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# QUANTITATIVE EVALUATION OF IMPURITIES G AND S IN VALACICLOVIR HYDROCHLORIDE HYDRATE ACTIVE PHARMACEUTICAL INGREDIENT USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY ANALYTICAL METHOD

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**Keywords:**

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

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**ABSTRACT:** Current 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 is complimentary to the thin layer chromatography (TLC) method available 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 mode using Ascentis Express C18 (15cm X 4.6 mm) 2.7µm analytical HPLC column. A gradient system was employed 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. A method developed was validated considering the International Conference on Harmonization pharmaceutical guidelines. The quantitation limit found for Impurity G and Impurity S were 207.20ppm and 216.00 ppm.

**INTRODUCTION:** L-valyl ester hydrochloride salt of acyclovir is Valaciclovir. Chemical name of Valaciclovir is 2-[(2-amino-6-oxo-1, 6-dihydro-9H-purin-9-yl) methoxy] ethyl L-valinate **Fig. 1C**. Valaciclovir Hydrochloride Hydrate is widely used to kill simplex and zoster herpes virus. Viral thymidine kinase phosphorylated valaciclovir to acyclovir triphosphate and this help in inhibition of herpes viral DNA replication.

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The active ingredient Valaciclovir Hydrochloride, Hydrate has two process-related impurities, Impurity G and Impurity S. Impurity G is N, N -dimethylpyridin-4-amine with the molecular formula $C_7H_{10}N_2$ **Fig. 1A**. Impurity S is 2-[(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl) methoxy] ethyl N-[1,1-dimetylethoxy) carbonyl]-L-valinate. Molecular formula of impurity S is $C_{18}H_{28}N_6O_6$ **Fig. 1B**.

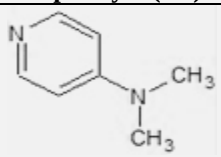
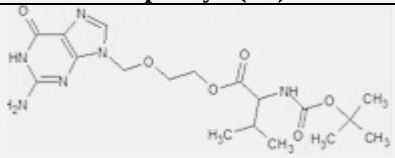
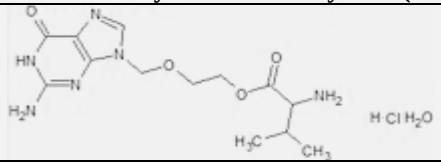
Impurity G is comparatively highly harmful, is destructive to the lungs and eyes, and gets absorbed through the skin [8]. Impurities S and G are official impurities of Valaciclovir Hydrochloride, Hydrate API as per European pharmacopeia. Chiral method development and method validation research article

is available for Valacyclovir drug and its associated constituent's guanine, acyclovir and unknown impurities are controlled using a high-performance liquid chromatograph method. 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 evaluating 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 public domain. p-Toluenesulfonic acid is a potent impurity and method developed to quantify the impurity in acyclovir drug substancesat trace or residual level by using API-4000 LC-MS/MS is also available.

## MATERIALS AND METHODS:

**Chemicals & Regents:** Procured Impurity G and Impurity S from authorised supplier 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 1: IMPURITY G, IMPURITY S AND VALACICLOVIR HYDROCHLORIDE HYDRATE STRUCTURES**

| Impurity G(1A) | Impurity S(1B) | Valaciclovir Hydrochloride Hydrate(1C) |
|---|---|---|
|  |  |  |

**Analytical Instrumentation:** Analytical instrument and analytical method parameter used for the impurity G and impurity S quantification method development in Valaciclovir Hydrochloride Hydrate API are given below in **Table 2.**

**TABLE 2: ANALYTICAL INSTRUMENT AND METHOD PARAMETER DETAILS**

| | |
|---|---|
| **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 |
| SamplerCooler Temperature | 5degrees 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 minutesfirst then 9.9 to 18 minutes. |
| Data Acquisition & Processing Software | Analyst 1.6.3 |

**Solutions Preparation for Standard and Sample:** Prepared different type of standard and sample solution to carry out the study as per given below. All the prepared solutions given below in **Table 3** were sonicated well before the analysis.

