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SIMULTANEOUS RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF NIMORAZOLE AND NORFLOXACIN IN TABLET DOSAGE FORM

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ABSTRACT

The estimation of Nimorazole and Norfloxacin in tablet dosage forms was developed and validated in a simple, rapid, accurate and robust reverse-phase high-performance liquid chromatography (RP-HPLC) method. In this technique, a Phenomenex C18 column was used, where the mobile phase was acetonitrile and 0.1% ortho-phosphoric acid buffer in a 30:70 v/v mixture and the UV detection wavelength of 290 nm. System suitability parameters such as theoretical plates, tailing factor, and resolution were established to be within the necessary limits, which proved that the method is suitable. It was found that there was linearity between a concentration of 80 and 120 percent and both Nimorazole and Norfloxacin had correlation coefficients of 0.9985 and 0.9968, respectively. Precision, accuracy and robustness were established using intraday and interday studies, recovery experiments and controlled variation of chromatographic conditions. The technique had a good recovery of both drugs with a range of 99.49 to 103.27. Pharmaceutical tablet assay had good results with Nimorazole at 100.51 and Norfloxacin at 100.73 of the label claim. The resulting method of RP-HPLC can be applied in routine quality control, stability studies, and regulatory analysis of Nimorazole and Norfloxacin in pharmaceutical deviations.

Key words: Nimorazole, Norfloxacin, RP-HPLC, Simultaneous Analysis

INTRODUCTION

Norfloxacin, on the other hand, is a broad-spectrum antibiotic belonging to the fluoroquinolone class, which is widely used to treat various bacterial infections (Millanao AR, *et al.* 2021). Fluoroquinolones like norfloxacin exert their antimicrobial effects by inhibiting bacterial DNA gyrase and topoisomerase IV, enzymes critical for DNA replication and cell division in bacteria. This action prevents the bacteria from replicating their DNA, ultimately leading to bacterial cell death. Norfloxacin has demonstrated efficacy against a variety of Gram-negative and Gram-positive bacteria, making it a valuable antibiotic in treating urinary tract infections

(UTIs). In addition to ensuring the quality of the final product, the accurate analysis of nimorazole and norfloxacin in pharmaceutical formulations is also essential for regulatory compliance (Giriraj P, Sivakkumar T. 2015). Regulatory bodies such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) require that pharmaceutical products undergo rigorous testing to ensure that they meet specific quality standards. This includes testing for the presence of impurities, degradation products, and ensuring that the drug content is within the acceptable range. Therefore, the development of reliable analytical methods for the simultaneous analysis of nimorazole and norfloxacin is essential for the pharmaceutical industry to meet these regulatory requirements and guarantee patient safety. Their combination in tablet dosage forms offers a potent and synergistic treatment approach, improving therapeutic

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outcomes in patients suffering from mixed infections. As the use of combination therapies increases, the accurate analysis of these drugs in pharmaceutical formulations becomes increasingly important (Rathod RH, *et al.* 2019). High-performance liquid chromatography provides a powerful tool for the precise quantification of these drugs, ensuring that they meet the necessary quality and regulatory standards.

This difference in polarity and solubility between the two drugs necessitates the use of advanced techniques, such as reverse-phase high-performance liquid chromatography (RP-HPLC), to achieve effective separation and quantification. RP-HPLC has proven to be one of the most reliable and versatile methods for analyzing pharmaceutical formulations containing multiple active ingredients, especially when these ingredients possess varying physicochemical properties, such as in the case of nimorazole and norfloxacin. The challenge lies in developing a chromatographic method that can effectively separate and quantify these two drugs in a combined tablet formulation, ensuring the accuracy, precision, and resolution required for routine quality control analysis (Regalado EL, *et al.* 2019). The development of an RP-HPLC method involves optimizing several critical chromatographic parameters, which must be carefully fine-tuned to achieve the desired separation.

METHODOLOGY

Determination of Melting point by Melting Point Apparatus:

Fill the capillary tube with the test samples of TINI and OFL respectively and insert the same into the sample holder. Turn heater knob to turn on. At temperatures of about 20° C and below what the substances are supposed to melt, turn the knob in the opposite direction (anticlockwise) to slow the rate at which they are heated. The heating rate should be adjusted to around 1°cpm (Young JC. 2013; White WP. 1909). Record the melting point/melting range of the substances.

SELECTION OF MOBILE PHASE

TRAIL-1

Preparation of buffer: Mix 0.1 gm of ammonium acetate in one litre of water and adjust the solution to pH 3.0 with dilute ortho-phosphoric acid.

Preparation of mobile phase: Add acetonitrile and buffer in a proportion of 60:40, filter using 0.45 µm membrane filter and aerate (Carr JE, *et al.* 2006).

