International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.9, No.05 pp 507-520, 2016

RP-HPLC Method Development and Cleaning Method Validation for the Analysis of Triclabendazole in Veterinary Pharmaceutical Dosage Forms

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Abstract : A new simple, selective, linear, precise and accurate RP-HPLC Cleaning method was developed and validated for rapid for the residual determination of triclabendazole by RP-HPLC in veterinary active pharmaceutical ingredients bulk drugs was developed and validated. Isocratic elution at a flow rate of 1.5ml per minute was employed on a waters symmetry C18 (250x4.6)mm, 5µmat 30ºc temperature. The mobile phase consisted of Acetonitrile: Water 70:30 (v/v). The UV detection wavelength was at 254nm.Linearity was observed in concentration range of 0.2-15ppm. The retention time for triclabendazole was 4.933 min. The method was validated as per the ICH guidelines. The proposed method can be successfully applied for routine analysis in bulk cleaning samples of triclabendazole in veterinary active pharmaceutical Ingredients.
Key words: Triclabendazole,Validation, Residual determination, RP-HPLC.

Introduction :

Triclabendazole **(MF:$C_{14}H_9Cl_3N_2OS$) (Fig. 1)** is a Verterinary Anthelmintic(Wormer) compound belonging to the chemical class of the benzimidazoles.The benzimidazole drugs share a common molecular structure, triclabendazole being the exception in having a chlorinated benzene ring but no carbamate group. Benzimidazoles such as triclabendazole are generally accepted to bind to beta-tubulin and prevent the polymerization of the microtubules of which they are part. Triclabendazole was initially only developed as an oral route drug, and displays high efficacy against both immature and adult liver flukes. Since late 1990s, triclabendazole became available as a generic drug, as patents expired in many countries. Many products were developed then. Among them, Trivantel 15, a 15% triclabendazole suspension, was launched by Agrovet Market Animal Health in the early 2000s. In 2009, the first triclabendazole injectable solution (combined with ivermectin) was developed and launched, also by Agrovet Market Animal Health. The product, Fasiject Plus, a triclabendazole 36% and ivermectin 0.6% solution, is designed to treat infections by *Fasciola hepatica* (both immature and adult liver flukes), roundworms and ectoparasites.It is abundantly used on sheep, goats and cattle, mostly in drenches, seldom in the form of tablets, boluses, etc.It is not used on pig, poultry, dos or cats.It is often used in mixtures, together with a broad-spectrum nematicide (e.g. abamectin, albendazole, Triclabendazole, ivermectin, levamisole) to add efficacy against roundworms.Never use products for livestock on dogs and cats, unless they are explicitly approved for both livestock and pets. Pets may not tolerate livestock formulations.[1-10]

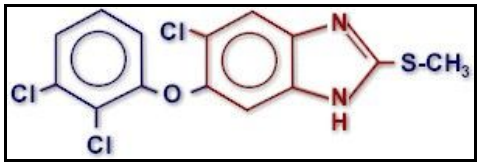


Fig. 1: Structure of Triclabendazole

IUPAC NAME :5-Chloro-6-(2,3-dichlorophenoxy)-2-(methylthio)-1H-benzimidazole **MF: $C_{14}H_9Cl_3N_2OS$**

Toxicity :

Despite being widely used as a dewormer in many species, toxicity has been reported. Triclabendazole is highly toxic to fish and moderately toxic to birds.Not being used in crop pesticides or in public hygiene, knowledge on its environmental fate and impact is very scarce. Nevertheless, it can be assumed that correctly used in livestock triclabendazole is unlikely to be detrimental for the environment, including coprophagous insects. Triclabendazole is not used in dogs or cats.Triclabendazole is used in human medicines. Triclabendazole is not used in crop pesticides.Triclabendazole is not used in public or domestic hygiene as a biocide. Click here for technical and commercial information on triclabendazole.[11-15]

Acute Toxicity and Tolerance of Triclabendazole

LD50 acute, rats, p.o.>5000 mg/kg,LD50 acute, rats, dermal >4000 mg/kg,LD50 acute, rabbits, p.o. 206 mg/kg. Safety margin Cattle: ~15,Sheep: ~20Therapeutic margin: 10 to 20.Cattle and sheep tolerate up to 100 mg/kg without toxic symptoms. In sheep, single doses >100 mg/kg caused loss of appetite and slightly increased blood levels of urea and alpha-globulins. A single dose of 200 mg/kg caused loss of appetite, transient weight loss and slight motor disturbances. At 50, 100 and 200 mg/kg a slight weight increase of the liver was recorded. As a general rule, sheep, goats and cattle tolerate triclabendazole very well[15-18]

Experimental

Chemicals and Reagents

All HPLC SOLVENTS used like Acetonitrile, ammonium acetate which are of HPLC grade were purchased from Thermo scientific (Qualigens) & E.Merck, Mumbai, India. Working standard of Triclabendazole was obtained from well reputed research laboratories.

Cleaning method for the residual determination :

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(Like Recator).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 GMP requirement .Now a days the regulatory authorties of all countries like U.S.Food and Drug Administration (FDA) strict regulation about the Clening Validation procedures.

