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Development and Validation of a *Dissolution method* with Isocratic High-Performance Liquid Chromatographic Determination of Nitazoxanide and Ofloxacin in Pharmaceutical Dosage form

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Abstract: A simple, specific, accurate and precise Isocratic reverse phase high pressure liquid chromatographic method has been developed for the simultaneous determination of Nitazoxanide and Ofloxacin from combined dosage form by reverse phase Separation was carried out on a columns containing different stationary phases, the final choice giving satisfactory resolution and run time was the 25 cm × 4.6 mm i.d, 5-µm particle; Phenomenex Luna C18 reversed-phase column. 2.0gm sodium dihydrogen phosphate and 5M of triethylamine are mixed into 500mL Milli Q water and pH was adjusted to 4.5 by orthophosphoric acid and diluted to 1000mL as a mobile phase at a flow rate of 1.2ml/min and detection at 246nm. The average retention times for amoxicillin (Internal standard), Nitazoxanide and Ofloxacin was found to be 3.11,5.28 and 7.31 min, respectively and recoveries from combined dosage form were between 98 and 102%. Quantification and linearity was achieved at 276 nm over the concentration range of 100-400 µg mL^{-1} for Nitazoxanide and 10-150 µg mL^{-1} for Ofloxacin. The method was validated for specificity, linearity, accuracy, precision, LOD, LOQ and robustness. The proposed method was optimized and validated as per the ICH guidelines. The method can be used for estimation of combination of these drugs in combined dosage form.

Key words: Nitazoxanide · Ofloxacin · RP-HPLC · *Dissolution*

INTRODUCTION

Ofloxacin (OF) is chemically 9-fluoro-2, 3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-Oxo-7H-pyrido (1, 2, 3-di)-1, 4-benzoxazine carboxylic acid. It is a fluoroquinolone derivative. It is used mainly as an antibacterial. It is official in USP [1], BP [2] and EP (European Pharmacopoeia) [3]. Literature survey reveals that Spectrophotometric [4], HPLC [5] and HPTLC [6] methods are available for determination of Ofloxacin from pharmaceutical preparations and biological formulation. Nitazoxanide (NT), N-(5-nitro-2-thiazolyl) salicylamide acetate [7,8] is a nitrothiazole derivative. It's chemical structure related to metronidazole. It is a broad spectrum antiprotozoal. It is not official in any Pharmacopoeias. The combination of Nitazoxanide and Ofloxacin is used in Diarrhea. Literature survey reveals that, Spectrophotometric [9] and RP-HPLC [10] method is available for estimation of NT in single dosage form. Author of the article and his research team has developed a HPLC method development in different pharmaceutical dosage form [11-22]. The proposed method overcomes many difficulties of tracing out lowest determination and quantification of related substances and its products. Also the affirmative points are; less instrument set up time by mean of simple isocratic elution which results into a negligible noise as compare to gradient methods. The reverse phase HPLC method was found to be simple and convenient for the simultaneous determination of the two drugs and results indicate high accuracy and precision.

MATERIALS AND METHODS

Materials: All experiments were performed using 'A' class volumetric glassware, sodium dihydrogen orthophosphate dihydrate (S.D. Fine Chem.), orthophosphoric acid, Using HPLC grade triethylamine, Methanol and highly pure HPLC grade Milli Q water (Millipore, Bedford, A, USA), mobile phase was prepared

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and employed for analysis. The mobile phase was filtered through 0.45µm PVDF filter (Millipore) and degassed under vacuum, prior to use. The tablet dosage form, Nitazete-O (claim: 500mg NT and 200mg OF) was procured from the local market. Acetonitrile and Phosphate buffer AR grade was obtained from Merck Limited, India.

