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"Analytical Method Development And Validation For Simultaneous Estimation Of Fipronil And Methoprene In Its Combined Dosage Form"

Mahesh Rathor[1], Saurabh Jain[2], Dr. Yogendra Singh[3]
Student[1], Associate Professor[2], Professor[3]
Department of Pharmacy
Address: Shri Ramnath Singh Mahavidyalaya (Pharmacy), Gormi, Bhind (M.P.)

ABSTRACT: This study aims to develop an analytical methodology for the concurrent estimation of Fipronil and Methoprene. This involved the creation and validation of both RP-HPLC and UV spectrophotometric methods suitable for analyzing these drugs in their pharmaceutical formulations. The UV spectrophotometric method employed a Q-absorption ratio approach. This particular method relies on absorbance measurements at two specific wavelengths: the iso-absorptive point (identified as 231 nm for Fipronil and Methoprene in methanol) and the λmax of Methoprene, one of the two components. The maximum wavelength was at 264nm which was of methoprene. Fipronil and Methoprene both showed linearity in the concentration range of 1–10 mg/ml. Using a C11 BDS column (100 mm 4.6 mm), the HPLC separation was accomplished. Employing a mobile phase made of acetonitrile and water in an 80:20 volume ratio. At a maximum wavelength of 254 nm, the analyte was detected while the mobile phase was flowing at a rate of 1.5 ml/min. In samples, the drug was eluted from the column after 3.1 minutes, and after 4.71 minutes. To estimate recovery and linearity, five different concentration levels of 5, 10, 15, 20, and 25 g/ml were used. The % RSD findings were found to be <2% for both the methods and the correlation coefficient was more than 0.999.

KEYWORDS:
Fipronil, Methoprene, uv spectroscopic Methoprene, High-Performance Reversed-Phase Liquid Chromatographic.

I. INTRODUCTION:

Fipronil and Methoprene are two active pharmaceutical ingredients (APIs) commonly used in a combined dosage form for the treatment of ectoparasitic infestations. Fipronil is a broad-spectrum insecticide and acaricide, while Methoprene is an insect growth regulator. The combination of these two drugs provides a synergistic effect, offering a more comprehensive solution for pest control.

The quality control of this combined dosage form requires a robust and reliable analytical method for the simultaneous estimation of Fipronil and Methoprene. Currently, there is a lack of validated analytical methods specifically designed for the simultaneous quantification of these two compounds in their combined formulation. This gap in the literature poses a challenge for pharmaceutical industries and regulatory bodies in ensuring the quality, safety, and efficacy of the product.

Analytical method development and validation are essential steps in the process of drug development, quality control, and manufacturing in the pharmaceutical industry [1]. This process, governed by guidelines from organizations like the International Council for Harmonization (ICH), USP General Chapter <1220>, and FDA Guidance for Industry, is essential for drug development and manufacturing [2,3].

The objective of this study was to develop and validate a simple, accurate, and cost-effective UV-Visible spectrophotometric method for the simultaneous estimation of Fipronil and Methoprene in their combined dosage form. The developed method was validated according to ICH guidelines, with a focus on parameters such as linearity, accuracy, precision, and robustness. The successful development of such a method will provide a valuable tool for routine quality control analysis of this important combination drug product.

II .MATERIALS AND METHODS

Materials

- **Active Pharmaceutical Ingredients (APIs):** Fipronil and Methoprene were procured from Sava Healthcare Ltd., Surendranagar, India.
- **Reagents and Solvents:** Analytical-grade reagents were used throughout the study. Acetonitrile (HPLC grade), Methopreneanol (HPLC grade), and HPLC-grade water were employed for method development and validation.

Instrumentation

The following instruments were utilized during the experimental work:

Table 1: Name of Instrument

Instrument	Manufacturer
FTIR Spectrometer	Bruker, Germany
Digital Weighing Balance	Mettler Toledo, Switzerland
UV-Visible Spectrophotometer	Shimadzu UV-1900, Japan
HPLC System	Shimadzu LC-2050C, Japan
Melting Point Apparatus	Veego, India

Preliminary Characterization

Solubility Test

The solubility of Fipronil and Methoprene was assessed by adding each drug (10 mg) to a 100 mL volumetric flask. Solvents were added incrementally (0.1 mL per addition) at room temperature with intermittent shaking until complete dissolution was observed. The volume of solvent required for dissolution was recorded and interpreted using standard descriptive solubility terms:

Table 2: Solubility data

Descriptive term	Part of solvent required per part of solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1000
Very slightly soluble	From 1000 to 10,000
Practically insoluble	10,000 and over

Melting Point Determination

The melting points of Fipronil and Methoprene were determined using a digital melting point apparatus (Veego, India). A small quantity of each drug was introduced into an open capillary tube, which was then placed in the device. The temperature at which the drug melted was recorded.

FTIR Spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy was employed to confirm the identity of the drugs. Sample pellets were prepared by triturating 1 mg of drug with 100 mg of potassium bromide (KBr) in an agate mortar and pestle to obtain a uniform mixture. The mixture was compressed using a KBr press at 20 psi for 10 minutes to form transparent discs. Pure KBr was used to record the baseline. The prepared discs were scanned using a Bruker FTIR spectrometer over the range of 200–400 cm^{-1}.

UV Spectroscopy Method Development:

Preparation of Stock Solutions

A stock solution of Methoprene was prepared by accurately weighing 10 mg of the active pharmaceutical ingredient (API) and transferring it into a 10 mL volumetric flask. The drug was dissolved in Methopreneanol and the volume was made up to 10 mL, yielding a final concentration **of 1000 µg/mL.**

Preparation of Working Standard Solutions

Aliquots from the stock solution were diluted to obtain working standard solutions of Methoprene and Fipronil in the concentration range of 2–10 µg/mL. These solutions were scanned using a UV-visible spectrophotometer in the range of 200–400 nm to determine the absorbance maxima and assess linearity.

Preparation of stock solutions for Linearity Studies

Aliquots were diluted to prepare concentrations of 2, 4, 6, 8, and 10 µg/mL for both drugs. Absorbance was measured between 200–400 nm.

Preparation of Mixed Standard Solutions

Mixed standard solutions containing both Methoprene and Fipronil were prepared by combining appropriate aliquots from their respective 100 µg/mL stock solutions. Final concentrations ranged from 2–10 µg/mL for each drug.

Analysis of Marketed Formulation

A commercial formulation labeled to contain 9.8 mg of Fipronil and 8.8 mg of Methoprene was analyzed. An amount equivalent to 50 mg of Fipronil was accurately weighed and transferred to a 100 mL volumetric flask. Methopreneanol was added, and the solution was sonicated for 15 minutes to ensure complete dissolution. The volume was made up to the mark with Methopreneanol and filtered through Whatman filter paper (0.45 µm). Sample solutions of 2, 4, 6, 8, and 10 µg/mL for both Fipronil and Methoprene were prepared by appropriate dilution of the stock solution. All analyses were performed in triplicate.

