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Stability-indicating Reverse phase-HPLC Method development and Method Validation for Quantitative determination of Degradation products in Molnupiravir

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Abstract:

Molnupiravir is an antiviral medication shown to be broad spectrum activity against RNA viruses, and potentially treating the COVID-19. In this study, the HPLC method for the quantification of degradation impurities (Trihydroxy and Isobutryryl Oxide Impurities) were developed and validated for Molnupiravir in Tablet dosage form. The specificity of the method was achieved in analytical column Sunfire C18 Column (250 mm x 4.6mm, 10µm) using a suitable mobile phase was 0.1% TFA buffer (pH 2.4 with acetic acid) and Methanol in the Isocratic more of 55:45 v/v. The flow rate is 0.9 mL/min. the injection volume is 5 µL, detection at 232 nm in UV and total run time is 8.0 minutes. The samples were made for forced degradation under hydrolysis, oxidation, thermal and photolytic conditions. The method was validated for specific, selective, linear, robust and accurate as per the ICH guidelines. The linearity of the method for Impurities and the analytes was found from 25 to 150 % concentration level with the correlation coefficient (r^2) > 0.999. The accuracy for DIs and the analytes was performed from 50 to 150% level concentration, and mean recovery was found from 98-102%. The analytical degradation and validated study results indicate its unstable nature in acidic, basic and peroxide conditions. Therefore, this method is simple, selective and sensitive, this method can be used in pharmaceutical research and development and quality control departments.

Keywords: Molnupiravir, HPLC, Degradation impurities, ICH guidelines, Forced degradation, Stability Indicating method.

1. Introduction:

Molnupiravir is an antiviral medication that has gained attention for its potential in treating COVID-19. Molnupiravir was discovered by scientists at Emory University and Ridgeback Biotherapeutics. Molnupiravir is a prodrug of the synthetic nucleoside analog β-D-N4-hydroxycytidine (NHC), which acts by introducing copying errors into the viral RNA during replication, leading to the accumulation of mutations and viral error catastrophe. It has shown broad-spectrum activity against several RNA viruses, including coronaviruses[1,2].

Molnupiravir is a nucleoside analogue that is N (4)-hydroxycytidine in which the 5'-hydroxy group is replaced by a (2-methylpropanoyl) oxy group. It is the prodrug of the active antiviral ribonucleoside analog N (4)-hydroxycytidine (EIDD-1931), has activity against a number of RNA viruses including SARS-CoV-2, MERS-CoV, and seasonal and pandemic influenza viruses. It is currently in phase III trials for the treatment of patients with COVID-19. It has a role as a prodrug, an anticoronaviral agent and an antiviral drug. It is a nucleoside analogue, an isopropyl ester and a ketoxime. It is functionally related to N (4)-hydroxycytidine[3,4].

Molnupiravir exhibits potent antiviral activity against SARS-CoV-2 in vitro and in vivo. Clinical trials have shown favorable safety profiles, although further studies are ongoing. Typical doses investigated in clinical trials for COVID-19 range from 200 mg to 800 mg orally twice daily for 5 days.

Molnupiravir well-absorbed orally. It distributes widely throughout the body, including to the site of viral replication. It undergoes rapid metabolism primarily via enzymatic processes. The elimination half-life is relatively short, typically ranging from hours to a few days. Primarily excreted via the urine.

Molnupiravir is chemically known as MK-4482. Its molecular formula is $C_{16}H_{14}F_3N_3O_4$. its IUPAC name is [2R,3S,4R,5R)-3,4-dihydroxy-5-[4-(hydroxyamino)-2-oxopyrimidin-1-yl] oxolan-2-yl] methyl 2-methylpropanoate. Its molecular weight is Approximately 383.3 g/mol. It exhibits moderate solubility in water, Typically, it is supplied as a solid substance, often in the form of capsules or tablets[5–7].

Molnupiravir has been granted Emergency Use Authorization (EUA) by several regulatory agencies for the treatment of COVID-19 in certain circumstances.

Ongoing research continues to explore its effectiveness against other RNA viruses and its potential for combination therapies.

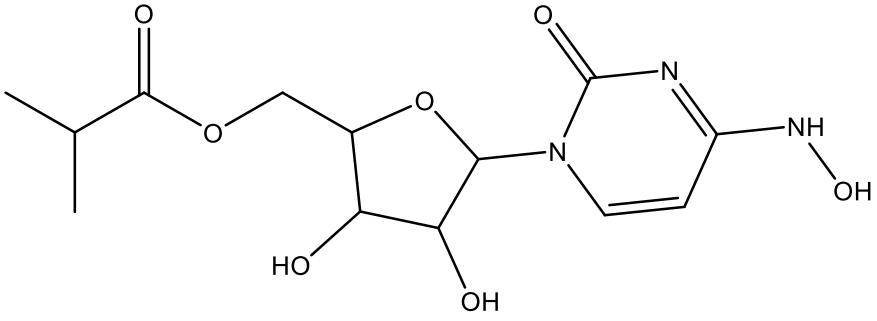

Figure 1: Representative Structure of Molnupiravir

literature survey indicated no chromatographic methods for determining degradation impurities (Trihydroxy and Isobutryryl Oxide Impurities) presented in the Molnupiravir. There are few reported literature on the quantification of Molnupiravir in chemical and biological matric. There are few reported literature on the quantification of metabolite generated from the biological matrices of Molnupiravir[12–18].

The objective of the current study was to create a simple, precise, linear, accurate, robust and stability-indicating assay method for identifying degrading impurities that are present in Molnupiravir. A proven quantitative analytical process called the stability-indicating assay and related impurity method typically involves forced degradation and validation experiments[19–22].

2. Materials and Methods:

2.1. Instrumentation:

The experiment was performed on a Waters HPLC 2695 with PDA detector integrated with Empower 2 Software equipped with Binary pumps, a PDA detector, an auto injector, a sample cooler, column heater. A pH meter from Digisun Electronics Hyderabad, India, to measure the buffer pH. Electronics Balance from Denver, India, was used. Vacuum microfiltration unit was used with 0.22µm PVDF filters from Millipore. Ultrasonicator from Labman India, was used for sonication of sample and standard. UV-VIS spectrophotometer integrated with UV win 6 Software from PG Instruments T60, India. Hot Air Oven from Servewell Instrument PVT LTD, Bangalore to study the stability of samples.

