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Forced Degradation Of Glecaprevir: Development And Validation Of Stability Indicating RP-HPLC Method And LC-MS Characterization Of Degradants

D. Upender Reddy*[1] , T. Ramamohan Reddy[2]

[1*]Research Scholar, Career Point University, Kota, Rajasthan.

[2]Research Supervisor, Career Point University, Kota, Rajasthan.

Abstract

Forced degradation studies of the drugs and drug products are vital in pharmaceutical quality management. Glecaprevir is an antiviral drug indicated for the treatment of Chronic HCV infection treatment. Glecaprevir was subjected to various stress (Hydrolytic, photolytic, thermal and oxidative) conditions according to the ICH guidelines. Degradation products were characterized by the tandem mass spectrometric method. A simple stability indicating RP-HPLC method was developed to determine Glecaprevir in the presence of its degradation impurities. Glecaprevir is stable in thermal, oxidative, and photolytic conditions. Degradation of the drug observed in Acid and Base hydrolytic conditions. Separation was achieved on BEH C18 column with 10mM formic acid aqueous solution and acetonitrile (30:70 % V/V) mobile phase in isocratic elution mode. The developed LC method is linear in the range of 20-200µg/ml. Degradation products were characterized by tandem mass spectral studies and the fragmentation pathways were proposed from the mass spectral analysis. The developed RP-HPLC method was validated according to the ICH guidelines.

Keywords: Glecaprevir, LC-MS/MS, Forced degradation, Method development, Validation.

1. INTRODUCTION

Globally, Hepatitis C is the reason for 3% deaths caused by viruses. Hence, the World Health Organization declared hepatitis a global health problem (Mahmoud et al., 2013; Global Hepatitis Report 2017; WHO CDS 2018; WHO IGO 2018). Glecaprevir (Figure 1) is one of the two APIs in Maviret, a fixed-dose combination along with Pibrentasvir indicated for the treatment of chronic hepatitis C virus (HCV) infection (Andreoni et al., 2021; Sandmann et al., 2019 Pawlotsky et al., 2018). Glecaprevir is an inhibitor of HCV NS3/4A protease necessary for the proteolytic cleavage of the HCV encoded polyprotein (Ng et al., 2017). In combination with Pibrentasvir, Glecaprevir is a useful therapy for patients who experienced therapeutic failure from other NS3/4A protease inhibitors. The ultimate goal of the combination treatment is to achieve sustained virologic response (SVR) and cure the patients from the infection (Asselah et., 2016). Chemically, Glecaprevir is (3aR,7S,10S,12R,21E,24aR)-7-tert-butyl-N-{(1R,2R)- 2-(difluoro methyl)-1-[(1-methyl cyclo propane-1-sulfonyl) carbamoyl] cyclopropyl} -20,20-difluoro-5,8-dioxo-2,3,3a,5,6,7,8,11,12,20,23,24a-dodecahydro-1H,10H-9,12-methanocyclo penta [18,19] [1,10,17,3,6]-trioxa diaza cyclononadecino-[11,12-b] quinoxaline-10-carboxamide corresponding to the molecular formula $C_{38}H_{46}F_4N_6O_9S$ with 838.87 molar mass.

Development of stability indicating assay method for the API and drug products is an essential part on the pharmaceutical quality control and ICH specified the respective guidelines in Q1A (R2) (ICH 2003). The ICH recommends the exposure of the drug to stress conditions of hydrolysis, oxidation, thermal and photolysis to generate information on degradation products liable to be formed under the influence of these conditions (Armenta et al., 2008; Koel et al., 2006). Previous reports are available majorly on the assay determination of Glecaprevir in API and various drug products using Liquid Chromatographic techniques (Kumar et al., 2018; Kanthale et al., 2019; Sreeram et al., 2018). There were no reports on the characterization of the forced degradation products of Glecaprevir using tandem mass spectral analysis.

Current analytical investigation studies the degradation behaviour of Glecaprevir under various stress conditions as per the ICH guidelines. Degradation products of Glecaprevir characterized by tandem mass spectrometric method and a stability indicating RP HPLC method established for Glecaprevir in the presence of its degradation products.

