RESEARCH ARTICLE

Stability-Indicating Liquid Chromatography Method for Rifaximin and LC-MS Characterization of its Potential Degradants

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Received: 28th July, 2023; Revised: 22nd November, 2023; Accepted: 13th December, 2023, 2023; Available Online: 25th December, 2023

ABSTRACT

Rifaximin (RFX) is a structural analog of rifampin that has been shown to be a gastrointestinal-selective antibiotic with broad-spectrum antibacterial properties, a pronounced safety profile, and limited drug interactions. Crohn's disease, diarrhea, hepatic encephalopathy, irritable bowel syndrome, and traveler's diarrhea caused by non-invasive diarrheagenic *Escherichia coli*, can all be treated with rifaximin. FDA has designated RFX as an orphan drug for the treatment of hepatic encephalopathy. There is a substantial need for analytical quality monitoring of Rifaximin to ensure the safety and efficacy of treatment. To generate degradants, forced degradation study of the drug was carried out in acid, alkali, oxidative, thermal, and photolytic stress conditions. The optimum separation of the drug, and its degradants was achieved on an Inertsil ODS-3V C18 column (250 X 4.6 mm X 5 µm) under gradient elution conditions using, 10 mM potassium dihydrogen orthophosphate (pH 5.0 ± 0.05) as mobile phase A, and acetonitrile as mobile phase B, at a flow rate of 1.0 mL/min. The run time was 41 minutes and the detection wavelength was 258 nm. The method was validated in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human use Guidelines. The drug was stable in neutral hydrolysis, photo and dry heat degradation conditions. The percentage degradation observed during acid, alkali hydrolysis and oxidative stress conditions were 70.46, 15.11 and 24.18, respectively. The degradants were investigated by LC-MS which indicated the presence of four (*m/z* 784.2, 744.5, 784.3, 753.8), three (*m/z* 744.5, 784.3, 753.8) and five (*m/z* 772.4, 838.4, 744.5, 753.8, and 801.9) degradants in acid, alkali and oxidative stress conditions, respectively. The structures of degradants were elucidated using Liquid chromatography–mass spectrometry (LC-MS) and Liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses.

Keywords: Rifaximin, Degradants, Liquid chromatography–mass spectrometry, Liquid chromatography tandem mass spectrometry, Method development, Impurity.

International Journal of Pharmaceutical Quality Assurance (2023); DOI: 10.25258/ijpqa.14.4.07

How to cite this article: Chaturvedi P, Chopra S, Desai P, Tauro S. Stability-Indicating Liquid Chromatography Method for Rifaximin and LC-MS Characterization of its Potential Degradants. International Journal of Pharmaceutical Quality Assurance. 2023;14(4):867-873.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Rifaximin (RFX) is a distinctive rifamycin with limited systemic absorption, increased fecal concentrations, reduced systemic toxicity, and limited drug interactions.[1] It works in the gastrointestinal tract and has few side effects.[2-4] It is used safely to treat a wide range of illnesses in children, the elderly, and enfeeble individuals. Rifaximin, therefore, reaches a huge target population.

A literature review for the quality control methods for RFX revealed the lack of analytical procedures for its quality control in research papers and most official compendia despite its relevance, versatility, and superiority over other medications. Also, researchers had expressed different opinions regarding the stability of the drug in various forced degradation conditions. Some other limitations observed in the methods reported for quality control of RFX were a selection of shorter run time with poor resolution, the use of low pH buffer which can decrease the column life and use of ion-pairing reagent in the mobile phase which necessitated the use of a dedicated column.[5-7] Bioanalytical methods, for the determination of RFX in plasma quantitatively, have also been reported. These methods, being specific for biological fluids, have limited application.[8,9] The high-performance thin layer chromatography (HPTLC) method has also been reported but it is not at par with HPLC, in terms of accuracy.[10]

Impurity profiling of RFX by LC-MS technique has also been conducted and this work reported that eleven impurities were produced from rifaximin API. However, the stress conditions in which these impurities were generated, were not mentioned in this research paper.[11]

