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STABILITY INDICATING RP HPLC METHOD DEVELOPMENT FOR ESTIMATION OF FAVIPIRAVIR IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Favipiravir is antiviral drug and nowadays it is part of treatment of covid 19. The aim of the research work was to develop a validated simple, sensitive, precise and specific high performance liquid chromatography method for estimation of favipiravir in bulk and pharmaceutical dosage form. The separation was carried out by using mobile phase consisting Methanol: Water (0.05% Triethylamine) 70:30. C 18 Column used with flow rate 0.8 ml/min.using UV detection at 360 nm. The Calibration curve of Favipiravir in concentration range 20-100 µg/ml. The correlation coefficient (r^2 = 0.9997) value indicated clear correlation coefficient between the validated compound concentration and their peak areas within test ranges. The detector response of favipiravir is directly proportional to concentration ranging from 10-100 µg/ml. The repeatability and intermediate precision expressed by % RSD, was less than 2%. The accuracy of method expressed as % recovery was satisfactory (99.7%). The drug was subjected to international conference on harmonization prescribed Hydrolytic, Oxidative, Photolytic and Thermal stress condition. The method was validated according to ICH guidelines. Result Showed that Favipiravir was stable in 0.5 N basic buffer and in 3% oxidative conditions, and some thermal and Photostability conditions, while it was more sensitive towards Acidic degradation. The result of the study showed that the proposed method is simple, rapid, precise, and accurate which is useful for the routine determination of favipiravir in bulk and pharmaceutical dosage form.

KEYWORDS: - RP-HPLC, Favipiravir, Forced degradation study, Method development.

INTRODUCTION

Favipiravir (Avigan) was developed by the Fujifilm Toyama Chemical Company in Japan. It selectively inhibits RNA dependant RNA polymerase (RdRP), an enzyme required for RNA viral replication inside human cells. It functions as a purine analogue and is incorporated instead of guanine and adenine. The incorporation of a single molecule of FAV terminates the elongation of viral RNA. Inside the cell, FAV is converted into its active phosphorylated form and is then recognized as a substrate by viral RdRP. It shows a broad spectrum of activity against different RNA viruses including influenza virus.[1] In 2014, FAV was approved in Japan for use in the outbreak of novel or recurrent influenza viral infections, where other antiviral drugs usually used in influenza are insufficiently effective. In Influenza, the beneficial effect has been attributed to decline in pulmonary viral load and TNF-alpha levels in the airways.[2] FAV was also used for the post exposure prophylaxis and treatment of patients with Ebola virus infections.[3] In December 2019, the first cases infected with COVID-19 virus (also known as SARS-Cov-2) were reported in Wuhan, China. Now this virus becomes pandemic all over the world. SARS-Cov-2 is a beta coronavirus which is enveloped positive strand RNA viruses like MERS (Middle East respiratory syndrome)-Cov and SARS (severe acute respiratory syndrome)-Cov.[4] For SARS-Cov-2, the viral genome codes for sixteen non-structural proteins (Nsps) required for virus replication and pathogenesis and four structural proteins.[5] Unfortunately, no specific therapeutic agent has been approved for the treatment of SARS-Cov-2 till now. However, a number of already existing antiviral drugs which have been proved to be safe and effective against other viruses are tested for their activity against the SARS-Cov-2. Special concern is given to RNA dependent RNA polymerase (RdRp) inhibitors. One of these drugs is favipiravir (FAV) which is known as T-705. Chemically, it is 6-fluoro-3-oxo-3,4- dihydropyrazine 2-carboxamide as shown in Fig. 1

According to the literature search, there are very few published high performance liquid chromatography (HPLC) methods for determining FVP assay and impurities in active pharmaceutical ingredients.[9,10] In those methods, a gradient HPLC mode was used for chromatographic separation. FVP is not officially available in any pharmacopoeia and there is still a need for validated HPLC methods to determine FVP in pharmaceutical formulations.

Chemical stability of pharmaceutical molecules is a matter of great concern as it affects the safety and efficacy of the drug product. The FDA and ICH guidelines state the requirement of stability testing data to understand how the quality of a drug substance and drug product changes with time under the influence of various environmental factor. Forced degradation is a process that involves degradation of drug products and drug substances at conditions more severe than accelerated conditions and thus generates degradation products that can be studied to determine the stability of the molecule. The ICH guideline states that stress testing is intended to identify the likely degradation products which further helps in determination of the intrinsic stability of the molecule and establishing degradation pathways, and to validate the stability indicating procedures used.

MATERIAL AND METHOD

- **Equipment**

Sr No.	Instrument	Company
1.	HPLC	The Agilent 1120 compact LC HPLC System Comprised of binary pump, column oven, Uv detector was used for the method development, forced degradation studies, and method validation. The y EZChrom Elite software was used for controlling instrument operation and processing data. Chromatographic separation was achieved C 18 column (Cosmosil) The injection volume was 20 µL.
2.	UV	Shimadzu 1900UV/VIS
3.	FTIR	Shimadzu
4.	Analytical Balance	LC/GC
5.	Ultrasonic Bath	Life care

- **Materials**

➢ **Standard drug**

Sr. no.	Drug	Gifted By
1.	Favipiravir	Blue Cross Ltd.

