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STUDY OF FORCED DEGRADATION BEHAVIOUR OF A NOVEL PROTEASOME-INHIBITING ANTICANCER DRUG BY LC-MS AND DEVELOPMENT OF A VALIDATED STABILITY-INDICATING ASSAY METHOD

Babita A. Agarwal ^{*1} and Santosh V. Gandhi ²

Department of Pharmaceutical Chemistry ¹, Marathwada Mitra Mandal's College of Pharmacy, Thergaon, Pune - 411033, Maharashtra, India.

Department of Pharmaceutical Chemistry ², A.I.S.S.M.S College of Pharmacy, Kennedy Road, Pune - 411001, Maharashtra, India.

Keywords:

Carfilzomib, Stress degradation, Stability indicating assay method, LC-MS, Degradation pathway

Correspondence to Author:

Babita A. Agarwal

Associate Professor,
Marathwada Mitra Mandal's
College of Pharmacy, Thergaon,
Kalewadi, Pune - 411033,
Maharashtra, India.

E-mail: babita_a_agarwal@yahoo.co.in

ABSTRACT: In the present study, comprehensive stress testing of Carfilzomib, a newly approved proteasome-inhibiting anticancer drug was carried out according to ICH guideline Q1A (R2). The drug was subjected to acid (0.1N HCl), neutral and alkaline (0.1N NaOH) hydrolytic conditions at 70 °C, as well as to oxidative decomposition at room temperature. Photolysis was carried out in 0.1N HCl, water and 0.1N NaOH at 40 °C. LC-PDA and LC-MS investigated the products formed under different stress conditions. The LC-PDA method that could separate all degradation products formed under various stress conditions involved a C18 column and a mobile phase comprising of ACN and phosphate buffer (pH 3). The flow rate and detection wavelengths were 1 ml/min and 220 nm, respectively. The developed method was found to be precise, accurate, specific and selective. It was suitably modified for LC-MS studies by replacing phosphate buffer with water, where pH was adjusted to 3.0 with formic acid. The drug showed instability in the solution state (under acidic, neutral, alkaline and oxidative stress conditions), but was relatively stable in the solid-state, except the formation of minor products under accelerated conditions. Primarily, maximum degradation products were formed in acid conditions, though the same were also produced variably under other stress conditions. LC-MS fragmentation studies characterized the products. Based on the results, a complete degradation pathway for the drug could be proposed. LC-ESI-MS/MS characterized the major stress degradation product, and its fragmentation pathway was proposed.

INTRODUCTION: Stability testing is nowadays the key procedural component in the pharmaceutical development program for a new drug as well as new formulation.

Drugs undergo physicochemical degradation upon storage. Pharmaceutical companies perform forced-degradation studies (stress testing) during pre-formulation to help in the selection of compounds and excipients for further development, to facilitate in salt selection or formulation optimization, and to produce samples for developing stability-indicating analytical methods.

Thus, stability testing of a drug under various temperature and humidity conditions is indispensable during the drug development process.

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A stability-indicating method is “a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product.” A stability-indicating method accurately measures the active ingredients, without interference from degradation products, process impurities, excipients, or other potential impurities. Stability testing guidelines issued by International Conference on Harmonization (ICH) and other international agencies^{1, 2, 3, 4, 5} require the reporting, identification, and characterization of degradation products (DPs). Tandem Mass Spectrometry (MSn) and LC coupled with Mass Spectrometry (LC-MS, LC-MS/MS) are becoming the most versatile techniques for characterization of pharmaceutical DPs and impurity profiling⁶.

Carfilzomib (CFZ **Fig. 1**) marketed under the trade name Kyprolis, developed by Onyx Pharmaceuticals is an anti-cancer drug acting as a selective proteasome inhibitor. Chemically, it is (2S)-N-((S)-1-((S)-4-methyl-1-((R)-2-methyl oxirane-2-yl)-1-oxopentan-2-yl)carbamoyl)-2-phenylethyl)-2-((S)-2-(2-morpholinoacetamido)-4-phenylbutanamido)-4-ethylpentanamide tetrapeptide epoxyketone and an analog of epoxomicin⁷. The U.S. Food and Drug Administration (FDA) approved it on 20 July 2012 for use in patients with multiple myeloma⁸. The epoxyketone moiety binds irreversibly to the N-terminal threonine of the chymotrypsin-like active site of the proteasome and disrupts its catalytic function⁹. Only two papers discuss LC-MS quantification of this drug in biological materials^{10, 11} and one UHPLC-UV-method for CFZ or its drug formulations¹² have been reported.

