1 INTRODUCTION:

Rifaximin, is a structural analogue of rifampicin. Chemically, it is a 2S,16Z,18E,20S,21S,22R, 23R,24R,25S,26S,27S,28E-5,6,21,23,25 pentahydroxy27-methoxy-2,4,11,16,20,22,24,26 octa- methyl -2,7-epoxypentadeca-[1,11,13] trienimino) benzofuro [4,5-e] pyrido [1,2 benzimidazole 1,15(2H)-dione,25-acetate [1]. The empirical formula is $C_{43}H_{51}N_3O_{11}$ and its molecular weight is 785.9. The chemical structure is as shown below:

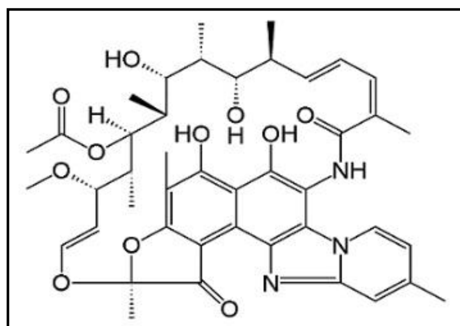


Figure 1: Structure of Rifaximin

Rifaximin is used as an antibiotic for the treatment of traveler's diarrhea[2-3] and heptic encdphalopathy[4] but not used for the systemic bacterial infection and for children below age of 12 years [5-7]. It is further developed for the treatment of hepatic encephalopathy [8] and crohn's disease [9]. It seems to have minimal impact on the normal intestinal microflora, so is being studied in a wide range of infective bowel disease such as *clostridium difficile* infection and diverticulitis. However it has been associated with cases of *clostridium difficile* in patients with risk of hepatic encephalopathy[10]. There are preliminary reports of its use in restless legs syndrome[11] and irritable bowel syndrome[12]. Rifaximin binds to the beta-subunit of bacterial DNA-dependent RNA polymerase and prevents catalysis of polymerization of deoxyribonucleotides into a DNA strand, thereby inhibiting bacterial RNA synthesis. In vitro studiesof rifaximin have demonstrated broad-spectrum coverage including gram-positive, gram-negative, and anaerobic bacteria as well as a limited risk of bacterial resistance [13]. Analysis of rifaximin is available in literature by different analytical and bioanalytical technique like spectrophotometric [14].

HPLC [15-17], HPLC-TMS [18-19], determination of rifaximin by LC-ESI-MS in human plasma [20] were reported in literature.

Pharmacokinetic[21]:

Absorption:

The mean pharmacokinetic parameters of rifaximin in 14 healthy subjects after a single oral 400-mg dose given as 2 x 200 mg doses under fed and fasting conditions. Rifaximin can be administered with or without food. Systemic absorption of rifaximin was low in both the fasting state and when administered within 30 minutes of a high-fat breakfast. 4C-Rifaximin was administered as a single dose to 4 healthy male subjects. The mean overall recovery of radioactivity in the urine and feces of 3 subjects during the 168 hours after administration was 96.94 ± 5.64% of the dose. Radioactivity was excreted almost exclusively in the feces (96.62 ± 5.67% of the dose), with only a small proportion of the dose (mean 0.32% of the dose) excreted in urine. Analysis of fecal extracts indicated that rifaximin was being excreted as unchanged drug. The amount of radioactivity in urine (<0.4% of the dose) suggests that rifaximin is poorly absorbed from the gastrointestinal tract and is almost exclusively and completely excreted in feces as unchanged drug. Mean rifaximin pharmacokinetic parameters were Cmax 4.3 ± 2.8 ng/mL and AUCt 19.5 ± 16.5 ng•h/mL with a median Tmax of 1.25 hours.

Systemic absorption of rifaximin (200 mg three times daily) was also evaluated in 13 subjects
with shigellosis on Days 1 and 3 of a three-day course of treatment. Rifaximin concentrations
and exposures were low and variable. There was no evidence of accumulation of rifaximin following repeated administration for 3 days (9 doses). Peak plasma rifaximin concentrations after 3 and 9 consecutive doses ranged from 0.81 to 3.4 ng/mL on Day 1 and 0.68 to 2.26 ng/mL on Day 3. Similarly, AUC0-last estimates were 6.95 ± 5.15 ng•h/mL on Day 1 and 7.83 ± 4.94 ng•h/mL on Day 3. Rifaximin is not suitable for treating systemic bacterial infections because less than 0.4% of the drug is absorbed after oral administration.

Distribution:

Animal pharmacokinetic studies have demonstrated that 80% to 90% of orally administered rifaximin is concentrated in the gut with less than 0.2% in the liver and kidney, and less than 0.01% in other tissues. In adults with infectious diarrhea treated with rifaximin 800 mg daily for three days, concentrations of rifaximin in stools averaged ~8000 µg/g the day after treatment ended.

Metabolism:

In vitro drug interactions studies have shown that rifaximin, at concentrations ranging from 2 to 200 ng/mL, did not inhibit human hepatic cytochrome P450 isoenzymes: 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4. In an *in vitro* hepatocyte induction model, rifaximin was shown to induce cytochrome P450 3A4 (CYP3A4), an isoenzyme which rifampin is known to induce. Two clinical drug-drug interaction studies using midazolam and an oral contraceptive containing ethinyl estradiol and norgestimate demonstrated that rifaximin did not alter the pharmacokinetics of these drugs.

Excretion:

Rifaximin is excreted primarily in the feces. After oral administration of 400 mg 14C-rifaximin to healthy volunteers, approximately 97% of the dose was recovered in feces, almost entirely as unchanged drug, and 0.32% was recovered in the urine.

2. LITERATURE REVIEW:

The literature review shows that various analytical methods were reported for its determination as API, pharmaceutical formulation. Brief details for the same are as under.

N..Bidyut, P.Amrutansu and M.M. Annapurna described Two methods, method A involving a visible spectroscopic method in double distilled water and method B involving a first derivative visible method in methanol. Analysis was performed at 437 nm and 474 nm for method A and B, respectively. Linearity ranges were found as 1 - 200μg $ml^{-1}$for method A and 2 - 100 μg ml^{-1} for method B. Developed methods were validated and showed good precision and accuracy. The proposed methods were successfully applied to the assay of rifaximin in pure and tablet dosage form. No interference was found from tablet excipients at the selected wavelengths and assay conditions.

T.Sudha,P.V.Hemalatha,V.R.Ravikumar,R.Jothi,and M. Radhakrishnan, described development and validation of RP-HPLC method for the estimation of rifaximin in bulk and in tablet dosage form. Sample was resolved on a Luna Phenomenax, C_{18} (150mm X 4.6 mm i.d., particle size 5μ) column. The mobile phase consisted of methanol l:10 mM phosphate buffer (70:30 v/v pH adjusted to 3.0 by using orthrophosphoric acid) was delivered at a flow rate of 1.2 ml/min at ambient temperature and the retention time was about 5.12 minutes. Studies were performed on an HPLC system equipped with a

UV/Visible detector at 293nm. The method is specific to Rifaximin and able to resolve the drug peak from formulation excipients. The calibration curve was linear over the concentration range of 5-30 µg/ml (R=0.9996).The results of analysis of formulation was found to be 100.31±0.5737. The lower limits detection for Rifaximin was found to be 0.0417 µg/ml and the quantification limit was about 0.1266µg/ml. The proposed method is applicable to routine analysis of Rifaximin in bulk and in tablet dosage form.

R. N. Rao, D. D. Shinde, and S. B. Agawane described Rapid determination of rifaximin in rat serum and urine by direct injection on to a shielded hydrophobic stationary phase by HPLC. Separation of rifaximin from biological matrix was achieved by direct injection of rat serum and urine onto a restricted-access medium, Supelco LC-Hisep, a shielded hydrophobic stationary phase, using acetonitrile:water:acetic acid (18:82:0.1 v/v/v) as a mobile phase. The linear range was 0.10–20 µg/mL ($r^2 > 0.999$, $n = 6$), intraday and interday variation was <6.10%. The limits of detection and quantification were 0.03 (signal-to-noise ratio >3) and 0.10 µg/mL (signal-to-noise ratio >10), respectively. The method was successfully applied to pharmacokinetic studies of rifaximin after an oral administration to rats.

K.N. Rao, S. Ganapaty and A.L. Rao described RP-HPLC determination of rifaximin in bulk drug and pharmaceutical formulation. The method was carried out using Chromosil Symmetry C18 (150 x 4.6 mm I.D., 5 m particle size) column and mobile phase comprised of phosphate buffer pH 4.0 and acetonitrile in proportion of ratio 40:60 v/v and degassed in ultrasonic water bath. The flow rate was 1.0 mL/min and the detection wavelength was at 292 nm. The linearity was observed in the range of 10-60 µg/mL with a correlation coefficient of 0.999. The retention time of Rifaximin was 2.963 min. The method was validated as per the ICH guidelines for its linearity, precision, accuracy, specificity, limit of detection, limit of quantitation and by performing recovery studies. The percentage recovery of the drug Rifaximin was 100.6% to 101.4% from the tablet formulation. The proposed method is suitable for the routine quality control analysis for the estimation of Rifaximin in bulk and pharmaceutical dosage form.

X. Zhang, J. Duan, K. Li, L. Zhou, and S. Zhai described sensitive quantification of rifaximin in human plasma by liquid chromatography-tandem mass spectrometry. Chromatographic separation was obtained on a RESTEK Pinnacle C18 column (50 mm × 2.1 mm, 5 µm) with a mobile phase consisted of ammonium acetate solution

(15 mM, pH 4.32) as buffer A and methanol as mobile phase B. Quantification was performed in positive mode using multiple reaction monitoring (MRM) of the transitions *m*/*z* 786.1 754.1 for rifaximin and *m*/*z* 268.3 116.1 for the IS. The assay has been validated over the concentration range of 0.5–10 ng/ml ($r = 0.9992$) based on the analysis of 0.2 ml of plasma. The assay accuracy was between 98.2% and 109%. The within-day and between-day precision was better than 3.9% and 8.9% at three concentration levels. The freeze–thaw stability was also investigated and it was found that both rifaximin and the IS were quite stable. This method provides a rapid, sensitive, specific and robust tool for the quantitative determination of rifaximin in human plasma, which is especially useful for the pharmacokinetic study of rifaximin.

B. R. Challa, M. R. Kotaiah, and B. R. Chandu developed a tandem mass spectrometry detection method for the determination of rifaximin in human plasma. He used 10 mM ammonium formate (pH 4.0) and acetonitrile (20:80) as a mobile phase on zorbax SB C18, 4.6*75mm, 3.5 µm column at 0.3 mL/min flow rate . Rifaximin and rifaximin D6 were detected with proton adducts at m/z 786.4 754.4 and 792.5 760.5 in multiple reaction monitoring positive mode respectively.

3. AIM OF PRESENT WORK

The above literature review reveals that there were many methods for the quantitative analysis of rifaximin as a drug saubstance as well as pharmaceutical dosage form, few methods are there which deals with bionalytical study and stability study. The aim of present work is to develop a shortest and optimized method for quantitative analysis of rifaximin and stability testing as well as for routine analysis. The aim and scope of the proposed work are as under.

3.1. To develop rapid RP-HPLC method for quantification of the drug substance with highest selectivity, precision and accuracy.