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The degradation of API as well as formulation when subjected to acidic, basic, thermal and oxidation stress conditions has also been reported.[12] The drug was stable in alkaline and oxidation conditions whereas during acidic and thermal degradation conditions, the degradation was found to be 99.52 and 98.06%, respectively. The extent of degradation in acid and thermal conditions was higher than the acceptable level. Also, it was evident from the literature that the drug is susceptible to degradation in alkaline and oxidative conditions.[11,13,14]

A comprehensive forced degradation study for RFX was performed and it was found that the degradation in alkaline, acidic, and oxidation conditions was 100, 38.44, 62.6%, respectively. The extent of degradation observed in stress conditions was higher than the accepted degradation level which necessitates the optimization of stress conditions.[15]

In some of the reported research works,[16-18] attempts were made to develop an assay method for RFX. The study focussed on the estimation of the drug and was not extended to develop stability-indicating assay method (SIAM) and also no impurity profiling was performed.

The behavior and stability of RFX pills were studied for six months, in temperature and humidity conditions, using UV, IR, HPLC, and turbidimetry techniques.[19] Also, one particular impurity of RFX, having *m/z* 802 was investigated and it was concluded that the structure of the impurity (*m/z* 802) is different from the impurity H mentioned in the European Pharmacopoeia (EU) monograph for RFX.[13]

After a thorough literature review and keeping in view the importance of stability-indicating assay methods and impurity profiling for drugs, the current research work was carried out with the goal of developing and validating an RP-HPLC technique for estimating RFX in the presence of its degradation products. Validation of the developed method was performed following the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q2 (R1) guidelines.[20] An LC-MS compatible method was developed for the separation of major degradants which were further analyzed by LC-MS/MS for structure determination.[21]

MATERIALS AND METHODS

Pure Sample of Drug Substance

Rifaximin (RFX) API was kindly gifted by Lupin Ltd., Tarapur, Maharashtra, India.

Chemicals and Reagents

Water, acetonitrile (ACN), methanol, ammonium formate, ammonium bicarbonate and potassium dihydrogen orthophosphate, of HPLC grade; sodium hydroxide (NaOH), hydrochloric acid (HCl) of laboratory-grade and hydrogen peroxide (H_2O_2) of analytical grade were procured from LOBA Chemie Pvt. Ltd., Colaba, Mumbai, India.

Instrumentation

The details of the instruments used in this work are as follows: weighing balance was of Mettler Toledo ME 203, Sonicator of INCO India, pH meter LABINDIA PICO$^+$ and UV-Vis Spectrophotometer (UV-1900) with UV Probe software was of Shimadzu. HPLC was Jasco High-Performance Liquid Chromatograph equipped with a quaternary gradient pump and degasser PU-2089 Plus, with 20 µL loop injector and UV-2075 plus detector. Data acquisition and data processing were carried out using ChromNAV software. The stationary phase used was an Inertsil ODS-3V - C18 column (250 X 4.6 mm, 5 µm). Hot air oven used was from Metalab. LC-MS/MS was carried out using Shimadzu Liquid Chromatograph-Mass Spectrometer (LC-MS-8040) with *m/z* range of 2 to 2000 and an MRM transition speed of 555 channels/sec. The ionization source in mass spectrometer was electrospray ionization (ESI).

Method Development

Rifaximin is a derivative of benzimidazole with log *p-value* of 4.8 and pKa value of 6.3. Because of its higher log P value, the RP-HPLC method was preferred with a bonded phase column, like a C18 stationary phase.

