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DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING REVERSE PHASE HPLC METHOD FOR THE DETERMINATION OF RELATED SUBSTANCES IN IXAZOMIB CITRATE DRUG SUBSTANCE

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ABSTRACT

A sensitive, precise, specific, linear and stability indicating a gradient reversed phase high performance liquid chromatography (RP-HPLC) method has been developed and validated for the determination of related substances of Ixazomib Citrate (IXM). The successful chromatographic separation of Ixazomib Citrate from its related substances was achieved on octadecyl silane chemically bonded to porous silica particles stationary phase i.e. Kromosil C18, 150 x 4.6mm, i.d., 5µm column maintained at 29°C and sample cooler: 5°C, using Orthophosphoric acid (OPA) as mobile phase A and mobile phase B composed a mixture of acetonitrile: methanol: isopropyl alcohol (800:120:80 v/v/v) respectively. Wavelength for UV detection: 225 nm, flow rate: 0.8 ml/min and injection volume: 10µl, diluent: mixture of acetonitrile and water in the ratio of (50:50) v/v. The performance of the method was validated according to the ICH guidelines for specificity, linearity, accuracy, precision, limit of quantification and limit of detection. Ixazomib was subjected to stress conditions of thermal, hydrolysis, peroxide and photolytic to observe the degradation products. Limit of detection of impurities were in the range of 0.003%–0.010% indicating the high sensitivity of the developed method. The experiment results are given in detail in this paper.

KEYWORDS: Ixazomib citrate, HPLC, Related substances, Development, Forced degradation and Validation.

1. INTRODUCTION

Ixazomib (trade name Ninlaro) is a drug for the treatment of multiple myeloma, a type of white blood cell cancer, in combination with other drugs. Common side effects include diarrhea, constipation and low platelet count. Ixazomib is used in combination with lenalidomide (Revlimid) and dexamethasone to treat multiple myeloma (cancer of the plasma cells in the bone marrow) that has worsened after treatment with other chemotherapy medications. Ixazomib is in a class of medications called proteasome inhibitors. It works by helping to kill cancer cells. Ixazomib comes as a capsule to take by mouth. It is usually taken with water on an empty stomach, at least 1 hour before or 2 hours after eating. It is taken on days 1, 8, and 15 of a 28 day treatment cycle. The chemical name of Ixazomib citrate is 2-[(1R)-1-[[2-[(2,5-dichlorobenzoyl) amino]acetyl] amino]-3- methyl butyl]-5-oxo-1,3,2-dioxaborolane-4,4-diacetic acid corresponding to the molecular formula $C_{20}H_{23}BCl_2N_2O_9$ and has a relative molecular mass of 517.12 g/mol.

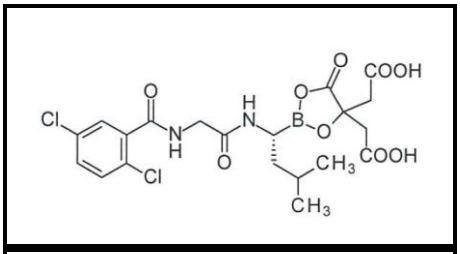
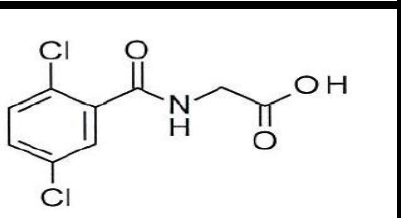
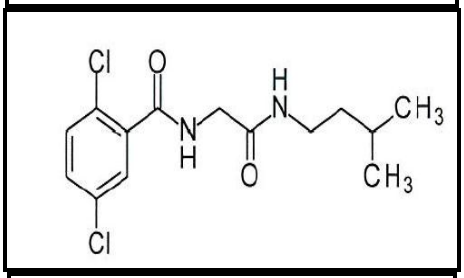
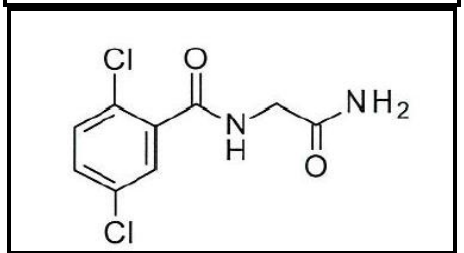
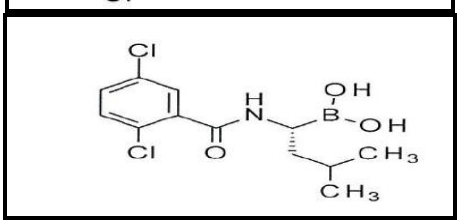
The active substance, Ixazomib citrate, is a pro-drug of Ixazomib. Under physiological conditions Ixazomib citrate rapidly hydrolyses to Ixazomib, which is a boronic acid of the general structure RB $(OH)_2$. Ixazomib citrate is a white to off-white non-hygroscopic powder, with a melting point ~ 231ºC (with decomposition). Based on its high solubility and low permeability, Ixazomib is a BCS Class 3 compound. Studies showed that Ixazomib is highly soluble across a broad aqueous pH range that includes the physiological pH range (1.2 to 6.85). The pKa and logP of Ixazomib citrate could not be determined due to the hydrolysis of Ixazomib citrate to Ixazomib in aqueous systems. The structure of Ixazomib citrate has been confirmed by IR spectroscopy, high resolution mass spectrometry, Elemental analysis, UV-Vis spectroscopy and single crystal X-ray crystallography. 1H and 13C-NMR and mass spectroscopy demonstrated that Ixazomib citrate exists as the cyclic citrate ester structure in anhydrous, aprotic solvents, and that the ester rapidly hydrolyzes to Ixazomib (boronic acid) in dilute aqueous solutions in the absence of excess citric acid. Supplementary NMR experiments confirmed the rapid kinetics of Ixazomib citrate hydrolysis and the favored Ixazomib equilibrium

once exposed to aqueous conditions. The structure of Ixazomib citrate contains one chiral centre.

The absolute stereochemistry of Ixazomib citrate at the single chiral centre has been unambiguously determined as R. Enantiomeric purity of the active substance is controlled by NP-HPLC and acceptance limit of this specification has been set at 0.15%. A number of polymorphic crystal forms of Ixazomib citrate were identified and characterized. One has been identified as the most thermodynamically stable form and was selected for development and commercial manufacture. This form has been demonstrated to be consistently manufactured by the proposed manufacturer using a controlled crystallization procedure. Polymorphism is controlled in the active substance specification by PXRD.

There are several process and degradation impurities of Ixazomib citrate, which originated during the synthesis process and as well as degradation during stability studies or on storage. Ixazomib citrate is a very novel and recently synthesized drug. It is not official in any Pharmacopoeia. A literature survey on Ixazomib related that, until now no analytical HPLC method for determination of Ixazomib citrate and its related substances is available. The reported methods are related to assay or single impurity determinations by HPLC methods. Hence, stability indicating RP-HPLC method has been developed for the quantification of impurities related to Ixazomib citrate.

The developed chromatographic method can resolve all these impurities with adequate resolution to achieve good chromatography and the optimized methodology have been validated to accomplish as per ICH guidelines. The chemical structures of Ixazomib citrate (IXM) and its related impurities are shown in Figure 1.