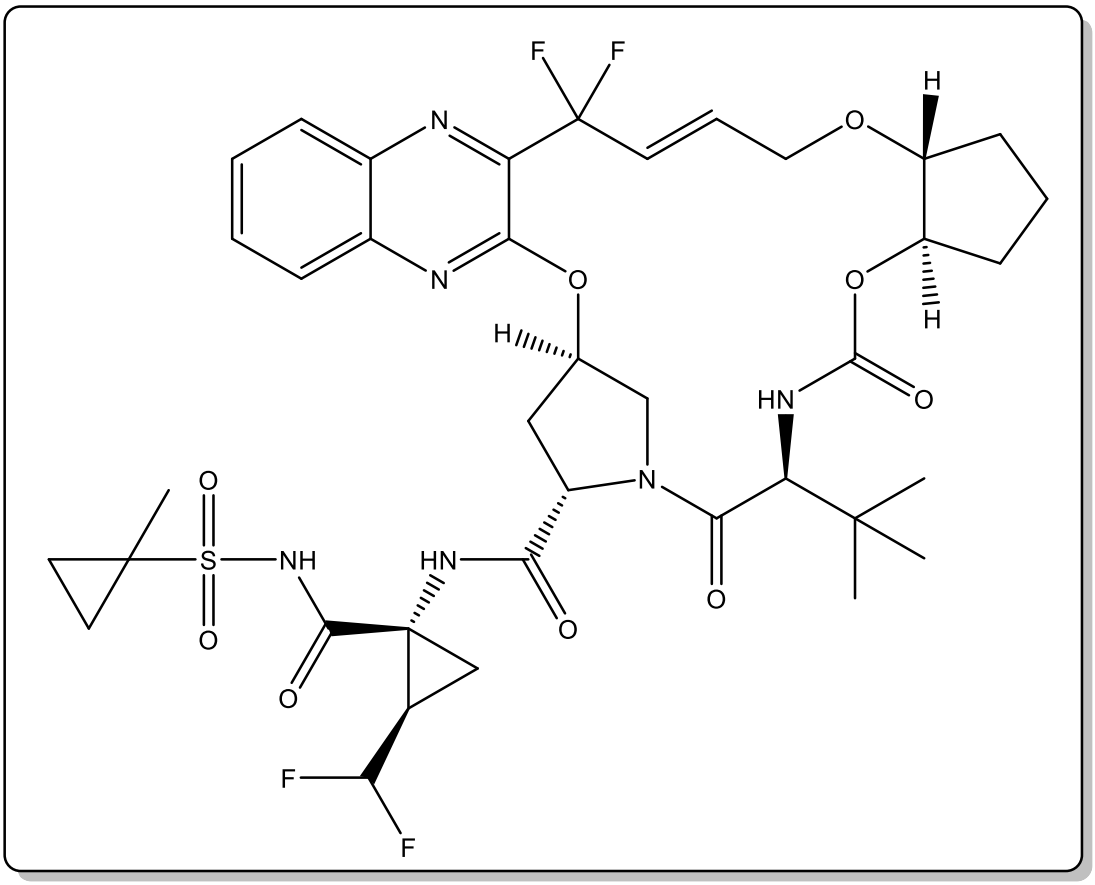

Figure 1: Structure of Glecaprevir

2. EXPERIMENTAL

2.1. Materials

Glecaprevir was kind gift sample provided by Santo Righello Private limited, Hyderabad, India. Analytical grade acetonitrile and formic acid were procured from Merck, India. Ultra-pure water was obtained by filtration with 0.22 µm membrane from Millipore. Hydrogen Peroxide, Sodium Hydroxide, Hydrochloric acid were procured from SD Fine Chem, Hyderabad, India.

2.2. Instrumentation

Shimadzu Nexera X2 (LC-30AD) chromatographic unit consisting of SPD-M20A (Prominence) diode array detector coupled with Shimadzu 8040 triple quadrupole mass analyzer was employed in the current analysis. Data acquisition and integration was carried out on LC-MS Lab Solutions software (Shimadzu Corp, Kyoto, Japan). Photodegradation was conducted in a photostability chamber equipped with UV lamp.

2.3. Forced degradation Study

ICH guidelines were followed in forced degradation experiments. Acid and base hydrolysis carried out in 0.1N HCl and 0.1N NaOH solutions, respectively, for 24hrs and neutral hydrolysis carried out in deionized water for 72 hours. All the hydrolysis experiments were conducted at room temperature. Oxidative degradation experiments were carried out with 0.3% H_2O_2 at room temperature for 2 days. Photodegradation was performed on a thin layer of solid drug exposed to UV light of 254nm for 24 hours in a photostability chamber. For thermal degradation, solid drug substance was spread about 1mm thickness in a Petri dish and kept at 80^0C for 72 hours. Drug sample concentration of 10mg/ml was employed for the acid, base, and oxidative stress experiments. For the LC analysis, sample aliquots were worked up and diluted with diluent then injected into the chromatographic system. Prior to the injection into the chromatographic system, all solutions were filtered using 0.22 µm membrane filters.

