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Implementation of green analytical principles to develop and validate the HPLC method for the separation and identification of degradation products of Panobinostat, and its characterization by using LC-QTOF-MS/MS and its *in-silico* toxicity prediction using ADMET software

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ABSTRACT

Panobinostat (PAN) is an inhibitor of histone deacetylase (HDAC) that has been granted approval by the US Regulator for the purpose of treating chronic lymphocytic leukemia. Stress studies were conducted on PAN to assess its inherent stability under physical (thermal and photolytic) and chemical conditions (acidic, basic, neutral, and oxidative) in diluent (water: ethanol 50:50 v/v). The developed HPLC method exhibits both selectivity and specificity towards PAN and its degradation products (DPs). PAN and DPs were resolved using a Waters Xbridge C18 3.0 μm (50 × 4.6 mm) column. The mobile phase consisted of a gradient program (0/15, 2/15, 6/25, 8/25, 10/70, 12/70, 14/90, 16/90, 18/15, and 20/15 (T min/%B)). using mobile phase A as 10 mM ammonium formate buffer (pH 3.0) and mobile phase B as Ethanol with 0.5 mL/min as flow rate, 3 μL as injection volume and at 277 nm as UV wavelength. The PAN exhibited instability under solution state conditions characterized by acidic, basic, and oxidative conditions. The developed chromatographic method has been expanded to include QTOF-MS/MS to characterize the DPs in positive ionization mode. The developed HPLC method has undergone validation in compliance with the ICH guideline Q2 (R1). The method found linear from 12 μg/mL (LOQ) to 300 μg/mL with $r^2 \geq 0.99$. The specificity of the method was assessed through the peak purity of analyte and DPs. The% recovery for analyte was fall in the range of 99.28 to 100.36 with 0.60 % RSD. The%RSD for the analyte in Method precision and intermediate precision is below 1.0. The sample and standard solutions are stable upto 48 h at room temperature. The Green Analytical Principles (GAP) applied to the analytical method using GAPI, AGREE and Eco-Scale tools to assess the Greenness of the analytical method. The PAN and DPs were applied with ADMET prediction software indicated the carcinogenicity, mutagenicity for PAN and only carcinogenicity for DPs. The reproductive toxicity for shown for PAN, DP-2 and DP-3. The DP-3 has shown the penetration of the blood-brain barrier. The method is suitable for the quantification of PAN and its DPs as both active pharmaceutical ingredient and formulations in quality control and stability studies for its regular use.

1. Introduction

Panobinostat lactate (PAN) chemically 3- [4- [2- (2-Methyl-1H-indole-3-yl)ethylaminomethyl] phenyl]−2(E) propenohydroxamic acid **(**Fig. 1**A),** which was approved by USFDA for the treatment of multiple myeloma, a relatively new class of anticancer agent [1]. Its mode of action is a histone deacetylase inhibitor (HDAC) [2]. Its route of administration is both oral and intravenous [3] PAN anhydrous appears as white to faintly yellowish or brownish powder. $C_{21}H_{23}N_3O_2.C_3H_6O_3$ is the chemical formula with lactate salt, and 439.51 (as lactate) is its molecular weight and 349.43 is its free base [4]. Chemically and thermodynamically PAN is stable crystalline form that devoid of polymorphism [5]. PAN is a basic drug substance with a pKa between 8.4 and 9.0 and it is barely soluble in water. According to research, the PAN exhibits optimal solubility in a pH 3.0 buffer, specifically Citrate buffer, at a concentration of approximately 5 mg/mL. Conversely, its solubility

