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Development and Validation of Stability Indicating RP-HPLC (PDA) Method for Estimation of Rifaximin and Ornidazole in Bulk and Combined Tablet Dosage Formulation

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Abstract : An isocratic stability indicating RP-HPLC(PDA) method was developed and validated for the determination of Rifaximin and Ornidazole in pharmaceutical dosage form. Isocratic elution was performed using the mobile phase Ammoniumformate buffer(pH 7.2) and Acetonitirile (55:45 v/v).Linearity was observed in the concentration range of 50-150µg ml^{-1} (0.999) for Rifaximin and 62.5 -875.5µg ml^{-1}(0.999) for Ornidazole. Rifaximin and Ornidazole were subjected to stress conditions of degradation such as acidic, alkaline, oxidation, photolytic and thermal degradation. The drug combination was found to be more sensitive towards acidic degradation. The method was validated as per ICH Guidelines. The recovery was in good agreement with the labeled amount in the pharmaceutical formulation. The proposed method is simple, precise, specific, accurate and robust for the determination of Rifaximin and Ornidazolein pharmaceutical dosage form.
Key words : RP-HPLC, Rifaximin, Ornidazole, Stability indicating, Validation, Quantification.

Introduction

Ornidazole(ONZ) is a 5-nitroimidazole derivative[1].Chemically ONZ is 1-(3-chloro-2-hydroxypropyl)-2-methyl-5 nitroimidazole**(Fig.1).**It is used in the treatment of amoebic dysentery, bacterial vaginosis, amoebiasis, giardiasis and trichomoniasis[2].

Rifaximin (RFX) is a benzimidazolederivative[3].Chemically it is 2S, 16Z, 18E, 20S, 21S, 22R, 24R,25S,27S,5,6,21,23,25-pentahydroxy-27-methoxy 2,4,11,16,20,22,24,26,-octa methyl-2,7-epoxy pentadeca-(1,11,13)trienimino)benzofuro(4,5-e)pyrido(1,2-a)-benzimidazole-1,15(2H)-dione,25acetate**(Fig.2).**It is used in the treatment of Travelers diarrhea caused by noninvasive strains of *Escherichia coli*[4].

Literature review reveals that UV method has been reported for estimation of ONZ[5] and UV and HPLC methods have been reported for estimation RFX[6-9]as a single component and few methods for combination with

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other drugs[10-12.] From the literature review it is evident thatno analytical method has been developed for estimation of ONZ and RFX in combined dosage form. The present workis aimed to developastability indicating HPLC method for simultaneous estimation of both the drugs in combined solid oral dosage form.

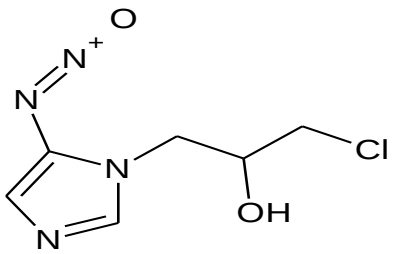


Fig.1: Structure of Ornidazole

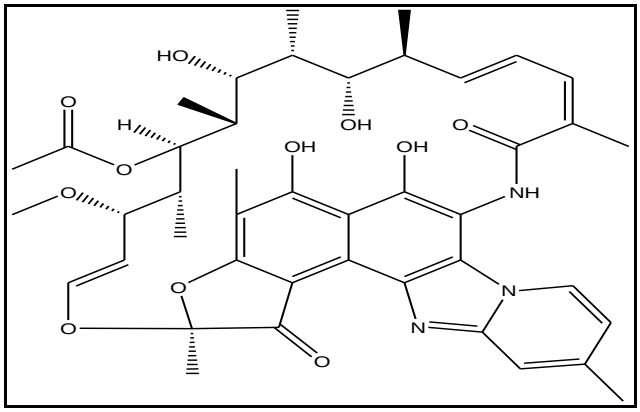


Fig.2: Structure of Rifaximin

Experimental

Chemical and Reagents : ONZ and RFX standard (Purity ≥99.0%) were obtained from SaimirraInnopharm Pvt.Ltd, India).All chemicals used were of Analytical grade.

Instrumentation : Chromatographic separation was achieved by using a Waters HPLC Model equipped with 2956 Photo iodide array detector.The instrumentation was controlled by using of Empower 3 software.

