DEVELOPMENT, VALIDATION AND FORCED DEGRADATION FOR QUANTIFICATION OF TECOVIRIMAT IN BULK AND DOSAGE FORMS BY UPLC-MS/MS

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ABSTRACT

UPLC has been used to find a selective and new way to test samples of Tecovirimat in bulk and formulations.The analytical method used phosphate buffer (pH: 7.0): acetonitrile, (75:25 %v/v), the cortecs C18 column and the analysis took five minutes.The flow of mobile phase through the column was 1 ml/min. The size of the sample was 10 µL. The detection was done with a photo diode array detector at a wavelength of 220 nm. At a retention time of 6.22min, Tecovirimat performed well. With a linear range of 1-1000ng/ml, the curve's r2 value of 0.9997 shows a very high degree of correlation. The LOD for Tecovirimat was 0.056ng/ml and the LOQ was 0.104ng/ml.The developed procedure was used for both the bulk and formulation. Accordance with ICH guidelines the drug product was subjected to forced degradation, in order to establish drug

substance and drug product quality as a function of time under the effect of different stressing situations, thanks to the stability-indicating capability of this approach.ICH guidelines say that Tecovirimat degradation was between 5-20%, but paracetamol's degradation was less than 20% in both acidic and basic conditions.

Keywords: Bulk drug, Forced degradation, MS, Quantification, Tecovirimat, UPLC, Validation,

1. INTRODUCTION

The medication tecovirimat can inhibit the envelope-wrapping protein (VP37) of the Orthopoxvirus. It is a white to off white crystalline solid with the chemical formula Benzamide, N [(3aR,4R,4aR,5aS,6S,6aS)-3,3a,4,4a,5,5a,6,6a-octahydro-1,3-dioxo4,6ethenocycloprop[f]isoindol-2(1H)-yl]-4-(trifluoromethyl).The formula is $C_{19}H_{15}F_3N_2O_3$, and the molecular weight is 376.13 g/moL [1-5].

The molecular structure is as follows:

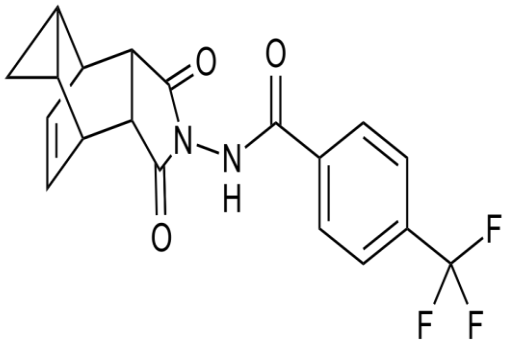

Figure .1. Chemical Structures of Tecovirimat

A search of the relevant literature finds that no procedure using this active moiety has been published. i.e. Using the UPLC technique, we were able to identify and quantify tecovirimat key components down to the ng/ml range in a very short amount of time, with high resolution and sharp peaks.Then, after considering and validating these factors, we were able to build a technique. The new technique successfully quantified and identified substances in the ng/ml concentration range.

2. MATERIALS AND METHODS:

Stainless steel Hypersil ODS-BPS, C18 were used to develop and test a method (250 x 4.6 mm, 3.5) column. The HPLC system has an Agilent 1200 Infinity series HPLC pump, a series PDA detector (Agilent 1200 infinity) and a Rheodyne injector with a fixed 20 L loop.The software used was Ezichrome Elite Compact. On a Shimadzu electronic scale, all of the drugs and chemicals were weighed (AX 200, Shimadzu Corp., Japan).

Sodium hydroxide (NaOH), hydrochloric acid (HCl), anhydrous Tetrabutyl ammonium hydrogen sulphate, Lobachemie Pvt. Ltd. was used to buy sodium dihydrogen phosphate and triethyl amine (Mumbai, India). Merck Specialist Pvt. Ltd. was used to buy HPLC-grade methanol (MeOH), acetic acid (AcOH), and acetonitrile (ACN) (Mumbai, India).All of the reagents and solutions were made with HPLC grade water from a Bio-Age Direct Ultra water purification system (Bio-Age Equipment and Services, Mohali, India).