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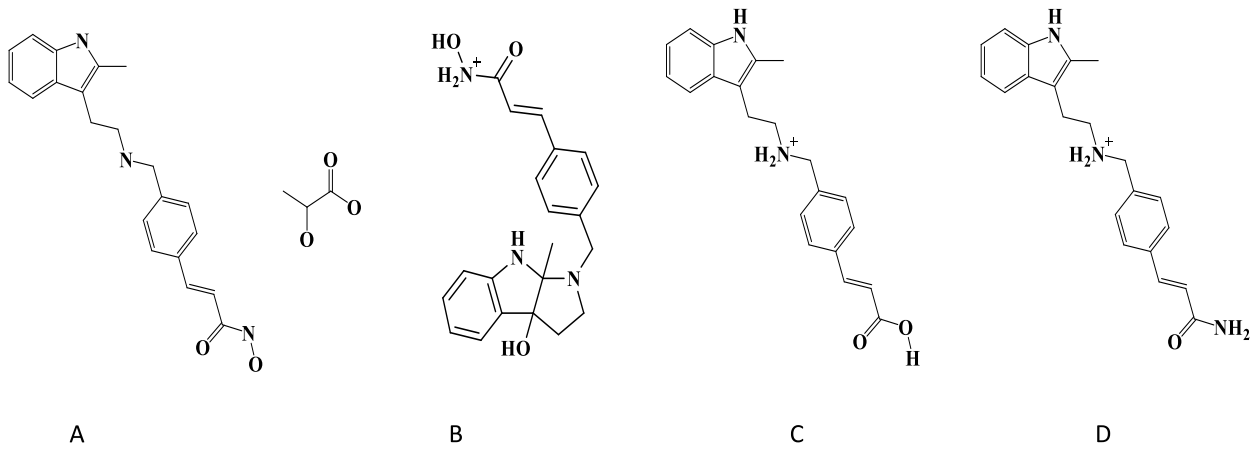

Fig. 1. Structure of (A) Panobinostat (PAN) and lactate (B) DP-1, (C) DP-2, (D) DP-3.

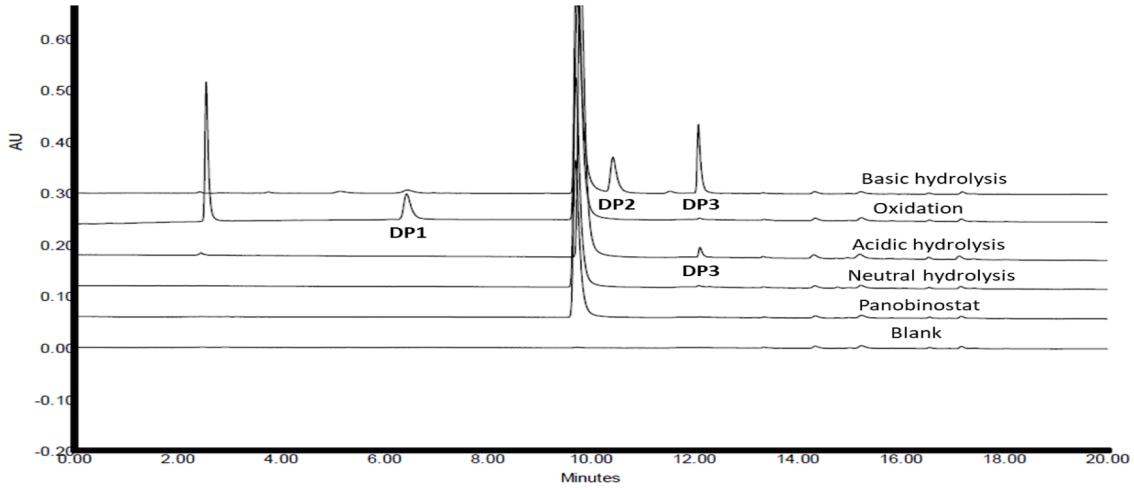

Fig. 2. The overlay of HPLC chromatogram of different degradation conditions of PAN.

Table 1
Optimized stress degradation conditions for PAN.

Stress condition	Concentration of stressor	Exposure conditions	Duration	Degradation Products formed
Acid Degradation	2 N HCl	80 °C	3 d	DP3
Base Degradation	3 N NaOH	80 °C	6 h	DP2 and DP3
Neutral hydrolysis	Water	80 °C	7 d	–
Oxidative degradation	30 % H_2O_2	RT	1 h	DP1
Thermal degradation		80 °C	2 d	–
Photolysis				–
UV (solid)	UV: 200 W h/m2	RT	7 d	–
UV (liquid)			7 d	–
FL (solid)	FL: 1.2 × 10^6 Lux. h	RT	7 d	–
FL (liquid)			7 d	–

UV: Ultraviolet; FL: Fluorescence; RT: Room Temperature; RH: Relative Humidity.

