REVERSE PHASE HPLC AND DERIVATIVE SPECTROPHOTOMETRIC METHODS FOR SIMULTANEOUS ESTIMATION OF FENBENDAZOLE AND NICLOSAMIDE IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

The present study narrates the developed and validated simple, reliable, sensitive, precise and accurate Spectrophotometric and RP-HPLC methods for the simultaneous estimation of Fenbendazole and Niclosamide in pharmaceutical dosage form. In the first order derivative method 0.1 N methanolic HCl was used as diluent. The zero crossing point wavelengths selected for the analysis were 226 nm and 317 nm for Fenbendazole and Niclosamide, respectively and RP – HPLC method has been developed using 1% methanolic HCl as diluent. Separations of drugs were achieved on L1 C18 100 A^0 column (250 x 4.6 mm, 5 µ) using 2 gm potassium dihydrogen phosphate and acetonitrile (70:30, v/v) as mobile phase with flow rate 1.0 mL/min. The detection wavelength was 290 nm. Validation of developed methods was done according to ICH Q2 (R1) guideline. Calibration curve was linear over the concentration range of 3-9µg/mL (Fenbendazole) and 10-30 µg/mL (Niclosamide) for spectrophotometric method and 24 - 39 µg/mL (Fenbendazole) and 80 – 130 µg/mL (Niclosamide) for RP – HPLC method. The developed RP-HPLC and derivative spectrophotometric method were successfully applied for the quantitative determination of cited drugs in pharmaceutical dosage form. The correlation coefficients (r^2) value greater than 0.995. Accuracy of methods were determined by recovery studies and it was found to be 98 to 102 %. The % RSD values for all the validation parameters were less than 2.0 % for both the methods. The developed UV and RP-HPLC methods were compared by t - test and it was found that t_{stat} value was less than $t_{critical}$ value for all. Hence there was no significant difference between the developed methods.

Keywords: Fenbendazole, Niclosamide, UV- Spectrophotometry, RP-HPLC, Validation

1. INTRODUCTION

Fenbendazole(FEN) is chemically methyl 5-(phenyl thio)-2-benzimidazolecarbamate (Figure 1) is a veterinary anthelmintic product belonging to the chemical class of the benzimidazoles.[1] It is soluble in methanolic HCl. FEN has a broad-spectrum of activity against gastrointestinal roundworms and lungworms.[2] It is official in Indian Pharmacopoeia(IP)[3], British Pharmacopoeia(BP)[4] and United States Pharmacopoeia (USP)[5]. Niclosamide (NIC) is chemically 5-chloro-N-(2-chloro-4-nitrophenyl)- 2 hydroxy benzamide (Figure 2) is a veterinary anthelmintic product belonging to the chemical class of salicylamide.[2] It is soluble in methanolic HCl. NIC is used to treat tapeworm infections. It is not used for the other types of worm infections like pinworms or roundworms. It is official in Indian Pharmacopoeia (IP)[6]. The combination of FEN and NIC is widely used as an anthelmintic.

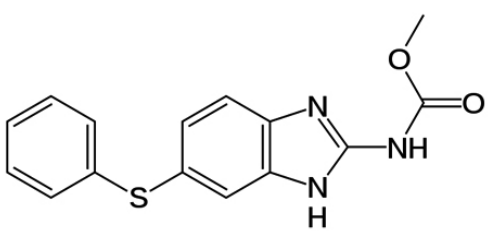

Figure 1: Structure of FEN

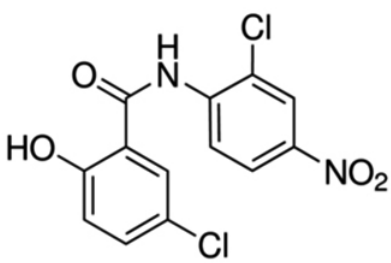

Figure 2: Structure of NIC

Literature review reveals that there are several analytical methods were reported, such as UV spectrophotometry[7-12], HPLC[13-17], HPTLC[18], LC-MS[19] for the estimation of FEN and NIC either individually and it's combination with other drug. However no method has been reported for simultaneous estimation FEN and NIC in pharmaceutical formulations. So, the aim of the present work was to develop accurate, precise and sensitive RP-HPLC and derivative spectrophotometric methods for the simultaneous estimation of Fenbendazole and Niclosamide in Pharmaceutical dosage form.