HPLC Method Development:

Selection of Solvent

Fipronil and Methoprene were found to be freely soluble in methopreneanol and acetonitrile (ACN). Stability studies were conducted by scanning 10 µg/mL solutions of each drug under UV spectroscopy. Both compounds remained stable at room temperature for 24 hours and under refrigerated conditions for 48 hours. Based on solubility and stability profiles, ACN and methopreneanol were selected as suitable solvents for HPLC analysis.

Selection of Wavelength

To determine the optimal detection wavelength, standard solutions of Fipronil and Methoprene (10 µg/mL each) were scanned in the range of 200–400 nm using a UV spectrophotometer. Overlaying the spectra revealed a common absorbance maximum at 231 nm, which was selected as the detection wavelength for simultaneous estimation.

Preparation of Standard Stock Solutions

Standard stock solutions were prepared by accurately weighing 10 mg each of Fipronil and Methoprene and dissolving them in mobile phase in separate 10 mL volumetric flasks to obtain concentrations of 1000 µg/mL (Stock A). From Stock A, 1 mL was diluted to 10 mL to prepare Stock B (100 µg/mL). Further dilutions were made from Stock B to obtain mixed standard solutions containing 20–100 µg/mL of each drug.

Selection of Mobile Phase

Various mobile phase combinations were evaluated to achieve optimal chromatographic performance, including peak shape, resolution, and theoretical plate count. Parameters such as buffer type, pH, and solvent ratio were systematically varied. The final mobile phase consisted of acetonitrile and water in an 80:20 v/v ratio, adjusted to the appropriate pH.

Optimization of Chromatographic Conditions

Chromatographic conditions were optimized using a Box-Behnken design approach. Key factors included mobile phase composition, pH, and flow rate. Separation was achieved using an Inertsil ODS-C11 column (250 mm × 4.6 mm, 5 µm particle size). The mobile phase was delivered at a flow rate of 1.5 mL/min, with acetonitrile serving as the diluent. Detection was performed at 231 nm.

Preparation of Working Solutions

Working standard solutions were prepared by accurately weighing 10 mg each of Fipronil and Methoprene and transferring them into separate 10 mL volumetric flasks. The drugs were dissolved by sonication for 20 minutes to yield stock solutions of 1000 µg/mL. These were further diluted to obtain the required concentrations for analysis.

Method Validation

The developed HPLC method for simultaneous estimation of Fipronil and Methoprene was validated in accordance with ICH Q2(R1) guidelines. Validation parameters included system suitability, specificity, linearity, precision, accuracy, robustness, and determination of LOD and LOQ.

System Suitability

System suitability was assessed by injecting six replicates of a mixed standard solution containing 10 µg/mL of Fipronil and 10 µg/mL of Methoprene. Parameters such as retention time, peak area, theoretical plates, and tailing factor were evaluated to ensure consistent system performance.

Specificity

Specificity was determined by comparing chromatograms of standard solutions (10 µg/mL) of Fipronil and Methoprene with those of the marketed formulation at the same concentrations. A blank chromatogram was also recorded. No interference from excipients or other components was observed, confirming the method's specificity.

Linearity

Linearity was evaluated over the concentration range of 20–100 µg/mL for both Fipronil and Methoprene. Aliquots of 0.2, 0.4, 0.6, 0.8, and 1.0 mL from the standard stock solution were diluted to 10 mL with mobile phase. Each concentration was injected in triplicate. Calibration curves were plotted for each drug, and regression equations were calculated. The correlation coefficients (R^2) were found to be >0.999, indicating excellent linearity.

Precision

Repeatability

Repeatability was assessed by injecting six replicates of a 10 µg/mL standard solution of each drug under identical chromatographic conditions. The %RSD of peak areas and retention times was calculated and found to be less than 2%, confirming method precision.

Intraday Precision

Three concentrations (20, 60, and 100 µg/mL) of Fipronil and Methoprene were analyzed in triplicate within the same day. The %RSD values were below 2%, indicating good intraday precision.

Interday Precision

The same three concentrations were analyzed over three consecutive days. The %RSD values remained below 2%, demonstrating consistent interday precision.

Accuracy

Accuracy was determined by recovery studies using the standard addition method. Known quantities of standard Fipronil (4, 8, and 12 µg/mL) and Methoprene (2, 4, and 6 µg/mL) were spiked into a sample solution containing 8 µg/mL of Fipronil and 50 µg/mL of Methoprene. Recovery was calculated using the regression equations from the calibration curves. The percentage recovery ranged within acceptable limits, confirming the accuracy of the method.

Robustness

Robustness was evaluated by analyzing three concentrations (8, 24, and 40 µg/mL for Fipronil; 2, 6, and 10 µg/mL for Methoprene) under varied conditions. Flow rate was altered by ±0.1 mL/min and detection wavelength by ±2 nm. The %RSD values remained below 2%, indicating the method's robustness against minor changes in analytical conditions.

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

LOD and LOQ were calculated from the standard deviation of the y-intercepts and the slope of the calibration curves using the following formulas:

- **LOD** = 3.3 × (SD/Slope)
- **LOQ** = 10 × (SD/Slope)

The calculated values confirmed the method's sensitivity for detecting and quantifying low concentrations of both analytes.

Analysis of Marketed Formulation

Twenty tablets were weighed, powdered, and an amount equivalent to 40 µg/mL Fipronil and 10 µg/mL Methoprene was transferred to a 10 mL volumetric flask. The mixture was sonicated for 30 seconds, diluted, and filtered. A 1 mL aliquot was further diluted to 10 mL to obtain final concentrations. % Recovery was calculated to assess accuracy.

III RESULT AND DISCUSSION

Preliminary Test:

1. Solubility Test

Initial characterization of Fipronil and Methoprene was performed to confirm their identity and suitability for analytical method development. Both compounds were found to be *freely soluble* in methanol and acetonitrile, as per solubility classifications outlined in the Indian Pharmacopoeia.

2. Melting Point Determination

The observed melting points—198–200 °C for Fipronil and 24–26 °C for Methoprene—were consistent with reported literature values, confirming the purity of the reference standards.

3. FTIR Spectroscopy

FTIR spectroscopy was utilized to identify and confirm the functional groups present in Fipronil and Methoprene. Samples were prepared using the potassium bromide (KBr) disc method, wherein 1 mg of drug was mixed with 100 mg of dry KBr and triturated to a fine powder. The mixture was compressed into pellets at 20 psi for 10 minutes. Spectral scans were recorded in the range of 200–400 cm^{-1}, and baseline correction was performed using pure KBr.

3.1 IR Spectra of Fipronil

Although Fipronil is not officially listed in any pharmacopeia, its IR spectrum was analyzed based on known structural features. The observed wave numbers were matched with expected functional group vibrations.

