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" DEVELOPMENT AND VALIDATION OF LCMS/MS METHOD FOR SIMULTANEOUS ESTIMATION OF METRONIDAZOLE AND RIFAXIMINE IN PHARMACEUTICAL DOSAGE FORM AND CHARACTERIZE DEGRADANTS"

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ABSTRACT

A simple, rapid, precise and accurate Stability indicating LC-MS/MS method for simultaneous estimation of Metronidazole and Rifaximin in their combined dosage form has been developed. The separation was achieved by Agilent, Zorbax, C18, (150mm x 4.6mm), 5µm column and Buffer (Ammonium formate, pH4.0): Acetonitrile (75:25) as mobile phase, at a flow rate of 1 ml/min. Detection was carried out. Retention time of Rifaximin and Metronidazole were found to be 4.9 min and 2.6 min respectively. The method has been validated for linearity, accuracy and precision. Linearity observed for Metronidazole 2-6µg/ml and for Rifaximin 1-3 µg/ml. Developed method was found to be accurate, precise and rapid for simultaneous estimation of Metronidazole and Rifaximin their Combined Dosage Form. The drug was subjected to stress condition of hydrolysis, oxidation, photolysis and Thermal degradation, Considerable Degradation was found in Alkali and Oxidation degradation. The proposed method was successfully applied for the simultaneous estimation of both the drugs in commercial combined dosage form.

KEY WORDS: Metronidazole, Rifaximin, Stability indicating LC-MS/MS Method, Validation.

1 INTRODUCTION

Rifaximin is newly approved semi-synthetic non-systemic antibiotic derived from a naturally occurring chemical, rifamycin that is produced by Streptomyces Mediterranean bacterium. WHO's one of the essential antiprotozoal and antibiotic Metronidazole is nitroimidazole class drug used particularly for anaerobic bacteria and protozoa. The drug acts by inhibiting synthesis of nucleic acid and thus DNA gets disrupt in the cell of bacteria. For this disruption it is necessary for Metronidazole to be partially reduced and because this reduction usually anaerobic cells get killed. Most of the cases of acute diarrhoea are mixed in origin wherein both bacteria and protozoa are most commonly responsible. Combination an anti-protozoal and an anti-bacterial serve as a blanket cover for both protozoa and bacteria in diarrhoea of bacterial, protozoal or mixed etiology. Various methods are reported for the analysis of individual drug and in combination with other drugs by HPLC but no Stability indicating HPLC

method reported for these drugs in combined dosage form. Therefore, it was thought worthwhile to develop Stability indicating HPLC methods for analysis of Rifaximin and Metronidazole in Combined Pharmaceutical dosage form.

2 Methodology:

2.1 Preparation of standard solutions

Preparation of Diluent

The diluent containing water and acetonitrile is prepared in the ratio of 50:50.

Preparation of Standard Stock Solution of Rifaximin

Accurately weighed separately quantity of 20.0 mg Rifaximin API were transferred into 100 ml volumetric flask and dissolved in diluent using ultra sonication and diluted up to mark to give a stock solution having concentration of 200µg/ml Rifaximin.

Preparation of Standard Stock Solution of Metronidazole

Accurately weighed separately quantity of 40 mg Metronidazole API were transferred into 100 ml volumetric flask and dissolved in diluent using ultra sonication and diluted up to mark to give a stock solution having concentration of 400µg/ml Metronidazole.

Preparation of Working Standard Solution of Rifaximin

From above Standard Stock Solution of Rifaximin, 1 ml was taken in to 100 ml volumetric flask and was made up to the mark with the diluent to get 2.0 µg/ml of Rifaximin.

Preparation of Working Standard Solution of Metronidazole

From above Standard Stock Solution of Metronidazole, 1 ml was taken in to 100 ml volumetric flask and was made up to the mark with the diluent to get 4.0 µg/ml of Metronidazole.

Combine Preparation of Working Standard Solution of Rifaximin and Metronidazole

Take 1ml from rifaximin stock solution and 1ml from metronidazole stock solution into 100ml volumetric flask and make up the volume with diluent to get 2.0 µg/ml of Rifaximin and 4.0 µg/ml of Metronidazole.

Preparation of Mobile Phase

Prepare 10mM ammonium formate buffer in water having pH 4.0 and Acetonitrile the ratio of 25:75.

