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Research Article

METHOD DEVELOPMENT AND VALIDATION OF CLEANING PROCEDURE FOR FENBENDAZOLE RESIDUAL DETERMINATION IN MANUFACTURING

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ABSTRACT

Objectives: Cleaning validation is the methodology used to assure that a cleaning process removes residues of the active pharmaceutical ingredients of the product manufactured in a piece of equipment. All residues are removed to predetermined levels to ensure the quality of the next product. Today, manufactured is not compromised by waste from the previous product and the quality of future products using the equipment, to prevent cross contamination, and as a good manufacturing practices requirement. The main aim of the study was to develop and validate a new simple, precise, and accurate reverse phase high-performance liquid chromatographic (HPLC) method for fenbendazole (FBZF) residual determination in veterinary active pharmaceutical ingredient manufacturing.

Methods: The method was developed using the isocratic solvent system, HPLC grade acetonitrile, and Mill-Q water in the ratio of 60:40 (volume/volume), and 1% hydrochloric methanol is used as diluent. Successful elution of the FBZF was achieved on Waters Symmetry C-18 column with 250 mm×4.6 mm internal diameter and 5 µm particle size.

Results: The method validation was successfully applied for routine analysis for cleaning/residual samples. The developed reverse phase liquid chromatography method was validated with respect to specificity, linearity, accuracy, precision, and high sensitivity with detection limits and quantification limits ranging from 0.2 ppm to 15 ppm.

Conclusion: The present developed and validated method is run successfully for FBZF residual determination of cleaning samples in veterinary active pharmaceutical ingredient manufacturing.

Keywords: Fenbendazole, Residual determination, Reversed phase high-pressure liquid chromatography and cleaning validation.

INTRODUCTION

Fenbendazole (FBZF) (Fig. 1) is a veterinary anthelmintic (Wormer) compound belonging to the chemical class of the benzimidazoles. FBZF is one of generic anthelmintics most used on livestock worldwide. For livestock, it is available in the form of drenches, feed activates, bouls, tablets, pills, etc., and slow release devices, all for oral administration [1]. There is no classic injectable or pour-ones with FBZF, excepting some drenches that can are approved for intraruminal injection in some countries [2]. It is used mainly on veterinary parasites controlled gastrointestinal and respiratory roundworms (=nematodes) and tapeworms in sheep, goats, pig poultry, dogs, and cats. It has a broad spectrum of activity against gastrointestinal round worms and lung worms of livestock including adults and L4-larvae of the most important species (e.g., of the genus bunostomum, hemonchus, ostertagia teladorsagia, trichostrongylus, cooperia, nematodirus, chabertia, oesophagostomum, trichuris, dictyocaulus, muellerius, etc.) as well as arrested larvae of several species [3]. It is also effective against most livestock tapeworms and the major parasitic roundworms (e.g., ancylostoma, toxocara, trichuris, uncinaria). Based on recent studies, this is information there is acceptance of an active ingredient by the most influential regulatory bodies such as EMA, USFDA, and AUS for use on livestock [4].

Toxicity

Despite being widely used as a dewormer in many species, toxicity has been reported. Birds (storks, pink pelicans, vultures, pigeons, and doves) and reptiles (vipers, turtles, and tortoises) have shown toxicity associated with bone marrow suppression, intestinal crypt cell necrosis, and distal villi sloughing [1]. Abortions in domestic ruminants have been associated with concurrent use of anti-trematode therapeutic agents [2]. Never use products for livestock on dogs and cats, unless they are explicitly approved for both livestock and pets [3]. Pets may not tolerate livestock formulations. It is obvious that veterinary products are not intended for and should never be used on humans [4].

METHODS

Chemicals

Reference standard of FBZF and cleaning samples was obtained from well-reputed research laboratories and characterized by the use of liquid chromatography (LC)/mass spectrometry, nuclear magnetic resonance, and infrared. All reagents used were of analytical reagent grade unless stated otherwise. Mill-Q water and high-performance liquid chromatographic (HPLC) grade acetonitrile were purchased from Thermo Scientific (Qualigens) and Merck, Mumbai, India. The solutions and the mobile phase prepared were stored at room temperature. The LC system was equipped with quaternary gradient pumps with autosampler and column oven, autoinjector connected to a variable wavelength programable ultra violet-visible detector all were controlled by open lab software and manufactured by Agilent Technologies with model number 1200 series.

