ORIGINAL RESEARCH

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE DETERMINATION OF NITAZOXANIDE AND OFLOXACIN IN COMBINED PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT:

A simple, rapid, specific and reproducible reverse phase HPLC method was developed and validated for the simultaneous separation and estimation of the Nitazoxanide and Ofloxacin from the commercially available tablet dosage form. The chromatography was carried out using a combination of 0.863% (w/v) ammonium dihydrogen orthophosphate buffer and Acetonitrile (45:55 ratio (v/v)) at a flow rate of 1.0 ml/min and was monitored at 240 nm wavelength. The method was statistically validated by the study of linearity, accuracy, precision, limit of detection, limit of quantification, recovery and robustness. The retention time of Ofloxacin and Nitazoxanide were 2.099 $\pm$ 0.010 and 5.623 $\pm$ 0.03 minutes respectively. The calibration curve showed the excellent linearity over a concentration range of 3.125 µg to 0.5 mg/ml for Nitazoxanide and 1.25 µg to 0.2 mg/ml for Ofloxacin with correlation coefficients of 0.99999 and 0.99998 respectively. The proposed method can be used for the simultaneous estimation of Ofloxacin and Nitazoxanide in the combined dosages forms.

KEY WORDS: Nitazoxanide, Ofloxacin, RP-HPLC, Method validation

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INTRODUCTION:

The synthetic nitrothiazole benzamide compound Nitazoxanide is chemically 2-(Acetyloxy)-N-(5-nitro-2-thiazolyl) (Figure I, according to PubChem) benzamide and was initially developed and commercialized as an oral antiparasitic agent. This compound is reported to be active against *Taenia crassiceps* cysticerci[1], *Trichinella spiralis*[2], Fasciola[3] and Leishmania[4 - 6] under the helminth parasites and *Blastocystis spp.*[7, 8] *Cryptosporidium spp.*[9 - 14] Giardia[15], *Entamoeba histolytica*, *Trichomonas vaginalis*, *Acanthamoeba castellanii*[16], *Neospora canimum*[17], and *Toxoplasma gondii*[18] under the protozoan parasite.

Recent researches showed the activity of this molecule and its active metabolite Tizoxanide against a broad range of obligate and facultative anaerobic gram positive and gram negative bacteria which includes *E. coli*[19, 20] *M. tuberculosis*[21 - 23], *H. pylori*[24 - 26] *C. difficile*[26 - 29] and *C. jejuni*[26, 30, 31]. Furthermore the scientist Rossignol described this molecule as "a first-in-class broad-spectrum antiviral agent" in 2014[32] and now it is found to be active against influenza[32, 33], parainfluenza, Sendai virus (SeV), Respiratory syncytial virus (RSV) A-2, coronavirus[34], Hepatitis B and C virus[35 - 40], Dengue -2 (New Guinea strain), Yellow fever virus, *J. encephalitis* virus (JEV)[41], human immunodeficiency virus (HIV)[35, 36, 42 - 44], Simian rotavirus and Human rotavirus[45]. The latest addition in this list is the chikungunya virus[46]. But this agent may also be active against a wide variety of other viruses due to its ability to stimulate the potent and multifaceted antiviral immune responses[42]. This molecule is also reported to have its potentiality to be used for the treatment of different types of cancers[47 - 52].

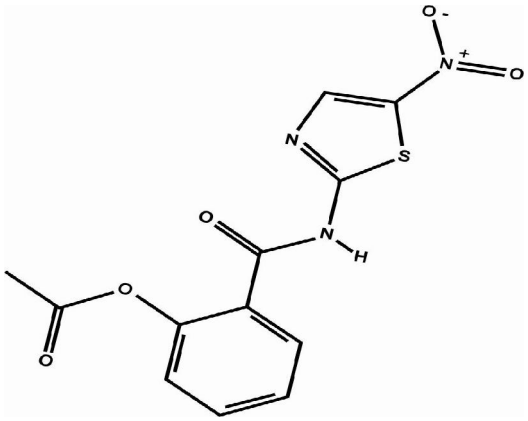

Figure I: Structure of Nitazoxanide according to PubChem.

