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RP-HPLC Analytical Method Development and Validation for Simultaneous Estimation of two Drugs Nitazoxanide, Ofloxacin and its Pharmaceutical Dosage Forms

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Abstract: Establishment of a single analytical method for estimation of individual drug from a multi-drug composition is a very challenging task. A rapid, simple and precise HPLC method was developed for the separation and estimation of two drugs Nitazoxanide and Ofloxacin from bulk drug mix and pharmaceutical dosage forms. The estimation was carried out using Luna C18 (250mm x 4.6mm, 5µm) column; mobile phase consisting of Acetonitrile and buffer at pH 4; the flow rate of 1.5ml/min and ultraviolet detection at 280 nm. Both drugs were properly resolved having run time of 3.7 min and 1.5 min for Nitazoxanide and Ofloxacin, respectively. The method was validated as a final verification of method development with respect to Precision, Linearity, Accuracy, Ruggedness and Robustness. The validated method was successfully applied to the commercially available pharmaceutical dosage form, yielding very good and reproducible result.
Key words: Nitazoxanide, Ofloxacin HPLC, LC Method development and validation.

1. INTRODUCTION

Nitazoxanide is used to treat diarrhea in children and adults caused by the protozoa Cryptosporidium or Giardia. Protozoa are suspected as the cause when diarrhea lasts more than seven days. Nitazoxanide is in a class of medication called antiprotozoal agent. It works by stopping the growth of protozoa that causes diarrhea. It is chemically 2-(acetolyloxy)-*N*-(5-nitro-2-thiazolyl) benzamide and its structure is as shown in Figure 1. Ofloxacin is a class of antibiotic called fluoroquinolones that stops bacterial multiplication by inhibiting the reproduction of the genetic material (DNA). It is chemically (*RS*)-7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo [7.3.1.0^{5,13}]trideca-5(13),6,8,11-tetraene-11-carboxylic acid and its structure is as shown in Figure 1. Both these drugs are used in the medication for children and they are prescribed either individually or in combination. The use of these drugs in combination was approved by DCGI, Govt of India. Keeping in view of their medical importance, these drugs were selected for the experimental work. As both these drugs are widely used in combination in the market, their medical importance is enhanced. Hence, it was found encouraging to develop a single Rapid method by which both the drugs could be estimated simultaneously. For individual estimation of each drug several methods[1-6] are available in the literature.

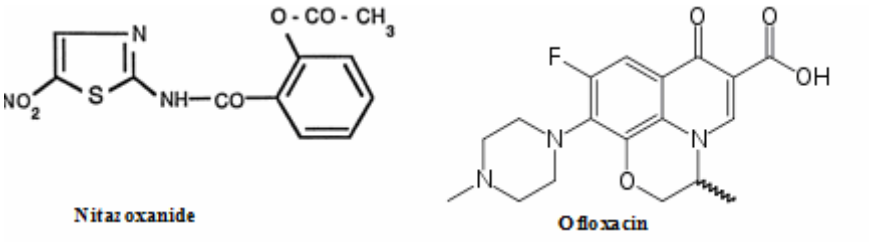


Figure 1: Structure of Nitazoxanide and Ofloxacin

There are couple of methods[7-10] available for the simultaneous estimation of both the drugs but they have their own limitations.

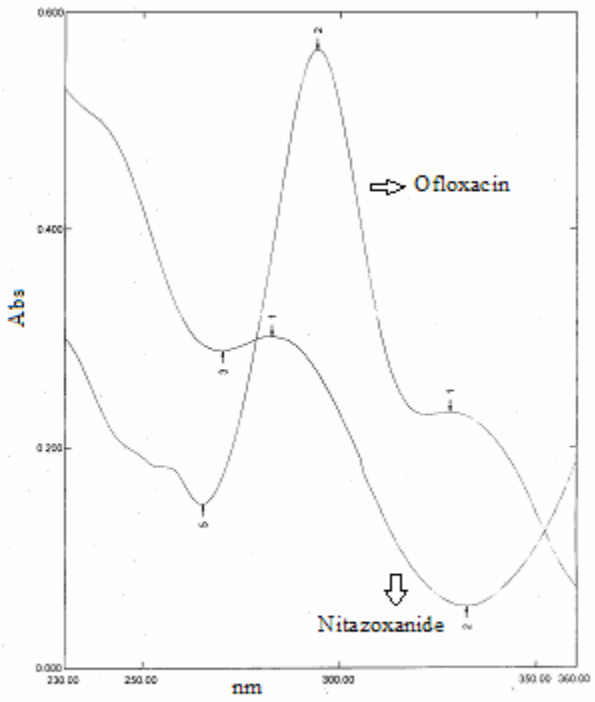
Through this article, we have tried our best to contribute, in the field of basic Pharmaceutical Analytical Chemistry, a very fast and user friendly method for the simultaneous estimation of Nitazoxanide and Ofloxacin using reverse phase-HPLC method in bulk drug mix and pharmaceutical dosage forms.

