METHOD DEVELOPMENT AND VALIDATION FOR QUANTIFYING CHIRAL ENANTIOMERS IN AFOXOLANER API USING RP-HPLC METHOD

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Abstract

A new antiparasitic drug called afoxolaner targets the glutamate and gamma-aminobutyric acid receptors of acarine insects. The isoxazoline class of chemicals has been used as an active pharmaceutical ingredient in medications that veterinarians have prescribed to treat dogs for flea and tick infestations. There is a racemic combination of afoxolaner, which has a chiral center at the isoxazoline ring. To confirm that afoxolaner is a racemic mixture as shown by specific rotation and to ascertain the enantiomeric purity of single enantiomer samples, a normal phase chiral high performance liquid chromatography analytical method has been devised. The optimized procedure used a HypersilODSC18 column with a 150 *4.6 mm I.D of 5µ diameter kept at 35°C. Acetonitrile/MeOH (90/10, v/v) was used as the mobile phase for the gradient elution analysis of the samples. The UV calibration has shown 266 nm as detecting wavelength. The resolution and selectivity factors were 5.0 and 1.54, respectively and the two enantiomers were successfully separated in less than 10 minutes. The analytical method was properly validated for its intended usage in accordance with ICH criteria. A simple rapid and chiral phase RP HPLC technique was developed for enhanced separation of Afoxolaner which is sensitive and ecofriendly. Thus the method developed was found to be validated as per ICH guidelines.

Keywords: Afoxolaner; Antiparasitic; Chiral HPLC; ICH

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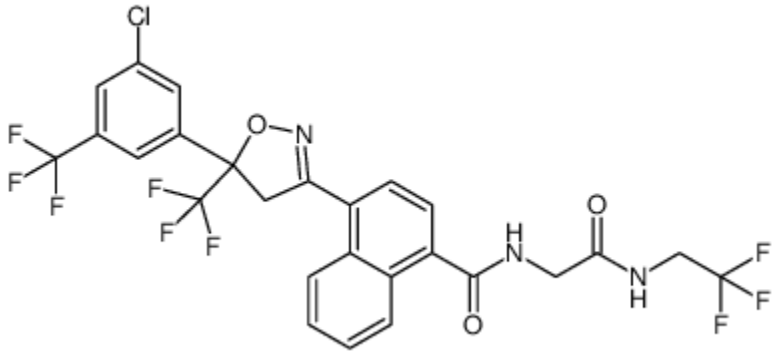
1. Introduction

The pharmaceutical industry has long employed single enantiomers of chiral chemicals that are physiologically active in their medication formulations. The pharmacology, toxicology, and metabolic activity of each enantiomer of a chiral molecule may differ in a biological system[.]Thus, it is crucial for the discovery and development of new drugs to identify and separate the enantiomer that is responsible for the intended therapeutic effect. Enantiomeric separation can be accomplished using chromatography, which is a quick and highly sensitive analytical technique[2,12]. Enantiomer separation and quantitation have been accomplished using a variety of chromatographic techniques, including as GC, LC, supercritical fluid chromatography, and HPLC[3,14] A trial-and-error methodology is used to build HPLC methods, together with the analytical scientist's understanding of separation science. Trial-and-error method development is generally quite time-consuming because it necessitates performing a number of experiments before a reasonable chromatographic separation for analyte peaks in a particular sample is attained[4,11]. Afloxoner is a Antiparasitic agents(insecticide and acaricide) present in NeXguard Spectra formulation which is used to treat infestations with fleas, ticks, demodectic and sarcoptic skin infestations used as prophylactic in dogs as veterinary medicine. Also the drug is actively used in dos for diseases caused by heart worm, gut worm and lung worms[5]. It is a white odorless crystalline powder (M.W 625.9, M.P. 152°C - 156°C) which is very soluble in N, N - Dimethylformamide, soluble in methanol and sparingly soluble in glacial acetic acid, very slightly soluble in chloroform, practically soluble in water[6]. The drug is given as a chewable tablet in a dose of 2.5-6.3mg/kg body weight once in a month which is found to have 99% protein binding[6]. The Afoxolaner(Figure 1) (the active ingredient of NEXGARD) and other isoxazolines chemical with insecticidal and tickicidal efficacy are non- competitive GABA (gamma-amino butyric acid) receptor antagonists, much more selective for GABA receptors in insects or ticks, than for those in mammals, including humans. The drug acts by binding to chloride channels in nerve and muscle cells, which block the transmission of neuronal signals. Affected parasites are paralyzed and die[15]. This mechanism exists not only in insects but also in mammals and other vertebrates. However the binding affinity of afoxolaner to GABA receptors of invertebrates is much higher than to GABA receptors in vertebrates[15]. Hence found to be significantly less toxic to mammals than to insects and other pests[16].

Figure 1: Structure of the drug Afoxolaner

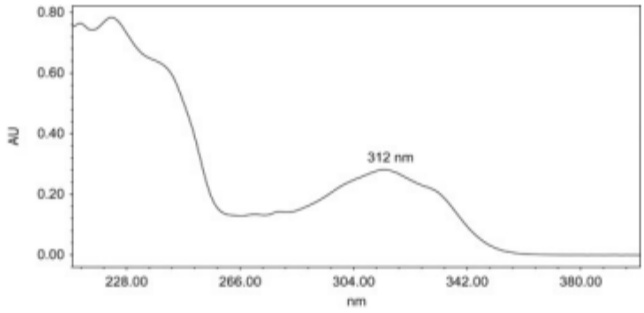


2. Materials And Methods

All the HPLC grade and analytical reagent grade chemicals were procured from W& D Chemsell, Excise colony," X'road, Subedari ,Hanamkonda-506001,Warangal Dist, Telangana State. The chemicals used for the study were Distilled water, HPLC grade reagents are Water, Acetonitrile, Methanol and Triethylamine. The Analytical grade reagents are Glacial acetic acid and Trifluoroaceticacid. The Drug Afoxolaner was purchased from W & D Chemsell, Excise colony,"X'road, Subedari, Hanamkonda-506001,Warangal Dist,Telangana State.

Determination of absorption maxima by UV/Vis Spectrophotometry: Precisely weigh100mg of drug into100ml volumetric flask. To this add 90ml and 10ml of diluents(acetonitrile90:10methanol) and sonicate it and further make up the volume with diluent. Dilute 1ml to 10ml. similarly prepare the solutions from 10 -100µg/ml concentration.
The peak was observed at 266nm as observed in UV spectrophotometer

Figure 1: UV graph of Afoxolanerin methanol



Method development

Method optimization

Preparation of mobile phase: Precisely estimated 90ml of Acetonitrile and 10ml of methanol(90:10). The HPLC grade were degassed in an ultrasonic water bath for 10 minutes and afterward shifted through 0.45µnylon filter under vacuum filtration.

Standard preparation: Precisely weigh 50mg of Afoxolaner and move in to 50ml volumetric flask. Add about 10ml of solvent mixture sonicate to dissolve. Cool the solution to room temperature and dilute to volume with solvent combination. Pipette out 1ml of above solution into a 10ml volumetric jar and adjust the volume with diluent to make up to 100µg/ml concentration. From this standard stock solution the dilution are made.

Improved chromatographic conditions

Column	-Hypersil OD SC18(150x4.6mm,5µ)
Flow rate	-1.6ml/min
Wavelength	-235nm
Column temperature	-35℃
Injection volume	-10µl
Runtime	-5min

Percentage Assay

Infuse 10µL of the standard sample into the chromatographic frame work and measure the area of Afoxolaner peaks and as certain the % Assay by utilizing the formulae.

$$\text{Assay\%} = \frac{AT \times WS \times DT \times P \times Avg\ Wt}{AS \times DS \times WT \times 100 \times \text{Label claim}} \times 100 \quad \text{---(I)}$$

Where:

AT = average area counts of sample preparation.