Chromatography Conditions: The HPLC system consisted of a solvent delivery module Agilent 1100 Series Isocratic pump equipped with 20 µl loop and G1365B Multi Wavelength Detector. Integration was achieved by using the software Chemstation. Separation was carried out on a columns containing different stationary phases, the final choice giving satisfactory resolution and run time was the 25 cm × 4.6 mm i.d, 5-µm particle; Phenomenex Luna C18 reversed-phase column.2.0gm sodium dihydrogen phosphate and 15ml of triethylamine are mixed into 500mL Milli Q water and pH was adjusted to 5.2 by orthophosphoric acid and diluted to 1000 mL The mixture is filtered through 0.45µm filter and used as buffer solution. The combination of buffer solution with methanol (50:50v/v) was used as a diluent (diluting solution) while preparing analytical solutions. The mobile phase was delivered at a flow rate of 1.5 mL/min with maintaining the column temperature at 35°C. The detection was achieved at 246 nm by injecting 20µL of sample and standard aliquots, prepared using diluent, with the above chromatographic conditions and after partition equilibration, well shaped peaks were separated.

Preparation of Standard and Sample Solution Calibration Graphs: Standard stock solution of Nitazoxanide (50.0 mg/ml) and Ofloxacin (10.0 mg/ml) was prepared in methanol as diluent. To study the linearity range of each component, serial dilutions were made to obtain working standards in the concentration range of Nitazoxanide (100-400 µg/ml) and Ofloxacin (10-150 µg/ml). A graph was plotted as concentration of drugs versus peak area response and results were found linear for both analytes. From the standard stock solution, a mixed standard solution was prepared containing Nitazoxanide (500 µg/ml) and Ofloxacin (100 µg/ml). The system suitability test was performed from five replicate injections of mixed standard solution.

Sample Preparation: Twenty tablets were weighed and finely powdered. The average weight of tablets was determined with weight of 20 tablets. A portion of powder equivalent to the weight of one tablet was accurately weighed into 100 ml A-grade volumetric flask and 70 ml diluent was added. The volumetric flasks were sonicated for about 20min to effect complete dissolution of the Nitazoxanide and Ofloxacin; the solutions were then made up to volume with diluent. The solution was filtered through 0.45 µm nylon filter. The aliquot portion of the filtrate was further diluted to get final concentration of 500 µg/ml of Nitazoxanide and Ofloxacin100 µg/ml.

Dissolution Studies: For the dissolution study [23-24] of Nitazoxanide and Ofloxacin analysis was done by using above chromatographic conditions. For this study standard solution of Nitazoxanide and Ofloxacin was prepared in dissolution media. For sample preparation an intact tablet was dissolved in 0.1 N HCl media (RPM 100). Sample was collected in dissolution vials after 2 hrs and then decanted the 0.1 N HCl media and the 4.0 pH phosphate buffer media was loaded and set RPM 100. Samples were collected in dissolution vials after different time intervals and filtered through 0.45 µm filter. Equal volumes (20 µL) of these solutions were injected into the chromatograph by auto sampler and peak areas were measured.

Stability of Analytical Solutions: Spiked test preparation were prepared and kept on bench top (25°C±2) and analyzed initially (0 day), after 1 day and after 2 days by injecting single injection of each set of spiked test preparation into liquid chromatography and chromatograms were recorded. Difference in result of impurity A, impurity B, Impurity C, unknown individual impurity and total impurities was determined at each time interval against respective initial result. Single and total impurities found well within the limit and the maximum difference observed was 0.67 and 0.45, for known and unknown impurities respectively. Initially acetonitrile and water in different ratios were tried. But in that, both drugs showed peak broadening and the resolution was very less. So acetonitrile was replaced by methanol, THF and water was replaced with various buffers with different pH and concentration. Hence methanol: THF: acetate buffer pH 3.6 (74:16:10v/v) was suitable to get resolved and sharp peak. Methanol was the organic modifier of choice giving symmetrical narrow peaks and good Resolution. The effect of changing the ratio of organic modifier on the selectivity and retention times of the test solutes was investigated using mobile phases containing concentrations of 60-40% methanol.

