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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR NITAZOXANIDE IN POWDER FOR SUSPENSION DOSAGE FORM

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ABSTRACT

A simple, precise, accurate, rapid and reproducible RP-HPLC method has been developed for the determination of Nitazoxanide in powder for suspension dosage form. Chromatography was carried out on an ODS C18 column (250 x 4.6 mm x 5 µm length), using a mixture of acetonitrile and 0.005 mol L^{-1} tetra n- butyl ammonium hydrogen sulphate (54:46 v/v) as the mobile phase at a flow rate of 1 mL/min and the detection was done at 240 nm. The method produced linear responses in the concentration range from 50 to 150 µg/mL of Nitazoxanide with correlation coefficients of 0.999, accuracy of 98.90% and precision of 0.811%. The method was found to be reproducible for analysis of the drug in suspension dosage form. The results of the analysis were tested and validated statistically for various parameters according to ICH guidelines and recovery studies confirmed the accuracy of the proposed method.

INTRODUCTION: Nitazoxanide, N-(5-nitro-2-thiozoly) salicylamide acetate is an antiamoebic and anhtelmintic agent. It is indicated for amoebiasis, helminthiasis, giardiasis, fasciasis, trichomoniasis and cryptosporidiosis, including those with AIDS or HIV infections [1].

Nitazoxanide is used in many areas of the world, especially in Central and South America, as a broad-spectrum parasiticidal agent in adults and children [2].

Nitazoxanide is not official in any of the pharmacopoeia. It found mentioned in Martindale, The Complete Drug Reference [3]. Very few analytical methods like RP-HPLC and spectrophotometric methods are reported in literature for the quantitative estimation of Nitazoxanide in bulk drug and pharmaceutical dosage forms [4-7].

Therefore, it was thought worthwhile to develop simple, precise, accurate RP-HPLC method for determination of Nitazoxanide in powder for suspension dosage form.

MATERIALS AND METHODS:

Drugs and Chemicals: HPLC grade acetonitrile (Merck, German) and tetra n- butyl ammonium hydrogen sulphate(A.R grade, Scharlau, Spain) water for HPLC (PALL life sciences, India) were used for preparing the mobile phase. All other reagents used were HPLC grade.

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Pure Nitazoxanide (Glenmark Pharmaceuticals Pvt. Ltd., India) used as working standard without further purification. A commercial Nitazoxanide suspension (Nitazox, Incepta Pharmaceuticals Ltd; Batch No. 11003; Mfg Date 07/2011, Exp Date 06/2013) was purchased from local market.

Instruments: A SHIMADZU SPD-20Av UV-Visible detector model, an ODS reverse phase column (250 x 4.6 mm x 5 µm length), SIL 20 AC HT auto sampler, CTO-10 AS vp column oven, LC-20 AT isocratic single pump with software LC solution of version 1.2 high pressure liquid chromatographic instrument was employed in the study.

Preparation of Mobile phase: A freshly prepared 54:46 v/v mixture of acetonitrile and 0.005 mol L^{-1} tetra n-butyl ammonium hydrogen sulphate (By taking 1.7 g of tetra n- butyl ammonium hydrogen sulphate in 1000-mL of water) was used as the mobile phase. Mixed them and filtered through a filter having a nominal pore size not greater than 0.45 µm.

Preparation of Standard Stock Solution: About 100 mg of Nitazoxanide working standard was dissolved in mobile phase and diluted up to 100 mL. Filter and collect filtrate.

Preparation of Analytical Standard Solution: Dilute 5 mL of this solution to 50 mL with mobile phase to make the concentration 100 µg/mL. Filter through a filter having a nominal pore size not greater than 0.45 µm.

Preparation of sample solution: Take about 5 g of reconstitute sample in a 100-mL volumetric flask and add 60 mL of mobile phase. Mix for 15 minutes in an ultrasonic bath. Cool the sample to room temperature. Finally add mobile phase q.s. to 100 mL. Filter and collect the filtrate. Dilute 5 mL of this solution to 50 mL with mobile phase. Filter through a filter having a nominal pore size not greater than 0.45 µm. The stock solution was further diluted with mobile phase to get required concentration in linearity range. All solutions were stored at room temperature; these solutions were shown to be stable during the period of study.