AS =average area counts of standard preparation.

WS =Weight of working standard taken in mg.

P =Percentage purity of working standard

LC =Label claim of tablet mg/ml.

UV calibration method

The mobile phase's pH or ionic strength were not anticipated to have a substantial impact on the retention and/or separation of enantiomers under RP chromatography because this chemical is not ionizable. The choice of appropriate HPLC columns, optimization of the organic modifier compositions in the mobile phases, and fine-tuning of the final elution profile were therefore the key focuses of method development. The UV spectra of afoxolaner revealed absorption maxima at 222 and 312 nm. To reduce interference from lower-wavelength-absorbing solvents and/or additives, a detection wavelength of 312 nm was used for the study. Based on the known UV response of afoxolaner, the analytical concentration of the afoxolaner sample solution was chosen to be 0.8 mg/mL in acetonitrile-water (80+20, v/v).

Validation of developed RP-HPLC method:

Validation: The Laying out documentation proof, which gives a high level of confirmation that particular cycle, will reliably deliver an item meeting its foreordained detail also, quality ascribes

The accompanying boundaries were considered for the analytical method validation of Afoxoloner in bulk Dosage form.

Accuracy:

For precision assurance, three distinct focuses were arranged independently for example 50%, 100%and150% for the analyte and chromatograms are recorded for something very similar.

Preparation of 50%solution: Around 25mg of Afoxolaner was gauged and moved to 50ml volumetric flask. Add 50ml of mobile phase, Sonicate for10min & filter through 0.45µl on filter leave up to the imprint with same dissolvable. Further 3ml of above arrangement was weakened to10ml with the diluents to get 50 percent

Preparation of 100% solution: Around 50mg of Afoxolaner was gauged and moved to 50ml volumetric flask. Add 50ml of mobile phase, Sonicated for10min & filtered through0.45µl on filter and leave up to the imprint with same

dissolvable. Further 3ml of above arrangement was weakened to10ml with the diluents to get 100 percent.

Preparation of 150% solution: Around 75mg of Afoxolaner was pipette and moved to 50ml volumetric flask. Add 50ml of mobile phase, Sonicate for10min & filter through0.45µ nylon filter leave up to the imprint with same dissolvable. Further 3ml of above arrangement was weakened to10ml with the diluents to get 150%. The average of six replicates should be within the acceptable limits of 98-102%.

Precision: Precisely weigh and move 50mg of Afoxolaner working standard into a 50 ml clean dry volumetric flask add diluents and sonicate to dissolve it completely and make volume sufficient with a similar dissolvable. Further pipette 3 ml of the above stock arrangement into a 10ml volumetric flask and weaken sufficient with diluent. Then the standard arrangement was infused for multiple times and estimated the region for every one of the six infusions in HPLC. The %RSD for the area of six recreate infusions was viewed as inside the predefined limits. The method is acceptable if RSD of six replicates is NMT than 2%.

Linearity and Range: Suitable aliquot of standard Afoxolaner stocks arrangement was taken in 50 ml volumetric flask and resultant solution was sufficiently diluted to obtain final concentration of Afoxolaner. This arrangement was infused into chromatographic frame work. The chromatograms were acquired and peak area was determined for each concentration of drug solution. Calibration curvewas constructed by plotting peak area against applied concentrations. The slope, intercept and correlation coefficient(R^2) were also determined. The Linearity of the analytical method for assay by injecting the linearity solutions prepared in the range of 100µg to 500µg of test concentration, into the chromatograph, covering minimum 5 different concentrations. The response is recorded as peak with concentration vs peak response of drug .

Standard Preparation: Precisely weigh 50 mg of Afoxolaner and move in to 50ml volumetric flagon. Add around 10 ml of dissolvable combination sonicate to disintegrate. Cool the solution for room temperature and weaken to volume with dissolvable mixture (Stock solution). the concentration ranges of the drug afloxoner are prepared form 100µg/ml-500µg/ml using sequential dilution method. Further the linearity is repeated over six replicates which should have a.R^2 value of 0.999 proving the APi purity.

Robustness:

As part of the Robustness, conscious change in the temperature, variety was had to assess the effect on the strategy. Robustness of measure strategy is exhibited by changing the flow rate for 1.4 ml/min and 1.8ml/min rather than 1.6ml/min by infusing the 6 recreate infusions of standard in 1.4ml/min also.1.8ml/min flow rate and found that system suitability parameters are passed. By changing the column temperature for 30℃ and 40℃ instead of 35℃ by infusing the 6 replicate injections of standard in 30℃ and 40℃ temperature and found that system suitability parameters are passed.

Solution stability: The standard solution should be examined over 24-48hrs period under normal laboratory and potency of solution should be determined by comparison to freshly prepared standards. The stability results should be in the acceptable limits of %RSD of NMT 2% ranging from 24 - 48hrs.

Selectivity:

Blank preparation: Dilute the stock of 5ml of diluents into 50ml volumetric flask and make up the volume with mobile phase.

Placebo Preparation: Weigh precisely around 50mg of placebo powder in a 50ml volumetric flask add 50ml diluent to it, sonicate for 20 minutes and cool, in the wake of cooling make up to the volume. Further weaken 0.5 ml of this solution for 10ml with diluent and infuse into the chromatogram.

Standard Preparation: Precisely gauge 25mg of Afloxoner and move into 25ml volumetric flask. Add around 10ml of dissolvable blend sonicate to break down. Cool the solution for room temperature and weaken to volume with dissolvable blend. Move 1ml of above arrangement in to a 10 ml volumetric flask what's more, weaken to volume with diluent. There should be No peak should be observed due to blank and placebo at retention time of Afoxolaner peak.

Ruggedness (Intermediate Precision):

Intermediate Precision communicates with in research center variety as on various days or with various experts with in a similar research center. The replicates of six are observed for no standard deviation in ruggedness of 6 different example arrangements of same bunch by an alternate investigator utilizing an alternate HPLC system. The assay qualifies the ICH limits if the Mean RSD is within the acceptance limits of relative standard deviation NMT 2%.

Limit of Detection (LOD)

The detection of limit is by the investigation of tests with known concentration of analyte and by laying out that base level at which the analyte can

dependably identified.

The LOD was determined by utilizing the formulas

LOD=3.3xSD/b----------(II)

Where,

SD- standard deviation of the peak area of the drugs.

b– Slope of calibration curve.

Limit of Quantification:

The quantification limit is examination of test with known convergences of analyte and by laying out the least level at which the analyte can be measured with satisfactory exactness and accuracy.