3.2. Forced Degradation Study to confirm the stability of the drug substance.

3.3. Perform analytical method validation for the proposed method as per ICH guideline [22].

4. METHOD DEVELOPMENT

4.1 Mobile phase and column selection:

The chromatographic method for the determination of rifaximin is finalized after Many experimental trials. During experimental trails different column and organic solvent (i.e. methanol, acetonitrile etc.) used and at last accepted result obtained using following chromatographic condition.

Chromatographic condition:

- Buffer : 0.1 % Trifluoro aceticacid in water
- Mobile phase : Buffer:Acetonitrile (40:60)
- Column : Waters symmetry C8(100 x 4.6mm), 5µm
- Temperature : 30°C
- Flow rate : 1mL/min
- Wavelength : 220 nm
- Runtime : 12 minute

4.2 Detection wavelength Selection:

Based on literature and drug information uv analysis performed using methanol as a blank. Uv analysis performed from 200nm to 400nm wavelength. From the spectrum maximum absorbance observed at 220 nm, 234nm and at 293 nm. From the literature review and theoretical information wavelength =220 selected for proposed method .

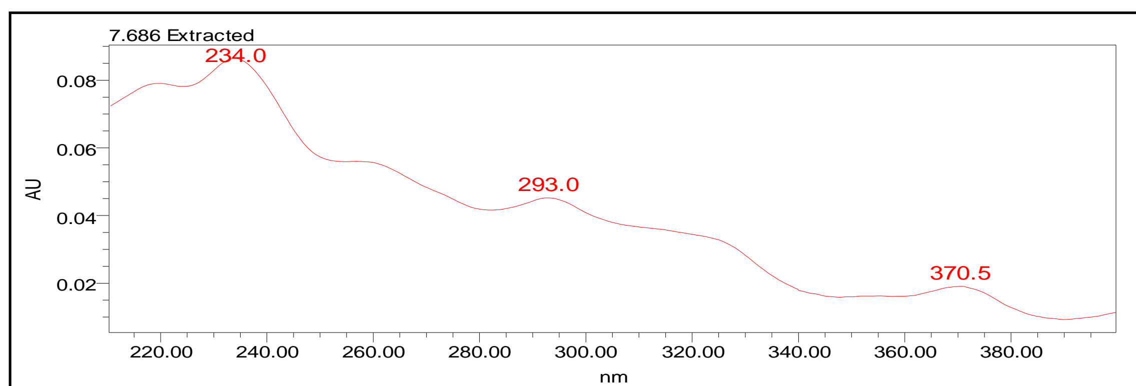


Figure 2:- UV spectrum of rifaximin

5. METHOD VALIDATION:

Chromatographic Conditions:

Instrument	: Waters HPLC with empower software
Column	: waters symmetry C8 (100 x 4.6 mm), 5µm
Flow rate	: 1 mL/min
Mobile phase	: Buffer:Acetonitrile (40:60)
Oven temperature	: 30°C
Wave length	: 220 nm
Injection volume	: 20µL
Run time	: 15 min

Mobile Phase Preparation:

1mL of Trifluoro aceticacid(TFA) added to 1Ltr of HPLC grade water. Mix well and sonicate to degass.

Diluent Preparation:

Water: Methanol (50:50) used as a diluents.

Blank Preparation:

Diluent is used as a blank

Standard Preparation:

To prepare a stock solution (500 µg/mL) for assay analysis, weigh accurately about 50 mg rifaximin reference standard and transfer into 100 mL volumetric flask. Add 70 mL of diluents to dissolve the substance by sonication for one minute and dilute it up to the mark with diluents.

Pipette out 5 mL of above standard stock solution, transfer into 50 mL volumetric flask and dilute it up to the mark with diluent. The concentration obtained is 50 µg/mL of rifaximin.

Test Preparation:

To prepare a stock solution (500 µg/mL) for assay analysis, weigh accurately about 50 mg rifaximin sample and transfer into 100 mL volumetric flask. Add 70 mL of diluents to dissolve the substance by sonication for one minute and dilute it up to the mark with diluents.

Pipette out 5 mL of above standard stock solution, transfer into 50 mL volumetric flask and dilute it up to the mark with diluent. The concentration obtained is 50 µg/mL of rifaximin.

Procedure:

Inject blank followed by five replicated injection of standard preparation. Asymmetry of first injection of standard should not more than 2.0. Related standard deviation of replicate standard preparation should not more than 2.0 %. If system suitability pass than make duplicate injection of Sample preparation.

5.1 Specificity Study:

The evaluation of the specificity of the method was determined against diluent and stress (forced) degradation. The specificity of the method toward the drug was established by means of the interference of the blank preparation and degradation products against drug during the forced degradation study.

Blank preparation:

Diluent is used as a blank.

Standard preparation:

To prepare a stock solution (500 µg/mL) for assay, weigh accurately about 50 mg rifaximin reference standard and transfer into 100 mL volumetric flask. Add 70 mL of diluents to dissolve the substance by sonication for one minute and dilute it up to the mark with diluents.

Pipette out 5 mL of above standard stock solution, transfer into 50 mL volumetric flask and dilute it up to the mark with diluent. The concentration obtained is 50 µg/mL of rifaximin.

Test preparation:

To prepare a stock solution (500 µg/mL) for assay, weigh accurately about 50 mg rifaximin sample and transfer into 100 mL volumetric flask. Add 70 mL of diluents to dissolve the substance by sonication for one minute and dilute it up to the mark with diluents.

Pipette out 5 mL of above standard stock solution, transfer into 50 mL volumetric flask and dilute it up to the mark with diluent. The concentration obtained is 50 µg/mL of rifaximin.

5.1.1 Forced Degradation Study:

Stress study was carried out by application of chemical and physical forced degradation. To perform forced degradation study, the drug content 50 mg in 100mL volumetric flask was employed for acidic, alkaline and oxidant media and also for thermal and photolytic conditions. After the degradation treatments were completed, the stress content were allowed to equilibrate to room temperature and diluted with diluent to attain 500 µg/ml concentrations, further 5 mL sample solution diluted to 50mL to attained 50 µg/ml concentration of rifaximin. Pattern of stress (degradation) conditions and preparation for same was described as under:

Acid Degradation:

Acidic degradation study was performed by heating the drug content in 30 ml of 1 N HCl at about 80° C for 1 hour and after cooling to room temperature it was neutralized with 1 N NaOH solution. Further solution was diluted to achieve concentrations 50 µg/ml with diluent.

***Alkali* Degradation:**

Alkaline degradation was performed by heating the drug content in 1 M NaOH at around 80˚ C for 1 hour and then the mixture was neutralized with 1 M HCl. It was further diluted with diluent to achieve 50 µg/ml concentrations.

Oxidative Degradation Study:

Oxidative degradation study was performed at room temperature for by adding 3% H_2O_2 in the drug content for 30 min then diluted to 50µg/ml with diluent.

Thermal Degradation Study:

Thermal degradation study was performing by keeping powdered drug content at around 80˚C for 1 hour. After this it was allowed to come at room temperature.

***Photolytic Degradation Study*:**

Photolytic degradation study was performed by exposing drug content in sun-light for 48 hour, further it diluted to 50 µg/ml using diluent.

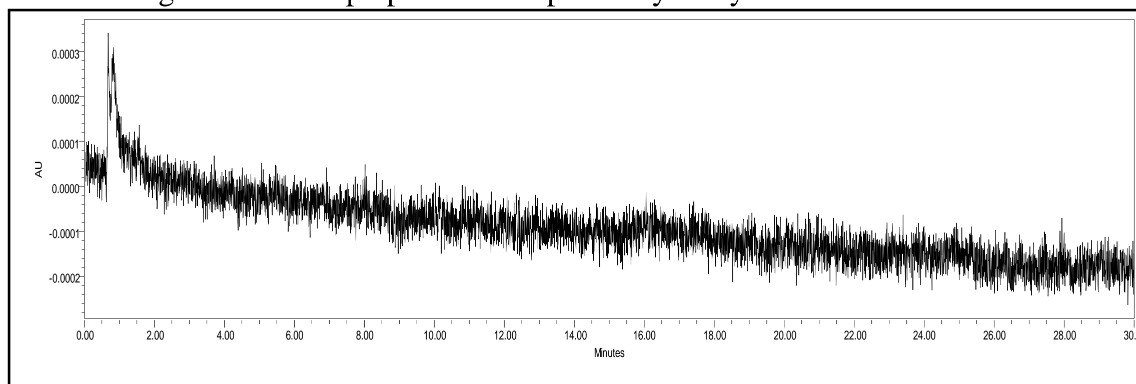
Blank preparation:

Blank preparation was also performed for all degradation to identify the peaks which arise due to blank. Blank was subjected under same all stress condition as that of sample.

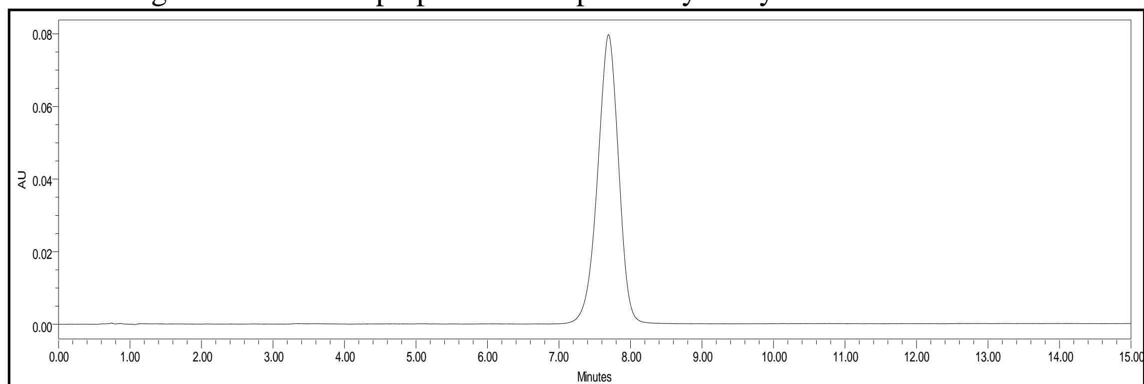
Table-1: Chromatographic sequence for Specificity study is represented through

No.	Description	Injection Replicate	Remarks
1	Blank	1	As such
2	Standard Preparation	5	
3	Test Preparation	2	
4	Bracketing Standard	1	
5	Blank preparation of acidic stress	1	Acidic Forced Degradation
6	Test preparation of acidic stress	2	
7	Bracketing Standard	1	
8	Blank preparation of alkali stress	1	
9	Test preparation of alkali stress	2	Alkali Forced Degradation
10	Bracketing Standard	1	
11	Blank preparation of oxidative stress	1	
12	Test preparation of oxidative stress	2	
13	Bracketing Standard	1	Oxidative Forced Degradation
14	Blank preparation of thermal stress	1	
15	Test preparation of thermal stress	2	
16	Bracketing Standard	1	
17	Blank preparation of photolytic stress	1	Thermal Forced Degradation
18	Test preparation of photolytic stress	2	
19	Bracketing Standard	1	

