Volume 3, Issue 3, 2024, Page 817-833

International Journal of Pharma Research and Technology

Development and Validation of Stability Indicating High-Performance Thin-Layer Chromatography Method for Estimation of Favipiravir by Quality by Design Approach

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Abstract

The objective of this study was to apply Quality by Design (QBD) concept for development and validation of stability indicating high-performance thin-layer chromatography (HPTLC) method for estimation of Favipiravir in bulk and marketed tablet formulations. Box-Behnken design was applied to optimize chromatographic conditions and to determine their effect on retardation factor (Rf) value. The developed chromatographic method was validated for linearity, specificity, range, accuracy, LOD and LOQ, precision and robustness as per ICH guidelines. This study was carried out by using the mobile phase containing toluene-ethyl acetate-methanol-formic acid in the ratio of (7:2:1:0.5 v/v). Scanning and densitometric analysis were done at 240 nm. The densitogram of favipiravir was obtained at Rf value of 0.49±0.02. The correlation coefficient was found to be 0.9913 in the concentration range of 2000-12000 ng/band. The percentage recovery was found to be between 98.37 – 100.20%. The LOD and LOQ were found be 20.50 ng/band and 62.13 ng/band respectively. For stability study, favipiravir was subjected to acid, base, oxidation, heat and photo-degradation studies. The developed method has advantages in being robust and able to determine the model drugs and degradation products with sensitivity, selectivity using simple mobile phase.

Keywords: Favipiravir, HPTLC, Quality by Design, Box-Behnken design, Stability indicating method, Validation.

Introduction

In the recent times 2019, a new pandemic of coronavirus disease (COVID-19) has spread across the world. The Chinese government alerted the WHO by the anonymous cause of pneumonia cases. These cases later identified as covid-19 with SARS COV-2 as the causative agent [1] Coronavirus infection was labelled as pandemic by the WHO in March 2020. Nearly 229,508,037 cases have been diagnosed as of March 1, 2022, and 4,707,821 people had died as a result of the pandemic. The worldwide pandemic has prompted researchers to focus their efforts on producing treatments or vaccines to slow or stop the spread of the disease [2]

Due to a lack of appropriate medical therapy and vaccines, COVID-19 has become a global health emergency. COVID-19 is treated or prevented using a variety of therapeutic and preventative techniques, including oxygen therapy, breathing support, convalescent plasma therapy, cell therapy, vaccinations, and medicines [3] World-wide up to date the categories of coronavirus vaccine includes vector vaccines, nucleic acid vaccines, subunit vaccines, live virus and inactivated virus vaccines. In this global health emergency some of the licensed vaccines played major role that are sputnik-v (Russia), Moderna (USA), Coronavac (China), Janssen Covid -19 (USA), Covaxin (India) [4] For the treatment of covid -19 many drugs have been used by the worldwide like Hydroxychloroquine, Remdesivir, Tocilizumab, Lopinavir, Ritonavir, etc [5]

Favipiravir (FAV) has recently been listed in many countries management guidelines as a progressing candidate in the treatment of covid-19. Firstly FAV was developed and licensed as anti-influenza agent by Japan [6] its chemical nomenclature is 6-fluro-3-hydroxypyrazine-2-carboxamide shown in Figure No 1. It works as an antiviral agent by selectively inhibiting the viral RNA dependent RNA polymerase (RdRp) enzyme, which is required for viral genome transcription and replication [7-8]

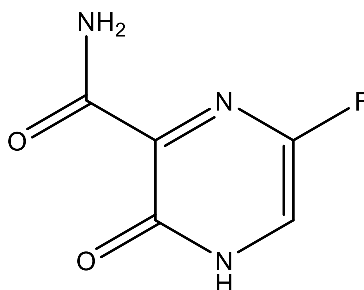

Figure No. 1: **The chemical structure of favipiravir**

According to a literature review reveals that variety of analytical methods employed for estimation of Favipiravir either alone or in combination with other drugs by using UV spectrophotometric method, HPLC and RP-HPLC method with UV, DAD and PDA detector, LC-MS/MS, UPLC etc. has been reported.[9-12] However, no High performance thin layer chromatography technique using AQBD approach to estimation based on favipiravir in active pharmaceutical ingredient and its medicinal dosage forms was yet reported in the literature. The present study describes a simple, sensitive, robust, effective and economical analytical technique for determination of favipiravir in active pharmaceutical ingredient and its medicinal forms using AQbD approach. Based on ICH standards, the proposed method was developed and validated.

Experimental

Instrumentation

The instruments used for laboratory analysis were an HPTLC system (CAMAG, Muttenz, Switzerland) with Linomat 5 semiautomatic applier, Micro syringe (100µl capacity,

Hamilton), TLC Scanner III of CAMAG, twin-trough developing chamber (10 cm × 10 cm), an ultraviolet cabinet with dual wavelength UV lamps, winCATS version 1.4.0 software, electronic analytical balance (Shimadzu AUX-220; Kyoto, Japan). Design expert version 13.0.5.0 64-bit software and Microsoft Excel were used for data analysis.

