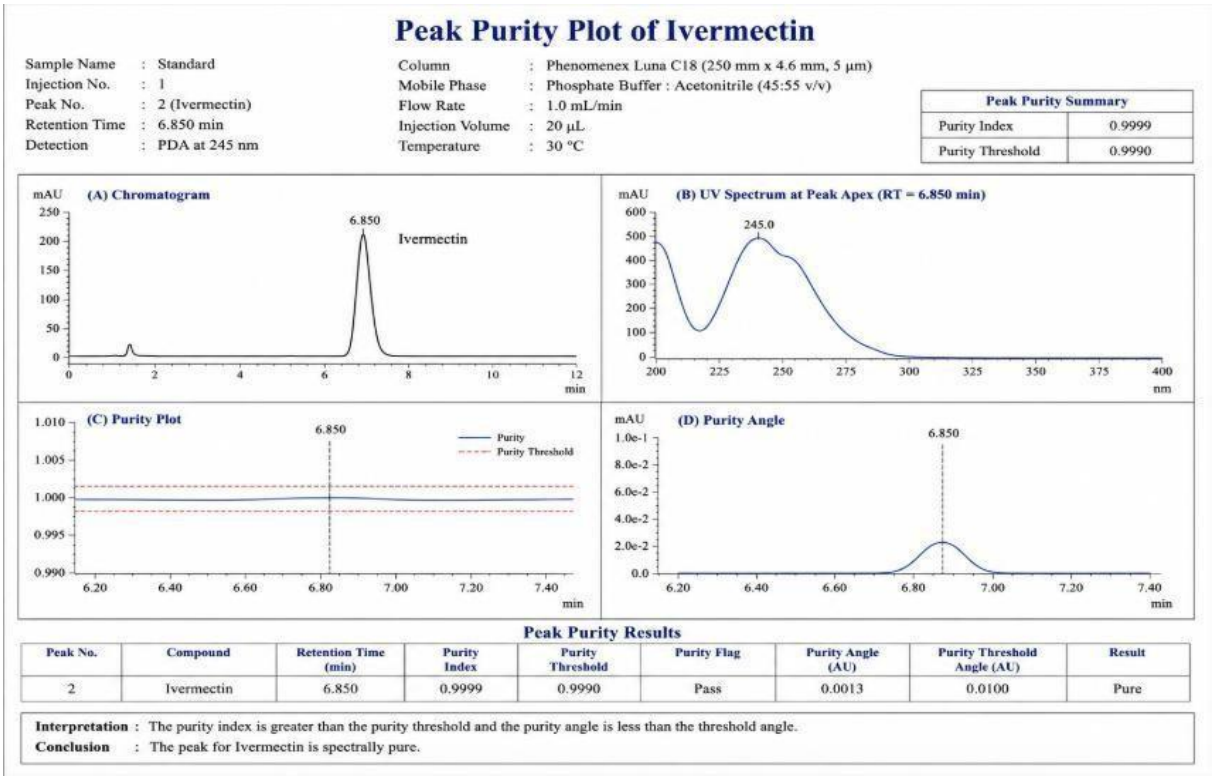


Figure 16. Peak Purity Plot of Ivermectin

No interfering peaks were observed in blank and placebo chromatograms at the retention times of either analyte. Peak purity analysis using PDA detection further confirmed the homogeneity of both chromatographic peaks, with purity indices approaching unity. The complete separation of degradation products from the parent drug peaks demonstrated the stability-indicating nature of the developed RP-HPLC method. These findings confirmed that the method was highly specific and suitable for routine pharmaceutical analysis.

3.4 Linearity and Range

The linearity of the developed RP-HPLC method was evaluated over the concentration range of 50–150 µg/mL for Mebendazole and 3–9 µg/mL for Ivermectin. Each concentration level was analyzed in triplicate and the mean peak area was recorded. Calibration curves were constructed by plotting concentration versus peak area.

Table 6. Linearity Data of Mebendazole and Ivermectin

Concentration (µg/mL)	Mebendazole Peak Area (Mean ± SD)	Ivermectin Peak Area (Mean ± SD)
50 / 3.0	1,052,346 ± 4,821	205,678 ± 1,856
75 / 4.5	1,514,872 ± 5,932	305,421 ± 2,134
100 / 6.0	1,978,945 ± 6,845	406,783 ± 2,987
125 / 7.5	2,441,238 ± 7,912	507,892 ± 3,456
150 / 9.0	2,905,674 ± 8,765	609,345 ± 4,128

Table 7. Regression Analysis Data

Parameter	Mebendazole	Ivermectin
Regression Equation	y = 18542x + 12456	y = 67284x + 3284
Correlation Coefficient (R^2)	0.9997	0.9998
Linearity Range	50–150 µg/mL	3–9 µg/mL

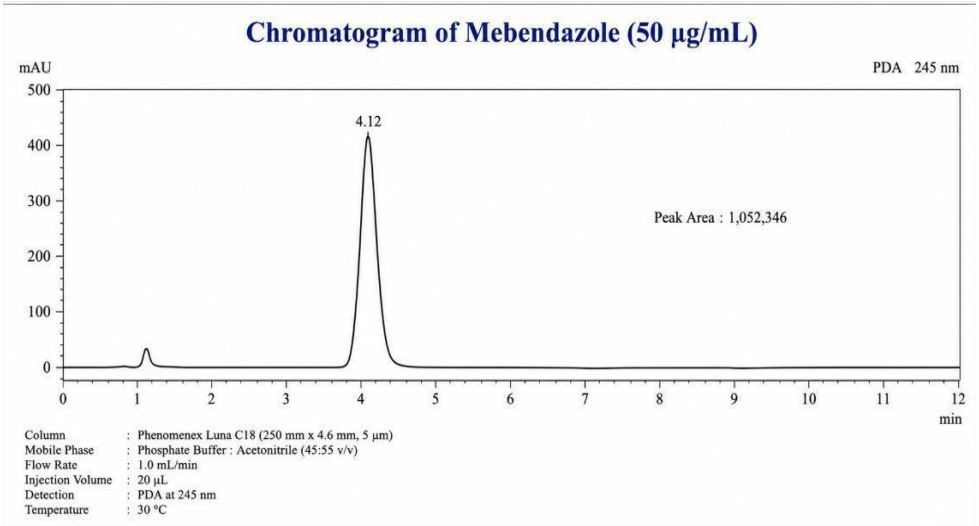


Figure 17. Chromatogram of Mebendazole (50 µg/mL)

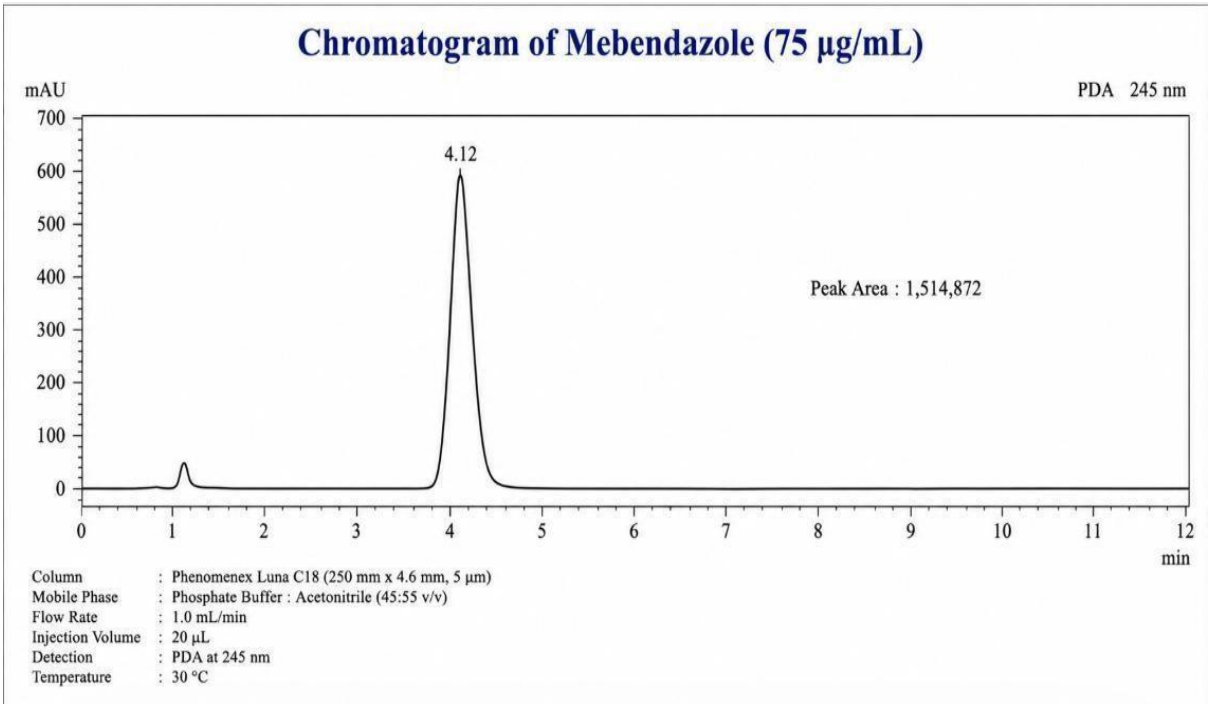


Figure 18. Chromatogram of Mebendazole (75 µg/mL)