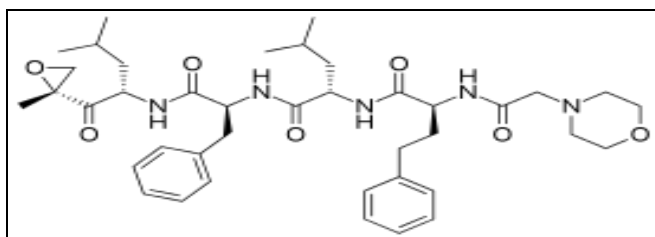


FIG. 1: STRUCTURE OF CARFILZOMIB

A comprehensive LC and LC-MS study of the degradation behavior of CFZ under various ICH prescribed stress conditions has been lacking. So, it was decided to carry out forced decomposition studies according to the ICH requirements and

develop a selective and validated stability-indicating HPLC method. An integral aim of the study was to identify new degradation products, if any, and to postulate the complete degradation pathway of the drug.

EXPERIMENTAL:

Reagents: Carfilzomib **Fig. 1** (CFZ, purity $\geq 98\%$) was obtained from Euroasian Chemicals Pvt. Ltd., (Mumbai, India) and was used without further purification. Acetonitrile (HPLC grade) was procured from SD Fine Chemicals (Mumbai, India). Analytical reagent grade (AR) hydrochloric acid, sodium hydroxide pellets, hydrogen peroxide solution (30%) were purchased from S. K. Enterprises (Pune, India). Double distilled and sonicated water was used throughout. Buffer materials and all other chemicals were of AR grade.

Instrumentation: An HPLC system used for analysis of stressed samples was an HPLC system of Agilent Technologies, Japan, LC-1260 Infinity model connected to PDA (Photodiode array, model no. G1315D 1260 DAD VL with 220 VA. A computer recorded chromatograms and treated with the aid of the LC Open Lab Software solution. Agilent 5 TC-C18 column (150 mm: 4.6 mm id, 5 μm) was used to perform the separation. The mobile phase was filtered through the Millipore glass filter assembly attached with a vacuum pump. In all studies, separations were achieved on 5 TC C-18 (150 mm: 4.6 mm id, 5 μm) column (Thermo Scientific, Japan). Carousel six stage reaction station was used for generating hydrolytic DPs. The thermal degradation study was performed using a high precision hot air capable of controlling the temperature within ± 2 $^{\circ}\text{C}$. Photodegradation study was carried out in a photostability chamber.

A pH meter (Equiptronics, Mumbai, India) was used to check and adjust the pH of the buffer solution. Also, sonicator (Citizon, Mumbai, India) and precision analytical balance (Shimadzu Aux 220) were used in the present studies. The HPLC-MS analyses were carried out on an LC-MS/MS analysis was performed using a Nexera2 LC system (Shimadzu Corporation, Kyoto, Japan) connected to a triple quadrupole mass spectrometer (LC-MS 8040; Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source.

Chromatographic separation was performed at 40°C using a SUPELCO SIL LC-18-S HPLC analytical column (5- μ m particle size, 25 cm length \times 4.6 mm inner diameter; Sigma-Aldrich).

Stress Decomposition Studies: Stress studies were carried out under the conditions of dry heat, hydrolysis, oxidation, and photolysis, as mentioned in ICH Q1A(R2)¹³. The approach suggested by Singh and Bakshi¹⁴ was adopted for these studies. A minimum of four samples were generated for every stress condition, *viz.*, the blank solution stored under normal conditions, the blank subjected to stress in the same manner as the drug (Carfilzomib), zero time sample containing the drug (which was stored under normal conditions), and the drug solution subjected to stress treatment. Hydrolytic decomposition of Carfilzomib was carried out in 0.1N HCl, water and 0.1N NaOH at a drug concentration of 2 mg ml⁻¹ at 80 °C till