2. EXPERIMENTAL

2.1 Chemicals and Reagents:

FEN and NIC reference standard were procured from Pharmanza (India) Pvt. Ltd, Cambay, Gujarat. The marketed suspension (Fensamide) used contains 30 mg FEN and 100mg NIC and was manufactured by Pharmanza (India) Pvt. Ltd. Analytical grade hydrochloric acid(HCl) ,acetonitrile, methanol and potassium dihydrogen phosphate were procured from Loba chemicals. WFI water used for HPLC System.

2.2 Equipments, instrumentation and software:

UV-Visible double beam spectrophotometer with a matching pair of 1 cm quartz cuvettes (Shimadzu UV-1800, Shimadzu Corporation, Kyoto, Japan), connected to a computer loaded with Shimadzu UV - PC version 3.42 software was used to record the absorption spectra of solutions. The spectral band width was 0.5 nm. An integrated HPLC system, LC 20AT from Shimadzu Corporation, Japan was used for the chromatographic separation of FEN and NIC. The HPLC system was comprised of a binary gradient pump and manual sampler, column oven and UV detector. PC-installed LC solution software was used to record and integrate the chromatograms. Electronic weighing balance (Shimadzu AUX 200) was used for weighing the samples.

2.3 Spectrophotometric conditions for First Order Derivative Methods[20-21]:

2.3.1 Experimental condition:

According to the solubility characteristics, the common solvent for the both drugs was found to be 0.1 N methanolic HCl. The selected wavelengths for the analysis were 266 nm (ZCP of FEN) and 317 nm (ZCP of NIC), respectively.

2.3.2 Preparation of stock solutions:

Accurately weighed and transferred FEN (10 mg) and NIC (10 mg) into two different 100 mL and 10 mL volumetric flask, respectively. The volume was made up to the mark with 0.1 N methanolic HCl. The final concentration of FEN and NIC were 100 (µg/mL) and 1000 (µg/mL), respectively.

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2.3.3 Construction of calibration curve:

Working standard solutions having 3 µg/ml of FEN and 10 µg/ml of NIC were scanned in range of 200- 400 nm to determine the detection λ for both the drugs. The recorded UV spectra of was transformed to 1st order derivative spectra, with smoothing factor (Δλ) =4 and multiplying the entire spectra by a constant scaling factor 10 to obtain zero crossing points (ZCP) for simultaneous estimation of FEN and NIC (Figure 3). The ZCP for FEN and NIC was obtained at 262 nm and 317 nm, respectively. So, the estimation of NIC was carried out by measuring amplitudes at 266 nm (ZCP of FEN) and for FEN at 317 nm (ZCP of NIC)**.** From working solution of FEN, aliquots of 0.3, 0.45, 0.6, 0.7 and 0.9 mL were taken and transferred in 10 mL volumetric flask and volume was made up to mark with 0.1 N methanolic HCl to get a series of final concentration of 3 - 9 µg/mL for FEN and working solution of NIC, aliquots of 0.1, 0.15, 0.2, 0.25 and 0.3 mL were taken and transferred in 10 mL volumetric flask and volume was made up to mark with 0.1 N methanolic HCl to get a series of final concentration of 10 - 30 µg/mL for NIC. The absorbance of resulting solutions were measured at 266 nm and 317 nm. (Figure 3)

