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Research Article

**DEVELOPMENT AND VALIDATION OF ANALYTICAL  
METHOD FOR SIMULTANEOUS ESTIMATION OF  
METRONIDAZOLE AND NALIDIXIC ACID BY RP-HPLC****Kommuri Hari Krishna Reddy\*, Godasu Suresh kumar, Dr. P. Viahnu priya.**<sup>1</sup> Department of Quality Assurance, Sri Indu Institute of Pharmacy, Sheriguda (V),  
Ibrahimpattanam, Telangana., 501510.**Abstract:**

*A simple, rapid, precise, and reproducible Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Metronidazole and Nalidixic Acid in bulk and pharmaceutical dosage forms. Chromatographic separation was carried out using a Phenomenex Luna C18 column (4.6 × 150 mm, 5 μm) with a mobile phase consisting of Methanol and Water (70:30 v/v), delivered at a flow rate of 1 mL/min. The column temperature was maintained at 35 °C, the detection wavelength was set at 230 nm, and the injection volume was 10 μL with a total run time of 10 minutes.*

*Both drugs were well resolved with sharp, symmetrical peaks, and the method was validated as per ICH guidelines. The results demonstrated excellent linearity, accuracy, precision, specificity, and robustness, with recovery studies confirming accuracy and %RSD values within acceptable limits (<2%). The developed method proved to be sensitive and free from interference of excipients.*

**Keywords:** RP-HPLC, Metronidazole and Nalidixic Acid, simultaneous estimation, validation, Phenomenex Luna C18 column, ICH guidelines.

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## 1. INTRODUCTION:

### 1.1 INTRODUCTION

Pharmaceutical analysis comprises those procedures necessary to determine “identity, strength, quality and purity of the drug substances and drug products. Pharmaceutical analyst plays a major role in all quality controlling divisions of industry. Analytical chemistry involves separating, identifying, and determining the relative amounts of components in a sample matrix. The number of new drugs is constantly growing. This requires new methods for controlling the quality. Modern pharmaceutical analysis must need the following requirements<sup>1</sup>.

1. The analysis should take a minimal time.
2. The accuracy of the analysis should meet the demands of the Pharmacopoeia.
3. The analysis should be performed with a minimal cost.
4. Precision and selectivity of the selected method should be good.

### 1.2 Typical Instrumental Techniques<sup>2,3</sup>:

The methods of estimation of drugs are divided into physical, chemical, physicochemical and biological ones of them, physical and physicochemical methods are used mostly. Physical methods of analysis involve the studying of the physical properties of a substance. They include determination of the solubility, transparency or degree of turbidity, colour density or specific gravity (for liquids), moisture content, melting, freezing and boiling points. Physicochemical methods are used to study the physical phenomenon that occurs as a result of chemical reactions. Among the physicochemical methods are optical refractometry, polarimetry, emission and fluorescent methods of analysis, photometry including photolorimetry, spectrophotometry, nephelometry and turbidometry, electrochemical (potentiometry, amperometry, coulometer, polarography) and chromatography (column, paper, thin layer, gas, high performance liquid) methods are generally preferable.

Methods involving nuclear reactions such as nuclear magnetic resonance (NMR) and paramagnetic resonance (PMR) are becoming more popular. The combination of mass spectroscopy with gas chromatography is one of the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures, which are based on complex formation, acid-base and precipitation and redox reactions. Titrations in non-aqueous media and complexometry have been widely used in pharmaceutical analysis whenever the existing amounts are in milligram level and the interference is negligible. The methods (LC-MS,<sup>4</sup> HPLC, GLC, NMR and Mass Spectroscopy) of choice for assay involve sophisticated equipment that are very costly and pose problems of maintenance. Hence, they are not in the reach of most laboratories and small-scale industries, which

produce bulk drugs and pharmaceutical formulations.

