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RP-HPLC Method Development and Validation for Nitroxynil in Active Pharmaceutical Ingredient Manufacturing.

V Sunil Kumar Yelamanchi[1], Useni Reddy Mallu[2] , I V Kasi Viswanath[1], G. Narishma muruthy[3]

[1] *Department of Chemistry, K.L.University, Green Fields, Vaddeswaram, Guntur, A.P, India.*
[2] *Celltrionpharm,inc .Seoul, South Korea .*
[3] *Head of quality Control , , Hetero labs limted, Andhra Pradesh.*

Abstract

Cleaning procedures should be monitored at appropriate intervals after validation to ensure that these procedures are effective when used during routine production. Visual inspection can allow detection of Cross contamination concentrated in small areas that could otherwise go undetected by sampling and/or analysis. The process of providing documented evidence that the cleaning methods employed with in the facility consistently controls potential carryover of product cleaning agents and extraneous material into subsequent product to a level that is below predetermined levels and as a Good manufacturing practices (GMP) requirement. The main scope of the study was to developed & Validated a new simple, precise and accurate Reverse Phase High Performance Liquid Chromatographic (RPHPLC) method for Nitroxynil (NXYF/01A) residual determination in veterinary active pharmaceutical ingredient manufacturing. The method was developed by using the isocratic solvent system, HPLC grade acetonitrile and 40 volumes of 0.1% Orthophospharic acid in Milli-Q water in the ratio of 60:40 (volume/volume) and Acetone is used as diluent. Successful elution of the Nitroxynil (NXYF/01A) was achieved on Nucleosil C18 column with 250x4.6 mm internal diameter and 5µm particle size (or) Equivalent . The method validation was successfully applied for routine analysis for cleaning/residual samples. The developed Reverse phase liquid chromatography (RP-LC) method was validated with respect to specificity, linearity, accuracy, precision and high sensitivity with detection limits and quantification limits ranging from 0.28 ppm to 0.88 ppm.

Key words: Nitroxynil (NXYF/01A) , Residual determination, Reversed phase High Pressure Liquid Chromatography and cleaning validation.

INTRODUCTION

Nitroxynil (NXYF/01A) is a low spectrum flukicide and nematicide not very much used on cattle, sheep and goats. It is available mostly in the form of injectables, often in combination with ivermectin.In the EU is the only flukicide with an established MRL for bovine and ovine milk, i.e. it can be used on dairy animals respecting the withholding period established for each particular product.It is not used in dogs or pets. Nitroxinil is highly effective against adult liver flukes (Fasciola hepatica) and against late immature stages (> 6 weeks) in cattle.It is also effective against a few gastrointestinal roundworms (e.g. Bunostomum spp, Haemonchus spp, Oesophagostomum spp, and Parafilaria bovicola) as well as against myiases caused by the sheep nasal bot fly (Oestrus ovis).In contrast with many other anthelmintics (e.g. imidazothiazoles, benzimidazoles, tetrahydropyrimidines), nitroxinil has a residual effect, i.e. it not only kills the parasites present in the host at the time of treatment, but protects against re-infestation for a period of time (up to several weeks) that depends on the dose and the specific parasite.Nitroxinil is not effective against rumen flukes (Paramphistomum spp), other roundworms such as lungworms (e.g. Dictyocaulus spp) or eyeworms (e.g. Thelazia spp), tapeworms and other external parasites. Nitroxinil is an uncoupler of the oxidative phosphorylation in the cell mitochondria, which disturbs the production of ATP, the cellular "fuel". This impairs the parasites motility and probably other processes as well. [1-4].