Chemicals and Reagents

Favipiravir standard was procured from Taj Mahal Vision Chemicals Pvt, Ltd. (Malad, Mumbai, India). Commercially available tablets Fabiflu® 200mg (Glenmark pharmaceuticals) were purchased from the local pharmacy. Aluminium plates Pre-coated silica gel 60 F_{254} (10cm × 10cm) with 0.25 mm thickness procured from Merck, Germany. Solvents of Analytical reagent (AR) like Methanol and Toluene are procured from Loba Chemie Pvt. Ltd (Mumbai, India), Ethyl acetate from Research- Lab Fine Chem. Industries (Mumbai, India), Formic acid from Sisco Research Laboratories Pvt. Ltd. (New Mumbai India). Hydrogen chloride, Hydrogen peroxide and Sodium Hydroxide were purchased from Merck Pharmaceuticals (India).

Preparation of Solutions

Preparation of Standard Working Solution

The bulk powder of FAV was weighed exactly about 200mg and added to 10 ml volumetric flask. It was mixed in small volume of methanol then sonicated for 15 minutes at room temperature to dissolve. Further methanol was used to get final volume up to the required level. An aliquot of 1 ml of above mentioned solution was added into 10 ml volumetric flask then mixed up to the mark with methanol to get the desired concentration of 2000 μg/ml.

Preparation of Sample Solution

Twenty Favipiravir (Fabi-flu®) medications have been carefully weighed and crushed in a clean and dry mortar. After that, the tablet powdered equivalent to 200 mg of FAV was then exactly weighed then added to 10 ml volumetric flask. It was mixed in small volume of methanol then sonicated for 15 minutes at room temperature to dissolve. The final volume was brought up to required level by using methanol. An aliquot of 1 ml of above mentioned solution was added into 10 ml volumetric flask then mixed up to the mark with methanol to get the desired concentration of 2000 μg/ml.

Preliminary Trials

Various preliminary trials were carried out for identification of effective mobile phase for development of HPTLC method. Solvents of Analytical reagent (AR) like methanol, toluene, ethyl acetate, ammonia, chloroform, formic acid were found potential for better resolution of chromatogram.

Optimization of chromatographic conditions

The CAMAG Linomat 5 applier and micro-syringe was used for spotting standard drug samples in a pattern of bands width of 6 mm on aluminum plates pre-coated with silica gel 60 F_{254} (10cm × 10cm). For linear ascending development of TLC plates, a twin trough glass chamber was used. The mobile phase utilized for separation was made up of toluene-ethyl acetate-methanol-formic acid in

the proportion of (7:2:1:0.5 v/v/v/v). The prepared mobile phase was maintained for 15 minutes at room temperature (25 ± 2ºC) for optimal chamber saturation. The chromatogram was developed up to 80 mm height and dried with the help of air dryer.

Selection of Wavelength for Detection

The working standard solution of FAV was spotted about 3 µL on pre-coated plates with the aid of linomat applicator. The chromatogram was developed in twin trough chamber that contained mobile phase and dried using dryer. For wavelength detection, the produced chromatograms were scanned in the 200-800 range.

AQbD Approach for Method Development

Box-Behnken Design (BBD) for Response Surface Modeling

In this study, the chromatographic conditions of the HPTLC method were optimized using a Box-Behnken design (BBD). Using design expert software, the BBD was used for response surface modeling to find out the correlation between critical method variables and retardation factor. The critical method parameters (CMP's) were selected as band length, saturation time and distance of solvent front to evaluate main effect, quadratic effect and interaction effect on retardation factor (Rf) [13].

Experimental Design for Method development

The method performance was optimized based on CMP's utilizing a three-factor Box Behnken design (BBD) at three levels, namely low (-1), intermediate (0) and high (+1). The design matrix as per the BBD which suggests 17 experimental runs shown in Table No 1. All experimental runs were performed in laboratory using standard concentration 6000 ng/band and resolution for each run was recorded. The relationship between critical method attributes and variables was investigated by entering all response variables against their corresponding experimental runs. The response surface model was optimized and validated for the development of design space and control strategy of robust HPTLC method [14-15].

Method Validation

The developed method was validated according to the Q2 (R1) regulations of the International Council for Harmonization (ICH). The validation parameters utilized were linearity, specificity, range, accuracy, precision, LOD and LOQ, robustness, etc. [16-19]

Linearity

The linearity of the developed method for the estimation of FAV was assessed using various concentration of the standard stock solution ranging from 2000-12000 ng/band. It were spotted on the TLC plate, developed in the twin trough chamber containing mobile phase, dried using dryer and analyzed at 240 nm as described under chromatographic conditions. The peak area versus respective concentration was plotted to establish the calibration curve of FAV.

Table No. 1: List of experimental runs and results for response surface analysis by BBD

		Factor 1	Factor 2	Factor 3	Response
Std	Run	Band length (A)	Saturation time (B)	Solvent front (C)	R_f (FAV)
		(mm)	(min)	(mm)	
12	1	6	20	90	0.48
6	2	8	15	70	0.42
7	3	4	15	90	0.43
4	4	8	20	80	0.46
8	5	8	15	90	0.47
11	6	6	10	90	0.41
2	7	8	10	80	0.40
5	8	4	15	70	0.34
3	9	4	20	80	0.45
9	10	6	10	70	0.33
10	11	6	20	70	0.44
16	12	6	15	80	0.49
13	13	6	15	80	0.49
17	14	6	15	80	0.49
15	15	6	15	80	0.49
14	16	6	15	80	0.49
1	17	4	10	80	0.35

Precision

Precision of the developed method was achieved by conducting repeatability and intermediate precision study. Six replicates of a sample containing 6000 ng/band of FAV was used to apply the sample and peak area was evaluated. Three replicates of standard solution were applied and analyzed at three time interval on the same day for intra-day precision, and three replicates of standard solution were applied and analyzed on three subsequent days for inter-day precision. The results of precision were calculated in terms of %RSD.

