RESEARCH PAPER

HPTLC Method Development and Validation for Simultaneous Estimation of Rifaximin and Metronidazole Benzoate in Combined Tablet Dosage Form

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ABSTRACT
Rifaximin is a non-systemic antibiotic with low oral bioavailability. It is soluble in methanol, chloroform, acetone and ethyl acetate. Metronidazole is a nitroimidazole derivative which have broad spectrum of amoebicidal activity against protozoa and some anaerobic bacteria. Practically, this drug is insoluble in water but soluble in Ethanol (13 mg/ml) and very freely soluble in (DMSO) (55 mg/ml). The combination of Rifaximin and Metronidazole has shown great evidence of eradication in case of H.pylori infection and in traveller diarrhoea. This Combination is exerting maximum effect at dose of 200 mg of Rifaximin and 400 mg of Metronidazole. High-Performance Thin-Layer Chromatography (HPTLC) is a form of thin-layer chromatography (TLC) that provides superior separation power using optimized coating material, novel procedures for mobile-phase feeding, layer conditioning and improved sample application. The method was developed using mobile phase toluene: chloroform: methanol: n-butanol (4:3:3:0.5 v/v/v), stationary phase silica gel G F254 (Precoated TLC plates) at the wavelength of 311 nm. Analytical method validation establishes documented evidence that the procedure adopted for a test is fit for the intended purpose in terms of quality, reliability and consistency of results.
Conclusion: HPTLC method was developed for estimation of Rifaximin and Metronidazole from their pharmaceutical dosage form. The developed method was validated and found to be simple, precise, accurate, and robust, as it separates components with good chromatographic criteria. All results were found satisfactory so, development and validation method can be applied to the tablet dosage form.
Keywords: Rifaximin, Metronidazole, HPTLC, Analytical validation, Estimation
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INTRODUCTION

Metronidazole is an antimicrobial agent and Rifaximin is an antibiotic. The Structure of Metronidazole and Rifaximin are shown in Figure 1 and Figure 2. They are used in H-pylori infection. This combination has shown great evidence of eradication in case of H-pylori infection and in traveller diarrhoea also. [1-6]

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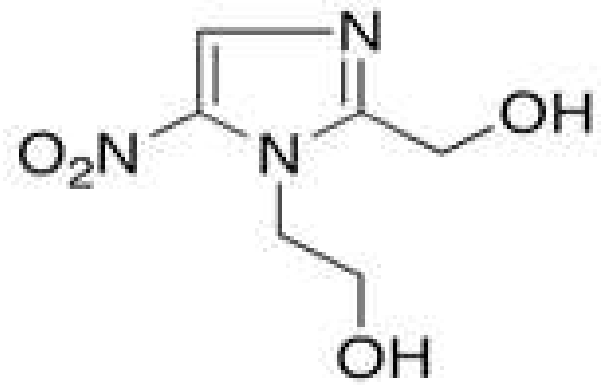

Figure 1: Structure of Metronidazole

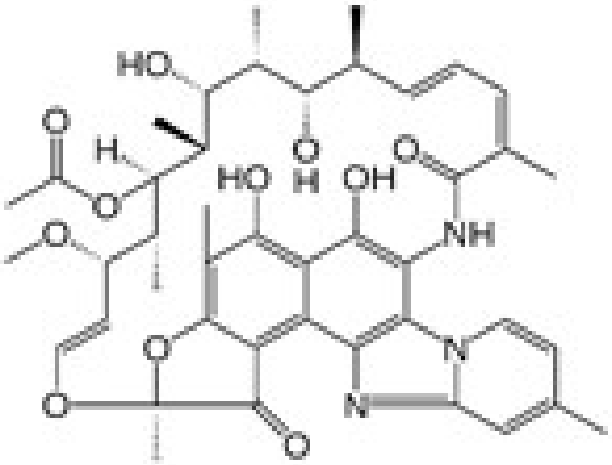

Figure 2: Structure of Rifaximin

A review of the literature has revealed a limited number of HPTLC methods for the determination of Rifaximin and Metronidazole alone or in combination with other drug components. To date, no High-Performance Thin Layer (HPTLC) chromatographic method has been reported for the determination of these agents from their combined pharmaceutical formulation.[7-14] Therefore, the objectives of this study are to develop and optimize an HPTLC method for the separation of Rifaximin and Metronidazole, validate the optimized HPTLC method for their separation, and apply the developed and validated method for the estimation of Rifaximin and Metronidazole from their combined tablet dosage form.