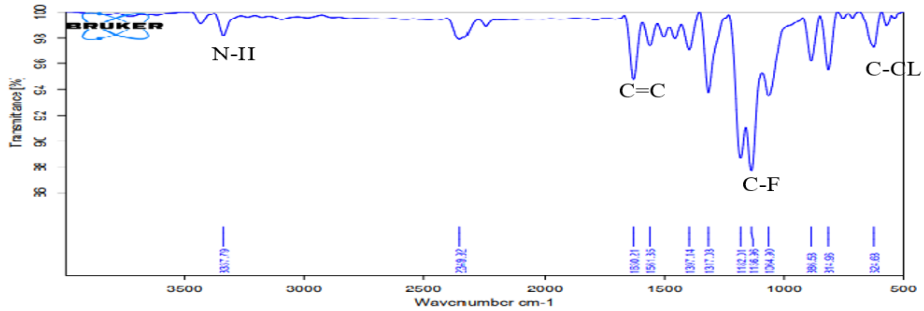


Figure 1: Observed IR spectra of Fipronil

Table 3: Observed Wave Numbers and Corresponding Functional Groups for Fipronil

Functional Group	Reported Wave No. (cm^{-1})	Observed Wave No. (cm^{-1})
C=C Stretching (Aromatic)	1740–1730	1630.21
N–H Stretching (2° Amine)	3500–3100	3337.79
C≡N Stretching	2260–2240	2349.92
C–Cl Stretching	785–540	624.63
C–F Stretching	1400–1000	1317.03

Discussion: The IR spectrum of Fipronil revealed characteristic peaks corresponding to its functional groups, including aromatic C=C, secondary amine N–H, nitrile C≡N, and halogen stretches (C–Cl and C–F). These findings support the structural identity of the compound.

3.2 IR Spectra of Methoprene

Methoprene was similarly analyzed using FTIR spectroscopy. The observed wave numbers were compared with literature-reported values for expected functional groups.

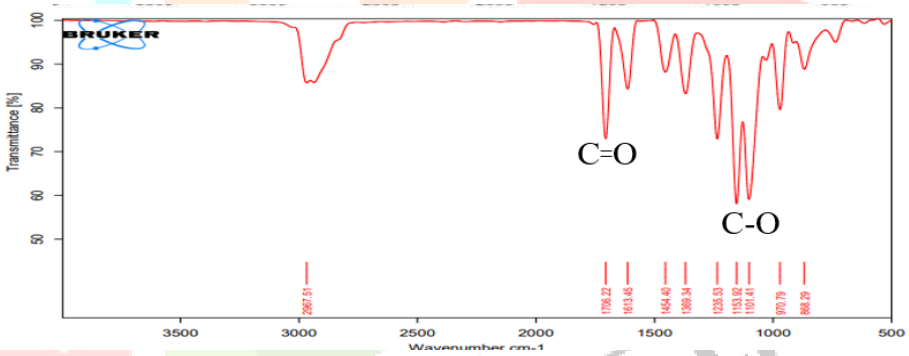


Figure 2: Observed IR spectra of Methoprene

Table 4: Observed Wave Numbers and Corresponding Functional Groups for Methoprene

Functional Group	Reported Wave No. (cm^{-1})	Observed Wave No. (cm^{-1})
C–H Stretching (Aromatic)	900–690	868.29
C=C Stretching	1740–1720	1613.45
C–O Stretching	1300–1000	1235.53
C=O Stretching (2° Alcohol)	1725–1700	1613.45

Discussion: The IR spectrum of Methoprene confirmed the presence of aromatic C–H, C=C, C–O, and carbonyl (C=O) groups, consistent with its known chemical structure.

Conclusion

The FTIR spectral analysis of Fipronil and Methoprene demonstrated the presence of expected functional groups, validating the identity of the procured reference standards. The observed wave numbers aligned well with reported literature values, confirming the authenticity of the samples.

4. Selection of Wavelength

To determine the optimal wavelength for UV Spectrophotometric analysis, standard solutions of Fipronil and Methoprene (10 µg/mL) were scanned individually in the range of 200–400 nm. An overlay of the absorption spectra revealed a common maximum absorbance at 264 nm.

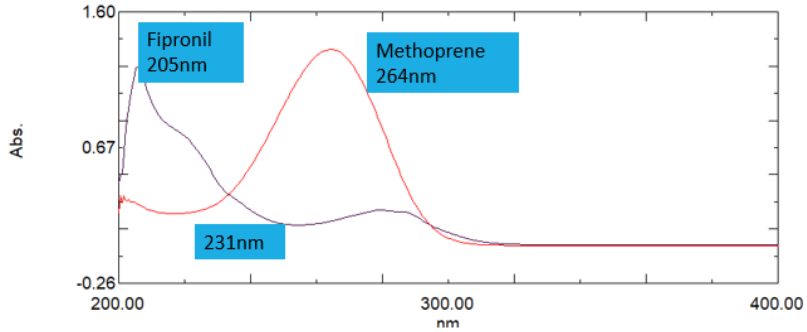


Figure 3: Overlay spectra of both the drug

Discussion: The wavelength of 264 nm was selected for further analytical procedures, as it represents the point of maximum absorbance for both Fipronil and Methoprene, ensuring optimal sensitivity and accuracy in quantitative analysis.

5. UV Method Developments

5.1 Solvent Selection

Solubility screening was performed to identify a suitable solvent for UV analysis. Both Fipronil and Methoprene showed good solubility in methanol and acetonitrile. Methanol was selected due to its superior solubilizing capacity and compatibility with UV spectrophotometry.

5.2 Standard Stock Preparation and Spectral Analysis

Stock solutions (1000 µg/mL) of each drug were prepared in methanol and diluted to obtain working standards (1–10 µg/mL). UV scans (200–400 nm) revealed Methoprene's λmax at 264 nm and an isoabsorptive point at 231 nm for both drugs. These wavelengths were selected for quantitative analysis. Overlay spectra confirmed minimal spectral overlap, supporting simultaneous estimation.

5.3 Wavelength Selection and Calibration

For High-Performance Liquid Chromatography (HPLC) with UV detection, selecting an appropriate wavelength is critical for ensuring sensitivity and selectivity. Separate UV scans of standard solutions of Fipronil and Methoprene (10 µg/mL each) were conducted in the 200–400 nm range. Overlaying the spectra revealed that both compounds exhibited significant absorbance at 231 nm, making it a suitable detection wavelength for simultaneous estimation.

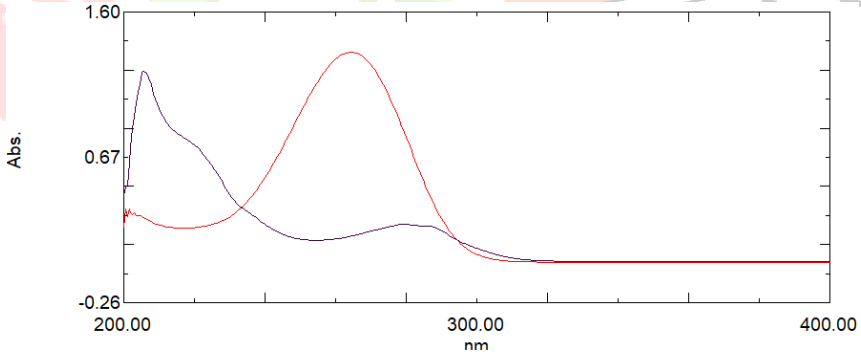


Figure 4: Absorbance at the detection wavelength

6. Validation

6.1 Linearity and Range

Linearity was assessed for both Methoprene and Fipronil over the concentration range of 2–10 µg/mL. Calibration curves were constructed by plotting absorbance against concentration at two selected wavelengths: 205 nm and 264 nm. The data demonstrated a strong linear relationship, confirming the method's suitability for quantitative analysis.

