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Simultaneous Determination of Impurities and Degradation Product in Fluralaner Antiparasitic Drugs Using Reverse Phase High-Performance Liquid Chromatography Method

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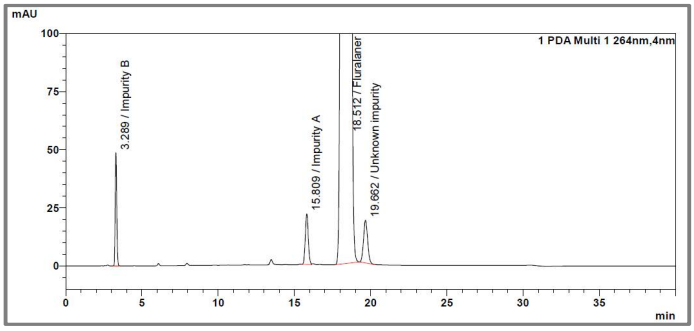
ABSTRACT

A simple, selective and fast Reversed-Phase High-Performance Liquid Chromatographic (RP-HPLC) approach for identifying related compounds in fluralaner, an Active Pharmaceutical Ingredient (API), has been developed and validated. The chromatographic system consisted of LC-2030C 3D with PDA detector and auto-sampler. The chromatographic separation was performed by gradient elution method using Hypersil BDS C_{18} column (250 mm×4.6 mm, 5 µm) as a stationary phase and mobile phase comprising of phosphate buffer (pH 4.0), acetonitrile (60:40) v/v in phase A and acetonitrile and methanol (50:50) v/v as phase B at a flow rate of 1.0 mL min^{-1}.Using an ultraviolet absorbance detector, the samples were detected and quantified at 264 nm.The method was found selective and a peak of the fluralaner was well separated with other impurities. For fluralaner the proposed method is linear (r^2 = 0.999), accurate (with 99.0% recovery) and precise (%RSD<2%). The method has been utilized to determine related substances in commercial products and was found to be accurate within a limit. .

Graphical Abstract:



Impurity spike chromatogram.

Keywords: Fluralaner, Impurity, Method validation, RP-HPLC.

INTRODUCTION

Fluralaner is a veterinary medicine that belongs to a unique class of chemicals known as isoxazolines, which are antiparasitic treatments with insecticidal and acaricidal activity. Fluralaner inhibits the arthropod nervous system by blocking ligand-gated chloride channels, including the gamma-aminobutyric acid (GABA) receptor and the L-glutamate receptor [**1-6**].

Fluralaner has the chemical formula $C_{22}H_{17}Cl_2F_6N_3O_3$ and a molecular weight of 556.3 g mol^{-1}. It was approved by the US Food and Drug Administration in May 2014, the EU in February 2014, and Australia in January 2015 for the treatment and prevention of ticks and fleas [**7-10**]. Figure 1 shows the structure of the fluralaner.

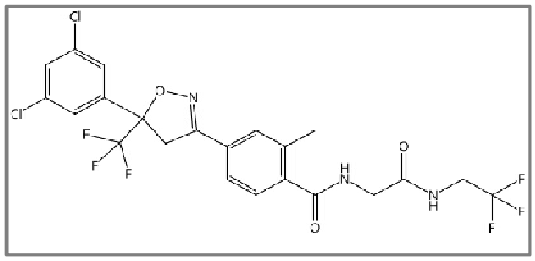


Figure 1. Chemical structure of fluralaner.

An examination of the literature revealed a controlled field experiment assessing the safety and effectiveness of various drugs and fluralaners on dogs against flea and tick infestation, in addition to research on the plasma pharmacokinetic profile of the drug [**11-14**]. According to a controlled trial carried out in the United States, fluralaner topical solutions are also found effective in treating feline flea infections in cats [**15-18**].The presence of impurities may lead to adverse effects or reduced effectiveness of the drug. However, the extent and nature of these effects can vary depending on the specific impurities involved and their concentrations. No complete chromatographic method was available in the updated monograph for the quantitative analysis of fluralaner; however, some methods for quantitative analysis studied in the laboratory were documented in the literature for the drug; but they were not compatible with separation of all impurities [**19, 20**]. Hence, we made attempts to develop an easy, simple, rapid, precise and accurate reverse phase chromatographic (RP-HPLC) method for estimating related substances as impurities (Figures 2 and 3) in fluralaner API.