2.2. Chemicals, reagents, and standards:

AR grade Potassium dihydrogen ortho phosphate, Ortho-phosphoric acid, sodium dihyrogen Ortho phosphate and Trifluoroacetic acid (TFA) was procured from Rankem, India. HPLC grade Acetonitrile, Methanol and Water was procured from Merck, Mumbai, India. Molnupiravir Tablets purchased from the local Pharmacy store.The Drug Substance (Molnupiravir) and the impurities were obtained as a gratis sample from Mylan Laboratories Ltd.

2.3. Chromatographic conditions:

The buffer was prepared for 0.1% TFA buffer adjusted pH to 2.4 with acetic acid in HPLC grade water and filtered through a 0.22µm membrane filter. HPLC mobile phase was composed of 0.1% TFA buffer is in A channel and Methanol is in B channel in Isocratic mode in the ration of 55:45 v/v. The selectivity was achieved using Sunfire C18 Column (250 mm x 4.6mm, 10µm). the flow rate of 0.9 mL/min was employed. The HPLC column temperature and sample temperature were set at 30°C and 25°C respectively. The analytes were detected at 232 nm. The injection volume is 5.0 µL and the total run time is 8.0 minutes. Water and Methanol (60:40) v/v is used as diluent.

2.4. Preparation of the Molnupiravir Standard Stock Solution:

Weighed 1 mg of Molnupiravir working Standard accurately and transferred into 50 ml clean dry volumetric flasks, added about 30ml of diluent, sonicated for 10 minutes, dissolved, and made up to the volume with diluent and mixed well. (20µg/ml Molnupiravir).

2.5. Preparation of the Molnupiravir Standard Solution:

Transferred 5 ml of Molnupiravir standard stock solutions into a 100 ml volumetric flask and made up to the volume with diluent and mixed well. (1µg/ml Molnupiravir)

2.6. Preparation of the Impurity stock solution:

Weighed accurately 1 mg each of Molnupiravir – Impurity A (Trihydroxy Impurity) and Molnupiravir – Impurity B (Isobutyryl Oxime) transferred into 50 ml clean dry volumetric flasks, added about 20ml of diluent, sonicated for 10 minutes and made up to the volume with diluent and mixed well. (20µg/ml impurity).

2.7. Preparation of the Molnupiravir stock solution:

Weighed accurately 0.5mg each of Molnupiravir standard and transferred into 10 ml clean dry volumetric flasks, added about 5ml of diluent, sonicated for 10 minutes and made up to the volume with diluent and mixed well. (50µg/ml impurity).

2.8. Preparation of the Linearity stock solution:

Transferred 5.0ml from the impurity stock solution and 2.0 ml of Molnupiravir stock solutions into a 10 ml volumetric flask and made up to with diluent and mixed well. For Lineairty solution preparation refer Table 1.

Sr.No	Level	Dilution (From Linearity Stock Solution)	Con. of Trihydroxy. (ppm)	Con. of Isobutyryl Oxime (ppm)	Con. of MolnupiravirAPI (ppm)
1	25%	0.25 ml to 10ml	0.25	0.25	0.25
2	50%	0.5 ml to 10 ml	0.50	0.50	0.50
3	75%	0.75 ml to 10ml	0.75	0.75	0.75
4	100%	1.0 ml to 10ml	1.00	1.00	1.00
5	125%	1.25 ml to 10ml	1.25	1.25	1.25
6	150%	1.50 ml to 10ml	1.50	1.50	1.50

Table 1: Linearity Solution Preparation from 25 to 150% level.

2.9. Preparation of the Sample solution (Drug Substance):

Weighed accurately and transferred about 100 mg of Molnupiravir drug substance into 100ml clean and dry volumetric flask and added about 60 ml of diluent, sonicated for 10 minutes, and made up to the volume with diluent and mixed well. (1000µg/ml Molnupiravir).

2.10. Preparation of the Sample solution (Drug Product):

Calculate the average weight of 10 tablets and crush to fine powder. Weigh accurately powder equivalent to 20 mg of Molnupiravir into a 20 mL volumetric flask and about 10 mL of diluent, sonicate for 10 minutes with intermittent shaking. Attain to room temperature. Dilute up to the volume with diluent and mix well.

2.11. Preparation of Spiked solution for Precision

Pipette 5ml of Impurity stock solution into a 10ml of volumetric flask, dilute to volume with diluent, mix well.

2.12. Preparation of the Spiked Sample solution for Precision:

Calculate the average weight of 10 tablets and crush to fine powder. Weigh accurately powder equivalent to 20 mg of Molnupiravir into a 20 mL volumetric flask. Add 2.0 ml of Spiking

solution for Precision and about 10 mL of diluent, sonicate for 10 minutes with intermittent shaking. Attain to room temperature. Dilute up to the volume with diluent and mix well.

2.13. Preparation of Spiked solution for Accuracy

Pipette 5ml of Impurity stock solution into a 10ml of volumetric flask, dilute to volume with diluent, mix well.

2.14. Preparation of the 50% Spiked Sample solution for Accuracy:

Calculate the average weight of 10 tablets and crush to fine powder. Weigh accurately powder equivalent to 20 mg of Molnupiravir into a 20 mL volumetric flask. Add 1.0 ml of Spiking solution for Accuracy and about 10 mL of diluent, sonicate for 10 minutes with intermittent shaking. Attain to room temperature. Dilute up to the volume with diluent and mix well.

2.15. Preparation of the 100% Spiked Sample solution for Accuracy:

Calculate the average weight of 10 tablets and crush to fine powder. Weigh accurately powder equivalent to 20 mg of Molnupiravir into a 20 mL volumetric flask. Add 2.0 ml of Spiking solution for Accuracy and about 10 mL of diluent, sonicate for 10 minutes with intermittent shaking. Attain to room temperature. Dilute up to the volume with diluent and mix well..