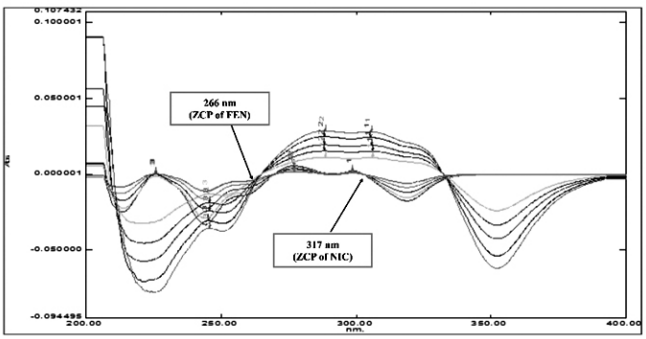

Figure 3: First Order Derivative spectra of FEN (3-9 **µg/mL**) and NIC (10 - 30 µg/mL)

2.4 Analysis of marked formulation by Derivative spectrophotometric method:

1.06 g. of suspension [contains 30mg (FEN) and 100mg (NIC)] was weighed and transferred into 50 mL volumetric flask. The volume was made up to the mark with 0.1 N methanolic HCl. Aliquot of 0.1 mL was withdrawn from the above flask and diluted up to 20mL with 0.1 N methanolic HCl to get final concentration of 3 µg/mL for FEN and 10 µg/mL for NIC.

2.5 Chromatographic conditions:

2.5.1 Experimental condition:

Chromatographic separation was performed on a Shimadzu's HPLC (LC-2010-HT), Phenomenex L1 HPLC analytical C18 100 A^0 column (250 * 4.6 mm, 5 µ) with mobile phase consisting of a mixture of buffer (2 g potassium dihydrogen phosphate) and acetonitrile (70:30 v/v) and flow rate of 1.0 mL/min. The UV detection was performed at 290 nm for FEN and NIC. (Figure 4)

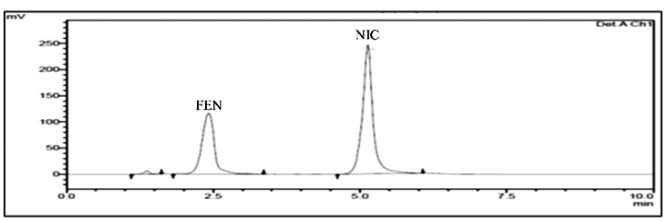

Figure 4: Optimized Chromatogram of FEN and NIC in buffer and acetonitrile (70:30 v/v) and flow rate of 1.0 mL/min at 290 nm

2.5.2 Construction of calibration curve:

Accurately weighed and transferred 15 mg and 50 mg of FEN and NIC into 50 mL volumetric flask and 40 mL 0.1N methanolic HCl was added and sonicated for 20 min. Then volume was made up to 50 mL with same diluent to make stock solution of 300 µg/mL of FEN and 1000 µg/mL NIC. Aliquot of 0.8,0.9,1.0,1.1,1.2 and 1.3mL was withdrawn from the above flask and transferred in 10 mL volumetric flask and volume was made up to mark with 0.1N methanolic HCl to get a series of final concentration of 24 – 39 µg/mL of FEN and 80 - 130 µg/mL of NIC.

2.6 Analysis of marked formulation by RP-HPLC method:

1.06 g. of suspension [contains 30mg (FEN) and 100mg (NIC)] weighed and transferred into 50 mL volumetric flask. Then make up volume up to the mark with 0.1 N methanolic HCl. Aliquot of 1 mL was withdrawn from the above flask and diluted up to 20 mL with 0.1 N methanolic HCl to get final concentration of 30 µg/mL for FEN and 100 µg/mL for NIC.

3.0 RESULTS AND DISCUSSION

3.1 Optimization of spectrophotometric conditions:

The proposed method is based on spectrophotometric first order derivative spectrophotometric method for the simultaneous estimation of FEN and NIC in UV region using 0.1N methanolic HCl as solvent. For first order derivative method convert normal spectrum into first derivative spectrum with smoothing factor (Δλ) =4 and multiplying the entire spectra by a constant scaling factor 10 to obtain zero crossing points (ZCP) for simultaneous estimation of FEN and NIC. 266 nm (ZCP of FEN) and 317 nm (ZCP of NIC) were selected for FEN and NIC, respectively.