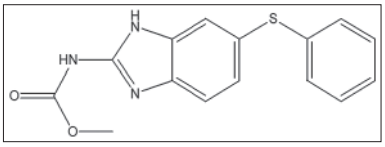

Fig. 1: Structure of fenbendazole

Selection of suitable mobile phase, diluent and wavelength

The mobile phase for the analysis of cleaning method validation for residual determination of FBZF was set by injecting different ratios of acetonitrile HPLC grade water, and 1% Hydrochloric methanol (1 ml concentrated HCl in 100 mL of methanol) is used as diluent. The selected mobile phase ratio was acetonitrile:HPLC grade water is 60:40 (ml/ml). Similarly, for the selection of diluent, we tried the standard into different solvents such as water, methanol, and mobile phase 1% hydrochloric methanol. Finally, the selected diluent was 1% hydrochloric methanol. Similarly, for the wavelength selection, we tried at different nanometers (nm) but at 254 nm it gives a higher response. The selected mobile phase, diluent, and wavelength have given a sharp peak with low tailing factor 1.0 (<2) [9].

Instrumentation and analytical chromatographic conditions

The chromatographic analysis of the cleaning method validation for residual determination of FBZF was carried out on Agilent high-pressure liquid chromatography Model-1200 series containing quaternary pump, variable wavelength programable ultra violet-visible detector, and autoinjector with up to 1-1000 µl loop, column oven modules. Chromatographic analysis was performed using Waters Symmetry C-18 column with 250 mm×4.6 mm internal diameter and 5 µm particle size. Sartorius electronic balance with model NumberCP-225D was used for weighing.

Isocratic elution with, acetonitrile, high-pressure liquid chromatography grade water 60:40 (ml/ml) was selected with a flow rate of 1.0 ml/minutes and injection volume 10 µl. The detection wavelength was set at 254 nm with a runtime of 10 minutes. The mobile phase was prepared freshly, and it was degassed by sonication for 5 minutes before use. The column was equilibrated for at least 10 minutes with the mobile phase flowing through the system. The column oven module and the high-pressure liquid chromatography system were kept at 30°C temperature [5-8].

Preparation of standard solutions

Pure standards of FBZF were used as external standards in the analysis. Different concentrations of the standards were used based on the range required to plot a suitable calibration curve. About 100 mg of the standard FBZF was accurately weighed and transferred into 100 ml volumetric flask and make up with sufficient diluent. The volumetric flask containing standard solution was sonicated for 10 minutes. Take 1 mL of above solution into the 100 mL volumetric flask and make up to the mark with diluent, and it is used as standard. Similarly, different concentrations of these standards were analyzed using the same chromatographic conditions, and a calibration curve was generated. The sample recorded chromatogram is in (Fig. 2).

Diluent

Used diluent (1% methanol hydrochloric acid solution) as blank.

DISCUSSION

Optimization of the chromatographic conditions

The nature of the sample, its molecular weight and solubility decide the proper selection of the stationary phase. The drug FBZF being non-polar is preferably analyzed by reverse phase columns and accordingly C18 column was selected. So, the elution of the compound from the column was influenced by polar mobile phase. The concentration of the acetonitrile and high-pressure liquid chromatography grade water was optimized to give symmetric peak with short runtime based on asymmetric factor and peak area obtained. Different mobile phases were tried but the selected mobile phase in the Waters Symmetry C-18 column with 250 mm×4.6 mm internal diameter, and 5 µm particle size has given a sharp peak with tailing factor 1.00 (<2) at retention time 4.693 minutes and the chromatographic run time is 10 minutes with the mobile phase acetonitrile, HPLC grade water 60:40 (ml/ml) [8]. The retention time of FBZF was found to be 4.693 minutes which indicates a good baseline. The % of relative standard deviation (RSD) values for accuracy and precision studies obtained were less than 2 (2%) which revealed that developed method was accurate and precise. The system suitability and validation parameters are given in (Table 1). The high percentage of recovery of FBZF was found to be 90 (90.22%) indicating that the proposed method is highly accurate. Proposed liquid chromatographic method was applied for analysis of cleaning method validation for residual determination of FBZF [5-8].

Method validation procedure

After the completion of high-pressure liquid chromatography method development, the objective of the method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in International Conference on Harmonization (ICH) guidelines. The method was validated for system suitability, precision, specificity, linearity, limit of detection (LOD) and limit of quantification (LOQ), and recovery [9].

Table 1: System suitability and precision parameters

Injection number	Area of FBZF 10 ppm standard solution	Retention time	Tailing factor
1	5914939	4.740	1.00
2	5895555	4.730	0.96
3	5859784	4.717	1.00
4	5839105	4.707	1.03
5	5841227	4.700	1.03
6	5831793	4.693	1.05
Average	5863734	4.715	1.01
Standard deviation	34010.32413	0.0	NA
% RSD	0.58%	0.38	NA
Acceptance criteria	NMT 5.0%	NMT 1.0%	Tailing <2

