Printed in the Republic of Korea

ANALYTICAL SCIENCE & TECHNOLOGY

Vol. 38 No. 5, 290-304, 2025

https://doi.org/10.5806/AST.2025.38.5.290

Green HPLC method development and validation for quantification of fostamatinib with kinetics, LC-MS/MS characterization and *in-silico* assessment of forced degradation products

Rajya Lakshmi Nimmagadda★, Sri Indupriya Gunji, Praveen Kumar Rampatoti, Sakitha Kolli, Sai Praveen Kumar Naik Gonavath, Bhavya Suryadevara, and Hindu Bai Vadithya

A M Reddy Memorial College of Pharmacy, Petlurivariplaem, Narasaraopet – 522601, Palnadu Dist., Andhra Pradesh, India

(Received July 9, 2025; Revised September 8, 2025; Accepted September 19, 2025)

Abstract: The present study focused to propose a green, stability-indicating HPLC method for the quantification of fostamatinib along with its degradation kinetics, LC-MS/MS-based characterization, and *in-silico* toxicity evaluation of forced degradation products. In order to reduce the environmental impact, a green mobile phase of 0.1 % aqueous formic acid and ethanol in 30:70 (*v/v*) was employed and this composition eliminate the need for conventional toxic solvents such as acetonitrile and methanol. The chromatographic separation was achieved on a Shim-pack GIST C18 column (250 × 4.6 mm, 5 μm) under isocratic conditions with 0.7 mL/min flow and detection at 238 nm. These conditions elute fostamatinib with 4.4 min retention time with 10 min run time. The method shows excellent linearity within 15-90 μg/mL with sensitive LOD (0.15 μg/mL) and LOQ values (0.5 μg/mL) concentrations. The forced degradation studies of fostamatinib reveal 23.56 % degradation in acid stress and generate five distinct DPs at specific retention times. The kinetic analysis confirms that the degradation follows first-order degradation with a rate constant of 0.0226 h^{-1} and a half-life of 30.61 hours. The DPs were structurally characterized using LC-MS/MS, with DP 1 have the lowest molecular weight (*m/z* 183.2044), and DP 4 and DP 5 shows complex phosphate and nitrogen-based fragmentation similar to fostamatinib. The *In-silico* toxicity analysis using ProTox-II shows that DP 1 exhibit the highest toxicity, while DP 5 show the lowest acute toxicity but still required attention due to multiple toxicological activities. The green HPLC method developed by utilizing ethanol and aqueous buffer as mobile phase solvents was demonstrated high environmental sustainability and was confirmed by AGREE (0.81) and GAPI tools. Overall, this method was validated, eco-friendly, and effective for routine quantification of fostamatinib that ensure both analytical reliability and environmental safety.

Key words: fostamatinib, HPLC analysis, *in-silico* study, LC-MS/MS characterization, stress degradation compounds

★ Corresponding author
Phone : +91 72288095994
E-mail : rlakshmi.pharma9@gmail.com

1. Introduction

In recent years, the pharmaceutical industry has witnessed a significant paradigm shift toward environmentally analytical methods for quantification of pharmaceutical compounds. This is due to the increase in global concerns over environmental pollution, health hazards posed by hazardous chemicals, and need to comply with stricter environmental regulations.[1] Due to this, the principles of Green Analytical Chemistry (GAC) have become significant importance in modern pharmaceutical research because these principles promote the adoption of eco-friendly, cost-effective, and sustainable analytical methodologies. HPLC is one of the most widely employed analytical tools for quantitative and qualitative analysis is also takes substantial reform to align with GAC principles. The traditional HPLC methods often involves usage of toxic organic solvents such as acetonitrile and methanol, which pose significant environmental and health risks. Moreover, these methods not only consume high solvent and energy but also increase their ecological footprint. Hence, to develop a robust and environment friendly method is a critical need in pharmaceutical analysis.[2]

Fostamatinib (*Fig.* 1) is a spleen tyrosine kinase (SYK) inhibitor drug that emerges as a promising therapeutic agent in treating chronic immune thrombocytopenia (ITP), rheumatoid arthritis, and other autoimmune conditions. It is a prodrug that rapidly converts in to its active metabolite tamatinib (R-406) which inhibits SYK which is a key mediator in immune cell signaling.[3] It reduces the destruction of platelets by blocking SYK and thereby treats ITP. The decrease in white blood cell count, chest pain, abdominal pain, fatigue, increase in blood pressure, respiratory infection, diarrhea, rash, nausea and dizziness are the most common adverse effects reported during the usage of fostamatinib.[4]

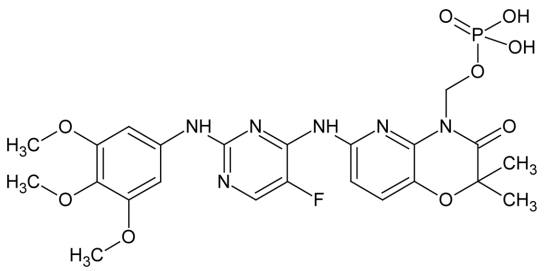

Fig. 1. Structure of fostamatinib.

Owing to its clinical utility and pharmacological importance, it is imperative that fostamatinib be quantified accurately and precisely in drug products to verify therapeutic efficacy as well as patient safety. Moreover, to determine its behavior towards degradation in stressed conditions, e.g., acid hydrolysis, base, peroxide, thermal and UV was extremely crucial to evaluate chemical stability, to forecast shelf-life, and to verify compliance with International Conference on Harmonisation (ICH) guidelines on stability testing. Among various stress degradation studies, acidic degradation emerges as important because many pharmaceutical compounds are susceptible to hydrolytic cleavage under acidic environments during manufacture, storage, or *in-vivo* conditions. Consequently, investigation of the acid degradation kinetics and characterization of degradation products (DPs) is an integral part of a comprehensive stability-indicating method development.

The comprehensive literature review reveals the existence of only one reported UPLC–MS/MS method for the quantification of the active metabolite of fostamatinib (R406) in rat plasma.[5] However, no method reported for the quantification of fostamatinib in pharmaceutical formulations and any study characterize its DPs. To fulfill this gap identified in literature, the present research aims to develop and validate a green HPLC method for the quantification of fostamatinib in the presence of its acid degradation products. The DPs were characterized by utilizing advanced analytical techniques such as LC-MS/MS.[6] Further, the toxicity concerns of the DPs were addressed by incorporating *in-silico* toxicity prediction tools, such as ProTox-II.

2. Materials and Methods

2.1. Standards and reagents

The high-purity reference standards of fostamatinib (purity 99.25 %) along with commercially available fostamatinib tablets (TAVALISSE® 100 mg) were procured from Simson Pharma Limited, Mumbai, Maharashtra, India. HPLC-grade methanol and water as well as buffer chemicals used for HPLC analysis were obtained from Merck Life Science Private Limited, Mumbai, India. The reagents required for stress degradation study that includes analytical grade hydrochloric acid (HCl), NaOH, and H_2O_2, sourced from Merck Life Science Private Limited.