is lowest at a neutral pH, measuring at 0.3 mg/mL at pH 6.8 [6]. The solubility of PAN is contingent upon the pH level.PAN inhibits HDAC enzyme activity is both potently and non-selectively. The transcription activation was led by increasing the acetylation of histone proteins. The accumulation of acetylated histone proteins may lead to cell cycle arrest or apoptosis in cancer cells. It works effectively in combination with Dexamethasone and Bortezomib [7–10]. For patients with advanced cancer, the oral PAN is readily absorbed with T_{max} (the time to peak concentration) within 2.0 h, C_{max} is 2.5 h, the absolute bioavailability is 21 %, the bound to plasma protein is 90 %, the oral clearance is 160 L/h, terminal clearance half-life is 37 h, the strong potential interactions show in strong sensitive CYP2D6 substrates (CytochromeP), CYP3 A inhibitor, strong CYP3 A inducers [11–13]. The general (≥20 %) adverse events include decreased appetite, diarrhoea, vomiting, nausea, fatigue, pyrexia, and peripheral oedema. The haematological laboratory abnormalities include (≥ 60 %) include thrombocytopenia, lymphopenia, leukopenia, neutropenia, anaemia [7,14].

The stability studies for the formulations and drug substances (APIs -Active Pharmaceutical Ingredients) are an important aspect in the drug discovery to forecast the stability nature of the drug [15]. These studies can aid with packaging materials selection, storage conditions and handling. Furthermore, in the identification of DPs (Degradation

Table 2
Elemental composition of Panobinostat (PAN) and its degradation products (DP1-DP3).

Compound	Molecular Formula	Calculated *m/z*	Observed *m/z*	Error[a] (ppm)	MS/MS fragment ions[b]
PAN	$C_{21}H_{24}N_3O_2^+$	350.1863	350.1860	−0.86	350, 333, 176, **158**, 143, 130, 115
DP1	$C_{21}H_{24}N_3O_3^+$	366.1812	366.1806	−1.64	366, 348, 319, 205, 176, 158, **144**, 130
DP2	$C_{21}H_{23}N_2O_2^+$	335.1754	335.1754	0.00	335, 318, 190, **158**, 143, 130, 115
DP3	$C_{21}H_{24}N_3O^+$	334.1914	334.1912	−0.60	334, 317, 170, **158**, 143, 132, 115

[a] The unit parts per million indicates (measured mass-calculated mass).
[b] Bold indicates base peak.

Products), which aid in the establishment of degradation pathways, its nature of the molecules, its properties and inherent stability of the drug [16]. As a regulatory requirement, the formed DPs or impurities at or above 0.1 % should be investigated and characterized using hyphenated techniques like LC-MS/MS (Liquid Chromatography-Mass Spectrometry), NMR (Nuclear Magnetic Resonance) etc. in both formulations and APIs [17,18]. The Green Analytical principles were developed by Galuszka et al. These principles aim to minimize or eliminate the utilization of harmful or hazardous substances. The objective of this study is to minimize the utilization of organic solvents while assessing the environmental sustainability of the existing approach. Several metric tools exist for evaluating the level of environmental sustainability, such the Green Analytical Procedure Index (GAPI), National Environmental Method Index (NEMI), Analytical GREEness (AGREE), and analytical Eco-scale [19–24].

An outright literature search revealed that UPLC-MS/MS (Ultra Performance Liquid Chromatography tandem Mass Spectrometry) methods are published for determination of PAN in mouse plasma. Reported the bioanalytical method for PKPD (pharmacokinetic and pharmacodynamics) and metabolism studies of the PAN in animals [25–27]. To the best of our knowledge, there is currently no literature available that details the analytical method development and method validation of PAN and its degradation products (DPs) under stress conditions using a HPLC (High Performance Liquid Chromatography) method, nor are there extensive stress study experiments of PAN employing LC-ESI-QTOF-MS/MS (Liquid Chromatography - Electron Spray Ionization -Quadrople -Time of Flight -Coupled Mass Spectrometry) in accordance with ICH Q1A guidelines (International Conference on Harmonization) [28] to study its degradation products (DPs). For the identification and characterization of DPs the instrument which is relatively high resolution and mass accuracy, the MS-Q-TOF (Mass spectrometry-Quadrupole-Time-Of-Flight) was used [29].

