





An effective HPLC method for evaluation of process related impurities of Letemovir and LC-MS/MS characterization of forced degradation compounds

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Abstract: This study aimed to propose a straightforward and highly sensitive HPLC method for the evaluation of letermovir, coupled with an examination of the stress degradation nature of letermovir. Chromatographic separation analytes were attained using a Phenomenex Luna C18 column (250 mm × 4.6 mm; 5 μm id) maintained at 35 °C. The mobile phase comprises 0.1% phosphoric acid, methanol, and acetonitrile in 45:25:30 (v/v) facilitated isocratic elution at 0.8 mL/min with 234 nm wavelength. Under the proposed conditions, retention times were determined to be 9.58 min for letermovir and 11.10 min, 4.18 min, 6.30 min, and 13.26 min for impurities 1, 2, 3, and 4, respectively. The method achieved a sensitive detection limit of 0.009 with 0.05–0.2 μg/mL as the linear range for impurities. Other validation tests met acceptable criteria for letermovir and impurities. Additionally, stress degradation tests were conducted following ICH Q1A (R2) guidelines, subjecting the drug to various stress conditions. LC-MS/MS analysis identified five degradation products (DPs), of which DP 1, 4, and 5 were formed due to acid stress, whereas DP 2, 3 and 5 were formed due to peroxide, base and UV stress respectively. The possible structure of DPs was assessed by the interpretation and correlation of mass fragment data. The validation test produces satisfactory results supporting the suitability of the method for regular analysis of letermovir and its impurities. Moreover, the method is applicable for evaluating the degradation mechanism of letermovir.

Keywords: Letermovir; impurities; HPLC method optimization; stress degradation products; LC-MS/MS. © 2023 ACG Publications. All rights reserved.

1. Introduction

Letermovir is an antiviral medication belonging to the viral terminase inhibitors used to prevent cytomegalovirus (CMV) infection in adult patients who have undergone hematopoietic stem cell transplantation. CMV is a common virus that can cause serious infections, particularly in humans with

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weakened immune systems who are undergoing stem cell transplantation [1]. Letemovir works by inhibiting the replication of CMV, thereby reducing the risk of CMV infection in susceptible individuals. The drug is typically administered as a once-daily oral tablet or as an intravenous (IV) infusion, depending on the patient's condition and specific circumstances [2]. Letemovir is generally prescribed as part of a comprehensive antiviral prophylaxis regimen for individuals at risk of CMV infection post-transplant. Side effects such as nausea, diarrhoea, vomiting, cough, headache, tiredness, stomach pain, swelling in arms and legs are possible during the use of letemovir [3]. The structure of letemovir is presented in Figure 1.

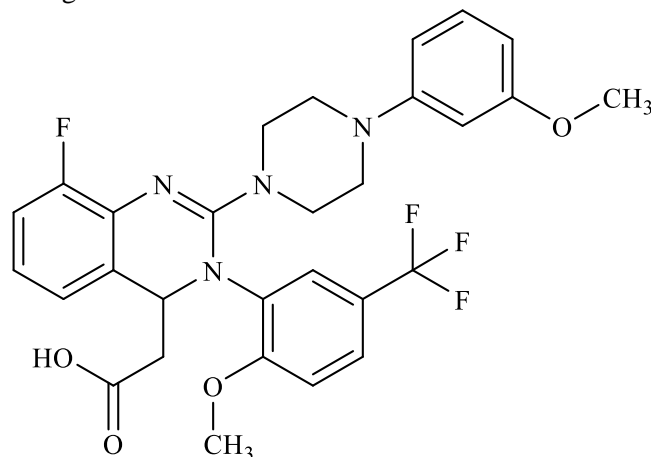


Figure 1. Molecular structure of letemovir

A medicinal formulation product contains not only active pharmaceutical ingredients but also excipients [4]. In certain situations, either the active ingredient or the excipients may not be entirely pure, potentially containing other substances from various sources, such as synthesis, excipient, residual solvent, or degradation products. These undesired components, aside from the API and excipients, are termed impurities [5].

In pharmaceutical products, when the presence of impurities is expected, it becomes essential to identify and characterize those with an efficient analytical method. This systematic process is commonly referred as impurity profiling, which serves as a comprehensive approach to identifying unknown impurities and elucidating their chemical structures. This process is crucial in ensuring that impurities in pharmaceutical substances are recognized and quantified within acceptable limits, thus preventing potential toxicological effects on the human body [6].

The detection and quantify trace amounts of impurities in impurity profiling process needs a very sensitive and efficient analytical procedure. Given that impurities may exist in very small quantities within drug substances, conventional methods with lower sensitivity and accuracy are inadequate for quantification [7]. Moreover, many impurities share structural similarities with the parent drug molecule, emphasizing the need for advanced hyphenated analytical techniques. HPLC and LC-MS/MS are versatile techniques significantly utilized for assessing trace-level impurities in pharmaceutical products [8].

The possible analytical procedures reported for quantifying letemovir were assessed by conducting a literature review. One analytical HPLC method was published for the evaluation of letemovir in degradation samples [9] and enantiomeric purity [10]. Bio-analytical methods reported for quantification of letemovir in biological samples were employed with HPLC [11], UPLC [12] and LC-MS/MS [13]. The literature review suggests the absence of analytical methods for analyzing the impurities of letemovir. Furthermore, no author has characterized the stress degradation compounds of letemovir. This paper addresses these gaps by presenting an optimized HPLC method for the evaluation of process-related impurities of letemovir and LCMS/MS was utilized for characterizing DPs. The availability of impurities was considered for the selection of impurities, and impurities 1, 2, 3 and 4 were finalized in this study (Figure 2). The systematic details of impurities including name, molecular formula and mass were provided in the supplementary note.