Rifaximin has shown to be almost insoluble in water but is easily soluble in acetonitrile, methanol, and acetone. Based on the solubility data mobile phase was optimized. Considering, that nearly all of the pH-related variation in retention of the drug happens for pH values within 1.5 units of the drug's pKa, efforts were made to choose a buffer that will maintain the mobile phase pH within 1.5 units of the drug's pKa, which is between pH 4.8 and 7.8.[22] It was observed that mobile phase of pH 5 gave the best resolution along with a number of theoretical plates (NTP), retention and tailing factor. Identification of wavelength maxima (λ_{max}) was carried out by scanning 20 µg/mL solution of RFX over the wavelength range of 200 to 400 nm on Shimadzu UV-1900 spectrophotometer; the software used was Lab Solutions. The wavelength maxima (λ_{max}) obtained for RFX was 258 nm. As the degradants and API depicted great variation in their retention factor, k, hence, gradient mode of elution was preferred.[23,24]

Force degradation study

Forced degradation studies were performed to predict drug stability and for the obtain degradants for the purpose of structure elucidation.[25]

Acid hydrolysis

In 5 mL of 1000 µg/mL RFX solution was transferred into a 100 mL round bottom flask. In I5 mL 0.1 M HCL solution was added to it and refluxed at 60°C for 15 minutes. In 1-mL of the solution from the aliquot was pipetted out after 0, 15, 30 minutes and diluted to 10 mL. The solutions were kept at room temperature for cooling, neutralized with 0.05 M NaOH solution and diluted to get final concentration of 50 µg/mL. These solutions were then injected into HPLC.

Alkali hydrolysis

In 5 mL of 1000 µg/mL RFX solution was transferred into a 10 mL volumetric flask. To it 5 mL of 0.05 M NaOH solution was added and this solution was kept at room temperature for 15 minutes. In 1-mL of solution from the aliquot was pipetted out after 0 and 15 minutes and transferred into separate 10 mL

volumetric flasks, neutralized with 1-mL of 0.025 M HCL and the volume made up to 10 mL with diluent to get 50 µg/mL solution and injected into HPLC.

Oxidation degradation

In 5 mL of 1000 µg/mL RFX solution was transferred into a 100 mL round bottom flask. 5 mL of 3% H_2O_2 solution was added to it and refluxed at 80˚C for one hour. Then 1-mL of solution from the aliquot was pipetted out after 0, 15, 30, 45 and 60 minutes and transferred into separate 10 mL volumetric flasks. The samples were cooled at room temperature and the volume made up with diluent to get 50 µg/mL solutions. Further, these solutions were injected into HPLC.

- *Neutral hydrolysis*

In 5 mL of 1000 µg/mL RFX solution was transferred into a 100 mL round bottom flask. In 5 mL of double distilled water was added to it and the solution was refluxed at 80˚C for 1-hour. In 1-mL of solution from the aliquot was pipetted out after 0, 15, 30, 45 and 60 minutes and transferred into separate 10 mL volumetric flasks. The samples were cooled at room temperature and the volume made up with diluent to get 50 µg/mL solution and injected to HPLC.

Photodegradation

Approximately, 1000 µg/mL of RFX solution was kept in sunlight. 0.5 mL of the aliquot was pipetted out after 1, 2, 3, 4 hours and transferred to separate 10 mL volumetric flasks and the volume was made up with diluent to get 50 µg/mL solution and injected to HPLC.

Thermal degradation

RFX in the solid state was heated at 80˚C temperature in a hot air oven for seven days. Thereafter, 50 µg/mL solution of this degraded API was prepared with diluent and analyzed by HPLC.

Optimized Stability-indicating Assay Method

All the degraded samples were mixed and diluted to get final concentration of 50 µg/mL. This was done to check the suitability of the method for a maximum challenging sample. The method was then optimized to separate RFX and its degradation products obtained in different stress conditions.

Validation of the Developed Stability Indicating HPLC Method

According to the ICH Q2 (R1)[20] guideline for validation of analytical procedures, the developed SIAM was validated for the following validation parameters.

Linearity

A sequence of dilutions containing 40, 60, 80, 100 and 120 µg/mL RFX were prepared from its stock solution (1000 µg/mL). The chromatograms were recorded, and a standard calibration curve was plotted between the concentration (µg/mL) of RFX and the response (peak area) obtained for each concentration.

