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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF OFLOXACIN AND METRONIDAZOLE IN BULK AND DOSAGE FORM USING RP-HPLC

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Abstract:
*A new method was established for simultaneous estimation of Ofloxacin and Metronidazole by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Ofloxacin and Metronidazole by using Agilent C18 5µm (4.6*250mm) column, flow rate was 1ml/min, mobile phase ratio was Methanol: ACN (70:30%v/v), detection wave length was 238nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, PDA Detector 996, Empower-software version-2.The retention times were found to be 2.443 mins and 2.918 mins. The % purity of Ofloxacin and Metronidazole was found to be 100.7% and 101.4% respectively. The system suitability parameters for Ofloxacin and Metronidazole such as theoretical plates and tailing factor were found to be 1.7, 2114.5and 1.7, 2931.0 the resolution was found to be 8.0.The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study for Ofloxacin and Metronidazole was found in concentration range of 1µg-5µg and 100µg-500µg and correlation coefficient (r2) was found to be 0.999 and 0.999, % mean recovery was found to be 100% and 100.5%, %RSD for repeatability was 2.0 and 2.0, % RSD for intermediate precision was 1.5 and 1.1 respectively. The precision study was precise, robust, and repeatable. LOD value was 2.95 and 3.04, and LOQ value was 9.87 and 10 respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Ofloxacin and Metronidazole in API and Pharmaceutical dosage form.*

Keywords: *Agilent C18, Ofloxacin and Metronidazole, RP-HPLC method.*

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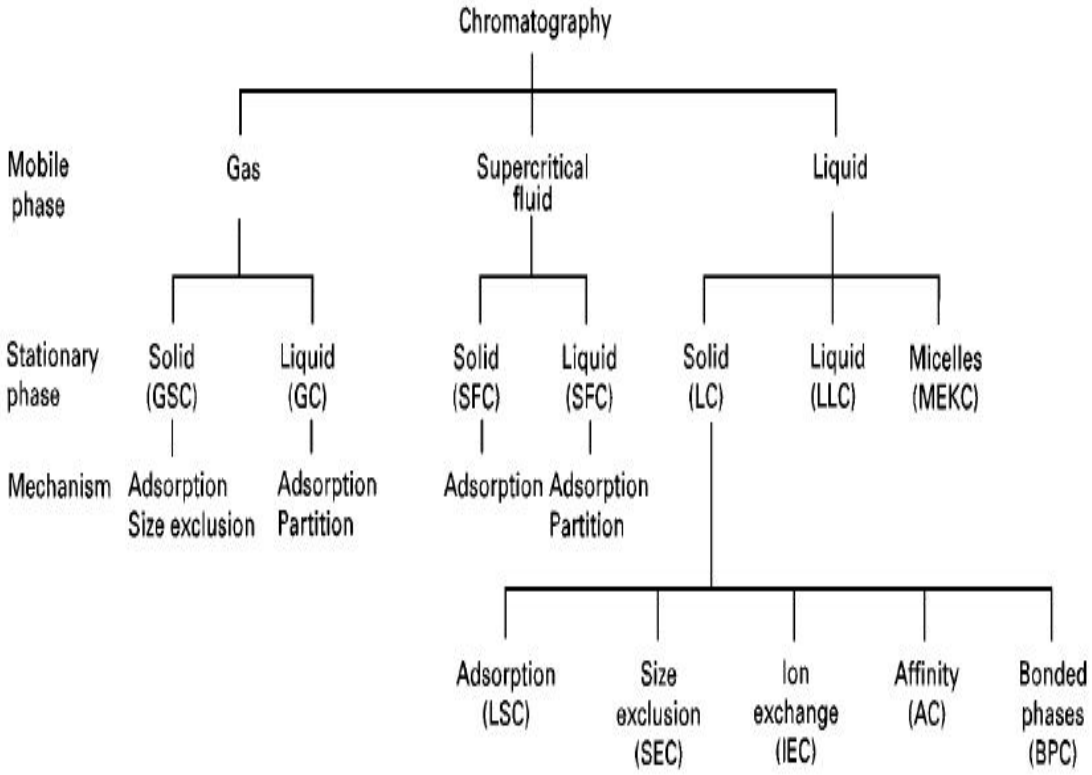
INTRODUCTION:

Chromatography is the most powerful and versatile technique available to the modern analyst. In a single step process it can separate a mixture into its individual components and simultaneously provide a quantitative estimate of each constituent. Samples may be gaseous, liquid or solid in nature and can range in complexity from a simple blend of two enantiomers to a multi component mixture containing widely differing chemical species. The word chromatography means "color writing" which is a way that a chemist can test liquid mixtures. While studying the coloring materials in plant life, a Russian botanist, M.S.Tswett invented chromatography in 1902 [1-5].

Chromatography is defined as a separation process that is achieved b y distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase[6,7].

Types of Chromatography

The mobile phase could be either a liquid or a gas, and accordingly we can subdivide chromatography into Liquid Chromatography (LC) or Gas Chromatography (GC). Apart from these methods, there are two other modes that use a liquid mobile phase, but the nature of its transport through the porous stationary phase is in the form of either (a) capillary forces, as in planar chromatography (also called Thin-Layer Chromatography, TLC), or (b) electro osmotic flow, as in the case of Capillaryy Electro Chromatography (CEC) [8-10].



Types of Chromatography

High Performance Liquid Chromatography

The acronym HPLC, coined by the late Prof. Csaba Horvath for his 1970 Pittcon paper, originally indicated the fact that high pressure was used to generate the flow required for liquid chromatography in packed columns. In the beginning, pumps only had a pressure capability of 500 psi. This was called high pressure liquid chromatography, or HPLC.

New HPLC instruments could develop up to 6,000 psi of pressure, and incorporated improved injectors, detectors, and columns. With continued advances in performance during this time (smaller particles, even higher pressure), the acronym HPLC remained the same, but the name was changed to high performance liquid chromatography.

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.

- Speed(min)
- Greater sensitivity
- Improved resolution (wide variety of stationary phases)
- Reusable columns
- Needs a small sample with a high accuracy and precise
- Easy sample recovery, handling and maintenance.
- Reproducibility of +/- 1% (not so for LC)
- Non-destructed sample during operation compared to GC.
- Controls and automates chromatography instrumentation
- Provides data management, security features, and reporting and instrument validation.

Materials

Ofloxacin, Metronidazole Potassium dihydrogen, Water and Methanol for HPLC, Acetonitrile for HPLC, Ortho phosphoric Acid.

METHODOLOGY:

HPLC Method Development

Method development for simultaneous estimation of Oflaxacin and Metronidazole in Pharmaceutical dosage forms includes the following steps:

1. Selection of detection wavelength (λ_{max})
2. Selection of column
3. Selection of mobile phase
4. Selection of flow rate
5. Preparations and procedures

1. Selection of Detection wavelength:

10 mg of Ofloxacin and Metronidazole was dissolved in mobile phase. The solution was scanned from 200-400 nm the spectrum was obtained. The overla y spectrum was used for selection of wavelength for Ofloxacin and Metronidazole. The isobestic point was taken as detection wavelength.

2. Selection of column:

Column is selected based on solubility, polarity and chemical differences among Analytes [Column: Agilent C18 (4.6 x 250mm, 5µm]

3. Selection of mobile phase:

Methanol: ACN (70:30%v/v) has been selected as mobile phase. If any buffer selected buffer pH should be between 2 to 8. If the buffer pH is below 2 siloxane linkages are cleaved. If the buffer pH is above 8 dissolution of silica takes place. pH controls the elution properties by controlling the ionization characteristics. It also decreases the retention and improves separation. Good Response, Area, Tailing factor, Resolution will be achieved.