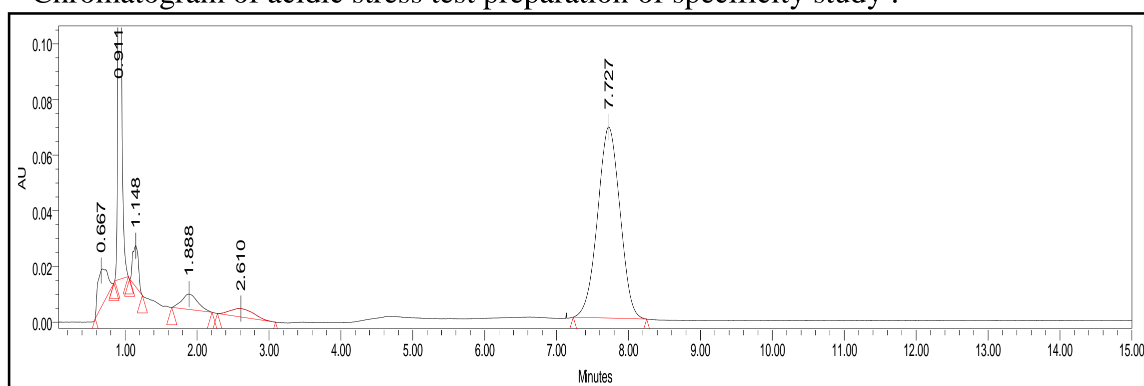
Chromatogram of blank preparation of specificity study:



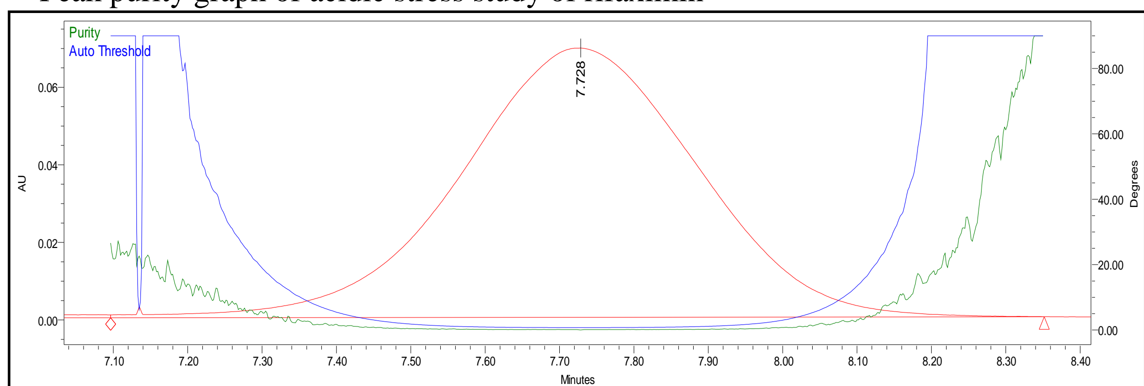
Chromatogram of standard preparation of specificity study:



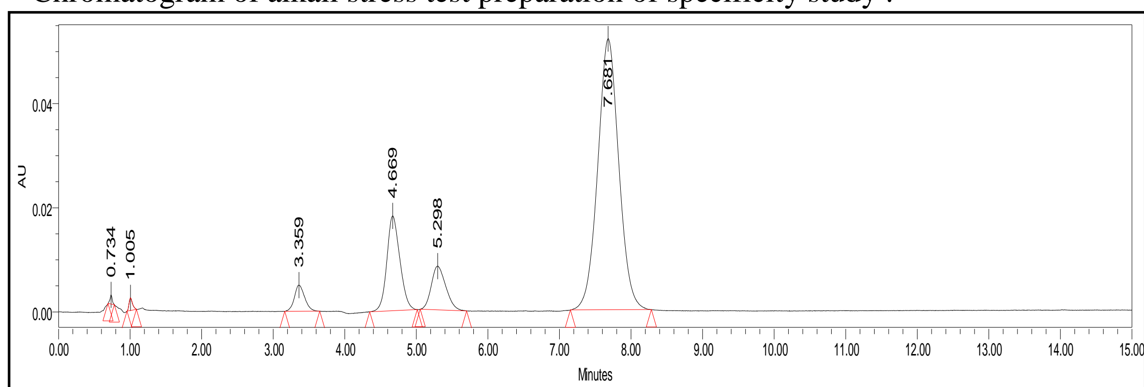
Chromatogram of acidic stress test preparation of specificity study :



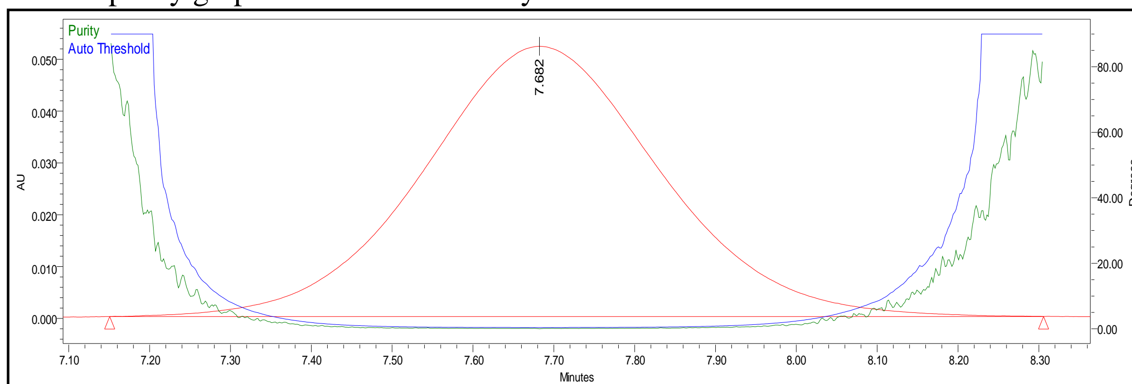
Peak purity graph of acidic stress study of rifaximin



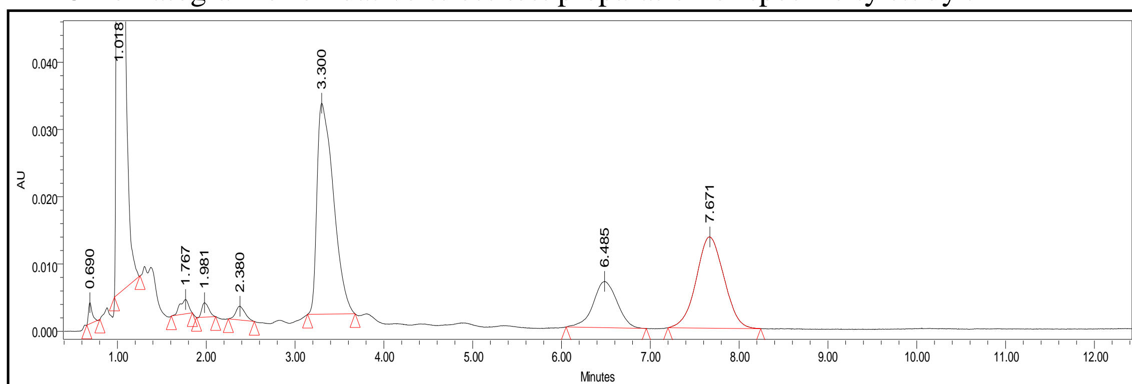
Chromatogram of alkali stress test preparation of specificity study :



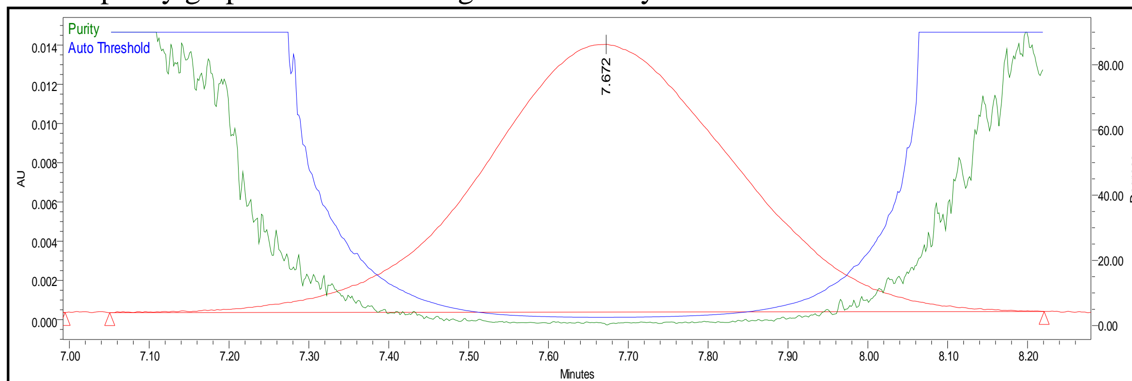
Peak purity graph of alkali stress study of rifaximin



Chromatogram of oxidative stress test preparation of specificity study :



Peak purity graph of oxidative degradation study of rifaximin



Observations:

(1) Any interference was not observed from blank to the peak of interest, in addition to this peak purity was also within the acceptance criteria proved by the photo diode detector.

(2) From the above chromatogram it can be conclude that there is no interference of any degradation product to the peak of interest and impurity has been generated by each stress condition.

Table-2: Degradation result of stress condition

Degradation Condition	Time	Peak Purity		% Degradation
		Purity Threshold	Purity Angle	
Acidic	1 Hrs at 80°C	1.2	0.38	12%
Basic	1Hrs at 80°C	0.59	0.30	27%
Oxidative	30 min at RT	4.27	1.72	82%
Thermal	1 hrs	1.32	0.74	0.4%
Photolytic	48 hrs	0.68	0..39	0.3%

5.2 Linearity and Range:

The linearity plot was prepared with 8 concentration levels (20, 30, 40, 50, 60, 70 and 80 µg/ml of Rifaximin). These concentration levels were respectively corresponding to 40, 60, 80, 100, 120, 140 and 160% of standard solution concentration. The peak areas vs. concentration data were evaluated by linear regression analysis.

Standard solution preparation:

Weigh accurately 50.03mg of rifaximin standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 500.3 µg/ml of Rifaximin.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.03 µg/ml of Rifaximin.

Standard Solution Preparation:

Stock Solution:

Weigh accurately 50.08mg of rifaximin standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 500.8 µg/ml of Rifaximin.

Linearity Level 1 (40%):

2 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 20.03 µg/ml of Rifaximin.

Linearity Level 2 (60%):

3 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 30.05 µg/ml of Rifaximin.

Linearity Level 3 (80%):

4 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 40.06 µg/ml of Rifaximin.

Linearity Level 4 (100%):

5 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 50.08 µg/ml of Rifaximin.

Linearity Level 5 (120%):

6 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 60.10 µg/ml of Rifaximin.

Linearity Level 6 (140%):

7 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 70.11 µg/ml of Rifaximin.

Linearity Level 7 (160%):

8 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 80.13 µg/ml of Rifaximin.

For each linearity level, the solution was injected in duplicate. Linearity was evaluated by linear regression analysis.

Linearity Level 8 (180%):

9 ml of stock solution was pipette out and transferred into 50 ml volumetric flak. The solution was diluted to volume with diluent. The concentration obtained is 90.14 µg/ml of Rifaximin.

For each linearity level, the solution was injected in duplicate. Linearity was evaluated by linear regression analysis.

