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RP-HPLC Method for Simultaneous Estimation of Nitazoxanide and Ofloxacin in Bulk Drug and in Pharmaceutical Formulation

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Abstract : A simple, precise, accurate, rapid and reproducible reverse phase high performance liquid chromatographic procedure was developed for simultaneous determination of nitazoxanide and ofloxacin in tablet dosage form was carried on an Inert ODS-3V (250X4.6 nm, 5micro) column using a mobile phase consisting of methanol and buffer (20:80 v/v) with a pH3.3 ± at a rate of 1.5 ml/min and the detection was carried out at 230 nm. The linearity was found to be in the range of 5-60 µg/ml and 2-24 µg/ml Nitazoxanide and Ofloxacin respectively with (r^2=0.9990, and r^2=0.9994). The peaks obtained were sharp having clear baseline separation with a retention time of 9.093 ± 0.03 and 5.946 ± 0.03 min for Nitazoxanide and Ofloxacin respectively. The results of the analysis were validated statistically and recovery studies confirmed the accuracy of the proposed method.
Key word s: Nitazoxanide, Ofloxacin, RP-HPLC, Simultaneous Estimation.

1. Introduction

Nitazoxanide (NTZ), Chemically, 2-(Acetyloxy)-N-(5-nitro-2-thiazolyl) Benz amide is a drug that fights protozoan infections other than malaria. It treats diarrheacaused by *Cryptosporidium parvum* and *Giardia lamblia*. In children over one year of age, adolescents, and adults, Nitazoxanide reduces the duration of diarrhea and helps in the elimination of protozoa from the body.It is freely soluble in Dimethyl formamide, Methanol, Acetonitrile, sparingly soluble in Acetone [1]. The chemical name of Ofloxacin (OFX) is 9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido(1,2,3-di)-1,4-benzoxazine-6-carboxylic acid. It belongs to a group of broad-spectrum antibiotics called the quinolones.Ofloxacin is a broad-spectrum antibioticthat is active against both Gram-positive and Gram-negative bacteria. It functions by inhibiting DNA gyrase, a type II

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topoisomerase, and topoisomerase IV, which is an enzyme necessary to separate replicated DNA, thereby inhibiting cell division. It is slightly soluble in water, freely soluble in acetonitrile, methanol and glacial acetic acid [2].

Literature survey revealed that there are several methods such as High-Performance Liquid Chromatography, UV spectroscopy and Colorimetric have been reported for individual drugs as well as in combination with other drugs in formulation[3-11].But the reported methods wereexpensiveand less sensitive. Hence, an attempt has been made to develop simple, accurate, sensitive, rapid and economic method for simultaneous estimation of Nitazoxanide and Ofloxacin from their combined dosage forms using High Performance Liquid Chromatography technique. These methods can also be applied for estimation of pure drug sample from bulk.

2. Experimental

2.1 Materials and methods

The working standard which is of Pharmaceutical grade NTZ and OFX was obtained as a gift sample from Karnataka Antibiotic and Pharmaceuticals Limited, Karnataka, India. The tablet dosage form (Inflobid NXT, Label claim: 200 mg OFX and 500 mg NTZ) was procured from the local market Intra Labs India Pvt Ltd, Bangalore - 560026. All chemicals used were of HPLC grade and were purchased from Spectrochem, Mumbai, India.

2.2 Instrument used:

LC system [Agilent 1100 series (Chemstation)] used consist of pump (L-7100 double reciprocating pump) with universalRheodyne loop injector of injection capacity 20 µl gradient system. Detector consists of UV-detector, L-7400 (190-666 nm), Shimadzu; the reversed phase column used was Inert ODS-3V (250X4.6 nm, 5micro), at ambient temperature. Among the several mobile phases used for the simultaneous estimation of NTZ and OFX, mobile phase consisting of methanol and buffer (20:80 v/v) with a pH3.3 ±1 was found to be most suitable and was filtered through 0.45 µm Nylon filter paper.