2. EXPERIMENTAL SECTION

2.1. Method Development

Both the drugs were scanned by UV, individually, in a wavelength range of 200-400 nm and maxima for each drug was measured. The maxima for Nitazoxanide was found to be 284.60nm, 330.60nm and 269.60nm whereas, for Ofloxacin maxima were found at 328.00nm, 294.20nm and 226.40nm. The corresponding UV spectrum graphs of the drugs Nitazoxanide and Ofloxacin are as shown in Figure 2. To optimise the UV maxima, various HPLC experiments were performed at different wavelengths starting from 240nm to 290nm. It was observed that both peaks were resolving at all six wave numbers namely, 240nm, 250nm

260nm, 270nm, 280nm and 290nm but experiment of HPLC run at 280 nm has been found to be better with respect to resolution of the peaks and balanced area acquisition of both drugs. Hence, wavelength of 280 nm was finalized for the data acquisition in HPLC for the simultaneous estimation of both the drugs. At this wavelength the retention time for Ofloxacin was 1.6 min and for Nitazoxanide 3.7 min. Respective graphs are represented in Figure 3.
While optimizing the mobile phase pH, the resolution pattern was studied at different pH starting from neutral pH , as it is beneficial for Human and instrument safety. The peaks got merged and no separation of peaks observed for Nitazoxanide and Ofloxacin. The pH was increased to 7.5 and here also we observed similar merged peaks only. We reduced the pH of mobile phase to 5 and here we observed that separation has started taking place for both the peaks (Nitazoxanide and Ofloxacin) but the resolution was not good at all. Then the experiments have been performed at pH 4 also. The peaks of Nitazoxanide and Ofloxacin were nicely resolved at pH 4. Respective graphs are represented in Figure 4. Such experiment has been performed to contribute about the effect of pH during HPLC analysis.
Various experiments to optimize the HPLC pump flow rate was done and ultimately optimized the flow rate of 1.5ml/min to get the better resolution and rapid analysis of the drug combination. The representative chromatogram is shown in Figure 5. The finalized method of analysis has been summarized in section 2.3.



	Nitazoxanide	Ofloxacin
1	284.60nm	328.00nm
2	330.60nm	294.20nm
3	269.60nm	226.40nm

