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Development and Validation of A High Performance Liquid Chromatography (HPLC) Method for Determination of Fipronil and Pyriproxyfen in A Veterinary Pharmaceutical Product

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ABSTRACT
In the present study, an analytical HPLC method was developed for the estimation of Fipronil, Pyriproxyfen, Benzyl alcohol, Butylhydroxyanisole (BHA) and Butylhydroxytoluene (BHT) and related substances from a veterinary pharmaceutical product. The method showed linearity in the range of 0.44-200% with a correlation coefficient of 0.99. The method was validated for different validation parameters such as: specificity (peak identification and interference study), precision, accuracy, linearity, robustness, limit of detection (LOD) and limit of quantitation (LOQ), stability of standard solutions and sample solution. The results were found to be within the acceptance limits as per ICH guidelines.

Keywords: Fipronil, Pyriproxiyfen, Benzyl alcohol, BHA, BHT, HPLC, validation.

INTRODUCTION

Fipronil, chemically known as (*RS*)-5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfinyl)-1H-pyrazole-3-carbonitrile, is a substance that belongs to the phenylpyrazole family and is broadly used as an insecticide. It has a molecular structure as shown in Figure 1.

Pyriproxyfen, chemically known as 4-phenoxyphenyl(*RS*)-2-(2-pyridyloxy)-propylether-2-[1-(4-phenoxy- phenoxy)propan-2-yloxy]pyridine, is a pyridine-based pesticide which is effective on a variety of arachnoda. It has a chemical structure as shown in Figure 2.

Benzyl alcohol is a widely used as preservative/solvent to its low toxicity and its polarity. Benzyl alcohol is completely soluble in alcohol and diethyl ether and partially soluble in water.

Butylhydroxyanisole (BHA) is an antioxidant used as a food additive, also known as E320.

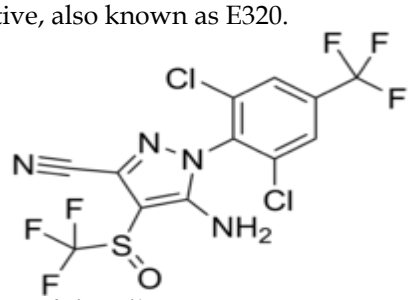

Fig. 1: Structure of Fipronil

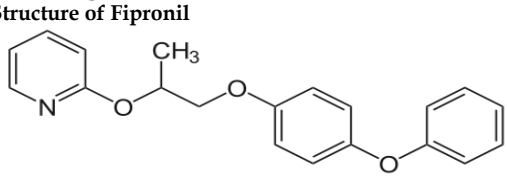

Fig. 2: **Structure of Pyriproxyfen**

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Butylhydroxytoluene (BHT) is a lipophile chemical derivative of phenol and is used as an antioxidant. BHT is widely used as a food additive (E321) in cosmetics, pharmaceuticals, rubber and embalming fluid for its antioxidant properties.

Several papers have been published proposing HPLC methods for the identification, assay and control for Fipronil using UV methods. [1-3] The published papers on HPLC methods for Pyriproxyfen were few [4] and this would be an alternative to those already published. However, there was no indicating high-performance liquid chromatography method for their estimation in European Pharmacopoeia, USP, and BP or in a pharmaceutical product, with excipients and in combination with another active substance. Hence the present work was aimed to develop and validate a HPLC method that would be able to estimate all of the above mentioned compounds at once. The method allows the estimation of the five compounds in a relatively short time, leading to an efficient use of time and resources.

The method was validated for accuracy, precision, specificity, robustness and quantitation limits according to ICH Guidelines. [5-11]

MATERIALS AND METHODS

Chemicals and reagents

Fipronil and Pyriproxyifen were supplied by Dr. Ehrenstorfer GmbH, BHA was supplied by Andres Pintaluba, BHT from Brenntag, Polska and Benzyl alcohol from INEOS chloroToluenes Ltd. Acetonitrile and distilled water, which were of HPLC grade, were also used. A pharmaceutical product solution was used as the test solution and Diethylene glycol monoethyl ether was used as the sample solvent.

Apparatus

Agilent 1260 Infinity series fitted with an inbuilt degasser, DAD detector 1260, autosampler and thermostat was used for developing the method. The column used was Zorbax SB C_{18}, 4.6×250mm, 5µm**.**

Chromatographic conditions

Chromatographic separation was achieved at 25°C on a reversed phase column using a mobile phase consisting of Acetonitrile (A) and Ultrapurified water (B). Gradient elution was performed slowly from 0 to 7 minutes 47-65% A, 7-12 minutes 65-75% A, 12-20 minutes 75% A, 20-22 minutes 75-47% A. The flow rate was kept at 2 ml/min and detection was performed at 278, 275, 254, 210 nm. The injection volume was 0.75µL in all HPLC runs.