2.3 Preparation of standard stock solution:

100 mg each of standard Nitazoxanide and Ofloxacin was weighed accurately and transferred to two separate 100 ml volumetric flasks. Both the drugs were dissolved in 50 ml of methanol with shaking and then volume was made up to the mark with methanol to obtain final concentration of 1000 µg/ml of each component (stock 'A' solution). These stock solutions were filtered through 0.45 µm Nylon filter paper.

Selection of analytical wavelength:

By appropriate dilution of each standard stock solution with methanol, various concentrations of Nitazoxanide and Ofloxacin were prepared separately. Each solution was scanned using double beam UV visible spectrophotometer L-7400 in the "Spectrum mode" between the range of 400 nm to 200 nm and their spectra was overlaid. From the overlain spectra of Nitazoxanide and Ofloxacin, 230.0 nm was selected as analytical wavelength for Multicomponent analysis using HPLC method.

2.4Chromatographic condition:

The mobile phase containing methanol and buffer in the ratio of 20:80 v/v was selected as the optimum composition of mobile phase, because it was found that this solvent system resolved both the components ideally. The flow rate was set to 1.5 ml/min and UV detection was carried out at 230 nm. The mobile phase and samples were degassed by ultrasonication for 20 min and filtered through 0.45 µm Nylon filter paper. All determinations were performed at constant column temperature (25^0C).

A study to establish the interference of the blank was conducted. Diluent was injected into the chromatographic conditions and the blank chromatograms were recorded. Chromatogram of blank solution (Fig.1). The average retention times for Nitazoxanide and Ofloxacin was found to be 9.093 ± 0.03min and

5.946 ± 0.03 min, respectively (Fig. 2 &4).

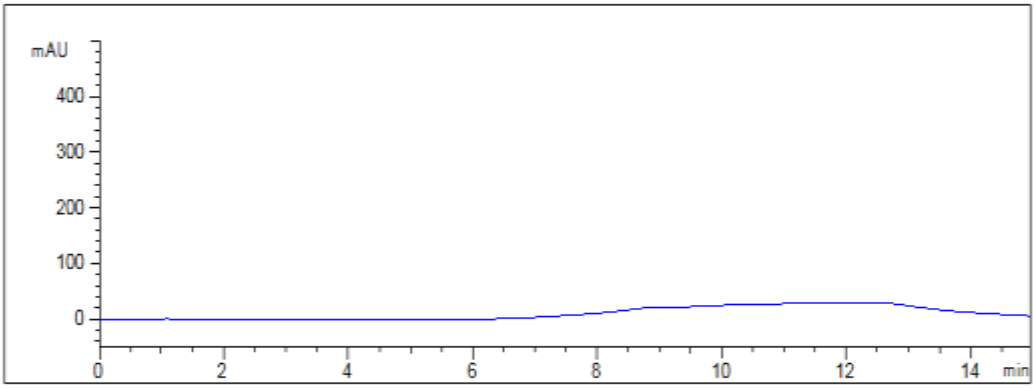

Fig: 1. Chromatogram for Blank.

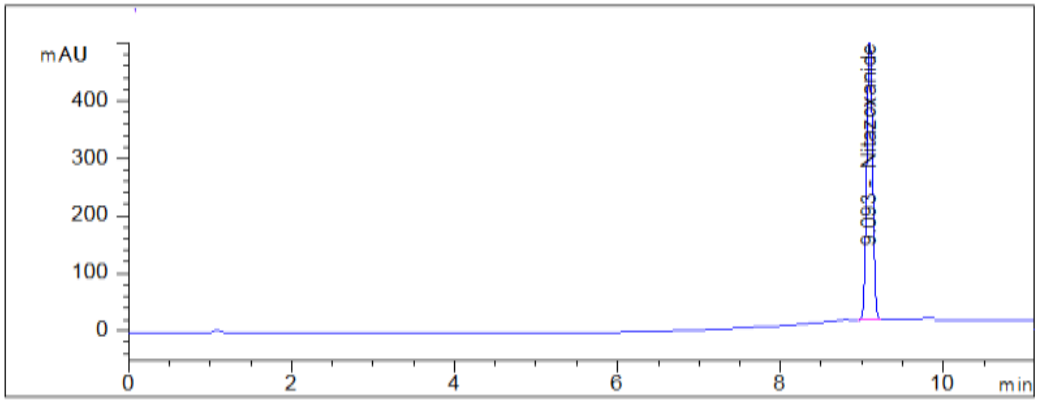

Fig: 2. Chromatogram for Nitazoxanide.