Hence, the aim is to determine the degradation behaviour of PAN which includes: (i) to optimize and resolved all the DPs from the drug peak and to carry out stress studies in both solution and solid state following ICH Q1A (R2) and Q1B guidelines (International Conference on Harmonization-Quality Guidelines) [28,30], (ii) to analyze the stressed sample by HPLC, (iii) to identify and characterize the DPs employing high resolution mass spectrometry (iv) to propose degradation pathways and mechanistic explanation for DPs and (v) to validate the chromatographic method according to ICH Q2 guidelines [30]. (vi) Utilizing ADMET software to predict the ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) properties, risks and toxicity natures for the drug [31–37].

2. Materials and methods

2.1. Chemicals and reagents

As a gratis sample, the drug PAN (Purity >98 %) was obtained from MSN Laboratories Ltd. Hyderabad, Telangana, India. Analytical reagent (AR) grade sodium hydroxide (NaOH), Ammonium Formate (NH_4COOH), hydrochloric acid (HCl) and formic acid (HCOOH) were acquired from SD Fine Chemicals, located in Mumbai, India. Ethanol was obtained from Honeywell (Hyderabad, India). Hydrogen peroxide (H_2O_2) was purchased as AR grade from Spectrochem (Mumbai, India). For the preparation of sample solutions and mobile phases Milli-Q water was used (Millipore water system Millipore technologies, United States).

2.2. Instrumentation

To perform the experiment, the Agilent 1290 HPLC 2695 series (Agilent Technologies, USA) equipped with photodiode array (PDA) detector, degasser, quaternary gradient pump, autosampler with sample temperature controller, column compartment with temperature controller. For processing the raw data used Empower 3 software. To achieve the good separation between the PAN and DPs the column

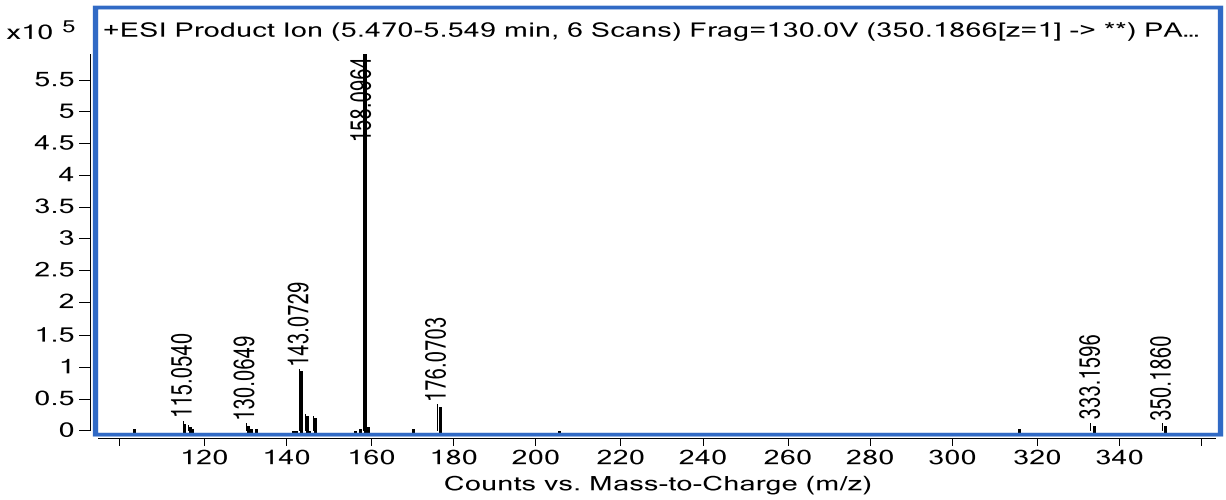

Fig. 3. Mass spectrum of Drug (PAN) using QTOF-MS/MS.