The LOQ was determined by utilizing the formulas

LOQ=10xSD/b----------(III)

Where,

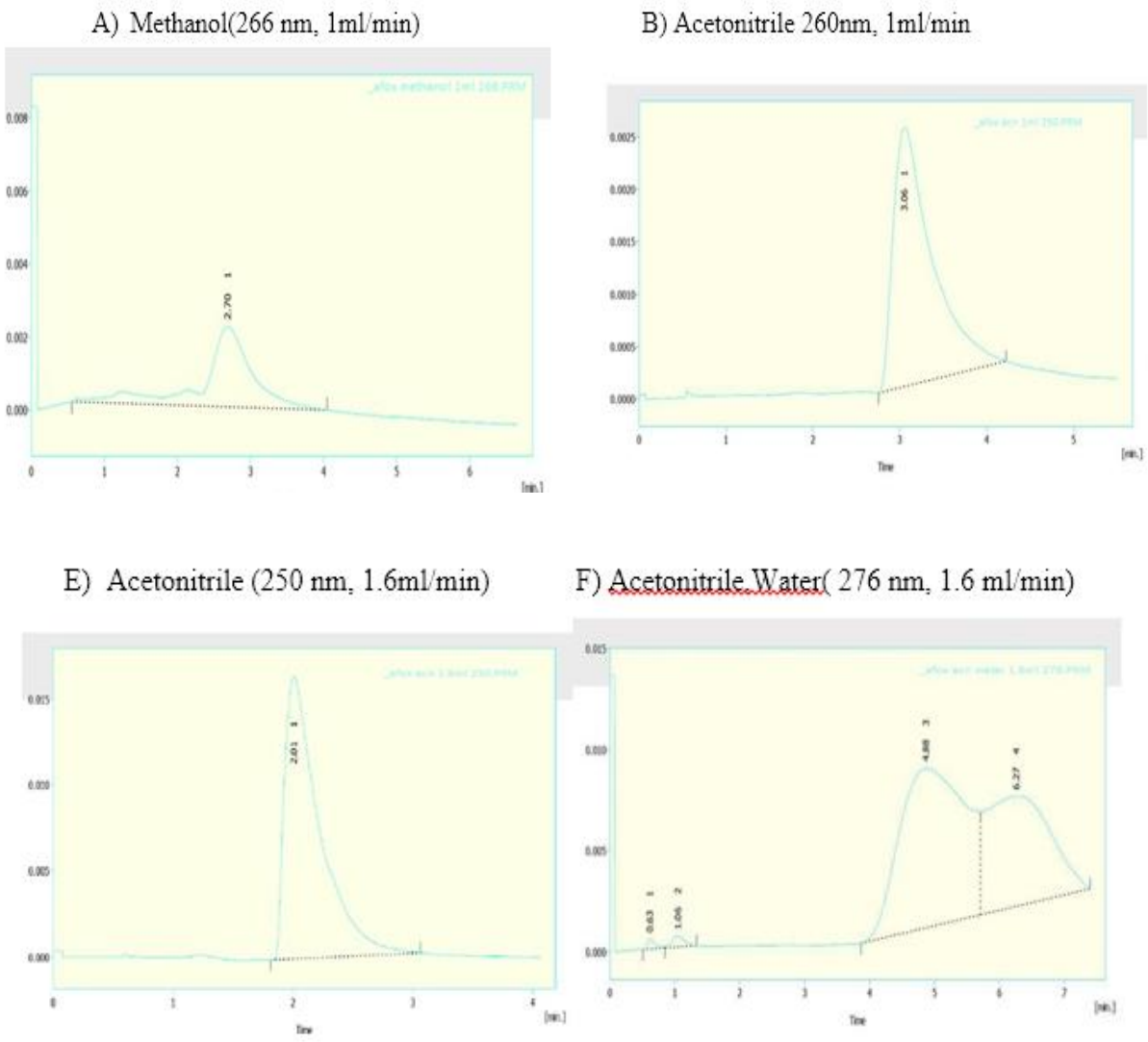
SD-standard deviation of the peak area of the drug,

b- slope of calibration curve

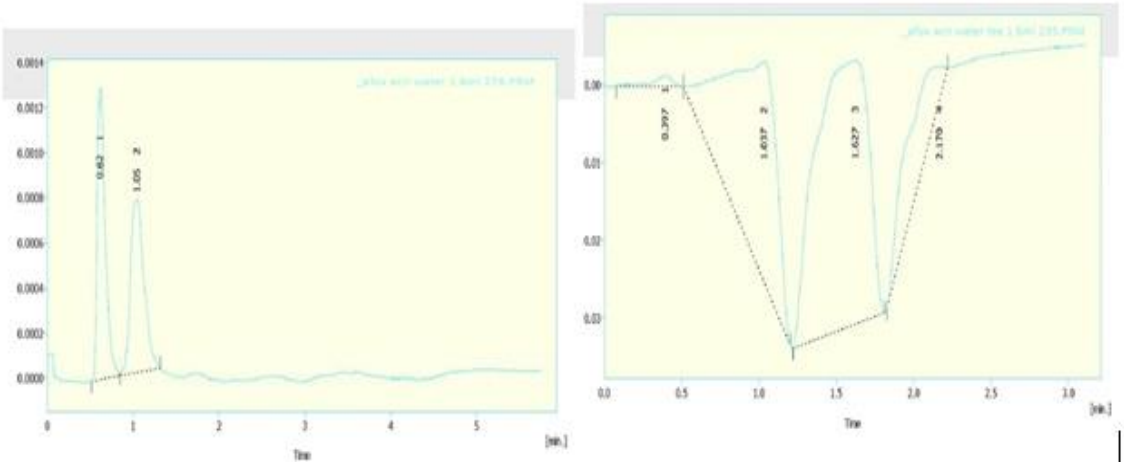
Method development and optimization of chromatographic parameters: Selection of mobile phase in different trial with the following chromatographic conditions was operated in various trials to optimize the mobile phase(Figure 2). The injection volume is 10µl

Mobile phase	: Acetonitrile, methanol and water
Flowrate	: 1-3 ml/min
Column	: Hypersil$ODSC_{18}$(150×4.6mm,5µ)
Detector wavelength	: 250-266 nm
Injection volume	: 10µl

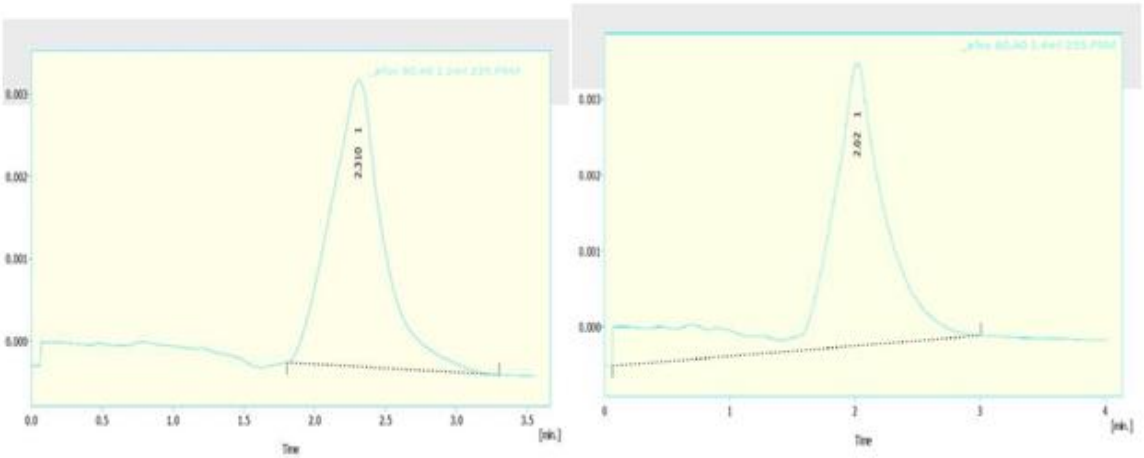
Figure 2: trials of various HPLC separation method for mobile phase optimization