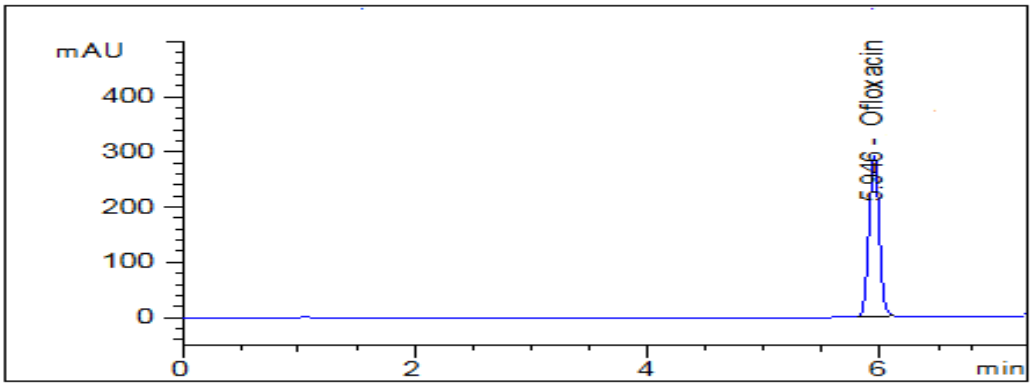

Fig: 4. Chromatogram for Ofloxacin.

2.5 Selection of analytical concentration range and preparation of calibration curve for Nitazoxanide and ofloxacin:

<u>Nitazoxanide</u>:

Appropriate aliquots were pipetted out from the standard stock solution (100 µg/ml) in to a series of 10 ml volumetric flasks. The volume was made up to the mark with the methanol to get a set of solutions having the concentration range, ranging from 5, 10, 20,30,40,50,60 µg/ml of Nitazoxanide.

Ofloxacin:

Appropriate aliquots were pipetted out from the standard stock solution (100 µg/ml) in to a series of 10 ml volumetric flasks. The volume was made up to the mark with the mobile phase to get a set of solutions having the concentration range, ranging from 2, 4, 8, 12, 16, 20, 24 µg/ml of Ofloxacin.

Triplicate dilutions of each of the above-mentioned concentrations were prepared separately and from these triplicate solutions 20 µl of each concentration of the drug were injected into the HPLC system two times separately and their chromatograms were recorded under the same chromatographic conditions as described above.

Both the drugs follow the Beer's & Lambert's law in the concentration range of 5-60 µg/ml for Nitazoxanide and 2-24 µg/ml for Ofloxacinand Peak areas were recorded for all the peaks and a standard calibration curve of AUC against concentration was plotted. (Table. 1 and Fig. 3 for Nitazoxanide and Table. 2 and Fig 5 for Ofloxacin respectively) and Statistical data of Nitazoxanide and Ofloxacin at 230 nm by HPLC method (Table. 3).

Table: 1. Result of calibration curve for Nitazoxanide at 230 nm by HPLC Method.

Sl. No.	Concentration (µg/ml)	Area Under Curve
1.	5	318.99
2.	10	429.32
3.	20	593.28
4.	30	765.86
5.	40	938.29
6.	50	1108.36
7.	60	1256.84