Effect of pH: The effect of changing the pH of the mobile phase on the selectivity and retention times of the test

solutes was investigated using mobile phases of pH ranging from 2.0-6.8. Shows that a pH of 4.5 was the most appropriate one giving well-resolved peaks and highest no. of theoretical plates. There were always asymmetric and broad peaks of Nitazoxanide and Ofloxacin at pH values > 5.0.

Effect of Flow Rate: The effect of flow rate on the formation and separation of peaks was studied by varying the flow rate from 1-1.5; a flow rate of 1.5 mL min^{-1} was optional for good separation and resolution of peaks in a reasonable time.

Effect of Temperature: The effect of Temperature on the formation, separation and resolution was studied by varying the Temperature from 22-30 °C; we found that at lower Temperatures the peaks are not well resolved.

Method Development: The HPLC method was validated in terms of precision, accuracy and linearity according to ICH guidelines. Assay method precision was determined using nine independent test solutions. The intermediate precision of the assay method was also evaluated as inter-day and intra-day precision. The accuracy of the assay method was evaluated with the recovery of the standards from excipients. Three different quantities (low, medium and high) of the authentic standards were added to the placebo. The mixtures were extracted and analyzed using the developed HPLC method. Linearity test solutions were prepared as described in Formulation analysis. The Limit of Detection (LOD) and Limit of Quantification (LOQ) for analytes were estimated by injecting a series of dilute solutions with known concentration. Values of LOD and LOQ were calculated by using σ (standard Deviation of response) and b (Slope of the calibration curve) and by using equations, LOD = (3.3 x σ)/ b and LOQ = (10 x σ)/ b. To determine the robustness of the method, the final experimental conditions were purposely altered and the results were examined. For method development and optimization, retention factor (k) was calculated by using parameters tR (retention time) and tM (elution time of the solvent front) and by using the equation k = (t R-t M)/ tM.

Validation of the Method: Recovery studies were carried out to study the accuracy of the proposed method and ascertained by standard addition method. A known amount of drug was added to preanalysed tablet powder, at three level and the percentage recoveries were calculated. Precision was found to be lower than 1%. Ruggedness of the proposed method was determined by analysis of aliquots from homogenous slot by different analysts using similar operational and environmental conditions.

Linearity: The linearity of an analytical method is its ability to elicit test results that are directly, or by well defined mathematical transformation, proportional to the concentration of analyse in samples within a given range. Linearity test solutions for the method were prepared by diluting stock solutions to the required concentrations. The solutions were prepared at six concentration levels starting from LOQ to 0.95%. The peak area versus concentration data was treated by least-squares linear regression analysis. Linearity test solutions for the related substance method were prepared. The % RSD value for the slope and Y-intercept of the calibration curve was calculated.

Specificity: The specificity of the method was determined by analyzing standard drug and test samples. The spot for Nitazoxanide and Ofloxacin in the samples was confirmed by comparing the RF and spectrum of the spot to that of a standard. The peak purity of Nitazoxanide and Ofloxacin was determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E).

RESULTS AND DISCUSSION

The method was validated, in accordance with ICH guidelines, for linearity, range, accuracy, precision, LOD and LOQ, specificity, ruggedness and robustness [ICH-Q2B, 2006]. For the RP-HPLC, chromatographic conditions were optimized to get best resolution and peak shape. The selection of mobile phase was based on peak parameters; (symmetry, theoretical plates, capacity factor and tailing factor) ease of preparation and cost. The optimum wave length for detection and quantification was 305 nm, at which good detector response was obtained with symmetrical peaks. Limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.174µg/ml and 0.21µg/ml and 0.034µg/ml and 0.08µg/ml respectively. The signal to noise ratio is 3 for LOD and 10 for LOQ. The selection of mobile phase was based on peak parameters; (symmetry, theoretical plates, capacity factor and tailing factor) ease of preparation and cost. A symmetrical peak with good separation (Rt of Nitazoxanide 5.28 min and 7.31 min Ofloxacin) was obtained with C-18 column and mobile phase.

