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DEVELOPMENT AND VALIDATION OF A NOVEL HPLC METHOD FOR IMPURITY PROFILING IN FIDAXOMICIN FILM-COATED TABLETS WITH LC-MS CHARACTERIZATION

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ABSTRACT

A novel reverse-phase high-performance liquid chromatography (RP-HPLC) approach was created and validated for the quantification of impurities in 200 mg film-coated tablets of Fidaxomicin. This method effectively distinguishes Fidaxomicin from its degradation products. The validation was executed in accordance with the International Council for Harmonization (ICH) guidelines, which included parameters such as specificity, precision, linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), and robustness. Five impurities associated with degradation were identified and characterized using the appropriate impurity standards. Chromatographic separation was achieved on an X Bridge C18 column (250 × 4.6 mm, 5 µm), utilizing a mobile phase system that consisted of mobile phase A (phosphate buffer, pH 7, and methanol in an 80:20 ratio) and mobile phase B (100% acetonitrile). The flow rate was established at 1.0 mL/min, with an injection volume of 10 µL, a run time of 50 minutes, and detection conducted at a wavelength of 237 nm. The structures of the five degradation impurities were elucidated through LC-MS analysis. In conclusion, the RP-HPLC method developed is robust, specific, stability-indicating, and suitable for routine quality control and stability evaluation of Fidaxomicin film-coated tablet formulations.

Keywords: Fidaxomicin tablet, Accuracy, Linearity, Method Validation, and Reverse Phase HPLC.
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INTRODUCTION

Fidaxomicin is known as a macrolide antibiotic, used to treat Clostridium difficile-associated diarrhoea (CDAD).[1] It works by killing bacteria or preventing their growth. Chemically it is [(2R,3S,4S,5S,6R)-6-[[(3E,5E,8S,9E,11S,12R,13E,15E,18S)-12-[(2R,3S,4R,5S)-3,4-dihydroxy-6,6-dimethyl-5-(2-methylpropanoyloxy)oxan-2-yl]oxy-11-ethyl-8-hydroxy-18-[(1R)-1 hydroxyethyl]-9,13,15-trimethyl-2-oxo-1-oxacyclooctadeca-3,5,9,13,15-pentaen-3-yl]methoxy]-4-hydroxy-5-methoxy-2-methyloxan-3-yl] 3,5-dichloro-2-ethyl-4,6-dihydroxybenzoate. Its molecular formula is $C_{52}H_{74}Cl_2O_{18}$, and the monoisotopic mass is 1058.[2] Fidaxomicin (DIFICID) was developed by Optimer Pharmaceuticals Inc. and received FDA approval on May 27, 2011, for CDAD.[3] According to BCS, it is a class IV drug (Low soluble and Low permeable).[4] Fidaxomicin appears as a white to off-white powder, with a melting point ranging from 165 °C-169 °C.[5] It is freely soluble in methanol, methylene dichloride and sparingly soluble in isopropyl alcohol and slightly soluble in water. The compound has a pKa value of 5.87 (strongly Acidic). Chemical structure of Fidaxomicin is represented in Fig.-1.

The impurity profile of a pharmaceutical product is a critical aspect of its quality evaluation. Ineffective control of impurities can pose significant safety risks in clinical applications.[6] For instance, cephalosporins are known to exhibit a relatively high incidence of allergic reactions,[7] which has been attributed to the presence of polymeric impurities. Traditional gel filtration chromatography methods, such as those using Sephadex G-10, have shown limited efficiency in detecting these polymerised species in cefotiam hydrochloride.[8] High-performance gel permeation chromatography (HPLC-GPC) with TSK-GEL G2000SWXL columns has emerged as a superior technique for detecting and controlling polymeric

impurities in cephalosporins. This method offers improved separation efficiency, faster analysis, and greater sensitivity, ultimately reducing allergic reactions.[9]