4. Selection of flow rate:

Flow rate selected was 1ml/min

Flow rate is selected based on

1. Retention time
2. Column back pressure
3. Peak symmetry
4. Separation of impurities

Method Validation

Accuracy

Preparation of standard solution (Metronidazole and Ofloxacin):

Accurately weighed 10 mg of Metronidazole and 10mg of Ofloxacin working standard were transferred into a 10mL and 100ml of clean dry volumetric flasks.

About 7mL and 70ml of Diluents are added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further 0.3ml and 0.3ml of the above stock solution was pipetted into a 10ml

volumetric flask and diluted upto the mark with diluents.

Precision

Repeatability:

Preparation of standard stock solution:

Accurately 10 mg of Metronidazole and 10mg of Ofloxacin working standard were weighed and transferred into a 10mL and 100ml of clean dry volumetric flasks and about 7mL and 70ml of Diluant was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further it was pipette (0.3ml and 0.3ml) into a 10ml volumetric flask and diluted up to the mark with diluents.

Procedure:

The standard solution was injected for five times and the areas for all five injections in HPLC were measured. The %RSD for the area of five replicate injections was found to be within the specified limits.

Acceptance criteria

- The % RSD for the area of five standard injections results should not be more than 2.

B) Intermediate Precision (Ruggedness):

To evaluate the intermediate precision (also known as ruggedness) of the method, precision was performed on different days by using different make column of same dimensions.

Specificity

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The specificity was performed by injecting blank.

LOD:

LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

LOQ:

LOQ's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula. Again, the standard deviation of the response can be determined based on the standard deviation of y- intercepts of regression lines.

Linearity

Preparation of stock solution:

Accurately 10 tablets were weighed & crushed in mortar and pestle and weight equivalent to 10 mg of Metronidazole and Ofloxacin (marketed formulation) sample were transferred into a 10mL clean dry volumetric flask and about 7mL of Diluant was added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution)

Robustness:

As part of the robustness, deliberate change in the flow rate, mobile phase composition was made to evaluate the impact on the method.

a) The flow rate was varied at 0.8ml/min to 1.2 ml/min. Standard solution 3ppm of Ofloxacin and 300 ppm of Metronidazole was prepared and analyzed using the varied flow rates along with method flow rate.

b) The organic composition in the mobile phase was varied from 65% to75 % standard solution 3 µg/ml of Ofloxacin and 300 µg/ml of Metronidazole were prepared and analyzed using the varied mobile phase composition along with the actual mobile phase composition in the method.

RESULTS:

Optimized Chromatogram is Obtained by Following Conditions

Trial-5:

Chromatographic conditions:

Column : Agilent C18 5µm (4.6*250mm)

Mobile phase ratio : Methanol: ACN (70:30%v/v)