TRAIL-2

Preparation of mobile phase: Mix acetonitrile and water in the ratio of 50:50, filter through a 0.45 µm membrane filter, and degas.

TRAIL-3

Preparation of mobile phase: Prepare a mixture of acetonitrile and methanol (80:20), filter through a 0.45 µm membrane filter, and degas.

TRAIL-4

- 1. Preparation of Buffer:** Dissolve 1% w/v ortho-phosphoric acid in 1000 mL of water.
- 2. Preparation of mobile phase:** Add acetonitrile and buffer in the proportion of 50:50 and then filter through a 0.45 µm membrane filter and finally degas.

TRAIL-5

- 1. Preparation of Buffer:** Dissolve 0.1% w/v ortho-phosphoric acid in 1000 mL of water.
- 2. Preparation of mobile phase:** Mix acetonitrile and buffer in the ratio of 30:70, strain through a 0.45 µm membrane filter, and produce a gas.

Choice of column

Selection of Column

A Phenomenex C₁₈ column (25 cm 4.6 mm i.d., 5 µm particle size) was chosen simply because it is a robust, reproducible, and reliable RP-HPLC column (Medina A, Magan N. 2012). The column was stable at the desired pH and temperature giving good peak symmetry. Particle size of 5 µm provides a good trade-off between efficiency and performance.

Choice of Mobile Phase

The mobile phase was optimized and consisted of a binary system of acetonitrile/0.1 percent ortho - phosphoric acid (30:70 v/v). In light of the pK_a values of Metronidazole (4.7 ± 0.2) and Ofloxacin (3.8 ± 0.2), this composition was effective in terms of selectivity and provided an appropriate interaction with the analytes. The greater difference in the combination 7 of Tinidazole (TINI) and Ofloxacin (OFL) in the combined dose form allowed its adequate separation using the buffer.

Choice of Solvent

The analytes proved to be well soluble in the mobile phase; hence the choice of the solvent. Its miscibility with the mobile phase was great, which increased the overall chromatographic performance.

Choice of Wavelength for Detection

UV spectrophotometric analysis of the analytes in solvent showed isobestic point at 290 nm hence, the choice of this wavelength as the detection wavelength to simultaneously analyze TINI and OFL.

Preparation of Mobile Phase

- Buffer Preparation:** Dissolve 1.0 g of ortho-phosphoric acid in sufficient water to make 1000 mL.
- Mobile Phase Mixture:** Mix 30 parts of acetonitrile and 70 parts of 0.1% ortho-phosphoric acid buffer.

Sonicate for 15 minutes, cool to room temperature, and filter through a 0.45 μm membrane filter.

Preparation of Metronidazole Standard Stock Solution-I (1000 $\mu\text{g/mL}$)

In a 50 mL volumetric flask, accurately weighed 50 mg of the Metronidazole working standard. Prepare a concentration of 1000 $\mu\text{g/mL}$ in freshly prepared mobile phase and bring the volume to the mark.

Preparation of Ofloxacin Standard Stock Solution-I (1000 $\mu\text{g/mL}$)

Accurately weigh 50 mg of Ofloxacin working standard into a 50 mL volumetric flask. Dissolve in freshly prepared mobile phase and make up the volume to obtain a concentration of 1000 $\mu\text{g/mL}$.

Preparation of Standard Solution-II

Pipette 6.0 mL of Metronidazole standard stock solution and 2.0 mL Ofloxacin standard stock solution into a clean, dry 50 mL volumetric flask. Dilute to the mark with mobile phase to obtain concentrations of 120 $\mu\text{g/mL}$ (Metronidazole) and 40 $\mu\text{g/mL}$ (Ofloxacin). Filter the solution on a 0.45 μm membrane filter.

Preparation of Sample Solution

Weigh 20 tablets and get the mean weight. Crush the tablets to a fine powder. Measure an amount, equivalent to half the weight of the tablet, into a clean, dry volumetric flask of 100 mL. Add 50 mL mobile and sonicate 15 minutes. Bring to mark with diluent. Dilute 2 mL of this solution to 50 mL with the diluent and filter using a 0.45 μm membrane filter prior to analysis.

Analytical Method Validation System Suitability Parameters

The suitability to USP plates was estimated by calculating parameters like USP theoretical plate count, USP tailing factor, resolution and %RSD. These were determined by injection of 3 replicate samples of Nimorazole and Norfloxacin standards (120 and 40 $\mu\text{g/mL}$) (Yuwono M, Indrayanto G. 2005).

Specificity

The specificity was distinguished by comparing blank and standard solutions chromatograms. No interference of the blank was detected by the time Nimorazole and Norfloxacin are detected indicating the specificity of the method.