A capsule formulation containing Tecovirimat 200mg was procured from in local pharmacy store. All chemicals used for this research work was AR grade.

Preparation of standard solution

In a 10 ml volumetric flask, 10 mg of tecovirimat was weighed before 5 ml of acetonitrile was added and sonicated for three minutes. Next, create up to 10ml of the diluent to get the 1000 mcg/ml concentration.

Preparation of Buffer (pH: 7.0)

In a 1000 mL beaker, 7 g of sodium hydrogen phosphate and 1.5 g of tetra butyl ammonium hydrogen sulphate were precisely weighed and combined. Trimethylamine was used to alter the pH to 7.0 after adding 1000 cc of water and stirring with a glass rod to dissolve the salts.

Preparation of mobile-phase: 750ml Mixed to make a buffer, use 250 ml of acetonitrile for every 1000 ml of mobile phase.

Preparation of Reagents and Solutions: Using HPLC grade water, all of the reagents were made to be about the same strength. The stress samples were mixed with the diluent to make a solution with a strength of 0.1% w/v.

*0.1 N HCl Solution:*Using a 10 ml volumetric pipette, a 10 ml aliquot of 1 N HCl solution was placed into a 100 ml volumetric flask. Enough water was added to bring the volume up to the mark, and the mixture was stored at room temperature for 24 hours.

*0.1 N NaOH Solution:*Using a 10 ml volumetric pipette, 10 ml of 1 N NaOH solution was transferred to a 100 ml volumetric flask. It was brought up to the correct volume with water, and the solution was stirred and let to sit at room temperature for 24 hours.

*Oxidative and Thermal Degradation:*Around 0.1 g of the medication was diluted in 100 ml of 30% H2O2 and left out at room temperature for 24 hours to undergo oxidative breakdown. The solid medication was placed in amber vials and heated in a hot air oven at 50 degrees Celsius for 24 hours to test its stability to heat.

*Photolytic Degradation:*The medication was exposed to light in a photo-stability chamber after being spread out as a thin layer in a Petri-dish. The distance between the samples and the light source was 9 inches. In the sample location, the intensity of the fluorescent and UV light was 5200 lux hours. Ten days of exposure to light was given to the samples.

Each sample of deteriorated drug solution for HPLC analysis was diluted with the diluent by a factor of up to ten. Before diluting, the acid and alkali hydrolyzed solutions were mixed with the same amount of NaOH and HCl of the same strength to neutralize them. A 1 mg ml1 solution of each sample was made, and then diluted with diluent up to 10 times, for examination of solid medication subjected to heat and photolytic degradations. Before being injected into the HPLC, each diluted sample was filtered via a membrane filter (0.45 m, 13 mm).

Method optimization Consideration:

A literature search revealed no high-resolution, high-sensitivity analytical techniques that could quickly, accurately, and without interferences identify and quantify the components in the formulation and bulk at a low concentration and with a small sample size. By modifying the buffer composition in addition to the solvent and column temperatures and detector wavelengths, instrument technique may be optimized.

Optimization of Chromatographic conditions:

Phosphate buffer (pH: 7.0): acetonitrile, (75:25%, v/v) with a cortecs c18 column provided the optimum chromatographic conditions after many attempts (100 x 4.6 mm, 2.7). The Tecovirimat was set up with a flow rate of 1 ml, a detection wavelength of 220nm, a column temperature of 25°C, and a sample compartment temperature of 10°C.

3. ANALYTICAL METHODVALIDATION

The developed method was tested with a strict limit to show that it works.

Precision:

Method precision was evaluated using a limit of 2% RSD for the retention duration and area NMT after it had been validated for specificity and system appropriateness. The intermediate precision was validated the next day using a different column and a 2% RSD limit for retention time and area. The next day, a different column was utilized to verify that the estimated intermediate accuracy was really accurate.

Linearity:

Robustness:

To test how well the method works, the optimized parameters for the mobile phase composition, column temperature, and flow rate had to be changed.