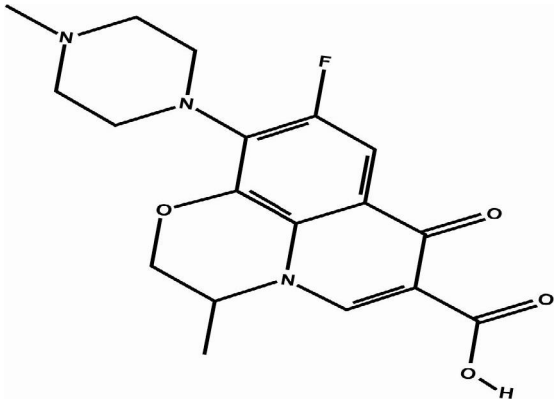

Figure II: Structure of Ofloxacin according to PubChem.

The use of this drug in combination is already approved by the Drug Controller General of India (DCGI), Govt of India and 16 brands of Generic Nitazoxanide are found to be listed currently in the Medindia's database (http://www.medindia.net) but the combination is only reported with Ofloxacin. Chemically Ofloxacin is a fluoroquinolone derivative (RS)-7-fluoro-2-methyl-6-(4 methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo [7.3.1.05,13] trideca-5(13), 6, 8, 11-tetraene-11- carboxylic acid (Figure II, according to PubChem) and shows the antibacterial activity by inhibiting the replication of the genetic material (DNA). So it will be active against a wide variety of gram-positive and gram-negative organism and is being used for the treatment of urinary tract infection, conjunctivitis, gonorrhoea, respiratory tract infection and skin infection.

A number of methods were reported for the simultaneous determination of Nitazoxanide and Ofloxacin in the combined formulations. These include HPLC[53 - 56], electrochemical[57] and spectrophotometric method[58 - 61], but they have their own limitations. We have tried to develop a simple, rapid, precise, specific, accurate and sensitive reverse phase HPLC method for the simultaneous determination of these components in this study.

MATERIALS AND METHODS:

Reagents and chemicals:

Working Standard Nitazoxanide and Ofloxacin IP were procured from Ind-Swift Limited, Industrial growth Centre, Phase-1, Samba (J&K), India. Combined formulation tablet dosage form NETAZOX-OF (claim: 500mg Nitazoxanide and 200mg Ofloxacin IP) (Mfd. By Ind-Swift Limited, Industrial growth Centre, Phase-1, Samba (J&K), India) was purchased from the local market. HPLC grade Acetonitrile and other chemicals were procured from Fisher Scientific, Mumbai, India and in house produced Mili-Q water (spec. ELGA of Veolia Water Systems of UK, model ULTRA IONIC) was used throughout the experiments.

Equipments: The equipments which were used in the present study were given in the Table I.

Table I: List of equipments.

Sl. No.	Name Of the Instruments	Make	Model
1.	HPLC with Total Chrome Software (Version 6.3.2)	Perkin Elmer	Flexar
2.	Electronic balance	Mettler Toledo	ME204E
3.	pH meter	Eutech	pH 700
4.	UV spectrophotometer	Perkin Elmer	LAMBDA 750
5.	Bath sonicator	Oscar	Microclean -103
6.	Water Purification Systems	ELGA of Veolia Water Systems of UK	ULTRA IONIC

Chromatographic conditions: The chromatographic conditions were given in the Table II.

Table II: Chromatographic conditions.

Column	Reverse phase C18 (Agilent ZORBAX Eclipse XDB-C18 column, 250mm x 4.6mm, particle size 0.5µm)
Detection wavelength	UV at 235 nm
Detection wavelength	UV at 240 nm
Detection wavelength	UV at 245 nm
Flow Rate (a)	1.20 µl/minute
Flow Rate (b)	1.00 µl/minute
Flow Rate (c)	0.80 µl/minute
Injection Volume	20 µl
Run time	8 minutes
Column oven temperature	25°C
Elution	Isocratic
Mobile phase (a)	Buffer : Acetonitrile = 40 : 60
Mobile phase (b)	Buffer : Acetonitrile = 45 : 55
Mobile phase (c)	Buffer : Acetonitrile = 50 : 50

Preparation of Buffer solutions:

8.63 g of ammonium dihydrogen orthophosphate was weighed and transferred into a 1000 ml of standard flask. 500 ml of Mili-Q water was added and sonicated for 20 minutes to dissolve it. pH was adjusted to 3.0 by adding 10 % (v/v) phosphoric acid. Next the solution was diluted to 1000 ml by adding the same Mili-Q water to prepare 0.863% (w/v) buffer solution and filtered before use through 0.45 µ membrane filtered.

