RESEARCH ARTICLE

DEVELOPMENT AND VALIDATION OF THE STABILITY INDICATING LIQUID CHROMATOGRAPHIC METHOD FOR RIFAXIMIN -AN ANTIBIOTIC

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ABSTRACT

An isocratic reversed-phase high-performance liquid chromatographic method was developed and validated for the determination of rifaximin. The samples were analyzed by high-performance liquid chromatography (HPLC). Chromatographic separation was achieved on a C18 column using an aqueous tetra butyl ammonium hydrogen sulphate: methanol (10:90, v/v), with flow rate 1.0 mL/min (UV detection at 454 nm). Linearity was observed over the concentration range of 1.0–200 µg/mL with regression equation 15407x +6677 (R^2 = 0.9999). The LOQ was found to be 0.786µg/mL and the LOD was found to be 0.238µg/mL. Rifaximin was subjected to stress conditions of degradation in aqueous solutions including acidic, alkaline, oxidation, photolysis and thermal degradation. The forced degradation studies were performed by using HCl, NaOH, H2O2, thermal and UV radiation. Rifaximin is more sensitive towards acidic conditions in comparison to oxidation and very much resistant towards alkaline, thermal and photolytic degradations. The method was validated as per ICH guidelines. The RSD for intra-day (0.28-0.55) and inter-day (0.68-0.81) precision were found to be less than 1 %. The percentage recovery was in good agreement with the labelled amount in the pharmaceutical formulations and the method is simple, specific, precise, robust and accurate for the determination of Rifaximin in pharmaceutical formulations.

Keywords: RP-HPLC; Rifaximin; Antibiotic; Stability-indicating; Validation.

INTRODUCTION

Rifaximin (Fig 1) (RFX) is a newer antibiotic, used for the treatment of patients (more than 12 years of age) with traveller's diarrhoea caused by non-invasive strains of Escherichia coli[1]. Rifaximin[2] is benzimdazole derivative and chemically it is a 2S,16Z,18E,20S,21S,22R,23R,24R,25S, 26S, 27S, 28E-5,6,21,23,25-pentahydroxy- 27-methoxy-2,4,11, 16, 20,22, 24, 26, - octamethyl-2,.7-epoxypentadeca- [1,11,13] trienimino) benzofuro [4,5-e] pyrido [1,2-a]-benzimidazole-1,15(2H)- dione, 25-acetate. It is a structural analog of Rifampin. RFX is a product of synthesis of Rifamycin, an antibiotic with low gastrointestinal absorption and good antibacterial activity[3]. Rifaximin binds to the beta-subunit of bacterial DNA-dependent RNA polymerase and prevents catalysis of polymerization of deoxyribonucleotides into a DNA strand. As a result, bacterial RNA synthesis is inhibited. In vitro studies of RFX have demonstrated broad-spectrum coverage including Gram-positive, Gram-negative, and anaerobic bacteria as well as a limited risk of bacterial resistance[4]. Furthermore, RFX does not bind to RNA polymerase in eukaryotic cells, thus human cell production is not affected. Compared with other antibiotics, RFX has a lower rate of fecal pathogenic eradication, so depletion of normal gastrointestinal flora is reduced[5]. Methods reported for the determination of RFX in pharmaceutical dosage forms and biological fluids include RPHPLC[6-8], LC-MS[9-12] and spectrophotometric[13-14] methods have been developed for the determination of RFX in pharmaceutical formulations and biological fluids. Impurity profiles of Rifaximin were also studied by using Diagnostic fragment-ion-based extension strategy (DFIBES) and derivative resolution of UV spectra [15-16]. In the present work we developed simple, rapid, precise and accurate robust liquid chromatographic method for the determination of RFX tablets. Previous reported methods have from one or other disadvantages and therefore the authors have developed a novel stability indicating liquid chromatographic method which was validated as per ICH guidelines[17].

