Original Article

Orthogonal Analytical Method Development And Validation Using LCMS Technique For The Accurate Estimation Of Valaciclovir Hydrochloride Hydrate Related Substances

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Abstract

Existing work makes known that Liquid Chromatography-Mass Spectrometry (LC-MS) method development and validation for the impurity G (N, N -dimethylpyridin-4-amine) and impurity S (2-[(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl) methoxy] ethyl N-[1,1-dimetylethoxy) carbonyl]-L-valinate) in Valaciclovir Hydrochloride Hydrate active pharmaceutical ingredient (API). The developed LC-MS analytical method considering is an orthogonal approach and is complimentary to the thin layer chromatography (TLC) method accessible for impurity G and impurity S quantitation in the Valaciclovir Hydrochloride Hydrate API monograph.

Impurity G and Impurity S were determined by the LC-MS method in Q1 Multiple ion/SIM mode using Ascentis Express C18 (15cm X 4.6 mm) 2.7µm analytical HPLC column. A gradient system was applied for the elution of analytes using acetonitrile (Eluent B) and 0.01M Ammonium Formate, LC-MS compatible volatile buffer, pH 3.0 (Eluent A) in distinct compositions. The gradient system (T/%B) was applied as 0.01/5, 4.00/5, 7.50/80, 10.00/80, 12.50/5, and 20.00/5. The method developed was validated considering the International Conference on Harmonization pharmaceutical guidelines. The quantitation limit found for Impurities G and S were 207.20 ppm and 216.00 ppm.

Index Terms— Impurity G, Impurity S, Valaciclovir Hydrochloride Hydrate API, LC-MS, Q1 Multiple Ion, ICH guidelines

INTRODUCTION

L-valyl ester hydrochloride salt of acyclovir is Valaciclovir. The chemical name of Valaciclovir is 2-[(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl) methoxy] ethyl L-valinate (Figure C). Valaciclovir Hydrochloride Hydrate is widely used to kill simplex and zoster herpes viruses. Viral thymidine kinase phosphorylated, valaciclovir to acyclovir triphosphate, and this help in inhibition of herpes viral DNA replication. The active ingredient Valaciclovir Hydrochloride, Hydrate has two process-related impurities, Impurity G and Impurity S. Impurity G is N, N -dimethyl pyridine-4-amine with the molecular formula C7H10N2 (Figure A). Impurity S is 2-[(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl) methoxy] ethyl N-[1,1-dimetylethoxy) carbonyl]-L-valinate. The molecular formula of impurity S is C18H28N6O6 (Figure B). Impurity G has comparatively high harmfulness, is destructive to the lungs and eyes, and gets absorbed through the skin [8]. Impurities S and G are official impurities of Valaciclovir Hydrochloride, Hydrate API as per European pharmacopeia.

A chiral method development and method validation research article is available for the Valacyclovir drug and its associated constituent's guanine, acyclovir, and unknown impurity using a high-performance liquid chromatograph. The Valacyclovir and its associated impurities were well separated in the published method. The HPLC method is also published for assay and purity determination of Acyclovir and Valacyclovir.

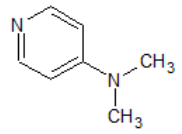
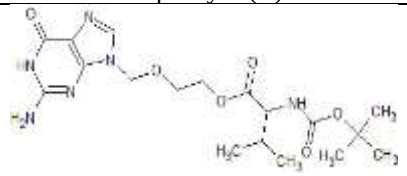
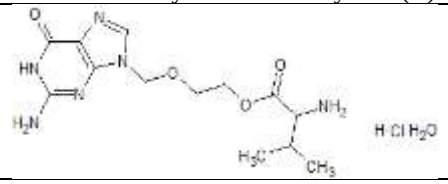
Literature for the evaluation of antiviral drugs acyclovir and valacyclovir with their impurity guanine using micellar electrokinetic chromatography (MEKC) is available. Literatures available related to Valaciclovir Hydrochloride Hydrate are less in number, but Acyclovir-related literatures are available in the public domain. p-Toluenesulfonic acid is a potent impurity and method developed to quantify the impurity in acyclovir drug substances at trace or residual levels by using API-4000 LC-MS/MS.