➢ **Chemicals**

Sr. no.	Solvent	Grade
1.	Methanol	HPLC Grade
2.	Water	HPLC Grade
3.	NaOH	AR Grade
4.	HCL	AR Grade
5.	Triethylamine	AR Grade

➢ **Marketed formulation used**

Sr No.	Ingredient	Label Claim	Company Name
1.	Favipiravir	Favipiravir 200 mg	Lupin Ltd.

- **Sample Preparation & Stock solution**

1.Preparation of mobile phase:

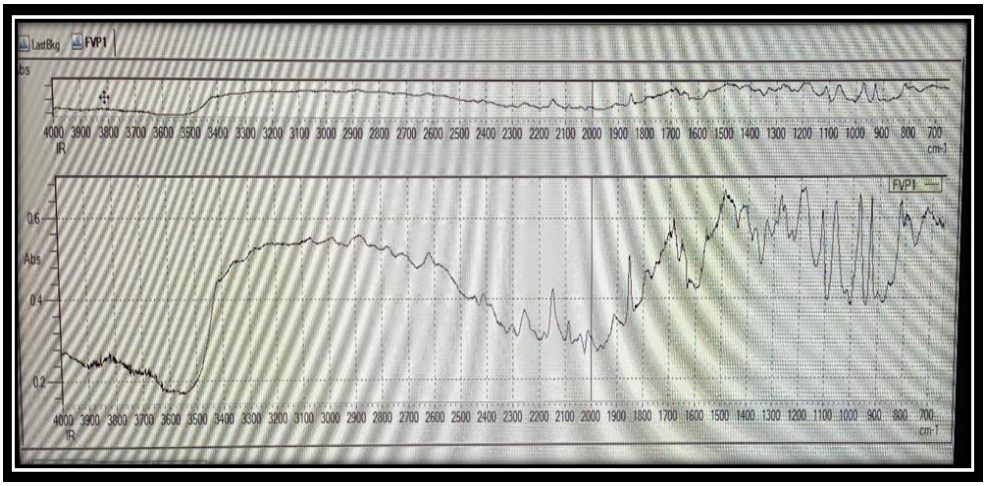
- HPLC grade Methanol: HPLC grade Water by adding 0.05% Triethylamine (70:30v/v) which was filtered through 0.45 µm membrane filter and sonicated on ultrasonic bath for 15 min.

1.Preparation of standard stock solution:

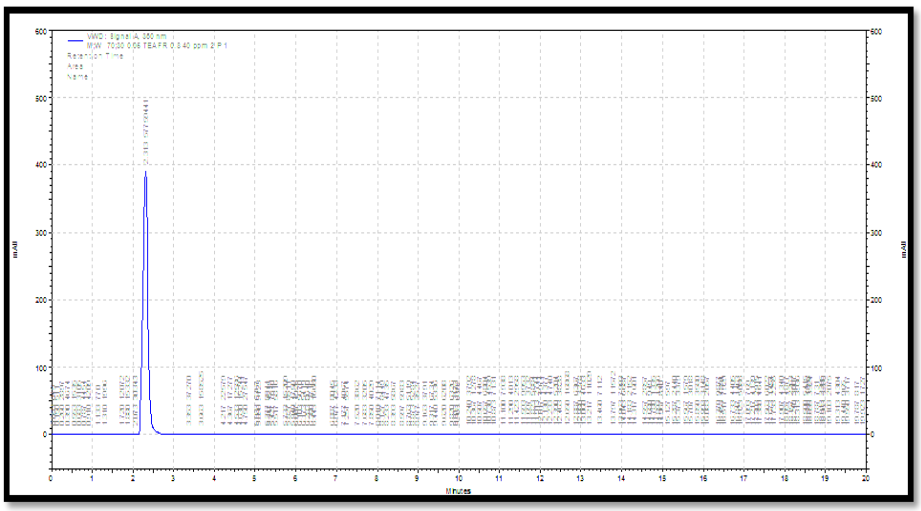
- Favipiravir standard stock solution was prepared by transferring 10 mg of Favipiravir working standard into a 100 ml volumetric flask, approximately 25 ml of methanol (HPLC Grade) was added and sonicated for 20 min. the volume was made up to 100 ml with HPLC grade water to get the concentration of 100 µg/ml. This solution was filtered through a 0.45µm pore size nylon 66 membrane. The subsequent dilutions were prepared by diluting stock solution with the Double Distilled water.

Preparation of Sample solution:

- Take 0.5 ml from stock solution (100ppm) Dilute it with 10 ml it will become 5 ppm. According to that take (1,2,3,4,5,6,7,8,9,10ml) Stock solution to prepared (10,20,30,40,50,60,70,80,90,100ppm) Solution respectively.



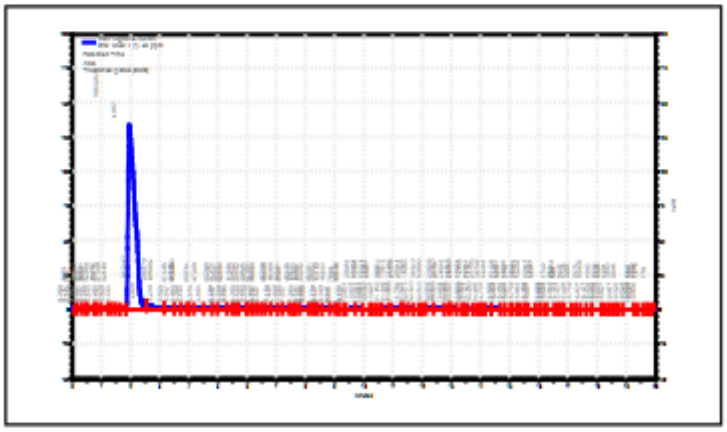
IR Spectra of Favipiravir



Original chromatogram of Favipiravir

- **Optimization of chromatographic conditions: -** Optimization of mobile phase was performed based on resolution, peak area and retention time. During the method development and validation we encountered some problems that were successfully solved The mobile phase Acetonitrile (A): Water (B) used with different ratio at different flow rate was tried.