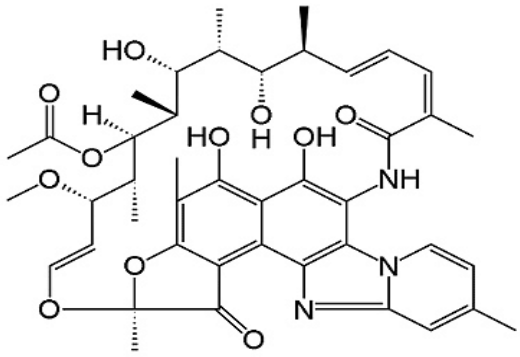


Figure 1: Chemical structure of Rifaximin (RFX)

EXPERIMENTAL

Chemicals and solutions

Rifaximin standard (purity ≥ 99.0 %) was obtained from Torrent Pharmaceuticals Limited, India). Methanol (HPLC grade), Tetra butyl ammonium hydrogen sulphate (Spectrochem Pvt. Ltd.) Sodium hydroxide (NaOH) and hydrochloric acid (HCl) and Hydrogen peroxide (H2O2) were obtained from Merck (India). Rifaximin is available (Label claim: 200 mg) with brand names RCIFAX (Lupin, India) and TORFIX (Torrent Pharmaceuticals, India). All chemicals were of analytical grade and used as received.

HPLC instrumentation and conditions

Chromatographic separation was achieved by using a Lichrocart / Lichrosphere 100 C-18 (250 mm × 4.6 mm i.d., 5 µm particle size) column of Shimadzu Model LC-Class-Vp version 6.12 SPI, equipped with UV-VIS detector Model SPD-10A maintained at 25 ºC. Isocratic elution was performed using 10 mM TBAHS and methanol (90:10, v/v). The overall run time was 5 min. and the flow rate was 1.0 mL/min. 20 µl of sample was injected into the HPLC system.

The mobile phase was prepared by accurately weighed and transferred 3.3954 grams of tetra butyl ammonium hydrogen sulphate (10mM TBAHS) (pH 3.37) in to a 1000 mL volumetric flask, dissolve and diluted to volume with HPLC grade water.

Rifaximin stock solution (1000 µg/mL) was prepared by accurately weighing 25 mg of Rifaximin in a 25 mL amber volumetric flask and making up to volume with mobile phase. Working solutions for HPLC injections were prepared on a daily basis from the stock solution in a solvent mixture of TBAHS and methanol (10:90 v/v) (mobile phase). Solutions were filtered through a 0.45 µm membrane filter prior to injection.

Analysis of marketed formulation (Tablets)

Twenty tablets from each brand of marketed formulations (RCIFAX and TORFIX) were procured from the medical store, weighed and crushed to a fine powder. Powder equivalent to 25 mg Rifaximin was accurately weighed and dissolved in mobile phase in a 25 ml volumetric flask. The volumetric flask was sonicated for 30 min and filtered through 0.45 µm nylon filter before injection. The filtrate obtained from the extracted marketed formulations (RCIFAX and TORFIX) was diluted as per the requirement and 20 µL was injected in to the HPLC system for conducting the assay.

Forced Degradation Studies/Specificity

The study was intended to ensure the effective separation of RFX and its degradation peaks of formulation ingredients at the retention time of RFX. Separate portions of drug product and ingredients were exposed to the following stress conditions to induce degradation. Forced degradation studies were performed to evaluate the stability indicating properties and specificity of the method[18]. All solutions for use in stress studies were prepared at an initial concentration of 1 mg/mL of RFX and refluxed for 30 min at 60 ºC. All samples were then diluted in mobile phase to give a final concentration of 20 µg/mL and filtered before injection.

Acid and alkali degradation studies

Acid decomposition was carried out in 0.1 M HCl at a concentration of 20 µg/mL RFX and after refluxation for 30 min at 60 ºC in thermostat the stressed sample was cooled, neutralized and diluted with mobile phase. Similarly stress studies in alkaline conditions were conducted using a concentration of 20 µg/mL in 0.1 M NaOH and refluxed for 30 min at 60 ºC in thermostat. After cooling the solution was neutralized and diluted with mobile phase.

Oxidation

Solutions for oxidative stress studies were prepared using 3% H2O2 at a concentration of 20 µg/mL of RFX and after refluxation for 30 min at 60 ºC on the thermostat the sample solution was cooled and diluted accordingly with the mobile phase.