Linearity and Range

Linearity was determined in the concentration range of 80-120%. A calibration curve was made with the concentration of the x-axis and corresponding peak area of the Y-axis. The statistical parameters e.g. correlation coefficient, slope, intercept were calculated. The range of

the analysis was obtained by using the highest and lowest values that showed linearity.

Precision

- **Intraday Precision (Repeatability):** Repeatability was assessed by performing nine determinations at three concentration levels in triplicate. %RSD values were calculated.
- **Interday Precision:** Precision was further evaluated over three different days ($n=6$). The %RSD of the results confirmed reproducibility.

Robustness

Robustness was studied by introducing deliberate variations, including changes in flow rate (± 0.5 mL/min), organic phase composition of the mobile phase (± 10 mL), and detection wavelength (± 1 nm). The method remained unaffected by these small changes.

Accuracy

Accuracy was determined through recovery studies at three concentration levels:

- **50% solution:** 2 mL of sample stock solution I with 2.5 mL each of Nimorazole and Norfloxacin stock solution I, diluted to 50 mL with mobile phase.
- **100% solution:** 2 mL of sample stock solution I with 5 mL each of Nimorazole and Norfloxacin stock solution I, diluted to 50 mL with mobile phase.
- **150% solution:** 2 mL of sample stock solution I with 7.5 mL each of Nimorazole and Norfloxacin stock solution I, diluted to 50 mL with mobile phase.

For each level, the amount added and amount recovered were calculated. Individual and mean recovery values were obtained for both Nimorazole and Norfloxacin.

Applicability of Validated Method by RP-HPLC Assay of Formulation

A sample of twenty tablets was weighed to enable determination of the mean weight. Tablets were powdered and a quantity equal to half of the average weight of a single tablet placed in a 100 mL volumetric flask [6]. A volume of about 60 mL of mobile phase was added and the solution was mixed by sonication for 15 minutes. 100 mL mobile phase was added to the volume till a total of 100 mL and then left to settle down after 10 minutes. The solution was then cooled and 2.0 mL was pipetted into a 50 mL volumetric flask, filled to mark with mobile phase and filtered through a 0.45 μm membrane filter to generate a 120 $\mu\text{g/mL}$ solution of Nimorazole and 40 $\mu\text{g/mL}$ solution of Norfloxacin.

The stock was diluted to make aliquots containing medium concentrations of the standard curve (120 $\mu\text{g/mL}$ Nimorazole and 40 $\mu\text{g/mL}$ Norfloxacin). Chromatographic separation was carried out with the help

of a C18 ODS column (25 cm x 4.6 mm, 5 μ m), at room temperature. A mobile phase was used in a flow rate of 1.0 mL/min and was detected at 290 nm. The volume of

injection was 20 μ L. Peak areas were measured and concentrations of analytes were calculated with the help of the standard calibration curve.