Figure 2 : UV Spectrum graph

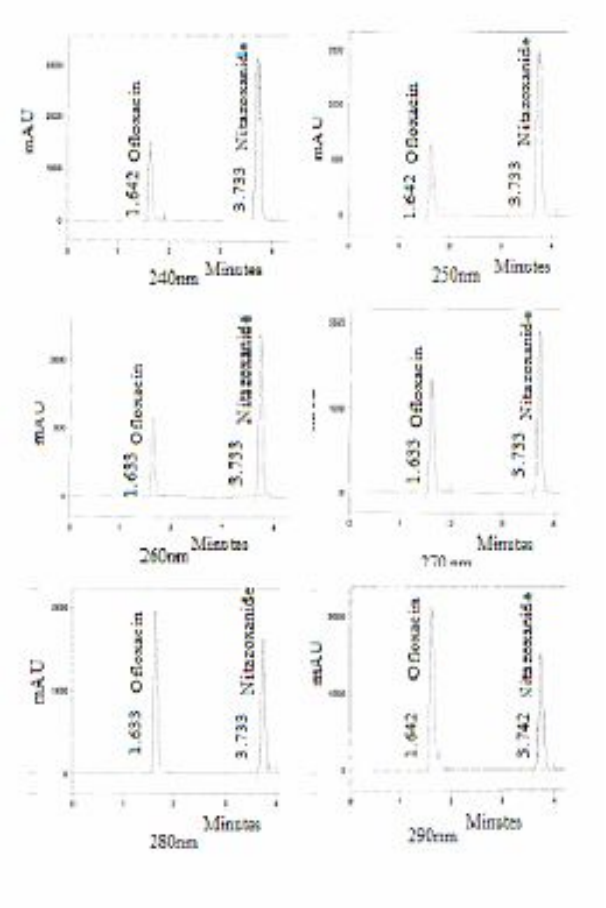


Figure 3: Chromatograms at different Wave lengths

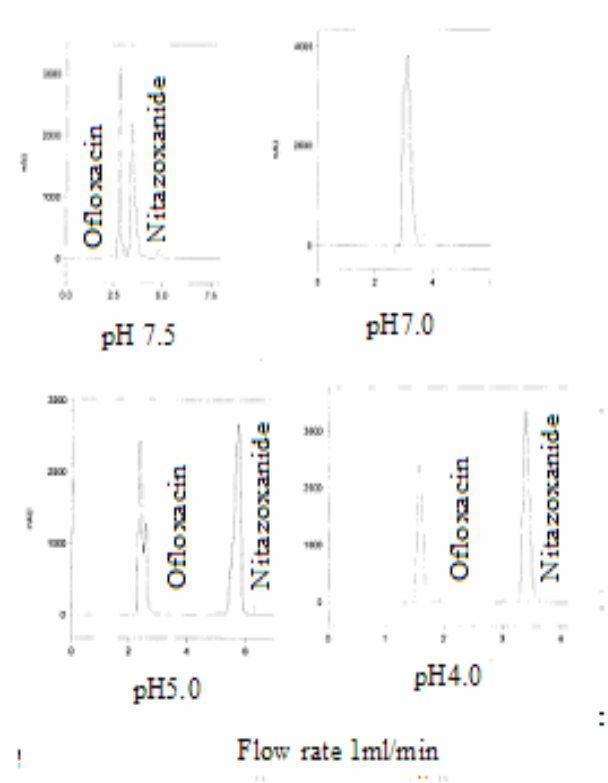


Figure 4: Chromatograms at different pH values

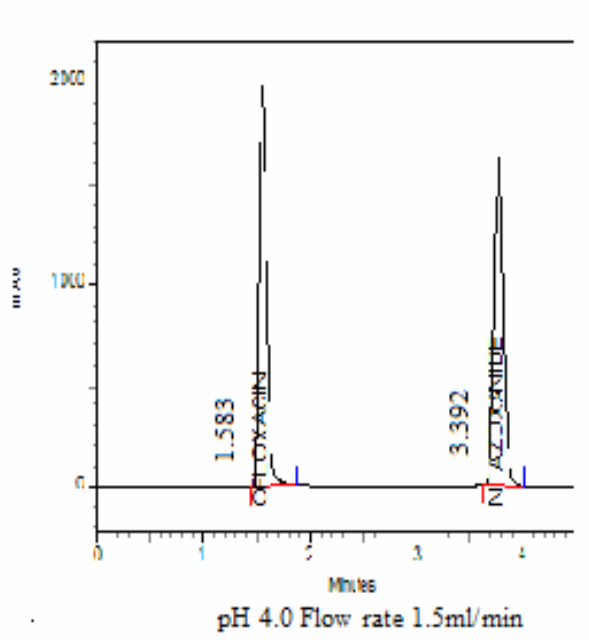


Figure 5: Chromatogram at an increased Flow rate of 1.5ml/min

2.2. Materials, Reagents and Chemicals

Nitazoxanide and Ofloxacin Standards were obtained from Startech Labs. Combination drug Tablets used for the experiment, NITA-O, was manufactured by Alembic Limited and Zenflox NT was manufactured by Windlas Biotech Limited. HPLC grade Acetonitrile, Orthophosphoric acid and Sodium hydroxide were obtained from Merck, Darmstadt, Germany.

2.3. Equipments

UV Visible spectrophotometer used was Shimadzu, model UV-2450. The HPLC instrument used was Schimadzu make, model-LC-2010 CHT. Class VP Software was used for data acquisition.

2.4. Chromatographic conditions

The Chromatographic column, Luna C18 (250mm x 4.6mm, 5µm) column was used as a stationary phase. Mobile phase was prepared with Buffer and Acetonitrile (40:60). Buffer was prepared by dissolving 1ml of Orthophosphoric acid in 1000 ml of water. The pH was adjusted to 4.0 with 1N Sodium hydroxide solution. Injection volume was 20µL. The pump flow rate was 1.5ml/min. The column temperature was maintained at 25ºC. The eluent was detected at 280 nm. The run time was 10 min.