MATERIALS AND METHODS

HPTLC of Camag Switzerland, applicator linomat 5, scanner TLC scanner 3, UV spectrometer UV-1800, Shimadzu Japan with software WinCATS was used. Methanol, n butanol, chloroform, acetonitrile, ammonia, toluene and diethylamine - LR grade, Water - HPLC grade, Merck India Ltd. Mumbai, was used. A commercial tablet formulation RIFAXIGYL M was purchased from local market.

Ratio of mixture for the development of HPTLC method

The marketed formulation of Rifaximin and Metronidazole is available in a dosage of 200 mg and 400 mg, respectively. In order to prepare a solution containing both Metronidazole and Rifaximin at the same dosage, a concentration of 4000 µg/ml and 2000 µg/ml, respectively, was utilized. The selection of methanol as the diluent was based on the favorable solubility properties of both compounds.

Selection of analytical wavelength:

Mandatory requirements for selection of analytical wavelength in HPTLC with UV detection are that both drugs should give adequate response and linearity at selected wavelength, shown in figure 3 and 4. Results are shown in table 1.

Table 1: Identification of drugs by UV

Name of Drug	Reported absorption maxima	Observed absorption maxima
Metronidazole	310 nm	311 nm
Rifaximin	292 nm	293nm

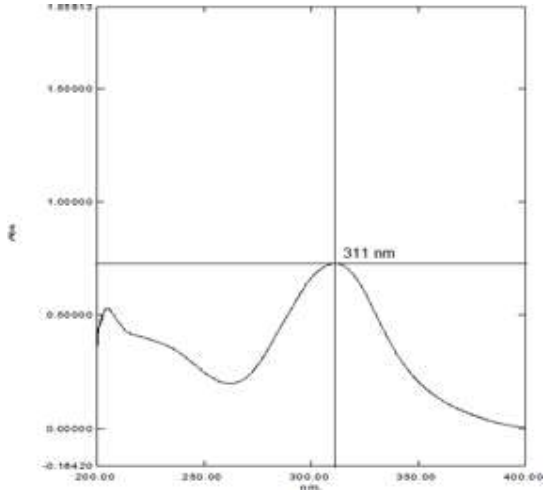

Figure 3: UV spectrum of Metronidazole sample (10 µg/ml)

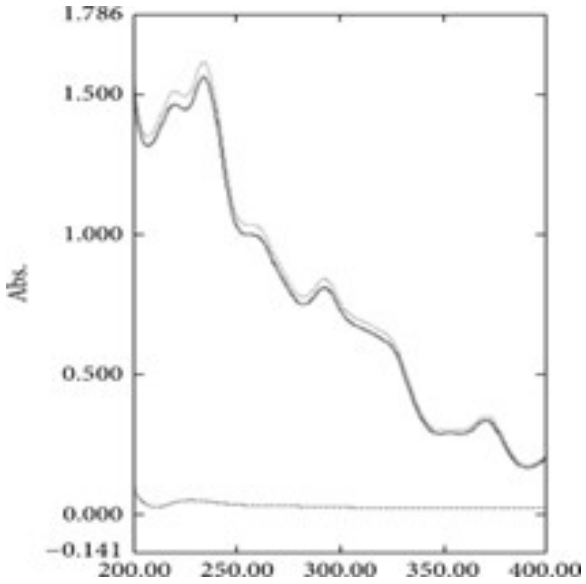

Figure 4: UV spectrum of Rifaximin in methanol (10 µg/ml

Selection of Mobile phase

The selection of the mobile phase components and their ratio was based on a thorough consideration of the solubility, polarity, and relevant literature pertaining to both drugs. The drugs were dissolved in methanol and subsequently applied to the plate. Methanol was chosen due to its rapid vaporization upon application under a nitrogen stream. After experimentation with various mobile phase systems, an optimal system was selected based on the resolution achieved between the compounds.

Selection of stationary phase:

The separation of Metronidazole and Rifaximin was accomplished using thin-layer chromatography (TLC) plates that were pre-coated with silica gel G60 F254 on an aluminium support (E. Merck). The silica gel particles had a size of 2 µm and the sorbent layer had a thickness of 0.2 mm. The plates were provided in a size of 20 × 20 cm, which were subsequently cut into appropriate sizes for method development (10 × 10). The initial method development was conducted using TLC plates with a size of 2 × 8 cm.