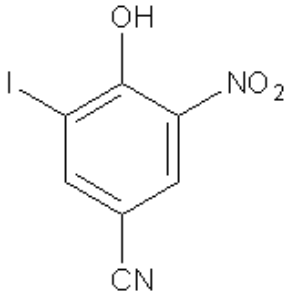

Fig. 1: Structure of Nitroxynil (NXYF/01A)
Molecular Formula:C 7 H 3 IN 2 O 3
4-hydroxy-3-iodo-5-nitrobenzonitrile

Toxicity

In cattle and sheep s.c. doses ≥40 mg/kg caused toxic symptoms, LD50 acute, rats, p.o. 170-450 mg/kg (various salts)In cattle, s.c. doses ≥55 mg/kg caused fatalities in adult cattle and calves. (Usual therapeutic dose is 7 to 13 mg/kg). The therapeutic index for cattle and sheep is ~4.As a general rule, at therapeutic doses nitroxinil is well tolerated by cattle and sheep. Toxic Symptoms caused by Dicyclanil Nitroxinil Intoxication symptoms are similar to those of other uncouplers of oxidative phosphorylation. The include tachycardia (excessive heart rate), rapid breathing, hyperventilation, fever, increased excitability. Nitroxinil Side Effects, Adverse Drug Reactions (ADRs) and Warnings, Swelling and other skin reactions can develop at the injection site. Nitroxinil residues in milk can negatively affect cheese production. Contaminated hair is stained

yellow. Antidote and Treatment of Dicyclanil Intoxication There is no antidote for nitroxinil poisoning. Treatment consists in supportive and symptomatic measures. [1-4].

MATERIALS AND METHODS

Chemicals : Reference standard of Nitroxynil (NXYF/01A) and cleaning samples was obtained from well reputed research laboratories and characterized by use of LCMS, NMR and IR . All reagents used were of analytical reagent grade unless stated otherwise. Milli.Q-water, HPLC grade acetonitrile, 0.1% Orthophospharic acid was purchased from Thermo scientific (Qualigens) & Merck, Mumbai, India. The solutions and the mobile phase prepared were stored at room temperature. The Liquid Chromatography system was equipped with quaternary gradient pumps with auto sampler and column oven , auto injector connected to a variable wave length programmable ultra Violet visible detector all were controlled by open lab software and Manufactured by Agilent technologies with model .no: 1200 series. [7-14].

Selection of suitable mobile phase, diluent & wave length : The mobile phase for the analysis of cleaning method validation for residual determination of Nitroxynil (NXYF/01A) (NXYF) was set by injecting different ratios of acetonitrile & 0.1% Orthophospharic acid in HPLC Grade water and acetone is used as diluent .The selected mobile phase ratio was Acetonitrile: 0.1% Orthophospharic acid in HPLC Grade water is 60:40(ml /ml). Similarly for the selection of diluent we tried the standard into different solvents like water, methanol, mobile phase & Acetone . Finally the selected diluent was Acetone. Similarly for the wave length selection we tried at different nanometres (nm) but at 237 nm it gives higher response. The selected mobile phase, diluent & wave length has given a sharp peak with low tailing factor 1.0 (<2). [7-14].

Instrumentation and analytical chromatographic conditions : The chromatographic analysis of the cleaning method validation for residual determination of Nitroxynil (NXYF/01A) (NXYF) was carried out on Agilent High Pressure Liquid Chromatography Model -1200 series containing quaternary pump, variable wave length programmable ultra violet visible detector and auto injector with up to 1µl-1000µl loop, column oven modules . Chromatographic analysis was performed using Nucleosil C18 column with with 250 x 4.6mm internal diameter and 5µm particle size (or) Equivalent. Sartorius electronic balance with model.no.CP-225D was used for weighing.
Isocratic elution with, acetonitrile, 0.1% Orthophospharic acid in HPLC Grade water 60:40 (ml /ml) was selected with a flow rate of 1.0 ml min-1 and injection Volume 10 µl (µL –micro.litre). The detection wavelength was set at 237 nm with a runtime of 10 min. The mobile phase was prepared freshly and it was degassed by sonication for 5 min before use. The column was equilibrated for at least 10 min with the mobile phase flowing through the system. The column oven module and the High pressure liquid chromatography system were kept at 27ºC temperature [7-14].