Instrumentation and analytical conditions

Selection of suitable mobile phase

The mobile phase for the analysis of Cleaning Method Validation for Residual Determination of TRICLABENDAZOLEwas set by injecting different ratio'sof Acetonitrile (Make-MERCK. SF8SF80771), HPLC Grade Water (Make-MERCK) and Methanol is used as diluent. The selected mobile phase ratio wasAcetontrile: HPLC Water is 70:30(v/v) .The selected mobile phase has given a sharp peak with low tailing factor(1.03) i.e. <2.[20.21]

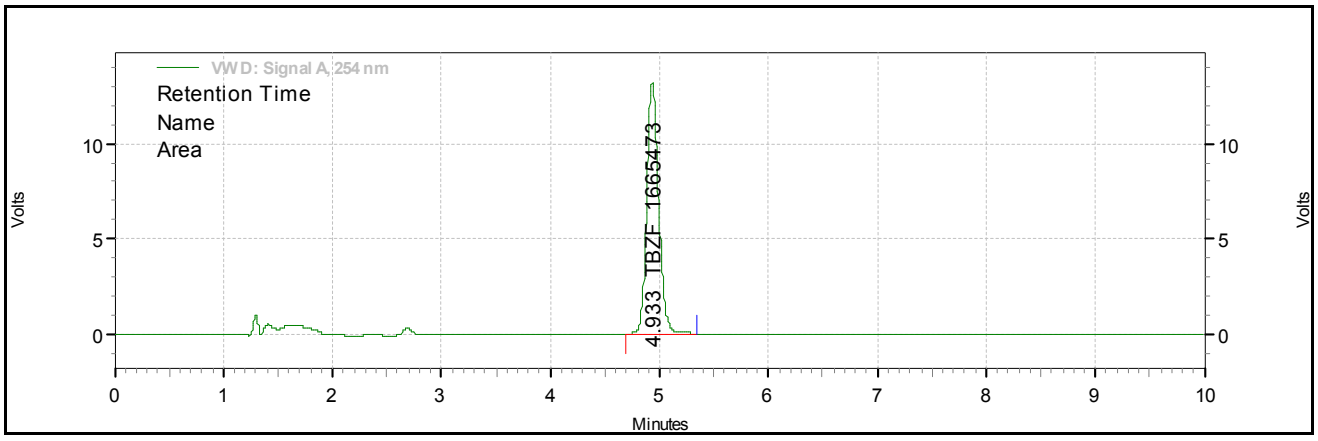


Fig. 2: Standard Chromatogram

The analysis of the drug was carried out on Agilent HPLC Model -1200 series containing Quarternary pump, variable wave length programmable UV/visible detector and Auto injector with up to 1µl- 1000µl loop, Column oven Modules.Chromatographic analysis was performed using Waters symmetry C18 (250x4.6)mm, 5µmat 30ºc temperature. Sartorius electronic balance (CP-225D) was used for weighing. Isocratic elution with ,Acetonitrile,HPLC Grade water 70:30 (v/v) was selected with a flow rate of 1.5 ml per minute.The detection wavelength was set at 254 nm with a runtime of 10 min. The mobile phase was prepared freshly and it was degassed by sonicating for 5 min before use. The column was equilibrated for at least 30min with the mobile phase flowing through the system. The column and the HPLC system were kept at 30ºc temperature. Preparation of Stock, working standard solutions and Sample solutions .100mg of TRICLABENDAZOLE was weighed and transferred (working standard) into a 100ml volumetric flask. The diluent methanol was added and sonicated to dissolve it completely and made up to the mark with the same solvent. Further 1ml of the above stock solution was pipetted into a 100ml volumetric flask and diluted up to the mark with diluent. The contents were mixed well and filtered through Ultipor N66 Nylon 6, 6 membrane sample filter paper. The calibration curve was plotted with the concentrations of the 15 to 0.2 ppm working standard solutions. Calibration solutions were prepared and analyzed immediately after preparation.

Method Validation procedure

After the completion of HPLC 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 ICH guidelines. The method was validated for System suitability, Precision, Specificity, Linearity, Limit of detection and Limit of quantification,Recovery.[19,23-31]

System Suitability& Precision Parameter

To verify that analytical system is working properly and can give accurate and precise results, the system suitability & Precision parameters are to be set.System suitability &Precision tests were carried out on freshly prepared 10 ppm standard solutions of Triclabendazole and it was calculated by determining the standard deviation of Triclabendazole standards by injecting standards in six replicates at 10 minutes interval .The values of % RSD prove that the method is accurae&precise and acceptance criteria is not more than 5.0% for absorbance response ,not more than 1.0% for retention time.The values were recorded inTable1.

10 ppm standard preparation:Weigh about 50.13 mg of Triclabendazolestandard into a 50mL 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.

Table1: System suitability &Precision parameters

Injection No.:	Area	Retention time	Tailing Factor
1	1665473	4.993	1.18
2	1663443	4.993	1.17
3	1672495	4.933	1.17
4	1663714	4.933	1.18
5	1655816	4.937	1.16
6	1654722	4.933	1.18
Average	1662730	4.715	1.17
Standard deviation	6644.111	0.0	0.008
% RSD	0.40	0.38	NA
Acceptance criteria	NMT 5.0%	NMT1.0%	NMT 2.0%

From the above tabulated data, it can be concluded that the system suitability & Precision parameters meets the requirements of method validation.

SpecificityParameter

Specificity is the ability of 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 Triclabendazole and it was determining by injecting blank, blank with swab stick and specify solution (standard solution) for Triclabendazole material at 0.01 mg/mL.