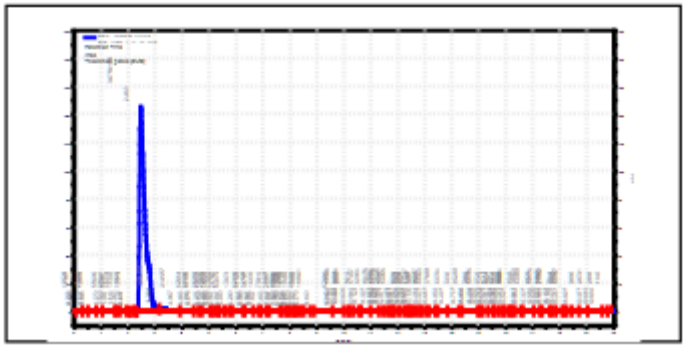
Sr. no.	Acetonitrile: Water (A: B)	Retention Time with flow rate 0.7 ml/min	Retention Time with flow rate 1ml/min
1.	90:10	1.9	2.50
2.	80:20	1.9	2.35
3.	70:30	1.8	1.94
4.	60:40	1.8	1.87
5.	50:50	1.7	1.83
6.	40:60	1.7	1.80



Chromatogram (Acetonitrile: Water)

The mobile phase Methanol (A): Water (B) used with different ratio at different flow rate was tried.

Sr. No.	Methanol: Water (A: B)	Retention Time with flow rate 0.8 ml/min	Retention Time with flow rate 1ml/min
1.	90:10	2.223	2.073
2.	80:20	2.200	2.073
3.	70:30	2.660	2.110
4.	60:40	2.743	2.123
5.	50:50	2.540	2.211
6.	40:60	2.320	2.122
7.	30:70	2.220	2.141
8.	20:80	2.100	2.132



Chromatograph (Methanol: Water)

Methanol: Water (70:30v/v) at flow rate 0.8 ml/min was found to be satisfactory ang gave symmetric and well resolved peak for favipiravir. For increase sharpness and avoid tailing 0.05% triethylamine was used in water at pH 6.

The Chromatogram was recorded at 360 nm as spectrum of favipiravir showed maximum response at this wavelength.

Chromatogram showed symmetrical peaks with good shapes for favipiravir and resolution of standard drug was satisfactory.

Retention time for favipiravir was found to be 2.66 min.

The validation parameter and all force degradation study were observed and performed by using this mobile phase are reported.

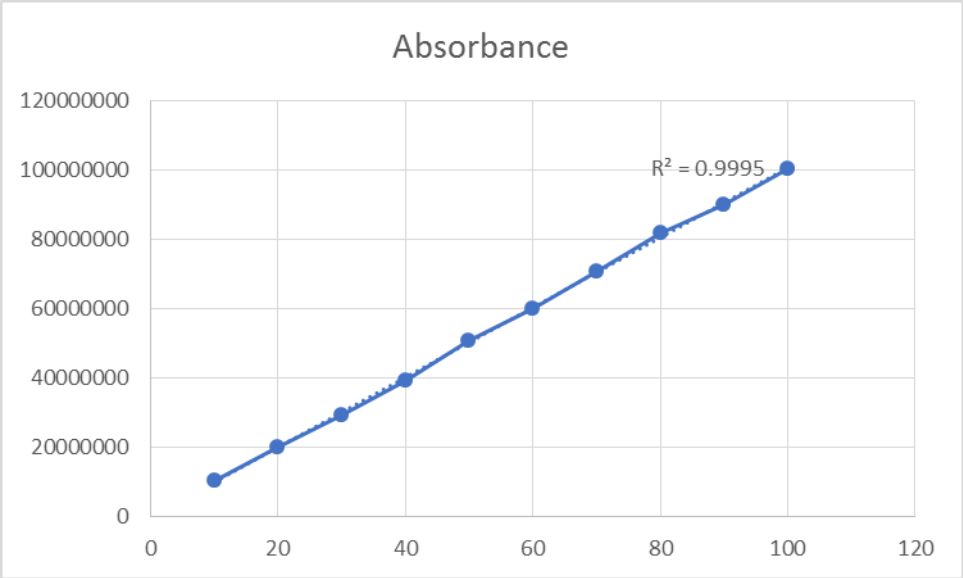
The experiments were performed with isocratic elution. The binary mobile phase consisted of a mixture of Methanol : Water (0.05% Triethylamine) in the ratio of 70:30 V/V. which was filtered through a membrane filter. The effluents were degassed before running at a flow rate

of 0.8 mL min^{-1}. The column temperature was ambient at 27 °C. The 20 µL volume of sample was injected per run and effluents were detected using UV detector at λ = 360 nm.

- **Validation method: -** The developed method was validated for as per ICH Q2 (R1) guidelines[21] for various parameters such as accuracy, precision, linearity, robustness, limit of detection (LOD), limit of quantitation (LOQ), and stability.