MATERIALS AND METHODS:

Chemicals & Regents:

Procured Impurity G and Impurity S from authorized suppliers of pharmacopeial impurities. Ammonium Format of LCMS grade and acetonitrile were purchased from Honeywell, India. Arranged Valaciclovir Hydrochloride Hydrate API sample from Pharma manufacturer, India.

Table No. 1

Impurity G (A)	Impurity S (B)	Valaciclovir Hydrochloride Hydrate(C)
		

Analytical Instrumentation:

The analytical instrument and analytical method parameters used for the impurity G and impurity S quantification method development in Valaciclovir Hydrochloride Hydrate API are given below in Table No.2.

Table No. 2

Liquid Chromatograph	
Pump Details	Shimadzu LC-20AD Pump
Detector Details	Shimadzu SPD-20A Detector
Auto Sampler	Shimadzu SIL-20AC/HT
Colum Thermostat	Shimadzu column Thermostat CTO-10ASvp
Chromatography Method Details	
Eluent A	0.01M ammonium Formate, LCMS compatible volatile buffer, pH 3.0
Eluent B	Acetonitrile (Cyanomethane)100% v/v
Analytical HPLC Column	Ascentis Express Octadecylsilane (15cm × 4.6mm) 2.7µm
Flow Rate	1.0mL/min, Flow Splitter used, Pass 0.5mL/min into the MS source
Thermostat temperature	15 degrees Celsius
Sampler Cooler Temperature	5 degrees Celsius
The Injection amount	5.0µl
System Runtime	20.0Minutes
Mass Spectrometer Parameter	
Mass Spectrometer	AB Sciex API 4000 model (Made in Singapore)
Ionization Probe	Electrospray ionization
Ionization Mode	Positive
Scan Type	Q1 Multiple Ions
Impurity G Molecular Mass Details	123.2 Dalton in Positive Mode
Impurity S Molecular Mass Details	425.2 Dalton in Positive Mode
Declustering potential	50 V
Entrance potential	10V
Curtain gas flow	35 (PSI)
Ion Spray Voltage(V)	5500V
Ion Source Gas 1	30 Nebulization pressures (PSI)
Ion Source Gas 2	50 Nebulization pressures (PSI)
Valco Valve Details	Venting was given from 4.1 to 7.4 minutes first then 9.9 to 18 minutes.
Data Acquisition & Processing Software	Analyst 1.6.3

Solutions preparation for Standard and sample:

Prepared different types of standard and sample solutions to carry out the study as given below. All the prepared solutions given below in Table No. 3, were sonicated well before the analysis.

Table No. 3

Solvent Mixture	Water: Ethanol mixture (20:80 *v/v*).
Stock solution Preparation	
0.52mg/mL stock solution of impurity G and 0.54mg/mL stock solution Impurity S prepared in solvent mixture	
Diluent	Acetonitrile: Mobile phase A (10:90)
0.05 mg/mL stock solution preparation	
Transferred 1.0ml of stock solution of impurity G and impurity S into 10mL volumetric flask and then diluted to 10mL with diluent to prepare 0.05mg/mL solution.	
0.001 mg/mL stock solution preparation	
Transferred 1.0ml of 0.05mg/mL solution into 50mL volumetric flask and then diluted to 50mL with diluent to prepare 0.001mg/mL solution.	
Standard solution preparation	
Transferred 0.50ml of 0.001 mg/mL solution into 10ml volumetric flask and then diluted to 10ml with diluent. The concentration of impurity G and Impurity was 518ppm and 540ppm in standard solution with respect to Valaciclovir Hydrochloride, Hydrate API concentration.	
Sample solution Preparation	
Prepared Valaciclovir Hydrochloride Hydrate sample solution at 0.1 mg/mL by dissolving a suitable quantity of sample in the diluent.	
Recovery studies solution preparation	
Prepared LOQ level-1, 50% level-2, 100% level-3, and 150% level-4 accuracy solution for impurities G and S with respect to Valaciclovir Hydrochloride, Hydrate API concentration for recovery studies by diluting the impurities stock solution with the essential amount of diluent.	
Linearity Study solutions preparation	
Prepared Linearity solutions for impurity G at 207.20, 259.00, 414.40, 518.00, 621.60, and 777.00 ppm and for impurity S at 216.00, 270.00, 432.00, 540.00, 648.00 and 810.00 ppm by diluting the stock solution with the requisite amount of diluent.	

