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Refining the Analysis of Impurity Profile in Favipiravir Using RP-HPLC: Method Development and Validation

P.Sathya Sowmya*1, N. Harikrishnan[2]

*Research Scholar, DR. M.G.R. Educational and Research Institute, Velappanchavadi, Chennai-600077, India.

[2]N. Harikrishnan, Principal, DR. M.G.R. Educational and Research Institute, Velappanchavadi, Chennai-600077, India.

Corrosponidng Author : Mail Id: nicpsowmi@gmail.com.

Mobile No:

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ABSTRACT

An HPLC method was innovatively devised and rigorously validated for assessing degradation impurities Favipiravir, a pivotal medication for SARS-CoV-2 treatment endorsed for emergency use across numerous nations. This method, characterized by its specificity, accuracy, linearity, robustness, simplicity, and capacity to indicate stability, effectively separated all impurities using a Symmetry Shield RP-18 column and a meticulously crafted gradient mobile phase. Chromatographic parameters were precisely optimized: flow rate set at 1.2mL/min, UV detection at 220 nm, injection volume of 10 µL, and column temperature maintained at 45°C. The linear characteristics of the method, along with its sensitivity, accuracy, and the simplicity of the mobile phase composition, indicate that it is highly suitable for quantifying impurities that are related to Favipiravir. Remarkably, validation outcomes demonstrated recoveries ranging from 99.7% to 102.7% and an exceptional linearity with r2 values between 0.9995 and 0.9998 for all identified impurities. Method precision, boasting relative standard deviations below <2%, further attested to its robustness. Systematic degradation investigations were performed under a range of biological and environmental stress conditions, highlighting the elements susceptibility to biochemical pressures. The developed method demonstrates precision and the ability to detect and quantify both known and unknown impurities effectively. The method's linear characteristics, sensitiveness, exactness, and simplicity of the mobile phase composition indicate that it is highly suitable for quantifying contaminants related to Favipiravir.

Keywords: Favipiravir related impurities, RP-HPLC, ICH guidelines, stability.

INTRODUCTION

A purine analog (Favipiravir (T-705) originally invented by Toyama Chemical located in Japan, functions as an inhibitor of RNA-dependent RNA polymerase. With its chemical name being 6-fluoro-3-hydroxyproline-2-carboxamide, it has undergone extensive clinical trials as

an antiviral agent[1]. Its efficacy against viral infections led to its licensing in several countries for treating mild to moderate cases of COVID-19. Favipiravir's (Fig No.1) mode of action to inhibit the activity of viral RNA polymerase[2] thereby impeding viral replication underscores its significance as a therapeutic option in combating viral diseases, particularly in the context of the ongoing COVID-19 pandemic[3].

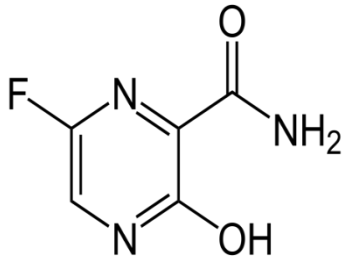

Fig 1: Structure of Favipiravir

Ensuring the validity of analytical methods is essential to guarantee accurate outcomes and enhanced reproducibility in multiple HPLC processes, which is pivotal for advancing new dosage formulations. The validation process provides evidence regarding various factors, including accuracy, precision, clarity, sensitivity, resilience, and limit quantification. According to the ICH guideline, the key aim of validating an analytical method is to prove its appropriateness for its intended use. Consequently, it has become imperative in the advancement of pharmaceutical formulations to submit validation data to regulatory bodies during the drug development registration phase (USP 40–NF 35 & ICH Guideline).[4,5].

Understanding the impurity profile plays a pivotal role in drug substance synthesis, offering vital insights into the quality, efficacy, toxicity, efficacy, and safety of the drugs. The domain of study encompasses diverse parameters, including the LODs and LOQs, along with the identification of inorganic and organic impurities linked to both bulk drugs and finished products. This information is indispensable for ensuring the integrity and regulatory compliance of pharmaceutical formulations[6].