Ixazomib citrate (IXM)	2-[(1R)-1-[[2-[(2,5-dichlorobenzoyl) amino]acetyl]amino]-3- methylbutyl]-5-oxo-1,3,2-dioxaborolane-4,4-diacetic acid. Mol. weight **:** 517.12 g/mol Mol. formula**:** $C_{20}H_{23}BCl_2N_2O_9$	
IXM/Impurity-A	N-(2, 5-Dichlorobenzoyl) glycine (or) 2-[2(2, 5-dichlorobenzoyl) amino] acetic acid. Mol. weight **:** 248.06 g/mol Mol. formula**:** $C_9H_7Cl_2NO_3$	
IXM/Impurity-B	2,5-Dichloro-*N-[*2-[(3-methylbutyl)amino]2-oxoethyl] benzamide Mol. weight**:** 317.21g/mol Mol. formula**:** $C_{14}H_{18}Cl_2N_2O_2$	
IXM/Impurity-C	*N*-(2-Amino-2-oxoethyl)-2,5-dichlorobenzamide. Mol. weight : 247.08 g/mol Mol. formula : $C_9H_8Cl_2N_2O_2$	
IXM/Impurity-D	[(1*R*)-1-[(2, 5-dichlorobenzoyl) amino]-3-methyl-butyl] boronic acid Mol. weight: 303.98 g/mol Mol. Formula : $C_{12}H_{16}BCl_2NO_3$	

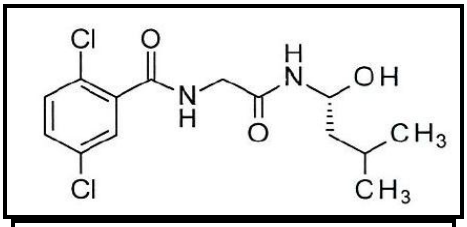
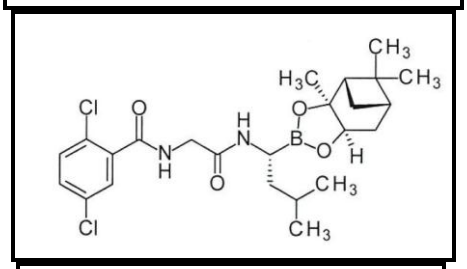
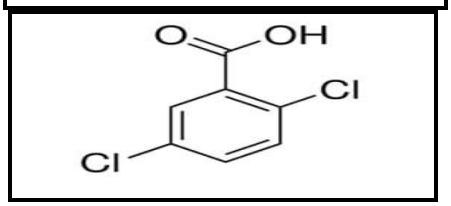
IXM/Impurity-E	(2,5-dichloro-*N*-[2-[[(1*R*)-1-hydroxy-3-methyl-butyl]amino]-2-oxo-ethyl] benzamide Mol. weight: 333.21g/mol Mol. formula : $C_{14}H_{18}Cl_2N_2O_3$	
IXM-I (Intermediate stage)	2, 5-Dichloro-*N*-[2-[[(1*R*)-1-[(3a*S*, 4*S*, 6*S*, 7a*R*)-hexahydro-3a,5,5-trimethyl-4,6-methano-1,3,2-benzodioxaborol-2-yl]-3-methylbutyl]amino]-2-oxoethyl] benzamide Mol. weight: 495.25g/mol Mol. formula : $C_{24}H_{33}BCl_2N_2O_4$	
2, 5-Dichlorobenzoic acid (Key starting material)	2,5-Dichlorobenzoic acid Mol. weight: 191.01/mol Mol. formula : $C_7H_4Cl_2O_2$	

Figure 1: Chemical structures of Ixazomib and its impurities.

2. MATERIALS AND METHODS

2.1. Chemicals, reagents, standards and samples

The investigated samples of Ixazomib citrate drug substance, its related impurities and Ixazomib peak identification mixture standard (PIM) for system suitability were arranged from Natco Research Centre (A division of Natco Pharma Ltd., Hyderabad). HPLC grade of Acetonitrile and Methanol were procured from Merck, India and Orthophosporic acid (~88%) and isopropyl alcohol were procured from Rankem and pure milli-Q water was used with the help of Millipore purification system (Millipore®, USA).

2.2 Instrumentation and methodology

The HPLC system used for method development, method validations as well as forced degradation studies on Waters Alliance 2695 separation module equipped with 2996 photo diode array detector and UV detector with Empower data handling system i.e. Empower 3 software, Build No: 2154 [Waters Corporation, MILFORD, MA 01757, USA] was used. HPLC column: Kromosil C18, 150 x 4.6mm, i.d., 5µm column maintained at 29°C and sample cooler: 5°C, using Orthophosphoric acid (OPA) as mobile phase A and mobile phase B composed a mixture of acetonitrile: methanol: isopropyl alcohol (800:120:80 v/v/v) respectively. Wavelength for UV detection: 225 nm, flow rate: 0.8 ml/min and Injection volume: 10µl, diluent: mixture of acetonitrile and water in the ratio of (50:50) v/v and data acquisition time: 65 minutes. Retention time of Ixazomib: about 21 minutes. The pump is in gradient mode and the program is as follows: Time (min)/ A (v/v): B (v/v); T0.01/80:20, T3/80:20, T15/65:35, T25/65:35, T35/45:55, T45/25:75, T55/25:75, T57/80:20, T65/80:20.

2.3 Preparation of solutions

2.3.1 Reference solution

Weigh about 15 mg of IXM standard into a 20 mL volumetric flask, add 5mL of diluent to dissolve and sonicate it, then make up to volume with diluent. Further transfer 100 µL of above solution into a 100mL volumetric flask, and add diluent and mix the solution, then make up to the mark with diluent.

2.3.2 System suitability solution

Weigh about 7.5 mg of IXM/Peak identification mixture standard into a 10 mL volumetric flask, add 5mL of diluent to dissolve and sonicate it, then make up to volume with diluent.

2.3.3 Evaluation of system suitability

Inject diluent as blank followed by six replicate injections of reference solution and one system suitability solution into the HPLC system and record the chromatograms. The system is suitable for analysis if and only if,

1. Relative standard deviation for IXM peak not more than 10.0 %.
2. Resolution between IXM and IXM/impurity-E peaks in system suitability solution should be not less than 1.5.
3. Theoretical plates for IXM peak in system suitability solution should be not less than 5000
4. USP tailing for IXM peak in system suitability solution should be not more than 2.0.

2.3.4 Test sample

Weigh about 15 mg of test sample into a 20 mL volumetric flask , add 5mL of diluent to dissolve and sonicate it, then make up to volume with diluent (0.75mg/mL).

2.3.5 Procedure

Inject test solution into the chromatograph and record the chromatogram. Disregard the peaks due to blank and disregard the peak at RT about 2.0 minutes due to citric acid. The retention time for IXM peak is about 21.0 minutes. The relative retention times for other components w.r.t to IXM are shown in Table 1.

Table 1: Retention time and Relative retention time of compounds.

S. No.	Component	Relative Retention Time about (RRT)	Relative Response factor (RRF)
1.0	IXM/impurity-C	0.45	2.38
2.0	IXM/impurity-A	0.64	2.27
3.0	IXM/impurity-E	1.15	1.65
4.0	2,5-dichlorobenzoic acid	1.27	2.28
5.0	IXM/impurity-D	1.36	1.14
6.0	IXM/impurity-B	1.67	1.93
7.0	IXM-I	2.39	0.92
8.0	IXM	1.00	1.00

Note

1. Compare the known impurities of test solution against the impurities of system suitability solution.
2. Consider the known impurities RRF values as per the above table, for unknown impurity as 1.0.
3. Calculate each specified and unspecified impurity in the test solution with respect to Ixazomib peak in reference solution.

Calculation and reporting

$$\%\ \text{w/w of impurity} = \frac{A_i}{A_s} \times \frac{C_s}{C_t} \times \frac{1}{RRF} \times P$$

Where,

A_i = Area of impurity in test solution

A_s = Average area of Ixazomib in reference solution.

C_s = Concentration of reference solution, mg/mL.

C_t = Concentration of test solution, mg/mL.

P = Purity/Potency of standard.

RRF = Relative response factor

Note

1. For Establishment of RRF Values, used reference solution-1& reference solution-2 solutions.
2. IXM-I performed single injection and injected separately, due to the hydrolysis of pinanediol ester of IXM-I, it produce Ixazomib only and results are discussed below.

1. RESULTS AND DISCUSSION

1.1. Method Validation

3.1.1 System suitability

The percentage relative standard deviation (% RSD) for each component from reference solution-2 except IXM-I peak. IXM-I area consider from reference solution-1 and theoretical plates, tailing and resolution from system suitability solution. The system suitability results are tabulated in Table 2. Typical representative HPLC chromatograms are shown in Figure 2 to Figure 5.