Table 1: Results from Assay of the Nitazoxanide and Ofloxacin

Drug	Label claim (mg) n=6	Amount Found in mg	Drug Concentration (%)	SD	COV (%)	SE
Nitazoxanide	500	501.05	101.05	0.04	0.754	0.21
Ofloxacin	200	200.23	100.23	0.31	0.154	0.11

S.D-standard deviation; COV-coefficient of variance; S.E-standard error; n-No, of replicates

Table 2: System suitability parameters

Property	Nitazoxanide	Ofloxacin
R'	5.28	7.31
T_f	1.17	1.23
K'	0.65	0.38
N	7693	9873
R_s	-	3.52

Rt, retention time; *Tf*, tailing factor; *k'*, capacity factor; *N*, number of theoretical plates *R*s, resolution

Table 3: Results of recovery studies

Drugs	Amount taken (µg m L^{-1})	Amount added % µg m L^{-1}		Recovery (%, ±S.D)	COV (%)
Nitazoxanide	500	80	60	00.02	0.187
	100	75	100.11	0.275	
	120	90	100.04	0.493	
Ofloxacin	200	80	20	100.01	0.321
	100	35	99.87	0.713	
	120	50	99.96	0.653	

S.D., standard deviation; COV, coefficient of variance

Table 4: Results from determination of intra-day and inter-day precision and LOD and LOQ

		Inter-day precision (COV, %)				
Drugs	Intra-day precision (COV, %)	Day [1]	Day [2]	Day [3]	LOD ng mL^{-1}	LOQngmL^{-1}
Nitazoxanide	2.321	1.995	1.653	1.488	0.416	0.278
Ofloxacin	1.053	0.874	0.693	0. 439	0.137	0.174

A Mean from six determinations COV, coefficient of variance; LOD, limit of detection; LOQ, limit of quantitation

Table 5: Dissolution parameters and HPLC Condition Nitazoxanide and Ofloxacin

Dissolution parameters:	
Medium	Phosphate buffer pH 6.8, 0.1 N HCL buffer pH-1.2 and Water.
Volume	900 mL
Apparatus	Paddle
RPM	100
Temperature	37 ± 0.5°C
Time	2hrs

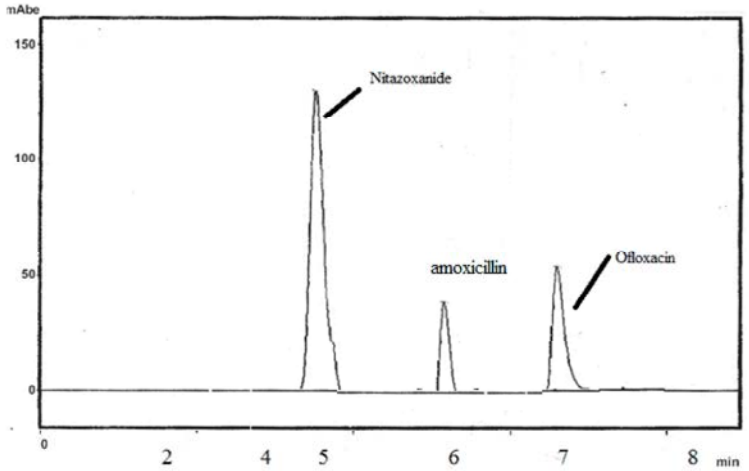

Fig. A: Typical Chromatogram showing the peaks of Amoxicillin (internal standard) Nitazoxanide (5.28 min) and Ofloxacin (7.31min)