2.16. Preparation of the 150% Spiked Sample solution for Accuracy:

Calculate the average weight of 10 tablets and crush to fine powder. Weigh accurately powder equivalent to 20 mg of Molnupiravir into a 20 mL volumetric flask. Add 3.0 ml of Spiking solution for Accuracy and about 10 mL of diluent, sonicate for 10 minutes with intermittent shaking. Attain to room temperature. Dilute up to the volume with diluent and mix well.

2.17. Preparation of Oxidative degradation sample solution:

Calculate the average weight of 10 tablets and crush to fine powder. Weighed accurately powder equivalent to 20 mg of Molnupiravir into a 20 mL volumetric flask and added about 10 ml of diluent and sonicated for 10 minutes to dissolve and added 5 mL of 3% Hydrogen peroxide (H_2O_2) solution to the sample containing solution. The resultant solution was kept for 30 minutes at 60°C on a hot water bath. Finally, made up to the volume with diluent and mixed well. (1000µg/ml Molnupiravir). Injected 1.0 µl of the solution into HPLC and recorded the stability of the sample.

2.18. Preparation of Acid degradation sample solution:

Calculate the average weight of 10 tablets and crush to fine powder. Weighed accurately powder equivalent to 20 mg of Molnupiravir into a 20 mL volumetric flask and added about 10 ml of diluent and sonicated for 10 minutes to dissolve and added 5 mL of 1N Hydrochloric acid (HCl) solution to the sample containing solution. The resultant solution was kept for 30 minutes at 60°C on a hot water bath. Finally, made up to the volume with diluent and mixed well. (1000µg/ml Molnupiravir). Injected 1.0 µl of the solution into HPLC and recorded the stability of the sample.

2.19. Preparation of Alkali degradation sample solution:

Calculate the average weight of 10 tablets and crush to fine powder. Weighed accurately powder equivalent to 20 mg of Molnupiravir into a 20 mL volumetric flask and added about 10 ml of diluent and sonicated for 10 minutes to dissolve and added 5 mL of 1N sodium hydroxide (NaOH) solution to the sample containing solution. The resultant solution was kept for 30 minutes at 60°C on a hot water bath. Finally, made up to the volume with diluent and

mixed well. (1000µg/ml Molnupiravir). Injected 1.0 µl of the solution into HPLC and recorded the stability of the sample.

2.20. Preparation of Thermal degradation sample solution:

Molnupiravir tablets placed on the Petri dish and kept in an hot air oven at 105°C for 6h. After 6 hrs calculate the average weight of 10 tablets and crush to fine powder. Weighed accurately powder equivalent to 20 mg of Molnupiravir into a 20 mL volumetric flask and added about 10 ml of diluent and sonicated for 10 minutes to dissolve, and finally made up to the volume with diluent and mixed well. (1000µg/ml Molnupiravir). Injected 1.0 µl of the solution into HPLC and recorded the stability of the sample.

2.21. Preparation of Photo stability degradation sample solution:

The Molnupiravir Tablets was placed in the Photo stability chamber exposing UV light and Visible light at 1.2 million Lux hours and 200-watt hours/minutes respectively. After exposed, calculate the average weight of 10 tablets and crush to fine powder. Weighed accurately powder equivalent to 20 mg of Molnupiravir into a 20 mL volumetric flask and added about 10 ml of diluent and sonicated for 10 minutes to dissolve, and finally made up to the volume with diluent and mixed well. (1000µg/ml Molnupiravir). Injected 1.0 µl of the solution into HPLC and recorded the stability of the sample.

Preparation of Neutral degradation sample solution:

calculate the average weight of 10 tablets and crush to fine powder. Weighed accurately powder equivalent to 20 mg of Molnupiravir into a 20 mL volumetric flask and added about 10 ml of diluent and sonicated for 10 minutes to dissolve, and finally made up to the volume with diluent and mixed well. (1000µg/ml Molnupiravir). Injected 1.0 µl of the solution into HPLC and recorded the stability of the sample.

3.0 Results and Discussions:

3.1. Method Development:

This study aimed to develop a quantification of degradation products and Molnupiravir in pharmaceutical dosage form (Tablets). Waters Allience HPLC system equipped with DAD (Liquid Chromatography equipped with a Diode array detector) and UV as detector, the method was developed to provide the suitability of routine stability studies and QC analysis. The method was optimized to improve the resolution between DPs, symmetrical peak shape, Isocratic mode. To achieve the criteria many experiments were performed to optimize the column, diluent, and mobile phases. **Trail 1**: The initial HPLC method development was initiated using an Isocratic mode using mobile phase with 0.1% orthophosphoric acid and Methanol (50:50) using the Symmetry C18 column (4.6mm x 250mm, 5.0µm) with a flow rate of 1.0 mL/min. The impurity Isbobutyl Oxime peak shape was distorted. **Trail 2**: For the second trial a Isocratic mode using mobile phase with 0.1% orthophosphoric acid pH adjusted to 3.5 and Methanol (65:35) using the Zorbax XDB C18 (4.6 x 250mm, 5.0µm) with a flow rate of 1.0 mL/min. The impurity Isbobutyl Oxime peak shape was distorted. **Trail 3**: For the third trial a Isocratic mode using mobile phase with 0.1% TFA pH adjusted to 2.4 and Methanol (90:10) using the column**,** Sunfire C18 Column (250 mm x 4.6mm, 10µm). with a flow rate of 0.9 mL/min. The impurity Isbobutyl Oxime peak retention time is too long. **Trail 4**: For the fourth trial a Isocratic mode using mobile phase with 0.1% TFA pH adjusted to 2.4 and