Table 2: System suitability results.

Injection	IXM/Imp-A	IXM/Imp-B	IXM/Imp-C	IXM/Imp-D	IXM/Imp-E	2,5-DCBA	IXM	IXM-I
Inj-1	42225	33534	41034	19399	26943	42201	11895	15469
Inj-2	42792	33920	41563	19650	27393	42847	11789	---
Inj-3	42927	33946	41696	20028	27588	42367	11959	---
Inj-4	43070	34000	41914	20321	27226	43047	11812	---
Inj-5	42904	33973	41827	19729	27338	43368	12307	---
Inj-6	42833	33639	41658	19946	27748	43101	11727	---
Mean	42792	33835	41615	19846	27373	42822	11915	15469
%RSD	0.69	0.58	0.75	1.63	1.03	1.05	1.75	---

Observed Results

1) % RSD for each component and IXM is in between 0.58 to 1.75.
2) USP plate count for IXM peak in system suitability solution is 48488.
3) USP tailing for IXM peak in system suitability solution is 1.30.
4) Resolution between IXM and IXM/impurity-E peaks are 7.45 in system suitability solution.

Acceptance criteria

1) % RSD for each component and IXM peak not more than 10.0 in reference solution-2
2) USP plate count for IXM peak in system suitability solution should be not less than 5000
3) USP tailing for IXM peak in peak system suitability solution should be not more than 2.0.
4) Resolution between IXM and IXM/impurity-E peaks in system suitability solution should be not less than 1.5.

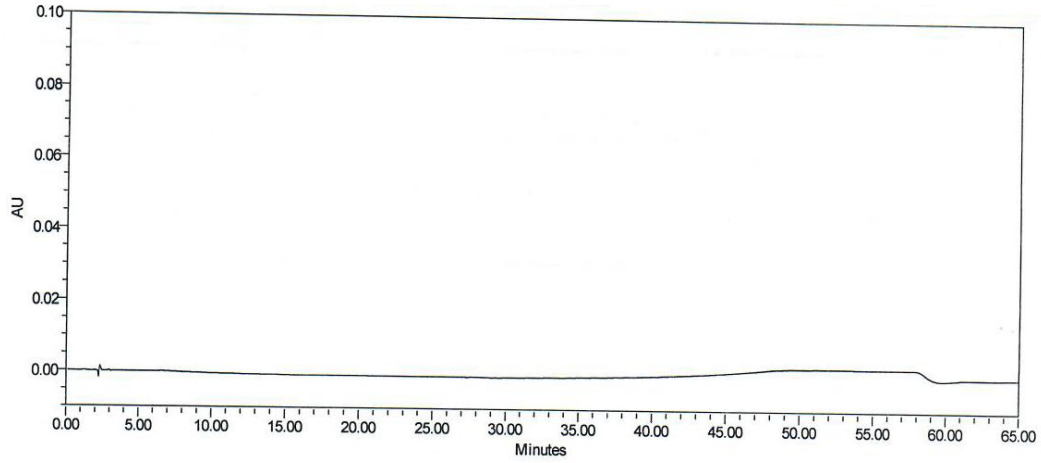


Figure 2: A typical representative HPLC chromatogram of Blank.

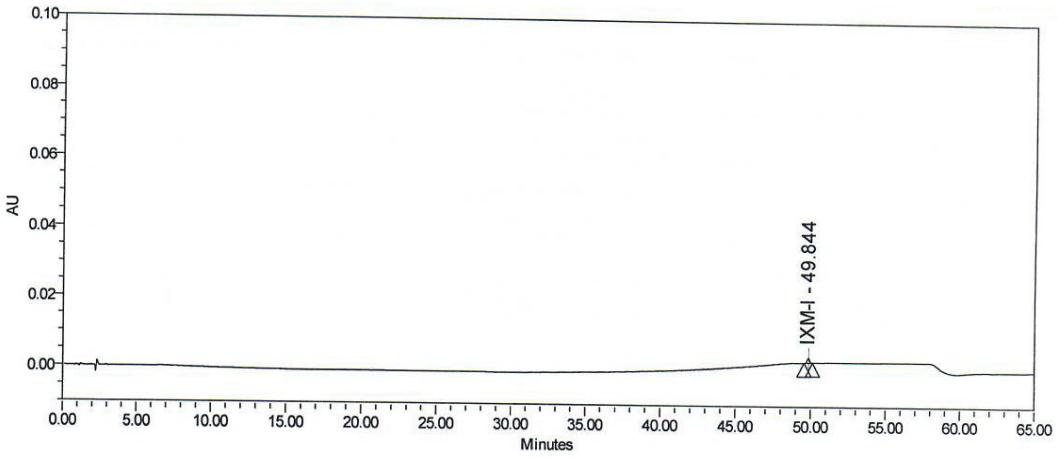


Figure 3: A typical representative HPLC chromatogram of Reference Solution-1.

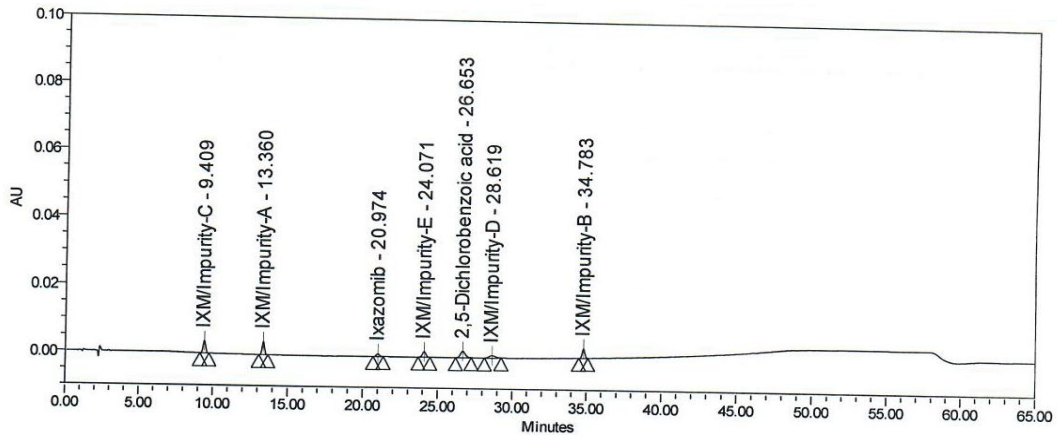


Figure 4: A typical representative HPLC chromatogram of Reference Solution-2.

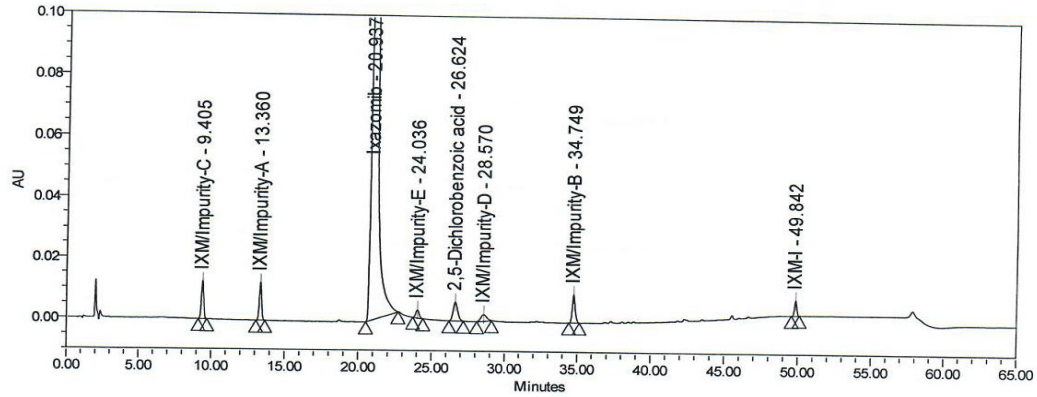


Figure 5: A typical representative HPLC chromatogram of System suitability solution.