LOQ:

The sample was introduced into the system at 10% of the desired concentration, with the S/N ratio set to be less than 10. Using the 3:1 acceptance criterion and a minimum detectability of five out of six injections from the same concentration, the LOQ concentration was injected into the assay to assess the detectability of the various concentration preparations.

Standard calibration

The tecovirimat stock solution (1.0mg/mL) was produced in Methanol as per industry standards. The standard stock solution was kept at 2–8ºC in the fridge until analysis. Analytical standards were prepared from a stock solution prepared (1.0 mg/mL) and diluted in diluent (mobile phase) to yield concentrations of 1ng/ml to 1000ng/ml; these solutions were stored in the refrigerator at 2–8ºC until testing.

Method validation

The method that was made was tested over a range of concentrations from 1.0ng/ml to 1000ng/ml. In the validation section, we looked at the system's suitability, LOQ, linearity, precision and accuracy, selectivity and specificity, matrix effect, and recovery.

System suitability:

To make sure that the optimized method was giving the same results every time, Six injections of the standard were performed with the desired retention time RSD of % in consideration.

Selectivity and Specificity:

To validate the method's superiority in terms of selectivity and accuracy, 100 ng/ml of Tecovirimat at a concentration of 100 ng/ml was injected in triplicate. Next, a blank was inserted to demonstrate that the technique did not have a carryover problem. Specificity is limited by meeting the requirements for system appropriateness, and there was no R_T shift for any of the three preparations.

Linearity:

Calibration standards were made to get a linearity range of 1, 2, 10, 50, 100, 200, 400, 800, and 1000ng/ml, and they were tested five times on five different days.

Precision:

After the method was optimized, it was tested for its precision to show how close the measurements from a single homogeneous sample were to each other, with a limit of 2% RSD for the area NMT. The next day, we used the same 2% NMT area RSD tolerance to verify the intermediate accuracy.

Accuracy and Recovery:

Quality control standards were made with four levels: 80%, 100%, 120%, and a limit of NLT98% Recovery to show that they were accurate.

Robustness:

The method was tested by making small changes to the composition of the mobile phase, the temperature and flow rate of the column, and the pH of the buffer.

Lower LOQ:

Six Lower level of quantification standards were used to measure the parts, since the LOQ has a S/N ratio of 10 and the LOD has a S/N ratio of 3.

Filter Compatibility:

To make sure that the filters were compatible, the assay was done on the samples that went through the PVDF and Nylon filters.

Assessment of stability:

The optimized concentrations of the standards were injected for up to 72 hours to find out how stable the standards and the mobile phase were at the same time, with a 2% RSD limit for the area NMT.

4. RESULTS AND DISCUSSION

Method development:

UPLC was chosen as the technique of choice on the path to developing a simple and easily applicable method for determination of Tecovirimat in bulk and dosage form samples. Sample extraction parameters were optimized in a methodical and systematic manner throughout the technique developed process for chromatographic conditions.

Different brands of UPLC C18 columns were used to separate the Tecovirimat. Initial separation was done with isocratic elution of 20mM ammonium formate, and acetonitrile was chosen as the mobile phase. Different combinations were tried, but the response was low.A mobile phase made of phosphate buffer (pH-7): acetonitrile (75:25% v/v) gave the best response, but the shape of the peaks was bad.

After a number of tests, a phosphate buffer (pH: 7.0) mobile phase. The best peak shape was found when the cortecs C18 column was used with acetonitrile (75:25, v/v).The Tecovirimat worked well when the temperature of the column was 25°C, the temperature of the sample compartment was 10°C, the flow was 1 ml, and the detection wavelength was 220nm.

Method validation:

At the time of Tecovirimat main component elution times, there was no interference seen. The level of concentration that can be found is 0.1ng/ml (Fig.2 and 3).

Linearity was shown as the area of a peak on the y-axis and the concentration of Tecovirimat in ng/ml on the x-axis. Over a linearity range of 1 to 1000ng/ml, calibration curves for Tecovirimat were found to be accurate and precise every time. The coefficient of correlation was higher than 0.9979. The standard deviation was less than 15%, and the average accuracy was between 99.46 and 103.24%. The results were shown in Figure 4.