Several chromatographic methods have been established for the quantification of favipiravir, each offering unique features and applications. Marzouk et al. (2022) introduced a HPLC-DAD method capable of determining favipiravir in the occurrence of its forced degradation products[7]. Mikhail et al. (2021) developed an HPLC method employing a C18-RP (stationary phase) and a solvent-free system (mobile phase), eluted by isocratic method[8]. In their study, Buldk (2021) employed isocratic HPLC-UV to conduct a stability assessment of favipiravir, with particular emphasis on minimizing interference from typical impurities and excipients[9]. Nadendla and Patchala (2021)[10]reported an HPLC-PDA method, to the marketed Favipiravir tablets. Abdul (2022) focused on developing and validating methods for stability indicators[11]. Forced degradation stability indicating studies were reported by Saranjit et al., (2013)[12]; Nazifa et al., (2021)[13] and Srinivasa et al., (2021)[14]. Bio-analytical method of the drug favipiravir spiked in the human plasma is reported and validated by Pallavi et al., (2021)[15] and Rezk et al., (2021)[16]. Only one of the published articles by Vemuri et al. (2023)[17] presented an HPLC method designed to detect degradation impurities in film-coated tablets of favipiravir. Additional research is required to explore how effectively an HPLC technique can precisely evaluate the correlation and degradation impurities of favipiravir. Consequently, it is essential to create a fresh method that indicates stability to accurately analyze and estimate favipiravir and its connected impurities.

EXPERIMENTAL METHODOLOGY

Acquisition of Materials: The core substances for this investigation, namely Favipiravir and its associated impurities including Heptyl, N-Methyl, Dimethyl, as well as $C_7H_7NO_5$ and Nitro diol, were sourced from MSN Laboratories Pvt. Ltd., located in Hyderabad, India. To maintain the analytical accuracy of this research, CH_3CN, KH_2PO_4, H_3PO_4 acid, HCl, NaOH, and 30% H_2O_2 of analytical reagent evaluation from Rankem, New Delhi, India. For all aqueous solutions required in the experiments, Milli-Q water was utilized, which was purified using a Millipore purification system (USA), to guarantee maximal purity.

Instrumentation and Analytical Procedures: For the instrumental analysis, this study utilized the Agilent 1200 series liquid chromatography (LC) systems, which are equipped with both diode array and variable wavelength detectors, provided by Agilent Technologies. EZChrom Elite software was utilized for both data collection and analysis. The homogeneity of the chromatographic peaks was thoroughly examined utilizing the diode array detector (DAD) of the Agilent 1200 series, ensuring the adherence to strict methodological standards. This meticulous approach to instrumentation and analytical procedure underpins the integrity and precision of the research outcomes.

METHODOLOGY FOR CHROMATOGRAPHIC ANALYSIS

Optimization of Chromatographic Conditions: Optimizing chromatographic parting involved employing a Regularity Shield RP-18 column, which measured 250 mm in length and 4.6 mm in diameter, with a particle size of 5 µm. To enhance the chromatographic process, a gradient elution technique was implemented. The first mobile phase, labelled as Mobile Phase-A, was formulated by mixing 2.72 g of KH_2PO_4 and 2.0 g of anhydrous $C_8H_{17}SO_3Na$ acid in 1000 mL of Milli-Q water to create a custom-prepared buffer solution. The pH was then adjusted to approximately 3.0 using a dilute solution of orthophosphoric acid. Mobile phase-B comprised a blend of acetonitrile and water in a 90:10 v/v ratio. I have revised the manuscript for grammatical accuracy, spelling, punctuation, and clarity while ensuring originality in the content. The High-Performance Liquid Chromatography (HPLC) gradient program was meticulously established as follows: starting at 0.01 minutes with 45% of mobile phase-B, increasing to 85% at 20 minutes, maintaining this proportion until 37 minutes, then reducing back to 45% at 37.5 minutes and maintaining through to 45 minutes. The experimentation used a flow rate of 1.2 mL/min and a queue temperature of 45°C. The detection wavelength of 220 nm was optimal, and an injection volume of 10 µL was specified. In addition, a diluent composed of acetonitrile and water in a 9:1 v/v ratio was used, while methanol was employed for needle washing to maintain precision and prevent any potential cross-contamination issues.

Chromatographic Gradient Program: The gradient system was detailed in Table No. 1, which elucidates the time-dependent modulation of the mobile phases A and B throughout the chromatographic process.