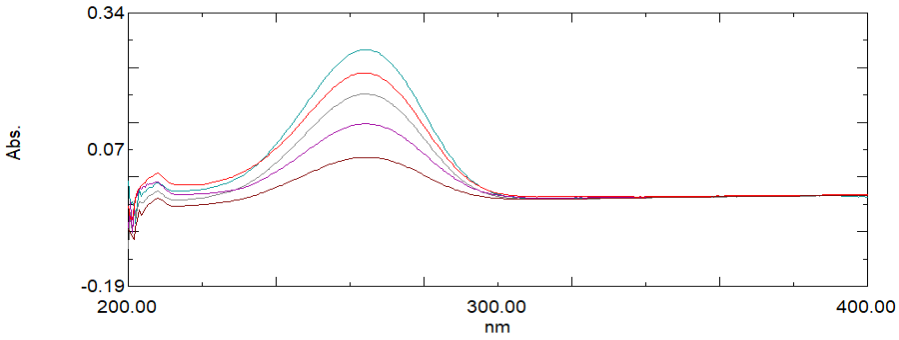


Figure 5: Linearity Spectra of Fipronil

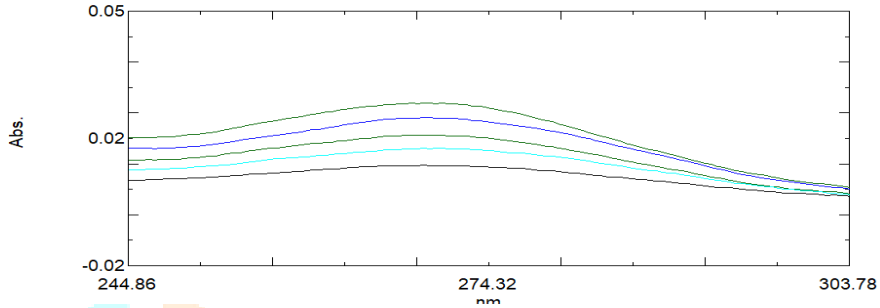


Figure 6: Linearity Spectra of Methoprene

Table 5: Linearity Data for Methoprene and Fipronil

Sr. No.	Concentration (µg/mL)	Absorbance at 205 nm (Methoprene)	Absorbance at 264 nm (Fipronil)
1	2	0.175	0.016
2	4	0.357	0.033
3	6	0.507	0.053
4	8	0.658	0.070
5	10	0.825	0.089

Discussion: The calibration curves for both drugs showed excellent linearity, with correlation coefficients (R^2) close to 1. This confirms the method's reliability for quantifying Methoprene and Fipronil within the tested range.

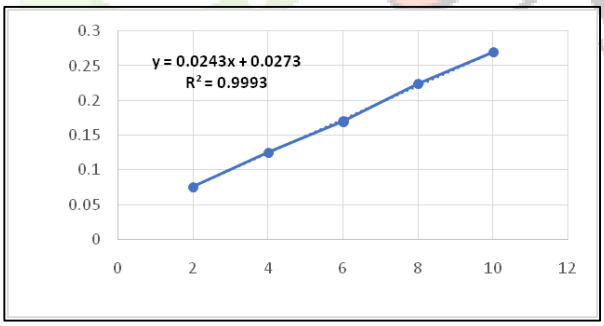


Figure 7: calibration curves for both drugs

6.2 Accuracy

Accuracy was evaluated through recovery studies at three concentration levels: 50%, 100%, and 150%. Known amounts of standard were spiked into pre-quantified sample solutions, and the percentage recovery was calculated.

Table 6: Recovery Study for Fipronil and Methoprene

Drug	Level (%)	Sample Amount (µg/mL)	Standard Spiked (µg/mL)	Total Amount (µg/mL)	Recovered Amount (µg/mL)	% Recovery ± SD
Fipronil	50	5	2.5	7.5	7.46	99.46 ± 0.34
	100	5	5	10	10.6	100.6 ± 0.38
	150	5	7.5	12.5	12.48	99.84 ± 0.45
Methoprene	50	5	2.5	7.5	7.48	99.73 ± 0.67
	100	5	5	10	9.94	99.40 ± 0.89
	150	5	7.5	12.5	12.46	99.68 ± 0.45

Discussion: The recovery values for both Fipronil and Methoprene ranged from 98% to 101%, indicating high accuracy of the developed method. The %RSD values were within acceptable limits, confirming consistency across replicates.

6.3 Precision

6.3.1 Repeatability

Repeatability was assessed by analyzing six replicates of a 1 µg/mL solution of both Fipronil and Methoprene. The absorbance values were recorded, and %RSD was calculated.

Table 7: Repeatability Data for Fipronil and Methoprene

Concentration (1:1µg/ml)	Fipronil (205 nm)	Methoprene (264 nm)
1	0.2351	0.0361
2	0.2344	0.0364
3	0.2357	0.0363
4	0.2351	0.0364
5	0.2349	0.0362
6	0.2351	0.0364
Mean	**0.2350**	**0.0363**
Standard Deviation (SD)	**0.0004**	**0.0001**
Relative Standard Deviation (%RSD)	**0.177**	**0.333**

Discussion: The %RSD values for both drugs were below 2%, indicating excellent repeatability and precision of the developed UV Spectrophotometric method.

6.3.2 Intraday Precision

Intraday precision was evaluated by analyzing standard solutions of Fipronil and Methoprene at three time intervals (0 hr, 1 hr, and 2 hr) on the same day. The %RSD values were calculated to assess method consistency.

Table 8: Intraday Precision Data

Drug	Conc. (µg/mL)	0 hr	1 hr	2 hr	Mean	SD	%RSD
Fipronil (205 nm)	5	0.2351	0.2348	0.2311	0.2336	0.0022	0.953
	10	0.3946	0.3942	0.3911	0.3933	0.0019	0.487
	15	0.6026	0.5982	0.5835	0.5947	0.0100	1.114
Methoprene (264 nm)	10	0.0335	0.0327	0.0324	0.0328	0.0005	1.330
	15	0.0521	0.0511	0.0501	0.0510	0.0009	1.261
	20	0.0761	0.0755	0.0731	0.0749	0.0012	1.730

Discussion: All %RSD values were below 2%, indicating excellent intraday precision and stability of the method.

6.3.3 Intermediate Precision

A. Interday Precision

Interday precision was assessed by analyzing standard solutions over three consecutive days. The %RSD values were calculated to evaluate reproducibility.

Table 9: Interday Precision Data

Drug	Conc. (µg/mL)	Day 1	Day 2	Day 3	Mean	SD	%RSD
Fipronil (205 nm)	5	0.2351	0.2311	0.2265	0.2336	0.0022	0.953
	10	0.3946	0.3839	0.3811	0.3865	0.0071	1.543
	15	0.6026	0.5991	0.5855	0.5973	0.0090	1.516
Methoprene (264 nm)	10	0.0335	0.0330	0.0323	0.0328	0.0005	1.330
	15	0.0521	0.0445	0.0438	0.0516	0.0006	1.245
	20	0.0761	0.0741	0.0739	0.0747	0.0012	1.628

Discussion: All %RSD values were within acceptable limits, confirming the method's reproducibility across days.