Precision and accuracy

The developed method was assessed for repeatability and intermediate (intra and inter-day) precision and accuracy was assessed by spiking and recovery study. The test concentration was taken as 50 µg/mL and the samples were spiked at concentrations of 80, 100 and 120% of the test concentration to assess the accuracy. The %recovery and coefficient of variation was calculated to measure accuracy, while the coefficient of variation was used to determine precision.

Limit of detection and Limit of quantitation

The method used for estimating the limit of detection (LoD) and the limit of quantitation (LoQ) was by determining the standard deviation (SD) of the response obtained for various concentrations and slope obtained for the calibration curve. Serial dilutions of RFX were prepared to obtain solutions of concentration 0.1, 0.3, 0.5, 0.7, 0.9 µg/mL, in triplicate. The linearity of these samples was assessed by plotting the calibration curve.

Specificity and selectivity

All the degraded samples were mixed and diluted to get final concentration of 50 µg/mL. This was done to check the suitability of the method for a maximum challenging sample. The chromatogram obtained (Figure 1). was then integrated.

LC-MS/MS studies on acid, alkali and oxidative degradation samples of RFX

Fractions of major degradation products (DPs) for each stressed condition sample were collected chromatographically and outsourced to an external facility for identification using LC–MS and further LC-MS/MS.

A separate MS-compatible LC method was developed using the mobile phase ammonium formate buffer pH 7 ± 0.05 and ACN (60:40), the stationary phase was a Finepeak SIL C18 column, 250 X 4.6 mm, 5 µm kept at ambient temperature, and the detection wavelength was 258 nm. The mobile phase flow rate was 0.8 mL/min, and the injection volume was 20 µL.

RESULT AND DISCUSSION

The separation of RFX and its degradants was achieved on the optimized method. The finalized stationary phase was Inertsil ODS-3V, C18 column (250 X 4.6 mm X 5 µm), Mobile phase A and B were 10 mM potassium dihydrogen orthophosphate (pH 5.0 ± 0.05) and acetonitrile, respectively. The flow rate of the mobile phase was 1-mL/min and the injection volume was 20 µL. The selected wavelength of detection (λ_{max}) was

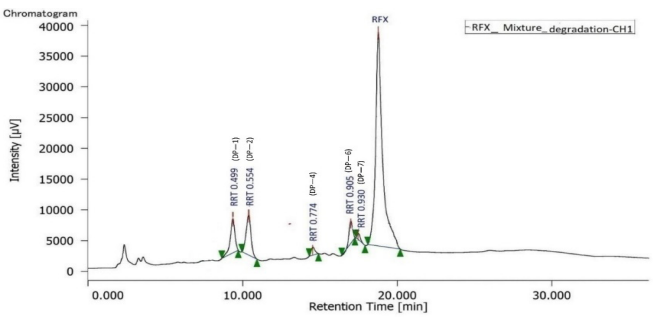

Figure 1: Chromatogram obtained for RFX and its degradants in final chromatographic conditions

258 nm. Separation was carried out in gradient mode (0/70:30, 15/45:55, 25/40:60, 32/60:40, 40/70:30) with a total run time of 41 minutes. The method was validated as per the ICH Q2 (R1) guidelines.[20]

Force Degradation Study

Forced degradation studies were performed to predict drug stability, develop SIAM and for the obtaining degradants for the purpose of structure elucidation.

Acid Hydrolysis

Four degradation products (DPs), DP-3, DP-4, DP-5 and DP-6 were observed at RRT of 0.69, 0.79, 0.86 and 0.89, respectively. Amongst these, DP-4 was significantly high in concentration, hence, to investigate its structure the sample was further degraded to obtain a high concentration of DP-4 (56.45 %). The chromatogram obtained is shown in Figure 2.

Alkali Hydrolysis

DP-4, DP-5 and DP-6 at RRT of 0.79, 0.86 and 0.89 respectively were obtained in alkali degraded sample of RFX. The representative chromatogram obtained is shown in Figure 3.