2.2. Apparatus and chromatographic conditions

The HPLC system equipped with UV was utilized for the analysis of fostamatinib. A Shimadzu system that includes a quaternary pump, auto-injector, vacuum degasser, and SPD 10Avp UV-Visible detector was utilized in this study. This setup was connected to a computer operating with N2000 software for instrument control, data acquisition, and analysis. The chromatographic separation was achieved using a Shim-pack GIST C18 analytical column with dimensions of 250 × 4.6 mm and a particle size of 5 μm at room temperature. The 0.7 mL/min isocratic flow of 0.1 % aqueous formic acid and ethanol in 30:70 (*v/v*) as mobile phase. The column eluents were recorded through UV detector at 238 nm and the analysis was completed within a total run time of 10 min.

The MS/MS analysis was performed on Waters SYNAPT G2-Si high-definition quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters, USA) equipped with an electrospray ionization (ESI) source at positive ion mode. The mass spectrometer was calibrated using sodium formate solution and real-time mass correction was performed using Leucine Enkephalin (m/z 556.2771 in positive mode. Data acquisition was carried the MS-E mode that alter between low-energy (collision cell off) and elevated-energy (20–40 eV ramp) conditions to generate both precursor and fragment ion information in a single injection. The capillary voltage of 3.0 kV, cone voltage of 35 V, source temperature of 120 °C, desolvation temperature of 350 °C, desolvation gas (nitrogen) flow of 800 L/h, and cone gas flow of 50 L/h were utilized as optimized ESI source parameters during the analysis. The data acquisition and processing were performed through Waters MassLynx 4.1 software with integrated UNIFI software platform for accurate mass measurement, elemental composition analysis, and structural elucidation.

The compatibility of chromatographic system with the mass spectrometer was ensured by utilizing a post-column split. This split ensures that the Q-TOF instrument receives approximately one-fifth of the total eluent flow (≈1:5 split ratio) and thereby prevent overloading of the ion source. The injection volume was reduces to 5 μL to maintain optimal peak shape and to minimize ion suppression effects in the ESI source. The retention times (RTs) and relative retention times (RRTs) of analyte peaks obtained in the LC-UV chromatogram were directly compared with those observed in the MS chromatograms to identify and confirm the compounds.

2.3. Preparation of solutions

A fostamatinib stock solution (1000 μg/mL) was prepared in HPLC-grade ethanol and stored at 0 °C for stability. The working standards (15–90 μg/mL; Levels 1–6) were obtained by serial dilution with deionized water. Calibration standards were prepared separately at each level, and 20 μL was injected into the HPLC–UV system under optimized conditions. Each concentration was analyzed in triplicate to assess precision and accuracy.

2.4. Assay sample preparation

One tablet of Tavalisse® with a label indicating a content of 100 mg of fostamatinib was opened with care, and its contents were weighed accurately. The tablet powder equivalent to one tablet weight was transferred to a 50 mL volumetric flask, extracted with 30 mL of ethanol–water (1:1) using 5 min ultrasonication, and diluted to volume with the same solvent. The solution was filtered through ordinary filter paper followed by a 0.22 μm membrane filter.

Then aliquot was further diluted to obtain a test solution of fostamatinib at 60 μg/mL and this solution was analyzed in the proposed method.

2.5. Forced degradation

The chemical stability of fostamatinib was assessed by forced degradation studies performed under diverse ICH-recommended stress conditions to discover possible DPs and establish the stability-indicating potential of the analytical technique developed.[7,8] The stress tests mimic extreme environmental conditions to learn about the degradation behavior of the drug, providing invaluable information for formulation development and determination of shelf-life. For every study, 1 mL of an fostamatinib stock solution with high concentration (1000 μg/mL) was exposed to particular stress conditions. The samples were neutralized if necessary and diluted in deionized water in 10 mL volumetric flasks to have a final concentration of 100 μg/mL for examination. For acid hydrolysis, 50 mg of fostamatinib was subjected to 50 mL of 0.1 N hydrochloric acid and incubated in a water bath at 60 °C for 24 hours and then further incubated for 12 hours at room temperature. In acid degradation, 1 mL of HCl solution (2 M) was added to fostamatinib solution, and the mixture was heated at 60 °C in a water bath for 30 min followed by incubating at room temperature for 12 hours. In alkaline degradation, 1 mL of NaOH solution (0.1 M) was added to fostamatinib solution, and the sample was left at room temperature for 12 hours. The impact of oxidative stress on fostamatinib was assessed by subjecting 1 mL of standard solution to 1 mL 30 % (*v/v*) peroxide. Then the stressed sample was incubated at 60 °C for 2 hours in a water bath and then cooled to room temperature. The degradation of fostamatinib under neutral conditions was assessed by mixing 1 mL of standard solution to 1 mL of deionized water. This mixture was heated at 80 °C in a water bath for 30 minutes and then cooled to room temperature. 1 mL of stock solution of fostamatinib to natural sun light for 4 hours in a 10 mL volumetric flask with lid whereas in thermal degradation, the fostamatinib powder was heated in an air oven for 2 hours at 100 °C.

2.6. Kinetics investigation

A degradation kinetics experiment was conducted to study the time-dependent decomposition of fostamatinib under acidic stress conditions.[9] In the process, 1 mL aliquots of stock solution of fostamatinib were acid hydrolyzed and incubated at room temperature. Samples were taken at regular intervals to monitor the progress of degradation. All the reactions were halted directly using neutralization to prevent further degradation, and the resulting solution was diluted to 10 mL using deionized water to prepare it HPLC analysis suitable. All time-point samples were analyzed using the established HPLC–UV method. Concentration of fostamatinib at every interval was determined using the calibration curve regression equation. A graph of concentration versus time was generated, and the data were fitted to obtain the degradation kinetics, e.g., reaction order and rate constant. The kinetic study provided useful information on the degradation behavior of fostamatinib, which will assist in its shelf-life prediction as well as the formulation of recommendations on its storage and handling conditions.

2.7. *In-silico* toxicity studies

An *in-silico* toxic prediction approach was employed to evaluate the potential toxicity of fostamatinib DPs. The experiment was carried out through the use of ProTox-II, which is a popular web-based tool that estimates toxicological properties based on the molecular structure of compounds.[10] ProTox-II estimates the LD_{50} value (mg/kg body weight), a dose required to kill 50 % of an administered population, and an estimate of acute toxicity in a quantitative format. Apart from LD_{50}, ProTox-II gives to every compound one of six toxicity classes, ranging from Class 1 (highly toxic) through to Class 6 (non-toxic), which corresponds to possible health risks upon exposure. These predictions provide valuable information concerning safety profiles of fostamatinib and DPs, especially under environmental stress conditions or poor storage conditions. This assists in minimizing formulation hazards as well as decision-making during

shelf-life establishment and product formulation[11,12]

2.8. Statistical analysis

All statistical calculations and data processing were carried out using the built-in tools available in Microsoft Excel 365. Furthermore, the toxicity of the DPs was assessed using the ProTox-II web server.