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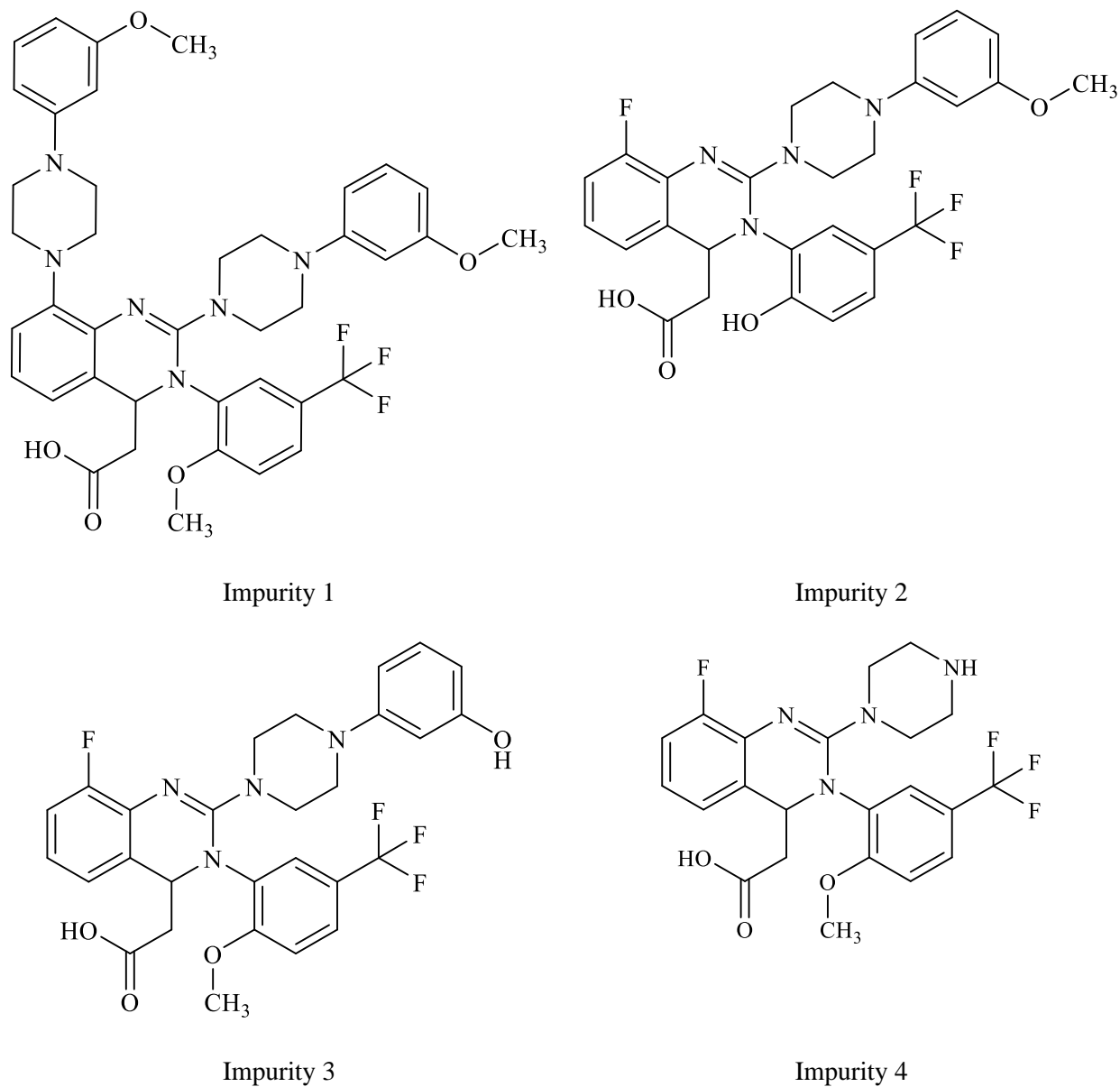


Figure 2. Impurities of letermovir in the study

2. Experimental

2.1. Chemicals and Reagents

Letermovir (98.75%) API, its impurities 1 (98.25 %), 2 (98.55 %), 3 (99.40 %) and 4 (99.10 %) along with its marketed formulations were brought from MSD pharmaceuticals India private limited, Mumbai, Maharashtra, India. Methanol (HPLC grade), acetonitrile (HPLC grade), water (milli-Q[®]), membrane filter (0.2 μ) and analytical grade chemicals including sodium hydroxide (NaOH) and

hydrochloric acid (HCl), hydrogen peroxide, formic acid and sodium acetate were brought from Merck chemicals, Mumbai.

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2.2. Instrument Configuration

The investigation was conducted on HPLC instrument (1100 series, Agilent, Japan). This system was outfitted with quaternary pump (G1311 A model), temperature programmable auto-sampler (G1329A model) featuring a 0.1–1500 μL injection volume, and ultraviolet (UV) detector (G1314 A model). Integration of chromatograms was achieved using Agilent ChemStation software. Additionally, MS experiment was conducted on LC-MS/MS system (Alliance 2695 series, Waters, Japan) with quadrupole mass detector and MassLynx software.

2.3. Solutions Preparation

2.3.1. Standard and Impurity

Letermovir pure drug, impurities at a concentration of 1 mg/mL (1000 $\mu\text{g/mL}$) were prepared independently with the same procedure. This involved precisely weighing 25 mg of the analyte and placing it in a 25 mL flask with 15 mL of methanol. The flask was kept on sonicator for 2 min to dissolve compounds in solvent and un-dissolved compounds were removed by filtration. Flasks were filled to the mark to achieve 1000 $\mu\text{g/mL}$ concentration of analytes separately. An appropriate dilution was made to achieve the desired concentration of analytes during analysis.

2.3.2. Formulation

The formulation solution was prepared using a tablet formulation that included 240 mg of letermovir under the brand Prevymis[®]. To prepare the formulation solution, precisely 10 mg equivalent of tablet powder taken in a 10 mL flask contain 5 mL methanol. The flask underwent sonication to ensure complete dissolution of the formulation in solvent, and flask filled till mark with same solvent. The un-dissolved formulation excipients were eliminated by filtration and 1000 $\mu\text{g/mL}$ concentration of letermovir formulation solution was obtained. An appropriate dilution was made to achieve desired concentration of analytes during analysis.

2.4. Method Development

The iso-absorption wavelength obtained from UV-visible spectrophotometer was finalized as optimum wavelength for detecting impurities along with letermovir. Best resolution for the analytes was verified on Inertsil ODS (GL, Lifesciences), Luna C18 (Phenomenex), Symmetry C18 (Waters) and Baker bond C18 (Avantor) columns of 250 mm and 100 mm length. Mobile phase was confirmed by optimizing various pH modifiers including acetate, phosphate buffer with wide pH ranges and flow rates. The condition that elutes best resolution with permissible system suitability was validated.

2.5. Method Validation

Validation studies as per ICH guidelines [14-16] were performed to evaluate method applicability for its intended purpose. Additionally, insights from relevant methodologies documented in the literature [17-24] were considered during the validation process.

2.6. Stability Studies

The drug's stability was evaluated under various conditions, including photolytic, dry heat, oxidative and hydrolytic (acid, base) stress. An ultraviolet (UV) detector was configured to record absorbance and LC-MS/MS technique employed to characterize structure of DPs. To investigate the impact of hydrolytic, oxidative, and photolytic conditions, a letermovir solution with a known targeted concentration was prepared in HPLC grade methanol.

Degradation experiments were conducted by introducing 10 mg of the drug into 50 mL volumes HCl (0.1 N) and NaOH (0.1 N) solutions respectively in acid and base induced degradation studies. These solutions were left undisturbed for 6 hours at room temperature in darkness to eliminate potential degradation caused by light exposure. The oxidative degradation was induced by introducing 10 mg of letermovir into 10 mL of 5% (v/v) hydrogen peroxide and left in darkness for 6 hours. The resulting solution (1 mL) was then diluted to 10 mL with methanol and subjected to same treatment described in acid and base study. Subsequently, each stressed solutions (1 mL each) were neutralized and diluted to 10 mL using methanol. Photo-stability was induced by exposing the solid form drug to 200 Whm⁻² UV light in a photolytic chamber. Whereas the solid form drug was exposed to 70 °C temperature in a air oven for inducing thermal degradation. Then they were brought to standard concentration and analyzed in this study method.