Accuracy

The accuracy (% recovery) of the method was obtained by calculating recovery of FAV by spiking 80%, 100%, and 120% of standard concentration to sample as per the ICH guidelines. For that, the standard FAV was spiked as 4800, 6000 and 7200 ng/band with the dosage form that contained 6000 ng/band of FAV. The results of accuracy were calculated in terms of % recovery and %RSD.

LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) of developed method of FAV were determined from the standard deviation of the y-intercept and slope of

calibration curves, using formulae as given below:

$$LOD = 3.3\ \sigma/S$$

$$LOQ = 10\ \sigma/S$$

Where, σ is the standard deviation of the response and *S* is the slope of the calibration curve.

Specificity

The developed method was assessed for specificity by analyzing standard drug and sample at the same time. The presence of FAV in the sample was confirmed by comparing the chromatograms retention factor with the standard.

Robustness

The robustness of the new method was assessed by making small deliberate changes in method parameters using 6000 ng/band standard solution. The method parameters utilized are mobile phase composition, mobile phase volume, saturation time, spotting to development time, development to scanning time. The effect of various parameters on retention factor and peak area was evaluated by calculating %RSD.

Forced degradation study

Acid degradation study

Acid degradation of FAV was performed by taking 200 mg of drug in 10 ml volumetric flask containing 3 ml 0.1 N HCL and maintained with temperature 37±2°C for 30 min. Further the volume was made up to the mark using methanol and HPTLC analysis was carried out.

Base degradation study

Base degradation of FAV was performed by taking 200 mg of drug in 10 ml volumetric flask containing 3 ml 0.1 N NaoH and maintained with temperature 37±2°C for 30 min. Further methanol was used to get final volume up to the required level and analyzed by HPTLC method.

Oxidative degradation study

Oxidative degradation of FAV was carried out by taking 200 mg of drug in 10 ml volumetric flask containing 3 ml 3% H_2O_2 and maintained with temperature 37±2°C for 30 min. Further methanol was used to get final volume up to the required level and analyzed by HPTLC method.

Heat degradation study

Heat degradation of FAV was performed by directly placing accurately weighed 200 mg of standard powder drug in an oven at 60°C for 30 min. Further the exposed drug was diluted in methanol to prepare appropriate dilution and HPTLC analysis was carried out.

Photolytic degradation study

Photolytic degradation of FAV was performed by directly placing accurately weighed 200 mg of standard powder drug in petri plate and exposed to UV light in UV chamber for 24 hrs. Further the exposed drug was diluted by using methanol to get final volume up to the required level and analyzed by HPTLC method.

Results and discussion

Preliminary method development studies

Based on literature reviewed, preliminary investigations were performed in order to develop the HPTLC method for estimation of favipiravir. Various combination of mobile phases ranging from non-polar to polar solvents were used namely methanol, ethyl acetate, toluene, chloroform, formic acid, n-butanol, acetic acid, etc. Mixture of toluene-ethyl acetate-methanol-formic acid in the proportion of (7:2:1:0.5 v/v/v/v) was selected for initial with better Rf values and maximum sensitivity. The use of formic acid was confirmed to be essential to obtain sharp peaks. Finally, saturation time of 15 min, development distance of 80 mm, band width of 6 mm and volume of mobile phase 10 ml was used for the HPTLC technique development. The retention factor of FAV was obtained to be 0.49 ± 0.02 respectively shown in Figure No 2.

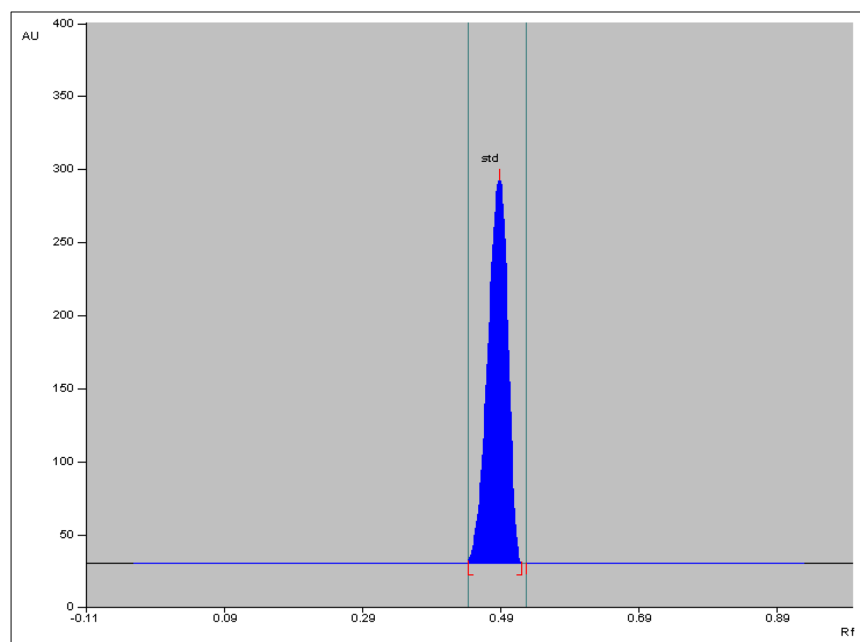

Figure No. 2: Densitogram showing retention factor of favipiravir

Selection of Wavelength for detection

The spots of standard FAV and FAV degradation products were scanned in the range of 200-800 nm using a CAMAG TLC Scanner III. The highest absorbance of standard FAV and degradation products in the UV spectrum was 240 nm. As a result, 240 nm was chosen as the measurement wavelength for FAV estimation.