2.4. Standard Solution

Glecaprevir standard solution of 1mg/ml concentration was prepared from the 10mg of API in water. Working standard solution of 100µg/ml concentration was prepared from the prepared standard solution. These working standard solutions were freshly prepared before the chromatographic analysis.

2.5. Calibration Curve

Working standard solution of Glecaprevir was diluted to obtain a concentration range of 20–150 µg/ml. 20µl of each concentration was injected into LC in a triplicate manner. The peak area response against each concentration tested was plotted in calibration graph and the regression equations

2.6. Chromatographic and Mass Spectrometric conditions

The Glecaprevir and its degradation products are separated on BEH C18 (100mm*4.6mm*1.5 µm) column. The mobile phase consists of 10mmol of formic acid aqueous solution and acetonitrile (30: 70 %V/V). The analysis was performed in isocratic elution mode with a flow rate of 1 ml/min. Column oven and Auto sampler temperatures were maintained at 35^0C.

Characterization of degradation products by mass detection was processed using Shimadzu 8040 triple quadrupole mass spectrometer with electrospray ionization in positive mode. The mass parameters were optimized by tuning the LC-MS/MS system for all the degradation products by introducing the degradation sample into LC mobile phase. The flow rate of was decreased to 0.3ml/min while the sample was introduced into the mass spectrometer. The optimized mass conditions were: ion source gas-1 was 30psi, ion-spray voltage was 3500V, curtain gas was 20 psi, de-solvation temperature at 400℃, ion source gas-2 was 35 psi. Good resolution was achieved by utilizing same column and mobile phase as in LC-separation and the structural identification of every degradation product was processed with LC-MS fragmentation process.

2.7. Method Validation

The developed LC technique was validated as per the guidelines of ICH-Q2(R1). The validation parameters selectivity, accuracy, Specificity, precision, and linearity were selected for the method validation (ICH Q2A; ICH Q2B).

3. RESULTS AND DISCUSSION

The present study aims to investigate the degradation behaviour of the Glecaprevir using LC-MS/MS technique and method development for the Glecaprevir estimation along with its degradants.

3.1. Optimization of Chromatographic and Mass Spectrometric conditions

Achievement of good resolution between the Glecaprevir and its degradation products with symmetric peak shapes is of prime importance in the method development process. The composition of mobile phase is the critical parameter to ensure the resolution and peak symmetry. Mobile phase system is selected based on solubility and pka of the Glecaprevir and compatibility with mass detector. Acetonitrile and Methanol provided good solubility for the Glecaprevir. For peak resolution, numerous mobile phase systems with varying compositions were investigated in both isocratic and gradient elution modes. Initially, methanol and formic acid aqueous solution mixture provided low peak response and peak purity. Acetonitrile and formic acid aqueous solution provided good peak response and resolution with symmetrical peaks. Various stationary phases (Luna C18, Inertsil ODS C18, and BEH C18) were employed to resolve the drug and its degradation products. A better resolution was observed on BEH C18 (100mm*4.6 mm*5 µm) column in isocratic elution mode with 10mmol formic acid and acetonitrile (30:70 V/V) mobile phase. LC run for 15mins with the mobile phase flow rate of 1ml/min and the detection was observed at 265nm. Method development chromatograms of acid and base degradation were given in Figures 2 and 3. A typical optimized LC chromatogram of Glecaprevir and its degradation products is shows that Glecaprevir retained at 4.5mins with no interference from its degradation products.

