WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 7.421
Volume 8, Issue 2, 1234-1343 Research Article ISSN 2278 – 4357

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

K. N. Rajanikanth[1], Dr. V. Kiran Kumar[2], Dr. VVSS. Appala Raju[3], Dr. Macharla Venkata Ramana[4], Dr. T. Shyam and Dr. N. Appala Raju*[6]

[1]HOD, Dept. of Phamaceutical Chemistry, Chalapathi Institute of Pharmaceutical Sciences, Chalapathi Nagar, LAM, Guntur 522 034 Andhra Pradesh, India.
[2]Principal, Dept. of Pharmaceutical Analysis, Sri Kakatiya Institute of Pharmaceutical Sciences, Unikicherla, Warangal, Telangana State, India.
[3]Department of Chemistry, Faculty of Pharmacy, MAHSA University, 59100 Kuala Lumpur, Malaysia.
[4]Faculty of Pharmacy, Lincoln University College, Kelanjaya, Petaling Jaya, Solangor Darul Ehsan, Malaysia.
[5]Principal, Dept. of Pharmacognosy, MRM College of Pharmacy, Chinthapaliguda(V), lbrahimpatnam(M), R.R. Dist. Telanagana State, India.
[6]Professor, Dept. of Pharmaceutical Chemistry, Sultan-Ul-Uloom College of Pharmacy, Banjara Hills, Hyderabad-500034, Telangana State, India.

Article Received on
20 Dec. 2018,
Revised on 11 Jan. 2019,
Accepted on 01 Feb. 2019
DOI: 10.20959/wjpps20192-12868

***Corresponding Author**
Dr. N. Appala Raju
Professor, Dept. of Pharmaceutical Chemistry, Sultan-Ul-Uloom College of Pharmacy, Banjara Hills, Hyderabad-500034, Telangana State, India.

ABSTRACT

During the preclinical study of the new antiviral drug, Letermovir, a sensitive liquid chromatography tandem mass spectrometry method was newly developed to study the pharmacokinetics of Letermovir in rat plasma and subcutaneous tissue samples. Although Letermovir was unstable in the rat plasma and subcutaneous tissue samples, pretreatment with EDTA and phosphoric acid (4 %) inhibited its degradation. The lower limits of quantification (LLOQ) for Letermovir were fully validated as 5 ng/ml in plasma and 5 ng/g in tissue with acceptable linearity, intra- and inter-assay precisions, and accuracy. Measurement of Letermovir concentration in plasma after intravenous injection *via* LC-MS/MS yields plasma concentration-time curves (AUC 0-∞) with areas of 667 ng•min/ml and an elimination half-life ($t_{1/2}$) of 4.8 min. The concentration of Letermovir in the subcutaneous tissue samples was 13.1 µg/gm tissues at 30 minutes after a single dermal application (1 mg/ml, 50 µl) to a full-thickness excisional wound. Here, a highly sensitive and specific LC-MS/MS assay with a

lower limit of quantification of 5 ng/ml was developed and validated to quantify Letermovir in rat plasma. This method is useful for pharmacokinetic studies of the peptide drugs in rats.

KEYWORDS: LC-MS/MS, Pharmacokinetics, Plasma, Quantitation, Subcutaneous Tissue.

INTRODUCTION

letermovir, an inhibitor of the CMV DNA terminase complex, and is administered orally or by intravenous infusion. {(4S)-8-Fluoro-2-[4-(3-methoxyphenyl)-1-piperazinyl]-3-[2-methoxy-5- (trifluoromethyl) phenyl]-3,4-dihydro-4-quinazolinyl} acetic acid (Letermovir, previously known as AIC246, chemical structure showed in (Figure 1) is a new highly potent anti-cytomegalovirus (CMV) agent with a novel mechanism of action targeting the viral terminase subunit pUL56[1,2], a component of the terminase complex involved in viral DNA cleavage and packaging that has no equivalent target enzyme in the human body.[3] Letermovir has a molecular formula of $C_{29}H_{28}F_4N_4O_4$ and a molecular weight of 572.55. Letermovir is very slightly soluble in water. Letermovir is indicated for prophylaxis of cytomegalovirus (CMV) infection and disease in adult CMV seropositive recipients [R+] of an allogeneic hematopoietic stem cell transplant (HSCT).As such; Letermovir also provides a potential new treatment option for patients infected with CMV strains that are resistant to approved antiviral drugs.[4] Initial clinical data on the use of Letermovir in a patient infected with a multidrug-resistant CMV strain who had multiorgan CMV disease appear to support in vitro data.[5–10] Letermovir was effective in reducing the incidence of CMV infection in recipients of allogeneic hematopoietic-cell transplants. The highest dose (240 mg/day) had the greatest anti-CMV activity, with an acceptable safety profile (3). Because of these attractive characters, Letermovir has already been employed as one possible candidate for human clinical trials.[11] This method has been developed exclusively to study the pharmacokinetic parameters by non-compartmental design, which offers the good selectivity and specificity. Also, the present method is sensitive in terms of LOD and LOQ by LC method with MS-detection.