Preparation of standard solutions:
Pure standards of Nitroxynil (NXYF/01A) 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 50mg of the standard Nitroxynil (NXYF/01A) was accurately weighed and transferred in to 50ml volumetric flask and make up with sufficient diluent. Volumetric flask containing standard solution was sonicated for 10minutes. 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 analysed using the same chromatographic conditions and a calibration curve was generated.

RESULTS & DISCUSSIONS

It is required to Validated analytical methods having sensitivity to detect residues or contaminants should be used. The detection limit for each analytical method should be sufficiently sensitive to detect the established acceptable level of the residue or contaminant. The method's attainable recovery level should be established. Residue limits should be practical, achievable, verifiable, and based on the most deleterious residue. Limits can be established based on the minimum known pharmacological, toxicological, or physiological activity of the API or its most deleterious component. The degree of analytical validation performed should reflect the purpose of the analysis and the stage of the API production process.

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 Nitroxynil (NXYF/01A) being non-polar is preferably analysed 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 0.1% Orthophospharic acid in 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 the selected mobile phase in the Nucleosil C18 column with with 250 x 4.6mm internal diameter and 5µm particle size (or) Equivalent particle size has given a sharp peak with tailing factor 1.00 (<2) at retention time 5.593min and the chromatographic run time is 10min with the mobile phase acetonitrile, 0.1% Orthophospharic acid in HPLC Grade water 60:40 (ml/ml) [8].The retention time of Nitroxynil (NXYF/01A) was found to be 5.593min, which indicates a good base line. The % of relative standard deviation (R.S.D) 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 Nitroxynil (NXYF/01A) was found to be 89 (88.68%) indicating that the proposed method is highly accurate. Proposed liquid chromatographic method was applied for analysis of cleaning method validation for residual determination of Nitroxynil (NXYF) [7-14].

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 Harmonisation (ICH) guidelines. The method was validated for system suitability, precision, specificity, linearity, limit of detection and limit of quantification, recovery [7-14].

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 Nitroxynil (NXYF/01A) and it was calculated by determining the standard deviation of Nitroxynil (NXYF/01A) standards by injecting standards in six replicates at 10 minutes interval .The values of %RSD prove that the method is accurate & precise and acceptance criteria is 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). [7-14].

NXYF 10 ppm standard preparation: Weigh about 50.25 mg of Nitroxynil (NXYF/01A) standard 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.

Table-1: System suitability & Precision parameters

Injection No.:	Area of NXYF/01A	Tailing Factor
1	5524466	Tailing Factor
2	5508071	0.98
3	5535212	1.00
4	5516104	1.03
5	5524128	1.04
6	5538940	1.03
Average	5524487	1.06
Standard deviation	11522.2	1.01
% RSD	0.21	NA
Acceptance criteria	NMT 5.0%	Tailing<2.

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

Specificity Parameter: 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. [7-14].

Specificity tests were carried out on above prepared 10 ppm standard solution of Nitroxynil (NXYF/01A) and it was determining by injecting blank, blank with swab stick and specify solution (standard solution) for Nitroxynil (NXYF/01A) material at 10 ppm Standard Solution.

Table2: Specificity Parameters

Peak name	RT
Blank	No peak
Blank with swab stick	No peak
Standard solution	5.593

From the above data (Table 2), Proves that method is specific that is there is no interference of blank peaks in Nitroxynil (NXYF/01A) 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 sample within a given range [7-14].

The developed method has been validated as per International Conference on Harmonisation (ICH) guidelines the Standard solutions of Nitroxynil (NXYF/01A) in the mass concentration range of 0.2 ppm to 15 ppm was 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 Nitroxynil (NXYF/01A) was obtained by plotting the peak area ratio versus the applied concentrations of Nitroxynil (NXYF/01A) . The linear correlation coefficient was found to be 1.0 (0.999). The Values & Calibration curve were recorded in (Table 4) & (Fig. 2).