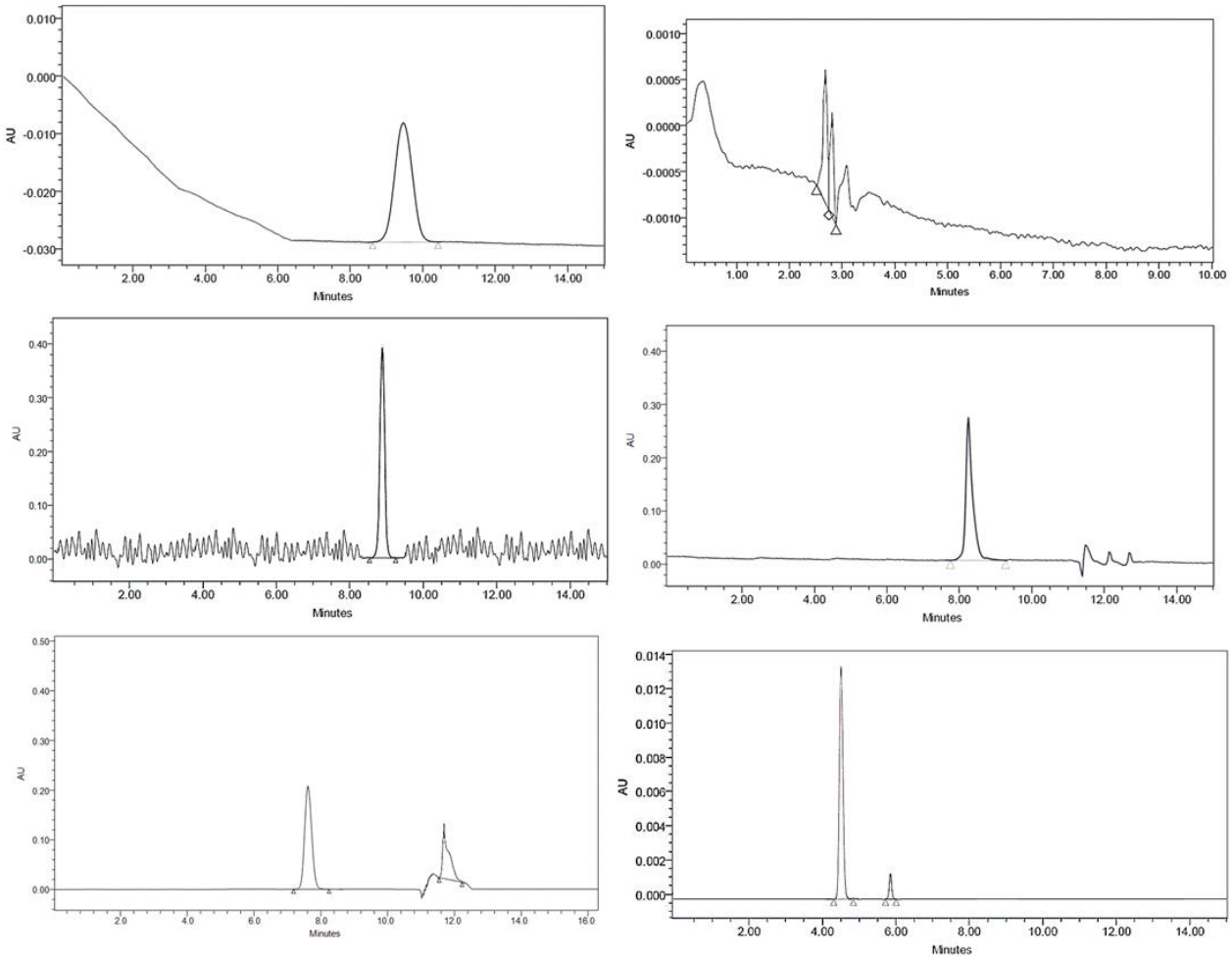

Figure 2: Trial and Optimized Chromatograms of Acid degradation.

3.2. Degradation Behaviour

Forced degradation of Glecaprevir under various conditions revealed stability of the drug towards thermal, photolytic, oxidative, and neutral hydrolytic conditions. Glecaprevir displayed considerable degradation in acidic and basic hydrolytic test conditions. Under acid hydrolytic conditions, 7 percent of Glecaprevir degraded after 24 hours. In basic hydrolytic conditions, 11 percent of Glecaprevir degraded after 24 hours. Percentage degradation suggests that Glecaprevir is relatively unstable in the basic medium. Two primary degradation products, DP-1, and DP-2 were observed in the acid and base degradation conditions, respectively. LC-MS/MS analysed the degradation products of acid and base hydrolysis, and the total ion chromatograms of both DPs were given in Figure 4. Mass analysis of both degradants displayed the same molecular ion at m/z 589 in their respective total ion chromatograms. It revealed that the same degradation product formed in acid and base hydrolytic conditions. The m/z values of all degradants and their fragment ions were enumerated in Table 1. The mass spectrum of the molecular ion and its degradants m/z values indicate that Glecprevir hydrolysed to its subsequent acid form (m/z 589) and corresponding amine in both hydrolytic conditions (Figure 5).