Preparation of standard and stock solution

Standard stock solution of Rifaximin (2000 µg.mL-1): Accurately weighed 20 mg drug dissolved in 10 mL methyl alcohol (2000 µg.mL-1).

Standard stock solution of Metronidazole (4000 µg.mL-1): Accurately weighed 40 mg drug dissolved in 10 mL methyl alcohol (4000 µg.mL-1).

Preparation of standard working solution of binary mixture of Rifaximin+Metronidazole = 200 + 400 µg.mL^{-1}: 1.0 mL from Rifaximin Stock Solution and 1.0 mL from Metronidazole Stock Solution make up to 10mL with mobile phase Rifaximin + Metronidazole = 200 + 400 µg.mL^{-1}

Preparation of Formulation Solution: Tablet powder, which is equivalent to Rifaximin (20mg) and Metronidazole (40mg), was introduced into a volumetric flask (10ml). The flask was then filled with methanol to raise the volume to 10ml, resulting in a stock solution containing 2000 µg/ml of Rifaximin and 4000 µg/ml of Metronidazole. The solution was sonicated for 10 minutes and filtered through a 0.45 µm Whatman filter paper.

To obtain a concentration of Rifaximin + Metronidazole = 200 + 400 µg/ml, 1ml of the above filtrate was diluted to 10ml. Subsequently, 1 microliter (Rifaximin + Metronidazole= 200 + 400 ng/band) of the resulting solution was applied to an HPTLC plate for analysis.

Optimized Chromatographic Conditions

Mobile Phase	Toluene: Chloroform: Methanol: n-butanol (4:3:3:0.5 v/v/v)
Stationary phase	Silica Gel G F254 (Precoated TLC plates)
Detection wavelength	311nm
Chamber saturation time	20 minutes
Plate dimension	10*10 cm
Rf value	0.52 For Metronidazole and 0.81 for Rifaximin
Band width and space between two bands	7 mm
Spraying rate	159 nL/sec
Slit dimension	4*0.10 mm
Lamp	Deuterium
Scanning speed	20 mm/sec

Preparation of calibration curve:
A combination of rifaximin 20 mg and metronidazole 40mg is blended and subsequently diluted with methanol in a 10ml volumetric flask, resulting in a concentration of Rifaximin 2000 mcg/ml and metronidazole 4000 mcg/ml. A subsequent dilution was performed by adding 1ml of the previous solution to 10 ml of methanol, resulting in a concentration of Rifaximin 200 mcg/ml and metronidazole 400 mcg/ml, shown in figure 5,6,7.

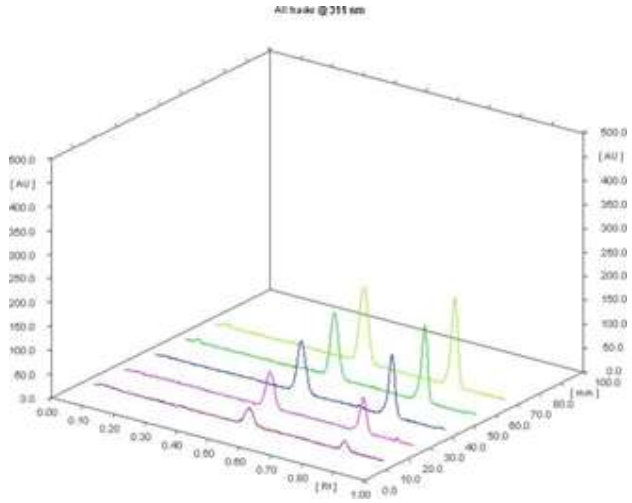

Figure 5: Overlain chromatogram of all the tracks at 311 nm

Table 2: Linearity Data of Metronidazole by HPTLC method

Sr No	Concentration (ng/Band)	Peak Area ± SD	RSD
1	**0**	0	**-**
2	**400**	718.44 ± 12.07	**1.68**
3	**800**	1224.64 ± 20.20	**1.42**
4	**1200**	2038.9 ± 26.70	**1.31**
5	**1600**	2714.5 ± 35.38	**1.30**
6	**2000**	3374.6 ± 46.51	**1.38**
Linear regression Equation			**y = 1.6911x - 12.59**
Regression coefficient (r^2)			**0.9978**