Table No. 1: Chromatographic Gradient System

Time (min)	Mobile phase-A (%)	Mobile phase-B (%)
0	55	45
20	15	85
37	15	85

37.5	55	45
45	55	45

This table presents the dynamic changes in the composition of the mobile phases over the course of the analysis, which is fundamental for achieving optimal separation of the analytes under investigation. This methodological development ensures precise and reliable chromatographic separation, which is critical for the subsequent analysis and interpretation of the data.

METHOD DEVELOPMENT AND VALIDATION

Preparation of Standard Solutions: A carefully prepared standard stock solution with a concentration of 100 µg/mL was established, integrating Impurity C_2H_6O, $C_6H_9NO_5$, $C_6H_9NO_4$, C_7H_{16}, and N-Methyl Impurity using a suitable diluent. To validate the process, distinct solutions with a level of 1 µg/mL for each impurity were accurately created by diluting the stock solution with the specified diluent.

Preparation of Sample Solutions: A precise quantity of 10 milligrams of the specimen was meticulously weighed and transferred into a 10-milliliter volumetric flask. The specimen was dissolved and then diluted with the diluent until the targeted volume was reached, leading to the attainment of the final concentration.

Method Validation: The method validation for High-Performance Liquid Chromatography (HPLC) followed the guidelines outlined by the International Council for Harmonization (ICH), particularly those detailed in document Q2(R1), which focuses on analytical procedure validation.

A) Linearity: The linearity of the method for Favipiravir and its impurities was evaluated by preparing stock solutions and diluting them to various concentrations ranging from 7.33% to 150%. These standards were chromatographed thrice under optimized conditions for 45 minutes. The data regarding mean peak areas with respect to concentration was examined through a least squares linear regression analysis in order to assess linearity within the specified range. In order to ensure the integrity of the method, the calibration curve was analyzed to determine the correlation coefficient, slope, and intercept.

B) Precision: Precision of the method was assessed by conducting intra-day and inter-day variability studies. Intra-day precision was determined by analysing the standard solution multiple times within one day, while inter-day precision was evaluated over three consecutive days.

C) LOD and LOQ: The LOD and LOQ for Favipiravir and its contaminated level were calculated using the calibration curve, applying the formulas LOD = $3.3\sigma/S$ and LOQ = $10\sigma/S$, where σ represents the standard deviation of the response and S denotes the slope of the calibration curve.

D) Accuracy: The accuracy was assessed through recovery experiments conducted at LOQ, 50%, 100%, and 125% of the analyte concentration, with the objective of achieving recoveries between 85% and 115%, indicative of the method's accuracy.

E) Robustness: Robustness was assessed by deliberately altering chromatographic conditions, including mobile grade column temperature (±5°C), pH (±0.2),), flow rate (±0.12 mL/min). The outcomes are presented as %RSD.

F) Ruggedness: Ruggedness was tested through the preparation and analysis of six separate impurity stock solutions and analysing sample solutions at 100% impurity level using two different HPLC systems and columns, under similar operational conditions, by two analysts.

G) Stress Studies: Stress studies were conducted on Favipiravir solutions under various conditions to assess the method's ability to distinguish Favipiravir from its degradation products, demonstrating the method's specificity and stability-indicating nature.

H) Relative Response Factors and Retention Times: The establishment of relative response factors (RRFs) and relative retention times (RRTs) involved the preparation of various solutions and calculating these parameters at three different concentrations to ensure consistent analytical response.

I) Solution Stability: Solution stability was assessed over 48 hours, with significant observations made regarding impurity levels over time, highlighting the importance of using freshly prepared solutions for analysis.

This comprehensive validation process ensures that the method is reliable, reproducible, and suitable for the analytical quantification and qualification of Favipiravir and its related impurities.