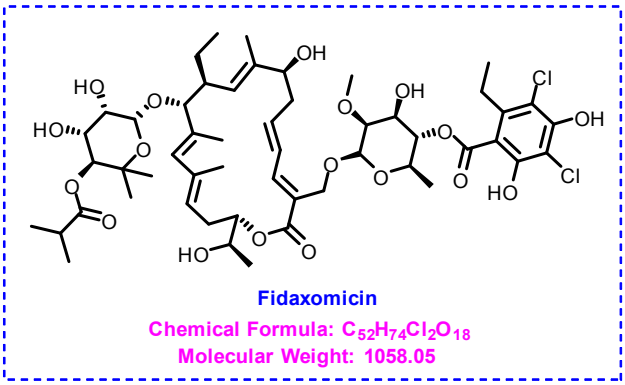

Fig.-1: Chemical Structure of Fidaxomicin

Quality by Design (QbD) is a systematic framework that uses knowledge management and risk assessment to develop and optimise pharmaceutical processes. It has become a key paradigm in establishing robust analytical methods within the pharmaceutical industry. Various factors can influence the performance and reliability of chromatographic procedures.[10] For example, Teng Chen et al. applied the QbD approach to optimise the chromatographic purification of saponins from Panax notoginseng extract, demonstrating its effectiveness in enhancing process understanding. The QbD strategy facilitates a deeper comprehension of process parameters, enabling the development of methods that are both controllable and adaptable. Overall, the application of QbD enhances the efficiency of analytical method development and ensures the generation of high-quality, reliable analytical data.[11] High-performance liquid chromatography (HPLC) methods for the separation and determination of impurities in Fidaxomicin have been reported in the literature. However, degradation products such as Fidaxomicin D and E have not yet been characterised. The presence of these degradation impurities may affect the clinical efficacy and increase potential toxicity. According to the guidelines of the International Council for Harmonisation (ICH) on the registration of pharmaceuticals for human use, impurities present at levels exceeding 0.1% must be structurally identified. Therefore, structural characterisation of Fidaxomicin impurities is essential to ensure the drug's quality, safety, and efficacy. HPLC-MS is commonly used for drug impurity characterization, but typically requires volatile mobile phases. The official USP method for Fidaxomicin, however, uses a non-volatile mobile phase, which, when adapted for MS, can compromise selectivity and sensitivity, and alter impurity elution order compared to the original HPLC chromatogram. A literature review revealed that Fidaxomicin does not currently have an official monograph in any of the widely recognised pharmacopoeias. Moreover, existing chromatographic methods for its quantification in bulk substances and pharmaceutical formulations are limited. This highlights the necessity for developing a new reverse-phase chromatographic method that is simple, rapid, accurate, and precise for the estimation of Fidaxomicin in tablet dosage forms. A novel, ICH Q2 (R1) compliant HPLC method was developed using a Quality by Design (QbD) approach to determine impurities in Fidaxomicin film-coated tablets, improving upon the official monograph. This method effectively resolved Fidaxomicin from forced degradation impurities, five of which were characterized by LC-MS in positive ionisation mode.

EXPERIMENTAL

Chemicals and Reagents

Fidaxomicin 200 mg film-coated tablets (Brand name: Dificid) provided by TEVA Pharmaceutical Industries Ltd., Fidaxomicin reference substance purchased from TEVA Pharmaceutical Industries Ltd., Fidaxomicin Impurity A, Fidaxomicin Impurities A, B, C, D and E were purchased from TLC Pharma Labs. Acetonitrile and methanol were used in analytical grade purchased from Merck. Sodium dihydrogen phosphate monohydrate (analytical grade) was purchased from Merck. Ammonia solution (Emparta grade), hydrochloric acid (analytical grade), 30 % hydrogen peroxide solution (analytical grade),

and sodium hydroxide analytical grade. HPLC grade water (filtered by a Millipore Milli-Q-Gradient purification system).

Instrumentation

HPLC Apparatus

Chromatographic separation was performed at 30°C using a Waters Alliance 2695 separations module with a Waters 2487 dual absorbance detector and an X-bridge C18 250 x 4.6mm, 5µm column. The mobile phase comprised (A) 0.1 M sodium dihydrogen phosphate in H_2O (pH 7.0 with ammonia) and methanol (80:20), and (B) acetonitrile. A gradient was applied: 0-15 min, 30-40% B; 15-25 min, 40-45% B; 25-40 min, 45% B; 41 min, 30% B; 50 min, 30% B. Detection was at 237 nm using an Agilent diode array detector. The flow rate was 1.0 $mL \cdot min^{-1}$, with a 5 µL injection volume. Data was processed with Waters Empower software.

LC-MS

A trap-free one-dimensional Nexera-XR liquid chromatograph (Shimadzu) was used, featuring two binary pumps (LC-30AD), an autosampler (SIL-30AC), a column thermostat (CTO-20A), and a diode-array detector (SPD-M20A). Chromatographic separation in the first dimension matched the conditions in Section 2.2.1, with a mobile-phase flow rate of 1.0 mL $min^{-1}$and a 5 µL injection volume.

UV-Visible spectrophotometer

Fidaxomicin solution (10 µg/mL) in mobile phase was scanned from 200-400 nm, showing a maximum absorbance (λmax) at 237 nm.

Preparation of Mobile Phase

Buffer preparation: Dissolve 1.38 g of sodium dihydrogen phosphate monohydrate in 1000 mL of water. Adjust the pH to 7.0 ± 0.05 using diluted ammonia solution.

Mobile Phase-A: Mix Buffer: Methanol in the ratio of 80:20 %v/v and filter through a 0.45µm PVDF membrane filter and degass.

