A green analytical method for estimation of letermovir in degradation studies

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**ABSTRACT**
The present established analytical method depicts identification of degradation products of letermovir with the aid of U.P.L.C-M.S/M.S. Letermovir was subjected to hydrolysis, oxidation, photolysis and thermal pressure, according to I.C.H conditions. The analyte shows degradation in oxidative and photolysis stress conditions. The analyte elution was finished on an intersil ODS column (150×4.6 m.m; 5 μ.m) with polar eluent contains water and methanol (20:80%, v/v) in an isocratic elution mode at a flow rate of 0.6 m.L/min. The eluents have been monitored through the use of a U.P.L.C-M.S/M.S and linearity range was obtained from 50-500 ng./m.L. The method was found to be stable and precision study was expressed in %R.S.D, ranged from 0.43 to 1.69. The developed method was validated as per I.C.H guidelines. No prior approach became made concerning degradation behavior of letermovir. The multiple reaction monitoring transitions were set at 573.31 >> 313.01 (m/z) and 288.10 >> 176.09 (m/z) for Letermovir and Tenofovir disoproxil respectively. The calibration curve was linear over the range of 50-550.00 ng/mL with lower limit of quantification validated at 50 ng/mL. The inter and intra-day accuracy values were below 15% at all quality control levels. The within run and between run precisions were within 15%, while accuracy ranged from 97.07 to 101.98. The degradation products were characterized by comparing their collision induced dissociation mass spectral data with that of Letermovir and the most possible degradation and fragmentation pathways were identified.

**INTRODUCTION**

Letermovir (LV) (Figure 1), (4S)-2-eight-fluoro-2-[4-(3-methoxyphenyl) piperazin-1-yl]-three-[2-methoxy-5(trifluoromethyl)phenyl]-3,4-dihydro quinazolin-four-ylacetacid (Lischka et al., 2010), is a highly potent anti-cytomegalovirus agent with a novel mechanism action, (Goldner et al., 2011) which targets the viral terminate complex and active against virus resistant to DNA polymerase inhibitors (Kaul et al., 2011; Marschall et al., 2012) Letermovir was effective in reducing the incidence of CMV infection in recipients of allogeneic hematopoietic-cell transplants (Chemaly et al., 2014). The highest dose (240 mg/day) had the greatest anti-CMV activity, with an acceptable safety profile (Tremblay et al., 2014; Pilorgé et al., 2014). The I.C.H-Q1-A (R-2) (ICH, 2003) recommends the stability of new drug substance or product exposure to stress conditions to generate degradation products to be formed with the influence of stress conditions (Armenta et al., 2008).
Figure 1: A) Letermovir (LV) B) Tenofovir disoproxil (TVIS)

(Koel and Kaljurand, 2006) During development of new analytical methods it is important to consider development of “Eco friendly” method to avoid environmental hazards (Alfonsi et al., 2008; Keith et al., 2007).

From our expertise, none of the methods has been stated for determination of letermovir and its degeneration of products by means of L.C.-M.S-M.S. The literature revealed very few analytical methods available methods for the estimation of letermovir, which include letermovir enantiomeric purity by HPLC (Zhang et al., 2016) and estimation in biological fluids (Rajanikanth et al., 2019). Based on literature, it is important to research the analytical method to analyze the degradation behavior of letermovir upon exposure to I.C.H., recommended stress parameters making use of an optimized L.C.-M.S-M.S (ICH, 2003).

MATERIALS AND METHODS

Materials

Letermovir (CHEMARC), Tenofovir disoproxil (CHEMARC). QSight® Triple Quad UPLC-ESI-MS/MS system (Perkin Elmer) Combined with QSight LX50 UHPLC.

Methods

Preparation of standards

Separate stocks of Letermovir (1.0 m.g/m.L) and Tenofovir disoproxil (Internal Standard) (1.0 m.g/m.L) were prepared by dissolving 10 m.g each substance in 10 m.L of Methanol. Series of different concentrations (50 to 550 ng./m.L) of Letermovir (LV) were prepared from LV stock solution (1000/µg/m.L) in eluent using 100.00 ng./m.L of Tenofovir disoproxil as internal standard.

Analytical Validation of the method
System suitability
Six replicate injections of aqueous standard 100% level (100.0 ng./m.L) along with internal standard (100.0 ng./m.L) were injected in to U.P.L.C-M.S/M.S and %RSD was calculated.

Linearity and Range
The Letermovir was assessed at 50% to 150% of the target concentration at different levels in the range of 50.0 ng./m.L to 550.0 ng./m.L for linearity study.

Precision study
The intra and Inter day precision study was assessed by analyzing six replicates of three different standard samples (200, 300 and 400 ng./m.L) and %CV was calculated.