3.1.2 Specificity

Specificity is the ability to assess unequivocally of the analyte in the presence of components which may be expected to be present. For determination of specificity, injection of blank, all individual impurities solutions were prepared and injected to confirm the retention times. The solutions of Ixazomib citrate drug substance (Control Sample) and Ixazomib citrate spiked with known related substances at specification level (Spiked Sample) were prepared and injected into HPLC. Peak purity was established by using Empower Software. The specificity results are tabulated in Table 3. A typical representative HPLC chromatogram of Ixazomib citrate drug substance spiked with all impurities is shown in Figure.6.

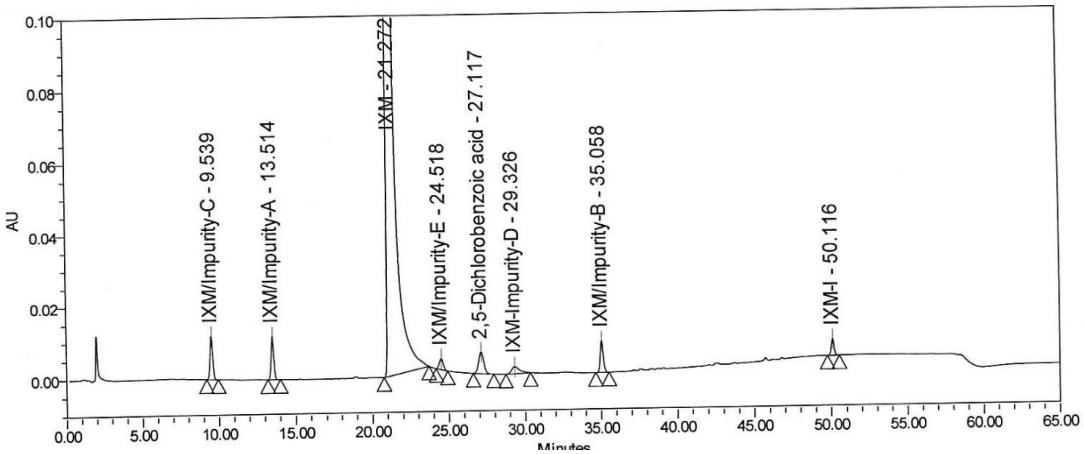


Figure 6: A typical representative HPLC chromatogram of Ixazomib citrate drug substance spiked with all Impurities.

Table 3: Specificity of impurities from system suitability solution.

Peak Name	Retention Time (RT) (Minutes)	Relative retention time (RRT)	Peak Purity	
			Purity angle	Purity Threshold
IXM/impurity-C	9.539	0.448	0.147	0.492
IXM/impurity-A	13.514	0.635	0.307	0.513
Ixazomib	21.272	1.00	0.318	0.460
IXM/impurity-E	24.518	1.153	0.384	1.169
2,5-DCB	27.117	1.275	0.228	0.628
IXM/impurity-D	29.326	1.379	1.057	1.879
IXM/impurity-B	35.058	1.648	0.375	0.607
IXM-I	50.116	2.356	0.407	0.960

3.1.3 Limit of Detection (LOD)/ Limit of Quantification (LOQ)

The limit of detection and limit of quantification is determined by calculating the signal to noise ratio method. By comparing test results from samples with known concentrations of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. These are determined from the formula S/N ratio is 3:1 for LOD and 10:1 for LOQ respectively. The LOD and LOQ results are

tabulated in Table 4. The typical representative HPLC chromatograms of Blank, LOD and LOQ experiment are shown in Figure 7-10.

Table 4: Limit of detection and Quantification for Ixazomib citrate (IXM) and its impurities.

Component	LOQ			LOD		
	Concentration (mg/ml)	Signal to noise ratio (S/N)	LOQ (%)	Concentration (mg/ml)	Signal to noise ratio (S/N)	LOD (%)
IXM/impurity-A	0.00007722	16.8:1	0.010	0.00002548	6.1:1	0.003
IXM/impurity-B	0.00007573	12.7:1	0.010	0.00002499	4.2:1	0.003
IXM/impurity-C	0.00007659	15.6:1	0.010	0.00002527	5.6:1	0.003
IXM/impurity-D	0.00026800	10.9:1	0.036	0.00008844	3.4:1	0.012
IXM/impurity-E	0.00011115	10.9:1	0.015	0.00003668	3.9:1	0.005
2,5-DCBA	0.00011987	12.0:1	0.016	0.00003956	4.3:1	0.005
Ixazomib	0.00015656	10.7:1	0.021	0.00005166	3.5:1	0.007
IXM-I	0.00015240	14.5:1	0.020	0.00005029	4.5:1	0.007

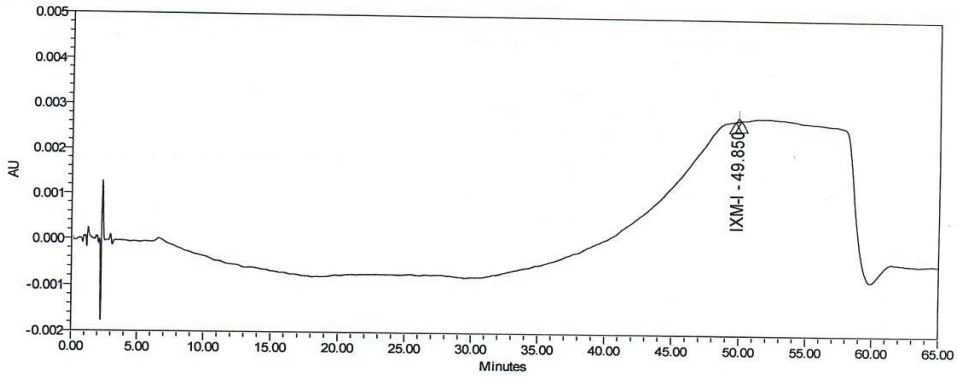


Figure 7: A typical representative HPLC chromatogram of LOD Reference solution-1.

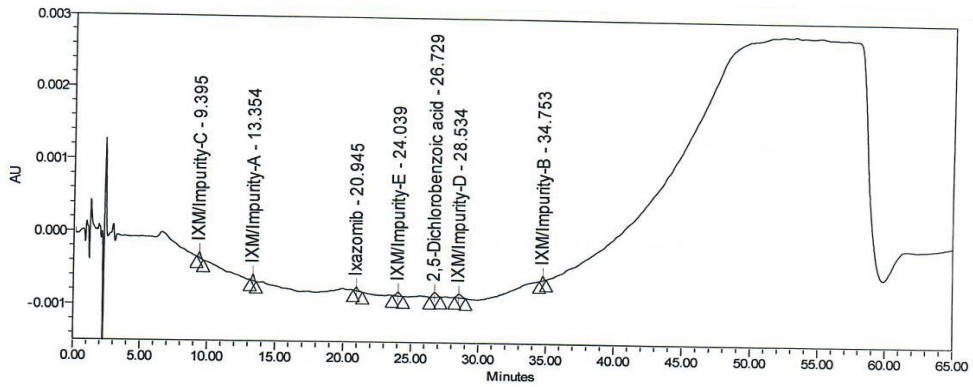


Figure 8: A typical representative HPLC chromatogram of LOD Reference solution-2.