Preparation of Mobile phase solutions:

The buffer and Acetonitrile was mixed in the ratio of 40 : 60 (v/v), 45 : 55 (v/v) and 50 : 50 (v/v).

Preparation of standard solutions:

Standard solution of 0.5 mg/ml of Nitazoxanide and 0.2 mg/ml of Ofloxacin was prepared by transferring 50 mg working standard of Nitazoxanide and 20 mg working standard of Ofloxacin in 100 ml standard flask and was diluted upto the mark with the mobile phase. This standard solution was treated as 100 % for various experimental purposes and was sonicated for 20 minutes followed by filtration through 0.45µ membrane filter before use.

Preparation of sample solution and analysis:

Twenty tablets were weighed and crushed to prepare a homogeneous mixture. Accurately weighed tablet powder equivalent to 250 mg of Nitazoxanide and 100 mg of Ofloxacin was transferred to a 50 ml of volumetric flask and 30 ml of mobile phase was added. The solution was sonicated for ten minutes and volume was made up to 50 ml with mobile phase. The solution was mixed and filtered through 0.45 µm membrane filter. The 10 ml of filtrate was further diluted to 100 ml with the same mobile phase. The resulting concentration of the Nitazoxanide and Ofloxacin would be 0.5 mg/ml and 0.2 mg/ml respectively. This sample was taken as 100%. Next the 75 % and 50 % concentrations were prepared accordingly by diluting with mobile phase. Replicate injections were analyzed under optimized chromatographic conditions as mentioned earlier. 20 µl of each standard and sample dilutions were injected and chromatogrammed. All the injections were repeated in triplicate. After that, the content of the Nitazoxanide and Ofloxacin was calculated by comparing the mean peak area of the sample with the mean peak area of the standard solution.

METHOD DEVELOPMENT:

Both the drugs were individually scanned in a wavelength range of 200-400 nm in the mobile phase. Nitazoxanide showed the two absorbance maxima at 240 and 346 nm whereas, Ofloxacin showed the two absorbance maxima at 226 and 295 nm. The UV absorption patterns are shown in Figure III. The isosbestic absorption for both the components was found to be present at 240, 252 and 326 nm. The isosbestic absorption was found much lower at 252 nm compared with 240 nm. So the absorbance at 252 nm was not selected. The wavelength of 240 nm was selected for the data acquisition in HPLC due to the resolution of the peaks and balanced area acquisition for the simultaneous estimation of the component drugs. The respective graph of chromatogram is represented in Figure IV.

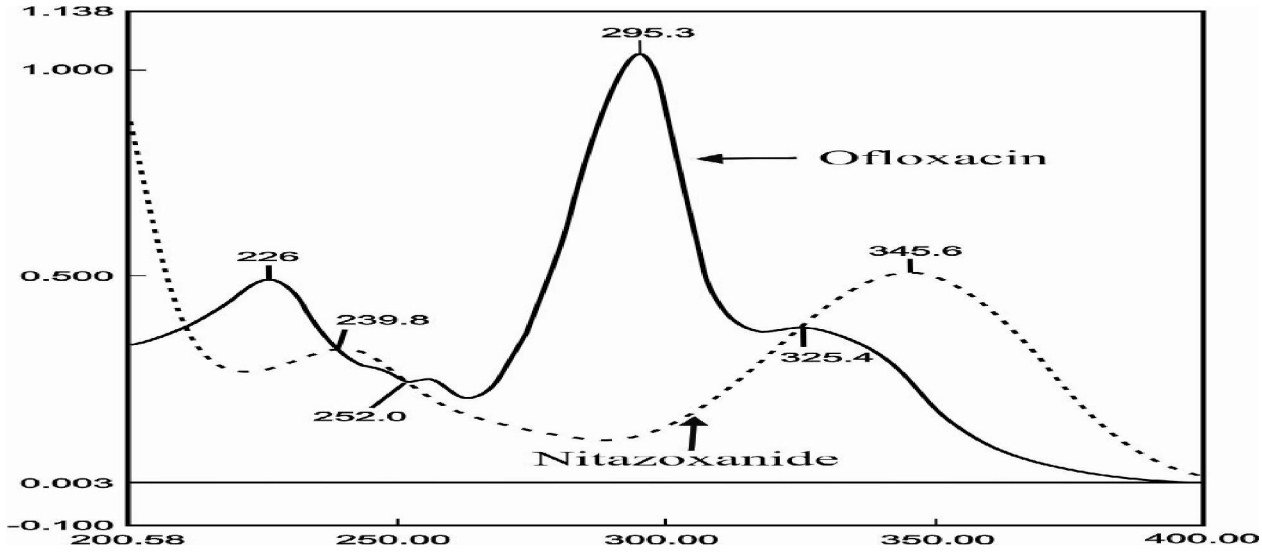

Figure III: UV absorption patterns of Ofloxacin and Nitazoxanide. Solid line depicted the absorption pattern of Ofloxacin and dotted line depicted the absorption pattern of Nitazoxanide.