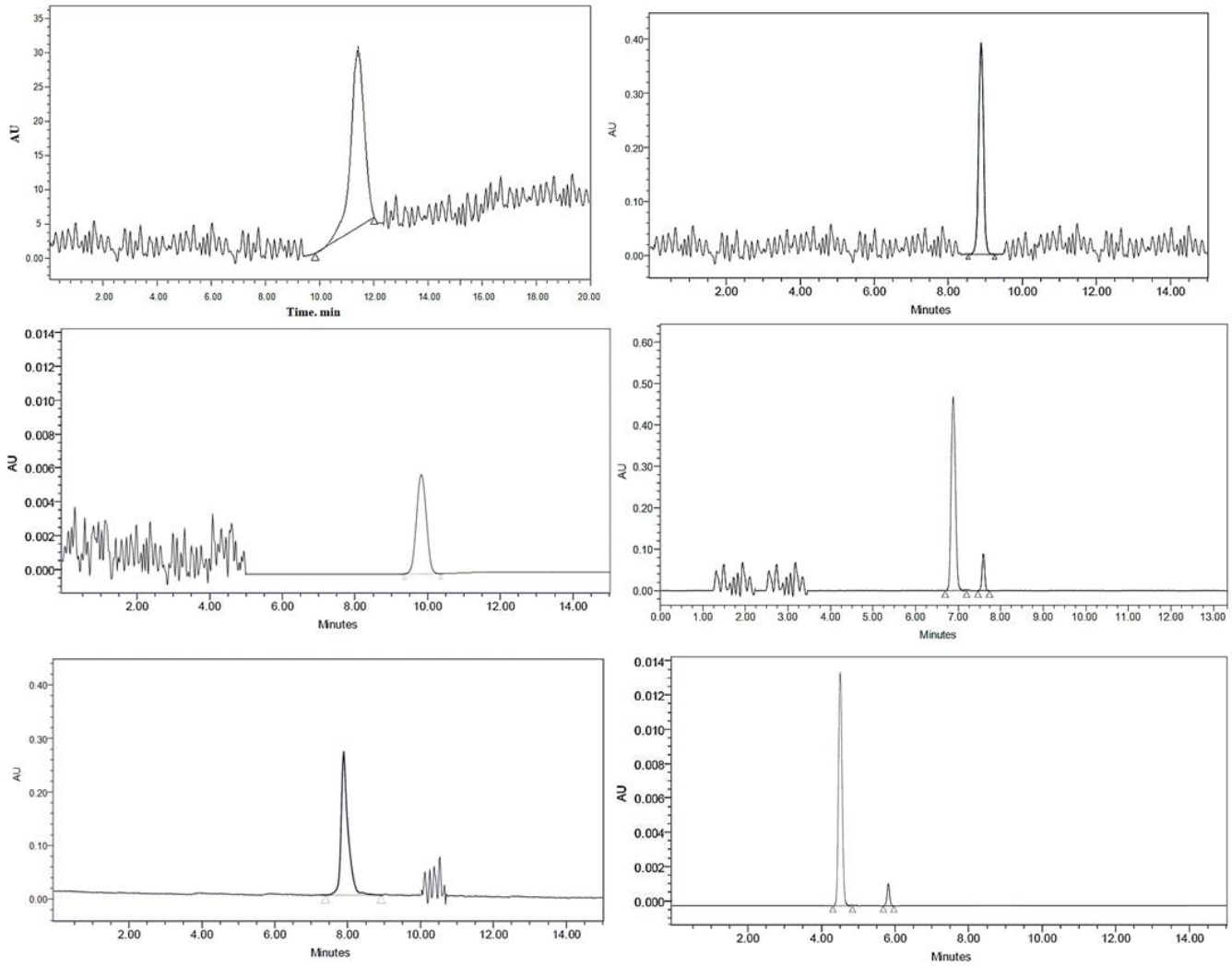

Figure 3: Trial and Optimized Chromatogram of Base degradation.