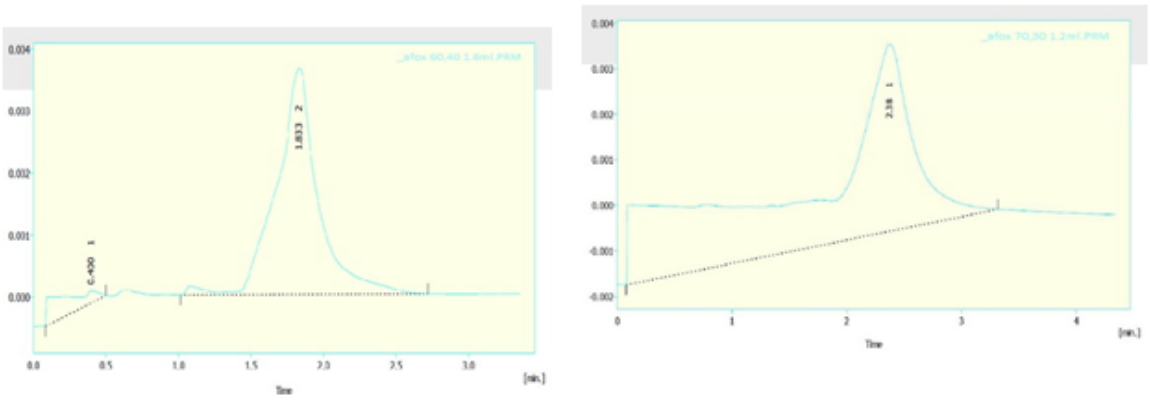
G)Acetonitrile.Water (276 nm, 1.8ml/min) H) Acetonitrile, methanol, triethanol amine (235b nm , 1.6ml/min)



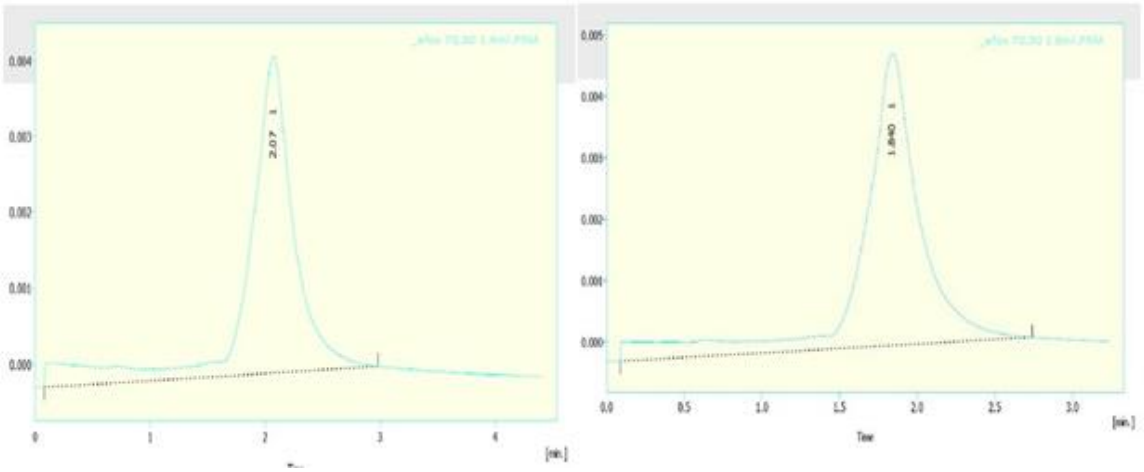
J) Acetonitrile, Methanol (60:40)(1.2ml/min, 235nm) J) Acetonitrile, methanol (60:40)(235nm, 1.4 ml/min)



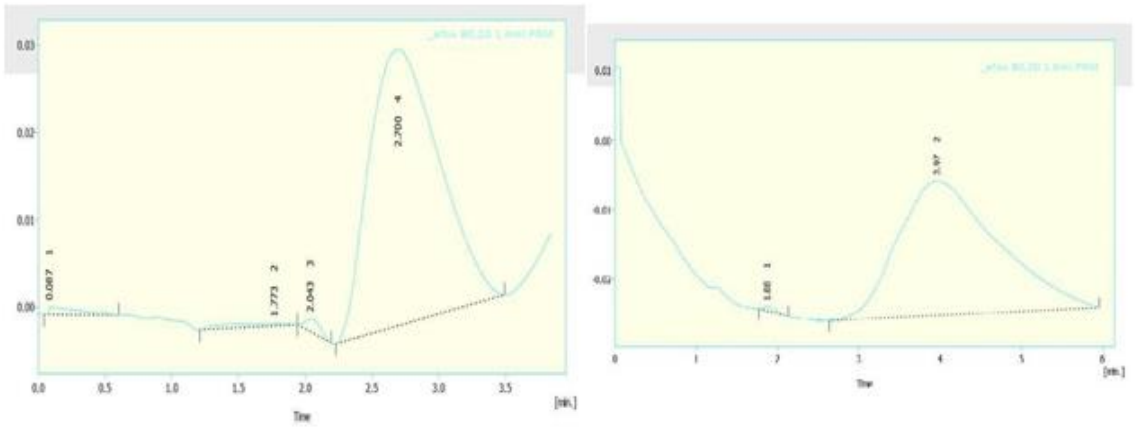
K) Acetonitrile, Methanol (60:40)(235 nm, 1.6ml/min) L) Acetonitrile, Methanol (70:30)(1.2 ml/min)



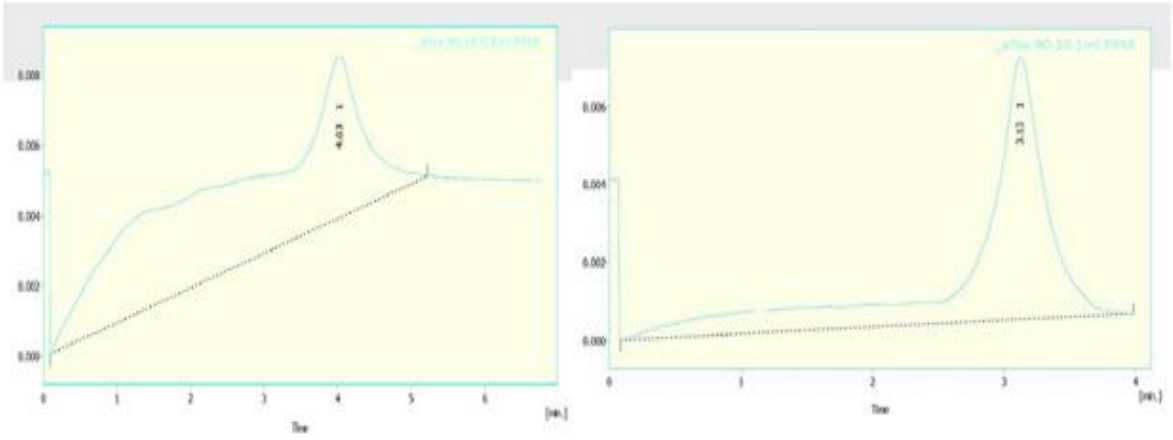
M) Acetonitrile, Methanol (70:30(235 nm,1.4ml/min) N) Acetonitrile, Methanol (70:30)(235 nm, 1.6ml/min)