Oxidation Degradation

As evident from Figure 4, the drug showed desirable degradation (DP-1, DP-2, DP-4, DP-6 and DP-7 at RRT of, 0.49, 0.55, 0.79, 0.89 and 0.93, respectively) by refluxing it at 80˚C for 1-hour with 3% H_2O_2.

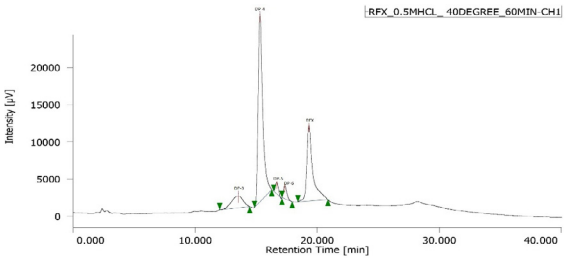

Figure 2: Acid degradation chromatogram

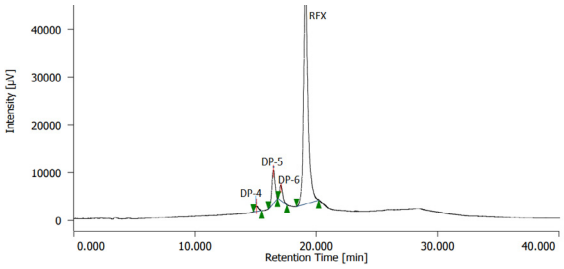

Figure 3: Alkali hydrolysis degradation chromatogram

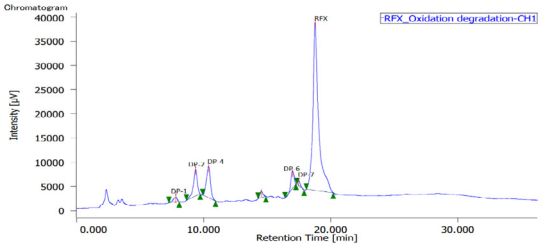

Figure 4: Oxidation degradation chromatogram

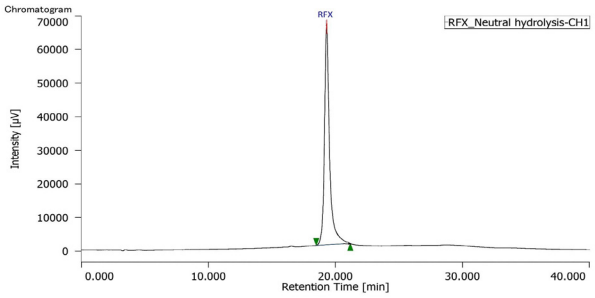

Figure 5: Neutral hydrolysis degradation chromatogram

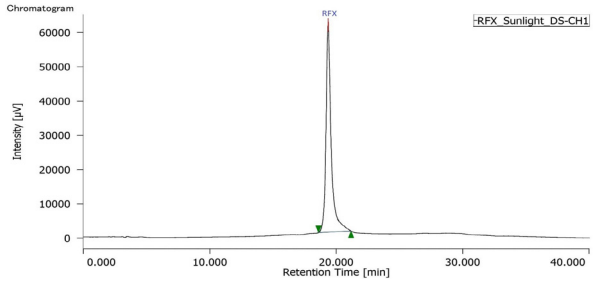

Figure 6: Photodegradation chromatogram

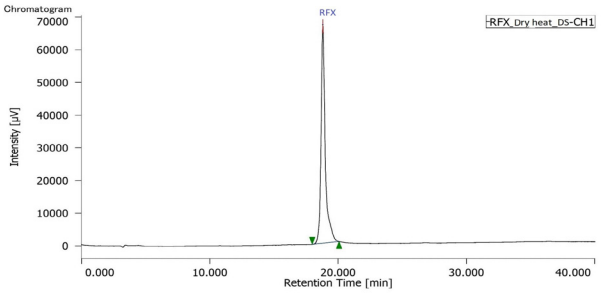

Figure 7: Dry heat degradation chromatogram

Neutral Hydrolysis

The drug was found to be stable in the neutral hydrolytic condition (Figure 5).