Table 3: Sequence of Linearity and range study

No.	Description	Injection Replicate
1	Blank	1
2	Standard Preparation	5
3	Linearity level-1 (40%)	2
4	Linearity level-2 (60%)	2
5	Linearity level-3 (80%)	2
6	Linearity level-4 (100%)	2
7	Linearity level-5 (120%)	2
8	Linearity level-6 (140%)	2
9	Linearity level-7 (160%)	2
10	Bracketing Standard	1

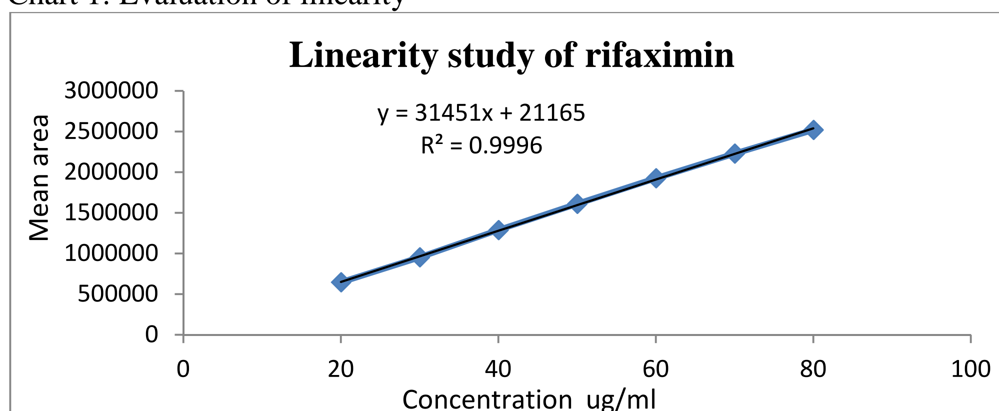
Table 4: Summary of Linearity and range study

Observation				
Data for Standard Preparation				
Replicate	Area		Standard Weight	50.08
1	1601287		Standard Potency	99.27
2	1617662			
3	1612469			
4	1609947			
5	1615378			
Average	1611348.6			
Stdev	6335.43			
%RSD	0.39			
Data for Leniarity Level Preparation				
Linearity Level	Replicate	Area	Mean Area	
Level-1 (40%)	1	642114	643200	
	2	644286		
Level-2 (60%)	1	951803	951796	
	2	951789		
Level-3 (80%)	1	1285223	1286216	
	2	1287209		
Level-4 (100%)	1	1611295	1608504	
	2	1605713		
Level-5 (120%)	1	1924556	1923729	
	2	1922902		
Level-6 (140%)	1	2223966	2225440	
	2	2226914		
Level-7 (160%)	1	2514336	2516992	
	2	2519648		

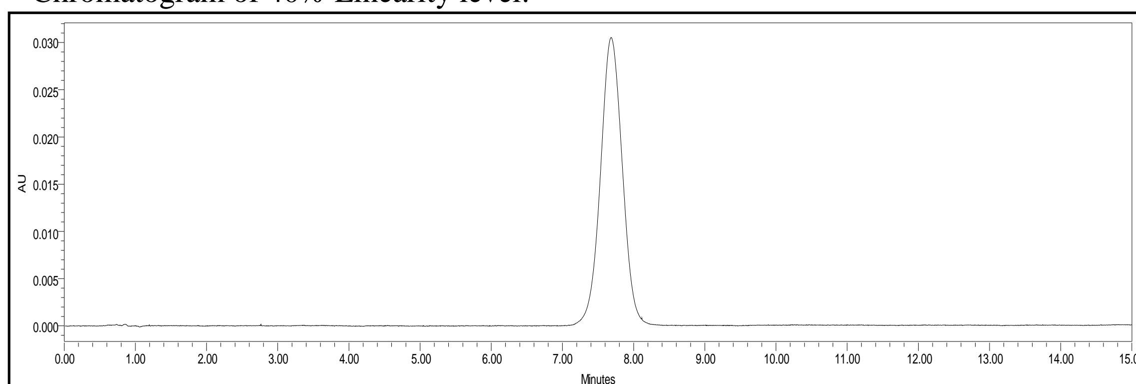
Table-5: Summary of linearity study

Linearity Level	% of Level	Concentration (µg/ml)	Mean Area
1	40	20	643200
2	60	30	951796
3	80	40	1286216
4	100	50	1608504
5	120	60	1923729
6	140	70	2225440
7	160	80	2516992
Correlation Co-efficient			0.999
Slope			31451
Intercept			21165

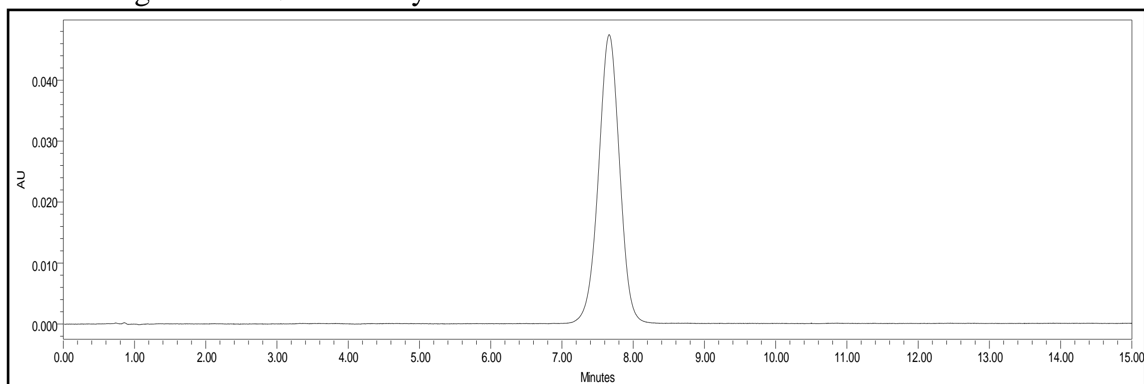
Chart 1: Evaluation of linearity



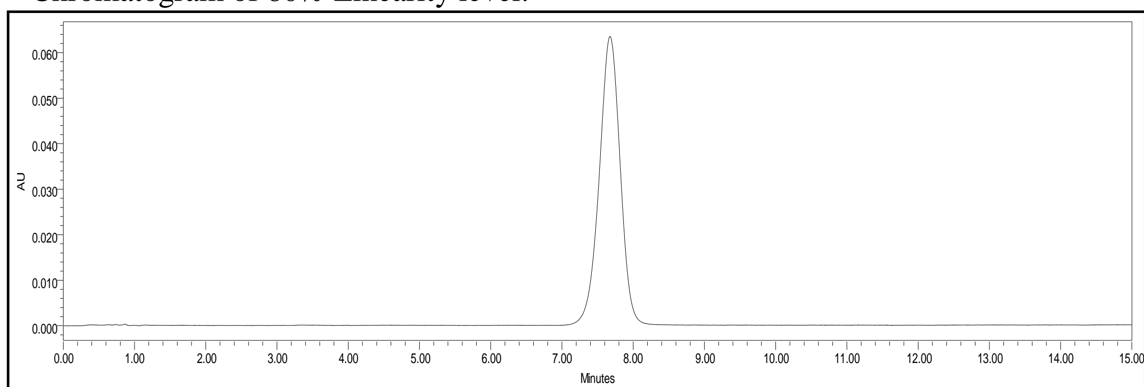
Chromatogram of 40% Linearity level:



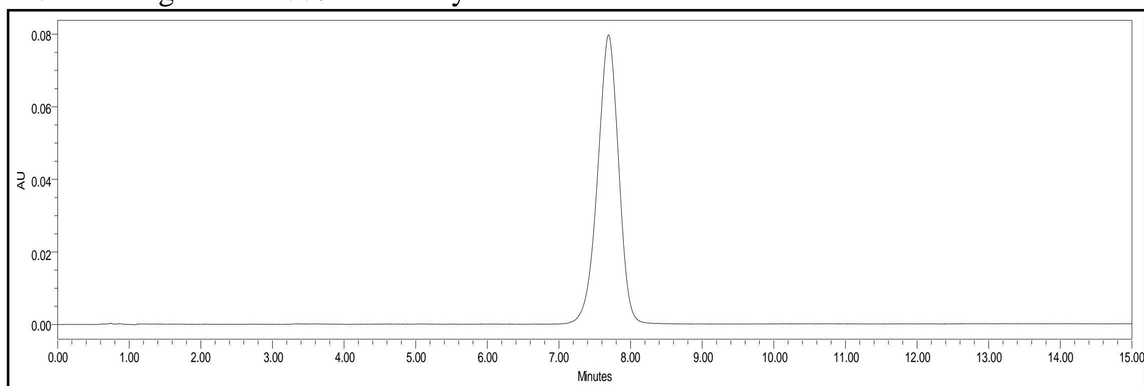
Chromatogram of 60% Linearity level:



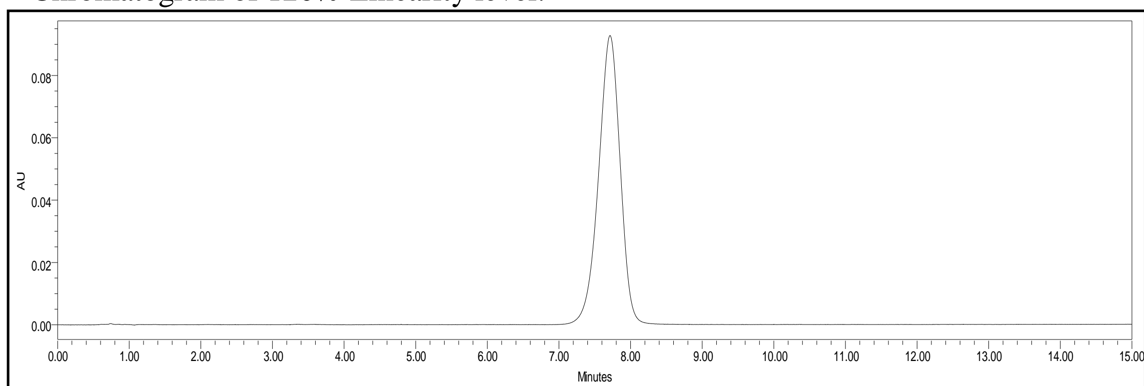
Chromatogram of 80% Linearity level:



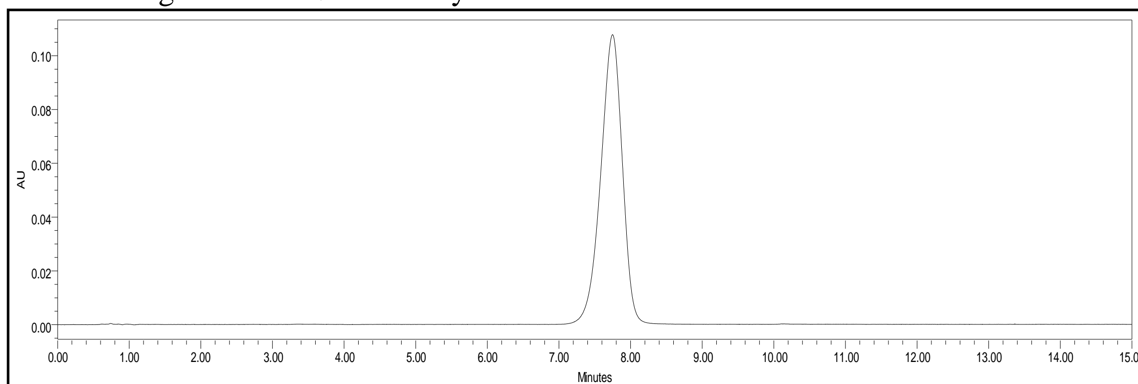
Chromatogram of 100% Linearity level:



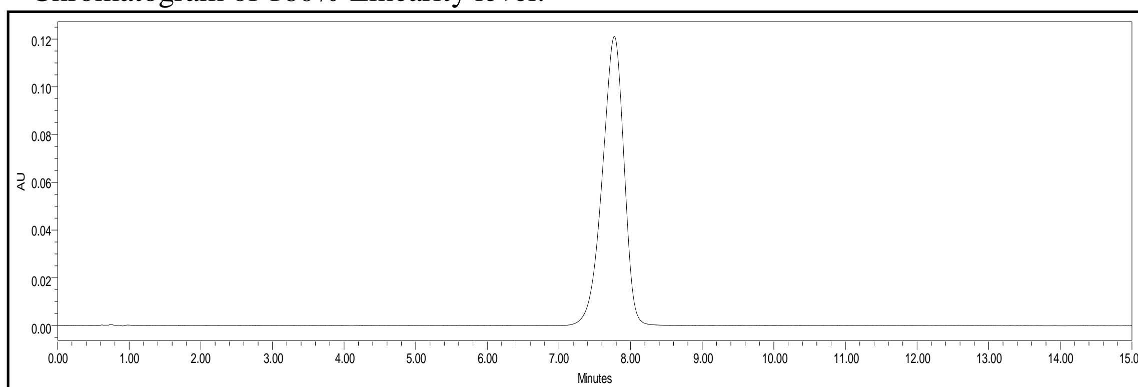
Chromatogram of 120% Linearity level:



Chromatogram of 140% Linearity level:



Chromatogram of 160% Linearity level:



Correlation coefficient of the linearity study was found to $R^2 = 0.999$ with linear regression equation y = 31451x + 21165, which proves the method is highly linear over the working range 20 – 80 µg/ml.

5.3 Limit of detection and Limit of quantification:

LOD is the lowest amount of the drug content which can be detected by the proposed method while LOQ is the lowest amount which can be quantified by the method. The guideline suggest minimum signal to noise ratio (S/N) more than 3.3 for LOD and more than 10 for LOQ. On the basis of linearity data theoretically it can be also calculated by the given formula,

LOD = 3.3 /S LOQ = 10 /S

Where = Residual Standard Deviation of regression line and S = Slope of regression line.

LOQ value is precised by six replicate injections and checked for linear response with respect to other linearity levels by extended linearity curve.

For LOD and LOQ study, blank, standard preparation, LOD preparation and LOQ preparation was prepared as under:

Blank preparation:

Diluent was used as blank.

Standard preparation:

Stock solution: 25.07 mg of Rifaximin reference standard was accurately weighed and transferred into 50 ml volumetric flask. 30 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 501.4 µg/ml of Rifaximin.

LOD and LOQ solution:

Solution-A: 25.00 mg of Rifaximin reference standard was accurately weighed and transferred into 50 ml volumetric flask. 30 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 500 µg/ml of Rifaximin. 5 ml of this solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.0 µg/ml of Rifaximin. 5 ml of this solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 5.0 µg/ml of Rifaximin. This solution is designated as Solution-A.

LOD preparation:

1 ml of above Solution-A was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 0.1 µg/ml of Rifaximin. 5 ml of this solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 0.01 µg/ml of Rifaximin.

LOQ preparation:

3 ml of above Solution-A was pipette out and transferred into 50 ml volumetricflask followed by diluted to volume with diluent. The concentration obtained is 0.3 µg/ml of Rifaximin. 5 ml of this solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 0.03 µg/ml of Rifaximin.

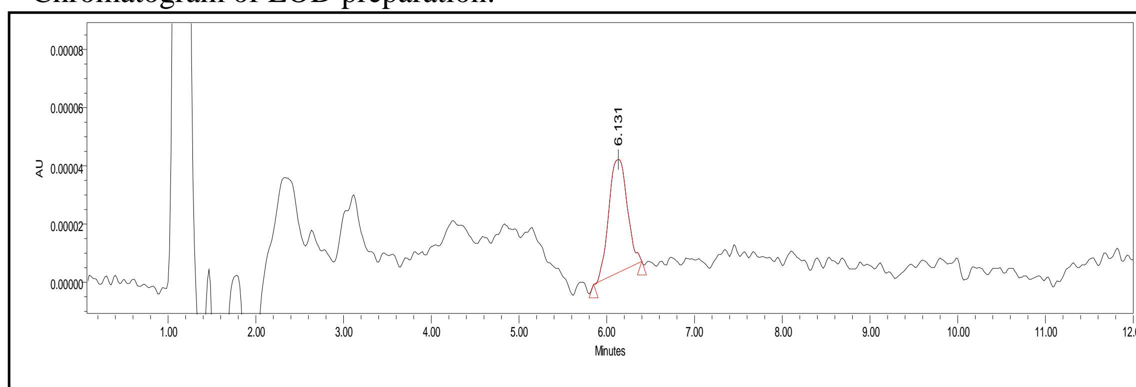
Table 6: Sequence of LOD and LOQ study

No.	Description	Injection Replicate
1	Blank	1
2	Standard Preparation	5
3	Blank	1
4	LOD	2
5	LOQ	6
6	Bracketing Standard	1

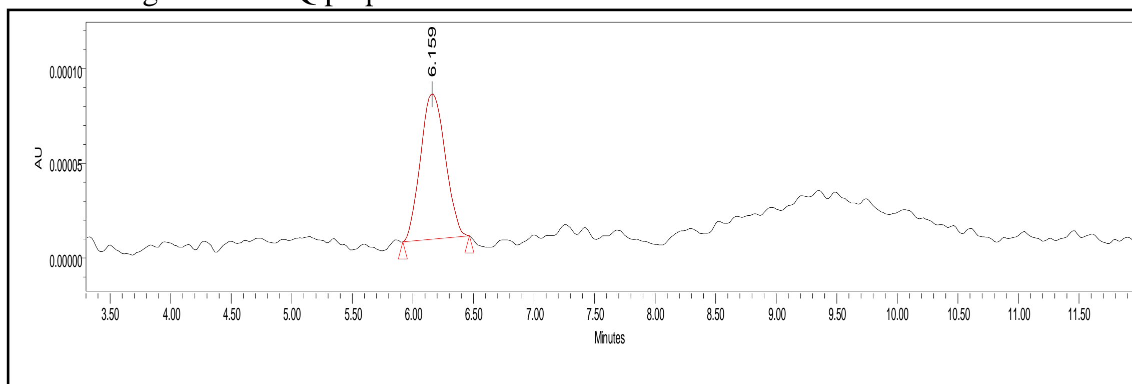
Table 7: Summary of LOQ study

Observation			
Data for Standard Preparation		*Data for LOQ Preparation*	
Replicate	Area	Replicate	Area
1	1601287	1	2837
2	1617662	2	2934
3	1612469	3	2841
4	1609947	4	2908
5	1615378	5	2947
Average	1611348.6	6	3017
Stdev	6335.43	Average	2914
%RSD	0.39	STDEV	68.40
		%RSD	2.35

Chromatogram of LOD preparation:



Chromatogram of LOQ preparation:



LOQ of the analytical method can evaluated by establish linearity up to LOQ value. Hence linearity study is extended to LOQ value,

Chart 2: Confirmation of LOQ value by extended linearity study upto LOQ level

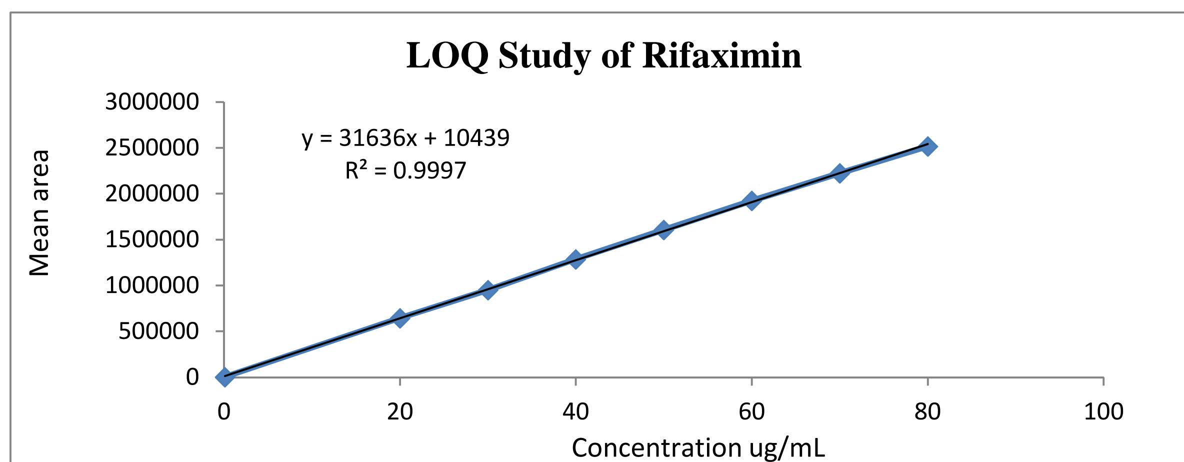


Table 8: Summary of LOQ study by evaluating linearity upto LOQ concentration

Linearity Level	% of Level	Concentration (µg/ml)	Mean Area
1	LOQ	0.09	2914
2	40	20	643200
3	60	30	951796
4	80	40	1286216
5	100	50	1608504
7	120	60	1923729
8	140	70	2225440
9	160	80	2516992
Correlation Co-efficient			0.999
Slope			31636
Intercept			10439

All the results of LOD and LOQ data were within the acceptance criteria, hence it can be conclude that the LOD and LOQ of the method was 0.03 µg/ml and 0.09 µg/ml respectively which correspond to 0.06% and 0.18% of working concentration.

5.4 Precision Study:

Precision study was established by evaluating method precision and intermediate precision study. Method precision of the analytical method was determined by analyzing six sets of Sample preparation. Assay of all six replicate Sample preparations was determined and mean % assay value, standard deviation, % relative standard deviation and 95% confidence interval for the same was calculated.

Intermediate precision of the analytical method was determined by performing method precision on another day by another analyst using different make of raw materials under same experimental condition. Assay of all six replicate Sample preparations was determined and mean % assay value, standard deviation, % relative standard deviation and 95% confidence interval for the same was calculated. Overall assay value of method precision and intermediate precision was compared and % difference and overall % relative standard deviation was calculated.

For method precision, blank, standard preparation and six sets of test preparations was prepared as per method as under:

Blank preparation:

Diluent was used as blank.

Standard preparation:

Stock solution: weigh accurately 50.04 mg of Rifaximin reference standard and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 500.4 µg/ml of Rifaximin.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.04 µg/ml of Rifaximin.

Test preparation:

weigh accurately 50.06 mg of Rifaximin reference standard and transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask and the standard substance was dissolved by sonication for one minute. Then, volume of the flask was make up with diluent. The concentration obtained is 500.6 µg/ml of Rifaximin.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.04 µg/ml of Rifaximin.

The same procedure was used for preparing the six Test preparation Sets. Same approach was applied for the intermediate precision study on the second day with different analyst.

