http://www.e-journals.in
DOI:10.7598/cst2014.830

Chemical Science Transactions
2014, **3(2),** 566-575

RESEARCH ARTICLE

Development and Validation of Stability-Indicating Liquid Chromatographic Assay for Rifaximin (An Antibiotic) in Bulk and Pharmaceutical Dosage Forms

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Received 31 January 2014 / Accepted 19 February 2014

Abstract: An isocratic reversed-phase high-performance liquid chromatographic method was developed and validated for the determination of Rifaximin. Chromatographic separation was achieved on a C_{18} column using an aqueous tetra butyl ammonium hydrogen sulphate (10 mM) (pH 3.37): acetonitrile (40:60, v/v), with flow rate 1.2 mL/min (UV detection at 441 nm). Linearity was observed in the concentration range of 0.1–200 µg/mL (R^2 = 0.9999). The limit of quantitation was found to be 0.0794 µg/mL and the limit of detection was found to be 0.0241 µg/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, H_2O_2, 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 method is simple, specific, precise, robust and accurate for the determination of Rifaximin in pharmaceutical formulations.

Keywords: RP-HPLC, Rifaximin, Stability-indicating, Validation, ICH

Introduction

Rifaximin[1] (RFX) is benzimdazole derivative and chemically it is a 2S,16Z,18E, 20S,21S,22R,23R,24R,25S,26S,27S, 5, 6, 21, 23, 25- penta hydroxy-27-methoxy-2,4,11, 16, 20,22,24,26,- octa methyl-2,. 7-epoxy penta deca-[1,11,13]trienimino) benzofuro[4,5-e] pyrido[1,2-a]-benzimidazole-1, 15 (2*H*)- dione,25acetate. It is a structural analog of Rifampin. Rifaximin (Figure 1) 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[2].

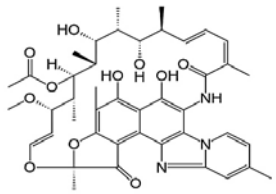


Figure 1. Chemical structure of Rifaximin

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 RP-HPLC[6-9], LC-MS[10-13] and spectrophotometric[14-15] 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[16-17]. 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[18].

Experimental

Rifaximin standard (purity ≥ 99.0%) was obtained from Torrent Pharmaceuticals Limited, India). Acetonitrile (HPLC grade), sodium hydroxide (NaOH) and hydrochloric acid (HCl) and Hydrogen peroxide (H_2O_2) were obtained from Merck (India). Rifaximin is available (Label claim: 200 mg) with brand names RCIFAX (Lupin) and TORFIX (Torrent). All chemicals were of analytical grade and used as received.

Preparation of buffer solution

The mobile phase was prepared by accurately weighing and transferring 3.3954 g of tetra butyl ammonium hydrogen sulphate (TBAHS) (10 mM) (pH 3.37) in to a 1000 mL volumetric flask, dissolving and diluting to volume with HPLC grade water.

Preparation of rifaximin stock solution

Rifaximin stock solution (1000 µg/mL) was prepared by accurately weighing 25 mg of RFX in a 25 mL volumetric flask with mobile phase. Working standard solutions were prepared on a daily basis from the stock solution in a solvent mixture of TBAHS (pH 3.37) and acetonitrile (40:60, v/v). Solutions were filtered through a 0.45 µm membrane filter prior to injection.

Instrumentation and chromatographic conditions

Chromatographic separation was achieved by using a Shimadzu Model CBM-20A/20 Alite HPLC system, equipped with SPD M20A prominence photodiode array detector with C18 (250mm × 4.6mm i.d., 5 µm particle size) column maintained at 25 ºC. Isocratic elution was

performed using tetra butyl ammonium hydrogen sulphate (TBAHS) (pH 3.37) and acetonitrile (40:60, v/v). The overall run time was 10 min and the flow rate was 1.2 mL/min. 20 µL of sample was injected into the HPLC system.

Forced degradation studies

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 [19]. 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 80 ºC. All samples were then diluted in mobile phase to give a final concentration of 10 µg/mL and filtered before injection.