Mobile phase-B: 100% Acetonitrile.

Diluent Preparation

A diluent was prepared by mixing water and acetonitrile in a 50:50 (v/v) ratio.

Preparation of Standard Solution

To prepare a 0.0005 mg/mL Fidaxomicin standard solution, begin by weighing 40 mg of Fidaxomicin and transferring it to a 100 mL volumetric flask. Add 60 mL of diluent, sonicate to dissolve, then bring the volume to 100 mL with diluent, mixing thoroughly to achieve a 0.4 mg/mL stock solution. Next, dilute 5 mL of this stock solution to 50 mL with diluent, mix well, then further dilute 5 mL of that solution to 100 mL with diluent, mixing well, resulting in a 0.002 mg/mL solution. Finally, dilute 5 mL of the 0.002 mg/mL solution to 20 mL with diluent and mix thoroughly to obtain the 0.0005 mg/mL standard solution.

Preparation of Sample Solution

Transfer 360 mg of powdered Fidaxomicin 200 mg film-coated tablets (equivalent to 200 mg Fidaxomicin) to a 200 mL flask. Add 75 mL of water and sonicate for 15 minutes. Add 75 mL of acetonitrile and sonicate for 30 minutes. Dilute to volume with diluent and mix thoroughly. Centrifuge at 4000 rpm for 5 minutes. Filter through a 0.45µm syringe filter, discarding the first 5 mL of filtrate.

Impurity-A Stock Solution Preparation

2.7094 mg of Impurity-A was weighed, transferred to a 50 mL volumetric flask, dissolved in 35 mL of diluent with sonication, and brought to volume with diluent.

Impurity-B Stock Solution Preparation

Precisely 2.61 mg of Impurity-B was weighed and transferred to a 50mL volumetric flask. Approximately 35 mL of diluent was added, and the mixture was sonicated until dissolved, then diluted to volume with the same diluent.

Impurity-C Stock Solution Preparation
2.62 mg of Impurity-C was precisely weighed into a 50 mL volumetric flask, dissolved in approximately 35 mL of diluent using sonication, and then diluted to the mark with the same diluent.

Impurity-D Stock Solution Preparation
Accurately weigh 2.66 mg of Impurity-D into a 50 mL volumetric flask. Add approximately 35 mL of diluent, sonicate until dissolved, and then dilute to the mark with the same diluent.

Impurity-E Stock Solution Preparation
Accurately weigh 2.60 mg of Impurity-D and transfer to a 50 mL volumetric flask. Add approximately 35 mL of diluent and sonicate until completely dissolved. Dilute to the mark with the same diluent.

Preparation of Sample Solutions for Forced Degradation Studies
Acid Degradation
To evaluate acid-induced degradation, 360 mg of powdered Fidaxomicin 200 mg film-coated tablets (equivalent to 200 mg Fidaxomicin) was dissolved in 20 mL of 0.01 N HCl, sonicated for 30 minutes, and then left at room temperature for another 30 minutes. The solution was neutralised with 0.01 N NaOH, diluted to volume with a diluent, filtered through a 0.45 µm membrane, and analysed by HPLC.

Base Degradation
To evaluate base-induced degradation, 360 mg of Fidaxomicin 200 mg film-coated tablet powder (which corresponds to 200 mg of Fidaxomicin) was precisely measured and placed into a 200 mL volumetric flask. Subsequently, 20 mL of 0.01 N NaOH was added, and the mixture was sonicated for 30 minutes before being allowed to sit on the benchtop for an additional 30 minutes. The resulting mixture was neutralized using 0.01 N HCl and then diluted to the desired volume with the diluent. Finally, the solution was filtered through a 0.45 µm membrane filter and analysed using HPLC.

Oxidative Degradation
To assess oxidative degradation, 360 mg of powdered Fidaxomicin 200 mg film-coated tablets (200 mg Fidaxomicin equivalent) was combined with 20 mL of 10% H_2O_2 solution in a 200 mL volumetric flask. After 10 minutes of sonication and 30 minutes at room temperature, the mixture was diluted to volume with diluent, filtered through a 0.45 µm membrane, and analysed by HPLC.

Thermal Degradation
A 360 mg Fidaxomicin sample was thermally degraded at 105 °C for 3 hours in a hot air oven. The resulting solution was filtered (0.45 µm) and analysed by HPLC.

RESULTS AND DISCUSSION

Selection of the Concentration of Sodium Dihydrogen Phosphate Solution
To optimize chromatographic separation of Fidaxomicin and its degradation impurities, sodium dihydrogen phosphate buffer solutions (0.005 M, 0.01 M, 0.05 M) were tested. Higher buffer concentrations improved peak resolution, column efficiency, and sensitivity. However, concentrations exceeding 0.02 M caused salt precipitation with acetonitrile, leading to mobile phase instability. Thus, 0.01 M sodium dihydrogen phosphate was chosen for its optimal separation performance, solubility, and mobile phase compatibility.