Table 1: m/z values of the fragmented ions of DPs

Degradation	m/z	Molecular formula
Acid and Base DP (DP-1 & 2)	589.55	$C_{29}H_{35}F_2N_4O_7^+$
	563.60	$C_{28}H_{37}F_2N_4O_6^+$
	337.26	$C_{17}H_{19}F_2N_2O_3^+$
	223.15	$C_{11}H_9F_2N_2O^+$

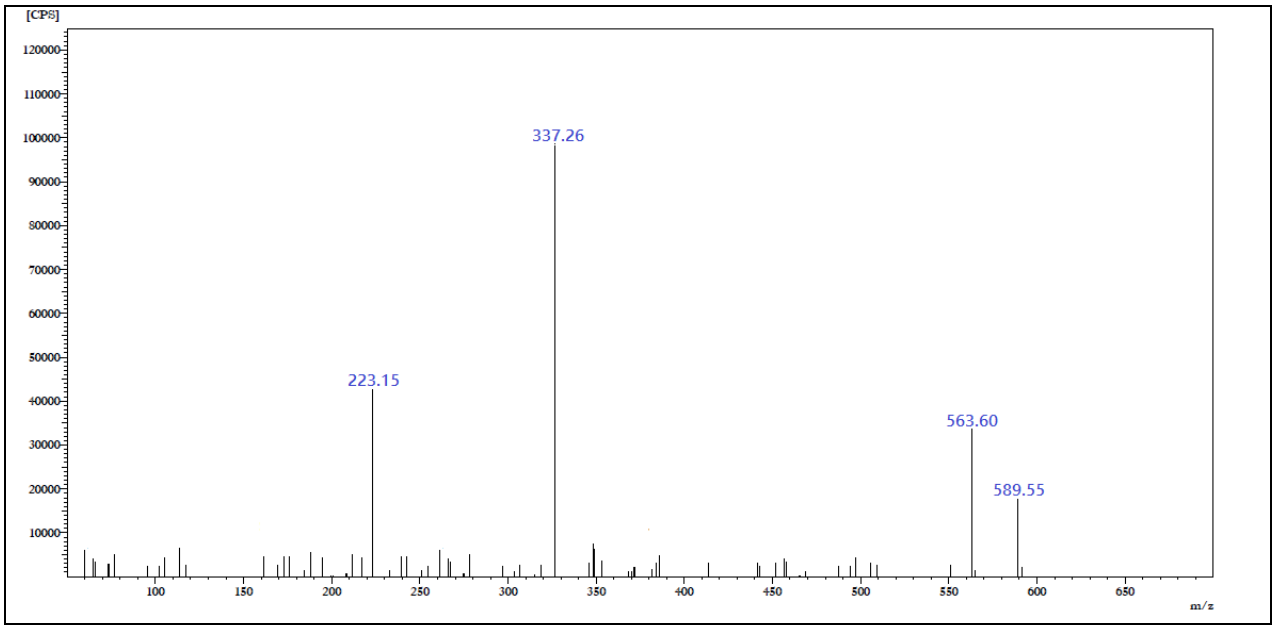

Figure 4: Total Ion Chromatogram of DP-1 & 2

3.3. Validation of HPLC Method

3.3.1. Specificity

The Specificity of the method was established by obtaining the resolution between the Glecaprevir and its degradation products. Satisfactory resolution without any interference at the retention time of Glecaprevir and its degradation products determined the Specificity of the developed method. Specificity chromatograms are depicted in Figure 2 and 3.

3.3.2. Linearity

Linearity of the method for determining Glecaprevir was established by analyzing the six different concentrations of the drug in the range of 20-150µg /ml. By plotting each concentration under analysis against their average peak area response, the linearity of the methods was determined, and regression equations were calculated. The calibration curve disclosed the linearity of the developed method over the tested concentration range, and r^2 values found to be 0.9997. Results of the linearity study were enumerated in Table 2.

