Original Research Article

Development and validation of UPLC-MS / MS method for obtaining favipiravir tablet dosage form and evaluation of its behavior under forced conditions

ABSTRACT

Aims: Favipiravir (FVP) is a drug developed against RNA viruses. It is a drug that is used actively in the treatment of coronavirus. In vitro and in vivo investigations have shown that it inhibits the virus. In this study, a recovery study from tablets was carried out by developing the UPLC-MS/MS method of favipiravir, which is used extensively in pandemic conditions. In addition, stability studies of favipiravir agent under forced conditions were conducted. The validated method is selective, robust, simple and applicable for tablet analysis. C18 (4.6 mm × 50 mm, 2.7 µm) column was used as the stationary phase and water-methanol (80-20 v/v) containing 0.1% formic acid was used as the mobile phase. UPLC optimization; It was conducted at a wavelength of 222 nm and a flow rate of 0.8 mL/min at 40 °C, retention time was 1.155 min. The electrospray jet stream ionization source was analyzed using mass spectrometry in negative ion mode. The molecular peak for Favipiravir was [M-1] 155.9, and the daughter ion determined 112.6. The stability test method was carried out in accordance with the ICH procedure. Reaction and degradation rates of the active substance under various forced conditions (acidic, basic, oxidative, UV light and thermal conditions) were investigated. The products formed by the decomposition of the active substance under stress conditions were determined by mass spectroscopy.

***Keywords**: Drug analysis, degradation products, ICH, stability-indicating*

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1. INTRODUCTION

Favipiravir is an antiviral drug with molecular formula $C_5H_4FN_3O_2$ (6-fluoro-3-hydroxypyrazıne-2-carboxamide) with molecular mass 157.1 and molecular shape shown in Fig. 1. [1]. Despite the fact that Favipiravir was licensed for use as a novel flu treatment in China on February 15-2020, clinical trials for COVID-19 treatment are still underway. Favipiravir is an RNA-dependent RNA polymerase (RdRp) inhibitor of a new kind. [2]. Favipiravir can inhibit the replication of flavi-, alpha-, filo-, bunya-, arena-, neuro-, and other RNA viruses in addition to its anti-influenza virus activities. [3]. There is no LC-MS/MS investigation on the determination of favipiravir from a pharmaceutical dosage form in the literature. Only one study with the HPLC method [4] and an UPLC-MS/MS study on detection from human plasma [5] are found in the literature. Determination of drug molecules and purities in commercial pharmaceutical forms is important from due to a toxicological and pharmacological point of view. Advanced methods are needed to monitor purity and related spoilage products during the preparation of pharmaceutical forms [6]. The impurity profile and stability of a drug substance are crucial in the safety assessment and manufacturing process.[7]. Stress tests are conducted to obtain information about the degradation products and degradation mechanisms of active substances under forced conditions

The stability of the active ingredient in the formulation under storage conditions is very important for the use of medicinal drugs. As a result, the active ingredient's degradation behavior is critical in determining the shelf life of a pharmaceutical product. Knowing the active ingredient's breakdown products can aid in preventing the creation of unwanted by-products that arise during storage. The impurity profile and stability of a drug substance are also important in the safety assessment and production process. Stress tests are performed to obtain information about the degradation products and degradation mechanisms of drug agents under forced conditions [8-10].

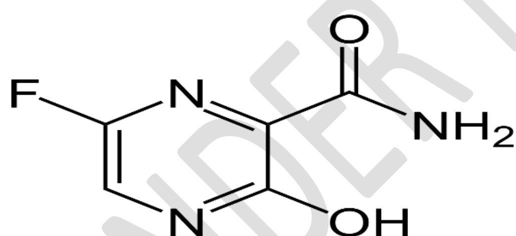

Fig. 1. Favipiravir molecule

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2. MATERİAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

