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Simultaneous High-Performance Liquid Chromatographic Determination of Nitazoxanide and Ofloxacin in Tablet Formulation

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Abstract

A simple, rapid, and precise method was developed for the quantitative simultaneous determination of nitazoxanide and ofloxacin in new tablet formulation. Chromatographic separation of the two drugs was achieved on an Ymc pack-AM $C_{18,}$ 25-cm analytical column using mobile phase consisting of 10 mmol L^{-1} dipotassium hydrogen phosphate:acetonitrile (65:35, v/v) finally the pH of the mobile phase was adjusted to 7.0 using o- phosphoric acid. The instrumental settings are flow rate of 1 mL min^{-1}, column temperature at 30^{o}C, and detector wavelength of 254 nm. The internal standard method was used for the quantification. Caffeine was used as an internal standard. The method validated for linearity, accuracy, precision, limit of detection, limit of quantification and robustness. The calibration curve shows excellent linearity over the concentration ranges of ofloxacin and nitazoxanide 20-200 µg mL^{-1} and 8-80 µg mL^{-1}, respectively. The separation was completed less than 6 minutes. The proposed method can be used for the quality control of formulation products.

Kewords:

Nitazoxanide, Ofloxacin, RP-HPLC, Tablet formulation, Method validation

1. Introduction

Nitazoxanide is chemically 2-(Acetyloxy)-N- (5-nitro-2-thiazolyl) benzamide and ofloxacin is chemically 9-Fluoro-2, 3-dihydro-3 methyl-10 (4-methyl-1-piprazinyl)-7-oxo-7H-pyrido [1, 2, 3-de]-1,4-benzoxazine-6-carboxylic acid (Fig.1). A combination of 200 mg of ofloxacin and 500mg of nitazoxanide is available commercially as tablets (Netazox-OF). An ofloxacin and nitazoxanide combination is indicated to antibacterial and antiprotozoal activity. The combination of nitazoxanide and ofloxacin is antiparasitic and antibacterial which is effective against a wide variety of protozoa, helminthes and gram-negative organisms. Oral bioavailability is good and well tolerated, with mild gastrointestinal side effects. Used in Giardia intestinalis-induced diarrhea in patients [1]. This new combination was recently developed by pharmaceutical companies. In the process of development, fast and reliable analytical method is required for the simultaneous determination of both drugs in this compound formulation.

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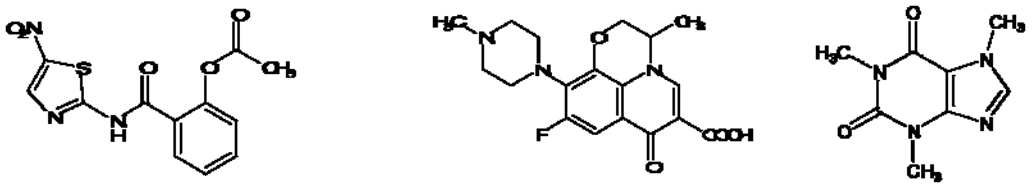

(a) (b) (c)

Fig.1. Structure of : (a) Nitazoxanide, (b) Ofloxacin and (c) Caffine

The literature survey revealed that several methods had been reported for the individual estimation of nitazoxanide and ofloxacin, which includes spectrophotometric method [2] for the estimation of nitazoxanide in tablets and method [3-5] is high-performance liquid chromatography (HPLC) and HPTLC methods reported in the literature for the estimation of nitazoxanide in plasma and pharmaceutical dosage form respectively. The reported method [6-8] is for the estimation of ofloxacin in combination with other drugs in plasma, serum and in tablets by high-performance liquid chromatography (HPLC) [9-12]. However, no reference has been found for simultaneous determination of ofloxacin and nitazoxanide in pharmaceutical preparations. LC with UV detection is often preferred in ordinary laboratories because of its wide suitability and availability. The reported LC Methods for the individual determination of the drugs cannot be easily applied for the simultaneous determination of both drugs in formulation owing to their large differences in physical and chemical properties such as polarity and solubility. If the reported individual methods were applied for the analysis of the tablets containing nitazoxanide and ofloxacin, it would require double time for analysis, as compared with the method would not be rapid, less expensive, or economical, whereas the simultaneous determination of the ingredients of the tablets would save analysis time and also economy.