Chromatographic conditions

Mobile phase	: Ammoniumformate buffer (pH 7.2) and Acetonitirile (55:45 v/v)
Column	: C_{18}, 250 x 4.6mm,5µm particle size
Column temperature	: 25°C
Flow rate	: 1.0ml/minute
Load	: 20µl
Run time	: 15 minutes

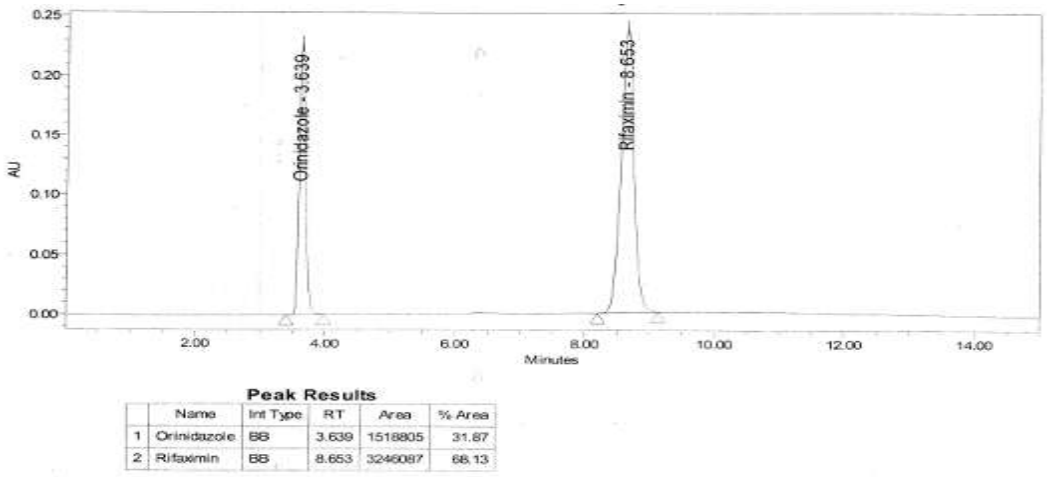
Preparation of solutions

Diluent : Acetonitirile: Water (40:60)v/v

Buffer : The Ammonium formate buffer(pH 7.2) was prepared by mixing 3.16g of Ammonium formate in a 1000ml volumetric flask with HPLC grade water and adding 2 drops of Ammonia.

Standard preparation : RFX stock solution (200µg ml^{-1}) and ONZ stock solution (250µg ml^{-1}) was prepared by accurately weighing 20mg of RFX and 25mg of ONZ in a 100ml volumetric flask and making up to volume with diluent.5ml of the above solution was further diluted to 10ml with diluent.

Method : 20µL of standard solution was injected into the HPLC system.The retention time of ONZ and RFX were found to be 3.6 minutes and 8.6 minutes respectively. The chromatogram showing the retention time of ONZ and RFX is shown in **Fig.3.**



Peak Results

	Name	Int Type	RT	Area	% Area
1	Ornidazole	BB	3.639	1518805	31.87
2	Rifaximin	BB	8.653	3246087	68.13

Fig-3.Chromatogram showing R_t of ONZ and RFX

Quantification of Pharmaceutical Formulation

20 tablets were weighed and powdered.1.3g of powdered drug was weighed accurately, transferred into a 200mL volumetric flask, about 120mL of diluent was added and sonicated for about 10 min. It was allowedto cool to room temperature, diluted with diluent to volume and mixed. The resulting solution was passed through a membrane filter of 0.45-µm pore size. 5 ml of this solution was diluted to 100 ml with diluent. The chromatogram was recorded and the amount of drug was calculated.

Method Validation

Linearity : The stock solution was diluted suitably to get various concentrations.20µL of each solution was injected into the HPLC system and the peak area of the various dilutions recorded. The analytical curve was constructed by by plotting peak area versus concentration.

Limit of Quantification and Limit of Detection : The limit of Quantification(LOQ) and limit of detection(LOD) were based on the standard deviation of the response and the slope of the constructed calibration curve.

Precision : Method Precision of the assay was evaluated by carrying out 6 independent assays of test sample (100 µg ml^{-1} of RFX and 120µg ml^{-1} of ONZ) (n = 3) against a qualified reference standard.The % RSD at three different concentration levels was calculated.The Intermediate Precision study was performed on different days and different instruments and the % RSD was calculated.