2.1. Materials

Chromatographic analyzes were performed on Agilent 1200 infinity UPLC device with DAD detector, and mass spectroscopy analyzes were performed on Agilent 6460 triple quadrapole ms/ms device. Perkin Elmer Lambda-35 device was used as UV spectroscopy. Memmert brand oven and Bandelin brand ultrasonic bath were used in the analysis. In this work, analytical grade compounds were employed without further purification. Hydrochloric acid (37%, Sigma-Aldrich), sodium hydroxide (Sigma-Aldrich), hydrogene peroxide (30%, Sigma-Aldrich), formic acid (99%, Sigma-Aldrich) and HPLC-grade methanol (≥99.9%, Sigma-Aldrich) were used. Tobio Novelfarma provided the FVP active

ingredient and tablets (favira, 200 mg) which were obtained using a Milli-Q system (Millipore) with conductivity less than 18.2 S cm^{-1}. (Istanbul, Turkey).

2.2. Preparation of Standard solutions

100 mg of active ingredient was carefully weighed and dissolved in roughly 20 mL of ultrapure water before being transferred to a 100 mL measuring flask. The volume of the stock solution was increased to 100 mL using ultrapure water to obtain 1 mg mL^{-1}. The resulting stock solution was filtered through a 0.45 µm filter and sonicated. The stock solution was further diluted with deionized water before being fed into the system for analysis to obtain the needed standard solution concentration. (1-10 µg mL^{-1}).

2.3. Preparation of Sample solution

Five FVP tablets were sensitively weighed before being transferred to a dry, clean mixture and pounded into a fine dust. The average weight of five tablets was 0.280 grams. Following that, 200 mg faripiravir (70 mg) tablet powder was placed to a 100 mL volumetric flask. 100 mL deionized water was added to the flask, which was then shaken for 10 minutes to completely disperse the components. After 30 minutes of sonication, the mixture was diluted to volume with ultrapure water to obtain a 500 µg mL^{-1} solution, which was then filtered through a 0.45 µm filter.

2.4. Determination of λ_{max}

A UV spectrophotometer was used to scan a standard solution (10 g mL^{-1}) between 200 and 800 nm. (Perkin Elmer Lambda-35 UV-VIS spectrophotometer). The UV spectra of standard solution was used to calculate *λ* max.

2.5. Chromatographic conditions

On a Poroshell column, chromatographic analysis was done. 120 EC-C18 (4.6 mm × 50 mm, 2.7 µm). The mobile phase contained of methanol and H_2O with formic acid % 0.1 (20:80, v/v). Before analysis, a 0.45 µm membrane filter was used to filter and degas the mobile phase, which was then pumped at 0.8 mL min^{-1}. The column's temperature has been set to 40 °C. The run time was 2 minutes under these situations.

2.6. Mass spectroscopy conditions

The MS analysis was performed on UPLC-MS/MS instrument (Agilent 6460, USA) equipped with electrospray jet stream ionization source (AJS ESI). The data acquisition was under the control of Masshunter (Agilent, USA). First, the mass spectrometer was optimized for FVR and the daughter ion, fragmentor voltage and collusion energy were determined. Analyses were performed in positive and negative ion modes. But ionization was good in negative ion mode. Multiple reaction monitoring mode (MRM) was used to operate the MS/MS system. The typical MS parameters in negative ion mode were fragmentor voltage which was set at 80 V; cone gas flow 12 L/h; collusion energy 20 eV; capillary voltage at 3.5 kv; source temperature at 400 °C; desolvation gas flow at 11 L/min; and desolvation

UNDER PEER REVIEW

temperature at 300 °C. As a result of mass spectrometry analysis, favipiravir molecular mass peak was found to be [M-1] 155.9 and main degradation product was 112.9.

2.7. Method validation

Analytical validation of the method was performed according to the ICH procedure. Q2 (R1) criteria [11,12]. System applicability, linearity, the limit of detection (LOD), the limit of quantification (LOQ), accuracy, specificity, precision, and robustness were among the validation parameters.

2.8. System suitability

The system appropriateness parameters (RSD% for retention time, RSD% for peak area, tailing factor and plate that is theoretical) were investigated after five replicate injections of standard solution (10 µg mL^{-1}) into the UPLC-MS/MS system.