Methanol (70:30) using the column**,** Sunfire C18 Column (250 mm x 4.6mm, 10µm). with a flow rate of 0.9 mL/min. The impurity Isbobutyl Oxime peak retention time is too long. **Trail 5**: For the fifth trial an Isocratic mode using mobile phase with 0.1% TFA pH adjusted to 2.4 and Methanol (65:35) using the column**,** Sunfire C18 Column (250 mm x 4.6mm, 10µm). with a flow rate of 0.9 mL/min. The impurity Isbobutyl Oxime peak retention time is about 6.2 minutes. The trail chromatograms are shown in **Figure 2**. **Trail 6**: For the sixth trial an Isocratic mode using mobile phase with 0.1% TFA pH adjusted to 2.4 and Methanol (55:45) using the column**,** Sunfire C18 Column (250 mm x 4.6mm, 10µm). with a flow rate of 0.9 mL/min. The analyte and DP peak shape was improved, and the plate count was above 5000. Hence the method was optimized and above conditions are considered as final method. The final optimised chromatograms of standard solution are shown in the **Figure 3.**

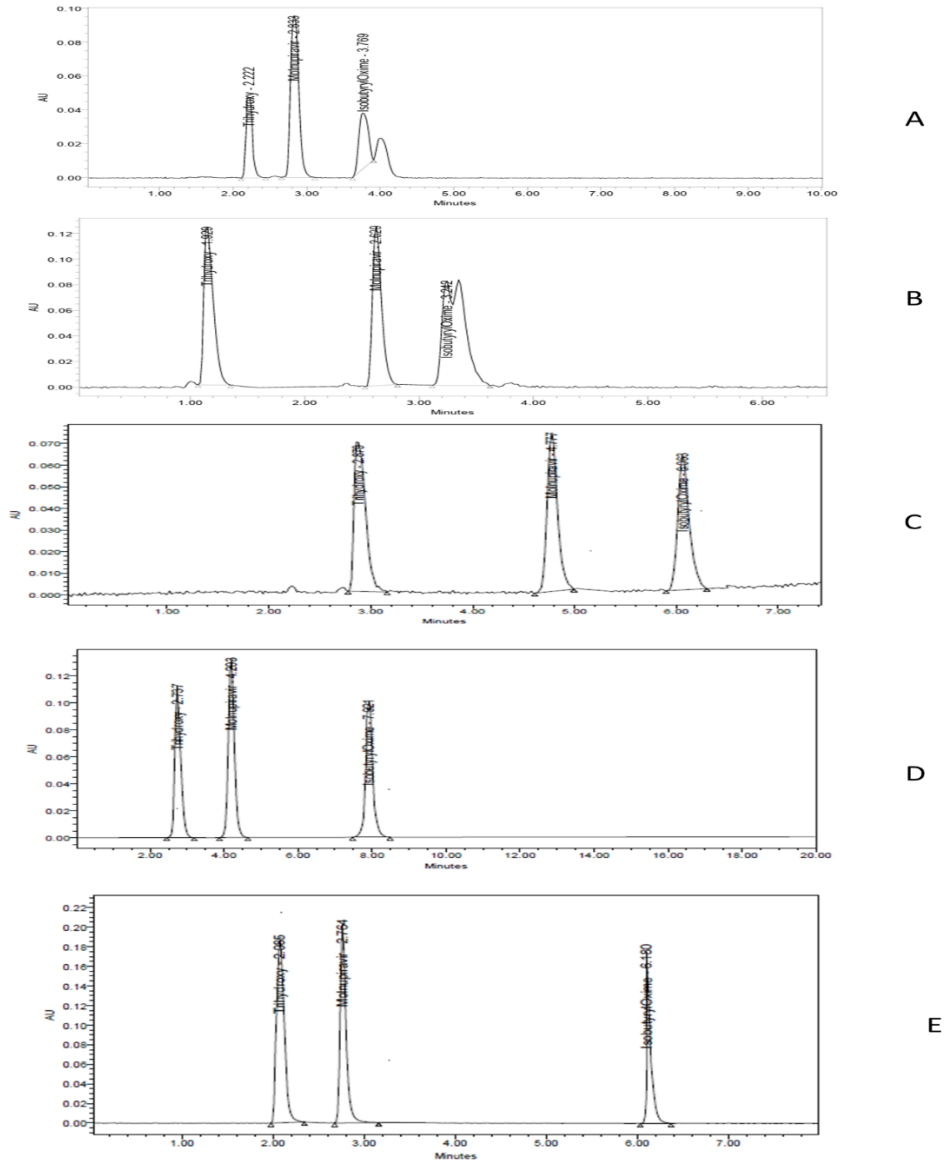

Figure 2: Representative chromatograms of Trail 1 (A), Trail 2 (B), Trail 3 (c), Trail 4 (D), Trail 5 (E),

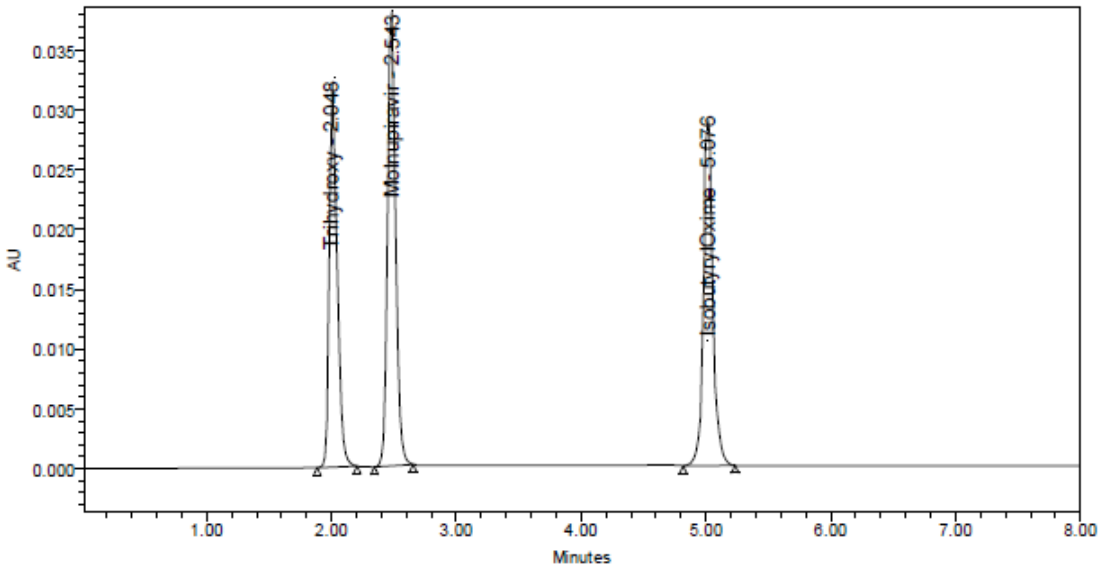

Figure 3: Representative chromatograms of standard solution (Optimized condition)

3.2. Method Validation

The analytical method validation on HPLC method was performed (in terms of System suitability, Specificity, Sensitivity, Accuracy, Precision, Linearity, Range, Robustness, and Solution stability) in accordance with ICH guidelines[23–25].