Response surface modelling by BBD

For the development of a robust HPTLC method, the Box-Behnken design was employed for response surface modelling to identify relationship between independent variables and resolution. The design expert software (trial version) used gives seventeen experimental runs for BBD shown in Table No 1. All the experimental runs were performed in lab and the obtained result were

entered against the respective run for response surface assessment. The model F-value in the ANOVA table was 209.99 indicating that the linear model is significant shown in Table No **2.** The model terms with P-values less than 0.0500 are considered significant. In this case A, B, C, AB, AC, BC, A^2, B^2, C^2 are significant model terms. The model terms are not significant if respective values are higher than 0.1000. In this case, band width, saturation time and solvent front have significant main effects on response. All types of first order interactions are significant. The terms in the quadratic formula are all significant. The following mathematical model is utilized for response optimization based on the response surface model and 3D contour plots shown in Figure No 3-5analysis:

Resolution = 0.4900 + 0.0187 × (band width) + 0.0425 × (saturation time) + 0.0287 × (solvent front) − 0.0100 × (band width) × (saturation time) − 0.0175 × (band width) × (solvent front) − 0.0100 × (saturation time) × (solvent front) − 0.0412 × $(\text{band width})^2$ − 0.0337 × $(\text{saturation time})^2$ − 0.0413 × $(\text{solvent front})^2$

For given levels of each element, this equation in terms of factors can be used to make predictions about the response. For each factor, the levels should be indicated in the original units. Because the coefficient are scaled to suit the units of each element and the center of the design space, this equation should not be used to evaluate the relative impact of each factor.

The Adjusted R^2 of 0.9916 is reasonably close to the Predicted R^2 of 0.9410; that the difference is less than 0.2. The signal to noise ratio is determined by Adeq precision. It is preferable to have a ratio of more than four. The obtained ratio is 40.745 shows an adequate signal.

Table No.2: ANOVA table for response surface analysis

Source	Sum of squares	df	Mean square	F-value	p-value	Significance
Model	0.0472	9	0.0052	209.99	<0.0001	**Significant**
A-Band length	0.0028	1	0.0028	112.50	<0.0001	Significant
B-Saturation time	0.0144	1	0.0144	578.00	<0.0001	Significant
C-Solvent front	0.0066	1	0.0066	264.50	<0.0001	Significant
AB	0.0004	1	0.0004	16.00	0.0052	Significant
AC	0.0012	1	0.0012	49.00	0.0002	Significant
BC	0.0004	1	0.0004	16.00	0.0052	Significant
A^2	0.0072	1	0.0072	286.58	<0.0001	Significant
B^2	0.0048	1	0.0048	191.84	<0.0001	Significant
C^2	0.0072	1	0.0072	286.58	<0.0001	Significant
Residual	0.0002	7	0.0000			
Lack of fit	0.0002	3	0.0001			
Pure error	0.0000	4	0.0000			
Cor total	0.0474	16				

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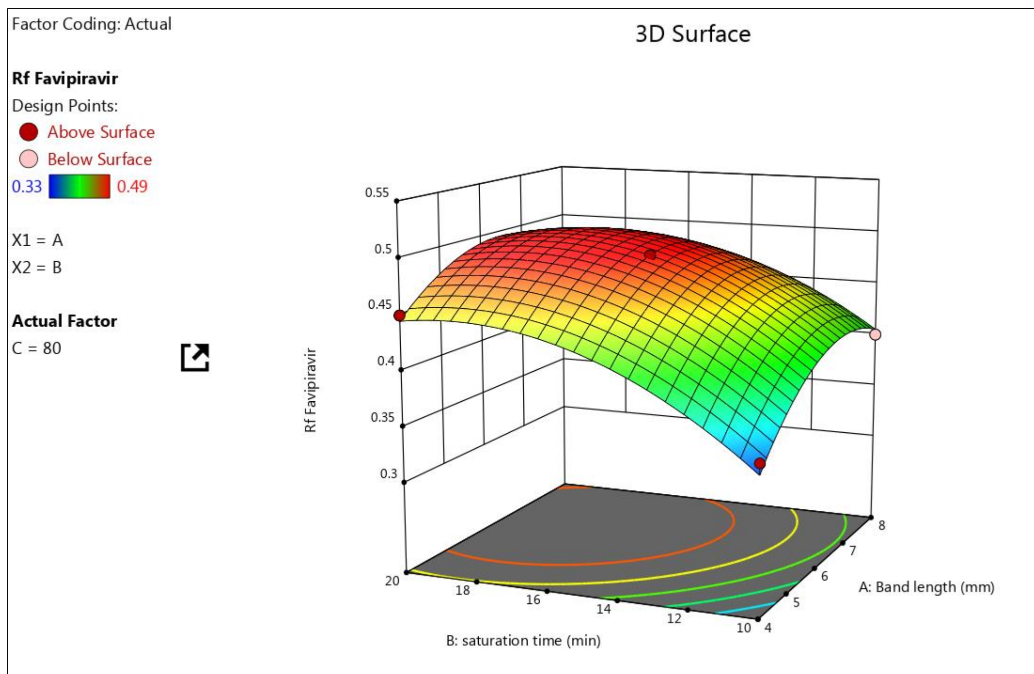