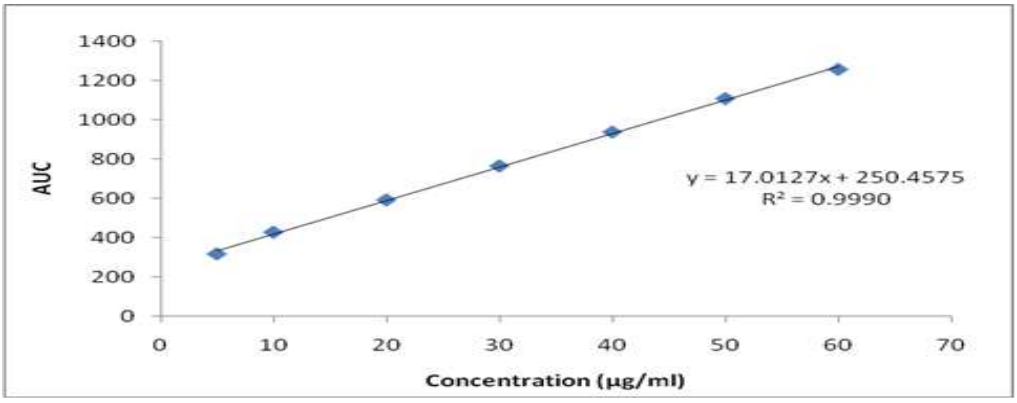

Fig: 3. Calibration curve of Nitazoxanide at 230 nm by HPLC Method.

Table: 2. Result of calibration curve for Ofloxacin at 230 nm by HPLC Method.

Sl. No.	Concentration (µg/ml)	Area Under Curve
1.	2	148.21
2.	4	345.28
3.	8	702.35
4.	12	998.26
5.	16	1347.96
6.	20	1697.49
7.	24	2062.06

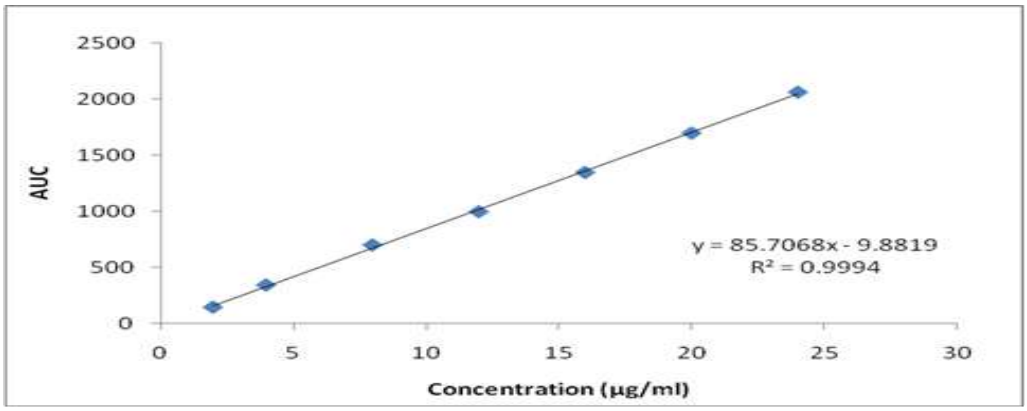

Fig: 5. Calibration curve of Ofloxacin at 230 nm by HPLC Method.

Table: 3. Statistical data of Nitazoxanide and Ofloxacin at 230 nm by HPLC method.

Parameter	**NTZ**	**OFL**
Linear Range (µg/ml)	5-60	2-24
Slope	17.0127x	85.7068x
Intercept	250.4575	9.8819
Limit of Detection (µg/ml)	0.9	0.3
Limit of Quantification (µg/ml)	2.7	0.9

Analysis of tablet formulation:

Twenty tablets of Nitazoxanide and Ofloxacin in combination were weighed individually and their average weight was determined. The tablets were then crushed to fine powder and powder equivalent to 50 mg of Nitazoxanide and 20 mg of Ofloxacin was weighed and transferred to 100 ml volumetric flask and dissolved in sufficient quantity of methanol. The contents were ultrasonicated for 15 minutes and the final volume was made up to the mark with methanol (20%).

The above-prepared solution was then filtered through 0.45 µm Nylon filter paper and was used as standard stock solution. Appropriate aliquot was pipetted out from the standard stock solution and was further diluted with the methanol (20%) to obtain a mixture containing 50 µg/ml of Nitazoxanide and 20 µg/ml of Ofloxacin. Six different mixtures containing 50µg/ml of Nitazoxanideand20µg/ml of Ofloxacin were prepared as above from the standard stock solution. A 20-µl volume of each sample mixture was injected in to the sample injector of HPLC system and their chromatograms were recorded under the same chromatographic conditions as described above. The area of each peak was determined at 230 nm and the amount of drug present in the sample mixture was determined.(Fig. 6 and Table 4& 5).

