Biological Forum – An International Journal **15(4): 844-853(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

HPLC Method Development, Validation, and Degradation Study of Fosetasmvir by: A Comprehensive Analytical Investigation

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(Received: 15 February 2023; Revised: 14 March 2023; Accepted: 20 March 2023; Published: 18 April 2023)
(Published by Research Trend)

ABSTRACT: This study aimed to quantify Fostemsavir and investigate its breakdown patterns using HPLC and LC-MS techniques. Fostemsavir was obtained as a free sample from Spectrum Laboratory Pvt. Ltd. Solvents and other substances were purchased from S.D. Fine Chemical and the Research Lab. Reliable and precise results were ensured by using high-quality reagents and suitable filtration methods. A Chromatographic Phenomenon C18 column (250 4.6 mm, 5 µm) was employed with a 70:30 methanol: phosphate buffer solvent ratio. UV radiation was used to measure the absorbance of a 10 µg/ml Fostemsavir solution in methanol, with a maximum absorbance observed at 278 nm. The method was validated using ICH standards and demonstrated accurate identification, quantification, and detection of breakdown products. This approach is suitable for routine laboratory analysis and quality control of Fostemsavir.

Keywords: HPLC, Fosetasmvir, Forced degradation, LC-MS, ICH.

INTRODUCTION

Chemically compound name for 1H pyridin-1-yl-methoxy) phosphonic acids is C25H26N7O8P, with its molecular mass is 583.498 g/mol. In water-based solutions with a pH of higher than 3.7, it is solvable to a concentration in excess of 250 mg/ml. It is solid in terms of its physical composition. Rakobia is one of the brand names for the drug fostemsavir. Fosteremsavir is the name of the phosphonooxy methyl prodrug of temsavir, a new HIV-1 adhesion blocker. It is an antiviral therapy drug for HIV-1. Temsavir, a first-in-class HIV-1 adhesion blocker that attaches to the virus surface the glycoprotein 120, is the active component of the drug fostemsavir (Moore *et al.*, 2019).

Chronic ART (antiretroviral therapy) and the resulting extension of life may raise the risk of chronic damage to the liver and kidneys in HIV-1 positive individuals. This could be caused by a variety of elements, namely the direct impacts of the virus, negative side effects from antiretroviral medications, as well as potential coinfection consequences. Temsavir is a prodrug that comes before the HIV-1 adhesion blocker fostemsavir, a first-in-class medication (Gartland *et al.*, 2021). it functions adhering to gp120, viral protein charge of anchoring HIV-1 to the CD4 receptors on host cells. By inhibiting this replenishment, fostemsavir prevents the virus from entering and infecting the host cells (Sivagami B *et al.*, 2018; Jain *et al.*, 2011). Fostemsavir achieves this without damaging the host cells. Fostersavir is a novel HIV treatment because it targets the early phases of the viral replication cycle. It offers an alternative for patients with few treatment alternatives or who have built up resistance to other types of antiretroviral drugs (Peerzade *et al.*, 2019).

Structure of Fostemsavir: (Stewart *et al.*, 2021**)**

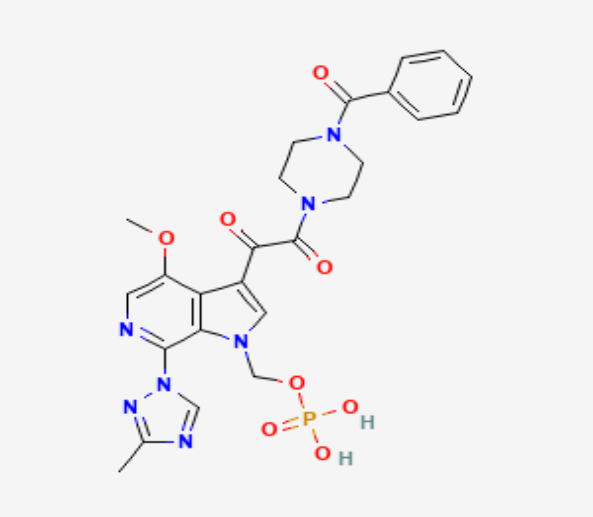

Fig. 1. Structure of Fostemsavir.

MATERIALS AND METHODS

Instrumentation**:** India employed a Schimadzu type LC 2030 plus HPLC apparatus. This research strategy was established as well as validated using a featured detector. The process used a Phenomenon C18 column (250 4.6 mm, 5 m), a moving phase with a methanol: phosphate buffer solution ratio of 70:30, an ultrasonicator (Pci-Analytics), a pH metre (Mettler Toledo), and an electronic balance (Mettler Toledo) that were all properly calibrated to aid in the validation procedure.

Chemicals and Reagents: Methanol (Manufactured by S.D. Fine Chem), Distilled water (Milli Q) (In house production) Phosphate Buffer, Formic Acid

Preparation of Buffer Solution: 1 mL of formic acid is combined with 1 litre of HPLC-grade water, which is then passed through a 0.45 m membrane filter.

Wave Length (λmax)

Using a photo diode detector, the wavelength of the medications' maximal absorbance in a solution of phosphate buffer with Methanol that has been pH-adjusted to 6 (70:30% v/v) was scanned in the range of 200–400 nm.