2.7. LC-MS/MS Characterization of DPs

The DPs observed in stress study chromatogram were identified through analysis using LC-MS/MS. The detected eluents were directed towards mass detector obtaining mass spectra of individual DP. For this process, it is important to ensure that 40% of the eluents were directed into mass detector with the assistance of splitter. The resultant mass spectra and mass fragmentation pattern was carefully scrutinized for predicting its structure.

2.8. Method applicability:

The proposed analytical HPLC method was examined to detect and measure impurities in letermovir tablet formulations. The prepared formulation sample was utilizing for testing the proposed method by directly analysing it, as well as by spiking it with known concentrations of the studied impurities. The resulting chromatograms and their responses were then used to assess the applicability of the method.

2.9. Assessment of Uncertainty

The uncertainty budget of the proposed method was evaluated by adopting EURACHEM guide and literature reported procedures [25-29]. The uncertainty arises due to standard compounds purity ($u_{standard}$), weighing of samples along with validation results that includes calibration curve slope ($u_{calibration}$), precision ($u_{repeatability}$) and recovery ($u_{recovery}$) were evaluated in the proposed method. The combined uncertainty ($u_{combined}$) that arise due to the combination of all these factors were assessed using the formula:

$$u_{combined} = \sqrt{(u_{standard})^2 + (u_{calibration})^2 + (u_{repeatability})^2 + (u_{recovery})^2}$$

The expanded uncertainty ($U_{Expanded}$) was evaluated at 95% confidence level with coverage factor (k) equal to 2.

3. Results and Discussions:

There is not any established analytical method in literature for determining process related impurities of letermovir prompted the development of a straightforward HPLC technique. As the impurities and letermovir are polar compounds, the optimization of the method involved exploring different column configurations including Inertsil ODS, Luna C18, Symmetry C18 and Baker bond C18 columns of different lengths.

To enhance the separation of analytes, various mobile phase compositions within a suitable pH range were examined. This optimization process included experimenting with different solvent compositions and buffer strengths. A thorough investigation of various buffers with different pH ranges was conducted to ensure effective resolution of these components. The optimal separation of letermovir impurities was achieved using a Phenomenex Luna C18 column (250 mm × 4.6 mm; 5 μm id) at 35 °C.

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Isocratic elution was employed with a mixture of 1% phosphoric acid, methanol, and acetonitrile in 45:25:30 (v/v) having pH of 4.9 at 0.8 mL/min. The detection wavelength was set at 234 nm, determined to be the most effective based on the observation that the detector response was optimal compared to determinations at other wavelengths for all analytes. Throughout the analysis, the column oven temperature (35 °C) and sample volume (20 µL) remained constant.

Under the established conditions, the peaks representing letermovir and its impurities exhibited a symmetrical shape, with a resolution exceeding 2 between adjacent peaks. The retention times were determined to be 9.58 min for letermovir, 11.10 min, 4.18 min, 6.30 min, and 13.26 min for impurities 1, 2, 3, and 4, respectively. Figure 3 represents chromatograms achieved and Table 1 represents results achieved in system suitability study. Analysing the chromatograms verified the method specificity for separating and analysing process-related impurities of letermovir.

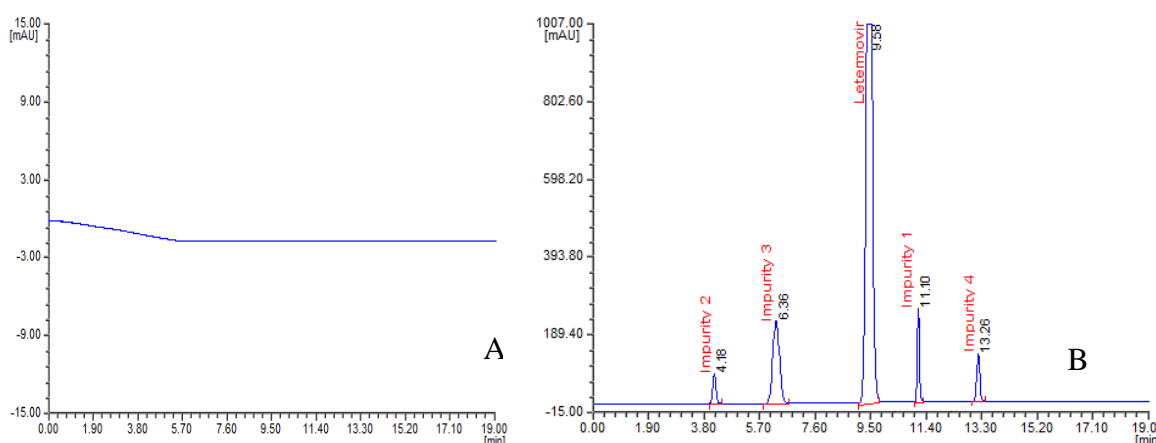


Figure 3. System suitability chromatograms in the optimized method

Placebo solution chromatogram spiked with no analytes (A) and spiked with known strength analytes (B)

Table 1. System suitability results in the optimized method

S No	Parameter	Results				
		Letermovir	Impurity 1	Impurity 2	Impurity 3	Impurity 4
1	t_R (min)	9.58	11.10	4.18	6.36	13.26
2	Relative retention time	--	1.16	0.44	0.66	1.38
3	Relative response factor	--	0.081	0.038	0.067	0.050
4	Resolution	11.21	6.57	--	7.58	9.47
5	Tail factor	1.08	0.99	1.02	0.94	0.96
6	Theoretical plates	13925	15460	6037	8425	16857

Signal-to-noise (S/N) protocol employed to assess method sensitivity, with results presented in terms of detection and quantification limit. Detection limit confirmed as 0.015 µg/mL, and quantification limit as 0.050 µg/mL for impurities. These findings underscore the method's heightened sensitivity in detecting impurities. The letermovir standard solution, comprising 0.1% of each impurity, was precisely prepared for evaluation of calibration range. A well-fitted calibration curve was successfully generated within 50-200 µg/mL for letermovir and 0.05-0.2 µg/mL for impurities with significantly high correlation coefficient (r^2). The %RSD of areas response for impurities and letermovir were consistently below the specified limits in all precisions (intraday, interday, LOQ level) and ruggedness tests performed. This outcome indicates the method's commendable precision. Table 2 presents the linearity and precision results achieved in the study.

Table 2. Linearity and precision study results in the optimized method

Parameter	Results				
	Letermovir	Impurity 1	Impurity 2	Impurity 3	Impurity 4
Linearity					
Range ($\mu\text{g/mL}$)	50 - 200	0.05-0.2	0.05-0.2	0.05-0.2	0.05-0.2
Intercept	11484	1536.8	758.76	144.73	3199.6
Slope	9837.3	801787	370263	664903	474396
r^2	0.9996	0.9988	0.9988	0.9994	0.9992
Precision [§]					
Intraday	0.50	0.42	0.53	0.57	0.62
Day 1 precision	0.25	0.46	0.97	0.53	0.54
Day 2 precision	0.41	0.76	0.40	0.19	0.41
LOQ level	--	0.97	0.85	1.32	1.21

[§]average results (n=6)

The method accuracy was evaluated by conducting recovery experiment. Standard (QC) concentrations were intentionally spiked at three (50%, 100%, 150%) levels corresponding to target concentration. These spiked samples, prepared in three replicates, were then analysed for quantification of letermovir and its impurities. The calculated analyte concentrations were subsequently compared to the nominal concentrations, allowing us to calculate the % recovery. The results revealed mean % recovery values for all three levels, based on three replicates, comfortably fell within the acceptance limit of 98 % to 102 %, as outlined in regulatory guidelines. These findings affirm the method's accuracy as it consistently delivers results that align with the expected analyte concentrations. Results were summarized in Table 3.