3. Results and Discussion

To conduct a comprehensive kinetic degradation study, it is necessary to employ a stability-indicating chromatographic technique that can efficiently separate fostamatinib from its DPs. The selected technique should not only allow precise quantitation of the parent drug, but also permit simultaneous detection of DPs, thereby providing robustness and reliability of the analysis. Such a two-way analytical approach dramatically enhances the suitability of the method in biological matrices and increases its potential for pharmacokinetic and clinical applications. In addition, DPs identified through LC–MS/MS analysis must then undergo extensive structural characterization, which is a critical consideration in terms of determining their chemical nature and potential for transformation routes. To validate these findings, *in-silico* prediction of toxicity was performed to establish the possible toxicological risks associated with the DPs detected, thus establishing a complete picture of the stability and safety profile of the drug.3.1.

3.1. Optimization of chromatographic conditions

The objective of this study was to develop a simple, robust, and MS-compatible HPLC method for the quantification of fostamatinib in pharmaceutical formulations. The method was designed to ensure high sensitivity, acceptable system suitability, compatibility with LC–MS detection in alignment with GAC principles by using environmentally friendly solvents such as ethanol and formic acid.

The detection wavelength of fostamatinib was selected based on UV spectral analysis of the analyte. A full-range UV scan reveals a maximum absorbance (λ_{max}) at 238 nm and hence was chosen as the optimal wavelength for chromatographic detection. This wavelength provides adequate sensitivity and specificity for fostamatinib without interference from the mobile phase components.

The initial trials for method development were carried on Phenomenex C18 (250 mm, 5 μm) column using of 20 % aqueous ethanol at 1.0 mL/min as mobile phase. However, this composition results multiple poor resolved peaks with low intensity indicate inadequate retention and separation (*Fig.* 2A). The same mobile phase was evaluated on an X-Bridge Phenyl C18 column (250 mm, 5 μm) column. There is no significant improvement was observed, and the chromatographic performance remains suboptimal. Subsequently, the ethanol concentration in the mobile phase was increases to 50 % to improve peak retention and shape. The resultant peak was asymmetrical and broad indicates insufficient column interaction and poor system suitability (*Fig.* 2B). These findings suggest the need for an acid modifier to enhance peak shape and stability.

Further, formic acid, a volatile and MS-friendly buffer was introduced in the mobile phase to improve chromatographic performance. A mobile phase consisting of 0.05 % formic acid and ethanol in 75:25 (*v/v*) was evaluated on Shim-pack GIST C18 (250 mm, 5 μm) column. This modification results a broad, split peak but with a stable and clear baseline, indicates partial improvement in separation efficiency (*Fig*. 2C).

The strength of formic acid was increases to 0.1 % and the same composition of organic solvent was studied to address the issue of peak splitting and to enhance peak shape. This composition yields single peak with a clear baseline but the peak remains broad with poor theoretical plates and tailing factor suggest the suboptimal system suitability (*Fig*. 2D). Hence, further optimization of chromatographic conditions was pursued using the same solvent components.

The last optimized technique employed a Shim-pack GIST C18 Column (250 × 4.6 mm, 5 μm) under an isocratic mobile phase consisting of 0.1 % aqueous formic acid and ethanol in 30:70 (*v/v*) ratio at a flow

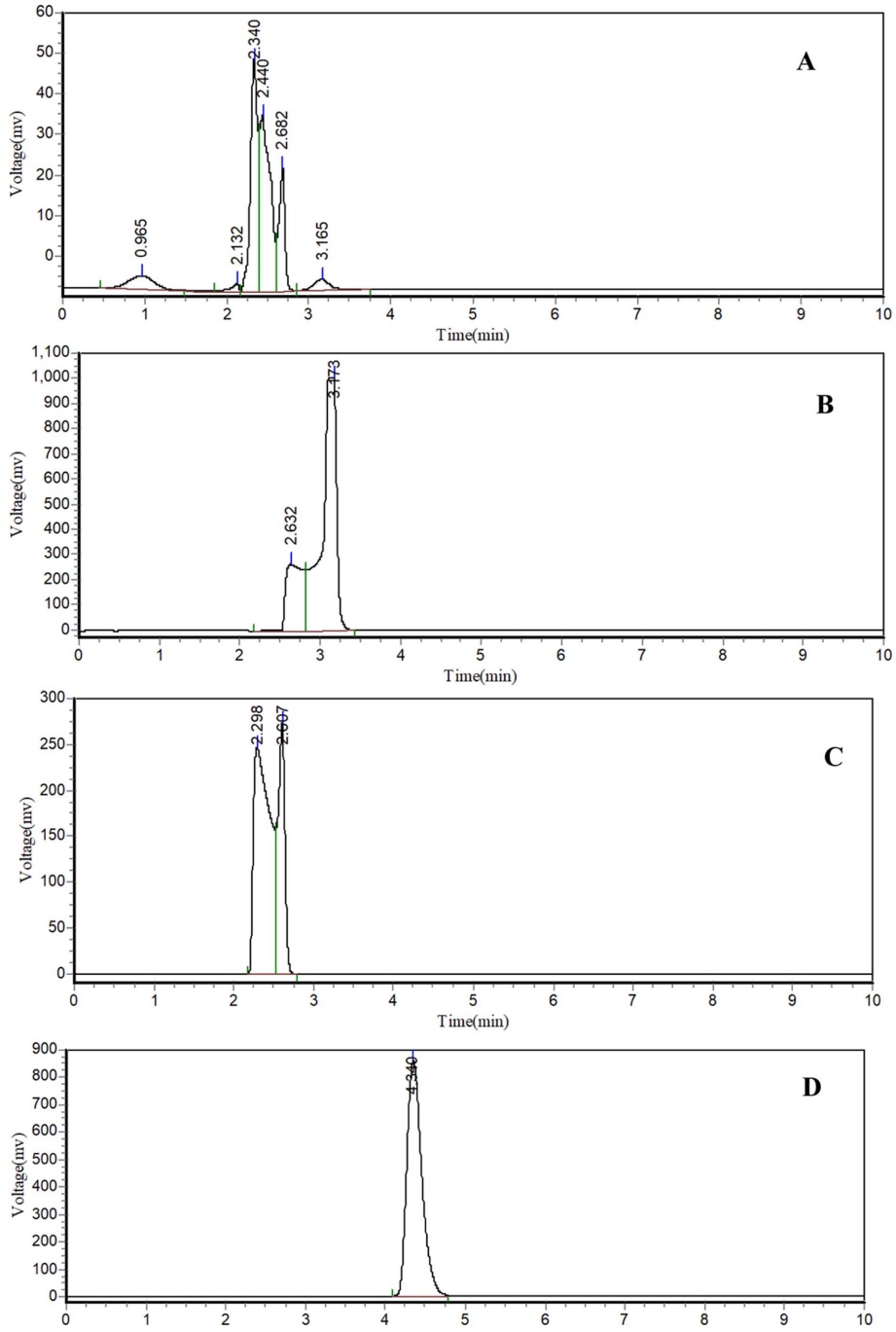

Fig. 2. Chromatograms observed in the optimization of method for the analysis of fostamatinib

rate of 0.7 mL/min. Detection was performed at 238 nm. The overall run time was 10 min, with retention times of 4.432 min for fostamatinib (*Fig.* 3). System suitability parameters proved the reliability of the method with high theoretical plate count of 14930 (> 2000) and tailing factor of 0.94 (< 2) in accordance