Thermal Degradation Study

For thermal stress testing, the drug solution (20 µg/mL) was heated in thermostat at 80 ºC for 30 min, cooled and used.

Photo stability

The drug solution (20 µg/mL) for photo stability testing was exposed to UV light for 4 hour UV light (365 nm) chamber and analyzed.

Method validation

The method was validated for the following parameters: system suitability, linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, selectivity and robustness.

Linearity

Linearity test solutions for the assay method were prepared from a stock solution at different concentration levels of the assay analyte concentration (1, 2, 5, 10, 20, 50, 100, 120, 150, 180 and 200 µg/mL). 20 µL of each solution was injected in to the HPLC system and the peak area of the chromatogram obtained was noted. The solutions extracted from the marketed formulations were injected in to the HPLC system and the peak area of the chromatograms was noted. The analytical curve was evaluated on three different days. The peak area vs. concentration data was analyzed with least squares linear regression. The slope and y-intercept of the calibration curve was reported.

Precision

The intra-day precision of the assay method was evaluated by carrying out 9 independent assays of a test sample of RFX at three concentration levels (10, 20 and 50 µg/mL) (n=3) against a qualified reference standard. The %RSD of three obtained assay values at three different concentration levels was calculated. The inter-day precision study was performed on three different days i.e. day 1, day 2 and day 3 at three different concentration levels (10, 20 and 50 µg/mL) and each value is the average of three determinations (n=3). The % RSD of three obtained assay values on three different days was calculated.

Accuracy

The accuracy of the assay method was evaluated in triplicate at three concentration levels (80, 100 and 120%), and the percentage recoveries were calculated. Standard addition and recovery experiments were conducted to determine the accuracy of the method for the quantification of RFX in the drug product. The study was carried out in triplicate at 18, 20 and 22 µg/mL. The percentage recovery in each case was calculated.

Robustness

The robustness of the assay method was established by introducing small changes in the HPLC conditions which included wavelength (452 and 456 nm), percentage of methanol in the mobile phase (88 and 92%) and flow rate

(0.9 and 1.1 mL/min). Robustness of the method was studied using six replicates at a concentration level of 20 µg/mL of RFX.

Sensitivity/Limit of quantification (LOQ) and limit of detection (LOD)

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 (n=3), as described in International Conference on Harmonization guidelines Q2 (R1)[16]. Sensitivity of the method was established with respect to limit of detection (LOD) and LOQ for RFX. LOD and LOQ were established by slope method as mentioned below.

$$LOD = \frac{3.3 \times \text{standard deviation of y-intercept}}{\text{Slope of the calibration curve}}$$

$$LOQ = \frac{10 \times \text{standard deviation of y-intercept}}{\text{Slope of the calibration curve}}$$

LOD and LOQ were experimentally verified by injecting six replicate injections of each impurity at the concentration obtained from the above formula.

Solution stability and mobile phase stability

The solution stability of RFX in the assay method was carried out by leaving both the sample and reference standard solutions in tightly capped volumetric flasks at room temperature for 48 h. The same sample solutions were assayed at 12 h intervals over the study period. The mobile phase stability was also assessed by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions at 12 h intervals up to 48 h. The prepared mobile phase remained constant during the study period. The % RSD of the RFX assay was calculated for the mobile phase and solution stability experiments. An additional study was carried out using the stock solution by storing it in a tightly capped volumetric flask at 4 ºC.

RESULTS AND DISCUSSION

The present proposed method is more simple, precise and accurate in comparison to the reported methods in the literature (Table 1). No stability indicating liquid chromatographic method was reported earlier. The linearity range for the methods reported in the literature was narrow and some of the methods are applicable only for bio analytical determination of Rifaximin. The present developed method is more sensitive and can be used in a wide concentration range for the determination of Rifaximin in pharmaceutical formulations. Satisfactory resolution was achieved with use of a mixture of TBAHS and methanol (10:90 v/v) (Fig. 2A) and C18 column was adopted for the analysis as it has provided a better separation of the analytes. UV detection was carried out at 454 nm. The present stability-indicating method for the determination of RFX in pharmaceutical formulations is specific because the drug peak was well separated even in the presence of degradation products. Overall, the data demonstrated that the excipients and the degradation products did not interfere with the RFX peak, indicating the selectivity of the method. The complete separation of the analytes was accomplished in less than 5 min and the method can be successfully applicable to perform long-term and accelerate stability studies of RFX formulations.