**TABLE 3: STANDARD AND SAMPLE SOLUTION PREPARATION DETAILS**

| | |
|---|---|
| **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 solutioninto 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 solutioninto 10ml volumetric flask and then diluted to 10ml with diluent. 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 suitablequantity of drug substance in the diluent | |
| **Recovery studies solution preparation** | |
| Prepared LOQ level-1, 50% level-2, 100% level-3 and 150% level-4 accuracy solution forimpurities 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, 621.60and777.00 ppm and for impurity S at 216.00, 270.00, 432.00, 540.00, 648.00and810.00 ppm by diluting the stock solution with 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 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 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. Mobile phases of multiple compositions were verified with different mobile phase flow rate. 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 it later confirmed the gradient method. Method development trials were taken using Multi reaction monitoring (MRM) mode, but the response of impurity and accuracy was not found in an acceptable range. Finally, looking at the analytical method development study data, selected an eluent A (0.01M Ammonium Formate LCMS compatible volatile buffer, pH 3.0) an eluent B (Acetonitrile (100 *v/v*)) with gradient run. Gradient programme was provided in **Table 4**.

## RESULTS AND DISCUSSION:

**Advantage of LC-MS Method over TLC Method:** Thin layer chromatography (TLC) method was available for Impurities G and S determination in the European pharmacopeia monograph of Valaciclovir Hydrochloride Hydrate API, and the limit of impurity G and impurity S are 0.05% or 500ppm with respect to Valaciclovir Hydrochloride Hydrate API sample concentration.

Thin layer chromatography plate image **Fig. 1** as per the monograph method, is given below.

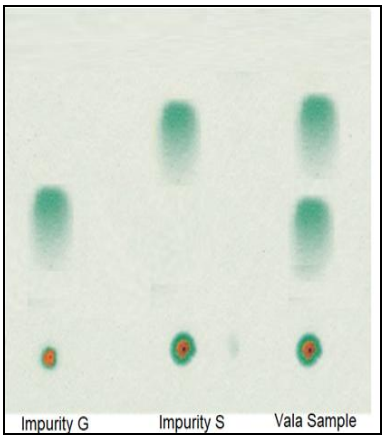



**FIG. 1: IMPURITY G AND IMPURITY S TLC PLATE**

TLC quantitation method showing reproducibility issues. The length of the TLC plate is restricted and therefore achieved restricted separation quality. Time consumption is a big concern associated with the TLC method. Looking at the disadvantage connected with the present method available in 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. A method developed using LC-MS was selective and sensitive compared to the available European pharmacopeia TLC method. It 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 the mobile phase flow rate was 1.0ml. Impurity G and Impurity S were well separated in LCMS method and proved orthogonality compared to the 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 **Fig. 2-6**.