3.3. System Suitability:

It is evaluated by injecting 6 replicate injections of Molnupiravir standard solution according to the United States Pharmacopeia (USP) recommendations. The peak asymmetry, theoretical plates, and %RSD for main peak areas were calculated. The results are shown in **Table 2**.

3.4. Specificity and Forced degradation studies:

The analytical method was evaluated for the specificity by injecting the blank and as such sample prepared at the specified concentration (1000µg/mL) and Standard solution (1µg/mL). The method was found specific as there is no interference observed in blank and placebo chromatograms at the main peak and DPs retention time, The representative chromatogram of blank, placebo, unspiked and spiked sample were shown in **Figure 4**. The Mass balance, % degradation and peak purity at various degradation conditions are given for Molnupiravir in the **Table 3**. The specificity of the method is also evaluated using forced degradation studies following ICH Q1A and Q1B guideline. The sample degradation was performed as per the experimental conditions in **Table 3**.

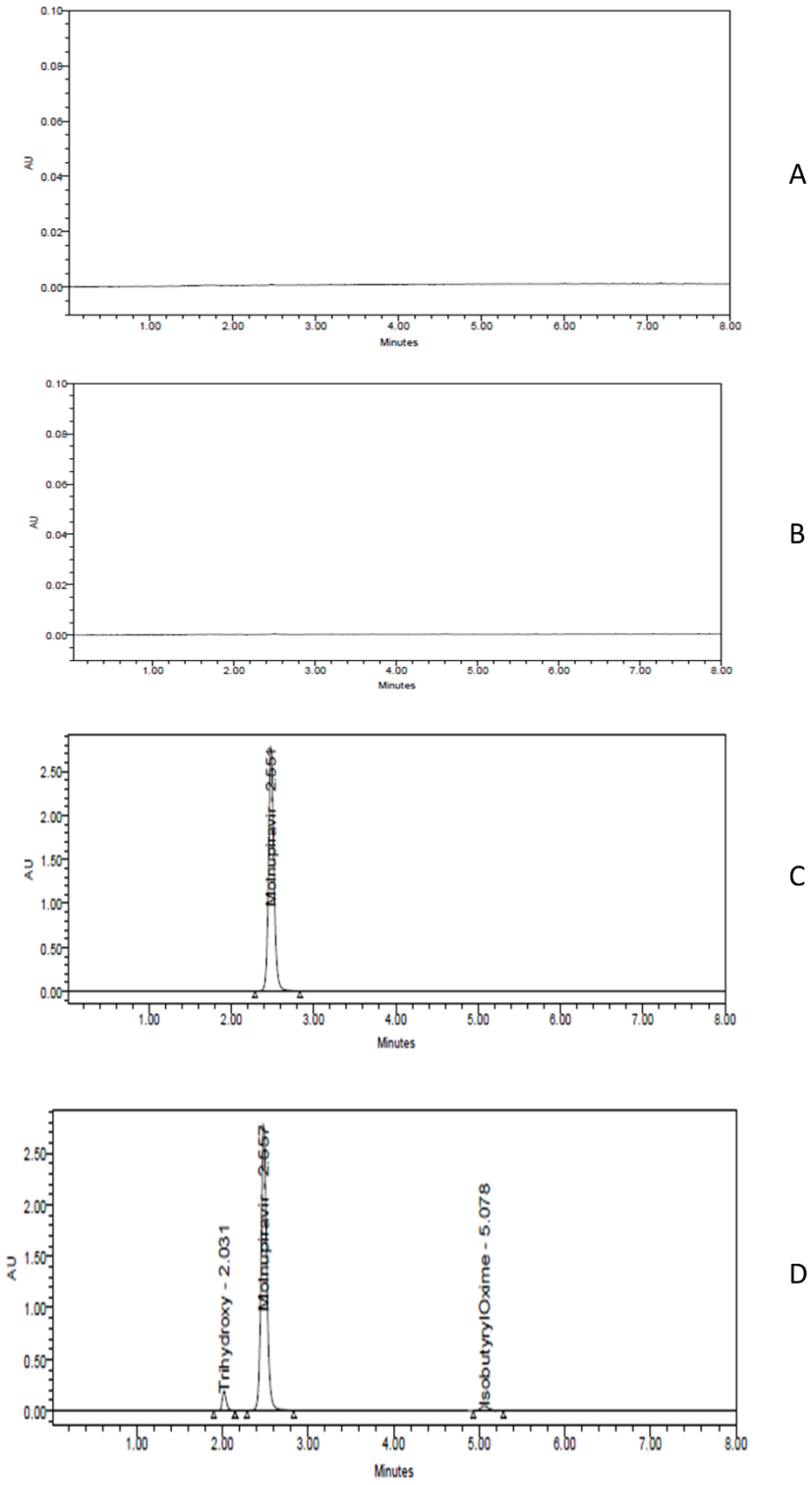

Figure 4: Representative chromatograms of Blank (A), Placebo (B), Unspiked Sample (C) and Spiked Sample (D).

3.4.1 Acidic Degradation:

The obtained chromatogram shows significant degradation under the basic condition. The representative chromatogram shown in **Figure 5**. The results of the percentage assay, percentage degradation, mass balance and peak purity of Molnupiravir are in **Table 3**.

3.4.2 Base Degradation:

The obtained chromatogram shows significant degradation under the basic condition. The representative chromatogram shown in **Figure 5**. The results of the percentage assay, percentage degradation, mass balance and peak purity of Molnupiravir are in **Table 3**.