Table 1: Comparison of the performance characteristics of the present HPLC method with the published methods

S.No	Method /Reagent	λ(nm)	Linearity	Remarks	Ref
1.	(HPLC) Methanol : phosphate buffer (70:30, v/v)	293	5-30	Very narrow linearity range	6
2.	(HPLC) Acetonitrile : Ammonium acetate (85:15, v/v)	-	5-50	Very narrow linearity range	7
3.	(HPLC) Acetonitrile : water : Acetic acid (18:82:0.1, v/v)	-	0.1-20	Rat serum and urine	8
4.	(LC-ESI/MS/MS) Acetonitrile : Acetic acid (Gradient mode)	-	$(0.5\text{-}10)10^{-3}$	Rat serum	9
5.	(LC-MS) Ammonium acetate : Methanol (pH-4.32)	-	$(0.5\text{-}10)10^{-3}$	Human Plasma	10
6.	(LC-MS) Ammonium formate : Acetonitrile (20:80, v/v)	-	$(0.2\text{-}200)10^{-4}$	Human Plasma	11
7.	(LC-ESI/MS)	-	$(0.1\text{-}10)10^{-4}$	Dried blood spots	12
8.	(Spectrophotometry) $Fecl_3$+MBTH(Method A) Alkaline borate buffer (pH 12) (Method B)	637 296	5-25 5-25	Very narrow linearity range	13
9.	(Spectrophotometry) Water (Method A) Methanol (Method B)	437 474	1-200 2-100	Colorimetric method	14
10.	(HPLC) TBAHS : Methanol (10:90, v/v)	454	1-200	Wide linearity range, stability indicating method	Present Work

HPLC method development and optimization

Initially the stressed samples were analyzed using a mobile phase consisting of TBAHS: Acetonitrile (45:55, v/v) at a flow rate of 1.0 mL/min. Under these conditions, the resolution and peak symmetry were not satisfactory and two peaks were observed, so the mobile phase was changed to TBAHS: methanol (45:55 v/v) with a flow rate of 1.0 mL/min in which tailing was observed and the retention time of the drug peak was more than 10 mins. Therefore mobile phase containing TBAHS: methanol (90:10, v/v) was chosen as the best chromatographic response for the entire study where the drug peak was well resolved with good symmetry and sharpness.

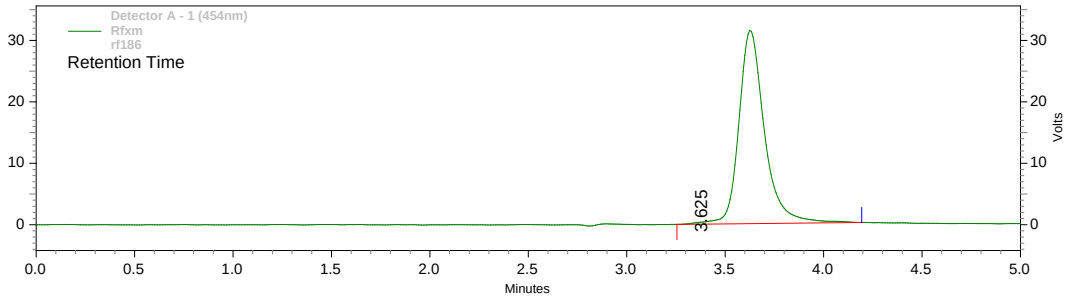
Method validation

System suitability

The system suitability test was performed to ensure that the complete testing system was suitable for the intended application. The parameters measured were peak area, retention time, tailing factor, capacity factor and theoretical plates. In all measurements the peak area varied less than 2.0%, the average retention time was 3.625 minutes. The capacity factor was more than 2, theoretical plates were 7786 (more than 2000) and tailing factor was 1.21 (less than 2) for the RFX peak. The LOQ was found to be 0.786 µg/mL and the LOD was found to be 0.238 µg/mL.