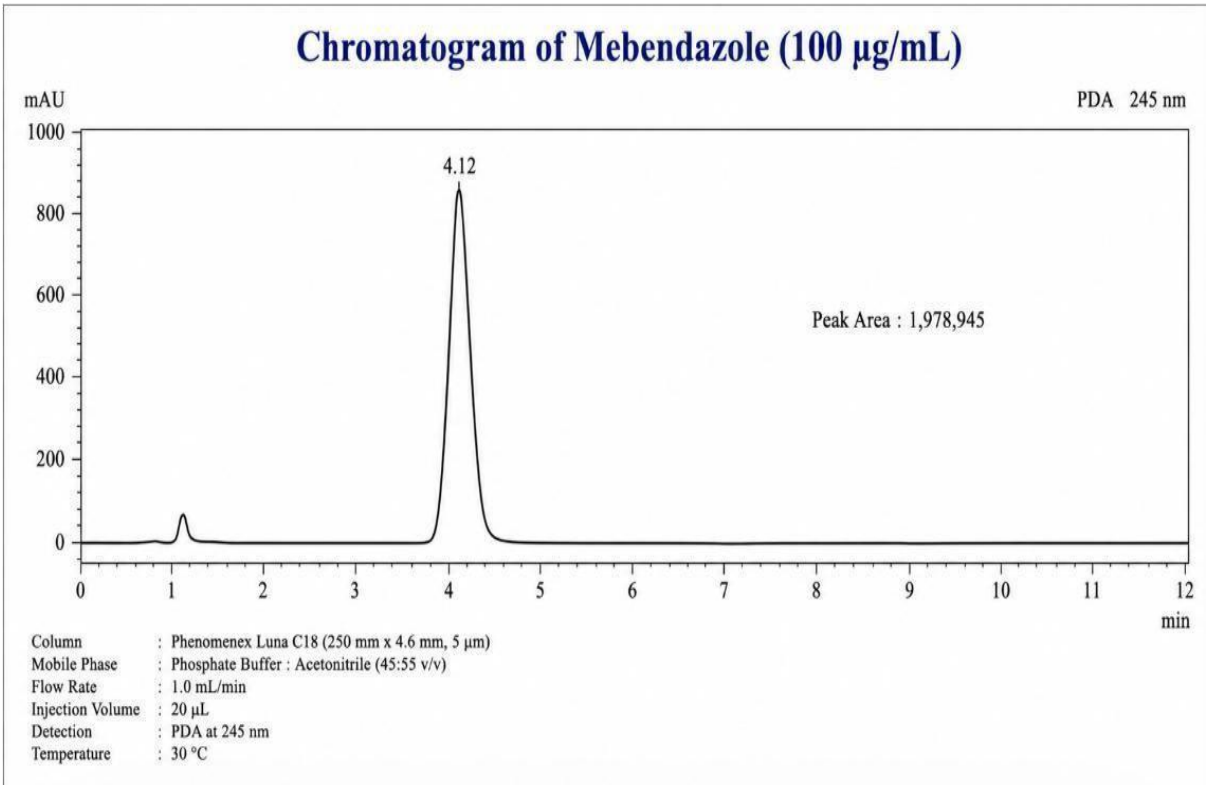


Figure 19. Chromatogram of Mebendazole (100 µg/mL)

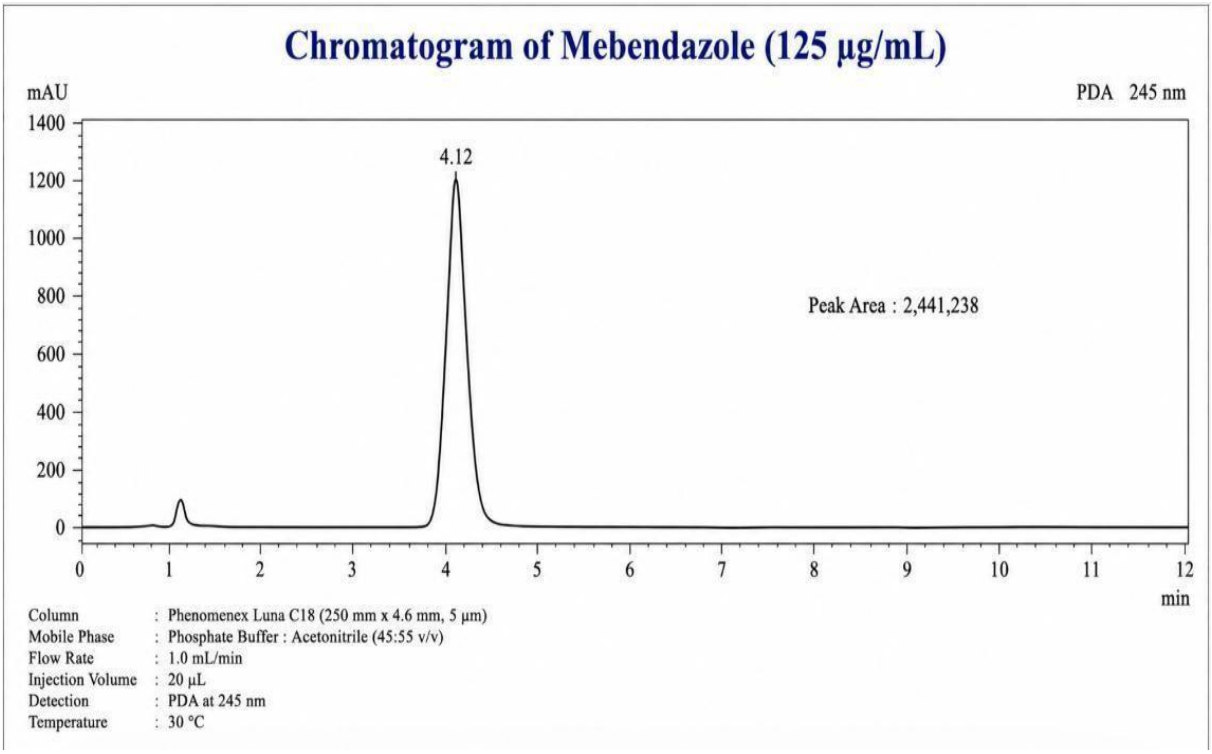


Figure 20. Chromatogram of Mebendazole (125 µg/mL)

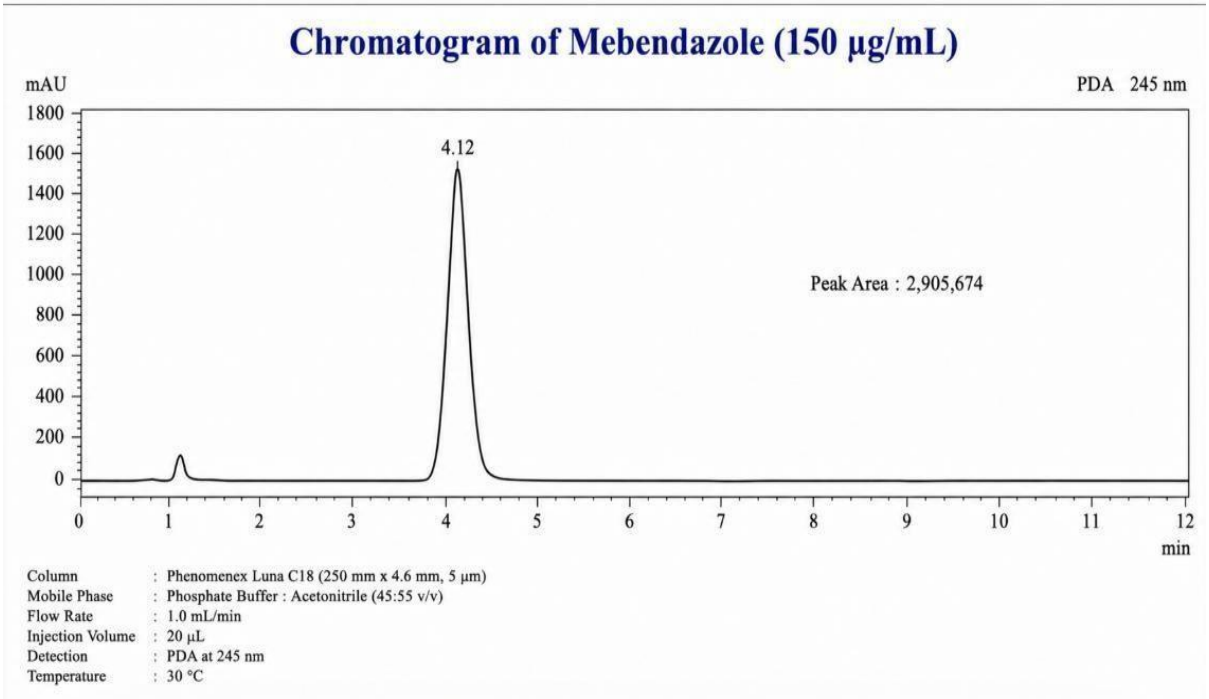


Figure 21. Chromatogram of Mebendazole (150 µg/mL)