Table2:Specificity parameters

Peak name	RT
Blank	No peaks
Blank with swab stick	No peaks
Standard solution	4.993

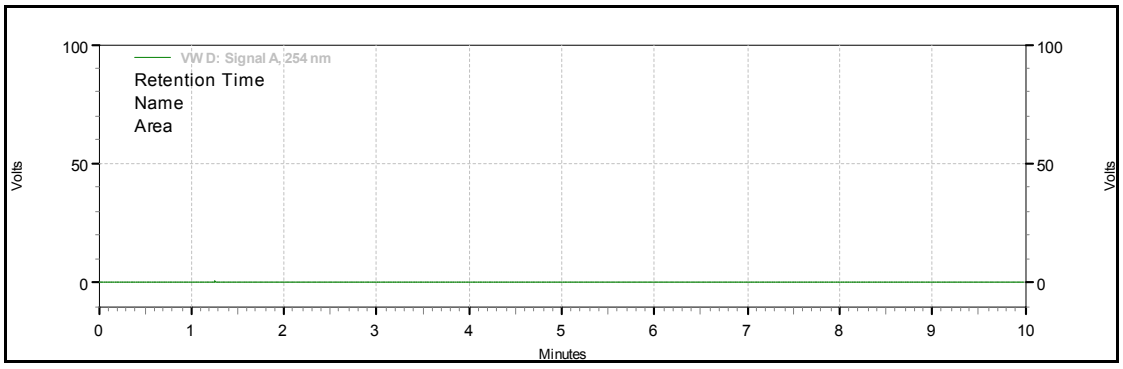


Fig. 3: Blank With Swab Stick

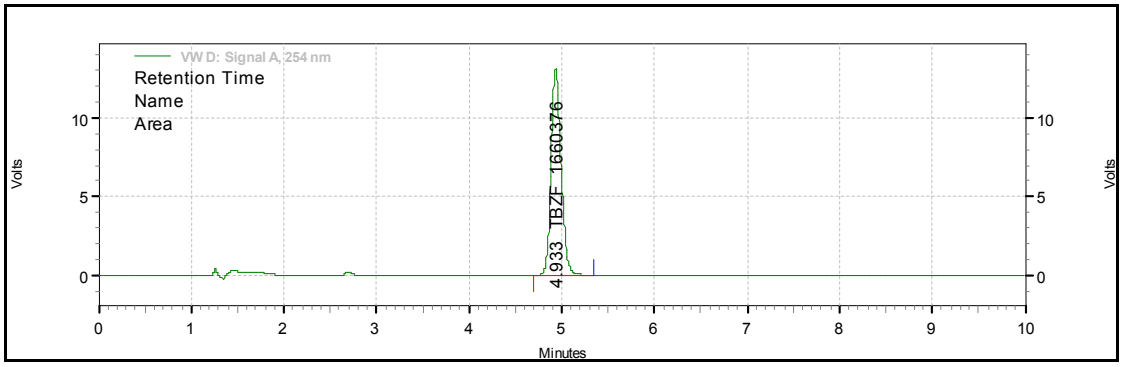


Fig. 4: 10ppm Standard Chromatogram

From the above data Table2, Proves that method is specific tahatis there is no interference of blank peaks in TriclabendazoleStandard 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 sample within a given range.

The developed method has been validated as per ICH guidelines the Standard solutions of Triclabendazolein the mass concentration range of 0.2 ppm to 15 ppm was injected into the chromatographic system. The chromatograms were developed and the peak area was determined for each concentration of the drug solution. Calibration curve of Triclabendazolewas obtained by plotting the peak area ratio versus the applied concentrations ofTriclabendazole. The linear correlation coefficient was found to be 0.999.The Values & Calibration curve were recorded in**Table3**&**Fig. 5,6,7,8,9,10,11,12&13,14**.

Preparation of TriclabendazoleStock solution &Linearity Solutions:

Weighed 100.24mg of working standard into 100 mL volumetric flask dissolved and diluted up to the mark with diluent.Preparation of different levels of concentrations.

