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Estimation of Fluralaner from Bulk and Dosage Form By Novel RP-HPLC Technique

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Abstract— *Fluralaner is a systemic insecticide and acaricide that is administered orally or topically. It is a veterinary drug and a member of the novel chiral isooxazoline class of antiparasitic drugs possessing promising insecticidal, acaricidal activity. The work aims to develop a simple, accurate, precise, and reproducible RP-HPLC method for the evaluation of fluralaner in bulk and liquid dosage form. The primary objective is to give an overview of the mechanism of RP-HPLC of fluralaner and explain the basis of the retention mechanism and the secondary is to achieve high-speed separation without any loss of reproducibility. The chromatographic analysis was performed isocratically by using Zorbax Eclipse XDB- C18 (4.6 mm X 150 mm, 5 µm) column. The mobile phase consisting of acetonitrile and 0.1% Formic acid, with a ratio of 70:30 v/v, was passed through the column maintained at 40°C with a flow rate of 1 ml/min. Approximately 20 µl of the solution was injected and the analyte was eluted at 265 nm. The method was validated as per ICH Q2 R1 guidelines. The retention time of the fluralaner was around 5.2 min. The Percentage RSD of each parameter was found within the limit. The recovery of the fluralaner was determined as 100.06 %. LOD and LOQ values of the fluralaner were found to be 2 µg/ml and 6 µg/ml respectively. The method was linear over the range of 10-90 µg/ml with a regression coefficient of 0.9997. All the verification parameters were within the range according to ICH guidelines.*

Keywords— *Fluralaner, antiparasitic, RP-HPLC, method development, validation.*

I. INTRODUCTION

Fluralaner (CAS 864731-61-3), a veterinary drug is a member of the novel chiral isooxazoline class of antiparasitic drug possessing promising insecticidal, acaricidal activity. fluralaner acts by inhibiting ligand-gated chloride channels both gamma-aminobutyric acid receptor and L-glutamate receptor that inhibits arthropod nervous system. The IUPAC name of fluralaner is 4-[5-(3,5-dichlorophenyl)-5-(trifluoromethyl)-4H-1,2-oxazol-3-yl]-2-methyl-N-[2-oxo-2-(2,2,2-trifluoroethylamino)ethyl]benzamide.Molecular formula and molecular weight of fluralaner are$C_{22}H_{17}Cl_2F_6N_3O_3$ and 556.3g/mol respectively. The structure of fluralaner is shown in Figure 1.[1-4] Fluralaner was approved for the treatment and prevention of ticks and fleas by U.S. Food Drug and Administration in May 2014, EU approved the drug in February 2014 and Australia approved it in January 2015.[5-7]

Literature survey revealed a plasma pharmacokinetic profile study of fluralaner on dogs and a controlled field study comparing the efficacy, safety of other drugs and fluralaner on dogs against fleas and tick's infection. A controlled USA study highlights the use of fluralaner topical solutions in controlling feline flea infections in cats.[8-10] No chromatographic method was found during the literature survey for quantitative analysis of the drug. Hence attempts were made to develop a simple, rapid, precise, and accurate reverse phase chromatographic (RP-HPLC) method to estimate fluralaner in the liquid dosage form. Fig 1: Chemical structure of Fluralaner.

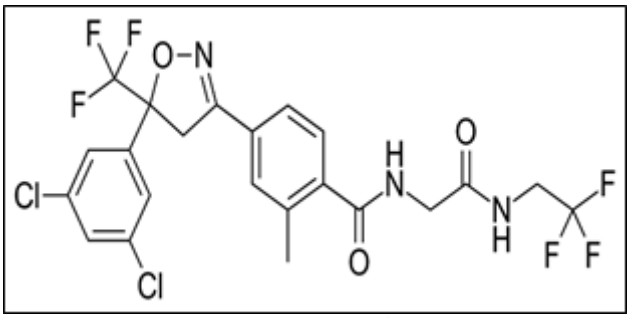

Fig. 1: Chemical structure of Fluralaner.