RESULTS AND DISCUSSION

In this extensive investigation, we formulated and validated a reverse-phase high-performance liquid chromatography (RP-HPLC) technique for measuring Favipiravir and its related impurities, adhering closely to the guidelines outlined by the International Council for Harmonization (ICH). The methodological framework was established with an emphasis on simplicity, accuracy, precision, rapidity, specificity, sensitivity, and selectivity. For the methodological foundation of RP-HPLC, a detection wavelength of 220 nm was judiciously selected. A thorough exploration of different organic modifier compositions, buffer pH level adjustments, and column oven temperature calibrations resulted in the successful chromatographic separation. This was achieved via a meticulously designed gradient program that balanced different ratios of buffer and acetonitrile. The optimized chromatographic peaks for Favipiravir and the diluent solution's chromatograms (referenced as figures 2 and 3) indicated that Favipiravir had a retention time of 10.804, with the theoretical plates numbering at 31109. The calculated tailing factor was 1.72, coupled with a signal-to-noise ratio of 63.45, which collectively reside well within established acceptance criteria.

The validation of this RP-HPLC method included assessing one-dimensionality, exactness, accuracy, LOD, LOQ, and robustness as parameters. A pivotal phase of optimization honed in on a buffer pH of 3, which notably enhanced resolution. This phase was critical for refining peak shapes and achieving distinguishable resolutions between Favipiravir, its 0.15% related impurities, and adjacent impurities. The achieved resolution values between known impurities and the Favipiravir peak exceeded the minimal requirement of 1.5, demonstrating substantial separation efficiency (referenced as figure 4). Linearity was scrutinized through an analysis of solutions across concentrations ranging from 7.33% to 150%, with the resultant linear regression values for Favipiravir-related impurities spanning 0.9995 to 0.9998. This linearity

curve underscored a direct correlation between increasing concentrations and absorbance, affirming the method's efficacy for Favipiravir and its impurities analysis (referenced as figures 5, 6, and 7).

Precision, a testament to the method's reproducibility, was validated by scanning Favipiravir-related impurities at 0.15% solutions at 220 nm, both intraday and over three distinct days. The method's precision was affirmed, with the percentage relative standard deviation (%RSD) consistently under 2%, aligning with predefined criteria (referenced as figure 8). Accuracy, gauged through recovery experiments at 50%, 100%, and 125% levels, yielded recovery percentages ranging from 99.7% to 102.7%. These low %RSD values vouch for the method's accuracy and reproducibility, with validation outcomes echoing these findings.

Robustness of the method was assessed by introducing minor modifications to the mobile phase composition, solvent system pH, and flow rate parameters. The %RSD observed for robustness fell within the acceptable range of 1.01% to 1.99%. Ruggedness validation encompassed operational system variations, column types, and analyst involvement. The method exhibited remarkable ruggedness, evidenced by minimal %RSD values, corroborating its consistency across different operational settings.

Forced degradation studies were undertaken to evaluate the method's efficacy in identifying Favipiravir's degradation products under various stress conditions. These studies also sought to delineate the drug's stability profile, facilitating preemptive formulation adjustments to mitigate potential instabilities. The peak purity analysis further validated the method's specificity, with purity angle values for stress samples falling below the purity threshold, indicating analyte peak homogeneity. Enhancing the analytical precision of the method involved establishing relative retention times (RRTs) and relative response factors (RRFs) for both Favipiravir and its impurities. Solution stability assessments were conducted over 48 hours, affirming the test sample solutions' integrity up to 24 hours, with less than 2% deviation in assay percentages, thereby underscoring the method's reliability for analytical pursuits.

This meticulous validation process not only fortifies the developed method's standing as an analytical benchmark but also underscores the significance of adopting a holistic approach to method development and validation in chromatographic studies.