Accuracy : Accuracy of the proposed method was checked by carrying out recovery experiments. The accuracy of the method was evaluated in triplicate at three concentration levels (50,100 and 150%) and the percentage recoveries were calculated.

Robustness : The Robustness of the method was established by introducing small changes in the HPLC conditionswhich included Mobile phase ratio,Wavelength, Flow rate,Column temperature and pH.Robustness of the method was studied using six replicates at a concentration level of 100µg ml^{-1} of RFX and 125µg ml^{-1} of ONZ.

Solution Stability : The solution stability of RFX and ONZwas checked for upto48 hrs.The same sample solutions were assayed at 12hrs intervals over the study period.The mobile phase stability was also assessed by assaying the freshly prepared sample solution against freshly prepared reference standard solution at 12 hrs interval upto 48 hrs.The prepared mobile phase remained constant during the study period.

Forced Degradation studies

The study was intended to ensure the effective separation of RFX and ONZ in presence of its degradation products.Force degradation studies were performed to evaluate the stability indicating propertiesof the method.All solutions for use in stress studies were prepared at a final concentration of 100µg ml^{-1} RFX and 125µg ml^{-1}ONZ.

Acid degradation : Acid decomposition was carried out in 0.1M HCl at a concentration of 100µg ml^{-1} RFX and 125µg ml^{-1} ONZ at 50°C. The stressed sample was cooled, neutralized and diluted with diluent.

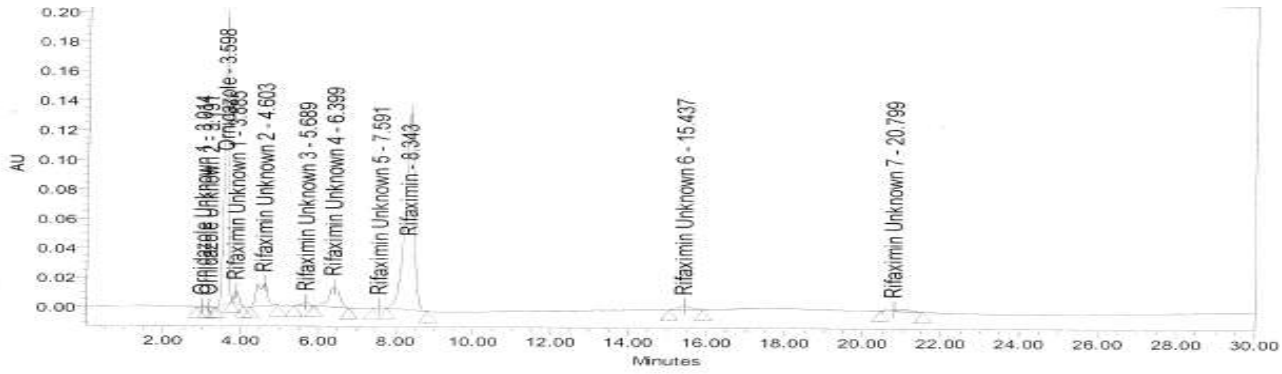
Alkali degradation : Alkaline decomposition was carried out in 0.1M NaOH at a concentration of 100µg ml^{-1} RFX and 125µg ml^{-1} ONZ at 50°C. The stressed sample was cooled, neutralized and diluted with diluent.

Oxidation : An oxidative stress study wascarried out using 3% H_2O_2 at a concentration of 100µg ml^{-1} RFX and 125µg ml^{-1}ONZ. The sample solution was cooled and diluted with the diluent.

Thermal Degradation : Thermal stress testing was done by heatingthe drug solution(100µg ml^{-1} RFX and 125µg ml^{-1} ONZ) in thermostat at 50°C for 15 minutes.

Photolytic Degradation : The drug solution (100µg ml^{-1} RFX and 125µg ml^{-1} ONZ) was exposed to UV light (365nm) for 15 minutes.