II. MATERIALS AND METHODS

Chemical and Reagents:

Fluralaner reference standard with defined potency and EXZOLT™ (10mg/ml)liquid formulation was obtained from the Central Drug Testing Laboratory, Mumbai. Acetonitrile (HPLC grade) from Merk life science Pvt. Ltd, Formic acid 98% (AR grade) from Molychem were used. Ultra-purified HPLC grade distilled water was obtained from the Milli-Q® system (Millipore, Milford, MA, USA) water purification unit.

Instrumentation:

Perkin Elmer UV/VIS Spectrometer Lambda 25 connected to a computer loaded with software Perkin Elmer UV Win Lab was used for all the spectrophotometric measurements. The chromatography was performed on Perkin Elmer Flexar HPLC using software Tc Nav/Ver 6.3.2 with LC instrument control. Precision parameter to be performed on the different instruments was done on Thermo Scientific Ultimate 3000 system using chromeleon 7.4.2 software. Sartorius Analytical Balance was used for all the weighing.

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Determination of wavelength of maximum absorbance:

Fluralaner standard 10 mg was weighed accurately transferred to the 100.0 ml volumetric flask and the volume was made up to the mark with mobile phase (100 µg/ml). The aliquot portion of the standard stock solution of the fluralaner was diluted appropriately with diluent to obtain a solution of 10 µg/ml concentration. Then the above solution was scanned in the range of 400.0 nm to 220.0 nm. Fluralaner showed maximum absorbance at 265.0 nm as shown in Figure 2. So, the suitable wavelength selected for the analysis of the fluralaner was 265 nm.

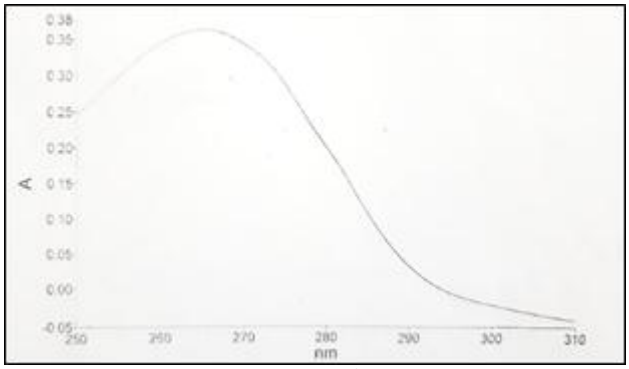

Fig. 2: UV Spectra of Fluralaner.

Preparation of Mobile Phase:

Acetonitrile and 0.1% formic acid in the ratio of 70:30 v/v were used as a mobile phase for the present study. Two different ports were used for running of mobile phase in isocratic form. Formic acid (0.1 % v/v) was prepared by dissolving 1 ml of 98% Formic acid into 1000 ml of HPLC grade water. The mobile phase was vacuum filtered through 0.45µm high flow nylon membrane filters purchased from Axiva Sichem Pvt. Ltd and was sonicated and degassed using an ultra sonicator.

Preparation of Standard solution:

A standard stock solution of 100 µg/ml was prepared by accurately weighing 10 mg of Fluralaner and transferring it into a 100 ml volumetric flask. To this add 50 ml of mobile phase and dissolve the drug properly by sonicating for about 5 min, make up the solution to 100 ml with the mobile phase. Working standard solution of concentration 50 µg/ml of fluralaner was obtained by pipetting out 10 ml of stock solution and transferring it into 20ml volumetric flask and making up to the mark with the mobile phase. All solutions were filtered through a 0.45 membrane filter and sonicated to degas.

Preparation of sample solution:

An amount of sample equivalent to 10 mg/ml of fluralaner in the liquid formulation was accurately weighed and transferred in six different 200 ml volumetric flasks followed by the addition of 140 ml of acetonitrile. The mixture was then subjected to sonication for 15 min, frequently subjected to vortex for the complete dissolution of the drug. The solution was cooled to room temperature and further volume was made up to the mark with 0.1% formic acid to obtain a solution of concentration 50 µg/ml. All solutions were filtered through a 0.45 membrane filter and sonicated to degas.

Method Validation Studies:

The developed RP-HPLC method was validated for system suitability testing, specificity, linearity, precision, accuracy and recovery, LOD, LOQ, and robustness parameters according to ICH guidelines[13].