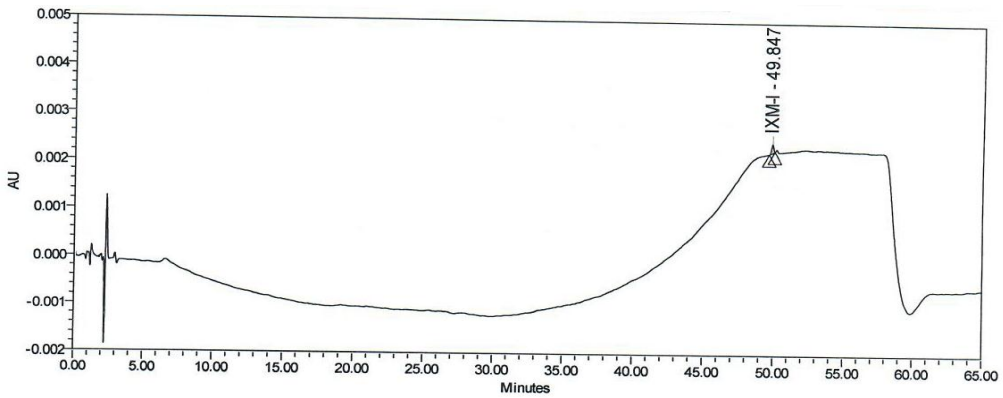


Figure 9: A typical representative HPLC chromatogram of LOQ Reference solution-1.

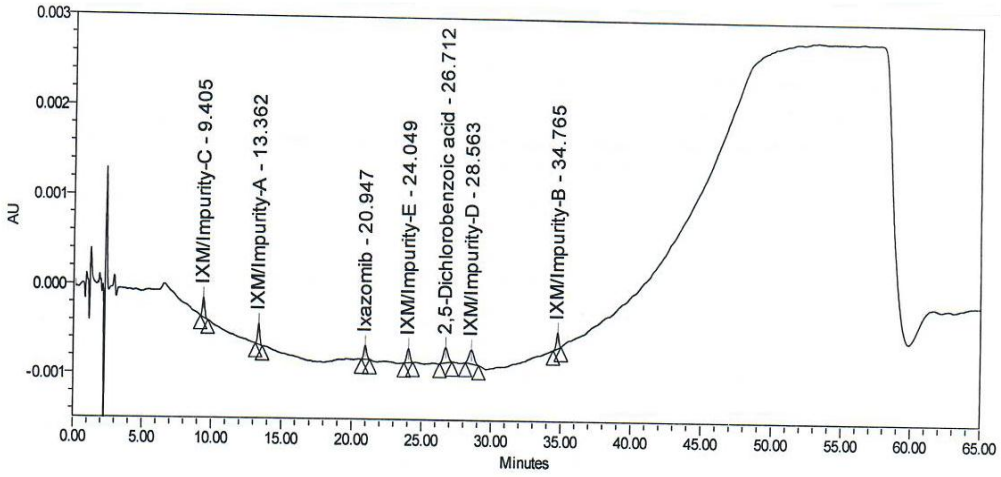


Figure 10: A typical representative HPLC chromatogram of LOQ Reference solution-2.

3.1.4 Linearity

A series of solutions were prepared using Ixazomib citrate and its impurities at concentration levels from LOQ to 150% of specification level and each solution was injected and calculated the statistical values like slope, intercept and correlation coefficient from linearity plot drawn for concentration versus area.

Linearity graphs Figure 11-17 and Tables 5-11 are shown below. The statistical values are presented in Table 12.

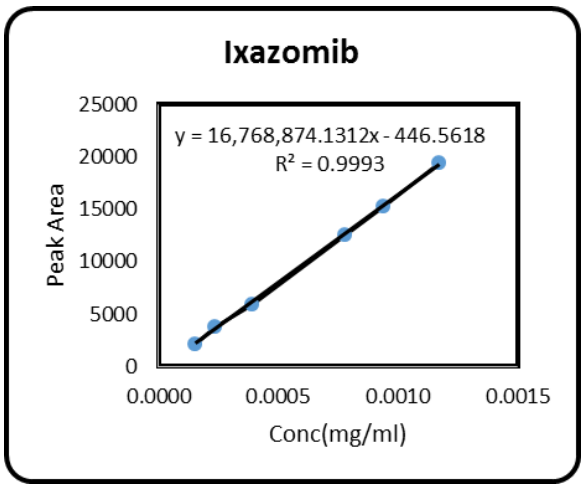


Figure 11: Linearity graph for Ixazomib citrate.

Table 5: Linearity Table for Ixazomib citrate.

Level	Concentration (mg/mL)	Average Peak area
LOQ	0.0001566	2114
30%	0.0002348	3784
50%	0.0003914	5937
100%	0.0007828	12508
120%	0.0009394	15302
150%	0.0011742	19371

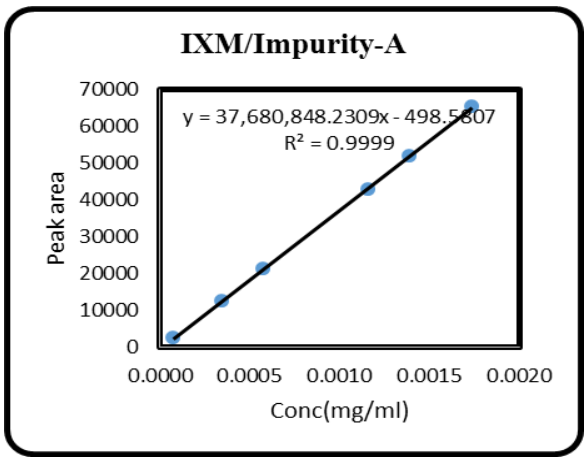


Figure 12: Linearity graph for IXM/Impurity-A.

Table 6: Linearity Table for IXM/Impurity-A.

Level	Concentration (mg/mL)	Average Peak area
LOQ	0.0000772	2597
30%	0.0003475	12474
50%	0.0005792	21415
100%	0.0011583	42755
120%	0.0013900	51824
150%	0.0017375	65259

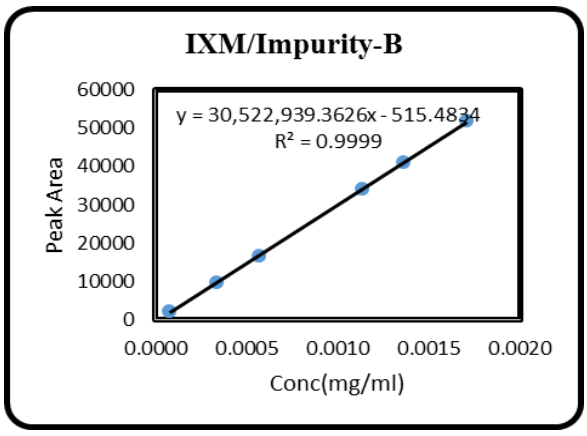


Figure 13: Linearity graph for IXM/Impurity-B.

Table 7: Linearity Table for IXM/Impurity-B.

Level	Concentration (mg/mL)	Average Peak area
LOQ	0.0000757	2097
30%	0.0003408	9665
50%	0.0005680	16761
100%	0.0011360	34010
120%	0.0013631	40983
150%	0.0017039	51729

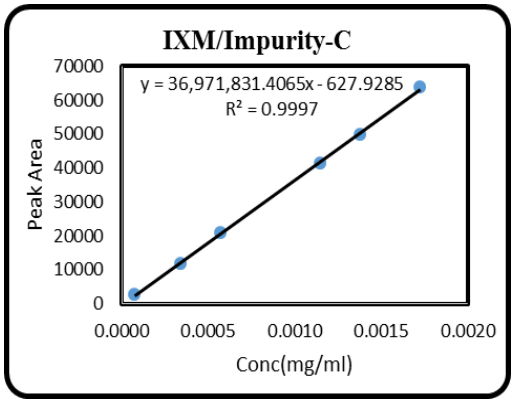


Figure 14: Linearity graph for IXM/Impurity-C.

Table 8: Linearity Table for IXM/Impurity-C.

Level	Concentration (mg/mL)	Average Peak area
LOQ	0.0000766	2570
30%	0.0003447	11878
50%	0.0005744	20705
100%	0.0011489	41394
120%	0.0013786	49976
150%	0.0017233	63679

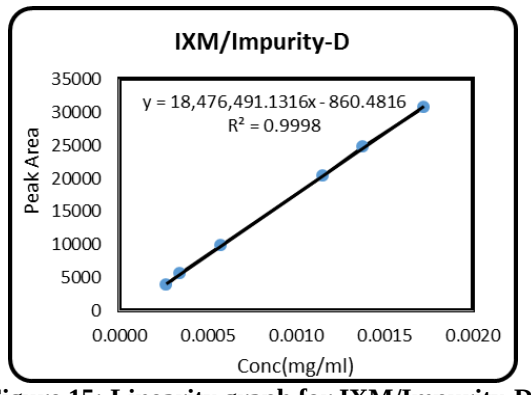


Figure 15: Linearity graph for IXM/Impurity-D.