2.9. Linearity

Six standard solutions in the concentration range of 1-10 µg mL^{-1} were used to prepare the standard calibration. Each standard solution was chromatographed three times for two minutes in optimum chromatographic conditions. To test the linearity of the method, regression analysis was performed based on the peak area versus the concentration data.

2.10. Precision

To prove the precision of the method, intraday analyzes and interday analyzes on five different days were performed. Studies on the interday and on intraday were completed with five injections at three different concentrations, 1, 4, and 10 µg mL^{-1}.

2.11. Accuracy

In order to prove the accuracy of the method, a recovery study was carried out using the standard addition method. Favipiravir recovery study was carried out by adding 80%, 100%, 120% of the amount found in the pure drug in three different concentrations to the samples.

2.12. Specificity

Specificity is that the method shows an analytical response in the presence of the sample. To prove the specificity of the method, the mobile phase solution, sample solution, and standard solution were compared with the chromatogram.

2.13. LOD and LOQ

UNDER PEER REVIEW

These values were calculated using the standard deviation (s) and regression line slope (m), as shown in the equations below.

LOD = 3.3 * s/m

LOQ = 10 * s/m

2.14. Robustness

The robustness of the method was demonstrated by testing the robustness study of chromatographic conditions with minor systematic changes in settings. Among the adjustments are different mobile phase flow rates (0.1 mL min^{-1}), methanol ratio in the mobile phase (±1%), and column temperatures (±2 °C). After each alteration, a sample was injected. By comparing the modified conditions to the original chromatographic settings, the method's robustness was established.

Analysis of marketed formulations

A total of 2 mL of the drug's homogenous solution was put to a 100 mL balloon. To make a 10 μg mL^{-1} solution, fill to the mark with ultrapure water. Filtration using a 0.45 μm membrane filter was used to analyze the samples.

2.15. Solution Stability

The stability of the solutions was tested during the day. The peak area and retention time of the sample and reference solutions were measured every 8 hours within 24 hours. All solutions were kept at room temperature (25 °C) and stored in the dark.

3. RESULTS AND DISCUSSION

3.1. Determination of λmax

The spectrophotometer had to be calibrated to zero first. Scanning in the range of 200 to 800 nm was used to determine the maximum absorption wavelength of favipiravir solution (10 μg mL^{-1}). Using the UV spectrum of the standard solution, the wavelength at which favipiravir exhibited the maximum absorbance was discovered to be 222 nm Fig. 2.

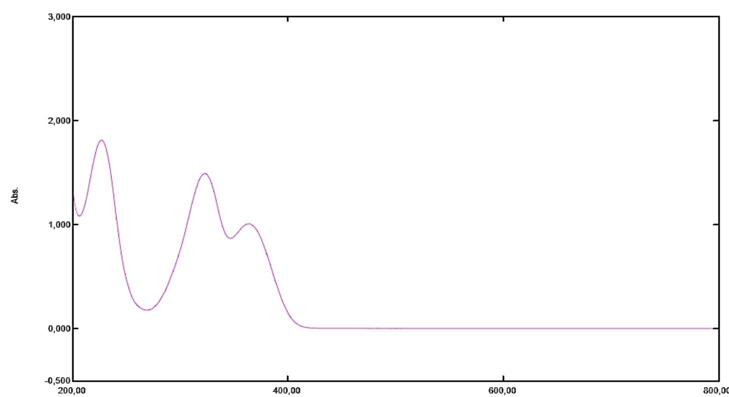

Fig. 2. UV spectrum (Standard solution, 10 μg mL^{-1})

3.2 Method Validation

UNDER PEER REVIEW

At the start of the study, different mobile phases were attempted, but none of them could fulfill the essential system parameters. Stronger theoretical plates (> 2000) and the highest tailing factor (<1.0) of the mobile phase then composed of water-methanol (80:20, v / v) containing 0.1% formic acid. The analysis was performed at 40°C, which is a cost-effective temperature with numerous benefits such as improved chromatographic peak shape, higher column efficiency, and low column pressure. The average retention time was 1.155 minutes under the specified chromatographic conditions.

