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DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR COMBINED ANALYSIS OF METRONIDAZOLE AND OFLOXACIN

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ABSTRACT

A combination drug most commonly refers to a fixed-dose combination (FDC), which is a formulation including two or more active pharmaceutical ingredients (APIs) combined in a single dosage form, which is manufactured and distributed in certain respective fixed doses. Analytical chemistry is the study of the separation, identification, and quantification of the chemical components of natural and artificial materials. Qualitative analysis is performed to establish composition of natural/synthetic substances. These tests are performed to indicate whether the substance or compound is present in the sample or not. Quantitative analytical techniques are mainly used to quantify any compound or substance in the sample. Metronidazole works by entering the bacterial or protozoal cell and disrupting its DNA synthesis. Ofloxacin acts on DNA gyrase and Toposiomerase IV, enzymes which, like human Topoisomerase, prevents the excessive super coiling of DNA during replication or transcription. A simple, accurate, precise method was developed for the simultaneous estimation of the Metronidazole and Ofloxacin in pharmaceutical dosage form HPLC method can be used for routine drug analysis of Metronidazole and Ofloxacin in pharmaceutical dosage form.

KEY WORDS: Ofloxacin, Metronidazole, Fixed-dose combination (FDC),HPLC High-performance liquid chromatography(HPLC) etc.

INTRODUCTION

A pharmaceutical drug is any chemical substance formulated or compounded as single active ingredient or in combination of other pharmacologically active substance, it may be in a separate but packed in a single unit pack as combination product intended for internal, or external or for use in the medical diagnosis, cure, treatment, or prevention of disease. [1, 2]

A combination drug most commonly refers to a fixed-dose combination (FDC), which is a formulation including two or more active pharmaceutical ingredients (APIs) combined in a single dosage form, which is manufactured and distributed in certain respective fixed doses.[3-5] Terms like "combination drug" or "combination drug product" can be common shorthand for a FDC product (since most combination drug products are currently FDCs), although the latter is more precise if in fact referring to a mass-produced product having a predetermined combination of drugs and respective dosages (as opposed to customized poly pharmacy via compounding). [6]

Analytical chemistry is the study of the separation, identification, and quantification of the chemical components of natural and artificial materials.Development of analytical methods is to achieve the final goal of ensuring the quality of drug substances and drug products must be implemented in conjunction with an understanding of the chemical behavior and physicochemical properties of the drug substances. [7] Qualitative analysis is performed to establish composition of natural/synthetic substances. These tests are performed to indicate whether the substance or compound is present in the sample or not. [8] Quantitative analytical techniques are mainly used to quantify any compound or substance in the sample. Validation is defined as “Establishing

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documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes". Metronidazole is used to treat various infections caused by bacteria and protozoa. Metronidazole works by entering the bacterial or protozoal cell and disrupting its DNA synthesis. [9] Ofloxacin acts on DNA gyrase and Toposiomerase IV, enzymes which, like human Topoisomerase, prevents the excessive super coiling of DNA during replication or transcription. [10] By inhibiting their function, the drug thereby inhibits normal cell division. Ofloxacin is a broad-spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria.

MATERIALS AN METHODS

List of Chemicals and Reagents:

All the chemicals and reagents were of high purity, procured from various sources listed in Table.

Table 1: List of chemicals and reagents

S.No	Chemicals Used	Source
1	Water HPLC grade	In-House Specification (Axon Drugs Pvt Ltd.,)
2	Double distilled water	In-House Specification (Axon Drugs Pvt Ltd.,)
3	Hydrochloric Acid	Merck Pvt Ltd, Mumbai (AR Grade)
4	Sodium Hydroxide	Merck Pvt Ltd, Mumbai (AR Grade)
5	Ammonium acetate	Fischer Chemicals, Mumbai (AR Grade)
6	Methanol	Merck Pvt Ltd, Mumbai (HPLC Grade)
7	Ortho phosphoric acid	Rankem(HPLC Grade)
8	Acetonitrile	Finar (HPLC Grade)
9	Potassium bromide	Fischer Chemicals

List of Active Pharmaceutical Ingredients:

Table 2: List of active pharmaceutical ingredients

S.No	API Used	Source
1	Metronidazole	Axon Drugs Pvt Ltd., Chembarambakkam
2	Ofloxacin	Axon Drugs Pvt Ltd., Chembarambakkam

List of Instruments:

Table 3: List of instruments

Name of the instrument	Make	Model
Calibrated electronic balance	Ragwad	ACGC-1800
Calibrated melting point apparatus	Sunbim	-
Calibrated pH meter	Digi sun	-
Calibrated UV-Visible spectrophotometer	Shimadzu	UV-1800
High performance liquid chromatography	Shimadzu	Prominence -i

Determination of Melting point by Melting Point Apparatus:

Fill the capillary tube with the testing samples of TINI and OFL respectively and place it in the sample holder. Switch 'ON' the 'HEATER KNOB'. When the temperature reaches within about 20˚C below the expected melting point of the substances, reduce the rate of heating by turning the Knob anti- clockwise. [11] Adjust heating rate suitably to about 1˚C per minute. Note the temperature of the Melting point or Melting range when the substances melts.

SELECTION OF MOBILE PHASE

TRAIL-1

1. Preparation of Buffer: 0.1 % Ammonium acetate buffer was dissolved in 1000ml of water and adjusts the pH to 3.0 using diluted O-phosphoric acid.

2. Preparation of mobile phase: Filtered and degassed mixture of acetonitrile and buffer in the ratio of 60:40 and filter through 0.45 micron membrane filter

TRAIL-2

Preparation of mobile phase: Filtered and degassed mixture of acetonitrile and water in the ratio of 50:50 and filter through 0.45 micron membrane filter.

TRAIL- 3

Preparation of mobile phase: Filtered and degassed mixture of acetonitrile and methanol in the ratio of 80:20 and filter through 0.45 micron membrane filter.

TRAIL-4

1. Preparation of Buffer:

1 %w/v of O-phosphoric acid was dissolved in 1000ml of water.

2. **Preparation of mobile phase:** Filtered and degassed mixture of acetonitrile and buffer in the ratio of 50:50, and filter through 0.45 micron membrane filter.

TRAIL-5

1. Preparation of Buffer: 0.1 %w/v of O-phosphoric acid was dissolved in 1000ml of water.

2. **Preparation of mobile phase:** Filtered and degassed mixture of acetonitrile and buffer in the ratio of 30:70 and filter through 0.45 micron membrane filter.

Table 4: Trials for Optimization of Method

Parameters	TRAIL-1	TRAIL-2	TRAIL-3	TRAIL-4	TRAIL-5
Mobile phase	Acetonitrile: Ammonium acetate buffer	Acetonitrile :Water	Acetonitrile :Methanol	Acetonitrile: 1% O-Phosporic acid Buffer	Acetonitrile: 0.1%O-Phosporic Acid buffer
Ratio	60: 40	50: 50	80:20	50:50	70:30
Column	Inertsil ODS ColumnC18(250*4.6*5µ)	Inertsil ODS C18(250*4.6 *5µ)	Column C8 (15 0*4.6* 5µ)	Symmetry C8 (150*4.6* 3.5µ)	C_{18},(250 x 4.6mm,5µ BDS)
Flow rate	1ml/min	1ml/min	1ml/min	1ml/min	1ml/min
Injection Volume	20µl	20µl	20µl	20µl	20µl
Column oven	Ambient	Ambient	Ambient	Ambient	Ambient
Run time	20 min	20min	20min	20 min	20 min
Inference	Interferences (ghost peaks)	No elution	Interferences (ghost peaks)	Broad peak, Theoretical plates <2000	Symmetric peak, Tailing factor, Theoretical plates within limits

Choice of column

- Phenomenex C_{18} (25 cm × 4.6 mm i.d., 5-µm particle size) was selected as the column owing to its robustness, reproducibility and reliability among diverse RP-HPLC columns.
- This column was found to be stable at the desired pH and temperature.
- It offers good peak symmetry. Columns with 5µm particle size give the best compromise of efficiency.

Choice of mobile phase

- The preferred mobile phase binary mixture is acetonitrile: 0.1% Ortho Phosphoric acid (30:70) pKa of Metronidazole (4.7 ± 0.2), Oflaxacin (3.8 ±0.2) which ensures greater selectivity and interaction with the analyte.
- 0.1% w/v Ortho Phosphoric acid buffer separates the TINI and OFL in combined dosage form.

Choice of solvent

- Owing to free solubility of the analyte in mobile phase it is used as solvent as it accomplishes enhanced miscibility with mobile phase.

Choice of wavelength for detection

- Analysis of the analyte in solvent by UV Spectrophotometry revealed the isobestic point of the TINI and OFL were found to be 290 nm.