Table 3. Accuracy results in the optimized method

Parameter	Results				
	Letermovir	Impurity 1	Impurity 2	Impurity 3	Impurity 4
50 % level [§]					
Prepared ($\mu\text{g/mL}$)	125	0.125	0.125	0.125	0.125
Recovered ($\mu\text{g/mL}$)	124.08	0.125	0.122	0.125	0.125
% Recovery	99.33	99.64	97.87	99.66	99.67
% RSD	1.81	0.35	1.90	0.88	0.88
100 % level [§]					
Prepared ($\mu\text{g/mL}$)	150	0.150	0.150	0.150	0.150
Recovered ($\mu\text{g/mL}$)	147.76	0.150	0.149	0.150	0.150
% Recovery	98.54	100.21	99.31	99.98	100.03
% RSD	1.39	0.66	0.48	0.60	0.68
150 % level [§]					
Prepared ($\mu\text{g/mL}$)	175	0.175	0.175	0.175	0.175
Recovered ($\mu\text{g/mL}$)	174.16	0.174	0.175	0.174	0.175
% Recovery	99.52	99.50	99.72	99.62	99.75
% RSD	0.99	1.02	0.69	0.80	0.78

[§]average results (n = 3)

The robustness in HPLC impurity analysis lies in its ability to ensure the reliability, consistency, and compliance of analytical method. A robust method enhances the quality control process in the pharmaceutical industry, contributing to the overall safety and efficacy of pharmaceutical products. The study conducted both positive and negative change in composition and pH of mobile phase as well as

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detector wavelength. As presented in Table 4, no significant variations in chromatographic results including system suitability were noticed when analysis was performed with nominal alteration in the proposed method proved that method was robust.

Table 4. Robustness test results

S No	Changed condition	Parameter	Results observed				
			Letermovir	Impurity 1	Impurity 2	Impurity 3	Impurity 4
1	MP 1	% change	0.53	0.95	0.73	0.67	0.49
		t _R	9.51	11.19	4.18	6.30	13.26
		N	13831	15374	5996	8453	16899
2	MP 2	% change	0.08	1.26	0.22	1.13	0.25
		t _R	9.58	11.11	4.11	6.35	13.28
		N	14044	15345	5985	8353	17025
3	pH 1	% change	0.25	0.35	0.42	1.05	0.13
		t _R	9.55	11.13	4.15	6.32	13.27
		N	13831	15404	6028	8446	17008
4	pH 2	% change	0.35	1.29	0.15	0.95	0.25
		t _R	9.56	11.16	4.12	6.34	13.21
		N	13845	15375	6052	8453	16743
5	WL 1	% change	0.44	0.43	0.38	0.33	0.35
		t _R	9.59	11.14	4.13	6.38	13.27
		N	13874	15344	6097	8488	17002
6	WL 2	% change	0.43	0.35	0.75	0.48	0.37
		t _R	9.53	11.17	4.19	6.39	13.25
		N	13852	15407	6091	8500	16743

Mobile phase altered as 1% phosphoric acid, methanol, and acetonitrile in 45:20:35 in MP (mobile phase) change 1 and 45:30:25 in MP change 2; mobile phase pH altered as 4.8 (pH 1) and 5.0 (pH 2); 229 nm in wavelength (WL) change 1 and 239 nm in WL change 2; [§]average results (n=3)

Stress degradation experiment was undertaken to assess the method's capability to separate degradation compounds. Among the stress tests conducted, very nominal degradation of 96.26% and 96.91%, respectively was noticed in thermal and UV light conditions suggest that letermovir was stable in these two stress conditions. Notably, base degradation showed a high % degradation at 9.65%. Chromatograms from this study (Figure 4B) exhibited well-resolved degradation products (DPs) at t_R of 5.60 min, denoted as DP 3. In peroxide degradation (Figure 4B), a distinct degradation product at t_R of 5.05 min, identified as DP 2, displayed a % degradation of 94.80%. Acid degradation resulted in a % assay of letermovir at 91.49% with of 99.01% mass balance. The chromatogram distinctly resolved two DPs at t_R of 2.02 min and 5.05 min, designated respectively as DP 1 and DP 4. The purity of letermovir peak in stress studies was evaluated by employing PDA detector and results affirms that the peak was pure in all stress studies. The mass balance of chromatographic results in stress study was evaluated and noticed a very high mass balance of 97.89 - 99.24% range. There is no considerable degradation, significantly high mass balance with very high peak purity suggest the stability-indicating ability of method. Table 5 provides a detailed presentation of results and Figure 4 represents the stress degradation chromatograms of letermovir.

Table 5. Forced degradation results of letermovir

Condition	% degradation [§]	% assay [§]	% Mass balance [§]	Remark
Acid	8.51	91.49	99.01	DP 1 & 4 were noticed
Base	9.65	90.35	97.89	DP 3 was noticed
Peroxide	5.2	94.8	98.62	DP 2 was noticed
Thermal	3.74	96.26	99.24	No degradation was noticed
UV light	3.09	96.91	98.59	DP 5 was noticed

[§]average of n=3; mass balance = sum of all peaks including DPs, impurities and standard