Figure No.3: 3D contour plot showing the relationship of (A) band width and (B) saturation time with resolution

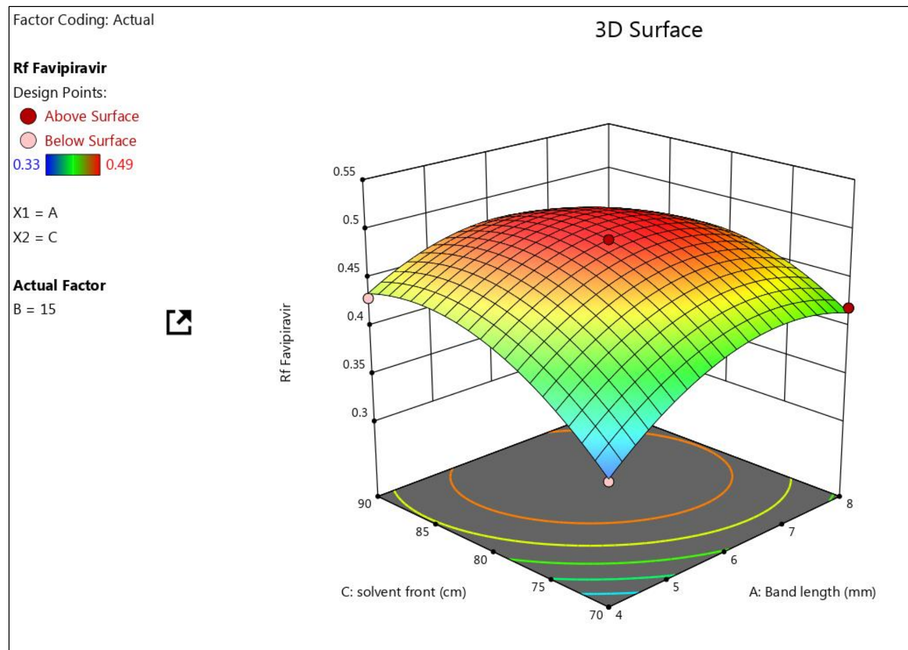

Figure No.4: 3D contour plot showing the relationship of (A) band width and (C) solvent front with resolution

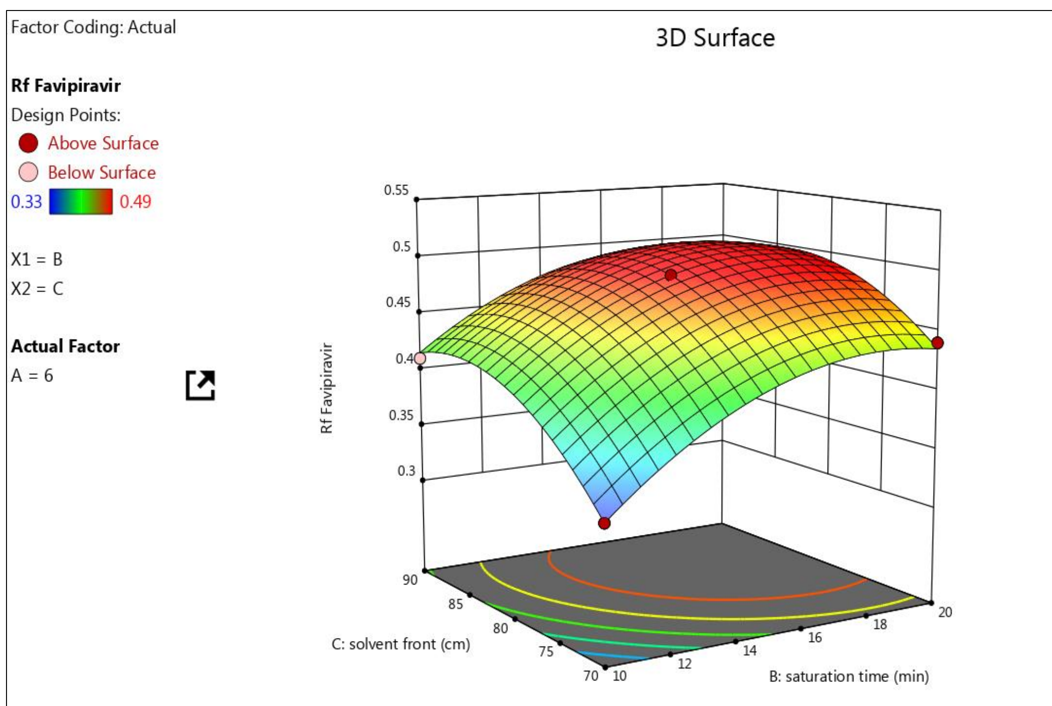

Figure No. 5: 3D contour plot showing the relationship of (C) solvent front and (B) saturation time with resolution

Optimization and Validation of Response Surface Model

The response surface linear model was used to improve the experimental parameters for the desired resolution, and the suggested experimental runs were carried out in the lab to ensure the validation of model. The predicted responses were in agreement with the resolutions of all performed experiments, indicating that the model was accurate in identifying design space for the development of HPTLC method.

