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Method Development, Validation and Forced Degradation Studies by Normal Chiral Phase Chromatography of Afoxolaner in Bulk and Pharmaceutical Dosage Forms

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ABSTRACT

The chiral chromatography has valuable application in quantification and identification of chiral compounds. Several methods including HPLC have been reported to be effective. HPLC is a sensitive technique that helps to identify the chiral centers in Direct and Indirect way. An indirect way of chiral analysis is more emerging involves analyte Derivitization resulting in two stereoisomer separation using reverse phase column in HPLC. The direct method which is used widely involves chiral based stationery phases or in mobile phase to derive the enantionmers. The HPLC technique was optimized by selection of suitable mobile phase, wavelength and flow rate by trial and error method and also with the forced degradation studies including the method validation by HPLC with ICH(Q2B) stability guidelines. The Afoxolaner is the veterinary drug widely used as antiparasitic drug, an Ectoparasiticide; widely used in cattle and poultry to relive of parasites ticks and mites. The present investigation involves the method development and validation of Afoxolaner by RP-HPLC for the determination chiral enantionmers and also determined that the API Afoxolaner is a racemic mixture with specific rotation, and enantiomeric purity of single enantiomer sample. The percentage of individual enantiomers found in formulations, mean, standard deviation and correlation coefficient for the formulations were calculated and are reported. The results obtained show a good agreement for the formulations with the label claim. The developed HPLC methods for the chiral separation of enantiomeric drug in afoxolaner was found to be simple, rapid, precise, accurate, specific and QC friendly with less elution time (10 min) which can be applied in industries, drug testing laboratories and R & D units for bioequivalence studies.. The method is also considered QC friendly as it is robust, uses isocratic mobile phase with both enantiomers eluting in <10 minutes, and employs commonly used solvents as mobile phase.

INTRODUCTION

A Stability indicating methods is a quantitative analytical procedure used to detect decrease in the amount of the active pharmaceutical ingredient (API) present due to degradation [1]. The FDA guidelines clearly specify the stability protocol for validation of the developed method that conveniently measures the potential interference in the drug such as degradative products, impurities, process impurity and excipient. The assay procedures by the FDA recommend [2] the HPLC stability studies are quantitative and routinely operated to separate and quantify the analytes of particular interest. The stability testing involves the determination of drug substance and the degradation products time to time along with the amount. This result is very crucial[3] to determine the safety of drug which is a mandate by FDA agency and therefore is a practice widely used which is well designed and validated. The Dissolution is prime requisite of a drug to act at the receptor and the test of dissolution is important emerging tool in the pharmacy available to determine the drug absorption at systemic level. Thus the determination of dissolution is used to assess the batch –to- batch consistency and amount of the drug release form the solid dosage form[4]. This enhances the process evaluation during the formulation development. The LC(Liquid chromatography) is routinely used to routinely to separate and quantitate the analytes in the sample drug. In QC the method validation protocol is prime and important step for the analytics. The confirmation of the process in the analytical validation procedure applied in the technique is highly suitable to determine the reliability of the applied method. The key aspects of the drug such as shelf life and dissolution play a prominent role in the assay or the stability study[5]. The ICH guidelines of stability testing based protocol is followed worldwide. Literature reports revealed that very few analytical methods have been reported for the drug Afoxolaner in human plasma and using the GC, MS or Liquid–liquid extraction which analyzed for crystal structure Elucidation, and determination in formulations by voltammeter, polarography[6]. But there is no method has been developed for quantification for its formulation by HPLC along with dissolution testing[7]. The main purpose of this investigation is to develop and validate simple, precise, sensitive and accurate stability indicating reversed phase HPLC for sensitive studies. Therefore, the present study has been undertaken in order to develop a new, simple, reproducible valid table, transferable[8], robust, reliable, accurate and precise individual methodology for the assay and drug release through the dissolution of the drug Afoxolaner in dosage forms by using RP(Reverse phase).-HPLC[9].

Afoxolaner (commonly called as Afoxolaner, es-Afoxolaner) with molecular formula[10]$C_{26}H_{17}ClF_9N_3O_3$, Figure 1 belongs to the chemical class of isoxazolines is popular antiparasitic agent against used in veterinary medicine in pets against external parasites (fleas, ticks lice, mites etc.). The drug is available in the form(Table 1) of chewable tablets with dose of 2.5-6.3 mg/kg bodyweight[11]. The drug is active ingredient of NEXGUARD including the other isoxazolines with action in insecticidal and ticks eliminating efficacy by functioning as selective and non competitive GAB[12] Antagonist in ticks and insects than in the mammals including the humans. The drugs mainly binds to the chloride channels in the muscle and nerves sites and elicit the action by blocking the transmission in nerve and muscle cells thereby resulting in the antiparasitic action where the parasites will become[13] paralysed and ultimately die. The mechanism is supported not only in the insects but also in the mammals and other invertebrates. The binding affinity of the drug in invertebrates is much high when compared to the mammals, thus the significant toxicity is less in mammals than in the insects[14]. Thus there exists a need to develop the method that can rapidly quantity the duration and its metabolites in different fluids by a rapid, effective and low cost method like HPLC[15].

The present investigation deals with the methods development and validation of chiral enantiomers of Afoxolaner by RP-HPLC based on standard ICH and WHO guidelines for safety and efficacy. This would contribute a sensitive rapid method in QC laboratory for affordable and effective quantification of Afoxolaner.

Figure 1: Structure of Aflaxolaner (IUPAC name 1-Naphthalenecarboxamide, 4 - ((5R) - 5 - (3 - Chloro -5 - (trifluoromethyl) - phenyl) - 4,5 - dihydro -5 - (trifluoromethyl) -3 - isoxazolyl) -N - (2 - oxo - 2-((2,2,2 - trifluoroethyl) amino) ethyl)

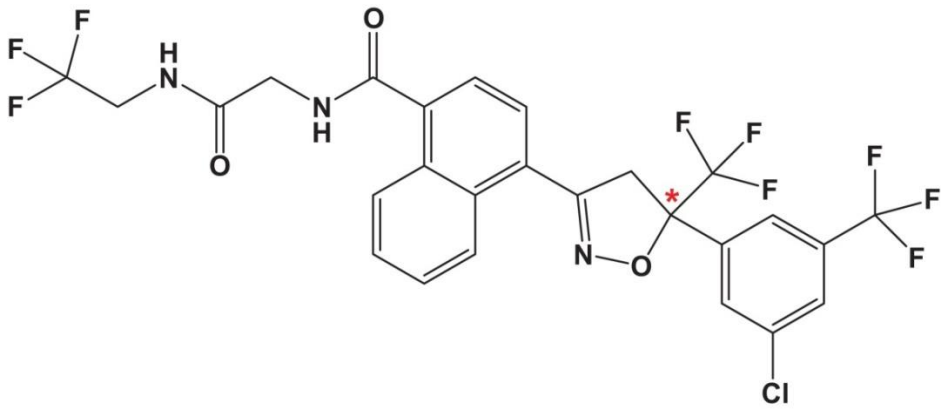

Table 1: Physical properties of the drug

S. No	Property	Value
1.	Molecular Weight	625.9
2.	Appearance	White or of - white powder or crystalline powder,
3.	Odour	Odourless
4.	Melting point	152°C - 156°C
5.	Solubility	Sparingly soluble in glacial acetic acid, Very slightly soluble in chloroform, Soluble in methanol

EXPERIMENTAL

Chemicals: All the chemicals and API used were of laboratory analytical grade and high purity. The API Afoxolaner was procured as gift sample from the Hetero labs, Hyderabad.