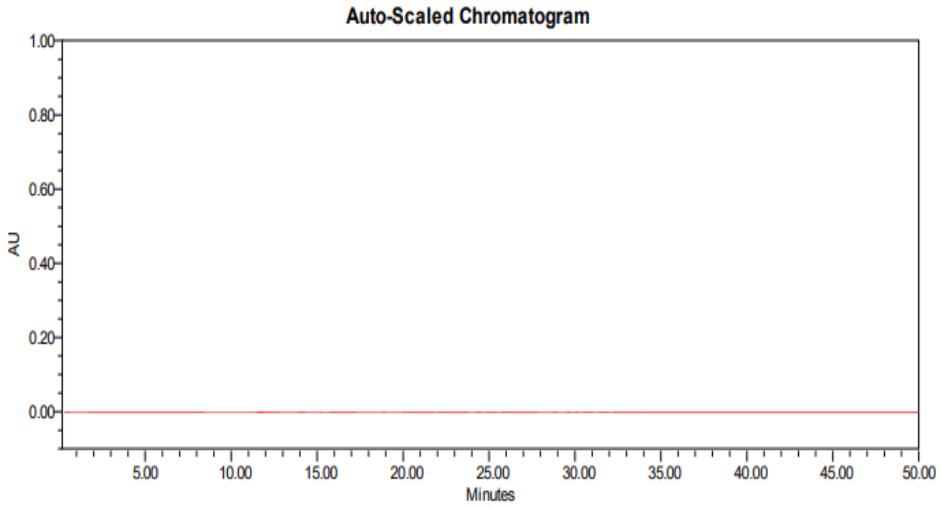

Fig 2: Chromatogram of the diluent

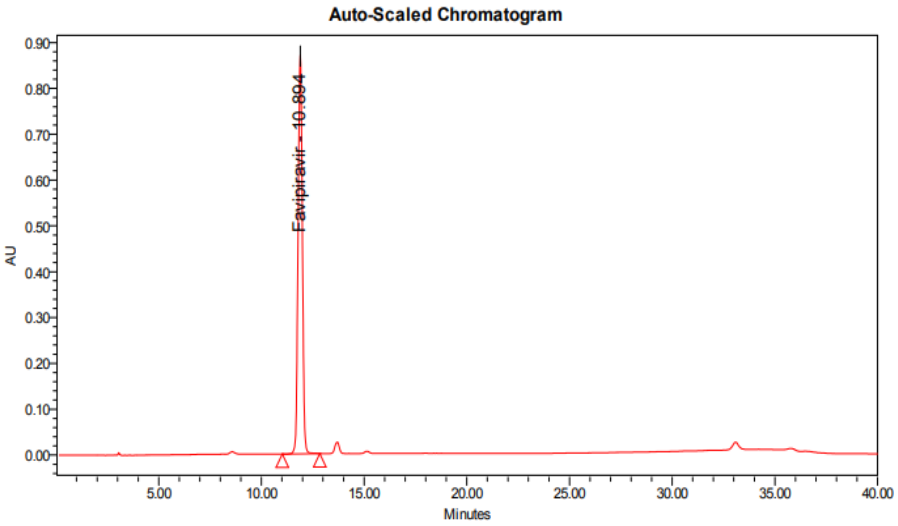

Fig 3: Chromatogram of the standard Favipiravir

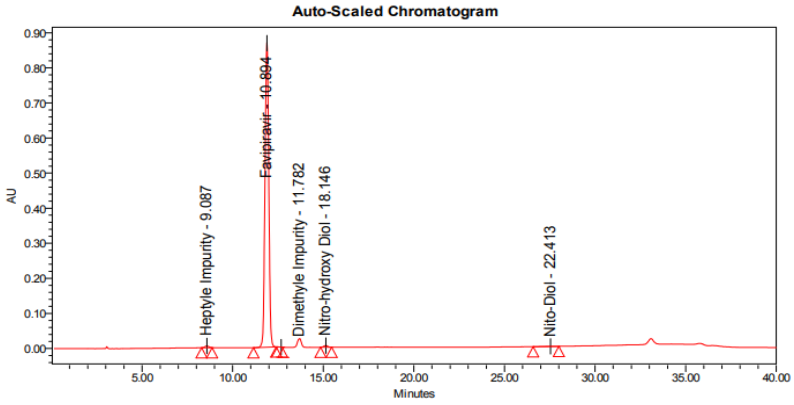

Fig 4: Chromatogram of 0.15% of impurities spiked to Favipiravir

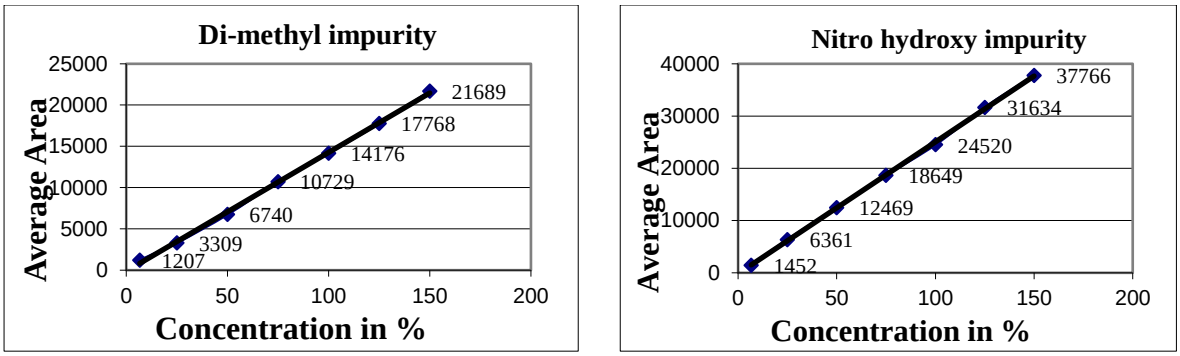

Fig 5: Linear calibration curve of Di-methyl and Nitro hydroxy impurity

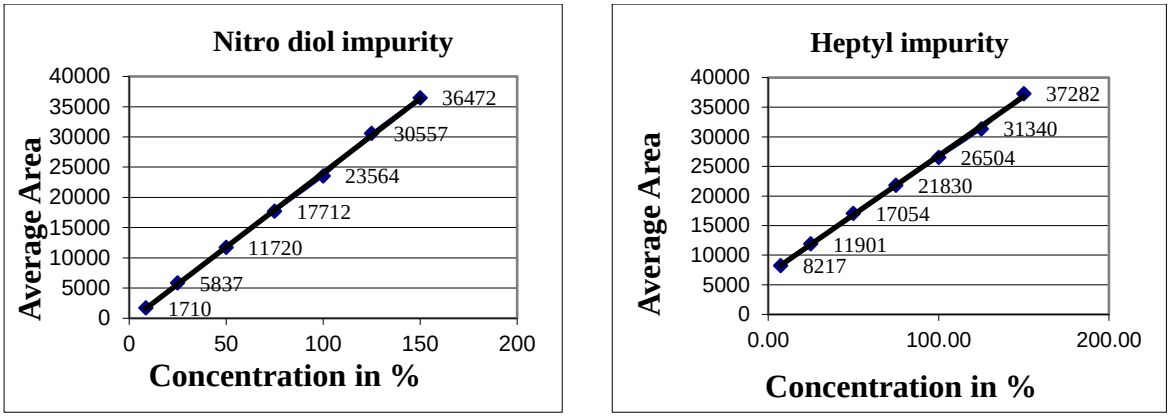

Fig 6: Linear calibration curve of Nitro diol and Heptyl impurity

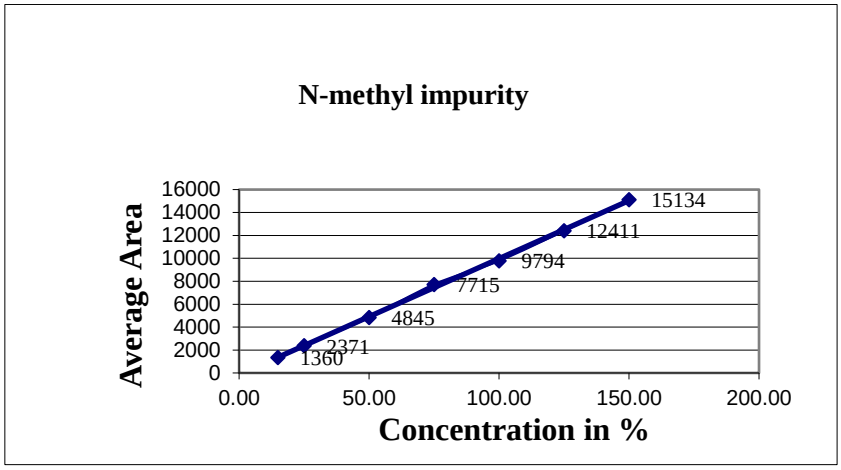

Fig 7: Linear calibration curve of N-methyl impurity

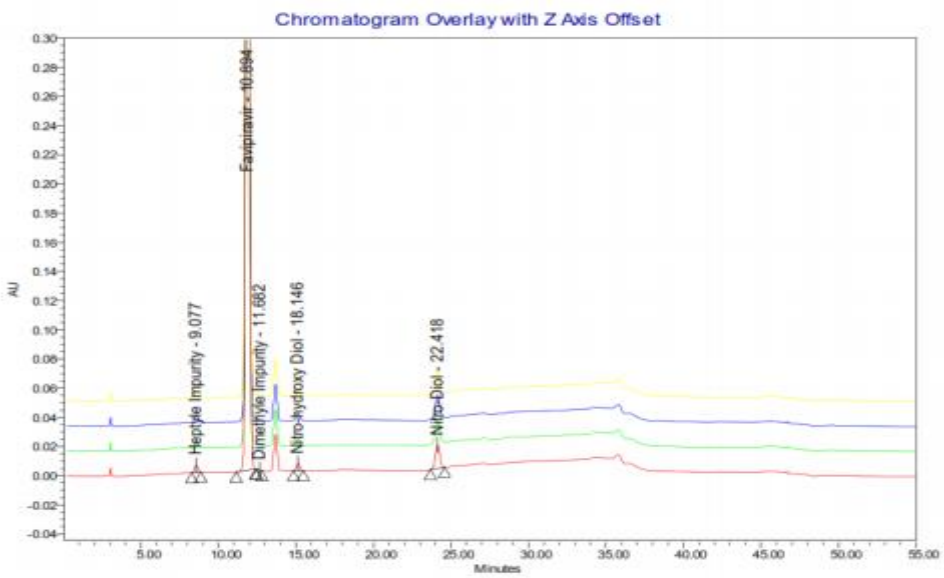

Fig 8: Overlay chromatogram of repeated injection of 0.15% Impurities spiked with Favipiravir

Table 2: Assessment of Favipiravir and Related Impurities: Validation Data