System Suitability Testing:

System suitability parameters were evaluated and analyzed to check the system performance by injecting a standard preparation (six replicates) of a working concentration of 50 µg/ml and blank preparation (single injection) into the HPLC. The chromatograms were recorded to evaluate SST parameters like %RSD of RT, N, Theoretical plates.

Specificity:

For specificity solutions of blank, a standard of 50 µg/ml and a sample of 50 µg/ml were injected and their chromatograms were recorded as represented in Figure 3,4,5 respectively.

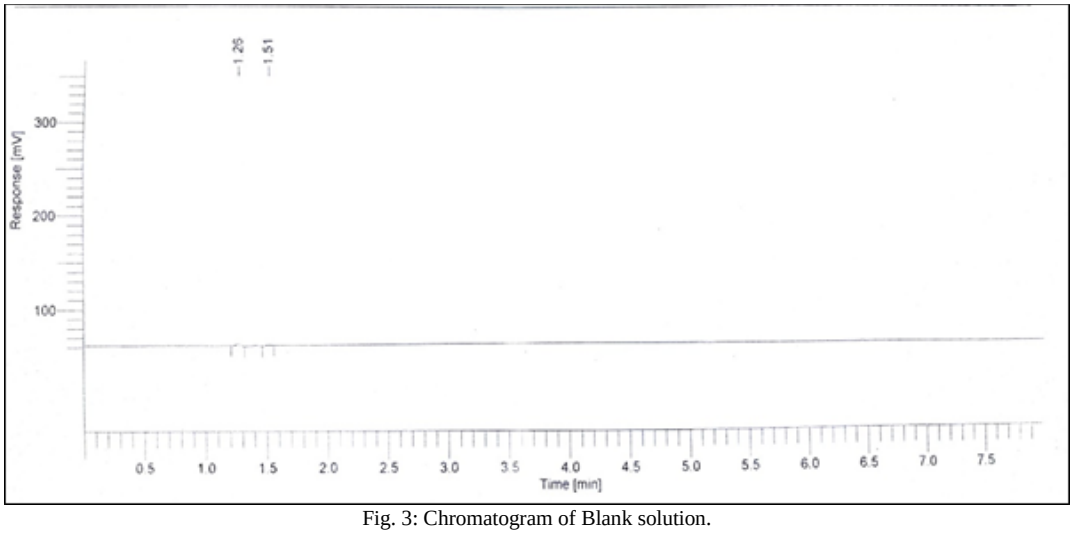

Fig. 3: Chromatogram of Blank solution.

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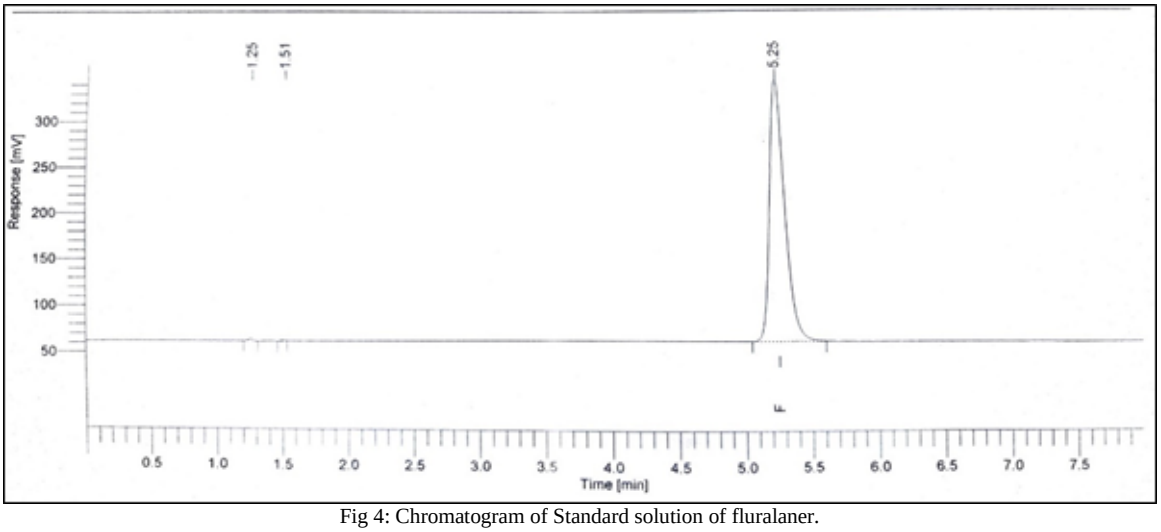

Fig 4: Chromatogram of Standard solution of fluralaner.

