IJBPAS, June, 2026, 15(6): 2533-2543

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

ANALYTICAL METHOD DEVELOPMENT, VALIDATION AND STABILITY-INDICATING ASSAY OF FAVIPIRAVIR A SARS COV-2 MOLECULE BY RP-HPLC USING ANALYTICAL QUALITY BY DESIGN APPROACH

NAYAK A[1*], SOWMYA CY[1], SANGEETHA G[1], DEVIKA HG[1] AND DAS P[2]

1: Department of Quality Assurance, Krupanidhi College of Pharmacy, 12/1, Chikka Bellandur Carmelaram Post, Varthur Hobli, off Sarjapur Road, Bangalore – 560 035, India

2: Department of Pharmaceutical Chemistry, Krupanidhi College of Pharmacy, 12/1, Chikka Bellandur, Carmelaram Post, Varthur Hobli, off Sarjapur Road, Bangalore – 560 035, India

***Corresponding Author: Dr. Anjali Nayak; E Mail: anjaliangel84.pharma@gmail.com**

Received 20th May 2025; Revised 5th June 2025; Accepted 4th Sept. 2025; Available online 1st June 2026

https://doi.org/10.31032/IJBPAS/2026/15.6.10194

ABSTRACT

Objectives: The present studies outline the structured development and validation of analytical quality by design based reversed-phase high-pressure liquid chromatographic system for estimation of favipiravir that is simple, robust, sensitive, and economical.

Methods: The method optimization involved custom design to enhance performance by selecting Solvent composition, column length, run time. Utilizing a Thermo C18, 150×4.6mm, 5µm column in an isocratic elution mode with mobile phase KH_2PO_4 buffer and methanol, (50:50% v/v) with flow rate 1 ml/min at pH 4.2, detection at 225 nm using a UV/Visible detector, utilization of High-performance liquid chromatography enabled improved drug separation.

Results: The use of JMP software was utilized for custom design to screen and optimize critical method parameters. Favipiravir retention time (Rt) was found to be 3.160 minutes. It was discovered that the method's linearity in the range of 50-150 µg/ml had a r2 of 1. The method LOD and LOQ are computed to be 0.08 µg/ml and 0.2 µg/ml, respectively. According to the precision study, there was <2% percentage RSD values found. Recovery was determined to be 100%, verifying the procedure efficacy.

Conclusion: The proposed studies utilized analytical quality by design (AQbD) methodology to optimize chromatographic conditions for the routine analysis of Favipiravir. A Favipiravir in bulk form was estimated to be using a RP-HPLC technique, developed and validated and sstability studies were performed according to ICH criterias.

Keywords: Coronavirus, Custom Design, RNA polymerase, Influenza, ICH

INTRODUCTION

Coronavirus disease (COVID-19) rapidly spread and became a pandemic that impacted nearly every country on the planet. According to World Health Organization (WHO) reports, the case fatality rate of COVID-19 varies from 1% to 7% **[1]**. Since the spread of COVID-19 started to affect the planet, numerous treatment methods have been proposed by countries.

Favipiravir targets enzymes like RNA-dependent RNA polymerase (RdRp) for viral genome transcription and replication, potentially inhibiting influenza A and B replication. It has shown potential in treating avian influenza and may be a substitute for neuramidase inhibitor-resistant strains. Favipiravir is also being investigated for treating lethal pathogens like Ebola-virus, Lassa-virus, and COVID-19 **[2]**.

Favipiravir is an analogue of pyrazine (6-fluoro-3-hydroxypyrazine-2-carboxamide).

it is an antiviral drug produced through Toyama Chemical for the treatment of influenza, specifically targets and inhibits RNA polymerase enzyme in RNA viruses, arresting viral replication. It exhibits arena-, alpha-, filo-, bunya-, flavi-, and norovirus antiviral activity. It has been authorized for COVID-19 treatment in China following the novel Covid infection outbreak **[3, 4]**.

METHODOLOGY

MATERIALS AND METHODS

Chemicals and Reagent

H_2PO_4, KH_2PO_4, HCl, NaOH, H_2O_2, and methanol were acquired from Merck India Pvt. Ltd, Mumbai, India and from SD Fine-Chem Limited, India. Favipiravir was sourced from Sunlight Sciences, Hyderabad, India.