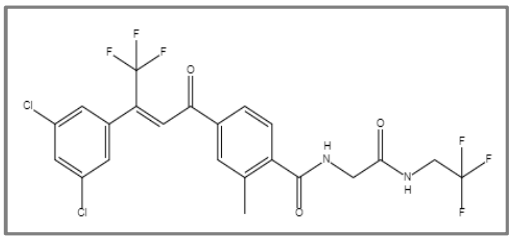


Figure 2. Impurity A.

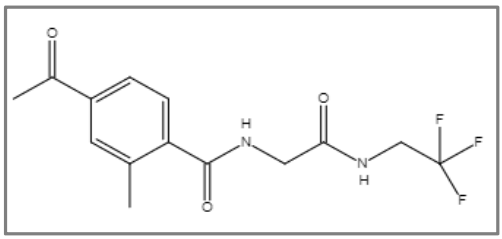


Figure 3. Impurity B.

MATERIALS AND METHODS

Chemicals and Reagents: Fluralaner reference standard and other impurities with defined potency were obtained from the NGL Fine Chem, R&D Division, Mumbai. Acetonitrile (HPLC grade), methanol (HPLC grade) and potassium dihydrogenorthophosphate 98% (AR grade) received from

Merck were used. The Milli-Q® system (Millipore, Milford, MA, USA) water purification unit provided ultra-purified HPLC grade water.

Instrumentation: Chromatography was carried out on a Shimadzu HPLC with the software Lab Solutions/Ver 6.83 and LC instrument control. The intermediate precision parameters were measured using different instruments with the same model number and software. All solutions were prepared using a Radwag analytical balance and a pH meter made by Horiba.

Preparation of Mobile Phase: For the current study, acetonitrile and 10 mM potassium phosphate buffer (pH 4.0) in the ratio of 40:60 (v/v) were used as a mobile phase A. Phosphate buffer at pH 4 is often considered optimum for solubility, stability and ionization of analytes. Whereas acetonitrile and methanol were prepared mobile phase B in a ratio of 50:50 (v/v) respectively. After the mobile phase was vacuum-filtered by means of 0.45µm nylon membrane filters, it was sonicated using an ultrasonicator and then degassed. To conduct the mobile phase in gradient form, two separate ports were used.

Preparation of the Standard solution: By properly weighing 50 mg of each fluralaner, impurity A, and impurity B and placing it into a 50 mL volumetric flask, a standard stock solution of 1 mg mL^{-1} was created. To this, 25 mL of diluent containing 80% (v/v) acetonitrile in water was added and then sonicated for about 5 minutes to dissolve the drug and impurities properly. Thereafter the final volume of solution made up to 50 mL with the help of diluent. This standard stock solution is used to prepare different linearity levels and spike solutions in various concentrations to determine accuracy.

Preparation of sample solution: A 10 mg mL^{-1} sample of fluralaner was precisely weighed and put into six different 10 mL volumetric flasks before being diluted. All of the solutions were injected to ensure the method's repeatability.

Preparation of spike sample solution: To determine the impurity content of the drug, weighed and then transferred 10 mg mL^{-1} in five different 10 mL volumetric flasks. Each flask was then filled with a known concentration of standard solution at a different level, followed by the addition of diluent. All the solutions were injected for accuracy study.

Method Validation Studies: According to ICH criteria, the developed RP-HPLC method has been verified for robustness parameters, LOD, LOQ, specificity, linearity, precision, accuracy, and recovery, as well as system suitability testing [**21**].

Assessment of System Suitability: In order to assess and evaluate the applicability of the system, blank preparation (single injection) and an impurity mixture (100% linearity level) were injected into the HPLC. To assess system appropriateness criteria including resolution, theoretical plates, and tailing factor, the chromatograms were recorded.

Specificity and selectivity: The absence of any interference during retention periods of interest peaks is known as specificity. It was determined by examining a sample that had been spiked with all possible contaminants. To investigate specificity, a series of blank, 0.1 mg mL^{-1} fluralaner, impurity A and B, and spiked concentrations were injected and their chromatograms were recorded, and no endogenous interference was observed at the retention time of analyte and internal standard peak, the impurity spike chromatogram, as shown in figure 4.

Linearity: Standard solutions were prepared for fluralaner, impurity A, and impurity B at eight distinct concentration levels, ranging from LOQ to 30 µg mL^{-1} (150% of the specified limit), respectively. They were examined to determine the linearity of each impurity. The approach was found to be appropriate for the quantitative determination range of LOQ to 150% of the specification limit using linearity regression analysis. The graph was plotted, and the least squares method was

used to determine the linear regression of peak response versus analyte concentration. The slope and intercept values of the calibration curves have been calculated.