Linearity

The typical chromatogram for RFX obtained from the standard solution and extracted marketed formulation was shown in Fig. 2A & 2B. The calibration curve for RFX was linear over the concentration range of 1.0–200 µg/mL. The data for the peak area of the drug in corresponds to the concentration was treated by linear regression analysis (Table 2) and the regression equation for the calibration curve (Fig. 3) was found to be y = 15407x +6677 with correlation coefficient of 0.9999 which is nearly equals to unity.



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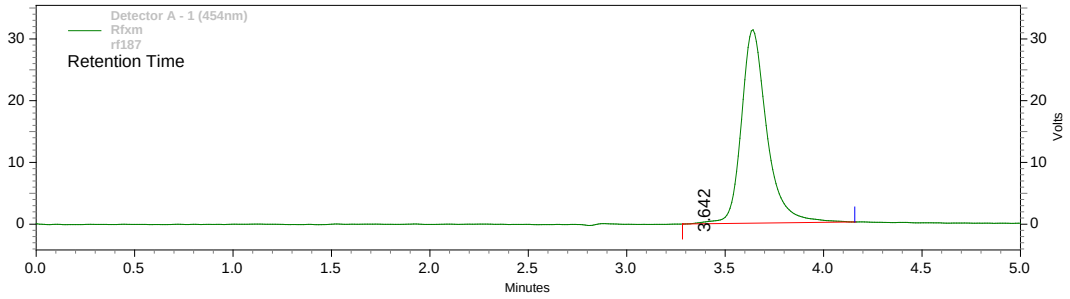

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Figure 2: Representative chromatograms of Rifaximin (20 µg/mL) [A], RCIFAX ®® (200 mg) [B]

Table 2: Linearity of Rifaximin

Conc.(µg/ml)	*Mean area ± SD	*RSD(%)
1	15924 ± 46.18	0.29
2	31985 ± 115.15	0.36
5	82579 ± 255.99	0.31
10	155168 ± 356.89	0.23
20	301638 ±1417.69	0.47
50	802375 ± 2808.31	0.35
100	1557275 ± 4204.64	0.27
120	1878632 ± 6011.62	0.32
150	2300850 ± 17026.29	0.74
180	2788630 ± 20078.14	0.72
200	3069481 ± 24862.79	0.81

Mean of three replicates

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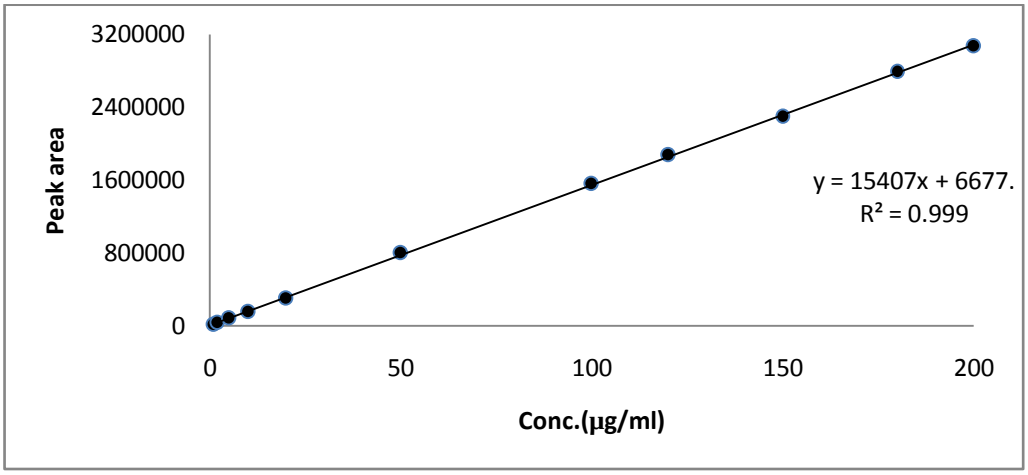


Figure 3: Calibration curve of Rifaximin

Precision

The precision of the method was determined by repeatability (intra-day precision) and intermediate precision (inter-day precision) of the RFX standard solutions. Repeatability was calculated by assaying three samples of each at three different concentration levels (10, 20 and 50 µg/mL) on the same day. The inter-day precision was calculated by assaying three samples of each at three different concentration levels (10, 20 and 50 µg/mL) on three different days. The % RSD range was obtained as 0.28-0.55 and 0.68- 0.81 for intra-day and inter-day precision studies respectively (Table 3).