Analytical Method development Details:

To obtain sensitivity, recovery, and separation between impurity G and impurity S along with Valaciclovir Hydrochloride Hydrate API, several analytical columns like Waters Xbridge C18 (150mm X 4.6mm, 5.0µm), Ascentis Express Octadecylsilane (10cm X 4.6mm, 2.7µm), Ascentis Express Octadecylsilane (50mm X 4.6mm, 2.7µm), Inertsil ODS (25cm X 4.6 mm,5.0µm) were evaluated during method development trials along with the different type of mobile phase A. The recovery of impurity G and impurity S was found within the acceptance range on Ascentis Express Octadecylsilane (15cm × 4.6mm, 2.7µm) analytical column, with as an eluent A (10mM Ammonium Formate LCMS Compatible buffer, pH 3.0) and as an eluent B (Acetonitrile) along with gradient system. On Ascentis Express C18 (15cm X 4.6mm, 2.7µm), injected impurity G, impurity S, and sample solution using MRM (Multi reaction monitoring) mode by keeping transition 123.20 -> 107.20 for impurity G and transition 425.20 -> 369.30 for impurity S. Responses for impurity G and impurity S were found satisfactory but the accuracy was not found within the acceptable range. Mobile phases of multiple compositions were verified with different mobile phase flow rates. 0.02% Trifluoracetic acid and 0.1% Formic acid in water as an eluent A and Methanol as an eluent B were used in another method development trial. The isocratic method was tried to set initially but later confirmed the gradient method for the analysis. Finally, looking at the analytical method development study data, selected an eluent A (0.01M Ammonium Formate LCMS compatible volatile buffer, pH 3.0) and an eluent B (Acetonitrile (100 v/v)) with gradient run. The gradient program was provided in Table 4.

RESULTS AND DISCUSSION:

Advantage of LC-MS method over TLC method:

The thin layer chromatography (TLC) method was available for Impurity G and Impurity S determination in the European pharmacopeia monograph of Valaciclovir Hydrochloride Hydrate API, and the limit of impurities G and S are 0.05% or 500ppm with respect to Valaciclovir Hydrochloride Hydrate API sample concentration.

Thin layer chromatography plate image as per monograph method is given below.

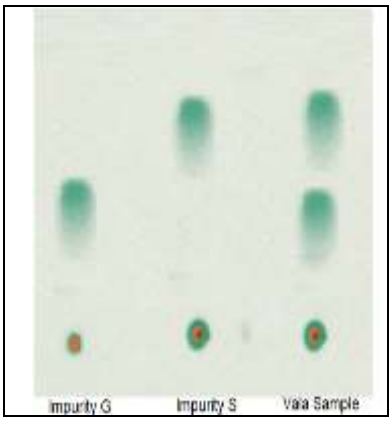


Figure 1: Impurities G and S TLC Plate

The available TLC quantitation method as per the monograph had reproducibility issues. Achieved restricted separation of impurities on the TLC plate due to the restricted length of the TLC plate. Time consumption was a big concern associated with the TLC method. Looking at the disadvantage connected with the present method available in the monograph, the current LC-MS analytical method was developed by testing different column chemistry stationary phases to obtain significant separation of the impurity G and impurity S with Valaciclovir Hydrochloride Hydrate API and recovery within the acceptance criteria.

Method developed using LC-MS was selective and sensitive as compared to available European pharmacopeia TLC method and can be used for exact evaluation of impurity G and impurity S in valaciclovir Hydrochloride hydrate antiviral drug.

LC-MS method run time was 20 minutes and mobile phase flow rate is 1.0ml. Impurity G and Impurity S was well separated in LCMS method and proving orthogonality compared to available TLC method.