Chromatographically circumstances. as the stationary phase, we used Column C18 (250 x 4.6mm, 5). The chromatography system was set to operate in an isocratic mode, and the mobile phase was made up of Methanol: Phosphate Buffer that had been pH-adjusted (70:30%v/v). With ambient temperature and an on-going flow rate of 1 mL/min, isocratic technique for separating was used. A 278nm detection wavelength was utilised. (Pathade *et al.*, 2019).

Standard Stock Solution Preparation

Accurately measure 10 mg of the reference standard for Fosetasvir in a 100 ml volumetric flask, add 70 ml of methanol, mix it up, and finally fill up to the required level with methanol. As a result, 100 µg/ml standard stock solutions for fosetasvir were created.

Method Validation (Kotecha *et al*., 2018; ICH 2007).

The developed approach was confirmed (Q2) in accordance with ICH guidelines, and the criteria "specificity, accuracy, precision, linearity, robustness, Limit of Detection (LOD) and Limit of Quantification (LOQ)" were assessed.

Linearity: Chromatograms of six linear concentrations 3, 6, 9, 12, 15, and 18 µg/ml were created from the stock solution. Individual mean peak areas were determined using chromatograms, and the average of the peak areas at each concentration were used to construct the calibration curve. The drug's coefficient of correlation was found to be 0.9991. Table 1 detailed the fostemsavir linearity data, while Fig 2 showed a calibration curve.

Specificity: Additionally, example the chromatograms of stressed samples under different pressure settings showed that fostemsavir was clearly separated from its breakdown products, illuminating the technique's selectivity. There were no peaks at the absorption period for fostemsavir in the HPLC chromatograms obtained for the blank mixture as well as the blank solution exposed to breakdown circumstances. In accordance the results of the HPLC chromatograms obtained for the blank, placebo, standard, and also sample solution chromatograms shown in Fig 3, the fostemsavir signal was unaffected by diluents as well as the placebo.

Table 1: Linearity data of fostemsavir.

	Concentration(µg/mL)	**Region**
sample	3	78141
	6	107920
	9	149923
	12	179227
	15	218296
	18	255892
Equation	y = 11897x + 39981	
Regression	0.9991	

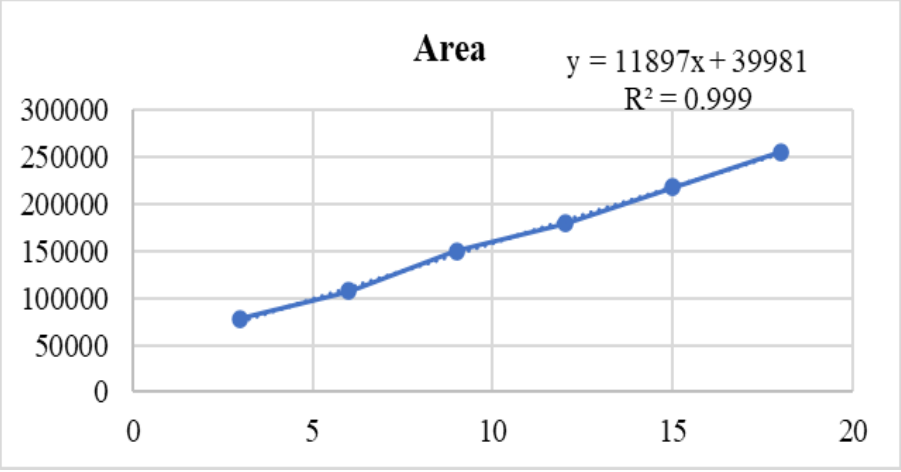

Fig. 2. Calibration curve of fostemsavir.

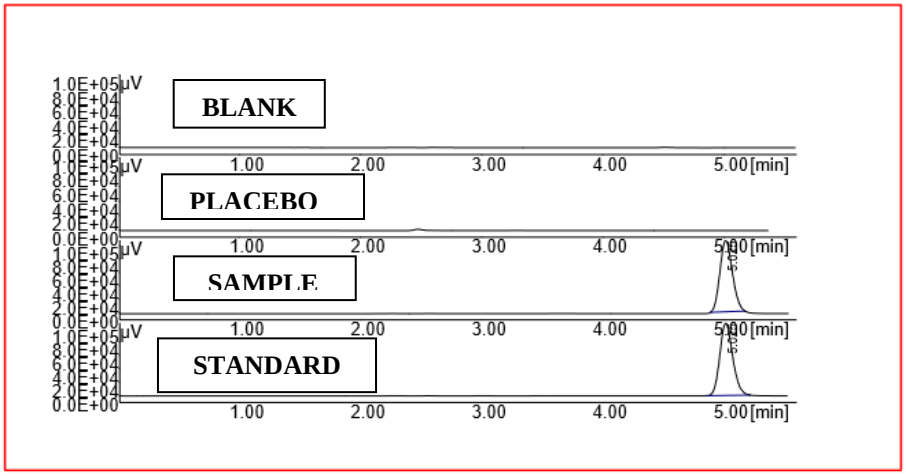

Fig. 3. Chromatogram of specificity for fostemsavir.