Selection of the pH value of Sodium Dihydrogen Phosphate Solution
Mobile phase pH (6.8, 7.0, and 7.2) did not significantly affect the chromatographic separation of Fidaxomicin and its impurities. A pH of 7.0 (measured in 0.001 N sodium dihydrogen phosphate solution) was chosen for subsequent analyses to ensure mobile phase stability.

Selection of Flow Rate
To optimize chromatographic separation, various flow rates (0.8, 1.0, and 1.2 $mL \cdot min^{-1}$) were tested. A flow rate of 1.0 $mL \cdot min^{-1}$ was found to be optimal; deviations led to distorted peak shapes for Fidaxomicin and its impurities, indicating reduced separation efficiency.

Influence of Gradient Elution Program

Initially, a sodium dihydrogen phosphate buffer (pH 7.0) was used as the aqueous phase, providing good resolution for Fidaxomicin and its degradation impurities. However, the preliminary gradient elution program yielded suboptimal separation and low resolution among certain impurity peaks. To improve this, the gradient profile was optimised by adjusting the mobile phase composition's rate of change, specifically by slowing the increase of the organic component. This enhanced analyte-stationary phase interaction, leading to a more stable baseline, better peak shape, and increased sensitivity for minor impurity detection. The optimised gradient ultimately produced a robust and reliable chromatographic method for analysing Fidaxomicin and its related impurities.

Influence of Injection Volume

Injection volume significantly impacts chromatographic separation. Volumes of 5, 10, and 20 µL were tested, revealing that larger injections led to broader peaks, lower resolution, and decreased sensitivity. Thus, 10 µL was chosen as the optimal injection volume.

Influence of Column Temperature

The optimal column temperature for chromatographic separation was determined to be 30 °C. Temperatures outside of 30 °C, specifically 25, 35, and 40 °C, resulted in peak distortion and reduced separation efficiency for Fidaxomicin and its degradation products.

Method Validation

The developed chromatographic method was validated in accordance with ICH guidelines by evaluating parameters such as system suitability,[13] specificity, linearity,[14] precision,[15] accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness.[16]

System Suitability

HPLC system suitability tests confirmed its performance and reliability. Six replicate injections of 30 µg/mL Fidaxomicin standard and a single blank injection were performed. Chromatograms were recorded, and column efficiency and peak symmetry were assessed via tailing factor and theoretical plate calculations. All parameters met acceptable limits, validating the chromatographic system for analysis. The system suitability results are presented in Table-1.

Table-1: Results of System Suitability Parameters

S. No.	Name	Rt (Min)	Area(AU)	USP Tailing	USP Plate Count
1	Fidaxomicin	27.545	2986034	1.2	31143

Specificity

To evaluate the specificity of the method, injections of the blank, standard drug solution (30 µg/mL), and sample solution (30 µg/mL) were performed. The chromatogram of the blank showed no peaks at the retention time of Fidaxomicin, while well-resolved peaks were observed in both the standard and sample chromatograms at the same retention time. This indicates that there was no interference from excipients or diluents, confirming the specificity of the method. The specificity results are summarised in Table-2, and the chromatograms of the blank, standard, and sample solutions are shown in Fig.-2 to Fig.-4.

Table-2: Specificity Result of Fidaxomicin and its Impurities

Name	Retention Time (t_R)	Area(AU)	Resolution	USP Tailing	USP plate count
Fidaxomicin	25.970	2986034	3.56	1.2	31143
Fidaxomicin-A	22.654	123306	3.41	1.02	26783
Fidaxomicin-B	36.534	132875	11.11	1.21	49970
Fidaxomicin-C	27.770	153573	3.04	1.29	34272
Fidaxomicin-D	20.827	129190	28.18	1.06	25655
Fidaxomicin-E	29.554	141819	2.97	1.19	38036

Linearity of Response

The detector response to varying concentrations of Fidaxomicin 200 mg film-coated tablets and their impurities demonstrated satisfactory linearity. As shown in Table-3 and Fig.-5, the coefficient of determination for Fidaxomicin and five impurities ranged from 0.9835 to 1.0000.