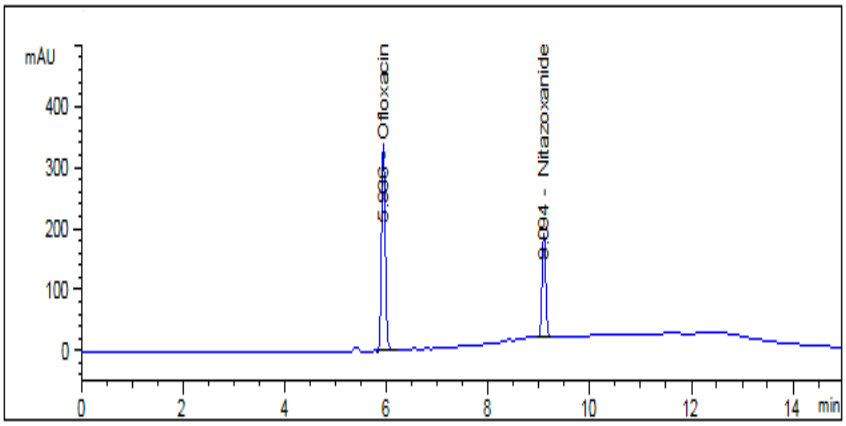

Fig: 6. Chromatogram of mixture of Nitazoxanide and Ofloxacin.

Table: 4. Assay results of tablet formulation.

Sr. No.	Amount present in (mg/tab)		Amount obtained in (mg/tab)		Label Claim %	
	NTZ	OFL	NTZ	OFL	NTZ	OFL
1	50	20	49.97	19.98	99.94	99.90
2	50	20	49.86	19.89	99.72	99.45
3	50	20	50.02	19.99	100.02	99.96
4	50	20	49.99	20.01	99.98	100.05
5	50	20	49.95	20.09	99.90	100.45
6	50	20	49.89	19.89	99.80	99.45

Table: 5. Statistical validation data for Tablet Formulation.

Components	Mean*	Standard Deviation*	Co-efficient of Variation*
NTZ	99.87	0.1136	0.1137
OFL	99.89	0.3821	0.3825

*n = 6

2.6 Method Validation[12-14]

Validation of an analytical method is the process to establish by laboratory studies that the performance characteristic of the method meets the requirements for the intended analytical application. Performance characteristics are expressed in terms of analytical parameters.

Accuracy

Procedure for determination of Accuracy:

Recovery studies were carried out by applying the method to drug sample present in tablet dosage form to which known amount of Nitazoxanide and Ofloxacin corresponding to 80%, 100% and 120% of label claim was added (standard addition method). In 80% recovery study, amount of standard added is 40 mg of Nitazoxanide and 16 mg of Ofloxacin (i.e., 80% addition). In 100% recovery study the amount of standard added is 50 mg of Nitazoxanide and 20 mg of Ofloxacin (i.e., 100% addition). In 120% recovery study the amount of standard added is 60 mg of Nitazoxanide and 24 mg of Ofloxacin (i.e., 120% addition). After the addition of the standards the contents were transferred to 100 ml volumetric flask and dissolved in 50 ml

methanol and the content was kept in ultrasonicator for 10 min. Finally, the volume was made up to the mark with methanol. The solution was filtered through Whatmann filter paper No.41. The accuracy of Nitazoxanide and Ofloxacin and statistical validation data were determined (Table 6 & 7).

Table: 6. Determination of Accuracy of Nitazoxanide and Ofloxacin.