Table 3: Linearity different levels of concentration

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

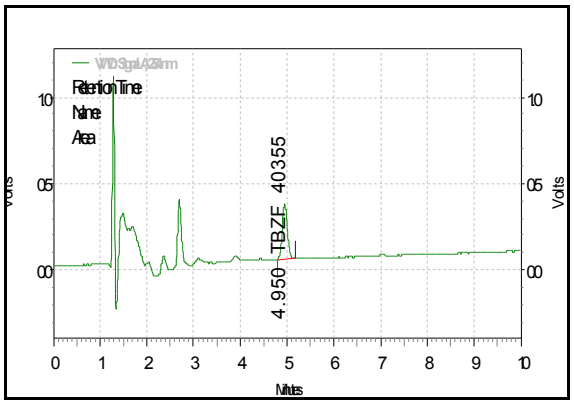


Fig. 05: 0.2PPM Standarad

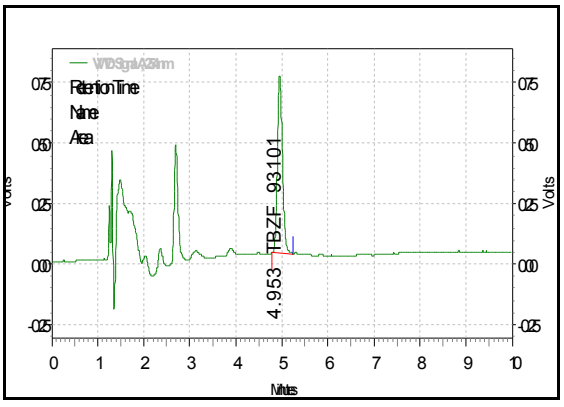


Fig. 06:0.5PPM Standarad

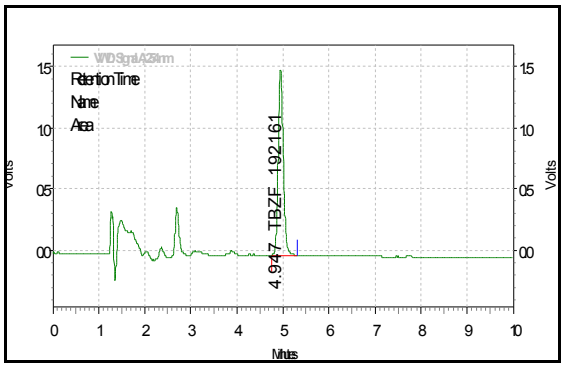


Fig. 07:1.0 PPM Standard

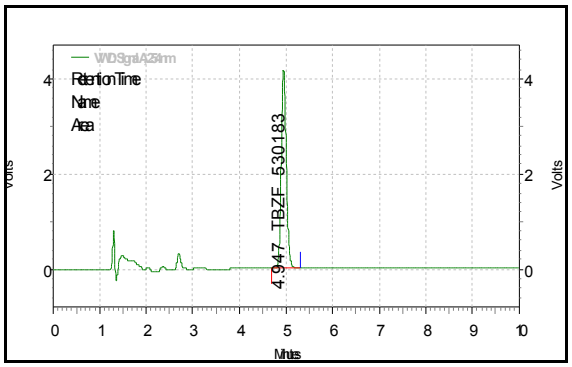


Fig. 08:3.0 PPM Standard

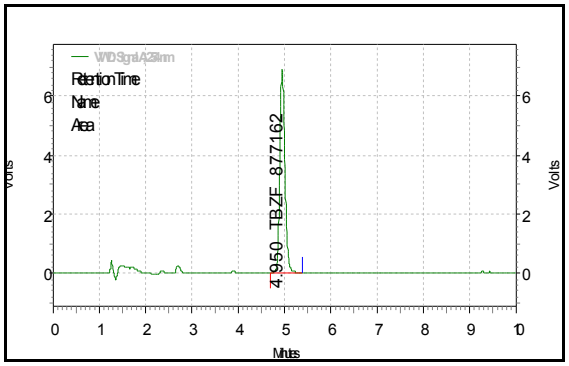


Fig. 09:5.0 PPM Standard

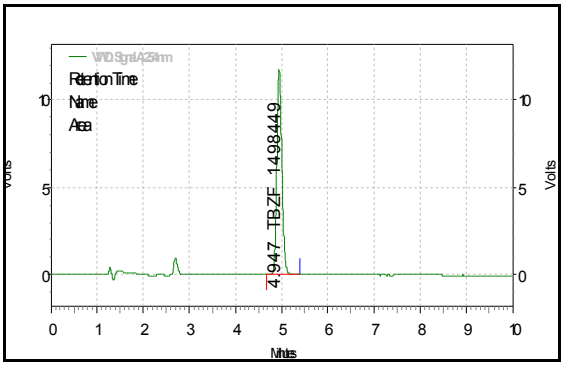


Fig. 10:8.0 PPM Standard

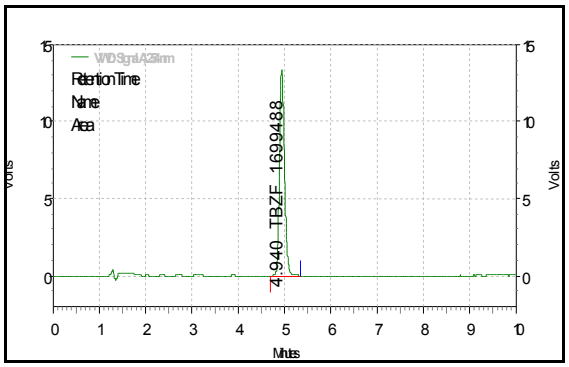


Fig. 11:10.0PPM Standard

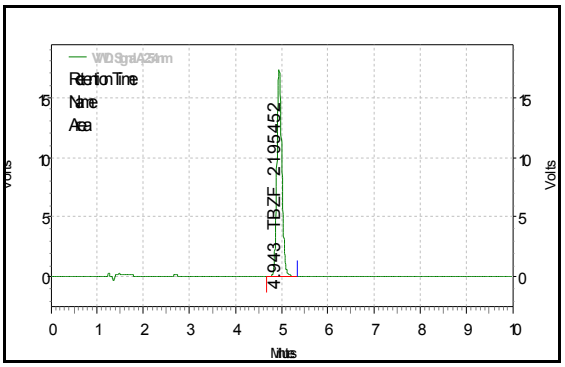


Fig. 12:13.0 PPM Standard

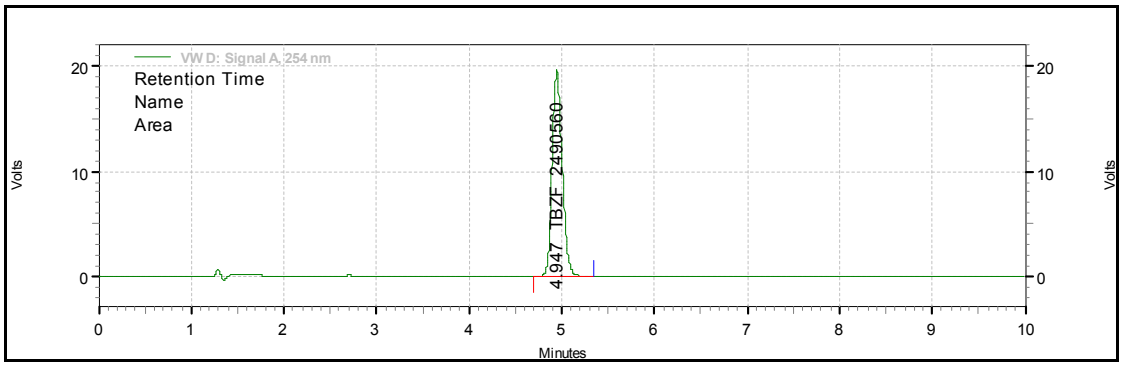


Fig. 13:15.0 PPM Standard

Table 4: Linearity parameter

Trial number	Actual concentration(ppm)	Area response
1	0.2	40355
2	0.5	93101
3	1.0	192161
4	3.0	530183
5	5.0	877162
6	8.0	1498449
7	10.0	1699488
8	13.0	2195452
9	15.0	2490560
Slope		166904742.1
Correlation coefficient		0.999288
Regression Coefficient		0.998576