The present paper describes a rapid, precise and accurate LC method for the simultaneous determination of ofloxacin and nitazoxanide in the tablet formulation. The developed method was validated in terms of selectivity, linearity, precision and accuracy.

2. Experimental

2.1. Materials and Reagents

Nitazoxanide and ofloxacin were obtained as gift samples from Lupin pharmaceutical Ltd. (Mumbai, India), dipotassium hydrogen phosphate; orthophosphoric acid and acetonitrile (HPLC grade) were purchased from Qualigens Fine Chemicals (Mumbai, India). Caffeine was obtained (as gift sample) from Merck Laboratories Ltd., (Mumbai, India). The 0.45 - Pump nylon filter was (purchased) from Advanced Micro devices Pvt. Ltd., (Ambala Cantt, India). The (Netazox-OF) tablets of the combination of nitazoxanide and Ofloxacin were purchased from local market. Double -distilled water was used throughout the experiment. Other chemicals used were analytical or HPLC grade

2.2. Chromatographic Conditions

A chromatographic system (Shimadzu, Japan) consisting of quaternary solvent delivery pump, a degasser, an auto- injector, column oven and photodiode array detector, 10A-VP series with LC-10 software. A Ymc pack-AM $C_{18,}$ (4.6 x 250 mm, 5 um, Ymc technology, Japan) column was used for separation. The column is end capped and carbon content of 17%.The instrumental settings were a flow of 1 mL min^{-1}., the analysis was

performed at 30°C. The injection volume was 10 µL. The peak purity was checked with the photodiode array detector from 10A-VP. Detection was performed at 254 nm.

2.3. Mobile Phase

The Mobile Phase consisted of 10 mM dipotassium hydrogen phosphate buffer and acetonitrile in the ratio 65.35 (v/v). The pH of the mobile phase was adjusted to 7.0 with orthophosphoric acid. The mobile phase was premixed and filtered through a 0.45 µm nylon filter and degassed.

2.4. Standard Stock Solutions

Standard solutions were prepared by dissolving the drugs in the diluents and diluting them to the desired concentration. Diluents used for the standards and sample was prepared as follow: diluent A was composed of methanol and aceonitrile in the ratio of 50:50(v/v) and diluent B was composed of water and actonitrile in the ratios of 65:35 (v/v).

A 50-mg sample of nitazoxanide was accurately weighed, transferred in a 50-mL volumetric flask, and dissolved with the diluent A (1000 µg mL^{-1}).

A 20-mg sample of ofloxacin was accurately weighed, transferred in a 50-mL volumetric flask, and dissolved with diluent A (400 µg mL^{-1}).

A 20-mg sample of caffeine was accurately weighed, transferred in a 50-mL volumetric flask, and dissolved with diluent A (400 µg mL^{-1}).

2.5. Mixed Standard Solution

A mixed standard solution was prepared from these stock solutions by transferring 5 mL of nitazoxanide standard solutions, 5 mL of ofloxacin by transferring standard solution, and 2.5 mL of caffeine standard solution in a 50 mL volumetric flask and diluted with diluent B. This solution contained 100 µg mL^{-1} of nitazoxanide, 40 µg mL^{-1} of ofloxacin and, 20 µg mL^{-1} of caffeine.

2.6. Calibration Curve Solutions

The calibration curve solutions containing 20-150 µg mL^{-1} of nitazoxanide, 8-60 µg mL^{-1} of ofloxacin, and 20 µg mL^{-1} of caffeine in each calibration level were prepared.

A 50-mg sample of nitazoxanide was accurately weighed, transferred in a 50-mL volumetric flask, and dissolved with the diluent A (1000 µg mL^{-1}). A 20-mg sample of ofloxacin was accurately weighed, transferred in a 50-mL volumetric flask, and dissolved with diluent A (400 µg mL^{-1}). A 20-mg sample of caffeine was accurately weighed, transferred in a 50-mL volumetric flask, and dissolved with diluent A (400 µg mL^{-1}). A mixed calibration curve solution was prepared from these stock solutions.