The visible Spectrophotometric methods which fall in the wavelength region 400-800 nm and fluorimetric methods (may fall in UV & Visible regions) are very simple, cheap and easy to carry out estimations of drugs in bulk form and their formulations. The limitations of many colorimetric or fluorimetric methods of analysis lie in the chemical reactions upon which the procedures are based rather than the instruments available. Many of the reactions involve colour or fluorescence of a drug are quite selective or can be rendered selective through the introduction of masking agents, control of PH, use of solvent extraction technique, adjustment of oxidation states or by prior removal of interfering ingredients with the aid of chromatographic separation.

1. This is preferably followed by general methodology for UV-Visible and HPLC method developments.

2. Followed by literature of drugs used in Analysis

### 1.3 INTRODUCTION TO HPLC

Russian botanist Tswett invented chromatography as a separation technique. He describes in detail the separation of pigments, the colour substances by filtration through column, followed by developments with pure solvents.

High-performance liquid chromatography (HPLC)<sup>5</sup> is the fastest growing analytical technique for analysis of drugs. Its simplicity, high specificity and wide range of sensitivity make it ideal for the analysis of many drugs in both dosage forms and biological fluids.

According to IUPAC, chromatography<sup>6</sup> is a physical method of separation in which components will be separated or distributed between stationary and mobile phases. The importance of chromatography is increasing rapidly in pharmaceutical analysis for the exact differentiation, selective identification and quantitative determination of structurally closely related compounds. Another important field of application of chromatographic methods is the purity testing of final products and the intermediates. The reasons for the popularity of the method is its sensitivity, its ready adaptability to accurate quantitative determinations, its suitability for separating non-volatile species or thermally fragile ones and its wide spread applicability to substances that are of prime interest to the industry. Sensitive detectors have transformed liquid column chromatography into high speed, efficient, accurate and highly resolved method of separation.<sup>8-12</sup>

The HPLC is the method of choice in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of

detection is low and also it offers the following advantages.<sup>13-14</sup>

- ❖ Speed (many analysis can be accomplished in 20 min or less)
- ❖ Greater sensitivity (various detectors can be employed)
- ❖ Improved resolution (wide variety of stationary phases)
- ❖ Reusable columns (expensive columns but can be used for many analysis)
- ❖ Ideal for the substances of low viscosity
- ❖ Easy sample recovery, handling and maintenance.
- ❖ Instrumentation leads itself to automation and quantification (less time and less labour)
- ❖ Precise and reproducible
- ❖ Integrator itself does calculations.

Validation involves laboratory investigations that the method is suitable and reliable for the intended application.<sup>15</sup>

#### EXPERIMENTAL METHODS:

##### INSTRUMENTS AND CHEMICALS USED

HPLC WATERS Alliance 2695 separation module, Software: Empower 2, 996 PDA detector.

pH meter Lab India

Weighing machine Sartorius

Volumetric flasks Borosil

Pipettes and Burettes Borosil

Beakers Borosil

Metronidazole provided by Sura Pharma labs

Nalidixic Acid provided by Sura Pharma labs

Water and Methanol for HPLC LICHROSOLV (MERCK)

##### HPLC METHOD DEVELOPMENT:

##### TRAILS

##### Preparation of standard solution:

#### RESULTS AND DISCUSSION:

##### (Optimized chromatogram) (Standard):

Temperature : 35°C

Column : Phenomenex Luna C18 (4.6 x 150mm, 5µm)

Mobile phase : Methanol and water (70:30 v/v)

Flow rate : 1ml/min

Wavelength : 230 nm

Injection volume : 10 µl

Run time : 10 min

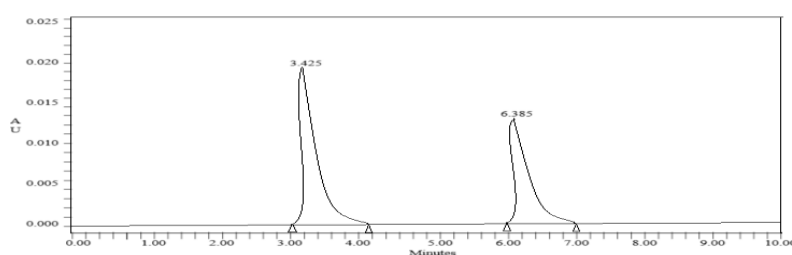


Figure7.5: Optimized Chromatogram (Standard)