RSD: Relative standard deviation

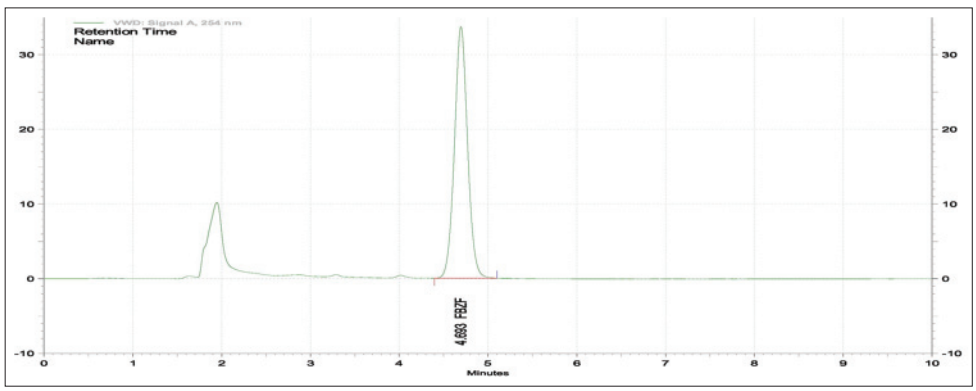

Fig. 2: 10 ppm standard chromatogram

System suitability and precision parameter

To verify that analytical system is working properly and can give accurate and precise results, the system suitability and precision parameters are to be set. System suitability and precision tests were carried out on freshly prepared 10 ppm standard solutions of FBZF, and it was calculated by determining the SD of FBZF standards by injecting standards in six replicates at 10 minutes interval. The values of %RSD prove that the method is accurate and precise, and acceptance criteria are not more than 5 (5.0%) for absorbance response, not more than 1 (1.0%) for retention time. The values were recorded in (Table 1).

FBZF 10 ppm standard preparation

Weigh about 50.13 mg of FBZF standard into a 50 mL volumetric flask dissolve and diluted volume with diluent. Take 1 mL of above solution into the 100 mL volumetric flask and make up to the mark with diluent.

From the Table 1, it can be concluded that the system suitability and precision parameters meet the requirements of method validation.

Specificity parameter

Specificity is the ability of the analytical method to assess the analyte in the presence of components that may be expects to be present such as impurities, degradation products, and matrix components.

Specificity tests were carried out on above prepared 10 ppm standard solution of FBZF, and it was determining by injecting blank, blank with swab stick, and specify solution (standard solution) for FBZF material at 10 ppm standard solution.

From the Table 2, Fig. 3 proves that method is specific that is there is no interference of blank peaks in FBZF standard solution.

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional to the concentration of analyte in the sample within a given range [9].

The developed method has been validated as per ICH guidelines; the standard solutions of FBZF in the mass concentration range of 0.2 ppm to 15 ppm were injected into the chromatographic system [9]. The chromatograms were developed, and the peak area was determined for each concentration of the drug solution. Calibration curve of FBZF was obtained by plotting the peak area ratio versus the applied concentrations of FBZF. The linear correlation coefficient was found to be 1.0 (0.999). The values and calibration curve were recorded in Table 4 and Fig. 4.

Preparation of FBZF stock solution and linearity solutions

Weighed 100.12 mg of working standard into 100 mL volumetric flask dissolved and diluted up to the mark with diluent. Preparation of different levels of concentrations.

From the Table 4, it is clear that the area response of FBZF versus concentration in ppm of FBZF is linear in the range of interest. The correlation coefficient and regression coefficient calculated from the regular plot are >0.999. Hence, the method is linear for the residual determination of FBZF.

LOD and LOQ

LOD is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions [9].

LOQ is the lowest amount of analyte in a sample that can be quantitated with acceptable precision, under the stated experimental conditions.

Table 2: Specificity parameters

Peak name	RT
Blank	No peaks
Blank with swab stick	No peaks
10 ppm standard solution	4.753

RT: Retention time

Table 3: Linearity different levels of concentrations

Concentration in ppm	Stock solution to be added	Volume make up to
0.2	0.02	100
0.5	0.05	100
1.0	0.10	100
3.0	0.30	100
5.0	0.50	100
8.00	0.80	100
10.0	1.00	100
13.0	1.30	100
15.0	1.50	100

Table 4: Linearity parameters

Trial number	Actual concentration (ppm)	Area response
1	0.2	223073
2	0.5	343692
3	1.0	838731
4	3.0	1775030
5	5.0	2894229
6	8.0	4705246
7	10.0	5954186
8	13.0	7505160
9	15.0	8575858
Slope		567172223.3
Correlation coefficient		0.999681792
Regression coefficient		0.999363686