Validation of the Developed method: The developed method for the determination of Nitazoxanide was validated as per ICH guidelines (ICH 2005).

System Suitability Test: Before starting validation parameters, System Suitability must be established by injecting 20 µL each for six replicate injections of system suitability solution prepared as analytical standard solution. Using six peak areas, calculate the Relative Standard Deviation (RSD %), mean tailing factor, theoretical plate and asymmetry factors (**Table 1**).

Linearity and Range: Appropriate dilutions of standard stock solution (50-150µg/ml) were assayed as per the developed method for Nitazoxanide. To establish linearity of the proposed method, seven separate series of solutions of Nitazoxanide were prepared from the stock solutions and analyzed (**Table 2**).

Precision: Precision was done by (i) repeatability or intra-assay precision and (ii) intermediate precision.

i) **Repeatability (intra-assay precision):** Repeatability was determine from six test samples by Injecting 20 µL of each sample. Duplicate injection was made for each concentration level (**Table 3**).

ii) **Intermediate precision:** A second analyst performed the same experiment as repeatability experiment. For determination of method precision, analyst 1 repeatability (n=6) was combined with analyst 2 precision (n=6) and expressed as method precision (n=12) (**Table 4**).

Accuracy: To check the accuracy of developed method and to study the interference of formulation additives analytical recovery experiments were carried out by standard addition 80%, 90%, 100%, 110% and 120% of the label claim. Accuracy was conducted by adding known amounts of Nitazoxanide to the sample matrix and five different concentrations of test sample were prepared. Duplicate injections were made for each concentration level (**Table 5**).

Robustness: The robustness of this validation was conducted by changing two different parameters (Temperature: 30◦C and 40◦C and Flow rate: 1 ml/min and 1.2 ml/min) of the method by using the same concentration of test sample of repeatability sample (**Table 6**).

Specificity: Specificity will be determined by injecting separately blank, placebo, standard and sample solution of Nitazoxanide in duplicate (**Fig. 2, 3, 4, 5**).

RESULTS AND DISCUSSION: The present study demonstrates a sensitive, precise and accurate HPLC method for the analysis of Nitazoxanide in powder for suspension dosage form.

System Suitability: Chromatograms were automatically integrated and visually inspected for an acceptable integration. The relative standard deviation of the peak areas (RSD 0.196%), the mean tailing factor (1.315), theoretical plates and asymmetry factors for six system suitability injections were calculated. The system suitability parameters were within the limits.

TABLE 1: RESULT OF SYSTEM SUITABILITY TEST

Replicate 1	Peak area	Tailing Factor	Theoretical plate	Asymmetry factor	Remarks
	4328869	1.315	6350.628	0.981	
2	4347516	1.316	6356.241	0.992	
3	4350277	1.316	6368.805	0.994	Passed
4	4344485	1.316	6366.125	1.101	
5	4353161	1.312	6369.245	1.103	
6	4344548	1.314	6371.253	1.115	

Linearity and Range: A good linear relationship (r=0.999) was observed between the concentration of Nitazoxanide and the respective ratio of peak areas. The regression curve was constructed by linear regression fitting and its mathematical expression was y=42330x+34698 (where y is the ratio of peak areas of the drug to that of reference standard and x is the concentration of Nitazoxanide) (**Fig. 1**). The lower limit of quantitation (LLOQ) was defined as the lowest concentration within the linear range (50.75µg/mL). The upper limit of quantitation (ULOQ) was defined as the highest concentration within the linear range (152.25µg/mL) (Table 2).