Acidic and alkaline degradation studies

Acid decomposition was carried out in 0.1 M HCl at a concentration of 1.0 mg/mL RFX and after refluxation for 30 min at 80 ºC the stressed sample was cooled, neutralized and diluted with mobile phase. Similarly stress studies in alkaline conditions were conducted using a concentration of 1.0 mg /mL in 0.1 M NaOH and refluxed for 30 min at 80 ºC. After cooling the solution was neutralized and diluted with mobile phase.

Oxidation degradation studies

Solutions for oxidative stress studies were prepared using 3% H_2O_2 at a concentration of 1 mg/mL of RFX and after refluxation for 30 min at 80 ºC on the thermostat the sample solution was cooled and diluted accordingly with the mobile phase.

Thermal degradation studies

For thermal stress testing, the drug solution (1 mg/ mL) was heated in thermostat at 80 ºC for 30 min, cooled and used.

Photolytic degradation studies

The drug solution (1 mg/ mL) for photo stability testing was exposed to UV light for 4 h 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 (0.1-200 µg/mL) of the assay analyte concentration and 20 µL of each solution was injected in to the HPLC system and the peak area of the chromatogram obtained was noted. The calibration curve was plotted by taking the concentration on the x-axis and the corresponding peak area on the y-axis. The data was treated with linear regression analysis method.

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 (439 and 443 nm), percentage of acetonitrile in the mobile phase (58 and 62%) and flow rate (1.1 and 1.3 mL/min). Robustness of the method was studied using six replicates at a concentration level of 20 µg/mL of RFX.

Analysis of marketed formulations

The content of 25 tablets (each containing 100 mg of RFX) was mixed and quantity equivalent to 25 mg of drug weighed accurately and dissolved in mobile phase in a 25 mL volumetric flask, sonicated and filtered. The filtrate was diluted as per the requirement and 20 µL solution of each of marketed formulations (RCIFAX and TORFIX) was injected in to the HPLC system for conducting the assay.

Results and Discussion

A reversed-phase liquid chromatographic technique was developed to quantitate Rifaximin in pharmaceutical dosage forms. No stability indicating liquid chromatographic method was reported earlier. A detailed comparative study of the previously published methods with the present method was discussed in Table 1. Satisfactory resolution was achieved with use of a mixture of TBAHS and acetonitrile (40:60, v/v) (Figure 2) and C18 column was adopted for the analysis as it has provided a better separation of the analytes. UV detection was carried out at 441 nm (PDA detector).

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

Method /Reagent	λ nm	Linearity, µg/mL	Comments	Ref.
(HPLC) Methanol: phosphate buffer (70:30, v/v)	293	5-30	Very narrow linearity range (UV/visible detector)	[6]
(HPLC) Acetonitrile: Ammonium Acetate (85:15, v/v)	236	5-50	Very narrow linearity range (UV/visible detector)	[7]
(HPLC) Acetonitrile: water: acetic acid (18:82:0.1, v/v/v)	-	0.10–20	rat serum and urine	[8]
(HPLC) Sodium acetate: Acetonitrile (pH 4.0) (35:65, v/v)	441	1.0-300	Wide linearity range Stability indicating method (PDA detector)	[9]

(LC-ESI/MS/MS) Acetic acid: Acetonitrile (gradient mode)	-	(0.5–10) 10^{-3}	rat serum	[10]
(LC –MS) Ammonium acetate: methanol (pH 4.32)	-	(0.5-10) 10^{-3}	Human Plasma	[11]
(LC –MS) Ammonium formate: acetonitrile (20:80, v/v)	-	(0.2-200) 10^{-4}	Human Plasma	[12]
(LC-ESI-MS)	-	(0.1–10) 10^{-3}	dried blood spots	[13]
(Spectrophotometry) $FeCl_3$ + MBTH Alkaline borate buffer	637 296	5-25 5-25	Very narrow linearity range	[14]
(Spectrophotometry) Water Methanol	437 474	1-200 2-100	Colorimetric methods	[15]
(HPLC) TBAHS: Acetonitrile (40:60, v/v)	441	0.1-200	Wide linearity range Stability indicating method (PDA detector)	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^{-1}. Under these conditions, the resolution and peak symmetry were not satisfactory and two peaks were observed, so the mobile phase was changed to TBAHS (pH 3.37): acetonitrile (40:60, v/v) with a flow rate of 1.2 mL min^{-1} under which peaks were well resolved with good symmetry and sharpness. Therefore, mobile phase containing TBAHS (pH 3.37): acetonitrile (40:60, v/v) was chosen for the best chromatographic response for the entire study.