Author of the article and his research team has developed a HPLC method development in different pharmaceutical dosage form [11-22]. For the construction of calibration curves, seven calibration standard solutions were prepared over the concentration range. Linearity was determined for Nitazoxanide in the range of 100-400 µg mL^{-1} and for Ofloxacin 10-150 µg mL^{-1}. The correlation coefficient ('r^2') values were > 0.998 (n = 6). Typically, the regression equations for the calibration curve was found to be y = 0.4724 x-5.8736 for Nitazoxanide, y=3.4276x-9.06511 for Ofloxacin. The precision of repeatability was studied by replicate (n=6) analysis of tablet solutions. The precision was also studied in terms of intra-day changes in peak area of drug solution on the same day and on three different days over a period of one week. The intra-day and inter-day variation was calculated in terms of percentage relative standard deviation. The peak area ratios of standard and sample solutions were calculated. The assay procedure was repeated for 6 times and mean peak area, mean peak area ratio, mean weight of standard drugs, mean weight of sample taken for assay were calculated. The percentages of individual drugs found in formulations, mean and relative standard deviations in formulation were calculated. The result of analysis shows that the amount of drugs present in the formulation has a very good correlation with the label claim of the formulation. The accuracy of the method was determined by recovery experiments. A known quantity of the pure drug was added to the pre-analyzed sample formulation at 80%, 100% and 120% levels. The recovery studies were carried out 6 times of each level and the percentage recovery and mean of the percentage recovery were calculated. From the data obtained, it was observed that the recoveries of standard drugs were found to be accurate and within the specified limits. The slope, intercept and correlation coefficient values were also calculated. The correlation coefficient of Nitazoxanide and Ofloxacin were found to be 0.9999 and 0.9996 respectively. The calibration curves were plotted as peak area Vs concentration of the standard solutions. The calibration graph shows that linear response was obtained over the range of concentrations used in the assay procedure. The developed method was used for the assay of commercially available tablets and six replicate determinations were performed.

CONCLUSION

hence, the chromatographic method developed for Nitazoxanide and Ofloxacin were found to be simple, precise, accurate and cost effective and it can be effectively applied for routine analysis in research institutions, quality control department in industries, approved testing laboratories, bio-pharmaceutical and bio-equivalence studies and in clinical pharmacokinetic studies in near future.

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REFERENCES

1. United State Pharmacopoeia, 2006. 24 National formulary 29, Asian[Edn.,] USP Conv. Inc. Rockville, USA., pp: 1574.
2. British Pharmacopoeia, 2002. Published by the London Stationary Office under department of health 4th Edn., 1: 1247-48.
3. European Pharmacopoeia, 2004. Published in accordance with convention on the elaboration of European Pharmacopoeia. The E.P. is Published by the directorate for the Quality of the Medicines of the council of European EDQM, 5th Edn., 2: 2131.
4. Kasture, V.S., A.D. Bhagat, N.C. Puro, P.S. More and N.K. Bhandari, 2004. Spectrophotometric Method for Simultaneous Estimation of Ofloxacin and Ornidazole in Tablet Dosage Form. Indian. Drugs., 41: 51-53.
5. Kraas, E. and A. Hirrle, 1986. Determination of Ofloxacin in biological fluids using HPLC with flurimetric detection, Fresenius. J. Ana. Chem., 324: 354-357.
6. Gandhimathi, M., T.K. Ravi and S.V. Nilima, 2006. Validated high performance thin layer chromatography method for simultaneous estimation of Ofloxacin and ornidazole in tablet dosage form, Indian. J. Pharm. Sci., 68: 838-840.
7. The Merck Index an Encyclopedia of chemicals, 2000. drugs and biological, 13rd edition Merck and co. Inc., White house station, New Jersey, 1177.
8. Reynolds, J.E.F., 2002. In Martindale, the extra Pharmacopoeia, 33rd Edn., The Royal Pharmacopoeial society, London, pp: 1229-45.
9. Kapse, G.K., G. Prabhakar and S.R. Appala, 2006. Spectrophotometric methods for the estimation of nitazoxanide in pharmaceutical formulation, Indian. J. Pharm. Sci., 68: 403-406.
10. Kalta, R.R., R. Sharma and S.C. Chaturvedi, 2008. Simultaneous RPHPLC Determination of Nitazoxanide and Ofloxacin in Combined Tablet Dosage Form. Indian. J. Pharm. Sci., 70(4): 491-494.