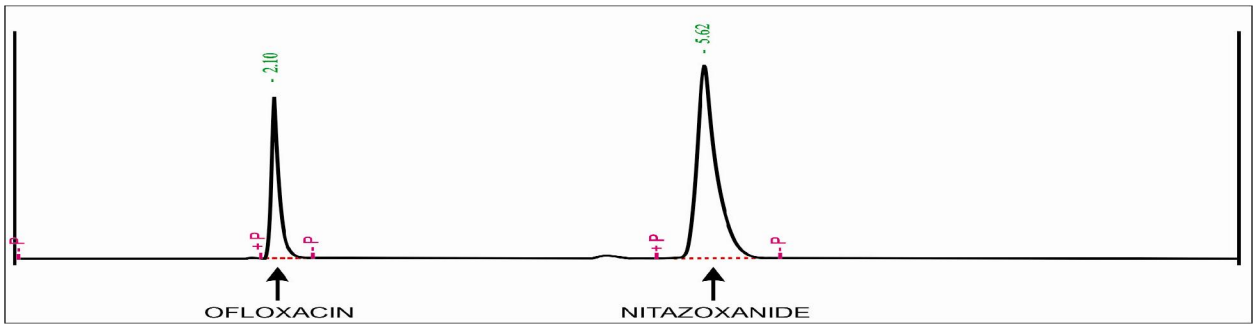

Figure IV: Typical RP-HPLC Chromatogram of Ofloxacin and Nitazoxanide. The Retention time is 2.10 for Ofloxacin and 5.62 for Nitazoxanide.

Validation of the RP-HPLC method:

System suitability:

This integral part of liquid chromatographic methods was done by injecting the 100% concentrated standard solution having 500 µg/ml of Nitazoxanide and 200 µg/ml of Ofloxacin for six times into the chromatographic system. Retention times, number of obtained theoretical plates (N), resolution and calculated tailing factors (T) are tabulated in Table III.

Table III: System Suitability Parameters. (n = 6)

Parameters	Ofloxacin	Nitazoxanide
Retention Time (min.)	2.099 ± 0.010	5.623 ± 0.03
Theoretical plates (N)	5535.32 ± 200.87	6747.49 ± 239.12
Tailing factor (T)	1.69 ± 0.019	1.57 ± 0.01
Resolution	18.24 ± 0.35	

Linearity:

Eight concentrations of the standard mixture 100%, 50%, 25%, 10%, 5%, 2.5%, 1.25% and 0.625% were injected in triplicate and the corresponding chromatograms were recorded. The calibration curves were constructed by plotting the mean peak areas against the concentration of the corresponding components. The Correlation coefficient(r) for each drug was calculated and the corresponding linearity parameters are tabulated in Table IV. The representative curves for both the components are given in Figure V(a) and V(b).