2.7. Preparation of Sample

Ten tablets were weighed and finely powdered. A quantity of powder equivalent to one tablet containing 500 mg of nitazoxanide and 200 mg of ofloxacin was transferred in a 200-mL volumetric flask. To this flask, 100 mL of diluent A was added, and the solution was sonicated for 10 min with intermittent shaking. An accurately measured volume of 10 mL acetonitrile was added to the flask and mixed well. Further sonication was performed for another 25 min with intermittent shaking. The solution was cooled to ambient temperature. An accurately measured volume of 10 mL methanol was added to the flask, and centrifuged at 10,000 rpm for 10 min. From the centrifuged solution, 5 mL of clear solution was transferred

into a 50-mL volumetric flask, and 2.5 mL of internal standard solution was added into it and diluted to volume with diluent B.

3. Results and Discussion

3.1. Method Development

The primary target in developing this LC method is to achieve simultaneous determination of nitazoxanide and ofloxacin in combined pharmaceutical dosage form under common conditions that are applicable for the routine quality control of this product in ordinary laboratories. Taken in to account the instability of nitazoxanide in strong acidic and basic media, a mobile phase with weakly acidic or neutral pH value is preferred. The optimal pH value was found to be 7.0. To achieve this number of stationary phase like C8, C18, CN and NH2 were employed and different combination of mobile phases were employed. In C18 stationary phase using ammonium acetate and phosphate buffer at different pH the resolution between nitazoxanide, ofloxacin and caffeine was achieved but broad peak shape of ofloxacin was obtained having tailing factor about 2.5. To minimize tailing effect, further NH2 and CN columns were tried but it has been observed that (tailing factor 2.4) but resolution between caffeine and ofloxacin decreased and in case of CN stationary phase peak shape of ofloxacin was improved but peak of caffeine, ofloxacin and nitazoxanide was eluted at 2.9, 4.1 and 18.5 respectively.

Finally used high carbon loading, double end capped C18 (Ymc pack-AM $C_{18,}$ 25-cm) column. Mobile phase was selected in terms of its components and proportions. This work began with a binary mixture of acetonitrile and 10mM dipotassium hydrogen phosphate in the ratio of 50:50 (v/v) at different pH 8.0 to pH 5.5. It was observed that at 50% aqueous composition containing dipotassium hydrogen phosphate pH-8.0 peak of caffeine, ofloxacin and nitazoxanide was eluted at 1.8, 3.1 and 14.5 respectively, while at pH 5.5 of mobile phase resolution between caffeine and ofloxacin was reduced. Finally a mobile phase consisting of mixture of 10mM dipotassium hydrogen phosphate and acetonitrile in the ratio of 65:35 (v/v) at pH 7 was adopted, which produces good resolution and reasonable retention and acceptable for both drug and internal standard caffeine and the chromatographic analysis time was less than 10 min. The tablet matrix was also determined to see any interference from them existed. No significant peak from matrix was observed in chromatograms, indicating no interference from the formulation matrix. A typical chromatogram for a tablet sample solution is shown in Fig 2 and 3. The retention time is 3.27 for Caffeine, 3.9 for ofloxacin and 6.39 for nitazoxanide, respectively. The run time is less than 10 min.

3.2. Specificity

The specificity of the method was checked by a peak purity test of the sample preparation using photodiode array detector. The peak purity for the peaks of nitazoxanide, and ofloxacin was observed to be 995, and 998 at wavelength 254 nm, which shows that the peaks of analyte were pure and also formulation excipients were not interfering with the analyte peaks.