Instruments: All the instruments and apparatus were precalibrated, cleaned and sterilized prior to the use and were of laboratory analytical grade.

Assay Method Development-UV Spectroscopic Method

Preparation of mobile phase: Accurately measured a volume of 1000 ml of Acetonitrile (100%) Filtered and degassed for 2mins. The diluent solution is prepared using the buffer (pH:3) along with Methanol (molar ratio 50:50)

Chromatographic conditions

Column : Peerless Basic C_{18}100×4.6mm,1.8µ

Flow rate: 1.0ml/min

Column oven temperature: 30^0 c

Injection volume: 20 µl

Run time : 10 mins

Buffer Preparation: Weighed and dissolved about 2.72 g of Potassium dihydrogen phosphate and 1.74 g of dipotassium hydrogen phosphate in1000 mL of Milli Q water & mixed and sonicated for 10 mins.

Preparation of mobile phase: Accurately measured a volume of 400ml of pH 6.8 Phosphate buffer and mixed with 600ml of Acetonitrile (40:60), filtered and degassed for 2 mins.

Preparation of mobile phase

S. no	Trail	Mobile phase preparation		Diluent(Buffer pH 3)
		Phosphate buffer pH 6.8	Acetonitrile: methanol	
1.	1	-	1000 ml ACN:0	Phosphate buffer and methanol(50:50)
2.	2	-	400ml 600 ml	Phosphate buffer and methanol(50:50
3.	3	200ml	600 ml 200 ml	Phosphate buffer and methanol(50:50
4.	4	250ml	500 ml 250ml	Phosphate buffer and methanol(50:50
5.	5	300ml	400ml 300 ml	Phosphate buffer and methanol(50:50

Preparation of standard stock solution: Accurately weighed and transferred about 51 mg of Afoxolaner working standard into100 mL volumetric flask, add about 40 ml Methanol, sonicated to dissolve the material completely and add 20ml of acetonitrile and dilute to volume with methanol and mixed.

Preparation of standard solution: Pipette 5 mL of above standard stock solution into a 50 mL volumetric flask dilute to volume with Diluents and mixed.

Test preparation Weighed and transferred 5 tablets into a 100 mL volumetric flask added about 70 ML of diluent, sonicated for about 30 minutes with intermediate shaking, dilute to volume with diluent and mixed. The above solution was centrifuged for 10 min in centrifuge tubes at 2500 rpm; 3mL of the centrifuged sample solution was diluted to 50mL with diluent and used as the test preparation Validation of assay method.

Validation Approach

System Suitability

The standard solution was prepared by using Afoxolaner working standard as per test method and was injected ten replicate injections into the HPLC system. The system suitability parameters were valuated from standard chromatograms by calculating the percentage RSD from ten replicate injections for Afoxolaner retention time and peak areas.

Precision

The precision determination in an analytical method gives the closeness of values (Degree of scattered) in a series of measurements obtained of the results predicted during the multiple samplings of the method under the given conditions.

Repeatability in a Method Precision determines the expression in the given operating conditions over a hour interval of time. The replicates are prepared in six number and estimated as per the test protocol. The individual assay value along with the mean and %RSD is recorded and estimated.

Specificity

The Specificity of analytical method is ability to measure specifically the analyte of interest without interference from blank and placebo.

Placebo Preparation: The placebo is determined to check the interference of the results. The placebo equivalents are weighed to five tablets in a 100 ml volumetric flask and added with 20 ml acetonitrile and 50 ml of methanol. Further sonicated for some time and finally made up with volume using methanol. Then 3ml of above solution is made to 100ml with diluents. This is injected and interference is estimated.

Check for interference from forced degradation studies:

1. Acid Degradation: Weigh accurately transfer five tablets into 100ml volumetric flask, add 25ml of 0.1N HCl and sonicated with shaking to disappear the tablet completely and place into water bath at 70^0C for about 30minutes.Add 25ml of 0.1NaoH for neutralization. The volume is made up in a ratio of 10ml of acetonitrile :40ml of Methanol. 3ml of solution is pipetted and made up with 100ml of diluent.

2. Base Degradation Weigh accurately transfer five tablets into 100ml volumetric flask, add 25ml of0.1N Sodium Hydroxide and sonicated with shaking to disappear the tablet completely and replaced into water Bath at 70^0C for about 30minutes. 0.1 N HCl of 3ml is added to above solution and subjected to neutralization. The volume is made up in a ratio of 10ml of acetonitrile: 40ml of Methanol. 3ml of solution is pipette out and made up with 100ml of diluent.

3. Oxidative Degradation: Accurately weighed five tablets are transferred into 100ml volumetric flask, and 25ml of 2% of Hydrogen peroxide solution and for suitable interval, place in to water bath at 40^0C for about 30 minutes and makeup the volume with10ml of acetonitrile and 40mlof Methanol. 3ml of solution is pipette out and made up to100ml with diluent.

4. Hydrolytic Degradation Accurately weighed and transferred five tablets into 100ml volumetric flask, then 25ml of water is added and placed in the volumetric flask in the water bath for about 30 mins. Then the volume is made up to volume with 10ml of acetonitrile and 40ml of Methanol. From this pipette out 3ml and make up to 100ml with diluent.

5. Photolytic Degradation the Tablet powder (covered with aluminum foil) was exposure in photostability chamber as per guidelines and over all illumination of NLT-1.2 million lux hour and an integrated near UV energy of NLT- 200 watt hour/Sq.m. Weigh accurately five tablets and crush them into powder. Tablet powder of 3900mg of exposed samples was transfer into 100ml volumetric flask add 20ml of Acetonitrile and 50ml of methanol and sonicated for some time and make up the volume with methanol, from this further dilute 3ml to 100mlwith diluents. Prepare standard and sample solution by suitable degradation method and injected into HPLC system and evaluate the peak purity.

Linearity: The linearity of analytical method is its ability to elicit thet results that are directly, by well defined mathematical transformation, proportional to the concentration of analyte in simple within a given working range. A series of solutions are prepared using Afoxolaner working standard at concentration levels from 25% to 150% of target concentrations (25%, 50%, 75%, 100%,125%,150%) measure the peak area response of the solutions.