Method validation

The typical chromatogram for RFX obtained from the extracted marketed formulation was shown in Figure 2. The calibration curve for RFX was linear over the concentration range of 0.1–200 µg/mL (Table 2). The data for the peak area of the drug in corresponds to the concentration was treated by linear regression analysis and the regression equation for the calibration curve (Figure 3) was found to be y = 17604x - 4629.7 with correlation coefficient of 0.9999 which is nearly equals to unity.

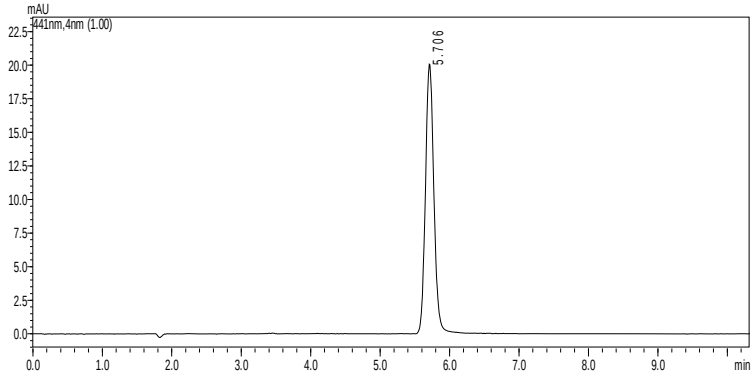


Figure 2. Representative chromatograms of Rifaximin (10 µg/mL)

Table 2. Linearity of Rifaximin

Conc. µg/mL	*Mean peak area ± SD		% RSD
0.1	1634	±7.205	0.441
0.5	8192	±26.378	0.322
1	16823	±106.657	0.634
5	84664	±416.546	0.492
10	169783	±599.333	0.353
20	349845	±1913.652	0.547
50	856082	±4126.315	0.482
100	1770119	±10160.483	0.574
150	2608725	±19095.867	0.732
200	3533933	±23748.029	0.672

**Mean of three replicates*

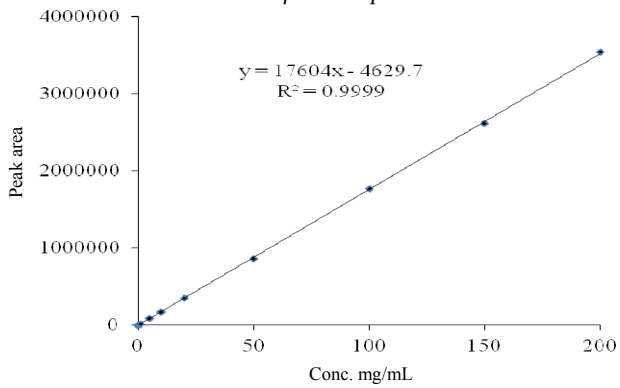


Figure 3. Calibration curve of Rifaximin

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 0.2-0.4 and 0.42-0.83 for intra-day and inter-day precision studies respectively (Table 3).

Table 3. Precision and accuracy study of Rifaximin

Conc. µg/mL	Intra-day precision	Inter-day precision	
	*Mean peak area ± SD (%RSD)	*Mean peak area ±SD (%RSD)	
10	169017.00±669.06 (0.4)	168866±974.334 (0.57)	
20	349191.33±688.28 (0.2)	327740±1378.17 (0.42)	
50	854368.67±2868.32 (0.34)	847364±7060.75 (0.83)	
	Accuracy		
Conc. µg/mL	*Mean peak area ± SD (% RSD)	Drug found µg/mL	*Recovery %
18	307472.7±2062.288 (0.66)	17.72	98.49
20	343376±1598.613 (0.45)	19.76	98.83
22	377945.7±2065.988 (0.54)	21.73	98.78

**Mean of three replicates*

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 3. The assay was repeated over three consecutive days. The resultant %RSD was in the range 0.45-0.66 (<2.0%) with a recovery 98.49-98.83%.

The % RSD value of assay determined for the same sample under original conditions and robustness conditions was less than 2.0% (1.09-1.38) indicating that the developed method was robust (Table 4).

