Method Development and Validation for The Simultaneous Estimation of Milbemycin Oxime and Lufenuron in Bulk and Tablet Dosage Forms By RP-HPLC

P V MADHAVI LATHA[1], NAGAMANI BOLLA[2], M SWAPNA[3], P UMA DEVI[4]
[1, 2, 3, 4]*Viswanadha Institute of Pharmaceutical Sciences*

Abstract*— In the present study a simple, accurate and precise reverse phase high performance liquid chromatographic method has been developed and validated for the simultaneous estimation of Milbemycin oxime and Lufenuron in bulk and tablet dosage form. The method was developed using Waters HPLC system on a Inertsil – C18, ODS column (150 x 4.6 mm, 5µ) using Methanol and Water in the ratio of 70:30 v/v as mobile phase in isocratic elution mode at a flow rate of 1.0ml/min with a load of 20µl. The detection was carried out at 353nm. The retention time of Milbemycin Oxime and Lufenuron were found to be 3.005 min and 5.291 min respectively. The developed method was validated with respect to linearity, robustness, precision and accuracy and was successfully applied for the simultaneous quantitative determination of Milbemycin Oxime and Lufenuron in the tablet dosage form.

Index Terms- Milbemycin oxime, Lufenuron, RP-HPLC

I. INTRODUCTION

Milbemycin oxime + Lufenuron (brand name Sentinel®) is heartworm disease preventive that also treats internal parasites (e.g., hookworms, roundworms, and whipworms) and controls fleas in dogs. Milbemycin oxime and lufenuron are classified as an anthelmintic and an insect development inhibitor. Milbemycin oxime works by opening chloride channels in the neurons and myocytes of invertebrates, which causes the cells to hyperpolarize and blocks signal transfer. This process leads to paralysis and death of the parasite. Lufenuron is classified as an insect development inhibitor (IDI). Lufenuron's mechanism of action is interference with chitin synthesis, polymerization, and deposition. It is administered orally with food or may be injected.

II. MATERIALS AND METHODS

Preparation of standard stock solution: The solution was prepared by dissolving 100 mg of accurately weighed Milbemycin oxime and 100mg Lufenuron in diluent, in two 100.0 ml volumetric flasks separately and sonicated for 20 min (1000µg/ml).

Preparation of sample stock solution: Twenty tablets were weighed and the average weight of each tablet was calculated, and a quantity of tablet powder equivalent to 100 mg of Milbemycin Oxime and 100mg Lufenuron were weighed and dissolved in 100 ml of diluent with the aid of ultra sonication for 20 min to furnish a sample stock solution.

Preparation of working standard solution: From the above standard stock solution 4 ml from each solution was taken into a 100 ml volumetric flask then made up the volume with diluents and sonicated for 10 min and filtered through 0.45µm membrane filter.

Preparation of sample working solution: The sample stock solution was filtered through a 0.45 µm nylon syringe filter and 4 ml of the filtrate was diluted into 100 ml volumetric flask to give a sample solution containing 40µg/ml Milbemycin Oxime and 40µg/ml Lufenuron.

III. RESULTS AND DISCUSSION

Method validation: Validation parameters includes specificity, linearity, range, accuracy, precision, repeatability, intermediate precision, limit of detection, limit of quantification, robustness.

Specificity: Specificity is the ability to assessing equivocally the analyte in the presence of components which may be expected to be present. Typically these components include impurities, degradants, matrix etc. Blank solution and standard solutions of Milbemycin Oxime (40µg/ml) and Lufenuron (40µg/ml) were injected into the HPLC system. The peak purity data of Milbemycin Oxime and Lufenuron were compared. There should not be any interference at the retention time of the main peaks.

