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Stability Indicating HPLC Method for Simultaneous Estimation of Rifaximin and Metronidazole Hydrochloride Tablet Dosage Form

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ABSTRACT:

A simple, rapid, economical, precise and accurate Stability indicating RP-HPLC method for simultaneous estimation of Metronidazole and Rifaximin in their combined dosage form has been developed. A reverse phase high performance liquid chromatographic method was developed for the simultaneous estimation of Metronidazole and Rifaximin in Their Combined Dosage Form has been developed. The separation was achieved by LC- 20 AT C18 (250mm x 4.6 mm x 2.6 µm) column and Buffer (Potassium Phosphate, pH 5.0): Acetonitrile (60:40) as mobile phase, at a flow rate of 1 ml/min. Detection was carried out at 233 nm. Retention time of Rifaximin and Metronidazole were found to be 3.713 min and 6.107 min respectively. The method has been validated for linearity, accuracy and precision. Linearity observed for Metronidazole 10-30 µg/ml and for Rifaximin 5-15 µg/ml. Developed method was found to be accurate, precise and rapid for simultaneous estimation of Metronidazole And Rifaximin In Their Combined Dosage Form. The drug was subjected to stress condition of hydrolysis, oxidation, photolysis and Thermal degradation, Considerable Degradation was found in Thermal 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 RP-HPLC Method, Validation.

INTRODUCTION:

Rifaximin is newly approved semi-synthetic non-systemic antibiotic derived from a naturally occurring chemical, rifamycin that is produced by Streptomyces mediterranei 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 of an anti-protozoal and an anti-bacterial serves 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.

METHADOLOGY:

- **Preparation of standard solutions**

(A) Rifaximin standard stock solution: (100µg/mL)

A 10 mg of Rifaximin was weighed and transferred to a 100mL volumetric flask. Volume was made up to the mark with methanol.

(B) Metronidazole standard stock solution: (200µg/mL)

A 20 mg of Metronidazole was weighed and transferred to a 100mL volumetric flask volume was made up to the mark with methanol

(C) Preparation of standard solution of binary mixtures of Rifaximin (10 µg/mL) and Metronidazole (20µg/mL)

Take 1 mL from the Rifaximin stock solution and 1mL from Metronidazole stock solution and transferred to 10 mL volumetric flask and volume made up to the mark by mobile phase which was used in particular trials.

- **Selection of wavelength:**

Standard solution of Rifaximin (10 µg/mL) and Standard solution of Metronidazole (20µg/mL) were scanned between 200-400 nm using UV-visible spectrophotometer. Both solutions were scanned between 200-400 nm.

- **Chromatographic separation:**

Standard solutions of 5-15 µg/ml of Rifaximin and 10-30 µg/ml of Metronidazole were injected in column with 20 µl micro-syringe. The chromatogram was run for appropriate minutes with mobile phase Buffer (pH 5.0): Acetonitrile 60:40. The detection was carried out at wavelength 233 nm.

- **Chromatographic conditions:**

Column: C_{18} (25 cm × 0.46 cm) Hypersil BDS
Mobile Phase: Buffer(pH 5.0):Acetonitrile- 60:40
Flow Rate: 1.0 ml/min
Detection Wavelength: 233 nm
Runtime: 10 min
Injection volume: 20.0 µl

- **Stability Indicating Method**

A. Acid degradation

Acid decomposition studies were performed by transferring one ml of stock solution to 10 ml of volumetric flask. Two ml of 0.1 N HCl solutions was added and mixed well and put for 5 hrs at RT. Then the volume was adjusted with diluent to get 10µg/ml for Rifaximin and 20 µg/ml for Metronidazole.

B. Base degradation

Base decomposition studies were performed by transferring one ml of stock solution to 10 ml of volumetric flask. Two ml of 0.1 N NaOH solutions was added and mixed well and put for 3 hrs at RT. Then the volume was adjusted with diluent to get 10µg/ml for Rifaximin and 20µg/ml for Metronidazole.

C. Oxidative degradation

Oxidation decomposition studies were performed by transferring one ml of stock solution to 10 ml of volumetric flask. Two ml of 3% H_2O_2 solutions was added and mixed well and put for 6 hrs at RT. Then the volume was adjusted with diluent to get 10µg/ml for Rifaximin and20µg/ml for Metronidazole.