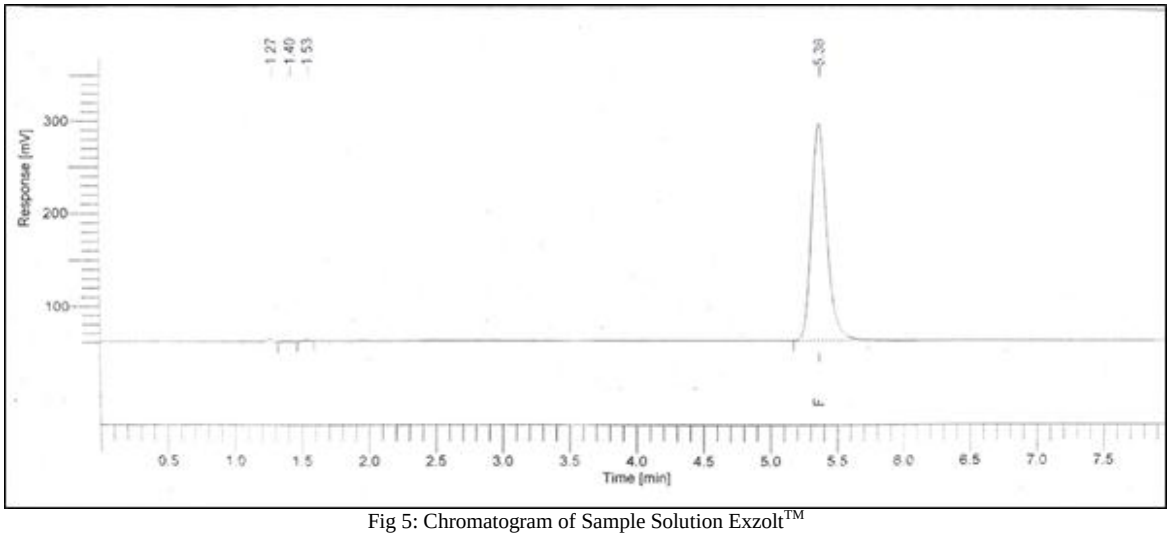

Fig 5: Chromatogram of Sample Solution Exzolt™

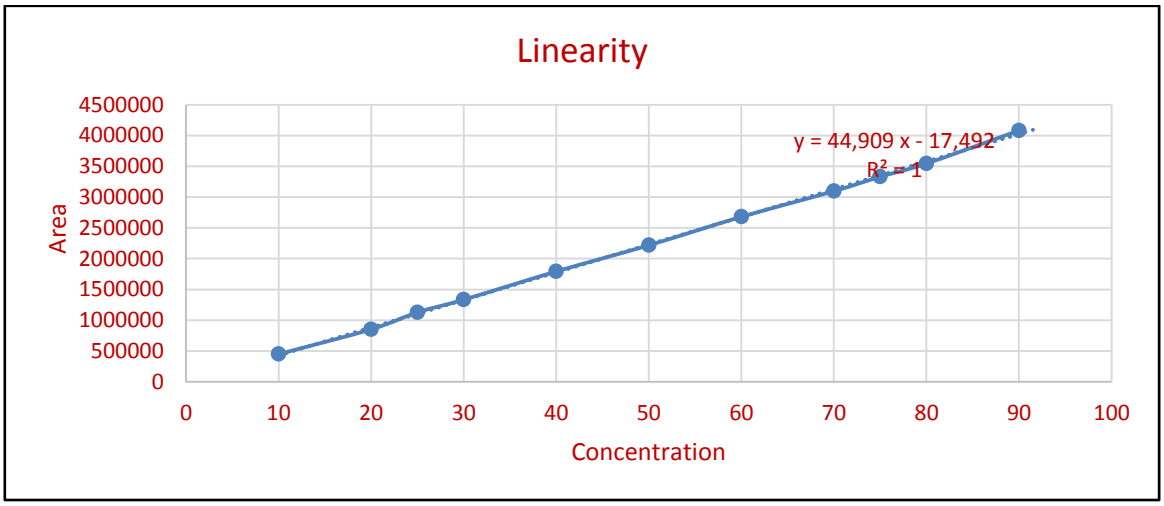

Fig. 6: Linearity graph of Fluralaner.

Linearity:

The linearity of the method was obtained within the concentration range of 10-90 µg/ml for the fluralaner. The linearity graph was plotted by taking the concentration of the drug on the X-axis and the corresponding peak area on the Y-axis as shown in Figure 6.

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Precision:

"The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample".

System Precision:

This was performed by injecting six replicate injections of a standard solution (50 µg/ml). The average, SD, %RSD of six replicate injections was calculated and reported.

Method Precision (Assay Repeatability):

This was performed by injecting six replicate injections of standard solution (50 µg/ml) and six sample preparations of fluralaner (50 µg/ml) in triplicates into the HPLC system. Its % Assay, average, SD, %RSD were calculated and reported.

Intermediate Precision:

This was performed on two different days, two different analysts, and different HPLC instruments. Five replicates of standard solution (50 µg/ml) and three sample preparation (50 µg/ml) in triplicates were injected into the HPLC system. Its % Assay, average, SD, %RSD were calculated and reported.

Accuracy and Recovery (Standard addition method):

Accuracy is a measure of how close is the experimental value to the true value. Accuracy was determined by the method of standard addition method, by calculating % mean recovery of the sample at four different levels 100, 110, 120, 130%. At each level, three determinations were performed, % recovery and % RSD were taken into consideration.

LOD and LOQ:

Limit of detection (LOD) and limit of quantification (LOQ) of fluralaner were determined from the calibration curve method using the following formulas:

$LOD = 3.3 \times \propto/s$, $LOQ = 10 \times \propto/s$

Where $\propto$ is the Standard deviation of the response of the regression line and s is the slope obtained from the calibration curve After calculating, solutions of desired concentration for LOD and LOQ were prepared and injected. The chromatograms obtained were recorded as represented in Figure 7,8.

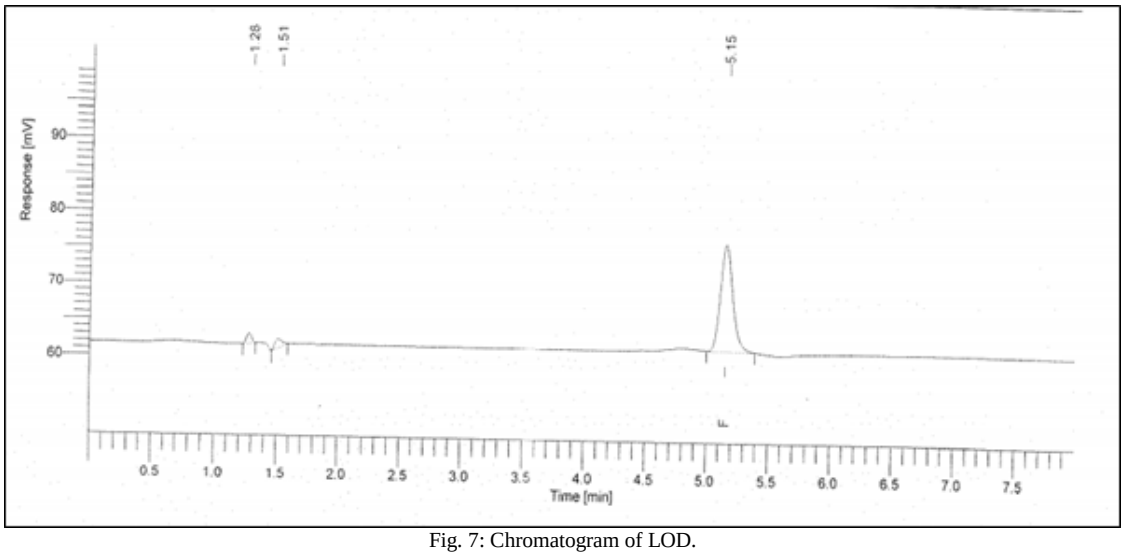

Fig. 7: Chromatogram of LOD.

