RESEARCH ARTICLE

# Method Development and Validation for Famciclovir and Valacyclovir by using UPLC and its Degradents are Characterized by LC-MS/MS

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**ABSTRACT**

An easy receptive technique was studied to evaluate valacyclovir and famciclovir in the tablet formulation using ultra-performance liquid chromatography (UPLC**)**. This method involves the separation by chromatographic technique with a Luna $C_{18}$ column (100 mmxv 2.6 mm, 1.6 µ). OPA (Ortho phosphoric acid, 0.1%) and 30:70 v/v acetonitrile act as a mobile phase with a flow rate of 1 mL/min, using an ambient temperature. The UV spectral data has recorded at about 230 nm. Using these conditions, we got good linearity results in the range of 10–150 µg/mL of valacyclovir and 2.5–37.5 µg/mL of famciclovir using the UPLC technique. With the help of the assay method mentioned above, the results of the other validation UPLC parameters like accuracy, degradation studies system precision, robustness, and method precision were present within the allowed limits as per the guidelines given by international council for harmonization (ICH).

**Keywords:** Famciclovir, LC-MS/MS, UPLC, Valacyclovir, Validation.

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## INTRODUCTION

The sensitivity of certain drugs for degradation may vary as per their chemical reactivity, structures, and nature of formulations. This sensitivity can lead to various physico-chemical changes.[1] Ultra-performance liquid chromatography (UPLC**)** technique covers the liquid chromatographic separations by implementing the column enclose particles smaller than 2.5–5 µm size usually used in high-performance liquid chromatography (HPLC).[2] To treat herpes zoster[3] or herpes simplex,[4] an antiviral drug[5] called Valacyclovir was issued. It includes the infections of oral and genital herpes simplex. Reduction of this virus may prevent the transmission from people with severe infection to uninfected people[6]. The usual dosage is 1000 mg orally for 7 days, 3 times/day. This drug is also effective in treatment[7] and high-risk kidney transplantation.[8,9] Vomiting and headache are mild symptoms, whereas severe side effects include kidney problems.[10]

Valacyclovir exhibits promising results in treating herpes B virus[11,12] and in treating infectious mononucleosis.[13] This drug is a choice for preventing cytomegalovirus following organ transplantation and treatment of herpes viruses in immune-compromised people[14] which is undergoing cancer chemotherapy.[15]

Due to the lack of any effect, this drug is generally not used for Bell's palsy.[16] Vomiting, nausea, headache, and diarrhea are the common symptoms of this drug. Confusion, vertigo, dizziness, agitation, edema, sore throat, abdominal pain, and rashes are infrequent side effects.[17] Other symptoms include seizures, coma,[18] leukopenia, neutropenia, ataxia, fatigue, encephalopathy, anorexia, psychotic symptoms, and toxic epidermal necrolysis.[19-23]

For various infections of different herpes viruses, and generally for herpes zoster, an antiviral drug famciclovir is used. This is a form of a prodrug of penciclovir which has advanced oral bio-availability. This drug is available in the trade name famvir in the market. This drug is indicated for the herpes simplex virus 2 (commonly called genital herpes) treatment[24] and herpes zoster (shingles),[25] and for the repression of recurring episodes of the herpes simplex virus 2[26] and for the treatment of herpes labialis virus (cold sores) in immune-competent patients.[27] Also, in treating patients with recurrent episodes of herpes simplex virus in human immunodeficiency virus (HIV), this drug has been used.[28] Some common side effects are headaches, stomach upset, and mild fever.