D. Photo Degradation

Photo decomposition studies were performed by transferring one ml of stock solution to 10 ml of volumetric flask. This solution was kept under UV light in UV chamber for 18 hrs. Then the volume was adjusted with diluent to get 10µg/ml for Rifaximin and 20µg/ml for Metronidazole.

Validation of RP-HPLC method

1) Linearity

The linearity for Rifaximin and Metronidazole were assessed by analysis of combined standard solution in range of 5-15µg/ml and 10-30µg/ml respectively, 5,7.5,10,12.5,15 ml solutions were pipette out from the Stock solution of Rifaximin (100 µg/ml) and Metronidazole (200 µg/ml) and transfer to 100 ml volumetric flask and make up with mobile phase to obtain 5,7.5,10,12.5 and 15µg/ml and 10,15,20,25 and 30µg/ml for Rifaximin and Metronidazole respectively ,In term of slope, intercept and correlation co-efficient value the graph of peak area obtained verses respective concentration was plotted.

2) Precision

Results should be expressed as Relative standard deviation (RSD) or coefficient of variance:-

A. Intra-day precision

Standard solution containing (5,10,15µg/ml) of Rifaximin and (10,20,30µg/ml) of Metronidazole were analyzed three times on the same day and % R.S.D was calculated.

B. Inter-day precision

Standard Standard solution containing (5, 10, 15µg/ml) of Rifaximin and (10.20.30 µg/ml) of Metronidazole were analyzed three times on the different day and % R.S.D was calculated.

3) Repeatability

Standard solution containing Rifaximin (10µg/ml) and Metronidazole (20µg/ml) was injected six times and areas of peaks were measured and % R.S.D. was calculated.

4) Accuracy

For Rifaximin

5 µg/ml drug solutions was taken in three different flask label A, B and C. Spiked 80%, 100%, 120% of standard solution in it and diluted up to 10ml. The area of each

solution peak was measured at 233 nm. The amount of Rifaximin was calculated at each level and % recoveries were computed.

For Metronidazole

10 µg/ml drug solutions was taken in three different flask label A, B and C. Spiked 80%, 100%, 120% of standard solution in it and diluted up to 10ml. The area of each solution peak was measured at 233 nm. The amount of Metronidazole was calculated at each level and % recoveries were computed.

5) LOD and LOQ

The LOD was estimated from the set of 3 calibration curves used to determination.

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) intercepts was calculated.

1) 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. pH of Mobile phase was changed (± 0.2) 5.2 and 4.8.
3. Ratio of Mobile phase was changed (±2) Buffer: Acetonitrile (62:38) and Buffer: Acetonitrile (58:42)

2) Analysis of formulation

Tablet powder equivalent to 10 mg of Rifaximin and 20 mg of Metronidazole was transferred to a 100 ml volumetric flask, add 60 ml of Mobile phase and shake well and made up volume up to the mark with mobile phase. The solution was filtered through Whatman filter paper no. 42 and first few drops of filtrate were discarded. 1 ml of this solution was diluted to 10 ml with mobile phase. The solution was injected 20 µl. The areas of resulting peak were measured at 233 nm.

RESULT AND DISCUSSION:

a) 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 performingin terms of efficiency, stability and reproducibility. C_{18} column is least polar compare to C_4 and C_8 columns. Here, A 250 x 4.6 mm 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.

b) Selection of wavelength:

Standard solution of Rifaximin (10µg/mL) and Standard solution of Metronidazole (20µg/mL) were scanned between 200-400 nm using UV-visible spectrophotometer. Wavelength was selected from the overlay spectra of above solutions. Both Rifaximin and Metronidazole show reasonably good response at 233 nm.

Figure 1 ***Overlain UV Spectrum of Rifaximin and Metronidazole showing selection of wavelength detection***

c) Selection of Mobile Phase

Standard solutions of 5-15 µg/ml of Rifaximin and 10-30µg/ml of Metronidazole were injected in column with 20 µl micro-syringe. The chromatogram was run for appropriate minutes with mobile phase Buffer (pH 5.0): Acetonitrile 60:40. The detection was carried out at wavelength 233 nm. The chromatogram was stopped after separation achieved completely. Data related to peak like area, height, retention time, resolution etc were recorded using software.

After considering the varying combinations of various mobile phases, Buffer (pH 5.0): Acetonitrile 60:40 was finalized as it was showing good peak shapes and a significant amount of resolution.