The degradation behavior of the selected combined dosage form in acid condition is shown in **Fig.4.**



Peak Results

	Name	Int Type	RT	Area	% Area
1	Ornidazole Unknown 1	BB	3.014	3204	0.07
2	Ornidazole Unknown 2	BB	3.191	3492	0.08
3	Ornidazole	BB	3.598	1428942	32.61
4	Rifaximin Unknown 1	BB	3.885	71734	1.64
5	Rifaximin Unknown 2	BB	4.603	282107	6.44
6	Rifaximin Unknown 3	BB	5.689	26589	0.61
7	Rifaximin Unknown 4	BB	6.399	266834	6.09
8	Rifaximin Unknown 5	BB	7.591	15482	0.35
9	Rifaximin	BB	8.343	2173904	49.62
10	Rifaximin Unknown 6	BB	15.437	54518	1.24
11	Rifaximin Unknown 7	BB	20.799	54713	1.25

Fig-4.Chromatogram showing acid degradation of ONZ & RFX

Results and Discussion

1. System Suitability

Acceptance Criteria : The relative standard deviation for the areas of Rifaximin and Ornidazole from replicate injections of standard solution is not more than 2.0%, Tailing factor is not more than 2.0, the column efficiency of Ornidazole and Rifaximin peak is not less than 2500 theoretical plates and Resolution is not less than 5.0. The results of system suitability parameters is shown in table-1.

Table 1. Results of System Suitability Parameters

S. No	Ornidazole			Rifaximin			
	Peak area	**Theoretical plates**	**Tailing factor**	**Peak area**	**Theoretical plates**	**Tailing factor**	**Resolution**
1.	1519260	6847	1.190	3244260	9242	0.999	18.22
2.	1524725	6753	1.207	3246087	9308	0.994	18.36
3.	1518805	6750	1.203	3254698	9265	0.995	18.32
4.	1521828	6969	1.195	3245408	9321	0.995	18.18
5.	1519186	6945	1.193	3249752	9353	0.993	18.22
6.	1524806	7033	1.190	3254465	9331	0.993	18.24
Average	**1521435**	**6883**	**1.196**	**3249112**	**9303**	**0.995**	**18.257**
% RSD	0.18%			0.14%			

Remarks : The system suitability parameters are within the limits.

2. Specificity

Acceptance Criteria : Any peak eluting from the placebo solution should not interfere with the retention time of Ornidazole and Rifaximin. The results of specificity is shown in table-2

Table 2. Results of Specificity

Injection	**Response of the peak with Retention time**	**Influence of placebo**
1.Blank	No peaks observed	-
2.Placebo	No peaks observed	-
3.Standard solution	Peak due to Ornidazole and Rifaximin eluted at a Retention time of 3.636 minutes and 8.650 respectively	-
4.Test solution	One major peak observed at a retention time of 3.631 minutes and 8.655 respectively which corresponds to Ornidazole and Rifaximin peak in standard solution.	No influence of placebo

Remarks: There is no interference of placebo in the analysis of Ornidazole and Rifaximin.

3. Accuracy

Acceptance Criteria : The recovery at various levels is between 98.0% and 102.0% of added value. The RSD for Recovery of triplicate samples at various levels is not more than 2.0%.

The results of accuracy study is furnished in table-3.

Table 3. Results of Accuracy study

	Ornidazole					
Level	**Amount Recovered 'mg'**	**Added Value 'mg'**	**% Recovery**	**Average**	**SD**	**%RSD**
50%	255.00	251.79	101.28	100.63%	0.92	0.91%
50%	250.72	251.79	99.57			
50%	254.37	251.79	101.03			
100%	499.96	499.45	100.10	100.40%	0.44	0.44%
100%	500.37	499.45	100.18			
100%	503.96	499.45	100.90			
150%	746.76	745.08	100.23			

150%	751.20	745.08	100.82	100.39%	0.38	0.38%
150%	745.96	745.08	100.12			
Rifaximin						
50%	202.96	201.24	100.86	100.25%	0.80	0.79%
50%	199.93	201.24	99.35			
50%	202.34	201.24	100.55			
100%	400.31	401.25	99.77	100.29%	0.62	0.61%
100%	401.80	401.25	100.14			
100%	405.14	401.25	100.97			
150%	600.64	599.62	100.17	100.24%	0.37	0.37%
150%	603.39	599.62	100.63			
150%	599.06	599.62	99.91			

Remarks: The recovery at various levels and %RSD for Recovery of triplicate samples at each level passes the acceptance criteria.

4. Precision

4.1 System Precision:

Acceptance Criteria : The relative standard deviation for the areas of Rifaximin and Ornidazole from replicate injections of standard solution is not more than 2.0%, Tailing factor is not more than 2.0, the column efficiency of Ornidazole and Rifaximin peak is not less than 2500 theoretical plates and Resolution is not less than 5.0. The results of system precision is shown in table-4.