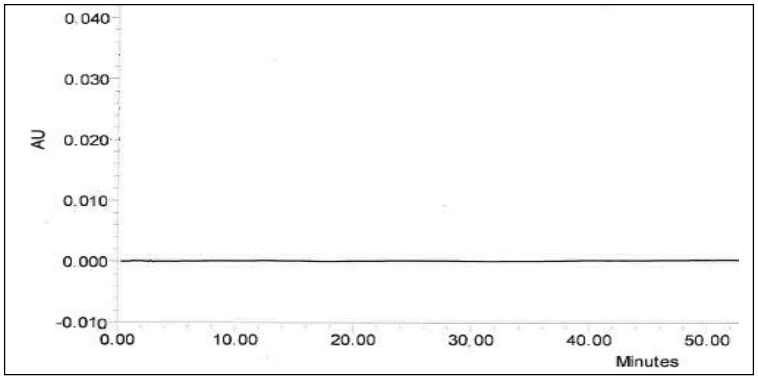

Fig.-2: Chromatogram of Blank Under Optimized Conditions

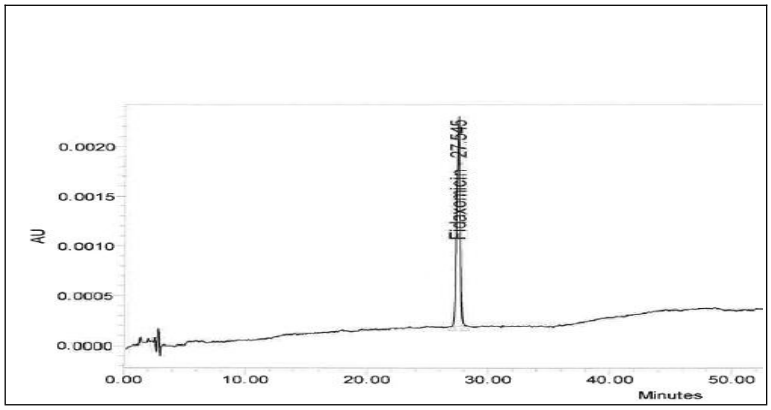

Fig.-3: Chromatogram of Standard (Fidaxomicin) under Optimized Conditions

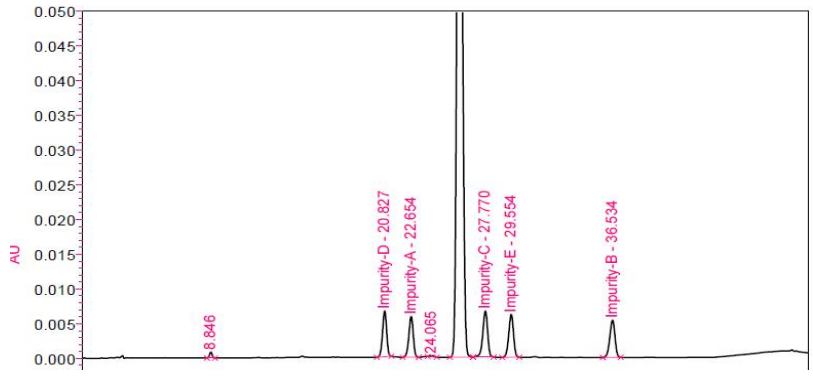

Fig.-4: Chromatogram of Fidaxomicin and its Impurities Under Optimized Condition

Table-3: The linearity of Fidaxomicin and its Impurities

Fidaxomicin/Imp.	Concentration range (mg/mL)	R^2	Correction factor
Fidaxomicin	0.01-3.098	0.9859	NA
Fidaxomicin Imp. A	0.01-3.079	0.9864	1.19
Fidaxomicin Imp. B	0.01-2.947	0.9857	1.09
Fidaxomicin Imp. C	0.01-3.122	0.9839	0.96
Fidaxomicin Imp. D	0.01-3.217	0.999	1.07
Fidaxomicin Imp. E	0.01-3.013	0.9980	1.01

Accuracy

Method accuracy was assessed through recovery studies at 50%, 100%, and 150% of the target concentration, with three replicates per level. Percentage recovery and per cent relative standard deviation (% RSD) were calculated. All results were within acceptable limits, confirming the method's accuracy and reliability. Results are in Table-4.

Table-4: The Results of the Accuracy and Recovery

Sample Name	% Level	Amount Added (mg/mL)	Amount Found (mg/mL)	% Recovery	% RSD
Fidaxomicin	50%	0.0020515	0.002161	105.35	1.72
	100%	0.0039452	0.004062	102.96	
	150%	0.0063123	0.006723	106.51	
Fidaxomicin A	50%	0.0003135	0.0003221	102.74	1.65
	100%	0.0069665	0.0071251	102.28	
	150%	0.0104498	0.0110189	105.45	
Fidaxomicin B	50%	0.0002970	0.0003050	102.7	0.67
	100%	0.0019802	0.0020106	101.54	
	150%	0.0029702	0.0030144	101.49	
Fidaxomicin C	50%	0.0002966	0.0003143	106.0	1.87
	100%	0.0039540	0.0043149	109.1	
	150%	0.0059310	0.0065099	109.8	
Fidaxomicin D	50%	0.0003056	0.0003116	101.96	1.93
	100%	0.0040430	0.0041402	102.40	
	150%	0.0058713	0.0062012	105.62	
Fidaxomicin E	50%	0.0003086	0.0003093	100.23	0.95
	100%	0.0040320	0.0040521	100.50	
	150%	0.0058920	0.0062087	105.38	