MATERIALS AND METHODS

Reagents: Synthetic LETERMOVIR was purchased from ILS, Inc. (Tokyo, Japan). An internal standard (purity>99%) containing LLeucine-13C6 and 15N was purchased from Sigma-Aldrich (Hokkaido, Japan) and used without further purification. All organic solvents were of LC/MS grade and were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan) and

Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HPLC grade water was obtained using a water purification system (Auto pure WR600G, Yamato Scientific Co., Ltd, Tokyo, Japan).

LC-MS/MS Analysis: The HPLC system (LC20AD series, Shimadzu Co., Kyoto, Japan) consisted of a binary pump, auto-sampler, column oven maintained at 40°C and X Bridge C18 column (2.1 mm x 150 mm, 3.5 µm, Waters, MA, USA). The mobile phase was a mixture of solvent A (0.2% formic acid in MilliQ water) and solvent B (0.2% formic acid in acetonitrile) and used a flow rate of 0.3 ml/min. The following gradient was used for the plasma analyses: hold at 10% B for 0.2 min, increase linearly to 30% B over 2.6 min, then increase to 90% B over 0.2 min, hold for 1.3 min, decrease to 10% B over 0.1 min, hold for 1 min, increase to 90% B for 0.1 min, hold for 1.2 min, decrease to 10% B for 0.1 min, and hold for 2 min. The following gradient was used for the subcutaneous tissue samples: hold at 10% B for 0.2 min, increase linearly to 30% B over 2.8 min, then increase to 90% B over 0.1 min, hold for 0.5 min, decrease to 10% B for 0.1 min, and hold for 1.8 min. The detection was performed using a QTRAP5500 mass spectrometer (Applied Biosystems Sciex, Framingham, MA, USA) with an ESI source operating in the positive ion mode. Multiple reaction monitoring (MRM) of Letermovir used the m/z 534.1 → 217.2 transition. The following optimized MS conditions were used: curtain gas (nitrogen), gas 1 (air) and gas 2 (air) in portions of 30, 30 and 30, respectively; ion spray voltage of 4500 V; source temperature of 600°C; declustering potentials of 126 V for Letermovir and 126 V for IS; collision energies of 29 eV (m/z 573.1 → 383.1) for Letermovir and 29 eV (m/z 534.4 → 217.2) for IS.

Optimization of Stability in Plasma

Letermovir samples (nominal concentrations of 10 and 800 ng/ml) were prepared in blank rat plasma (EDTA). The samples were mixed after adding three times their mass of 4% phosphoric acid. The short-term matrix stability was assessed by analyzing the samples after storing at -80°C, 4°C and room temperature for 24 hours.

Pretreatment of Plasma Samples

A 5-µL aliquot of a Letermovir working solution in 50% methanol/H_2O at various concentrations (5 – 1000 ng/ml) was mixed with 1500 µL phosphoric acid (4%). Next, 495 µL of rat plasma and 10 µL of the internal standard solution (5000 ng/ml) were added. The solution was vortexed for 10 s and centrifuged at 10,000 x *g* for 5 min. The supernatant was transferred to an Oasis HLB µElution plate that had been previously activated by successive

applications of 0.3 ml methanol and 0.3 ml Milli-Q water. This plate was then washed with 0.3 ml of Milli-Q water. The peptide adsorbed onto the surface of the plate was eluted with 150 µL of acetonitrile- water-trifluoro acetic acid (75:25:1). A 10 µL aliquot was transferred to the LC-MS/MS system.