Accurately weigh and transfer 10 mg of Metronidazole and Nalidixic Acid working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 2.25ml of the above Metronidazole and 0.45ml of the Nalidixic Acid stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

##### Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

##### Mobile Phase Optimization:

Initially the mobile phase tried was Methanol: Water, Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Methanol and water in proportion 70:30 v/v respectively.

##### Optimization of Column:

The method was performed with various columns like C18 column, X- bridge column, Xterra. Phenomenex Luna C18 (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

#### VALIDATION

##### PREPARATION OF MOBILE PHASE:

##### Preparation of mobile phase:

Accurately measured 700ml (70%) of HPLC Methanol and 300ml of Water (30%) were mixed and degassed in a digital ultrasonicator for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

##### Diluent Preparation:

The Mobile phase was used as the diluent.

**Table7.5: Optimized Chromatogram (Standard)**

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count	USP Resolution
1	Metronidazol	3.425	2391746	39726	1.2	9028	
2	Nalidixic Acid	6.385	194627	8497	1.1	7398	7.4

**Observation:**

This trial shows improper separation sample peaks, baseline and show very less plate count in the chromatogram. So it's required more trials to obtain good peaks.

From the above chromatogram it was observed that the Metronidazole and Nalidixic Acid peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

**Optimized Chromatogram (Sample)**

Temperature : 35°C

Column : Phenomenex Luna C18 (4.6 x 150mm, 5µm)

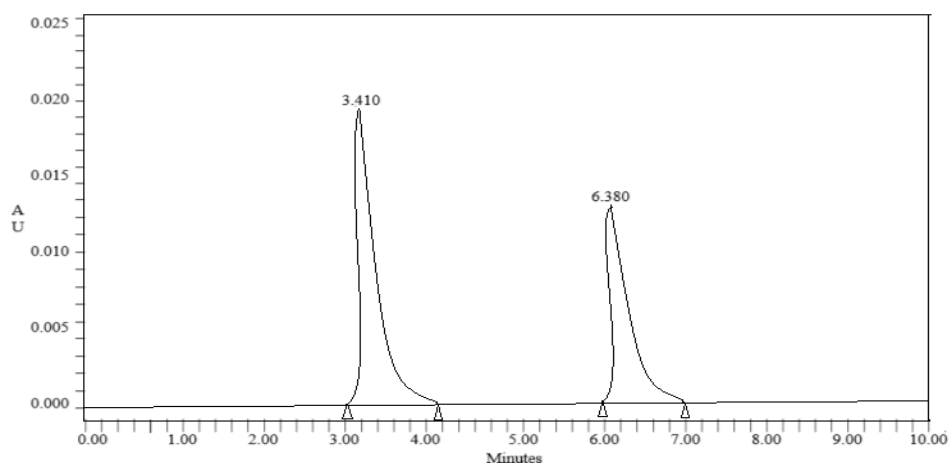
Mobile phase : Methanol and water (70:30 v/v)

Flow rate : 1ml/min

Wavelength : 230 nm

Injection volume : 10 µl

Run time : 10 min

**Figure7.6: Optimized Chromatogram (Standard)****Table7.6: Optimized Chromatogram (Sample)**

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count	USP Resolution
1	Metronidazole	3.410	2381649	391846	1.2	9472	
2	Nalidixic Acid	6.380	1910575	8104	1.1	8936	7.5

**Acceptance criteria:**

- Resolution between two drugs must be not less than 2
- Theoretical plates must be not less than 2000
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