Methods

The study utilized UV/Visible detector Model No. (2489) equipment and a Waters Alliance HPLC 2695 system, using Empower 3 for developing and validating HPLC methods. Custom Design was utilized with the JMP PRO 14 program to enhance the approach's performance by selecting components and evaluating responses **[5]**.

Preparation of Solutions

KH_2PO_4 buffer (0.1 M) preparation

Weigh 1.70 g of KH_2PO_4 in a 1000 ml flask, dissolve and dilute with water, this solution was filtered by using a 0.45µ membrane filter and sonicated for 10 minutes **[6].**

Mobile phase preparation

Following 10-minutes degassing period in an ultrasonicator, a mixture of 500 ml (50%) of KH_2PO_4 buffer and 500 ml (50%) was subjected to vacuum pressure filtering through a 0.45 µm filter **[7]**.

Diluent preparation

The diluent utilized was mobile phase.

Preparation of stock standard solutions

The 10 mg working standard of Favipiravir was transferred to a 10 ml volumetric flask, diluent was added, and the flask was sonicated for 30 minutes before adding diluent to achieve different concentrations **[8]**.

Method development- Optimized chromatographic conditions

To optimize the final method, initial trials are required. Chromatographic separation was achieved using a Thermo C18, 150×4.6mm,5µm column at 25°C, with 0.1N KH_2PO_4 and methanol were mixed 50:50 (v/v) as mobile phases and pumped at a flow rate of 1 ml/min at 225 nm, the UV detector was set **[9]**.

Experimental design

The method was optimized using custom design, considering buffer concentration, run time, and column length. Different solvent composition ranges were chosen, with lower and upper limits of 30 and 70, run time of 5- 10 minutes, and column lengths of 150 and 250. JMP PRO 14 was used to generate and analyze a study design consisting of 12 runs., as depicted in **Table 2 [10, 11]**.

Table 1: Design Summary of Custom Design

CMPs	Units	Lower limit	Upper limit
Buffer concentration in mobile phase	%	50	70
Column length	-	150	250
Run time	minutes	5	10

CQA: Retention time, Theoretical plates, Tailing factor

Table 2: Method runs by Custom Design

Sl.no	Factors			Responses		
	Buffer concentration in mobile phase	Column length	Run time	Retentiontime	Tailing factor	USP Plate count
1	70	250	5	4	1.2	7077
2	60	150	7.5	3.4	1.7	4246
3	50	250	10	3.8	1.3	4821
4	50	150	5	3.1	1.5	4198
5	70	250	5	3.1	1.3	6245
6	70	150	10	2.1	1.6	4540
7	60	150	7.5	3.1	1.7	5182
8	50	250	5	3.6	1.1	5269
9	70	150	10	2.5	1.7	5315
10	50	150	5	3	1.6	4997
11	70	250	10	3.4	1.4	6123
12	50	250	10	3.6	1.3	4486

Method Validation

It is a crucial process in the pharmaceutical field to assure that a particular technique is appropriate according to its original purpose

and produces reliable and accurate results. It involves a series of tests and assessments to confirm the performance, robustness, and reliability of the analytical method.

Linearity and Range

Linearity of Favipiravir, checked with concentrations between 50 and 150 g/mL administered via injection. The graph indicates that the R2 value was determined to be 1, which means that Favipiravir maintains linearity throughout the range of 50% to 150% **[7, 8]**.

Precision

5 ml of stock solution was pipetted out and diluted, which gives 100µg/ml, 6 vials were prepared and injected, with acceptance criteria ranging from 97-103% and a 2% relative standard deviation between 6 replicas **[9]**.

Accuracy

The drug was added to samples at different concentrations- 50%, 100%, 150% in order to verify accuracy, and the percentage recovery was calculated by comparing measured and expected amounts **[12].**

Robustness

The robustness of the system was investigated by adjusting its pH (±0.2), temperature (± 2 ◦C), flow rate (± 10 ◦C), and mobile phase composition (±5%) **[13]**.

System suitability

The system has passed parameters for tailing, acceptable peak area, Rt, plate count found to be more than 2500, with a tailing factor of 1.20 percent and a retention time of 3.160 minutes and a RSD of 0.2%. The total number of USP plates was 4198 **[14]**.