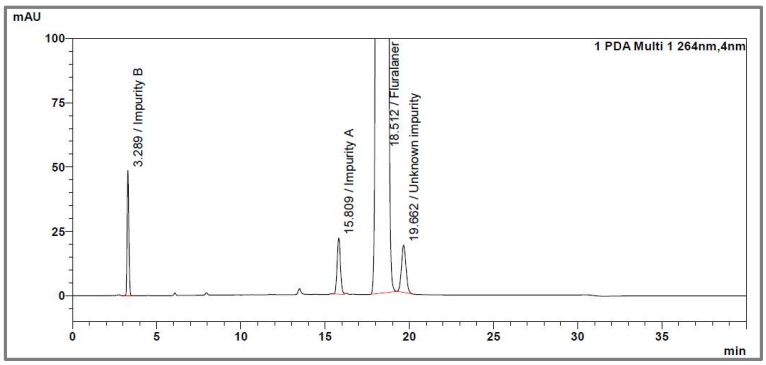


Figure 4. Impurity spike chromatogram.

Precision: The term precision refers to "the degree of agreement between individual test results obtained when the method is applied to multiple sampling of a homogeneous sample." Precision of the system: This was achieved by sequentially injecting a standard solution (20 µg mL^{-1}) six times. Six repeating injections were averaged, SD and percent RSD were calculated and reported. Method Precision:In order to accomplish this, the HPLC instrument was injected with six replicate injections of the standard solution (20 µg mL^{-1}) and six replicated sample preparations of fluralaner (10 mg mL^{-1}). Its percent impurity, average, standard deviation, and percent RSD were determined and reported. Intermediate Precision: This was carried out twice, using two different HPLC instruments and analyzers. The HPLC system was injected with five duplicates of the standard solution (20 µg mL^{-1}) and sample preparation (10 mg mL^{-1}). The impurity percentage, average, standard deviation, and percent relative standard deviation (%RSD) were computed and reported.

Accuracy and Recovery (Standard addition method): The degree of accuracy determines how closely the experimental value matches the true value. The usual addition method was used to calculate accuracy by computing the sample's mean recovery at five distinct levels (LOQ, 25, 50, 100, and 150%). Single determinations were performed and percent recovery was calculated at each level.

Limit of detection (LOD) and quantification (LOQ): LOD and LOQ for fluralaner and both impurities were measured by injecting a series of diluted solutions with known concentrations at corresponding signal-to-noise ratios (S/N) of 3:1 and 10:1 respectively. After calculating, LOD and LOQ solutions of the desired concentration were made and injected. The obtained chromatogram is shown in figure 5.

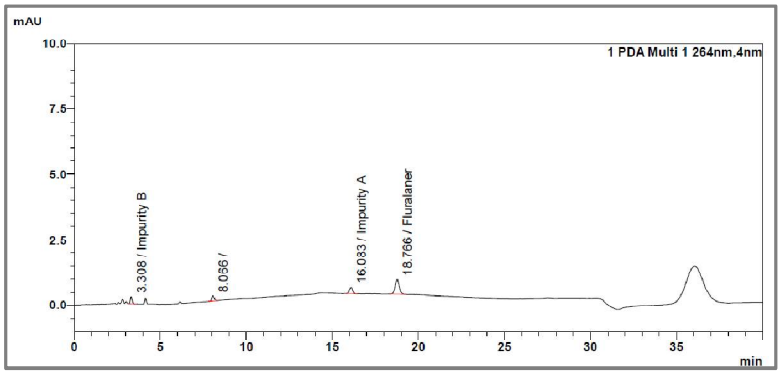


Figure 5. Peak at LOQ level.

Robustness: The robustness of the method was demonstrated by examining the impact of slight but considerable changes in specific analytical parameters altering selectivity or quantitative results. This was accomplished by altering the flow rate (± 0.1 mL min^{-1}), the mobile phase pH (±0.1) and the composition of mobile phase A (±10%). Sample preparations containing 10 mg mL^{-1} were made and injected alongside standard solutions containing 20 g mL^{-1} under different chromatographic conditions. Its resulting parameters were calculated and reported. The results showed that there were no statistically significant variations in the system suitability characteristics of any of the key peaks or contaminants, proving the method's reliability.