10M ammonium formate buffer preparation (pH 4.0): Take 630.0mg of ammonium formate buffer and dissolve in 1000ml LCMS grade water. Adjust the pH 4.0 with formic acid. Mix well and degas by sonication.

Preparation of Sample Stock Solution of Rifaximin and metronidazole

The average weight of 10 tablets was determined and was ground in a mortar. Stock solution was prepared by dissolving tablet powder equivalent to 20.0 mg of Rifaximin and 40 mg of metronidazole was transferred to 100ml volumetric flask. Then 50 ml diluent was added and sonicated for 5 mins to ensure complete solubilization of drug. After sonication, volume was made up to the mark with diluent. Filter the stock solution with 0.45µ Millipore filter and the final filtrate is collected as sample stock solution.

Preparation of Sample Working Solution of Rifaximin and metronidazole

From above Sample Stock Solution of Rifaximin and Metronidazole, 1 ml was taken in to 100 ml volumetric flask and was made up to the mark with the diluent to get 2.0 µg/ml of Rifaximin and 4.0 µg/ml Metronidazole.

Table 1 : Chromatographic Conditions of LC-MS/MS

Instrument	Liquid chromatography Mass spectrometer (API-2000) equipped with auto sample, auto injector, column oven, ion source ESI electron spray ionizer with Q1 and collision energy.		
Ion Source setting		***Scan setting***	
Ion source	ESI	Polarity	Positive ion
Curtain Gas	30psi	Scan type	MRM
Ion Spray Voltage	5500	Scan time	1-10 min
Temperature	400°C	Declustering Potential	90
Ion Source Gas(GS1)	50psi	Focusing Potential	500
Ion Source Gas(GS2)	50psi	Entrance Potential	10
Scan type	Rifaximin	MRM:(Q1)786.800 Da and (Q3) 727.500 Da	
	Metronidazole	MRM:(Q1)172.100 Da and (Q3) 126.400 Da	

- **Chromatographic condition:**

Column	:	Agilent, Zorbax, C18, (150mm x 4.6mm), 5µm			
Flow rate	:	1.0 mL/min	Injection volume	:	20 µL
Column oven temperature	:	35 °C	Run time	:	10 min
Column oven compartment	:	Ambient	Mode	:	Isocratic
Rifaximin R.T	:	About 4.9 min			
Metronidazole R.T	:	About 2.6 min			

3 Stability Indicating

Method.

3.1 Acid degradation : Acid decomposition studies were performed by transferring one ml of stock solution to 100 ml of volumetric flask. 1 ml of 0.1 N HCl solutions was added and mixed well and put for 4 hrs at 60°C. Then the volume was adjusted with diluent to get 2µg/ml for Rifaximin and 4 µg/ml for Metronidazole.

3.2 Base degradation : Base decomposition studies were performed by transferring one ml of stock solution to 100 ml of volumetric flask. 1 ml of 0.1 N NaOH solutions was added and mixed well and put for 4 hrs at 60°C. Then the volume was adjusted with diluent to get 2µg/ml for Rifaximin and 4µg/ml for Metronidazole

3.3 Oxidative degradation : Oxidation decomposition studies were performed by transferring 1 ml of stock solution to100 ml of volumetric flask. Two ml of 3% H2O2 solutions was added and mixed well and put for 5 hrs at 60°C. Then the volume was adjusted with diluent to get 2µg/ml for Rifaximin and 4µg/ml for Metronidazole.

3.4 Photo degradation : Put about 100.0 mg of Rifaximin and 100 mg of Metronidazole standard into petridish and place the petridish into photo stability chamber for 5 days. After 5 days weigh and transfer about 20.0mg of rifaximin powder and 40.0 mg Metronidazole into a 100ml volumetric flask and make up volume with diluent. Transfer 1.0ml each solution into a 100ml volumetric flask and make up volume with diluent to get 2µg/ml for Rifaximin and 4µg/ml for Metronidazole.

3.5 Thermal Degradation

Put about 100.0mg of Rifaximin and 100 mg of Metronidazile standard into petridish and place the petridish into hot air oven at 100^0C for 5 days. After 5 days weigh and transfer about 20.0mg of rifaximin powder into a 100ml volumetric flask and 40.0 mg of Metronidazole in to 100 ml volumetric flask and make up volume with diluent. Transfer 1.0ml each solution into a 100ml volumetric flask and make up volume with diluent to get 2µg/ml for Rifaximin and 4µg/ml for Metronidazole.