Table 3: Intra-day and inter-day precision studies of Rifaximin

Sample No.	Conc. (µg/ml)	Intra-day precision		Inter-day precision	
		Mean ± SD	%RSD	Mean ± SD	%RSD
1.	10	154143 ± 423.2004	0.28	156349 ± 1063.1732	0.68
2.	20	311654 ± 1277.7814	0.41	310347 ± 2296.5678	0.74
3.	50	803462 ± 4419.041	0.55	798976 ± 6471.7056	0.81

Mean of three replicates

Because the stability of standard solutions can also affect the robustness of analytical methods, the stability of standard solutions of the drug substance used in this method was tested over a long period of time. One portion of a standard solution was kept at room temperature and the other portion was stored under refrigeration at approximately 4ºC and the content of these solutions was regularly compared with that of freshly prepared solutions. No change in drug concentrations were observed for solutions stored under refrigeration. But it is recommended that the sample and standard solutions must therefore, be freshly prepared in amber colour flasks to protect from light.

Accuracy

The method accuracy was proven by the recovery test. A known amount of RFX standard (10 µg/mL) was added to aliquots of samples solutions and then diluted to yield total concentrations as 18, 20 and 22 µg/mL as described in Table 4. The assay was repeated over 3 consecutive days. The resultant % RSD was in the range 0.15-0.36 (<2.0 %) with a recovery 98.70-99.71 %.

Table 4: Accuracy-recovery study of Rifaximin by standard-addition method

Sample No	Spiked Concentration(µg/ml)	*Measured Concentration(µg/ml)	Recovery* (%)	%RSD
1.	16(80%)	15.91	99.44	0.15
2.	20(100%)	19.74	98.70	0.23
3.	24(120%)	23.93	99.71	0.36

Mean of three replicates

Robustness

The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability for routine analysis [16]. The robustness of the method was evaluated by assaying the same sample under different analytical conditions deliberately changing from the original condition. The detection wavelength was set at 452 and 456 nm (± 2 nm), the ratio of percentage of TBAHS: Methanol in the mobile phase was applied as 8:92 and 12:88 (± 2 %, v/v), the flow rate was set at 0.9 and 1.1 mL/min (± 0.1 mL/min). The results obtained (Table 5) from assay of the test solutions were not affected by varying the conditions and were in accordance with the results for original conditions. The % RSD value of assay determined for the same sample under original conditions and robustness conditions was less than 2.0% (0.35-0.78) indicating that the developed method was robust.

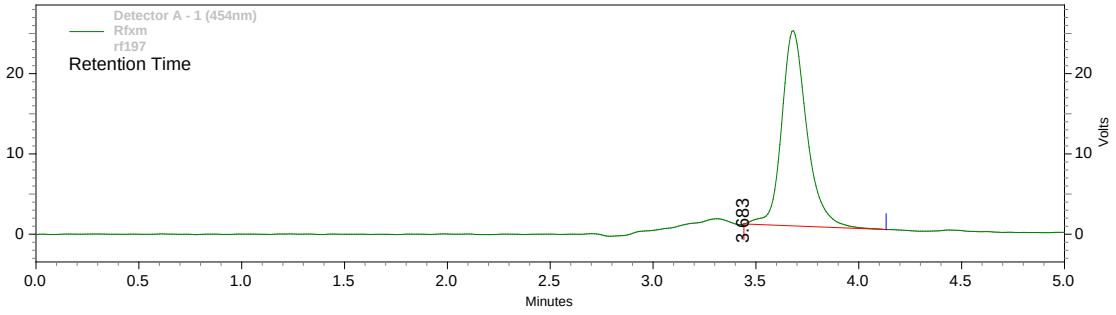
Table 5: Robustness study of Rifaximin

Parameter	Condition	*Mean peak area	Statistical analysis *Mean ± SD (%RSD)
Flow rate	0.9	306828	309074 ± 1976.69 (0.64)
	1.0	309845	
	1.1	310549	
Detection wave length(nm)	452	307855	308613 ± 1076.35 (0.35)
	454	309845	
	456	308139	
Mobile phase composition (TBAHS:Methanol,v/v)	8:92	314243	312640.67 ± 2429.74 (0.78)
	10:90	309845	
	12:88	313834	