Accuracy: The recovery rate of fostemsavir was calculated using the conventional combination approach; therefore, every phase should have a percentage recovery of 98.0 to 99.78. We determined the standard deviation, average area, and percent RSD because the mean values of the medicine's estimated and actual dosages were found to be remarkably similar. These results are shown in Table 2 along with the percentage RSD.

Precision: Six analyses were conducted using a standard stock solution containing the target analytes to evaluate the intra-day precision. Similar to intra-day precision, inter-day accuracy was evaluated utilising replicate analyses over a three-day period using the same concentrations of all analytes. The % RSD less than 2.0 and was shown in Table 3.

LOD: The standard deviation as well as slope was used to determine the detection limit, which was found to be 0.59 µg/ml

LOQ: The standard deviations as well as slope of the response were used to determine the quantification limit, which was found to be 1.34µg /ml.

Robustness: The stability of an analytical technique, which assesses its capacity to remain undisturbed by subtle but deliberate changes in technique variables, provides an indication of its reliability in usual circumstances. By assessing the sample solution containing 10 gmL^{-1} of fostemsavir under various variations of the technique variables, like flow rate as well as mobile phase composition, which are shown in Table 4, robustness was assessed.

Table 2: Accuracy studies on Fostemsavir.

Concentration Mixture (µg/mL)	Calculated conc. (µg/mL)	Standard Deviation	Relative Standard Deviation (%)	Accuracy (%)
10+8 18(µg/mL) 80%	17.91	81121.04	0.9592	98.99
	17.90	81121.04	0.9592	98.99
	17.89	81011.28	0.9435	97.92
10+10 20(µg/mL) 100%0	19.91	92166.02	0.9023	99.37
	19.98	92135.02	0.9188	99.7
	19.48	92145.99	1.0028	99.6
10+12 22(µg/mL) 120%	21.99	17073.21	0.9901	99.78
	21.98	17048.09	0.9861	99.34
	21.99	17079.00	1.0119	99.78

Table 3: Precision studies of Fosetasmvir.

Sample	Intraday		Interday	
Injection	**Area**	**RSD (%)**	**Area**	**RSD (%)**
1	357142.75	1.32	352537	1.34
2	351285		358729	
3	355478		359271	
4	362371		359271	
5	364444.5		358796	
6	359141		367542	

Table 4: Change in Flow rate.

Parameter	Condition	Retention time
Flow rate	0.9 mL	5.0
	01 mL	4.9
	1.1 mL	4.8
Mobile phase composition	Methanol: Phosphate buffer (68:22 % *v/v*)	4.8
	Methanol: buffer Phosphate (70:30 % *v/v*)	4.9
	Methanol: Phosphate buffer (72:28 % *v/v*)	4.8

Analysis of Formulation: The analysis of the pharmaceutical samples that had been separated did not show any alteration in the duration of retention. Excipients, which are typically contained in tablet formulations, had no impact on the outcomes. The amount of the drug was found to be 101.10% with a percent relative standard deviation of 0.0079%, as shown by the TABLE. It was concluded as a result that fostemsavir had not broken down in commercial formulations. The technique's suitability for the routine analysis of fostemsavir in commercial formulations, as shown in Fig. 4 and Table 5, was demonstrated by the % RSD value.

Forced Degradation Studies for Fostemsavir

Research on pressure degradation was done, putting the chromatographic analysis at risk. In settings that are bitter, alkaline in nature oxidative, as well as photolytic, fostemsavir was found to have substantial impacts.

Acid Degradation: Samples for deterioration were made. The representative chromatogram of fostemsavir under acid degradation is given in Fig 5 and the percentage degradation is specified in Table 6 the results for various combinations.

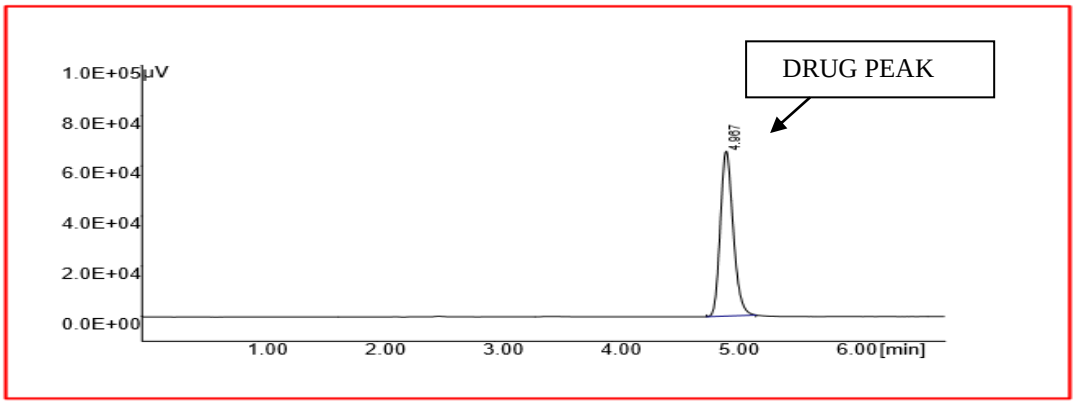

Fig. 4. Chromatogram of formulation of fostemsavir.