4 VALIDATION OF LC-MS/MS METHOD

4.1 Specificity

The blank solution, working standard solution and working sample solution of Rifaximin and metronidazole is injected in to the LC-MS/MS system. The chromatogram of standard and sample has no interference with the chromatogram of blank.

4.2 Linearity and Range

The linearity for Rifaximin and Metronidazole were assessed by analysis of standard solution in range of 1.0-3.0 µg/ml for Rifaximin and 2.0-6.0 µg/ml Metronidazole respectively. 0.5, 0.75, 1.0, 1.25, 1.50 ml solutions were pipette out from the Stock solution of Rifaximin and metronidazole and transfer to 100 ml volumetric flask and make up with diluent to obtain 1.0, 1.5, 2.0, 2.5 and 3.0 µg/ml for Rifaximin and 2.0, 3.0, 4.0, 5.0 and 6.0 µg/ml for metronidazole respectively. In term of slope, intercept and correlation co-efficient value is obtained. The graph of peak area obtained verses respective concentration was

plotted.

Acceptance criteria: Value of r^2 should be more than 0.99 should be less than 1.0.

4.3 Precision

4.3.1 Repeatability

Standard solution containing Rifaximin (2.0 µg/ml) and metronidazole (4.0 µg/ml) was injected six times and areas of peaks were measured and % R.S.D. was calculated.

Acceptance criteria: % RSD of Area should not be more than 2.0%

4.3.2 Intraday Precision

Standard solution containing (1.0, 2.0, 3.0 µg/ml) of Rifaximin and (2.0, 4.0, 6.0 µg/ml) Metronidazole were analyzed three times on the same day and % R.S.D was calculated.

Acceptance criteria: % RSD of Area should not be more than 2.0%

4.3.3 Interlay Precision

Standard solution containing (1.0, 2.0, 3.0 µg/ml) of Rifaximin and ((2.0, 4.0, 6.0 µg/ml) Metronidazole were analyzed three times on different day and % R.S.D was calculated.

Acceptance criteria: % RSD of Area should not be more than 2.0%

Accuracy

0.5 µg/ml drug solutions were taken in three different flask label A, B and C. Spiked 80%, 100%, 120% of standard solution in it and diluted up to 100ml. The area of each solution peak was measured. The amount of Rifaximin and metronidazole was calculated at each level and % recoveries were computed.

Acceptance criteria

% Recovery (individual) at each level should be between 98.00% and 102.00%

4.4 Limit of Detection and Limit of Quantitation

The LOD was estimated from the set of 3 calibration curves used to determination method linearity. The LOD may be calculated as,

LOD = 3.3 × (SD/Slope)

Where, SD = Standard deviation of Y-intercepts of 3 calibration curves.

Slope = Mean slope of the 3 calibration curves.

The LOQ was estimated from the set of 3 calibration curves used to determine method linearity. The LOQ may be calculated as,

LOQ = 10 × (SD/Slope)

Where, SD = Standard deviation of Y-intercepts of 3 calibration curves.

Slope = Mean slope of the 3 calibration curves.

4.5 Robustness

Following parameters were changed one by one and their effect was observed on system suitability for standard preparation.

1. Flow rate of mobile phase was changed (± 0.2 ml/min) 0.8 ml/min and 1.2 ml/min.
2. Ratio of Mobile phase was changed (±2) Buffer: acetonitrile (23:77) and Buffer: Methanol (27:73).

Acceptance criteria

- % RSD for the analyte peak should not be more than 2.0%

4.6 Analysis of Market Formulation

Take tablet powder equivalent to 200.0 mg rifaximin and 400.0 mg of metronidazole was transferred to a 100 ml volumetric flask, shake for 15 minutes and made up volume up to the mark with diluent. The solution was filtered through 0.45µ Millipore filter and first few drops of filtrate were discarded. 1 ml of this solution was diluted to 100 ml with diluent. The solution was injected 20 µl into the LC-MS/MS system. The areas of resulting peak were measured.