Accuracy (Recovery) The accuracy of analytical method is the closeness of sample results obtained by that method to the true value. The true value is that result which would be

observed in the absence of error. Accuracy may often be expressed as present recovery by assay of known, added amounts of analyte. Accuracy is a measure of the exactness of the analytical method that is true for all practical purpose. Determine accuracy over the range 25%, 50%, 75%, 100%, 125% & 150% of the working concentration. Added calculated amount of Afoxolaner working standard or APIionplacebotoattain25%,50%, 75%, 100%, 125% & 150%.

Recovery preparations:

Level 1:(25%) Weighed and transfer above 42.5mg of Afoxolaner into 100ml Volumetric flask, add 3780 mg of placebo add 20ml Acetonitrile and 80ml of Methanol and shake well and sonicated for some time. Pipette out 3ml of above solution and makeup thevolume100ml with diluent.

Level 2:(50%) Weighed and transfer above 85mg of Afoxolaner into 100ml Volumetric flask, add 3780 mg of placebo add 20ml Acetonitrile and 80ml of Methanol and shake well and sonicate for some times. Pipette out 3ml of above solution and makeup thevolume100ml with diluent.

Level 3:(75%) Weighed and transfer above127.5mg of Afoxolaner into 100mlVolumetric flask, add 3780 mg of placebo add 20ml Acetonitrile and 80ml of Methanol and shake well and sonicate for sometimes. Pipette out 3ml of above solution and make up the volume 100mlwith diluent.

Level 4:(100%) Weighed and transfer above 170mg of Afoxolaner into 100ml Volumetric flask, add 3780 mg of placebo add 20ml Acetonitrile and 80ml of Methanol and shake well and sonicate for some times. Pipette out 3ml of above solution and makeup thevolume100ml with diluent.

Level 5:(125%) Weighed and transfer above 212.5mg of Afoxolaner into100mlVolumetric flask, add 3780 mg of placebo add 20ml Acetonitrile and 80ml of Methanol and shake well and sonicate for sometimes. Pipette out 3ml of above solution and make up the volume 100mlwith diluent.

Level 6:(150%) Weighed and transfer above 255mg of Afoxolaner into 100ml Volumetric flask, add 3780 mg of placebo add 20ml Acetonitrile and 80ml of Methanol and shake well and sonicate for some times. Pipette out 3ml of above solution and makeup thevolume100ml

with diluent. Prepare three preparations for each level and inject e each preparation in triplicate. Calculate the amount found and percentage recovery at each level and calculate the mean percentage recovery and %RSD.

Robustness:

a **Effect of variation in mobile phase composition**: A study was conducted to determine the effect of variation in organic phase composition in mobile phase. The Standard solution prepared as per the test method was injected into HPLC system using various mobile phase compositions. The system suitability parameters were evaluated and found to be within the limits.

b. **Effect of variation of flow rate:** A study was conducted to determine the effect of variation in flow rate. Standard solution prepared as per the test method was injected into HPLC system using flow rate0.9ml/min and 1.1ml/min. The system suitability parameters were evaluated and found to be within the limits for0.9ml/min and 1.1ml/min flow. Afoxolaner were resolved from all other peaks and the retention was compared with those obtained for mobile phase having flow rate 1.0ml/min.

c. **Effect of variation in pH:** A study was conducted to determine the effect of variation in PH. The Standard solution prepared as per the test method was injected into HPLC system using 6.6&7. The System suitability parameters were evaluated and found to be within the limits for pH 6.6 and 7. Afoxolaner were resolved from all other peaks and retention time were comparable with those obtained for mobile phase having pH 6.8.

d. **Effect of variation in Temperature:** A study was conducted to determine the effect of variation in Temperature. The Standard solution prepared as per the test method was injected into HPLC system at 28^{o}C & 32^{0}C temperature. Similarly sample solution was chromatographed at 30^{o}C temperature. Afoxolaner were resolved from all other peaks and retention times were comparable with those obtained mobile phase having 30^{o}C temperature.

Filter validation

To demonstrate robustness of assay method, carry out filter validation using two different filter prepare test solution in triplicate. Centrifuge and filter different portions of the test preparation and inject into the HPLC system along with unfiltered standard. Calculate the percentage assay.

Ruggedness: Intermediate Precision was observed on a day and also on different day, by a other analyst, by other column with the using same lot of sample as specified under repeatability. Bench top stability of mobile phase, standard and sample preparation also evaluated. Repeated the procedure followed for method precision on a different day, different analyst, using different HPLC system and by different column by using same with lot of sample calculate individual assay value mean assay value, %RSD, overall l% RSD for determining intermediate precision.

Dissolution

Detection Method And Selection Of Wavelength Known concentrations of Afoxolaner working standard was taken and dissolved in Methanol such that the standard solution contains about 51 ppm. Placebo & blank solutions also prepared. All these solutions were scanned between 200-400 nm using UV visible spectrophotometer.

Optimization Of Mobile Phase Mobile Phase Composition

Trail: 1

Buffer Preparation: Weighed and dissolved about 2.72g of Potassium dihydrogen phosphate and 1.74g of Di-Potassium hydrogen phosphate in 1000 mL of MilliQ water & mixed and sonicated for`10 minutes.

Preparation of mobile phase: Accurately measured a volume of 300ml of pH6.8 Phosphate buffer and mixed with 400ml of Acetonitrile 300ml of methanol(30:40:30) .filtered and degassed for 2 mins.

Chromatographic conditions

Column	:	ProntosilHC_{18}100×4.6mm,3µ
Flow rate	:	1.5ml/min
Column oven temperature	:	Ambient
Injection volume	:	50µl
Runtime	:	10 mins

Trail: 2

Buffer Preparation:

Preparation of pH3.0 buffer 7ml of triethylamine is mixed with 1000ml of water and pH was adjusted with ortho-phosphoric acid. Preparation of mobile phase accurately measured a volume of 400ml of pH3 Phosphate buffer and mixed with 600ml of Acetonitrile (40:60) .filtered and degassed for 2mins.

Chromatographic conditions

Column	:	ProntosilHC_{18}100×4.6mm,3µ
Flow rate	:	1.5ml/min
Column oven temperature	:	Ambient
Injection volume	:	50µl
Runtime	:	10mins

Trail: 3

Buffer Preparation:

PreparationofpH3.0buffer 7ml of triethyl amine is mixed with 1000 ml of water and pH was adjusted with ortho phosphoric acid.

Preparation of mobile phase Accurately measured a volume of 200ml of pH3 Phosphate buffer and mixed with 600ml ofAcetonitrile,200ml of Methanol in the ratio of (20:60:20) filtered and degassed for 2mins.