11. Sharma, S., M.C. Sharma, D.V. Kohli and S.C. Chaturvedi, 2010. Isocratic Reverse Phase HPLC estimation method of Torsemide and Spironolactone in pharmaceutical combined dosage form. J. Optoel. and Biomed. Mate-Rapid Communications., 4: 234-237.
12. Sharma, S., M.C. Sharma, D.V. Kohli and S.C. Chaturvedi, 2010. Study of stressed degradation behaviour of Pioglitazone hydrochloride in bulk and pharmaceutical formulation by HPLC assay method. J. Optoel. and Biomed. Mate., 1: 17-24.
13. Sharma, S., M.C. Sharma and S.C. Chaturvedi, 2010. Development and validation of isocratic reversed-phase HPLC method for simultaneous estimation of torsemide and spironolactone in bulk and pharmaceutical combined tablet dosage form. J. Optoel. and Biomed. Mate-Rapid Communications, 4(3): 427-430.
14. Sharma, S., M.C. Sharma and S.C. Chaturvedi, 2010. Simultaneous estimation of Rosuvastatin calcium and Ezetimibe in tablet dosage form by reverse phase high performance liquid chromatography. Optoel. and Biomed. Mate-Rapid Communications, 4: 238-241.
15. Sharma, M.C. and S. Sharma, 2011. A Facile and Isocratic RP-HPLC Method and Spectroscopy for Simultaneous estimation of Ramipril in Formulation: dissolution method. Intl. J. Chem. Tech. Res., 3(2): 992-996.
16. Sharma, M.C. and S. Sharma, 2011. Isocratic RP-HPLC Method for Simultaneous Estimation of Paracetamol and Lornoxicam in combined tablet Dosage Form and its Dissolution Assessment. Intl. J. Chem. Tech. Rese., 3(2): 997-1002.
17. Sharma, M.C. and S. Sharma, 2011. Dissolution Studies and RP-HPLC Method for the Simultaneous Determination of Satranidazole and Ofloxacin in Pharmaceutical Dosage Form. Intl. J. Chem. Tech. Rese., 3(2): 1003-1007.
18. Sharma, S., M.C. Sharma, R. Sharma and A.D. Sharma, 2011. High Performance Liquid Chromatographic Assay method for the determination of Paracetamol and Caffeine in Tablet Formulation-*in vitro dissolution studies*. J. Pharma. Res., 4(5): 1559-1561.
19. Sharma, S., M.C. Sharma and A.D. Sharma, 2011. Application to Dissolution Assessments; A Facile and Rapid procedure of Atenolol and Hydrochlorothiazide in Pharmaceutical Formulation by RP-HPLC assay Method. Drug. Invention. Today, 3(6): 100-101.
20. Sharma, S., M.C. Sharma and A.D. Sharma, 2011. Facile and Rapid Simultaneous Estimation of Dexrabeprazole and Domperidone by RP-HPLC assay Method in Combined Dosage Form-In Application Dissolution Assessment. Drug. Invention. Today, 3(6): 98-99.
21. Sharma, S., M.C. Sharma and A.D. Sharma, 2011. Study of stressed degradation behavior of Amlodipine Besylate and development of Reverse phase HPLC assay method. J. Pharma. Res., 4(5): 1579-1581.
22. Sharma, M.C. and S. Sharma, 2011. Stability Indicating RP-HPLC Method for Determination and Validation of Repaglinide in Pharmaceutical Dosage Form. Intl. J. Chem. Tech. Rese., 3(2): 210-216.
23. Morre, J.W. and H.H. Flanner, 1996. Mathematical comparison of dissolution profiles. Pharm. Techno., 6: 64.
24. Shah, V.P., Y. Tsong, P. Sathe and R.L. Williams, 1999. Dissolution profile comparison using similarity factor, f_2. Dissolution. Techno., 6: 15.