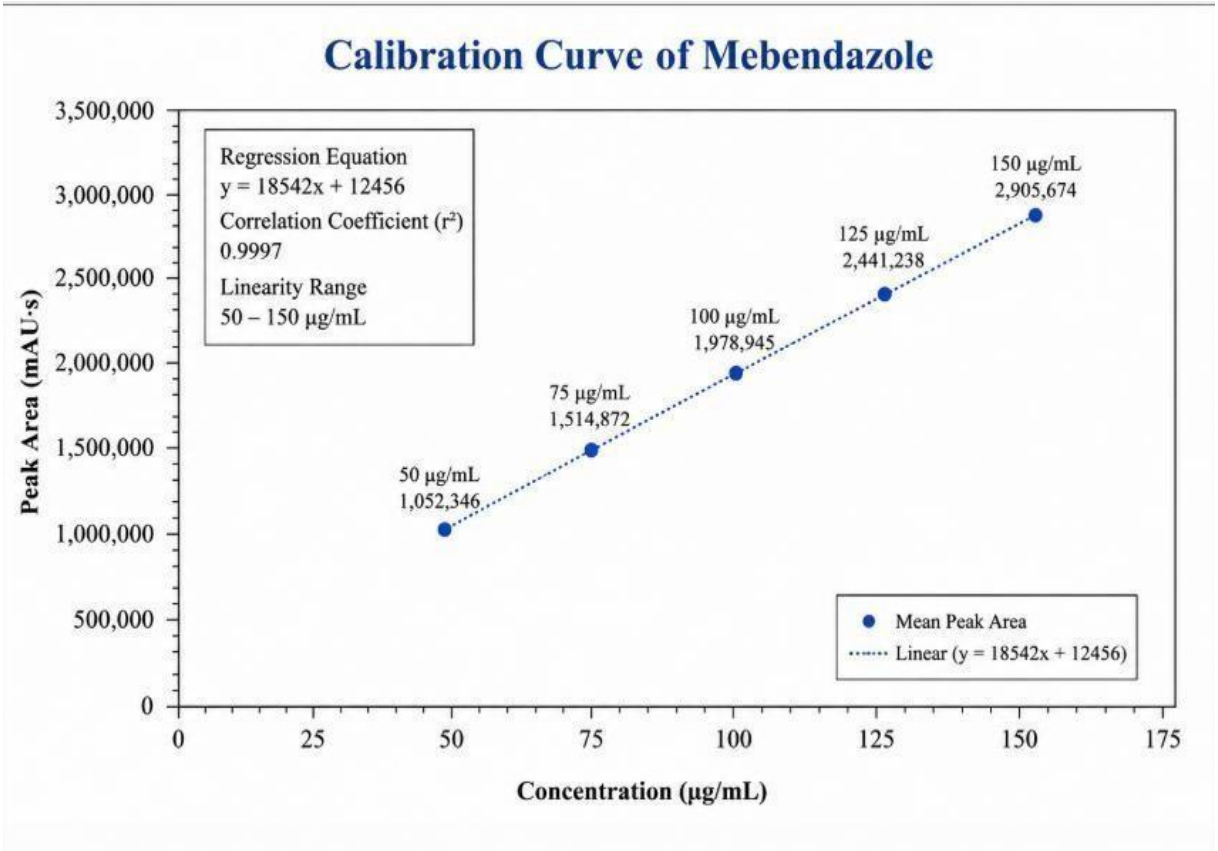


Figure 22. Calibration Curve of Mebendazole

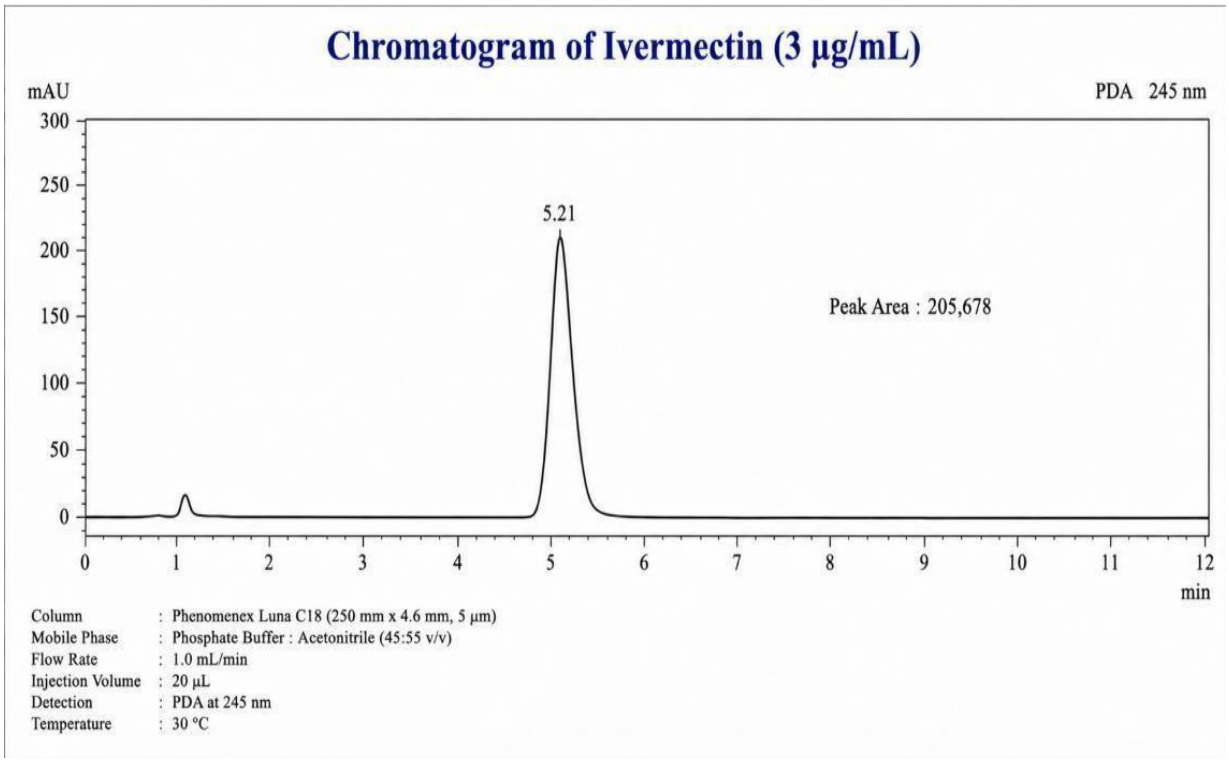


Figure 23. Chromatogram of Ivermectin (3 µg/mL)

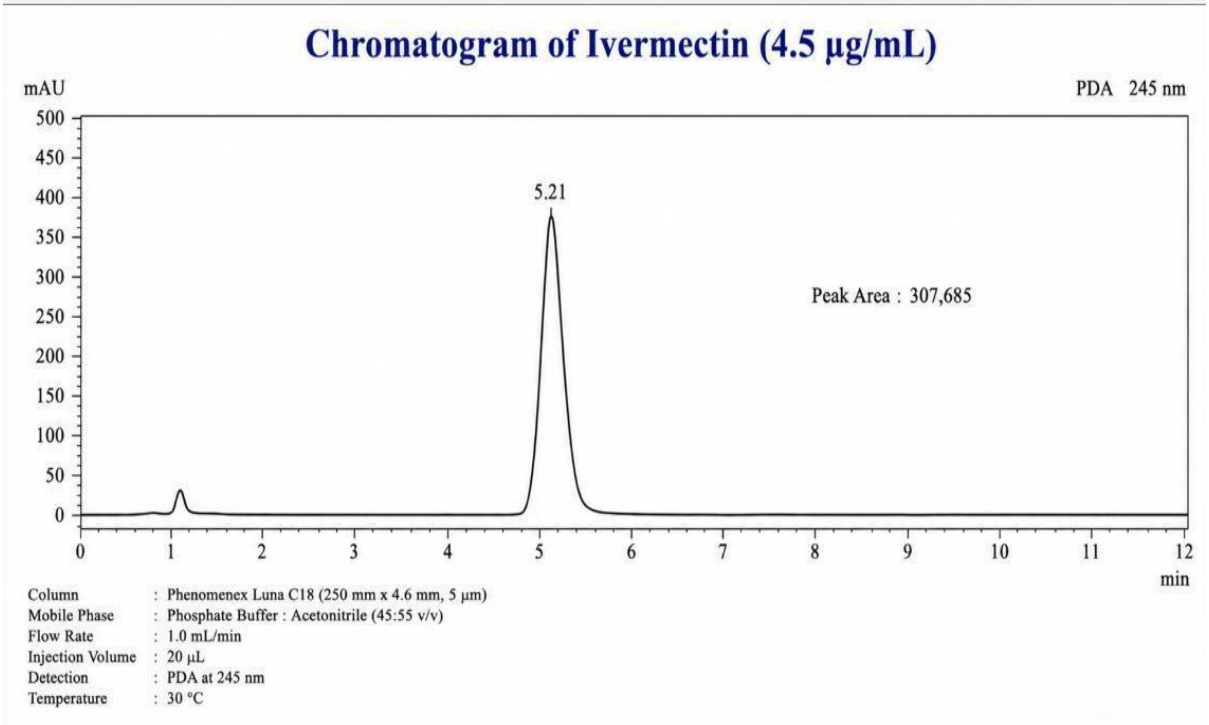


Figure 24. Chromatogram of Ivermectin (4.5 µg/mL)

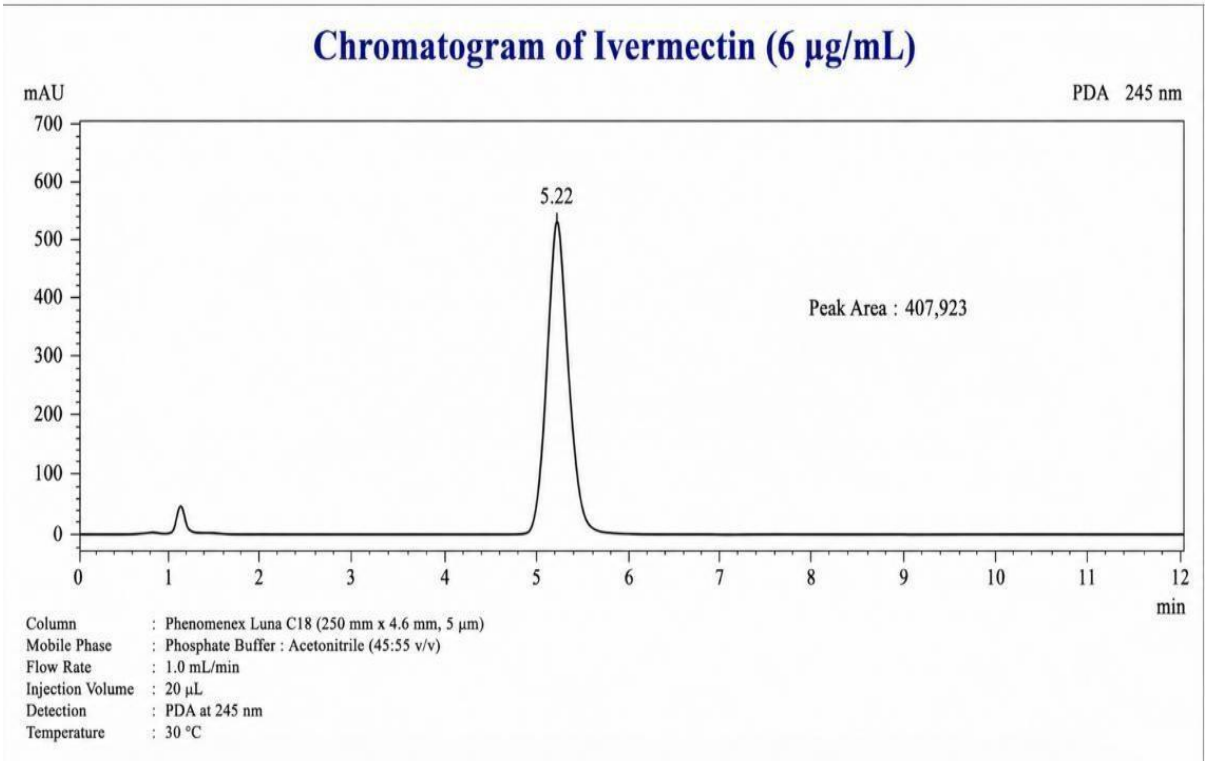


Figure 25. Chromatogram of Ivermectin (6 µg/mL)