**VALIDATION****Blank:**

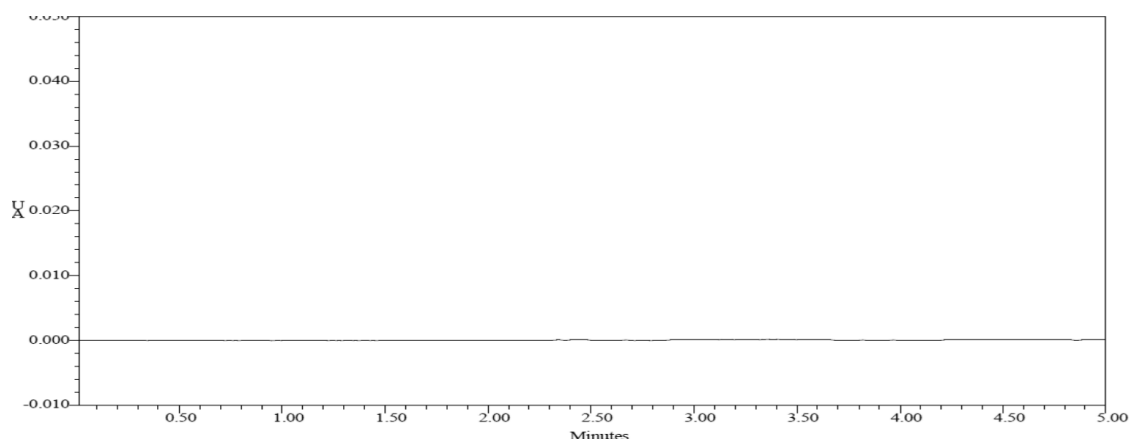


Fig7.7: Chromatogram showing blank (mobile phase preparation)

System suitability:

Table:7.7 Results of system suitability for Metronidazole

S.No	Peak Name	RT	Area ( $\mu\text{V}\cdot\text{sec}$ )	Height ( $\mu\text{V}$ )	USP Plate Count	USP Tailing
1	Metronidazole	3.422	2391746	394171	8952	1.2
2	Metronidazole	3.419	2391647	381946	9561	1.2
3	Metronidazole	3.415	2381647	391746	6572	1.2
4	Metronidazole	3.420	2385631	386562	6452	1.2
5	Metronidazole	3.424	2385635	389164	7452	1.2
<b>Mean</b>			2387261			
<b>Std. Dev.</b>			4363.771			
<b>% RSD</b>			0.182794			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is suitable.

Table:7.8 Results of system suitability for Nalidixic Acid

S.No	Peak Name	RT	Area ( $\mu\text{V}\cdot\text{sec}$ )	Height ( $\mu\text{V}$ )	USP Plate Count	USP Tailing
1	Nalidixic Acid	6.381	198362	7917	5272	1.1
2	Nalidixic Acid	6.379	197486	7486	6291	1.1
3	Nalidixic Acid	6.376	198354	7859	6184	1.1
4	Nalidixic Acid	6.382	197352	7926	7145	1.1
5	Nalidixic Acid	6.384	198453	7946	6946	1.1
<b>Mean</b>			198001.4			
<b>Std. Dev.</b>			535.1774			
<b>% RSD</b>			0.27029			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is suitable.

**SPECIFICITY**

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

**Assay (Standard):**

Table:7.9 Peak results for assay standard

**Metronidazole**

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Metronidazole	3.424	2397162	397161	1.2	9472
2	Metronidazole	3.420	2394721	389173	1.2	9745
3	Metronidazole	3.415	2389461	391723	1.2	8917

**Nalidixic Acid**

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Nalidixic Acid	6.384	198462	7811	1.1	8492	7.49
2	Nalidixic Acid	6.382	198472	8193	1.1	8916	7.52
3	Nalidixic Acid	6.376	198735	7972	1.1	9372	7.44

**Assay (Sample):**

Table:7.10 Peak results for Assay sample

**Metronidazole**

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Metronidazole	3.419	2391741	381612	1.2	9472
2	Metronidazole	3.415	2389166	391746	1.2	8927
3	Metronidazole	3.424	2361731	381634	1.2	9017