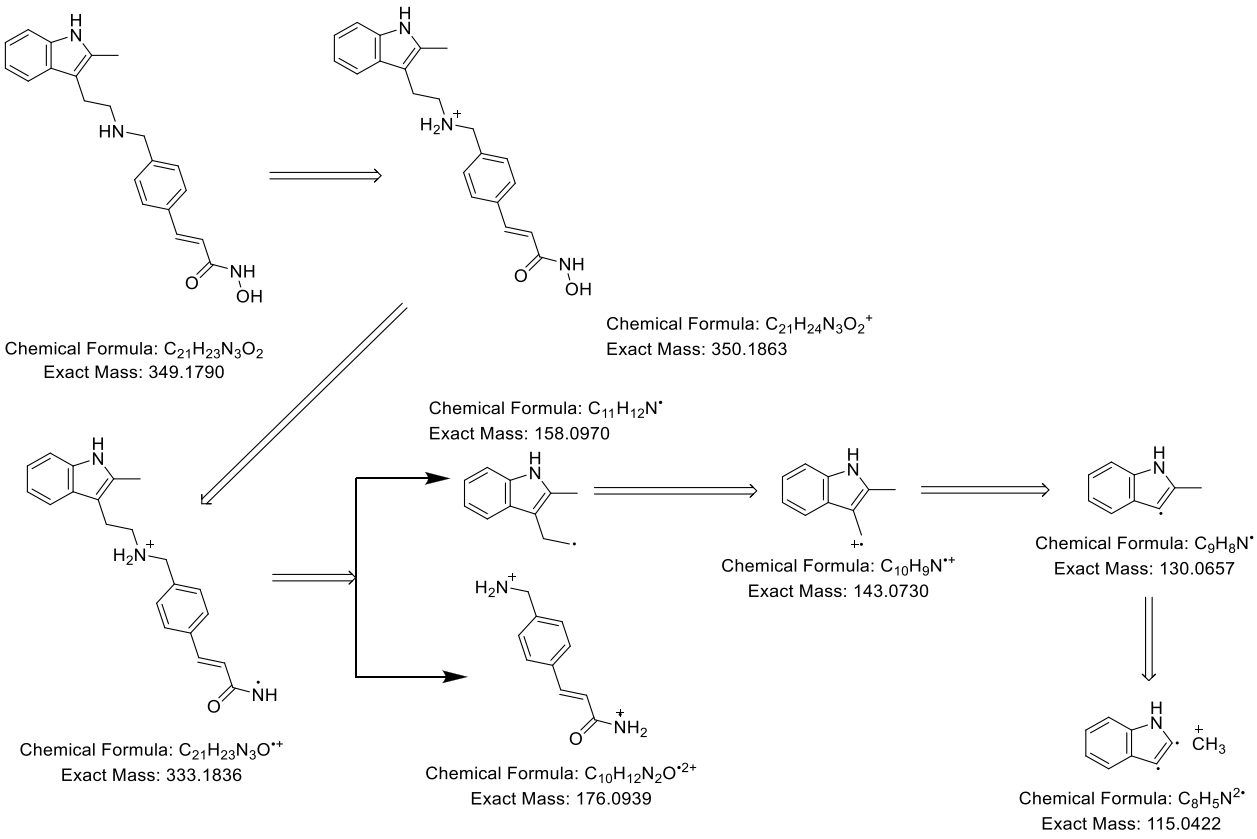

Fig. 4. Proposed degradation pathway of drug (PAN).

Waters X bridge C18 3.0 μm (50 × 4.6 mm) was used with mobile phase A as 10 mM NH_4COOH buffer adjusted to pH 3.0 with HCOOH and Ethanol as mobile phase B with gradient elution mode. The mobile phase flow rate is 0.5 mL/min and injection volume are 3 μL. The final optimized chromatograms were obtained at a wavelength of 277 nm.

To identify and elucidate the DPs, LC-MS/MS 1290 series (Liquid Chromatography-Tandem Mass Spectrometry) (Agilent Technologies, USA) coupled with quadrupole-time of flight (Q-TOF) 6540 series. For ionization, the Electron spray ionization (ESI) was used. The positive mode ESI source was used to get the ionization of drug molecules. Mass Hunter Workstation software was used for the data acquisition. The operating source condition for mass spectrometry includes fragmentation energy was 180 V, capillary at 3600 °C, skimmer 60v. For drying, nebulizing gas at (45 psi) and the nitrogen was used (360 °C, 12 L/min). As a collision gas, the ultrahigh pure nitrogen was used.

For pH measurement, pH meter (Mettler Toledo, Switzerland) was used. To dissolve the samples, an ultrasonic device from Oscar Ultrasonic Pvt Ltd. located in Andheri East, Mumbai, Maharashtra, India was utilized. The thermal stability study was conducted using a hot air oven (Oswarld Scientific Pvt. Ltd., India) that provides digital temperature control with a range accuracy of 2 %. The ICH Q1B compliant photostability chamber was used to perform the photostability experiment (Newtronic Life Care Sciences Equipment Pvt. Ltd., India). The chamber was with temperature sensors, better heat transfer and capable of controlling humidity in the range of ± 2 % RH.