Detection wavelength : 238nm

Flow rate : 1ml/min

Injection volume : 10µl Column temperature : Ambient

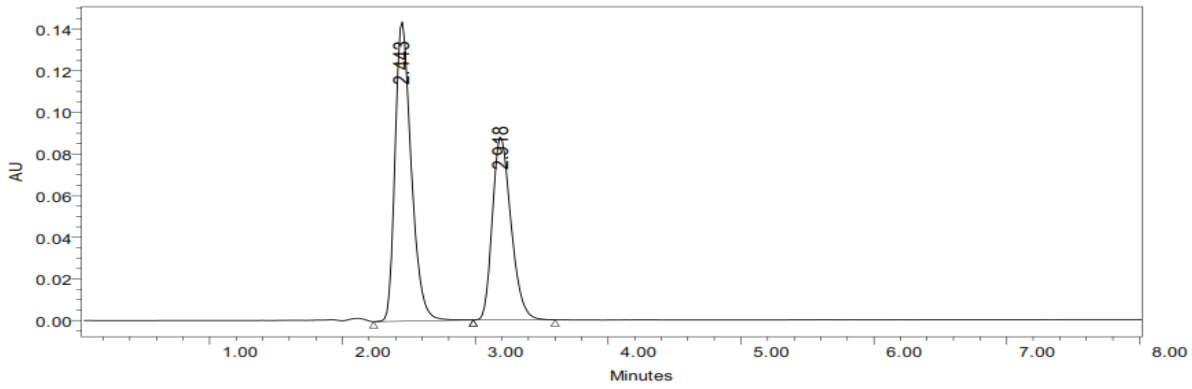


Fig. 1: Chromatogram of Trail-5

Table 1: Details of Trail-5

S.No	Peak name	Rt	Area	Height	USP Plate count	USP Tailing	USP Resolution
1	Metronidazole e	2.918	946124	155429	5105	1.3	8.1
2	Ofloxacin	2.443	111541	13239	3788	1.4	

Validation Parameters

Assay

The assay study was performed for the Ofloxacin and Metronidazole. Each three injections of sample and standard was inject into chromatographic system.

Table 2: Details of Oflaxacin and Metronidazole (Sample)

		RT	Area	Height
1	Ofloxacin	2.405	1355413	163926
2	Ofloxacin	2.419	1357013	163581
3	Metronidazole	3.867	463759	40317
4	Metronidazole	3.958	463128	39386
Mean				
Std. Dev.				
% RSD				

Table 3: Details of Ofloxacin and Metronidazole (standard)

	PeakName	RT	Area	Height	USP Plate Count	USP Tailing
1	Ofloxacin	2.318	1333112	164078	2114.9	1.7
2	Ofloxacin	2.379	1355521	164511	2127.0	1.7
3	Metronidazole	3.535	462181	44873	2931.4	1.7
4	Metronidazole	3.749	465519	41056	2697.1	1.7

Accuracy

Table 4: Details of Accuracy 50 %

	Peak Name	RT	Area	Height
1		2.346	702873	86026
2	Ofloxacin	2.351	704987	85549
3	Ofloxacin	2.360	702008	84196
4	Metronidazole	3.639	239401	21744
5	Metronidazole	3.668	239865	21909
6	Metronidazole	3.692	239948	21382
Mean			471513.5	
Std. Dev.			253899.3	
% RSD			53.8	

Table 5: Details of Accuracy 100 %

	Peak Name	RT	Area	Height
1	Ofloxacin	2.372	1390018	163987
2	Ofloxacin	2.378	1385589	165904
3	Ofloxacin	2.472	1419041	163460
4	Metronidazole	3.728	480779	42641
5	Metronidazole	3.772	480218	41532
6	Metronidazole	4.122	480338	37644
Mean			939330.5	
Std. Dev.			502815.3	
% RSD			53.5	

Table 6: Details of Accuracy 150 %

	Peak Name	RT	Area	Height
1	Ofloxacin	2.462	2206281	251287
2	Ofloxacin	2.500	2199166	252406
3	Metronidazole	4.096	733144	59726
4	Metronidazole	4.252	734279	56682
Mean			1468217.4	
Std. Dev.			848139.6	
% RSD			57.8	

The accuracy results for Oflaxacin

Table 7: Accuracy results of Ofloxacin

%Concentration (at specification Level)	**Area**	**Amount added(m)**	**Amount found(m)**	**% Recovey**	**Mean Recovery**
50%	702873	5	5.10	101.8%	
100%	1390018	10	9.99	99.9%	
150%	2206281	15	14.9	99.1%	

Acceptance Criteria:

- The % Recovery for each level should be between 98.0 to 102.0%.