- **Linearity: -** and range the five series of standard solutions were selected for assessing linearity range. The calibration curve was plotted using peak area versus concentration of the standard solution and the regression equations were calculated. The least squares method was used to calculate the slope, intercept and correlation coefficient.

Sr. No.	Concentration	Area		
		Linearity 1	Linearity 2	Linearity 3
1.	10 ppm	11976289	9547875	10495739
2.	20 ppm	21953203	19338813	19959637
3.	30 ppm	31602858	29409799	29307157
4.	40 ppm	43543493	40586064	39307181
5.	50 ppm	54903287	50944464	50900134
6.	60 ppm	66874569	61791950	60155017
7.	70 ppm	77675567	71763343	70567681
8.	80 ppm	88460569	80935658	81895704
9.	90 ppm	98698128	90717366	90083584
10.	100 ppm	109615992	102622895	100321241
Correlation Coefficient (r^2)		0.9995	0.9996	0.9995
Mean		0.9995		



- **Precision: -** The precision of the developed method was studied by performing interday and intraday variations. Intraday variations were studied by consecutively injecting the standard and sample solutions for six times on the same day. Interday variations were studied by estimating the drugs present in the multicomponent dosage forms on three different days. Six injections of standard and sample solutions were made every day. The amount of each drug, percentage content, standard deviation, and percentage coefficient of variation were calculated.

Sr. No.	Concentration	Intraday Precision (Area)		
		2 hr.	4 hr.	6 hr.
1.	40 ppm	59496962	59882229	59480300
2.	40 ppm	57759441	58937989	58038778
3.	40 ppm	59523330	60042944	59805956
4.	40 ppm	58692397	59320923	58620689
5.	40 ppm	59849199	58576791	59226044
6.	40 ppm	59061711	59252143	59252143
	N	6	6	6
	Mean	59063840	59335503	59070652
	S. D	689800.2	506656.5	582065.3
	RSD	1.16%	0.85%	0.98%

Sr. No.	Concentration	Interday precision (Area)		
		Day 1	Day 2	Day 3
1.	40 ppm	55338948	55078530	58369547
2.	40 ppm	56853200	55754558	58654360
3.	40 ppm	55809757	55526109	57369136
4.	40 ppm	55246395	56736181	58924856
5.	40 ppm	56578761	55845297	58172893
6.	40 ppm	56151915	56711528	58248697
	N	6	6	6
	Mean	55996496	55942033.8	58289915
	S. D	652230.27	661200.20	484000.4
	RSD	1.16%	1.18%	0.83%

- **Accuracy:-** The accuracy of the RP-HPLC method was evaluated by selecting three different concentrations lower quantitation limit (LQC), medium quantitation limit (MQC), and higher quantitation limit (HQC). In each concentration, a minimum of six injections were given and the amount of the drugs present, percentage recovery, and related standard deviation were calculated. The percentage recovery was calculated using the formula (2): Percentage recovery test/std into 100. Where a is the amount of the

sample drug, b is the amount of sample drug and the standard drug and c is the amount of standard drug added.

Sr. No	Conc. (ppm)	Area	Found conc. (ppm)	Add conc. (ppm)	Area	Found Conc. (ppm)	% Recovery	% RSD
1.	30	39516254	29.25	24	72360081	53.57	99.20	0.42
2.	30	39926545	29.55	24	72421234	53.61	99.28	
3.	30	40022346	29.63	24	72921164	53.98	99.96	
4.	30	41123456	30.44	30	81000038	59.96	99.93	0.57
5.	30	39926661	29.55	30	80199143	59.37	98.95	
6.	30	39118023	28.96	30	80991256	59.96	99.95	
7.	30	39512456	29.25	36	89100032	65.96	99.93	0.28
8.	30	40127612	29.70	36	89141185	65.99	99.90	
9.	30	41120021	30.44	36	88622958	65.60	99.43	

- **LOD and LOQ:-** The LOD and LOQ of Favipiravir was determined by injecting progressively lower concentrations of the standard solutions into the HPLC column using the optimized chromatographic conditions in accordance with 3.3 s/n and 10 s/n criteria, respectively, where s/n indicates signal-to-noise ratio.
- LOD = 3.3×SD/S and
- LOQ = 10×SD/S

Sr. No.	Parameter	Value
1.	Slope	1310010.1
2.	S. D	689800.2
3.	LOD	1.73 µg/ml
4.	LOQ	5.26 µg/ml

- **Robustness: -** For demonstrating the robustness of the method, slight variations in the optimized conditions were done and the standard solution was injected. The variations made were ±5% in the ratio of

- Methanol in the mobile phase, ±0.1 mL/min in the flow rate, ±5 C in the column temperature, and ±5 nm in the wavelength. The separation factor, retention time and peak asymmetry were calculated.