Table 5: Analysis of formulation for fostemsavir.

Quantity each tablet(mg)	Quantity Found (mg)	Percent Found	Average percentage	±Standard deviation	%Relative standard deviation
182	183.09	100.40	101.10	0.80	0.0079
182	185.46	101.30			
182	183.03	101.61			

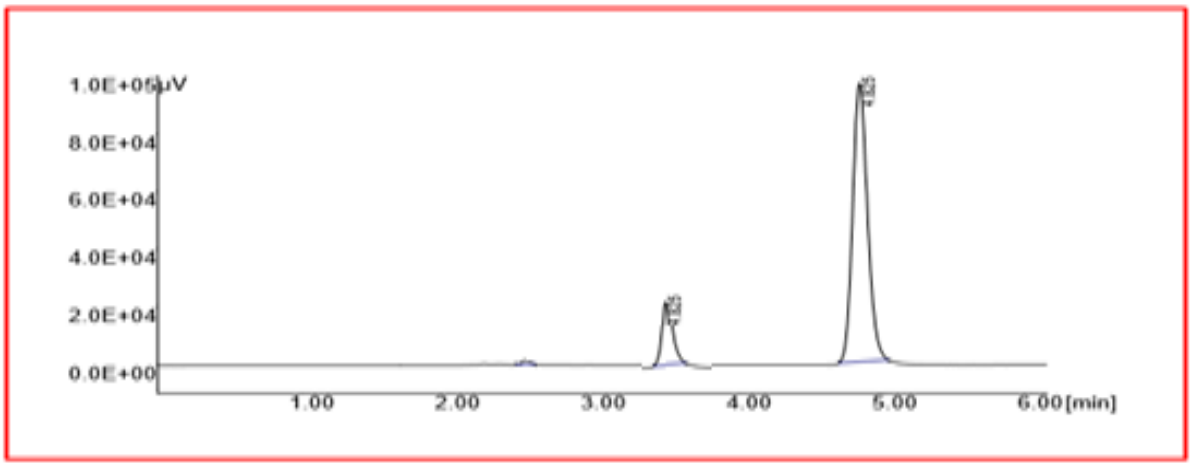

Fig. 5. A Chromatogram of fostemsavir that has been treated with acid (0.1N Hydrochloric at 60°C).

Table 6: Acid degradation results of fostemsavir.

Sr	Stressed condition	Number of degradants form	RT of degradation product	Area		% Degradation
				Unstressed	Stressed	
01	0.1 *N* HCl, 60^0**C** reflux for 15 min.	01	3.49	194258	170630	12.16

After being exposed to 0.1 N HCl for 15 minutes at 60°C, fostemsavir displays one degradants. When injected for chromatographic examination, the treated sample of fostemsavir exhibits an extra peak at 3.49 min and undergoes 12.16% degradation.

Alkali Degradation: For 15 minutes at 60°C, fostemsavir is allowed to reflux with 0.1 N sodium hydroxide solutions. When the above-mentioned sample solution was injected for HPLC analysis, the results showed the creation of an extra peak that indicated fostemsavir degradation in an alkaline environment. The highest value for the breakdown of the product was obtained at a time frame for retention of 2.5 min, and fostemsavir's breakdown rate is 16.70%. Fig. 6 and Table 7 both show the deterioration peak and percent degradation, respectively.

Oxidative Degradation: When the medication was exposed to 3% H_2O_2 for 48 hours in the dark, one degradants was discovered. There was an extra peak with retention duration of 2.5 minutes when the stressed sample was heated for 5 minutes and tested after cooling. Oxidative degradation depicted in Fig. 7 and result of degradation in Table 8.

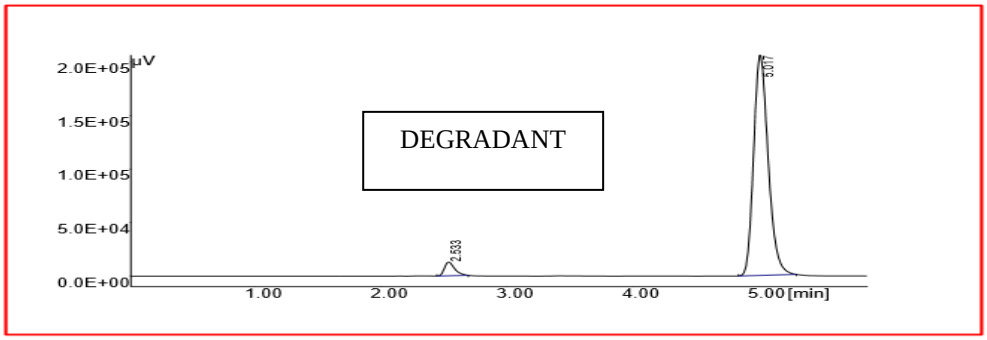

Fig. 6. Chromatography of fostemsavir after alkali treatment (0.1N NaOH at 60°C for fifteen minutes).

Table 7: Alkali degradation results of fostemsavir.