Pretreatment of Subcutaneous Tissue Samples

A 5 µL aliquot of a Letermovir working solution in 50% methanol/H2O at various concentrations (5 - 1000 ng/g tissue) was mixed with 750 µl phosphoric acid (4%). Next, 250 µl of a sub-cutaneous tissue sample (20% homogenate) and 10 µl of an internal standard solution (5000 ng/ml) were added to the above mixture. The solution was vortexed for 10 s and centrifuged at 10,000 x *g* for 5 min. The supernatant was transferred to an Oasis HLB µElution plate that had been activated by successive applications of 0.3 ml of methanol and 0.3 ml of Milli-Q water. The plate was then washed with 0.4 ml of Milli-Q water. The adsorbed peptide was eluted on the surface using 150 µl of acetonitrile-water-trifluoroacetic acid (75:25:1). A 10 µl aliquot was transferred to the LC-MS/MS system.

Method Validation

The method validation evaluated the specificity, linearity, lower limit of quantitation (LLOQ), precision, accuracy, and extraction recovery. The specificity was evaluated based whether an interference peak eluted at the same retention time as for the analytes in a blank sample. The calibration curves were constructed over the Letermovir concentration range from 5 to 1000 ng/ml for plasma and from 5 to 1000 ng/g for tissue. The intra-assay precision and accuracy were evaluated by replicating five QC samples on the same day. The inter-assay precision and accuracy were evaluated by analyzing QC samples across three separate days. The extraction recovery was determined by comparing to a blank plasma or subcutaneous tissue sample containing analytes added after the pretreatment.

Method Application for a Pharmacokinetic Study in Rats

Male SD rats weighing 250-350 g were used for the following pharmacokinetic studies. Letermovir (200 µg/kg) was dissolved in saline and administered to the rats *via* a single i.v. bolus injection. Blood samples were collected 2, 5, 10, 15 and 30 min after dosing. Letermovir (1 mg/ml for 50 µl/ rat) was transdermal administered throughout the full depth of the rat skin. Blood samples were collected after 0.5, 1, 2, 4, 8 and 24 hours. Subcutaneous tissue samples were collected after 0.5, 1 and 24 hours. The Letermovir concentrations in

both the plasma and subcutaneous tissues were measured *via* LC-MS/MS. The study was discussed and approved by the Ethics Committee.

LC-MS/MS Analysis

Fig. **1A** shows a typical positive ion ESI mass spectrum for Letermovir. The ion peaks at m/z 445.0, 534.1, 667.2 and 889.3 correspond to the 6+, 5+, 4+, 3+ ions, respectively. The 5+ ion showed the highest sensitivity. The product ion spectrum for the 5+ ion is shown in Fig. **1B**. High collision energies were found to yield intense product ions. Typical SRM chromatograms were obtained using the blank (0 ng/ml) and the lowest calibration standard (5 ng/ml) in rat plasma (Fig. **2**). No interfering peaks from endogenous substances were observed at the Letermovir retention times.

Optimization of Short-term Stability in Plasma

The short-term stability of Letermovir in plasma was assessed by analyzing the samples (10 and 800 ng/ml) after storing at -80°C, 4°C, and room temperature for 24 hours. The normal plasma concentrations (nominal concentrations of 10 and 800 ng/ml) were below the limit of quantification rat plasma. Adding 4% phosphoric acid to the plasma sample stabilized the Letermovir. This method development study demonstrated that LC-MS/MS was sufficiently suitable for detecting Letermovir in 4% phosphoric acid/plasma (3/1, v/v) to proceed with the method validation.

Pretreatment of the Plasma and Subcutaneous Tissue Samples

Solid-phase extraction (SPE) was used as the sample preparation method. During the method development, a variety of SPE cartridges were investigated for both the plasma and subcutaneous tissue sample pre-treatments. Oasis HLB µElution plates provided the highest recovery amongst the SPE cartridges tested.

Method Validation: The specificity was determined by analyzing blank plasma samples from three male rats and three female rats and the pooled plasma of different rats. The assay was linear across the range from 5 to 1000 ng/ml (Table **2**). The lower limit of quantification (LLOQ) in rat plasma was determined to be 5 ng/ml. The within-run CVs were 5.4, 8.9, 6.0 and 5.4% (n=5 for each) for the 5, 10, 50 and 800 ng/ml LLOQ respectively (Table **3**). The between-run CVs were 7.4, 8.4, 5.5 and 4.9% (n=15), respectively, for these samples (Table **4**). The within-day and between-day validation parameters meet the FDA Good Laboratory Practice criteria for an analytical method validation.