Figure 2 ***Chromatogram of Rifaximin and Metronidazole in Buffer (pH 5.0): Acetonitrile (60:40 v/v) (Flow rate-1.0 ml/min) (Final)***

d) System suitability test

The mobile phase Buffer (pH 5.0): Acetonitrile (60:40) was selected because it was found to ideally resolve the peaks with retention time (RT) 3.713 min and 6.107 min for Rifaximin and Metronidazole respectively and the same is shown in fig. 3

Figure 3 ***Chromatogram of Rifaximin and Metronidazole in Buffer (pH 5.0): Acetonitrile (60:40v/v) (Flow rate-1.0 ml/min (Final)***

❖ **Observed values for system suitability test:**

1. Resolution (Rs): Resolution was observed 9.825, depicted in Table 1.

2. Column efficiency (N): Number of plates observed for Rifaximin and Metronidazole were 6714 and 6376 respectively, depicted in Table 1.

3. Symmetry factor (S): Tailing factor observed for Rifaximin and Metronidazole were 1.375 and 1.439 respectively, depicted in Table 1.

Table 1 ***Results for system suitability test.***

Parameters	Data observed	
	Rifaximin	Metronidazole
Theoretical plates	6714	6376
Symmetry	1.375	1.439
Resolution	9.825	

e) Stability indicating method for simultaneous estimation of Rifaximin and Metronidazole done by RP-HPLC

Figure 4 ***Metronidazole and Rifaximin Standard for stability***

Figure 5 ***Metronidazole and Rifaximin sample***

Calculation for Stability:

Table 2 ***Rifaximin and Metronidazole std for stability***

Drugs	Area
Rifaximin	1562.410
Metronidazole	3537.78

Table 3 ***Rifaximin % Degradation***

Rifaximin				
Parameter	**Standard**		**Sample**	
	Area	**% Degradation**	**Area**	**% Degradation**
Acid	1237.01	20.83	1233.37	21.06
base	1258.93	19.42	1254.18	19.73
Oxidation	1161.49	25.66	1124.32	28.04
Photo	1179.45	24.51	1213.25	22.35

Table 4 ***Metronidazole % Degradation***

Metronidazole				
Parameter	**Standard**		**Sample**	
	Area	**% Degradation**	**Area**	**% Degradation**
Acid	2652.15	25.03	2633.59	25.56
base	2824.17	20.17	2806.63	20.67
Oxidation	2467.828	30.24	2406.465	31.98
Photo	2786.432	21.24	2755.239	22.12

Validation of RP-HPLC method

Linearity:-

Figure 6 Linearity data for Metronidazole

Figure 7 Linearity data for Rifaximin

2. Precision:-

Table 5 ***Intraday precision data for estimation of Rifaximin***

	Rifaximin		
SR. NO.	**Conc. (µg/ml)**	**Area Mean ± S.D. (n=3)**	**% R.S.D**
1	50	765.712±1.176	0.1535
2	100	1517.763±3.810	0.2510
3	150	2283.195±15.149	0.6635

Table 6 ***Intraday precision data for estimation of Metronidazole***

	Metronidazole		
SR. NO.	**Conc. (µg/ml)**	**Area Mean ± S.D. (n=3)**	**% R.S.D**
1	50	1761.403±6.8040	0.3862
2	100	3492.040±11.002	0.3150
3	150	5250.840±24.9254	0.4764

Table 7 ***Intraday precision data for estimation of Rifaximin***

	Rifaximin		
SR. NO.	**Conc.(µg/ml)**	**Area Mean ± S.D. (n=3)**	**% R.S.D**
1	50	756.305±4.9383	0.6529
2	100	1513.43±8.0481	0.5317
3	150	2260.461±22.5805	0.9989

Table 8 ***Intraday precision data for estimation of Metronidazole***

	Metronidazole		
SR. NO.	**Conc. (µg/ml)**	**Area Mean ± S.D. (n=3)**	**% R.S.D**
1	50	1747.377±5.4336	0.3109
2	100	3481.259±10.6596	0.3092
3	150	5223.842±30.8321	0.5902

Repeatability:-

Table 9 ***Repeatability data for Rifaximin***

	Rifaximin			
Sr No.	Conc (µg/ml)	Area	Mean ± S.D (n=6)	% R.S.D
1.	100	1514.329 1508.290 1512.866 1517.429 1512.885 1515.771	1513.595±3.1353	0.2071