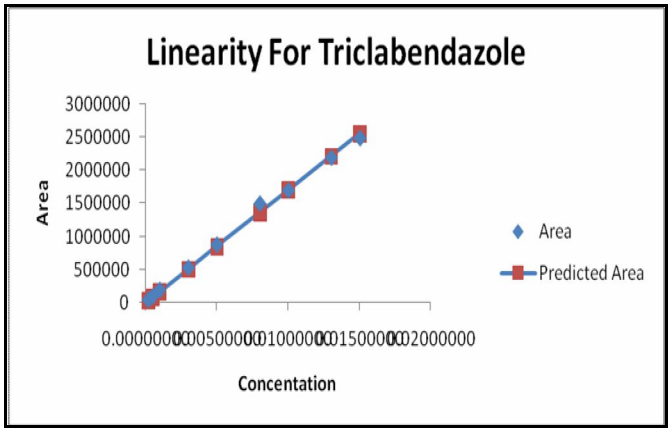


Fig. 14: Calibration curve for Linearity

Table 5: Residual output for Linearity parameters

RESIDUAL OUTPUT		
Observation	**Predicted Y**	**Residuals**
1	34082.78902	6272.210976
2	85206.97256	7894.02744
3	170413.9451	21747.05488
4	511241.8354	18941.16464
5	852069.7256	25092.2744
6	1363311.561	135137.439
7	1704139.451	-4651.451196
8	2215381.287	-19929.28655
9	2556209.177	-65649.17679

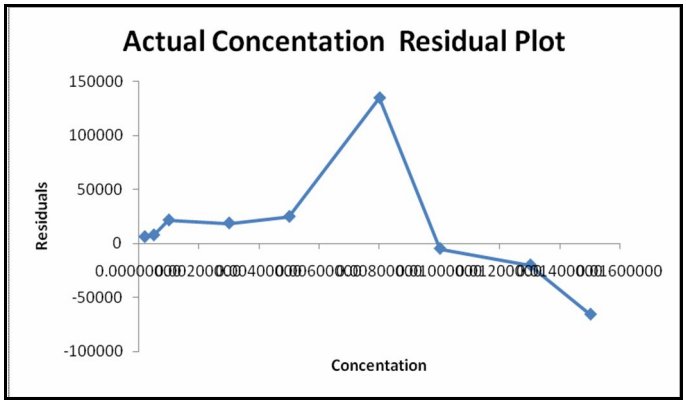


Fig. 15 : Residual Plot for Linearity parameters

From the above data, it is clear that the area response vs concentration in mg/mL of Triclabendazole is linear in the range of interest. The correlation coefficient and regression coefficient calculated from regular plot is greater than 0.999.Hence the method is linear for the residual determination of Triclabendazole.

Limit of Detection & Limit of Quantification:

Limit of detection is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions.

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

LOD = (3.3 X Residual standard deviation) / slope.
LOQ=(10X Residual standard deviation) / slope .

Acceptance criteria:

The % RSD for area response of Triclabendazole six replicates at LOQ level should be NMT 10.0%.

Performed a regression analysis of the linearity data with concentration vs mg/mL on X-axis.Calculated the residual standard deviation of the Y data. Calculated the slope of the linearity curve generated with concentration on X-axis and area response on Y-axis from Table3,Table4 & Fig. 4

Calculate the LOD/ LOQ as follows:

Table6: LOD&LOQ Theoretical Results

Triclabendazole	
Theoretical LOD in mg/mL	1.1ppm
Theoretical LOQ in mg/mL	3.2ppm

Preparation of LOD Solution:

0.11mL of Triclabendazole stock solution taken into 100 mL volumetric flask and diluted up to the mark with diluent. Injected in triplicate. The LODExperimentalResults are recorded in **Table6 & Fig.16, 17 &18**.