2.5. Preparation of Standard solution

Standard solution of 1mg/ml of Nitazoxanide and 0.4mg/ml of Ofloxacin (treat this as 100 % for

various experimental purpose) was prepared by taking 50mg of Nitazoxanide and 20 mg of Ofloxacin in 50ml standard flask and diluted upto the mark with mobile phase.

2.6. Preparation of Linearity solutions

For Linearity 150%, 125%, 100%, 75%, & 50% solutions were prepared.

150% Solution was prepared by using 150mg of Nitazoxanide and 60mg of Ofloxacin was dissolved in 100ml for 150% solution. 20.83 ml of 150% solution was taken in a 25ml standard flask and make up with mobile phase for 125% solution. 16.67 ml of 150% solution was taken in a 25ml standard flask and make up with mobile phase for 100% solution. 12.5ml of 150% solution was taken in a 25ml standard flask and make up with mobile phase for 75% solution. 8.33ml of 150% solution is taken in a 25ml standard flask and make up with mobile phase for 50% solution.

2.7. Sample preparation for Accuracy

Five different solutions were prepared for performing the accuracy studies. The first solution was prepared by dissolving 10mg each of Nitazoxanide and Ofloxacin in 25 ml standard flask and make up the solution with 50% linearity solution. The second solution was prepared by dissolving 10mg each of Nitazoxanide and Ofloxacin in 25 ml standard flask and make up the solution with 75% linearity solution. The third solution was prepared by dissolving 10mg each of Nitazoxanide and Ofloxacin in 25ml standard flask and make up the solution with 100% linearity solution. The fourth solution was prepared by dissolving 10mg each of Nitazoxanide and Ofloxacin in 25 ml standard flask and make up the solution with 125% linearity solution. The fifth solution was prepared by dissolving 10mg each of Nitazoxanide and Ofloxacin in 25 ml standard flask and make up the solution with 150% linearity solution.

2.8.Preparation of Sample solution for Batch Analysis

Two commercial samples were used for batch analysis.Ten tablets were weighed and their average weight were calculated. The average weight was found to be 414.19mg and 425.63mg of NITA-O and Zenflox NT, respectively . The tablet was crushed to a homogeneous mixture and 41.45 mg of NITA-O tablet and 41.85mg of Zenflox NT tablet has been dissolved in 50ml each of the mobile phase. To extract the drug in solution, sonicated for 5 minutes followed by cyclomixing for 5 minutes. The resulting solution was filtered by using Millipore syringe filter (0.42µ). The resulting clear solution was injected in HPLC in duplicate as per the developed method.

2.9. Analytical Method Validation

2.9.1. Specificity of the method

The terms selectivity and specificity are often used interchangeably. Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. This parameter was performed to know the Retention Time of each drug in a mixture and in the sample to understand if any drug-drug interaction or drug-excipient interaction is present.

2.9.2. System Suitability

System suitability test is used to verify if the resolution and reproducibility of the chromatographic systems are adequate for the analysis to be done. The tests are based on the fact that the equipment, electronics, samples to be analyzed constitutes an integral system that can be evaluated as such. The limits for system suitability were set for Theoretical plates, Resolution, Asymmetry.

2.9.3. Linearity

Five concentrations of the standard mixture, 50%, 75%, 100%, 125% and 150% were injected and chromatogram was recorded. A graph was plotted for the concentration of the corresponding drug versus Area. The Correlation coefficient(r) for each drug was calculated.

2.9.4. Accuracy

To determine the accuracy in sample preparation method of standard additions was made for measuring the recovery of the drugs. To the known standard solution concentrations of the drug (50%, 75%, 100%, 125%, and 150%) was added. Five different solutions were prepared as mentioned in section 2.7. The accuracy was expressed as the percentage of the analytes recovery.

2.9.5. Method Precision

It is very important that the method developed should be precise. Six replicates of the sample prepared from the commercial tablets were injected and Assay was calculated to measure the repeatability of retention times and peak area of standard and sample.

2.9.6. Robustness

To verify the robustness of the method, the analysis was done under variable flow rates. The flow rate as per the developed method is 1.5ml/min. This has been purposely changed to 1.3ml/min and 1.7ml/min and the chromatogram was obtained.