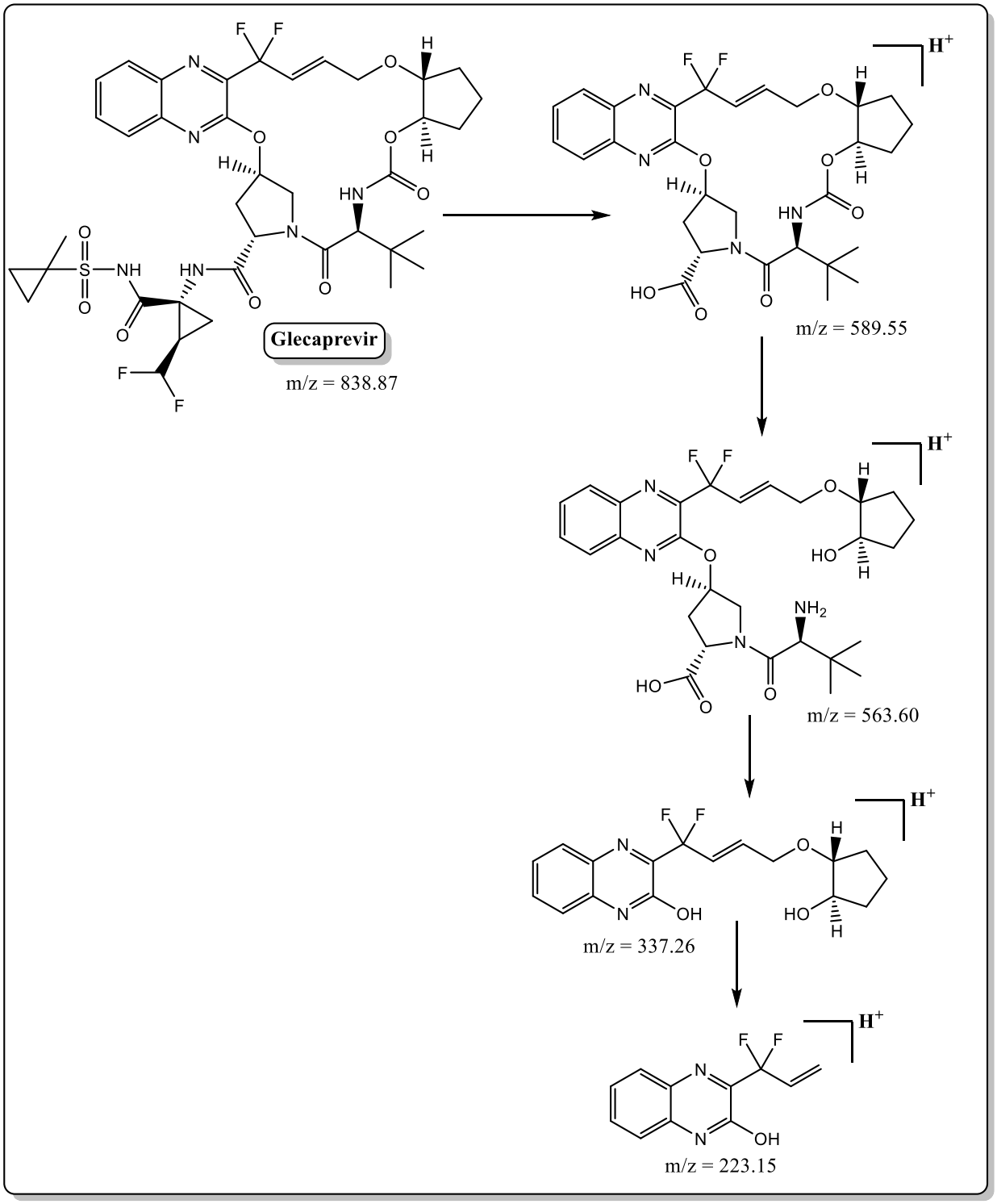

Figure 5: Fragmentation pathway of DP-1&2

Table 2: Results of Linearity study of Glecaprevir

S. No	Level	Glecaprevir	
		Concentration	Peak area
1	20%	20µg/ml	65231

2	50%	50µg/ml	169824
3	75%	75µg/ml	246071
4	100%	100µg/ml	325618
5	150%	150µg/ml	488023
6	200%	200µg/ml	659874
Slope			**0.0003x**
Intercept			**-0.2252**
r2			**0.9997**