3.3. Linearity

It was made by diluting FVP, with ultrapure water to obtain calibration points in the 1-10 μg mL^{-1} concentration range. Each prepared standard solution was injected three times under the specified chromatographic conditions. Linear regression analysis was used to test the linearity of the method in the UPLC-MS system in the concentration range of 1-10 μg mL^{-1}. Fig. 4 shows the calibration curve based on the concentration of the standards versus the peak area. The least squares approach was used to obtain the correlation coefficient, intersection, and slope of the regression. The relationship between the mean peak area, Y(n=3), and the concentration, X, was linear, as described by the equation Y = a + bX. As indicated in Table 1, the slope, intercept, and correlation coefficient (r) were 19.825, -7957, and 0.9999, respectively. Overlay chromatogram of FVP standard solutions (1, 2, 4, 6, 8, 10 μg mL^{-1}) was demonstrated in Fig. 3. Mass specturum was showed in Fig. 5.

Table 1. Statistical data (Calibration curve, FVP)

Parameter	**Value**
Linearity range (μg mL^{-1})	1 - 10
Slope	19.825
Intercept	-7957
Correlation coefficient	0.9999
LOD/ LOQ (μg mL^{-1})	0.30/0.80

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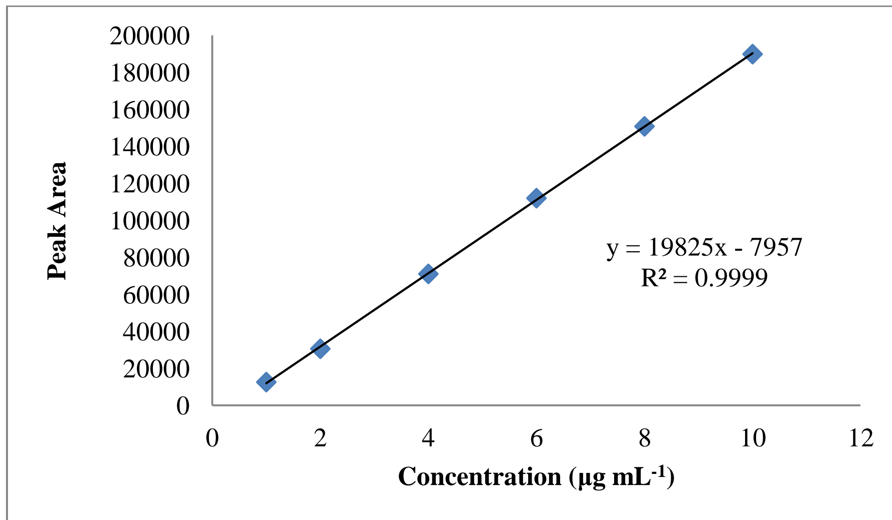

Fig. 3. Calibration curve UPLC-MS/MS system

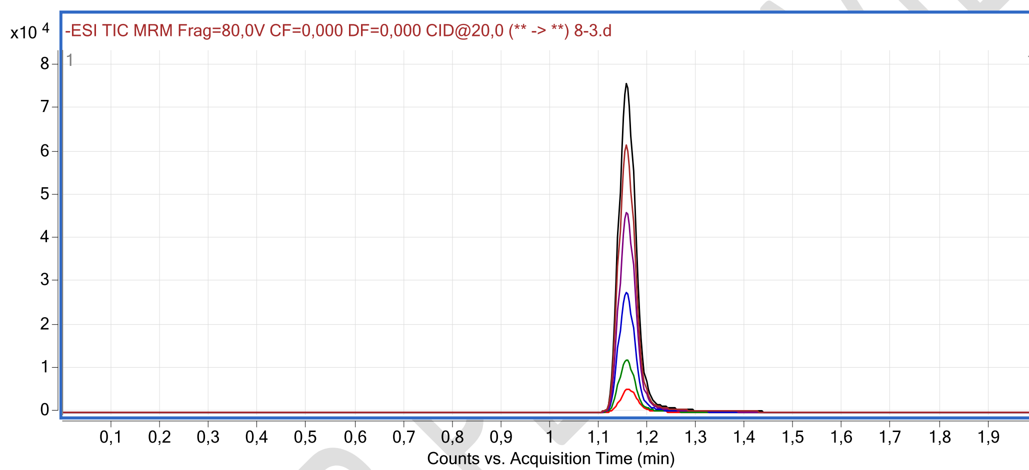

Fig. 4. Overlay chromatogram (Standard solutions, 1, 2, 4, 6, 8, 10 µg mL^{-1})