Method Validation:

The method specificity was verified by infusing the diluent, individual impurities and Valaciclovir Hydrochloride Hydrate API sample and the chromatograms of connected solutions are available in Figure 2-5. The diluent chromatogram in Figure 2 showed that no interfering peak was observed at the retention times of Valaciclovir Hydrochloride Hydrate API as well as impurities. Impurities G and S, xtracted chromatograms of in Figure 2-5 displayed that Impurities G and S eluted at the retention times of 2.70 min and 8.18 min respectively. Established method chromatograms display absent of any interfering peak at Valaciclovir Hydrochloride Hydrate API and impurities G and S retention time. Developed analytical method was able to distinct impurities G and S with each other and with Valaciclovir Hydrochloride Hydrate drug.

Figure 2: Xtracted ion chromatogram (XIC), Blank for Impurity G and Impurity S

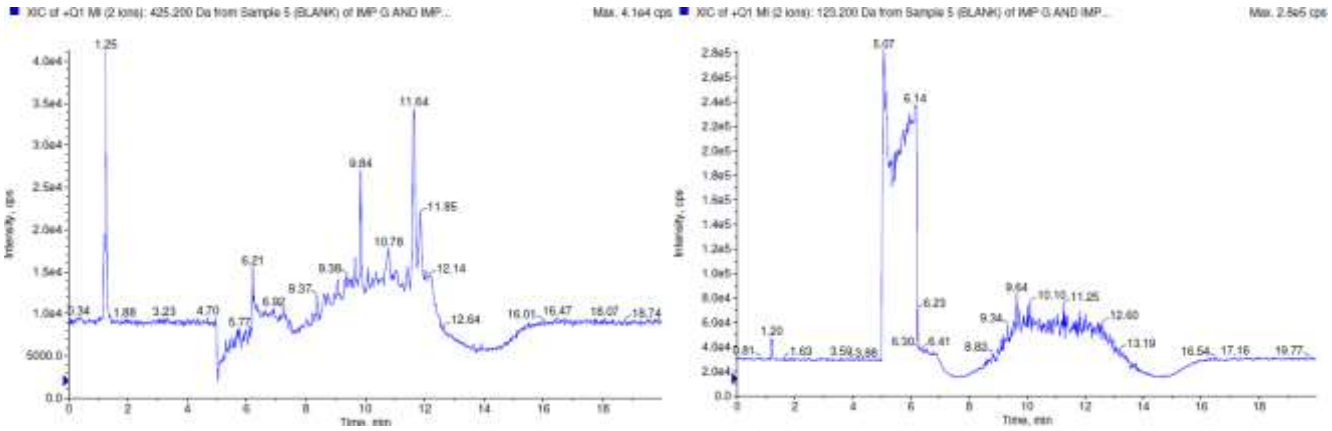


Figure 3: Impurity G- Xtracted ion chromatogram (XIC)

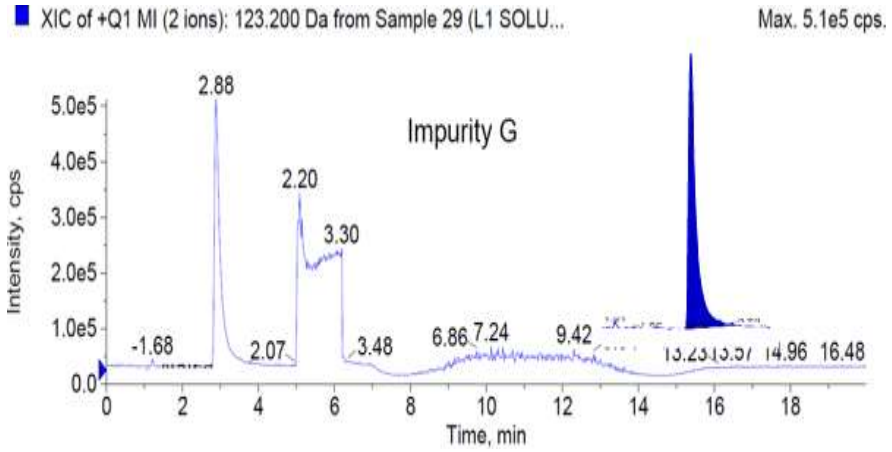


Figure 4: Impurity S - Xtracted ion chromatogram (XIC)