sufficient degradation (~20% of the initial amount) of the drug was attained. For oxidative stress studies, Carfilzomib was dissolved at a concentration of 2 mg ml⁻¹ in 3% H₂O₂ and 20 mg/ml in 30% H₂O₂ and kept for 8 days at room temperature. Photolytic studies in solution were carried by dissolving Carfilzomib in 0.1N HCl, water and 0.1N NaOH, each at a concentration of 2 mg/ml, and exposing the solutions at by exposure to a UV lamp in a chamber set at accelerated conditions of temperature and humidity (40 °C/75% Rh). The parallel blank set was kept in the dark for comparison. Studies were also conducted on the solid drug, which was heated at 50 °C for 21 days in a dry bath. The same was also exposed to ICH prescribed dose of light in a photostability chamber set at 40 °C/75% Rh in 1 mm layer in a Petri-plate. The optimized stressed conditions are enlisted in **Table 1**.

TABLE 1: OPTIMIZED STRESS CONDITIONS FOR THE DRUG

Stressors	Hydrolytic at 80 °C			Oxidative at R.T.	Photolytic UV light at 40 °C/75% RH Solid	Thermal at 50 °C
	Acid	Neutral	Base			
Concentration of stressor	0.1N HCl	H ₂ O	0.1N NaOH	3% H ₂ O ₂	-	-
Duration	3h	3h	2h	2h	21 days	21 days

Sample Preparation for HPLC and LC-MS Analysis: The stressed samples of acid and base hydrolysis were neutralized with NaOH and HCl, respectively to obtain 1000 μ g/ml solutions. Neutral hydrolysis, thermal and photolytic samples were diluted with mobile phase to obtain concentration at 1000 μ g/ml solutions. The oxidative stress sample was diluted with mobile phase to obtain a 100 μ g/ml solution. All the prepared samples were passed through a 0.45 μ m membrane filter before HPLC and LC-MS analyses.

Development of LC-MS Method and Characterization of Degradation Products: To characterize degradation products by LC-MS studies, the developed method was modified by replacement of phosphate buffer with water and adjusting the pH to 3 by formic acid. Rest of the parameters were the same. Satisfactory separation of degradation products was achieved using a C18 column (150 mm: 4.6 mm id, 5 μ m). The optimized peak is shown in **Fig. 2**. The obtained m/z values in positive ESI mode were compared to the molecular weights (mol. wt.) of the degradation products.

The fragmentation pattern was also investigated. Based on the mol. wt. and the fragmentation pattern, the presence of known degradation products was confirmed and also, structures could be proposed for the unknowns. The degradation pathway was outlined based on the results.

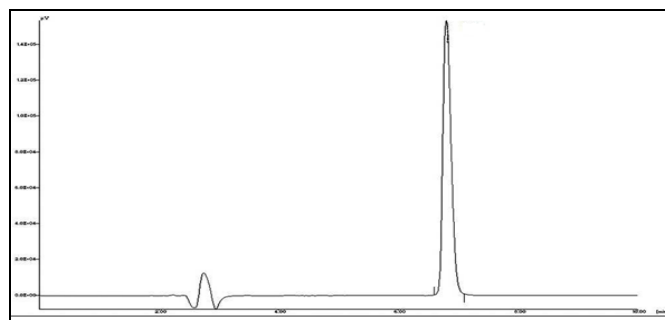


FIG. 2: CHROMATOGRAM OF STANDARD CARFILZOMIB UNDER OPTIMISED CONDITION

RESULTS AND DISCUSSION:

LC-MS Conditions: The main aim of the present research work was to separate CFZ and its DPs. A 5 TC-C18 column (150 mm: 4.6 mm id, 5 μ m) was found to be suitable for this analysis after having tried with different columns. During the optimization process on above- mentioned column,

several conditions with various mobile phases like methanol/water and acetonitrile/water in different proportionalities were tried in an isocratic mode. The peaks corresponding to DPs did not resolve completely, and tailing was observed. To get acceptable separation between the drug and its DPs, acetonitrile, and phosphate buffer was used. Further, studies were carried out using varied proportions of acetonitrile (A), phosphate buffer (B). The pH of the buffer, flow rate and composition of the mobile phase were systematically varied to optimize the method. To detect drug and DPs with sufficient peak intensity, the wavelength at 220 nm was chosen. Finally, a mobile phase consisting of A, B (pH 3.0) (0.01M, 50:50% v/v) at a flow rate of 1.0 ml/min and PDA detection at 220 nm, in an isocratic mode gave good separation of drug and its DPs. The advantage of the method was simple and rapid. Validation of the optimized LC method was done concerning various parameters outlined in ICH guideline Q1A (R2)¹³ and was extended to LC-MS studies.

LC-MS Studies: LC-MS/MS studies were carried out in + APCI ionization mode in the mass range of 50-2000 amu. High purity helium was used as carrier gas, and nitrogen was used as a nebulizer. Mass parameters were optimized to the following values: R_f loading: 80%; capillary voltage: 80 volts; syringe volume: 250 µl; spray chamber temperature: 50 °C; nebulizer pressure: 35 psi; drying gas temperature: 300 °C; drying gas pressure: 10 psi; vaporizer gas temperature: 350 °C; vaporizer gas pressure: 20 psi; spray shield voltage: ± 600.0 volts.

Evaluation of Method Validation Parameters:

Specificity: Specificity is the ability of the analytical method to measure the analyte concentration accurately in the presence of all potential DP. The specificity was determined by subjecting CFZ to stress degradation under various conditions. The DP was well separated, peak purity assessment was carried out on the stressed samples of CFZ by using diode-array-detector, and the specificity was also established by subjecting the degradation sample to LC-MS analysis using the same method. The mass detector also showed an excellent mass purity for CFZ and its DP which unambiguously proves the specificity of the method.

Linearity: Linearity test solutions were prepared from a stock solution at six concentration levels of analyte (20, 40, 60, 80, 100 and 120 µg/ml). The peak area versus concentration data was performed by least squares linear regression analysis. The calibration curve was drawn by plotting CFZ average area for triplicate injections and the concentration expressed as a percentage. Linearity was checked over the same concentration range for three consecutive days. Good linearity was observed in the concentration range from 20 to 120 µg/ml of CFZ. The data were subjected to statistical analysis using a linear regression model; the linear regression equation and correlation coefficient (r²) were $y = 28528x + 99441$ and 0.9999 respectively. These results indicate estimable linearity. The LOD and LOQ for CFZ were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively. The LOD and LOQ were 6.80 µg/ml and 20.61 µg/ml respectively.

Precision: Repeatability and intermediate precision studies verified the precision of the method. Repeatability studies were carried out by analyses of three different concentrations of the drug in hexaplicate on the same day. Intermediate precision of the method was checked by repeating the studies on three different days. Additionally, the developed HPLC method was checked through separation studies on the mixture of reaction solutions on a different chromatographic system on a different day. The results of repeatability and intermediate precision experiments are shown in **Table 2**.

The developed method was found to be precise as the RSD values for repeatability, and intermediate precision studies were < 1.5. Separation of the drug and its DP in a mixture of stressed samples was found to be similar when analyses were performed on a different chromatographic system on different days.

TABLE 2: PRECISION STUDIES

Concentration taken (µg/ml)	Measured concentration (µg/ml) ± S.D.	RSD (%)
40	40.15 ± 0.58, 1.25	40.42 ± 0.69, 1.37
60	60.75 ± 0.59, 0.98	59.38 ± 0.60, 0.75
80	80.54 ± 0.64, 1.05	80.79 ± 0.45, 1.18

Accuracy: Accuracy of the method was assessed employing the standard addition method at three different levels (80%, 100%, 120%). The mixtures

were analyzed in triplicate and the percentage of added drug obtained from the difference between peak areas of unfortified and fortified samples of CFZ. The HPLC area responses for accuracy determination are depicted in **Table 3**. Good recoveries (99.46 ± 0.23) of the spiked drugs were obtained at each added concentration, indicating that the method was accurate.