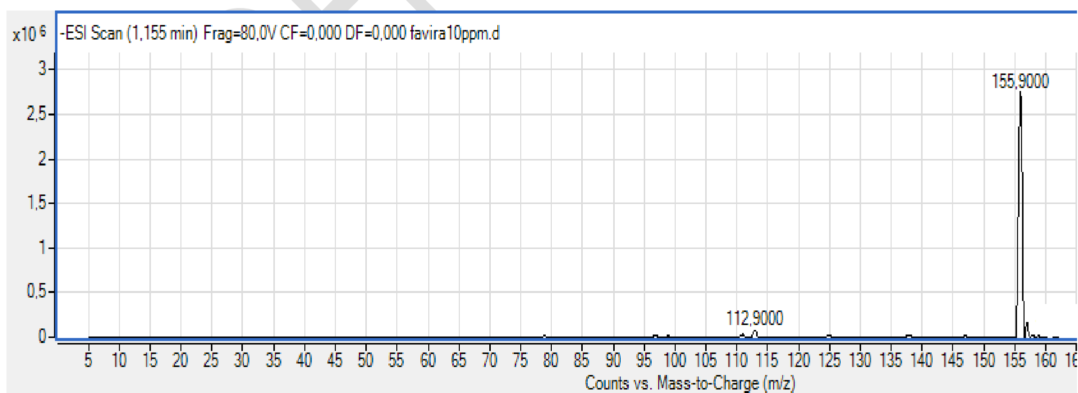

Fig. 5. Mass spectrum FVR. Molecular peak 155.9 and daughter ion 122.9

PEER REVIEW

3.4. Specificity

The method's specificity was determined by comparing the chromatograms produced for FVP standard, tablet, and blank solutions. The analysis of the chromatograms is shown in Fig. 6, 7 and 8 indicates that peaks because of excipients do not occur in pharmaceutical formulations.

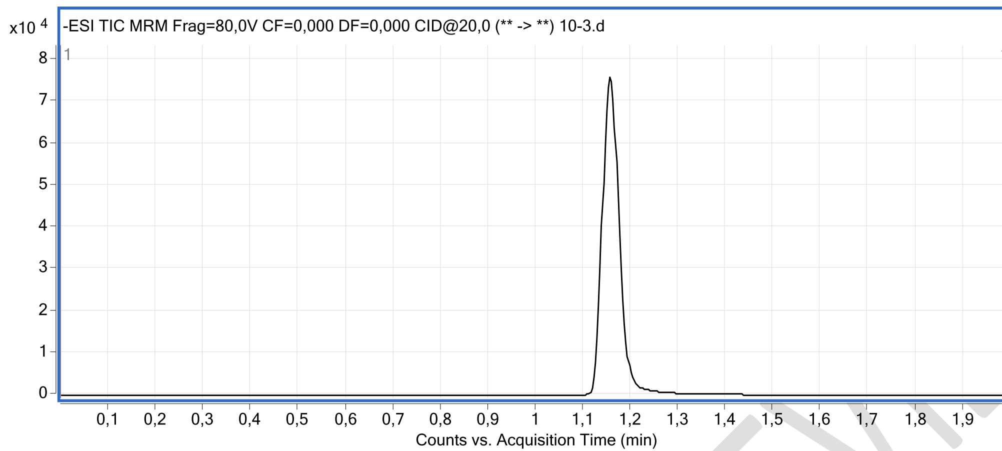

Fig. 6. Chromatogram (Standard solution, 10 µg mL^{-1})