Diluent: dissolution medium

Chromatographic conditions

Column	:	ProntosilHC_{18}100×4.6mm,3µ
Flow rate	:	1.5ml/min
Column oven temperature	:	Ambient

Injection volume	:	50µl
Runtime	:	10mins

Trail: 4

Buffer Preparation:

Preparation of pH3.0 buffer 7ml of triethylamine is mixed with 1000ml of water and pH was adjusted with ortho phosphoric acid.

Preparation of mobile phase Accurately measured a volume of 300ml of pH 3 Phosphate buffer and mixedwith400mlofAcetonitrile,300ml of Methanol in the ratio of (30:40:30) .filtered and degassed for 2 mins.

Diluent: dissolution medium

Chromatographic condition

Column	:	ProntosilHC$_{18}$100×4.6mm,3µ
Flow rate	:	1.5ml/min
Column oven temperature	:	Ambient
Injection volume	:	50µl
Runtime	:	10min

Solubility studies: Solubility of the drug can studied in different medium like water, 0.1 HCL, pH4.5 Phosphate buffer, pH 6.8 Phosphate buffer.

Selection of dissolution parameters: Dissolution parameters like Medium, Apparatus, RPM, Time points, Medium volume can be selected from Office of Generic Drugs. It can be optimized with different trails and using similarity factor.

Preparation of solutions

Preparation of pH 3.0 buffer: Weighed and dissolved about 2.72 g of Potassium dihydrogen phosphate and 1.74 g of Di Potassium hydrogen phosphate in 1000 mL of Milli Q water & mixed well. And pH was adjusted with ortho-phosphoric acid.

Preparation of mobile phase Mixed pH3 buffer: methanol: Acetonitrile in the ratio 30:30:40(v/v) filtered and degassed for 10mins.Preparation of standard stock solution Accurately weighed and transferred about 50 mg of Afoxolaner working standard into 100 ML volumetric flask, add about 70ml of methanol, sonicated to dissolve the material completely, dilute to volume with methanol and mixed. Preparation of standard solution Pipette 4 mL of above standard stock solution into a 50ml volumetric flask dilute to volume with dissolution medium(0.1NHCL+0.5%SLS) and mixed. Test preparation: Weighed and transferred each individual tablet to respective dissolution vessels containing 900 mL of dissolution medium (0.1NHCL+0.5%SLS), and started the run. The samples are collected at specific time point.

Validation Of Dissolution Method

Validation procedures

System Suitability: The standard solution was prepared by using Afoxolaner working standard as per the test method and was injected ten times into the HPLC system. The system suitability parameters were evaluated from standard chromatograms by calculating the percentage RSD from ten replicate injections for Afoxolaner retention time and peak areas.

Precision: The precision of an analytical method is the closeness of agreement (Degree of scattered) between series of measurements obtained from multiple samplings of the same homogeneous sample under the prescribed conditions.

Repeatability (Method Precision) Repeatability expresses the precision under the same operating condition over a short interval of time Prepare and analysis six replicate sample preparation as per method. Calculated individual % Dissolution value, %RSD and 95% confidence interval and recorded.

Specificity: Specificity of analytical method is ability to measure specifically the analyte of interest without interference from blank and placebo.

Check for interference from placebo:

Placebo Preparation: Weigh placebo equivalent to one tablet, transfer in to dissolution vessel and add 900 ml of dissolution medium (0.1NHCL+0.5%SLS), and started the run. The samples are collected at specific time point. (24^{th}hour). Inject the sample and check the interference.

Linearity: The linearity of analytical method is its ability to elicit test results that are directly, by well-defined mathematical transformation, proportional to the concentration of analyte in simple within a given working range. A series of solutions are prepared using Afoxolaner working standard at concentration levels from 25% to 150% of target concentrations (25%, 50%, 75%, 100%, 125%, 150%). Measure the peak area response of the solutions.

Accuracy (Recovery): The accuracy of analytical method is the closeness of sample results obtained by that method to the true value. The true value is that result which would be observed in the absence of error. Accuracy may often be expressed as present recovery by assay of known, added amounts of analyte. Accuracy is a measure of the exactness of the analytical method that is true for all practical purpose. Determine accuracy over the range 25%, 50%, 75%, 100%, 125% & 150% of the working concentration. Add calculated amount of Afoxolaner working standard or API in placebo to attain 25%, 50%, 75%, 100%, 125% & 150%.

Recovery Preparations:

Level 1:(25%): Weighed and transfer about 8.5mg of Afoxolaner and add 756 mg of placebo, transfer in to dissolution vessel and add 900 ml of dissolution medium (0.1NHCL+0.5%SLS) and started the run. The samples are collected at specific time point (24^{th}hour).

Level 2:(50%): Weighed and transfer about 17mg of Afoxolaner and add 756 mg of placebo, transfer in to dissolution vessel and add 900 ml of dissolution medium (0.1NHCL+0.5%SLS),and started the run. The samples are collected at specific time point (24^{th}hour).

Level 3:(75%) Weighed and transfer about 25.5mg of Afoxolaner and add 756 mg of placebo, transfer in to dissolution vessel and add 900 ml of dissolution medium (0.1NHCL+0.5%SLS),and started the run. The samples are collected at specific time point(24^{th}hour).

Level 4:(100%) Weighed and transfer about 34mg of Afoxolaner and add 756 mg of placebo, transfer in to dissolution vessel and add 900 ml of dissolution medium (0.1NHCL+0.5%SLS) and started the run. The samples are collected at specific time point (24^{th}hour).

Level 5:(125%) Weighed and transfer about 42.50mg of Afoxolaner and add 756 mg of placebo, transfer in to dissolution vessel and add 900 ml of dissolution medium (0.1NHCL+0.5%SLS), and started the run. The samples are collected at specific time point(24^{th}hour).

Level 6:(150%) Weighed and transfer about 51mg of Afoxolaner and add 756 mg of placebo, transfer in to dissolution vessel and add 900 ml of dissolution medium (0.1NHCL+0.5%SLS) and column was run. The samples are collected at specific time point(24^{th}hour). Prepare three preparations for each level and injected each preparation in triplicate. Calculate the amount found and percentage recovery at each level and calculate the mean percentage recovery and %RSD.

1) Robustness:

Effect of variation of flow rate: A study was conducted to determine the effect of variation in flow rate. Standard solution prepared as per the test method was injected into HPLC system using flow rate 1.4ml/min and 1.6ml/min. The system suitability parameters were evaluated and found to be within the limits for 1.4ml/min and 1.6ml/min flow. Afoxolaner were resolved from all other peaks and there tension was compared with those obtained for mobile phase having flow rate1.5ml/min.

a) Effect of variation in pH: A study was conducted to determine the effect of variation in pH. Standard solution prepared as per the test method was injected into HPLC system using 2.8 and 3.2 The System suitability parameters were evaluated and found to be within the limits for pH 2.8 and 3.2. Afoxolaner were resolved from all other peaks and retention time were comparable with those obtained for mobile phase having pH 3.

b) Filter validation: To demonstrate robustness of dissolution method, carry out filter validation using two different filters. Prepare test solution in triplicate. Centrifuge and filter different portions of the test preparation and injection to the HPLC system along within filtered standard. Calculate the percentage dissolution.

c) Ruggedness: Intermediate Precision within laboratory precision on a different day, by a different analyst, by different column by using same lot of sample as specified under repeatability. Repeated the procedure followed for method precision on a different day, different analyst, using different HPLC system and by different column by using same lot of sample calculate individual assay value mean assay value, %RSD, overall %RSD. Find out

the difference in the assay value of intermediate precision. Perform dissolution on Afoxolaner drug substance in dissolution medium by adding 100 mg of Afoxolaner to dissolution vessel at RT with 150 rpm. The % dissolution of Afoxolaner was calculated.