B. Robustness

Robustness was evaluated by varying the detection wavelength ±1 nm. The %RSD values were calculated to assess the method's reliability under slight changes in analytical conditions.

Table 10: Robustness Data

Drug	Concentration (µg/ml)	204nm	205nm	206nm	Mean	SD	%RSD
Fipronil	5	0.2351	0.2373	0.2331	0.2351	0.0021	0.893
	10	0.3946	0.3850	0.3811	0.3869	0.0069	1.295
	15	0.6026	0.5979	0.5821	0.5942	0.0107	1.407
Methoprene	10	0.0335	0.0329	0.0341	0.0335	0.0006	1.691
	15	0.0521	0.0519	0.0537	0.0525	0.0009	1.476
	20	0.0761	0.0753	0.0779	0.0764	0.0013	1.342

Discussion: The method remained robust under minor wavelength variations, with %RSD values below 2%.

Instrument Variability

Precision was also tested across different instruments (1700, 1800, 1900 I series). The %RSD values confirmed consistency across platforms.

Table 11: Instrument Variability Data

Drug	Conc. (µg/mL)	Inst. 1 (1700)	Inst. 2 (1800)	Inst. 3 (1900 I)	Mean	SD	%RSD
Fipronil	5	0.2351	0.2411	0.2431	0.2397	0.0042	1.136
	10	0.3946	0.4012	0.4087	0.4015	0.0070	1.457
	15	0.6026	0.6178	0.6245	0.6149	0.0112	1.012
Methoprene	10	0.0335	0.0349	0.0351	0.0347	0.0005	1.690
	15	0.0521	0.0533	0.0545	0.0534	0.0010	1.483
	20	0.0761	0.0766	0.0771	0.0764	0.0008	1.144

Discussion: Instrumental precision was confirmed, with %RSD values well within acceptable limits.

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

LOD and LOQ were calculated using the standard deviation of the intercept and the slope of the calibration curve:

- **LOD** = 3.3 × SD / Slope
- **LOQ** = 10 × SD / Slope

Table 12: LOD and LOQ Data

Parameter	Fipronil (205 nm)	Methoprene (264 nm)
SD of Intercept	0.00531	0.0015
Mean Slope	0.0306	0.0037
LOD (µg/mL)	0.57	1.33
LOQ (µg/mL)	1.73	4.05

Discussion: The method demonstrated adequate sensitivity for both drugs, with low LOD and LOQ values suitable for trace-level detection.

6.5 Analysis of Marketed Formulations

The developed method was applied to analyze commercial formulations containing Fipronil and Methoprene. Drug content was quantified and % amount found was calculated.

Table 13: Analysis of Marketed Formulations

Drug	Drug content (mg)	Conc. (µg/ml)	% Amount found			Mean % Amount found
			1	2	3	
Fipronil	9.8mg	5	101.04	100.17	99.31	100.17±0.86
		10	101.56	100.69	99.83	100.69±0.86
		15	101.15	100.58	100.67	100.58±0.30
Methoprene	8.8 mg	4	100.40	101.35	100.89	100.36±0.40
		8	101.06	101.78	99.02	100.04±0.75
		12	100.11	101.09	100.34	100.21±0.54

7. HPLC Method Development

Selection of Mobile Phase:

A series of trials were conducted to optimize the mobile phase for simultaneous estimation of Fipronil and Methoprene using High-Performance Liquid Chromatography (HPLC). Various combinations of solvents, buffers, and pH adjustments were evaluated to achieve optimal peak resolution, retention time, and system suitability parameters.

Trial 1:

Observation: Sample and blank eluted at same retention time.

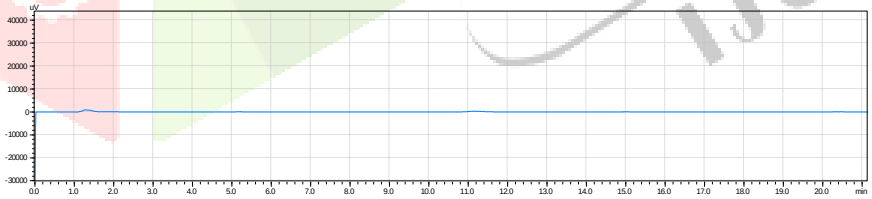


Figure 8: Mobile phase: Methopreneanol: Water (70:30 v/v) (80:20 v/v) at 264 nm

Trial 2:

Observation: Sample and blank eluted at same retention time.

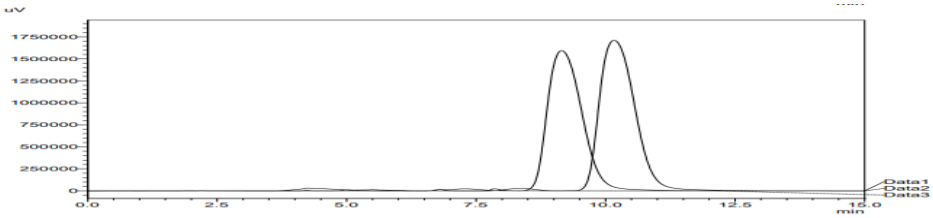


Figure 9: Mobile phase: Methopreneanol: Potassium dihydrogen orthophosphate Buffer (pH 4) (pH adjusted with Orthophosphoric acid) (60: 40 v/v) (70:30 v/v) at 264 nm

Trial 3:

Observation: Extra peaks before Fipronil; peak fronting in Methoprene

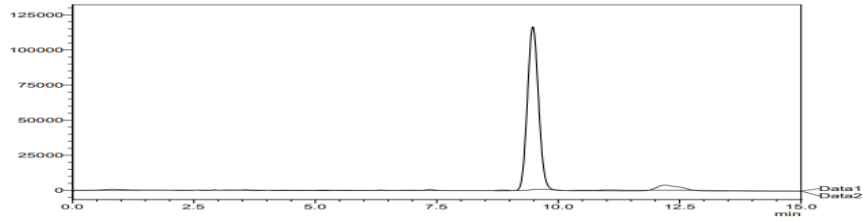


Figure 10: Mobile phase: Acetonitrile: Water (75:25 v/v) at 264 nm

Trial 4:

Observation: Peak broadening in Fipronil; peak splitting in Methoprene

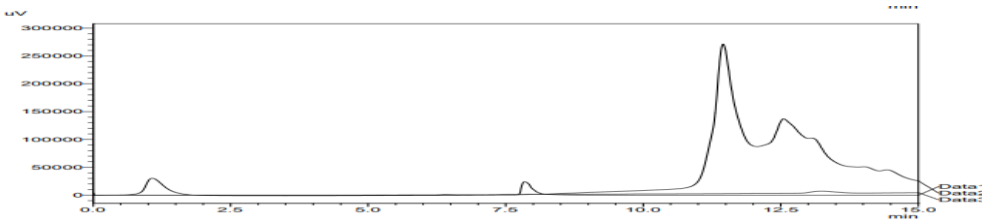


Figure 11: Mobile phase: Acetonitrile: Water (80: 20 v/v) at 264 nm

Trial 5:

Observation: Both drugs eluted; peaks not well resolved.