System and method precision Intra and inter batch %accuracy for Tecovirimat ranged from 99.63 to 100.38, 99.52 to 99.87, %CV is 0.20 to 4.88, and 0.22% to 0.93%. This showed that the developed method could be used again and again (Table.1).

The average percentage of recovery at each level was found to be 96.25%, 97.72%, and 95.25 percent, respectively. The mean percent recovery and percent CV of Tecovirimat levels are 96.24 percent and 1.02 percent, respectively (Table-2.0).

To show that the method was stable, it was tested with small changes to the flow, the composition of the mobile phase, the pH, and the temperature of the column. With only small changes to the method parameters, the results in Table 3 show that the method was able to produce accurate and consistent results.

By injecting the lower concentrations with the S/N ratio criteria, the LOQ and LOD could be found. The S/N ratio was 11.5 and the LOQ was 0.104ng/ml. The S/N ratio was 2.8 and the LOD was 0.056ng/ml (Table. 4 and 5).

The method works well for testing how stable the Tecovirimat standard drug solution is. When Tecovirimat in solution form is injected at different times, it does not change in a way that is important.All of the stability samples had pure drug peaks, and no chromatograms showed any extra peaks. So, it was found that the drug solution was stable for up to 72 hours (Table.6.0).LC-PDA chromatography of Tecovirimat standard solution showed no signs of impurities. Water, 0.1, 2, and 5 N solutions of HCl, and NaOH, respectively, kept the medication stable at 85 C for 8 hours.In HCl, alkaline, and peroxide mediums, the drug was degraded into one product. In solid form, the drug was not affected by heat or light. So, these results show that the drug can handle acid, base, and peroxide conditions (Table.8 and Fig. 5 to 9).

The method was used to figure out how much Tecovirimat was in the brand that was for sale. The assay for tranexamic acid was 99.96%, and the method had already been proven in bulk measurements for accuracy and linearity, among other things.The results showed that the method was a good way to figure out how much of a product was left in cleaning samples.