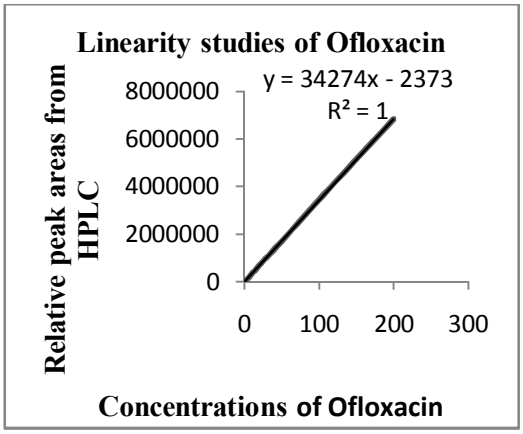

Figure V (a): Calibration curve of Ofloxacin

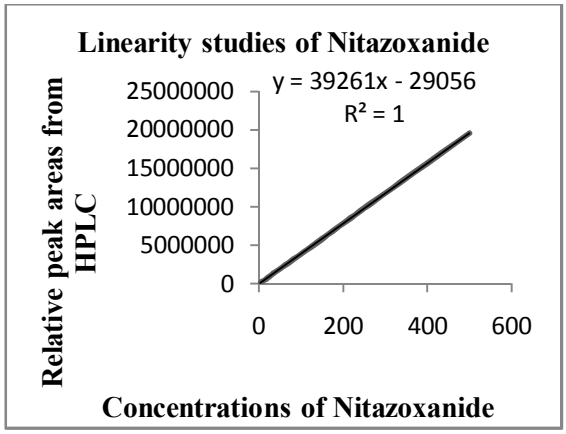

Figure V (b): Calibration curve of Nitazoxanide

Limit of Detection (LOD) and Limit of Quantitation (LOQ):

The LOD and LOQ for both the components were calculated using the standard deviation of the response (Sy) and the slope (S) of the calibration plots using the formula LOD = 3.3 (Sy/S) and LOQ = 10 (Sy/S). The results are given in Table IV.

Table IV: Results of Linearity. (n = 3)

Parameters	Ofloxacin	Nitazoxanide
Slope (S)	34274.10	39261.14
Correlation coefficient (R^2)	0.999986959	0.999991088
Linearity Range	0.00125 to 0.2mg/ml	0.003125 to 0.5 mg/ml
LOD (µg/ml)	1.27	2.63
LOQ (µg/ml)	3.85	7.96

Precision:

The repeatability of this method was verified by injecting 100% concentration (0.5 mg/ml of Nitazoxanide and 0.2 mg/ml of Ofloxacin) of the sample for six times on one day and the same procedure was repeated on another day. Next the percentages of RSD values for the areas were calculated and the results are tabulated in Table V

Table V: Results of Precision. (n = 6)

Components	1st set Precision (RSD)	2nd set Precision (RSD)
Ofloxacin	0.81	0.45
Nitazoxanide	0.88	0.77

Accuracy:

Recovery studies were carried out to ensure the reliability and accuracy of the method. This was done by adding the known quantity of the pre-analyzed sample to the standard solution having 50%, 75% and 100% concentration. The results are tabulated in Table VI.

Table VI: Results of Accuracy. (n = 6)

% of standard added	Ofloxacin				Nitazoxanide			
	Amount added (µg/ml)		Amount found (mg/ml)	% Recovery	Amount added (µg/ml)		Amount found (mg/ml)	% Recovery
	Std (mg/ml)	Test (mg/ml)			Std (mg/ml)	Test (mg/ml)		
50%	0.102	0.105	0.217	104.83	0.254	0.264	0.513	99.03
75%	0.153	0.104	0.267	103.89	0.381	0.260	0.645	100.62
100%	0.204	0.112	0.343	108.54	0.508	0.281	0.776	98.35

Robustness:

Robustness of the proposed method was verified by altering the flow rate, wavelength detection and mobile phase composition. For the slight change, a deviation of ± 2 was selected for the flow rate, ± 5 was selected for wavelength and 40:60 and 50:50 buffer and Acetonitrile were selected for the mobile phase. RSD, retention time, number of theoretical plates and resolutions are reported in Table VII (a) and VII (b) for Ofloxacin and Nitazoxanide respectively.

RESULT AND DISCUSSION:

The objective of this study was to develop a simple, rapid, specific and reproducible reverse phase HPLC method for the simultaneous separation and estimation of Nitazoxanide and Ofloxacin in commercially available tablet dosage form employing the most commonly employed RP C-18 column with the UV detection system. From the overlaid absorption spectrum of Nitazoxanide and Ofloxacin, it was found to consist there isoabsorptive points at 240 nm, 252 nm and 326 nm. The wavelength of 240 nm was selected for the balanced area acquisition. There is no interference between the two peaks at 240 nm (Figure IV). The retention times were found 2.099 ± 0.010 minutes for Ofloxacin and 5.623 ± 0.03 minutes for Nitazoxanide. The other chromatographic conditions were selected after a number of trials. 1.0 ml/min flow rate with buffer and Acetonitrile ratio of 45:55 was taken because the maximum resolution (18.527) as well as the maximum numbers of theoretical plates was observed in this flow rate for both of the components (Table VII (a) and Table VII (b)). This indicated the good level of peak separation for these components in these selected parameters.