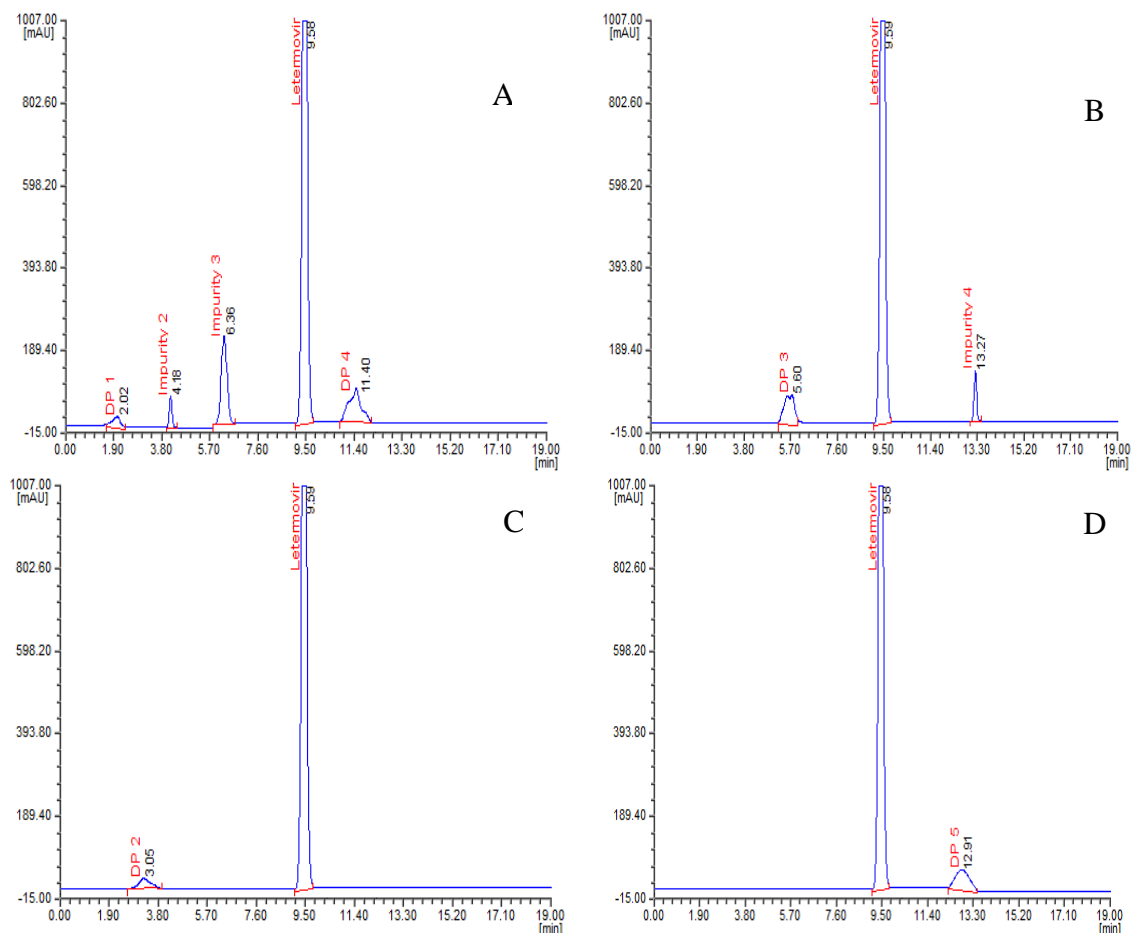


Figure 4. Stress degradation chromatograms of letemovir

A) DP 1 & 4 were noticed in acid degradation; **B)** DP 3 observed in base degradation; **C)** DP 2 identified in peroxide degradation; **D)** DP 5 visualized in UV Light degradation

3.1. Characterization of DPs by LCMS/MS

The structural characterization of DPs was performed using mass spectral analysis which was conducted in ESI positive ion trap MS/MS. The mass operating conditions were fine-tuned to maximize the detection of each mass fragment while minimizing noise and an average of 20-30 scans were performed in each analysis. The molecular ion $[M+H]^+$ of DPs was evaluated and detailed interpretation of mass fragments was undertaken to elucidate its fragmentation pattern.

The mass spectrum of DP 1 ($t_R = 2.02$ min) visualizes parent ion fragment at m/z of 289 $[M+1]^+$. The mass spectrum visualizes high intense fragment ion at m/z of 252 by losing H_2FO from parent ion and another fragment at 189 by loss of $C_4H_6NO_2$ from parent ion. In addition, very high intense product ion observed at m/z of 162 $[M+1]^+$ with molecular formula of $C_9H_6FN_2$ formed by the loss of $C_5H_7N_2O_2$ from parent ion. Based on mass spectral results and fragmentation pattern (Figure 5), DP 1 was confirmed as 2-(3,6-dihydropyrazin-1(2H)-yl)-8-fluoroquinazolin-4-yl] acetic acid with molecular formula of $C_{14}H_{13}FN_4O_2$ and 288 g/mol as molecular mass.

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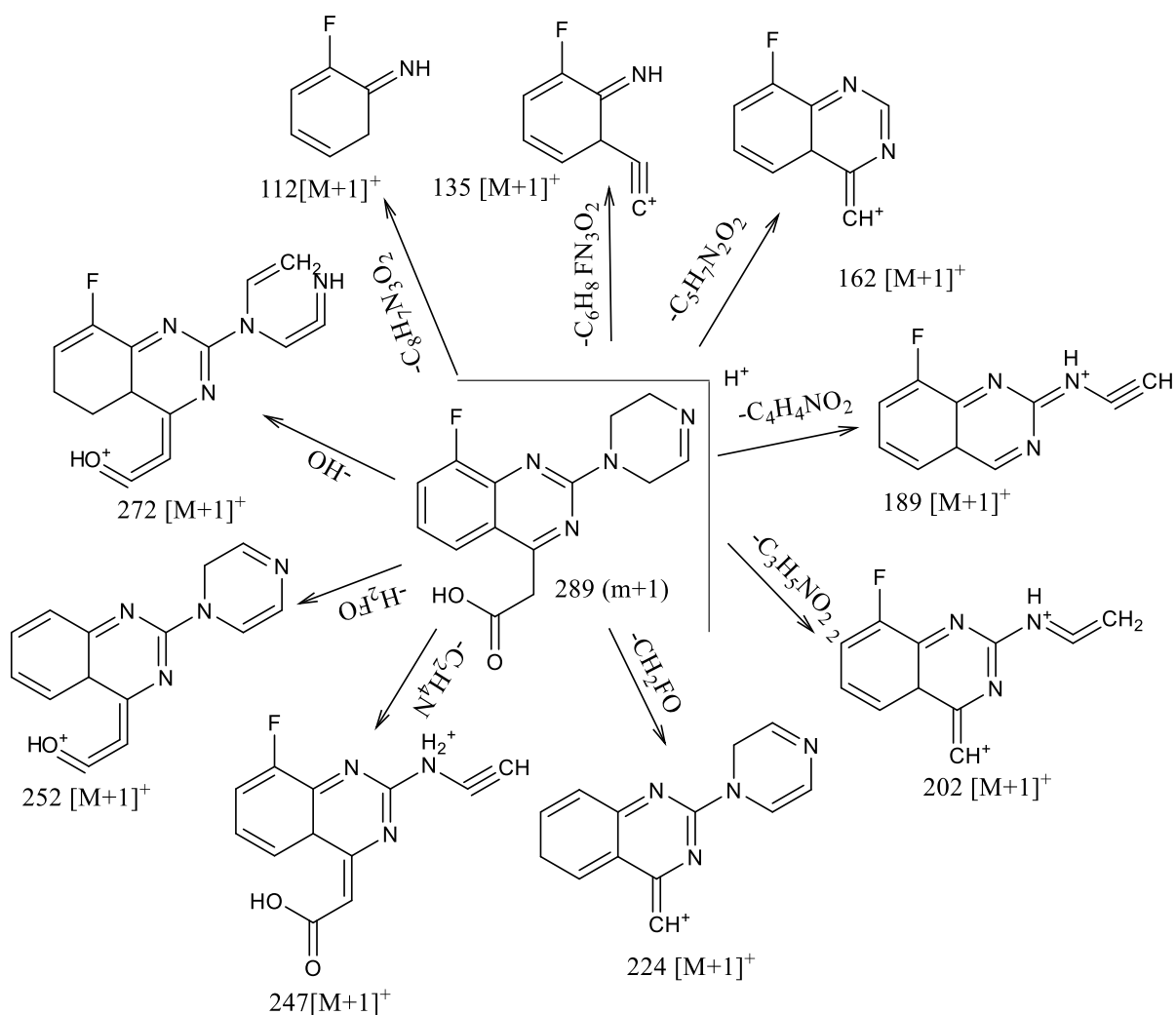