Mobile Phase ratio Methanol: water (65:35)			
Sr. no.	**Concentration**	**R.T**	**Area**
1.	40 ppm	2.250	55319474
2.	40 ppm	2.253	57171130
3.	40 ppm	2.250	56003238
4.	40 ppm	2.250	55985247
5.	40 ppm	2.257	55659005
6.	40 ppm	2.257	56936187
	N	6	6
	Mean	2.252	56179046.8
	SD	0.003430	725984.34
	RSD	0.15%	1.29%

Mobile Phase ratio Methanol: water (75:25)			
Sr. no.	Concentration	R.T	Area
1.	40 ppm	2.290	56387509
2.	40 ppm	2.293	54987765
3.	40 ppm	2.293	55822335
4.	40 ppm	2.297	56429616
5.	40 ppm	2.307	56930984
6.	40 ppm	2.300	55663982
	N	6	6
	Mean	2.296	56037031
	SD	0.006153	687361.45
	RSD	0.27%	1.23%

Flow Rate 0.7			
Sr. No.	**Concentration**	**R.T**	**Area**
1.	40 ppm	2.570	70775669
2.	40 ppm	2.523	72736768
3.	40 ppm	2.543	71735776
4.	40 ppm	2.527	71388006
5.	40 ppm	2.533	71428386
6.	40 ppm	2.523	72592399
	N	6	6
	Mean	2.5365	71776167.33
	SD	0.018063	756734.72
	RSD	0.71%	1.05%

Flow Rate 0.9			
Sr.No.	**Concentration**	**R.T**	**Area**
1.	40 ppm	1.957	55229231
2.	40 ppm	1.957	55838756
3.	40 ppm	1.957	55299441
4.	40 ppm	1.960	56361518

5.	40 ppm	1.957	56624501
6.	40 ppm	1.957	56831041
	N	6	6
	Mean	1.9575	56030748
	SD	0.0012247	680664.47
	RSD	0.06%	1.21%

Wavelength λmax =355			
Sr. No.	**Concentration**	**R.T**	**Area**
1.	40 ppm	2.207	55229231
2.	40 ppm	2.210	55838756
3.	40 ppm	2.210	55299441
4.	40 ppm	2.217	56361518
5.	40 ppm	2.213	56624501
6.	40 ppm	2.217	56831041
	N	6	6
	Mean	1.9575	56030748
	SD	0.0012247	680664.47
	RSD	0.06%	1.21%

Wavelength λmax =365			
Sr. no.	**Concentration**	**R.T**	**Area**
1.	40 ppm	2.250	55078530
2.	40 ppm	2.250	55754558
3.	40 ppm	2.255	55526109
4.	40 ppm	2.250	56736181
5.	40 ppm	2.255	55845297
6.	40 ppm	2.252	56711528
	N	6	6
	Mean	2.252	55942033.8
	SD	0.002236	661200.20
	RSD	0.09%	1.18%

- **Stability: -** The mobile phase, standard solution, and the sample solution were subjected to long-term (3 days) stability studies. The stability of these solutions was studied by storing the standard solution for 3 days and observing for changes in the separation, retention, and asymmetry of the peaks, which were then compared with the pattern of the chromatogram of freshly prepared solution.

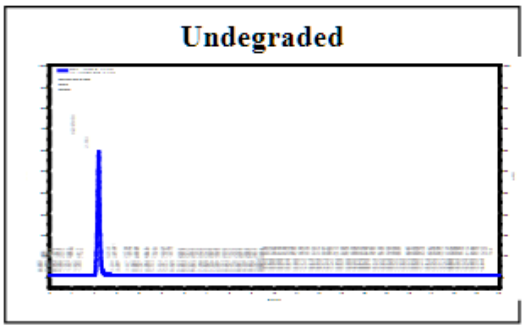
Sr. No.	**Parameters**	**Results**
1	Calibration range (µg/ml)	10-100 (µg/ml)
2	Detection Wavelength	360 nm
3	Mobile Phase	Methanol: Water (70:30)

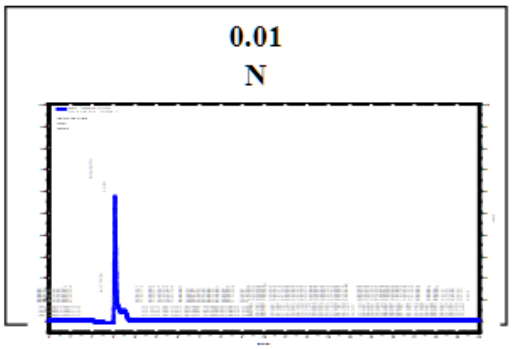
		With 0.05% TEA
4	Retention Time	2.34
5	Standard Deviation	652230.27
6	% RSD	1.16%
7	Correlation coefficient (r^2)	0.9997
8	Theoretical plates (N)	7599

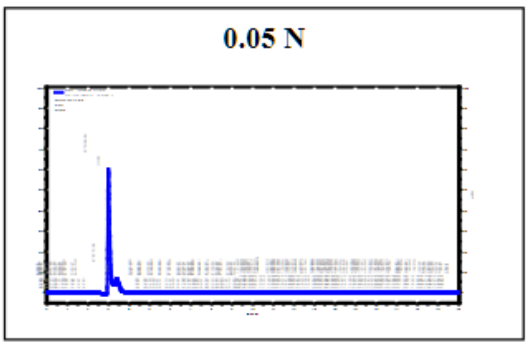
- **Forced degradation: -** Chemical stability of pharmaceutical molecules is a matter of great concern as it affects the safety and efficacy of the drug product. The FDA and ICH guidelines state the requirement of stability testing data to understand how the quality of a drug substance and drug product changes with time under the influence of various environmental factors. . Forced degradation is a process that involves degradation of drug products and drug substances at conditions more severe than accelerated conditions and thus generates degradation products that can be studied to determine the stability of the molecule. The ICH guideline states that stress testing is intended to identify the likely degradation products which further helps in determination of the intrinsic stability of the molecule and establishing degradation pathways, and to validate the stability indicating procedures used.