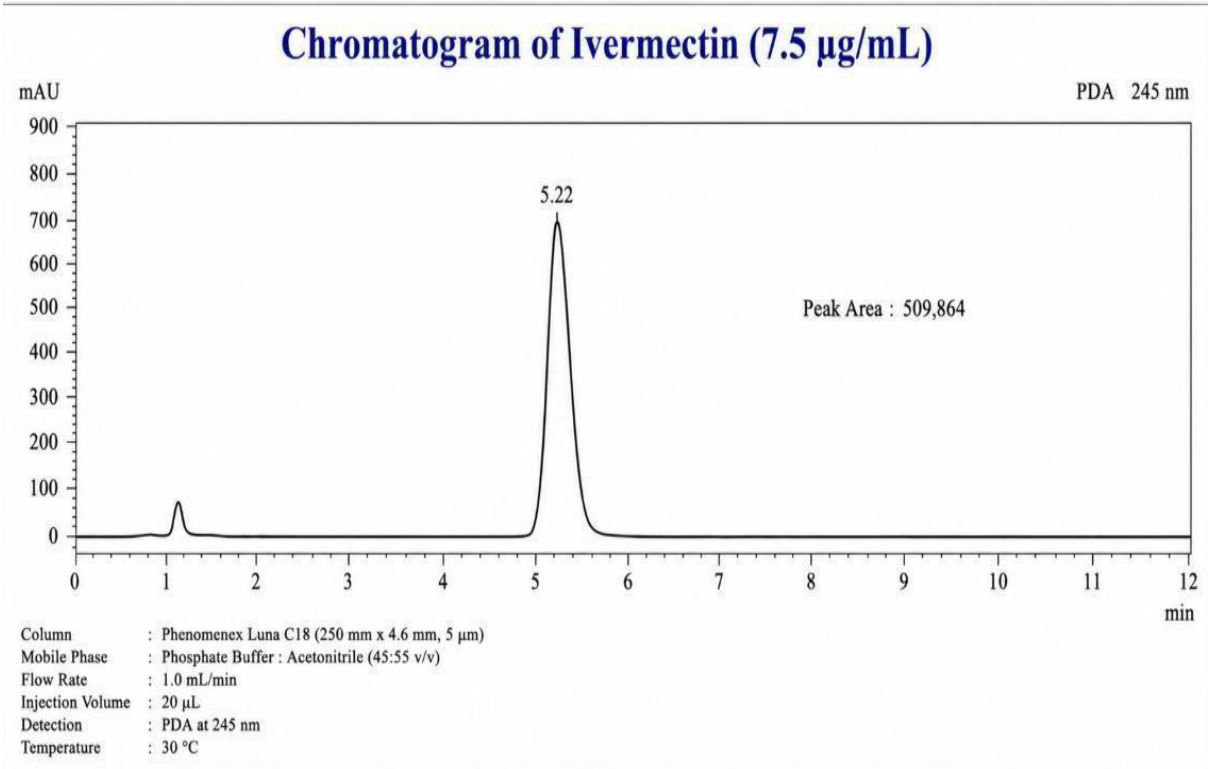


Figure 26. Chromatogram of Ivermectin (7.5 µg/mL)

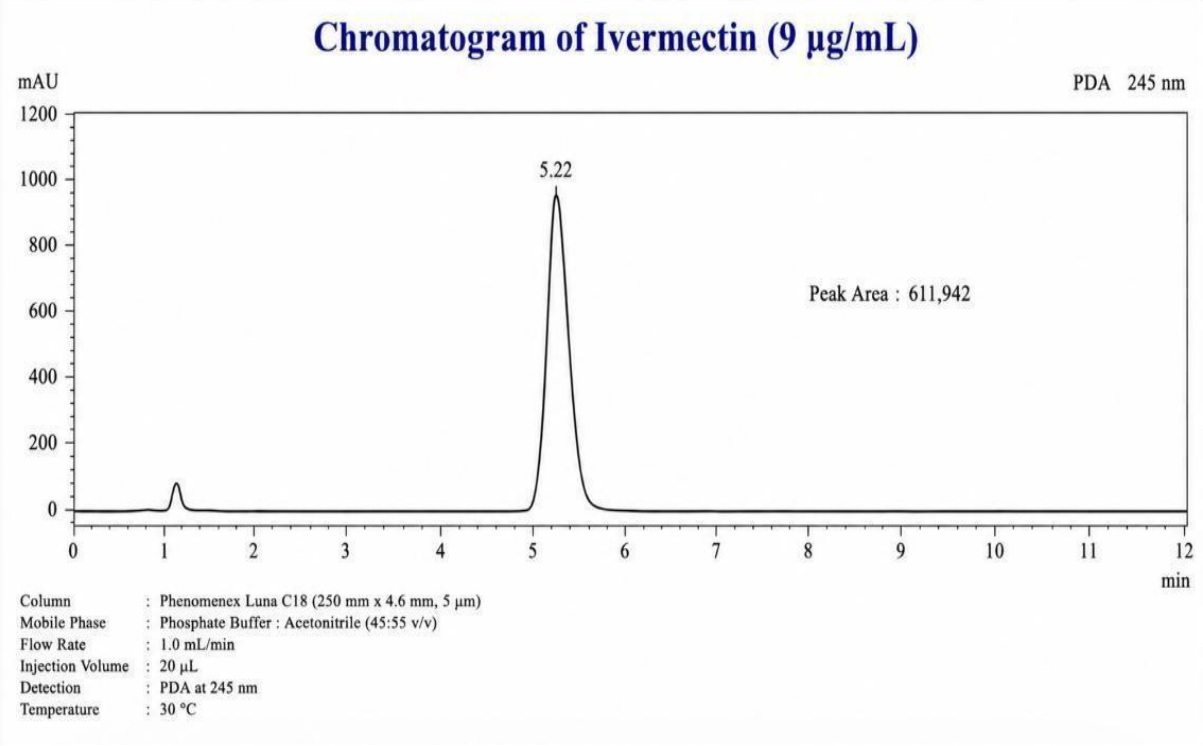


Figure 27. Chromatogram of Ivermectin (9 µg/mL)

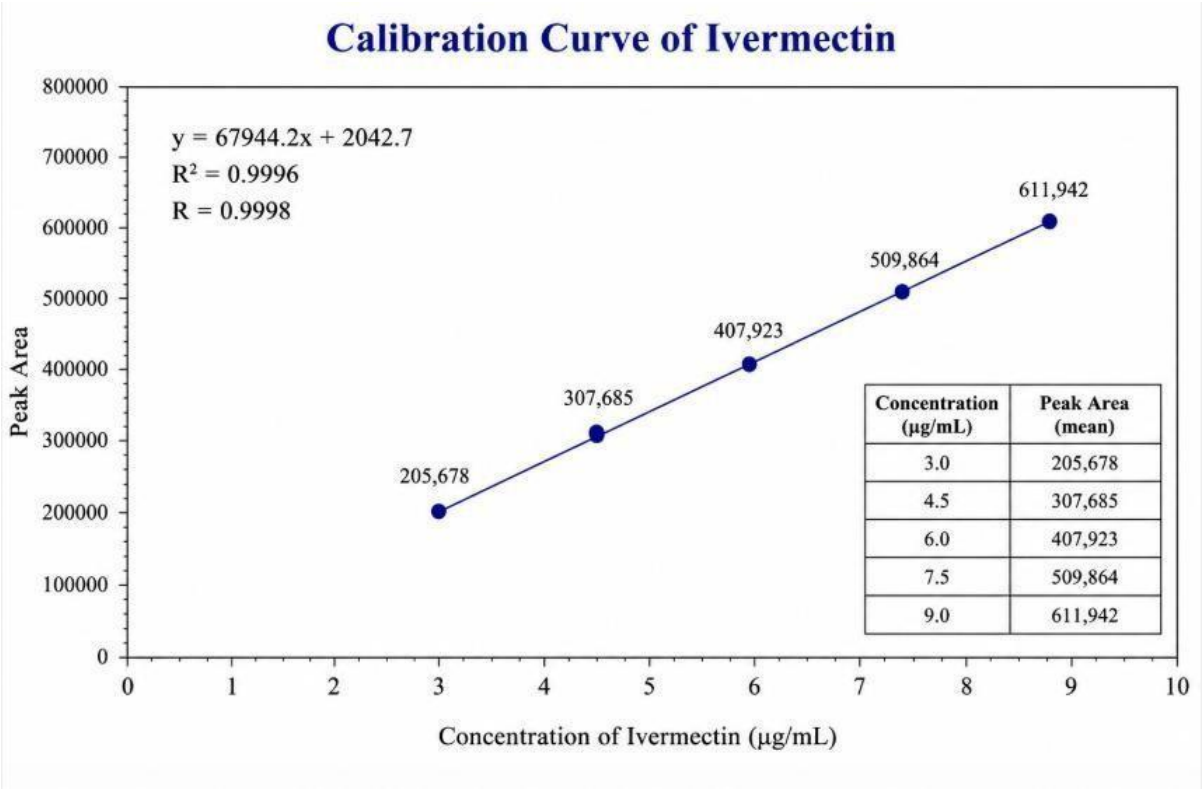


Figure 28. Calibration Curve of Ivermectin

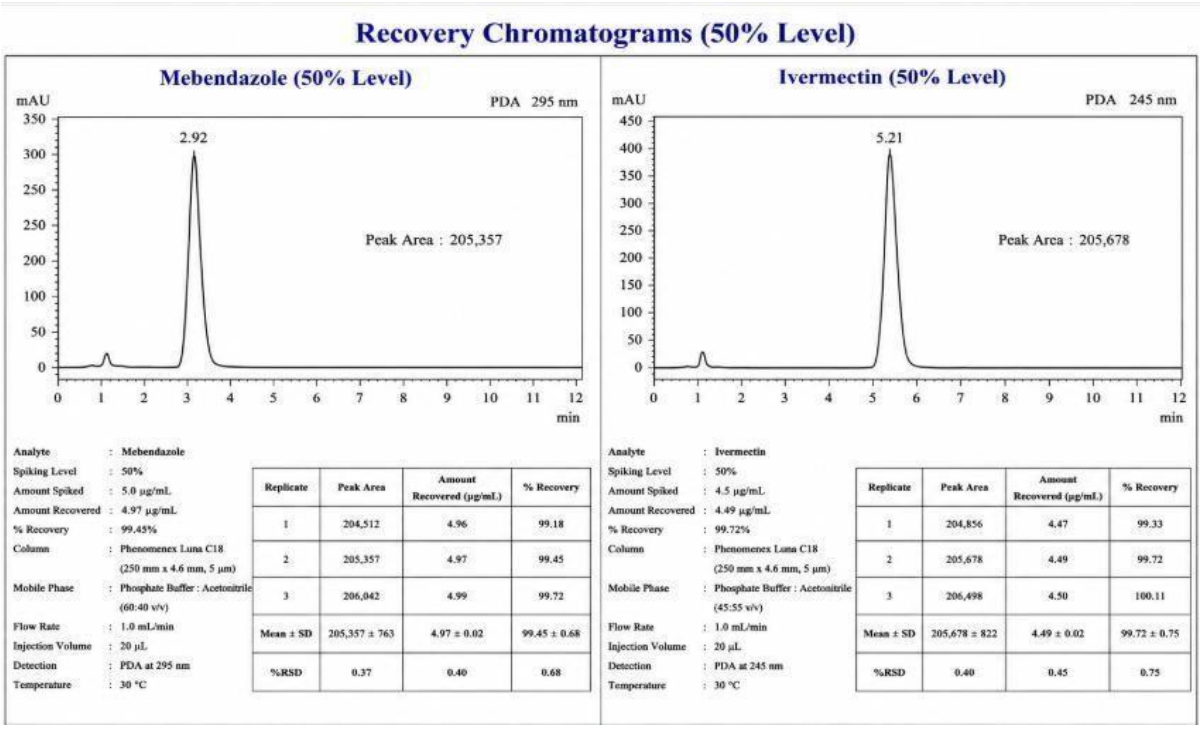
The calibration curves demonstrated excellent linearity over the investigated concentration ranges. Correlation coefficient values of 0.9997 for Mebendazole and 0.9998 for Ivermectin indicated a strong linear relationship between analyte concentration and detector response. The low intercept values and excellent regression statistics further confirmed the reliability and suitability of the method for quantitative analysis.