Figure 5. Proposed fragmentation profile of DP 1

The mass spectrum of DP 2 as shown in Figure 10B visualizes the presence of parent ion at m/z of 329 [M+1]⁺ confirms its mass as 328 g/mol. In spectrum, fragment ion noticed m/z of 298 (elimination of CH₃O from m/z 329), 194 (elimination of C₇H₄FN₂ from m/z 329) and 138 (elimination of C₁₁H₁₅N₂O from m/z 329). A high intense fragment noticed at m/z of 113 corresponds to C₆H₆FN which was formed due to elimination C₁₂H₁₄N₃O from m/z of 329 (Figure 6). Based on the fragmentation spectrum, compound was identified as *N*-(2-fluorophenyl)-4-(3-methoxyphenyl)piperazine-1-carboximidamide with molecular formula of C₁₈H₂₁FN₄O.

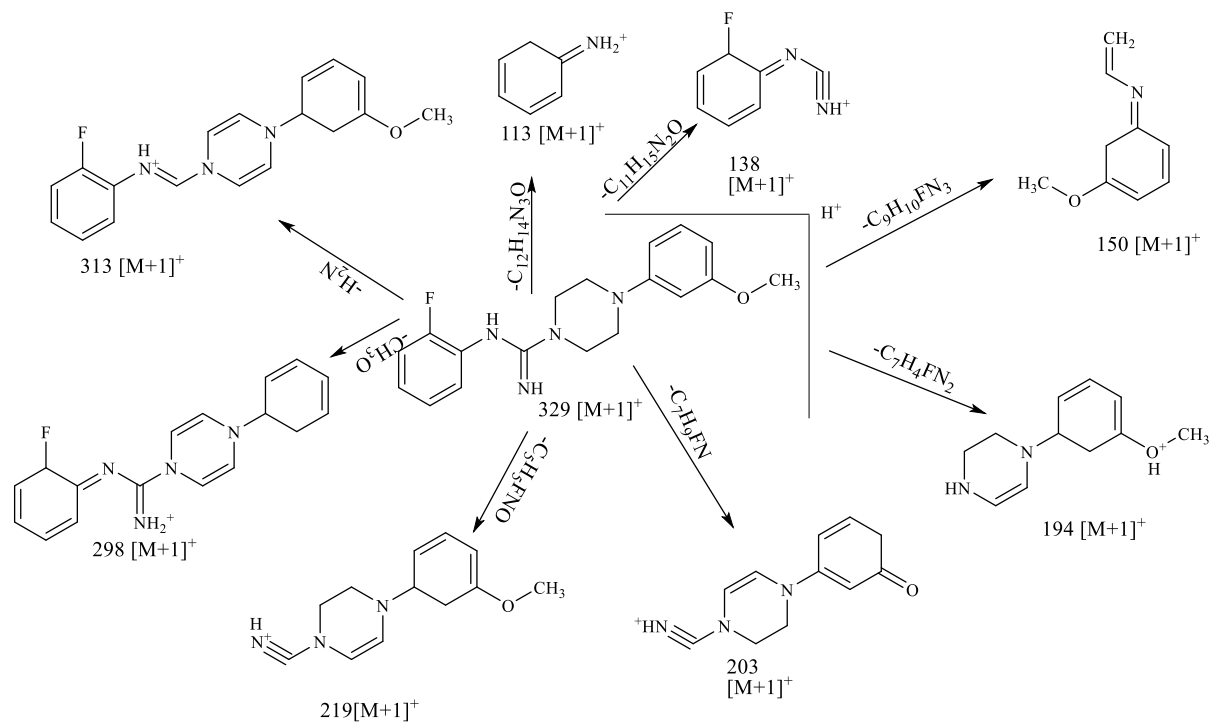


Figure 6. Proposed fragmentation profile of DP 2

Mass fragmentation spectrum of DP 3 was shown in Figure 10 C which clearly visualizes the parent ion fragment at m/z of 426 [M+1]⁺. Mass spectrum visualizes high intense fragment ion at m/z of 381 by losing CHO_2 group from parent ion and another fragment at 261 by lose of $C_9H_{10}FN_2$ from parent ion. The spectrum also show very high intense product ion at m/z of 191 [M+1]⁺ with molecular formula of $C_8H_7F_3NO$ formed by the loss of $C_{12}H_{12}FN_2O_2$ from parent ion (Figure 7). The compound was confirmed as 2-(dimethylamino)-8-fluoro-3-[2-methoxy-5-(trifluoromethyl)phenyl]-3,4-dihydroquinazolin-4-yl}acetic acid with molecular formula of $C_{20}H_{19}F_4N_3O_3$ and 425 g/mol as molecular mass. Proposed mass fragmentation profile of DP 3 was presented in Figure 7.