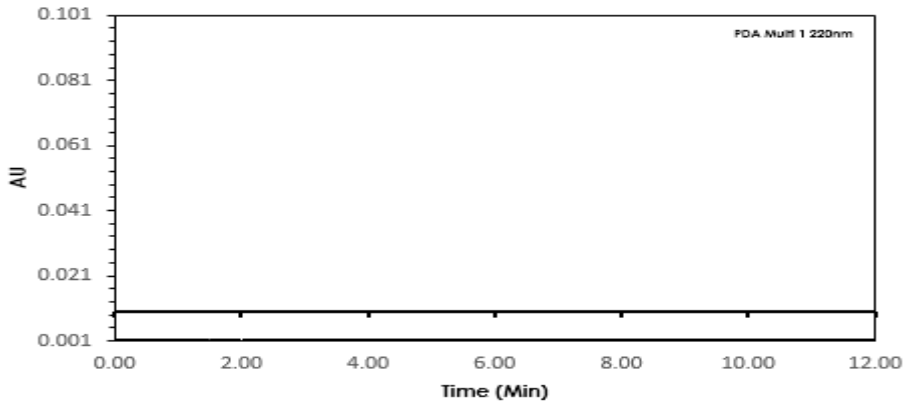

Figure.2. Chromatogram for mobile (Diluent) phase

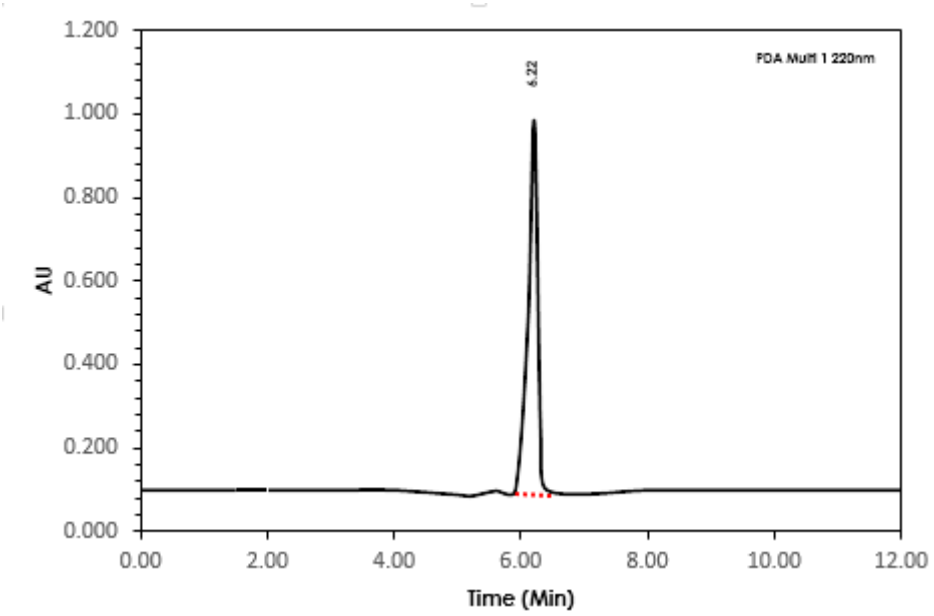

Figure.3. Standard chromatogram of Tecovirimat

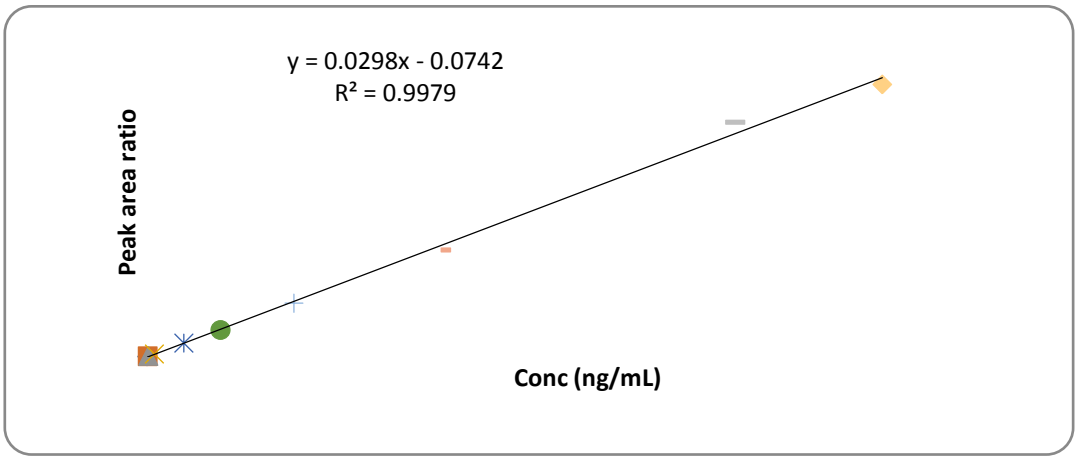

Figure.4. Linearity of Tecovirimat

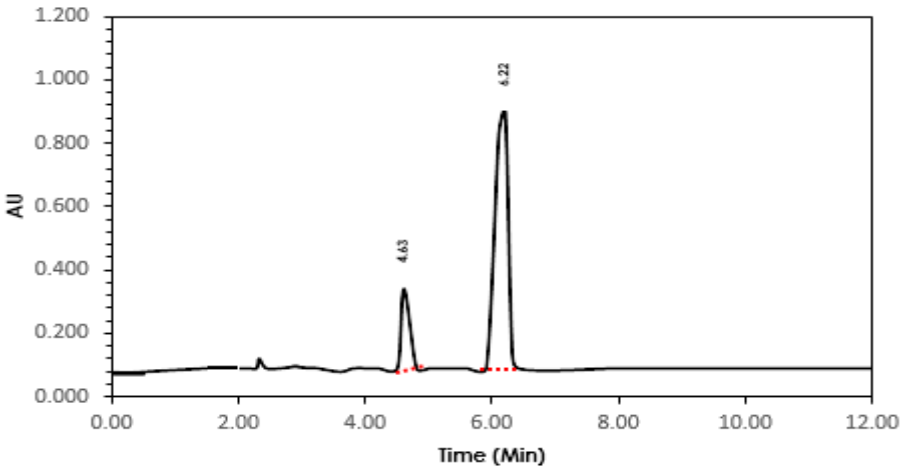

Figure.5. Chromatogram for acid degradation