2.3. Forced degradation study (Stress study)

The forced degradation studies was conducted by following the ICH Q1 A (R2) guidelines [28].

2.3.1. Acid degradation

The sample solution prepared at 1000 μg/mL concentration was used for the stress studies. About 100 mg of the drug was weighed and transferred in a 100 mL volumetric flask, added 50 mL of diluent, 5 mL of 2 N HCL solution was added and made reflux at 80 °C for 3 days. After 3 days 2 N NaOH solution was added to neutralize and makeup to the volume with the diluent (1000 μg/mL). Further transfer 5 mL of the stock solution into 20 mL with the diluent and mixed well. The degradation samples were filtered through a 0.22 μm PVDF (Poly Vinylidene Fluoride) membrane syringe filter and subsequently injected into the HPLC at a concentration of 250 μg/mL.

2.3.2. Base degradation

Weighed and transferred about 100 mg of the drug into a 100 mL volumetric flask, added 50 mL of diluent, 5 mL of 3 N NaOH solution was added and made reflux at 80 °C for 6 h. After 6 h 3 N HCl solution was added to neutralize and makeup to the volume with the diluent (1000 μg/mL). Further transfer 5 mL of the stock solution into 20 mL with the diluent and mixed well. The degradation samples were filtered through a 0.22 μm PVDF and injected into the HPLC at a concentration of 250 μg/mL.

2.3.3. Water degradation

Weighed and transferred about 100 mg of the drug into a 100 mL volumetric flask, added 50 mL of diluent and made reflux at 80 °C for 7 days. After 7 days makeup to the volume with the diluent (1000 μg/mL). . Further transfer 5 mL of the stock solution into 20 mL with the diluent and mixed well. The degradation samples were filtered through a 0.22 μm PVDF and injected into the HPLC at a concentration of 250 μg/mL.

2.3.4. Oxidative degradation

Weighed and transferred about 100 mg of the drug into a 100 mL volumetric flask, added 50 mL of diluent and 5 mL of 30 % H_2O_2 solution and kept at room temperature for 1 hour and makeup to the volume with the diluent (1000 μg/mL). Further transfer 5 mL of the stock solution