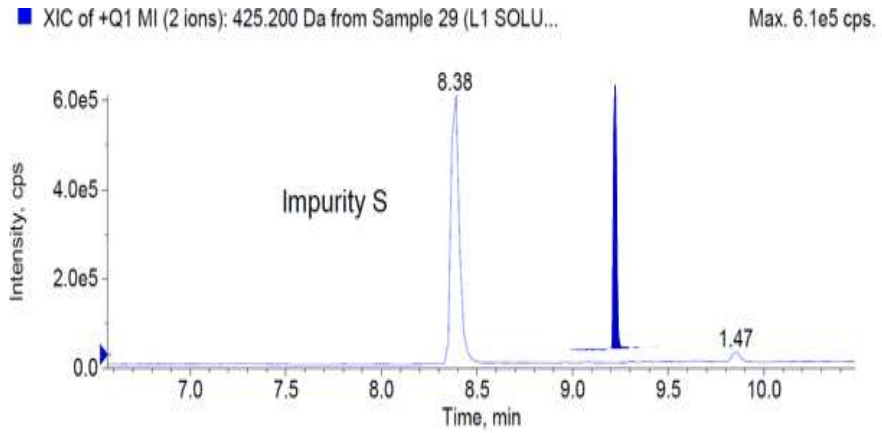
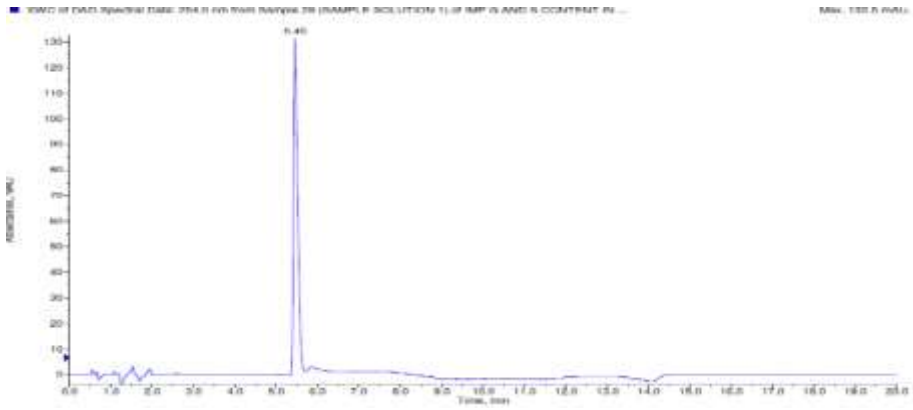


Figure 5: Valaciclovir Hydrochloride, Hydrate UV Chromatogram



The limit of detection (LOD) and lower limit of quantification (LLOQ) are determined for impurity G and Impurity S from signal to noise ratio. Prepared the lower concentrations of standard solutions to obtain a lower limit of quantification in this procedure. The lower limit of quantification of impurity G and Impurity S are 207.20ppm and 216.00ppm and LLOQ solutions of impurities G and S give a signal-to-noise ratio of 245.5 and 219.0 respectively.