The system suitability was carried out by using six injections of 100% standard solutions. The number of theoretical plates was detected 5535.32 and 6747.49 with the tailing factor 1.69 and 1.57 for Ofloxacin and Nitazoxanide respectively (Table III). The RSD values for the two components were found less than 1. According to the International Conference on Harmonisation (ICH) guideline, the number of theoretical plates (Efficiency) should be greater than 2000, tailing factor should be equal to or less than 2 and RSD value should be less than 1. This indicated that this RP-HPLC based method is fully validated according to the guideline of ICH.

In all the previous studies, the authors reported the linearity over a very narrow range of the concentrations. This method fist claimed the linearity over a broad range of concentrations having 0.00125 to 0.2 mg/ml for Ofloxacin and 0.003125 to 0.5 mg/ml for Nitazoxanide. This result claimed the applicability of this method for a wide strength of pharmaceutical formulations. The correlation coefficients were found 0.9999 and 0.99999 i.e. too much close to 1 (Table IV). This indicated that the assay results at different concentrations are more tightly clustered along a line i.e. the linearity over the range. The detection limit for Ofloxacin and Nitazoxanide were 1.27 µg/ml and 2.63 µg/ml whereas the quantitation limits were 3.85 µg/ml and 7.96 µg/ml, respectively. The RSD values were found to be 0.81 and 0.88 for first day and 0.45 and 0.77 for another day which indicated the reproducibility of this proposed method. (Table V)

Accuracy of the method was verified by performing the recovery studies by the standard addition method. The percent recovery of the standard added to the pre-analyzed sample was calculated and it was found to be 103.89 to 108.4 % for Ofloxacin and 98.35 to 100.62 % for Nitazoxanide. Percentage of recovery showed that the method is free from interference of the excipients, used in the formulation (Table VI).

The method was found to be robust after changing the conditions like detection wavelength (upto ± 5nm), flow rate (± 0.2 ml/min) and Buffer : Acetonitrile (± 5 %). The RSD values were found to be much lower than 1 and reported in Table VII (a) and (b). This lower RSD values indicated that the results were almost unaltered due to the small deliberate variation in the method parameters.

These optimized parameters were then employed for the quantitative estimation of the Ofloxacin and Nitazoxanide in the commercially available mixed formulation. It was found to be 96.46 to 104.36 % from the claimed amount of Ofloxacin and 94.62 to 97.67 % from the claimed amount of Nitazoxanide.

Table VII (a): Robustness for Ofloxacin.

Parameters (n=3)	Flow rate (ml/min)			Buffer : Acetonitrile			Wavelength		
	1.2	1.0	0.8	40:60	45:55	50:50	235	240	245
Retention Time (min.)	1.74	2.11	2.61	2.026	2.11	2.063	2.089	2.11	2.099
Theoretical plates (N)	4347.26	5714.19	4636.73	1983.38	5714.19	2217.31	4402.34	5714.19	4042.94
Tailing factor (T)	1.46	1.69	1.55	1.759	1.69	1.611	1.532	1.69	1.482
RSD	0.77	0.81	0.19	0.47	0.81	0.14	0.19	0.81	0.09

Table VII (b): Robustness for Nitazoxanide.

Parameters (n=3)	Flow rate (ml/min)			Buffer : Acetonitrile			Wavelength		
	1.2	1.0	0.8	40:60	45:55	50:50	235	240	245
Retention Time (min.)	4.68	5.65	6.86	4.050	5.65	6.796	5.597	5.65	5.667
Theoretical plates (N)	5258.39	6934.32	5334.26	5254.33	6934.32	6746.22	5284.64	6934.32	5031.66
Tailing factor (T)	1.14	1.57	1.49	1.555	1.57	1.361	1.428	1.57	1.448
RSD	0.82	0.88	0.11	0.61	0.88	0.42	0.23	0.88	0.01
Resolution	16.13	18.53	16.33	9.979	18.53	18.700	16.171	18.53	15.552

CONCLUSIONS:

The proposed method described a new RP-HPLC method for the determination of Nitazoxanide and Ofloxacin in combined tablet dosage form. The method gives good resolution with a short analysis time (<7 min). The developed method was validated with a holistic approach according to ICH guidelines and found to be simple, sensitive, accurate and precise. Therefore, the proposed method can be used for the routine analysis of these drugs in their combined dosage form.

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