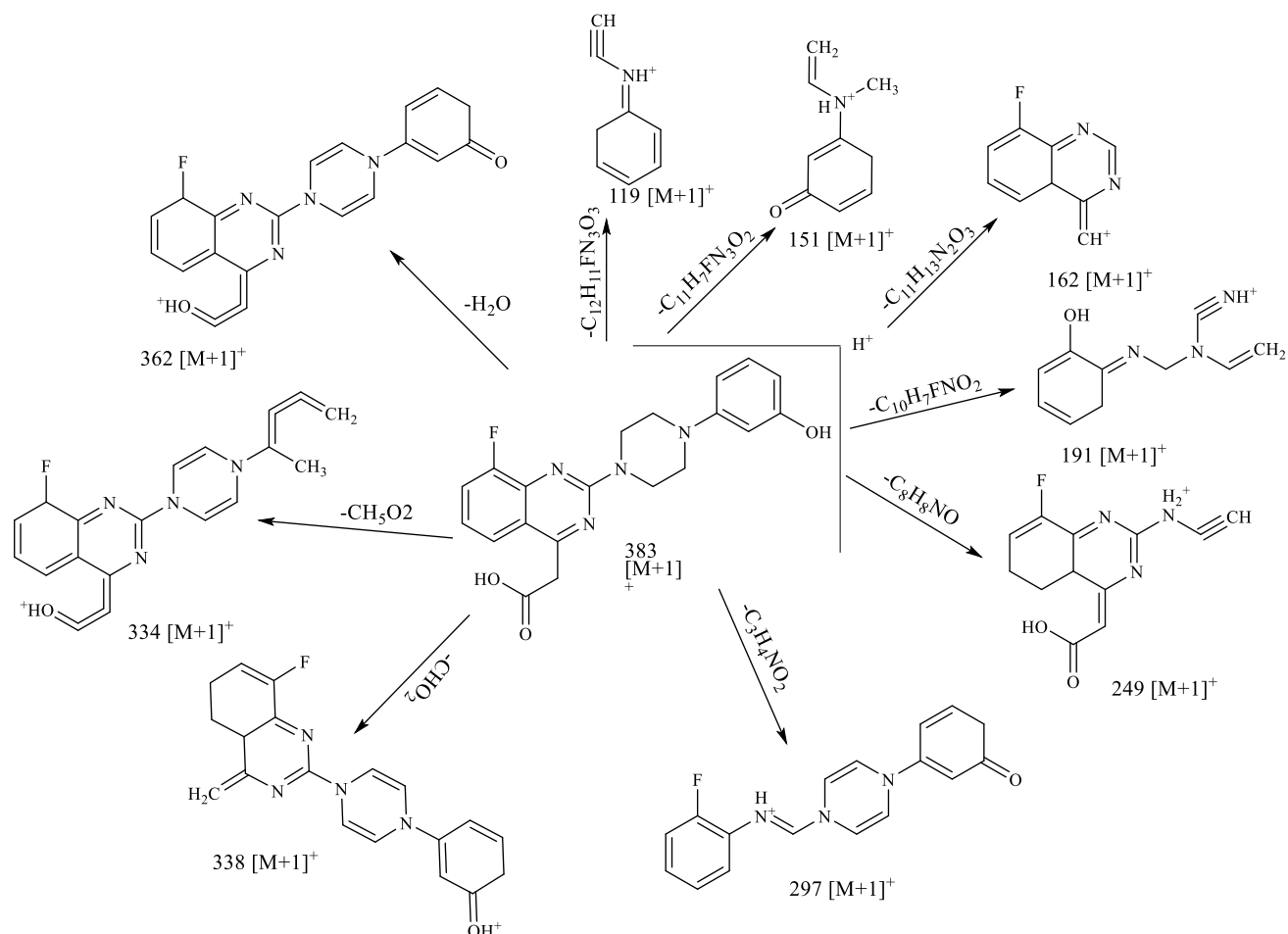


Figure 8. Proposed fragmentation profile of DP 4

Figure 10E shows the fragmentation spectrum of DP 5 which confirms the presence of parent fragment at m/z of 379 $[M+1]^+$ confirms its molecular mass as 378 g/mol. An abundant fragment ion noticed at m/z of 189 corresponds to $C_{14}H_{11}N_3$ due to elimination of $C_8H_7F_3NO$ from m/z of 378. The compound was confirmed as *N*-[2-methoxy-5-(trifluoromethyl)phenyl]-4-phenylpiperazine-1-carboximidamide with molecular formula of $C_{19}H_{21}F_3N_4O$. The molecular structure along with its mass fragmentation mechanism was presented in Figure 9.

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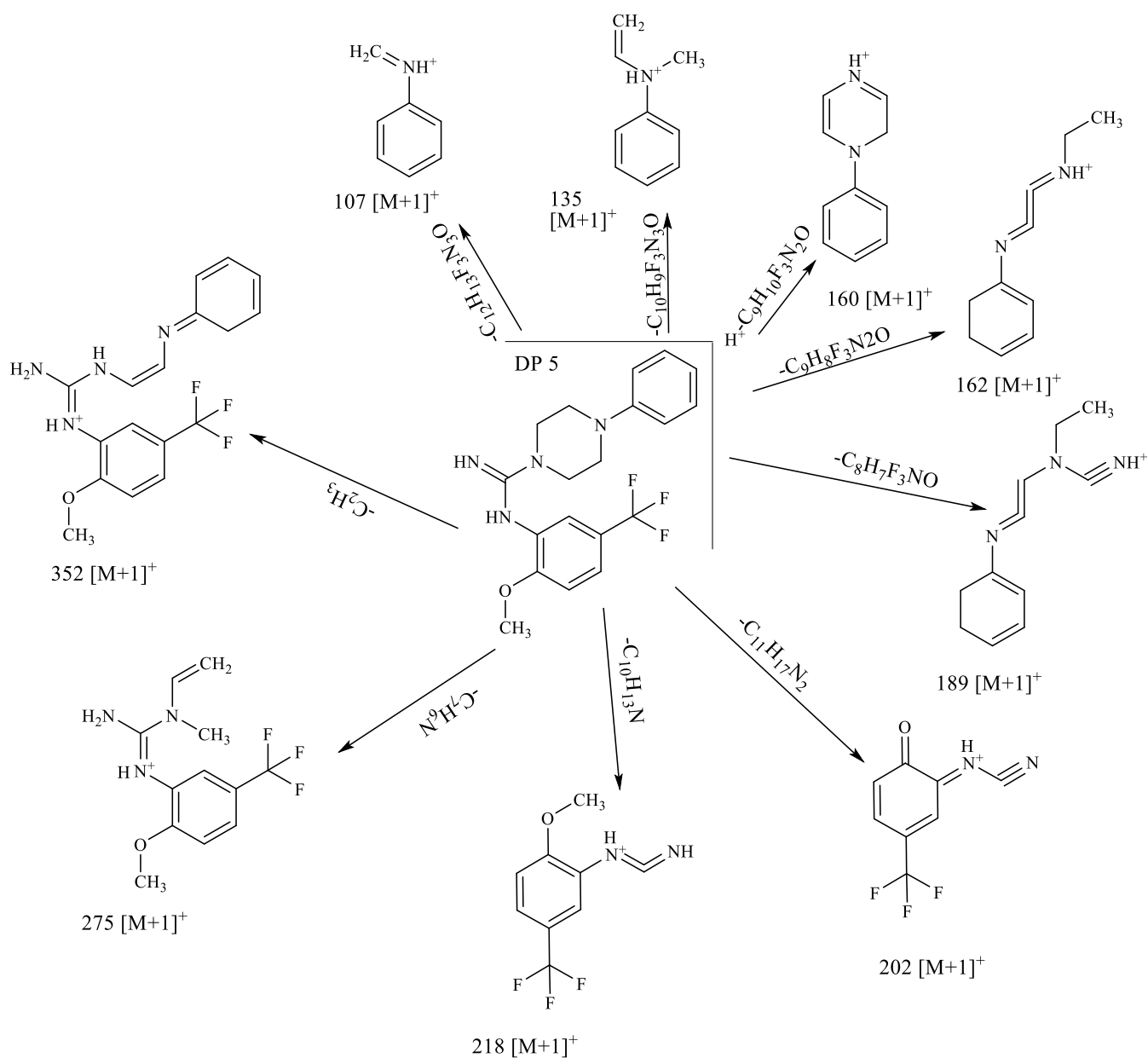