Photodegradation

The drug depicted stability in the photodegradation conditions (Figure 6).

Thermal Degradation

The drug did not show any degradation in the dry heat degradation condition (Figure 7).

Method Validation

Linearity of the method was assessed using calibration curve method which was found to be a straight line over the concentration range of 40 to 120 µg/mL. The least-square linear regression equation obtained was y = 47995x+67851 and the correlation coefficient (R^2) was 0.998. For the precision (repeatability) study, six replicates of RFX, at a concentration of 50 µg/mL, were injected and the %RSD was calculated on the response (peak area) obtained. The %RSD was 0.53. Intermediate precession was assessed by intra and interday precision study. The %RSD obtained for intraday studies was

Table 1: Summary of the degradation studies

Forced degradation condition		*(DP-1) 0.49 772.4*	*(DP-2) 0.55 838.4*	*(DP-3) 0.69 784.2*	*(DP-4) 0.79 744.5*	*(DP-5) 0.86 784.3*	*(DP-6) 0.89 753.8*	*(DP-7) 0.93 801.9*	*RFX*
0.1 M HCl reflux at 60˚C for 30 minutes.	% Degradation			8.914	56.449	2.292	2.804		29.541
0.05 M NaOH kept at room temperature for 15 minutes.				--	1.657	9.349	4.104		84.890
3% H_2O_2 reflux at 80˚C for 1 hour.		7.950	9.791		1.543		3.973	0.925	75.818

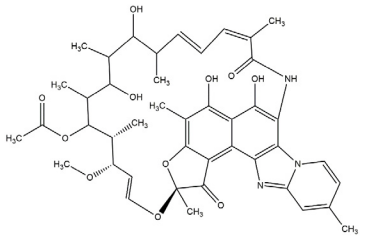

Figure 8: Rifaximin structure (*m/z* of 785.9)

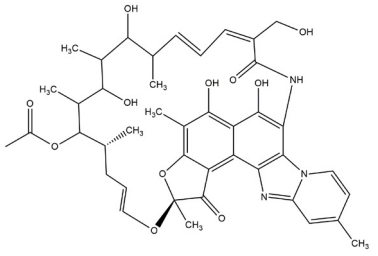

Figure 9: Rifaximin degradant 1 (*m/z* 772.4)

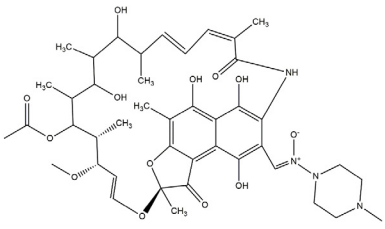

Figure 10: Rifaximin degradant 2 (*m/z* 838.4)

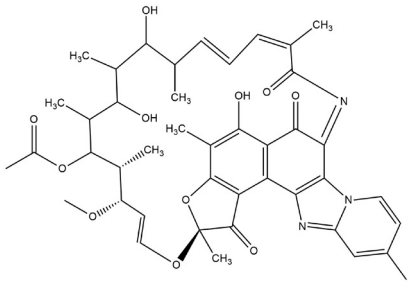

Figure 11: Rifaximin degradant 3 (*m/z* 784.2, EP Impurity G)

1.2. The %RSD obtained for the intermediate precision day 1, day 2 and day 3 studies were 1.5, 0.6 and 0.3, respectively. In all the cases, the % RSD values were below the acceptable limit of 2.0%.

The accuracy of the method was determined by the recovery studies. The mean %recovery for spiked samples, at 80, 100 and 120% of the test concentration, was 84.3, 88.4 and 88.4, respectively, which is within the acceptable range of 80 to 120%. LoD and LoQ were found to be 0.03 and 0.1060 µg/mL, respectively. This method can be used for the quantification of RFX in presence of its degradants.