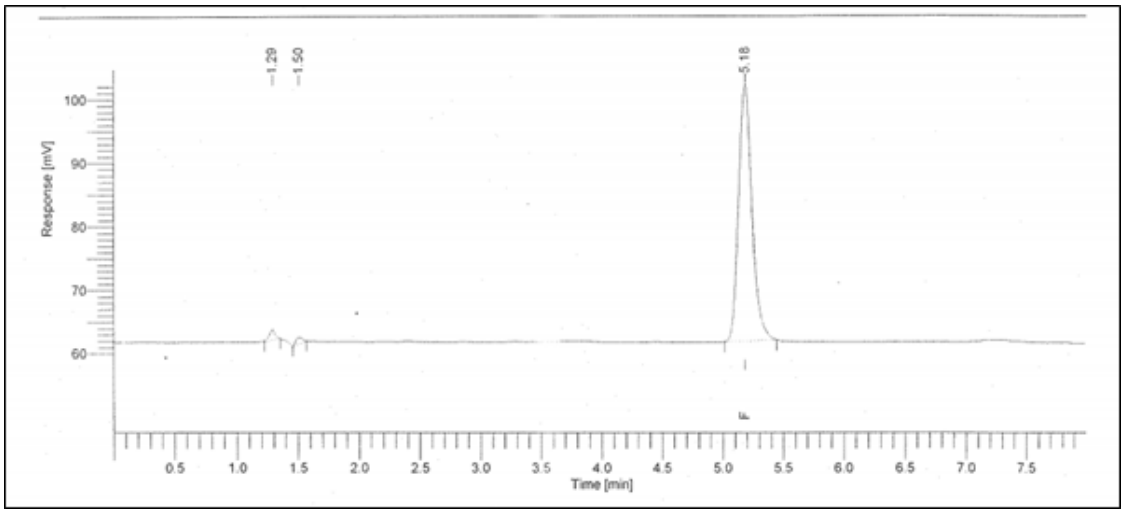

Fig. 8: Chromatogram of LOQ.

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Robustness:

It is defined as a small or deliberate change in the parameter that should not affect any method. This was performed by a change in flow rate (± 0.2 ml/min), change in the column temperature (±5 °C), change in wavelength (±2 nm), and change in column (Different column and HPLC instrument, Different column on same HPLC instrument). Sample preparations of 50 µg/ml was prepared and injected in triplicate along with five replicate injections of a standard solution of 50 µg/ml under different chromatographic conditions. Its % assay, average, SD, %RSD were calculated and reported.

Assay:

The optimized method was applied on EXZOLT oral solution having a label claim of fluralaner 10 mg/ml. The assay was performed on the above solution by injecting five replicate injections of standard preparation 50 µg/ml and six sample preparation 50 µg/ml in triplicate into the HPLC system. Its % assay, average, SD, %RSD were calculated and reported.

III. Result and Discussion

Method Optimization:

Molecular structure and solubility data show that fluralaner is a basic, non-polar drug. Zorbax Eclipse XDB column was selected for the retention of the fluralaner by considering the chemical structure of the molecule. 0.1 % Formic acid was finalized to use in the mobile phase by considering the nature of the fluralaner.