3.4.3 Hydrolysis (Neutral):

The obtained chromatogram shows significant degradation under the hydrolytic condition. The representative chromatogram shown in **Figure 5**. The results of the percentage assay, percentage degradation, mass balance and peak purity of Molnupiravir are in **Table 3**.

3.4.4 Peroxide Degradation:

The obtained chromatogram shows significant degradation under the oxidative degradation condition. The representative chromatogram shown in **Figure 5**. The results of the percentage assay, percentage degradation, mass balance and peak purity of Molnupiravir are in **Table 3**.

3.4.5 Thermal Degradation:

The obtained chromatogram shows no significant degradation under the thermal condition. The representative chromatogram shown in **Figure 5**. The results of the percentage assay, percentage degradation, mass balance and peak purity of Molnupiravir are in **Table 3**.

3.4.6 Photo Degradation:

The obtained chromatogram shows significant degradation under the Photo degradation condition. The representative chromatogram shown in **Figure 5**. The results of the percentage assay, percentage degradation, mass balance and peak purity of Molnupiravir are in **Table 3**.

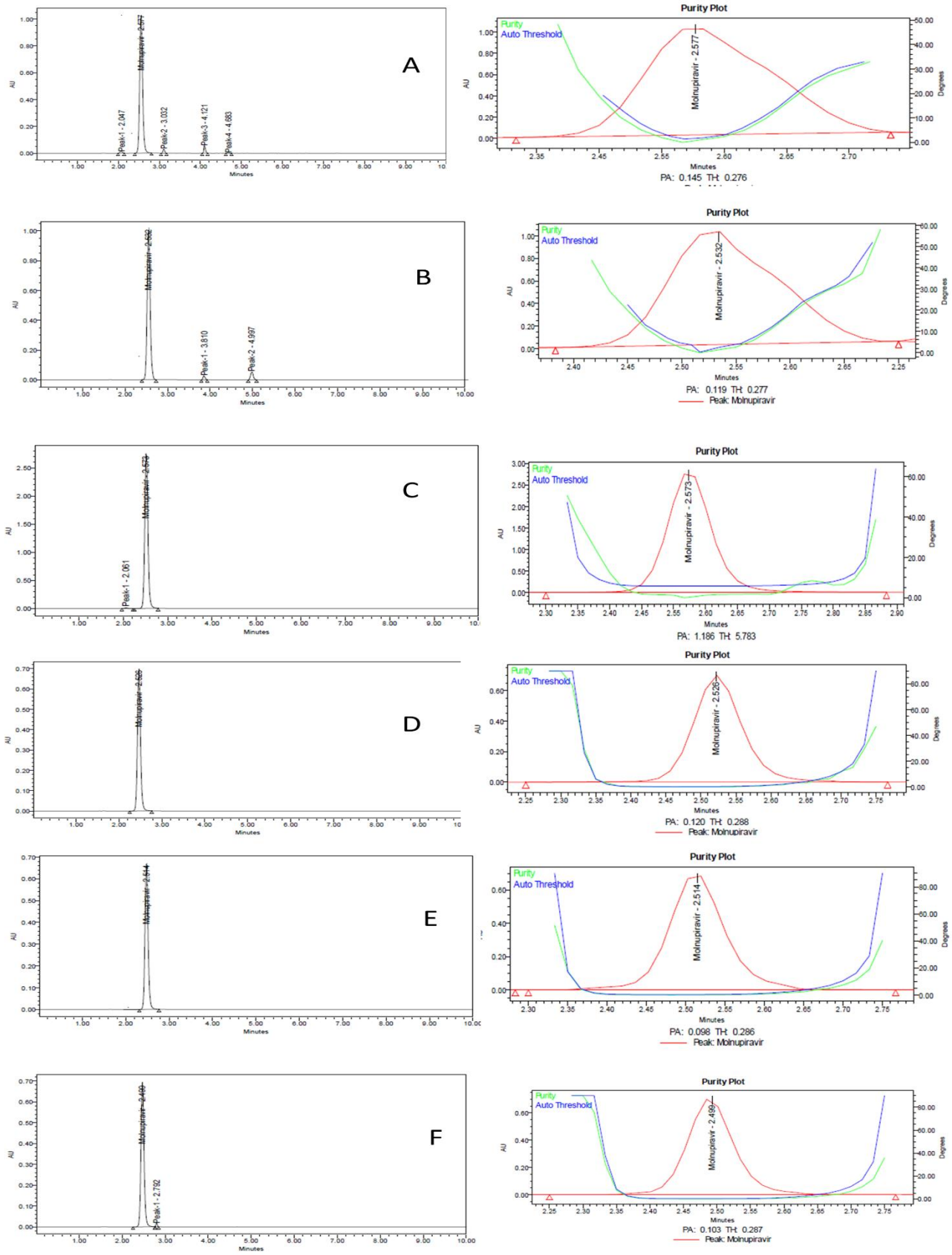

Figure 5: Representative chromatogram Acidic (A), Basic (B), Peroxide (C), Neutral (D), Thermal (E) and Photolytic (F) degradation

3.5. Linearity:

The analytical method was evaluated for the linearity by injecting the spiked standard solutions of Molnupiravir at concentrations ranging from 25 to 150% for more than 6 levels and 3 sets were prepared individually. The calibration curve was obtained by plotting a graph between the average peak areas of 3 sets and the concentrations of Molnupiravir. The obtained calibration curve showed a correlation coefficient greater than 0.9998 for Molnupiravir and the method is found to be linear**.** The results are tabulated shown in **Table 4**. The Linearity plots are represented in **Figure 6**.