Level of % recovery	Amount present (mg/tab)		Amount of standard drug added (mg)		Total amount recovered (mg)		% Recovery	
	NTZ	OFL	NTZ	OFL	NTZ	OFL	NTZ	OFL
80%	50	20	40	16	89.96	35.96	99.95	99.88
	50	20	40	16	89.99	35.94	99.98	99.83
	50	20	40	16	89.89	35.99	99.87	99.97
100%	50	20	50	20	100.01	39.96	100.01	99.96
	50	20	50	20	100.06	40.02	100.06	100.02
	50	20	50	20	99.99	39.99	99.98	99.98
120%	50	20	60	24	109.94	43.97	99.94	99.93
	50	20	60	24	110.01	43.92	100.01	99.89
	50	20	60	24	109.98	44.03	99.98	100.03

Table: 7. Statistical validation data for Accuracy determination.

Level of % recovery	Mean*		Standard Deviation*		Co-efficient of Variation*	
	NTZ	OFL	NTZ	OFL	NTZ	OFL
80%	99.93	99.89	0.0568	0.0763	0.0568	0.0568
100%	100.01	99.98	0.0404	0.0305	0.0403	0.0305
120%	99.97	99.95	0.0351	0.0721	0.0351	0.0721

*n = 3

Precision

Procedure for determination of Precision:

The precision of the analytical method was determined using the tablet sample. Tablet powder equivalent to 50 mg of Nitazoxanide and 20 mg of Ofloxacin was weighed and transferred to 100 ml volumetric flask and dissolved in 50 ml methanol and the content was kept in ultrasonicator for10 min. Finally, the volume was made up to the mark with methanol. The solution was filtered through Whatmann filter paper No.41.The above sample mixture was suitably diluted to obtain a sample mixture containing 50 µg/ml of Nitazoxanide and 20 µg/ml of Ofloxacin.

Intra-day Precision and Inter-day precision:

In intraday precision and inter-day precision, the sample mixture containing 50 µg/ml of Nitazoxanide and 20 µg/ml of Ofloxacin was analyzed six times at different time intervals in the same day. The concentration of the sample mixture was determined as per the procedure given for the tablet formulation by determining AUC at selected analytical wavelength 230 nm.The variation of the results within the same day on different days was analyzed and statistically validated respectively(Table 8 & 9).

Table: 8. Statistical validation data for Intra-day Precision.

Components	Mean*	Standard Deviation*	Co-efficient of Variation*
NTZ	99.98	0.1984	0.1984
OFL	100.04	0.1865	0.1864

*n = 6

Table: 9. Statistical validation data for Inter-day Precision.

Components	Mean*	Standard Deviation*	Co-efficient of Variation*
NTZ	99.88	0.7953	0.7962
OFL	100.19	0.6453	0.6440

*n = 3

Specificity and Selectivity

The specificity of the HPLC method was determined by complete separation of Nitazoxanide and Ofloxacin, with parameters like retention time (t_R), resolution (R_S) and tailing factor (T_f). Here tailing factor for peaks of Nitazoxanide and Ofloxacin was less than 2% and resolution was more than 1%. The average retention time ± standard deviation for Nitazoxanide and Ofloxacin were found to be (9.093 ± 0.03 and 5.946 ± 0.02) respectively for six determinations. The peaks obtained for Nitazoxanide and Ofloxacin were sharp and have clear baseline separation.

Robustness

The evaluation of robustness should be considered during the development phase and depends upon the type of procedure under study. It should show the reliability of analysis with respect to deliberate variations in method parameters. The solution containing 50µg/ml of Nitazoxanide and 20 µg/ml of Ofloxacin was injected into sample injector of HPLC three times under different parameters like deliberate variations in flow rate (Table 10) and column temperature (Table 11) were determined.

Table: 10. Robustness results for variations in flow rate (ml/min).

Method Parameter	Level	Retention Time		Tailing factor		Amount obtained %	
Flow Rate (ml/min)		NTZ	OFL	NTZ	OFL	NTZ	OFL
1.4	-0.1	9.345	6.126	1.12	1.90	100.54	98.90
1.5	0	9.093	5.946	1.13	1.91	100.04	100.02
1.6	+0.1	8.938	5.819	1.11	1.90	99.55	98.99

Table: 11. Robustness results for variations in column temperature.