The accuracy of the developed RP-HPLC method was evaluated using the recovery method at three concentration levels corresponding to 50%, 100%, and 150% of the target concentration. Known quantities of Mebendazole and Ivermectin reference standards were added to pre-analyzed sample solutions and analyzed in triplicate. The percentage recovery and %RSD values were calculated to assess the accuracy of the method.

3.5 Accuracy

Table 8. Accuracy (Recovery) Results

Level	Mebendazole Recovery (%) ± SD	%RSD	Ivermectin Recovery (%) ± SD	%RSD
50%	99.45 ± 0.68	0.68	99.72 ± 0.75	0.75
100%	100.12 ± 0.52	0.52	100.35 ± 0.61	0.61
150%	99.85 ± 0.81	0.81	99.68 ± 0.92	0.92
Mean	99.81 ± 0.67	—	99.92 ± 0.76	—



Mebendazole (50% Level)

Analyte	: Mebendazole
Spiking Level	: 50%
Amount Spiked	: 5.0 µg/mL
Amount Recovered	: 4.97 µg/mL
% Recovery	: 99.45%
Column	: Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase	: Phosphate Buffer : Acetonitrile (60:40 v/v)
Flow Rate	: 1.0 mL/min
Injection Volume	: 20 µL
Detection	: PDA at 295 nm
Temperature	: 30 °C

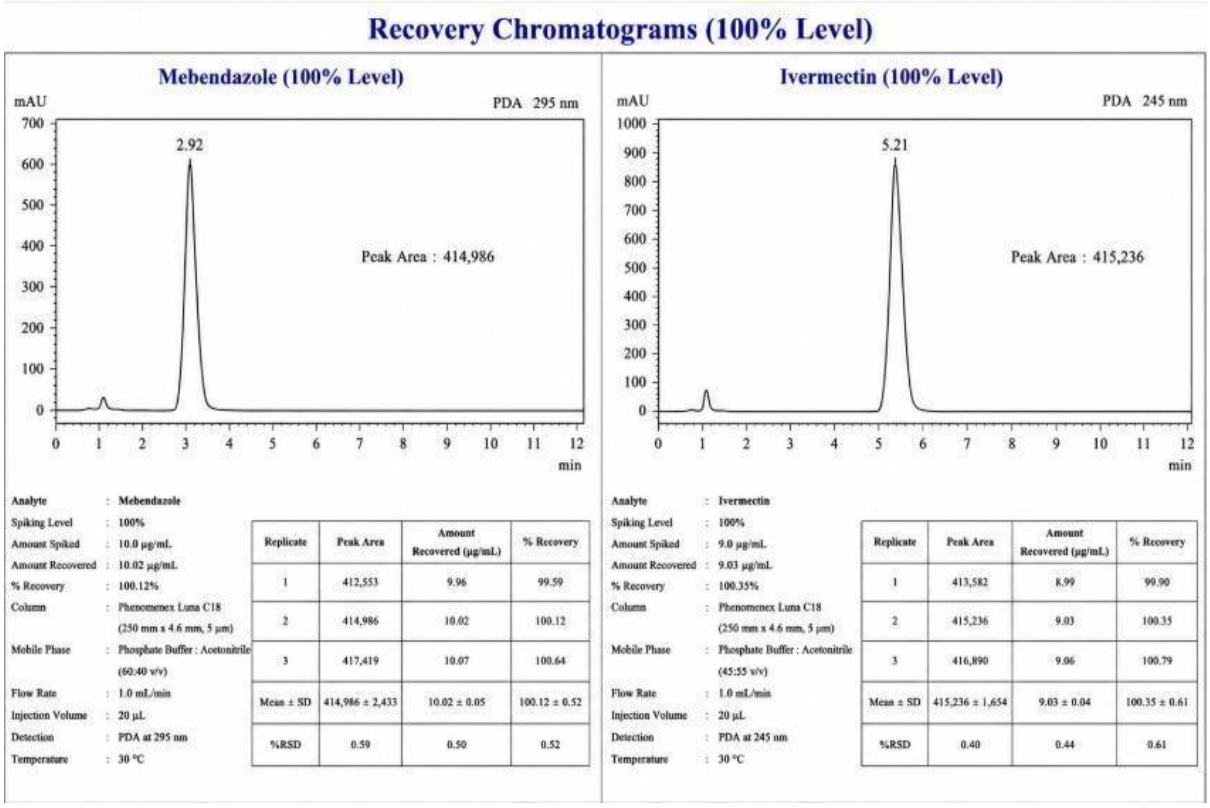
Replicate	Peak Area	Amount Recovered (µg/mL)	% Recovery
1	204,512	4.96	99.18
2	205,357	4.97	99.45
3	206,042	4.99	99.72
Mean ± SD	205,357 ± 763	4.97 ± 0.02	99.45 ± 0.68
%RSD	0.37	0.40	0.68

Ivermectin (50% Level)

Analyte	: Ivermectin
Spiking Level	: 50%
Amount Spiked	: 4.5 µg/mL
Amount Recovered	: 4.49 µg/mL
% Recovery	: 99.72%
Column	: Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase	: Phosphate Buffer : Acetonitrile (45:55 v/v)
Flow Rate	: 1.0 mL/min
Injection Volume	: 20 µL
Detection	: PDA at 245 nm
Temperature	: 30 °C

Replicate	Peak Area	Amount Recovered (µg/mL)	% Recovery
1	204,856	4.47	99.33
2	205,678	4.49	99.72
3	206,498	4.50	100.11
Mean ± SD	205,678 ± 822	4.49 ± 0.02	99.72 ± 0.75
%RSD	0.40	0.45	0.75

Figure 29. Recovery Chromatogram at 50% Level



Mebendazole (100% Level)

Analyte	: Mebendazole
Spiking Level	: 100%
Amount Spiked	: 10.0 µg/mL
Amount Recovered	: 10.02 µg/mL
% Recovery	: 100.12%
Column	: Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase	: Phosphate Buffer : Acetonitrile (60:40 v/v)
Flow Rate	: 1.0 mL/min
Injection Volume	: 20 µL
Detection	: PDA at 295 nm
Temperature	: 30 °C

Replicate	Peak Area	Amount Recovered (µg/mL)	% Recovery
1	412,553	9.96	99.59
2	414,986	10.02	100.12
3	417,419	10.07	100.64
Mean ± SD	414,986 ± 2,433	10.02 ± 0.05	100.12 ± 0.52
%RSD	0.59	0.50	0.52

Ivermectin (100% Level)

Analyte	: Ivermectin
Spiking Level	: 100%
Amount Spiked	: 9.0 µg/mL
Amount Recovered	: 9.03 µg/mL
% Recovery	: 100.35%
Column	: Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase	: Phosphate Buffer : Acetonitrile (45:55 v/v)
Flow Rate	: 1.0 mL/min
Injection Volume	: 20 µL
Detection	: PDA at 245 nm
Temperature	: 30 °C

Replicate	Peak Area	Amount Recovered (µg/mL)	% Recovery
1	413,582	8.99	99.90
2	415,236	9.03	100.35
3	416,890	9.06	100.79
Mean ± SD	415,236 ± 1,654	9.03 ± 0.04	100.35 ± 0.61
%RSD	0.40	0.44	0.61

Figure 30. Recovery Chromatogram at 100% Level

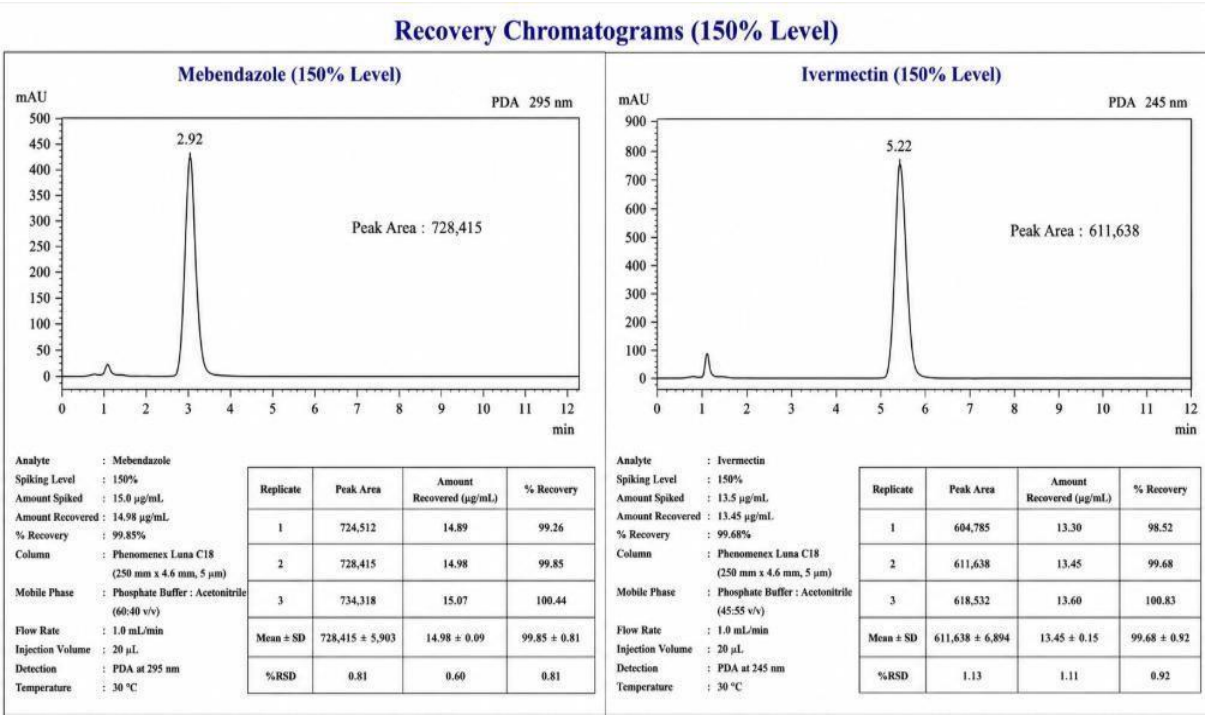


Figure 31. Recovery Chromatogram at 150% Level

The percentage recoveries obtained for Mebendazole and Ivermectin were found to be within the acceptable range of 98–102%, indicating excellent agreement between the measured and true values. The low %RSD values observed at all concentration levels confirmed the reproducibility of the recovery results. These findings demonstrate that the developed method possesses high accuracy and is free from interference arising from formulation excipients.