3.2 Optimization of chromatographic conditions:

The main criterion for developing an RP-HPLC method was being accurate, reproducible, robust, linear, free of interference from other excipients and convenient enough for routine use in quality control laboratories. The standard solution of FEN and NIC were scanned over the range of 200 nm to 400 nm wavelengths. The wavelength maxima of FEN (305 nm) and NIC (332 nm) are quite apart from each other and there was isobestic point observed. But at 306 nm they overlapped to each other. Based on above findings 290 nm was selected as detection wavelength and at the selected wavelength both drugs were showing quantifiable height and area.

Initially, the separation of all the peaks was studied by using a reversed-phase phenomenex L1 HPLC analytical C18 100 A^0, 250 x 4.6 mm, 5 µ particle size columns with isocratic elution. Optimization of the mobile phase was performed based on trials and errors method. In this method different mobile phase trials were tried in buffer with differ in ratio of the mobile phase. After that trial with buffer (2 g potassium dihydrogen phosphate): acetonitrile (70:30 % v/v) in this all two drugs are full fill all the criteria of system suitability test.

3.3 Solution stability study:

Solution stability was performed to check that the drugs were stable in solvent or not. The stability was performed by measuring the absorbance (for UV) and peak area (for HPLC) of the solution at different time intervals. It was observed that FEN and NIC were stable in solution form at 48 hours at refrigerator temperature.

3.4 Method validation:

The developed and optimized method was validated for system suitability, specificity, sensitivity [limit of detection (LOD) & limit of quantitation (LOQ)], linearity, precision [repeatability & intermediate precision], accuracy and robustness as per ICH Q2 (R1) guideline.[22]

3.4.1 System Suitability (for RP-HPLC):

System suitability is established to prove that suitability and reproducibility of the chromatographic system are adequate to perform an analysis. Single set of mixed standard solution was prepared as mentioned in the test method and six replicate injections of mixed standard preparation were injected and chromatogram was taken. Results were shown in Table 1.

Table 1: System Suitability Parameter for FEN and NIC by RP-HPLC Method

Parameters	Drugs	
	FEN	NIC
Retention time	2.56	5.21
Tailing Factor	1.12	1.10
Theoretical Plate	3015.16	4771.08
Peak area	1783721.50	3160125.83
Resolution	2.36	

3.4.2 Specificity:

The specificity of the method was determined by comparing the spectra (for UV) and chromatogram (for RP-HPLC) of the standard and sample solutions of FEN and NIC.

3.4.3 Sensitivity:

The sensitivity of the analytical method was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ) using following equations and result of sensitivity was shown in Table 9.

LOD = 3.3 σ / S and LOQ = 10 σ / S

Where, σ = standard deviation of y intercept of calibration curve (n = 6)
S = slope of a regression equation

3.4.4 Linearity:

The linear regression analysis obtained by plotting the absorbance (for UV) and peak area (for HPLC) of analyte vs. concentration shown correlation coefficients(r^2) greater than 0.995. The statistical results such as correlation coefficients (r^2), slope, intercept are reported in Table 9.

3.4.5 Precision:

The precision of the method was confirmed by repeatability and intermediate precision. Repeatability expresses the precision under the same operating conditions over a short interval of time. The repeatability was performed by the analysis of the formulation was repeated for six times with the same concentration. The amount of each drug present in the formulation was calculated as reported in %RSD. Results were shown in Table 2.