Table-9: Sequence of precision study

No.	Description	Injection Replicate
1	Blank	1
2	Standard Preparation	5
3	Test preparation (Set-1)	2
4	Test preparation (Set-2)	2
5	Test preparation (Set-3)	2
6	Test preparation (Set-4)	2
7	Test preparation (Set-5)	2
8	Test preparation (Set-6)	2
9	Bracketing Standard	1

Table-10: Observation of precision study

Observation:					
Data for Standard preparation					
Replicate	Area		Standard Weight	50.04	
1	1611734		Standard Potency	99.27	
2	1607255				
3	1610021				
4	1607785				
5	1609224				
Average	1609204				
Stdev	1794.50				
%RSD	0.11				
Data for Test preparation					
Set No.	Replicate	Area	Mean Area	Wt. of Sample	% Assay
1	1	1609641	1613584	50.06	99.58
	2	1617527			
2	1	1616883	1614715	50.04	99.61
	2	1612548			
3	1	1612277	1616655	50.08	99.65
	2	1621033			
4	1	1613337	1614719	50.01	99.67
	2	1616102			
5	1	1615311	1613908	50.02	99.6
	2	1612505			
6	1	1618449	1619578	50.05	99.89
	2	1620707			

% Assay calculation is as under:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{100}{W_2} x \frac{50}{5} xP$$

Where,

A_T = Average Area of Test Preparation.

A_S = Average Area of Standard Preparation.

W_1 = Weight of Working Standard (mg).

W_2 = Weight of Test Sample (mg).

P = Potency of Working Standard (%).

Table-11: Summary of Intermediate precision study

Observation				
Data for Standard preparation				
Replicate	Area		Standard Weight	50.03
1	1612299		Standard Potency	99.27
2	1618354			
3	1612928			
4	1612157			
5	1609551			
Average	1613058			
Stdev	3230.77			
%RSD	0.20			

Data for Test preparation

Set No.	Replicate	Area	Mean Area	Wt. of Sample	% Assay
1	1	1626535	1621006	50.12	99.58
	2	1615477			
2	1	1613084	1614675	50.06	99.31
	2	1616267			
3	1	1610119	1614037	50.01	99.37
	2	1617955			
4	1	1642579	1640375	50.11	100.79
	2	1638172			
5	1	1625311	1627357	50.03	100.15
	2	1629403			
6	1	1617212	1618744	50.04	99.6
	2	1620275			

% Assay calculation is as under:

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{100}{W_2} x \frac{50}{5} xP$$

Where,

A_T = Average Area of Test Preparation.

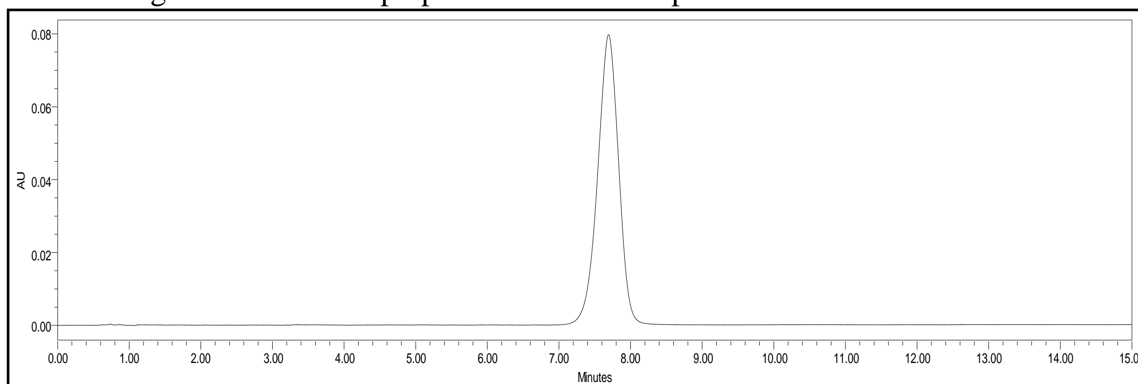
A_S = Average Area of Standard Preparation.

W_1 = Weight of Working Standard (mg).

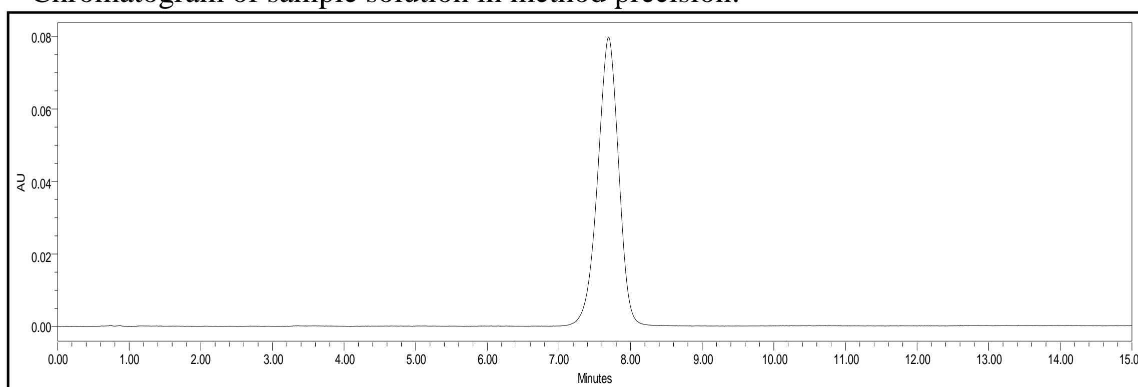
W_2 = Weight of Test Sample (mg).

P = Potency of Working Standard (%).

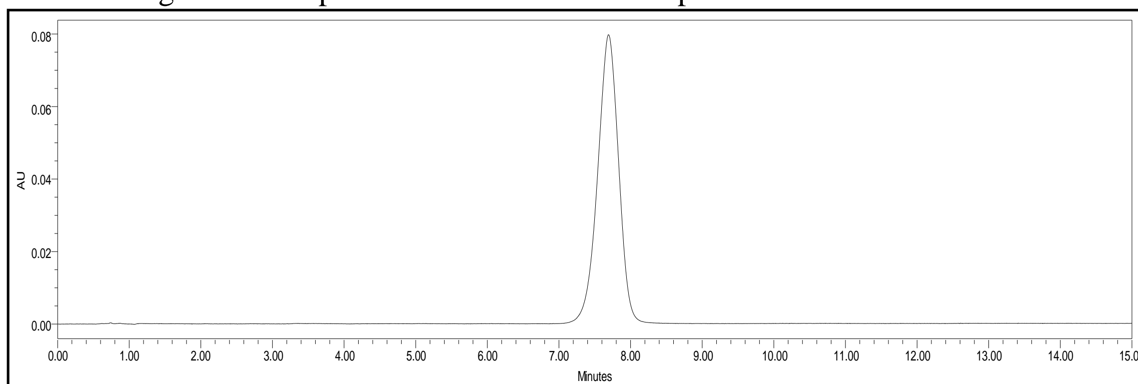
Chromatogram of Standard preparation in method precision:



Chromatogram of sample solution in method precision:



Chromatogram of sample solution in intermediate precision



Overall the data for the precision study suggest % Assay value for each Test Preparation is between 98 – 102% which is under the acceptance criteria while % RSD of all results are less than 2%. Hence from all the observation it can conclude that the proposed method is highly precise.

5.5 Accuracy Study:

This Experiment can be performed by the recovery test. Recovery of the method was evaluated at 3 different concentration levels (Generally corresponding to 50, 100 and 150% of test solution concentration) by addition of known amounts of standard to

placebo preparation. For each concentration level, 3 sets were prepared and injected in duplicate.

Blank preparation:

Diluent was used as blank.

Standard preparation:

Stock solution: Accurately weigh 50.12 mg of Rifaximin reference standard transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask, sonicate to dissolved and diluted up the mark with diluent. The concentration obtained is 501.2 µg/ml of Rifaximin.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.12 µg/ml of Rifaximin.

Accuracy level 1 (50%):

Test stock solution:

Acuurately weigh 25.23 mg of Rifaximin reference standard transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask, sonicate to dissolved and diluted up the mark with diluent. The concentration obtained is 252.3 µg/ml of Rifaximin.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 25.23 µg/ml of Rifaximin. The same procedure was applied for preparing the three sets.

Accuracy level 2 (100 %):

Test stock solution:

Acuurately weigh 50.26 mg of Rifaximin reference standard transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask, sonicate to dissolved and diluted up the mark with diluent. The concentration obtained is 501.4 µg/ml of Rifaximin.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.14 µg/ml of Rifaximin. The same procedure was applied for preparing the three sets.

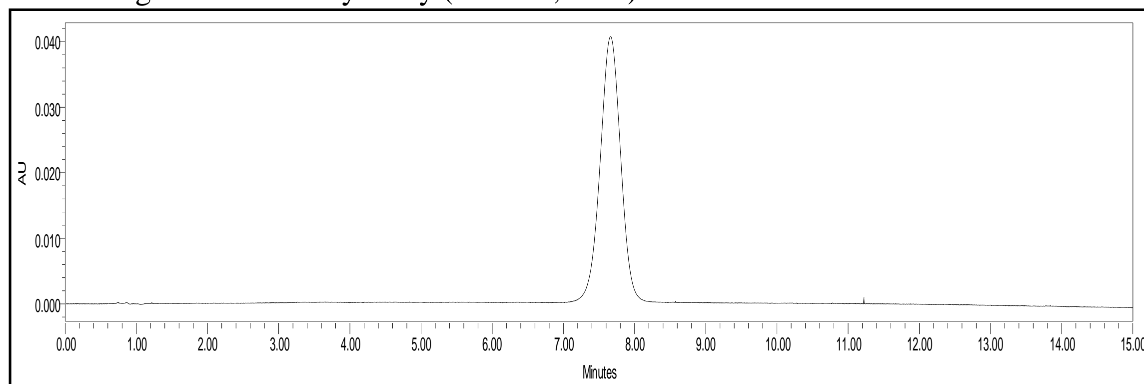
Accuracy level 3 (150 %):

Test stock solution:

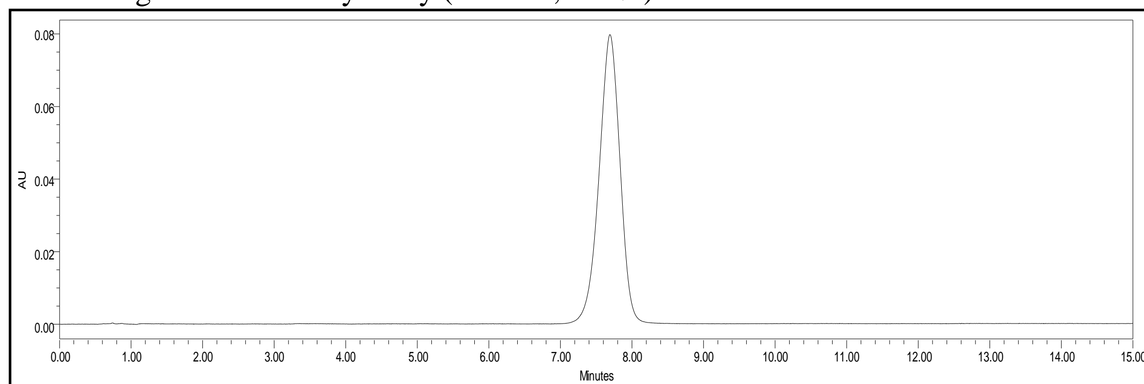
Acuurately weigh 75.07 mg of Rifaximin reference standard transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask, sonicate to dissolved and diluted up the mark with diluent. The concentration obtained is 750.7 µg/ml of Rifaximin.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 75.07 µg/ml of Rifaximin. The same procedure was applied for preparing the three sets.