5 RESULT AND DISCUSSION:

5.1 FORCED DEGRADATION STUDY

Rifaximin and Metronidazole standard was injected under various stress conditions. The optimized degradation condition is shown below.

Table 2: Different Degradation Conditions for Rifaximin

Sr. No.	Stress Type	Stress Condition
1	Acid Degradation	1 N HCl at 60°C for 4 hr.
2	Base Degradation	1 N NaOH at 60°C for 3 hr.
3	Oxidative Degradation	30.0 % H_2O_2 at 60°C for 5 hrs.
4	Thermal Degradation	105 ^oC for 5 days
5	Photolytic Degradation	UV for 5 days

Table 3: Different Degradation Conditions for Metronidazole

Sr. No.	Stress Type	Stress Condition
1	Acid Degradation	1 N HCl at 60°C for 4 hr.
2	Base Degradation	1 N NaOH at 60°C for 3 hr.
3	Oxidative Degradation	30.0 % H_2O_2 at 60°C for 6 hrs.
4	Thermal Degradation	105 ^oC for 5 days
5	Photolytic Degradation	UV for 5 days

Selection of Elution Mode:

Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also better performing in terms of efficiency, stability and reproducibility. C18 column is least polar compare to C4 and C8 columns. Here, A250x4.6mm column of 5.0µm particle packing was selected for separation of Rifaximin and Metronidazole. Isocratic mode was chosen due to simplicity in application and robustness with respect to longer column stability.

5.2 Observation

- The acidic ,Thermal and Photolytic degradation solution of rifaximin and metronidazole is infused into the mass spectrometer to identified and characterized acidic degradation product (i.e DP1).
- From the mass spectra, it is found that, Rifaximin and Metronidazole is not degraded under acidic condition.
- Hence, it was found that rifaximin and Metronidazole was stable under acidic conditions.

- The basic degradation solution of rifaximin is infused into the mass spectrometer to identified and characterized basic degradation product (i.e DP2) of rifaximin.
- From the mass spectra and fragmentation pathway, it is found that, Rifaximin is degraded under basic condition.
- The ESI-MS/MS spectra shows basic degradation product DP1 whose m/z ratio is obtained around 744.100 Da and its fragment ion peak is found whose m/z ratio is obtained around 584.200 Da, 635.100 Da and

714.700

- For MRM scan, Rifaximin basic degradation product (DP2) molecular mass (Q1) is 744.100 Da and its fragment mass (Q3) is 714.700 Da is selected

- The oxidative degradation solution of rifaximin is infused into the mass spectrometer to identified and characterized oxidative degradation product (i.e DP2) of rifaximin.
- From the mass spectra and fragmentation pathway, it is found that, Rifaximin is degraded under oxidative condition.
- The ESI-MS/MS spectra shows oxidative degradation product DP2 whose m/z ratio is obtained around 802.100 Da and its fragment ion peak is found whose m/z ratio is obtained around 743.100 Da, 677.700Da and 600.600 Da
- For MRM scan, Rifaximin oxidative degradation product (DP2) molecular mass (Q1) is 802.100 Da and its fragment mass (Q3) is 743.700 Da is selected

- The base degradation solution of Metronidazole is infused into the mass spectrometer to identified and characterized base degradation product (i.e DP1) of Metronidazole
- From the mass spectra and fragmentation pathway, it is found that, Metronidazole is degraded under base condition.
- The ESI-MS/MS spectra shows base degradation product DP1 whose m/z ratio is obtained around 194.600 Da and its fragment ion peak is found whose m/z ratio is obtained around 141.200 Da and 148.100Da
- For MRM scan, Metronidazole base degradation product (DP1) molecular mass (Q1) is 194.600 Da and its fragment mass (Q3) is 148.100 Da is selected.

- The oxidative degradation solution of Metronidazole is infused into the mass spectrometer to identified and characterized oxidative degradation product (i.e DP2) of Metronidazole
- From the mass spectra and fragmentation pathway, it is found that, Metronidazole is degraded under oxidative condition.
- The ESI-MS/MS spectra shows oxidative degradation product DP1 whose m/z ratio is obtained around 188.600 Da and its fragment ion peak is found whose m/z ratio is obtained around 157.800 Da and 142.600 Da
- For MRM scan, Metronidazole oxidative degradation product (DP1) molecular mass (Q1) is 188.600 Da and its fragment mass (Q3) is 157.800 Da is selected.