Figure 9. Proposed fragmentation profile of DP 5

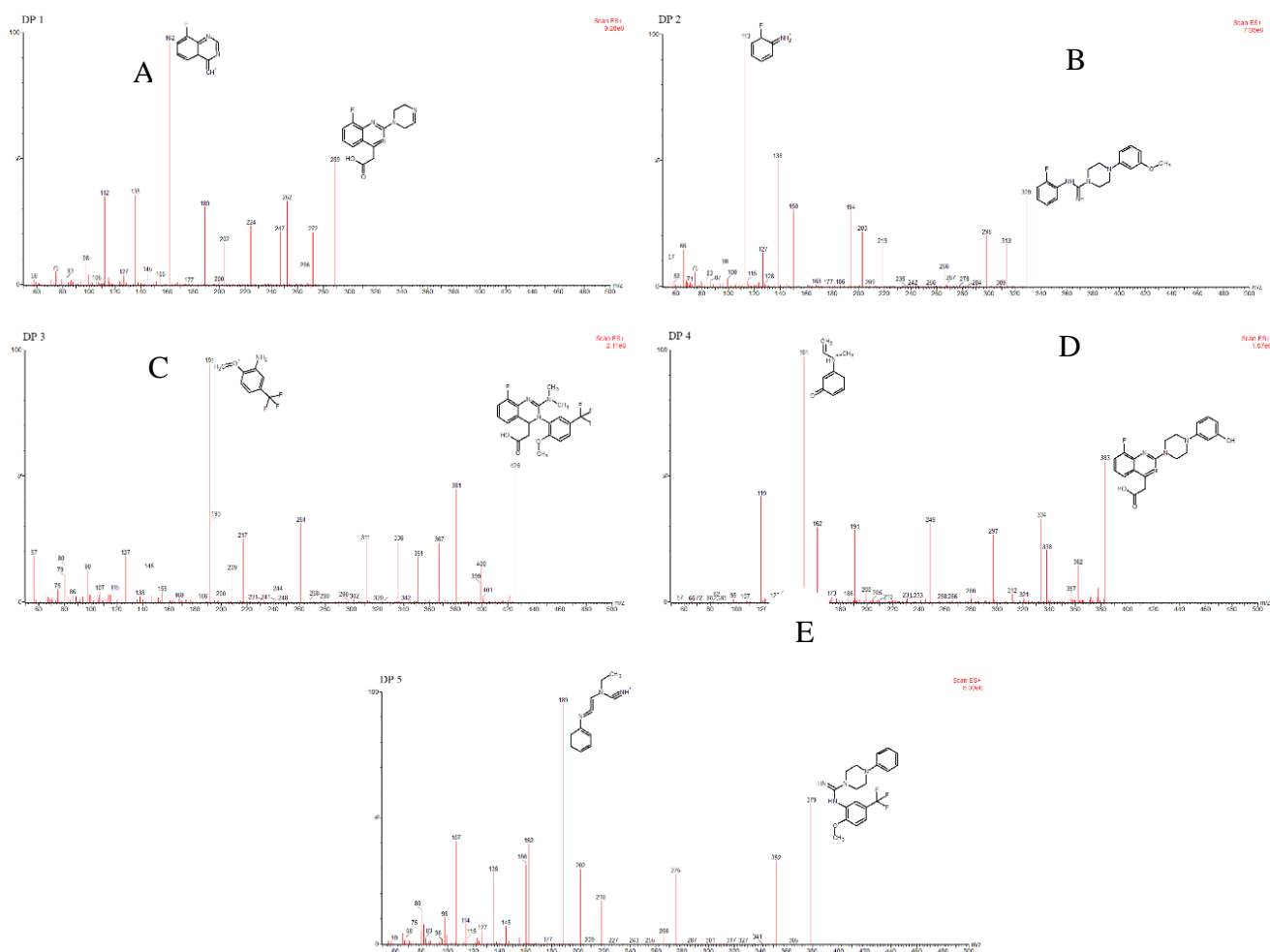


Figure 10. Mass spectra of letermovir DPs formed in this study

A) DP 1 at t_R of 2.02 min; **B)** DP 2 at t_R of 5.05 min; **C)** DP 3 at t_R of 5.60 min; **D)** DP 4 at t_R of 11.40 min; **E)** DP 5 at t_R of 12.91 min (see the enhanced spectra in supporting information)

The method proposed in this study was applied for evaluation of letermovir impurities in samples. The prepared sample solution directly and spiked with known strength impurities was evaluated in this study method. The direct analysed sample chromatogram doesn't show any peak corresponds to studied impurities whereas the spiked sample chromatogram clearly shows peaks for impurities. This suggests that the method has the capability for resolving the impurities in sample and hence can significantly applicable for evaluation of process related impurities of letermovir.

3.2. Uncertainty Assessment

The uncertainty assessment of proposed method was evaluated with 0.1 % impurities spiked standard solution of letermovir at 150 $\mu\text{g/mL}$ concentration. The uncertainty arise due to weighing was negligible and hence were not considered for calculating the combined uncertainty. The uncertainty results were tabulated in Table 6 and detailed calculation was given in the supplementary file attached to this manuscript. The acceptable uncertainty data was achieved in this proposed method suggest that the method was appropriate for the analysis of letermovir and its impurities.

Table 4. uncertainty assessment results of the proposed method

Analytes	Uncertainty (<i>u</i>) in %					
	<i>u</i> _{standard}	<i>u</i> _{calibration}	<i>u</i> _{recovery}	<i>u</i> _{repeatability}	<i>u</i> _{combined}	<i>U</i> _{expanded}
Letermovir	0.722	0.045	0.247	0.136	0.776	1.521
Impurity 1	0.393	0.009	0.488	0.531	0.821	1.609
Impurity 2	0.722	0.065	0.202	0.209	0.781	1.530
Impurity 3	0.433	0.028	0.151	0.224	0.511	1.002
Impurity 4	0.202	0.198	0.162	0.381	0.502	0.983

4. Conclusion

The present study introduces a convenient HPLC method for quantifying process-related impurities of letermovir. Furthermore, the suggested method is demonstrated to be applicable for characterizing stress degradation products using LC-MS/MS analysis. Letermovir underwent forced degradation studies, including exposure to acid, base, peroxide, thermal, and UV light conditions following the prescribed ICH guidelines. Under various stress conditions, a total of five DPs were formed and characterization five distinct DPs was achieved through LC-MS/MS analysis, with successful characterization in ESI positive mode. The DPs were identified as 2-(3,6-dihydropyrazin-1(2H)-yl)-8-fluoroquinazolin-4-yl]acetic acid (DP 1), N-(2-fluorophenyl)-4-(3-methoxyphenyl)piperazine-1-carboximidamide (DP 2), 2-(dimethylamino)-8-fluoro-3-[2-methoxy-5-(trifluoromethyl)phenyl]-3,4-dihydroquinazolin-4-yl]acetic acid (DP 3), {8-fluoro-2-[4-(3-hydroxyphenyl)piperazin-1-yl]quinazolin-4-yl]acetic acid (DP 4) and N-[2-methoxy-5-(trifluoromethyl)phenyl]-4-phenylpiperazine-1-carboximidamide (DP 5). The study outlined and discussed the most probable mechanisms and pathways leading from letermovir to the characterized degradation products. This comprehensive analysis is valuable for identifying process-related impurities and DPs present at trace level. The method developed is practical for estimating quality control samples of letermovir.

The findings achieved in this study were compared with methods reported in literature. In literature methods published only for quantification of letermovir in formulations and biological samples [9-14] using different analytical techniques. There is currently no established method for quantifying process-related impurities or characterizing the stress degradation products of letermovir. Therefore, this study represents the optimal choice for the identification and characterization of impurities and degradation products of letermovir.

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