Method Validation

The method validation parameters studied were selectivity/specificity, linearity (range of linearity, LOD, LOQ), precision (intra-day and inter-day), recovery, robustness and solution stability. [5-11]

Specificity

The specificity of the method was evaluated by force degradation of the samples. The samples were subjected to acid and alkaline-induced degradation, thermal degradation, hydrogen peroxide-induced degradation, UV and natural light exposure.

Detection limit and quantitation limit

LOQ was defined as the lowest concentration that could be determined with acceptable accuracy and precision. The LOD and LOQ were resulted from statistical calculations.

Linearity

The linearity of the method was determined by calibration curve. For the construction of the calibration curve, five calibration standard solutions were prepared for assay and five calibration standard solutions were prepared for purity and were injected three times.

Precision and repeatability

The precision and repeatability of the method were estimated by assaying six replicate samples on day-1 and day-2.

Robustness

The robustness of the method was evaluated by investigating the following operational parameters: length of chromatographic column, mobile phase composition, temperature of the chromatographic column, flow of mobile phase.

Preparation of Solutions

For active substance identification and assay: The reference solution for active substances identification and assay consisted of Fipronil 45000 ppm**,** Pyriproxifen 45000 ppm, Benzyl alcohol 40000 ppm, BHA 200 ppm and BHT 100 ppm. The sample solution consisted of undiluted finished pharmaceutical product.

For related substances: The reference solution for related substances consisted of Fipronil 135 ppm**,** Pyriproxyfen 135 ppm and for preservative Benzyl alcohol 120 ppm. The sample solution consisted of undiluted finished pharmaceutical product.

RESULTS AND DISCUSSION

Specificity: The proposed method showed that it can separate the chromatographic peaks of Fipronil, Pyriproxyfen, Benzyl alcohol, BHA and BHT from the peaks coresponding to the related substances. The method also showed that it can separate the peaks corresponding to the veterinary product's compounds from the chromatographic peaks of the degradation products of API's obtained after physical and chemical stress. Thus, the method is selective.

LOD and LOQ: Limit of Detection (LOD) and Limit of Quantitation (LOQ) were statistically determined. The results are presented in Table 1.

Linearity: The method showed to be linear in the range of 0.44-200%, with a correlation coefficient of 0.99.

Precision: In order to evaluate the method's capability to produce similar results on repetitive tests for nominal concentration, six individual samples from the Test Solution were tested separately. It showed a very low relative standard deviation for the samples. The results are shown in Table 4.

Table 1: Statistically determined LOD and LOQ of the five compounds in the assay solutions

	LOD (ppm)	LOQ (ppm)
Fipronil	6.94	23.15
Pyriproxyfen	7.16	23.87
Benzyl alcohol	9.09	30.31
BHA	13.78	45.94
BHT	5.99	19.97

Table 2: Statistically calculated parameters for all five compounds for the assay solutions

	Slope	Intercept	Correlation coefficient
Benzyl alcohol	128.724	192.7669	0.99
BHA	1.224	-2.5610	0.99
Fipronil	314.632	321.5840	0.99
Pyriproxifen	398.307	424.3133	0.99
BHT	0.280	-0.2153	0.99

Table 3: Statistically calculated parameters for the purity reference solution

	Slope	Intercept	Correlation coefficient
Benzyl alcohol	1.469	-5.6981	0.99
BHA	2.028	-8.2304	0.99
Fipronil	2.324	-6.6999	0.99
Pyriproxifen	2.324	-6.6999	0.99

Table 4: Precision results for the assay solutions

	Inter-day, %RSD	Intra-day, %RSD
Benzyl alcohol	0.46	0.8
BHA	1.21	1.7
Fipronil	0.32	0.5
Pyriproxyfen	0.28	0.6
BHT	1.2	1.9

Accuracy: The concentrations considered were 50%, 70%, 100% and 150%. The recovery ranged from 95-105 for Benzyl alcohol, 95-105 for BHA, 95-103 for Fipronil, 95-103 for Pyriproxyifen and 98-105 for BHT. Accuracy was evaluated then by recovery determination at different concentration levels. The concentrations used were 50%, 70%, 100% and 150%. The recovery ranged from 95-102 for Benzyl alcohol, 95-102 for Fipronil and 98-102 for Pyriproxyifen.

Robustness: Robustness was determined by analyzing the same standards at normal operating conditions and then slightly changing the length of chromatographic column, mobile phase composition, temperature of the chromatographic column and debit. The retention times of the five compounds were modified, but the separation was not affected by the changed chromatographic conditions.

The aim of this study was to develop a selective and sensitive HPLC method for the rapid detection of Fipronil, Pyriproxiyfen, assay of active substances, of preservative Benzyl Alcohol, antioxidants BHA, BHT and relative substances/degradation products from the pharmaceutical product in the same chromatographic conditions. The proposed method was found to be rapid, accurate, repeatable, specific and robust. This method was applied for the analysis of the drug product in marketed formulations and could be used for the routine analysis of the drug product.

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