4. Validation of the Method

4.1. Calibration and Linearity

An internal standard method was used for quantitative determinations. Linearity of the method was tested from 20% to 200% of the targeted level of the assay concentration (nitazoxanide, 100 µg mL^{-1} and ofloxacin 40 µg mL^{-1}) for both the analytes. Mixed standard

solutions containing 20-200 µg mL^{-1} of nitazoxanide, 8-80 µg mL^{-1} of ofloxacin, and 20 µg mL^{-1} of caffeine in each linearity level were prepared. Linearity solutions were injected in triplicate. The calibration graphs were obtained by plotting peak area ratio against the concentration of the drugs. The equations of the calibration curves for nitazoxanide and ofloxacin obtained were y = 5267.5x – 4505.5 and y = 5580.6x - 14477, respectively. The result shows that an excellent correlation exists between the peak area and concentration of each drug within the concentration range tested. The correlation coefficient for nitazoxanide and ofloxacin are 0.9994 and 0.9992, respectively. In the simultaneous determination, the calibration graphs were found to be linear in the aforementioned concentrations.

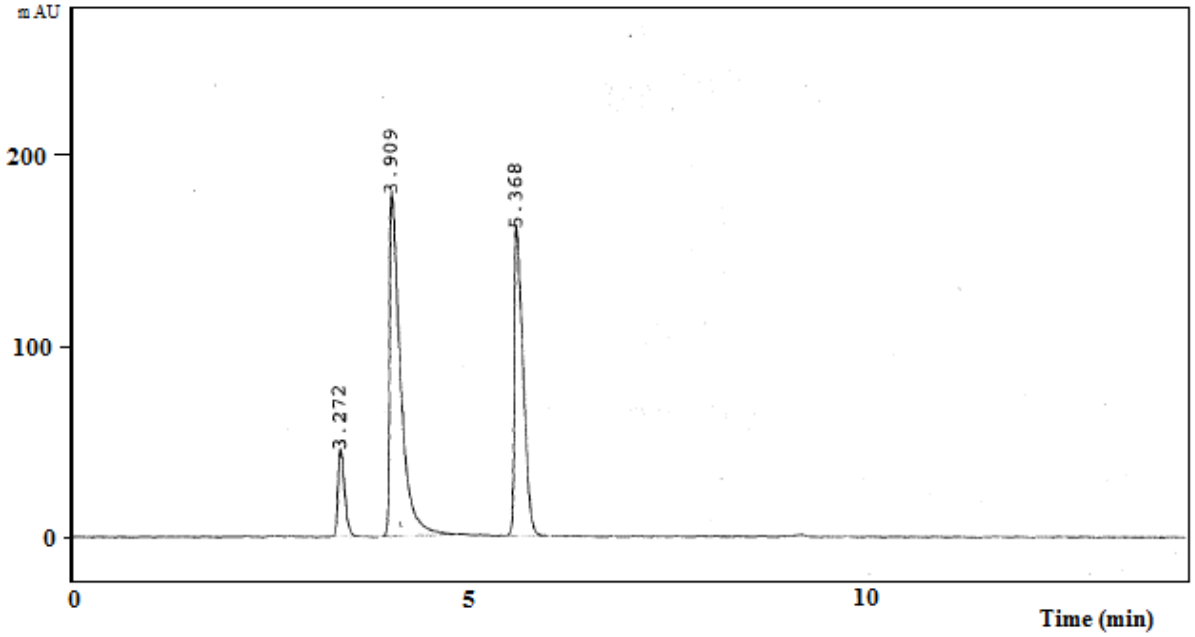

Fig. 2. A typical chromatogram of the tablet: caffeine (3.27 min), ofloxacin (3.9 min), and nitazoxanide (6.36 min)