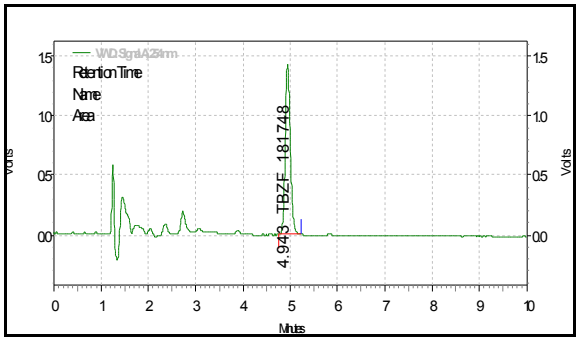


Fig. 16: LOD 1.1 ppm Solution-1

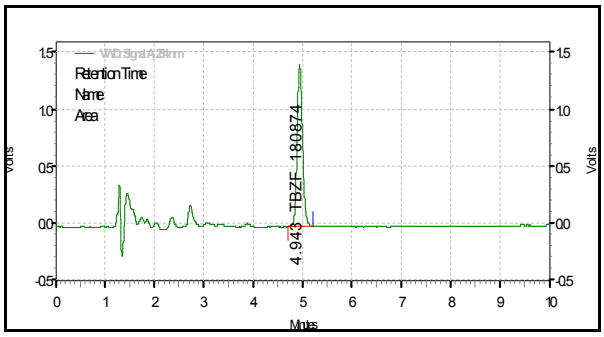


Fig. 17: LOD 1.1 ppm Solution-2

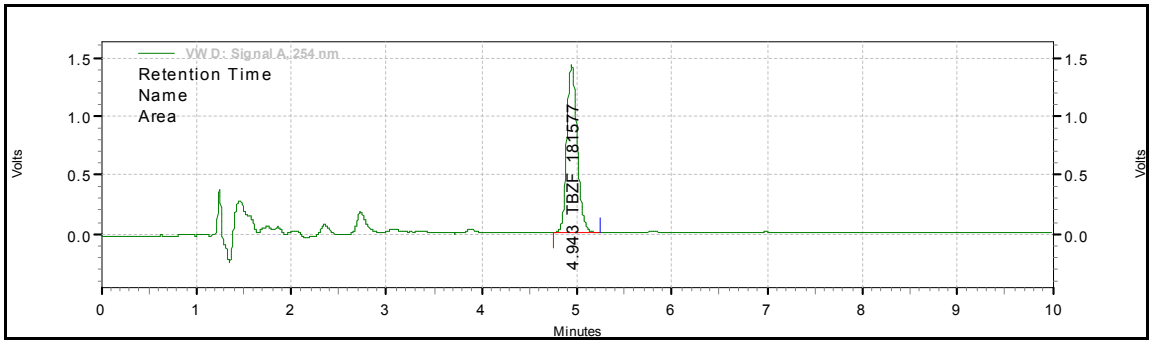


Fig. 18: LOD 1.1ppm Solution-3

Table7: LOD Experimental Results

Trial	Area response	S/N Ratio
1	181748	3.1
2	180874	3.2
3	181577	3.0

Preparation of LOQ Solution:

0.32 mL of Triclabendazole 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 **Table7** & **Fig.19, 20, 21, 22, 23 & 24**.