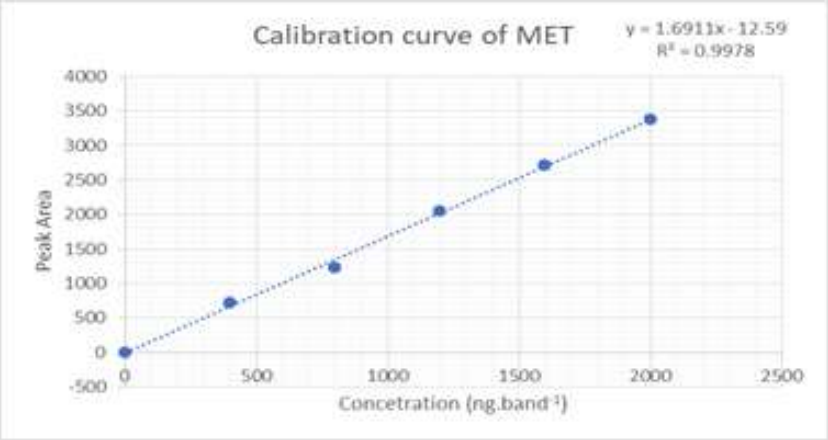

Figure 6: Calibration Curve of Metronidazole

Table 3: Linearity Data of Rifaximin by HPTLC method

Sr No	Concentration (ng/Band)	Peak Area ± SD	RSD
1	**0**		**-**
2	**200**	413.9 ± 07.49	**1.81**
3	**400**	1135.7 ± 19.06	**1.68**
4	**600**	1852.2 ± 20.44	**1.10**
5	**800**	2452.1 ± 27.95	**1.14**
6	**1000**	3084.6 ± 23.28	**0.75**
Linear regression Equation			**y = 3.1792x – 99.829**
Regression coefficient (r^2)			**0.996**

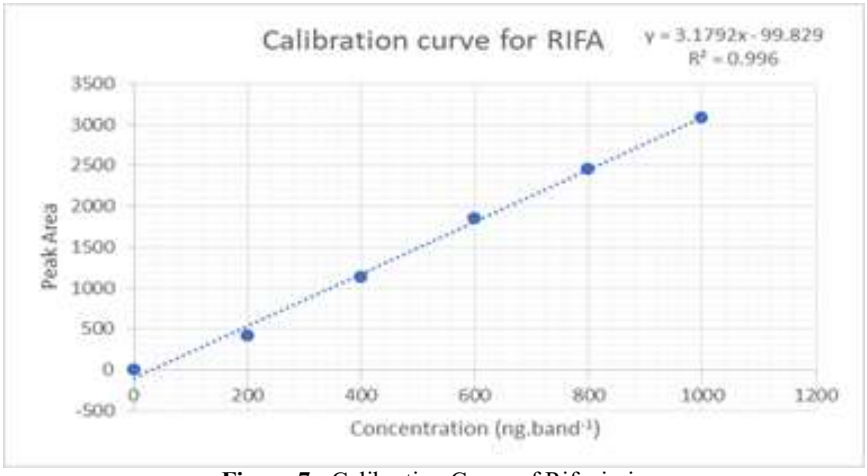

Figure 7: Calibration Curve of Rifaximin

METHOD VALIDATION

Accuracy (Recovery study)

Accuracy of the analytical method has been performed by spiking of sample (Formulation) with the standard. Spiking of the sample was performed at 50, 100 and 150 % of the target concentration (400 + 800 ng/Band). Concentration of standard solution was also kept at 4000 µg/ml of Metronidazole and 2000 µg/ml of Rifaximin (Solution B), given in table 4 and 5.

Table 4: Preparation of Accuracy Solution

Concentration of stock solution (Sample)	**2000 µg/ml of Rifaximin and 4000 µg/ml of Metronidazole**			
Volume taken from SS (mL)	1.00	1.00	1.00	1.00
Concertation corresponding to solution taken	2000+4000 µg/ml	2000+4000 µg/ml	2000+4000 µg/ml	2000+4000 µg/ml
Volume of Standard taken in mL (Solution B)	-	0.50 (1000+2000)	0.10 (2000+4000)	0.15 ml (3000+6000)
Diluent (Up to 10 ml)	Mobile phase	Mobile phase	Mobile phase	Mobile phase
Total concentration achieved (µg/ml)	200+400	300+600	400+800	500 + 1000
Identification	Unspiked	50 % Spiked	100 % Spiked	150 % Spiked
Volume applied in µL	2	2	2	2
Concentration in ng/band	400+800 ng/band	600+1200 ng/band	800+1600 ng/band	1000+2000 ng/band