Table-4 Chromatographic Conditions of LC-MS/MS for Forced Degradation Studies

Instrument	Liquid chromatography Mass spectrometer (API-2000) equipped with auto sample, auto injector, column oven, ion source ESI electron spray ionizer with Q1 and collision energy.		
Ion Source setting		***Scan setting***	
Ion source	ESI	Polarity	Positive ion
Curtain Gas	30psi	Scan type	MRM
Ion Spray Voltage	5500	Scan time	1-10 min
Temperature	400°C	Declustering Potential	90
Ion Source Gas(GS1)	50psi	Focusing Potential	500
Ion Source Gas(GS2)	50psi	Entrance Potential	10
Scan type	Rifaximin	MRM:(Q1)786.800 Da and (Q3) 727.500 Da	
	Metronidazole	MRM:(Q1)172.100 Da and (Q3) 126.400 Da	
	Rifa DP1	MRM:(Q1)744.100 Da and (Q3) 126.400 Da	
	Rifa DP2	MRM:(Q1)804.100 Da and (Q3) 743.700 Da	
	Metro DP1	MRM:(Q1)199.600 Da and (Q3) 148.100 Da	
	Metro DP2	MRM:(Q1)188.600 Da and (Q3) 157.800 Da	

- **Chromatographic condition:**

Column	:	Agilent, Zorbax, C18, (150mm x 4.6mm), 5µm			
Flow rate	:	1.0 mL/min	Injection volume	:	20 µL
Column oven temperature	:	35 °C	Run time	:	10 min
Column oven compartment	:	Ambient	Mode	:	Isocratic
Rifaximin R.T	:	About 4.9 min			
Metronidazole R.T	:	About 2.6 min			
Rifa DP1 R.T	:	About 5.0 min			
Rifa DP2 R.T	:	About 2.3 min			
Metro DP1 R.T	:	About 3.2 min			
Metro DP2 R.T	:	About 2.4 min			

6 METHOD VALIDATION

6.1 Specificity

The Chromatograms of Rifaximin and Metronidazole standards and Rifaximin and Metronidazole sample show no interference with the Chromatogram of Rifaximin and Metronidazole Blank, so the Developed method is Specific.

6.2 Linearity and Range

The linearity for Rifaximin and Metronidazole were assessed by analysis of standard solution in range of 1.0-3.0 µg/ml and 2.0 – 6.0 Rifaximin and Metronidazole respectively. Correlation co-efficient for calibration curve Rifaximin and Metronidazole was found to be 0.998 and 0.997 respectively.

The regression line equation for Rifaximin and Metronidazole are as following:

For Rifaximin and Metronidazole : **y = 80.48x – 19.12 and y = 9746x – 8899**

Table 5: Linearity Data for Rifaximin

Sr. No	Concentration (µg/ml)	Area
1	1	3767.166
2	1.5	5077.839
3	2	7095.703
4	2.5	8694.047
5	3	10693.216

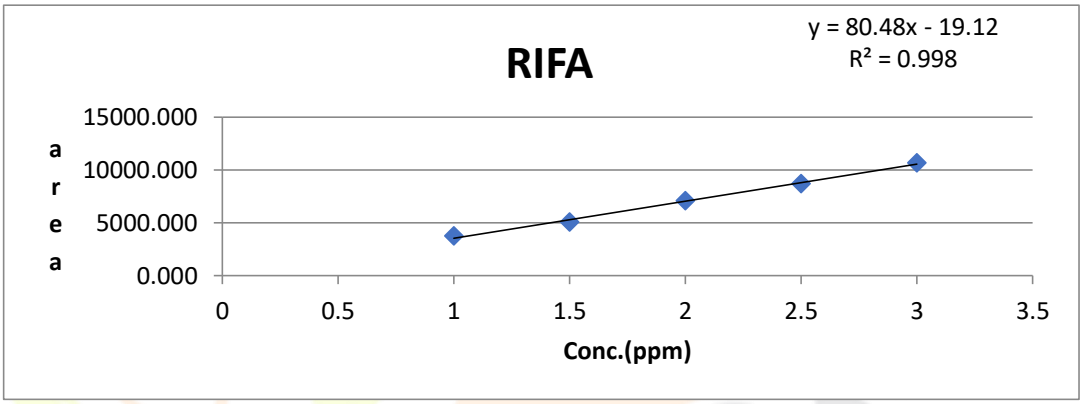

Fig. Calibration Curve of Rifaximin(1.0-3.0µg/ml)