Preparation of NXYF Stock solution & 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.

Table 3: Linearity different levels of concentrations

Concentration in ppm	Stock Solution to be added	Volume make up to
0.0002	0.02	100
0.0005	0.05	100
0.001	0.10	100
0.003	0.30	100
0.005	0.50	100
0.008	0.80	100
0.010	1.00	100
0.013	1.30	100
0.015	1.50	100

Table 4: **Linearity parameters**

Trial number	Actual concentration(ppm)	Area response
1	0.00020034	114240
2	0.00050085	279580
3	0.00100170	555657
4	0.00300510	1664357
5	0.00500850	2804488
6	0.00801360	4501785
7	0.01001700	5527730
8	0.01302210	7159260
9	0.01502550	8175712
	Slope	547087292.1
	Correlation coefficient	0.999945319
	Regression Coefficient	0.999890642

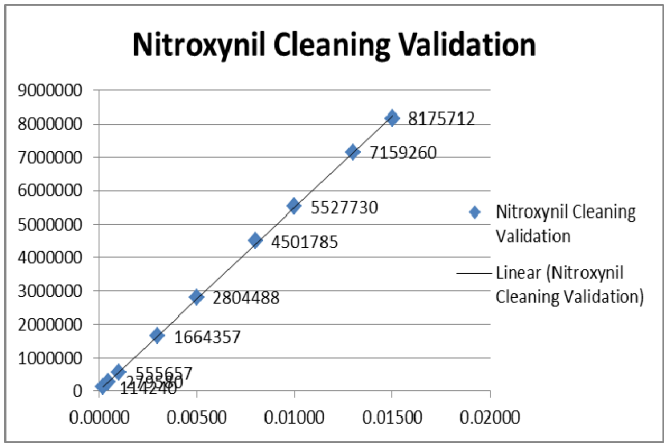

Fig. 2: Calibration curve for Linearity

Table 5: Residual output for Linearity parameters

RESIDUAL OUTPUT		
Observation	**Predicted Y**	**Residuals**
1	110142.2394	4097.760623
2	275355.5984	4224.401557
3	550711.1969	4945.803115
4	1652133.591	12223.40934
5	2753555.984	50932.01557
6	4405689.575	96095.42492
7	5507111.969	20618.03115
8	7159245.56	14.44049285
9	8260667.953	-84955.95328

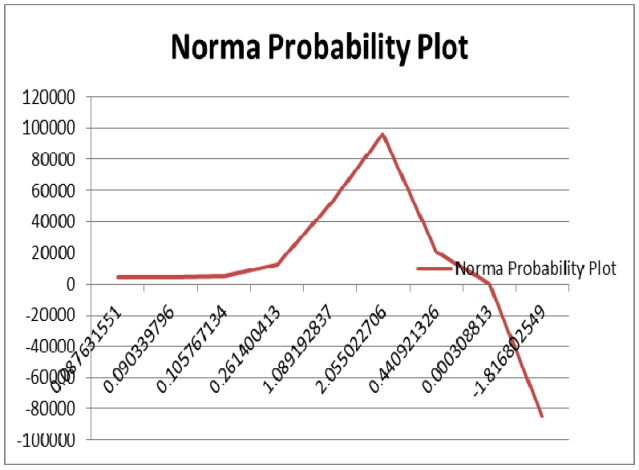

Fig. 3: Residual plot for linearity parameters

From the above data, it is clear that the area response of Nitroxynil (NXYF/01A) vs concentration in ppm of NXYF 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 Nitroxynil (NXYF/01A).

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[7-14].
Limit of quantification is the lowest amount of analyte in a sample that can be quantitated with acceptable precision, under the stated experimental conditions.

Limit of detection (LOD)
= (3.3 X Residual standard deviation) / slope.[9]
Limit of quantification (LOQ)
= (10X Residual standard deviation) / slope. [9]

Acceptance criteria:
The % of Relative standard deviation (R.S.D) for area response of Nitroxynil (NXYF/01A) six replicates at Limit of quantification (LOQ) level should be NMT 10 (10.0%).