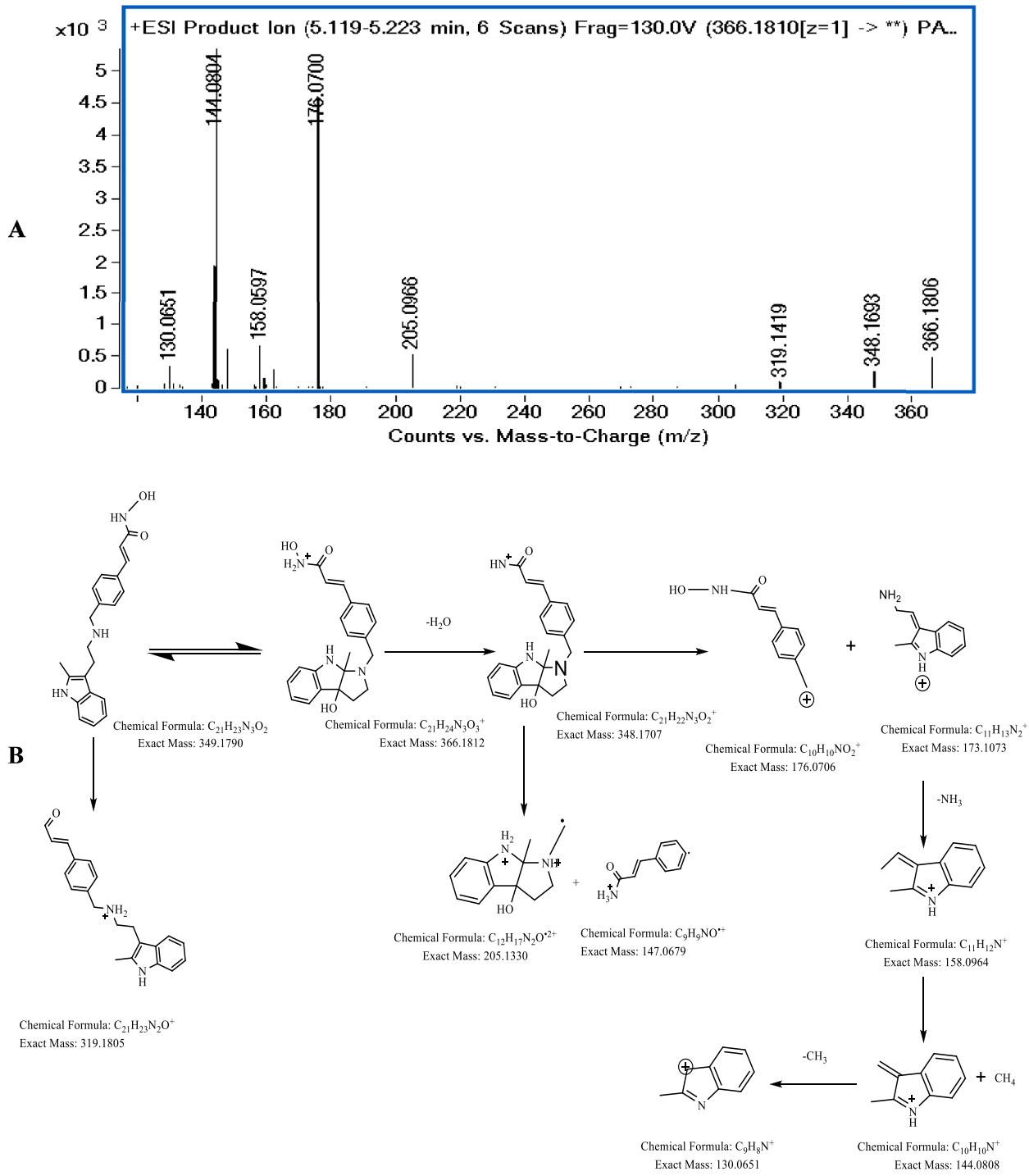

Fig. 5. Mass spectrum for DP1 using QTOF-MS/MS (A) and Proposed Degradation pathway of DP-1 (B).

into 20 mL with the diluent and mixed well. The degradation samples were filtered through a 0.22 μm PVDF and injected into the HPLC at a concentration of 250 μg/mL.

2.3.5. *Thermal degradation*

The drug PAN was subjected to thermal stress studies by being placed in a petri plate as a thin layer and exposed to a temperature of 80 °C for a duration of 2 days in a hot air oven. Weighed and transferred about 100 mg of the drug into a 100 mL volumetric flask, added 50 mL of diluent and makeup to the volume with the diluent (1000 μg/mL). Further transfer 5 mL of the stock solution into 20 mL with the diluent and mixed well. The degradation samples were filtered through a 0.22 μm PVDF and injected into the HPLC at a concentration of 250 μg/mL.

2.3.6. *Photo degradation*

The drug PAN was subjected to photo stress studies by being placed in a petri plate as a thin layer and exposed to UV–Visible light at 200-Watt hours/m^2 and 1.2 M Lux hours for a cycle. Weighed and transferred about 100 mg of the drug into a 100 mL volumetric flask, added 50 mL of diluent and makeup to the volume with the diluent (1000 μg/mL). Further transfer 5 mL of the stock solution into 20 mL with the diluent and mixed well. The degradation samples were filtered through a