Table 6: Linearity Data for Metronidazole

Sr. No	Concentration (µg/ml)	Area
1	2	27347.252
2	3	39105.84
3	4	49233.923
4	5	56225.988
5	6	67520.986

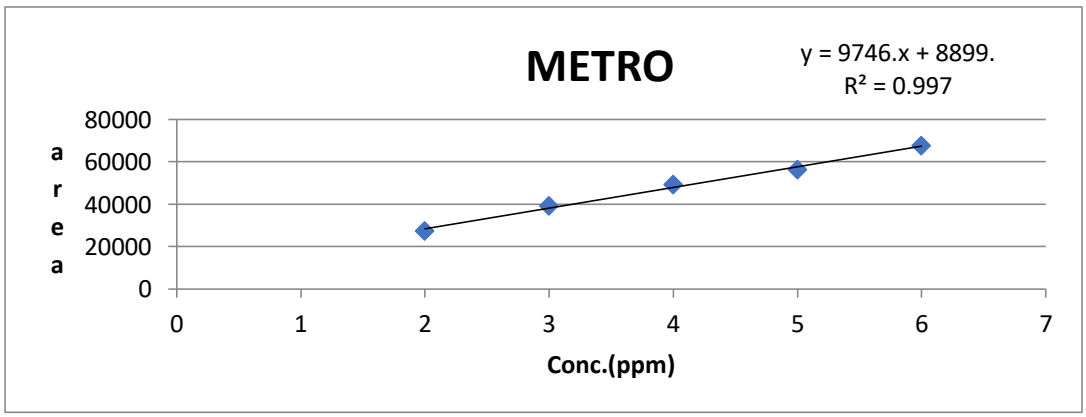

Fig. Calibration Curve of Metronidazole (2.0-6.0 µg/ml)

6.3 Precision

6.3.1. Repeatability

The data for repeatability of peak area measurement for Rifaximin and Metronidazole , based on six measurements of same solution of Rifaximin and Metronidazole are depicted in table 6.12 and 6.13 respectively. The % RSD for Rifaximin and Metronidazole was found to be 1.524 and 1.843 respectively.

Table 7: Repeatability Data for Rifaximin

Rifaximin				
Sr. No.	**Conc (µg/ml)**	**Area**	**Mean ± S.D (n=6)**	**% R.S.D**
1.	2.0	7041.361 7142.593 7003.592 7284.625 7042.393 7194.362	7118.154±108.516	1.524

Table 8: Repeatability Data for Metronidazole

metronidazole				
Sr. No.	**Conc (µg/ml)**	**Area**	**Mean ± S.D (n=6)**	**% R.S.D**
1.	4.0	48621.266 49204.632 50321.056 49751.669 50472.268 48221.563	49432.076±910.924	1.843

6.3.2. Intraday precision

The data for intraday precision for Rifaximin and Metronidazole is shown in table 6.14 and 6.15 respectively. The % R.S.D. for Intraday precision was found to be 0.948-1.439 for Rifaximin and 0.890 – 1.838 for Metronidazole .

Table 9: Intraday precision data for Estimation of Rifaximin

Sr. No.	**Rifaximin**		
	Conc. (µg/ml)	**Area Mean ± S.D. (n=3)**	**% R.S.D**
1	1	3804.309 ± 51.457	1.353
2	2	7088.873 ± 67.190	0.948
3	3	10383.820 ± 149.412	1.439

Table 10 : Intraday precision data for Estimation of Metronidazole

Sr. No.	Metronidazole		
	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	% R.S.D
1	2.0	28466.283 ± 523.342	1.838
2	4.0	50840.273 ± 483.169	0.950
3	6.0	67372.516 ± 599.920	0.890

6.3.3. Interday precision

The data for intraday precision for Rifaximin and Metronidazole is shown in table 6.16 and 6.17 respectively. The % R.S.D. for interday precision was found to be 0.517-1.583 for Rifaximin and 1.117-1.331 Metronidazole .