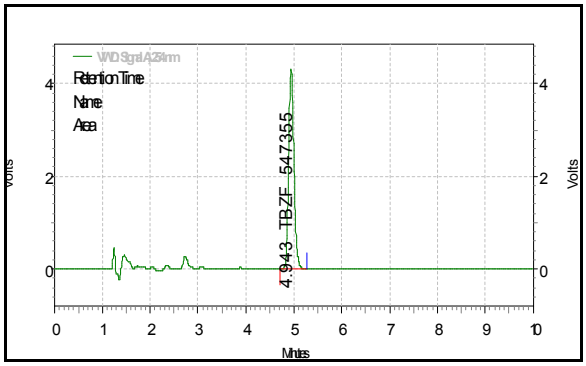


Fig. 19: LOQ 3.2 ppm Solution-1

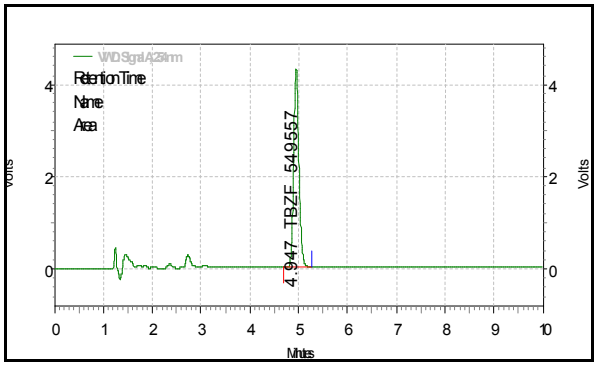


Fig. 20: LOQ 3.2 ppm Solution-2

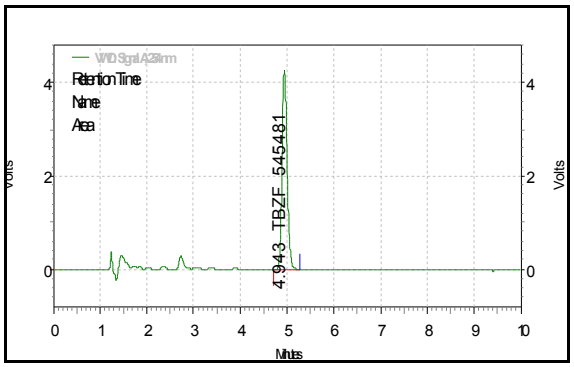


Fig. 21: LOQ 3.2 ppm Solution-3

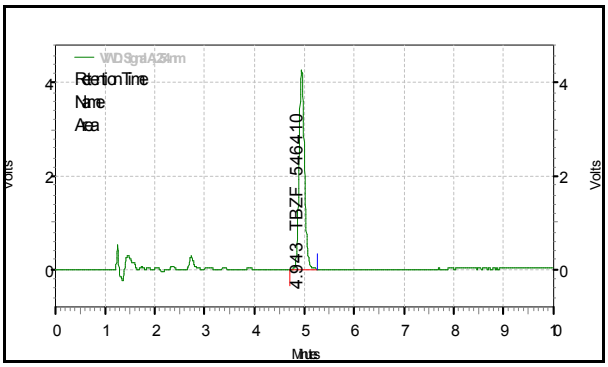


Fig. 22: LOQ 3.2 ppm Solution-4

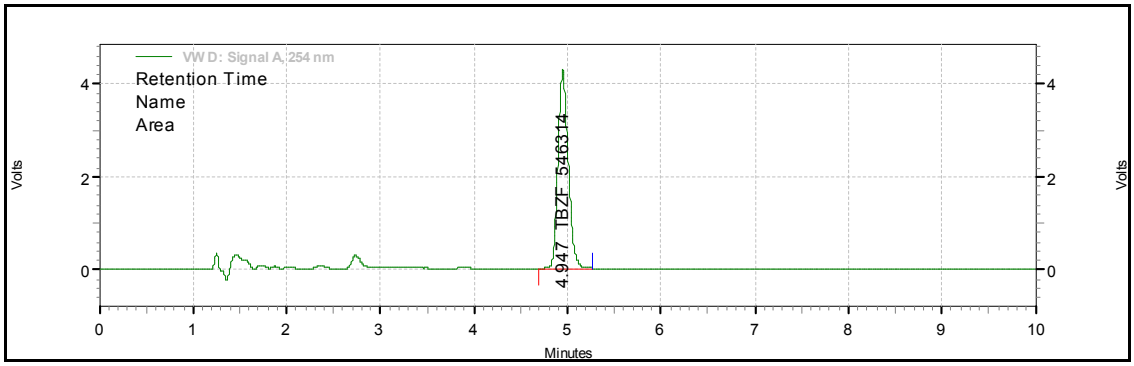


Fig. 23: LOQ 3.2 ppm Solution-5

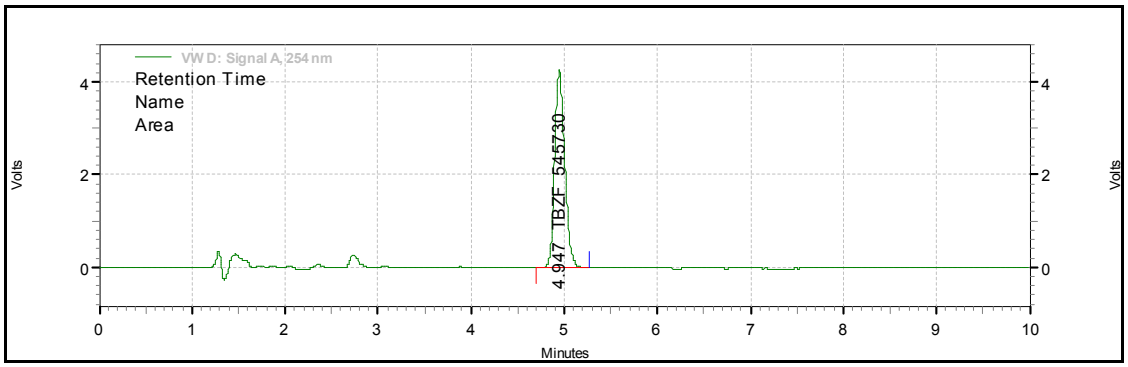


Fig. 24: LOQ 3.2 ppm Solution-6

Table8: LOD Experimental Results

LOQ Solution precision:

Injection number	Area response	S/N Ratio
1	547355	9.8
2	549557	9.7
3	545481	9.6
4	546410	9.8
5	546314	9.9
6	545730	10.0
% RSD	0.27%	10.0
Acceptance criteria	NMT 10.0%	9.8

From the above precision results it can be concluded that the cleaning method validation is precise at LOQ concentration (3.2 ppm) & LOD concentration (1.1ppm) level.

Recovery study:

To study of the reliability, suitability and accuracy of the method recovery experiments were carried out for Cleaning Method Validation for Residual Determination of Triclabendazoleare broadly classified into two stages.

1) Swab method 2) Rinse method.

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.

Preparation of spiking solutions :

Preparation of Rinsed spiking solution :

Weighed about100.38mg 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. Dissolve and dilute up to the mark with diluent, Mix well.

Rinse recovery study on stainless plate:

Select three cleaned and dried 10 x 10 cm surface area stainless steel plates. Spread 10 mL of spiking solution on dried 10 x 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 x 10 cm surface area stainless steel plate, by gentle swirling. Filter and inject into HPLC. Perform the excersie in triplicate.

Rinse recovery study on glass plate:

Select three cleaned and dried 10 x 10 cm surface area glass plate. Spread 10 mL of spiking solution on dried 10 x 10 cm surface 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 x 10 cm surface area glass plate, by gentle swirling. Filter and inject into HPLC. Perform the exercise in triplicate.

Table 9: % Rinse recovery Results

% Rinse recovery					
S. No.:	**Type**	**% Recovery**	**Mean % Recovery**	**SD**	**% RSD**
1	SS Plate	87.08	87.43%	0.88	1.00%
2		88.43			
3		86.79			
4	Glass plate	88.57	88.21%	1.09	1.24%
5		86.98			
6		89.07			

Finally Recorded the area of test sample in the Rinse recovery on stainless plate & 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.

Preparation of Swab spiking solution :

Weighed about100.59mg 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. Dissolve and dilute up to the mark with diluent. Mix well.

Swab recovery study on stainless plate:

Select three cleaned and dried 10 x 10 cm surface area glasplates. Spread 10 mL of spiking solution on dried 10 x 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 x 10 cm surface area glass plate, by gentle swirling. Filter and inject into HPLC. Perform the exercise in triplicate.

Finally Recorded the area of test sample in Swab recovery on stainless plate & glass plate in **Table 10.**

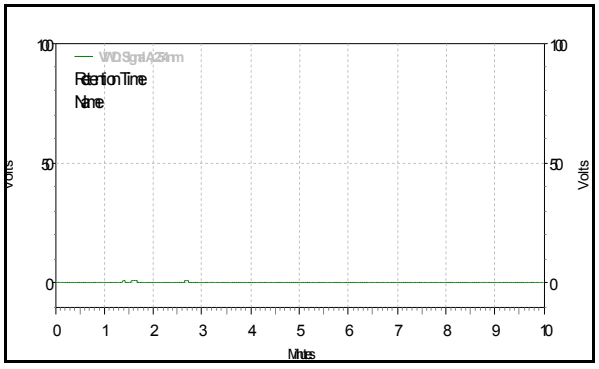
Table10: % Swab recovery Results

% Swab recovery					
S. No.:	**Type**	**% Recovery**	**Mean % Recovery**	**SD**	**% RSD**
1	SS Plate	89.22	89.21%	0.08	0.09%
2		89.29			
3		89.13			
4	Glass plate	89.16	89.23%	0.30	0.34%
5		88.97			
6		89.56			

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

Record of analysis For Triclabendazole cleaning samples:

A triplicate Triclabendazolecleaning samples are run Succesfully by using this method and The Experimental Results& Chromatograms are recorded in **Figs.25,26 ,27,28& 29**.