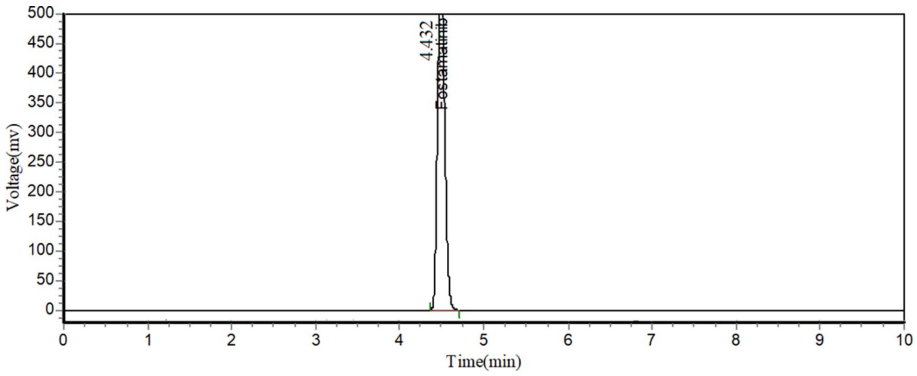

Fig. 3. System suitability chromatogram of fostamatinib in the optimized method.

with FDA acceptance criteria. These findings confirm the method's robustness and appropriateness for routine stability and quality control analysis of fostamatinib.

3.2 Method validation

The method developed for the analysis of fostamatinib was validated as per available literature and ICH guidelines[13,14] to ensure the reliability, accuracy, reproducibility and suitability for its intended purpose. The developed HPLC method for the quantification of fostamatinib demonstrates excellent linearity over 15–90 µg/mL with linear regression equation of Y = 37862x + 21815 (R^2 = 0.9996) indicates a strong linear relationship between peak area and analyte concentration. The precision study was conducted to assess the repeatability and reproducibility of the method. The % RSD values for intraday and interday precision were 0.873 % and 0.745 %, respectively and these values are within the acceptable limits of less than 2 % confirms the precision and consistency of the method. The method accuracy was evaluated through recovery studies at three different concentration levels (50 %, 100 %, and 150 %) and % recovery in the range between 98.99 % and 101.33 %, reflects method accuracy. The %RSD of each spiked level was 0.440 %, 0.729 %, and 0.439 %, respectively was further validate the method's reliability and reproducibility. The Ruggedness expressed in terms of %RSD and was found to be 0.699 % suggests that the method is sufficiently robust against variations in analytical conditions such as different analysts or instruments. The method robustness was further assessed by inducing small deliberate changes in analytical parameters. The % change in in this study was with 0.031 % to 0.210 % was noticed which was within the acceptable limit of less than 2 %. These findings clearly indicate that minor changes in chromatographic conditions do not significantly affect the performance of the method and thereby establishes its reliability and robustness for routine use. Furthermore, the assay of fostamatinib in pharmaceutical formulations using the developed method yield an assay of 98.62% and was in consistent with the expected content and meets the regulatory specifications. The observation of more than 98 % assay value supports the method's applicability for the routine analysis of fostamatinib in both bulk drug and dosage forms.

3.3. Forced degradation study and kinetics

Initially, the mild conditions like Base, thermal, sun light, peroxide and neutral water produces only partial degradation with no DPs in the chromatogram whereas acid conditions proved to be highly significant with five DPs identification. The acid degradation study under forced conditions in acid environment and degradation kinetics were studied to determine the stability profile of fostamatinib in acidic environment. The assay % of fostamatinib was evaluated in various time intervals from 2 to 12 hours, and the % drug remaining in each time interval was analyzed using the green HPLC method validated. The % assay was

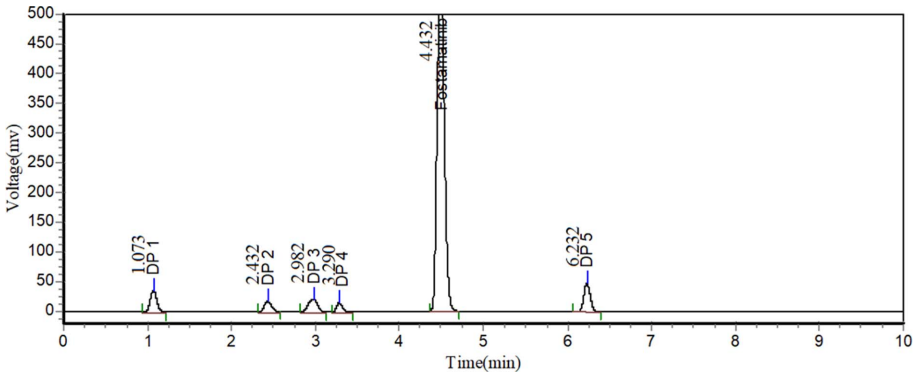

Fig. 4. Acid stress study chromatograms of fostamatinib in the optimized method.

Table 1. Stress degradation study results of fostamatinib in the optimized method

S No	Condition studied	% assay observed	% degradation observed	Retention of DPs identified	Name of given for identified DP
1	Acid	76.44	23.56	1.073	DP 1
				2.432	DP 2
				2.982	DP 3
				3.290	DP 4
				6.232	DP 5

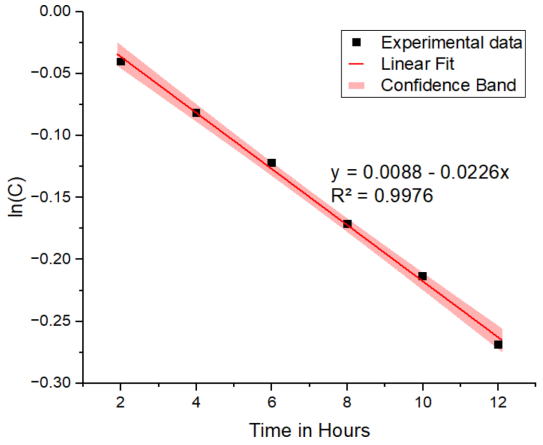

Fig. 5. First-order kinetics plot observed for fostamatinib in acid degradation study.

noticed to be 76.44 with a % degradation of 23.56. The degradation chromatogram (*Fig.* 4) show well resolved peaks correspond to DPs formed in acid stress study. The acid stress study results and the DPs formed in the study were summarized in *Table* 1.

The % assay values were converted to fractional concentration and further transformed into natural logarithmic values (ln C). These ln C values were utilized to assess the reaction order by constructing kinetic plot against time. A straight-line plot (*Fig.* 5) with high linear correlation was observed in the study indicates that the degradation of fostamatinib follows first-order kinetics. The slope of the linear regression line yields the rate constant (k) of 0.0226 h^{-1} and the half-life ($t_{1/2}$) of the drug in acidic condition was found to be about 30.61 hours. That is, 50 % of the drug would be degraded after 30.61 hours in acidic medium. The first-order kinetic behavior observed indicates that the rate of degradation of fostamatinib was proportional to the amount of drug remaining in the reaction mixture at any time. It was a critical finding to know the chemical stability of fostamatinib and gain important information to establish a stable and stability-indicating analytical procedure useful for pharmaceutical quality control and regulatory approvals.