We proposed a sensitive, fast and robust UPLC method. The factors affecting the proposed method's efficacy have been optimized, and the obtained method showed high selectivity and sensitivity. The literature survey shows that the elucidation of the structure of these drugs' degradation products (DPs)

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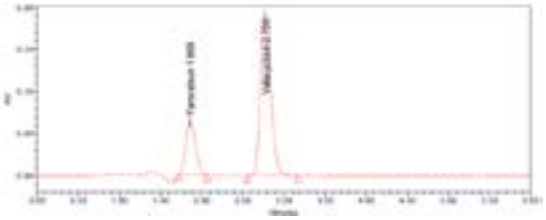


**Figure 1:** Standard chromatogram of UPLC.

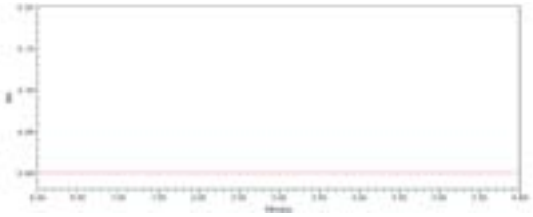


**Figure 2:** UPL blank chromatogram.

has received less attention. No papers on identifying their decomposition products using liquid chromatography–mass spectrometry (LC-MS) methods were found in the literature.

## MATERIALS AND METHODS

### Chemicals and Reagents

The HPLC mark chemicals like acetonitrile, orthophosphoric acid, and water have been procured from the Merck India Limited, Mumbai. APIs of valacyclovir and famciclovir with a purity of 99.9 %have been obtained from Cipla, Maharashtra.

### Instrumentation

*UPLC Conditions*

Waters acuity UPLC with the quaternary pump and a PDA detector having empowered 2.0 software has been used.

*MS/MS and LC Conditions*

The process chromatographic method involves a column of Luna $C_{18}$ (100x2.6 mm, 1.6 µ) with an ambient temperature. The isocratic elution with 30 % of OPA (0.1%) and acetonitrile of 70% was taken as a movable phase, with a 1-mL/min flow rate and a dose of volume 10 µL has been used in UPLC.

For the study of forced degradation, the mass spectrophotometer is connected with an HPLC based on the condition of placing a splitter previous to a source of employees' state insurance (ESI), permitting 35% of eluent into it. For the MS scan of valacyclovir and famciclovir, the standard conditions of operating source in a positive ESI mode were optimized. A fragmentary voltage has been set at about 80 V, the skimmer at 60 V, capillary at 3000 V, and for drying and nebulizing purpose, the nitrogen gas was used (45 psi). As a collision gas, highly filtered nitrogen gas has been used.

### Ordinary Solution Preparation

A standard solution containing valacyclovir (100 µg/mL) and famciclovir (25 µg/mL) has been prepared by dissolution of 100 mg of valacyclovir and 25 mg of famciclovir in a sufficient amount of the mobile phase and structure to the mark 100 mL. 5 mL of this prepared stock has been diluted to 50 mL with the diluent solutions.

### Validation of Method

The systematic UPLC technique has been confirmed by the evaluation of parameters like limit of detection (LoD), suitability of the system, limit of quantitation (LoQ), linearity, accuracy, robustness, etc., and results were observed in the prescribed range of requirements of ICH.

### Suitability of the System

For system performance checking, we would like to check the parameters like the percentage of relative variance, unique selling point united states pharmacopoeia (USP) plate count, and USP tailing.

### Linearity

The linearity has been checked with the standard solutions of valacyclovir and famciclovir in various concentration levels like 10, 25, 50, 100, 125, and 150%.

### Accuracy

The accuracy has been checked at three concentration levels of 50, 100, and 150%. Three injections got in each level by using this digital communication. %RSD and %recovery have been calculated.

### Precision

The precision includes:

- **Precision of the System:** The reference and standard solution of valacyclovir and famciclovir has been injected six times and calculated %RSD.
- **Precision Method:** Six individual standard solutions of valacyclovir and famciclovir have been injected, and %recovery and %RSD were calculated.
- **Intermediate Precision:** Inter-day precision study was managed for standard solutions of valacyclovir and famciclovir and calculated %recovery %RSD.

### Robustness

The method has been studied with a change in the flow of ± 20% and organic phase of ± 10%.