Limits of detection (LOD) and limits of quantitation (LOQ)

LOD is the low concentration level where the peak area is three times higher than baseline noise, indicating the smallest amount of the substance that can be reliably detected, where the signal-to-noise ratio is higher than 10 **[15]**.

Forced degradation studies

Acid stress

5 ml Favipiravir was diluted to 50ml, then withdrawn into 10 ml volumetric flask and sonicated for 30 minutes with 0.1 N HCL. The solution was then poured into an HPLC vial and injected into the system, ensuring accurate results **[16, 17]**.

Base stress

Favipiravir was pipetted out of stock solution in 5 ml increments and diluted to 50 ml. 1ml of solution was transferred to a 10ml volumetric flask, mixed with 0.1N NaOH, adjusted, and sonicated for 30 minutes. The solution was then injected into a HPLC vial, ensured accurate results **[16, 18].**

Hydrolysis

5 ml of Favipiravir was pipetted from stock solution and diluted to 50 ml, ml of appropriately diluted water was added after withdrawing 1 ml of the solution and

transferring it into a 10 ml volumetric flask and sonicated for 30 minutes. The solution was then poured into an HPLC vial and injected into the HPLC system **[12]**.

Peroxide

5 ml Favipiravir from stock solution was pipetted and diluted to 50ml, 1 ml of the solution was withdrawn and passed on to a 10ml volumetric flask. 1% H_2O_2 was incorporated, diluted, and sonicated for 30 minutes. The solution was then injected into an HPLC vial and analyzed using a HPLC system **[19]**.

Heat

5 ml Favipiravir was diluted from stock solution to 50ml, then 1ml was withdrawn into a 10ml volumetric flask, filled with diluent, and heated at 60°C for 30 minutes. The solution was then injected into a HPLC vial and analyzed **[20, 21]**.

Sunlight

5 ml Favipiravir was diluted from stock to 50 ml, then 1 ml of solution was added to a 10 ml volumetric flask and left in sunlight for six days. The solution was then injected into a HPLC vial and analyzed **[22]**.

RESULTS AND DISCUSSION

Analytical techniques indicate product quality and strength throughout its lifetime. HPLC method's primary aim is to isolate and quantify target analytes for contaminants and excipients. Identifying critical quality attributes (CQA) is crucial for ensuring quality of analytical method. JMP software (JMP pro 14 trial version) was used for optimization study and statistical analysis to develop the analytical RP-HPLC method, determining design space and control strategy through optimization study.

Design of experiment by custom design

Effect Summary

In **Figure 1**, it manifests that method is impacted by factors such as Buffer concentration column length and run time. A bar representing the buffer concentration, column length, and run time crosses the blue vertical line to indicate significance.

Model fit of specific HPLC technique responses

The model fit was evaluated using data from all 12 runs, enhancing method's robustness. In accordance with **Figure 2**, at a significance threshold of $p<0.01$, $R^2 = 0.90$ and $p = 0.0002$ for response retention time, $R^2 = 0.91$ and $p = 0.0001$ for response tailing factor, and $R^2 = 0.92$ and $p = 0.0025$ for response plate count, which were found to be statistically significant. The high R^2 values and low p values suggest that the model being evaluated is a good fit for the data **[6, 22]**.

Statistical optimization of selected responses of the HPLC method

Numerical optimization: The prediction profiler reveals a constant relationship between parameters and responses, revealing variability in extreme results. **Figure 3** shows a global desirability value

of 73.7%, indicating the model's validity for achieving desired goals for all three responses **(Table 3 and Figures 3)**.

Method validation

The RP-HPLC method was developed by combining mobile phases like methanol, orthophosphoric acid, and potassium dihydrogen phosphate (KH_2PO_4) in different proportions and run times. produced no distinct peak and retention time was longer. The final combination of KH_2PO_4 buffer and methanol produced a well-defined peak with a flow rate of 1ml/min and a wavelength of 225 nm. **[7, 23]**. The result of the method validation parameters is shown in **Table 4**.