Mean of three replicates

Selectivity/specificity

The specificity of the developed method was determined by injecting sample solutions (20µg/mL) which were prepared by forcibly degrading under such stress conditions as heat, light, oxidative agent, acid and base under the proposed chromatographic conditions. The stability indicating capability of the method was established from the separation of RFX peak from the degraded samples. The degradation of RFX was found to be very similar for both the tablets and standard. Typical chromatograms obtained following the assay of stressed samples are shown in Fig. 4 A-E.



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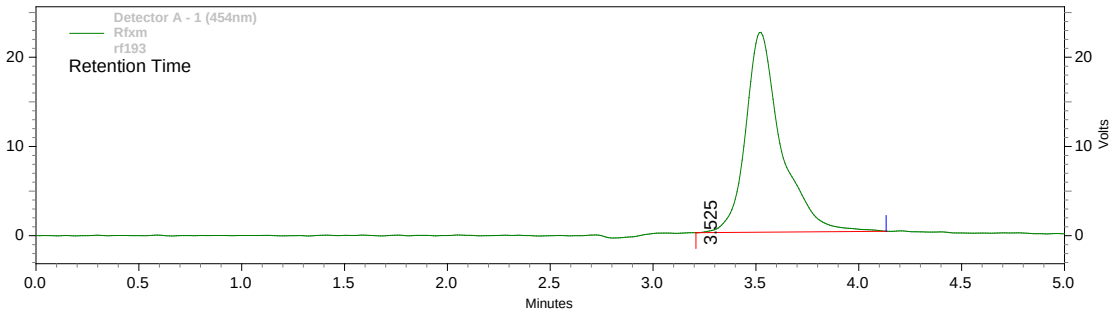

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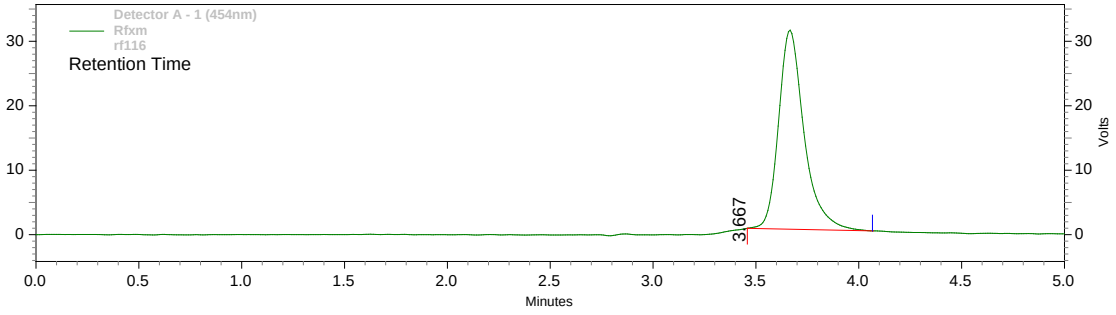

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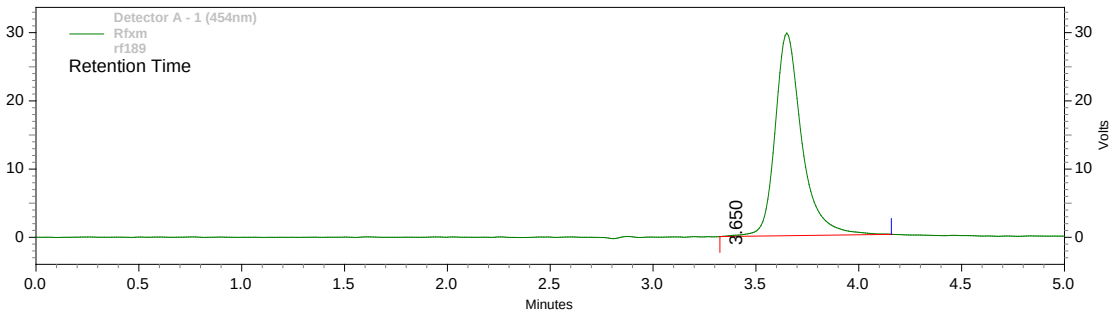