### Limit of Detection (LoD), Limit of Quantitation (LoQ)

The LoD is the small quantity of the analyte during the detection of the sample. In contrast, the LoQ is the amount of the analyte during the sample that is to be identified in tolerable accuracy and precision. LoD and LoQ for valacyclovir and famciclovir are calculated by progressive injection of the ordinary solutions of low concentration with the developed method called ultra performance liquid chromatography (Reversed-Phase-UPLC). LoQ and LoD have been determined, respectively at 3 s/n, and 10 s/n according to the ICH instructions.

### Stress Degradation

For the forced degradation method's chromatogram, the stress degradation does not interfere between obtained peaks. As per the ICH guidelines, this stress degradation process was carried out. We should separate the degradation peaks from

**Table 1:** HPLC results: Linearity.

| *Linearity* | *Valacyclovir* | | *Famciclovir* | |
|---|---|---|---|---|
| | *Conc. (µg/mL)* | *Area* | *Conc. (µg/mL)* | *Area* |
| Linearity 1 | 10.00 | 236501 | 2.50 | 17504 |
| Linearity 2 | 25.00 | 603257 | 6.25 | 45653 |
| Linearity 3 | 50.00 | 1205746 | 12.50 | 95687 |
| Linearity 4 | 100.00 | 2451068 | 25.00 | 191546 |
| Linearity 5 | 125.00 | 2825715 | 31.25 | 228167 |
| Linearity 6 | 150.00 | 3498601 | 37.50 | 280568 |
| Slope | 23187.74 | | 7462.49 | |
| Intercept | 22075.56 | | 134.12 | |
| CC | 0.99901 | | 0.9996 | |

**Table 2:** UPLC accuracy results: (a) Valacyclovir and (b) Famciclovir.

| *Valacyclovir* | | | |
|---|---|---|---|
| *S. no* | *Amount added (µg/mL)* | *Mean ± SD, %RSD* | *%Recovery* |
| 1 | 50 | 50.05 ± 0.21, 0.22 | 98.4 |
| 2 | 100 | 100.15 ± 0.62, 0.61 | 100.1 |
| 3 | 150 | 150.11 ± 0.61, 0.61 | 100.2 |
| *Famciclovir* | | | |
| *S. no* | *Amount added (µg/mL)* | *Mean ± SD, %RSD* | *%Recovery* |
| 1 | 12.5 | 12.59 ± 0.31, 0.31 | 99.7 |
| 2 | 25 | 25.05 ± 0.36, 0.36 | 100.2 |
| 3 | 37.5 | 37.58 ± 0.54, 0.54 | 98.9 |

one another, and the resolution among these peaks must be a minimum of 1.0. Hence, the purity of the shape of the main peak shall pass. Various stress types have carried out work of forced degradation to get a percentage degradation of about 20%.

## RESULTS AND DISCUSSION

An isocratic elution of valacyclovir and famciclovir involves the Luna $C_{18}$ column with the 1-mL per minute flow rate with an ambient temperature maintained in the column. The open policy agent (OPA) of 0.1% and acetonitrile (30:70 v/v) have been used as the mobile phase. The UV observation has been brought at 230 nm.

### Precision of the System

A standard solution of valacyclovir (100 µg/mL) and famciclovir (25 µg/mL) has been injected into the UPLC system. The resulting chromatogram was given in Figure 1. %RSD has been calculated with the use of the peak is as obtained by UPLC, and the results obtained were found within the acceptable limits.

### Specificity

The specificity is not to test the power of an assay technique to eliminate the effect of all interfering substances on valacyclovir and famciclovir peak results, particularly with comparing the blank sample with the chromatogram sample represented in Figure 2. This justified method showed that these drugs had been eluted without involving peaks created by the excipients within the products of the market.

### Linearity

The linearity of the tactic has been determined by preparing a typical solution with 100 µg/mL of valacyclovir and 25 µg/mL of famciclovir. Successive dilutions were carried out to the given dilutions at 10, 25, 50, 100, 125, and 150% of the selected concentrations. These were injected into UPLC. Calibration curves are shown to be linear in the concentration series of valacyclovir and famciclovir. The linearity values were tabulated in Table 1. The values of the coefficient of correlation of those analytes were 0.999. The calibration curve for valacyclovir and famciclovir using UPLC are shown in Figure 3(a, b).