Table 2: Repeatability Data for FEN and NIC by both the methods

Parameters	First order derivative method		RP-HPLC method	
	FEN	NIC	FEN	NIC
Concentration (µg mL)	3	10	30	100
SD[a]	0.0012	0.0016	12864.91	18837.05
%RSD[b]	0.853	0.310	0.72	0.59

a = Standard Deviation, b= Relative Standard Deviation

Table 3: Results of intraday and interday precision studies of FEN & NIC for both methods.

Parameters		First order derivative method			RP-HPLC method		
FEN							
Concentration (µg/mL)		3	6	9	24	30	36
Intraday Precision	SD[a]	0.0001	0.0002	0.0002	9890.85	11053.72	10018.15
	%RSD[b]	0.54	0.42	0.45	0.83	0.62	0.40
Interday Precision	SD[a]	0.0004	0.0005	0.0004	15526.9	17040.49	15245.71
	%RSD[b]	0.67	0.52	0.57	1.32	0.95	0.62
NIC							
Concentration (µg/mL)		3	6	9	24	30	36
Intraday Precision	SD[a]	0.0002	0.0004	0.0002	17746.8	24225.30	12599.13
	%RSD[b]	0.24	0.16	0.11	0.81	0.76	0.30
Interday Precision	SD[a]	0.0004	0.0005	0.0004	24173.4	27892.02	15666.22
	%RSD[b]	0.37	0.23	0.21	1.11	0.88	0.38

a = Standard Deviation, b= Relative Standard Deviation

The intraday and interday precision of the proposed methods were performed by analyzing the corresponding responses three times on the same day for intraday precision and over a period of three days for inter day with three different concentrations of standard binary mixture solutions. The results were reported in terms of % RSD. Each concentration was applied in triplicates and % RSD was calculated. The precision studies data are represented in table for FEN and NIC, respectively. Results were shown in Table 3.

3.5.6 Accuracy:

It is the closeness of test results obtained by that method to the true value. The accuracy of the method was carried out at three levels 80, 100 and 120 % of the working concentration of sample. From the total amount of drug found, the % recovery was calculated. This procedure was repeated for six times. The %RSD was calculated and results were shown in Table 4 & 5.

Table 4: %Recovery studies of FEN and NIC for first order derivative method

Parameters	First order derivative method					
	FEN			NIC		
Level	80	100	120	80	100	120
Sample conc.(µg/mL)	3	3	3	10	10	10
Amount of Std. added (µg/mL)	2.4	3	3.6	8	10	12
Total Conc. (µg/mL)	5.4	6	6.6	18	20	22
Found Conc. ±SD[a] (µg/mL)	5.39± 0.0015	6.00± 0.002	6.59± 0.0015	17.98± 0.0015	19.99± 0.0014	21.99± 0.0010
%RSD[b]	0.54	0.34	0.23	0.32	0.21	0.11
%Recovery	99.81	100.00	99.84	99.98	99.99	99.55

a = Standard Deviation, b= Relative Standard Deviation

Table 5: % Recovery studies of FEN and NIC for RP-HPLC method.

Parameters	RP-HPLC method					
	FEN			NIC		
Level (%)	80	100	120	80	100	120
Sample conc.(µg/mL)	15	15	15	50	50	50
\ Amount of Std. Added(µg/mL)	12	15	18	40	50	60
Total conc.(µg/mL)	27	30	33	90	100	110
Found conc.±SD[a] (µg/mL)	26.99± 16654.4	30.00± 12864.9	32.98± 17913.5	89.99± 25592.7	100.00± 18837.0	109.99± 14605.4
%RSD[b]	1.12	0.72	0.83	0.95	0.59	0.40
%Recovery	99.96	100.00	99.93	99.97	100.00	99.97

a = Standard Deviation, b= Relative Standard Deviation

3.5.7 Robustness (for RP-HPLC):

The robustness of an analytic procedure is a measure of its capacity to remain unaffected by small, but deliberate variation in method parameters. Robustness of the method was determined by small changes in flow rate, mobile phase ratio and wavelength of detection. Flow rate was changed to 0.9 ± 1.1 mL/min. The mobile phase ratio was changed to ± 2 % for all three components. Wavelength of detection was changed to 290 ± 2 nm. Results of robustness were shown in Table 6.