Method Validation

Linearity

The linearity of FAV showed good linear relationship between peak areas and concentration. The linearity range was found to give linear detector response In the concentration range of 2000- 12000 ng/band, the linearity range was observed to produce linear detection response. For FAV calibration curves, the coefficient of correlation (r^2) was observed to be 0.9913. 3D chromatograms for FAV are shown in Figure No 6 and 7.

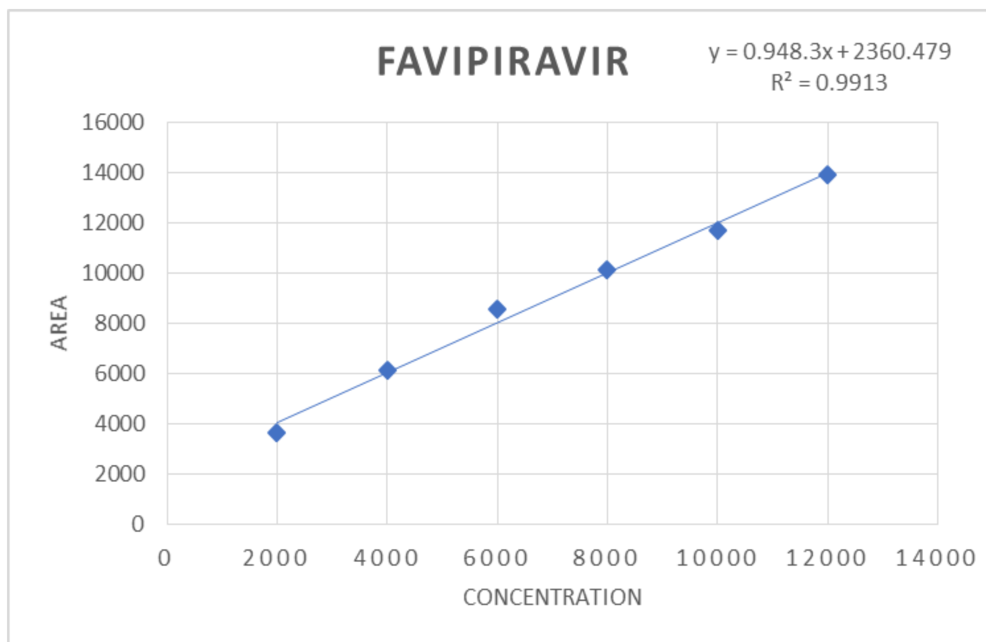

Figure No.6: Calibration curve of favipiravir

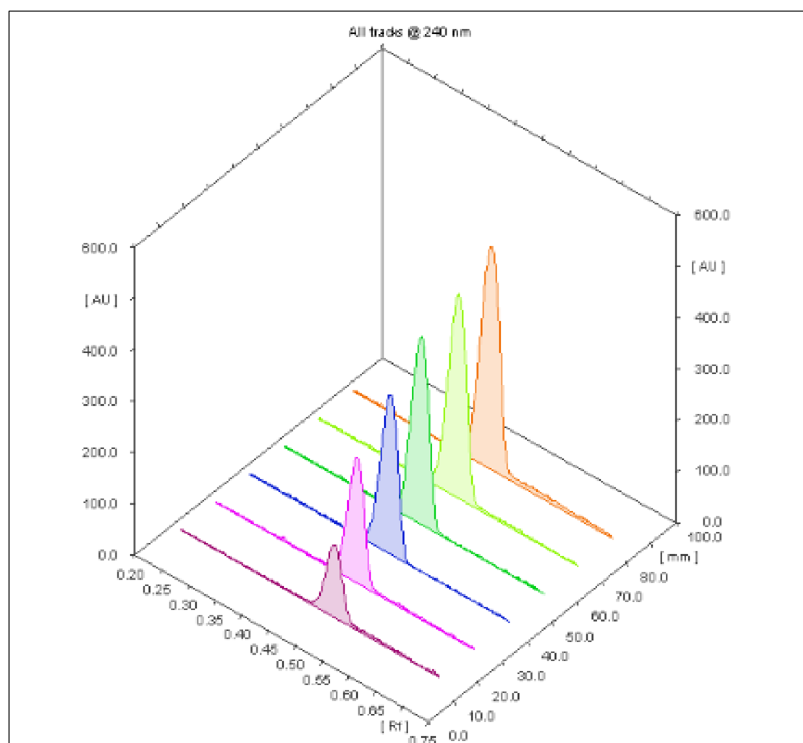

Figure No. 7: 3D densitogram of favipiravir linearity

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Precision

Repeatability and intermediate precision for the developed method were given in terms of relative standard deviation (%RSD) of the peak area. The %RSD for repeatability was established less than two indicates system precision and method precision. The finding that the %RSD for intra-day and inter-day precision was established less than two shows reproducibility, respectively shown in Table No 3

Table No.3: Summary of linear regression and validation data

Parameters	FAV
Linearity range	2000-12000 ng/band
Linear regression equation	y = 0.948.3x + 2360.479
Correlation coefficient (r^2)	0.9913
Limit of detection (LOD)	20.50 ng/band
Limit of quantification (LOQ) Repeatability (%RSD)*	62.13 ng/band 1.66
Intra-day (%RSD)	1.24
Inter-day (%RSD)	1.82
Specificity	Specific

*Mean of six determinant

Accuracy

The accuracy (% recovery) of the method was obtained by calculating recovery of FAV by spiking 80%, 100%, and 120% of standard concentration to sample as per the ICH guidelines. At all three levels, the %recovery was obtained between 98.37% and 100.2%. This value indicates that the method is accurate shown in Table No 4.