### Accuracy and Precision

The accuracy was decided by using recovery data that was administered in three kinds of concentration levels, *i.e.,* 50, 100, and 150%. APIs with 50, 100, 150 µg/mL of valacyclovir and 12.5, 25, 37.5 µg/mL of famciclovir have been prepared. According to the test procedures, the solutions of the test have been injected into three preparations for each spike level; hence, the assay has been done. Share recovery data have been found to be within 98 to 102% limits, and these results are given in Table 2.

### Precision

The precision of this method has been evaluated in the form of the method and the intermediate variations. The intraday studies were conducted by carrying out six repeated analyses of the sample solution of valacyclovir and famciclovir on equivalent conditions of the experiment. The intermediate tactic, precision, was administered in the laboratory by conducting the analysis with various analysts well as with

(a) (b)

**Figure 3:** UPLC linearity plots of (a) Valacyclovir (b) Famciclovir.

various instruments, the tactic is found to be precise, and the values of RSD were found to be greater than 2%. These selected drugs were recovered well 98 to 102 and they were formed at every attached concentration, which tells the accuracy of the tactic. The results are furnished in Table 3.

**Table 3:** UPLC precision results: (a) Valacyclovir (b) Famciclovir.

| *(a)* | | | |
|---|---|---|---|
| *S. no.* | *Added amount (µg/mL)* | *Area* | *%RSD* |
| *Method precisions* | | | |
| 1 | 100 | 2451991 | 0.36 |
| 2 | 100 | 2451387 | |
| 3 | 100 | 2435647 | |
| 4 | 100 | 2458475 | |
| 5 | 100 | 2455305 | |
| 6 | 100 | 2461250 | |
| *Intermediate precision results* | | | |
| 1 | 100 | 2451206 | 0.49 |
| 2 | 100 | 2451954 | |
| 3 | 100 | 2434567 | |
| 4 | 100 | 2454877 | |
| 5 | 100 | 2448512 | |
| 6 | 100 | 2425457 | |

| *(b)* | | | |
|---|---|---|---|
| *S. No.* | *Added amount (µg/mL)* | *Area* | *%RSD* |
| *Method precision results* | | | |
| 1 | 25 | 191365 | |
| 2 | 25 | 191143 | |
| 3 | 25 | 191650 | 0.47 |
| 1 | 25 | 191554 | |
| 2 | 25 | 190546 | |
| 3 | 25 | 193341 | |
| *Intermediate precision results* | | | |
| 1 | 25 | 191884 | |
| 2 | 25 | 191327 | |
| 3 | 25 | 191009 | 0.25 |
| 1 | 25 | 191567 | |
| 2 | 25 | 191256 | |
| 3 | 25 | 192368 | |

**Table 4:** UPLC results of robustness.

| *Change in parameter* | *%RSD of valacyclovir* | *%RSD of famciclovir* |
|---|---|---|
| Flow rate of (0.8 mL/min) | 1.14 | 1.09 |
| Flow rate of (1.2 mL/min) | 0.88 | 0.85 |
| Organic phase (77:23) | 0.74 | 0.69 |
| Organic phase (63:37) | 1.19 | 1.11 |

## LoD and LoQ

Calibration curve methods were used to calculate the LoQ and LoD separately.The detection and quantification limits of these compounds have been determined by injection of continuous lower accumulations of standard solutions with the developed method of RP-UPLC technique. The values of LOD for valacyclovir and famciclovir were detected as 0.125 µg/mL, 0.031 µg/mL, and s/n values are 7 and 4, respectively. The limit of detection data was shown as 0.413 µg/mL and 0.102 µg/mL, and the s/n values, respectively were 27 and 22.