Here different columns and different mobile phase solvents were used on a trial-and-error basis. Initial trials on HPLC were carried out on BDS Hypersil C18 (4.6 mm X 125 mm, 5 µm) column, with mobile phase Acetonitrile: 0.1% Formic acid in a ratio of 50:50 was run at a flow rate of 1 ml/min. But, in the chromatogram obtained poor peak shape was observed. Further trials were conducted on syncronis C8 (4.6 mm X 225 mm, 5 µm) column with the same mobile phase, variations were made in mobile phase proportion, injection volume, and concentration of the standard solution. But none of the conditions was acceptable due to unacceptable SST parameters. Then various HPLC trials were made with a mobile phase consisting of 0.1% formic acid and Acetonitrile with different ratios, different standard solution concentrations, and injection volume on Zorbax Eclipse XDB-C18 (4.6 mm X 150 mm, 5 µm). Chromatograms with acceptable peak shape and SST parameters were obtained on this column. The best peak was obtained with the following chromatographic conditions, standard preparations and sample preparations were separately injected and chromatographed.

Chromatographic conditions:

Column: Zorbax Eclipse XDB- C18 (4.6 mm X 150 mm, 5 µm)
Flow rate: 1 ml/min.
Column Temperature: 40°C.
Autosampler Temperature: 10°C ± 5.
Programming: Isocratic.
Wavelength: 265 nm.
Run time: 8 min.
Injection volume: 20 µl.
Diluent: Mobile phase.

System Suitability

Before starting sample analysis, the chromatographic system used for analysis must pass the SST limits. All the SST parameters like tailing factor should be less than 2, theoretical plates greater than 6000, and % RSD of peak areas less than 2. In the current method, all parameters were established within the limit which demonstrates that the values are reproducible. The results of system suitability studies are summarized in Table No. 1.

Table No.1: System suitability and system precision study of Fluralaner

Injection no	Area	Retention Time	Theoretical Plates	Tailing Factor
1	2219940	5.292	10267	1.170
2	2206304	5.301	10615	1.165
3	2240152	5.277	10252	1.166
4	2188620	5.273	10505	1.168
5	2210843	5.262	10507	1.179
6	2214739	5.252	10559	1.185
AVERAGE	2213433	5.276	10451	1.172
SD	16918.43584	0.01824	153.95	0.01
% RSD	0.76	0.35	1.47	0.69
LIMIT	NMT 2.0%	NMT 1.0%	NMT 2.0%	NMT 2.0%

SD= Standard Deviation.
% RSD= Percentage relative standard deviation.
NMT= Not more than.

Linearity:

Linearity was determined over the range of (10-90 µg/ml) for the fluralaner. Regression equation obtained was y = 44908.6x-17492.2. The method is having good linearity (r^2 = 0.99975). The linearity data is summarized in Table No. 2.

Table No. 2: Linearity data of Fluralaner.

Concentration (µg/ml)	Area
10	447144
20	851035
25	1127413
30	1333016
40	1791878
50	2217871
60	2679814
70	3097560
75	3331663
80	3546864
90	4083039

Precision:

System precision: The %RSD values were found to be within the limit that is less than 2%. The results are summarized in Table No 1.
Method Precision: The mean assay percentage results are summarized in Table No. 3 and are found to be within the limit.
*Intermediate Precision***:** The % assay, average, SD, %RSD for Day-1 and Day-2, Analyst-1 and Analyst-2 and HPLC-1 and

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HPLC-2 were found to be not more than 2%. The results are summarized in Table No. 4.

Table No. 3: Method Precision (Assay Repeatability) data of Fluralaner.

Sample No.	% Assay
1	99.56
2	99.18
3	99.38
4	99.17
5	99.39
6	99.18
AVERAGE	99.31
SD	0.145
%RSD	0.15
Limit	NMT 2%

SD= Standard Deviation.
% RSD= Percentage relative standard deviation.
NMT= Not more than.

Accuracy and Recovery:

Accuracy results at various levels of concentration are summarized in Table No. 5. For accuracy studies, the limit for percent means recovery is 98%-102%. From the results, it can be seen that the percent mean recovery is 100.06% which is within the limit, hence the method is accurate.

LOD and LOQ:

The present method can detect and quantify the analyte at a lower concentration. Values were estimated as following $\propto$ =28544.71, s =44908.57, LOD = 2 µg/ml, LOQ = 6 µg/ml.

Table No. 4: Intermediate precision data of Fluralaner.