Sr	Stressed condition	Number of degradants form	RT of degradation product	Area		% Degradation
				Unstressed	Stressed	
01	0.1 N NaOH 60^0**C** reflux for 15 min.	01	2.5	194258	161809	16.70

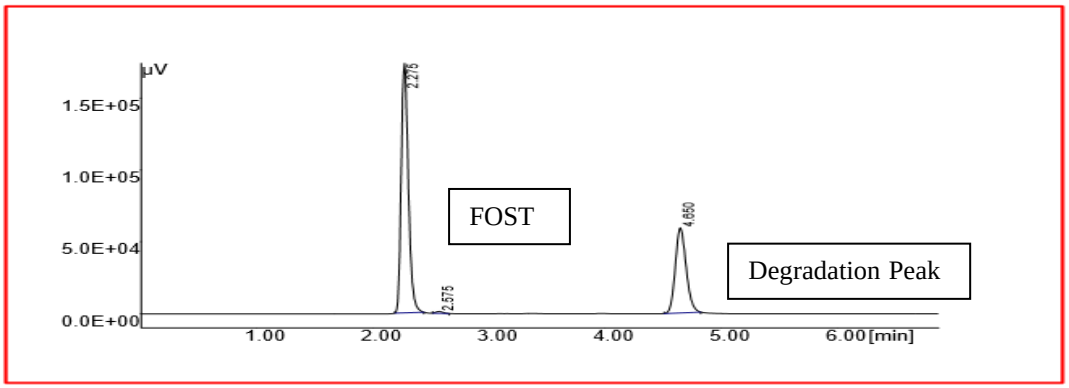

Fig. 7. Chromatogram of oxidative degradation studies of fostemsavir (3% H_2O_2 for 48hrs).

Table 8: Oxidative degradation results of fostemsavir

Sr. No.	Number of degradants form	RT of degradation product	Area		% Degradation
			Unstressed	Stressed	
01	01	2.5	194258	62033	68.06

Wet Heat Degradation: Fostemsavir does not exhibit a new peak in the chromatogram when subjected to wet heat degradation, which is when it is refluxed with water for 30 min at 60°C. Comparing the peak areas of stressed and unstressed done indicates no change in peak areas, providing additional confirmation. Thus, it was determined that the medication was stable under the testing conditions. Chromatogram of Wet heat degradation depicted in Fig. 8 and results of degradation study depicted in Table 9.

Dry Heat Degradation: Fostemsavir was kept under dry heat drug conditions for one hour at a temperature of 80 °C. Analysis of the strained sample revealed no deterioration. With a temperature increase to 100°C, the heating time was further prolonged to 2, 3, 4, and 5 hours. There was no evidence of degeneration. Chromatogram of Dry heat degradation depicted in Fig. 9 and results of fostemsavir degradation in Table 10.

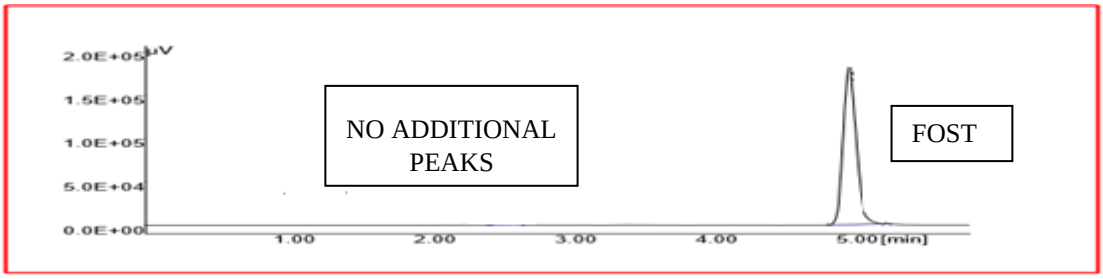

Fig. 8. Chromatogram of Wet heat degradation results of fostemsavir

Table 9: Wet heat degradation results of fostemsavir.

Sr	Stressed condition	Number of degradants form	RT of degradation product	Area		% Degradation
				Unstressed	Stressed	
01	Reflux with water 60^0C , for 30 min.	00	NA	194258	192988	NA

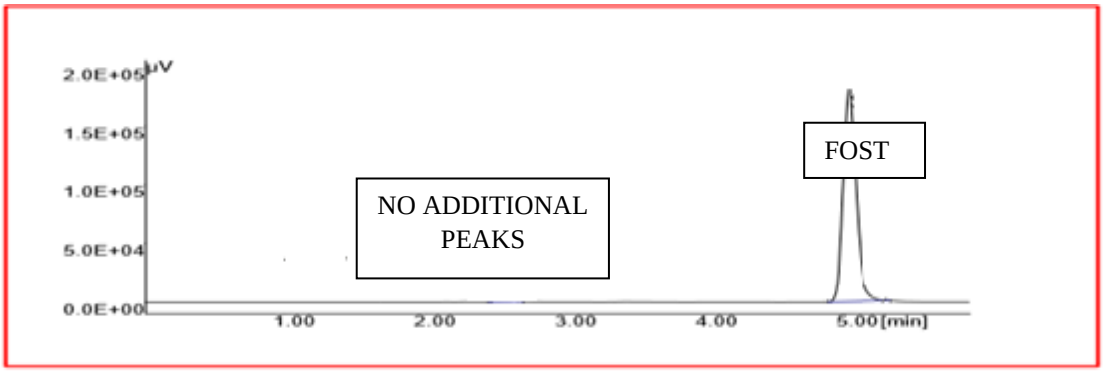

Fig. 9. Chromatogram of dry heat degradation results of fostemsavir

Table 10: Dry heat degradation results of fostemsavir.