Forced degradation studies

Favipiravir stability was tested in extreme environments like heat, light, acidity, basic conditions, peroxide, and water. High-temperature and acid stressed conditions led to noticeable degradation, with comparable degradation peaks. Details are summarized in **Table 5**. [Nayak A, 2023].

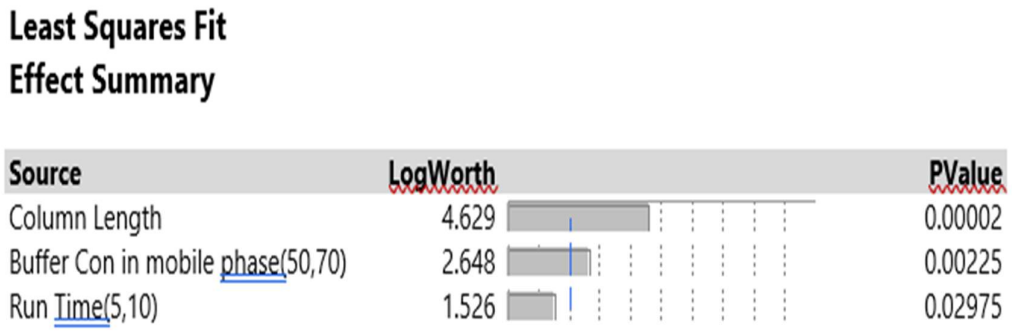

Figure 1: Effect summary

Table 3: Final optimized HPLC chromatographic condition

Sl. No	Parameters
Mobile Phase Composition	KH_2PO_4 (50%): Methanol (50%)
Flow rate	1ml/min
Temperature of the Column	25^0C

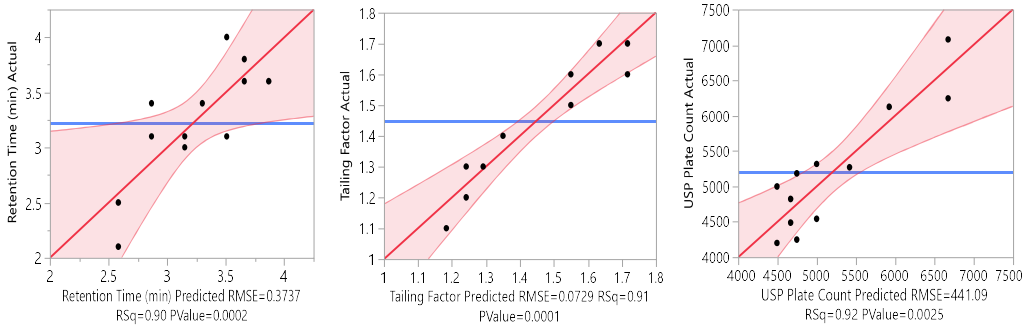

Figure 2: Actual vs Predicted plot for selected responses (R_t, tailing, Peak area) Prediction Profiler