Table 4. Results of System Precision

Ornidazole						
Test Parameter	**Test 1**	**Test 2**	**Test 3**	**Test 4**	**Test 5**	**Test 6**
RSD for the peak areas of Ornidazole	0.18%	0.41%	0.87%	0.19%	0.17%	0.19%
Column efficiency (No. of theoretical plates)	6883	6897	6913	6873	6846	6854
Tailing Factor	1.196	1.193	1.191	1.194	1.193	1.193
Rifaximin						
RSD for the peak areas of Rifaximin	0.14%	0.64%	0.78%	0.15%	0.22%	0.25%
Column efficiency (No. of theoretical plates)	9303	9205	9129	9140	9107	9046
Tailing Factor	0.995	0.994	0.994	0.998	0.999	1.002
Resolution	18.257	18.190	18.137	18.128	18.138	18.098

Remarks : The relative standard deviation for the areas of Ornidazole and Rifaximin from replicate injections of standard solution, the column efficiency, tailing factor and Resolution of Ornidazole and Rifaximin peak passes the acceptance criteria.

4.2 Method Precision

Acceptance Criteria : % RSD for the six assay determinations is NMT 2.0%. The results of Method precision is shown in table-5.

Table 5. Results of Method Precision

S.No	Content of Ornidazole mg/tablet	Content of Rifaximin mg/tablet
1	501.19	402.17
2	496.99	397.09
3	501.02	397.83
4	503.13	401.94
5	499.76	396.69
6	504.38	403.79
Average	**501.08**	**399.92**
RSD	**0.52%**	**0.77%**

Remarks: The % RSD is within the limit. Hence the method is precise.

5. Linearity

Acceptance Criteria : The correlation coefficient is not less than 0.995 and y-intercept is not more than ± 2.0%.The results of Linearity is shown in table-6.

Table 6. Results of Linearity

	Ornidazole		Rifaximin	
Sample ID	**Concentration**	**Area**	**Concentration**	**Area**
50% of operating concentration	62.5	761001	50	1621290
80% of operating concentration	100	1198513	80	2546882
100% of operating concentration	125*	1508739	100*	3226184
120% of operating concentration	150	1819900	120	3881900
150% of operating concentration	187.5	2241094	150	4786986

Report : On plotting the concentration against the area obtained, the graph is found to be linear in the range of 50%-150% of the operating concentration. The y-intercept and correlation coefficient are with acceptable limits.

6. Intermediate Precision

Analyst, Instrument, Laboratories and Day variability

Acceptance Criteria : The % RSD for the 6 assay values is NMT 2.0%. The overall % RSD for the two sets (Intermediate Precision and Precision) is NMT 2.0%.The results of Intermediate Precision is shown in table-7.

Table 7. Results of Intermediate Precision

S.No	Ornidazole 500mg mg/tablet			Rifaximin 400 mg mg/tablet		
	Inter day precision results	Intra day Precision results	Reproducibility	Inter day precision results	Intra day Precision results	Reproducibility
1	498.33	501.19	499.65	406.35	402.17	400.25
2	501.18	496.99	500.21	402.81	397.09	401.32
3	495.06	501.02	498.67	400.42	397.83	399.85
4	500.62	503.13	501.46	402.52	401.94	400.19
5	497.00	499.76	500.21	399.34	396.69	400.65
6	501.32	504.38	500.67	403.03	403.79	400.31
Avg	**500.49**	**501.08**	**500.15**	**402.41**	**399.92**	**400.43**
RSD NMT 2.0%	**0.52%**	**0.52%**	**0.19%**	**0.60%**	**0.77%**	**0.13%**
Overall RSD NMT 2.0%	**0.45%**			**0.60%**		

Remarks : Relative standard deviation between the assay values and overall Relative standard deviation between the two sets are within acceptable limits.

7. Robustness

Variations made in flow rate, mobile phase composition, wavelength, Column Temperature and pH of buffer.The results of Robustness is shown in table-8and 9.