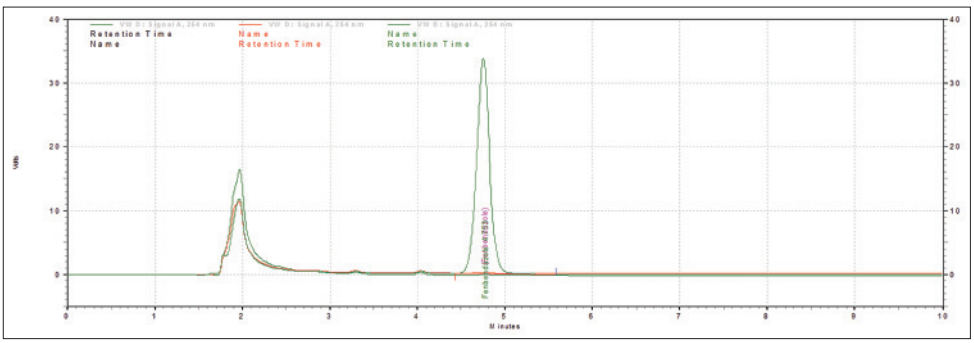

Fig. 3: Over laid chromatogram for specificity

Limit of detection (LOD)=(3.3×Residual standard deviation)/slope [9].

Limit of quantification (LOQ)=(10×Residual standard deviation)/slope [9].

Acceptance criteria
The % of RSD for area response of FBZF six replicates at LOQ level should be NMT 10 (10.0%).

Performed a regression analysis of the linearity data with concentration versus ppm on X-axis. Calculated the residual SD of the Y data. Calculated the slope of the linearity curve generated with concentration on X-axis and area response on Y-axis from Tables 4 and 5, Fig. 5.

Preparation of LOD solution
About 0.06 mL of FBZF stock solution taken into 100 mL volumetric flask and diluted up to the mark with diluent. Injected in triplicate. The LOD Experimental results are recorded in Table 7.

Preparation of LOQ solution
About 0.2 m of FBZF stock solution taken into 100 ml volumetric flask and diluted up to the mark with diluent. Injected in six replicates. The LOQ experimental results are recorded in Table 8.

LOQ solution precision
From the Table 8 precision results, it can be concluded that the cleaning method validation is precise at LOQ at concentration 2.0 ppm and LOD at concentration 0.6 ppm level.

Recovery study (or) accuracy
To study of the reliability, suitability, and accuracy of the method recovery experiments were carried out for cleaning method validation for residual determination of FBZF are broadly classified into two stages [9]:

1. Rinse method
2. Swab method.

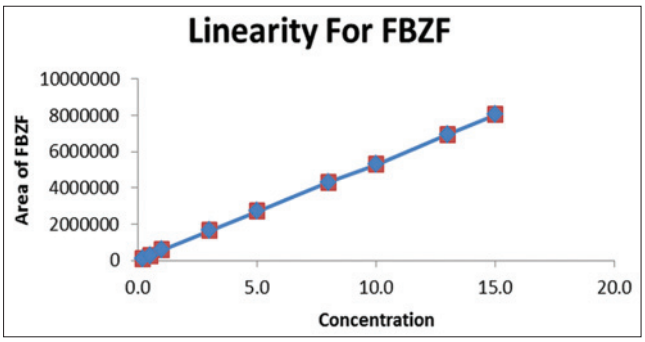

Fig. 4: Calibration curve for linearity

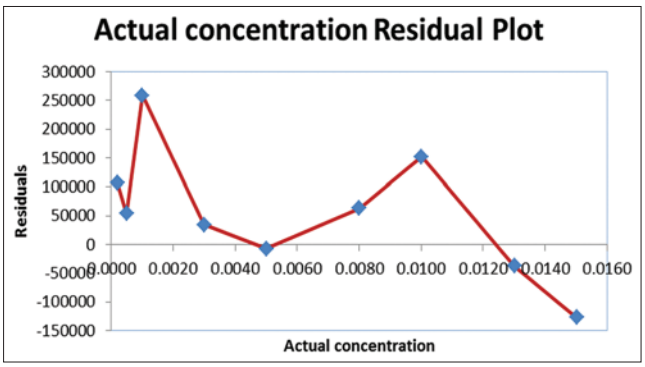

Fig. 5: Residual plot for linearity parameters

Rinse recovery
The rinse recovery of the sampling method shall be established by spiking a solution of known concentration on both stainless surface and glass plate. Recovery the spiked sample from the surface by rinsing the surface with the sampling agent [9].

Preparation of rinsed spiking solution
Weighed about 100.25 mg of the test sample and transferred into 100 mL volumetric flask. Dissolved and dilute up to the mark with diluent. Mixed well. Take 10 mL of the above solution into 100 mL volumetric flask. Dissolved and dilute up to the mark with diluent. Mixed well.