**Nalidixic Acid**

S. No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Resolution
1	Nalidixic Acid	6.379	198641	8174	1.1	9284	7.18
2	Nalidixic Acid	6.376	196547	8942	1.1	8974	7.44
3	Nalidixic Acid	6.384	194027	7294	1.1	9017	7.38

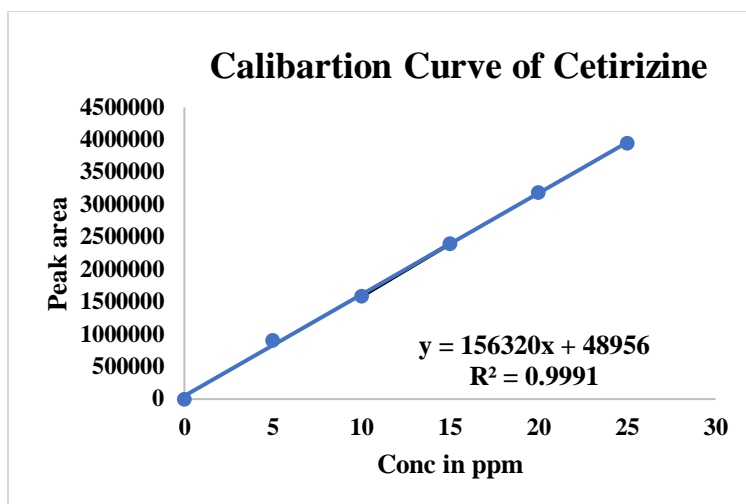
%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

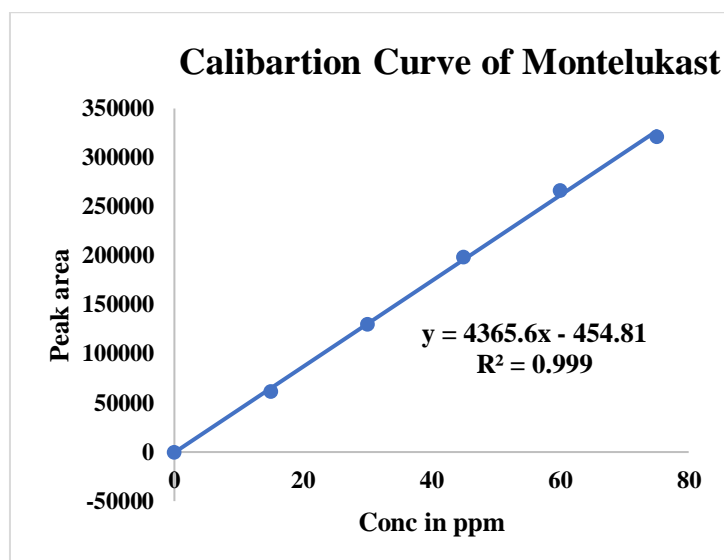
The % purity of Metronidazole and Nalidixic Acid in pharmaceutical dosage form was found to be 99.5%

**LINEARITY****CHROMATOGRAPHIC DATA FOR LINEARITY STUDY:****Metronidazole**

Concentration µg/ml	Average Peak Area
0	0
5	909889
10	1583641
15	2395378
20	3185089
25	3943725

**Nalidixic Acid**

Concentration µg/ml	Average Peak Area
0	0
15	61953
30	130213
45	198697
60	267002
75	321658

**REPEATABILITY****Table:7.11 Results of repeatability for Metronidazole:**

S. No	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Metronidazole	3.419	2397164	381741	8155	1.2
2	Metronidazole	3.420	2391741	371742	9174	1.2
3	Metronidazole	3.419	2371846	391746	7154	1.2
4	Metronidazole	3.415	2361748	391847	9917	1.2
5	Metronidazole	3.419	2371649	384622	9247	1.2
<b>Mean</b>			2378830			
<b>Std.dev</b>			14958			
<b>%RSD</b>			0.628797			