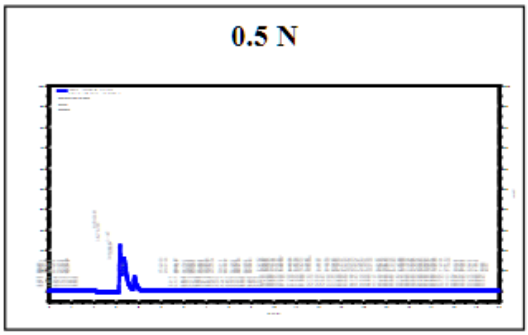
- **Hydrolytic conditions:** - Hydrolysis is one of the most common degradation chemical reactions over a wide range of pH. Hydrolysis is a chemical process that includes decomposition of a chemical compound by reaction with water. Hydrolytic study under acidic and basic condition involves catalysis of ionizable functional groups present in the molecule. Acid or base stress testing involves forced degradation of a drug substance by exposure to acidic or basic conditions which generates primary degradants in desirable range. The selection of the type and concentrations of acid or base depends on the stability of the drug substance. Hydrochloric acid or sulfuric acids (0.01–1 N) for acid hydrolysis and sodium hydroxide or potassium hydroxide (0.01–1 N) for base hydrolysis are suggested as suitable reagents for hydrolysis .If the compounds for stress testing are poorly soluble in water, then co-solvents can be used to dissolve them in HCl or NaOH. The selection of co-solvent is based on the drug substance structure. Stress testing trial is normally started at room temperature and if there is no degradation, elevated temperature (50–70 1C) is applied. Stress testing should not exceed more than 7 days. The degraded sample is then neutralized using suitable acid, base or buffer, to avoid further decomposition.

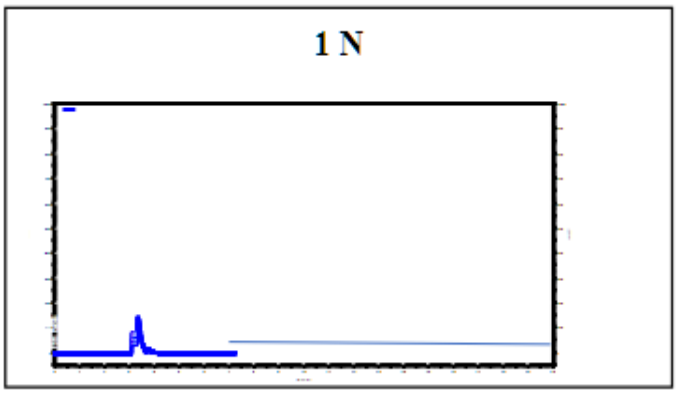
Sr. no.	Time (hr.)	Conc. (N HCL)	Area	% Degraded
1.	0 hr.	Undegraded	82095038	0%
2.		0.01 N	67189022	19%
3.		0.05 N	62982760	24%
4.		0.1 N	54129379	34%
5.		0.5 N	25913001	69%
1.	2 hr.	0.01 N	60426819	27%
2.		0.05 N	58179596	36%
1.	24 hr.	0.01 N	58179902	30%
2.		0.05 N	54129379	34%