Limit of detection and Limit of Quantification

The limit of detection (LOD) and limit of quantification (LOQ) of Fidaxomicin were determined using the calibration curve method, based on the following equations:

$$LOD = 3.3 \times (\sigma/S)$$

$$LOQ = 10 \times (\sigma/S)$$

The limits of detection (LOD) and quantification (LOQ) were determined using the standard deviation of the response (σ) and the slope of the calibration curve (S). To confirm method sensitivity, standard solutions at the calculated LOD and LOQ concentrations were injected into the HPLC system. The lowest concentration was chosen based on baseline noise, and the signal-to-noise (S/N) ratio was assessed to ensure it met the criteria for LOD (S/N ≈ 3:1) and LOQ (S/N ≈ 10:1). Results are presented in Tables-5 and 6.

Table-5: LOD Results of Fidaxomicin and its Impurities

Component	Concentration (mg/mL)	Concentration with respect to the sample solution (%)	Signal-to-noise ratio (USP/EP)
Fidaxomicin	0.0000774	0.008	5.59/5.59
Impurity-A	0.0000780	0.008	5.45/5.45
Impurity-B	0.0000760	0.008	4.82/4.82
Impurity-C	0.0000759	0.008	8.69/8.69
Impurity-D	0.0000769	0.008	7.59/7.59
Impurity-E	0.0000771	0.008	6.51/6.51

Table-6: LOQ Results of Fidaxomicin and its Impurities

Component	Concentration (mg/mL)	Concentration with respect to the sample solution (%)	Signal-to-noise ratio (USP/EP)
Fidaxomicin	0.0004033	0.04	200/200
Impurity-A	0.0003135	0.03	144/144
Impurity-B	0.0002970	0.03	61/61
Impurity-C	0.0002966	0.03	104/104
Impurity-D	0.0003056	0.03	102/102
Impurity-E	0.0003176	0.03	99/99

Robustness

To assess the robustness of the method for determining related substances in Fidaxomicin 200 mg film-coated tablets, deliberate variations were introduced. These variations included the stability of analytical solutions, detection wavelength, mobile phase flow rate, column oven temperature, and mobile phase buffer pH. The standard solution remained stable for 40 hours at bench-top conditions and 72 hours under refrigeration, while the sample solution was stable for 72 hours under both conditions. No significant variation was observed with minor changes in detection wavelength. However, small changes in mobile phase flow rate resulted in a minor but significant variation. Column oven temperature proved critical, as higher temperatures increased Impurity-C recovery. Consequently, the allowable column oven temperature range is 25-30°C. Results from these varied conditions are summarised in Tables-7 to 10.

Table-7: Stability of Analytical Solutions (Spiked sample solutions)

Parameter	Specification NMT	Limit (Difference from initial) NMT	Initial	30 hours		72 hours	
				Bench top	Refrigerator	Bench top	Refrigerator
Impurity-A (%)	0.7%	NMT 20%	0.77	0.76	0.76	0.73	0.74
Difference	NA				1.3		
Difference	NA		NA	1.3	1.3	5.2	3.9
Impurity-B (%)	0.2%		0.21	0.22	0.22	0.20	0.21
Difference	NA		NA	4.8	4.8	4.8	0.0
Impurity-C (%)	0.4%		0.43	0.43	0.43	0.41	0.42
Difference	NA		NA	0.0	0.0	4.7	2.3
Impurity-D (%)	0.2%		0.21	0.22	0.21	0.21	0.22
Difference	NA		NA	0.02	0.01	0.01	0.0
Impurity-E (%)	0.2%		0.20	0.21	0.22	0.21	0.22
Difference	NA		NA	0.01	0.02	0.01	0.0
Total impurities (%)	2.0%	NMT 15%	1.49	1.49	1.49	1.53	1.45
Difference	NA		NA	0.0	0.0	2.7	2.7

Conclusion

Standard solution is stable up to 40 hours on the bench top and 72 hours in refrigerated conditions. The sample solution is stable up to 72 hours on the bench top and in refrigerated conditions.