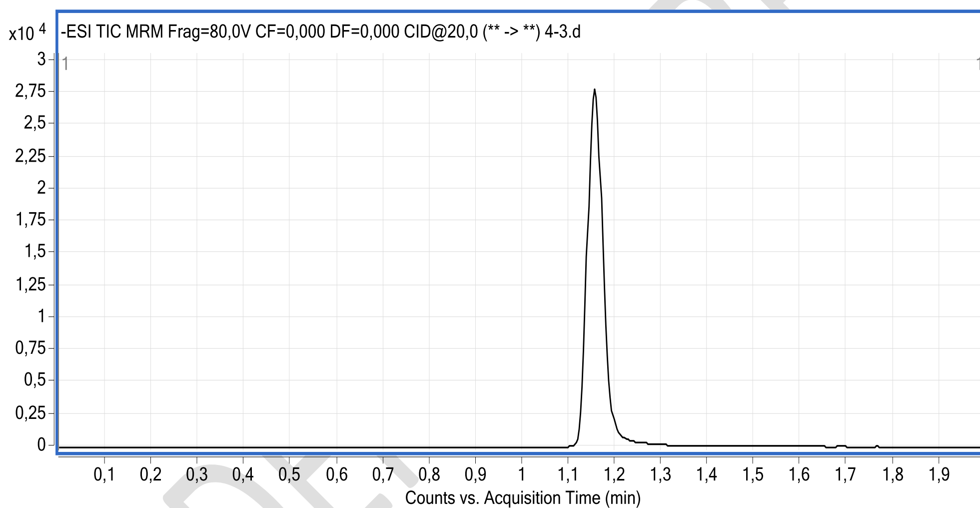

Fig. 7. Chromatogram (Sample solution, 4 µg mL^{-1}).

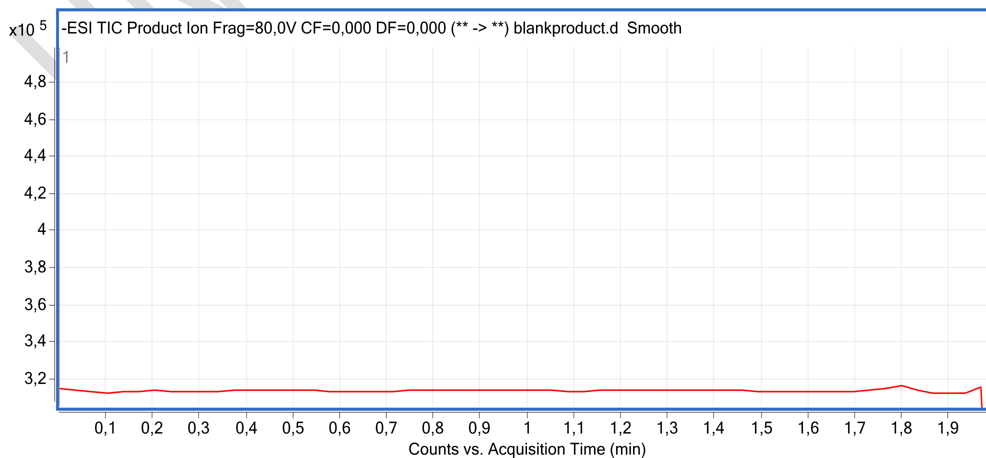

Fig. 8. Chromatogram (Blank solution)

3.5. Precision

The method's sensitivity was tested by injecting 1, 4, and 10 µg mL^{-1} doses five times on the same and five different days, with the findings shown in Table 2. At all concentrations studied, the RSD values for retention time and peak area were 0.54 and less than 2.0%, respectively. In these conditions, the approach was precise and can be utilized for the intended analysis.