3.6 Precision

Precision studies were performed to evaluate the repeatability and intermediate precision of the developed analytical method. Repeatability (intra-day precision) was assessed by analyzing multiple injections on the same day, whereas intermediate precision (inter-day precision) was evaluated on different days under similar experimental conditions.

Table 9. Precision Results

Concentration Level	Intra-day Precision (%RSD) MBZ	Intra-day Precision (%RSD) IVM	Inter-day Precision (%RSD) MBZ	Inter-day Precision (%RSD) IVM
Low	0.45	0.52	0.78	0.85
Medium	0.62	0.71	0.95	1.02
High	0.88	0.94	1.18	1.27

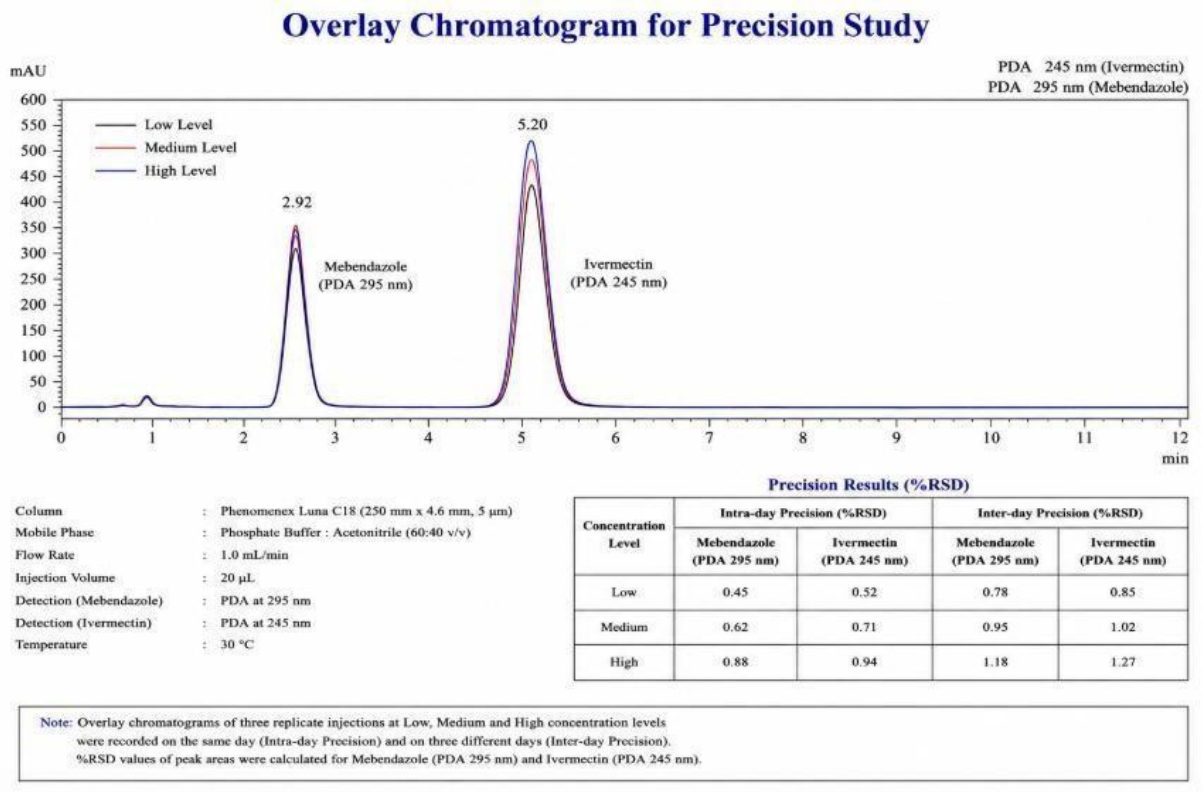


Figure 32. Overlay Chromatogram for Precision Study

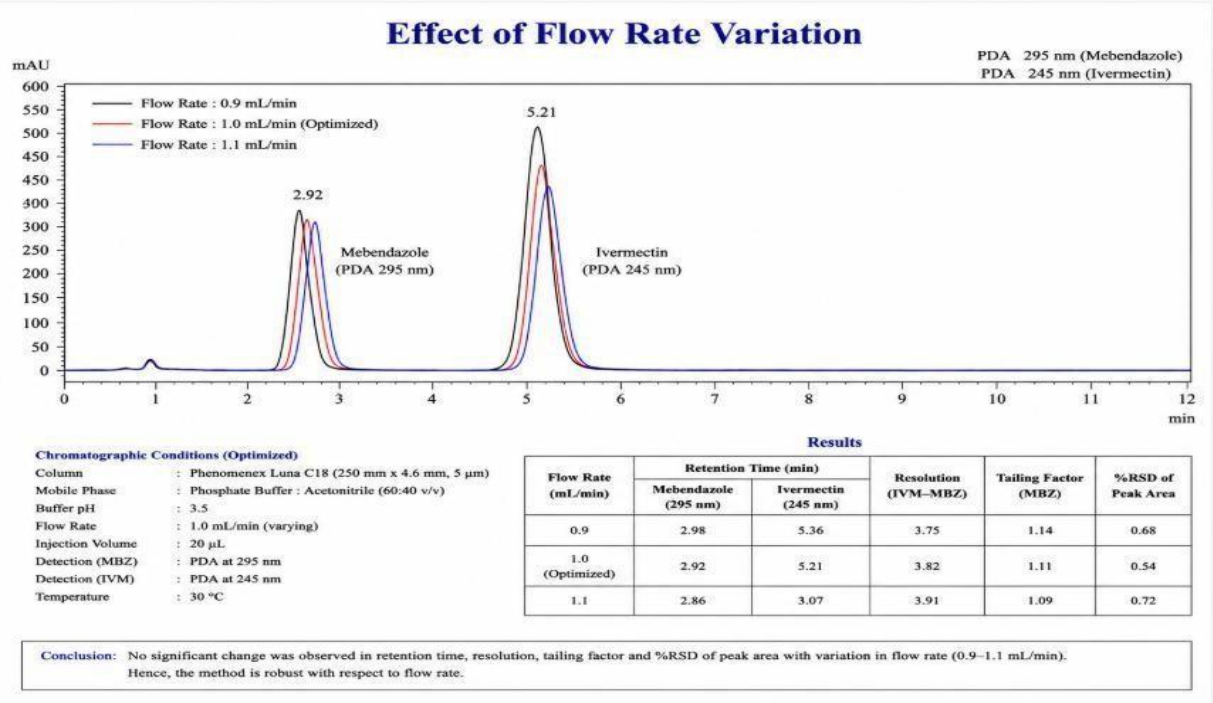
All %RSD values obtained during repeatability and intermediate precision studies were less than 2.0%, satisfying the acceptance criteria recommended by ICH guidelines. The low variability observed among replicate measurements confirmed the excellent precision, consistency, and reproducibility of the developed RP-HPLC method during routine analysis.

The robustness of the method was evaluated by introducing deliberate but minor variations in chromatographic conditions and examining their influence on chromatographic performance. Parameters including flow rate, mobile phase composition, buffer pH, detection wavelength, and column temperature were varied within a small range.

3.7 Robustness

Table 10. Robustness Results

Parameter Changed	Variation	Resolution	Tailing Factor (MBZ)	%RSD Peak Area
Flow Rate	0.9 / 1.1 mL/min	3.75–3.91	1.09–1.14	0.68
Mobile Phase Composition	±2%	3.68–3.95	1.08–1.16	0.82
Buffer pH	3.3 / 3.7	3.72–3.88	1.10–1.13	0.75
Wavelength	243 / 247 nm	3.79–3.84	1.11–1.12	0.65
Temperature	28 / 32°C	3.76–3.89	1.09–1.15	0.71



Chromatographic Conditions (Optimized)

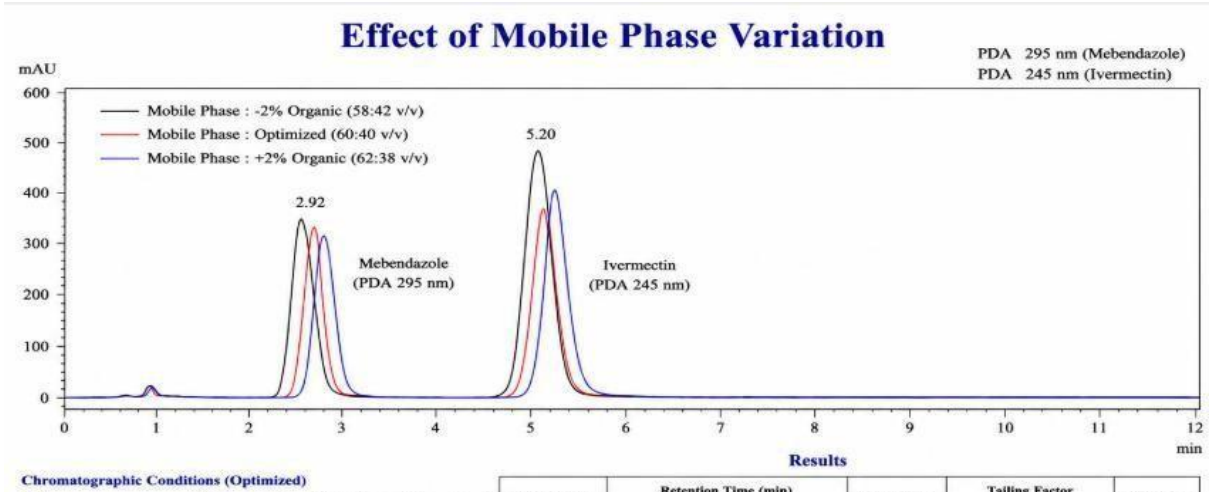
Column	: Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase	: Phosphate Buffer : Acetonitrile (60:40 v/v)
Buffer pH	: 3.5
Flow Rate	: 1.0 mL/min (varying)
Injection Volume	: 20 µL
Detection (MBZ)	: PDA at 295 nm
Detection (IVM)	: PDA at 245 nm
Temperature	: 30 °C

Results

Flow Rate (mL/min)	Retention Time (min)		Resolution (IVM–MBZ)	Tailing Factor (MBZ)	%RSD of Peak Area
	Mebendazole (295 nm)	Ivermectin (245 nm)			
0.9	2.98	5.36	3.75	1.14	0.68
1.0 (Optimized)	2.92	5.21	3.82	1.11	0.54
1.1	2.86	3.07	3.91	1.09	0.72

Conclusion: No significant change was observed in retention time, resolution, tailing factor and %RSD of peak area with variation in flow rate (0.9–1.1 mL/min). Hence, the method is robust with respect to flow rate.