Table 4. Robustness study of Rifaximin

Parameter	Condition	*Mean peak area	Statistical analysis	*Retention time
	1.1	356828	Mean = 352407.33	5.706
Flow rate	1.2	349845	SD = 3844.56	5.693
mL/min	1.3	350549	% RSD = 1.09	5.684
Detection	439	344281	Mean = 345433	5.692
wavelength nm	441	349845	SD = 3963.61	5.693
	443	342173	% RSD = 1.15	5.695
TBAHS:	38:62	344243	Mean = 349307.33	5.702
Acetonitrile	40:60	349845	SD = 48118.05	5.693
(v/v)	42:58	353834	% RSD = 1.38	5.684

**Mean of three replicates*

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 5.69 minutes. The capacity factor was more than 2, theoretical plates were more than 2000 and tailing factor was less than 2 for the RFX peak. The details were shown in Table 4. The peak purity index was found to be 1.0000. The LOQ was found to be 0.0794 µg/mL and the LOD was found to be 0.0241 µg/mL.

Analysis of commercial formulations (Tablets)

The proposed method was applied for the determination of RFX in tablets (RCIFAX and TORFIX) and the results show 98.48- 99.19% recovery (Table 5) indicates that the method is selective for the assay of RFX without interference from the excipients used in these tablets.

Table 5. Analysis of Rifaximin commercial formulation (Tablets)

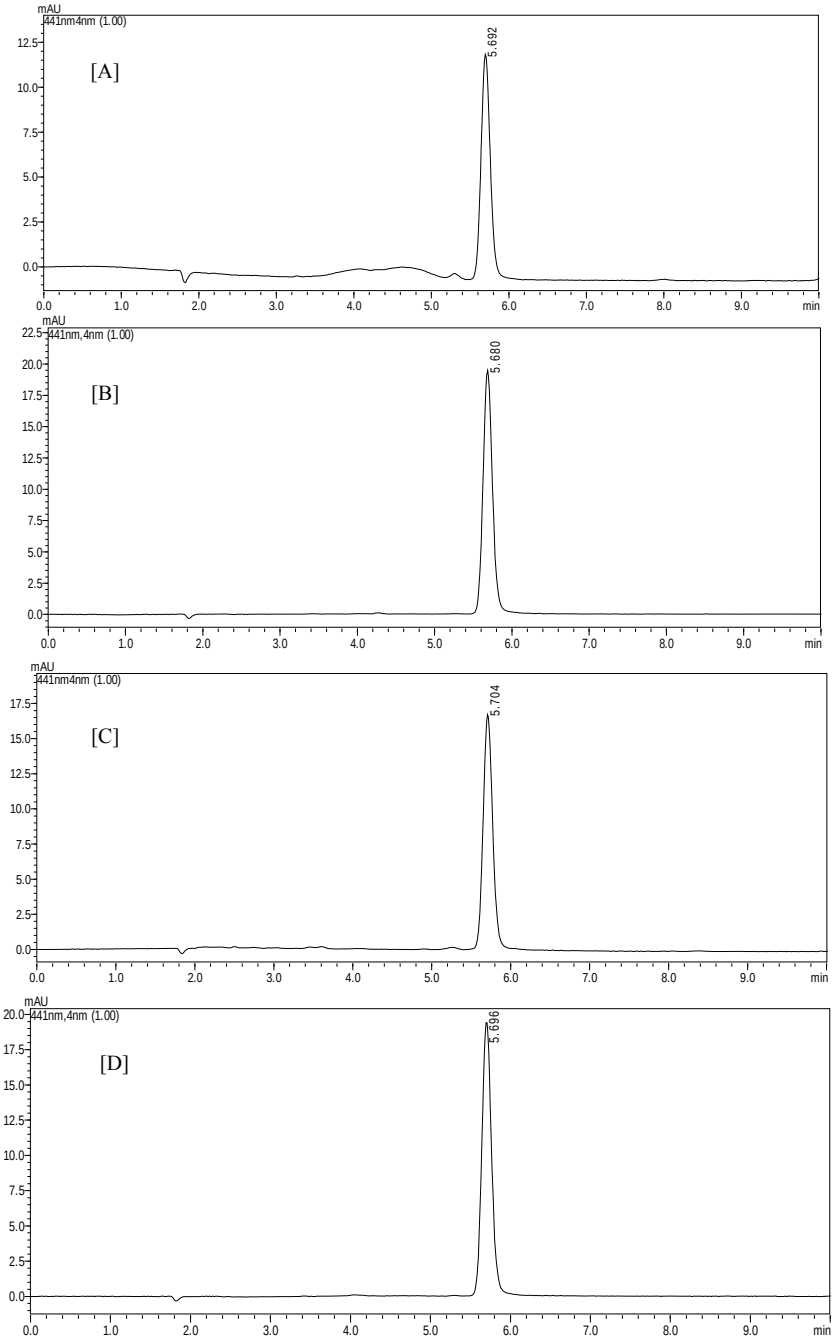
S No.	Formulation	Labeled claim mg	*Amount found mg	*Recovery %
1	RCIFAX®	200	196.96	98.48
2	TORFIX ®	200	198.38	99.19