Detection and quantification Limit
For Letermovir, the LOD and LOQ of was determined by calibration curve method. Solutions of Letermovir (LV) were prepared in linearity range and injected in triplicate. Average peak area ratio of three analyses was plotted against concentration.
Table 1: Linearity of Letermovir (LV)

| Linearity Level (%) | Nominal Conc. (ng./m.L) | Letermovir (LV) Mean Peak Area (n=3) | Tenofovir disoproxil (TVIS) Mean Peak Area (n=3) | *Mean Peak Area Ratio (n=3) |
|---------------------|--------------------------|-------------------------------------|-----------------------------------------------|---------------------------|
| 50                  | 50                       | 54321                               | 352173                                        | 0.154                     |
| 70                  | 150                      | 162902                              | 331431                                        | 0.492                     |
| 90                  | 250                      | 271611                              | 362141                                        | 0.750                     |
| 100                 | 350                      | 379232                              | 352153                                        | 1.077                     |
| 125                 | 450                      | 488781                              | 350211                                        | 1.396                     |
| 150                 | 550                      | 597533                              | 327415                                        | 1.825                     |

Correlation coefficient 0.9960
Y-Intercept -0.016458
Slope 0.00323
Standard Error -0.016458

*Results are mean of three measurements with six different concentration levels.

Table 2: Precision study of Letermovir (LV)

| Concentration (ng./m.L) | Within-run (Intra-day study) | Between-run (Inter-Day study) |
|-------------------------|------------------------------|------------------------------|
|                         | *Mean of measured Conc(n=6;ng./m.L;mean±S.D) | %C.V | %Accuracy | *Mean of measured Conc(n=6;ng./m.L;mean±S.D) | %C.V | %Accuracy |
| 200.00                  | 199.22±0.85                  | 0.43 | 99.61     | 199.74±1.15                  | 0.57 | 99.87     |
| 300.00                  | 298.08±1.55                  | 0.52 | 99.36     | 305.26±10.25                 | 1.69 | 101.98    |
| 400.00                  | 398.94±9.05                  | 1.27 | 99.73     | 388.26±1025                  | 1.64 | 97.07     |

*Results are mean of six measurements with three different concentration levels.

Table 3: Solution stability data of Letermovir & Tenofovir disoproxil

| Stability Sample | Ambient temperature | Refrigerated Temperature |
|------------------|----------------------|--------------------------|
|                  | %Difference at 0.0Hours | %Difference at 48.0Hours | %Difference at 0.0Hours | %Difference at 48.0Hours |
| Letermovir (300.00ng./m.L) | 0.00 | 1.36 | 0.00 | 1.24 |
| Tenofovir disoproxil (200.00ng./m.L) | 0.00 | 0.013 | 0.00 | 0.13 |

Robustness

Robustness was carried out by varying the method parameters like flow rate (±5%), Column temperature (±5%) and pH (±2%). Six replicate injections of aqueous standard 100% level (300.0 ng./m.L ) along with internal standard was injected in to U.P.L.C-M.S/M.S and %RSD was calculated.

Solution Stability

A portion of the freshly prepared standard solutions (LV-300.0 ng./m.L & TVIS-100.0 ng./m.L) were kept at ambient and refrigerated for 48 hr and analyzed by the proposed method by comparing with freshly prepared samples.

Filter validation (Filter Interference)

A Letermovir (LV) at concentration of 300.00ng./m.L solution and Tenofovir disoproxil (TVIS) solution at 100.00ng./m.L were prepared from fresh stock solutions. Some portion of Letermovir (LV) and Tenofovir disoproxil (TVIS) standard solutions (300.00ng./m.L and 100.00ng./m.L) was filtered through three different filters namely
0.45μm PVDF filter, 0.45μm PTFE and 0.45μm Nylon filter and some portion was centrifuged and injected into the UPLC-MS/MS system.

**FORCED DEGRADATION STUDIES**

**Preparation of unstressed sample**

Stock solution of Letermovir (1000 μg/mL) was prepared by dissolving an accurately weighed 10 mg of the sample and dissolved in 10 mL of the methanol in a volumetric flask. Letermovir (100 ng/mL) solution was prepared with eluent and injected into the UPLC-M.S/M.S System to study the unstressed sample.

**Table 4: Letermovir mass degradation profile**

| S No | Peroxide Degradation (m/z) ions | Photolytic Degradation (m/z) ions |
|------|---------------------------------|----------------------------------|
| 1    | 554                             | 471                              |
| 2    | 523                             | 457                              |
| 3    | 495                             | 381                              |
| 4    | 392                             | 325                              |
| 5    | 201                             | 162                              |
| 6    | 175                             | 135                              |
| 7    | 134                             | 51                               |
| 8    | Degradation product-I (554)     | Degradation product-II (471)     |

**Preparation of stressed or degradation sample solutions**

Weighed about 10 mg of Letermovir was taken in a 10 mL volumetric flask individually and dissolved 25 mL methanol and diluted with 0.1 N HCl, 0.1 N NaOH and 3% v/v H2O2 and water and set aside for 72 h at ambient temperature. For Thermal (Dry heat) and Photolytic Degradation 10 mg Letermovir was taken on Petri dish and exposed to heat (80ºC for 72 hrs) and UV radiation ((254 n.m) at 1.2 million lux-hours for 72 h). After degeneration, samples were diluted with eluent to get the final concentration 100 ng/mL and injected into UPLC-M.S/M.S system.