Sr	Stressed condition	Number of degradants form	RT of degradation product	Area		% Degradation
				Unstressed	Stressed	
01	100°**C**, for 01 HRS.	00	NA	194258	193992	NA

Photolytic Degradation: The medication powder was out in the sun for eight hours. Since no degradation was discovered after analysis of the stressed sample, the exposure duration was increased for 24 and 48 hours. Analysis of the stressed sample revealed no new peaks. Furthermore, there was no difference among the stressed fostemsavir sample's peak regions compared to those of the initial samples, demonstrating that there was no breakdown. Chromatogram of Photolytic degradation shown in Fig. 10 and results of fostemsavir degradation depicted in Table 11.

Thus, it was discovered that fostemsavir was unstable under photolytic, moist heat, as well as dry heat circumstances, but deteriorated in an alkaline, acidic, or oxidising environment.

Confirmation of Degradation Product

LC-MS analysis of the breakdown product was performed. Fig. 11 shows the mass spectra of the degradation products, whereas Fig. 12 and 13 show the LC-MS images of the acidic and alkaline degradation products, respectively.

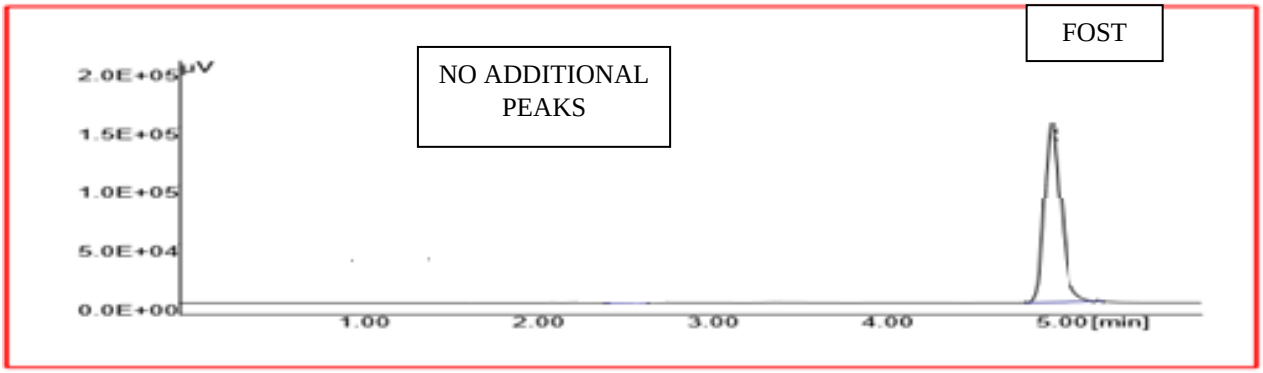

Fig. 10. Chromatogram of Photolytic degradation results of fostemsavir.

Table 11: Photolytic degradation results of fostemsavir.

Sr	Stressed condition	Number of degradants form	RT of degradation product	Area		% Degradation
				Unstressed	Stressed	
01	Sunlight 48 HRS.	00	NA	194258	194193	NA