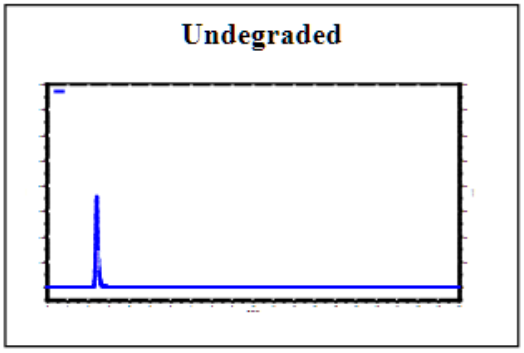


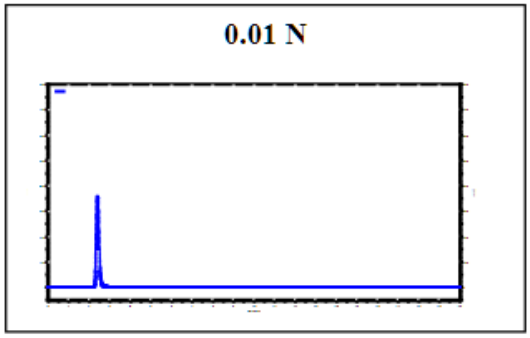


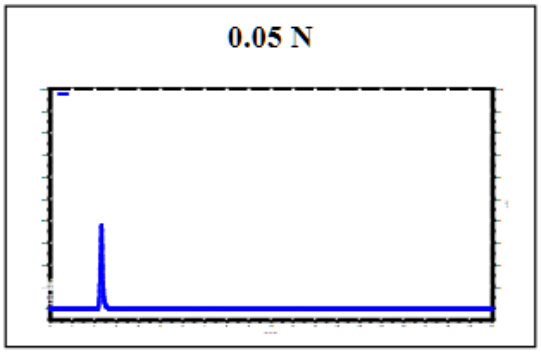


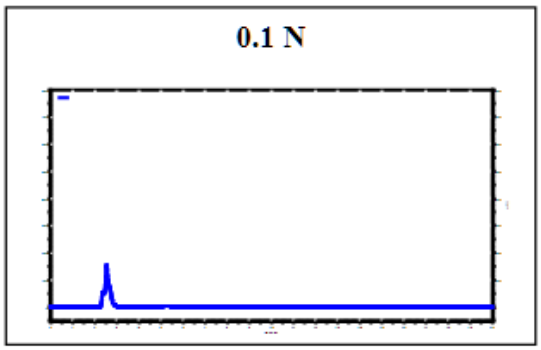


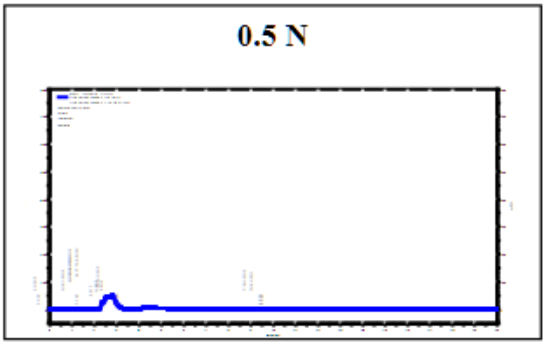
Sr.No.	Time (hr.)	Conc. (N NaOH)	Area	% Degraded
1.	0 hr.	Undegraded	85867394	0%
2.		0.01 N	82553537	4%
3.		0.05 N	72982171	16%
4.		0.1 N	70550192	18%
5.		0.5 N	44686275	48%
1.	2 hr.	0.01 N	79412168	8%
2.		0.05 N	70550192	18%
3.		0.1 N	66805727	22%
4.		0.5 N	40567259	53%
1.	24 hr.	0.01 N	76368468	12%
2.		0.05 N	69149849	20%
3.		0.1 N	65700634	24%

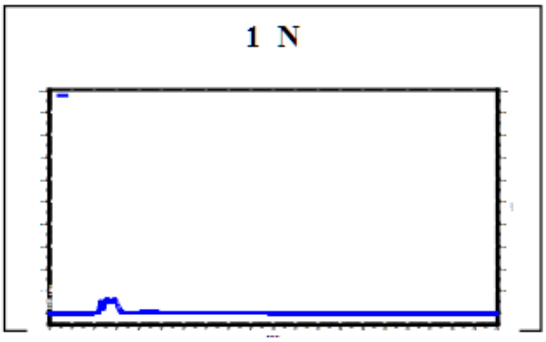






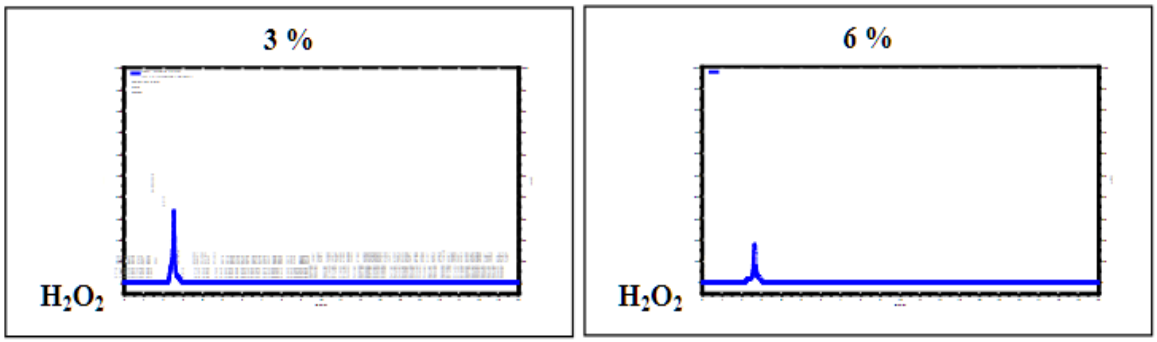






Sr No.	Time (hr.)	Conc.	Area	% degraded
1.	0 hr.	Undegraded	79412168	0%
2.	0 hr.	3%	65933464	17%
3.	2 hr.		58889481	26%
4.	24 hr.		57798660	28%
5.	48 hr.		46150931	42%
1.	0 hr.	6%	57940691	28%
2.	2 hr.		56177571	30%
3.	24 hr.		54104259	32%
4.	48 hr.		51167175	36%