Preparation of mobile phase

- **% w/v Ortho Phosporic Acid Buffer Preparation:** Dissolve 1.0 gm of Ortophosporic acid in sufficient water to produce 1000 ml.
- **Mobile Phase Mixture:** Mix 30 volumes of Acetonitrile and 70 volumes of 0.1 % Orthophosporic acid and ultra sound for 15 minutes cool to room temperature and filter the mobile phase through 0.45 micron membrane filter.

Assay

Preparation of Metronidazole standard stock solution- I (1000 µg/ml)

50 mg of Metronidazole working standard was accurately weighed into 50 ml volumetric flask and dissolved in freshly prepared mobile phase and made up to the volume to get concentration of 1000 µg/ ml.

Preparation of Oflaxacin standard stock solution- I (1000 µg/ml)

50 mg of Oflaxacin working standard was accurately weighed into 50 ml volumetric flask and dissolved in freshly prepared mobile phase and made up to the volume to get concentration of 1000 µg/ ml.

Preparation of standard solution- II

Transfer 6.0 ml of Metronidazole and 2.0 ml of Oflaxacin from standard stock solution I to clean, dry 50 ml volumetric flask, dilute to 50 ml with the mobile phase to get the concentration range of 120 and 40 µg/ ml of Metronidazole and Oflaxacin. Filter through 0.45 micron membrane filter.

Preparation of sample solution

Take 20 tablets and find the average weight and crush the tablets into a fine powder Transfer half the tablet weight of powdered sample into a 100 ml clean, dry volumetric flask, add 50 ml mobile phase and ultra sound for 15 minutes to dissolve, make up to the volume with diluents. [12] Dilute 2 ml of this solution to 50 ml with diluents. Filter through 0.45 micron membrane filter.

ANALYTICAL METHOD VALIDATION

System suitability parameters

System suitability parameters including USP Theoretical Plate Count, USP Tailing factor and Resolution, % RSD were assessed from 3 injections of Metronidazole and Oflaxacin standards (120 and 40 µg/ml).

Specificity

The interference of the blank with the chromatogram of Metronidazole and Oflaxacin was checked by recording and comparing the chromatograms of blank and that of Metronidazole and Oflaxacin.

Linearity and Range

Linearity for the concentration range 80%-120% was established by plotting concentrations on X- axis and corresponding peak area on Y- axis. Statistical parameters like correlation coefficient (R^2), line equation including slope (m), y- intercept (C) were determined. The specified range was derived from linearity studies by determining the difference between highest and lowest concentrations. [13]

Precision

Intraday precision (Repeatability)

Repeatability of the developed method was assessed by 9 determinations covering 3 concentrations each of 3 replicates. % RSD was calculated for the results obtained.

Interday precision

Variation in the results for the developed method was assessed 3 different days (n=6). % RSD was calculated for the results obtained.

Robustness

Typical variations including change in flow rate (± 0.5 ml of optimized flow rate), change in the organic phase composition of mobile phase (± 10 ml) and change in wavelength (± 1 nm) were assessed.

Accuracy

Preparation of 50% solution: Transfer 2 ml of sample (stock solution I) and each of 2.5 ml of Metronidazole and Oflaxacin working standard stock solution I into a 50 ml volumetric flask and diluted up to the mark with mobile phase.

Preparation of 100% solution: Transfer 2 ml of sample (stock solution I)and each of 5ml of Metronidazole and Oflaxacin working standard (stock solution I) into a 50 ml volumetric flask and diluted up to the mark with mobile phase.

Preparation of 150% solution: Transfer 2 ml of sample (stock solution I)and each of 7.5 ml of Metronidazole and Oflaxacin working standard (stock solution I) into a 50 ml volumetric flask and diluted up to the mark with mobile phase . Calculate the amount found and amount added for Metronidazole and Oflaxacin, also calculate the individual recovery and mean recovery values.

Applicability of Validated Method by RP- HPLC

Assay of Formulation

Weigh 20 tablets and note the weight, divide it by 20 to find its average weight, and crush the tablets into a fine powder.

Sample preparation:

Shake a quantity of the powdered tablets containing half the weight of its average weight with 60 ml of mobile phase and ultra sound for 15 minutes, dilute to 100.0ml with mobile phase and allow settling for 10 minutes. [14] The solution is cooled to room temperature and 2.0 ml of above

solution is transferred into a 50 ml volumetric flask and diluted to volume with mobile phase and filtered through 0.45 micron membrane filter. [15] This constitutes 120 µg/ ml of Metronidazole and 40 µg/ ml of Oflaxacin.