3.4. LC-MS/MS analysis

The LC-MS/MS analysis was performed to characterize the DPs formed in this study. The LCMS analysis of standard solution having fostamatinib

shows peaks corresponds to analytes in the study. The RRT of the identified peaks were in correlation with the LC analysis confirms the method suitability. The mass spectrum corresponds to fostamatinib display its characteristic mass fragments. The m/z value was noticed to be 581.4599 (m+1) and was found to be correlate with its molecular mass of 580 g/mol.

In acid degradation chromatogram display five DPs additional peaks and are named as DP 1, DP 2, DP 3, DP 4, and DP 5 based on the elution and retention time of the compound. DP 1 is observed at 1.073 min, DP 2 was identified at 2.432 mins, DP 3 at 2.982 mins, DP 4 at 3.290 mins, and DP 5 was observed at 6.232 minutes. In acid degradation fostamatinib was all five DPs shows different retention times there is no overlap of the compounds was observed, and fostamatinib was observed at 4.432 min of retention time.

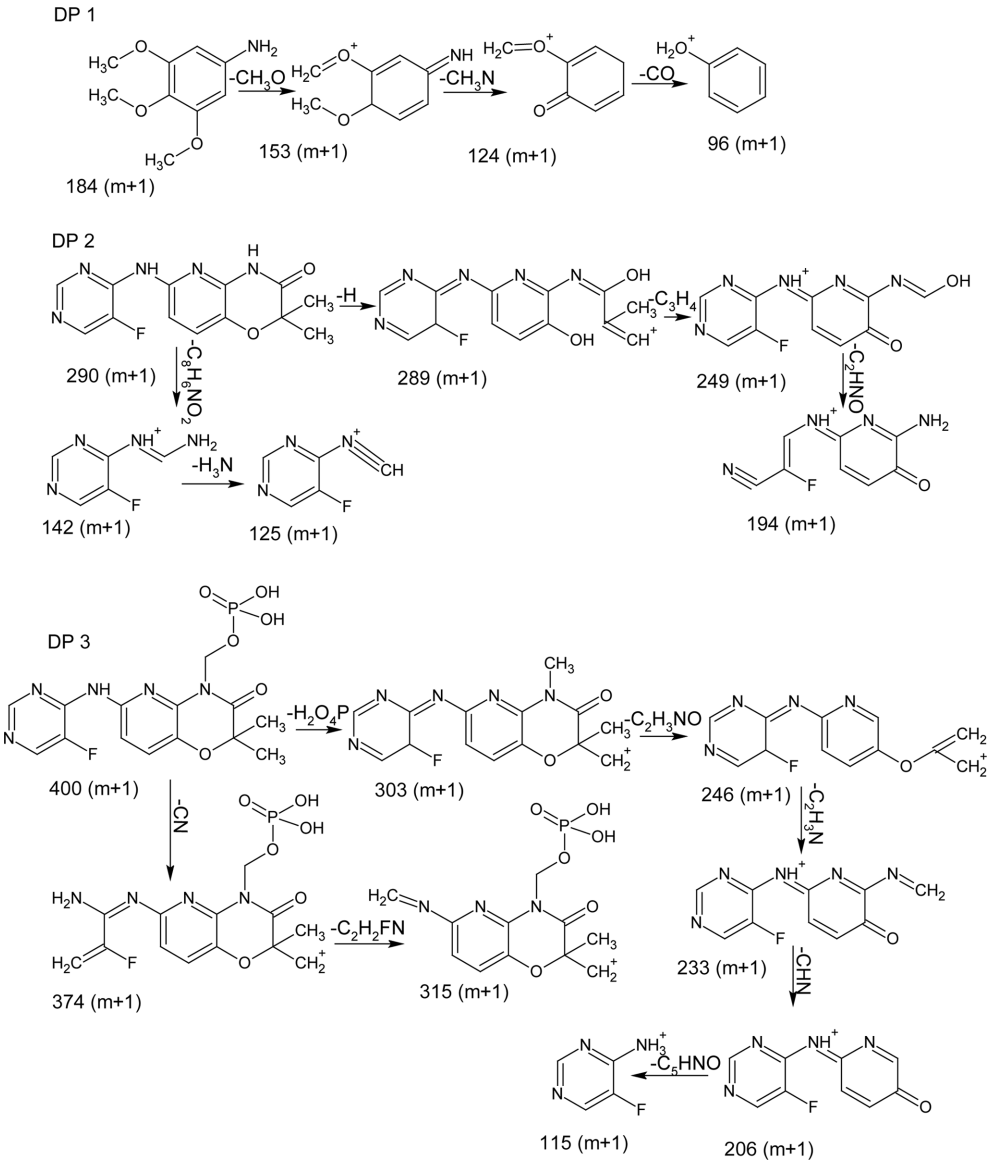

Fig. 6. Fragmentation mechanism of DP 1, DP 2, and DP 3.

3.4.1. DP 1 characterization

DP 1 is observed at 1.073 mins in acid degradation with molecular weight of 183.2044 *m/z*, and molecular formula is $C_9H_{13}NO_3$. DP 1 was found to be the lowest molecular weight of the all five DPs. DP 1 fragmentation pathway shows parent ion at 184.2044 $[M+H]^+$, and product ions observed only three products are possible. Parent ion of 184.2044 loss methoxy group likely known as methoxide which resemble as CH_3O group, and forms product ion with mass of 153.1699 $(C_8H_{10}NO_2)^+$, this formed product ion is by the loss of Methanimine group which is related to the gasoline and also looks like formaldehyde that is CH_3N group which is the combination of single Carbon, three Hydrogen groups and single Nitrogen, and forms precursor ion of 124.1287 with formula of $C_7H_7O_2^+$. The second formed product ion is loss Carbon monoxide which is single carbon and single Oxygen, and forms final product ion with 96.1186 molecular weight, and formula is $C_6H_7O^+$. By all this mechanism patter DP 1is identified as *3,4,5-trimethoxyaniline*. DP 1 fragmentation mechanism pattern is given in *Fig.* 6, mass spectrum is given in Supplementary *Fig.* S1, and fragmentation mechanism table is given in Supplementary *Table* S1.

3.4.2. DP 2 characterization

DP 2 is formed retention time is at 2.432 min with 289.2650 $(C_{13}H_{12}FN_5O_2)^+$. Main product ion loss single Hydrogen atom and forms precursor with molecular weight of 289.2565 and formula is $C_{13}H_{11}FN_5O_2^+$. Precursor ion was loss Cyclopropene, which is a cyclic alkene nothing but C_3H_4 and forms another product ion, resulted product ion is $C_{10}H_7FN_5O_2^+$ with mass of 249.1927, this precursor ion loss HC(=O)−C≡N which is simple organic compound group generally known as Formyl cyanide, and forms $C_8H_6FN_4O^+$ (194.1572). Parent ion of 289.2650 was forms another precursor ion by the loss of $C_8H_6NO_2$ and forms product ion with molecular formula $C_5H_6FN_4^+$ with 142.1257 mass, this formed product ion loss ammonia group which is nothing but NH_3 and forms final product ion with 125.0952 with molecular formula is $C_5H_3FN_3^+$. By all this pattern of mechanism DP 2 is identified as *6-[(5-fluoropyrimidin-4-yl)amino]-2,2-dimethyl-2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one* DP 2 fragmentation mechanism pattern is given in *Fig.* 6, mass spectrum is given in Supplementary *Fig.* S2, and fragmentation mechanism table is given in Supplementary *Table* S1.