The accuracy results for Metronidazole

Table 8: Accuracy results of Metronidazole

%Concentration(at specification level)	Area	Amount Added(mg)	Amount Found(mg)	% Recovery	Mean Recovery
50%	239401	5	5.0	101.3%	100.0%
100%	480779	10	9.94	99.4%	
150%	733144	15	14.8	99.2%	

Acceptance criteria:

The % recovery for each level should be between 98.0 to 102.0 %

Precision

Table 9: Precision results

	Peak Name	RT	Area	Height
1	Ofloxacin	2.282	1313235	163051
2	Ofloxacin	2.312	1326776	162363
3	Ofloxacin	2.344	1347962	163866
4	Ofloxacin	2.351	1368872	163893
5	Ofloxacin	2.358	1363598	161294
6	Metronidazole	3.433	458218	46160
7	Metronidazole	3.557	452495	45294
8	Metronidazole	3.623	453221	44163
9	Metronidazole	3.639	457145	43079
10	Metronidazole	3.704	458898	43930
Mean			900041.9	
Std. Dev.			468338.8	
% RSD			2.0	

Acceptance Criteria:

- The % RSD for the area of five standard injections results should not be more than 2%

The Method precision study was performed for the %RSD of Ofloxacin and Metronidazole was found to be 2.0 and 2.0 (NMT 2).

Intermediate Precision/Ruggedness

Table 10: Ruggedness results

	Peak Name	RT	Area	Height
1	Ofloxacin	2.381	1366825	164933
2	Ofloxacin	2.382	1379095	163608
3	Ofloxacin	2.384	1375825	164628
4	Ofloxacin	2.395	1364299	164510
5	Ofloxacin	2.412	1395271	163964
6	Ofloxacin	2.590	1393763	166747
7	Metronidazole	3.784	484545	41393
8	Metronidazole	3.797	484511	40825
9	Metronidazole	3.803	480804	40865
10	Metronidazole	3.845	485023	40309
11	Metronidazole	3.915	504952	39213
12	Metronidazole	4.607	485203	41640
Mean			933342.8	
Std. Dev.			465781.8	
% RSD			49.9	

Acceptance Criteria:
The % RSD for the area of five standard injections results should not be more than 2%.
The intermediate precision was performed for %RSD of Ofloxacin and Metronidazole was found to be 1.5 and 1.1 respectively (NMT 2).

Specificity

Table 11: Details of Standard Injection

	PeakName	RT	Area	Height	USP Plate Count	USP Tailing
1	Ofloxacin	2.318	1333112	164078	2114.9	1.7
2	Ofloxacin	2.379	1355521	164511	2127.0	1.7
3	Metronidazole	3.535	462181	44873	2931.4	1.7
4	Metronidazole	3.749	465519	41056	2697.1	1.7

The specificity test was performed for Ofloxacin and Metronidazole. It was found that there was no interference of impurities in retention time of analytical peak.

Linearity

Linearity Results

Table 12: Details of *Linearity Results*

	PeakName	RT	Area	Height
1	Ofloxacin	2.297	869216	109198
2	Ofloxacin	2.264	1148093	145069
3	Ofloxacin	2.308	1398858	164962
4	Ofloxacin	2.370	1676584	193291
5	Ofloxacin	2.322	1936686	238262
6	Metronidazole	3.458	296156	30269
7	Metronidazole	3.351	371946	39434
8	Metronidazole	3.488	452984	45638
9	Metronidazole	3.712	537383	50538
10	Metronidazole	3.535	617463	65483

Acceptance Criteria:
Correlation coefficient should be not less than 0.999

Robustness
As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A) Flow Rate:
The robustness was performed for the flow rate variations from 0.8 ml/min to 1.2ml/min. Standard solution 300 µg/ml of Metronidazole & 3µg/ml of Ofloxacin was prepared and analyzed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