RESULTS AND DISCUSSION

Melting point of Metronidazole and Ofloxacin

Table 5: Melting point determination

Sample	Observed MP (^{0}C)^n	Standard MP (^{0}C)
Metronidazole	127.5^{0}C	127^{0}C-128^{0}C
Ofloxacin	254.3^{0}C	250^{0}C -257^{0}C

The obtained limits are within the limit.

Method Development and Validation by Reversed Phase High Performance Liquid Chromatography

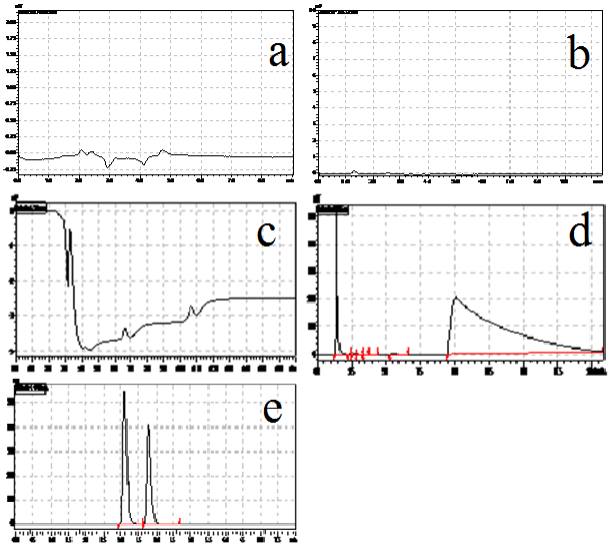

Figure 1: Trials for Optimization of RPHPLC Method
a. Trial 1; b. Trial 2; c. Trial 3; d. Trial 4; e. Trial 5

Optimized Parameters for RP- HPLC

Table 6: 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

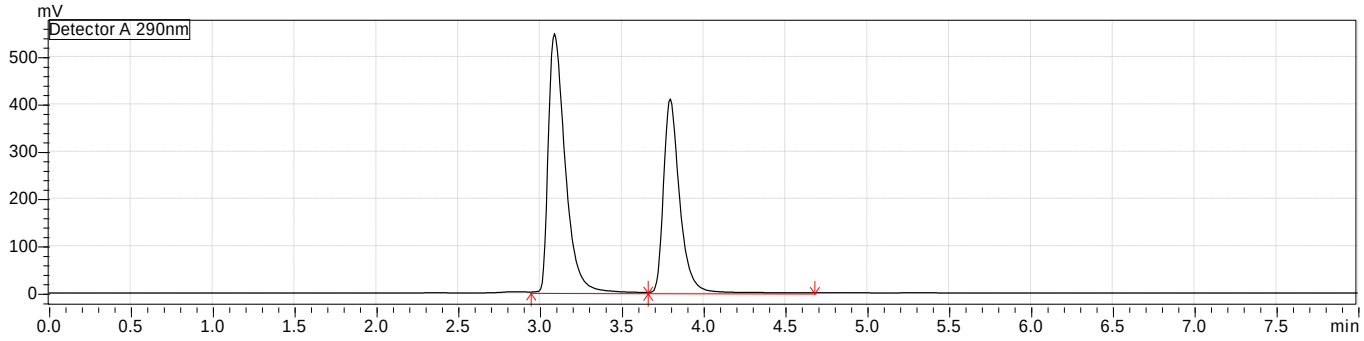

Figure 2: Chromatogram for Optimized Method

Table 7: Chromatogram Optimized parameters

Name	Peak	Ret. Time	Area	Height
Oflaxacin	1	3.251	3795244	553269
Metronidazole	2	3.842	2703614	419986

System Suitability Parameters

Table 8: System Suitability Parameters for Metronidazole

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 9: System Suitability Parameters for Oflaxacin