## Robustness

As per the ICH guidelines, small but deliberate variations have been made within the method parameters like changes within the flow (± 20%), and organic contents within the mobile phase (± 10%) to see the tactic capacity to remain unaffected. The tactic robustness is shown in Table 4 and was analyzed with the observation of results of the changed parameters on tailing factor, retention time, and percentage content by using the UPLC. The degree of the reliability of these consequences obtained by little intentional variation had shown that this tactic has been strong.

## Stability

For steadiness of the sample solutions assessment, they were analyzed for 24 hours initially at various periods. There is no observation of significant degradation during this period; hence, the mean deviation and the mean were not quite 5%. By proposing that these solutions have been stable for about 24 hours and that was enough for the entire UPLC analytical method.

## Forced Degradation

The suitability of the method for the degradation products the forced degradation process have been conducted. These studies help us understand the condition at which these drugs seem unstable to prevent the potential instabilities, and proper measures are often taken during formulations. These degradation samples were characterized by liquid chromatography–mass spectrometry (LCMS) and mass spectrometry (MS) spectra shown in Figure 4. The degradant values were tabulated in Table 5.

### *Acid Degradation*

Acid degradation of the said drugs has been studied in 1N HCl, 16.1% of valacyclovir, and 12.4% famciclovir. This degradation has been observed in UPLC and the formation of two degradation products.

### *Alkali Degradation*

Alkali degradation of the said drugs was studied in 1N NaOH, Valacyclovir (15.2%), and Famciclovir (13.5%) degradation has been noticed in UPLC and shows two degradation products.

### *Peroxide Degradation*

Peroxide degradation of these drugs has been studied in hydrogen peroxide of 30 and 3.7% of valacyclovir, and 15% of famciclovir degradation has been noticed in UPLC and a degradation product was formed.