Linearity: Linearity for the drugs Milbemycin Oxime and Lufenuron was determined by preparing the standard solutions at seven concentrations levels in six replicates in the range of 20-80µg/ml for Milbemycin Oxime and 20-80µg/ml for Lufenuron from stock solution. The linearity charts of Milbemycin Oxime and Lufenuron was shown in the figure no 2&3. The correlation coefficient was found to be 1.0 and 0.9999 for Milbemycin Oxime and Lufenuron respectively. Linearity results were tabulated in table 2.

Accuracy: Accuracy was performed by spiking known amounts of standard solution to sample solution at three different concentrations levels (50%, 100%, 150%) and there by analyzed for %RSD which should not be more than 2.0.The % recovery was calculated and the results was reported in table no. 3 & 4.

Precision: The precision of the analytical method was studied by injecting six replicates of standard containing 40µg/ml of Milbemycin Oxime and 40µg/ml of Lufenuron which were injected into HPLC system. The % RSD was calculated and the results were reported in the table no.5 & 6.

Limit of Detection (LOD) and Limit of Quantification (LOQ):
The limit of detection was defined as the concentration which yields a signal - to – noise ratio 3:1 where as the limit of quantification was calculated to be the lowest concentration that could be measured with signal - to – noise ratio10:1. LOD and LOQ were calculated from slope and standard deviation. The results were tabulated in table no. 7.

Robustness: The smallest deliberate changes in method like change in flow rate are made but there were no predictable changes in the results and are in the range as per ICH guidelines. Conditions like flow rate minus (0.8 ml/min), flow rate plus (1.2 ml/min) was maintained and sample were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. % RSD was found to be within the limits and results were tabulated in table no. 8.

Assay: Assay was conducted on marketed formulation and mean % assay was found. The results were tabulated in table no. 9.

Table 1: Chromatographic conditions for optimized method

Parameter	Conditions
Flow rate	1.0ml/min
Column	Inertsil – C18, ODS column, 150 x 4.6 mm, 5µ
Detector wave length	353nm
Column temperature	Ambient
Mobile phase	Methanol: Water (70:30 v/v)
Diluent	Acetonitrile : Water (50:50 v/v)
Injection volume	20µL
Elution type	Isocratic mode
Run time	10 min
Retention time	3.005 min for Milbemycin Oxime 5.291 min for Lufenuron

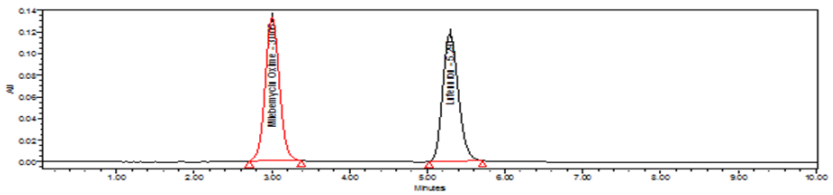

Figure 1: Optimized chromatogram

Table 2: Linearity results of Milbemycin Oxime and Lufenuron

Milbemycin Oxime		Lufenuron	
Conc (µg/ml)	Peak area	Conc(µg/ml)	Peak area
20	1674311	20	1212548
30	2511466	30	1818358
40	3348621	40	2424865
50	4145002	50	2993456
60	5022932	60	3636458
70	5860087	70	4242689
80	6697242	80	4849487