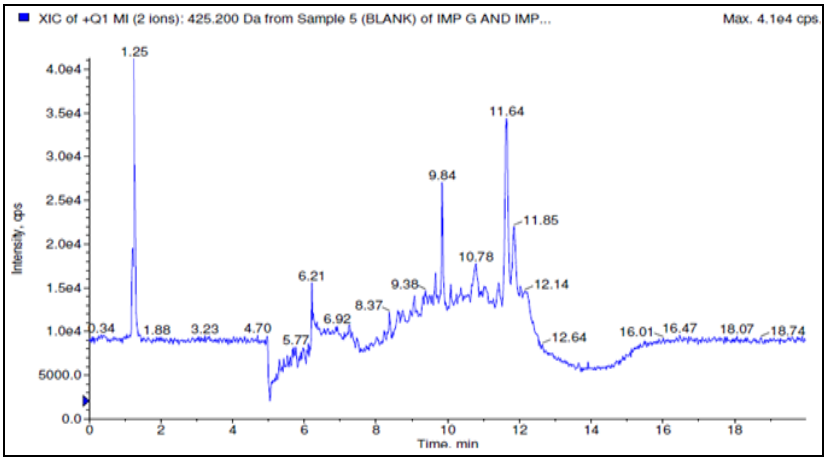



**FIG. 2: XTRACTED ION CHROMATOGRAM (XIC), BLANK-IMPURITY S**

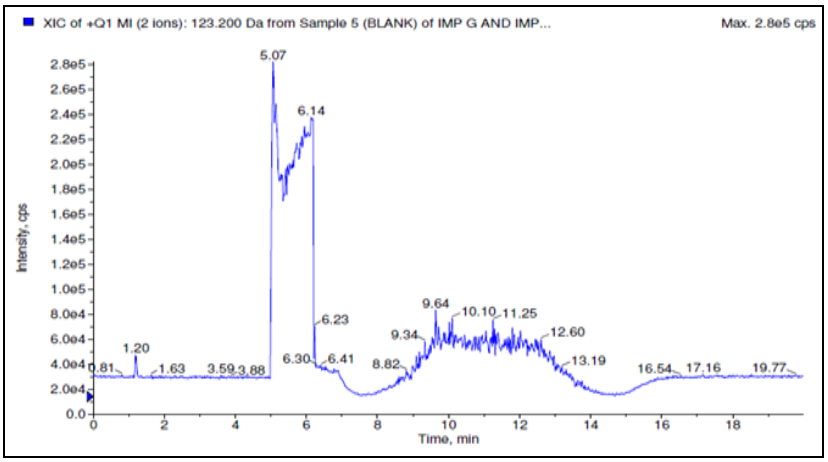



**FIG. 3: XTRACTED ION CHROMATOGRAM (XIC), BLANK- IMPURITY G**

The diluent chromatogram in **Fig. 2** & **Fig. 3** showed that no interfering peak was observed at the retention times of Valaciclovir Hydrochloride Hydrate APIas well as impurities. Impurities G and S, xtracted chromatograms of in **Fig. 4** and **Fig. 5** displayed that Impurities G and S eluted at the retention times of 2.88 min and 8.38 min, respectively. Established method chromatograms display the absent of any interfering peak at Valaciclovir Hydrochloride, Hydrate API, and impurities G and S retention time. A developed analytical method could distinguish G and S impurities with each other and with the main Valaciclovir Hydrochloride Hydratedrug.