Pharmacokinetic Study: A profile of the Letermovir concentration in plasma versus time after an intravenous injection is shown in (Fig. **3A**). The Letermovir concentration in the plasma rapidly decreased. The pharmacokinetic parameters for Letermovir are presented in (Table **5**). The area under the concentration to time curve ($AUC_{0-\infty}$) was 667 ng·min/ml and the obtained elimination half-life (t1/2) was 4.8 min. The Letermovir concentrations in plasma were below the limit of quantification after a single application of Letermovir (1 mg/ml, 50 µl) to a full-depth excisional wound. A profile of the subcutaneous tissue concentration versus time for SR- 0379 after a single dermal application (1 mg/ml, 50 µl) to a full-depth excisional wound is shown in (Fig. **3B**). The SR- 0379 concentration in the subcutaneous tissue samples was 13.1 mg/gm tissue at 30 minutes after the application. The concentration in the subcutaneous tissue gradually decreased.

DISCUSSION

In this study, we developed a method for the quantitative analysis of a functional peptide, Letermovir, in rat plasma and tissue using LC/MS with an ion trap.[10-12] These peptides are unstable in biological fluids, such as plasma and urine.[13,14] EDTA, a metalloproteinase inhibitor, can be added to the samples to prevent their degradation when determining the concentration.[15] In this study, the stability of Letermovir spiked into plasma was investigated. The results indicated that Letermovir was unstable in plasma even with EDTA. 4% phosphoric acid was added to improve the stability of analyte. Thus, a simple method for determining Letermovir in biological matrices was established. The objective of this study was to characterize the pharmacokinetics of Letermovir using this LC-MS/MS technology. The Letermovir concentration decreased rapidly following a single intravenous injection of 200 mg/kg into male rats, and an elimination half-life (t1/2) of 4.8 min was obtained. Letermovir has potential antiviral activities that are important to wound healing and is for indicated for prophylaxis of cytomegalovirus (CMV) infection and disease in adult CMV seropositive recipients [R+] of an allogeneic hematopoietic stem cell transplant (HSCT). The Letermovir concentration in rat plasma was below the limit of quantification after a single dermal application (1 mg/ml, 50 µl) to a full-depth excisional wound. Topical treatments have the advantages of avoiding adverse systemic effects while increasing concentration at the target site. In contrast, the Letermovir concentration in the subcutaneous tissue was 13.1 mg/g of tissue 30 min after its application (1 mg/ml, 50µ l) to a full-depth excisional wound. The concentration in the subcutaneous tissue gradually decreased. It was suggested that the Letermovir degraded locally. A highly sensitive and specific LC-MS/MS assay with a lower

limit of quantification (LLOQ) of 5 ng/ml was developed and validated to quantify Letermovir in rat plasma. Most bioanalytical assays for antiviral agents show an LLOQ between 0.5 and 10 ng/ml, for some lower-LLOQs especially for low molecular weight compounds. Lower LLOQ of Letermovir has achieved by using QTRAP5500 mass spectrometer. This method was fully validated and possessed acceptable linearity, accuracy, and both intra- and inter-assay precisions. Furthermore, this method is useful for performing pharmacokinetic studies in rats.

Table. 1: Stability of LETERMOVIR for 24hr on Each Condition.

Nominal Concentration (ng/ml)	Experimental Conditions		Observed Value (ng/ml)	RR %
10	Normal Plasma	RT	No Peak	
		4°C	2.91	29.1
		8°C	9.49	94.9
	4% Phosphoric acid/ Plasma (3/1, v/v)	RT	7.41	74.1
		4°C	9.87	98.7
		8°C	10.5	105.0
80	Normal Plasma	RT	31.5	3.98
		4°C	123	16.1
		8°C	868	105.8
	4% Phosphoric acid/ Plasma (3/1, v/v)	RT	725	92.8
		4°C	857	108
		8°C	898	112.2

Table. 2: Linearity of Calibration Curves for the Determination of LETERMOVIR Concentrations in Rat Plasma.