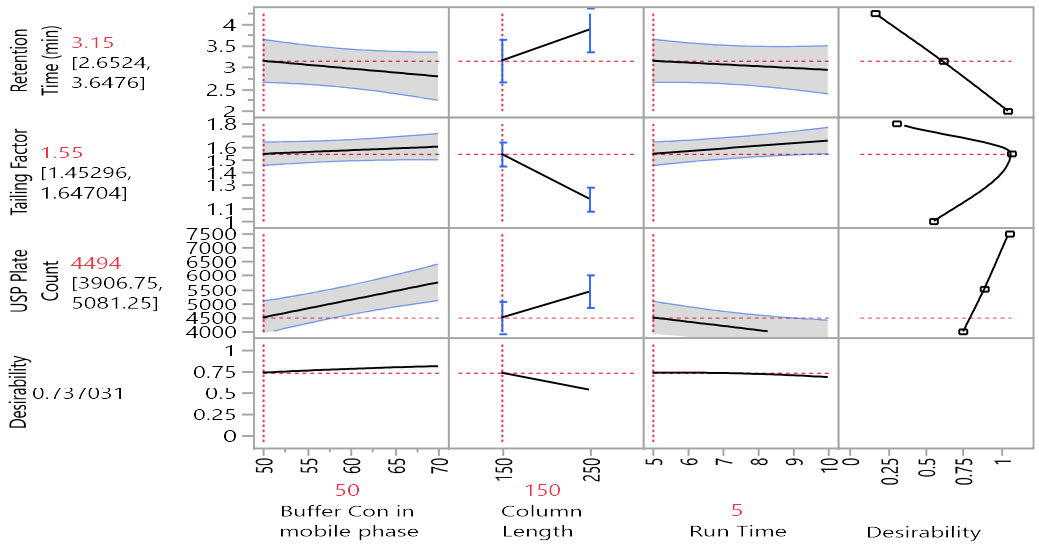

Figure 3: Prediction profiler with maximized desirability

Table 4: Results of Validation Parameter

Sl. No	Parameters	Results
1	Linearity	R^2=1
2	Accuracy	RSD%=0.3
3	Precision	RSD%=0.11
4	Retention time	3.160
5	Tailing factor	1.20
6	Plate count	4198

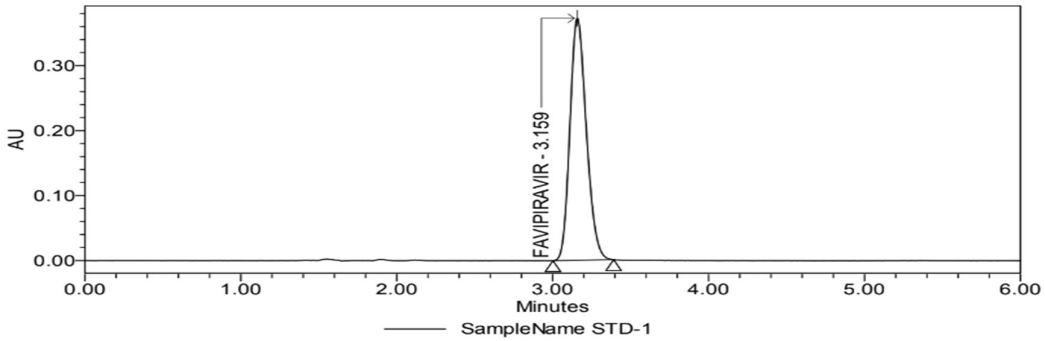

Figure 4: Chromatogram of the optimized method (standard chromatogram)

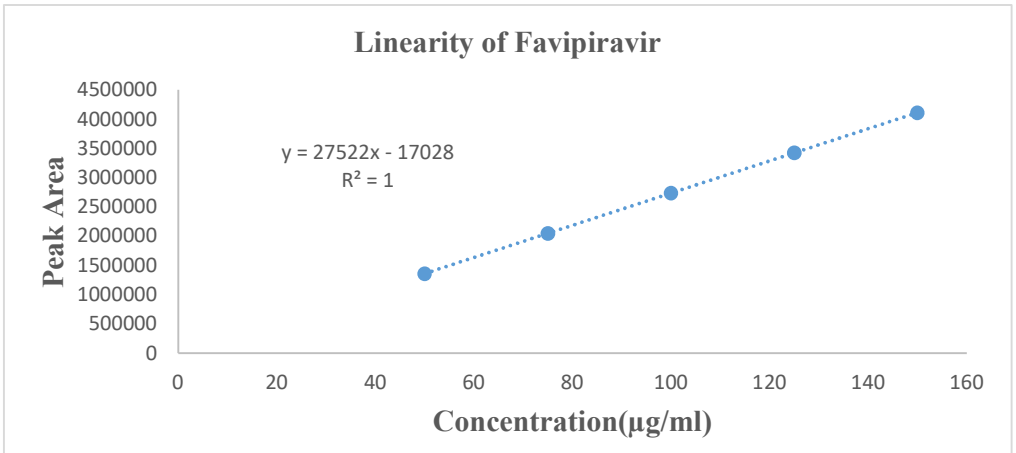

Figure 5: Linearity of Favipiravir

Table 5: Results of forced degradation study

Stress	Sample weight (mg)	Sample Area	% Assay	%Degraded
Acid stress	**100**	**2800225**	**90.45**	**9.55**
Base stress	**100**	**2916317**	**92.21**	**7.79**
Peroxide stress	**100**	**2996560**	**96.29**	**3.71**
Heat stress	**100**	**2877043**	**91.16**	**8.84**
Photo stress	**100**	**2980642**	**94.97**	**5.03**
Hydrolysis stress	**100**	**3142857**	**99.76**	**0.24**