Table 10 ***Repeatability data for Metronidazole***

	Metronidazole			
Sr No.	Conc (µg/ml)	Area	Mean ± S.D (n=6)	% R.S.D
1.	100	3491.047 3477.098 3421.438 3494.591 3484.053 3490.821	3476.508±27.6883	0.7964

4. Accuracy:-

Table 11 ***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 %	5	4	4.05	101.21	100.27±1.50
2		5	4	3.94	98.54	
3		5	4	4.04	101.06	
4	100	5	5	5.08	101.62	100.95±0.65
5	%	5	5	5.02	100.32	
6		5	5	5.05	100.92	
7	120	5	6	6.10	101.74	100.29±1.25
8	%	5	6	5.98	99.63	
9		5	6	5.97	99.50	

Table 12 ***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 %	10	8	8.05	100.64	99.99±0.70
2		10	8	8.00	100.06	
3		10	8	7.94	99.26	
4	100	10	10	10.12	101.19	101.46±0.32
5	%	10	10	10.14	101.39	
6		10	10	10.18	101.81	
7	120	10	12	11.96	99.63	100.68±0.95
8	%	10	12	12.18	101.49	
9		10	12	12.11	100.90	

5. LOD LOQ

Table 13 ***Limit of Detection data for Rifaximin and Metronidazole***

Rifaximin	Metronidazole
LOD = 3.3 x (SD / Slope)	LOD = 3.3 x (SD / Slope)
= 3.3 x (16.540/150.8)	= 3.3 x (62.267/173.88)
= 0.362 µg/ml	= 1.182µg/ml

Table 14 ***Limit of Quantitation data for Rifaximin and Metronidazole***

Rifaximin	Metronidazole
LOQ = 10 x (SD / Slope)	LOQ = 10 x (SD / Slope)
= 10 x (16.540/150.8)	= 10 x (62.267/173.88)
= 1.097µg/ml	= 3.581µg/ml

6. Robustness:

Table 15 ***Robustness data for Rifaximin***

SR NO.	Area atFlowrate (-0.2ml/min)	Area at Flow rate (+0.2ml/min)	Area at pH (-0.2)	Area at pH (+0.2)	Area at Mobilephase (-2)	Area at Mobile phase (+2)
1	1670.85	1358.85	1537.07	1482.66	1580.31	1424.21
2	1665.87	1348.05	1521.75	1475.28	1588.26	1431.40
3	1669.25	1356.16	1524.82	1482.72	1596.28	1434.29
%R.S.D	0.15	0.41	0.53	0.29	0.50	0.36

Table 16 ***Robustness data for Metronidazole***

SR NO.	Area at Flow rate (0.2ml/min)	Area at Flow rate (+0.2ml/min)	Area at pH (-0.2)	Area at pH (+0.2)	Area at Mobile phase (-2)	Area at Mobile phase (+2)
1	3852.13	3129.69	353 9.73	341 4.32	3643.15	3280.23
2	3840.61	3104.69	350 4.56	340 0.84	3661.53	3296.68
3	3828.30	3123.36	350 1.66	343 4.52	3680.00	3323.40
%R.S.D	0.31	0.42	0.60	0.50	0.50	0.66

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

Table 17 ***Analysis of marketed formulation***

Tablet	Label claim		Assay (% of label claim*) Mean ± S. D.	
	Rifaximin	Metronidazole	% Rifaximin	% Metronidazole
Rifaxigyl-M	200mg	400mg	98.66± 0.195	97.68± 0.195

6. RESULT

The assay results were comparable to labeled value of each drug in Tablet 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. SUMMARY

- RP-HPLC method was developed for simultaneous estimation Metronidazole and Rifaximin. In RP-HPLC method, good resolution and separation of two drugs was achieved. 0.05 M Sodium dihydrogen phosphate (pH 5):Acetonitrile (60:40 v/v) was used as mobile phase. Retention time of Rifaximin and Metronidazole were found to be 3.713 and 6.107 min respectively with a flow rate of 1 ml/min. The

proposed method was accurate and precise. Therefore proposed method can be used for routine analysis of Metronidazole and Rifaximin in tablets.

- Forced degradation study of Metronidazole and Rifaximin was performed by RP-HPLC method which includes Acid, Base, Oxidative, Photo and Thermal degradation. Results of degradation were found within limit.

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