Figure 2: Calibration Curve of Milbemycin Oxime

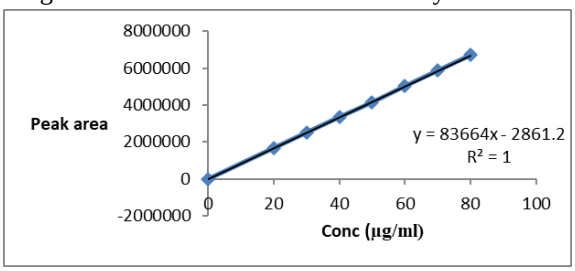

Figure 3: Calibration Curve of Lufenuron

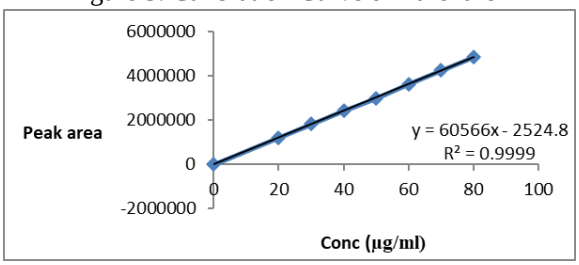

Table 3: Accuracy data of Milbemycin Oxime

% level	Amount spiked (µg)	Amount recovered (µg)	% Recovery	Statistical Analysis of % Recovery
50%	20	20.04	100.20	MEAN = 100.19 %RSD = 0.009
50%		20.03	100.18	
50%		20.03	100.19	
100 %	40	40.07	100.19	MEAN = 100.17
100 %		40.06	100.15	%RSD = 0.013
100 %		40.07	100.17	
150 %	60	60.10	100.17	MEAN = 100.17 %RSD = 0.013
150 %		60.10	100.17	
150 %		60.10	100.17	

Table 4: Accuracy data of Lufenuron

% level	Amount spiked (µg)	Amount recovered (µg)	% Recovery	Statistical Analysis of % Recovery
50%	20	20.04	100.23	MEAN = 100.28 %RSD = 0.060
50%		20.05	100.27	
50%		20.07	100.35	
100 %	40	40.01	100.03	MEAN = 100.15 %RSD = 0.155
100 %		40.03	100.08	
100 %		40.13	100.32	
150 %	60	60.07	100.13	MEAN = 100.12 %RSD = 0.039
150 %		60.04	100.07	
150 %		60.09	100.15	

Table 5: System Precision data of Milbemycin Oxime and Lufenuron

S. No	Peak area	
	Milbemycin Oxime	Lufenuron
1	3348900	2422124
2	3348201	2428654
3	3348578	2426902
4	3348907	2421845
5	3348577	2421109
Mean	3348528	2424415
SD	364.48	3131.126
% RSD	0.0108	0.129

Table 6: Method Precision data of Milbemycin Oxime and Lufenuron

S. No	Peak area	
	Milbemycin Oxime	Lufenuron
1	3347897	2421796
2	3348915	2429938
3	3347684	2420157
4	3348555	2423315
5	3349564	2424210
6	3348652	2426598
Mean	3348544	2424335
SD	385.27	3506.366
% RSD	0.0204	0.144

Table 7: LOD and LOQ data of Milbemycin Oxime and Lufenuron

Drug name	LOD (µg/ml)	LOQ (µg/ml)
Milbemycin Oxime	0.023	0.070
Lufenuron	0.026	0.080

Table 8: Robustness data of Milbemycin Oxime and Lufenuron

S. No	Drug name	Condition	Peak area	Tailing factor
1	Milbemycin Oxime	Flow rate(-) 0.8 ml/min	3318975	1.170
2		Flow rate(+) 1.2 ml/min	3382356	1.138
3	Lufenuron	Flow rate(-) 0.8 ml/min	2420777	1.078
4		Flow rate(+) 1.2 ml/min	2428606	1.076

Table 9: Assay data Milbemycin Oxime and Lufenuron

S. No	Peak area of Milbemycin Oxime	% Assay	Peak area of Lufenuron	% Assay
1	3348798	99.35%	2424333	99.70%
2	3349568		2424789	
3	3348271		2423789	
Mean	3348879		2424303	
SD	652.2829		500.6449	
%RSD	0.0194		0.0206	

CONCLUSION

The developed RP-HPLC method was validated as per ICH guidelines. All the system suitability parameters were within the range as stated by ICH guidelines. Interference peaks were not observed in blank, standard and sample chromatogram. Hence simple, precise and accurate, sensitive, specific and robust method was developed and validated. This can be used in quality control department with respect to routine analysis.

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