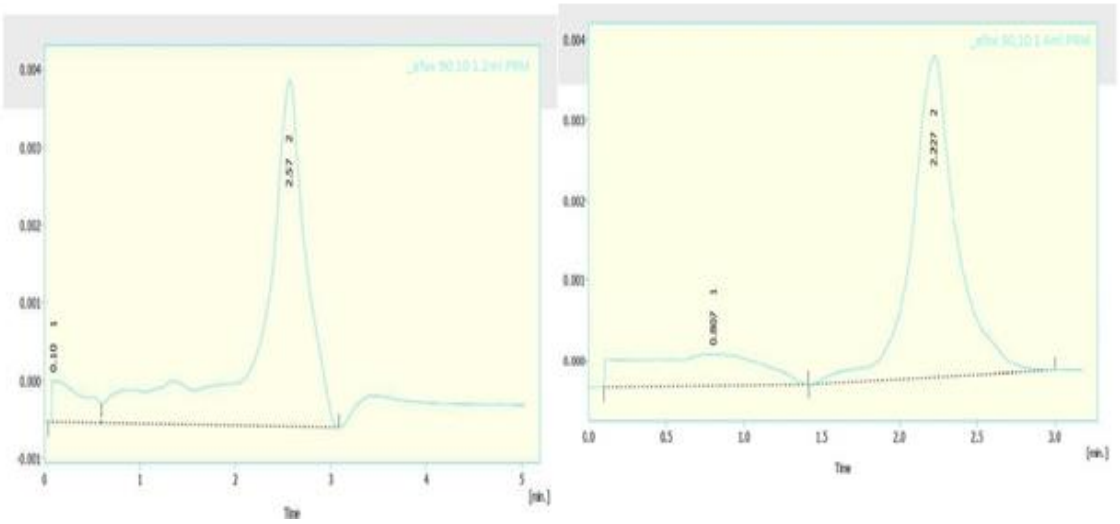
O) Acetonitrile, methanol (80:20) (235nm, 1.4ml/min) P) Acetonitrile, methanol (80:20)(235 nm, 1.6ml/min)



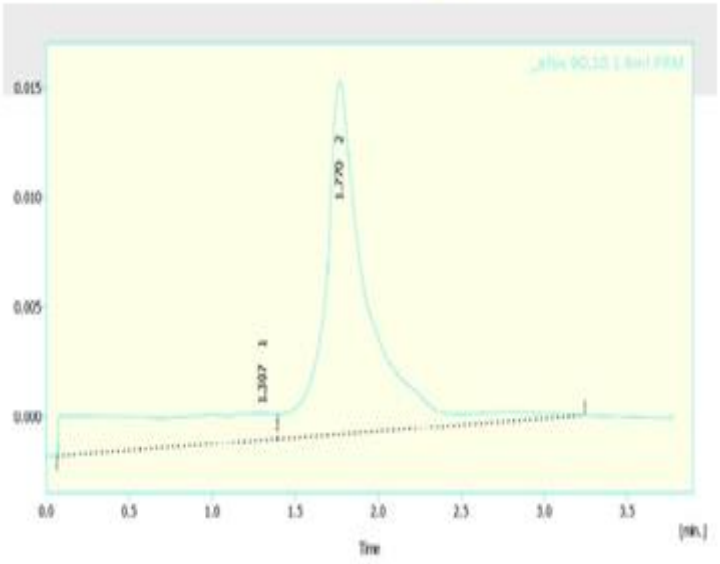
Q) Acetonitrile, methanol (90:10)(235nm, 0.8ml/min) R) Acetonitrile, methanol (90:10)(235 nm,1ml/min)



S) Acetonitrile, methanol (90:10)(235 nm, 1.2ml/min) T) Acetonitrile, methanol (90:10) (235 nm, 1.4 ml/min)



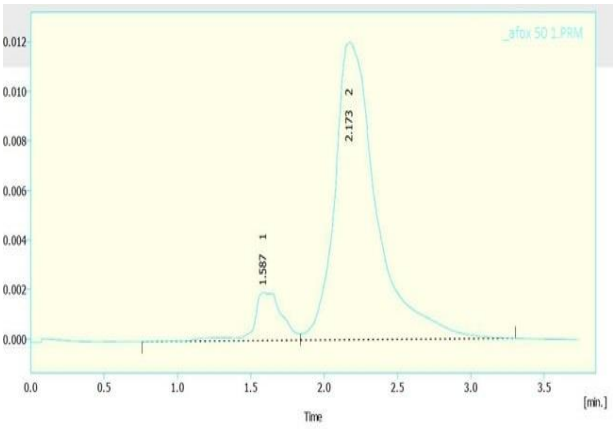
U) Acetonitrile, methanol (90:10)(235nm,1.6ml/min)



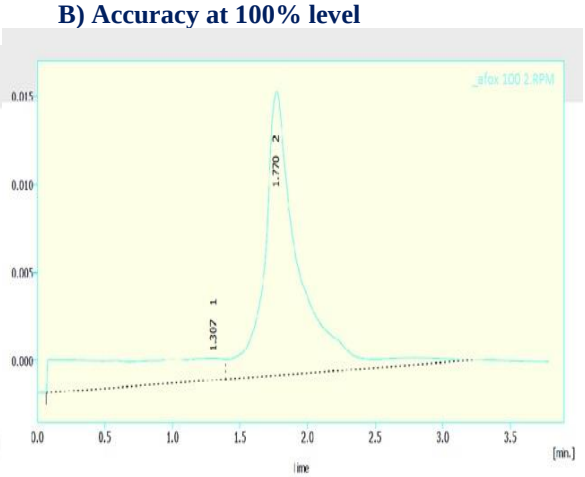
Validation

Accuracy

A) Accuracy at 50% level



B) Accuracy at 100% level



B) Accuracy at 150% level

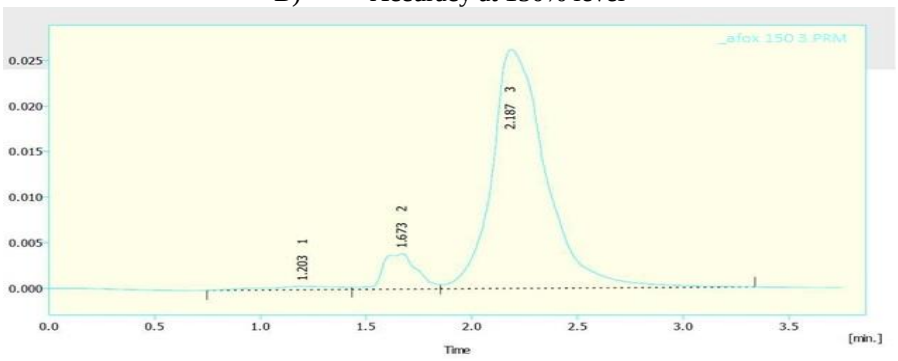


Table 1: Accuracy results of Afoxolner at various intervals

Concentration level	**Amount added(mg)**	**Amount found(mg)**	**%recovery**	**Average %recovery**
50%	25mg	25.5mg	102%	100.2%
	25mg	25.4mg	101.6%	
	25mg	24.3mg	97.2%	
100%	50mg	50.5mg	101%	100.9%
	50mg	50.45mg	100.9%	
	50mg	50.40mg	100.8%	
150%	75mg	75.5mg	100.6%	100.2%
	75mg	75.40mg	100.5%	
	75mg	74.65mg	99.53%	