RESULTS AND DISCUSSION

Melting point of Nimorazole and Norfloxacin

Table 1: Melting point determination

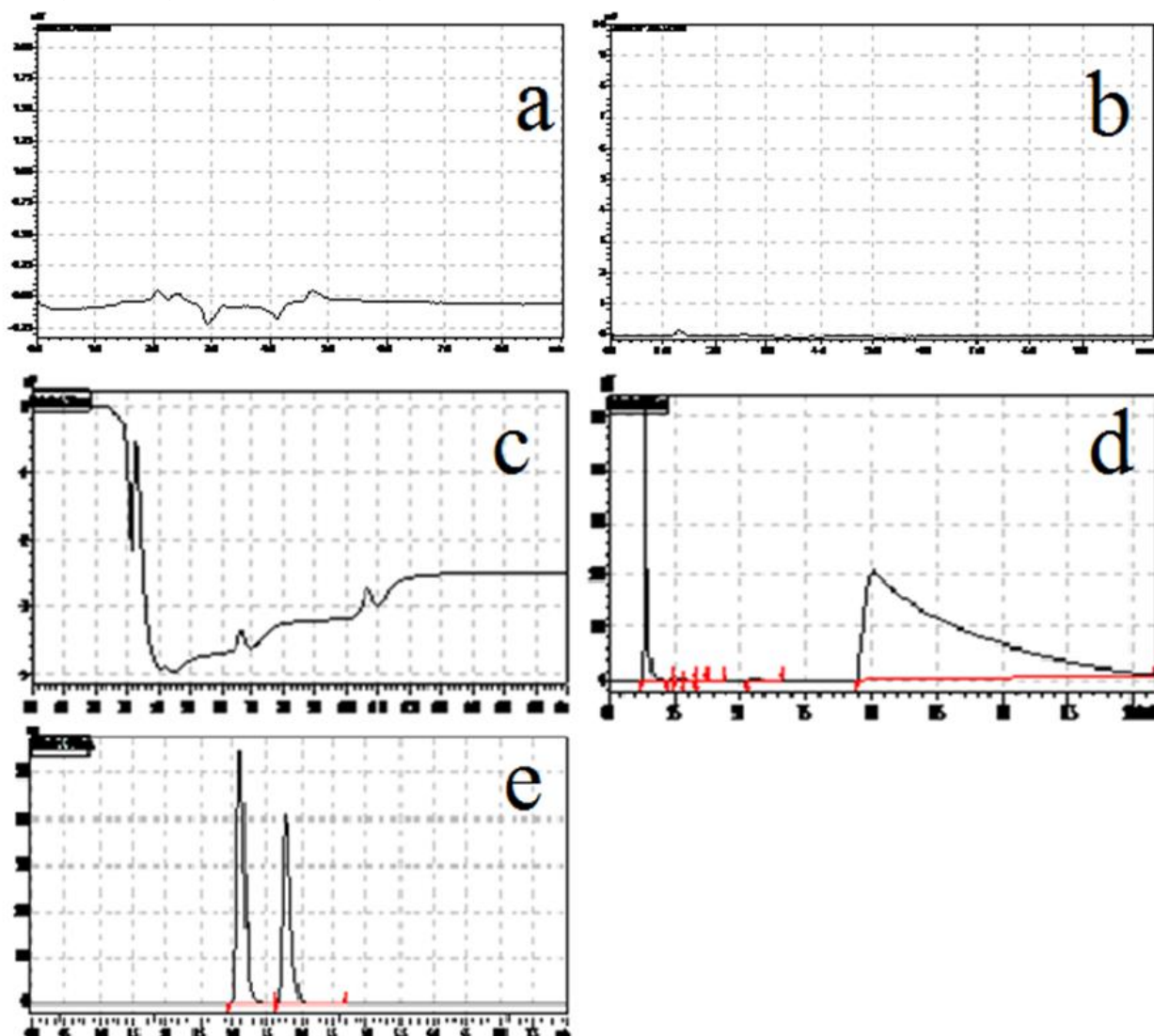
Sample	Observed MP ($^{\circ}$ C) ^a	Standard MP ($^{\circ}$ C)
Nimorazole	127.5 $^{\circ}$ C	127 $^{\circ}$ C-128 $^{\circ}$ C
Norfloxacin	254.3 $^{\circ}$ C	250 $^{\circ}$ C -257 $^{\circ}$ C

The obtained limits are within the limit.

METHOD DEVELOPMENT AND VALIDATION BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

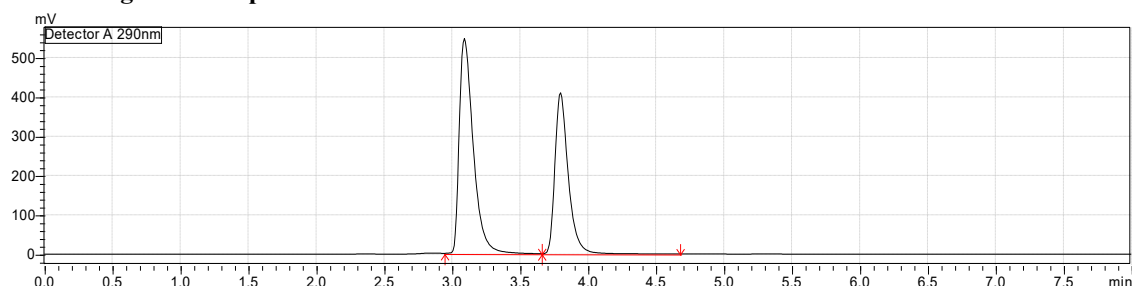
Figure 1: Trials for Optimization of RPHPLC Method

a. Trial 1; b. Trial 2; c. Trial 3; d. Trial 4; e. Trial 5



Optimized Parameters for RP- HPLC**Table 2: Optimized parameters for RP-HPLC method**

Column	Phenomenex C18 packed with Octadecyl silane
Mobile phase	Acetonitrile: 0.1 % Ortho phosphoric acid Buffer (30:70)
Solvent/ diluents	Mobile Phase
Flow rate	1.0 ml/ min
Injection volume	20µl
UV detection	290 nm

Figure 2: Chromatogram for Optimized Method**Table 3: Chromatogram Optimized parameters**

Name	Peak	Ret. Time	Area	Height
Norflaxacin	1	3.251	3795244	553269
Nimorazole	2	3.842	2703614	419986

System Suitability Parameters**Table 4: System Suitability Parameters for Nimorazole**

Inj.No	RT	Area	Theoretical Plates	USP Tailing Factor
1	3.865	3087184	7655	1.433
2	3.880	3086122	7652	1.436
3	3.861	3087244	7665	1.433
4	3.867	3086953	7653	1.441
5	3.870	3089213	7657	1.416
Mean		3087343	7657	1.431
SD		1137.39	5.193	0.009
% RSD		0.0425	0.069	0.659