Figure 6: Impurity G S/N ratio

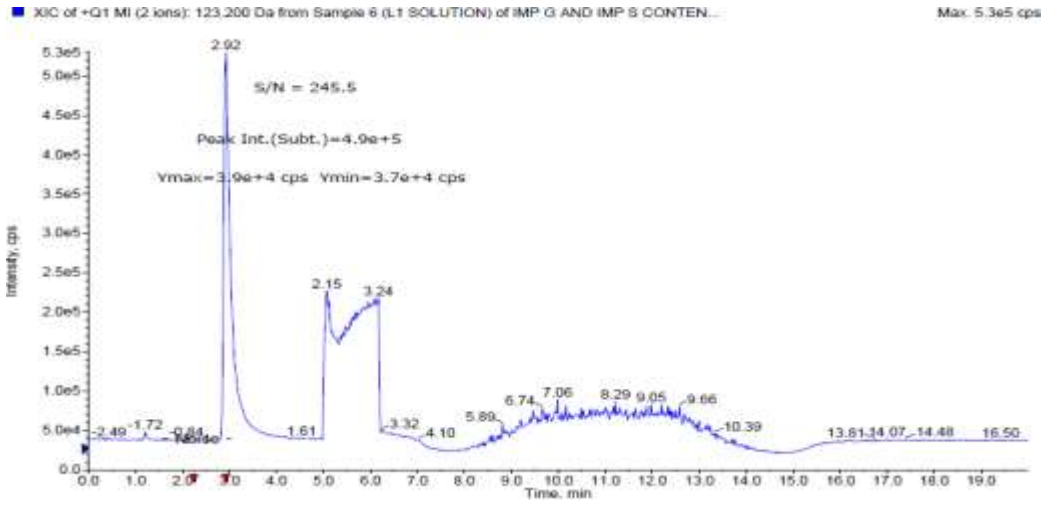


Figure 7: Impurity S S/N ratio

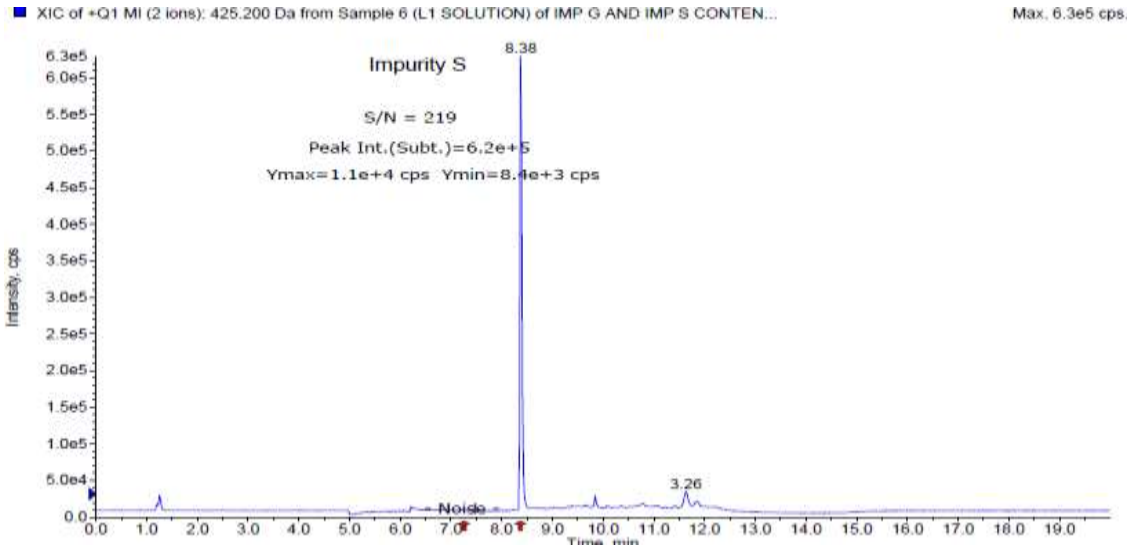


Table 4. Eluent Gradient run program

Time(minutes)	Eluent A (%)	Eluent B (%)
0.01	95.0	5.0
4.00	95.0	5.0
7.50	20.0	80.0
10.00	20.0	80.0
12.50	95.0	5.0
20.00	95.0	5.0

The developed analytical method linearity in Q1 Multiple ions scan type was established by injecting impurity G and impurity S at many levels of the concentrations between LLOQ and 150 % of the target concentration. The calibration curve was designed by drawing the chart between the peak response and impurity G concentration at 207.20, 259.00, 414.00, 518.00, 621.60, and 777.00 ppm and Impurity S at 216.00, 270.00, 432.00, 540.00, 648.00 and 810.00 ppm. Carried out linear least square regression study to get the slope, correlation coefficient values, and intercept. Linearity data were accessible in Table 5.

Table 5 Results of linearity for Impurities G and S

Linearity Level	Impurity G Concentration in ppm	Impurity G Area	Impurity S Concentration in ppm	Impurity S Area
Level 1	207.20	2263698	216.00	231327
Level 2	259.00	2750265	270.00	326601
Level 3	414.00	4525288	432.00	558096
Level 4	518.00	5577955	540.00	705811
Level 5	621.60	6567234	648.00	829608
Level 6	777.00	8691013	810.00	1137051
Correlation Coefficient(r^2)		0.9984	Correlation Coefficient(r^2)	0.9976
Slope		11096.19	Slope	1472.18
Intercept		-110971.06	Intercept	-84064.92

The spiking study was carried out to establish the accuracy of the newly analytically developed method by spiking the impurity G and Impurity S at LLOQ level, 100% and 150 % of the specification concentrations, with respect to the Valaciclovir Hydrochloride, Hydrate API sample concentration.