2.9.7. Ruggedness

To test the ruggedness of the method, the analysis was done on different days and different chemists to check for any changes in the chromatograph. The percentage RSD for the retention time and area was calculated.

2.9.8.Performance of the method on Commercial Samples

The method is said to be effective if it can be applied for the analysis of commercial tablets. For this purpose, performance test of the method has been conducted on two market samples NITA-O, manufactured by Alembic limited, Batch No 10321004 and Zenflox NT manufactured by Windlas Biotech Limited, Batch No ZNX35.

3. RESULTS AND DISCUSSION

After several experiments above method has been optimized. Very interestingly, by following our method, the elution pattern of both the drugs is entirely reverse than reported in one of the article[9] . In some of the articles[10] authors have tried to work at low pH mobile phase, which is not good with respect to the life of the stationary phase.

In the recent days, industries are looking for the methodology which can save sophisticated instruments and chemist's valuable time and as a result they can release their product analysis report within lesser time. This is the reason why people are more attracted towards UFLC (Ultra-Fast Liquid Chromatography) [11-14] methods, though most of the Pharmacopeia still have the HPLC methods.

Keeping all these points in mind, the current method has been developed and it is very fast and encouraging. The developed method was validated with a holistic approach according to ICH guidelines and details of findings are expressed in following lines :

3.1. Specificity of the method

The Retention times of the standard drugs individually were measured and it was found to be 3.750 min and 1.533min for Nitazoxanide and Ofloxacin, respectively. The drugs were mixed and injected for taking the chromatogram. Both drugs were resolved very nicely in the mixture. Retention time of both drugs in Standard mix was found to be 3.760 min and 1.542 min for Nitazoxanide and Ofloxacin, respectively. This indicates there is no drug- drug interaction. The pharmaceutical dosage form (Tablet) was then injected and the chromatogram was obtained. The Retention time of the drugs in the Dosage Form was found to be 3.750 min and 1.542 min for Nitazoxanide and Ofloxacin, respectively. There is no specific change in Retention time of both drugs, which indicates that there is no Drug-Excipient interaction. Respective HPLC chromatograms are represented in Figure 6.

The resolution between Nitazoxanide and Ofloxacin was 34.28 which indicates a very good separation. The asymmetry factor for Nitazoxanide and Ofloxacin were 1.24 and 1.03, respectively. Therefore, this is a suitable method for the simultaneous estimation of Nitazoxanide and Ofloxacin in drug mixture and Dosage forms.