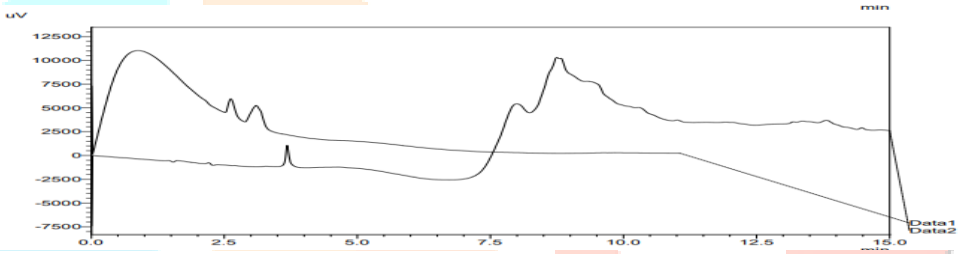


Figure 12: Mobile phase: Acetonitrile: Water (80: 20 v/v) (0.1 % orthophosphoric acid) at 241 nm

Trial 6:

Observation: Peak splitting in Fipronil; broadening in Methoprene.

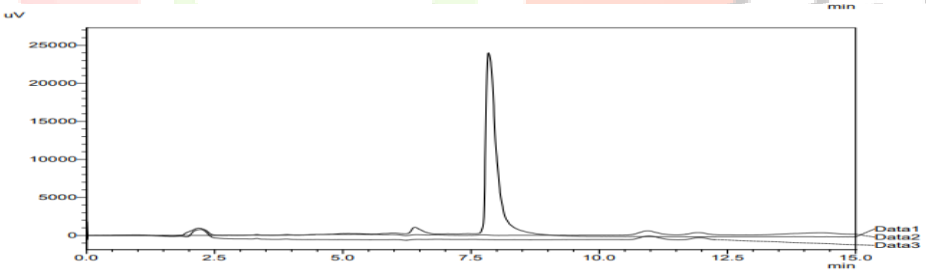


Figure 13: Mobile phase: Acetonitrile: Water (pH 4) (pH adjusted with Orthophosphoric acid) (80: 20 v/v) at 241 nm

Trial 7:

Observation: Broad peaks for both drugs; low theoretical plates.

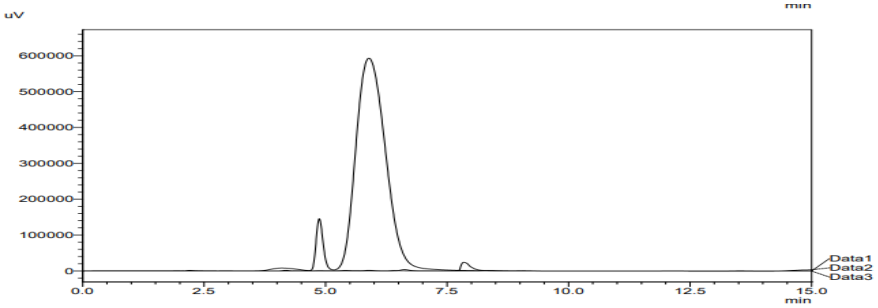


Figure 14: Mobile phase: Acetonitrile: Water (pH 6) (pH adjusted with Orthophosphoric acid) (80: 20 v/v) at 264 nm

Trial 8:

Observation: Poor peak shape for Fipronil; peak splitting in Methoprene

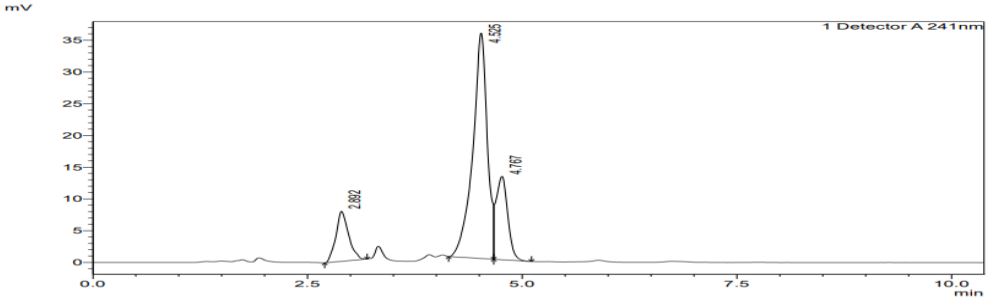


Figure 15: Mobile phase: Acetonitrile: Water (pH 6.5) (pH adjusted with Triethylamine) (80: 20 v/v) at 241

Trial 9 (Optimized Condition):

Observation: Optimized condition: well-resolved peaks, acceptable system suitability.

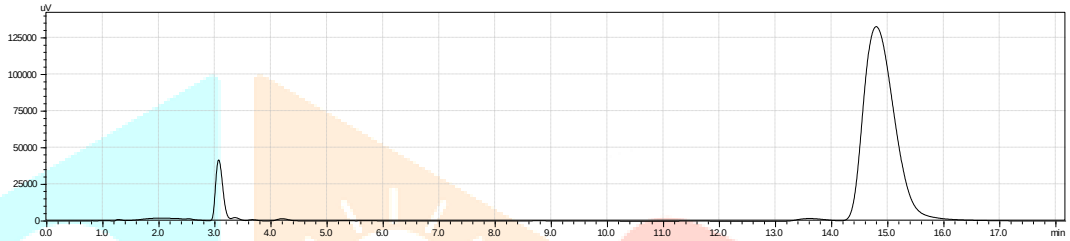


Figure 16: Mobile phase: Acetonitrile: water (80:20) at 264 nm

Optimized Chromatographic Conditions

Under Trial 9 conditions, both Fipronil and Methoprene were successfully eluted with sharp, symmetrical peaks and acceptable system suitability parameters.

Table 14: Optimized Chromatographic Conditions of HPLC

Drug	Retention Time (min)	Tailing Factor	Theoretical Plates	Resolution
Fipronil	3.7	1.265	6421	6.294
Methoprene	14.7	1.163	9943	—

Based on extensive literature review and experimental trials, various mobile phase compositions were tested using methanol, acetonitrile, water, and buffer systems at different pH levels. Among all trials, **Trial 9**—using **Acetonitrile: Water (80:20 v/v) at 264 nm**—provided optimal chromatographic performance. This mobile phase was selected for further method validation and analysis due to its superior resolution, acceptable tailing factors, and high theoretical plate counts.

8. Method Validation

Method validation was performed in accordance with ICH guidelines to ensure the reliability, accuracy, and reproducibility of the developed HPLC method for simultaneous estimation of Fipronil and Methoprene.

8.1 System Suitability

System suitability parameters were evaluated using six replicate injections of standard solutions. Parameters assessed included theoretical plates, retention time, tailing factor, and resolution.