Elements	LOD %	Limit of Quantitation (LOQ %)	Accuracy (% Recovery)	Linearity	System Precision (% RSD)	Method Precision (% RSD)
Favipiravir	0.0026	0.011	Not applicable	(Kindly fill)	(Kindly fill)	(Kindly fill)
Dimethyl Impurity	0.004	0.015	At LOQ:96.8, 50%:95.7,75%:101.6, 100%:100.6, 125%:100.9,150%:102.7	Correlation coefficient:0.99984; slope: 146; y-intercept: -372.1	0.54	3.14
Nitro hydroxy diol impurity	0.0027	0.011	At LOQ:99.4, 50%:98.6,75%:98.3, 100%:96.9, 125%:100.1,150%:99.5	Correlation coefficient:0.99978; slope: 253; y-intercept: -256.8	0.64	1.70
Nitro diol impurity	0.0028	0.013	At LOQ:102.2, 50%:96.2,75%:96.9, 100%:96.7, 125%:100.3,150%:99.7	Correlation coefficient:0.99975; slope: 246; y-intercept: -507.5	0.70	1.43
Heptyl impurity	0.0024	0.010	At LOQ:105.3, 50%:103.5,75%:100.4, 100%:98.4, 125%:97.8,150%:101.0	Correlation coefficient:0.99954; slope: 200; y-intercept: 6825.3	0.97	4.83
N-methyl impurity	0.0032	0.014	At LOQ:95.8, 50%:95.1,75%:100.9, 100%:96.1, 125%:97.4,150%:99.0	Correlation coefficient:0.99959; slope: 101; y-intercept: -145.7	1.04	5.22

Table 3: Analytical Validation of Favipiravir and Associated Impurity Levels

Name of the compound	**Robustness (%RSD)**								
	Fc at 1.08 ml/min	**Fc at 1.2 ml/min**	**Fc at 1.32 ml/min**	**CT at 40ºC**	**CT at 45ºC**	**CT at 50ºC**	**pH 2.8**	**pH 3.0**	**pH 3.2**
Dimethyl Impurity	1.46	1.98	1.76	1.99	1.44	1.33	1.37	1.95	1.09
Nitro hydroxy diol impurity	1.71	1.32	1.54	1.27	1.40	1.06	1.04	1.63	1.88
Nitro diol impurity	1.05	1.04	1.22	1.53	1.20	1.45	1.40	1.42	1.70
Heptyl impurity	1.13	1.01	1.09	1.26	1.11	1.05	1.94	1.04	1.42
N-methyl impurity	1.21	1.0	1.21	1.13	1.86	1.62	1.02	1.01	1.01