Table No.4: Recovery study of the method for favipiravir

Drug	Recovery level (%)	Initial amount (ng/band)	Amount added (ng/band)	% Recovery*	% RSD*
	80	6000	4800	100.2	0.64
Favipiravir	100	6000	6000	98.37	1.93
	120	6000	7200	99.16	0.69

*Mean of three determinant

Limit of Detection and Limit of Quantification

The new method's limit of detection (LOD) and limit of quantification (LOQ) for FAV were determined to be 20.50 and 62.13 ng/band, respectively, showing its sensitivity shown in Table No 3.

Specificity

The developed method for FAV was found to be specific as there is no any extra peak obtained other than the standard drug and sample shown in Figure No 8.

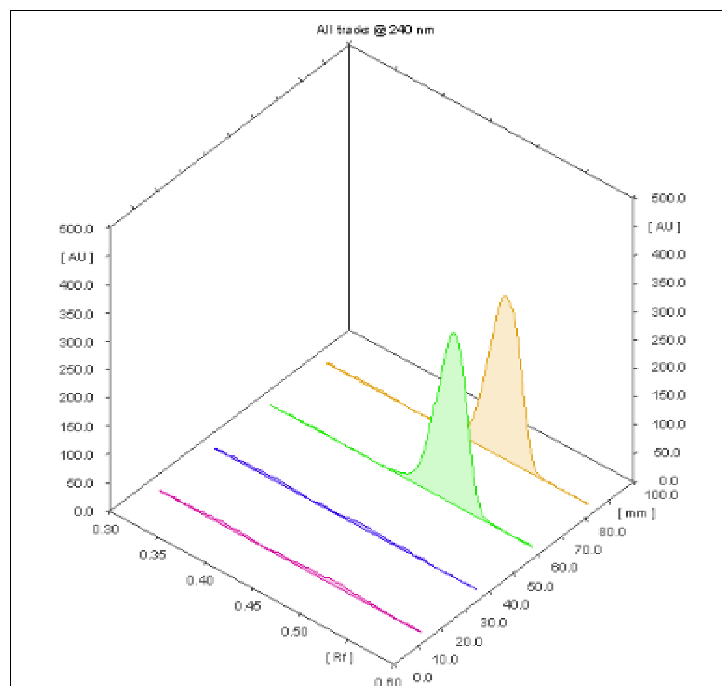

Figure No.8: 3D densitogram of favipiravir for specificity

Robustness

The developed method was found to be unaffected by small deliberate changes in method parameters. The value of % RSD less than 2% indicate that the developed method was robust shown in Table No 5.

Table No.5: Robustness study for the developed method

Parameter	S.D of peak area*	% RSD*
Mobile phase composition (±0.1mL)	76.73	0.90
Mobile phase volume (±1mL)	163.3	1.94
Saturation time (±5min.)	158.3	1.82
Spotting to development time (±5min)	147.59	1.71
Development to scanning time (±5min)	128.19	1.51

*Mean of three determinants

Analysis of forced degraded samples

FAV was exposed to degradation in variety of stressful conditions like acid, base, oxidation, dry heat and photo-degradation. It was found to be degrade in all mentioned stress conditions. The percentage assay of active substance and Rf value of degradation product are shown in Figure No 6. FAV was found to undergo acid degradation showing additional peaks at Rf values of 0.03, 0.32, 0.35, 0.53, and 0.77 shown in Figure No 9a. The densitogram of base degraded FAV showed additional peaks at Rf values of 0.02,

0.07, and 0.86 shown in Figure No 9b, the densitogram of oxidative degraded FAV showed additional peaks at Rf values of 0.03, 0.04, and 0.06 shown in Figure No 9c, the densitogram of heat degraded sample showed additional peaks at Rf values of 0.01, 0.04, and 0.45 shown in Figure No 9d. In photolytic degradation study FAV showed additional peaks at Rf values of 0.02 shown in Figure No 9e.

Table No. 6: Results of forced degradation study

Stress condition	Temperature and time	% Assay of active substance	Rf values of degraded substances
Acidic degradation (0.1 N HCl)	37±2°C for 30 min.	53.29	0.03, 0.32, 0.35, 0.53, 0.77
Base degradation (0.1 N NaoH)	37±2°C for 30 min.	84.32	0.02, 0.07, 0.86
Oxidative degradation (3% H_2O_2)	37±2°C for 30 min.	89.90	0.03, 0.04, 0.06
Dry heat degradation (Hot air oven)	60°C for 30 min.	81.78	0.01, 0.04, 0.45
Photolytic degradation (UV scanner)	254 nm for 24 hr.	96.28	0.02