Table 11: Interday Precision data for Estimation of Rifaximin

Sr. No.	Rifaximin		
	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	% R.S.D
1	1	4002.405 ± 20.692	0.517
2	2	7039.562 ± 111.420	1.583
3	3	11054.805 ± 106.341	0.962

Table 12: Interday Precision data for Estimation of Metronidazole

Sr. No.	Metronidazole		
	Conc. (µg/ml)	Area Mean ± S.D. (n=3)	% R.S.D
1	2.0	27811.541 ± 310.602	1.117
2	4.0	49876.839 ± 664.042	1.331
3	6.0	67310.682 ± 796.776	1.184

6.4.4. Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation at three level of standard addition. The results are shown in table 6.18 and 6.19 respectively. Percentage recovery for Rifaximin and Metronidazole was 100.179-100.852% and 99.635-100.291 respectively.

Table 13: Recovery Data for Rifaximin

Sr. No.	Conc. Level (%)	Sample amount (µg/ml)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	80 %	1	0.8	0.793	99.182	100.179 ± 0.888
2		1	0.8	0.807	100.887	
3		1	0.8	0.804	100.469	
4	100 %	1	1	0.997	99.729	100.852 ± 0.972
5		1	1	1.014	101.403	
6		1	1	1.014	101.423	
7	120 %	1	1.2	1.190	99.144	100.607 ± 1.418
8		1	1.2	1.224	101.975	
9		1	1.2	1.208	100.702	

Table 14: Recovery Data for Metronidazole

Sr. No.	Conc. Level (%)	Sample amount (µg/ml)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	80 %	2	1.6	1.620	101.240	99.635 ± 1.496
2		2	1.6	1.590	99.369	
3		2	1.6	1.573	98.295	
4	100 %	2	2	2.000	100.020	99.860 ± 0.519
5		2	2	2.006	100.281	
6		2	2	1.986	99.280	
7	120 %	2	2.4	2.412	100.509	100.291 ± 1.269
8		2	2.4	2.435	101.438	
9		2	2.4	2.374	98.927	

6.4.5. LOD and LOQ

Calibration curve was repeated for five times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were calculated as follows:

LOD = 3.3 * SD/slope of calibration curve

LOQ = 10 * SD/slope of calibration curve

Limit of Detection

Table 15: Limit of Detection Data for Rifaximin and Metronidazole

Rifaximin	Metronidazole
LOD = 3.3 x (SD / Slope) = 3.3 x (207.385/3493.664) = 0.196 µg/ml	LOD = 3.3 x (SD / Slope) = 3.3 x (1395.556/9746.762) = 0.472 µg/ml

Limit of Quantitation

Table 16: Limit of Quantitation Data for Rifaximin and Metronidazole

Rifaximin	Metronidazole
LOQ = 10 x (SD / Slope) = 10 x (207.385/3493.664) = 0.594 µg/ml	LOQ = 10 x (SD / Slope) = 10 x (1395.556/9746.762) = 1.432 µg/ml

6.4.6. Robustness

The effect of changes was found to be within the acceptance criteria as shown in table 6.22 and 6.23 respectively. The % RSD should be less than 2%.

Table 17: Robustness data for Rifaximin

Sr No.	Area at Flow rate (- 2.0 ml/min)	Area at Flow rate (+ 2.0 ml/min)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	8952.004	4863.352	7300.308	5258.53
2	9043.254	4722.654	7510.364	5310.246
3	8814.362	4874.236	7321.654	5243.723
% R.S.D	1.289	1.754	1.567	0.663

Table 18: Robustness data for Metronidazole

Sr No.	Area at Flow rate (- 2.0 ml/min)	Area at Flow rate (+ 2.0 ml/min)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
1	56382.667	33788.631	47279.323	34926.825
2	55102.356	34206.892	48462.043	33925.618
3	57215.699	34224.566	49024.982	34281.062
% R.S.D	1.893	0.724	1.846	1.476

6.4.7. Analysis of marketed formulation by developed method.

Applicability of the proposed method was tested by analyzing the commercially available Tablet formulation Rifaxigyl-M. The results are shown in table 6.24.