Chromatogram of accuracy study (Level-1, 50%)



Chromatogram of accuracy study (Level-2, 100%)



Chromatogram of accuracy study (Level-3, 150%)

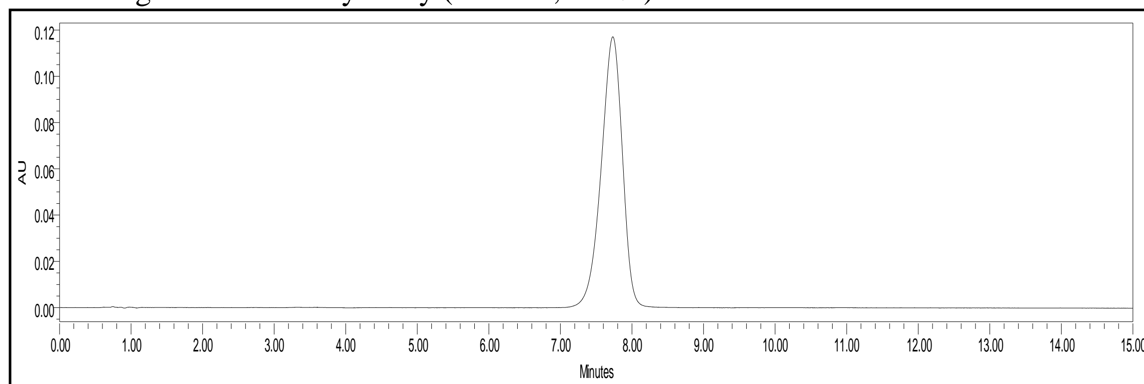


Table-12: Sequence of Accuracy Study

No.	Description	Injection Replicate
1	Blank	1
2	Standard Preparation	5
3	Accuracy level-1 preparation: Set-1	2
4	Accuracy level-1 preparation: Set-2	2
5	Accuracy level-1 preparation: Set-3	2
6	Accuracy level-2 preparation: Set-1	2
7	Accuracy level-2 preparation: Set-2	2
8	Accuracy level-2 preparation: Set-3	2
9	Accuracy level-3 preparation: Set-1	2
10	Accuracy level-3 preparation: Set-2	2
11	Accuracy level-3 preparation: Set-3	2
12	Bracketing Standard	1

Table-13: Observation of accuracy study

Observation

Data for Standard preparation

Replicate	Area			
1	1611487		Standard Weight	50.12
2	1612331		Standard Potency	99.27
3	1612214		Standard Conc.	50.12
4	1618926			
5	1609554			
Average	1612902			
Stdev	3546.10			
%RSD	0.22			

Data for Test preparation

Accuracy Level	Set No	Replicate	Area	Mean Area
1(50%)	1	1	808954	809026
		2	809098	
	2	1	804961	805486
		2	806011	
	3	1	798955	799049
		2	799143	
2(100%)	1	1	1612994	1613546
		2	1614098	
	2	1	1614722	1615798
		2	1616874	
	3	1	1610745	1609684
		2	1608623	
3(150%)	1	1	2397846	2396826
		2	2395806	
	2	1	2426251	2425146
		2	2424041	
	3	1	2444109	2443810
		2	2443511	

Table-14: Summary of accuracy study

Accuracy (Recovery) Study							
Accuracy Level	**Set No**	**Amount added (µg/ml)**	**Amount Found (µg/ml)**	**Recovery (%)**	**Average recovery**	**Std Dev.**	**% RSD**
I (50%)	1	25.23	25.14	99.64	100.04	0.70	0.70
	2	24.82	25.03	100.85			
	3	24.92	24.83	99.64			
II (100%)	1	50.26	50.14	99.76	99.94	0.58	0.58
	2	50.48	50.21	99.47			
	3	49.73	50.02	100.58			
II (150%)	1	74.91	74.48	99.43	100.02	0.57	0.57
	2	75.30	75.36	100.08			
	3	75.52	75.94	100.56			

Calculation formulas for recovery study are as under:

$$\text{Amount added } (\mu g/ml) = \frac{Wt.taken}{Volume\,1} x \frac{Volume\,2}{Volume\,3} x\,1000$$

Where, Volume = Dilution given for preparing the solution.

$$\text{Amount found } (\mu g/ml) = \frac{Mean\ area\ of\ test\ preparation}{Average\ area\ of\ standard\ preparation} x\ standard\ \text{conc.}$$

$$\%\ \text{Recovery} = \frac{Amount\ found}{Amount\ added} x\,100$$

From the all above data it has been proven that the % recovery is within the limit of 98 to 102 % this is in the limit of acceptance criteria and % RSD value of % recovery of replicate set is below 2 % .Hence this suggest that proposed method is highly accurate.

5.6 Robustness Study:

Robustness of the method was evaluated by assaying test solutions under slight but deliberate changes in analytical conditions, such as change in flow rate, change in proportions of Buffer-Acetonitrile (42:58 and 38:62,v/v), Change in temperature and change in column-lot.

5.6.1 Change in flow rate:

Blank preparation:

Diluent was used as blank.

Standard preparation:

Stock solution: Acuurately weigh 50.02 mg of Rifaximin reference standard transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask, sonicate to dissolved and diluted up the mark with diluent. The concentration obtained is 500.2 µg/ml of Rifaximin.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.02 µg/ml of Rifaximin.

Test preparation:

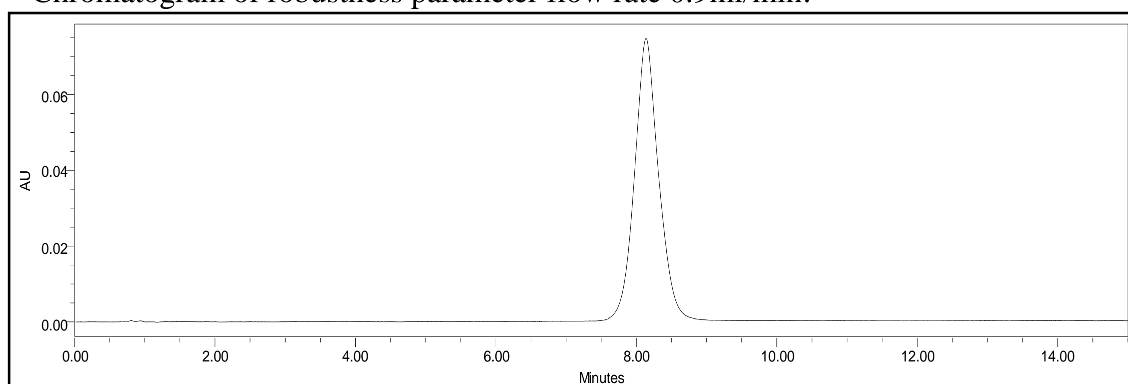
Acuurately weigh 50.04 mg of Rifaximin reference standard transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask, sonicate to dissolved and diluted up the mark with diluent. The concentration obtained is 500.4 µg/ml of Rifaximin.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.04 µg/ml of Rifaximin.

Table-15: Sequence for flow rate robustness study

No.	Description	Injection Replicate	Chromatographic Parameter
1	Blank	1	Flow rate: 0.9 mL/min
2	Standard Preparation	5	
3	Test Preparation	2	
4	Bracketing Standard	1	
5	Blank	1	Flow rate: 1.1 mL/min
6	Standard Preparation	5	
7	Test Preparation	2	
8	Bracketing Standard	1	

Chromatogram of robustness parameter flow rate 0.9ml/min:



Chromatogram of robustness parameter flow rate 1.1 ml/min:

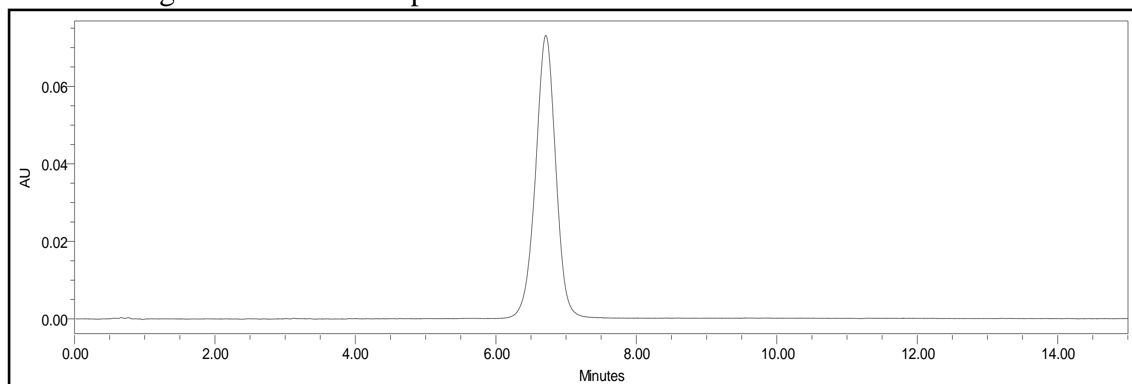


Table-16: Summary for flow change parameter of robustness study.

At 0.8 mL/min flow rate		At 1.2 mL/min flow rate	
Data for standard preparation		*Data for standard preparation*	
Replicate	Area	Replicate	Area
1	1624881	1	1589654
2	1625447	2	1581285
3	1622971	3	1590027
4	1619304	4	1589672
5	1617029	5	1584298
Mean	1621926	Mean	1586987
Std.dev.	3642.70	Std.dev.	3978.29
%RSD	0.22	%RSD	0.25
Data for Test preparation		*Data for Test preparation*	
Replicate	Area	Replicate	Area
1	1620938	1	1582591
2	1621270	2	1582737
Mean	1621104	Mean	1582664
Standard wt. (mg)	50.02	Standard wt. (mg)	50.02
Test wt. (mg)	50.04	Test wt. (mg)	50.04
%Assay	99.18	%Assay	98.96

The calculation formula for the determination of assay is,

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{100}{W_2} x \frac{50}{5} xP$$

Where,

A_T = Average Area of Test Preparation.

A_S = Average Area of Standard Preparation.

W_1 = Weight of Working Standard (mg).

W_2 = Weight of Test Sample (mg).

P = Potency of Working Standard (%).

5.6.2 Change in mobile phase composition:

In this experiment the test samples were analyzed at the mobile phase proportion of (Buffer:Acetonitrile) 42:58 and 38:62 v/v each and the results were observed in terms of assay value.

Blank preparation:

Diluent was used as blank.

Standard preparation:

Refer standard preparation under flow rate parameter of robustness study.