Rinse recovery study on stainless plate
Selected three cleaned and dried 10 cm×10 cm surface area stainless steel plates. Spread 10 mL of the spiking solution on dried 10 cm×10 cm surface area steel plates, taking utmost care to avoid any spillage. Dry the plate at room temperature. Using 100 mL of accurately measured diluent recover the test sample from 10 cm×10 cm surface area stainless steel plate, by gentle swirling. Filter and inject into high-pressure liquid chromatography. Performed the exercise in triplicate.

Rinse recovery study on glass plate
Select three cleaned and dried 10 cm×10 cm surface area glass plate. Spread 10 mL of the spiking solution on dried 10 cm×10 cm surface

Table 5: Residual output for linearity parameters

Residual output		
Observation	**Predicted Y**	**Residuals**
1	116044.4992	107028.5008
2	290111.248	53580.75204
3	580222.4959	258508.5041
4	1740667.488	34362.51226
5	2901112.48	−6883.479563
6	4641779.967	63466.0327
7	5802224.959	151961.0409
8	7542892.447	−37732.44686
9	8703337.439	−127479.4387

Table 6: Limit of detection and Limit of quantification Theoretical Results

Fenbendazole	
Theoretical LOD in mg/mL	0.6 ppm
Theoretical LOQ in mg/mL	2.0 ppm

LOD: Limit of detection, LOQ: Limit of quantification

Table 7: Limit of detection experimental results

Trial	Area response
1	387254
2	385337
3	385759

Table 8: Limit of quantification experimental results

Injection number	Area response
1	1190378
2	1187660
3	1190200
4	1185441
5	1184204
6	1183182
% RSD	0.26
Acceptance criteria	NMT 10.0%

RSD: Relative standard deviation

area glass plate, taking utmost care to avoid any spillage. Dry the plate at room temperature. Using 100 mL of accurately measured diluent recover the test sample from 10 cm×10 cm surface area glass plate, by gentle swirling. Filtered and inject into high-pressure liquid chromatography. Performed the exercise in triplicate. Finally recorded the area of the test sample in the rinse recovery on stainless plate and glass plate in Table 9.

Swab recovery

The swab recovery of the sampling method shall be established by spiking a solution of known concentration on stainless steel surface. Recover the spiked sample from the surface by swabbing the surface using swab stick with the sampling agent [9].

Preparation of swab spiking solution

Weighed about 100.67 mg of test sample taken into 100 mL volumetric flask dissolved and diluted with diluent. Further, 10 mL of this solution diluted to 100 mL with diluent. Take 10 mL of the above solution into 100 mL volumetric flask. Dissolved and dilute up to the mark with diluent. Mixed well.

Swab recovery study on stainless plate

Select three cleaned and dried 10 cm×10cm surface area glass plates. Spread 10 mL of the spiking solution on dried 10 cm×10 cm surface glass plates, taking utmost care to avoid any spillage. Dry the plate at room temperature.

Using 100 mL of accurately measured diluent recover the test sample from 10 cm×10 cm surface area glass plate, by gentle swirling. Filtered and inject into high-pressure liquid chromatography. Performed the exercise in triplicate. Finally recorded the area of the test sample in swab recovery on stainless plate and glass plate in Table 10 than calculate the % rinse recovery, % swab recovery below formula.

From the Table 10 results, it can be concluded that % of rinse and % of swab recovery on SS plate and the glass plate is consistently above 80.0 (80.0%). The values obtained above are in good agreement in terms reliability, suitability, and accuracy of the proposed method.

Solution stability and mobile phase stability

To determine the stability of sample solution, the mobile phase 10 ppm standard and cleaning sample solutions of FBZF were prepared and analyzed immediately after preparation and after different time intervals up to 24 hrs, while maintaining the sample cooler temperature at about 30 (30°C). The results from these studies indicated the standard and sample solution was stable at room temperature for at least 24 hrs. The sampled chromatograms are recorded as below in Figs. 6-11 [9].

Record of analysis for FBZF cleaning samples

Triplicate FBZF cleaning samples are run successfully using this method, and the experimental results and chromatograms are recorded in Figs. 12-16 [9].