**RESULTS AND DISCUSSION**

**LC-MS/MS Conditions**

For better separation and ionization, a mixture of methanol and water (80:20, v/v) and an “Inertsil ODS-3 C18 (250 mm × 4.6 mm, i.d., 5 μm)” column at flow of 0.6 mL/min was used. Due to compatibility with analyze chromatographic conditions Tenofovir disoproxil was selected as IS (internal standard). The chromatographic peaks were eluted at 5.68 for Letermovir and Tenofovir disoproxil for 5.88 min. Mass parameters were optimized positive ion mode with ion transitions of m/z 573.31 → 381.12 and 288.10 → 176.10 for LV and TVIS (Figure 2). In the proposed method, nontoxic, nor hazardous. The pH of the samples and the eluent is about neutral i.e., not corrosive. So according to these criteria, the proposed method passes the greenness profile.

**Method validation**

Precision of Instrument parameter can be defined as test to ensure that the instrument can generate precise results. In this method %RSD value obtained was less than 2%.

From Figure 3, it was observed at retention times of Letermovir and Tenofovir disoproxil in eluent no significant response. It can be concluded that the method is specific for estimation of Letermovir in presence of solvent. Linearity was found to be satisfactory with correlation coefficient 0.9960 over the concentration range of 50.0 to 550.0 ng/mL (Table 1 and Figure 4).

The precision study of the proposed method was evaluated at three different concentration levels. The %RSD and accuracy was found to be 0.43 to 1.27 and 99.36 to 99.73% for intraday precision study. Whereas, for interday precision study %RSD and accuracy was found to be 0.57 to 1.64 and 97.07 to 101.98% (Table 2). The detection and quantification limits were evaluated from calibration curve plotted in concentration range of 50.0 – 550.0 ng/mL. L.O.D and L.O.Q for this method were found to be 45.49 and 137.85 p.g/mL respectively. These values indicated that the method was sensitive to quantify the drug.

The %RSD of Letermovir was good under most conditions and didn’t show any significant change when the critical parameters were modified and the components (Analyte and IS) were well separated under all the changes carried out. Thus, the method conditions were robust and Stable (Table 3). The % difference values for Letermovir and Tenofovir disoproxil of different filter materials was found to be 0.17 to 1.39% and 0.45 to 0.94 and no significant interference was observed.

**Identification of major degeneration of product formed under stress conditions by U.P.L.C-M.S/M.S**

The fragmentation for the degradants was also carried out for Letermovir using product ion scan by U.P.L.C-M.S/M.S. In these stress studies, a total of two degradation products (D1 and D2) were observed for Letermovir in D1 (Peroxide) and D2 (Photolyic).
Table 5: Degradation products of Letermovir

| Stress Parameter          | Strength of stress | Temperature | Duration | %Degradation | Inference                |
|---------------------------|--------------------|-------------|----------|--------------|--------------------------|
| Acid Hydrolysis           | 0.1M HCl           | Ambient     | 72 h     | 0.00         | Degradation not observed |
| Basic Hydrolysis          | 0.1M NaOH          | Ambient     | 72 h     | 0.00         | Degradation not observed |
| Oxidative/Peroxide        | 3% v/v H2O2        | Ambient     | 72 h     | 100.00       | Complete degradation    |
| degradation               |                    | -           | 80ºC     | 0.00         | Degradation not observed |
| Thermal                   | UV lamp (254 nm) at 1.2 million lux hours | Ambient | 72 h     | 100.00       | Complete degradation    |
| Neutral (Water)           | Water              | Ambient     | 72 h     | 0.00         | Degradation not observed |

Under oxidative and photolytic degeneration, the percentage degeneration of the drug was 100% and no degradation occurred for acidic and basic hydrolysis after 72 h. Under oxidative degradation, the Letermovir was degraded after 72 h with the formation of molecular ion.

The degeneration products of oxidative and photolytic were analyzed by U.P.L.C-M.S/M.S, the total ion chromatograms of both degrades was showing molecular ions at m/z 554 and 471 indicating the presence of the degeneration product in both cases. The oxidation stress conditions were studied using 3% v/v H2O2 up to 72 h at room temperature. It was found that, 3% v/v H2O2 was effective in oxidizing the drug even after 72 h. The degradant products of molecular ion at m/z 554 and 471 were shown in Figures 5 and 6. No Acidic, Basic, neutral and thermal degeneration was observed for the solution and solid form of drug after exposure to the water, 80ºC, up to 72 h, respectively. It was confirm that drug was found to be stable solid as well as in solution forms under Acidic, Basic, neutral and thermal stress conditions. The respective degeneration of m/z values of all degradate and their fragmentation ions was represent in Tables 4 and 5 and Figures 5 and 6.