Table 9: Linearity Table for IXM/Impurity-D.

Level	Concentration (mg/mL)	Average Peak area
LOQ	0.0002680	3874
30%	0.0003446	5578
50%	0.0005743	9852
100%	0.0011486	20442
120%	0.0013783	24758
150%	0.0017228	30780

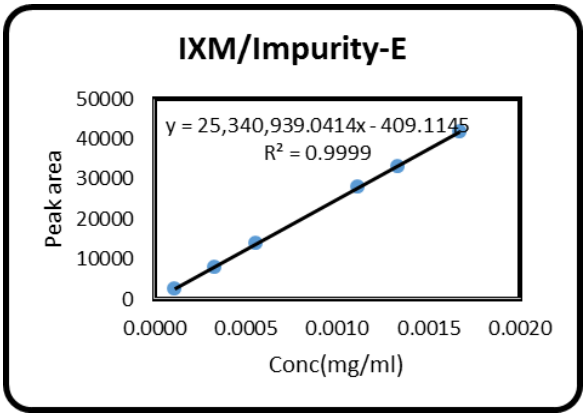


Figure 16: Linearity graph for IXM/Impurity-E.

Table 10: Linearity Table for IXM/Impurity-E.

Level	Concentration (mg/mL)	Average Peak area
LOQ	0.0001112	2454
30%	0.0003335	7795
50%	0.0005558	13868
100%	0.0011115	27900
120%	0.0013338	33273
150%	0.0016673	41821

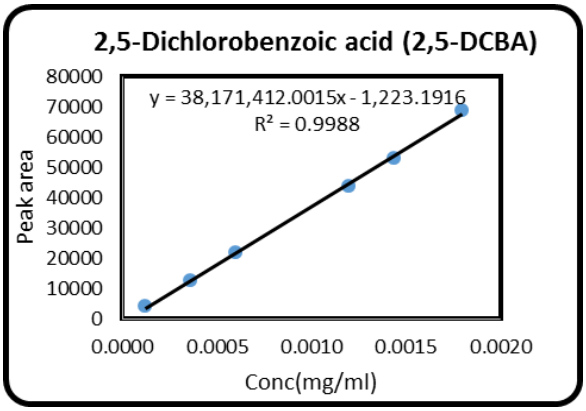


Figure 17: Linearity graph for 2, 5-DCBA.

Table 11: Linearity Table 2, 5-DCBA.

Level	Concentration (mg/mL)	Average Peak area
LOQ	0.0001199	3885
30%	0.0003596	12356
50%	0.0005993	21858
100%	0.0011987	43498
120%	0.0014384	52825
150%	0.0017980	68708

Table 12: Statistical evaluation of Linearity.

Component	Slope	Intercept	Correlation coefficient (R)	R^2	% Y-intercept	Relative Response factor (RRF)
IXM/Impurity-A	37680848.2309	-498.5807	0.9999	0.9999	-1.17	2.27
IXM/Impurity-B	30522939.3626	-515.4834	0.9999	0.9999	-1.50	1.93
IXM/Impurity-C	36971831.4065	-627.9285	0.9998	0.9997	-1.50	2.38
IXM/Impurity-D	18476491.1316	-860.4816	0.9999	0.9998	-4.21	1.14
IXM/Impurity-E	25340939.0414	-409.1145	0.9999	0.9999	-1.47	1.65
2,5-DCBA	38171412.0015	-1223.1916	0.9994	0.9988	-2.81	2.28
Ixazomib	16768874.1312	-446.5618	0.9996	0.9993	-3.57	1.00
IXM-I	14461153.7983	-421.9321	1.0000	0.9999	-2.64	0.92

3.1.5 Accuracy/Recovery

The accuracy of the method is determined by using the solutions containing Ixazomib citrate samples spiked with the respective impurities at approximately LOQ, 50%, 100% and 150% of the specification limit (w.r.t test concentration). The percentage recovery calculated should be in the range of 80 to 120, and at LOQ level the % recovery calculated should be in the range of 70-130. The percentage recovery values for all the impurities are calculated and tabulated in Table 13.

Table 13: Statistical evaluation of Recovery/Accuracy.

Name of the impurity /Compound	% Recovery or Accuracy			
	Spiked levels			
	LOQ Level	50% Level	100% Level	150% Level
IXM/impurity-A	94.5	98.9	99.4	101.2
IXM/impurity-B	91.1	100.0	99.5	99.4
IXM/impurity-C	95.3	99.9	99.7	100.9
IXM/impurity-D	91.0	87.1	96.1	100.9
IXM/impurity-E	88.2	95.0	94.5	97.0
2,5-Dichlorobenzoic acid	88.4	99.3	101.4	102.7
IXM-I	109.5	105.2	104.7	105.8

3.1.6 Stability of solutions

Standard solution and sample solution spiked with impurities were prepared and analyzed initially and at different time intervals for 24hrs by keeping the solutions at room temperature (~ 25°C) and refrigerator condition (~2-8°C) and found the solutions were stable.

3.1.7 Forced degradation

The degradation behavior of Ixazomib citrate has been studied by performing forced degradation studies. Ixazomib citrate was subjected to different stress conditions i.e. acid/base hydrolysis [2N HCl /90°C /9 hrs & 1.0N NaOH/ 70°C /30minutes, peroxide degradation under oxidative stress [0.5% v/v hydrogen peroxide (H_2O_2) solution initial & RT/3hrs], thermal degradation [70°C/24Hours], UV Solid and solution/24 hours and water hydrolysis [90°C/12Hours]. Peak purity of Ixazomib citrate peak was established by using PDA detector in these stress samples resulting purity angle should be less than purity threshold. The mass balance should be in the range of 95% to 105%. The forced degradation results are tabulated in Table 14. The typical representative HPLC chromatograms of forced degradation experiment are shown in Figure 18-26.

Table 14: Degradation study Results.

Name of the impurity	RRTs at	Control sample (%)	2N HCl	1N NaoH	0.5% H_2O_2	
			At 90°C 9hrs (%)	At 70°C 30 min (%)	Initial (%)	RT 3hrs (%)
IXM/impurity-C	0.448	0.04	0.10	0.66	0.05	0.05
IXM/impurity-A	0.635	0.02	5.69	6.23	0.03	0.04
IXM/impurity-E	1.153	ND	ND	ND	5.63	7.83
2,5-DCBA	1.275	ND	0.07	ND	ND	ND
IXM/impurity-D	1.379	ND	ND	ND	ND	ND
IXM/impurity-B	1.648	ND	ND	ND	0.10	0.21
IXM-I	2.356	ND	ND	ND	ND	ND
Unknown- 1	0.889	0.03	0.03	0.03	0.03	0.03
Unknown- 2	1.767	0.04	0.04	0.04	0.03	0.04

Unknown -3	1.997	0.05	0.05	0.04	0.04	0.05
Unknown- 4	2.000	0.05	0.04	0.04	0.04	0.04
Purity (%)	1.00	99.69	93.94	92.64	93.99	91.65
Mass balance	NA	100.0	101.94	100.44	99.92	99.90
Purity angle		0.433	0.330	0.319	0.341	0.406
Purity threshold		0.645	0.625	0.551	0.574	0.539

Table 15: Degradation study Results.