Table 5: Accuracy data of Metronidazole and Rifaximin

Accuracy Data for Metronidazole				
Level of Spiking	**Amount of Drug present**	**Amount of drug added**	**Amount of Drug recovered**	**% Recovery**
Unspiked	800	-	-	-
50 %	800	400	396.37 ± 0.75	99.09 ± 0.18
100 %	800	800	789.99 ± 4.36	98.74 ± 0.54
150 %	800	1200	1183.04.56 ± 2.63	98.58 ± 0.21
Accuracy Data for Rifaximin				
Unspiked	400	-	-	-
50 %	400	200	198.58 ± 0.63	99.29 ± 0.31
100 %	400	400	396.01 ± 1.93	99.01 ± 0.48
150 %	400	600	591.02 ± 3.05	98. 50 ± 0.51

Precision:

A standard working solution was prepared consisting of mixtures with concentrations of Metronidazole (ranging from 400 to 2000 ng/band) and Rifaximin (ranging from 200 to 1000 ng/band). The solution was injected into the column at a volume of 20 µL, utilizing optimized chromatographic conditions. Each standard mixture was injected five times and the peak area was monitored. The repeatability of each concentration was assessed by calculating the relative standard deviation (RSD), given in table 6 and 7.

Table 6. Repeatability data of Metronidazole by HPTLC method

Concentration	**400 ng. band^{-1}**	**800 ng. band^{-1}**	**1200 ng. band^{-1}**	**1600 ng. band^{-1}**	**2000 ng. band^{-1}**
Area 1	712.2	1410.2	2052.1	2731.4	3401.4
Area 2	724.6	1396.5	2011.2	2664.0	3307.3
Area 3	718.8	1435.4	2034.9	2712.5	3370.5
Area 4	702.4	1442.8	2018.8	2704.4	3362.6
Area 5	734.2	1438.3	2077.5	2760.0	3431.6
Mean	718.44	1424.64	2038.90	2714.45	3374.68
SD	12.07	20.20	26.70	35.38	46.51
RSD	1.68	1.42	1.31	1.30	1.38

(n = 5 determinations)

Table 7: Repeatability data of Rifaximin by HPTLC method

Concentration	200 ng. band^{-1}	400 ng. band^{-1}	600 ng. band^{-1}	800 ng. band^{-1}	1000 ng. band^{-1}
Area 1	412.2	1132	1840.3	2450.8	3089.3
Area 2	404.2	1149.2	1860.2	2470.2	3122.2
Area 3	418.4	1106.5	1872.5	2422.3	3078.5
Area 4	423.8	1134.8	1822.5	2488.6	3062.4
Area 5	410.9	1155.8	1865.3	2428.1	3070.4
Mean	413.90	1135.66	1852.16	2452.00	3084.56
SD	7.49	19.06	20.44	27.95	23.28
RSD	1.81	1.68	1.10	1.14	0.75

(n = 5 determinations)

Intraday Precision:

Mixtures that represent overall range (Rifaximin + Metronidazole = 200+400, 600+1200 and 1000+2000 ng/band) were analysed on the same day at different time intervals for intraday precision, given in table 8.

Table 8: Intraday Precision data for estimation of Rifaximin and Metronidazole

Sr. No.	Rifaximin			Metronidazole		
	Conc. (µg/ml)	Intraday Mean ± SD	RSD	Conc. (µg/ml)	Intraday Mean ± SD	RSD
1	200	415.63 ± 7.1	1.71	400	718.53 ± 6.2	0.86
2	600	1857.67 ± 16.25	0.87	1200	2032.73 ± 20.54	1.01
3	1000	3093.97 ± 26.21	0.85	2000	3398.52 ± 34.6	1.02

(n = 3 determinations)

Interday Precision:

Mixtures that represent overall range (Rifaximin + Metronidazole = 200+400, 600+1200 and 1000+2000 ng/band) were analysed on different days for interday precision, given in table 9.