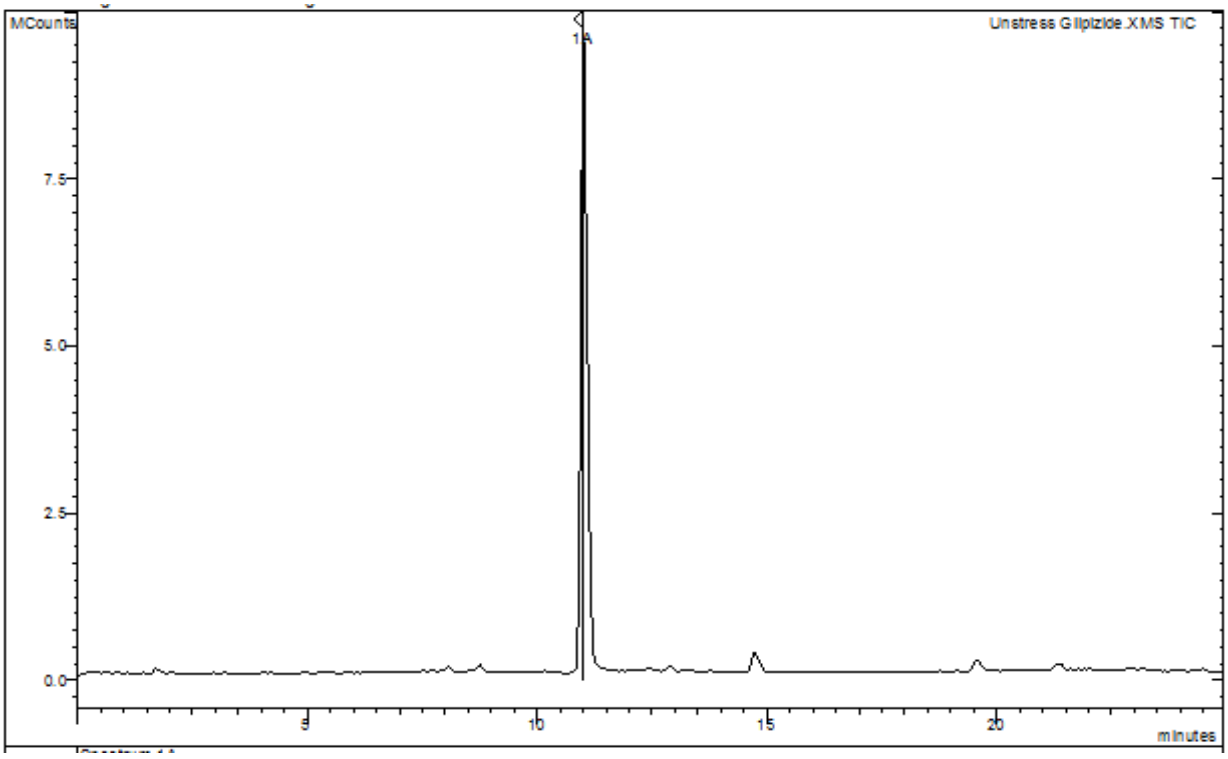

Fig. 11. LC-Mass spectra of fostemsavir.

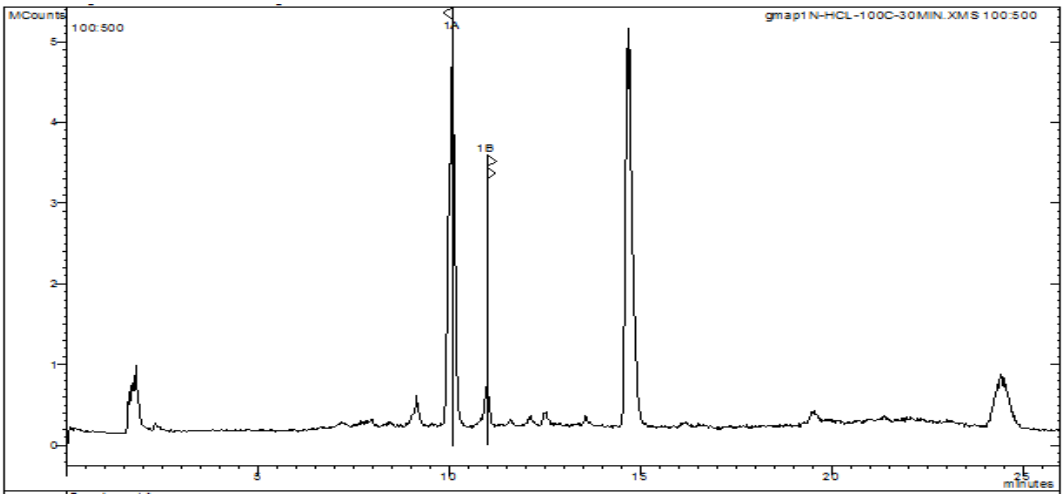

Fig. 12. MSD Chromatogram of fostemsavir and Stressed fostemsavir (15 min. at 100°C with 0.1 N Hf).

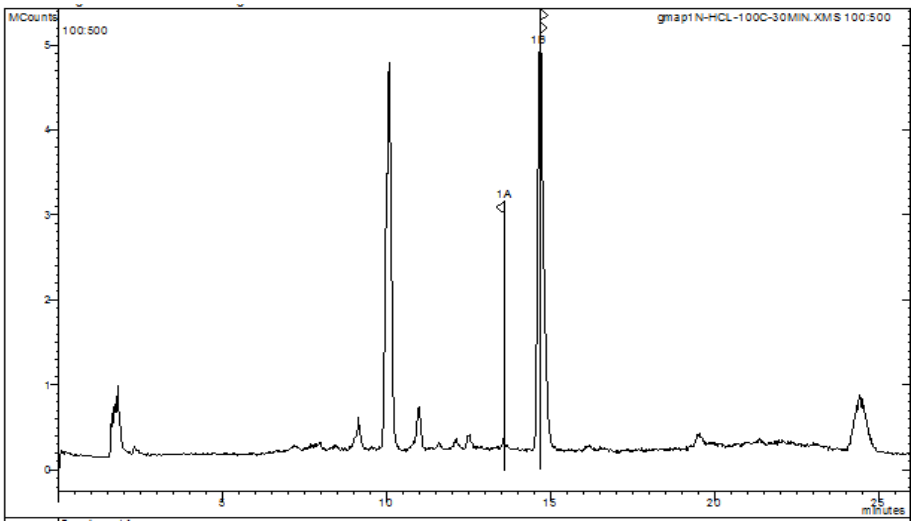

Fig. 13. MSD of stressed fostemsavir (0.1*N* NaOH 100°C for 15 min).

Characterization of degradation products by ^{1}H NMR Spectroscopy

By using 1H NMR spectroscopy, the acid degradation product was characterised. The substance was discovered to be water soluble. Fig. 14 below displays the NMR spectrum of the acid breakdown product Table 12.