Table 6: Robustness Study of FEN and NIC for RP- HPLC

Parameters	Actual condition	Change condition	%RSD[b]	
			FEN	NIC
Flow Rate ± 10%	1.0 ml/min	0.9	0.86	0.56
		1.1	0.76	0.57
Wavelength± 2nm	290 nm	288	0.76	0.54
		292	0.84	0.57
Mobile Phase Ratio± 2%	100.00%	98%	0.84	0.53
		102%	0.94	0.58

b= Relative Standard Deviation

3.8 Analysis of market formulation:

The validated UV spectrophotometric and RP-HPLC methods were used in the analysis of the marketed formulation FENSAMIDE with a label claim of 30 mg for FEN and 100 mg for NIC per Suspension. Results of assay were shown in Table7.

Table 7: Analysis of marketed formulation by both developed methods

Parameters	First Order Derivative Method		RP-HPLC Method	
	FEN	NIC	FEN	NIC
Found Mean Conc ± SD[a]	3.02 ± 0.0012	10.02 ±0.0015	30.02 ± 12864.91	100.04± 18837.05
%Assay	100.00	100.02	100.06	100.04
%RSD[b]	0.529	0.345	0.729	0.512

a = Standard Deviation, b= Relative Standard Deviation

3.9 Statically Comparison of UV and HPLC methods:

Statistical comparison of both the methods were carried out by applying t- test to the assay results of all two drugs obtained by developed methods. It was found that t_{stat} value was less than $t_{critical}$ value for all the two drugs. Hence there was no significant difference between the developed methods. Results were shown in Table 8.

Table No. 8: Results of t-test for FEN and NIC

Parameters	First Order Derivative Method	RP-HPLC method	First Order Derivative Method	RP-HPLC method
	FEN		NIC	
Mean	100.012	100.014	100.012	100.010
Variance	0.00037	0.00073	0.00037	0.00025
Observations	5.000	5.000	5.000	5.000
Hypothesized Mean Difference	0.000		0.000	
D_f	4.000		4.000	
t-Test	-0.534		0.196	
P(T<=t) two tail	0.621		0.854	
T Critical two tail	2.77		2.77	

Table No. 9: Summary of Validation Parameter of RP-HPLC and First Order Derivative Method

Parameters	First Order Derivative Method		RP-HPLC method	
	FEN	NIC	FEN	NIC
Linearity (µg/mL)	3 – 9	10– 30	24 – 39	80– 130
Repeatability (% RSD[b])	0.85	0.31	0.72	0.59
Correlation Coefficient(r^2)	0.9995	0.9992	0.999	0.999
Intraday precision (%RSD[b]) (n=3)	0.42-0.54	0.11-0.24	0.40-0.83	0.30-0.81
Interday precision (% RSD[b]) (n=3)	0.52-0.67	0.21-0.37	0.62-1.32	0.38-1.11
LOD (µg/mL)	0.42	0.58	2.846	5.673
LOQ (µg/mL)	0.97	1.10	6.749	9.936
% Assay	100.00	100.02	100.06	100.04
%Recovery	99.81-100.00	99.55 – 99.99	99.93 - 100.00	99.97 - 100.00

b= Relative Standard Deviation, LOD = Limit of Detection, LOQ= Limit of Quantification

4.0 CONCLUSION

First order derivative UV Spectrophotometric and RP - HPLC methods were successfully developed and validated for the simultaneous determination of FEN and NIC. The developed methods were found to be sensitive, accurate, precise, and robust. The UV and HPLC methods were not significantly different as per statistical analysis. This implies that the proposed UV and HPLC methods can be used for quality control analysis in pharmaceutical dosage form.

5.0 CONFLICT OF INTEREST

Authors have no conflict of interest

6.0 ACKNOWLEDGEMENT

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