3.4.3. DP 3 characterization

DP 3 is identified at 2.982 mins molecular formula of DP 3 is $C_{14}H_{15}FN_5O_6P^+$ and mass is 400.2709 *m/z*. Parent ion was loss two hydrogens and four oxygen atoms and resulted a product ion with mass of 303.2831 *m/z* with molecular formula of $C_{14}H_{13}FN_5O_2^+$, this product ion loss Glycolonitrile group generally called as hydroacetolnitrile and forms precursor ion with mass of 246.2318 with formula of $C_{12}H_{10}FN_4O^+$. Product ion of $C_{12}H_{10}FN_4O^+$ loss ethanenitrile group generally denoted as C_2H_3N and forms a product ion with mass of 233.1933 $(C_{10}H_7FN_5O^+)$, from this product ion again loss single molecule of Carbon, single molecule of Hydrogen and single molecule of Oxygen (CHN) and forms precursor ion with molecular formula of $C_9H_6FN_4O^+$ with mass of 206.1679 *m/z*. Product ion of 206.1679 *m/z* loss C_5HNO and forms smallest product ion with mass of 115.1004 *m/z* and its molecular formula is $C_4H_5FN_3^+$. Parent ion of 400.2709 *m/z* loss Cyanide group (CN) and forms precursor ion with mass of 374.2530 *m/z* $(C_{13}H_{15}FN_4O_6P^+)$, from this product ion loss Fluoroacetonitrile and forms precursor ion with mass of 315.2106 *m/z* with molecular formula is $C_{11}H_{13}N_3O_6P^+$. By all this pattern of mechanism DP 3 is identified *{6-[(5-fluoropyrimidin-4-yl)amino]-2,2-dimethyl-3-oxo-2,3-dihydro-4H-pyrido[3,2-b][1,4]oxazin-4-yl}methyl dihydrogen phosphate*. DP 3 fragmentation mechanism pattern is given in *Fig.* 6, mass spectrum is given in Supplementary *Fig.* S3, and fragmentation mechanism table is given in Supplementary *Table* S1.

3.4.4. DP 4 Characterization

DP 4 was observed at 3.290 mins in acid degradation process, identified DP 4 molecular weight 539.3797 *m/z* $(C_{20}H_{20}FN_6O_9P^+)$. Parent ion of $C_{20}H_{20}FN_6O_9P^+$ forms two product ions, one is formed by the loss of

C_5HO_3, and second product ion is formed by the loss of Dihydrogen phosphate generally denoted as H_2PO_4 and forms precursor ion are $C_{15}H_{19}FN_6O_6P^+$, and $C_{20}H_{18}FN_6O_5^+$. First product ion of $C_{15}H_{19}FN_6O_6P^+$ loss CH_5O_4P and forms $C_{14}H_{14}FN_6O_2^+$ (318.2978 *m/z*), this ion loss cyanate ester group (C_2H_3NO) and forms fragment ion with mass of 261.2464 *m/z* ($C_{12}H_{11}FN_5O^+$). Fragment ion of $C_{20}H_{18}FN_6O_5^+$ loss

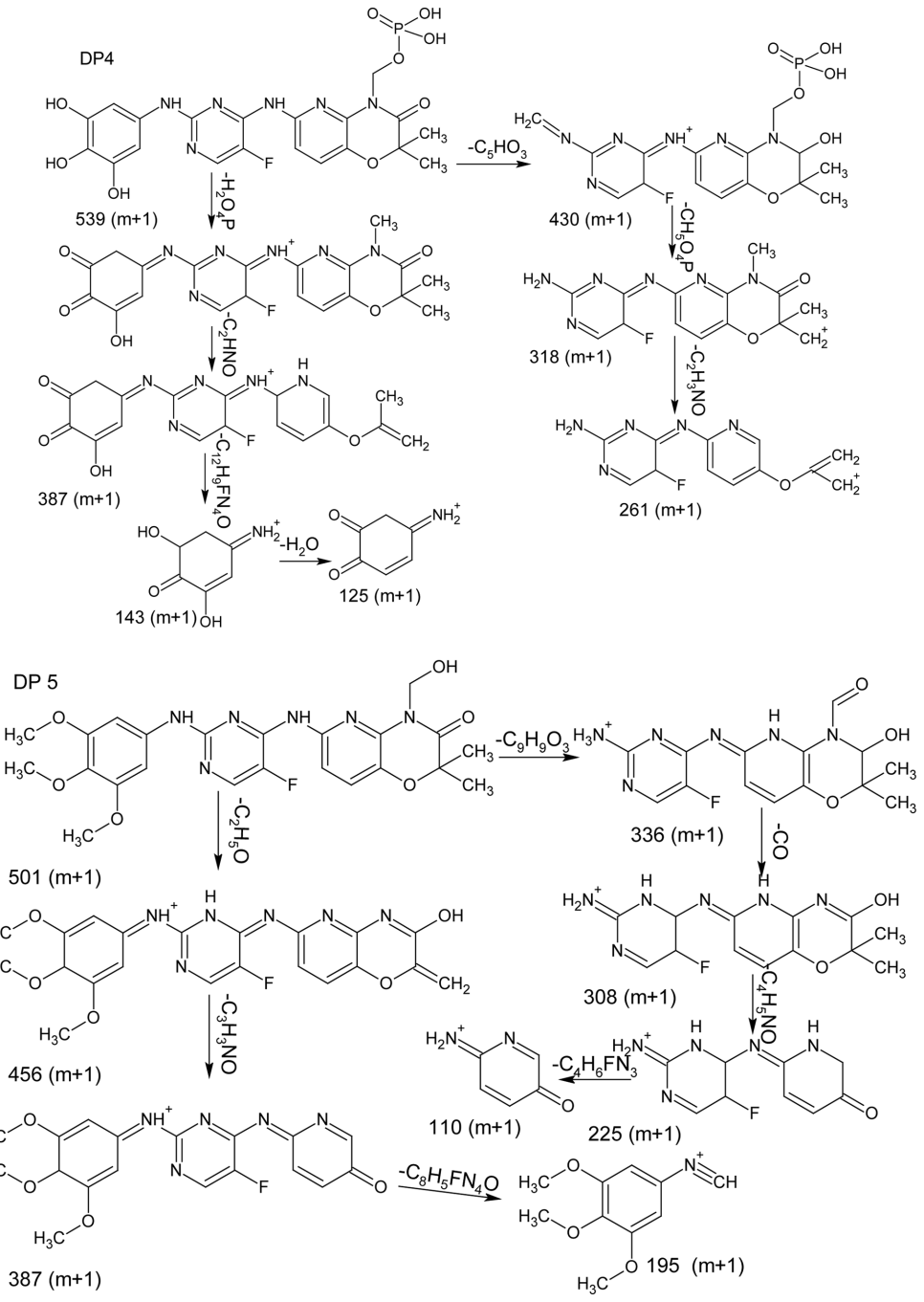

Fig. 7. Fragmentation mechanism of DP 4, and DP 5.