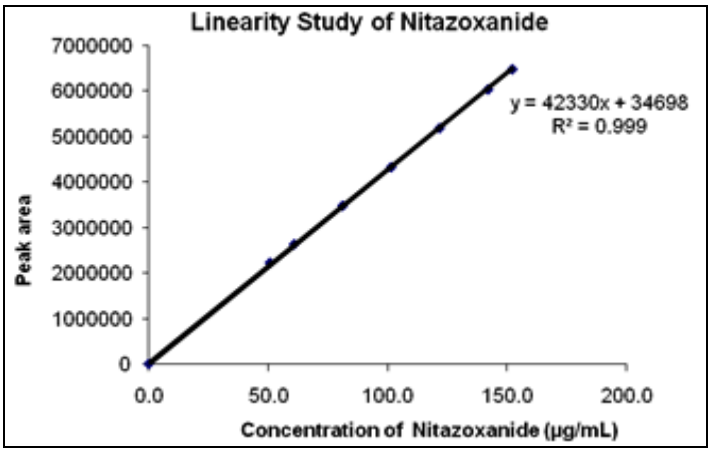

FIGURE 1: LINEARITY STUDY OF NITAZOXANIDE

TABLE 2: RESULT OF LINEARITY AND RANGE

% of Nominal value	Conc. of Std (µg/mL)	Peak areas	Statistical Analyses	Pass/ Remark
50%	50.75	2222181		
60%	60.90	2630322		
80%	81.20	3476512	Regression correlation coefficient, (R^2)= 0.999	
100%	101.50	4325304	y-intercept = 34698	**Passed**
120%	121.80	5183614	Slope of regression line = 42330	
140%	142.10	6036729		
150%	152.25	6478552		
		Lower limit of quantitation (LLOQ)		50.75 µg/mL
		Upper limit of quantitation (ULOQ)		152.25 µg/mL

PRECISION: The repeatability and intermediate precision study of the developed method demonstrate RSD 0.528% for analyst-1 and RSD 0.497% for analyst-2 where RSD value for 12 samples was 0.811% which was not more than 2.0 % that indicating the developed method has excellent repeatability and intermediate precision.

TABLE 3: RESULT OF REPEATABILITY

Sample	Peak area of Sample	Average peak areas of Sample	Assay, mg/5mL	%RSD
1	5190584	5211245	111.68	0.528
	5231905			
2	5055425	5053679	111.91	
	5051932			
3	5251532	5293769	110.76	
	5336006			
4	5309778	5249334	110.71	
	5188890			
5	5093833	5076747	110.58	
	5059660			
6	5227066	5263169	110.65	
	5299272			
Average of Assay			111.05	

TABLE 4: RESULT OF INTERMEDIATED PRECISION

	Analyst-1			Analyst-2		
Sample	Peak area of Sample	Average peak areas of Sample	Assay, mg/ 5mL	Peak area of Sample	Average peak areas of Sample	Assay, mg/ 5mL
1	5190584	5211245	111.68	5287463	5240301	110.03
	5231905			5193138		
2	5055425	5053679	111.91	5223708	5236540	110.48
	5051932			5249372		
3	5251532	5293769	110.76	5096563	5088698	109.11
	5336006			5080833		
4	5309778	5249334	110.71	5215001	5208637	109.89
	5188890			5202273		
5	5093833	5076747	110.58	5210861	5214528	109.14
	5059660			5218194		
6	5227066	5263169	110.65	5097818	5103739	109.43
	5299272			5109659		
	RSD for analyst-1		0.528 %	RSD for analyst-2		0.497 %
RSD for 12 sample			0.811 %			

Accuracy: The validity and reliability of proposed method was assessed by recovery studies by standard addition method. The mean of % recovery (98.90%) and % RSD (0.1%) were found to be within limit (NMT 2%) for the developed method. This result revealed that any small change in the drug concentration in the solution could be accurately determined by the developed analytical method.

TABLE 5: RESULT OF ACCURACY

% of Nominal Value	Peak area of Sample	Average peak area of sample	%Recovery
80%	3909943	3926061	98.83
	3942178		
90%	4452970	4451634	99.04
	4450298		
100%	5063615	5081514	98.84
	5099413		
110%	5544755	5554369	98.98
	5563982		
120%	5710603	5715053	98.82
	5719503		
Mean			98.90%
RSD			0.101%

Robustness: Robustness of the method was found out by testing the effect of deliberate changes in the chromatographic conditions and the corresponding peak areas. The factors selected for this purpose were temperature and flow rate. The method was found to be robust enough that the RSD of peak area, tailing factor were not apparently affected by variation in the chromatographic conditions.