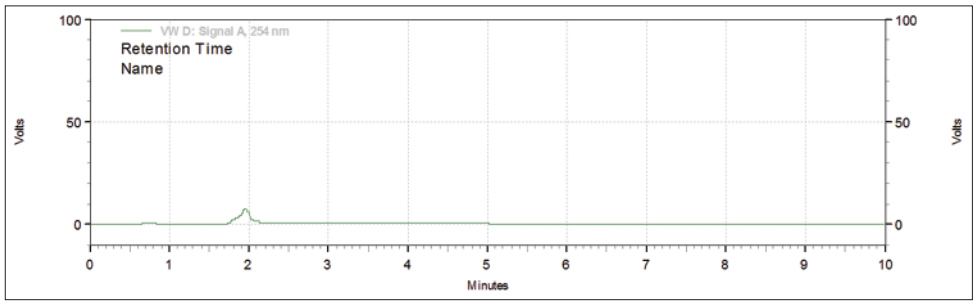

Fig. 6: Blank solution immediately prepared

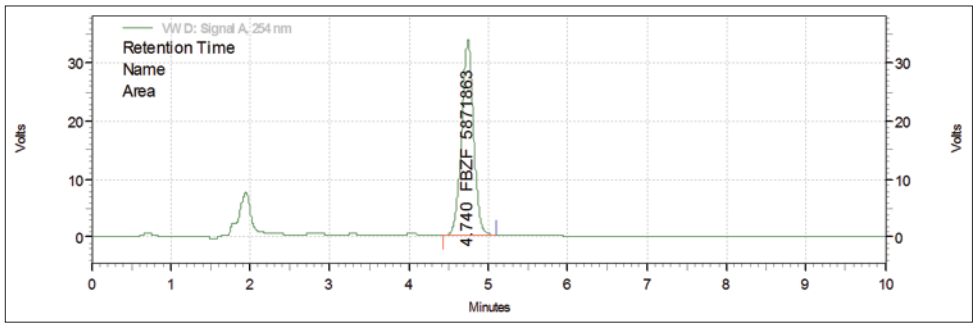

Fig. 7: Standard solution immediately prepared

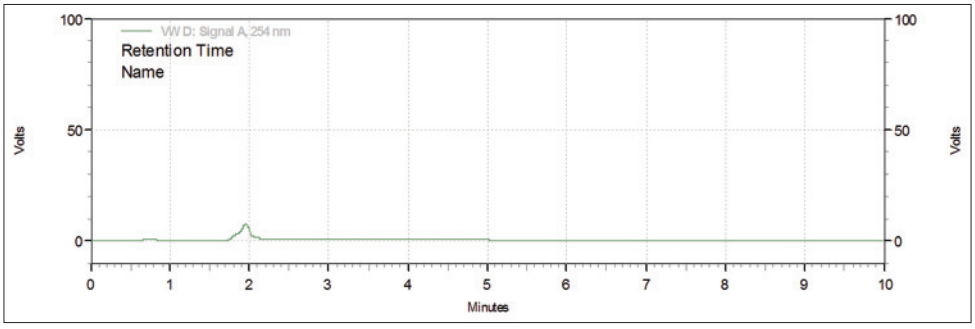

Fig. 8: Sample solution immediately prepared

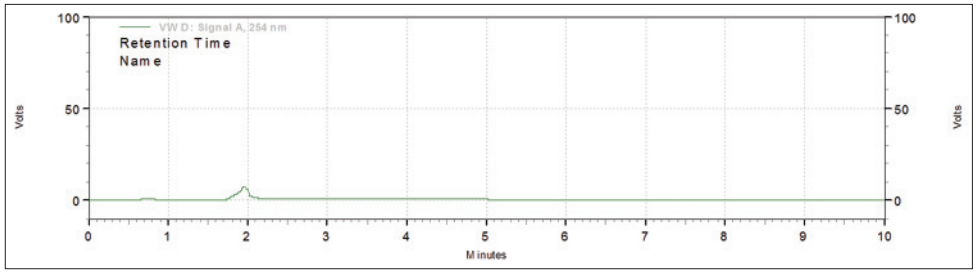

Fig. 9: Blank after 24 hrs injected chromatogram

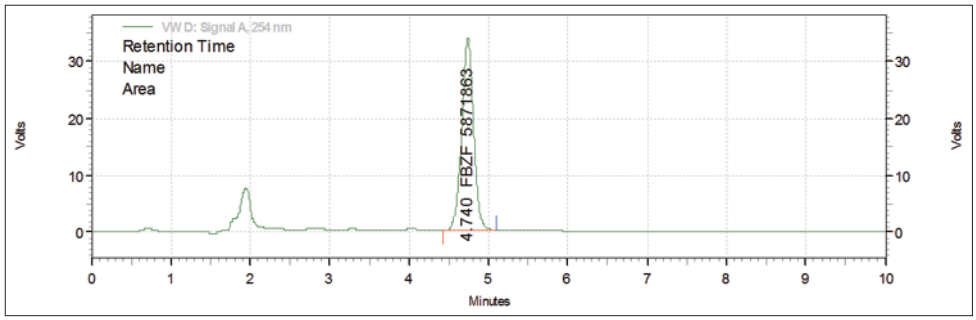

Fig. 10: Standard after 24 hrs

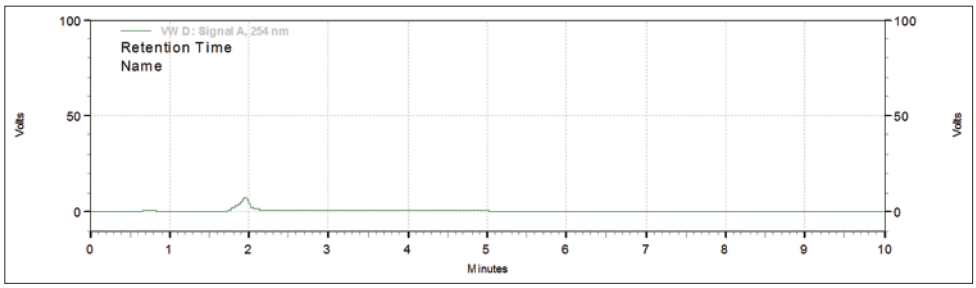

Fig. 11: Sample After 24hours

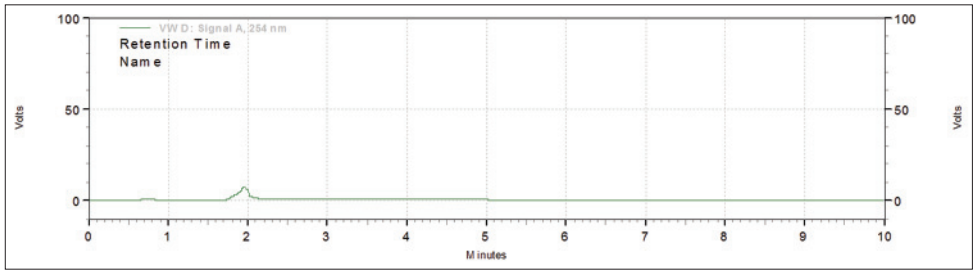

Fig. 12: Blank

Table 9: % Rinse recovery results

% Rinse recovery					
Serial number	**Type**	**% Recovery**	**Mean % recovery**	**SD**	**% RSD**
1	SS plate	86.90	86.89	0.03	0.04
2		86.92			
3		86.86			
4	Glass plate	87.20	87.00	0.20	0.22
5		86.81			
6		86.98			

RSD: Relative standard deviation, SD: Standard deviation

Table 10: % Swab recovery results

% Swab recovery					
Serial number	**Type**	**% Recovery**	**Mean % recovery**	**SD**	**% RSD**
1	SS plate	90.52	90.22	0.26	0.29
2		90.09			
3		90.04			
4	Glass plate	89.69	89.79	0.09	0.10
5		89.85			
6		89.84			

RSD: Relative standard deviation, SD: Standard deviation

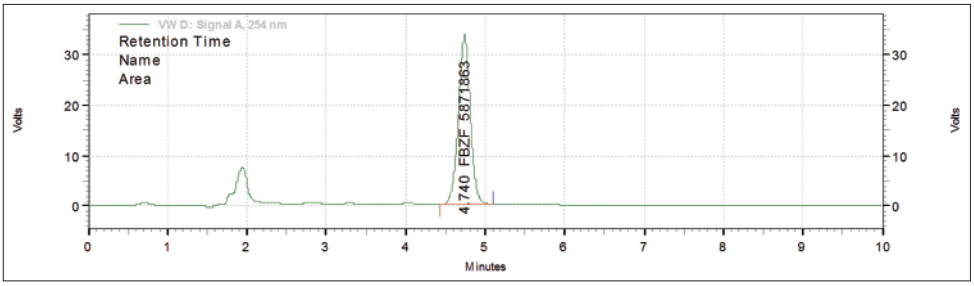

Fig. 13: 10 ppm standard chromatogram

Table 11: Summary and evaluation of results