**Acceptance criteria:**

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

**Table:7.12 Results of repeatability for Nalidixic Acid :**

S. No	Peak name	Retention time	Area( $\mu\text{V}\cdot\text{sec}$ )	Height ( $\mu\text{V}$ )	USP Plate Count	USP Tailing
1	Nalidixic Acid	6.379	198464	7291	6274	1.1
2	Nalidixic Acid	6.382	193643	7219	6592	1.1
3	Nalidixic Acid	6.379	196462	7194	6028	1.1
4	Nalidixic Acid	6.376	194644	8174	6927	1.1
5	Nalidixic Acid	6.379	198464	8653	5920	1.1
<b>Mean</b>			196335.4			
<b>Std.dev</b>			2190.191			
<b>%RSD</b>			1.115536			

**Intermediate precision:****Day 1:****Table:7.13 Results of Intermediate precision for Metronidazole**

S. No	Peak Name	RT	Area ( $\mu\text{V}\cdot\text{sec}$ )	Height ( $\mu\text{V}$ )	USP Plate count	USP Tailing
1	Metronidazole	3.419	2389572	395275	9375	1.2
2	Metronidazole	3.415	2391847	392175	9275	1.2
3	Metronidazole	3.424	2319472	312947	8265	1.2
4	Metronidazole	3.415	2306842	310585	6254	1.2
5	Metronidazole	3.419	2375972	310694	9028	1.2
6	Metronidazole	3.424	2396746	358373	8928	1.2
<b>Mean</b>			2363409			
<b>Std. Dev.</b>			39730.83			
<b>% RSD</b>			1.681082			

**Acceptance criteria:**

- %RSD of six different sample solutions should not more than 2

**Table:7.14 Results of Intermediate precision for Nalidixic Acid**

S. No	Peak Name	RT	Area ( $\mu\text{V}\cdot\text{sec}$ )	Height ( $\mu\text{V}$ )	USP Plate count	USP Tailing
1	Nalidixic Acid	6.379	197284	7194	8264	1.2
2	Nalidixic Acid	6.376	197849	7294	9174	1.2
3	Nalidixic Acid	6.384	196572	7147	9164	1.2
4	Nalidixic Acid	6.376	195028	7927	9733	1.2
5	Nalidixic Acid	6.379	199474	8238	9194	1.2
6	Nalidixic Acid	6.384	197482	7638	8973	1.2
<b>Mean</b>			197281.5			
<b>Std. Dev.</b>			1466.354			
<b>% RSD</b>			0.74328			

**Acceptance criteria:**

- %RSD of six different sample solutions should not more than 2

**Day 2:**

**Table:7.15 Results of Intermediate precision Day 2 for Metronidazole**

S.No	Peak Name	RT	Area ( $\mu\text{V}\cdot\text{sec}$ )	Height ( $\mu\text{V}$ )	USP Plate Count	USP Tailing
1	Metronidazole	3.424	2389562	391741	9264	1.2
2	Metronidazole	3.422	2381654	391047	9746	1.2
3	Metronidazole	3.419	2381946	391748	9816	1.2
4	Metronidazole	3.415	2391741	391746	9917	1.2
5	Metronidazole	3.420	2386452	381641	9742	1.2
6	Metronidazole	3.424	2374763	381645	9017	1.2
<b>Mean</b>			2384353			
<b>Std. Dev.</b>			6183.339			
<b>% RSD</b>			0.25933			

**Acceptance criteria:**

- %RSD of six different sample solutions should not more than 2

**Table:7.16 Results of Intermediate precision Day 2 for Nalidixic Acid**

S.No	Peak Name	RT	Area ( $\mu\text{V}\cdot\text{sec}$ )	Height ( $\mu\text{V}$ )	USP Plate Count	USP Tailing
1	Nalidixic Acid	6.384	197486	7582	6272	1.1
2	Nalidixic Acid	6.381	197486	7184	6174	1.1
3	Nalidixic Acid	6.379	196746	7456	5184	1.1
4	Nalidixic Acid	6.376	195862	7814	6194	1.1
5	Nalidixic Acid	6.382	196582	7194	6292	1.1
6	Nalidixic Acid	6.384	198463	7745	6191	1.1
<b>Mean</b>			197104.2			
<b>Std. Dev.</b>			903.542			
<b>% RSD</b>			0.458408			