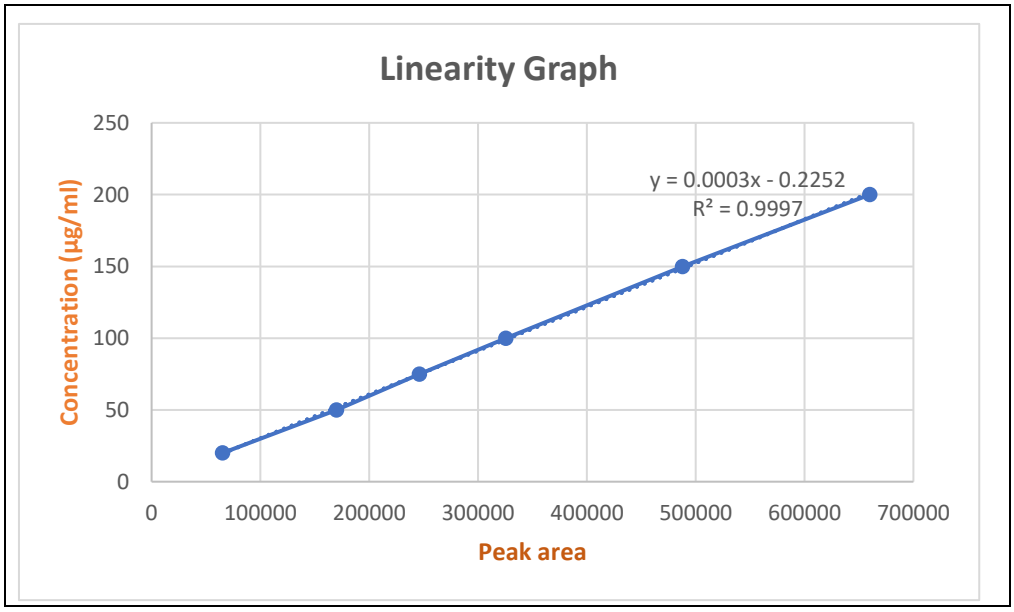

Figure 4: Linearity Plot of Glecaprevir

3.3.3. Limit of detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ of the developed method were determined by obtaining signal to noise ratio of 3:1 and 10:1, respectively. The limit of detection and quantification were estimated to be 0.1µg/mL and 0.4µg/mL, respectively, for Glecaprevir.

3.3.4. Accuracy

The accuracy of the developed method was checked by the percent recovery method. Recoveries of the three different concentrations of Glecaprevir samples were determined. The percentage recovery findings were found in the range of 99.74% - 103.08%, and the findings are shown in Table 4. All accuracy experiments were performed in triplicates.

Table 3: Results of Accuracy study of Glecaprevir

	50% level			**100% level**			**150% level**		
Amount Added (µg)	50	50	50	100	100	100	150	150	150

Amount found (µg)	51.06	49.87	50.85	103.08	101.32	99.92	149.74	152.96	153.04
% Recovery	102.12	99.74	101.7	103.08	101.32	99.92	99.82	101.97	102.02
AVG	102.12			103.08			99.82		
SD	1.270328042			1.583414033			1.255985669		
RSD	1.243956171			1.536102089			1.25825052		

3.3.5. Precision

The precision was estimated by %RSD of response. The precision data were shown in Table 4 and the %RSD was 2.26%, which is well below the acceptance criteria, indicating the precision of the developed method.

3.3.6. Robustness

Robustness was processed by creating slight variations in the flow(±0.1ml/min) and composition (±2%) of mobile phase and % RSD was evaluated for chromatographic parameters such as theoretical plates tailing factor with respect to the variations were tabulated. The findings were satisfactory and represented in Table 5.

Table 4: Results of Precision study of Glecaprevir

S. No	**concentration (ng/mL)**	**Peak area**
1	100µg/ml	328961
2	100µg/ml	330145
3	100µg/ml	324872
4	100µg/ml	321478
5	100µg/ml	311243
6	100µg/ml	330214
Avg		324485.5
SD		7340.762869
%RSD		2.262277627

Table 5: Results of Robustness Study

Change in parameter	**% RSD**
Flow (1.1 ml/min)	2.4
Flow (0.9 ml/min)	2.7
Acetonitrile (2% increase)	3.1
Acetonitrile (2% decrease)	2.9

4. CONCLUSION

Glecaprevir active pharmaceutical ingredient subjected to Hydrolytic, photolytic, thermal and oxidative stress conditions in accordance to the ICH guidelines. Degradation of the API observed in acid and base hydrolytic conditions. Degradation products were characterized by the tandem mass spectrometric method. A simple stability indicating RP-HPLC method was developed to determine Glecaprevir in the presence of its degradation impurities. Two degradation products, DP-1 & DP-2 were formed in acid and base hydrolytic conditions. Mass Spectral fragmentation of both DPs revealed a similar fragmentation pattern that indicates the same degradation product in acid and base hydrolysis.

Acknowledgment

None

Conflict of Interest

Authors disclose no conflicting interests with this work.

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