Validation parameter	Acceptance criteria	Results
System suitability	The % of RSD for the area response of FBZF peak obtained from six replicate injections of system suitability should be NMT 5.0%	System suitability parameter meets the criteria % of RSD=0.58%
Specificity	The peaks of blank should not interfere with FBZF peak	The peaks of blank do not interfere with FBZF peak
Linearity	The correlation coefficient and the regression coefficient between concentration and area response of FBZF should be NLT 0.995	The method is linear Correlation coefficient=0.99968 Regression coefficient=0.99936
LOD/LOQ	The RSD for area response of FBZF from six replicates at LOQ level should be NMT 10.0%	The RSD for area response of FBZF from six replicates at LOQ level=0.26%
Recovery study	Report the % rinse recovery if the % rinse recovery is less than 80.0% then incorporate the recovery factor to the analytical method	
Recovery study	Report the % swab recovery if the % swab recovery is less than 80.0% then incorporate the recovery factor to the analytical method	

Individual solutions

Peak name	Retention time (in minutes)
Blank	No peak
Blank with swab stick	No peak
System suitability solution	4.753

	FBZF (FBZF/01 A)
LOQ in mg/mL	0.0020 mg/mL (2.0 ppm)
LOD in mg/mL	0.0006 mg/mL (0.6 ppm)