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

Specificity

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

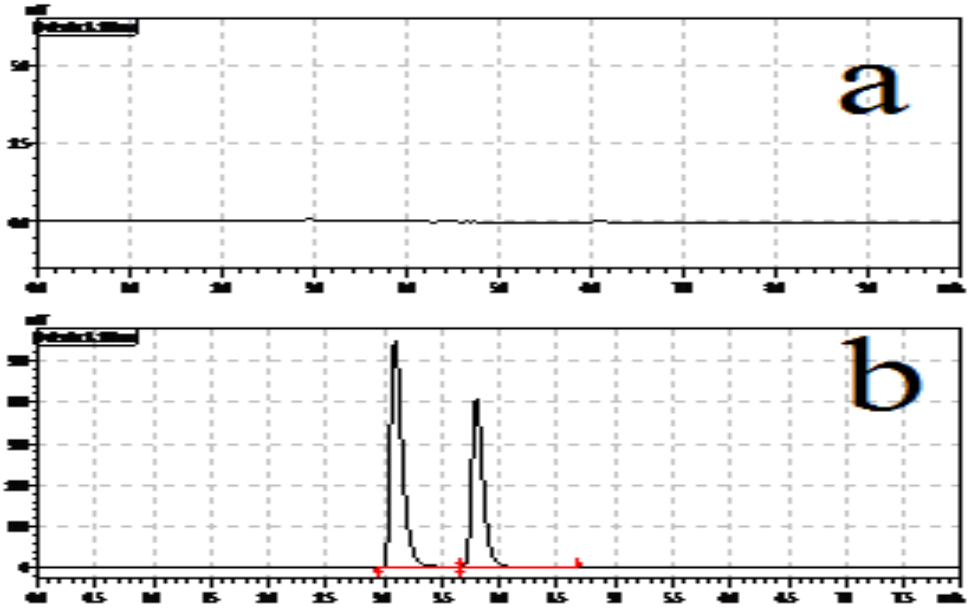

Figure 3: Chromatogram for Specificity

a. Blank; b. Formulation

Linearity and Range

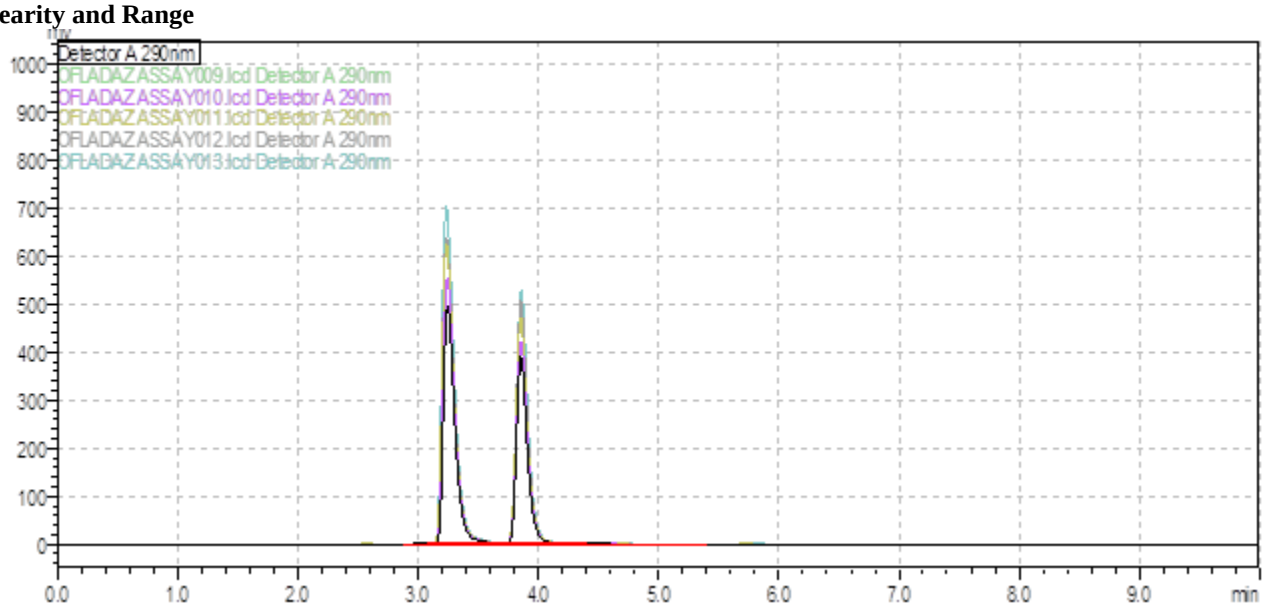

Figure 4: Linearity by RP-HPLC

Table 10: Linearity Profile by RP- HPLC

Concentration	Metronidazole Peak Area	Oflaxacin Peak area
80	2489336	3572519
90	2768577	4029736
100	3085337	4466338
110	3386046	4872843
120	3644620	5359196

Table 11: Summary of Regression by RP- HPLC

Parameters	Metronidazole	Oflaxacin
Linear equation	Y=28985.03x+145268.315	Y=43525.628x+14625.38
Correlation oefficient (R^2)	0.9985	0.9968

The calibration set was linear with regression coefficient of 0.9985 for Metronidazole and 0.9968 for Oflaxacin.