RESULTS AND DISCUSSION

Method Optimization: Fluralaner is a basic, non-polar drug based on molecular structure and solubility data. The chemical structure of the molecule led to the selection of the Hypersil BDS C18 column for fluralaner retention. Taking into account the characteristics of the fluralaner, phosphate buffer (pH 4.0) was chosen for use in the mobile phase. On a trial-and-error basis, different columns and mobile phase solvents were utilized. Initial HPLC studies were performed on an Inertsil ODS (4.6 mm × 250 mm, 5 m) column with a mobile phase A containing acetonitrile and phosphate buffer in a 60:40 ratio and flow rate of 1 mL min^{-1} [**19**]. However, the chromatogram showed poor peak shape and very little resolution between the fluralaner and unknown impurity near it. Further trials were conducted using the same mobile phase in a Zorbax Eclipse C18 (4.6 mm × 250 mm, 5 m) column, with modifications in mobile phase proportion, injection volume, and standard solution concentration [**20**]. However, none of the situations could be tolerated because of poor system suitability factors. Following that, several HPLC trials were performed on Hypersil BDS C18 (4.6 mm × 250 mm, 5 m) with a mobile phase containing 10 mM potassium dihydrogen phosphate buffer (pH 4.0) and acetonitrile with varying ratios, standard solution concentrations, and injection volumes. Chromatograms with suitable peak shapes and system suitability criteria were obtained in this column. The following chromatographic conditions led to the best peak being obtained: separate injection and chromatography of standard preparations and sample preparations.

Chromatographic conditions: HPLC separations were performed by using the column Hypersil BDS C18 (4.6 mm × 250 mm, 5 µm) make Thermo, using an ultraviolet absorbance detector at a wavelength of 264 nm. Mobile phase A comprised the mixture of 10 mM Potassium dihydrogen phosphate buffer (pH 4.0) and acetonitrile in the ratio (60:40) v/v and mobile Phase B was made up of a 50:50 v/v mixture of acetonitrile and methanol. The initial mobile phase composition was kept at 65% mobile phase A for two minutes at a flow rate of 1.0 mL min^{-1}. After that, it changed linearly to 50% (2–10 min) and was held for 17 minutes (10–27 min). After that, it was returned to the initial conditions within one minute (27–28 min) and then held for twelve minutes (28–40 min) for the chromatograph column equilibrium on ambient column oven temperature. The injection volume was of 10 µL.

System Suitability: Before beginning the sample analysis, the chromatographic system used for the analysis must pass the system suitability constraints. All SST parameters, including the resolution between the closed peak and fluralaner, should be larger than 1.5 and the tailing factor should be less than 2. All parameters in the present method were satisfied and within the allowed limit as per requirement.

Linearity: For the fluralaner, linearity was established within the range of (0.1 - 30 µg mL^{-1}). The obtained regression equation was y = 0.000214 × - 0.48808.The method is having good linearity (r^2 = 0.999). The linearity data is summarized in tables1, 2 and 3. The correlation coefficient was determined to be greater than 0.999.The linearity graph was created by plotting the drug concentration on the X-axis and the matching peak area on the Y-axis, as illustrated in figures 6, 7, and 8 respectively.

Table 1. Linearity of fluralaner

Concentration (µg mL^{-1})	Area
0.1008	9396
2.016	49039
5.04	118602
10.08	264650
16.128	368031
20.16	463805
24.192	566271
30.24	694770
Correlation coefficient	0.9990

Table 2. Linearity of impurity A

Concentration (µg mL^{-1})	Area
0.20156	3370
2.0156	31435
5.039	75798
10.078	143097
16.1248	233414
20.156	292914
24.1872	358079
30.234	442423
Correlation coefficient	0.9998

Table 3. Linearity of impurity B

Concentration (µg mL^{-1})	Area
0.15228	2089
2.0304	36378
5.076	87730
10.152	164923
16.2432	269260
20.304	336219
24.3648	412051
30.456	507931
Correlation coefficient	0.9998

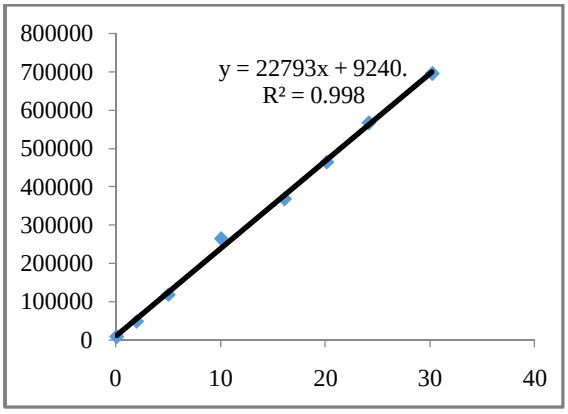


Figure 6. Linearity of fluralaner.