Method Parameter	Level	Retention Time		Tailing factor		Amount obtained %	
Columns Temperature		NTZ	OFL	NTZ	OFL	NTZ	OFL
24ºC	-1	9.182	5.985	1.11	1.92	99.75	100.56
25ºC	0	9.093	5.946	1.12	1.90	100.04	100.02
26ºC	+1	9.106	5.911	1.11	1.92	100.97	99.95

3. Result and Discussion

The objective of the proposed work was to develop simultaneous methods for the determination of Nitazoxanide and Ofloxacin, and to validate the methods according to USP and ICH guidelines and applying the same for its estimation in marketed formulations.

UV Spectrophotometric, and HPLC methods developed were found to be rapid, simple, precise, accurate and economic for routine estimation of Nitazoxanide and Ofloxacin simultaneously in commercial dosage forms.

In HPLC method, the conditions were optimized to obtain an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried, to separate title ingredients. Mobile phase and flow rate selection was based on peak parameters (height, tailing, theoretical plates, capacity or symmetry factor), run time, resolution. The system with methanol: buffer with 1.5ml.min^{-1} flow rate is quite robust. The optimum wavelength for detection was 230 nm at which better detector response for both the drugs was obtained. A study to establish the interference of the blank was conducted. Diluent was injected into the chromatographic conditions and the blank chromatograms were recorded. Chromatogram of blank solution.The average retention times for Nitazoxanide and Ofloxacin was found to be 9.093 ± 0.03min and 5.946 ± 0.03 min, respectively. System suitability tests are an integral part of chromatographic method. They are used to verify the reproducibility of the chromatographic system. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared stock solutions.

The calibration was linear in concentration range of 5-60µg/ ml and 2-24µg/mL, with regression 0.9990 and 0.9994, intercept 250.4575 and 9.8819and slope 17.0127 and 85.7068 for Nitazoxanide and ofloxacin respectively (Table 13). The low values of % R.S.D indicate the method is precise and accurate. The mean recoveries were found in the range of 99 – 102 %.

Sample to sample precision and accuracy were evaluated using six samples of five different concentrations, which were prepared and analyzed on same day. Day to day variability was assessed using six concentrations analyzed on three different days over a period of three days. These results showed the accuracy and reproducibility of the assay.

Robustness of the proposed method was determined by varying various parameters, the percentage R.S.D. reported was found to be less than 2 % (Table 12). The proposed method was validated in accordance with ICH parameters and the applied for analysis of the same in marketed formulations.

Table: 12. Statistical validation of robustness results for variations in method Parameters.

Method Parameters	**Mean***		**Standard Deviation***		**Co-efficient of Variation***	
	NTZ	**OFL**	**NTZ**	**OFL**	**NTZ**	**OFL**
Flow Rate (ml/min)	9.125	5.96	0.0152	0.0057	0.5390	0.1008
Column Temperature	9.127	5.94	0.0057	0.0057	0.2021	0.1008

Table: 13. Summary of validation and system suitability parameters of Nitazoxanide and Ofloxacin.

Parameters	**NTZ**	**OFL**
Linear range (µg/ml)	5-60	2-24
Slope	17.0127	85.7068
Intercept	250.4575	9.8819
Regression coefficient (r^2)	0.9990	0.9994
Limit of Detection (µg/ml)	0.9172	0.3232
Limit of Quantification (µg/ml)	2.779	0.9794
Retention time (min)	9.093 ± 0.03	5.946 ± 0.03
Tailing factor	1.11	1.90
Resolution factor	34.28	
Theoretical plate	13537	6568

4. Conclusion

A simple, economic, accurate and precise HPLC method was successfully developed using Methanol: Buffer as mobile phase. The peaks obtained were sharp with retention time of 9.093 min for Nitazoxanide and 5.948 min for Ofloxacin. The peaks were well resolved with a resolution factor of 34.28. The method was precisely applied to the tablet formulation and the results obtained were accurate and reproducible.

All the developed methods were statistically validated in terms of accuracy, precision, linearity and repeatability.

5. Acknowledgement

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