RESULT

Optimization of chromatographic conditions: The main purpose of this work is to develop a fast Normal Phase HPLC method to separate and quantitate the two enantiomers in Afoxolaner (racemic mixture). During method development work, several cellulose based chiral HPLC columns were selected on the basis of the chemical structure and location of the chiral center of the analyte and also on the characteristics of the chiral phase of the analytical columns. Three columns namely Prontosil® OJ-3, Prontosil® OD-H and Prontosil AD-3 were selected and screened by using low polarity mobile phase system. Figure 2 shows the overlaid chromatograms using these columns with mobile phase of *n*-Hexane/IPA (95:5, v/v). Best chiral selectivity and resolution was obtained on ProntosilAD-3 column. Therefore, Prontosil AD-3 column was selected for further optimization.

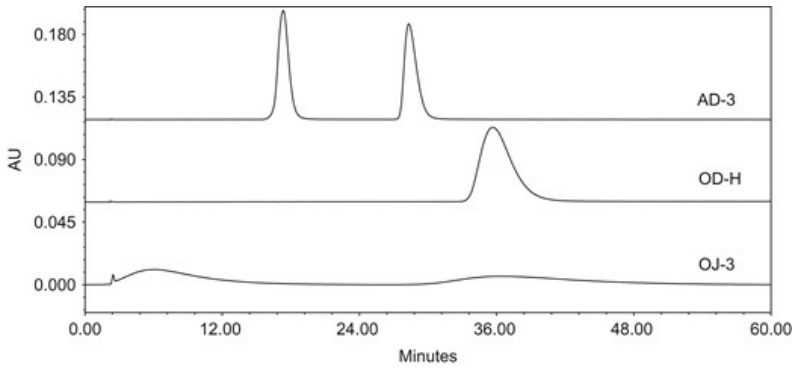

Figure 2:. Chromatograms of Afoxolaner (0.1 mg/mL) on various chiral HPLC columns with mobile phase *n*-Hexane/IPA (96:4, v/v); flow rate: 0.9 mL/min; wavelength: 333 nm; column temperature: 35.2°C; injection volume: 10 µL.

As indicated in Figure 3, the separation of the two enantiomers was obtained using isocratic elution by combination of *n*-Hexane and IPA with the ratios of 90:10 and 95:5. The result obtained was better and fast separation in less time along with *n*-Hexane/IPA (90:10, v/v). For optimization of the mobile phases. Thus, the effect of a variety of organic modifiers such as methanol, ethanol, hexanol, 1-butanol, cyclohexanol were investigated. The final optimized mobile phase was found to be *n*-Hexane/IPA/MeOH (88:10:1, v/v/v). Table 2 summarized the final chromatographic conditions.

The final method was then used for analysis of both Afoxolaner (racemic mixture) and E. 1 samples. Typical chromatograms of both Afoxolaner (racemate) and "E. 1" that had a low level of "E. 2" as the impurity are shown in Figure 3 and 4 respectively. Figure 3 chromatogram of Afoxolaner (racemic mixture) indicates the the peak area ratio of E. 1 and E. 2 as resulted to be 50:50. Figure 5 chromatogram of E. 1 clearly indicates the final chromatographic conditions. E. 1 along with E. 2 were found to be well separated with R_s = 5.4. The %peak area of E. 1 along with E. 2 was found to be 98.63% and 0.397%, respectively.

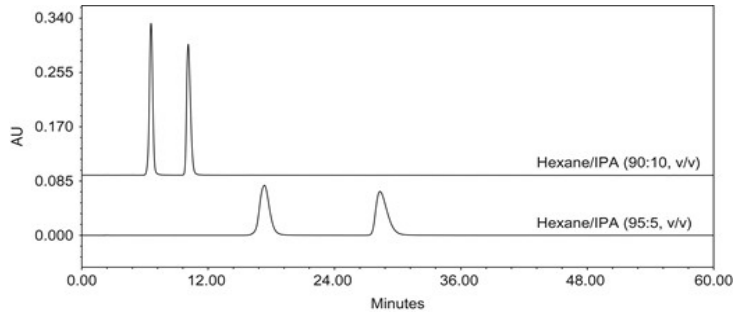

Figure 3: Chromatograms of Afoxolaner (0.8 mg/mL) on AD-3 column including various mobile phases; flow rate: 0.9 mL/min; wavelength: 333 nm; column temperature: 35°C; injection volume: 10 µL.

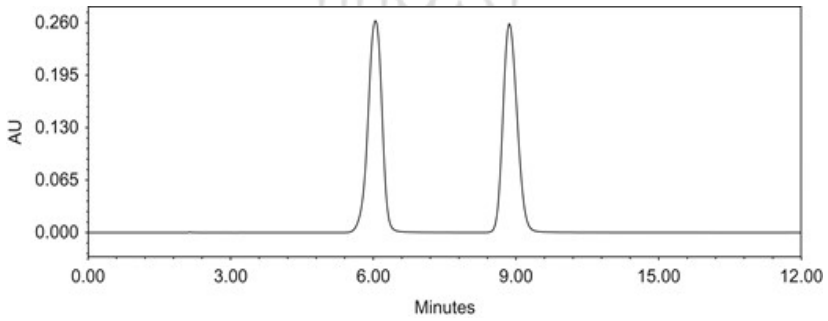

Figure 4: A typical chromatogram of 0.8 mg/mL Afoxolaner racemate analyzed using the final chromatographic conditions [Prontosil ® AD-3 150 × 4.6 mm, 3 µm; mobile phase = *n*-Hexane/IPA/MeOH (88:10:1, v/v/v); flow rate: 0.9 mL/min; wavelength: 333 nm; column temperature: 35°C; injection volume: 10 µL).