two carbon atoms, one Hydrogen atom, one Oxygen atom, and one Nitrogen atom (C_2HNO) and form fragment ion with formula of $C_{18}H_{17}FN_5O_4^+$, this formed ion loss $C_{12}H_9FN_4O$ and forms fragment with mass of 143.1320 *m/z* ($C_6H_8NO_3^+$). Last smallest product ion formed by the loss of water molecule (H_2O), with formula of $C_6H_6NO_2^+$ (*m/z* 125.1167). By all this pattern of mechanism DP 4 is identified *(6-((5-fluoro-2-((3,4,5-trihydroxyphenyl)amino) pyrimidin-4-yl)amino)-2,2-dimethyl-3-oxo-2H-pyrido [3,2-b][1,4]oxazin-4(3H)-yl)methyl dihydrogen phosphate*. DP 4 fragmentation mechanism pattern is given in *Fig.* 7, mass spectrum is given in Supplementary *Fig.* S4, and fragmentation mechanism table is given in Supplementary *Table* S2.

3.4.5. DP 5 Characterization

DP 5 is identified at 6.232 min, observed molecular mass is 501.4796 *m/z* and denoted formula is $C_{23}H_{25}FN_6O_6$. In DP 5 fragment ions are 456.4185 *m/z*, 386.3565 *m/z*, 336.3130 *m/z*, 308.3029 *m/z*, 225.2143 *m/z*, 195.2066 *m/z* and 110.1054 *m/z* with molecular formulas of $C_{21}H_{20}FN_6O_5$, $C_{18}H_{17}FN_5O_4$, $C_{14}H_{16}FN_6O_3$, $C_{13}H_{16}FN_6O_2$, $C_9H_{11}FN_5O$, $C_{10}H_{12}NO_3$, $C_5H_5N_2O$. DP 5 is identified *(6-((5-fluoro-2-((3,4,5-trihydro-xyphenyl)amino)pyrimidin-4-yl)amino)-2,2-dimethyl-3-oxo-2H-pyrido[3,2-b][1,4]oxazin-4(3H)-yl)methyl dihydrogen phosphate*. DP 5 fragmentation mechanism pattern is given in *Fig.* 7, mass spectrum is given in Supplementary *Fig.* S5, and fragmentation mechanism table is given in Supplementary *Table* S2.

3.5.s *In-silico* toxicity assessment of DPs

An *in-silico* toxicity analysis was conducted by employing computation programs to guarantee the safety of fostamatinib and its DPs. This research was crucial due to the DPs generated upon drug storage or metabolism, may have unidentified or even detrimental biological activities. This evaluation was conducted using freely accessible online platforms such as ProTox-II which is a machine learning-based model designated to predict experimental toxicity data of chemical compounds. This analysis includes the prediction of acute toxicity (LD_{50}), toxicity class, and various organ-specific and systemic toxicities (*Table* 2).

The DP 1 has the most toxicity potential of any of the DPs with an estimated LD_{50} of 681 mg/kg with class 4 toxicity. It was active for hepatotoxicity, neurotoxicity, nephrotoxicity, mutagenicity, cytotoxicity, and ecotoxicity suggests a wide range of toxic effects including high risk to liver and kidney function and genetic and cellular injury. The DP 2 also belongs to toxicity class 4 with a predicted LD_{50} of 1500 mg/kg and demonstrates to be active for hepatotoxicity,

Table 2. predicted *in-silico* toxicity results noticed for the DPs of fostamatinib identified in the study

S No	Parameters	Results noticed for				
		DP 1	DP 2	DP 3	DP 4	DP 5
1	Toxicity class	4	4	4	4	5
2	Predicted LD_{50}	681 mg/kg	1500 mg/kg	1500 mg/kg	1500 mg/kg	2935 mg/kg
3	Hepatotoxicity	Active	Active	Inactive	Inactive	Inactive
4	Neurotoxicity	Active	Active	Active	Active	Active
5	Nephrotoxicity	Active	Inactive	Active	Active	Active
6	Respiratory toxicity	Inactive	Active	Active	Active	Active
7	Cardiotoxicity	Inactive	Inactive	Inactive	Inactive	Inactive
8	Carcinogenicity	Active	Active	Inactive	Inactive	Inactive
9	Immunotoxicity	Inactive	Active	Active	Active	Active
10	Mutagenicity	Active	Inactive	Inactive	Inactive	Inactive
11	Cytotoxicity	Active	Inactive	Inactive	Inactive	Inactive
12	Ecotoxicity	Active	Active	Inactive	Inactive	Inactive
13	Clinical toxicity	Inactive	Active	Active	Active	Active
14	Nutritional toxicity	Inactive	Inactive	Active	Inactive	Inactive

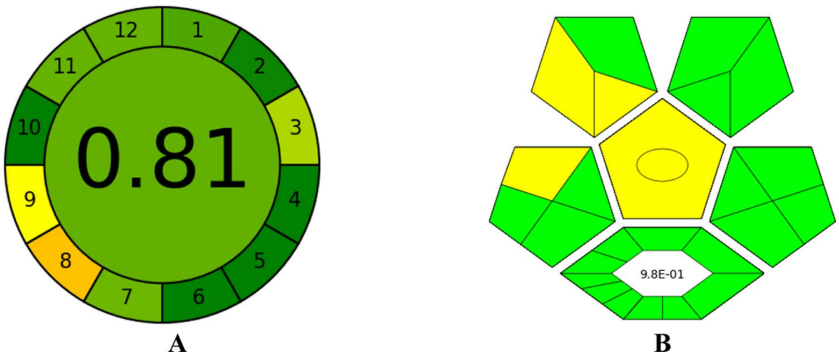

Fig. 8. Greenness assessment results for the proposed HPLC method for the analysis of fostamatinib based on AGREE (A) GAPI, (B) tools.

neurotoxicity, respiratory toxicity, carcinogenicity, immunotoxicity, ecotoxicity, and clinical toxicity. This compound was predicted to be carcinogenic and immunotoxic properties that may pose long-term health hazards.

The DP 3 and 4 also exhibits a calculated LD_{50} of 1500 mg/kg and were assigned into toxicity class 4. In contrast DP 5 was assigned under toxicity class 5 with a comparatively higher LD_{50} of 2935 mg/kg. This indicates the lower acute toxicity nature of the other DPs encountered in the study. This DP proved to be active against neurotoxicity, nephrotoxicity, respiratory toxicity, immunotoxicity, and clinical toxicity. It exhibits less acutely toxic, its effect on a number of significant toxicological parameters makes inclusion of it in regular degradation profiling and risk assessment necessary. In conclusion, these *in-silico* toxicity results highlight the need to not only examine degradation products for chemical structure and formation but also for their possible toxicological effects.