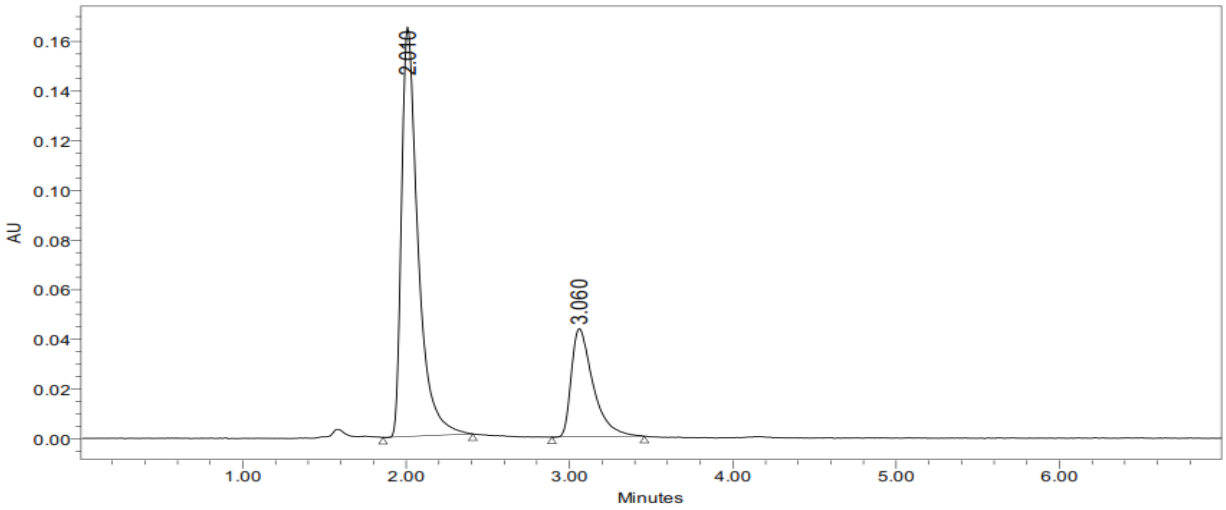


Fig.2: Chromatogram for Robustness more flow

Table 13: Details of Robustness more flow

	Peak Name	RT	Area	Height	USP Plate Count	USP Tailing
1	Ofloxacin	2.010	1150303	165118	2069.9	1.7
2	Metronidazole	3.060	402322	43574	2713.8	1.7

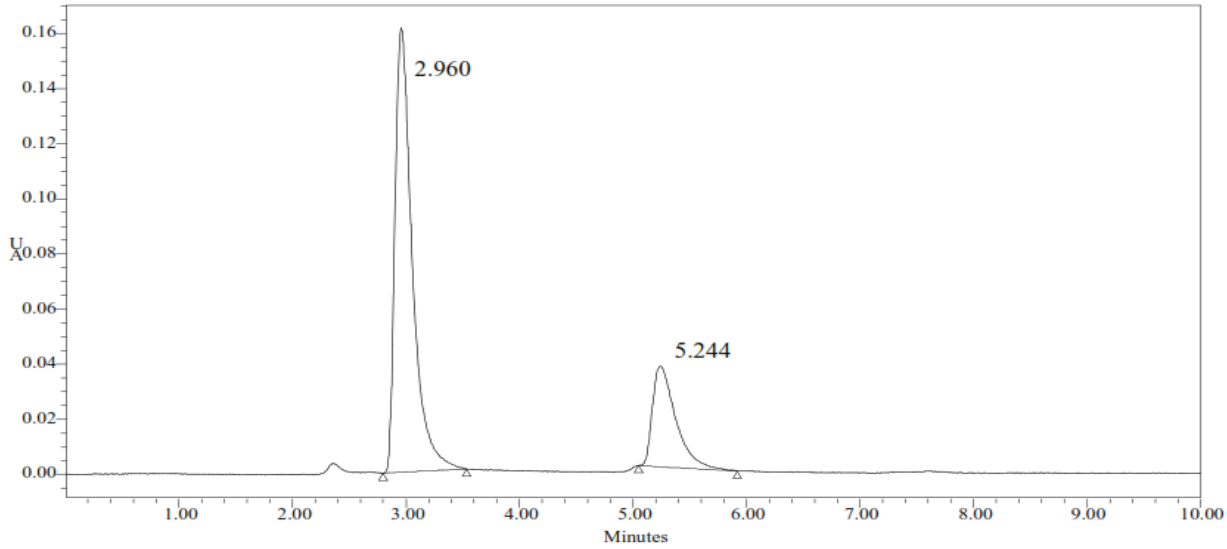


Fig. 3: Chromatogram for Robustness less flow

Table 14: Details of Robutness less flow

	PeakName	RT	Area	Height	USP PlateCount	USP Tailing
1	Ofloxacin	2.960	1690740	161204	2158.1	1.8
2	Metronidazole	5.244	519208	36602	3536.2	1.7

The results are summarized

On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±0.2ml/min.