Table 2. Precision data

1 ppm				
Injection No.	**Intraday Precision**		**Interday Precision**	
	Area	**% Assay**	**Area**	**% Assay**
1	12541	100,02	12595	100,45
2	12553	100,12	12602	100,51
3	12534	99,97	12517	99,83
4	12533	99,96	12593	100,44
5	12529	99,93	12475	99,50
Average	12538,0	100,00	12556	100,15
Std. Dev	9,43	0,08	57,20	0,46
% RSD	0,08	0,08	0,46	0,41

4 ppm				
Injection No.	**Intraday Precision**		**Interday Precision**	
	Area	**% Assay**	**Area**	**% Assay**
1	71070	100,01	71071	100,01
2	71028	99,95	71083	100,03
3	71052	99,98	71014	99,93
4	71109	100,06	71023	99,94
5	71066	100,00	70089	98,63
Average	71065,00	100,00	70856	99,71
Std. Dev	29,58	0,04	429,80	0,60
% RSD	0,04	0,04	0,61	0,54

UNDER PEER REVIEW

10 ppm				
Injection No.	**Intraday Precision**		**Interday Precision**	
	Area	**% Assay**	**Area**	**% Assay**
1	189843	100,02	189511	99,84
2	189873	100,03	189983	100,09
3	189822	100,01	189594	99,89
4	189873	100,03	189974	100,09
5	189644	99,91	189587	99,88
Average	189811,00	100,00	189730	99,96
Std. Dev	95,81	0,05	229,37	0,12
% RSD	0,05	0,05	0,12	0,11

3.6. Accuracy Study

A known amount of standard was added to the previously analyzed samples. (80%, 100% and 120%). The amount of recovery was calculated for favipiravir at these three concentrations. Table 3 summarizes the recovery information. In all analyses, RSD values were determined to be less than 2%. As a result, the approach is certain to be unaffected by excipients in pharmaceutical formulations.

Table 3. Recovery data

Spiked Level	**Amount added (µg mL^{-1})**	**Amount recovered (µg mL^{-1})**	**Recovery (%)**	**Average (%)**	**S.D.**	**RSD (%)**
	4	3,97	99,25			
80%	4	3,95	98,75	99,96	0,901	0,902
	4	3,90	97,5			
	5	5,01	100,2			
100%	5	4,98	99,6	99,93	0,503	0,504
	5	4,96	99,2			
	6	5,89	98,2			
120%	6	5,93	98,8	99,75	0,585	0,587
	6	5,96	99,3			

UNDER PEER REVIEW

3.7. Robustness

According to the analysis results, it was seen that the mobile phase flow rate and content had little effect on the chromatographic data of FVP. Changes in flow rate and acetonitrile content had little effect on retention time. The method did not alter significantly when the column temperature was changed. Table 4 shows the findings of this study, represented as a % RSD.

Table 4. Robustness data

Condition	**Variation**	**Assay %**	**SD**	**RSD %**
Mobile phase flow rate (0.8 mL min^{-1})	0.9 mL min^{-1}	99.86	0.60	0.60
	0.7 mL min^{-1}	99.94	0.62	0.62
Methanol ratio in mobile phase (20 %)	19 %	100.12	0.67	0.67
	21 %	99.96	0.71	0.71
Column temperature (40 ^{0}C)	38 ^{0}C	99.96	0.34	0.34
	42 ^{0}C	100.05	0.32	0.32

3.8 System Suitability

In the performance evaluation of the method, the suitability parameters of the system were evaluated. Tailing factor, retention time and theoretical plate number were found as 1.155, 5293, 1.1. These values found were at acceptable levels.

3.9. Solution Stability

The stability of the sample and reference solutions was tested during a 24-hour period. The peak area and retention time of standard and sample solutions were measured after eight hours of injection into the system. Over the course of 24 hours, no changes in standard concentrations were found. For standard solution, the % RSD for peak area (n = 3) was 0.323%, and the figure for retention time (n = 3) was 0.1150%. Table 5 summarizes the findings. In the tablet solution, there were no significant variations in active component concentration.

UNDER PEER REVIEW

Table 5. Standard solution stability (10 µg mL^{-1}).