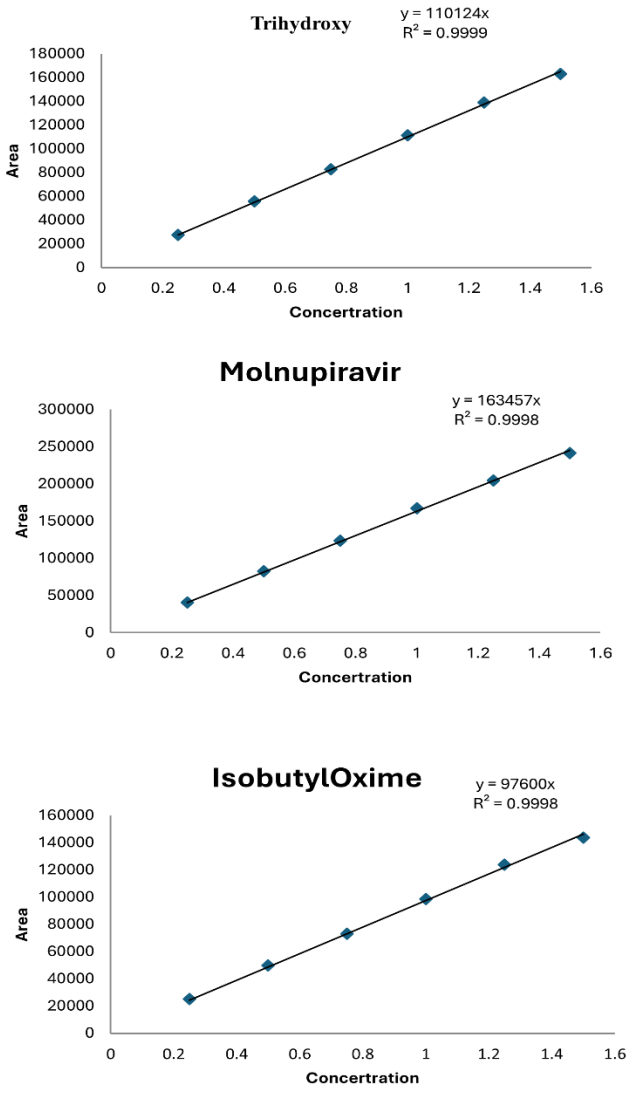

Figure 6: Representative Plots of Trihydroxy, Molnupiravir and IsobutylOxime.

3.6. DL and QL:

The DL and QL are defined as the lowest concentration of the analyte, where DL stands for Detection Limit, and QL stands for Quantification Limit. These was evaluated by using the Calibration plot. The calculated DL and QL for Molnupiravir are 0.38 ppm and 0.13 ppm respectively, the calculated DL and QL for Trihydroxy impurity are 0.01 ppm and 0.02 ppm

respectively and the calculated DL and QL for Isobutyloxime are 0.03 ppm and 0.01 ppm respectively injected into the HPLC. The precision results of QL are shown in **Table 5** . The % RSD of the peak areas of each analyte is not more than 10.0%.

3.7. Method Precision :

The analytical method was evaluated for method precision by analysing 6 different preparations of Molnupiravir spiked with the Trihydroxy and Isobutyloxime impurity at the specification level, the %RSD for impurities was calculated and the results are presented in **Table 6**. The results confirm that the method is precise for determining Molnupiravir by HPLC.

3.8. Accuracy:

The analytical method was evaluated to determine the accuracy of the method by using the standard addition method. The experiment was performed in triplicate at 50%, 100%, and 150% levels and the % recoveries were calculated. The % recovery values were in the range of 97.67 to 100.13 for Trihydroxy Impurity and % recovery values were in the range of 98.03 to 100.12 for Isobutryloxime impurity, which are within the acceptance criteria. The %RSD values of the recoveries obtained for all impurities were less than 1.0. The results are shown in **Table 7 and 8.**

3.9. Solution Stability:

The analytical method was evaluated for the solution stability of Molnupiravir, Trihydroxy Impurity and Isobutryloximie Impurity was determined by storing the samples in tightly capped volumetric flasks at 25°C and 2-8°C for 48 hrs. The % recovery of samples was calculated against freshly prepared sample solution. The results were found that Molnupiravir were stable at 2-8°C and 25°C after 48 hrs.

3.10. Robustness:

The analytical method was evaluated for the robustness by deliberate change in the experimental conditions and the system suitability data were recorded. The variables evaluated in the study were column temperature from 25°C to 35°C as Temperature Minus (TM) and Temperature Plus (TP) respectively, the Flow rate from 0.8 to 1.0 mL/min as Flow Minus (FM) and Flow Plus (FP) respectively and mobile phase organic phase change with ±10%. The results met the acceptance criteria, and the results are shown in **Table 9,10 and 11.**

4. Conclusion:

The optimized experimental and validated results confirm that the analytical method on HPLC can quantify degradation impurities, known impurities (Trihydroxy impurity and Isobutryloxime Impurity) and Molnupiravir using suitable stationary and mobile phases. The proposed analytical method was validated according to ICH Q2 guidelines. Molnupiravir is found to be susceptible to peroxide, acidic, basic degradation conditions but remained stable under thermal, photolytic and neutral forced degradation conditions. The methodology appears to be a specific, linear, accurate, precise robust, and stability-indicating method, according to the degradation and analytical validation. By employing HPLC, it is possible to quantify impurities and Molnupiravir. This method is shown to be specific and with lesser run time quantify both impurities and drug in drug product. This method is useful for the Quality Control Laboratories and the Stability Studies.

5. Acknowledgement
The author thanked the pharmaceutical company for providing the drugs, impurities and the resources for the work.

6. Conflict of Interest
The author has no conflict of interest to declare.

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Table 2: System suitability parameters and retention time results

	Trihydroxy Impurity	**Molnupiravir**	**Isobutyryl Oxime Impurity**
	112494	165134	98325
	113082	161271	99041
	114571	166412	98677
	109974	163857	98783
	112889	164540	98821
	111501	168166	98921
Average	**112419**	**164897**	**98761**
Standard Dev.	**1556.8**	**2340.7**	**247.1**
%RSD	**1.4**	**1.4**	**0.3**

Molnupiravir USP Theoretical Plate Count	6915
Molnupiravir USP Tailing Factor	1.2
Sample and impurity ID	**Retention time (min)**
Trihydroxy Impurity	2.048 min
Molnupiravir	2.543 min
Isobutryloxime Impurity	5.076 min

Table 3: Forced degradation conditions for Molnupiravir and Peak Purity data.