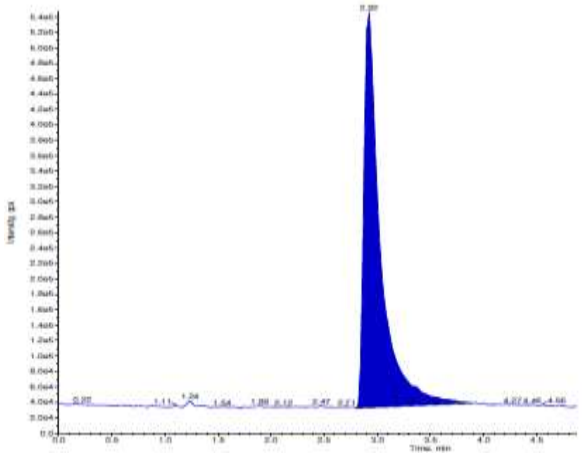


Figure 8: XIC of Impurity G LLOQ Spiked Sample

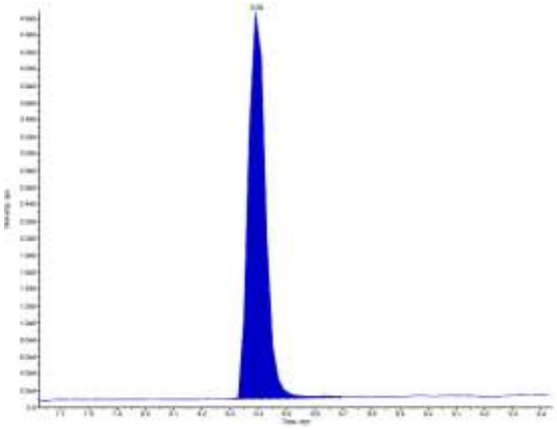


Figure 9: XIC of Impurity S - LOQ spiked sample

LLOQ level, Limit level, and 150% Level determination were carried out and corresponding data is presented in Table 7. The recovery of impurity G and Impurity S at three points (LLOQ level, 100 %, and 150 %) should be within the limit of 85.0 % to 115.0 % and the relative standard deviation should not be more than 10.0 %. Recovery values of 96.0 % to 98.3 % for impurity G and 98.6 % to 103.4 % for impurity S were obtained with % RSD 1.53 & 2.96 respectively.

Table 6 Results of Accuracy for Impurities G and S

Impurity Name	Impurity G			Impurity S		
Level	Theoretical Conc in ppm wrt Sm	Measured Conc in ppm wrt Sm	Recovery %	Theoretical Conc in ppm wrt Sm	Measured Conc in ppm wrt Sm	Recovery %
LLOQ (40%)	200.07	196.69	98.3	198.89	196.12	98.6
100%	248.05	245.69	98.8	246.59	241.64	98.0
150%	744.12	714.29	96.0	739.71	764.69	103.4
	% R.S.D.		1.53	% R.S.D.		2.96

A repeatability and ruggedness study were carried out to check method precision of the developed analytical method. Repeatability was checked by spiking specification level impurity G and impurity S, standard concentration in six freshly prepared sample solutions on the same day, and RSD of the content of impurity G and impurity S was checked. The relative standard deviation should be not more than 10.0 % and corresponding data is presented in Table 7. The relative Standard deviation of impurity G and impurity S obtained 1.9% and 2.1% respectively. LLOQ level precision was also checked and the % relative standard deviation of six replicate injections was 1.67 for impurity G and 3.92 for impurity S respectively. Connected data of LLOQ precision are presented in Table 8

Table 7 Results of Spike Precision for Impurities G and S

Injection	Impurity G Concentration obtained in sample	Impurity S Concentration obtained in sample
1	467.18	461.52
2	465.93	462.86
3	489.98	461.82
4	478.62	475.45
5	474.84	486.09
6	478.76	472.33
Mean	**475.885**	**470.012**
S.D.	**8.8361**	**9.84**
R.S.D.%	**1.9**	**2.1**