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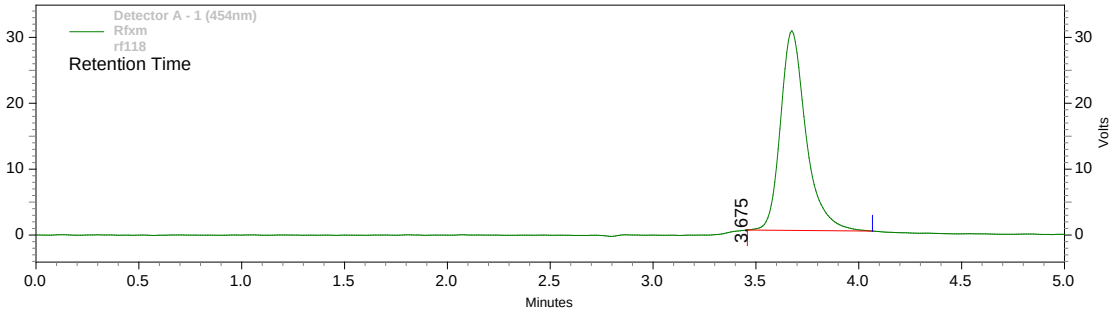

[E]

Figure 4: Representative chromatograms of Rifaximin (20 µg/mL) on acidic [A], alkaline [B], oxidative [C], thermal [D] and photolytic [E] degradations

Solution stability and mobile phase stability

The %RSD of the assay of RFX from the solution stability and mobile phase stability experiments was within 2%. The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the assays were stable up to 48 h at room temperature and up to 3 months at 4ºC.

Analysis of commercial formulations (Tablets)

The proposed method was applied for the determination of RFX in tablets (RCIFAX and TORFIX) and the results show 97.70- 98.76 % recovery (Table 6) indicates that the method is selective for the assay of RFX without interference from the excipients used in these tablets.

Table 6: Analysis of Rifaximin commercial formulation (Tablets)

Sample No.	Formulation	Labelled claim (mg)	*Amount found (mg)	*Recovery (%)
1.	RCIFAX	200	198.40	99.20
2.	TORFIX	200	199.51	99.76

*Mean of three replicates

Forced degradation studies

RFX standard and tablet powder were found to be quite stable under oxidation, alkaline and thermal degradation conditions. A slight decomposition was seen on exposure of RFX drug solution to acidic and photolytic degradations. During the acidic degradation, 6.63 % of the drug was decomposed. The benzimidazole group present in the RFX chemical structure may be responsible for the reported acidic degradation. The drug has undergone oxidative degradation (0.64 %) very slightly and alkaline (1.83 %) degradation slightly in comparison to other degradations (Table 7).

Table 7: Forced degradation studies of Rifaximin

Stress conditions	*Mean Peak area	*Drug recovered (%)	*Drug decomposed (%)
Standard Drug	304750	100	-
Acidic Hydrolysis	254070	83.37	6.63
Alkaline Hydrolysis	299165	98.17	1.83
Oxidative Degradation	302786	99.36	0.64
Thermal Degradation	289087	94.86	5.14
Photolytic Degradation	263373	86.42	13.58

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CONCLUSION

The proposed stability-indicating HPLC method was validated as per ICH guidelines and applied for the determination of RFX in pharmaceutical dosage forms. The chromatographic elution step is undertaken in a short time (< 4 min). The method was found to be accurate, precise, robust and specific as the drug peak elution did not interfere with any degradants during the forced degradation studies and therefore the drug is more resistant and at the same time the proposed method can be successfully applied to perform long-term and accelerated stability studies of Rifaximin formulations and biological fluids.

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