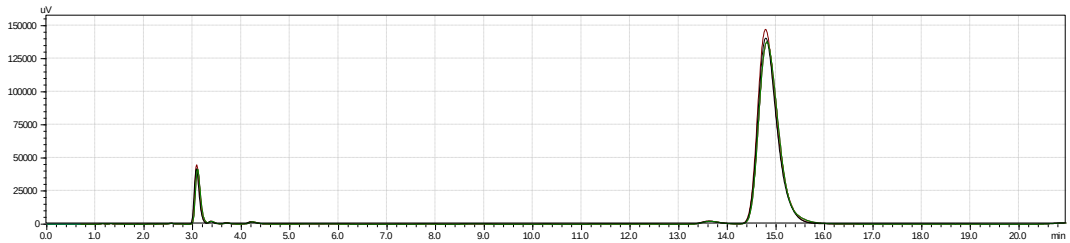


Figure 17: Chromatogram of System suitability of Fipronil and Methoprene

Table 16: System Suitability Data

Sr. No.	Theoretical Plates		Retention Time		Tailing Factor		Resolution
	Fipronil	Methoprene	Fipronil	Methoprene	Fipronil	Methoprene	
1	2471	5653	3.7	14.7	1.367	1.463	2.207
2	2476	5650	3.7	14.7	1.365	1.463	2.304
3	2470	5631	3.7	14.7	1.364	1.464	2.225
4	2472	5596	3.7	14.8	1.365	1.462	2.350
5	2471	5642	3.7	14.7	1.365	1.459	2.196
6	2473	5659	3.7	14.8	1.364	1.465	2.213

Acceptance Criteria:

- Theoretical Plates: > 2000
- %RSD for Retention Time: < 2%
- Tailing Factor: < 1.5
- Resolution: > 2

Discussion: The %RSD for retention time was found to be 0.2605 for Fipronil and 0.5683 for Methoprene, both within the acceptable limit of <2%. Theoretical plate counts exceeded 2000 for both drugs, indicating good column efficiency. Tailing factors were below 1.5, and resolution values were above 2, confirming adequate separation. These results validate the system's suitability for routine analysis.

8.2 Specificity

Specificity was assessed by comparing chromatograms of standard solutions, marketed formulations, and blank samples. The objective was to ensure no interference from excipients or other formulation components.

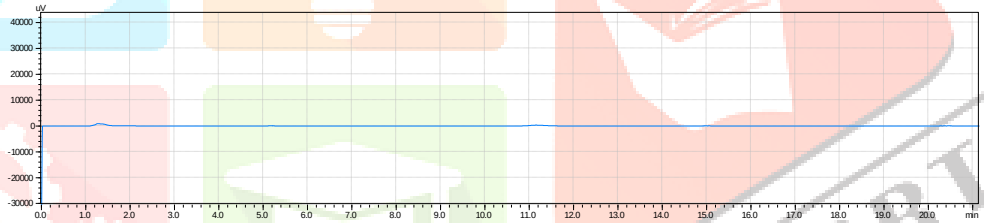


Figure 18: Chromatogram of Blank

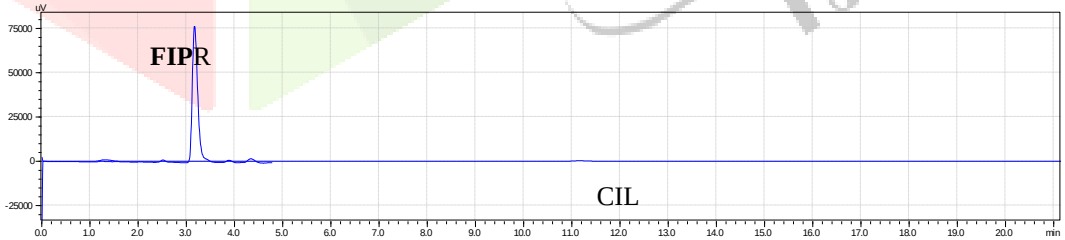


Figure 19: Chromatogram of Standard FIPR

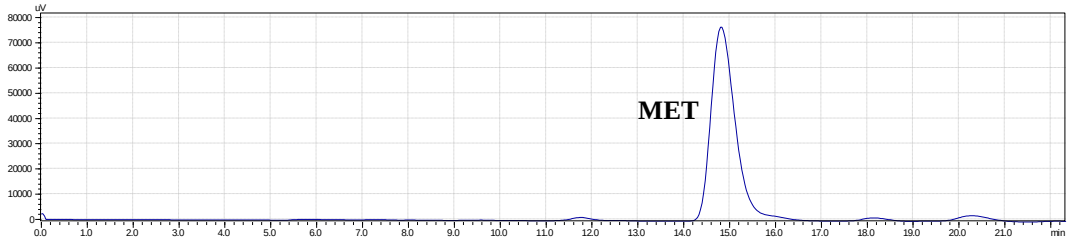


Figure 20: Chromatogram of Standard METHOPRENE

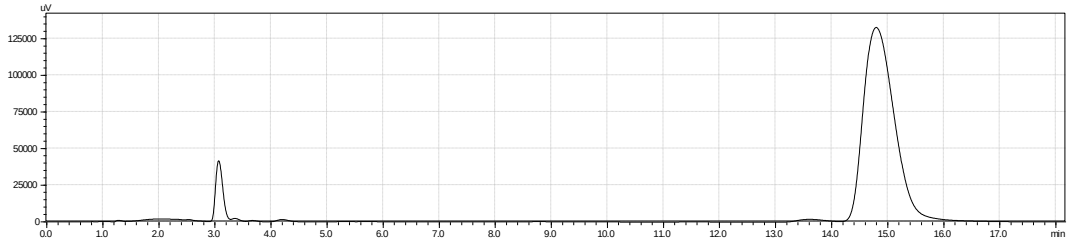


Figure 21: Chromatogram of Marketed formulation

Discussion: No interfering peaks were observed in the chromatograms of marketed formulations or blanks. The peaks corresponding to Fipronil and Methoprene were well-resolved and free from excipient interference, confirming the specificity of the method.

8.3 Linearity

Linearity was evaluated by plotting calibration curves of peak area versus concentration for both drugs. The concentration range tested was 20–100 µg/mL for Fipronil and Methoprene.

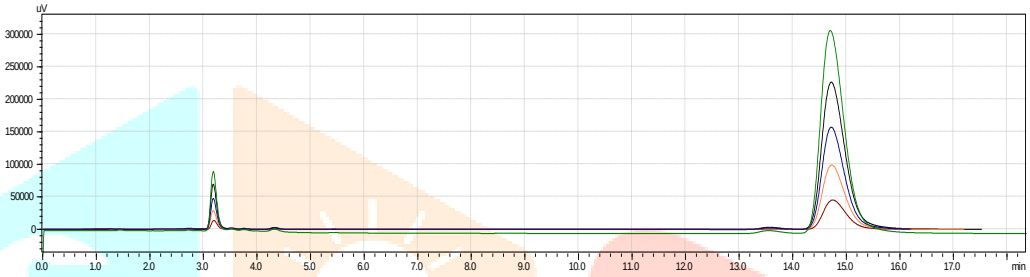


Figure 22: Chromatogram of System Linearity of Fipronil and Methoprene

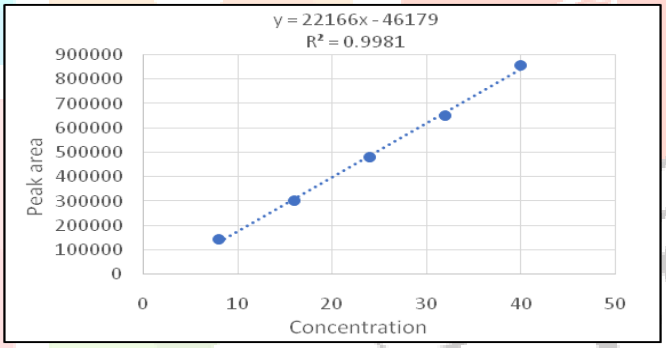


Figure 23: Linearity Graph of Conc. v/s Peak area

Discussion: The calibration curves demonstrated excellent linearity across the tested range, with correlation coefficients (R^2) close to 1. This confirms the method's capability to produce accurate and proportional responses over a wide concentration range.