Performed a regression analysis of the linearity data with concentration vs. ppm 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 (Table 4), (Table 5) & (Fig. 3).

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

Nitroxynil (NXYF/01A)	
Theoretical LOD in mg/mL	0.28ppm
Theoretical LOQ in mg/mL	0.88ppm

Preparation of Limit of detection (LOD) Solution: 0.028 mL of Nitroxynil (NXYF/01A) stock solution taken into 100 mL volumetric flask and diluted up to the mark with diluent. Injected in triplicate. The Limit of detection (LOD) Experimental Results are recorded in (Table 7).

Table 7: Limit of detection Experimental Results

Trial	Area
1	154627
2	155935
3	156618

Preparation of Limit of quantification (LOQ) Solution: 0.088 mL of Nitroxynil (NXYF/01A) stock solution taken into 100 ml volumetric flask and diluted up to the mark with diluent. Injected in six replicates. The Limit of quantification (LOQ) experimental results are recorded in (Table 8).

Limit of quantification Solution precision:

Table 8: Limit of quantification Experimental Results

Trial	Area
1	484677
2	487531
3	488111
4	488765
5	486990
6	486789
% RSD	0.29%
Acceptance criteria	NMT 10.0%

From the above precision results it can be concluded that the cleaning method validation is precise at Limit of quantification at concentration 2.0 ppm & Limit of detection (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 Nitroxynil (NXYF/01A) are broadly classified into two stages [7-14].

1) Rinse method 2) Swab 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 [7-14].

Preparation of rinsed spiking solution: Weighed about 100.15 mg of test sample and transfer 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 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 High pressure liquid chromatography. Performed 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. Filtered and inject into High pressure liquid chromatography. Performed the exercise in triplicate. Finally recorded the area of test sample in the rinse recovery on stainless plate & glass plate in (Table 9).

Table 9: % Rinse recovery Results

% Rinse recovery					
S. No.:	**Type**	**% Recovery**	**Mean % Recovery**	**SD**	**% RSD**
1	SS Plate	88.65%	88.68%	0.001	0.13%
2		88.58%			
3		88.80%			
4	Glass plate	88.15%	87.67%	0.005	0.68%
5		87.01%			
6		87.86%			

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. [7-14].

Preparation of Swab Spiking solution: Weighed about 100.69 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 x 10 cm surface area glass plates. 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. Filtered and inject into High pressure liquid chromatography. Performed the exercise in triplicate. Finally recorded the area of test sample in swab recovery on stainless plate & glass plate in (Table 10) than calculate the % rinse recovery, % swab recovery below formula,

Table 10: % Swab recovery results

% Swab recovery					
S. No.:	**Type**	**% Recovery**	**Mean % Recovery**	**SD**	**% RSD**
1	SS Plate	86.24%	86.76%	0.008	0.95%
2		86.32%			
3		87.71%			
4	Glass plate	87.54%	87.11%	0.004	0.51%
5		87.14%			
6		86.66%			

From the above results it can be concluded that % of rinse & % of swab recovery on SS plate and 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.

From the above chromatograms or figures observes that there is absence of Nitroxynil (NXYF/01A) content in triplicate bulk cleaning samples. Hence proved this method is applicable for Nitroxynil (NXYF/01A) bulk cleaning samples. [7-14]. Based on the above observed results the developed cleaning method validation for Nitroxynil (NXYF/01A) method is valid and run successfully the summary and evaluation of results are in (Table 11