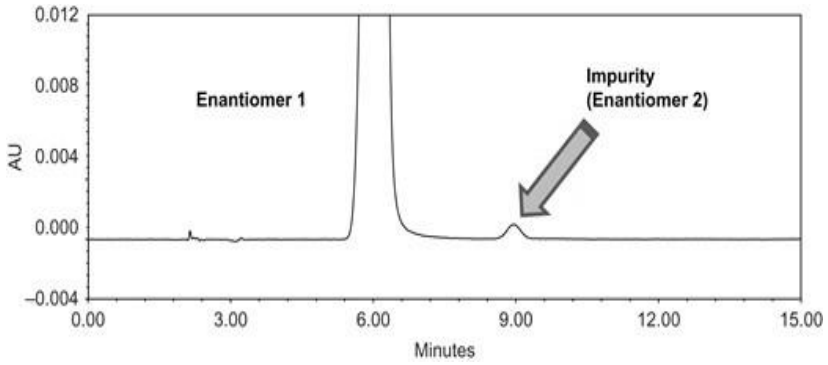

Figure 5: A typical chromatogram of 0.5 mg/mL "E. 1" analyzed (Prontosil® AD-3 150 × 4.6 mm, 3 µm; mobile phase = *n*-Hexane/IPA/MeOH (88:10:2, v/v/v); flow rate: 0.9 mL/min; wavelength: 333 nm; column temperature: 35.2°C; injection volume: 10 µL).

Table 2: Final Chromatographic Conditions of the Method

HPLC column	ProntosilAD-3 150 × 4.6 mm, 3 µm
Mobile phase	*n*-Hexane/IPA/MeOH (88:10:1, v/v/v)
Temperature	35°C
Flow rate	0.9 mL/min
Wavelength	333 nm
Injection volume	10 µl
Afoxolaner	0.9 mg/mL

Validation of method

To ensure that method is suitable for the intended purpose key method characteristics, i.e., specificity, accuracy, linearity, precision, DL (detection limit) and QL (quantitation limit) were evaluated. Results of these evaluations are summarized in sections below. System suitability requirements [blank baseline: no interfering peaks above 0.1% level at the retention time of two Afoxolaner enantiomers, 0.2% standard solution have signal-to-noise ratio (S/N) ≥10 for each enantiomer; standard solution preparation agreement (≤2.0%), resolution factor (≥1.2), injector agreement (difference between two injections ≤2.0%)] were met prior to performing the method validation experiments.

Specificity the method was specific identified by the no significant interference peaks (peak area >0.1%) observed at the RT levels of the two enantiomers in the blank solution. Further there was no evidence of co-eluant was observed during the determination of the peak purity analysis (purity angle <purity threshold using HPLC Empower Software) for the two enantiomers.

DL/QL evaluation The DL and QL of method were estimated for the sample using the dilution Afoxolaner (racemate) solution as standard. The estimated DL (S/N~3) was found to be 0.06 µg/mL (0.02%) for each enantiomer. The QL was set to (0.2 µg/mL; 0.05%) as S/N for "E. 1" and "E. 2" was 21 and 15, respectively. These results demonstrate the method is sensitive for Afoxolaner enantiomers detection and simultaneous estimation.

Linearity/accuracy the linearity profile of the Afoxolaner racemate with its "E. 1" is treated as API and "E. 2" is treated as impurity was found using the standard protocol. The "E. 1" was treated as API, its linearity was evaluated from 0.1% to 125% of the nominal concentration of 0.4 mg/mL. the regression data analysis of the data set was calculated and the correlation coefficient (r) of 1.0 was determined. The y-intercept was found to be ≤0.5% /100% level resulting the note that there is no significant bias for quantitation in case of "E. 1". The method is considered accurate for "E. 1" because as per ICH Q2B method's precision, linearity and specificity were indicated by method validation. Further the "E. 2" (as impurity), linearity and recovery were studied by preparing a series of solution with concentrations [0.2%, 0.5%, 1.0%, 2.5% and 5.0%] in "E. 1" (0.4 mg/mL). The solutions prepared were spiked with E. 2 into "E. 1" (0.4 mg/mL) solution at calculated ratios. At each level, duplicate injections were performed.

A linear least square analysis of the data gave correlation coefficient (r) of 0.999. The y-intercept obtained was ≤3.0% of the 100% level indicating that there is no significant bias for quantitation for "E. 2". The method is proven to be linear within the investigational range. The recoveries of "E. 2" at each level were also calculated and the results ranged from 98% to 104% meeting the acceptance criteria of 70–130%. The %RSD of recovery from five levels in the linearity/recovery study was calculated to be 2% meeting the acceptance criteria of ≤10%. These results demonstrate the method is linear and accurate for "E. 1" (as API) and "E. 2" (as an impurity).

Precision

The method precision was evaluated by analyzing six solutions of E. 1 at 100% level (0.4 mg/mL). The enantiomer purity value was calculated for each was determined as an average. The average enantiomer purity was 99.6% and %RSD = 0.0% (n = 6) thus met the criteria of %RSD ≤2%. These results demonstrate that the method is precise for determination enantiomer purity of the API samples.

DISCUSSION

Column and mobile phase screening

Polysaccharide-based HPLC columns were selected for the method development. As polysaccharide-based CSPs lack ionic sites, they are suitable for chromatography of a neutral compound such as Afoxolaner [16,17]. In addition, polysaccharide-based columns have been demonstrated to be very useful in separation of a wide spectrum of racemate in pharmaceutical industry due to their versatility and durability. Normal chromatographic conditions were selected for the method. The hydrogen bonding, dipole–dipole and (π–π) interactions which are considered critical for chiral recognition are known to be more effective under normal phase conditions[14]. Among the large number of available polysaccharide-based columns, Prontosil® OD, Prontosil AD and Prontosil® OJ columns are most commonly used for pharmaceutical analysis; therefore, these were selected for the method development. A detection wavelength of 333 nm was selected for analysis since Afoxolaner has λ_{max} around this wavelength (Figure 6). There is selection of higher wavelength minimizes potential interference from common solvent/reagents contaminants. At the initial stage of method development 0.1 mg/mL Afoxolaner reference standard (a racemic mixture) solution was used. To increase sensitivity of the method, the concentration was increased to 0.9 mg/mL later during method development. The HPLC columns utilized were ProntosilAD-3 (150 × 4.6 mm, 3 µm particle size), Prontosil® OD-H (150 × 4.6 mm, 5 µm particle size) and Prontosil® OJ-3 columns (150 × 4.6 mm, 3 µm particle size) columns.

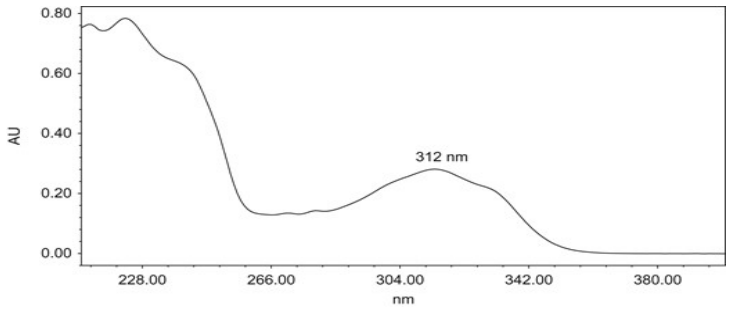

Figure 6: Typical UV spectrum of Afoxolaner in *n*-Hexane/IPA/MeOH (88:10:/2, v/v/v).