TABLE 3: RECOVERY STUDIES

Spiked concentration ($\mu\text{g/ml}$) (%)	Measured concentration ($\mu\text{g/ml}$) \pm S.D.	RSD	Recovery (%)
32	31.35 ± 0.12	0.384	99.17
40	39.69 ± 0.122	0.308	99.22
48	47.81 ± 0.165	0.34	99.60

Robustness: To determine the robustness of the method, experimental conditions were purposely altered. Three parameters selected were flow rate, the composition of mobile phase and solvent from different lots. The mobile phase flow rate was 1.0 ml/min which was changed to 0.9, and 1.1 ml/min and the effect was studied. The effect of mobile phase composition was analyzed by use of acetonitrile and phosphate buffer (pH 3.0; 0.01M) and in the ratio of 48:52 (v/v) and 52:48 (v/v). For all changes in conditions, the sample was analyzed in triplicate. When the effect of altering one set of conditions was tested, the other conditions were held constant at the optimum values. In all the calculated varied chromatographic conditions, no significant change in retention time and a tailing factor of CFZ was observed. The summary of results is shown in **Table 4**.

TABLE 4: ROBUSTNESS STUDIES

Chromatographic changes			
Factor ^a	Level	RT ^b	Tf ^c
A: Flow rate (ml/min)			
0.9	-1	7.0	1.22
1.0	0	6.8	1.21
1.1	1	6.5	1.11
Mean \pm S.D. (n = 3)		6.76 ± 0.25	1.18 ± 0.06
B: Percentage of acetonitrile in the mobile phase (v/v)			
3	-1	6.8	1.21
5	0	6.8	1.21
7	1	6.7	1.06
Mean \pm S.D. (n = 3)		6.76 ± 0.05	1.16 ± 0.08

^aTwo factors were slightly changed at three levels (1, 0, -1); each time a factor was changed from level (0), another factor remained at level (0). ^bRT: Retention time. ^cTf: tailing factor.

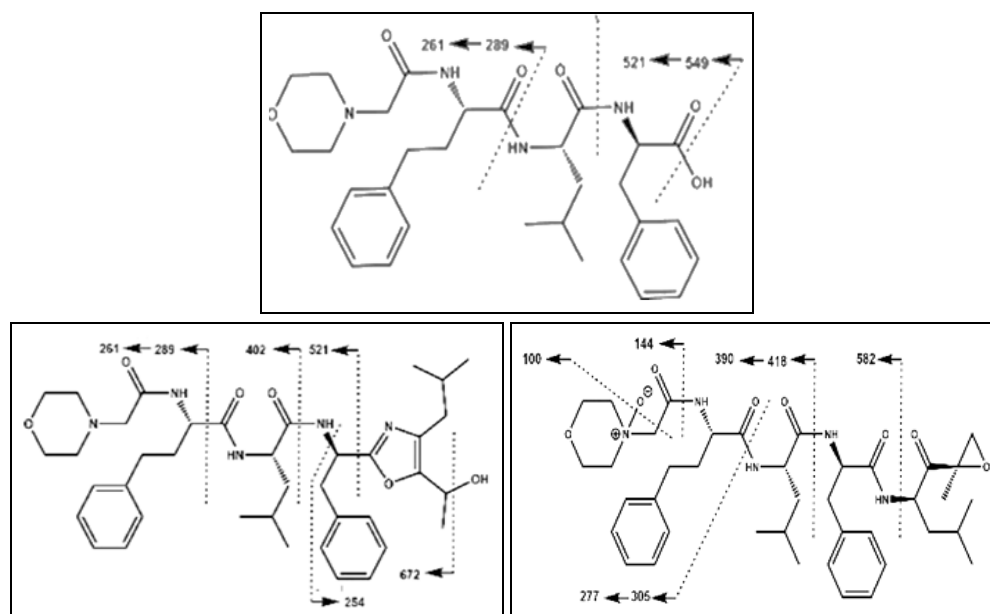
System Suitability Test: The system suitability parameters concerning theoretical plates, capacity factor, resolution factor, tailing factor were calculated and are given in **Table 5**. It could be seen from the table that all the peaks were well resolved.

TABLE 5: HPLC SYSTEM SUITABILITY PARAMETERS

Code	RT ^a	K	Rs	N	Tf
CFZ	6.8	2.2	1.8	12681	1.2

^aRT: retention time; K: capacity factor; Rs: USP resolution; N: number of theoretical plates; Tf: USP tailing factor.