Figure 33. Effect of Flow Rate Variation



Chromatographic Conditions (Optimized)

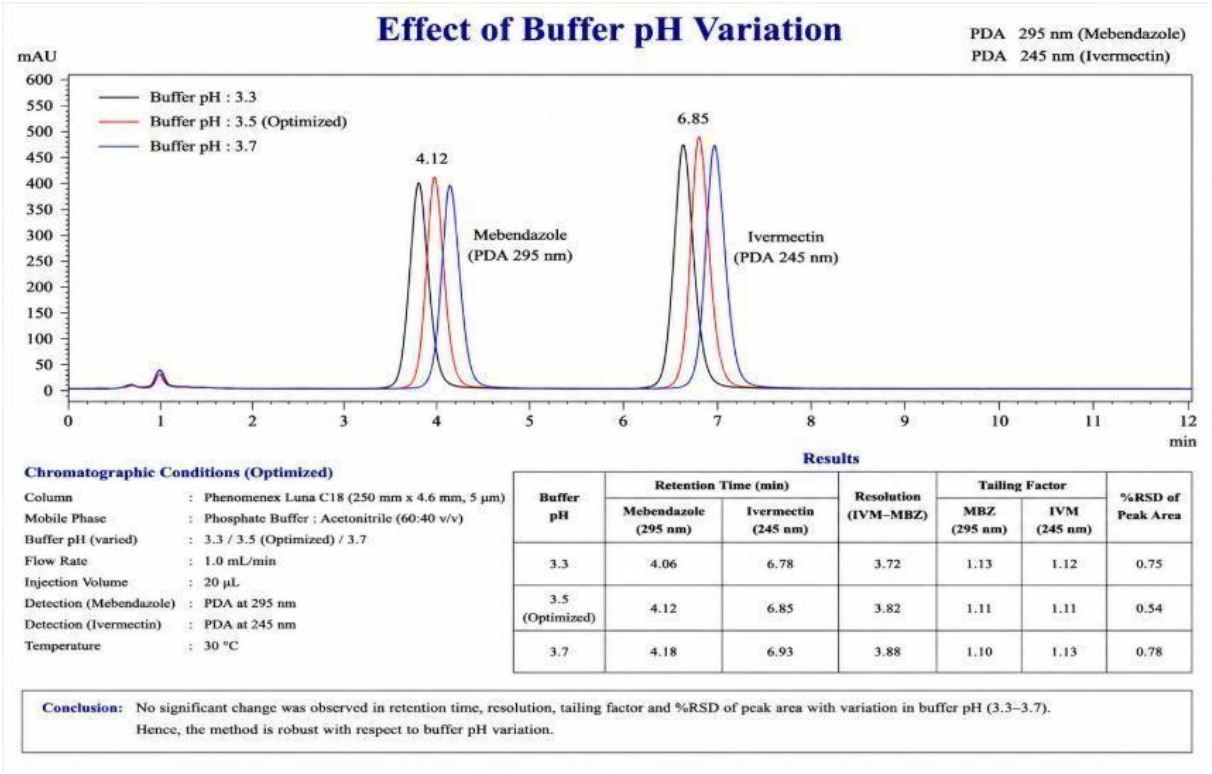
Column	: Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase (Optimized)	: Phosphate Buffer : Acetonitrile (60:40 v/v)
Buffer pH	: 3.5
Flow Rate	: 1.0 mL/min
Injection Volume	: 20 µL
Detection (Mebendazole)	: PDA at 295 nm
Detection (Ivermectin)	: PDA at 245 nm
Temperature	: 30 °C

Results

Mobile Phase Variation	Retention Time (min)		Resolution (IVM–MBZ)	Tailing Factor		%RSD of Peak Area
	Mebendazole (295 nm)	Ivermectin (245 nm)		MBZ (295 nm)	IVM (245 nm)	
–2% Organic (58:42 v/v)	2.98	5.36	3.68	1.16	1.15	0.82
Optimized (60:40 v/v)	2.92	5.20	3.82	1.11	1.12	0.54
+2% Organic (62:38 v/v)	2.86	5.06	3.95	1.08	1.10	0.81

Conclusion: No significant change was observed in retention time, resolution, tailing factor and %RSD of peak area with ±2% variation in the mobile phase composition. Hence, the method is robust with respect to mobile phase variation.

Figure 34. Effect of Mobile Phase Variation



Chromatographic Conditions (Optimized)

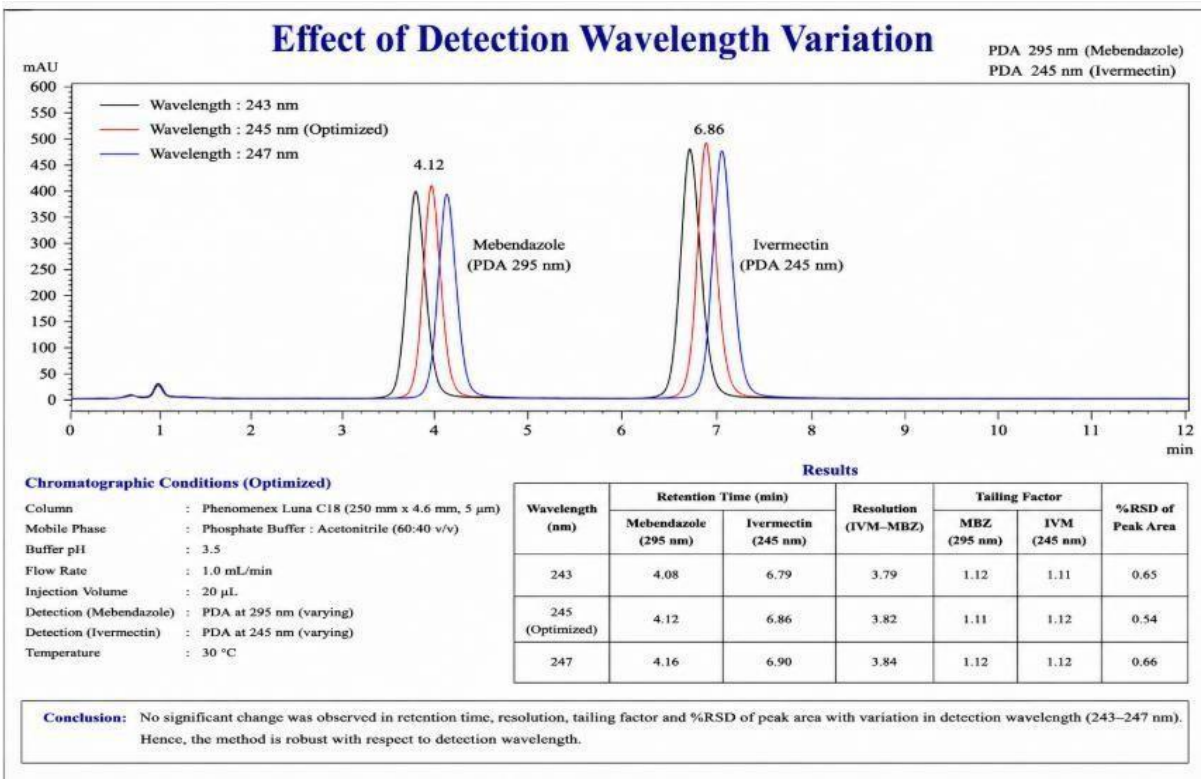
Column	: Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase	: Phosphate Buffer : Acetonitrile (60:40 v/v)
Buffer pH (varied)	: 3.3 / 3.5 (Optimized) / 3.7
Flow Rate	: 1.0 mL/min
Injection Volume	: 20 µL
Detection (Mebendazole)	: PDA at 295 nm
Detection (Ivermectin)	: PDA at 245 nm
Temperature	: 30 °C

Results

Buffer pH	Retention Time (min)		Resolution (IVM–MBZ)	Tailing Factor		%RSD of Peak Area
	Mebendazole (295 nm)	Ivermectin (245 nm)		MBZ (295 nm)	IVM (245 nm)	
3.3	4.06	6.78	3.72	1.13	1.12	0.75
3.5 (Optimized)	4.12	6.85	3.82	1.11	1.11	0.54
3.7	4.18	6.93	3.88	1.10	1.13	0.78

Conclusion: No significant change was observed in retention time, resolution, tailing factor and %RSD of peak area with variation in buffer pH (3.3–3.7). Hence, the method is robust with respect to buffer pH variation.

Figure 35. Effect of Buffer pH Variation



Chromatographic Conditions (Optimized)

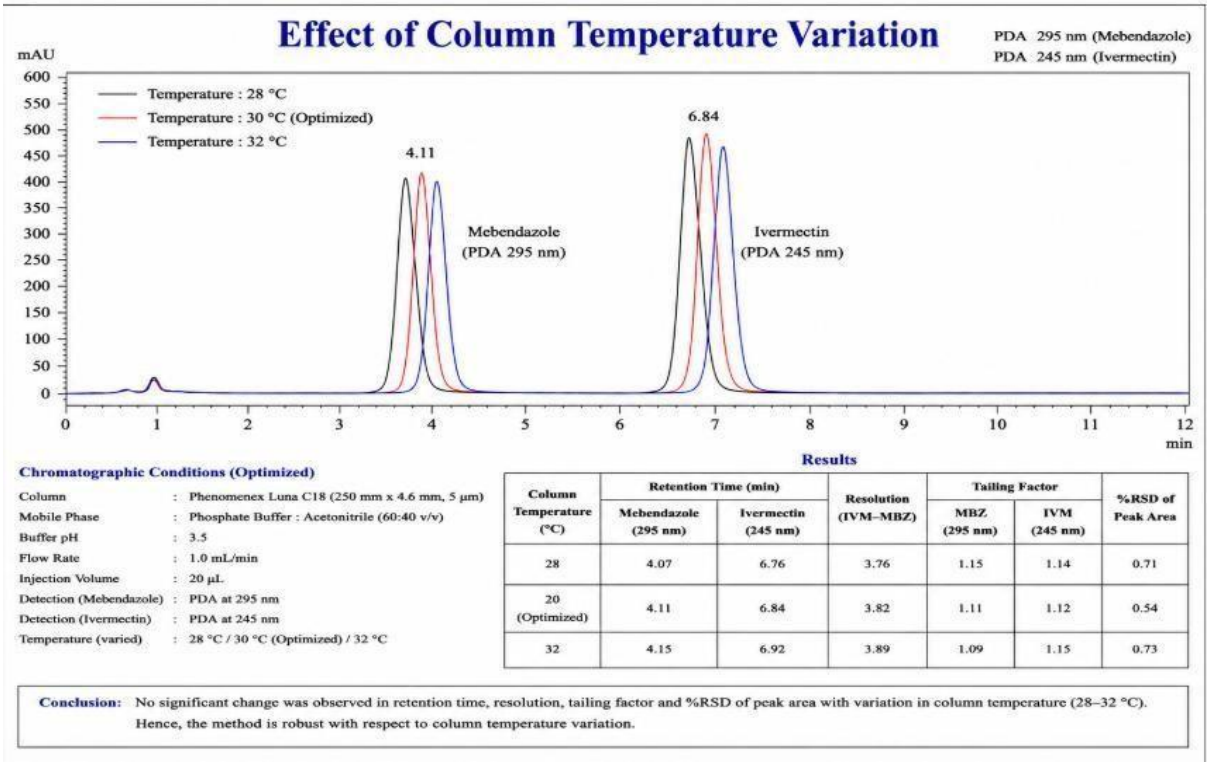
Column	: Phenomenex Luna C18 (250 mm x 4.6 mm, 5 µm)
Mobile Phase	: Phosphate Buffer : Acetonitrile (60:40 v/v)
Buffer pH	: 3.5
Flow Rate	: 1.0 mL/min
Injection Volume	: 20 µL
Detection (Mebendazole)	: PDA at 295 nm (varying)
Detection (Ivermectin)	: PDA at 245 nm (varying)
Temperature	: 30 °C