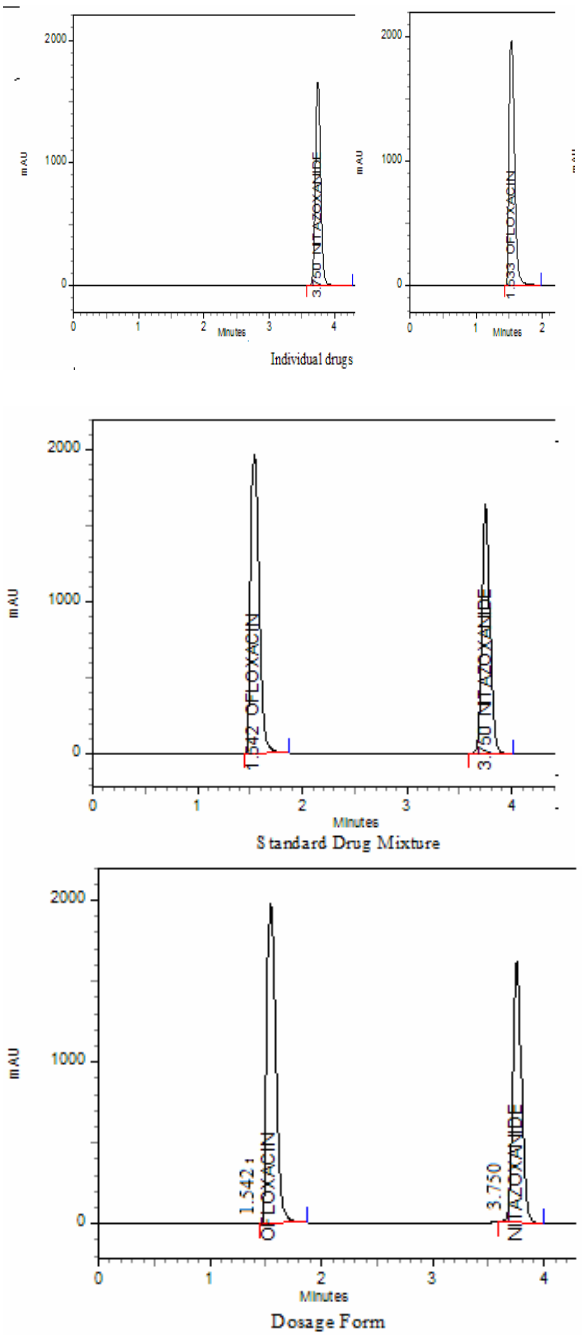


Figure 6: Chromatograms of Drugs

3.2. System Suitability

Five injections of the standard mix and two injections of the sample were injected for this purpose. The Resolution, Areas, Retention time, Theoretical plates values and peak Asymmetry were calculated for standard and sample solutions. Results obtained are given in following Table1.

Table 1: System suitability Results

Standard		Average	SD	% RSD
Nitazoxanide	Retention Time	3.763	0.004	0.13
	Area	8737421	22507.9	0.26
	Resolution	14.874	0.08	0.56
	Theoretical Plates	10333	-	-
	Asymmetry	1.15	-	-
Ofloxacin	Retention Time	1.542	0	0
	Area	11064903	19876.52	0.18
	Resolution	0	0	0
	Theoretical Plates	1676.006	-	-
	Asymmetry	1.482	-	-
Dosage form				
Nitazoxanide	Retention Time	3.758	0	0
	Area	8664919	15152.59	0.17
	Resolution	14.75	0.18	1.24
	Theoretical Plates	10049.16	-	-
	Asymmetry	1.095	-	-
Ofloxacin	Retention Time	1.542	0	0
	Area	11057507	16247.9	0.14
	Resolution	--	--	--
	Theoretical Plates	1676.93	-	-
	Asymmetry	1.495	-	-

3.3. Linearity

The correlation coefficient (r) obtained was calculated and it was found to be greater than 0.99 for Nitazoxanide and Ofloxacin, which is well within the acceptance criteria. The results are shown in Table 2. The concentration was found to be proportional to the area and the response of the detector was determined to be linear over the range of 0.2 to 0.6mg/ml for Ofloxacin and 0.5 to 1.5mg/ml for Nitazoxanide as shown in the Figure 7.

Table 2 : Linearity results

	Linearity Range	Correlation Coefficient
Nitazoxanide	0.5-1.5mg/ml	0.9998
Ofloxacin	0.2-0.6mg/ml	0.9969

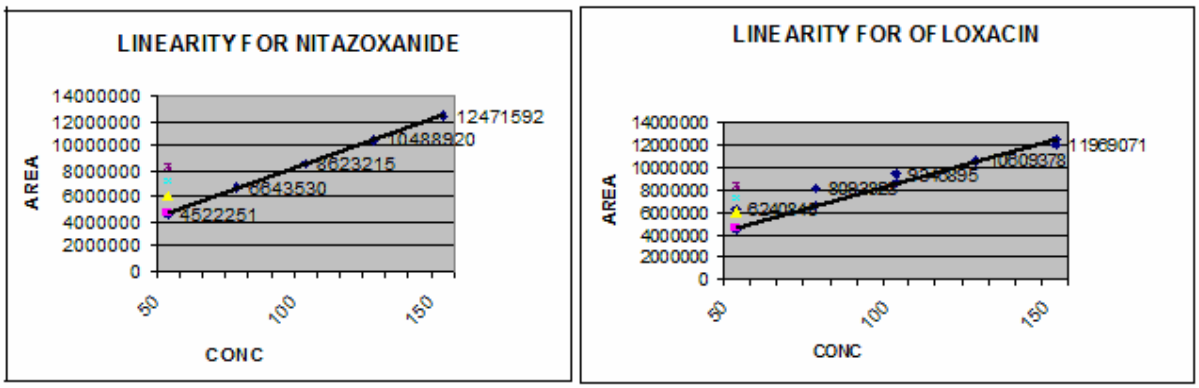


Figure 7 : Graphs Showing Linearity of the drugs

3.4. Accuracy

The results indicate that the recoveries are well within the acceptance range of 80 – 120 % , therefore, method is accurate and it can be used for the simultaneous estimation of Nitazoxanide and Ofloxacin. The percentage recovery of the results obtained are listed in Table 3.

3.5. Method Precision

The percentage RSD values for Area and Retention Time in precision study were calculated. The results as shown in Table 4 indicate that the method developed is precise.