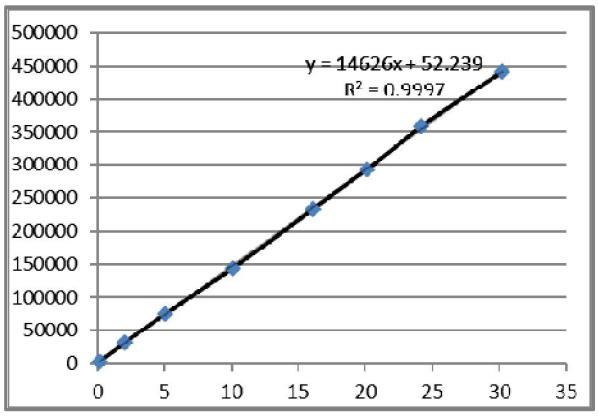


Figure 7. Linearity of impurity A.

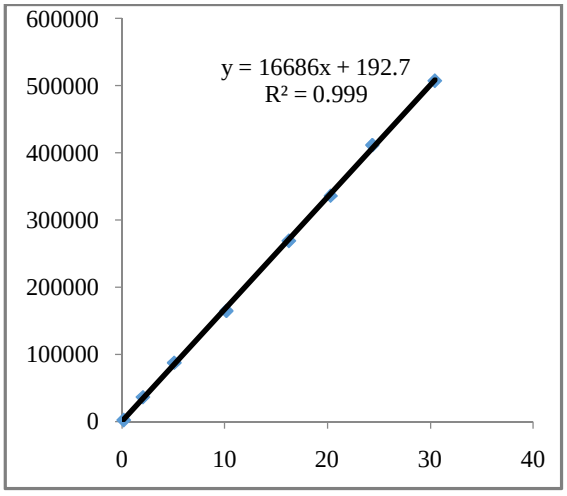


Figure 8. Linearity of impurity B.

Precision: The precision of the method for determining impurities associated to the fluralaner was examined for repeatability and intermediate precision. Repeatability was established by analyzing the fluralaner sample six times. The mean impurity percentage and %RSD values were found to be less than 2% of the allowable limit.

Accuracy and Recovery: Tables 4 and 5 describe the accuracy results at different concentration levels. At different levels of accuracy, the limit for percent mean recovery is 98-102%, and at the LOQ level, accuracy is greater than 90%. The findings show that the mean recovery is within the limit, indicating that the procedure is accurate.

Table 4. Accuracy for impurity A

Level	Amount found (µg mL^{-1})	Amount spiked (µg mL^{-1})	% Recovery
LOQ	0.02	0.02	100.05
25%	0.503	0.501	100.40
50%	0.9984	1	99.84
100%	2.014	2.001	100.65
150%	3.047	3.022	100.83

Table 5. Accuracy for impurity B

Level	Amount found (µg mL^{-1})	Amount spiked (µg mL^{-1})	% Recovery
LOQ	0.01	0.015	93.33
25%	0.508	0.504	100.79
50%	0.995	1.008	98.71
100%	1.997	2.015	99.11
150%	3.008	3.044	98.82

LOD and LOQ: The current method confirms the method's great sensitivity for drug analysis by being able to identify and quantify the analyte at lower concentrations. Table 6 shows the estimated values.

Table 6. Estimated values of LOD and LOQ for fluralaner, Impurity A and B

Peak	Slope	LOD (µg mL^{-1})	LOQ (µg mL^{-1})
Fluralaner	0.0000438	0.03	0.100
Impurity A	0.000068	0.07	0.202
Impurity B	0.0000599	0.05	0.152

Robustness: When robustness was checked, results fell within the limit of less than 2%, demonstrating the robustness of the developed procedure.

CONCLUSION

In summary, a logical approach was devised to separate and quantify fluralaner from probable impurities (Impurities A and B) in bulk drug samples using a speedy, easy-to-use, and precise RP-HPLC technology enhanced by UV detection. This created approach was proven to be robust, specific, linear, precise, accurate, and thoroughly validated in accordance with the ICH regulatory standards. As a result, the devised HPLC method can be easily utilized in the pharmaceutical industry for routine examination of the fluralaner medicinal ingredient.

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ABBREVIATIONS

RP-HPLC: Reversed Phase High-Performance Liquid Chromatography; API: Active Pharmaceutical Ingredient, 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; SST: System suitability, N: Theoretical plates; SD: Standard deviation; %RSD: Percentage relative standard deviation.

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