Table 15: System suitability results For Metronidazole (Flow rate)

S.No	Flow Rate(ml/min)	System suitability results	
		USP Plate count	USP Tailing
1	0.8	3536	1.7
2	1.0	2931	1.7
3	1.2	2713	1.7

***** Results for actual flow (1.0 ml/min) have been considered from Assay standard.

Table 16: System suitability results for Ofloxacin (Flow rate)

S.No	Flow Rate(ml/min)	System suitability results	
		USP Plate count	USP Tailing
1	0.8	2158	1.8
2	1.0	2114	1.7
3	1.2	2069	1.7

***** Results for actual flow (1.0ml/min) have been considered from Assay standard

Mobile Phase:

The Organic composition in the Mobile phase was varied from 70% to 60%. Standard solution 300 µg/ml of Metronidazole & 3µg/ml of Ofloxacin was prepared and analyzed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

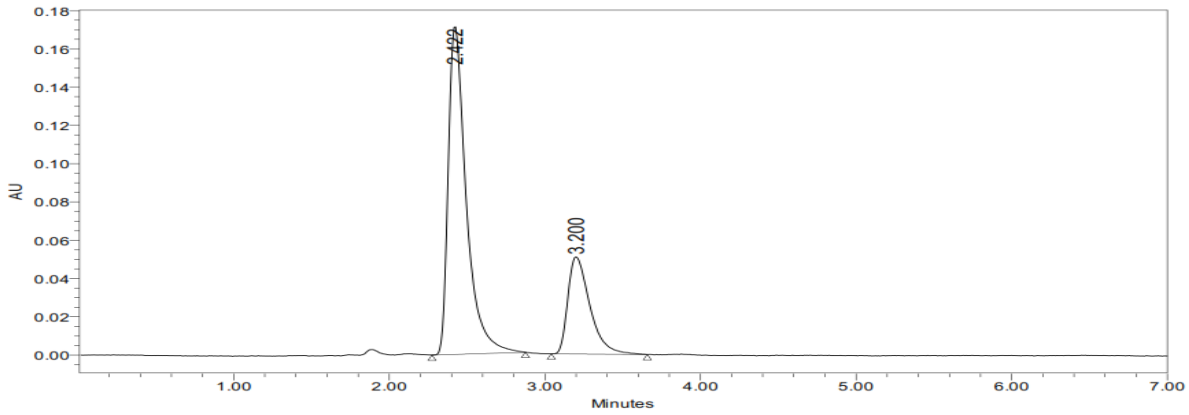


Fig. 4: Chromatogram for Robustness more organic

Table 17: Details of Robustness more organic

	Peak Name	RT	Area	Height	USP Plate Count	USP Tailing
1	Ofloxacin	2.422	1378798	171546	2358.0	1.7
2	Metronidazole	e3.200	499679	50843	2616.1	1.6