The isocratic mobile phase was preferred over the gradient was selected as the gradient phase was found to show slow equilibrium in the normal phase chromatography. *n*-Hexane / IPA were selected as non-polar and for the polar components respectively because these are most common solvents in normal phase chiral chromatography which are suitable for enantiomeric separation. Chromatographic analysis with *n*-Hexane/IPA (95:5, v/v) mobile phase and Prontosil® OD-3 column showed two broad peaks around 6 and 37minutes. With same mobile phase there was no separation of two enantiomers on Prontosil® OD-H column. Using this mobile phase combination, the best separation was achieved on ProntosilAD-3 column with a resolution factor (R_s) of 6.0 and selectivity (α)of 1.8 between the two enantiomers (Figure 1). However, the retention times of "E. 1" and "E. 2" peaks were ~17 and 28 minutes, respectively. To reduce analysis time, the mobile phase polarity was changed to *n*-Hexane/IPA (90:10,v/v). On ProntosilAD-3 column, the two enantiomer peaks eluted at 6 and 8 minutes, respectively with resolution of 5.6 (see Figure. On Chiral cel® OJ-3 column, two peaks eluted around 22 and 30 minutes, respectively with R_s of 1.6 and selectivity of 1.4. Both peaks showed peak tailing factor great than 1.5. No separation was achieved on Prontosil® OD-3 column under this mobile phase condition. As it has been reported in literature changing from one alcohol to another can affect chiral separation (including elution order), alcohols other than IPA were studied as well in combination with IPA. The other alcohols evaluated were methanol, ethanol, 1-butanol *n*-propanol, 2-butanol, *t*-butanol, 3-Methyl-1-butanol, Hexanol and cyclohexanol. These alcohols are different in properties like polarity, chain length and steric effects. The different butanols have multiple steric effect. The analysis was run on a Prontosil® AD-3 column with Afoxolaner standard solution (0.8 mg/mL) with *n*-Hexane/alcohol (95:5, v/v) as mobile phase. There was no clear separation achieved for other alcohols except 3-Methyl-1-Butanol. There is a selectivity factor for 3-Methyl-1-Butanol (α = 1.35) which was found to be much less than IPA (α = 1.89). Thus the IPA is the polar component of the mobile phase.

Effect of organic modifiers In normal phase chiral chromatography, there is routine used of organic modifiers [acids or alcohol] to improve column's HPLC selectivity. The addition of organic acid in the mobile phase is to minimize interactions with residuals silanols for better peak shape and resolution [10]. Also, as reported in literature [10] addition of small amounts of alcohols does affect column selectivity as well. Various alcohols were evaluated as organic modifier to the mobile phase. First, the *n*-Hexane/IPA (90:10, v/v) was modified to include 1% methanol. Addition of 1% methanol improved both resolution and selectivity compared to *n*-Hexane/IPA (90:10, v/v). There is a small amount of methanol alters the stearic environment around the chiral cavities thus favouring further differentiation of the two enantiomers. However, addition of more methanol (nearly 3%) did not improve resolution or selectivity (Table 3).

Table 3. Effect of MeOH as Organic Modifier in Mobile Phase using AD-3 Column

***n*-Hexane (%)**	**IPA (%)**	**MeOH (%)**	**RT (min)**		**Resolution (R_s)**	**Selectivity (α)**
			E. 1	**E. 2**		
90	10		6.1	8.3	5.9	1.39
88	10	2	6.3	9.0	8.2	1.54
87	10	3	5.6	7.4	7.0	1.25

(Afoxolaner conc: 0.1 mg/mL; flow rate: 0.9 mL/min; Wavelength: 333 nm; Column temperature: 35.2°C; Injection volume: 10 µL). Along with methanol, other alcohols were evaluated as the organic modifiers at 1% level. They are longer linear alcohols [ethanol, 1-butanol, hexanol], branched alcohols [*t*-butanol, cyclohexanol, diol (propylene glycol)].

Table 4 summarizes, methanol has the best selectivity. So based on the data *n*-Hexane/IPA/MeOH (88:10:2, v/v/v) was selected as the mobile phase for the final method.

Table 4: Effect of Various Alcohols as Organic Modifiers in Mobile Phase with AD-3 Column

***n*-Hexane (%)**	**IPA (%)**	**Organic modifier**	**RT (min)**		**Resolution (R_s)**	**Selectivity (α)**
			E. 1	**E. 2**		
88	10	1% MeOH	5.9	8.5	5.0	1.54
88	10	1% Ethanol	6.2	8.7	4.5	1.48
88	10	1% 1-Butanol	6.0	8.2	4.2	1.46
88	10	1% 2-Butanol	6.1	8.6	4.5	1.50
88	10	1% *t*-Butanol	6.3	9.0	4.5	1.50
88	10	1% Hexanol	6.0	8.3	4.1	1.45
88	10	1% Cyclohexanol	5.8	7.8	4.1	1.42
88	10	1% Propylene Glycol	5.8	8.0	4.3	1.47

(Afoxolaner concentration: 0.8 mg/mL; flow rate: 0.9 mL/min; Wavelength: 333 nm; Column temperature: 35°C; Injection volume: 10 µL).

Column temperature

The column temperature effect on the separation of the two enantiomers was studied at 25, 30, 35 and 40°C. At lower temperature (25°C) better separation was achieved (R_s: 6.6) compared to higher temperatures (35°C) (R_s: 5.0) and 40°C (R_s: 4.2). For rugged HPLC method, a well-controlled column temperature is crucial. Ideally, column temperature should be at least 10°C above or below the ambient temperature (~25°C) for better temperature control. Column temperature set around ambient is difficult to tightly control due to environmental fluctuations. Furthermore, 40°C is the highest recommended temperature for this type of column. Based on these rationales the column temperature of 35°C was selected for the final method.

Robustness study

The robustness of the method was demonstrated by showing the capacity of the method remained unaffected while deliberately changing HPLC parameters. Several key parameters, including %IPA in mobile phase, flow rate, detector wavelength, temperature and injection volume were varied around the procedural values. Resolution of the two enantiomers under each HPLC parameter variation was assessed against the procedural parameters. Except variation of %IPA in mobile phase, all other method robustness parameters were evaluated using Chrom-S-word Auto Robust, a computer software that tests method's robustness with minimal analyst intervention. Method robustness results are summarized in Table 5. In all varied conditions, the two enantiomers were well separated with a minimum resolution factor of 4 and runtime of <10 minutes. This shows method is robust as well as efficient and suitable for high throughput analysis in a QC laboratory.