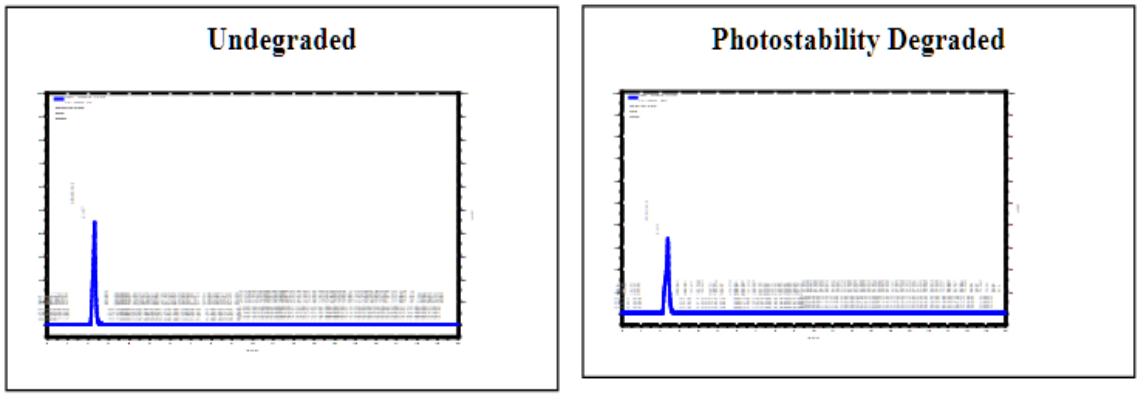
- **Oxidation conditions:** -Hydrogen peroxide is widely used for oxidation of drug substances in forced degradation studies but other oxidizing agents such as metal ions, oxygen, and radical initiators (e.g., azobisisobutyronitrile, AIBN) can also be used. Selection of an oxidizing agent, its concentration, and conditions depends on the drug substance. It is reported that subjecting the solutions to 0.1–6% hydrogen peroxide at neutral pH and room temperature for seven days or up to a maximum 20% degradation could potentially generate relevant degradation products. The oxidative degradation of drug substance involves an electron transfer mechanism to form reactive anions and cations. Amines, sulfides and phenols are susceptible to electron transfer oxidation to give N-oxides, hydroxylamine, sulfones and sulfoxide. The functional group with labile hydrogen like benzylic carbon, allylic carbon, and tertiary carbon or α-positions with respect to heteroatom is susceptible to oxidation to form hydro peroxides, hydroxide or ketone.



- **Photolytic conditions:** -The photo stability testing of drug substances must be evaluated to demonstrate that a light exposure does not result in unacceptable change. Photo stability studies are performed to generate primary degradants of drug substance by

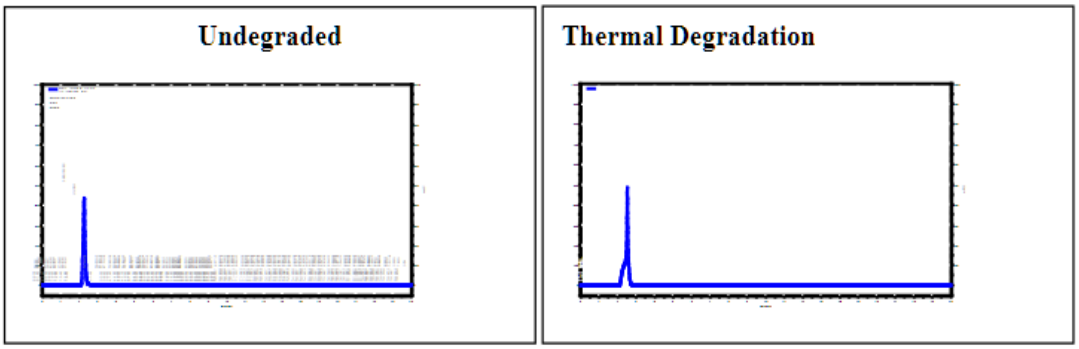
exposure to UV or fluorescent conditions. Some recommended conditions for photostability testing are described in ICH guidelines.[11] Samples of drug substance and solid/liquid drug product should be exposed to a minimum of 1.2 million lx h and 200 W h/m2 light. The most commonly accepted wavelength of light is in the range of 300– 800 nm to cause the photolytic degradation.[26,27]

Sr. No.	Time(hr.)	Area	%Degraded
1.	0 hr.(undegraded)	79412168	0%
2.	2 hr.	73041233	9%
3.	4 hr.	68889140	14%
4.	24 hr.	68572434	14%
5.	48 hr.	66627866	17%



- **Thermal conditions: -** Thermal degradation (e.g., dry heat and wet heat) should be carried out at more strenuous conditions than recommended ICH Q1A accelerated testing conditions. Samples of solid-state drug substances and drug products should be exposed to dry and wet heat, while liquid drug products should be exposed to dry heat. Studies may be conducted at higher temperatures for a shorter period.[22] Thermal degradation study is carried out at 40–80 1C.

Sr. No.	Time (hr.)	Area	%Degraded
1.	0 hr.(undegraded)	79412168	0%
2.	2 hr.	74921932	6%
3.	4 hr.	63828885	19%
4.	6 hr.	59825920	25%
5.	24 hr.	49763353	28%



CONCLUSIONS

A very quick, cost-effective, precise and accurate HPLC method for the determination of FVP has been developed and validated in compliance with ICH guidance Q2.

Besides the short run time (15 min), retention time (2.34) and flow rate of mobile phase (0.8 ml/min) made the method attractive because these features save analysis time and cost.

The developed method showed to be suitable technique to quantify the antiretrovirals and might be employed for quality control analysis as well as in further studies in other matrices, such as plasma.

The method was complete validated showing satisfactory data for all the method validation parameters tested.

In this study, Favipiravir was also subjected to stress studies under various ICH recommended conditions. Degradation products generated from forced degradation studies are potential degradation products that may or may not be formed under relevant storage conditions but they assist in the developing stability indicating method. It is better to start degradation studies earlier in the drug development process to have sufficient time to gain more information about the stability of the molecule.

This information will in turn help improve the formulation manufacturing process and determine the storage conditions. As no specific set of conditions is applicable to all drug products and drug substances and the regulatory guidance does not specify about the conditions to be used, this study requires the experimenter to use common sense.

The aim of any strategy used for forced degradation is to produce the desired amount of degradation i.e., 5–20% A properly designed and executed forced degradation study would generate an appropriate sample for development of stability indicating method.

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