CONCLUSIONS

In this study, the forced degeneration study on letermovir has been performed under I.C.H prescribed situations as to take a look at its degradation profile. An Eco friendly, sensitive, accurate, specific and reproducible isocratic stability-indicating U.P.L.C-M.S/M.S technique became evolved. The validation experiment proved that the developed method was linear, unique, specific and selective and further identified, the drug was sensitive to oxidative and photolytic conditions. This approach may be used for ordinary analysis of letermovir in Q.C laboratories without harming the environmental conditions.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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REFERENCES

Alfonsi, K., Colberg, J., Dunn, P. J., Fevig, T., Jennings, S., Johnson, T. A., Kleine, H. P., Knight, C., Nagy, M. A., Perry, D. A., Stefaniak, M. 2008. Green chemistry tools to influence a medicinal chemistry and research chemistry based organisation. Green Chem., 10(1):31–36.

Armenta, S., Garrigues, S., de la Guardia, M. 2008. Green Analytical Chemistry. TrAC Trends in Analytical Chemistry, 27(6):497–511.

Chemaly, R. F., Ullmann, A. J., Stoelben, S., Richard, M. P., Bornhäuser, M., Groth, C., Einsele, H., Silverman, M., Mullane, K. M., Brown, J., Nowak, H., Kölling, K., Stobernack, H. P., Lischka, P., Zimmermann, H., Rübsamen-Schaeff, H., Chamlpin, R. E., Ehninger, G. 2014. Letermovir for Cytomegalovirus Prophylaxis in Hematopoietic-
Cell Transplantation. *New England Journal of Medicine*, 370(19):1781–1789.

Goldner, T., Hewlett, G., Ettsicher, N., Ruebsamen-Schaeff, H., Zimmermann, H., Lischka, P. 2011. The Novel Anticytomegalovirus Compound AIC246 (Letermovir) Inhibits Human Cytomegalovirus Replication through a Specific Antiviral Mechanism That Involves the Viral Terminase. *Journal of Virology*, 85(20):10884–10893.

ICH 2003. Stability testing of new drug substances and products. In *International Conference on Harmonization. IFPMA*.

Kaul, D. R., Stoelben, S., Cober, E., Ojo, T., Sandusky, E., Lischka, P., Zimmermann, H., Rubsamen-Schaeff, H. 2011. First Report of Successful Treatment of Multidrug-Resistant Cytomegalovirus Disease with the Novel Anti-CMV Compound AIC246. *American Journal of Transplantation*, 11(5):1079–1084.

Keith, L. H., Gron, L. U., Young, J. L. 2007. Green Analytical Methodologies. *Chemical Reviews*, 107(6):2695–2708.

Koel, M., Kaljurand, M. 2006. Application of the principles of green chemistry in analytical chemistry. *Pure and Applied Chemistry*, 78(11):1993–2002.

Lischka, P., Hewlett, G., Wunberg, T., Baumeister, J., Paulsen, D., Goldner, T., Ruebsamen-Schaeff, H., Zimmermann, H. 2010. In Vitro and In Vivo Activities of the Novel Anti-cytomegalovirus Compound AIC246. *Antiviral Research*, 86(1):A23–A23.

Marschall, M., Stamminger, T., Urban, A., Wildum, S., Rubsamen-Schaeff, H., Zimmermann, H. 2012. In vitro evaluation of the activities of the novel anticytomegalovirus compound AIC246 (Letermovir) against herpesviruses and other human pathogenic viruses. *Antimicrobial Agents and Chemotherapy*, 56:1135–1137.

Pilorgé, L., Burrel, S., Aït-Arkoub, Z., Agut, H., Boutolleau, D. 2014. Human cytomegalovirus (CMV) susceptibility to currently approved antiviral drugs does not impact on CMV terminase complex polymorphism. *Antiviral Research*, 111:8–12.

Rajankanth, K. N., Kumar, V. K., Raju, V. A. 2019. Development And Validation Of A LC-MS/MS Method For The Determination Of Letermovir In Sprague Dawley Rat Plasma And Its Application To Pharmacokinetic Study. *World Journal Of Pharmacy And Pharmaceutical Sciences*, 8(2):1234–1343.

Tremblay, S., Dansereau, N., Balsitis, S., Franti, M., Lamorte, L. 2014. Development of a high-throughput human cytomegalovirus quantitative PCR cell-based assay. *Journal of Virological Meth-