LC-MS Characterization of Impurities

Forced degradation studies were carried out by employing acid, alkali, oxidative, thermal, and photolytic stress conditions on RFX drug substances. The drug was stable in neutral hydrolysis, photo and dry heat degradation conditions. However, significant degradation was observed during acid (70.46%), due to the presence of lactone ring, ester and hydroxyl group in RFX structure followed by base hydrolysis (15.11%) and oxidation (24.18 %) degradation conditions. The

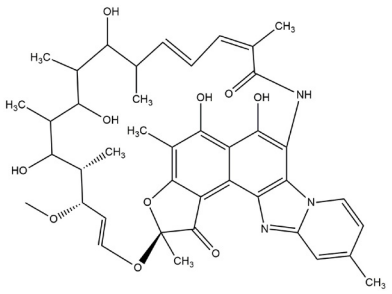

Figure 12: Rifaximin degradant 4 (*m/z* 744.5)

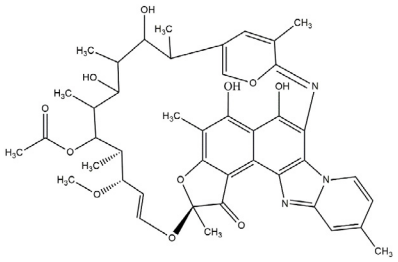

Figure 13: Rifaximin degradant 5 (*m/z* 784.3)

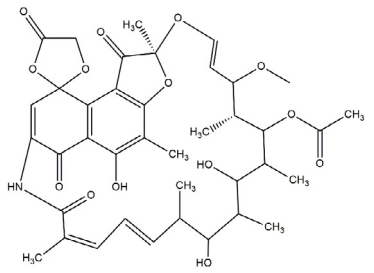

Figure 14: Rifaximin degradant 6 (*m/z* 753.8, EP Impurity F)

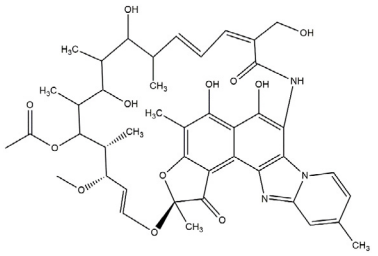

Figure 15: Rifaximin impurity 7 (*m/z* 801.9, EP Impurity H)

degraded samples were investigated by LC-MS. Four (*m/z* 784.2, 744.5, 784.3, 753.8), three (*m/z* 744.5, 784.3, 753.8) and five (*m/z* 772.4, 838.4, 744.5, 753.8, and 801.9) degradants were found in samples degraded in acid, alkali and oxidative stress conditions, respectively. The details of the degradation studies are summarized in Table 1.

All values of degradants 1-7 are reported as area % by HPLC

DP-1 (t_R = 9.3 min, RRT = 0.49, m/z = 772.4); DP-2 (t_R = 10.4 min, RRT = 0.55, m/z = 838.4); DP-3 (t_R = 13.5 min, RRT = 0.69, m/z = 784.2); DP-4 (t_R = 15.3 min, RRT = 0.79, m/z = 744.5); DP-5 (t_R = 16.7 min, RRT = 0.86, m/z = 784.3); DP-6 (t_R = 17.3 min, RRT = 0.89, m/z = 753.8), DP-7 (t_R = 17.5 min, RRT = 0.93, m/z = 801.9)

The structures of the generated degradants were elucidated with the help of LC-MS data which are depicted here in Figures 8 to 15.

CONCLUSION

A simple and novel HPLC approach for assessing the stability of RFX has been developed. The developed method has been validated and it meets the ICH criteria.[20] A total of seven degradants were generated during the forced degradation study which were further investigated by LC-MS for structural elucidation. It has been observed that a total of three generated degradants were reported in the European Pharmacopeia as Impurity G (Figure 11), F (Figure 14) and H (Figure 15), respectively. Remaining degradants 1 (Figure 9), 2 (Figure 10), 4 (Figure 12) and 5 (Figure 13) with *m/z* of 772.4, 838.4, 744.5 and 784.3 were the novel impurities generated during the stability studies.

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