Table 8. Results of Robustness (Ornidazole)

Parameters	Variation		RSD NMT 2.0%	Tailing Factor NMT 2.0	Column Efficiency NLT 2500 theoretical plates
Actual chromatographic conditions			0.18%	1.196	6883
Flow rate	Plus	1.2 mL/min	0.27%	1.138	5637
	Minus	0.8 mL/min	0.24%	1.228	7345
Mobile phase composition	Decrease in buffer	Buffer: Acetonitrile 43:57	0.34%	1.163	5884
	Increase in buffer	Buffer: Acetonitrile 47:53	0.24%	1.199	6472
Wavelength	Lower	273 nm	0.52%	1.183	6291
	Higher	277 nm	0.52%	1.182	6296
pH of buffer	Decrease	7.0	0.10%	1.058	3491
	Increase	7.4	0.53%	1.253	3575
Column Temperature	Decrease	23°C	0.72%	1.179	6291
	Increase	27°C	0.22%	1.181	6444

Table 9. Results of Robustness (Rifaximin)

Parameters	**Variation**		**RSD NMT 2.0%**	**Tailing Factor NMT 2.0**	**Resolution NLT 5.0**	**Column Efficiency NLT 2500 theoretical plates**
Actual chromatographic conditions			0.14%	0.995	18.257	9303
Flow rate	Plus	1.2 mL/min	0.31%	0.986	16.77	7274
	Minus	0.8 mL/min	0.31%	0.982	18.62	7345
Mobile phase composition	Decrease in buffer	Buffer: Acetonitrile 43:57	0.31%	0.989	16.94	7453
	Increase in buffer	Buffer: Acetonitrile 47:53	0.33%	0.986	18.05	8501
Wavelength	Lower	273 nm	0.44%	0.978	17.78	8101
	Higher	277 nm	0.40%	0.978	17.79	8107
pH of buffer	Decrease	7.0	0.23%	0.929	13.10	4126
	Increase	7.4	1.45%	1.111	9.20	3575
Column Temperature	Decrease	23°C	0.60%	0.964	17.74	8052
	Increase	27°C	0.20%	0.984	17.92	8274

Remarks : The system suitability parameters pass the acceptance criteria in all the above conditions. Based on the above results, it is concluded that the method is unaffected by small, deliberate variations in flow rate, mobile phase composition, wavelength, column temperature and pH of buffer.

8. Solution stability:

Acceptance Criteria : The deviation in area from the initial value is NMT 2.0%. The results of Solution stability is shown in table-10.

Table 10. Results of Solution stability

S.No	**Time (Hour)**	**Area response of Ornidazole**		**Area response of Rifaximin**	
		Standard solution % Deviation from the initial area	**Test solution % Deviation from the initial area**	**Standard solution % Deviation from the initial area**	**Test solution % Deviation from the initial area**
1.	Initial	-	-	-	-
2.	After 24 hours	0.67%	0.24%	0.72%	0.07%
3.	After 36 hours	0.72%	1.79%	0.39%	0.35%
4.	After 48 hours	0.71%	0.76%	1.15%	1.19%

Remarks : Since the deviation in area is less than 2% for a period of upto 48 hours in all the solutions, standard and test solutions are said to be stable upto 48 hours.

9. Filter integrity:

Acceptance Criteria : The deviation in area of the filtered samples from the membrane filtered sample of 0.45-µm pore size is NMT 2.0%. The results of Filter integrity is shown in table-11.

Table 11. Results of Filter integrity

S.No	Filter used	Deviation in area from Membrane filtered sample (Ornidazole)	Deviation in area from Membrane filtered sample (Rifaximin)
1	Centrifuge	0.91%	0.74%
2	PVDF, 0.45µm (Polyvinylidene fluoride)	0.63%	0.47%
3	Nylon, 0.45 µm	0.82%	0.72%
4	PTFE, 0.45 µm (Polytetrafluoroethylene)	0.71%	0.37%

Remarks : Centrifuge, PVDF 0.45µm (Polyvinylidene fluoride), Nylon 0.45 µm and PTFE 0.45µm (Polytetrafluoroethylene) are suitable for filtering the sample solutions.

10. Limit of detection and Limit of quantification

The results of Limit of detection and Limit of quantization is shown in table-12.

Table 12. Results of LOD and LOQ

S.No	Parameters	Ornidazole	Rifaximin
1.	LOD	0.179	0.01
2.	LOQ	0.543	0.03

Conclusion :

On evaluating the various parameters it is concluded that the results obtained meet the pre-established acceptance criteria. Hence the method adopted for the assay of Ornidazole and Rifaximin Tablets is validated and can be used for routine analysis and stability studies.

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