Table 2: Recovery of Afoxolaner

Amount added(mg)	**Amount found(mg)**	**Average % recovery**
50mg	50.2mg	100.4%

2) Precision

Intraday precision

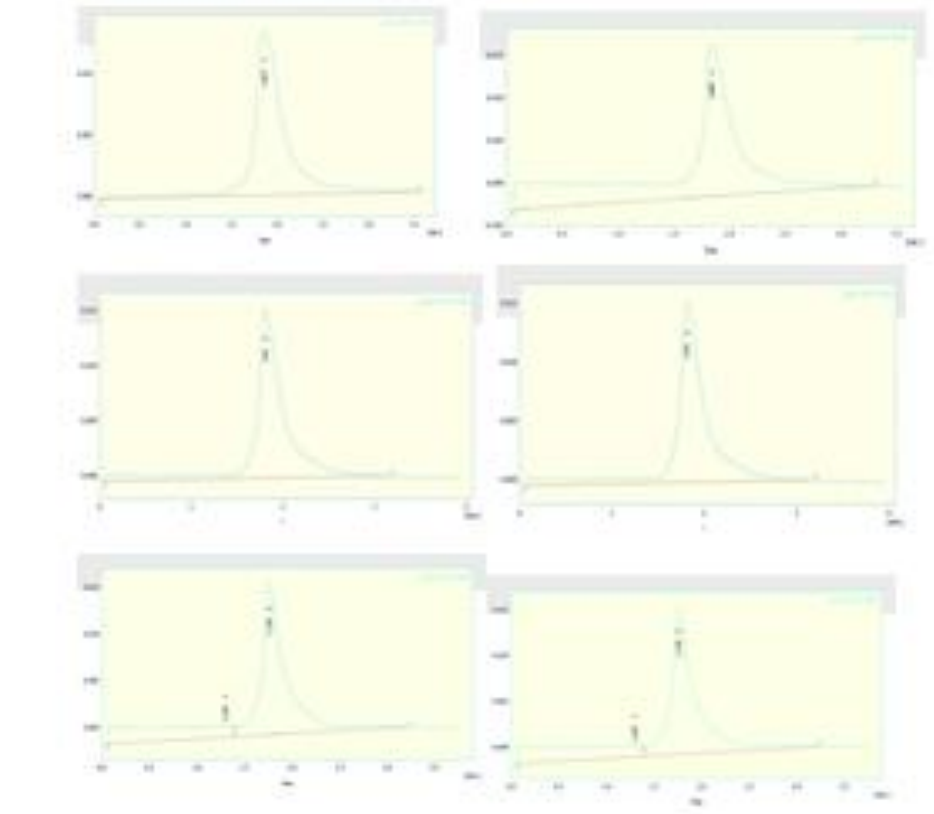


Table:2: Precision results of Afoxolaner for 6 injections

Sample No	**Peak area of Afoxolaner**
Injection1	284.421
Injection2	287.884
Injection3	291.462
Injection4	291.462
Injection5	291.514
Injection6	291.514
Mean	289.709
Standard deviation	2.96498
%RSD	1.02

Specificity

Figure 4A: Blank Chromatogram

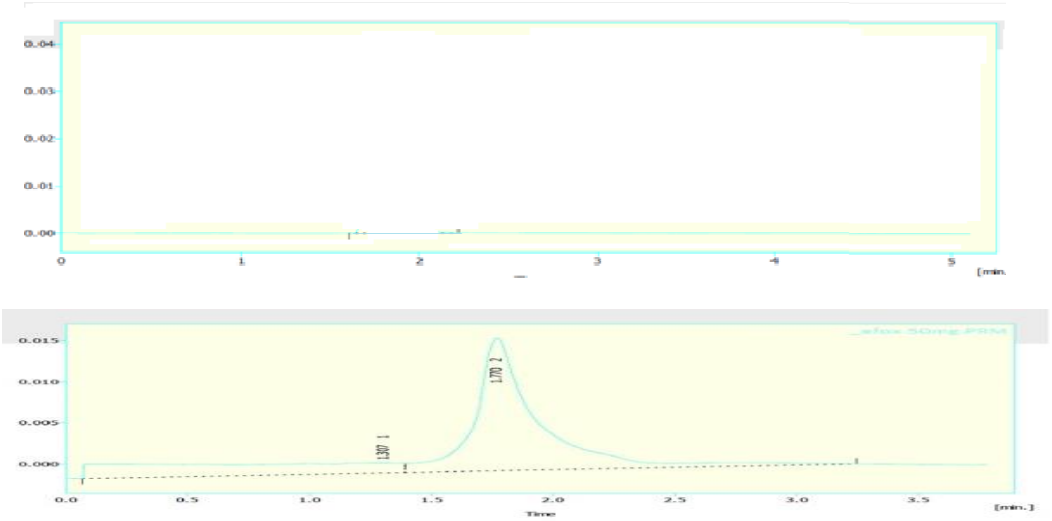


Figure4B: Chromatogram of Standard

Linearity and Range

Figure 5 : Calibration Graph of Afoxolaner

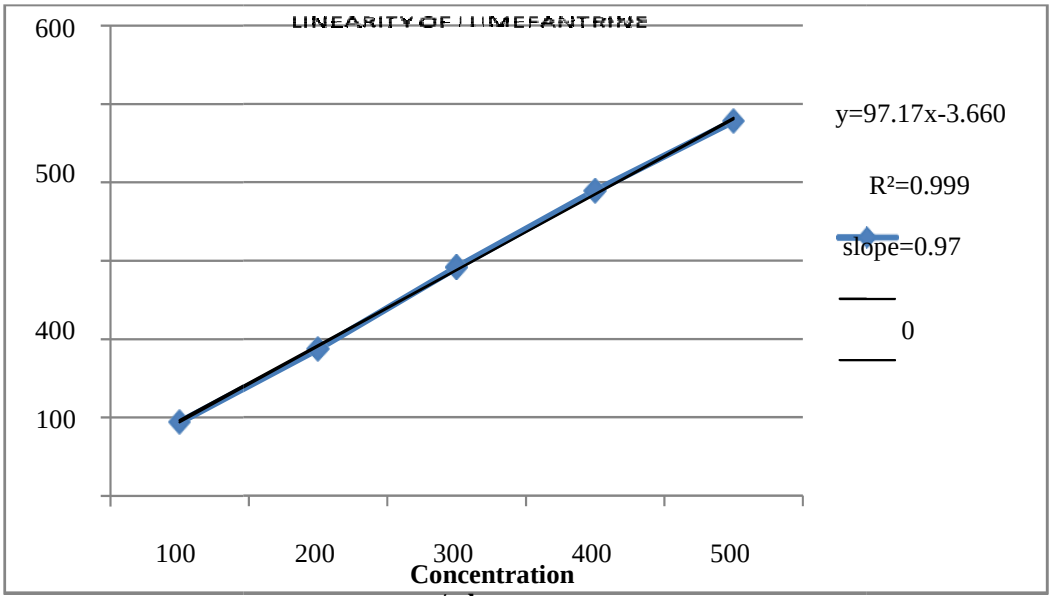


Figure 5.: Calibration Graph of Afoxolaner

Figure 6: Linearity graphs of Afoxolaner

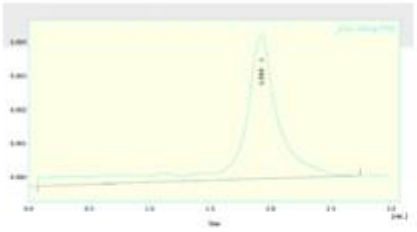


Figure 6A: Linearity at 100µg/ml

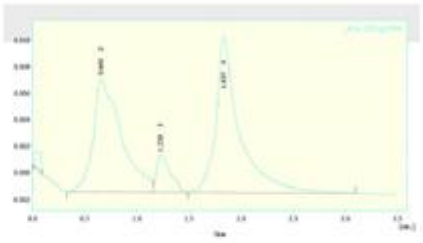


Figure:6B: Linearity at s200µg/ml

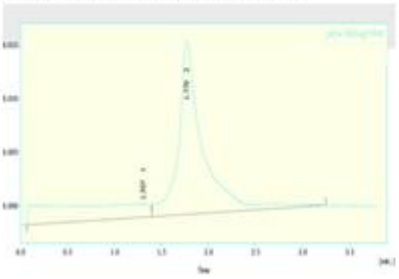


Figure 6C: Linearity at 300µg/ml

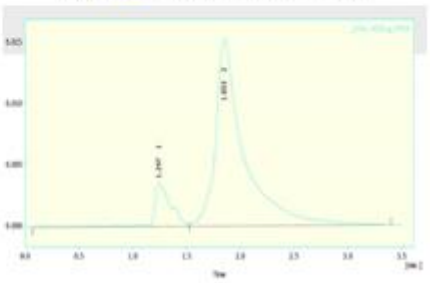


Figure 6D: Linearity at 400µg/ml

Table %: Linearity range of Afoxolaner at different concentrations

%level	**Concentration(µg/ml)**	**Peak area**
33	100	93.48
66	200	186.96
100	300	291.514
133	400	389.15
166	500	478.28
Y Intercept		96.18
Correlation co-efficient(r^2)		0.999
Slope		0.97
Linearity range	100-500	