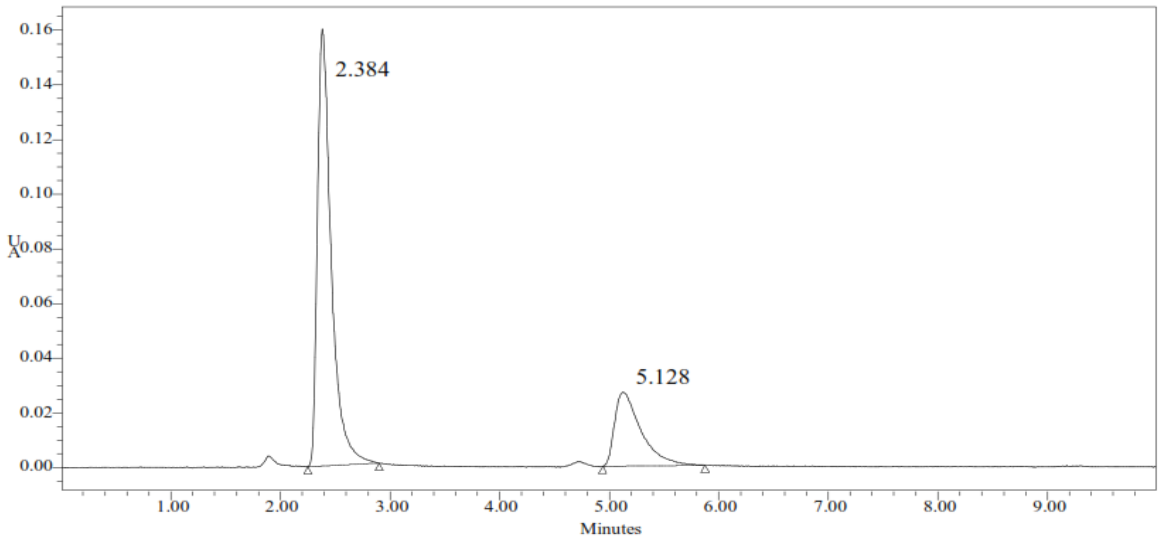


Fig 5: Chromatogram for Robustness less organic

Table 18: Details of Robustness les organic

	PeakName	RT	Area	Height	USP PlateCount	USP Tailing
1	Ofloxacin	2.384	1404976	159808	2910.4	1.8
2	Metronidazole	5.128	453297	27049	2840.1	1.7

The results are summarized. On evaluation of the above results, it can be concluded that the variation in 10% Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10

Table 19: System suitability results for Metronidazole (Mobile phase)

S.No	Changein Organic Composition in the Mobile Phase	System suitability results	
		USP Plate count	USP Tailing
1	10% Less	2910	1.8
2	Actual	2860	1.7
3	10% More	2358	1.7

***** Results for actual Mobile phase composition (55:45Buffer: Methanol) have been considered from Accuracy standard

Table 20: System suitability results for Ofloxacin (Mobile phase)

S.No	Changein Organic Composition in the Mobile Phase	System suitability results	
		USP Plate count	USP Tailing
1	10% Less	2540	1.7
2	Actual	2458	1.7
3	10% More	2616	1.7

*****Results for actual Mobile phase composition (55:45Buffer: Methanol) have been considered from Accuracy standard.

SUMMARY AND CONCLUSION:

A new method was established for simultaneous estimation of Ofloxacin and Metronidazole by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Ofloxacin and Metronidazole by using Agilent C18 5µm (4.6*250mm) column, flow rate was 1ml/min, mobile phase ratio was Methanol: ACN (70:30%v/v), detection wave length was 238nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, PDA Detector 996, Empower-software version-2.

The retention times were found to be 2.443 mins and 2.918 mins. The % purity of Ofloxacin and Metronidazole was found to be 100.7% and 101.4% respectively. The system suitability parameters for Ofloxacin and Metronidazole such as theoretical plates and tailing factor were found to be 1.7, 2114.5and 1.7, 2931.0 the resolution was found to be 8.0.The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study for Ofloxacin and Metronidazole was found in concentration range of 1µg-5µg and 100µg-500µg and correlation coefficient (r2) was found to be 0.999 and 0.999, % mean recovery was found to be 100% and 100.5%, %RSD for repeatability was 2.0 and 2.0, % RSD for intermediate precision was 1.5 and 1.1 respectively. The precision study was precise, robust, and repeatable. LOD value was 2.95 and 3.04, and LOQ value was 9.87 and 10 respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Ofloxacin and Metronidazole in API and Pharmaceutical dosage form.

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