Nominal Concentration in ng/mL	Back calculated value in in ng/mL	% RE	Back calculated value in in ng/mL	% RE	Back calculated value in in ng/mL	% RE
5	4.99	-0.2	5.89	17.8	4.86	-2.8
10	9.45	-5.5	10.67	6.7	10.82	8.2
20	20.9	4.5	18.18	-9.1	20.65	3.25
50	48.76	-2.48	44.76	-10.48	49.11	-1.78
100	99.7	-0.3	100.98	0.98	109.2	9.2
200	196.4	-1.8	199.16	-0.42	199.5	-0.25
800	802.4	0.3	826	3.25	811	1.375
1000	1022	2.2	1011	1.1	995.4	-0.46
Corr. Coeff.	0.989		0.9964		0.993	
Regression equation	y= 0.00421 *x* + 0.000198		*y* = 0.00489 *x* - 0.000198		*y* = 0.00401 *x* + 0.00139	
Weighting: $1/x^2$						

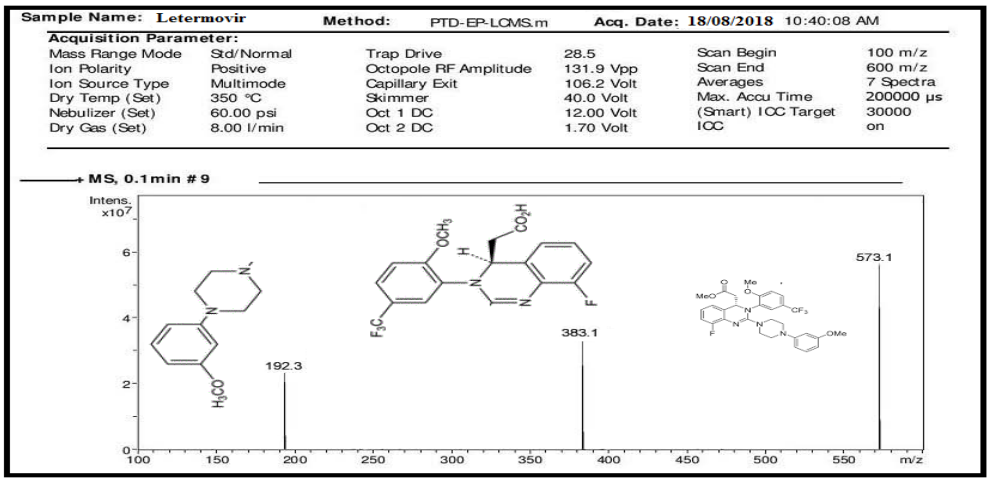

Figure. 2: Positive-ion electrospray mass spectra of Letermovir.

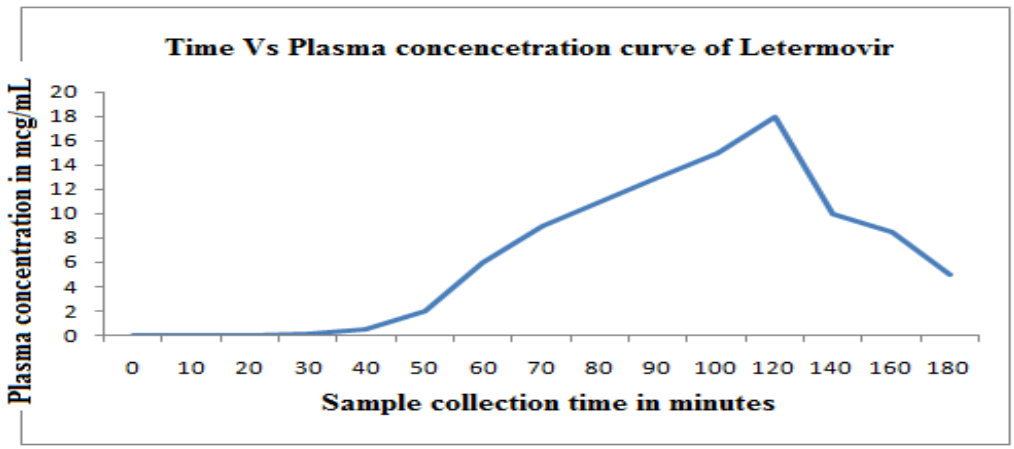

Figure. 3: Plasma concentration Vs time curve of Letermovir. (After oral administration of Letermovir (200 µg/kg) to the rats. N=4 per group).