Table-8: Effect of Change in Detection Wavelength (237 ± 2 nm)

Parameter	Acceptance criteria	Actual (237 nm)	Change-1(235 nm)	Change-2(239 nm)
		Spiked sample	Spiked sample	Spiked sample
Impurity-A (%)	NMT 0.7%	0.767	0.76	0.77
% Recovery	85% to 115%	111.1	109.4	111.0
Impurity-B (%)	NMT 0.2%	0.217	0.22	0.21
% Recovery	85% to 115%	109.0	113.0	108.3
Impurity-C (%)	NMT 0.4%	0.430	0.42	0.44
% Recovery	85% to 115%	109.1	106.5	113.4

Impurity-D (%)	NMT 0.2%	0.410	0.408	0.405
% Recovery	85% to 115%	106.1	105.5	104.2
Impurity-E (%)	NMT 0.2%	0.395	0.401	0.399
% Recovery	85% to 115%	104.3	105.2	104.8
Total impurities	NMT 2.0%	1.493	1.47	1.50

Conclusion

No significant variation observed in the results due to a small variation in detection wavelength.

Table-9: Effect of Change in Mobile Phase Flow Rate (1.0 ± 0.2 mL/min)

Parameter	Acceptance criteria	Actual (1.0 mL/minute)		Change-1 (0.8 mL/minute)		Change-2 (1.2 mL/minute)	
		Spiked	Retention time (min)	Spiked	Retention time (min)	Spiked	Retention time (min)
Impurity-A %)	NMT 0.7%	0.767	24.114	0.75	27.854	0.76	21.903
% Recovery	85% to 115%	111.1	-	106.0	-	108.5	-
Impurity-B %)	NMT 0.2%	0.217	37.504	0.22	41.893	0.21	35.002
% Recovery	85% to 115%	109.0	-	108.3	-	107.8	-
Impurity-C %)	NMT 0.4%	0.430	29.670	0.45	33.620	0.43	27.266
% Recovery	85% to 115%	109.1	-	113.5	-	108.3	-
Impurity-D %)	NMT 0.2%	0.390	29.554	0.402	33.521	0.410	27.152
% Recovery	85% to 115%	102.38	-	103.94	-	103.54	
Impurity-E %)	NMT 0.2%	0.410	36.544	0.395	31.254	0.405	34.215
% Recovery	85% to 115%	104.23	-	104.7	-	105.05	-

A small, significant variation was observed in the results due to a small variation in the mobile phase flow rate.

Table-10: Effect of Change in Column Oven Temperature (30 ± 5 °C)

Parameter	Acceptance criteria	Actual (30 °C)		Change-1 (25 °C)		Change-2 (35 °C)	
		Sample		Sample		Sample	
		Spiked	Retention time (min)	Spiked	Retention time (min)	Spiked	Retention time (min)
Impurity-A (%)	NMT 0.7%	0.767	24.114	0.78	26.859	0.769	21.562
% Recovery	85% to 115%	111.1	-	112.1	-	100.26	-
Impurity-B (%)	NMT 0.2%	0.217	37.504	0.22	41.118	0.219	32.025
% Recovery	85% to 115%	109.0	-	109.6	-	103.9	-
Impurity-C (%)	NMT 0.4%	0.430	29.670	0.43	33.028	0.426	27.052
% Recovery	85% to 115%	109.1	-	108.1	-	106.21	-
Impurity-D (%)	NMT 0.2%	0.390	29.554	0.401	32.02	0.398	27.054
% Recovery	85% to 115%	102.36	-	103.54	-	103.98	-
Impurity-E (%)	NMT 0.2%	0.410	36.544	0.392	38.245	0.402	35.21
% Recovery	85% to 115%	105.24	-	104.5	-	106.05	-

Column oven temperature is critical. During the higher column oven temperature change, % Recovery impurity-C was found on the higher side. Hence, the allowable column oven temperature is from 25°C to 30°.

Forced Degradation Studies

Characterisation of Fidaxomicin and Its Degradation Impurities by LC–MS

Fidaxomicin's forced degradation studies, conducted under acidic, basic, oxidative, photolytic, and thermal stress, evaluated the analytical method's stability-indicating capability. Degradation products were characterised using LC-MS, and mass balance was achieved, confirming no co-elution of impurity peaks with Fidaxomicin. Significant degradation (40.77%) occurred under basic conditions (0.01 N

NaOH, 10 min), and minor degradation (2.62%) under acidic conditions (0.01 N HCl, 10 min). Oxidative, thermal, aqueous, humidity, and photolytic stress caused no significant degradation. Impurity structures and degradation results are presented in Fig.-6 and Table-12, respectively.