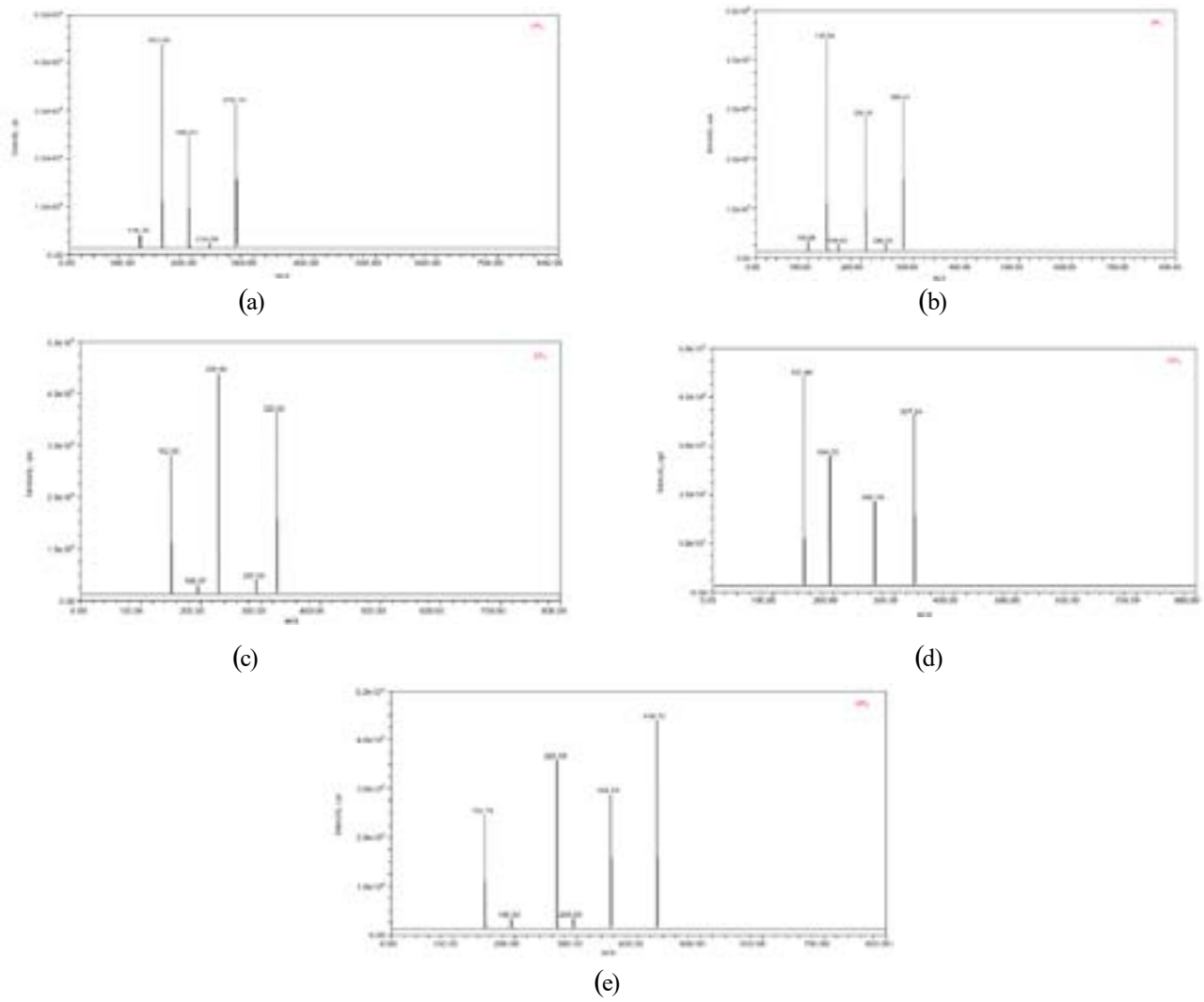



**Figure 4:** MS Spectra of (a) $DP_1$ (b) $DP_2$ (c) $DP_3$ (d) $DP_4$ (e) $DP_5$ (f) $DP_{6.}$

*Reductive Degradation*

Reductive degradation of these drugs has been studied in sodium bisulfate solution of 30, and 13.0% of valacyclovir and 2.8% of famciclovir degradation is found in UPLC, and a product of degradation has been obtained.

*Thermal Degradation*

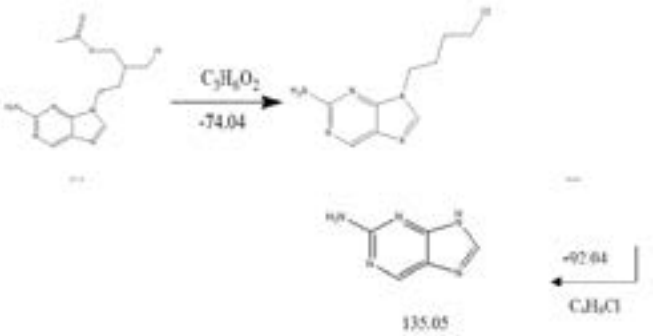
Thermal degradation of the compounds was carried out for 6 hours at 105°C. Degradation of 2.1% of Valacyclovir and 3.3% of Famciclovir has been found in UPLC, and no degradation products were obtained.

**Table 5:** Degradation results using UPLC.

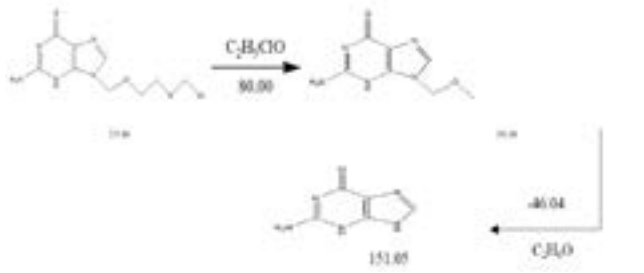
| *Degradation condition* | *Time/Temp* | *Valacyclovir* | | *Famciclovir* | | *No. of DPs formed* |
|---|---|---|---|---|---|---|
| | | *Deg%* | *Assay%* | *Deg%* | *Assay%* | |
| Acid | 3 hou, 60°C | 16.1 | 84.1 | 12.4 | 90.5 | Two |
| Alkali | 3 h, 60°C | 15.2 | 85.2 | 13.5 | 87.5 | Two |
| Peroxide | - | 3.7 | 96.8 | 15.0 | 83.6 | One |
| Reduction rs | 3 h, 60°C | 13.0 | 90.2 | 2.8 | 96.2 | One |
| Thermal | 24 hours, 105°C | 2.1 | 94.2 | 3.3 | 96.3 | No |
| Photolytic | UV-Vis light | 1.2 | 96.6 | 2.6 | 94.9 | No |
| Hydrolysis | 3 hours, 60°C | 1.9 | 97.5 | 1.5 | 97.2 | No |