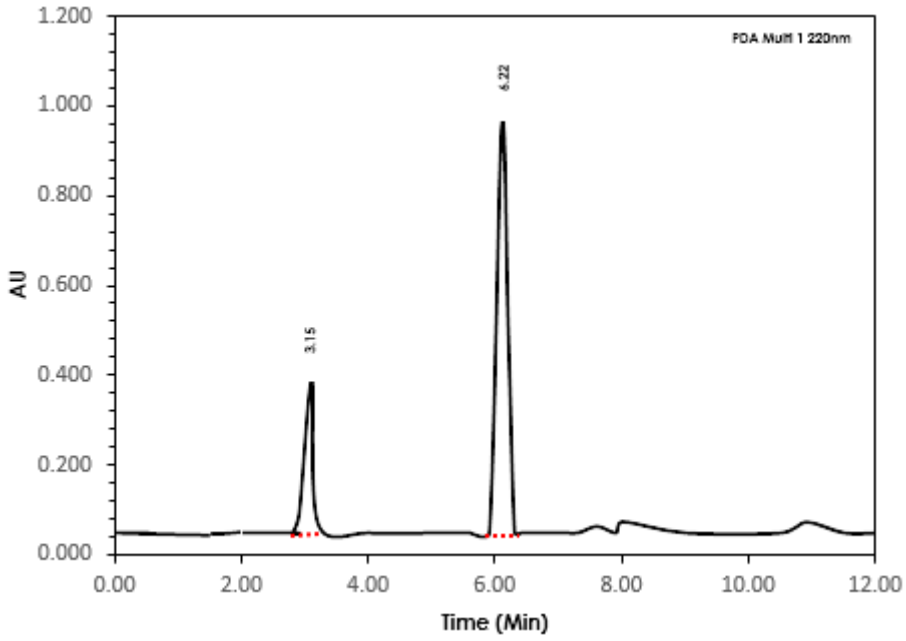

Figure.6. Chromatogram for base degradation

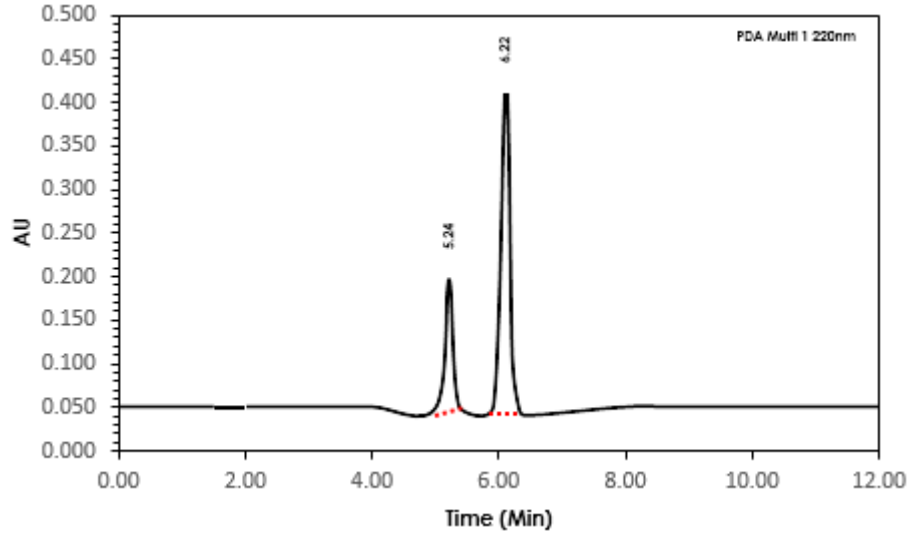

Figure.7. Chromatogram for peroxide degradation

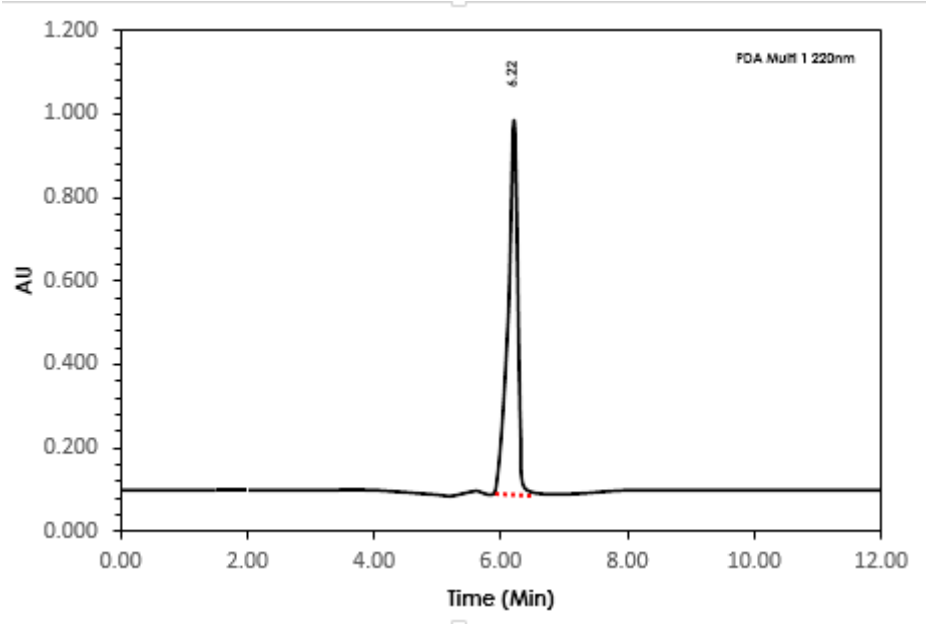

Figure.8. Chromatogram for photolytic degradation

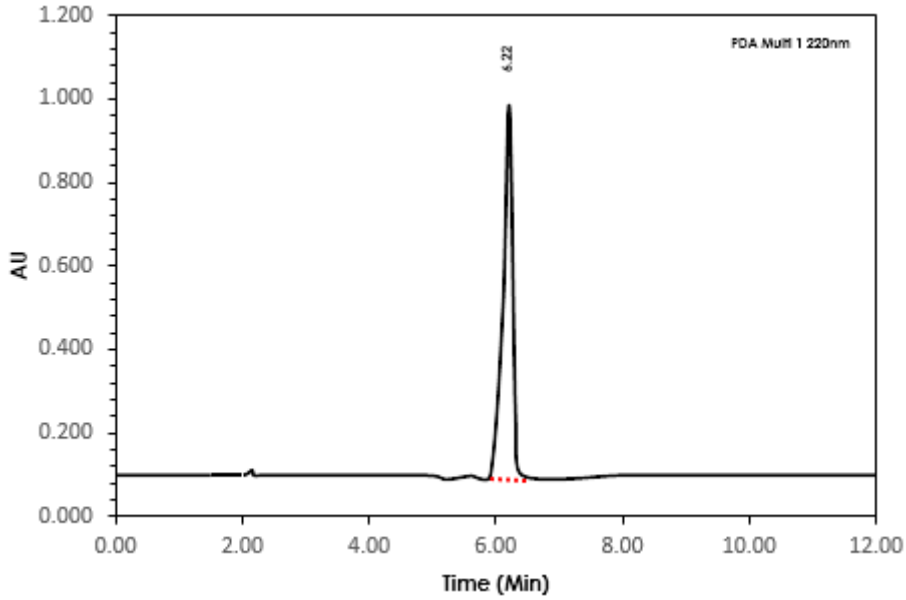

Figure.9. Chromatogram for thermal degradation

Table.1. Tecovirimat Precision and Accuracy

Standard Concentration (ng/ml)	(Intraday)			(Interday)		
	Concentration measured (ng/ml) (n=6; mean)	%CV	%Accuracy	Concentration measured (n=6;ng/ml; mean)	%CV	%Accuracy
1	1	4.88	100.38	0.97	0.22	99.52
500	499.69	0.20	99.94	492.95	0.93	98.59
800	797.05	0.29	99.63	798.97	0.67	99.87