Table 11: Summary and Evaluation of Results

<table>
<tr><th>Validation parameter</th><th>Acceptance criteria</th><th>Results</th></tr>
<tr><td>System suitability</td><td>The RSD for the area response of Nitroxynil (NXYF/01A) peak obtained from six replicate injections of system suitability should be NMT 5.0%</td><td>System suitability parameter meets the criteria.
RSD=0.21%</td></tr>
<tr><td>Specificity</td><td>The peaks of blank should not interfere with Nitroxynil
(NXYF/01A) peak</td><td>The peaks of blank do not interfere with Nitroxynil (NXYF/01A) peak.
<table>
<tr><th colspan="2">Individual solutions</th></tr>
<tr><th>Peak Name</th><th>Retention time(in minutes)</th></tr>
<tr><td>Blank</td><td>No peak</td></tr>
<tr><td>Blank with swab stick</td><td>No peak</td></tr>
<tr><td>System suitability solution</td><td>5.593</td></tr>
</table></td></tr>
<tr><td>Linearity</td><td>The correlation coefficient and the regression coefficient between concentration and area response of Nitroxynil (NXYF/01A) should be NLT 0.995</td><td>The method is linear
Correlation coefficient=0.99994
Regression coefficient=0.99989</td></tr>
<tr><td>LOD/LOQ</td><td>The RSD for area response of Nitroxynil (NXYF/01A) from six
replicates at LOQ level should be NMT 10.0%</td><td>The RSD for area response of Nitroxynil (NXYF/01A) from six replicates at LOQ level=
<table>
<tr><th></th><th>Fenbendazole (NXYF/01A)</th></tr>
<tr><td>LOQ in mg/mL</td><td>0.00028 mg/mL</td></tr>
<tr><td>LOD in mg/mL</td><td>0.00088mg/mL</td></tr>
</table></td></tr>
<tr><td>Recovery study</td><td>Report the % rinse recovery if the % rinse recovery is less than 80.0% then incorporate the recovery factor to the analytical method.</td><td>
<table>
<tr><th colspan="5">% Rinse recovery</th></tr>
<tr><th>Type</th><th>% Recovery</th><th>Mean % Recovery</th><th>SD</th><th>%RSD</th></tr>
<tr><td rowspan="3">SS plates</td><td>88.65%</td><td rowspan="3">88.68%</td><td rowspan="3">0.001</td><td rowspan="3">0.13%</td></tr>
<tr><td>88.58%</td></tr>
<tr><td>88.80%</td></tr>
<tr><td rowspan="3">Glass plates</td><td>88.15%</td><td rowspan="3">87.67%</td><td rowspan="3">0.005</td><td rowspan="3">0.68%</td></tr>
<tr><td>87.01%</td></tr>
<tr><td>87.86%</td></tr>
</table></td></tr>
<tr><td>Recovery study</td><td>Report the % swab recovery if the %swab recovery is less than 80.0% then incorporate the recovery factor to the analytical method.</td><td>
<table>
<tr><th colspan="5">% Rinse recovery</th></tr>
<tr><th>Type</th><th>% Recovery</th><th>Mean % Recovery</th><th>SD</th><th>%RSD</th></tr>
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<tr><td>86.32%</td></tr>
<tr><td>87.71%</td></tr>
<tr><td rowspan="3">Glass plates</td><td>87.54%</td><td rowspan="3">87.11%</td><td rowspan="3">0.004</td><td rowspan="3">0.51%</td></tr>
<tr><td>87.14%</td></tr>
<tr><td>86.66%</td></tr>
</table></td></tr>
</table>

Based on the above observed results the cleaning method validation for Nitroxynil (NXYF/01A) method is valid.

CONCLUSION

Analytical method was validated unless the method employed is included in the relevant pharmacopoeia or other recognized standard reference and also included the consideration of characteristics within the ICH guidance's on validation of analytical methods. The degree of analytical method validation performed should reflect the purpose of the analysis and the stage of the API production process. Finally proposed method is found to be specific for the Residual determination of Nitroxynil (NXYF/01A). The method is found to be linear in the range of interest. The sampling method is found to be precise for rinse and swab recovery. A System suitability test is established and recorded. Hence, this method stands validated can be used for routine line clearance samples.

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