DP-degradation product; Deg-degradation

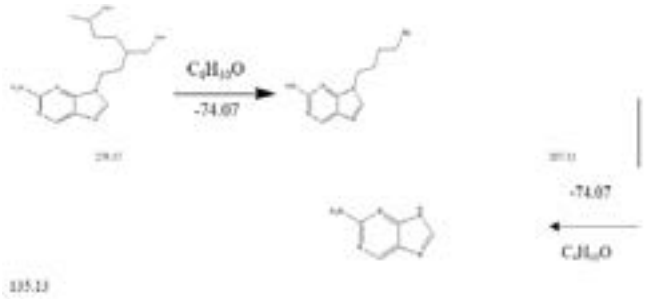
UV-vis light- (200 W h/$m^2$) and fluorescent light (1.2 milliion lux-h)



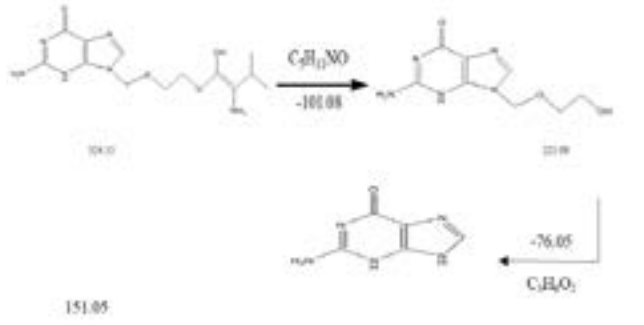
**Scheme 1:** Proposed fragmentation mechanism for Famciclovir ($DP_1$).



**Scheme 2:** The mechanism of Proposed fragmentation for Valacyclovir ($DP_2$).



**Scheme 3:** Proposed fragmentation mechanism for Famciclovir ($DP_3$).



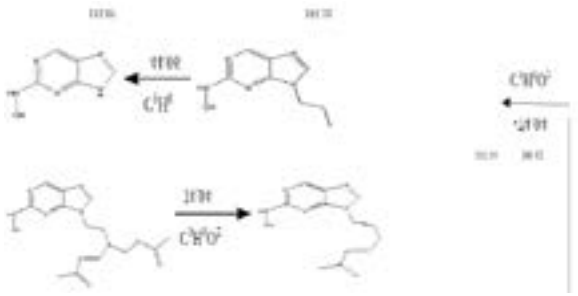
**Scheme 4:** The mechanism of proposed fragmentation for Valacyclovir ($DP_4$).

*Photolytic Degradation*

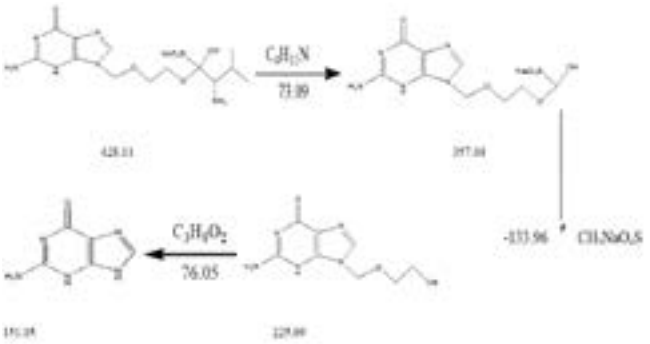
Standard was exposed for 12 hours in sunlight. Degradation of 1.2% of valacyclovir and 2.6% of famciclovirin UPLC has been observed, and there was no formation of degradation products.