Table 8 Results of Precision at LLOQ Level

Injection	Area of Impurity G in LLOQ Solutions	Area of Impurity S in LLOQ Solutions
1	2253187	217423
2	2267775	236221
3	2197894	243270
4	2274616	235322
5	2312591	225579
6	2276122	230144
Mean	2263697.50	231326.50
S.D.	37739.51	9058.77
R.S.D.%	1.67	3.92

Ruggedness was checked by spiking the specification level standard concentration of impurity G and impurity S in six freshly prepared sample solutions on different day and the commutative relative standard deviation of content of each impurity between spike precision and intermediate precision should be not more than 10.0 %. Connected data are presented in Table 9.

Table 9 Results of Ruggedness for Impurities G and S

Injection	Impurity G Concentration obtained in sample	Impurity S Concentration obtained in sample
1(Precision)	467.18	461.52
2(Precision)	465.93	462.86
3(Precision)	489.98	461.82
4(Precision)	478.62	475.45
5(Precision)	474.84	486.09
6(Precision)	478.76	472.33
1(Ruguddness)	496.26	517.10
2(Ruguddness)	497.90	526.81
3(Ruguddness)	522.21	518.63
4(Ruguddness)	491.53	520.05
5(Ruguddness)	515.95	528.40
6(Ruguddness)	502.98	515.28
Mean	490.18	495.53
S.D.	18.01	27.70
% R.S.D.	3.67	5.59

The method robustness was checked by doing intentional changes in flow rate, and mobile phase pH. The flow rate of the eluent in the method of analysis was 1.0mL, which was changed by 10% (0.90 to 1.00 mL/min). The mobile phase pH effect on the analysis was explored at 2.8 pH and 3.2 pH (Mobile phase pH changed by +0.2 units). All the changes in the above-mentioned parameters did not show any considerable changes in the separation of impurity G and impurity S from the Valaciclovir Hydrochloride Hydrate and on chromatographic performance.

To prove the stability of impurity G and impurity S solutions, specification level impurities solution spiked in the sample solution and kept at room temperature (25^{o}C) for 48 hrs. Solution stability was evaluated by calculating the percent relative standard deviation of the area of impurities G and S solution between 0 Hrs and 39 Hrs. The percent relative standard deviation of the area of impurities G and S solution should not be more than 20.0 %The data presented in Table 10 revealed that the solution of impurities G and S was steady up to 39.30 hrs at room temperature.

Table 10. Solution stability data of Impurities G and S

Conditions	Impurity G Area in PPM	Impurity S Area in PPM
At 0 hrs	8715433	664232
At RT for 39.30 hrs	7505262	583583
% R.S.D.	15.6	12.6

CONCLUSION

The accurate, sensitive, selective, specific analytical method developed for the quantification of impurities G and S in Valciclovir Hydrochloride, Hydrate API at 0.05% with respect to Valaciclovir Hydrochloride, Hydrate API sample concentration using liquid chromatograph mass spectrometer. Electrospray ionization source/probe was used in the positive mode of ionization. Also verified that the LC-MS method is more sensitive and effective than the TLC method for the quantification of impurities G and S.

Specificity, precision, linearity, accuracy, and solution stability studies were performed to validate the analytical method. The method specificity was proved by the acceptable resolution of impurities with the Valaciclovir Hydrochloride Hydrate API. This method linearity covered in the range of 207.20 ppm to 777.00 ppm with respect to Valaciclovir Hydrochloride Hydrate API sample for impurity G, 216.00 ppm to 810.00 ppm with respect to Valaciclovir Hydrochloride Hydrate API sample for impurity S with a coefficient correlation of 0.9984 & 0.9976 respectively. The method accuracy was confirmed by the recovery values in the range of 96.0 % to 98.3 % for impurity G, and 98.02 % to 103.4 % for impurity S with % RSD 1.53 & 2.96 respectively. This developed method is sensitive with a lower limit of quantification of 207.20 for impurity G and 216.00 ppm for impurity S.

CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this investigation**.**

ACKNOWLEDGMENTS

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