Sample	% Active remaining	% Total Impurities	% Total found (% w/w)	% Mass balance	Peak Purity
Unstressed sample	99.36	0.0	99.36	-	Pass
Light, solution, exposed, ($1.2X10^6$ lux hours and 200.25-watt hours/square meter of UV energy)	98.25	0.513	98.715	99.34	Pass
Hydrolytic at 60°C for 30 min	99.16	0.0	99.16	99.16	Pass
1N HCl at 60°C for 30 min.	92.1	7.26	99.579	99.58	Pass
1N NaOH at 60°C for 30 Mins.	95.6	4.68	98.877	99.48	Pass
3% Hydrogen Peroxide at 60°C for 30 min	97.5	2.41	99.156	99.42	Pass
Heat 105°C for 6 Hours.	99.14	0.0	99.14	99.14	Pass

Table 4: Linearity dilutions ranging from 25 to 150%.

%Level	Conc (ppm)	Trihydroxy Impurity	Molnupiravir	Isobutyryl Oxime Impurity
25	0.25	27702	40810	25338
50	0.50	55678	82993	50025
75	0.75	82670	123573	73063
100	1.00	111316	167413	98542
125	1.25	139065	204481	123816
150	1.50	162944	241512	143764
R^2		**0.9999**	**0.9998**	**0.9998**

Table 5: Precision at QL Level

Precision at QL			
	Trihydroxy Impurity	**Molnupiravir**	**IsobutyrylOxime Impurity**
	560	10267	518
	577	10029	512
	576	10409	512
	581	10125	517
	577	10295	505
	563	10193	510
Average	**572**	**10220**	**512**
Standard Dev.	**8.6**	**134.0**	**4.8**
%RSD	**1.5**	**1.3**	**0.9**

Table 6: Method Precision at Specification Level

Sample No.	**Sample-1**	**Sample-2**	**Sample-3**	**Sample-4**	**Sample-5**	**Sample -6**	**% RSD**
Impurity name	**% w/w**	**% w/w**	**% w/w**	**% w/w**	**% w/w**	**% w/w**	
Trihydroxy Impurity	0.09950	0.09940	0.09945	0.09931	0.09933	0.09967	**0.13**
IsobutyrylOxime Impurity	0.09968	0.09973	0.09988	0.09990	0.10008	0.09980	**0.14**

Table 7: Accuracy results for Trihydroxy Impurity

Trihydroxy Impurity						
% Spike level	**Amount found (%w/w)**	**Amount recovered (%w/w)**	**Amount added (%w/w)**	**% Recovery**	**Mean % Recovery**	**% RSD**
50	0.052	0.049	0.049	98.94	**98.87**	**0.1**
	0.052	0.049	0.049	98.91		
	0.052	0.049	0.049	98.76		
100	0.099	0.096	0.098	97.67	**99.10**	**1.3**
	0.101	0.098	0.098	100.01		
	0.101	0.098	0.098	99.62		
150	0.150	0.147	0.148	99.84	**99.02**	**1.7**
	0.146	0.143	0.148	97.08		
	0.151	0.148	0.148	100.13		

Table 8: Accuracy results for Isobutryloxime Impurity

	Isobutryloxime					
% Spike level	**Amount found (%w/w)**	**Amount recovered (%w/w)**	**Amount added (%w/w)**	**% Recovery**	**Mean % Recovery**	**% RSD**
50	0.053	0.049	0.050	98.77	**98.50**	**0.4**
	0.053	0.049	0.050	98.03		
	0.053	0.049	0.050	98.70		
100	0.103	0.099	0.100	98.69	**99.00**	**0.3**
	0.103	0.099	0.100	99.27		
	0.103	0.099	0.100	99.03		
150	0.154	0.150	0.150	100.12	**99.55**	**0.6**
	0.153	0.149	0.150	99.56		
	0.152	0.148	0.150	98.97		

Table 9: Robustness Study for Flow Variations

	FM	**FP**	**FM**	**FP**	**FM**	**FP**
	Trihydroxy		**Molnupiravir**		**Isobutyryl Oxime**	
	111904	109309	169613	168627	99744	99896
	111471	109568	169318	168673	98739	99646
	111897	108789	169322	168804	99358	99002
	111702	109724	169560	168923	99092	99442
	111089	109282	170299	168505	98543	99565
Average	**111613**	**109334**	**169622**	**168706**	**99095**	**99510**
STD.DEV	**342.1**	**356.1**	**401.5**	**161.6**	**480.1**	**329.1**
%RSD	**0.3**	**0.3**	**0.2**	**0.1**	**0.5**	**0.3**

Table 10: Robustness Study for Mobile Phase organic Variations

	MM	**MP**	**MM**	**MP**	**MM**	**MP**
	Trihydroxy		**Molnupiravir**		**Isobutyryl Oxime**	
	116573	112658	170623	169658	99690	98482
	120257	113594	170814	166838	99672	98512
	117979	116762	170091	169237	99250	98809
	119167	114169	170621	169086	98101	99283
	119816	113896	170323	169908	99473	99421
Average	**118758**	**114216**	**170494**	**168945**	**99237**	**98901**
STD.DEV	**1492.9**	**1533.1**	**285.8**	**1222.7**	**659.7**	**433.5**
%RSD	**1.3**	**1.3**	**0.2**	**0.7**	**0.7**	**0.4**

Table 11: Robustness Study for Column Temperature Variations

	TM Trihydroxy	**TP Trihydroxy**	**TM Molnupiravir**	**TP Molnupiravir**	**TM Isobutyryl Oxime**	**TP Isobutyryl Oxime**
	114301	129570	169567	167858	97773	99946
	116371	129806	169190	164418	98987	98592
	114046	130709	169784	168898	99807	99438
	117937	129951	169077	164369	97156	99046
	115762	129966	169130	168750	97383	99758
Average	**115683**	**130000**	**169350**	**166859**	**98221**	**99356**
STD.DEV	**1592.9**	**426.8**	**309.8**	**2285.3**	**1133.5**	**546.9**
%RSD	**1.4**	**0.3**	**0.2**	**1.4**	**1.2**	**0.6**