Table 5: Method for Robustness Study

Parameters	**Variation**	**Retention time (min)**		**Resolution (R_s)**
		E. 1	**E. 2**	
Normal	None	6.2	8.6	4.4
Column Temperature	−2°C	6.3	8.8	4.7
	+2°C	6.1	8.3	4.2
Flow rate	−0.1 (mL/min)	7.1	9.8	4.5
	+0.1 (mL/min)	5.5	7.6	4.4
Injection volume	−2 µl	6.2	8.6	5.0
	+2 µl	6.2	8.6	4.0
Wavelength	−2 nm	6.3	8.8	4.7
	+2 nm	6.3	8.8	4.7
%IPA	*n*-Hexane /IPA/ MeOH(91:8:1,v/v/v)	6.8	9.3	4.5

Parameters	Variation	Retention time (min)		Resolution (R_s)
		E. 1	E. 2	
	n-Hexane/IPA/MeOH (87:12:1, v/v/v)	4.6	6.0	3.5

(Afoxolaner concentration: 0.9 mg/mL; Prontosil® AD-3 150 × 4.6 mm, 3 µm).

CONCLUSION

An isocratic RP- HPLC method for analysis of Afoxolaner in pharmaceutical dosage form has been developed and validated. The Best separation was achieved on a Prontosilc_{18}h(100×4.6 mm) 3µm column using a mobile phase of composition of pH 3 buffer: ACN: Methanol(30:40:30) at a flow rate of 1.5mL/min. UV detection was performed at 333 nm. The method was validated for specificity, linearity, precision, accuracy, robustness, ruggedness & solution stability according ICH guidelines. The calibration plot was linear over the concentration range10-60ppm with correlation coefficient 0.9997. The accuracy was good and consistent for 6 intervals. The data validation shows that the RP-HPLC method is accurate, robust and posses an excellent linearity and precision characteristics. This method can be successfully used for the quantization of Afoxolaner as active substance, in dissolution studies and in tablet dosage forms. This HPLC method is simple, linear, accurate, precise, robust, sensitive and reliable for identification of Aflonazer drug and its formulation. The method can is used to verify that Afoxolaner is a racemic mixture as demonstrated by specific rotation, as well as to determine enantiomeric purity of single enantiomer samples. The method is also considered QC friendly as it is robust, uses isocratic mobile phase with both enantiomers eluting in 10min, and employs commonly used solvents as mobile phase.The method is linear over a wide range which is economical with easy preparation of mobile phase which determines the method suitable for quantification of Afoxolaner in bulk drugs and in pharmaceutical dosage forms without interference. Hence the method is reliable and efficient, ecofriendly and affordable can be used a small lab scale with high accuracy and precision.

Abbreviations

HPLC- High performance liquid chromatography

RP-Reverse phase

E.1 – Enantiomer 1

E. 2-Enantiomer 2

Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES

1. Carithers, D., Crawford, J., de Vos, C. *et al.* Assessment of afoxolaner efficacy against *Otodectes cynotis* infestations of dogs. *Parasites Vectors* **9**, 635 (2016). https://doi.org/10.1186/s13071-016-1924-4
2. Nguyen, L.A., He, H., Chuong, P.H; Chiral drugs: an overview; *International Journal of Biomedical Science*, (2006);2: 85-100
3. FDA(1992) Development of New Stereoisomeric Drugs
4. Beugnet, F., Crafford, D., de Vos, C., Kok, D., Larsen, D., & Fourie, J. (2016). *Evaluation of the efficacy of monthly oral administration of afoxolaner plus milbemycin oxime (NexGard Spectra®, Merial) in the prevention of adult Spirocerca lupi establishment in experimentally infected dogs. Veterinary Parasitology, 226, 150–161.* doi:10.1016/j.vetpar.2016.07.002
5. Drag, M., Saik, J., Harriman, J. & Larsen, D. (2014) Safety of orally administered afoxolaner in 8-week-old dogs. *Veterinary Parasitology*, **201**, 198– 203.
6. Dumont, P., Gale, B., Chester, S.T. & Larsen, D.L. (2014b) Curative and preventive efficacy of orally administered afoxolaner against *Ctenocephalides canis* infestation in dogs. *Veterinary Parasitology*, **201**, 212– 215.
7. Kunkle, B., Daly, S., Dumont, P., Drag, M. & Larsen, D. (2014a) Assessment of the efficacy of orally administered afoxolaner against *Rhipicephalus sanguineus sensu* lato. *Veterinary Parasitology*, **201**, 226– 228.
8. Letendre, L., Harriman, J., Drag, M., Mullins, A., Maliniski, T. & Rehbein, S. (2017) The intravenous and oral pharmacokinetics of afoxolaner and milbemycin oxime when used as a combination chewable parasiticide for dogs. *Veterinary Pharmacology and Therapeutics*, **40**, 35– 43.
9. Mitchell, E.B., Dorr, P., Everett, W.R., Chester, T.S. & Larsen, D. (2014a) Efficacy of afoxolaner against *Dermacentor variabilis* ticks in dogs. *Veterinary Parasitology*, **201**, 220– 222.
10. Kunath, A., Theil, F., & Jähnisch, K. (1996). Influence of the kind of the alcoholic modifier on chiral separation on a Chiralpak AD column. Journal of Chromatography A, 728(1-2), 249–257. doi:10.1016/0021-9673(95)00891-8
11. Letendre, L., Huang, R., Kvaternick, V., Harriman, J., Drag, M., & Soll, M. (2014). *The intravenous and oral pharmacokinetics of afoxolaner used as a monthly chewable antiparasitic for dogs. Veterinary Parasitology, 201(3-4), 190–197.* doi:10.1016/j.vetpar.2014.02.021
12. Shoop, W. L., Hartline, E. J., Gould, B. R., Waddell, M. E., McDowell, R. G., Kinney, J. B., … Confalone, P. N. (2014). *Discovery and mode of action of afoxolaner, a new isoxazoline parasiticide for dogs. Veterinary Parasitology, 201(3-4), 179–189.* doi:10.1016/j.vetpar.2014.02.020
13. Jinyou Zhuang, Satish Kumar, Abu Rustum, Development and Validation of a Normal Phase Chiral HPLC Method for Analysis of Afoxolaner Using a ProntosilAD-3 Column, *Journal of Chromatographic Science*, Volume 54, Issue 10, 30 November 2016, Pages 1813–1819, https://doi.org/10.1093/chromsci/bmw162

14. Stringham, R. W., & Yun, K. Y. (2006). Chiral separation of amines by high-performance liquid chromatography using polysaccharide stationary phases and acidic additives. *Journal of Chromatography A*, *1101*(1-2), 86-93.
15. Padivitage, N., Adhikari, S., & Rustum, A. M. (2021). Assay of afoxolaner and determination of its related substances in commercial bulk batches of afoxolaner by reversed-phase HPLC method based on a short octadecyl column. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, *1184*, 122984. https://doi.org/10.1016/j.jchromb.2021.122984