Name of the impurity	RRTs at	Control sample (%)	UV Solution 24 hrs (%)	UV Solid 24 hrs (%)	Heat at 70°C 24 hrs (%)	Water hydrolysis At 90°C 12 hrs (%)
IXM/impurity-C	0.448	0.04	0.16	0.10	0.05	0.07
IXM/impurity-A	0.635	0.02	0.30	0.02	ND	3.36
IXM/impurity-E	1.153	ND	0.19	ND	ND	ND
2,5-DCBA	1.275	ND	ND	ND	ND	ND
IXM/impurity-D	1.379	ND	ND	ND	ND	ND
IXM/impurity-B	1.648	ND	0.01	ND	ND	ND
IXM-I	2.356	ND	ND	ND	ND	ND
Unknown - 1	0.889	0.03	0.05	0.03	0.03	0.03
Unknown - 2	1.767	0.04	0.04	0.04	0.04	0.04
Unknown - 3	1.997	0.05	0.02	0.01	0.05	0.05
Unknown - 4	2.000	0.05	0.03	0.02	0.05	0.04
Purity	1.00	99.69	98.19	99.65	99.64	96.38
Mass balance	NA	100.0	99.92	99.53	99.44	102.63
Purity angle		0.433	0.578	0.337	0.464	0.328
Purity threshold		0.645	0.625	0.653	0.643	0.644

ND**:** Not detected, NA: Not applicable

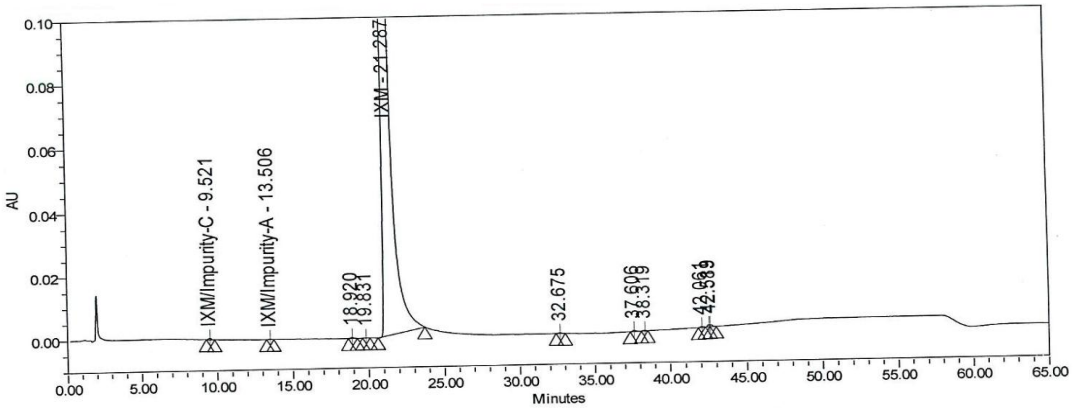


Figure 18: Typical chromatogram for control sample.

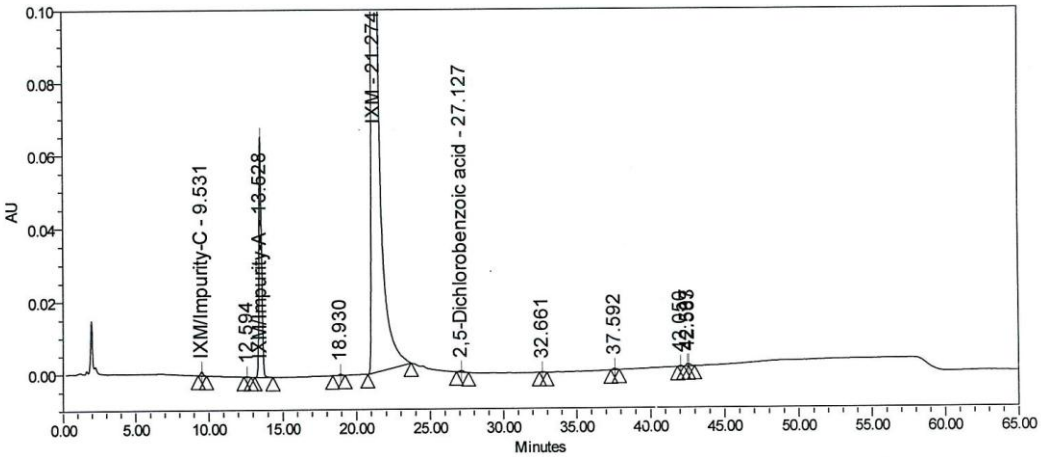


Figure 19: Typical chromatogram for 2.0 N HCl at 90°C 9 hrs.

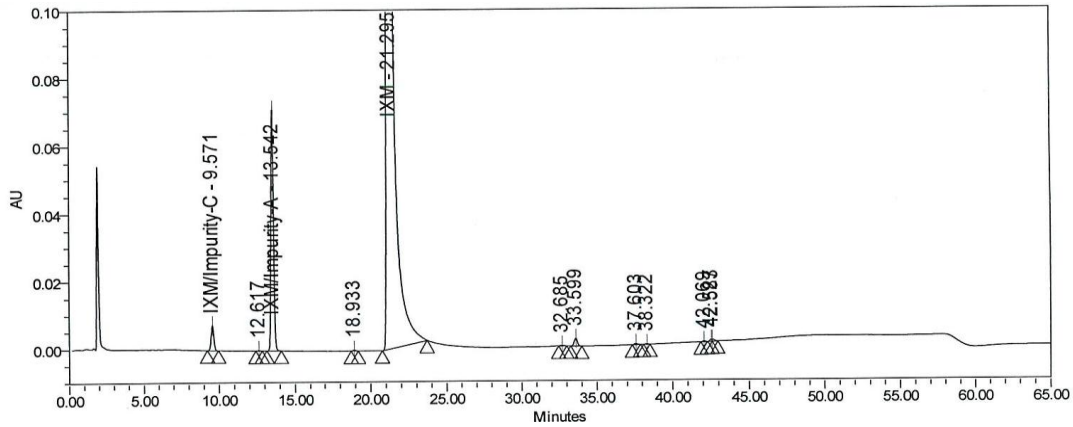


Figure 20: Typical chromatogram for 1.0 NaOH at 70°C 30 min.

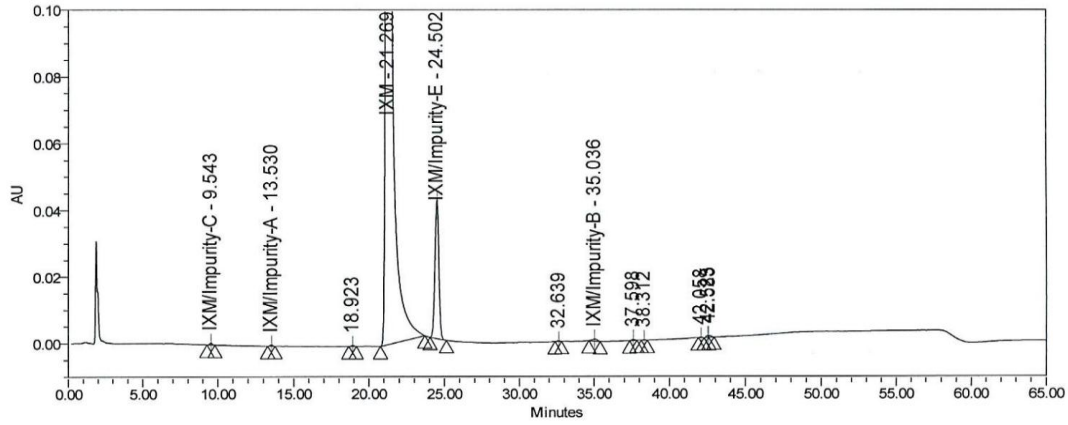


Figure 21: Typical chromatogram for 0.5% H_2O_2 initial.