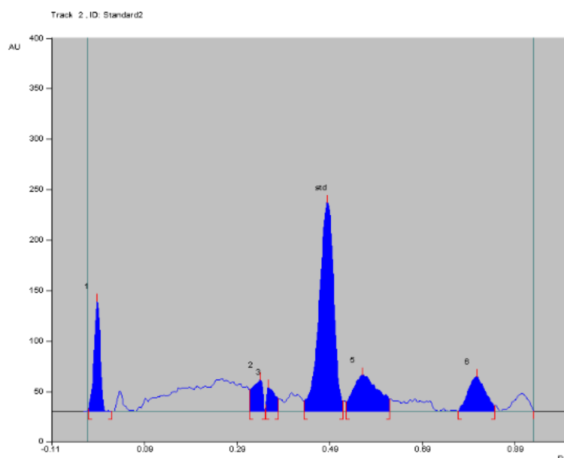

Figure (9a)

Densitogram of acid degraded sample

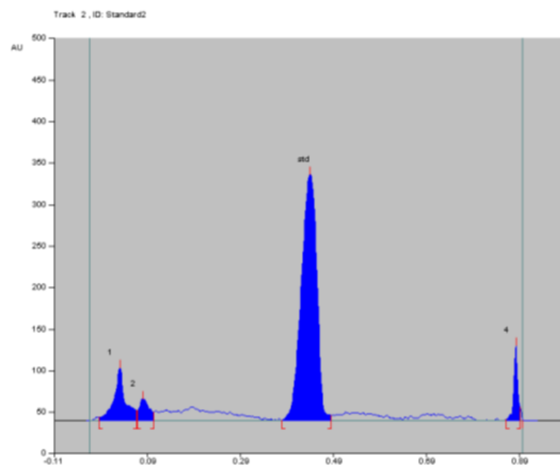

Figure (9b)

Densitogram of base degraded sample

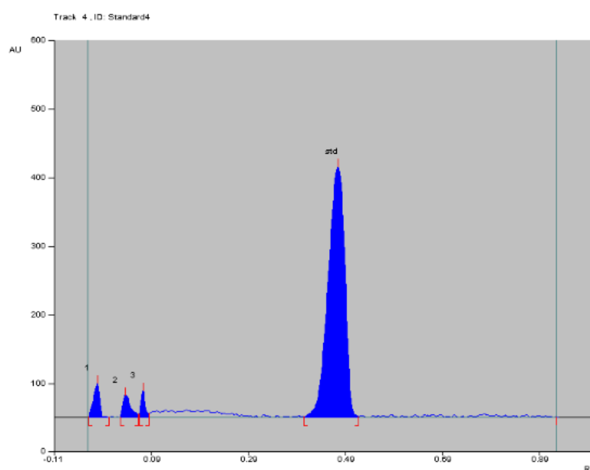

Figure (9c)

Densitogram of oxidative degradation sample

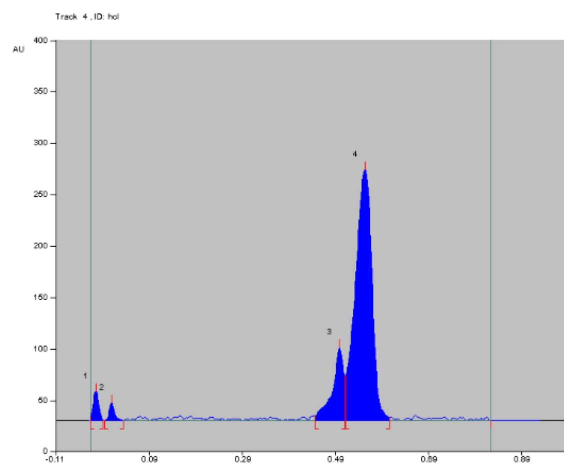

Figure (9d)

Densitogram of heat degradation sample

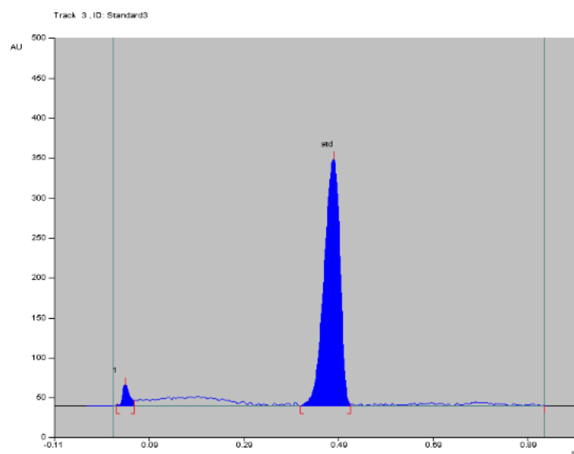

Figure (9e)

Densitogram of photo-degraded sample

Conclusion

Favipiravir is currently available for the treatment of most severe disease COVID-19. As there was no any reported method found so, HPTLC method was used to create and validate a stability indicating method for estimation of favipiravir utilizing a quality by design approach. The chromatographic parameters of the method were optimized using Box-Behnken design approach. The established analytical method was found to be simple, sensitive and rapid for determination of favipiravir. The established analytical method was validated as per ICH Q2 (R1) guidelines for linearity, specificity, range, precision, accuracy, LOD and LOQ, robustness. The stability-indicating characteristics were determined in accordance with the ICH guidelines. It provided the knowledge about the stability indicating capabilities of the product. This study

information will aid in improvement of the manufacturing process as well as the selection of storage conditions for the marketed formulation.

Acknowledgments

The author's would like to thank to Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune, and also Sinhgad Institute of Pharmacy, Narhe, Pune, for providing necessary infrastructural facilities to perform this study.

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