The linearity range for the six replicates of the drug was found to be within in the limits and the regression coefficient was found to be 0.999

1) Robustness

i) Change in flow rate

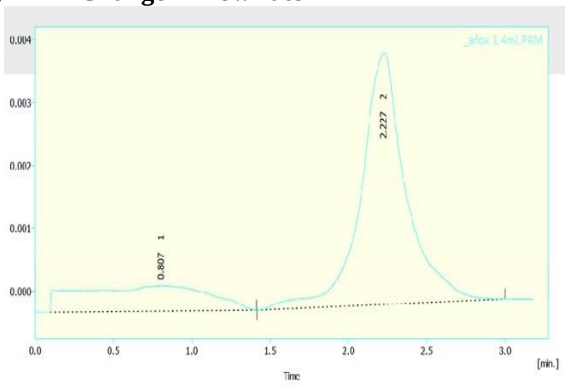


Figure 7A: Flow rate at1.4ml/min

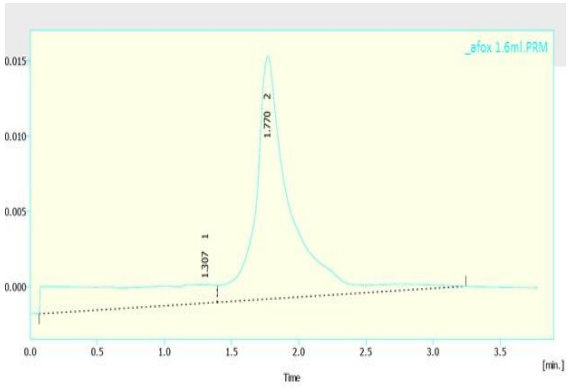


Figure 7B: Flow rate at 1.6 rate/min

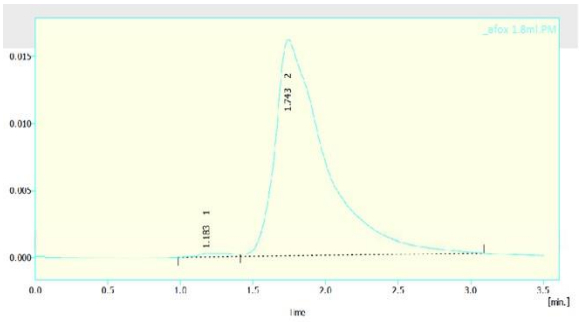


Figure 7C: Flow rate at 1.8ml/min

Table: 6: Robustness results of Afoxolaner

S.no	Flow rate	Peak area of Afoxolaner		Average	SD	%RSD
1	1.4ml/min	79.921	78.910	79.4155	0.7	0.88
2	1.6ml/min	291.514	289.512	290.513	1.41	0.48
3	1.8ml/min	389.150	387.45	388.3	1.20	0.30

.

Change in Column temperature

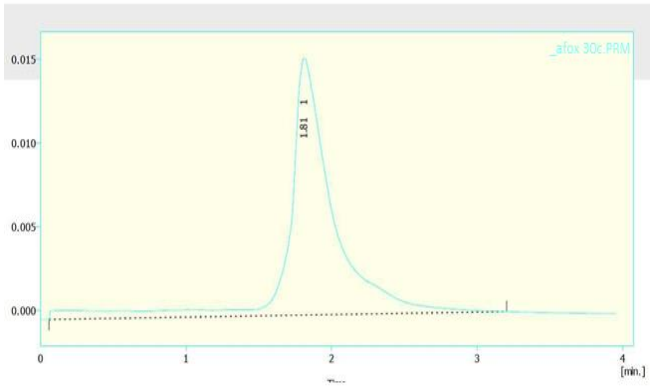


Figure 8A: Column temperature at 30^0C

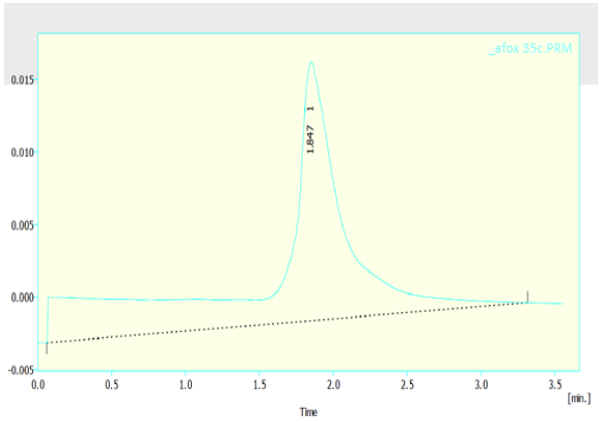


Figure 8B:Column temperature at 35ºC

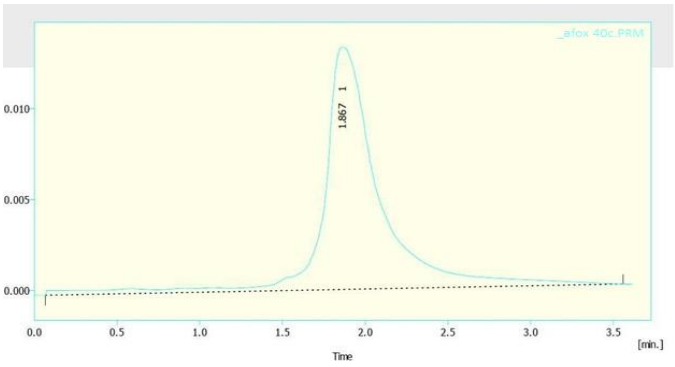


Figure 8C: Column temperature at 40ºC

Table 9: Robustness results of Afoxolaner

S.NO	Temperature	Peak area of Afoxolaner		Average	SD	%RSD
1	30ºC	291.462	285.452.	288.457	1.41	0.48
2	35ºC	287.884	285.882	286.883	1.415	0.49
3	40ºC	284.421	282.420	283.420	1.414	O.498

Figure 7: Ruggedness(Intermediate precision)