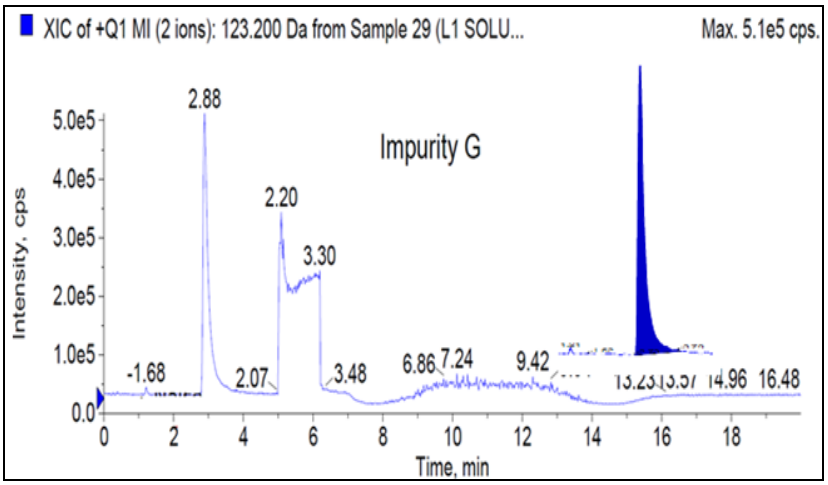



**FIG. 4: XTRACTED ION CHROMATOGRAM (XIC), IMPURITY G STANDARD**

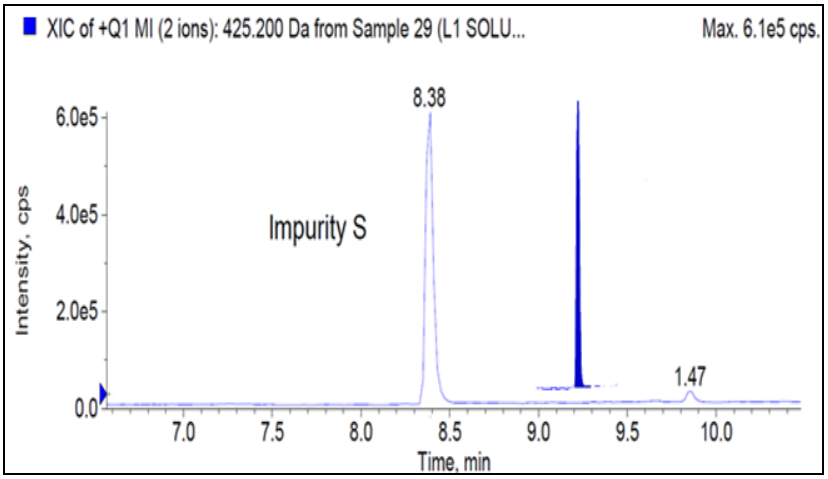



**FIG. 5: XTRACTED ION CHROMATOGRAM (XIC), IMPURITY S STANDARD**

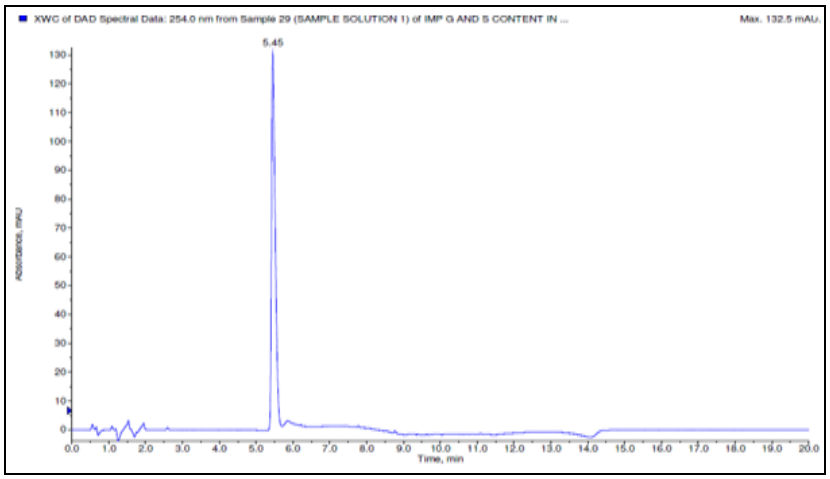



**FIG. 6: VALACICLOVIR HYDROCHLORIDE HYDRATE UV CHROMATOGRAM**

The limit of detection (LOD) and lower limit of quantification (LLOQ) determined for impurities G and S from signal to noiseratio. Prepared the lower concentrations of standard solutions to obtain lower limit of quantification in this procedure.

The lower limit of quantification of impurities G & S are 207. 20 ppm and 216.00 ppm and LLOQ solutions of impurities G and S give signal-to-noise ratios 245.5 and 219.0, respectively.

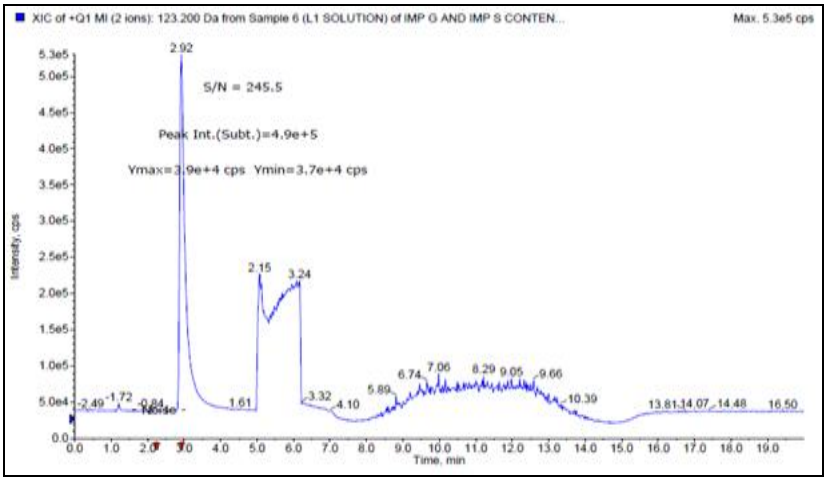



**FIG. 7: IMPURITY G S/N RATIO**

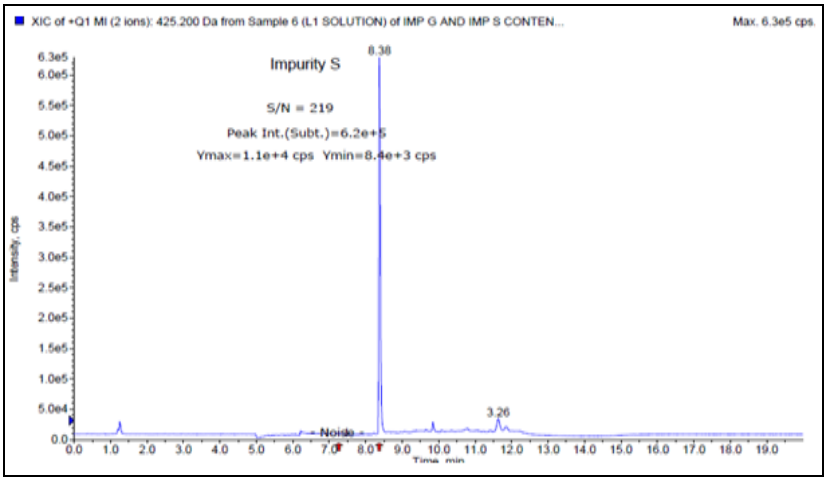



**FIG. 8: IMPURITY S S/N RATIO**

**TABLE 4: ELUENT GRADIENT 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**.

A spiking study was carried to establish the accuracy of the newly analytically developed method by spiking the impurities G and S at LLOQ level, 100% and 150 % of the specification concentrations, concerning the Valaciclovir Hydrochloride, Hydrate API sample concentration.