Table 5: System Suitability Parameters for Norflaxacin

Inj.No	RT	Area	Theoretical Plates	USP Tailing Factor
1	3.211	4412136	4902	1.357
2	3.214	4408331	4908	1.361
3	3.213	4409332	4904	1.349
4	3.217	4409332	4907	1.360
5	3.213	4408950	4898	1.346
Mean		4401696	4903	1.355
SD		1466.884	4.038	0.007
% RSD		0.0351	0.0854	0.5023

Acceptance criteria

- Theoretical Plates- NLT 2000; USP Tailing factor- NMT 2.0; % RSD- NMT 2.0; Resolution-NLT 2.0

The system suitability parameters were within limits and hence the parameters for the optimized method could be applicable for the method to be validated.

Specificity

The method was found to be specific since the interference of blank with the chromatogram of Nimorazole and Norfloxacin was not observed.

Figure 3: Chromatogram for Specificity
a. Blank; b. Formulation
Linearity and Range

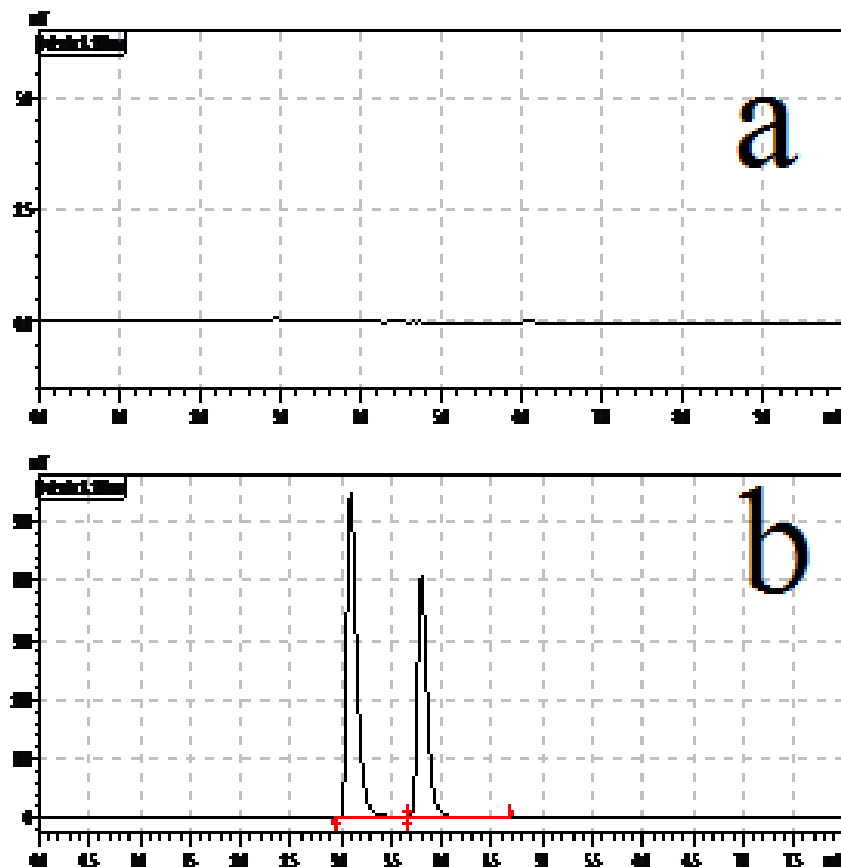


Figure 4: Linearity by RP-HPLC
Robustness

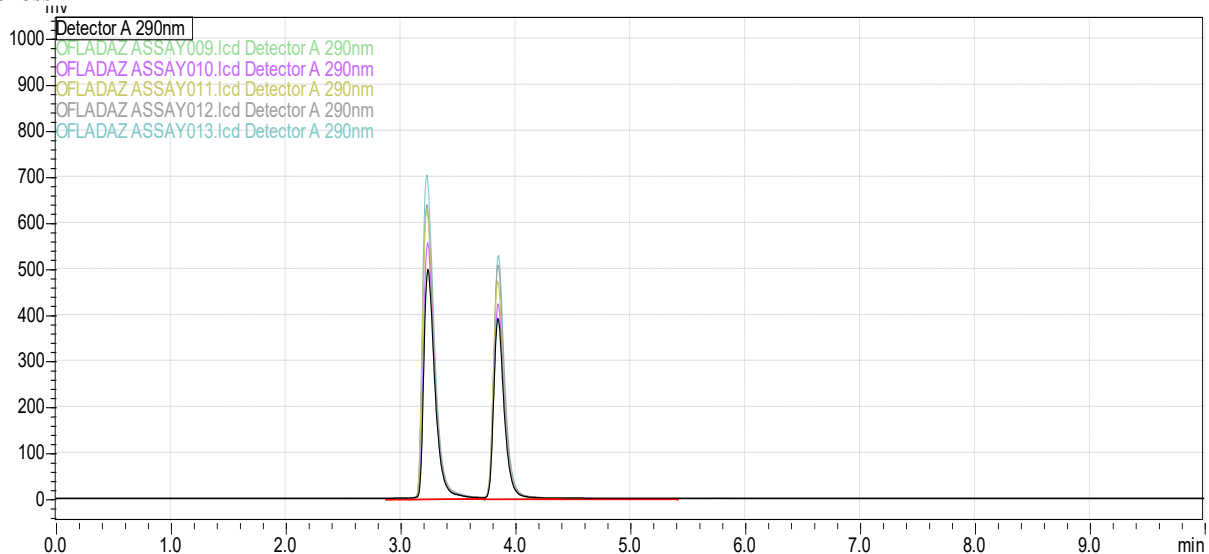
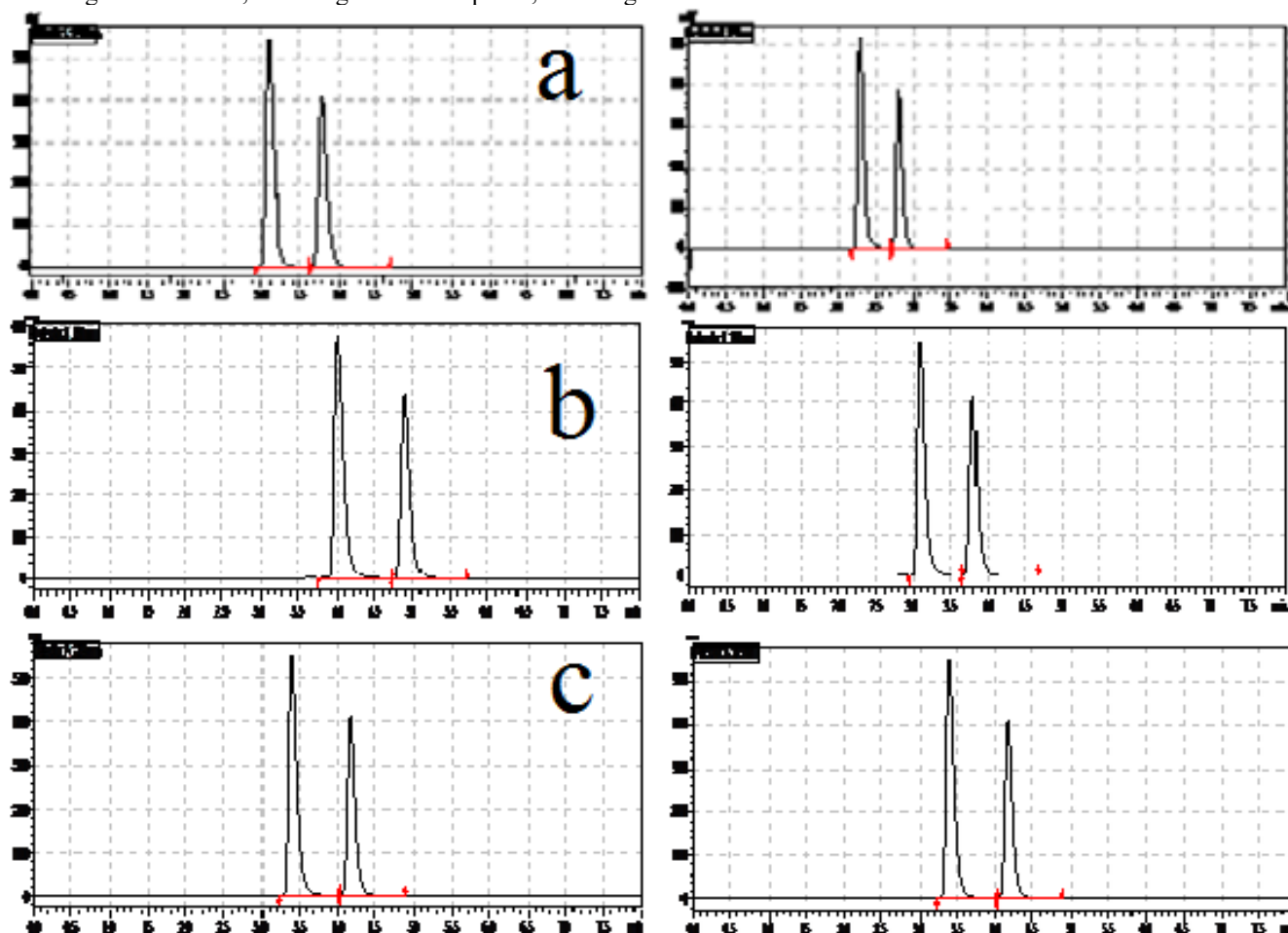