8.4 Precision

- **Repeatability:** %RSD values were 0.5535 for Fipronil and 1.3327 for Methoprene, indicating excellent repeatability.

Table 17: Data of Repeatability (n=6)

Conc. of Fipronil µg/ml	Peak area of Fipronil	Conc. of Methoprene µg/ml	Peak area of Methoprene
40	853141	10	481913
40	855485	10	489267
40	852217	10	481751
40	854233	10	489435
40	854232	10	483750
40	854233	10	477273
Mean	856206.8	**Mean**	487896.1
S.D.	4407.51	**S.D.**	5835.94
%RSD	0.5535	**%RSD**	1.3327

- **Intraday Precision:** %RSD values ranged from 0.0685 to 0.6062 across concentrations and time intervals, confirming stability and precision within a single day.

Table 18: Data of Intraday Precision (n=3)

Drug	Conc. µg/ml	Peak area (Day 1)	Peak area (Day 2)	Peak area (Day 3)	Mean	S.D.	%RSD
Fipronil	20	142841	142737	139130	141569.3	2113.16	0.4926
	60	478987	477505	475485	477325.7	1757.87	0.3682
	80	853141	852185	852514	852514.3	5421.94	0.0685
Methoprene	20	102836	101836	102976	102549.3	6211.71	0.6062
	60	289761	288241	287737	288579.7	1053.64	0.3651
	80	4819113	481751	471913	481859.1	5393.53	0.0216

- **Interday** Precision: Over three consecutive days, %RSD values remained below 2% for both drugs, demonstrating reproducibility.

Table 19: Data of Interday Precision (n=3)

Drug	Conc. µg/ml	Peak area (Day 1)	Peak area (Day 2)	Peak area (Day 3)	Mean	S.D.	%RSD
Fipronil	40	142841	145453	142887	143727	1494.93	1.0402
	50	478987	480311	488987	482761	5431.78	1.1251
	60	883141	851046	842233	858806	4942.37	1.1872
Methoprene	40	112836	103804	103762	103467	5471.15	0.5228
	50	289761	288399	286538	288232	1617.92	0.5613
	60	481913	484984	472963	486620	5703.77	0.3063

8.5 Accuracy

Recovery studies at 50%, 100%, and 150% levels yielded recoveries between 98.06% and 100.37% for both drugs. These results confirm the method's accuracy and reliability for quantitative analysis.

Table 20: Data of Accuracy study (n=3)

Drug	Level	Amount of sample µg/ml	Amount of Std. spike µg/ml	Total amount µg/ml	Amount found µg/ml	% Recovery ± S.D.
Fipronil	50%	9	4	13	11.45	98.16±0.0351
	100%		8	17	15.69	98.06±0.0458
	150%		12	21	19.69	98.45±0.0404
Methoprene	50%	8	2	10	5.95	99.16±0.0173
	100%		4	12	8.03	100.37±0.0115
	150%		6	14	9.63	98.36±0.0321

8.6 Robustness

Deliberate variations in wavelength (±1 nm) and flow rate (±0.1 mL/min) resulted in %RSD values below 2%, indicating that the method is robust and unaffected by minor operational changes.

Table 21: Data of Robustness for Wavelength (n=3)

Drug	Conc. µg/ml	240 nm	241 nm	242 nm	Mean	S.D.	%RSD
Fipronil	8	218454	218453	224852	220586	3694.17	1.6747
	24	380311	369153	377705	374389	5610.42	1.4985
	40	849470	854920	851147	845179	3734.57	1.6250
Methoprene	2	157804	157819	156915	156493	1564.15	0.9995
	6	268399	268399	270602	268541	1993.31	0.7422
	10	474984	484984	483270	483270	1551.07	0.3499

Table 22: Data of Robustness for Flow rate (n=3)

Drug	Conc. µg/ml	240 nm	241 nm	242 nm	Mean	S.D.	%RSD
Fipronil	20	218453	217804	223705	219987	3235.90	1.4709
	60	369153	360311	358674	362712	5637.23	1.5541
	80	859020	852395	841046	849453	7389.89	1.4869
Methoprene	20	157804	157762	156915	157493	5501.57	0.3184
	60	268399	265538	263154	265697	2626.11	0.9883
	80	484984	480409	514243	393212	2455.58	0.6244

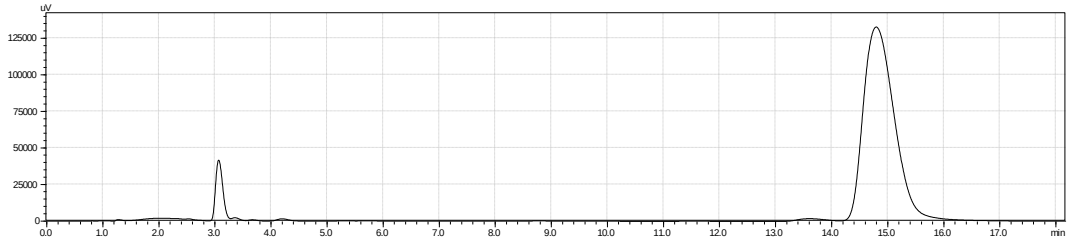
8.7 LOD and LOQ

Based on calibration curve data:

- **Fipronil:** LOD = 0.57 µg/mL; LOQ = 1.69 µg/mL
- **Methoprene:** LOD = 0.21 µg/mL; LOQ = 0.65 µg/mL

These values demonstrate the method's sensitivity for trace-level detection.

Analysis of Marketed Formulations



The developed method was successfully applied to synthetic mixtures and marketed formulations. The assay results showed drug content of **99.02% for Fipronil** and **99.63% for Methoprene**, with no interference from excipients. These findings confirm the method's applicability for routine quality control and regulatory compliance.

IV. CONCLUSION

RP-HPLC Methoprene was successfully developed to Simultaneous determination of Fipronil and Methoprene. This Methoprene was successfully applied for convention analysis of Fipronil and Methoprene in combined marketed tablet dosage form with results in compliance with the standards. The systematic approach was utilized for Methoprene development and to validate All the Validation parameter were found within the acceptance criteria according to ICH Q2(R2) guideline. The proposed Methoprene was simple, rapid, accurate, precise and specific and have the ability to determine Fipronil and Methoprene in the dosage form.

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