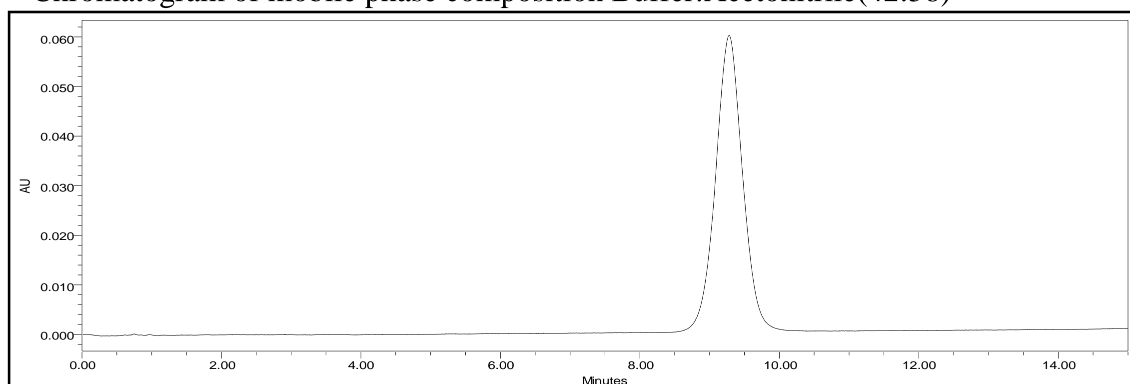
Test preparation:

Refer test preparation under flow rate parameter of robustness study.

Table-17: Sequence for change in composition robustness study

No.	Description	Injection Replicate	Chromatographic Parameter
1	Blank	1	Buffer:Acetonitrile (38:62)
2	Standard Preparation	5	
3	Test Preparation	2	
4	Bracketing Standard	1	
5	Blank	1	Buffe:Acetonitrile (42:58)
6	Standard Preparation	5	
7	Test Preparation	2	
8	Bracketing Standard	1	

Chromatogram of mobile phase composition Buffer:Acetonitrile(42:58)



Chromatogram of mobile phase composition Buffer:Acetonitrile (38:62):

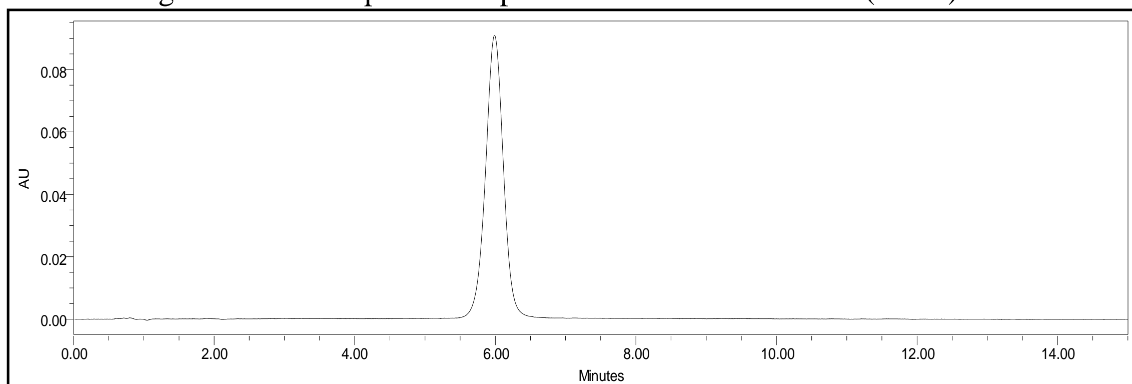


Table-18: Summary for change mobile phase composition.

Buffer:Acetonitrile (42:58)		**Buffer:Acetonitrile (38:62)**	
Data for standard preparation		*Data for standard preparation*	
Replicate	Area	Replicate	Area
1	1624881	1	1589654
2	1625447	2	1581285
3	1622971	3	1590027
4	1619304	4	1589672
5	1617029	5	1584298
Mean	1621926	Mean	1586987
Std.dev.	3642.70	Std.dev.	3978.29
%RSD	0.22	%RSD	0.25
Data for Test preparation		*Data for Test preparation*	
Replicate	Area	Replicate	Area
1	1620938	1	1582591
2	1621268	2	1582737
Mean	1621103	Mean	1582664
Standard wt. (mg)	50.02	Standard wt. (mg)	50.02
Test wt. (mg)	50.04	Test wt. (mg)	50.04
%Assay	99.18	%Assay	98.96

The calculation formula for the determination of assay is,

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{100}{W_2} x \frac{50}{5} xP$$

Where,

A_T = Average Area of Test Preparation.

A_S = Average Area of Standard Preparation.

W_1 = Weight of Working Standard (mg).

W_2 = Weight of Test Sample (mg).

P = Potency of Working Standard (%).

5.6.3 Robust Parameter: Change column lot:

In this parameter, column used in analytical method was changed to different lot. Sample was assayed by changing the lot of column.

Blank preparation:

Diluent was used as blank.

Standard preparation:

Stock solution: Acuurately weigh 50.18 mg of Rifaximin reference standard transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask, sonicate to dissolved and diluted up the mark with diluent. The concentration obtained is 501.8 µg/ml of Rifaximin.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.18 µg/ml of Rifaximin.

Test preparation:

Stock solution: Acuurately weigh 50.11 mg of Rifaximin reference standard transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask, sonicate to dissolved and diluted up the mark with diluent. The concentration obtained is 501.1 µg/ml of Rifaximin.

Pipette out 5 mL of above standard stock solution, transfer into 50 mL volumetric flask and dilute it up to the mark with diluent. The concentration obtained is 50.11 µg/mL of rifaximin.

Table-19: Sequence for change in composition robustness study

No.	Description	Injection Replicate	Chromatographic Parameter
1	Blank	1	Change column lot
2	Standard Preparation	5	
3	Test Preparation	2	
4	Bracketing Standard	1	

Chromatogram of robustness parameter column change

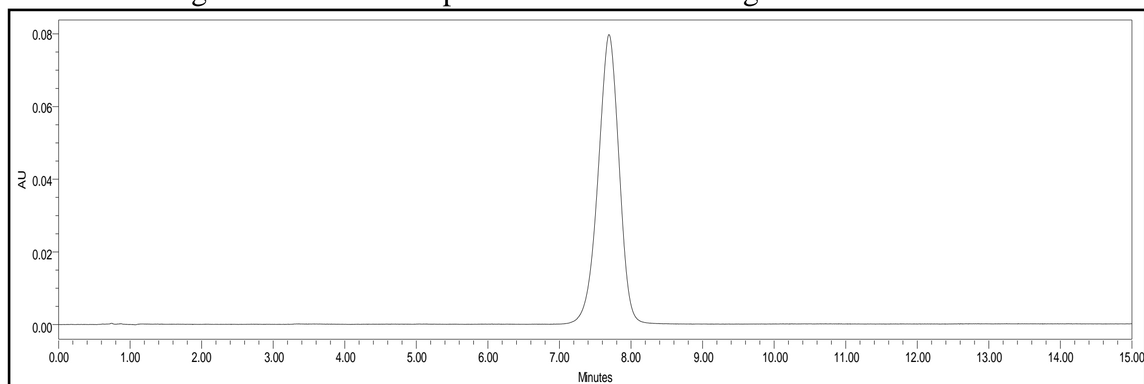


Table-20: Summary for column change parameter of robustness study:

Column lot change	
Data for standard preparation	
Replicate	Area
1	1609855
2	1611127
3	1617854
4	1609799
5	1616592
Mean	1613045
Std.dev.	3876.18
%RSD	0.24
Data for Test preparation	
Replicate	Area
1	1621185
2	1620201
Mean	1620693
Standard wt. (mg)	50.18
Test wt. (mg)	50.11
%Assay	99.88

The calculation formula for the determination of assay is,

$$\% \text{ Assay} = \frac{A_T}{A_S} x \frac{W_1}{100} x \frac{5}{50} x \frac{100}{W_2} x \frac{50}{5} xP$$

Where,

A_T = Average Area of Test Preparation.

A_S = Average Area of Standard Preparation.

W_1 = Weight of Working Standard (mg).

W_2 = Weight of Test Sample (mg).

P = Potency of Working Standard (%).

Table-21: Summary of robustness study

Summary of Robustness Study				
Robust Condition	**% Assay**	**Retention time (min.)**	**System Suitability**	
			Theoretical Plates	**Asymmetry**
Flow Change 0.9 ml/min	99.14	8.14	5845	1.12
Flow Change 1.1 ml/min	99.36	6.71	5533	1.14
MP Proportion Change (42:58)	99.18	9.28	6024	1.14
MP Proportion Change (38:62)	98.96	5.99	5492	1.12
Column Lot Change	99.88	7.70	5562	1.14

The data and the chromatogram given above suggest that there is no considerable influence of the change in flow rate, mobile phase composition and column lot change on the result of the analysis by this method or on chromatographic suitability of this method. Hence, it can be conclude from this experiment that the method is highly robust.

5.7 Solution stability study:

Solution stability period for the solutions of standard preparation and test preparation was evaluated. The solutions were stored at 5° C and ambient temperature without protection against light and tested at interval of 6, 12, 24, 36, and 48 h. The responses for the aged solution were evaluated using a freshly prepared standard solution.

Blank preparation:

Diluent was used as blank.

Standard preparation:

Stock solution: Acuurately weigh 50.10 mg of Rifaximin reference standard transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask, sonicate to dissolved and diluted up the mark with diluent. The concentration obtained is 501.0 µg/ml of Rifaximin.

Pipette out 5 mL of above standard stock solution, transfer into 50 mL volumetric flask and dilute it up to the mark with diluent. The concentration obtained is 50.10 µg/mL of rifaximin.

Test preparation:

Stock solution: Acuurately weigh 50.08 mg of Rifaximin reference standard transferred into 100 ml volumetric flask. 70 ml of diluent was added into the volumetric flask, sonicate to dissolved and diluted up the mark with diluent. The concentration obtained is 500.8 µg/ml of Rifaximin.

Pipette out 5 mL of above standard stock solution, transfer into 50 mL volumetric flask and dilute it up to the mark with diluent. The concentration obtained is 50.08 µg/mL of rifaximin.

Table-22: Summary of solution stability:

Time intervals	Absolute difference in assay for standard solution %		Absolute difference in assay for sample solution %	
	5°C	Room temperature	5°C	Room temperature
After 6 hours	0.23	0.37	0.25	0.35
After 12 hours	0.33	0.46	0.39	0.5
After 24 hours	0.47	0.86	0.79	0.97
After 48 hours	2.38	2.46	2.86	3.21

Solution stability time period for standard solution is 24 hours at 5°C and room temperature. Solution stability time period for test solution is 24 hours at 5°C and at room temperature.

5.8 System suitability:

A system suitability test for the chromatographic system was performed before each validation experiment. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate and % RSD of peak area were determined for same. Only after the system suitability results were in acceptance criteria the experiments were precede further.

The Theoretical plates should be more than 5000, Asymmetry should be less than 2.0 and % RSD should be less than 2.0. As the data suggest the system suitability was within the criteria in each validation experiment. Hence the system was found suitable to perform

the validation experiment which confirms the reliability of the data generated during the method validation.

Table-23: Summary of system suitability solution

Summary of System Suitability Test			
Experiment Name	**Theoretical Plates**	**Asymmetry**	**% RSD**
Specificity	5799	1.14	0.43
Linearity and Range	5638	1.16	0.39
LOD and LOQ	5974	1.15	0.39
Method Precision	5720	1.16	0.11
Int. Precision	5789	1.16	0.20
Accuracy	5827	1.17	0.22
Robustness	6024	1.14	0.24
Solution Stability	5471	1.14	0.62

6. CONCLUSION:

The surveillance and outcome obtained from each validation experiment including specificity, linearity and range, LOD and LOQ, precision, accuracy, robustness, solution stability and system suitability lies well inside the acceptance criteria. Since, all the results are with-in the limit, the developed Analytical method is considered as validated and suitable for anticipated use.

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