Figure 5: RPHPLC Chromatograms

a. Change in Flow rate; b. Change in mobile phase; c. Change in Detector

**Table 6: Robustness of the method for Nimorazole**

Parameter	Condition	System suitability parameters	
		Theoretical plates	USP Tailing factor
Change in flow rate (± 0.2 ml/ min)	0.8 ml/ min	7475	1.40
	1.2 ml/ min	7656	1.47
Change in organic phase composition (± 10 ml)	Methanol: Water (60:40)	7536	1.43
	Methanol: Water (80:20)	7656	1.45
Change in detector wavelength (± 2 nm)	292 nm	7583	1.45
	288 nm	7475	1.40

Table 7: Summary of Robustness for Norfloxacin

Parameter	Condition	System suitability parameters	
		Theoretical plates	USP Tailing factor
Change in flow rate (± 0.2 ml/ min)	0.8 ml/ min	4827	1.63
	1.2 ml/ min	4868	1.66
Change in organic phase composition (± 10 ml)	Methanol: Water (60:40)	4829	1.62
	Methanol: Water (80:20)	4826	1.65
Change in detector wavelength (± 2 nm)	292 nm	4837	1.64
	288 nm	4824	1.65

Accuracy:
Acceptance Criteria:

The % Recovery for each level should be between 98.0 and 102.0%. The accuracy data was found to be within limit.

Table 8: Accuracy Data for Nimorazole

Recovery levels	Accurate data for Nimorazole					
	Amount taken (µg/mL)	Amount added (µg/mL)	Area	Average area	Amount recovered (µg/mL)	% recovery
50%	100	50	4664469	4663588	150.92	100.61
	100	50	4662996			
	100	50	4663299			
100%	100	100	6160140	6158151	201.85	100.92
	100	100	6156172			
	100	100	6158142			
150%	100	150	7948540	7967092	249.21	103.27
	100	150	7926569			
	100	150	8026168			

Table 9: Accuracy Data for Norfloxacin

Recovery levels	Accurate data for Norfloxacin					
	Amount taken (µg/mL)	Amount added (µg/mL)	Area	Average area	Amount recovered (µg/mL)	% recovery
50%	100	50	6371034	6469512	149.51	99.67
	100	50	6469442			
	100	50	6568060			
100%	100	100	8418078	8415670	201.58	100.79
	100	100	8411806			
	100	100	8417127			
150%	100	150	10977532	10964401	248.73	99.49
	100	150	10448820			
	100	150	11466852			

ASSAY OF TABLETS BY RP- HPLC

Table 10: Common test used to level of significance

Formulation	Peak area	Label claim	Amount found	% Assay $\pm$ SD*
Nimorazole	3087184	600mg	603.06	100.51
	3086122			
	3087244			
Norfloxacin	4412136	200mg	201.47	100.73
	4408331			
	4409332			

Acceptance criteria: 95- 105%, Assay results were satisfactory and within limits

DISCUSSION

The present study was to develop and validate a simple, precise, accurate and robust RP-HPLC method to estimate the simultaneous estimation of Nimorazole and Norfloxacin in bulk and pharmaceutical formulations (Giriraj P, Sivakkumar T. 2015). The work consisted of a thoughtful assessment of melting points, optimization of methods, system suitability, linearity, precision, robustness, accuracy, and assay following the ICH guidelines. The findings prove that the method is

reproducible and can be used in quality control analysis. The melting points of Nimorazole and Norfloxacin were initially determined to confirm purity and identity of the drug substances (Bhattacharya B, *et al.* 2018). Nimorazole showed a melting point of 127.5°C, which corresponds well with its standard range of 127–128°C. Similarly, Norfloxacin displayed a melting point of 254.3°C, falling within the reported range of 250–257°C. These values proved the originality of the drugs used and showed that no major impurities were found. To undertake RP-HPLC

analysis, we undertook diverse mobile phase experiments with different proportions of acetonitrile, water, methanol, and buffer mixtures (Peltier R, *et al.* 2018).

The method specificity was determined through analysis of chromatograms, using blank and formulation samples (Yuwono M, Indrayanto G. 2005). There are no interfering peaks of excipients or the mobile phase at the retention times where Nimorazole and Norfloxacin are located, which proves that the method allows specifically quantifying both drugs in the presence of others. The Linearity was tested at concentration range of 80 to 120% of the target concentrations. The correlations found between concentration and peak area of the two drugs were strong with correlation coefficients (R_2) values of 0.9985 and 0.9968 respectively of Nimorazole and Norfloxacin. The linear regression equations were $y = 28985.03x + 145268.315$ for Nimorazole and $y = 43525.628x + 14625.38$ for Norfloxacin. These findings confirmed excellent linearity, suitable for quantitative analysis within the studied range.