Table. 4: PK Parameters of SR-0379 in Rat Plasma after Single oral Administration of Letermovir to Intact Skin Rats (Group 1, Dose: 200 µg/kg).

Kinetic Parameters	Results of Letermovir
AUC_{24h} (ng*h/mL)	1421.7
AUC_{inf} (ng*h/mL)	1710.239008
$AUMC_{24h}$(ng*h*h/mL)	162164.00
$AUMC_{inf}$ (ng*h*h/mL)	257393.50
Kel (h^{-1})	-0.187817427
Ka (h-1)	0.683
$T_{1/2}$ (h)	39.99
MRT(h)	150.50
Vd area (mL)	202455.22
Vd area (mL/kg)	1687126.81
Tmax (h)	2
Cmax (ng/mL)	19.00

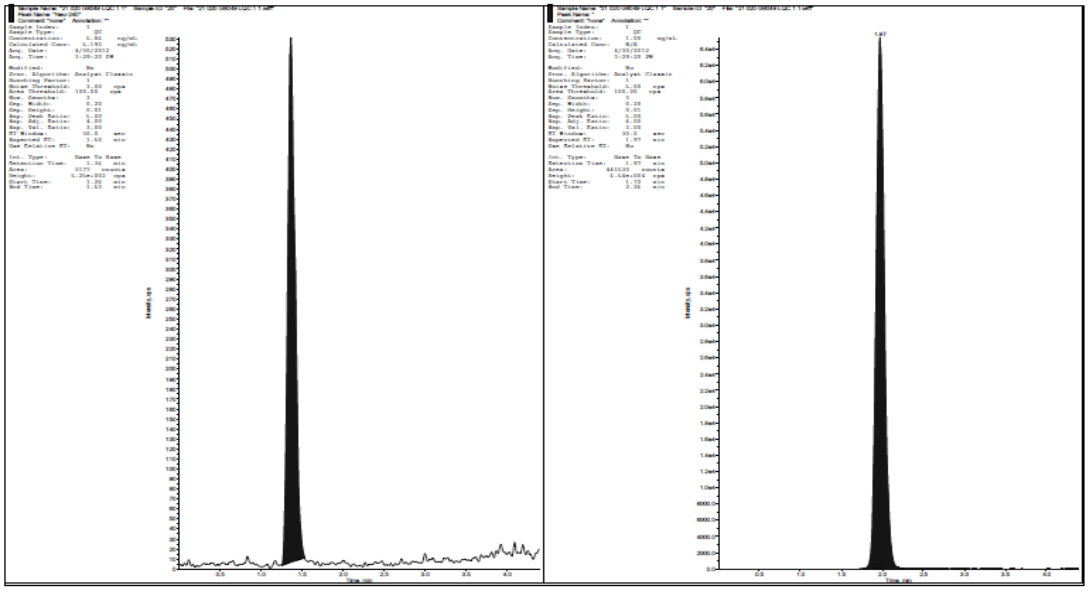

Figure. 4: Typical chromatogram of Letermovir at LQC level with internal standard level in human plasma.