Intraday Precision for Metronidazole

Table 12: Intraday Precision by Metronidazole

Conc. (%)	Peak area			Average	SD	% RSD
	Day 1	Day 2	Day 3			
80	2489336	2493169	2486868	2489791	3175	0.0053
100	3084253	3082794	3078785	3081944	2832	0.0045
120	3644620	3635753	3639292	3639888	4463	0.0059

Intraday precision for Oflaxacin (Repeatability)

Table 13: Intraday Precision of Oflaxacin

Conc. (%)	Peak area			Average	SD	% RSD
	Day 1	Day 2	Day 3			
80	3492814	3493139	3492690	3492881	232	0.0057
100	4475366	4476284	4477483	4476378	1061	0.0169
120	5459925	5457395	5459517	5458946	1358	0.0145

Robustness

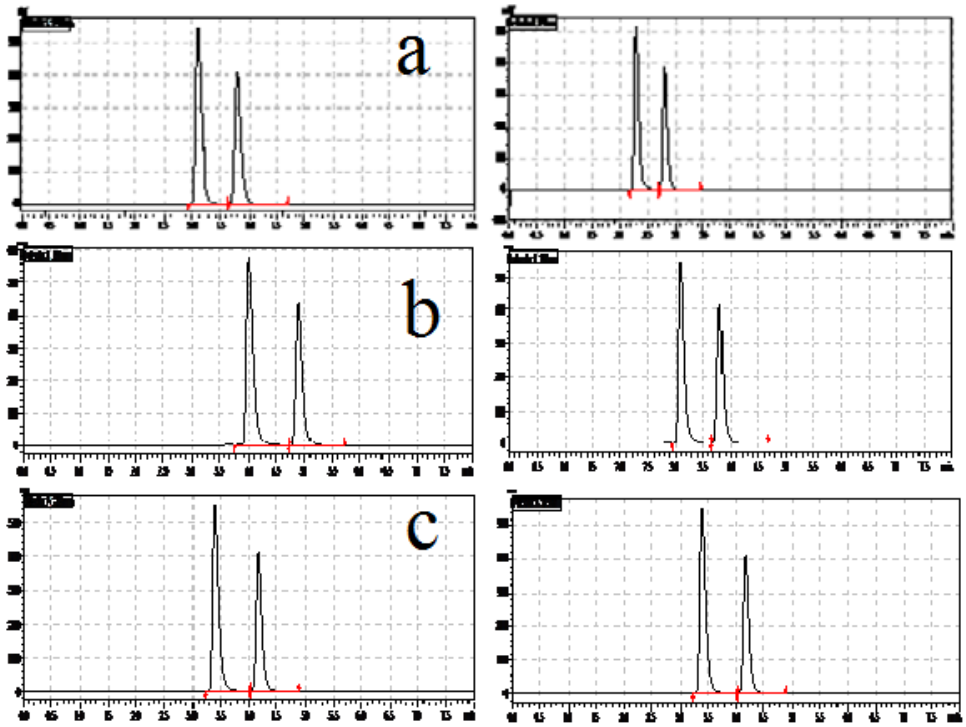

Figure 5: RPHPLC Chromatograms

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

Table 14: Robustness of the method for Metronidazole

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 15: Summary of Robustness for Ofloxacin

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 limits

Table 16: Accuracy Data for Metronidazole

Recovery levels	Accurate data for Metronidazole					
	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 17: Accuracy Data for Ofloxacin

Recovery levels	Accurate data for Ofloxacin					
	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 18: Common test used to level of significance

Formulation	Peak area	Label claim	Amount found	% Assay ± SD*
Metronidazole	3087184	600mg	603.06	100.51
	3086122			
	3087244			
Ofloxacin	4412136	200mg	201.47	100.73
	4408331			
	4409332			

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

CONCLUSION

A simple, accurate, precise method was developed for the simultaneous estimation of the Metronidazole and Ofloxacin in pharmaceutical dosage form. The developed method was validated based on ICH guidelines. The percentage recovery was obtained as 100.51%and 100.73% for Metronidazole and Ofloxacin respectively by RP-HPLC. The advantages lie in the simplicity of sample preparation and the low costs of reagents used. The proposed HPLC conditions ensure sufficient resolution and the precise quantification of the compounds. Results from statistical analysis of the experimental results were indicative of satisfactory precision and reproducibility. Hence, the proposed HPLC method can be used for routine drug analysis

of Metronidazole and Ofloxacin in pharmaceutical dosage form.

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