Time, hours	Peak area	Mean	SD	RSD %	Retention time (min)	Mean	SD	RSD %
8	190583 190584 190587	190584,6	1,69	0,0009	1,155 1,156 1,158	1,156	0,0012	0,108
16	190588 190583 190586	190585,6	2,05	0,0011	1,154 1,153 1,155	1,154	0,0008	0,071
24	190582 190586 190584	190584	1,63	0,0008	1,156 1,155 1,155	1,155	0,0005	0,041

3.10. Application of the method to the marketed tablets

FVP in pharmaceutical formulations has been effectively determined using the established and verified method. Table 6 shows the results of an analysis of a marketed favipiravir tablet. The acquired results are closely connected to the amount specified on the pill labels. This demonstrates the utility of the content evaluation method.

Table 6. Method application results

Formulation	Label claim (mg)	Amount of drug (mg)	% Assay ± SD
Favira tablet	200 mg	200.35 mg	100.18 ± 2.05

3.11. Degredation study

Degradation products study was carried out in accordance with ICH guidelines. The degradation products study was evaluated applying soft and hard conditions. Degradation studies; It was carried out under acidic, basic, oxidative, UV light and thermal conditions. While performing the decomposition procedure, from the stock solution to 10 ppm; It was diluted with 0.1-1 N HCl, 0.1-1 N NaOH, 3% -30% H_2O_2 solutions. In UV and thermal studies, dilution to 10 pmm was made with ultrapure water. In acidic, basic and oxidative studies, the solution was kept in an oven at 80 °C for 30 minutes. UV study was carried out at 254 nm wavelength for 6 and 24 hours at room temperature. Thermal decomposition was completed by waiting in the oven at 100 °C for 6 and 24 hours. The results of all degradation products are shown in Table 7. As can be seen clearly in the Fig. 9. of the study results;

UNDER PEER REVIEW

While favipiravir is unstable under basic, acidic and oxidative conditions, it has managed to remain stable under other conditions.

Table 7. Degradation products under stressed conditions

Forced Conditions	Degradation Products m/z
0.1 N HCl	125,0-112,9
1 N HCl	125,0-110,9-96,9-78,9
0.1 N NaOH	146,9-124,8-112,9-93,0-78,9
1 N NaOH	146,9-125,0-112,9-102,9-79,0-45,0
3% H_2O_2	130,9-112,9
30% H_2O_2	131,0-124,9-112,9-96,9-86,9
6 HOUR UV	124,9-112,9-96,9
24 HOUR UV	125,0-113,0-96,9
6 HOUR THERMAL	113,0
24 HOUR THERMAL	113,0

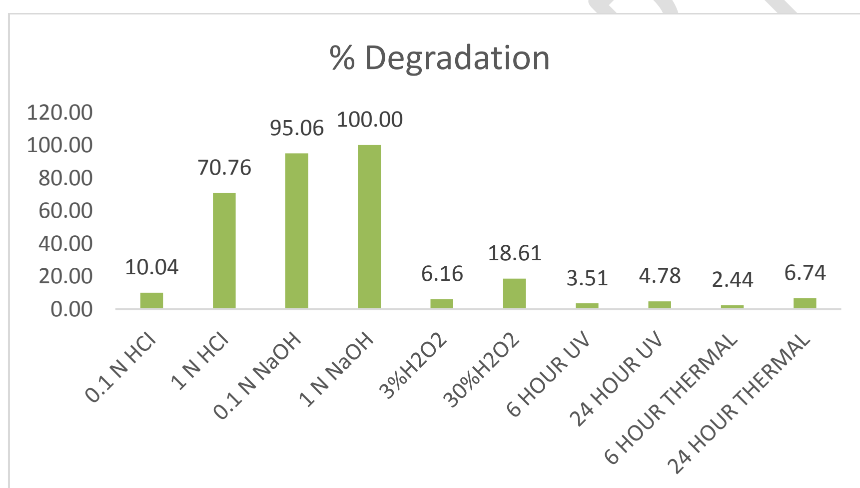

Fig. 9. Stability profiles under forced conditions

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4. CONCLUSION

The developed UPLC method is a fast, reliable and robust method for medical and chemical use. It differs from other studies in the literature with its speed and simplicity. Decomposition studies are important for chemical studies, drug production techniques and processes as well as for advanced studies, as they show under which conditions the active substance remains stable and decomposes.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

UNDER PEER REVIEW

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