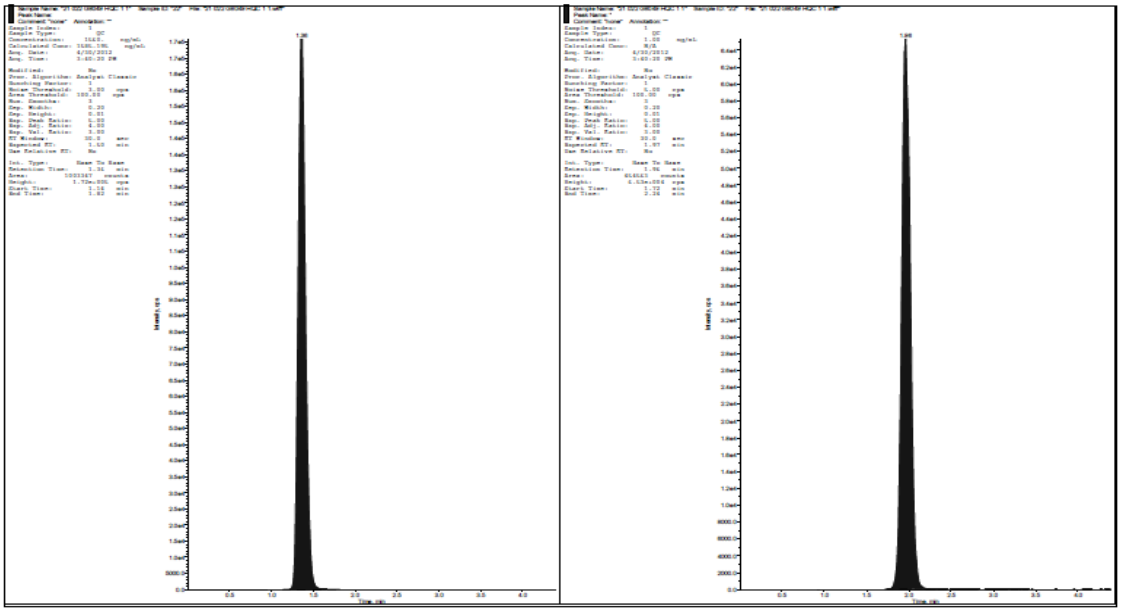

Figure. 5: Typical chromatogram of Letermovir at HQC level with internal standard level in human plasma.

REFERENCES

1. Lischka, P., Hewlett, G., Wunberg, T., Baumeister, J., Paulsen, D., Goldner, T., et al.; In vitro and in vivo activities of the novel anticytomegalovirus compound AIC246; Antimicrobial Agents and Chemotherapy, 2010; 54: 1290–1297.
2. Goldner, T., Hewlett, G., Ettischer, N., Ruebsamen-Schaeff, H., Zimmermann, H., Lischka, P.; The novel anticytomegalovirus compound AIC246 (Letermovir) inhibits

human cytomegalovirus replication through a specific antiviral mechanism that involves the viral terminase; Journal of Virology, 2011; 85: 10884–10893.

3. Chemaly, R.F., Ullmann, A.J., Stoelben, S., Richard, M.P., Bornhäuser, M., Groth, C., et al.; Letermovir for cytomegalovirus prophylaxis in hematopoietic-cell transplantation; The New England Journal of Medicine, 2014; 370: 1781–1789.
4. Marschall, M., Stamminger, T., Urban, A., Wildum, S., Rubsamen-Schaeff, H., Zimmermann, H., et al.; In vitro evaluation of the activities of the novel anticytomegalovirus compound AIC246 (Letermovir) against herpesviruses and other human pathogenic viruses; Antimicrobial Agents and Chemotherapy, 2012; 56: 1135–1137.
5. Kaul, D.R., Stoelben, S., Cober, E., Ojo, T., Sandusky, E., Lischka, P., et al.; First report of successful treatment of multidrug-resistant cytomegalovirus disease with the novel anti-CMV compound AIC246; American Journal of Transplantation, 2011; 11: 1079–1084.
6. Ljungman, P., Griffiths, P., Paya, C.; Definitions of cytomegalovirus infection and disease in transplant recipients; Clinical Infectious Diseases, 2002; 34: 1094–1097.
7. Tremblay, S., Dansereau, N., Balsitis, S., Franti, M., Lamorte, L.; Development of a high-throughput human cytomegalovirus quantitative PCR cellbased assay; Journal of Virological Methods, 2014; 195: 67–71.
8. Pilorgé, L., Burrel, S., Aït-Arkoub, Z., Agut, H., Boutolleau, D.; Human cytomegalovirus (CMV) susceptibility to currently approved antiviral drugs does not impact on CMV terminase complex polymorphism; Antiviral Research, 2014; 111: 6–12.
9. Goldner, T., Hempel, C., Ruebsamen-Schaeff, H., Zimmermann, H., Lischka, P.; Geno- and phenotypic characterization of human cytomegalovirus mutants selected in vitro after Letermovir (AIC246) exposure; Antimicrobial Agents and Chemotherapy, 2014; 58: 610–613.
10. Stoelben, S., Arns, W., Renders, L., Hummel, J., Mühlfeld, A., Stangl, M., et al.; Preemptive treatment of cytomegalovirus infection in kidney transplant recipients with Letermovir: results of a phase 2a study; Transplant International, 2014; 27: 77–86.
11. De Clercq, E.; A cutting-edge view on the current state of antiviral drug development; Medicinal Research Reviews, 2013; 33: 1249–1277.