Robustness testing was performed by introducing deliberate changes in chromatographic parameters such as flow rate (± 0.2 ml/min), organic phase composition (± 10 ml), and detection wavelength (± 2 nm) [14]. Accuracy was assessed through recovery studies at 50%, 100%, and 150% of target concentrations. The percentage recovery for Nimorazole ranged from 100.61% to 103.27%, while for Norfloxacin it ranged from 99.49% to 100.79%. All

values were within the acceptance limits of 98–102%, confirming the accuracy and reliability of the method for quantifying both drugs in bulk and formulation.

The devised and validated procedure was utilizing the assay analysis of commercial tablet preparations of Nimorazole (600 mg) and Norfloxacin (200 mg). The results of assay were found to be 100.51 in Nimorazole and 100.73 in Norfloxacin which falls under the acceptance criterion of 95-105 per cent. This shows that the procedure is applicable in routine estimation of such drugs in combined drug forms. The method is also sensitive and reproducible, which makes it potentially useful in studies concerning stability.

CONCLUSION

The simple, rapid, accurate, precise, specific and robust validated RP-HPLC method was able to estimate Nimorazole and Norfloxacin concomitantly. The acceptance criteria as recommended by the ICH guidelines were met by all the validation parameters and this is an indicator that the method is reliable and replicable. Thus, the proposed technique can be used in routine quality control, stability testing and regulatory analysis of pharmaceutical formulations of Nimorazole and Norfloxacin. It offers a quality analysis tool that is easy to use in academic studies research as well as in industrial pharmaceutical laboratories.

REFERENCE:

- Bhattacharya B, Mondal A, Soni SR, Das S, Bhunia S, Raju KB, Ghosh A, Reddy CM. Multidrug salt forms of norfloxacin with non-steroidal anti-inflammatory drugs: solubility and membrane permeability studies. *CrystEngComm*. 2018;20(41):6420-9.
- Carr JE, Kwok K, Webster GK, Carnahan JW. Effects of liquid chromatography mobile phases and buffer salts on phosphorus inductively coupled plasma atomic emission and mass spectrometries utilizing ultrasonic nebulization and membrane desolvation. *Journal of pharmaceutical and biomedical analysis*. 2006;40(1):42-50.
- Giriraj P, Sivakkumar T. A rapid-chemometrics assisted RP-HPLC method with PDA detection for the simultaneous estimation of ofloxacin and nimorazole in pharmaceutical formulation. *Journal of Liquid Chromatography & Related Technologies*. 2015;38(8):904-10.
- Giriraj P, Sivakkumar T. A rapid-chemometrics assisted RP-HPLC method with PDA detection for the simultaneous estimation of ofloxacin and nimorazole in pharmaceutical formulation. *Journal of Liquid Chromatography & Related Technologies*. 2015;38(8):904-10.
- Le Mapihan K, Vial J, Jardy A. Reversed-phase liquid chromatography column testing: robustness study of the test. *Journal of Chromatography A*. 2004;1061(2):149-58.
- Medina A, Magan N. Comparison of three different C18 HPLC columns with different particle sizes for the optimization of aflatoxins analysis. *Journal of Chromatography B*. 2012;889:138-43.
- Millanao AR, Mora AY, Villagra NA, Bucarey SA, Hidalgo AA. Biological effects of quinolones: a family of broad-spectrum antimicrobial agents. *Molecules*. 2021;26(23):7153.
- Peltier R, Bialek A, Kuroki A, Bray C, Martin L, Perrier S. Reverse-phase high performance liquid chromatography (RP-HPLC) as a powerful tool to characterise complex water-soluble copolymer architectures. *Polymer Chemistry*. 2018;9(46):5511-20.
- Rathod RH, Chaudhari SR, Patil AS, Shirkhedkar AA. Ultra-high performance liquid chromatography-MS/MS (UHPLC-MS/MS) in practice: analysis of drugs and pharmaceutical formulations. *Future Journal of Pharmaceutical Sciences*. 2019;5(1):6.

- Regalado EL, Haidar Ahmad IA, Bennett R, D'Atri V, Makarov AA, Humphrey GR, Mangion I, Guillaume D. The emergence of universal chromatographic methods in the research and development of new drug substances. *Accounts of chemical research*. 2019;52(7):1990-2002.
- White WP. Melting point methods at high temperatures. *American Journal of Science*. 1909;4(167):474-89.
- Young JC. True melting point determination. *Chem Educ*. 2013;18(2):208.
- Yuwono M, Indrayanto G. Validation of chromatographic methods of analysis. *Profiles of drug substances, excipients and related methodology*. 2005;32:243-59.