Forced Degradation Study: CFZ was stable in the neutral solution, where virtually no degradation was observed after 6 h at 70 °C. It was relatively stable in acidic media, where only increased temperature and high concentrations of HCl yielded appreciable degradation. CFZ readily degraded under alkaline, oxidative and photolytic stress.

**FIG. 3: STRUCTURE FRAGMENTATION**

The HPLC-PDA analysis of the samples exposed to stress conditions revealed 5 unknown peaks corresponding to potential degradation products. The fragmentation of the molecule is shown in **Fig. 3**.

Acidic Conditions: Two degradation product (DP I, RT 0.5, m/z at 567.31, DP II, RT 0.7, m/z at 756.41) was detected in the sample exposed to hydrogen peroxide in the acidic medium in all two peaks were seen in the chromatogram and the mass studies revealed them to be at 567.31 and 756.41 apart from the standard drug peak. Literature studies reveal two means by which the peaks might have been observed.

First is the peptide bond hydrolysis which breaks the molecule into two DPs and second could be due to epoxide hydrolysis in acidic medium. The covalent stability of peptide bonds is a critical aspect of biological chemistry and therapeutic protein applications. In addition to affecting atoms directly involved in bond making and breaking processes, the water network also has determining effects on the hydrolytic reaction, a fact by which the degraded products might have been formed in the stressed samples of acidic conditions. The proposed degradation pathway for the formation of acid degraded product is shown in **Fig. 4**.

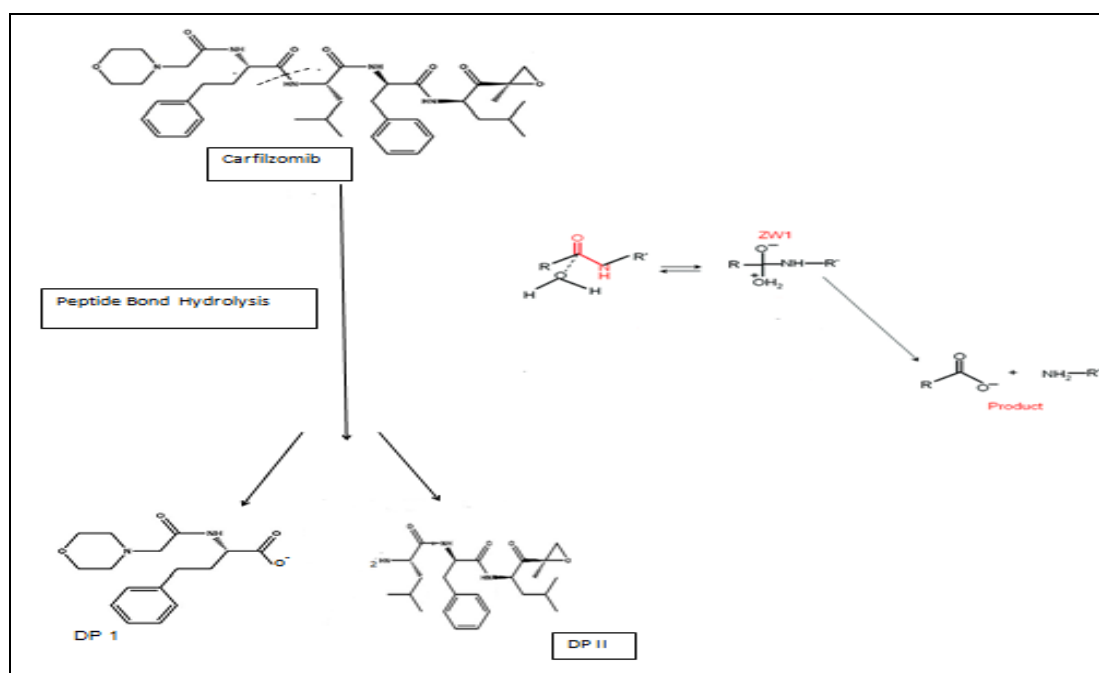


FIG. 4: DEGRADATION MECHANISM FOR ACIDIC CONDITION

Alkaline Conditions: The proposed mechanism of CFZ chemical decomposition to Alkaline degraded product (DP III, RT 1.2, m/z at 672.44, DP IV, RT 0.5, m/z 738.44) has been reported by Vit Sestak *et al.*,¹² which shows that the products might have been formed by base-catalyzed epoxide ring opening of CFZ [followed by retro-aldol condensation [and a base-mediated Robinson-Gabriel reaction yielding an oxazole ring.