Table 9: Intraday Precision data for estimation of Rifaximin and Metronidazole

Sr. No.	Rifaximin			Metronidazole		
	Conc. (µg/ml)	Inter-Day Mean ± SD	RSD	Conc. (µg/ml)	Inter-Day Mean ± SD	RSD
1	200	411.6 ± 7.12	1.73	400	723.67 ± 11.03	1.52
2	600	1853.43 ± 27.03	1.46	1200	2049.47 ± 29.44	1.44
3	1000	3096.67 ± 22.76	0.74	2000	3359.73 ± 47.93	1.43

LOD and LOQ

LOD and LOQ of the drug were calculated from signal-to-noise ratio (i.e., 3.3 for LOD and 10 for LOQ, given in table 10.

Table 10. Result for LOD and LOQ

Sr No	Drug	LOD	LOQ
1	Rifaximin	6.55 ng/band	19.85 ng/band
2	Metronidazole	19.85 ng/band	40.88 ng/band

Robustness

The study involved the systematic alteration of various parameters to assess the robustness of the method. The effects of these alterations were observed by comparing the results with those obtained from the standard preparation. Specifically, the following parameters were modified one at a time: i) chamber saturation time (± 5 min), with the optimized saturation time as the reference point, and ii) mobile phase composition (± 2 mL), with the optimized ratio as the reference point. For each alteration, three determinations of Rifaximin + Metronidazole = 600+1200 ng/band were carried out, and the relative standard deviation (RSD) was measured, given in table 11 and12.

Table 11: Robustness study for Metronidazole and Rifaximin

Sr No	Drug	Regression	Correlation	Slope	Intercept
1	Metronidazole	y = 1.6911x - 12.59	0.9978	1.6911	12.59
2	Rifaximin	y = 3.1792x – 99.829	0.996	3.1792	99.829

Table 12: Robustness data of Rifaximin and Metronidazole

Parameter	Level of Change	Area (n = 3)	
		Rifaximin	Metronidazole
Chamber saturation time	15 minutes	1842.5	2044.9
	20 minutes	1822.5	2038.8
	25 minutes	1875.3	2077.5
	Mean ± SD	1846.77 ± 26.66	2053.73 ± 20.81
	RSD	1.44	1.01
Toluene: Chloroform: Methanol: n-butanol (4:3:3:0.5 v/v/v)	6:2:2:0.5	1855.2	2101.3
	4:3:3:0.5	1854.5	2071.9
	4:4:2:0.5	1892.5	2059.8
	4:2:4:0.5	1845.3	2035.5
	Mean ± SD	1861.88 ± 20.91	2067.13 ± 27.35
	RSD	1.12	1.32

RESULT AND DISCUSSION

Results are in good agreement with label claim, which indicates that there is no interference in routinely used experiments. The proposed method is accurate and precise therefore the proposed method can be used for routine analysis of Rifaximin and Metronidazole in tablet dosage form, shown in table 13. Summary of validation parameters are given in table 14.

Table 13: Analysis of marketed formulation of Rifaximin and Metronidazole by proposed method

Drug	Labelled Amount (ng/band)	Amount found (ng/band)	RSD	% Assay	RSD
Rifaximin	200	199.196 ± 1.28	0.642	99.598 ± 0.64	0.642
Metronidazole	400	400.304 ± 4.063	1.014	100.076 ± 1.457	1.014

(n = 3 determinations)

Table 14: Summary of Validation Parameters

Parameter	Limit	Result		Conclusion
		Rifaximin	Metronidazole	
Linearity and Range	$R^2 > 0.995$	0.996 (200 – 1000 ng/Band)	0.9978 (400 – 2000 ng/Band)	Method was linear
Repeatability	RSD < 2	0.75 – 1.81	1.3 – 1.68	Method was repeatable
LOD	-	6.55 ng/Band	19.85 ng/Band	-
LOQ	-	13.49 ng/Band	40.88 ng/Band	-
Intraday Precision	RSD < 2	0.85 - 1.71	0.64 – 0.97	Method was precise
Inter-Day Precision	RSD < 2	0.74 - 1.73	1.43 - 1.52	Method was precise
% Recovery	98 - 102 %	98.5 – 99.29 %	98.58 – 99.09 %	Method was accurate
Robustness	The system suitability parameters were found well within the acceptance criteria.			
Assay	98 – 102 %	99.59 %	100.07 %	Pass

CONCLUSION

HPTLC method was developed for estimation of RIFAXIMIN and METRONIDAZOLE from their pharmaceutical dosage form. The developed method was validated and found to be simple, precise, accurate, and robust, as it separates components with good chromatographic criteria. All results were found satisfactory so, the development and validation method can be applied to the tablet dosage form**.**

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