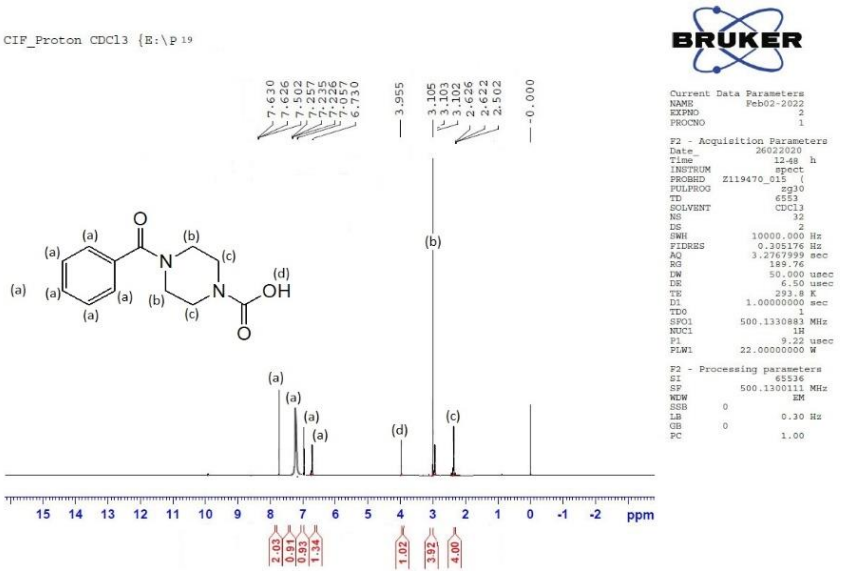

Fig. 14. 1H NMR spectrum of alkali degradation product.

Table 12. Interpretation of 1H NMR [400 MHz ∂, ppm water].

∂ ppm	Signal	No. of Hydrogen	Group
7.2-7.9	Multiplate	5 H	Aromatic
3.1	Triplet	2H	Two equivalent -CH_2
2.6	Triplet	2H	Two equivalent -CH_2
3.9	Singlet	1H	-OH

DISCUSSION

A simple, precise, and specific HPLC approach was developed to identify fostemsavir. The separation by chromatography was accomplished using a phase that is mobile made up of a mixture of Phenomene C18 column (250 4.6 mm, 5 m) with a limit of detection of 278 nm. For fostemsavir, linearity was seen in a concentration range of 3 to 18 mg/ml, and the medicine dosage evaluated using the advised methodology provided strong support for the label claim. The proposed method was validated. Recovery studies were conducted to assess the precision of the methods on three different levels. Excipients or additives are frequently found in medicinal products did not interfere, according to recovery trials. The repeatability analysis, which showed % RSD less than 2, showed that the approach was accurate. According to the International Council for Harmonization's (ICH) requirements, experimental breakdown tests were conducted, and the breakdown products that emerged were identified using liquid chromatography-mass spectrometry (LC-MS) and Fourier-transform infrared spectroscopy (FTIR). The major objective of our research was to develop a unique HPLC technique capable of accurately analysing fostemsavir and identifying its breakdown products utilising auxiliary LC-MS and FTIR techniques.

CONCLUSION

A validated HPLC method was developed for the quantification of the medication fostemsavir in compliance with ICH criteria. The technique performed satisfactorily for all validation metrics, including system appropriateness, method precision, accuracy, LOD and LOQ, and robustness. This tried-and-true method has many advantages, including shorter run times, cheaper costs, accessibility, good sensitivity, dependability, and reproducibility. The behaviour of the medications' degradation was investigated under a variety of stress conditions, including acid, basic, oxidation, reduction, photolytic, and thermal stress. The medications were shown to be unstable in situations involving acid, alkali, and oxidation, but stable in situations involving reduction, heat, and photolysis. The degradation products were further characterised using LC-MS and FTIR analyses.

FUTURE SCOPE

The purpose of the research was to compare an anti-HIV drug's forced breakdown behaviour as well as pinpoint its breakdown by-products using LC-MS analysis. To simulate breakdown, the medication was put under a variety of stress circumstances, including heat, acid, base, oxidation, reduction, as well as photolysis stress. LC-MS techniques were then used to characterise and identify the breakdown products.

Future research on the processes and pathways involved in the forced degradation of the anti-HIV medicine is part of the study's future focus. In-depth structural elucidation can also be applied to the discovered degradation products in order to comprehend how they develop and how they might affect the safety and effectiveness of the medicine. This information can help techniques for design for improving the formulation and stability of the medicine, preserving its quality throughout storage and use. In order to provide useful information for medication development and optimisation, additional studies can also investigate the kinetics of degradation and establish breakdown profiles under various environmental circumstances.

Acknowledgements. The authors are grateful to JJTU University for providing facilities to complete this research work.

Conflict of Interest. The author declares no conflict of interest for the present manuscript.

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How to cite this article: Aher B.O., Prakash S. and Bairagi V.A. (2023). HPLC Method Development, Validation, and Degradation Study of Fosetasmvir by: A Comprehensive Analytical Investigation. *Biological Forum – An International Journal*, *15*(4): 844-853.