3.6. Robustness

Due to deliberate change in the method, excellent performance of the method was observed. This indicates that the method is Robust. The results obtained are shown in Table 5.

Table 3: Results for Accuracy of the method

		Initial conc Area	Sol 1 Area	50% Area	Sol1-50% Area	%Recovery
Sol 1	Nitazoxanide	9229201	14254586	5127784	9126802	98.89
	Ofloxacin	9573259	15988642	6457546	9531096	99.56
		Initial conc Area	**Sol 2 Area**	**75% Area**	**Sol 2-75% Area**	**Recovery**
Sol 2	Nitazoxanide	9229201	16485546	7257719	9227827	99.99
	Ofloxacin	9573259	17744686	8247649	9497037	99.20
		Initial conc Area	**Sol 3 Area**	**100% Area**	**Sol 3-100% Area**	**Recovery**
Sol 3	Nitazoxanide	9229201	18499668	9229201	9270467	100.45
	Ofloxacin	9573259	19187986	9573259	9614727	100.43
		Initial conc Area	**Sol 4 Area**	**125% Area**	**Sol 4-125% Area**	**Recovery**
Sol 4	Nitazoxanide	9229201	19877564	10735585	9141979	99.05
	Ofloxacin	9573259	20414987	10820864	9594123	100.22
		Initial conc Area	**Sol 5 Area**	**150% Area**	**Sol 5-150% Area**	**Recovery**
Sol 5	Nitazoxanide	9229201	21856689	12643104	9213585	99.83
	Ofloxacin	9573259	21656338	12156095	9500243	99.24

Table 4: Method Precision results

	Retention Time		Area	
	Nitazoxanide	Ofloxacin	Nitazoxanide	Ofloxacin
1	3.695	1.501	8563271	10587458
2	3.697	1.498	8564315	10596371
3	3.698	1.5	8607243	10613872
4	3.699	1.512	8617425	10638541
5	3.698	1.511	8653482	10625874
Average	3.697	1.504	8601147	10612423
%RSD	0.44	0.44	0.44	0.2

Table 5: SD and RSD at different Flow rates

1.3ml/min	Standard %RSD	Sample %RSD
	Area	Area
Nitazoxanide	0.2	0.35
Ofloxacin	0.06	0.12
1.7ml/min	**Standard %RSD**	**Sample %RSD**
	Area	Area
Nitazoxanide	0.09	0.33
Ofloxacin	0.07	0.29

3.7. Ruggedness

Data acquired and % RSD of Area and Retention time was calculated for various trials and data tabulated in Table 6. Based on the data it is evident that the method is Rugged.

Table 6 : Assay and % RSD of the drugs on different days

DAY 1 & Analyst 1	Standard %RSD	
	Retention Time	**Area**
Nitazoxanide	0.8	1.03
Ofloxacin	0.5	1.14
DAY 2 & Analyst 2	**Standard %RSD**	
	Retention Time	**Area**
Nitazoxanide	0.6	1.01
Ofloxacin	0.5	0.94

3.8. Performance of the Method on Commercial Formulations

As per the label claim, both commercial tablets namely NITA-O and Zenflox NT contains 500mg of Nitazoxanide and 200mg of Ofloxacin. The quantification of both the drugs was done in both formulations and the results have been found to be in the range of 99.2 to 100.8 %. This indicates that the method developed by us can be used for the simultaneous estimation of Nitazoxanide and Ofloxacin in any of the pharmaceutical dosage forms. The results obtained are as shown below in the Table 7.

Table 7: Estimation of the drugs in commercial samples

NITA-O	Label claim	Acquired data	% recovery
Nitazoxanide	500 mg/tab	503.71 mg/tab	100.742%
Ofloxacin	200 mg/tab	198.49 mg/tab	99.245%
Zenflox NT	**Label claim**	**Acquired data**	**% recovery**
Nitazoxanide	500 mg/tab	504.28 mg/tab	100.856%
Ofloxacin	200 mg/tab	199.28 mg/tab	99.64%

4. CONCLUSION

A unique, user friendly, rapid and reproducible HPLC Method for simultaneous estimation of Nitazoxanide and Ofloxacin in Pharmaceutical dosages forms has been developed and validated as per ICH Guidelines. Therefore, this method can be used by the industries and academic institutions for their combination drug estimation, which is fast as well as safe.

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