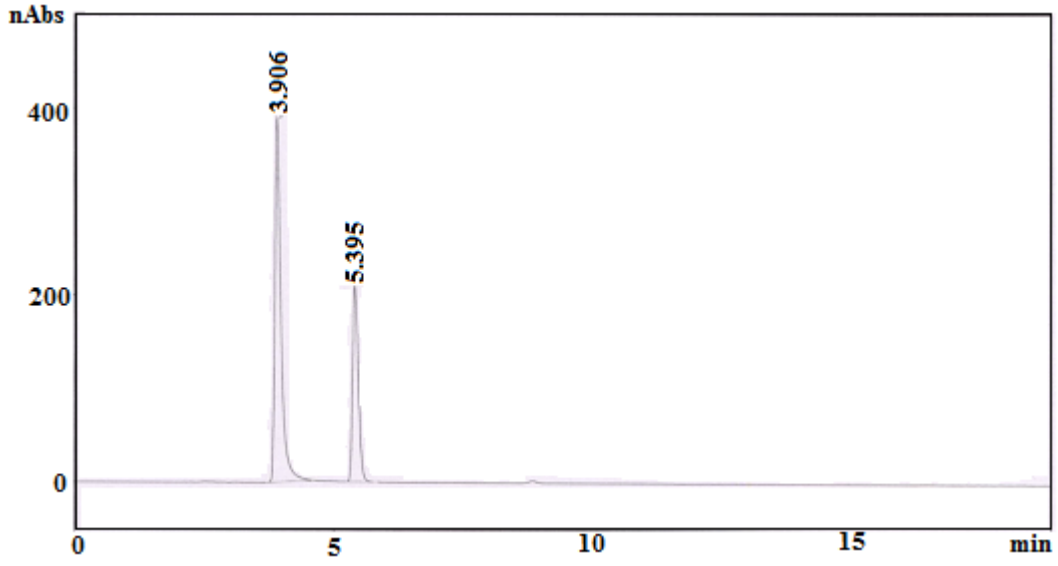

Fig.3. A typical chromatogram of the tablet: ofloxacin (3.9 min), and nitazoxanide (6.36 min)

4.2. Assay of Tablets

The validated LC method was applied to the determination of ofloxacin and nitazoxanide in tablets. Two batches of the tablets were assayed and the results shown in Table 1, indicating that the amount of each drug in tablet samples met the requirement.

Table 1. Assay Results of Active Ingredients in Tablets

Batch no.	Ingredient	Label value (mg)	Found (mg)*	% Label claim	%RSD
1	Nitazoxanide	500	501.99	100.398	0.78
	Ofloxacin	200	199.58	99.79	0.85
2	Nitazoxanide	500	503.45	100.69	0.67
	Ofloxacin	200	198.12	99.06	0.81

4.3. Precision

The system precision is measure of the method variability that can be expected for given analyst performing the analysis and was determined by performing five replicate analysis of the same working solution. The obtained relative standard deviation (R.S.D.) for ofloxacin and nitazoxanide was 0.62% and 0.48%, respectively.

The intraday precision of developed LC method was determined by preparing the tablet samples from same batches in nine determinations with three concentrations and three replicate each. The R.S.D.of the assay results, expressed as percentage of the label claim, was used to evaluate the method precision. The obtained R.S.D. values were 0.81% for ofloxacin and 0.74% for nitazoxanide. The inter-day precision was also determined by assaying the tablets in triplicate day for consecutive three days, which was found to be 0.87% for ofloxacin and 0.79% for nitazoxanide, respectively. The results indicated good precision of the developed method. The results are shown in Table 2.

Table 2. Result of precision of test method

Sample number	Assay of NTZ and OFLX as % of labeled amount			
	Analyst-1 (Intra-day precision)		Analyst-2 (Inter-day precision)	
	NTZ	OFLX	NTZ	OFLX
1	98.25	98.75	99.63	99.13
2	99.92	99.39	98.98	98.21
3	98.24	99.21	99.16	98.96
4	99.28	98.19	99.12	99.97
5	99.32	99.59	98.21	99.59
6	98.45	99.23	99.32	99.21
Mean	99.22	99.36	99.44	99.15
RSD	0.74	0.81	0.79	0.87

4.4. Accuracy (Recovery Test)

Accuracy of the method was studied by recovery experiments. The recovery experiments were performed by adding known amounts of the drugs in the placebo. The recovery was performed at three levels, 80%, 100% and 120% of the label claim of the tablet

(500mg of nitazoxanide and 200 mg of ofloxacin). Placebo equivalent to one tablet was transferred into a 200-mL volumetric flask, and the amounts of nitazoxanide and ofloxacin at 80%, 100% and 120% of the label claim of the tablet were added to it. The recovery samples were prepared as per the procedure mentioned, and then 5 mL of each of the solutions were transferred into a 50-mL volumetric flask; 2.5 mL of internal standard solution was added to it and diluted to volume with diluent B. Three samples were prepared for each recovery level. The solutions were then analyzed, and the percentage recoveries were calculated from the calibration curve. The recovery values for nitazoxanide and ofloxacin ranged from 101.25% to 100.97% and 100.52 - 100.87%, respectively (Table 3). The obtained results suggested the accuracy of developed method for the simultaneous determination of the two drugs in the formulation.