Table.2. Recovery data

Level of % recovery	Target Conc. (ng/mL)	Amount of drug spiked (ng/mL)	Nominal conc. (ng/mL)	Drug recovered (ng/mL)	% Recovery	Mean	SD	%RSD
				180.13	100.07			
				180.01	100.01			
80	100	80	180.00	180.25	100.14	100.07	0.07	0.07
				200.12	99.94			
				200.20	100.10			
100	100	100	200.00	200.10	100.05	100.03	0.08	0.08
				220.21	100.10			
				220.18	100.08			
120	100	120	220.00	210.85	95.84	98.67	2.45	2.49

Table 3: Robustness Data

Condition	Value	Tecovirimat	
		RT	Asymmetry
Flow	0.7 ml/min	6.21	1.051
	0.8 ml/min	6.21	1.012
	0.9 ml/min	6.22	1.011
Mobile Phase (Buffer: Acetonitrile)	80/20 v/v	6.251	1.048
	75/25 v/v	6.121	1.012
	70/30 v/v	6.101	1.001
P^H	6.8	6.212	1.21
	7.0	6.121	1.01
	7.2	6.212	1.05
Column Temperature	23	6.119	1.031
	25	6.122	1.012
	27	6.118	1.001

Table 4: LOQ

Drug Name	Area	LOQ	S/N ratio
Tecovirimat	4261	0.104ng/ml	11.5

Table 5: LOD

Drug Name	Area	LOD	S/N ratio
Tecovirimat	865	0.056ng/ml	2.8

Table 6: Filter Compatibility

Drug	PVDF Filter Assay 0.2µm	Nylon Filter Assay 0.2µm
Tecovirimat	100	99.3
Difference	0.7% For Tecovirimat	
Suitability	PVDF 0.2µM Filter	

Table 7: Stability of standard stock solution

Drug name	Tecovirimat	
Injection Time (Hours)	**RT**	**Response**
Initial	6.23	525528
12	6.22	525693
24	6.22	525489
36	6.21	525465
48	6.23	521634
72	6.21	521698
Mean	**6.22**	**524251**
SD	**0.00063**	**2004.14**
.%R.SD	**0.02**	**0.38**

Table 8: Degradation behavior of Tecovirimat

Drug name	Condition	Peak response	.% Degeneration
Tecovirimat	**Un Degraded**	524365	-
	Acid	14682	2.8
	Base	23072	4.4
	Thermal	0	0.0
	Photo	0	0.0
	Peroxide	40376	7.7

5. CONCLUSION

This paper presents the development and validation of a 1.0–1000ng/ml detection approach.The intra and inter batch precision (%CV) was below 15%, while the %accuracy was over 95%.The overall percentage recovery for Tecovirimat greater than 90%. This method's selectivity, sensitivity, precision, and accuracy make it appropriate for the current investigation.In conclusion, the method used in this study is easy and quick to use. It also has good accuracy, precision, selectivity, and stability.The method that was made and improved was validated according to ICH Q2B rules. All of the strict criteria that were set up front were met by the method.Success was had in identifying and quantifying the primary components of the commercially available formulation using the established approach. They were pleased with the outcomes that were achieved.The created approach had an extremely low LOQ and LOD, and when the LOQ precision was checked, the % RSD was determined to be under 1%.This method is also very specific because of the inherent selectivity and sensitivity of UPLC. It also has many benefits, such as being new, sensitive, cost-effective, fast, accurate, and using a small sample volume.

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7. CONFLICT OF INTEREST

The authors say that there are no competing interests.

8. FUNDING

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