*Hydrolysis Degradation*

Hydrolysis degradation of these drugs was observed in 3 mL of HPLC water, degradation of 1.9% of valacyclovir and 1.5%



**Scheme 5:** Suggested fragmentation mechanism for Famciclovir ($DP_5$)



**Scheme 6:** Proposed fragmentation mechanism for Valacyclovir ($DP_6$)

of famciclovir has been shown in the UPLC method, and formation of no products of degradation were found.

**Collision-induced Dissociation of Valacyclovir and Famciclovir**

In Scheme 1, the mechanism of fragmentation of famciclovir degradation product 1 (m/z-297), which has been observed under acid degeneration conditions, has been shown. The obtained spectrum shows an abundant product ion at the m/z-225 (losing $C_3H_6O_2$) and m/z-135 (losing $C_4H_9Cl$). The proposed method has been established by the experiments of MS/MS along with the mass measurements with accuracy.

Scheme 2 represents the mechanism of fragmentation of Valacyclovir degradation product 2 (m/z-273) observed in acid conditions. The obtained spectra exhibit abundant ions of the product at the m/z-195 (losing of $C_2H_5ClO$) and m/z-151 (losing of $C_2H_6O$). The experiments of MS/MS, along with the correct mass observations, explained the proposed scheme.

The mechanism of fragmentation of famciclovir degradation product 3 with m/z 279 that was observed under alkali conditions is shown in Scheme 3. The ions of the abundant product of the MS spectrum are displayed at the m/z-207 (losing of $C_4H_{10}O$) and m/z-135 (losing of $C_4H_{10}O$). The measurements of LC-MS/MS, along with exact mass calculations, establish this scheme.

The mechanism of fragmentation of valacyclovir degradation product 4 of m/z-324that has been observed at alkali degradation conditions is shown in Scheme 4. This spectrum shows the abundant ions of the product at the m/z-225 (losing of $C_5H_{11}NO$) and m/z-151 (losing of $C_3H_8O_2$). MS/MS studies and exact mass evaluation confirmed the suggested scheme..

The mechanism of the fragmentation of famciclovir degradation product 5 (m/z-337) that has been observed at peroxide degradation conditions is shown in Scheme 5. The abundant product ions displayed in the spectrum at the m/z-265 (losing of $C_3H_6O_2$), m/z-193 (losing of $C_3H_6O_2$), m/z-151 (loss of $C_3H_8$). The MS/MS method and exact mass evaluations have established the suggested scheme.

In Scheme 6 the mechanism of fragmentation for valacyclovir degradation product 6 (m/z-428) that has been observed at reduction degradation conditions is shown. The spectrum exhibits abundant ions of the product at the m/z-357) (losing of $C_4H_{11}N$), m/z-225 (losing of $CH_3NaO_4S$), and m/z-151 (losing of $C_3H_8O2$). The proposed scheme has been established by MS/MS experiment along with exact mass evaluations.

## CONCLUSION

In the present study a completely rapid, unique, economical, simple, sensitive, and easily available UPLC method has been developed for the simultaneous determination of valacyclovir and famciclovir in API form. There is no UPLC methods were reported for these drugs. We reported low prices, shorter run times, reliability, accessibility, reproducibility, and sensitivity during this method. The degeneration reactions of these drugs have been observed under acidic, basic, oxidation, neutral, reduction, photolytic, and thermal stress conditions. There was no degradation in neutral, thermal, and photolytic conditions. The degradation products have been identified as $[M+H]^+$ ion, and the structures proposed have been confirmed by the LC-MS/MS technique along with the exact mass evaluation. The RP-UPLC technique was supported as per the ICH guidelines.

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## CONFLICTS OF INTEREST

The authors have declared that there is no conflict of interest.

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