CONCLUSION

Analytical Method development by AQbD approach ensures the creation of an effective method for the desired purpose. The developed RP-HPLC method produced results that satisfied each of the validation parameter requirements. It was discovered that the precision RSD was less than 2%. Favipiravir retention period was discovered to be 3.160 minutes. The sensitivity of the approach is confirmed by LOQ. The percentage of acid and heat stressed degradation was found to be 9.55% and 8.84%. The recently designed RP-HPLC method was determined to be user-friendly, accurate, quick, precise, and affordable.

ACKNOWLEDGEMENT

The authors express gratitude to the Krupanidhi College of Pharmacy, Bangalore faculties and management for providing scientific resources and financial assistance.

REFERENCES

[1] Abu-Gharbieh E, Saber-Ayad M, Saleh MA. The rationale for potential pharmacotherapy of COVID-19. *Pharmaceuticals* **2020**, *13*, 96–105.

[2] Abdelnabi R, Delang, L, Neyts J. Favipiravir as a potential countermeasure against neglected and emerging RNA viruses. *Antiviral Res*. **2018**, *153*, 85–94.

[3] Adour L, Grib C, Grib L, Hamdache A,Mezrouai A, Zatout H. Forced Degradation Studies of Sofosbuvir with a Developed and Validated RP-HPLC Method as per ICH Guidelines. *Chromatographia*. 2021;84(12):1131–1140.

[4] Ahmad S, Baheti D, Bakhshi AR, Khairnar M, Tare H, Tare M. QBD approach to develop stability indicating RP-HPLC method development for Levosulpiride and Ilaprazole. *International journal of health sciences*. 2022;6(S5):7413–7429.

[5] Akshaya K, Ramya SS, Sudhakar B. Analytical Method Development and Validation for the Simultaneous Estimation of Bilastine and Montelukast by RP-HPLC. *Asian Journal of Research in Pharmaceutical Science*. 2023;13(2):79–84.

[6] Ali AH. High-Performance Liquid Chromatography (HPLC): A review. *Annals of Advances in Chemistry*. 2022;6:10–20.

[7] Ankalgi AD, Ashawat MS, Rana A, Singh N, Sharma S. Modern Trends in Analytical Techniques for Method Development and Validation of Pharmaceuticals: A Review. *Journal of Drug Delivery and Therapeutics*. 2021;11(1-s):121–130.

[8] Bagal D, Chachare A, Joshi A, Khadse S, Nagar A, Shirkhedkar A. Development and validation of stability-indicating RP-HPLC method for estimation of dalfampridine in bulk drug and tablet dosage form. *Future Journal of Pharmaceutical Sciences*. 2021;7(1):1–7.

[9] Bagul N, Kalyankar G, Prajapati PB. Implementation of DoE and Risk-Based Enhanced Analytical Quality by Design Approach to Stability-Indicating RP-HPLC Method for Stability Study of Bosutinib. *Journal of AOAC INTERNATIONAL*. 2021;104(6):1742–1753.

[10] Carlota RR, Nayak A, Reddy YV, Prathibha KR, Nirma T, Marbaniang D. RP HPLC-Based Method Development, Validation and Stability Indicating Assay of Levosulpiride in Bulk Drug Using Analytical Quality by Design Approach. Indian Journal of Science and Technology. 2023 Dec 20;16(46):4436-44.

[11] Chimakurthy J, Konidala SK, Samineni R, Yamarthy V. Development and Validation of Analytical Method for Estimation of Balofloxacin in Bulk and Pharmaceutical Dosage Form by RP-HPLC. *Research Journal of Pharmacy and Technology*. 2022;15(7):2992–2996.

[12] Chhalotiya UK. Development of Stability Indicating RP-HPLC Method for Determination of Levosulpiride Hydrochloride In Bulk And Pharmaceutical Dosage Form. *International Journal of Advances in Pharmaceutical Analysis*. 2012;2(2):41–46.