% Rinse recovery

Type	% Recovery	Mean % recovery	SD	% RSD
SS plates	86.90 86.92 86.86	86.89	0.03	0.04
Glass plates	87.20 86.81 86.98	87.00	0.20	0.22

% Swab recovery

Type	% Recovery	Mean % recovery	SD	% RSD
SS plates	90.52 90.09 90.04	90.22	0.26	0.29
Glass plates	89.69 89.85 89.84	89.79	0.09	0.10

RSD: Relative standard deviation, SD: Standard deviation, LOD: Limit of detection, LOQ: Limit of quantification, FBZF: Fenbendazole

From the above chromatograms or figures observes that there is the absence of FBZF content in triplicate bulk cleaning samples. Hence, proved this method is applicable for FBZF bulk cleaning samples.

RESULTS

As there is a growing demand of FBZF in veterinary active pharmaceutical ingredient market, it is required to develop fast, cost effective, stable, precise, and sensitive analytical cleaning method. The primary target in developing and validate this cleaning RP-HPLC method is to achieve the optimum resolution between products with other products to supply high purity of the drug, i.e., All residues are removed to predetermined levels to ensure the quality of the next product and also to prevent cross contamination and as a good manufacturing practices requirement. Based on the above-observed results, the developed cleaning method validation for FBZF method is valid and run successfully the summary and evaluation of results are in below Table 11.

CONCLUSION

A validated reversed phase high-pressure liquid chromatography cleaning method has been developed for cleaning method validation for

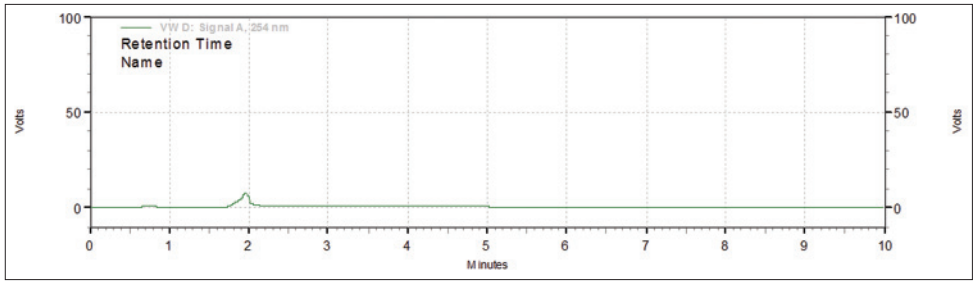

Fig. 14: 1st sample chromatogram

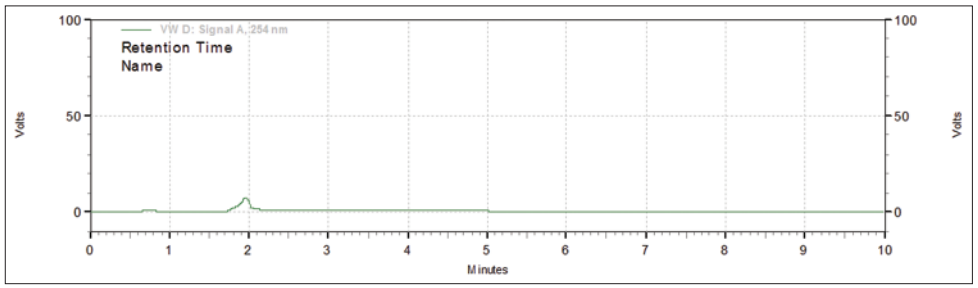

Fig. 15: 2nd sample chromatogram

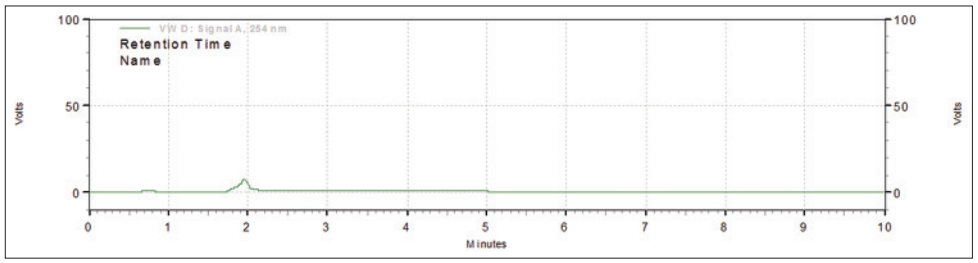

Fig. 16: 3rd sample chromatogram

residual determination of FBZF cleaning samples. The proposed method is simple, rapid, accurate, precise, and specific. Its chromatographic run time of 10 minutes allow the analysis of a large number of cleaning samples in a short period. Therefore, it is suitable for the routine analysis of FBZF cleaning samples in veterinary active pharmaceutical ingredients bulk drugs.

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