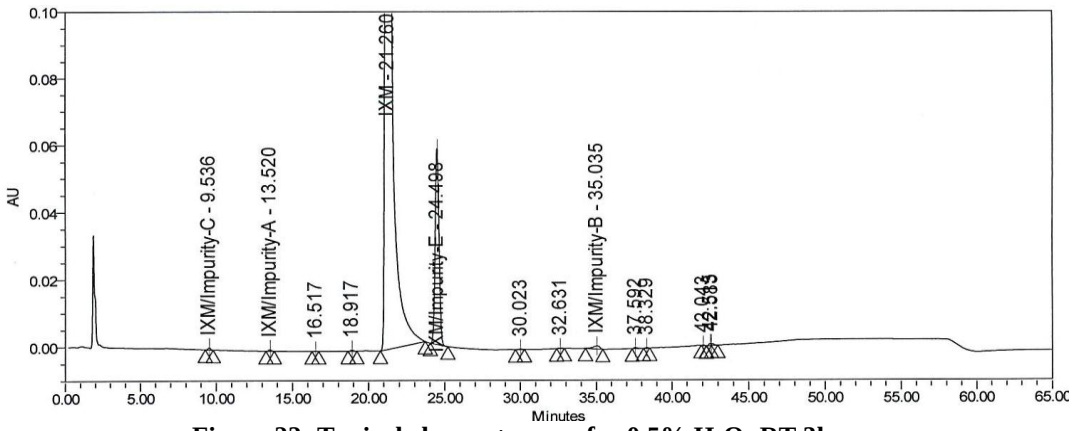


Figure 22: Typical chromatogram for 0.5% H_2O_2 RT 3hrs.

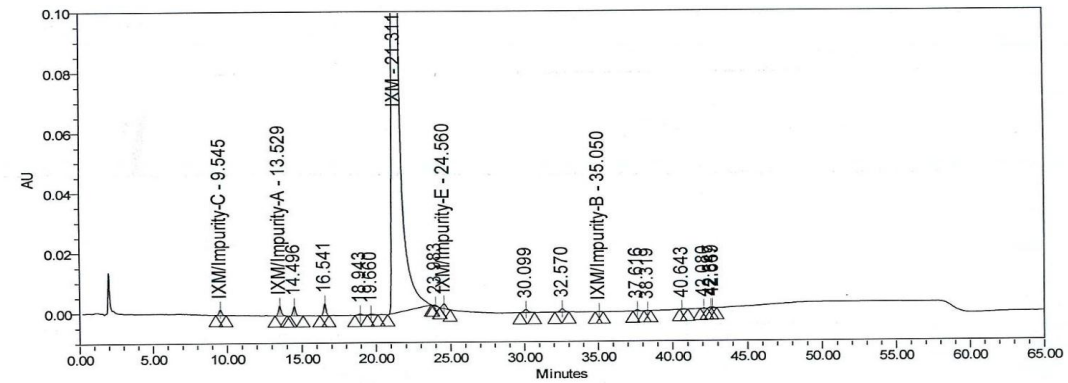


Figure 23: Typical chromatogram for Solution at UV 24 hrs.

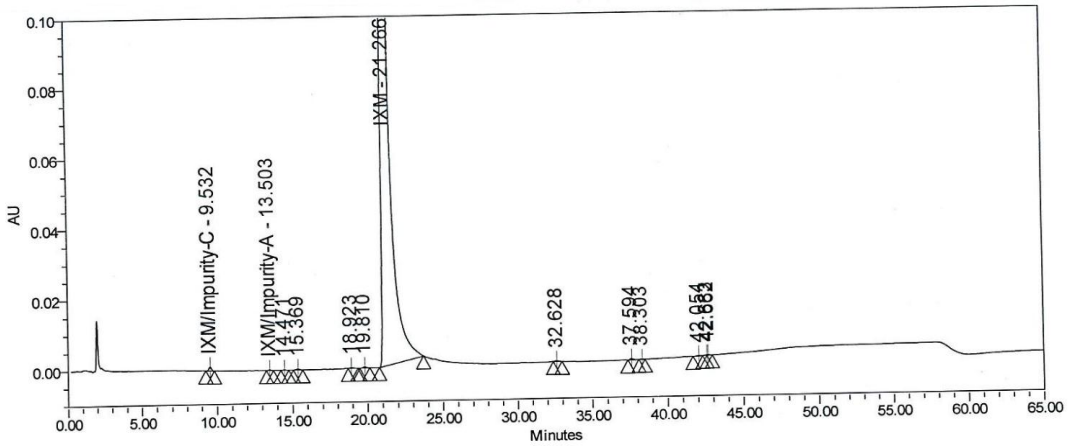


Figure 24: Typical chromatogram for Solid at UV 24 hrs.

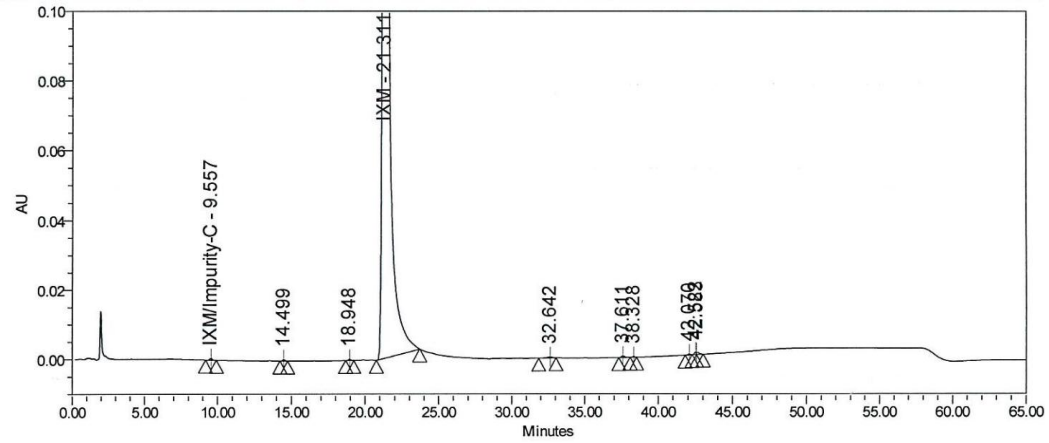


Figure 25: Typical chromatogram for Heat at 70 °C 24 hrs.

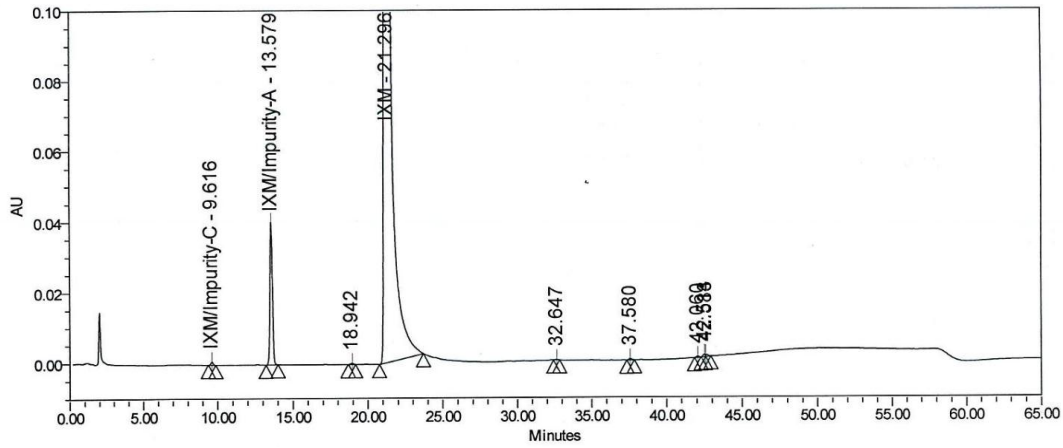


Figure 26: Typical chromatogram for water hydrolysis at 90 °C 12 hrs.

A reverse phase stability indicating HPLC method was developed and validated for the quantitative determination of the process and degradation impurities of Ixazomib citrate. The results obtained from validation experiments proved that the chromatographic method is well separated all impurities from drug substance. The present study will help the manufacturers and suppliers of Ixazomib citrate to quantify and quality the purity based on degradation data. Thus, it can be used for routine analysis, quality control and for determining quality during the stability studies of pharmaceutical analysis.

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