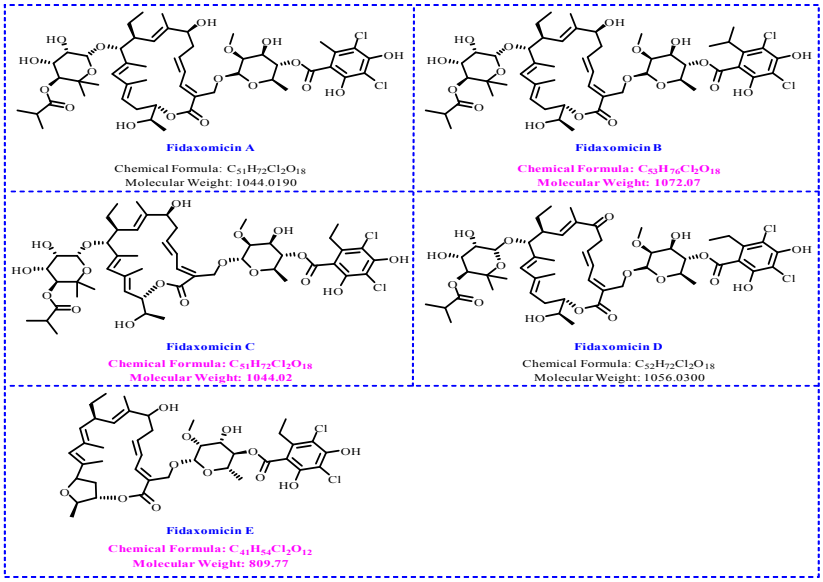

Fig.-5: Chemical Structure of Fidaxomicin Impurities

Fidaxomicin has a molecular weight of 1058.0460 g/mol, and the protonated molecular ion $[M + H]^+$ was recorded at m/z 1058.3, which confirms the integrity of the parent molecule under non-stress conditions. A number of degradation products were detected and identified through their molecular ions and fragmentation patterns. The molecular ion peaks that were observed corresponded closely with the calculated values, confirming the proposed structures of the degradation impurities. These findings highlight the sensitivity and specificity of the LC–MS method developed for the identification and characterisation of Fidaxomicin degradation products. The results from the LC-MS analysis are displayed in Table-11.

Table-11: ESI-MS Exact Mass Data and Theoretical Mass Data on $[M+H]^+$ of Five Unknown Impurities of Fidaxomicin Film Coated Tablets in the Positive Ion Mode

Impurity	$[M+H]^+$ (m/z)	Formula	Theoretical $[M+H]^+$ (m/z)
A	1044.3	$C_{51}H_{72}Cl_2O_{18}$	1044.3
B	1071.3	$C_{53}H_{76}Cl_2O_{18}$	1071.3
C	1044.1	$C_{51}H_{72}Cl_2O_{18}$	1044.1
D	1056.4	$C_{52}H_{72}Cl_2O_{18}$	1056.3
E	810.1	$C_{41}H_{54}Cl_2O_{12}$	809.77

Table-12: Summary of Forced Degradation Studies of Fidaxomicin

Name of degradation	Degradation conditions	% Assay	% Impurities	%Assay + % impurities	% Mass balance
Un-degraded / Initial / as such sample	NA	101.36	0.41	101.8	NA
Acid degradation	0.01N HCl, BT, 10 minutes	99.98	2.62	102.6	100.8
Alkali degradation	0.01N NaOH, BT, 10 minutes	60.17	40.77	100.9	99.1
Oxidation degradation	3% H_2O_2, BT, 30 minutes	101.14	0.50	101.6	99.8

UV degradation	200-watt hr /m^2	103.33	0.42	103.8	102.0
Visible degradation	1.2 million lux hours	103.23	0.52	103.8	102.0
Thermal degradation	105°C, 1 day	101.82	0.52	102.3	100.5
Humidity degradation	85% RH, 7 days	102.08	0.41	102.5	100.7

CONCLUSION

This study presents a novel HPLC method designed for the detection of impurities in Fidaxomicin film-coated tablets, aimed at improving the official monograph in the USP. The method was thoroughly validated in accordance with regulatory guidelines, demonstrating specificity, linearity, precision, and accuracy. Furthermore, five degradation impurities in Fidaxomicin film-coated tablets were characterized using liquid chromatography combined with high-resolution ion trap/time-of-flight mass spectrometry in positive ion mode. The complete fragmentation patterns of these five degradation impurities were analysed to extract structural information. The structures of the five degradation impurities in the Fidaxomicin film-coated tablet were deduced from the high-resolution MS data. This extensive characterization facilitated the identification of their chemical structures, providing vital insights into the degradation pathways of Fidaxomicin. The developed HPLC method, together with the structural elucidation of impurities, presents a robust and reliable approach for quality control and stability assessment of Fidaxomicin film-coated tablets, ultimately contributing to improved patient safety and product efficacy.

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CONFLICT OF INTEREST

All authors acknowledge that there are no conflicts of interest or financial interests linked to this research work.

AUTHOR CONTRIBUTIONS

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