Table 3. Results of the Recovery Tests for the Drugs

Level of addition (%)	Ingredient	Amount added (n = 3) (mg)	% Recovery	% Average recovery
80	Nitazoxanide	400	101.25 (0.45)	101.25(0.35)
	Ofloxacin	160	100.52 (0.37)	
100	Nitazoxanide	500	100.97 (0.24)	100.55 (0.29)
	Ofloxacin	200	100.78 (0.55)	
120	Nitazoxanide	600	101.23 (0.49)	100.95 (0.55)
	Ofloxacin	240	100.87 (0.31)	

4.5. Limit of Quantification and Limit of Detection (LOQ & LOD)

For determining the limit of detection (LOD) and limit of quantification (LOQ), the method based on the residual standard deviation of a regression line and slope was adopted (13). The limit of detection and limit of quantification can be determined with acceptable accuracy and precision, which can be established at signal-to-noise ratio 3 and 10, respectively. To determine the LOD and LOQ, a specific calibration curve was constructed using samples containing the analytes in the range of LOD and LOQ. The LODs for nitazoxanide and ofloxacin were 0.022 and 0.008 μg mL^{-1}, and the LOQs were 0.070 and 0.028 μg mL^{-1}, respectively.

4.6. Robustness

The robustness of a method is the ability of method to remain unaffected by small changes in parameters. To determine robustness of the method, experimental conditions were purposely altered and chromatographic resolution between ofloxacin and nitazoxanide were evaluated.

The flow rate of the mobile phase was 1.0 mL min^{-1}. To study the effect of flow rate on resolution of ofloxacin and nitazoxanide, it was changed 0.2 units from 1.0 to1.2 mL min^{-1}. The effect of change in percent Acetonitrile on resolution was studied by varying from -1 to +1% while the other mobile phase components were held constant. The effect of column temperature on resolution was studied at 20 ^{o}C and 25 ^{o}C instead of 30 ^{o}C while other mobile phase components were held constant. The robustness results are shown in Table 4.

Table 4. Robustness of the method

Parameter	USP Resolution between Ofloxacin and Nitazoxanide
Flow rate (mL min^{-1})	
0.8	7.5
1.0	6.8
1.2	6.2
Column temperature (^oC)	
25	7.25
30	6.93
35	6.76
Acetonotrile percentage in the mobile phase	
34	6.85
35	6.77
36	6.56

4.7. Solution Stability

The stability of the standard solutions and the sample solutions was tested at intervals of 24, 48 and 72 h. The stability of solutions was determined by comparing results of the assay of the freshly prepared standard solutions. The RSD for the assay results determined up to 75 h for nitazoxanide, ofloxacin and the caffeine internal standard were 0.65%, 0.34 %, and 0.39 %, respectively. The assay values were within ±2 % after 72 h. The results indicate that the solutions were stable for 72 h at ambient temperature.

Standard solutions were used, and the RSD of peak area ratio, column efficiency, resolution, and tailing factor of the peaks were calculated. The system suitability results are shown in Table 5.

Table 5. System Suitability Parameters

Parameter	Caffeine	Ofloxacin	Nitazoxanide
Theoretical plate (per column length)	6112	5509	12839
Resolution	3.29		6.74
Tailing factor	1.21	1.55	1.1
Capacity factor	1.15	1.23	1.26
% RSD		0.21	0.18

5. Conclusion

The proposed LC method is rapid and accurate for the simultaneous determination of ofloxacin and nitazoxanide in new formulation. It can be used in the quality control departments for the assay and dissolution of tablets of the combined pharmaceutical-dosage forms containing nitazoxanide and ofloxacin.

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