Oxidative Conditions: One degradation product (DPV, RT 2.2, m/z at 736.4288) was detected in the sample exposed to hydrogen peroxide. According to literature, N-oxides can be formed from tertiary amines by using hydrogen peroxide. The nitrogen atom acts as a nucleophile in this reaction by attacking an oxygen atom of the

peroxide resulting in the formation of hydroxy ammonium ion and hydroxide anion. Subsequently, the latter reacts with the hydrogen atom of the hydroxyl group at the nitrogen releasing the amine oxide. Tertiary amines are readily oxidized. Atmospheric oxygen is sufficient to oxidize aromatic amines to the corresponding amine oxides.

Therefore, other functional groups can be oxidized only with difficulty in the presence of amines. Hence the degraded peak which was seen in MS studies at 736.42 could be due to the formation of N-oxide. The peak could be of Carfilzomib N-Oxide. The proposed degradation pathway for formation of Carfilzomib N-oxide is shown in **Fig. 5**.

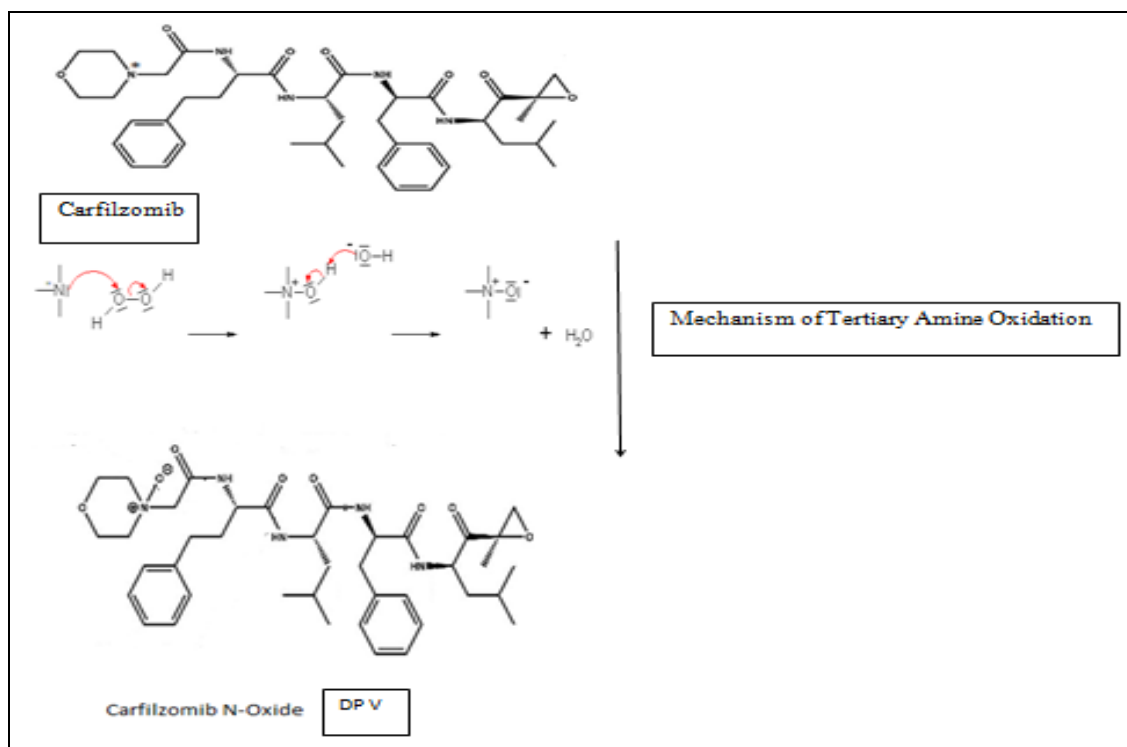


FIG. 5: DEGRADATION MECHANISM FOR OXIDATION CONDITION

Mass Chromatograms in the negative electron spray ionization (ESI) mode for the drug and the degraded products are shown in **Fig. 6**. The m/z

value of 719 for Carfilzomib corresponded to its molecular weight of 720, thus validating the output of the Mass Spectrometer.