LLOQ level, Limit level, and 150% Level determination were carried out, and corresponding data is presented in **Table 6**. The recovery of

impurity G and Impurity S at three points (LLOQ level, 100 % and 150 %) should be in the limit of 85.0 % to 115.0 %, and the relative standard deviation should be not 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 5: RESULTS OF LINEARITY FOR IMPURITY G AND IMPURITY 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 |

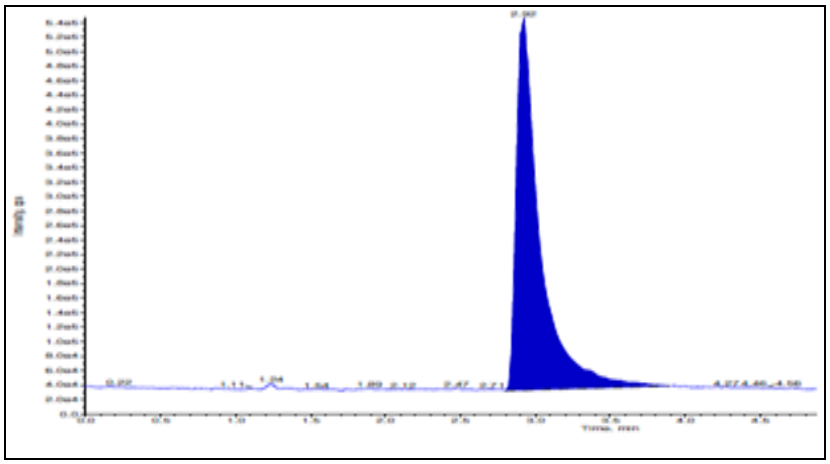


**FIG. 9: XTRACTED ION (XIC) OF IMPURITY G-LLOQ SPIKED SAMPLE**

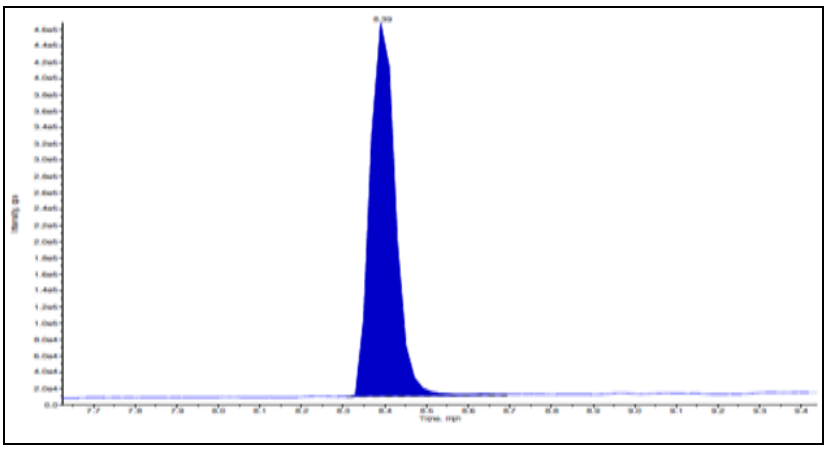


**FIG. 10: XTRACTED ION (XIC) OF IMPURITY S-LLOQ SPIKED SAMPLE**

**TABLE 6: RESULTS OF ACCURACY FOR IMPURITY G AN DIMPURITY S**

| Impurity Name | Impurity G | | | Impurity S | | |
|---|---|---|---|---|---|---|
| Level | Theoretical Conc in ppm wrtSm | Measured Conc in ppm wrtSm | Recovery % | Theoretical Conc in ppm wrtSm | Measured Conc in ppm wrtSm | 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 |

Repeatability and ruggedness study was carried out to check method precision of 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 content of impurity G and impurity S were checked. Relative standard deviation should be not more than 10.0 % and the 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 % 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: RSULTS OF SPIKE PRECISION FOR IMPURITY G AND IMPURITY 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 Sin 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 a different day, and the commutative relative standard deviation of the 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 IMPURITY G AND IMPURITY 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 |

Intentional changes in flow rate, mobile phase pH checked the method's robustness. 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 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 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 be not 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 IMPURITY G AND IMPURITY 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:** 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 concentartion using liquid chromatograph mass spectrometer. Electrospray ionization source/probe was used in positive mode of ionization. Also verified that LC-MS method is more sensitive and effective than TLC method for quantifying 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 207.20 ppm to 777.00 ppm concerning Valaciclovir Hydrochloride Hydrate API sample for impurity G, 216.00 ppm to 810.00 ppm concerning Valaciclovir Hydrochloride Hydrate API sample for impurity S with a coefficient correlation of 0.9984 & 0.9976 respectively. The recovery values confirmed the method accuracy in the range of 96.0 % to 98.3 % for impurity G, 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.

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