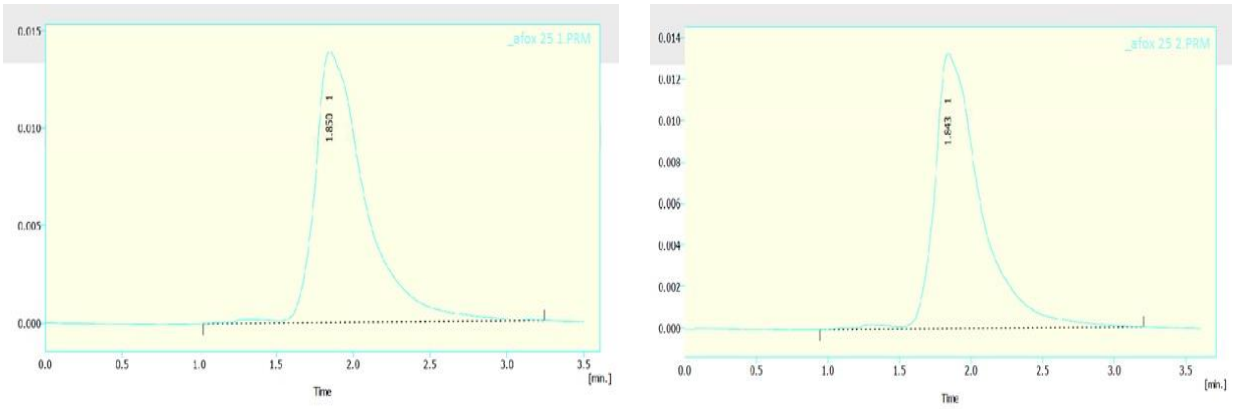


Figure 9A: Inter day precision of 1st injection Figure 9B: Inter day precision(2ndinjection)

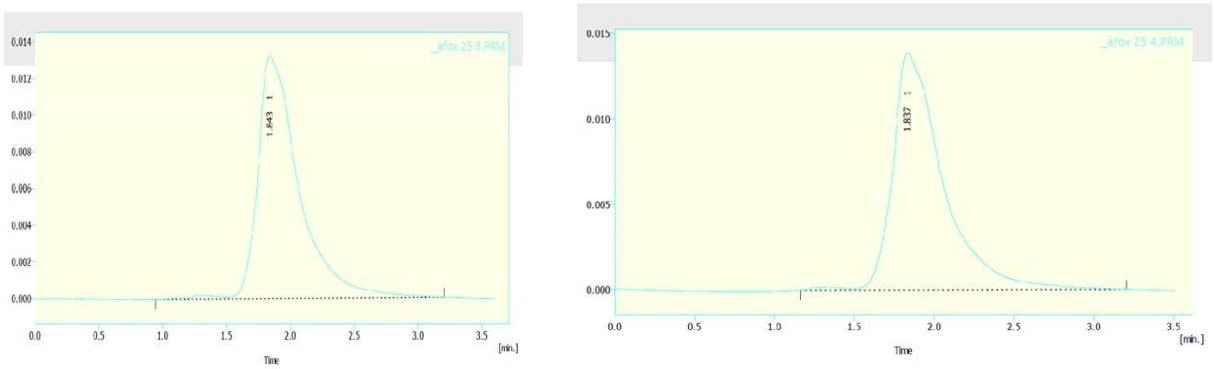


Figure: 9C: Inter day precision(3rdinjection) Figure 9D: Inter day Precision (4thinjection)

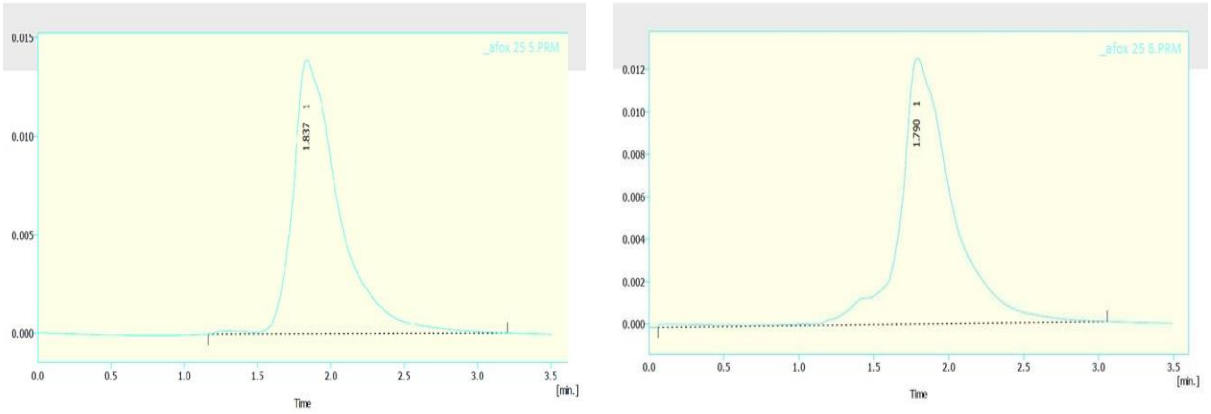


Figure: 9E: Inter day precision(5thinjection) Figure: 9F: Inter day precision(6thinjection)

Table 7: Ruggedness results of Afoxolaner

Name	**Peak area of Afoxolaner**
Ruggedness-(Day-1)-1	295.230
Ruggedness-(Day-1)-2	297.238
Ruggedness-(Day-1)-3	297.238
Ruggedness-(Day-1)-4	305.319

Ruggedness-(Day-1)-5	305.319
Ruggedness-(Day-1)-6	308.437
Average	301.4635
SD	5.530383088
%RSD	1.83

7) LOD&LOQ

Table:8: LOD & LOQ results of Afoxolaner

Parameters	Afoxolaner
LOD	10.0µg/ml
LOQ	30.5µg/ml

Table 9: Validation parameters

S.NO	**Parameter**	**Acceptance criteria**	**HPLC**
1	% Recovery	98-102%	100.4
2	Linearity range((µg/ml)	99-100%	100-500(µg/ml)
3	Correlation coefficient	NLT 0.999	0.999
4	No.of Theoreticalplates	NLT 2500	10877
5	Method precision	%RSD (NMT2%)	1.02
6	System precision	%RSD (NMT2%)	0.48
7	Intermediate precision	%RSD (NMT2%)	1.83
8	LOD	-	10.0(µg/ml)
9	LOQ	-	30.5(µg/ml)

Summary and conclusion

The Method development and validation of Afoxolaner was performed using the RP-HPLC method. The chromatographic conditions were set by using column Hypersil C18 (4.6 x 150mm, 5µm, Make: Analytical technologies) mobile phase as Acetonitrile, methanol (90:10) at a flow rate 1.6ml/min. Accuracy parameter is thought of as precise in the event that the average recovery is not under 98% and not over 102%. Precision parameter RSD of six replicate infusions ought to be NMT2%. The linearity range of Afoxolaner was viewed as 100-500µg/ml in HPLC. Linear regression was not more than 0.999. The values of %RSD was<2 Robustness of assay method is examined by changing the flow rate for 1.4ml/min and 1.8ml/min rather than 1.6ml/min by infusing the 6 replicate infusions of standard in 1.4ml/min and 1.8ml/min flow rate and viewed that system suitability parameters are passed. By changing the column temperature for 30℃ and 40℃ rather than 35℃ by injecting the 6 replicate infusions of standard in 30℃ and 40℃ temperature and viewed that system suitability parameters are passed. Ruggedness parameter RSD for the assay values of 6 sample preparations of same batch ought to be not over 2.0%. The LOD are determined by utilizing the formula LOD = 3 x SD/ b where, SD-standard deviation of the peak area of the drug, b-is slope of calibration curve passed. The% recovery varies in the range of 97-102%. LOD&LOQ values are found within the limits. These results show the method is accurate, precise, sensitive, economic &rugged. The HPLC method is more rapid. The proposed method is successfully applied to the bulk dosage form. The method was found to behaving suitable application in routine laboratory analysis with high degree of accuracy and precision.

Conflicts of interest

The authors declare no conflicts of interest

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