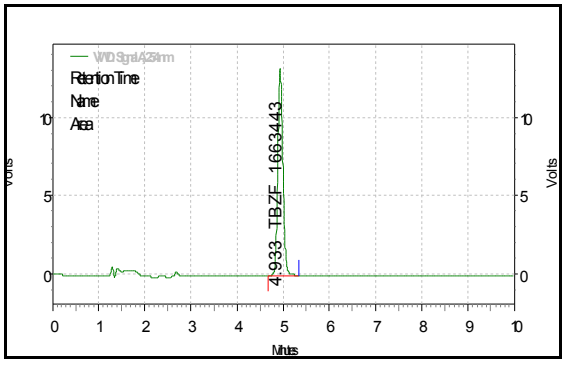
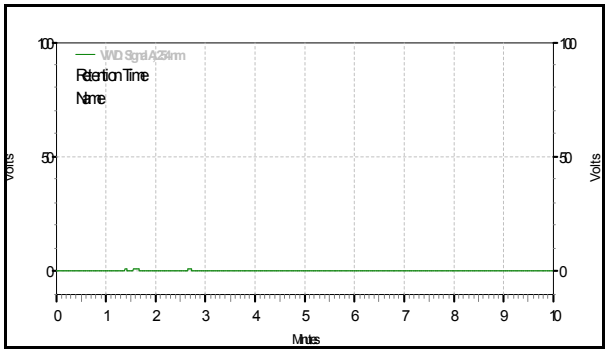


Fig. 25:BalnkFig. 26:10 ppm Standard Chromatogram



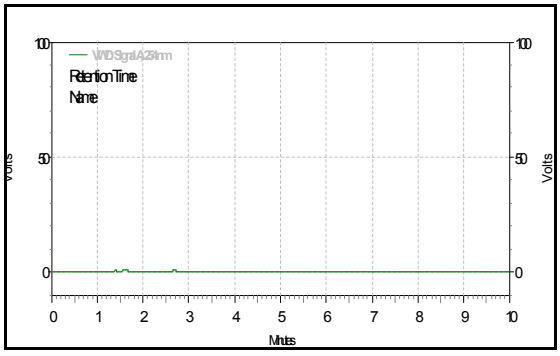


Fig. 27:1st Sample Chromatogram **Fig. 28:2nd Sample Chromatogram**

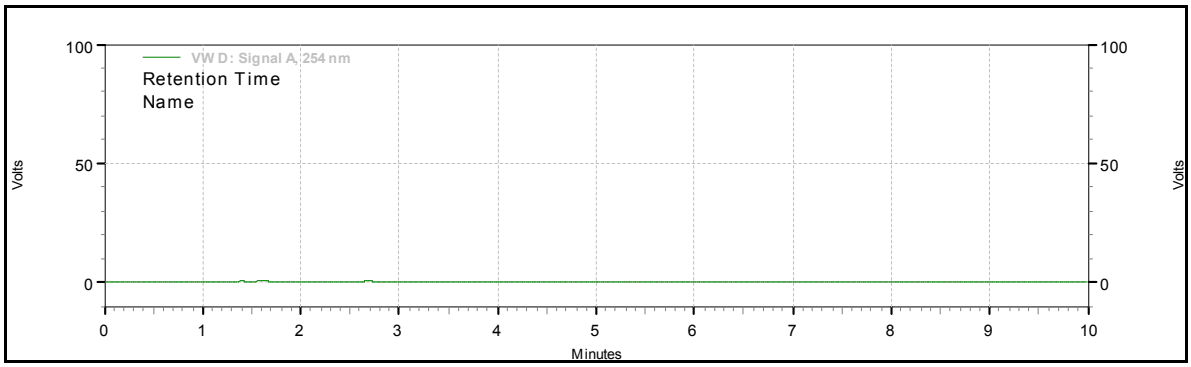


Fig. 29:3rd Sample Chromatogram

From the above chromatograms or Figures, it is observed that there is absence of triclabendazole content in triplicate bulk cleaning samples .Hence proved this metheod is applicable for triclabendazole bulk cleaning samples

Result and Discussion:

Optimization of the chromatographic conditions The nature of the sample, its molecular weight and solubility decides the proper selection of the stationary phase. The drug Triclabendazole 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 HPLC Grade Water were optimized to give symmetric peak with short run time based on asymmetric factor and peak area obtained. Different mobile phases were tried but satisfactory separation, well resolved and good symmetrical peaks were obtained with the mobile phase Acetonitrile, HPLC Grade Water70:30 (V/V). The retention time of TRICLABENDAZOLEwas found to be 4.933 min, which indicates a good base line. The RSD values for accuracy and precision studies obtained were less than 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 TRICLABENDAZOLE was found to be 80.0% indicating that the proposed method is highly accurate.

Conclusions:

A validated RP-HPLC method has been developed for Cleaning Method Validation for Residual Determination of TRICLABENDAZOLE Cleaning samples. The proposed method is simple, rapid, accurate, precise and specific.Its chromatographic run time of 10 min allows the analysis of a largenumber of Cleaningsamples in short period of time. Therefore, it is suitable for the routine analysis of Triclabendazole Cleaning samples in Veterinary Active pharmaceutical Ingredients bulk drugs.

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