Table 4: Analytical Validation of Favipiravir and its Impurity Profile

Name of the compound	Ruggedness					
	Different systems		Columns		Different Analyst	
	Agilent Technologies 1260 infinity series (%RSD)	Waters alliance 2695 (%RSD)	ARLC14012 (%RSD)	ARLC14030 (%RSD)	Analyst 1 (%RSD)	Analyst 2 (%RSD)
Dimethyl impurity	0.12%	0.14%	0.10%	0.15%	0.12%	0.14%
Nitro hydroxy diol impurity	0.12%	0.28%	0.15%	0.11%	0.14%	0.11%
Nitro diol impurity	0.15%	0.18%	0.2%	0.19%	0.17%	0.13%
Heptyl impurity	0.19%	0.23%	0.14%	0.19%	0.15%	0.12%
N-methyl impurity	0.18%	0.11%	0.17%	0.16%	0.19%	0.13%

Table 5: Peak Purity Analysis of Favipiravir under Stress Conditions

Name of the compound	Peak purity angle	Peak purity threshold
Acid decomposition	0.04	1.0
Base decomposition	0.06	1.0
Oxidation	0.02	1.0
Hydrolysis	0.08	1.0
Light decomposition	0.04	1.0
Photo degradation		
UV direct	0.03	1.0
UV indirect	0.06	1.0
LUX direct	0.08	1.0
LUX indirect	0.04	1.0
Thermal degradation	0.08	1.0

Relative Humidity	0.05	1.0

Table 6: "Relative Retention and Response Factors for Favipiravir Impurities"

Elements	RRT	RRF	RF
Dimethyl impurity	1.11	0.84	1.19
Nitro hydroxy diol impurity	1.77	0.91	1.10
Nitro diol impurity	2.20	0.98	1.02
Heptyl impurity	0.84	0.98	1.02
N-methyl impurity	1.06	0.78	1.28

Table 7: Percentage of impurities for Sample solution stability from "0" Hours to "48" Hours.

Duration in Hours	% of C_7H_{16}	% of CH_4	% of C_2H_6	% of $C_3H_7NO_5$	% of $C_3H_7NO_4$	% of Highest individual unspecified impurity	% of Total impurities
0^{th}	0.06	0.00	0.00	0.00	0.00	0.01	0.07
12^{th}	0.05	0.02	0.00	0.00	0.00	0.00	0.07
24^{th}	0.07	0.00	0.00	0.00	0.00	0.01	0.08
36^{th}	0.06	0.00	0.00	0.00	0.00	0.02	0.09
48^{th}	0.08	0.00	0.00	0.00	0.00	0.03	0.12

Table 8: Percentage of impurities for Mobile phase stability from "0" Hours to "48" Hours.

Duration in Hours	% of C_7H_{16}	% of CH_4	% of C_2H_6	% of $C_3H_7NO_5$	% of $C_3H_7NO_4$	% of Highest individual unspecified impurity	% of Total impurities
0^{th}	0.06	0.00	0.00	0.00	0.00	0.01	0.07

12th	0.05	0.00	0.00	0.00	0.00	0.01	0.06
24th	0.06	0.00	0.00	0.00	0.00	0.01	0.07
36th	0.06	0.00	0.00	0.00	0.00	0.01	0.07
48th	0.08	0.00	0.00	0.00	0.00	0.00	0.08

Conclusion

The HPLC method described in this research showcases specificity in quantifying impurities linked to Favipiravir, including Dimethyl Impurity, Nitro Hydroxy Diol Impurity, Nitro Diol Impurity, Heptyl Impurity, and N-methyl Impurity. Linear calibration curves were effectively generated for both Favipiravir and its associated impurities. The identified LOD and LOQ values meet acceptable standards. Furthermore, the %recovery outcomes obtained validate the method's reliability. The results achieved demonstrate reproducibility, affirming the appropriateness of this technique for detecting and measuring impurities in Favipiravir.

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