[13] Concentino V, Fachi MM, Junkert AM, Marson BM, Pontarolo R,Vilhena RO. Validation of analytical methods in a pharmaceutical quality system: An overview focused on HPLC methods. *Química Nova*. 2020;43(8):1190–1203.

[14] De Clercq E. New nucleoside analogues for the treatment of hemorrhagic fever virus infections. *Chem.–Asian J.* **2019,** *14*(22), 3962–8.

[15] Dhingra D, Gulati P, Kumar M, Pannu A,Pannu S,Thakur M. A recent review on developed analytical methods for detection of curcumin. *International journal of health sciences*. 2022;6(S7):173–194.

[16] Furuta Y, Komeno T, Nakamura T. Favipiravir (T-705), a broad-spectrum inhibitor of viral RNA polymerase. *Proc Jpn Acad Ser B Phys Biol Sci*. **2017**, *93*(7), 449-463.

[17] Gorad S, Jagdale A, Kasbale A, Kulkarni A, Tawde H, Tol T. Optimization of a liquid chromatography method for the analysis of related substances in daclatasvir tablets using design of experiments integrated with the steepest ascent method and Monte Carlo simulation. *Journal of Pharmaceutical and Biomedical Analysis*. 2020;178:112943.

[18] Gowen BB, Feldmann F, Feldmann H, Westover JB, Furuta Y, Hanley PW, Komeno T, Lovaglio J, Martellaro C, Rosenke K, Scott DP, Safronetz D, Saturday G. Use of Favipiravir to Treat Lassa Virus Infection in Macaques. *Emerg Infect Dis*. **2018,** *24*(9), 1696-1699.

[19] Mahapatra DK, Puranik MP, Soni MA. Analytical quality-by-design (AQBD) approach for the development and validation of RP-HPLC method for the estimation of lamotrigine in bulk and tablet formulation. *Journal of Medical pharmaceutical and allied sciences*. 2021;10(5):3591–3596.

[20] Gupta S, Mathur R, Mishra AP, Omar N, Verma P. A Review on Novel Analytical Method Development and Validation by RP-HPLC Method. *Indian Journal of Forensic Medicine & Toxicology*. 2021;15(4):3479–3486.

[21] Hayden FG, Shindo N. Influenza virus polymerase inhibitors in clinical development. *Curr Opin Infect Dis*. **2019**, *32*(2), 176-186.

[22] Hilurudeen MSM, Nagarajan NC, Ponraj PB, Santhanam MK. A Complete Roadmap of Analytical Quality by Design in Various Analytical Techniques. *Current Pharmaceutical Analysis*. 2023;19(3):184–215.

[23] Hema B, Nayak A, Sangeetha G, Rosita ruth Carlota, Maria Danish Alwin, Paramita Das. A new RP-HPLC Method Development, Validation and Stability-Indicating Studies of Epalrestat in Bulk Drug by Analytical Quality by Design Approach.Research J. Pharm. and Tech 2024; 17(9):4437-4442.

[24] Nayak A, Alwin RM, Sangeetha G, Sowmya CY, Reddy YV, Dang R. Stability Indicating Assay for the Determination of Bilastine in Bulk Drug and Method Development Validation by RP-HPLC Using Analytical Quality by Design Approaches. Indian Journal of Pharmaceutical Education and Research. 2024 May 27;58(2s):s582-90.

[25] Nayak A, Punith Kumar V. Novel method development and validation of rivaroxaban in bulk and pharmaceutical formulation by RP-HPLC method. *Int J Biopharm Pharm Sci*. 2023 Aug;12(8):3648-58.

[26] Pandey MM, Saha P. Design of Experiment (DoE)-Approach Based RP-HPLC Analytical Method Development and Validation for Estimation of Efavirenz in Bulk and Formulations. *Journal of Chromatographic Science*. 2022;60(1):35–44.

[27] Patel AM, Patel CN, Patel KK. A new simple RP-HPLC Method development, Validation and Forced degradation studies of Bilastine. *Asian Journal of Pharmaceutical Analysis*. 2021;11(3):183–187.

[28] Prabhu Padmavathi P, Das P, Panara K, Subrahmanyam EVS. Analytical method development and validation for estimation of drotaverine HCl in bulk and tablet formulation. *Asian J Biomed Pharm Sci*. 2013;3(22):12-6.