**Acceptance criteria:**

- %RSD of six different sample solutions should not more than 2

**ACCURACY:****The accuracy results for Metronidazole**

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	1217218	112.5	112.4	99.6	99.3
100%	2397141	225	225	100	
150%	3514547	337.5	332.5	98.5	

**Acceptance Criteria:**

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

**The accuracy results for Nalidixic Acid**

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	98598.67	22.5	22.4	99.9	99.6
100%	198359.7	45	45	100	
150%	291512.3	67.5	66.8	99	

**Acceptance Criteria:**

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

**LIMIT OF DETECTION****Metronidazole:****Result:**

$$=3.3 \times 39762 / 10421$$

$$=12.5 \mu\text{g/ml}$$

**Nalidixic Acid:****Result:**

$$=3.3 \times 5008 / 4365$$

$$=3.7 \mu\text{g/ml}$$

**Metronidazole:****Result:**

$$=10 \times 39762 / 10421$$

$$=38.1 \mu\text{g/ml}$$

**Nalidixic Acid:****Result:**

$$=10 \times 5008 / 4365$$

$$=11.4 \mu\text{g/ml}$$

**ROBUSTNESS****Table:7.20 Results for Robustness****Metronidazole**

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical	Tailing factor
Actual Flow rate of 1.0mL/min	2391746	3.418	9028	1.2
Less Flow rate of 0.9mL/min	2371831	3.424	7381	1.2
More Flow rate of 1.1mL/min	2218319	3.422	9311	1.1
Less organic phase (about 5 % decrease in organic phase)	2294821	3.419	7462	1.2
More organic phase (about 5 % Increase in organic phase)	2394811	3.422	6817	1.1

**Acceptance criteria:**

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

**Table:7.21 Results for Robustness****Nalidixic Acid**

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.1mL/min	194627	6.463	7398	1.1
Less Flow rate of 0.9mL/min	183738	6.384	6883	1.1
More Flow rate of 0.8mL/min	198373	6.381	9917	1.2
Less organic phase (about 5 % decrease in organic phase)	178471	6.379	8372	1.1
More organic phase (about 5 % Increase in organic phase)	189462	6.381	7716	1.2

**Acceptance criteria:**

The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

**8. SUMMARY AND CONCLUSION:**

A reliable, simple, and precise Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Metronidazole and Nalidixic Acid in bulk and pharmaceutical dosage forms. Chromatographic separation was achieved on a Phenomenex Luna C18 column (4.6 × 150 mm, 5

μm) using a mobile phase of Methanol and Water (70:30 v/v), maintained at a flow rate of 1 mL/min. The analysis was carried out at a column temperature of 35 °C, with detection at 230 nm using an injection volume of 10 μL. The method demonstrated efficient separation with sharp, symmetrical, and well-resolved peaks for both drugs within a run time of 10 minutes.

The developed method was validated according to ICH guidelines. It showed excellent linearity across the tested concentration ranges, with correlation coefficients close to 1.0 for both drugs. Accuracy was confirmed through recovery studies, yielding results within acceptable limits. Precision studies indicated low %RSD values (<2%), confirming reproducibility. The method also proved to be robust, specific, and sensitive, with no interference from excipients or impurities.

### CONCLUSION:

The proposed RP-HPLC method for the simultaneous determination of Metronidazole and Nalidixic Acid was found to be simple, rapid, accurate, and reproducible. The optimized chromatographic conditions ensured effective separation, excellent peak symmetry, and consistent retention times. Validation results proved that the method is accurate, precise, robust, and specific in accordance with ICH guidelines. Therefore, this method can be successfully applied for the routine quality control, formulation analysis, and stability testing of pharmaceutical dosage forms containing Metronidazole and Nalidixic Acid.

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