TABLE 6: RESULT OF ROBUSTNESS

Temp. (◦C)	Flow rate (mL/min)	% RSD of Peak Area	Tailing Factor	Theoretical plate
30	1	1.001	1.156	6350.628
30	1.2	1.131	1.161	6368.805
40	1	1.210	1.212	6299.372
40	1.2	1.225	1.219	6301.520

Specificity: Specificity of the analyte peak was determined from that of the vehicle and blank injection. The chromatograms of Blank injection, Placebo injection, Standard injection and Test sample injection used to justify the specificity of target analytes. Necessary chromatograms are presented below:

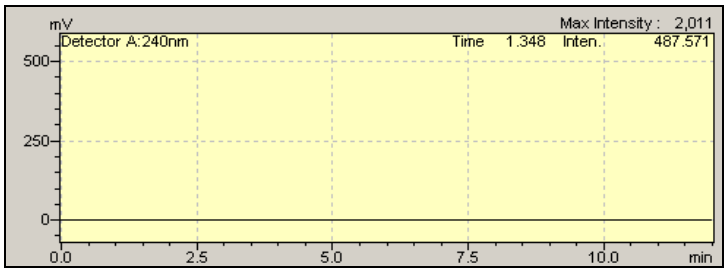

FIGURE 2: CHROMATOGRAM OF BLANK

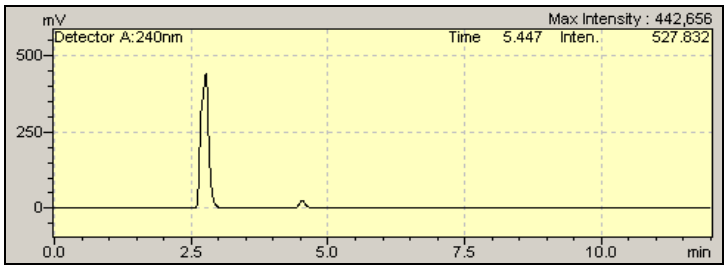

FIGURE 3: CHROMATOGRAM OF PLACEBO

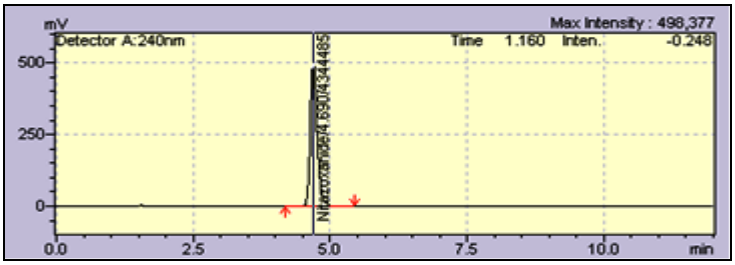

FIGURE 4: CHROMATOGRAM OF STANDARD

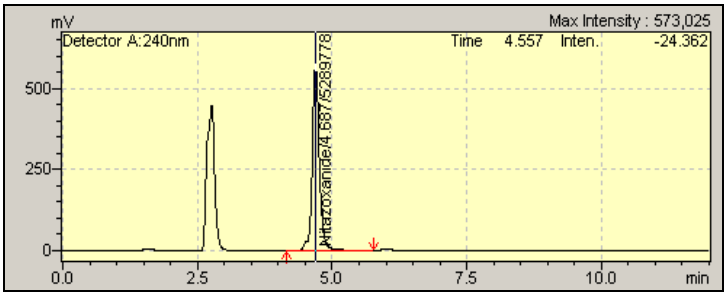

FIGURE 5: CHROMATOGRAM OF SAMPLE

CONCLUSIONS: The isocratic RP-HPLC method developed for quantitative determination of Nitazoxanide is precise, accurate, and selective. The method was completely validated and satisfactory results were obtained for all the method validation data tested. Percent of recovery shows that the method is free from interference of the excipients used in the formulation. Therefore, the proposed method can be used for routine analysis of Nitazoxanide in powder for suspension dosage form.

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