Table 19: Analysis of Marketed Formulation

Tablet	Label claim		Assay (% of label claim*) Mean ± S. D.	
	Rifaximin Metroni-	Metronidazole	% Rifaximin Metronida-	% Metronidazole
Rifaxigyl-M	200mg	400mg	100.054 ± 1.215	100.290± 1.896

The assay results were comparable to labeled value of each drug in combined dosage form. These results indicate that the developed method is accurate, precise, simple and rapid. It can be used in the routine quality control of dosage form in industries.

7. METHOD VALIDATION SUMMARY

Table 20: Summary of Validation Parameters of LC-MS/MS Method for Rifaximin and Metronidazole

Sr No.	Parameter		Rifaximin	Metronidazole
1	Specificity		Specific	specific
2	Linearity & Range		1-3	2-6
3	Regression equation		y = 80.48x – 19.12	y = 9746x + 8899
4	Correlation co-efficient (r^2)		0.998	0.997
5	Precision (% RSD)	Repeatability	1.524	1.843
		Interday	0.517-1.583	1.117-1.331
		Intraday	0.948-1.439	0.890 – 1.838
6	Accuracy (% recovery)		100.179-100.852	99.635-100.291
7	Limit of Detection(LOD)		0.196µg/ml	0.472
8	Limit of Quantification(LOQ)		0.594 µg/ml	1.432 µg/ml
9	Robustness (% RSD)		< 2.0 in each parameters	< 2.0 in each parameters
10	% Assay		100.054 ±1.215	100.290± 1.896

8 SUMMARY AND CONCLUSION

- There is no analytical work has been available regarding LC-MS/MS method for Rifaximin and Metronidazole in a literature. Data regarding behavior of drug in chromatographic conditions and other relevant analytical properties are not available.
- Rifaximin is an antibiotic used in the treatment of several gastrointestinal and liver disease. This activity will review the indications, mechanism of action, administration, safety profile, and contraindications for rifaximin. This activity will highlight the mechanism of action, adverse event profile, and other key factors (e.g., off-label uses, dosing, pharmacodynamics, pharmacokinetics, monitoring, relevant interactions) pertinent for the members of the interprofessional team.
- Metronidazole is the drug of choice in the chemotherapy of necrotizing ulcerative gingivitis.
- It is also used in high dosage in the chemoprophylaxis and treatment of many strictly anaerobic. Prolonged use has been advocated in the treatment of peridonititis.
- A novel attempt in a field of research has been made to develop and validate stability indicating analytical method via LC-MS/MS
- The objective of this study was to study the degradation behaviour of Rifaximin and Metronidazole under acidic, basic, oxidative, photolytic and thermal stress conditions as per prescribed International Conference on Harmonization (ICH) guidelines.
- Rifaximin was degraded under basic and oxidative stress condition and Metronidazole was degraded under basic and oxidative stress condition.
- A total of two degradation products (DPs) were characterized for rifaximin and two degradation products (DPs) were characterized for Saxagliptin, and their chromatographic separation was accomplished on Hypersil, BDS, C_{18}, (150mm x 4.6mm, 5µm) column using a mobile phase consisting buffer : acetonitrile (25:75)in a isocratic elution mode.
- The ion transitions were quantified in positive mode with MRM transition of 786.800→727.500 Da for Rifaximin and 172.100→126.400 Da for metronidazole.
- Retention time of Rifaximin and Metronidazole were found to be 4.9 min and 2.6 min respectively with a flow rate of 1.0 ml/min.
- Rifaximin and Metronidazole and its degradation products were characterized based on MRM scan mode and fragmentation patterns were obtained from ESI-MS/MS spectra.
- Structural elucidation of DPs of Rifaximin and Metronidazole was achieved by comparing their fragmentation patterns with that of Rifaximin and Metronidazole.
- The developed method has been validated as per ICH guideline by applying various validation parameters like specificity, linearity and range, accuracy precision and robustness.
- The LC-MS/MS method developed for the determination of Rifaximin and Metronidazole is found to be specific, linear, sensitive, precise, accurate and robust in nature.
- The method was successfully validated in terms of specificity, precision, linearity, accuracy and robustness as per ICH guidelines.

- It can be concluded that the proposed method can be used for routine analysis for estimation of Rifaximin and Metronidazole in its Pharmaceutical dosage form by LC-MS/MS.

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