**Mean of three replicates*

Forced degradation studies/selectivity/specificity

The specificity of the developed method was determined by injecting sample solutions (10 µg/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 Figure 4A-4E.



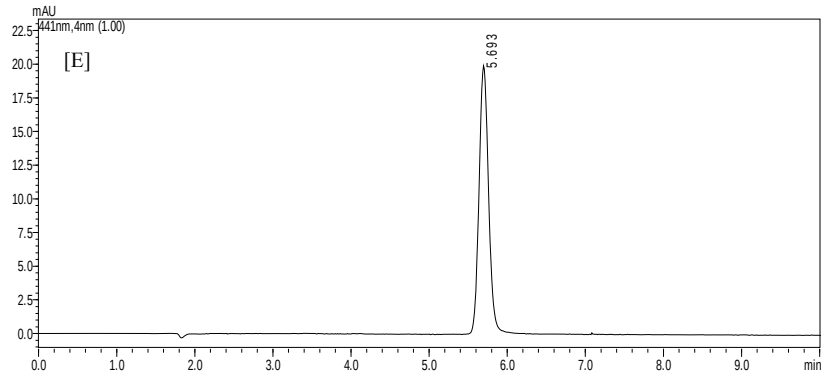


Figure 4. Representative chromatograms of Rifaximin (10 µg/mL) [A] Acidic [B] Alkaline [C] Oxidative [D] Thermal and [E] Photolytic degradation

RFX standard and tablet powder was found to be quite stable under dry heat conditions. A slight decomposition was seen on exposure of RFX drug solution to alkaline (2.21%), thermal (3.19%) and photolytic (0.51%) conditions. During the acidic degradation, 37.94% of the drug was decomposed. The benzimidazole group present in the RFX chemical structure may be responsible for the acidic degradation. As the imidazole moiety has basic character probably it may be responsible for major degradation of Rifaximin in acidic environment. The drug has even undergone oxidative degradation (16.21%) without any major degradant. Therefore it can be concluded that the drug is more sensitive towards oxidation and acidic conditions and the details of degradation were shown in Table 6. The system suitability parameters for the RFX peak shows that the theoretical plates were more than 2000 and the tailing factor.

Table 6. Forced degradation studies of Rifaximin

Stress Conditions	*Mean peak area	*Drug recovered %	*Drug decomposed %	Theoretical plates	Tailing factor
Standard Drug	169783	100	-	9481.309	1.154
Acidic degradation	105364	62.06	37.94	9484.383	1.168
Alkaline degradation	166028	97.79	2.21	9336.099	1.162
Oxidative degradation	142274	83.79	16.21	9429.806	1.153
Thermal degradation	164362	96.81	3.19	9401.145	1.158
Photolytic degradation	168913	99.49	0.51	9434.722	1.154

**Mean of three replicates*

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 4 ºC.

Conclusion

The proposed stability-indicating HPLC method was validated as per ICH guidelines and can be applied for the determination of Rifaximin in pharmaceutical dosage forms. The complete separation of the analytes was accomplished in less than 10 min and the method can be successfully applied for kinetic studies.

Acknowledgement

We are grateful to M/s GITAM University, Visakhapatnam, India for providing research facilities and Torrent Pharmaceuticals Limited, India for providing the gift samples of Rifaximin.

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