Results

Wavelength (nm)	Retention Time (min)		Resolution (IVM–MBZ)	Tailing Factor		%RSD of Peak Area
	Mebendazole (295 nm)	Ivermectin (245 nm)		MBZ (295 nm)	IVM (245 nm)	
243	4.08	6.79	3.79	1.12	1.11	0.65
245 (Optimized)	4.12	6.86	3.82	1.11	1.12	0.54
247	4.16	6.90	3.84	1.12	1.12	0.66

Conclusion: No significant change was observed in retention time, resolution, tailing factor and %RSD of peak area with variation in detection wavelength (243–247 nm). Hence, the method is robust with respect to detection wavelength.

Figure 36. Effect of Detection Wavelength Variation



Column Temperature (°C)	Retention Time (min)		Resolution (IVM–MBZ)	Tailing Factor		%RSD of Peak Area
	Mebendazole (295 nm)	Ivermectin (245 nm)		MBZ (295 nm)	IVM (245 nm)	
28	4.07	6.76	3.76	1.15	1.14	0.71
20 (Optimized)	4.11	6.84	3.82	1.11	1.12	0.54
32	4.15	6.92	3.89	1.09	1.15	0.73

Figure 37. Effect of Column Temperature Variation

The deliberate changes introduced into chromatographic conditions did not significantly affect peak shape, retention behavior, resolution, or precision. Resolution values remained greater than 2.0 and %RSD values remained below 2.0% under all conditions investigated. These results confirmed the robustness and reliability of the method and demonstrated its suitability for routine quality control analysis.

3.8 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The sensitivity of the developed RP-HPLC method was assessed by determining the limit of detection (LOD) and limit of quantification (LOQ) according to ICH recommendations. These parameters were calculated using the standard deviation of the response and the slope of the calibration curve.

Table 11. LOD and LOQ Values

Parameter	Mebendazole	Ivermectin
LOD (µg/mL)	0.28	0.09
LOQ (µg/mL)	0.85	0.27
Signal-to-Noise Ratio at LOQ	10.8	11.2

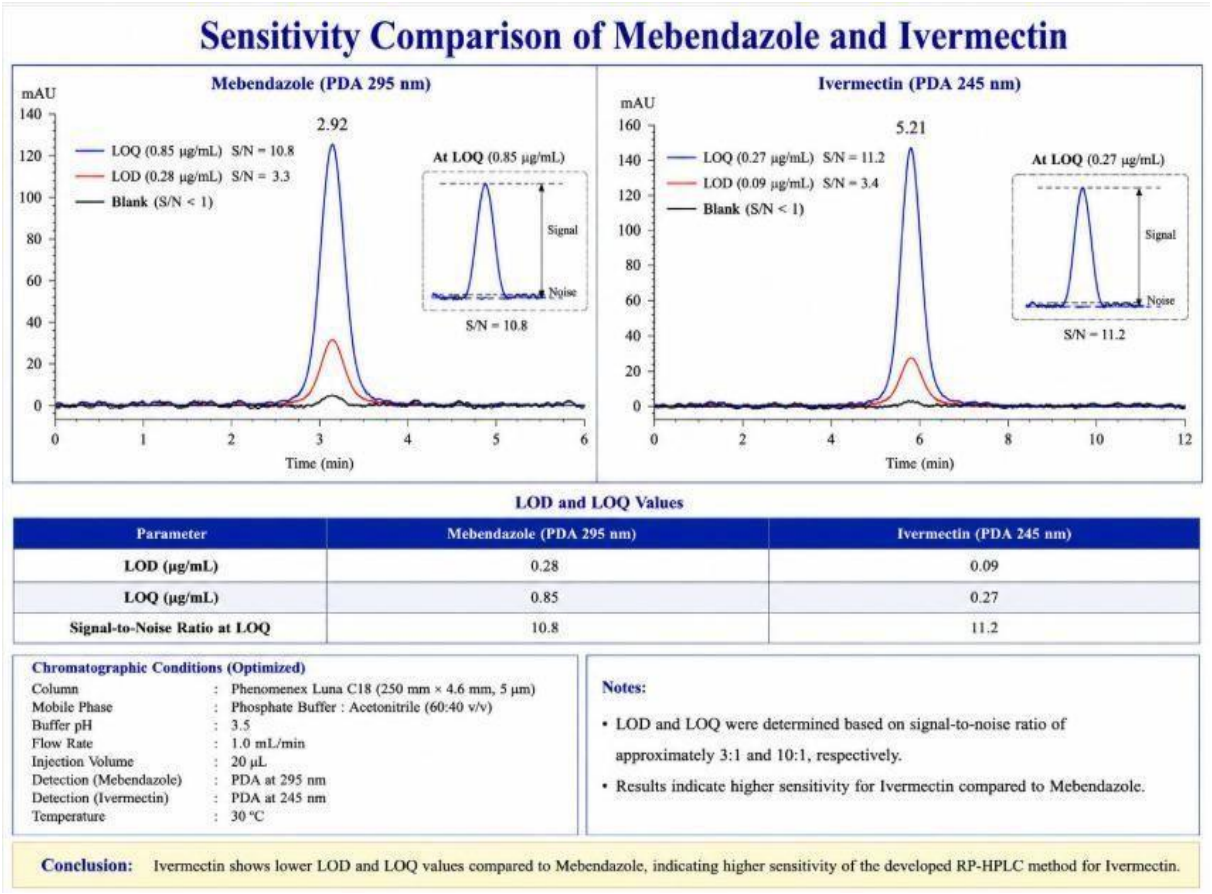


Figure 38. Sensitivity Comparison of Mebendazole and Ivermectin

The low LOD and LOQ values obtained for both analytes demonstrated the high sensitivity of the developed analytical method. The method was capable of detecting and quantifying very low concentrations of Mebendazole and Ivermectin with acceptable accuracy and precision. Such sensitivity makes the method suitable for routine quality control testing and stability studies.

3.9 Overall Discussion

The present investigation successfully established a simple, accurate, precise, robust, and stability-indicating RP-HPLC method for the simultaneous estimation of Mebendazole and Ivermectin in pharmaceutical dosage forms. Systematic optimization of chromatographic variables resulted in excellent chromatographic performance, with Mebendazole and Ivermectin eluting at retention times of 4.12 min and 6.85 min, respectively. Complete baseline separation was achieved with a resolution value greater than 3.8, demonstrating efficient chromatographic separation.

System suitability studies confirmed the adequacy and reproducibility of the chromatographic system, with theoretical plate counts exceeding 4500 and %RSD values below 1.0%. Validation studies performed according to ICH guidelines demonstrated excellent specificity, linearity, accuracy, precision, robustness, and sensitivity. The developed method exhibited outstanding linearity over the investigated concentration ranges with correlation coefficients greater than 0.999 for both analytes.

Accuracy studies produced recoveries close to 100%, while precision studies demonstrated

excellent repeatability and intermediate precision with %RSD values well below the acceptable limit of 2.0%. Robustness testing further confirmed that minor variations in chromatographic conditions did not significantly influence analytical performance. The low LOD and LOQ values highlighted the high sensitivity of the method and its capability to detect trace quantities of the analytes.

The absence of interference from excipients and the excellent peak purity results confirmed the specificity and selectivity of the method. Overall, the developed RP-HPLC method fulfilled all validation requirements and can be successfully employed for routine quality control analysis, method validation studies, and pharmaceutical research involving simultaneous estimation of Mebendazole and Ivermectin in combined dosage forms.

CONCLUSION

The present study successfully developed and validated a simple, rapid, accurate, precise, robust, and stability-indicating RP-HPLC method for the simultaneous estimation of Mebendazole and Ivermectin in pharmaceutical dosage forms. Systematic optimization of chromatographic parameters resulted in efficient separation of both analytes using a Phenomenex Luna C18 column with a mobile phase consisting of 0.1 M potassium dihydrogen orthophosphate buffer (pH 3.5) and acetonitrile (45:55, v/v). Under the optimized chromatographic conditions, Mebendazole and Ivermectin were eluted at retention times of 4.12 min and 6.85 min, respectively, with excellent peak symmetry and satisfactory resolution.

The developed method was validated in accordance with ICH Q2 guidelines and demonstrated excellent performance with respect to system suitability, specificity, linearity, accuracy, precision, robustness, limit of detection, and limit of quantification. The calibration curves exhibited outstanding linearity over the investigated concentration ranges, with correlation coefficient values greater than 0.999 for both analytes. Accuracy studies showed mean recoveries of 99.81% for Mebendazole and 99.92% for Ivermectin, while precision studies produced %RSD values below 2.0%, confirming the reliability and reproducibility of the method.

Specificity studies confirmed the absence of interference from excipients and other formulation components, whereas peak purity analysis demonstrated excellent selectivity of the method. Robustness evaluation revealed that minor deliberate variations in chromatographic conditions did not significantly affect analytical performance. Furthermore, the low LOD and LOQ values indicated high sensitivity of the developed method.

Overall, the validated RP-HPLC method proved to be simple, sensitive, economical, and stability-indicating, making it highly suitable for routine quality control analysis, assay determination, stability studies, and pharmaceutical research involving Mebendazole and Ivermectin in combined dosage forms. The developed method can therefore be confidently employed in pharmaceutical industries and quality control laboratories for reliable simultaneous estimation of these antiparasitic drugs.

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