3.6. Greenness assessment

The analytical HPLC method developed was systematically evaluated for its environmental sustainability using the principles of GAC. The environmental performance of the method was quantitatively assessed using the AGREE and GAPI tools. The AGREE tool yield a high overall score of 0.81 suggest the greenness of the proposed method (*Fig.* 8A). The method was purposefully designed to minimize environmental impact by replacing conventional toxic solvents such as acetonitrile and methanol with greener alternatives like ethanol, water and these solvents were utilized throughout the mobile phase and sample preparation. These solvents are known for their low toxicity, biodegradability, and reduced ecological footprint. These solvents were pumped at 0.7 mL/min enables reduced solvent consumption per run and also save the usage of energy. Further the green assessment through GAPI tool yield a highly favorable score of 0.98. The GAPI pictogram (*Fig.* 8B) reveals predominantly green zones indicate the low environmental risk across sample handling, reagent use, and instrumentation. Only a few yellow segments correspond to automation and in-line monitoring, suggest the minor opportunities for further greening. The central yellow pentagon, reflect the overall method greenness that reinforces the method's safety and environmental compatibility. Collectively, the method's design features and the greenness assessment results demonstrate that the procedure is not only analytically robust and cost-effective but also adheres strongly to green chemistry principles and make this method ideal choice for routine pharmaceutical analysis with minimal ecological impact.

This method enables the complete elution of fostamatinib along with its DPs within a short 10-minute run. This simultaneous resolution of the standard and all DPs in a single isocratic run not only enhances analytical efficiency but also significantly reduces solvent consumption and analysis time. The

exclusive use of ethanol, a low-toxicity and renewable solvent, in place of hazardous organic modifiers such as acetonitrile or methanol, further strengthens the greenness of the method by lowering environmental impact and waste disposal risks. Thus, the method offers a sustainable, rapid, and eco-friendly alternative without compromising chromatographic performance. Notably, no analytical method reported in the literature for the quantification of fostamatinib and makes this the first, best, and most sustainable choice for its reliable analysis.

4. Conclusion

This study successfully proposed a green, sensitive, and robust HPLC method for the quantification of fostamatinib that emphasizes the use of environmentally friendly solvents such as ethanol and water over traditionally used hazardous solvents like acetonitrile and methanol. The method, optimized with a Shim-pack GIST C18 column and isocratic elution was demonstrate excellent linearity (15–90 µg/mL), precision (RSD < 1 %), sensitivity (LOD 0.15 µg/mL), accuracy (98.62 %) and confirms its applicability for routine pharmaceutical analysis. The acid-induced degradation studies reveal 23.56 % degradation of fostamatinib with the formation of five distinct (DP 1–DP 5) and all these DPs were eluted at unique retention times. The kinetic model confirms the first-order degradation behavior of fostamatinib with a rate constant of 0.0226 h^{-1} and a half-life of 30.61 hours. The structural characterization of these DPs was accomplished via LC-MS/MS and provides detailed fragmentation pathways and insight into the degradation mechanism. The *In-silico* toxicity analysis using ProTox-II highlight the potential safety concerns. DP 1 was the most toxic that exhibit activity in multiple toxicity endpoints. DP 2 also shows significant carcinogenic and immunotoxic potential whereas DP 5 was less acutely toxic (class 5, LD_{50} = 2935 mg/kg). The method's environmental impact was assessed using AGREE and GAPI tools confirms its alignment with green analytical chemistry principles. The study is limited to focus on acidic degradation alone and future work should explore other ICH-recommended stress conditions and validate the method for use with biological samples. Additional experimental validation of *in-silico* toxicity findings and deeper structural studies using techniques like LC-QTOF or NMR are recommended for complete analysis of DPs. Overall, the developed method provides a reliable, eco-friendly, and comprehensive approach to analyse fostamatinib that offers significant advantages for sustainable pharmaceutical quality control and regulatory compliance.

Conflict of Interest

All authors declare there is no conflict of interest

Acknowledgments

The authors are thankful to the A M Reddy Memorial College of Pharmacy, Petlurivariplaem, Narasaraopet – 522601, Palnadu Dist., Andhra Pradesh, India.

References

1. Bairam Ravinder, Tatapudi Hemant Kumar, Pothula Vijay Srinivas, Akaram Likhitha, Tummala Sambasiva Rao, and Gorrepati Naveena, *Lett. Appl. NanoBioScience.*, **14**(1), 1-12 (2024). https://doi.org/10.33263/LIANBS141.048
2. S. S. Prasad, B. B. Kasimala, and V. R. Anna, *Rasayan J. Chem.,* **14**(4), 2183-2190 (2021). http://doi.org/10.31788/RJC.2021.1446426
3. M. Riccaboni, I. Bianchi, and P. Petrillo, *Drug Discov. Today.*, **15**(13-14), 517-530 (2010). http://doi.org/10.1016/j.drudis.2010.05.001.
4. J. R. Strich, M. J. Ramos-Benitez, D. Randazzo, S.R. Stein, A. Babyak, R. T. Davey, A. F. Suffredini, R.W. Childs, and D. S. Chertow, *J. Infect. Dis*., **223**(6), 981-984 (2020). http://doi.org/10.1093/infdis/jiaa789
5. E. Ezzeldin, Iqbal, M., Asiri, Y. A., Sayed, A. Y. A., and Alsalahi, R, *Molecules.,* **26**(15), 4663 (2021). https://doi.org/10.3390/molecules26154663
6. S. R. Tummala and K. P. Amgoth, *Turk. J. Pharm. Sci*., **19**(4), 455-461 (2022). https://doi.org/10.4274/tjps.galenos.2021.17702

7. V. B. Rajesh, S. R. Battula, M. V. N. R. Kapavarapu, and V. R. Mandapati, *Rasayan J. Chem.,* **15**, 2373-2381 (2022). https://doi.org/10.31788/RJC.2022.1547008
8. Bhupatiraju Rajesh Varma, Kasimala Bikshal Babu, Nagamalla Lavanya, and Sayed Fathima, *Anal. Sci. Technol.*, **37**(2), 98-113 (2024). https://doi.org/10.5806/AST.2024.37.2.98.
9. K. M. Amgoth and S. R. Tummala, *J. Res. Pharm.,* **26**(6), 1685-1693 (2022). https://doi.org/10.29228/jrp.259
10. Bhupatiraju Rajesh Varma, Peddi Pavani, Edla Subhashini, Rekha Kandula, and Kasimala Bikshal Babu, *Sep. Sci. Plus.,* **7**(9), e202400106 (2024). https://doi.org/10.1002/sscp.202400106
11. S. R. Tummala, N. Gorrepati, and H. K. Tatapudi, *Current Pharmaceutical Analysis,* **20**(8), 944-952 (2024). https://doi.org/10.2174/0115734129332940240919113159
12. Bhupatiraju Rajesh Varma, B. Srinivasa Kumar, Tangeti Venkata Swamy, Kandula Rekha, and Sayed Fathima, *Toxicol. Int*., **31**(2), 321-334 (2024). https://doi.org/10.18311/ti/2024/v31i2/36370
13. ICH guideline, stability testing of new drug substances and products Q1A(R2), 2003.
14. ICH guideline, harmonised tripartite guideline impurities in new drug substances Q3A(R2), 2006.