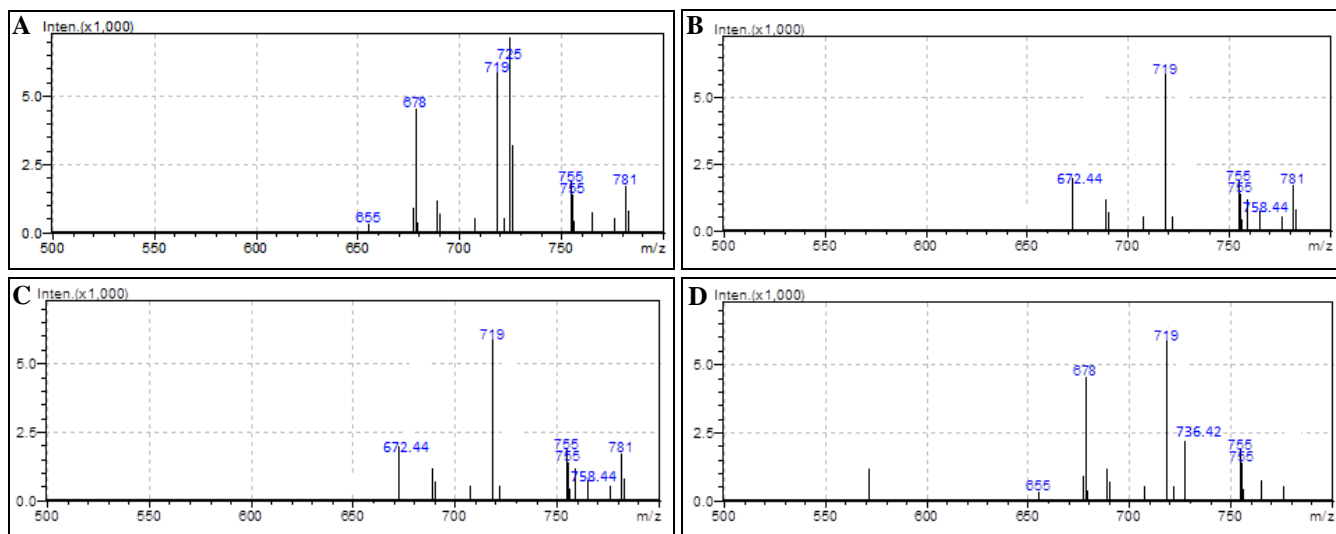


FIG. 6: MASS PROFILES FOR CARFILZOMIB (A), ACID DEGRADATION (B), ALKALINE DEGRADATION (C) AND OXIDATIVE DEGRADATION (D)

CONCLUSION: It was possible in this study to develop a stability-indicating LC assay method for Carfilzomib by subjecting the drug to ICH recommended stress conditions. The drug and degradation products got well separated from each other in an isocratic mode using a reversed-phase C18 column and mobile phase composed of acetonitrile and phosphate buffer (pH 3.0; 0.01M)

(50:50, v/v). The flow rate and detection wavelengths were 1 ml /min and 220 nm, respectively. The method proved to be simple, accurate, precise, specific and selective. It was easily transferable to LC-MS. Also, it could be successfully employed for analysis of the drug and degradation products in the marketed products. The stress studies and subsequent LC-MS analyses

showed that the drug was decomposed in acidic, alkaline, oxidative and photolytic conditions. The product was characterized through MS and IR spectral studies.

The mechanistic explanation for the formation of this product is postulated. It may be pertinent to add here that the stability-indicating method proposed in this paper may be used for the establishment of mass balance, provided consideration is given to the formation of equimolar quantities of products during degradation of the drug.

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CONFLICT OF INTEREST: The author declares no conflict of interest.

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