Sample No.	% Assay Day-1 HPLC-1	% Assay Day-2 Analyst -1	%Assay Analyst-2	% Assay HPLC-2
1	99.56	99.52	100.44	100.90
2	99.18	99.73	100.09	102.09
2	99.38	99.69	100.46	100.71
AVERAGE	99.37	99.65	100.33	101.23
SD	0.19	0.11	0.171	0.75
%RSD	0.189	0.110	0.17	0.739
LIMIT	NMT 2%	NMT 2%	NMT 2%	NMT 2%

SD= Standard Deviation.
% RSD= Percentage relative standard deviation.
NMT= Not more than.

Robustness:

By analyzing robustness, resulted values were found to be within the limit that is less than 2%, thus the developed method was proved to be robust. The results are summarized in Table No. 6.

Assay:

The results obtained show that the percentage recoveries were high and SD values are very low, which confirms that the method is suitable for routine analysis of fluralaner in its pharmaceutical preparation. The results are summarized in Table No. 7.

Table No.5: Accuracy data of Fluralaner.

% Level	Amount Spiked	%Amount Recovered	% Recovery	AVERAGE	SD	%RSD	%Mean Recovery
100	10.01	100.1	100.1	100.1	0.072	0.072	100.06
	10.01	100.1	100.1				
	10.02	100.2	100.2				
110	10.95	109.5	99.6	99.6	0.020	0.020	
	10.95	109.5	99.6				
	10.96	109.6	99.6				
120	12.00	120.0	100.0	99.9	0.088	0.088	
	11.99	119.9	99.9				
	11.97	119.7	99.8				
130	12.98	129.8	99.9	100.9	0.654	0.648	
	13.12	131.2	100.9				
	13.14	131.4	101.1				

SD= Standard Deviation.
% RSD= Percentage relative standard deviation.
NMT= Not more than.

Table No. 6: Robustness data of Fluralaner.

Parameter	Change in parameter (±)	% Assay Estimation	AVERAGE	SD	% RSD	LIMIT
Flow rate (±0.2 ml/min)	0.8	100.17	100.47	0.39	0.388	NMT 2%
	1	100.33				
	1.2	100.91				
Column temperature (±5°C)	25	99.00	98.70	0.45	0.452	
	30	98.92				
	35	98.19				
Wavelength (±2 nm)	263	100.71	100.38	0.69	0.690	
	265	100.8				
	267	99.58				
Different column and HPLC instrument)	-	100.15	99.96	0.50	0.496	
	-	100.33				
	-	99.40				
Different Column and same HPLC instrument	-	100.34	101.1	0.72	0.710	
	-	100.93				
	-	101.77				

SD= Standard Deviation.
% RSD= Percentage relative standard deviation.

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NMT= Not more than.

Table No. 7: Assay results of Fluralaner.

Sample No.	Weight of standard (mg)	Sample weight (equivalent to 10mg/ml)	Mean Area of standard at 265 nm	Area of sample at 265 nm	% Assay
1	10.17	903.93	2215555.2	1874895	99.56
2		914.00		1888641	99.18
3		920.36		1905550	99.38
4		920.48		1901809	99.17
5		906.43		1876931	99.39
6		906.51		1873061	99.18
Mean					99.31
± SD					0.145
% RSD					0.15

SD= Standard Deviation.
% RSD= Percentage relative standard deviation.
NMT= Not more than.

IV. CONCLUSION

The RP-HPLC method development was found to be simple, precise, rapid, accurate for the quantification of fluralaner in the liquid dosage form. The method was reliable in terms of system suitability, linearity, precision, accuracy and recovery, robustness and assay. All the verification parameters were within the range according to ICH guidelines. Hence the proposed RP-HPLC technique can be used for routine analysis in the pharmaceutical industry.

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ABBREVIATIONS

RP-HPLC: Reversed Phase High Performance Liquid Chromatography; LOD: Limit of detection; LOQ: Limit of quantification; ICH: International Council for Harmonization; AR: Analytical reagent; CAS: Chemical Abstracts Service; USA: United States of America; UV-VIS: Ultraviolet-visible spectrophotometry; RT: retention time; N: Theoretical plates; SD: Standard deviation; %RSD: Percentage relative standard deviation.

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