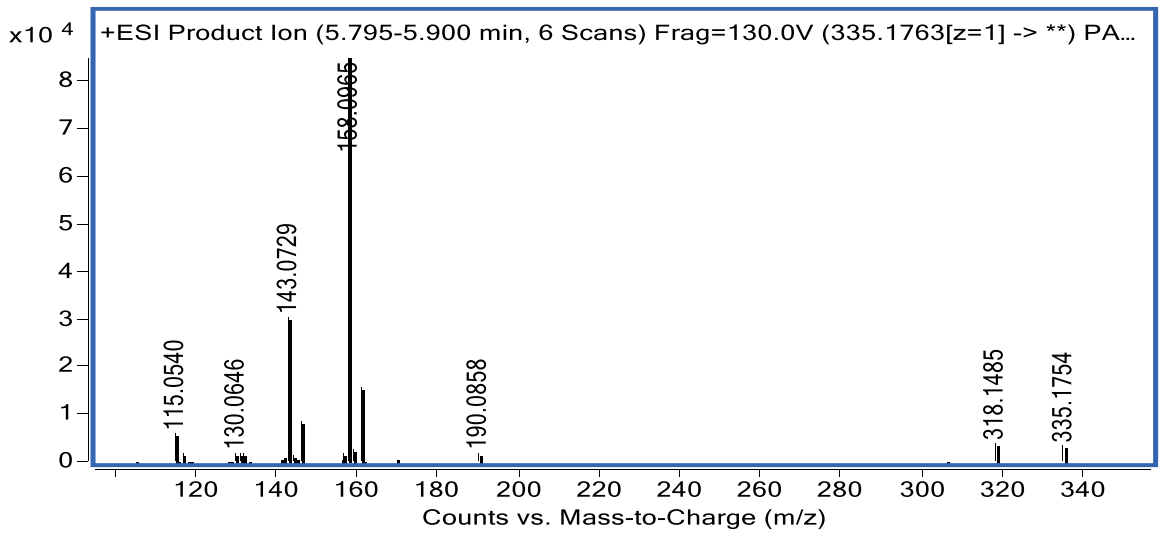

Fig. 6. Mass Spectrum for DP2 using QTOF-MS/MS.

0.22 μm PVDF and injected into the HPLC at a concentration of 250 μg/mL.

At regular intervals the stress samples were collected to identify the DPs formation. The stress studies of PAN were conducted under various hydrolytic conditions (acidic, basic, and neutral) as well as under oxidative (peroxide), thermal, and photolytic conditions to determine its inherent stability and degradation pathways [38].

2.4. Sample preparation

To prepare the sample stock solution of PAN at a concentration of 1000 μg/mL, approximately 100 mg of the drug was weighed and dissolved in a 100 mL volumetric flask using a diluent consisting of a 50:50 v/v mixture of water and Ethanol. To prepare the working standard (PAN 250 μg/mL), 5 mL of the stock solution was diluted with the diluent to a final volume of 20 mL. The samples were filtered through a 0.22 μm PVDF (Poly Vinylidene Fluoride) membrane syringe filter and subsequently injected into the UPLC at a concentration of 250 μg/mL.

2.5. Analytical method validation

The validation of the analytical method was carried out in accordance with ICH Q2 (R1) guidelines. Specificity and selectivity, linearity, precision, intermediate precision, accuracy, robustness, LOD (Limit of Detection), LOQ (Limit of Quantification) and solution stability were among the parameters used to evaluate the method.

2.6. Green analytical principles

The GAC metric tools encompass many key metrics, namely the Green Analytical Procedure Index (GAPI), the National Environmental Method Index (NEMI), the Analytical Eco-Scale, and the AGREE score. The evaluation encompasses several aspects, such as the composition of sample collections, method employed, reagents and solvents utilized, energy consumption, waste disposal procedures, considerations for health, safety, and environmental impact, and other relevant elements pertaining to utilization. The GAPI is a useful tool for assessing the level of environmental sustainability in relation to several factors such as sample preparation, procedures employed, and sample determination. The pictograph is depicted using a color scheme consisting of red, yellow, and green colors. The pictogram is comprised of six separate sections, namely: 1. Sample source, 2. Method type, 3. Sample preparation, 4. Reagents and chemicals employed, 5. Instrumentation, and 6. Symbol O indicates the qualitative or quantitative nature of the method. The NEMI framework is utilized as a visual representation to assess the environmental sustainability of the analytical method. This evaluation is conducted by considering key factors such as the persistence, bioaccumulation, and toxicity (PBT), hazards, waste generation, and corrosiveness associated with the method. The Analytical Eco-scale is a metric tool utilized for assessing the environmental sustainability of a given technique. It achieves this by calculating penalty points associated with various aspects of the method's ecological impact. The Eco-scale quantifies the cumulative penalty points derived from a comprehensive scale of 100. A method is deemed excellent if the cumulative penalty point exceeds 75 %. The AGREE metric is a technique utilized to quantify the level of environmental sustainability of a particular method, drawing upon the 12 principles of green analytical chemistry. The present study employed the AGREE, GAPI, and Eco-scale tools to evaluate the environmental sustainability of the analytical approach as theses tool are advanced and can calculate both qualitative and quantitative. The current method includes the total run time of 20 min, the flow rate of 0.5 mL/min. Each sample run requires the utilization of less than 6 mL of pH of 3.0 Ammonium formate buffer and 4 mL of Ethanol.

2.7. In-Silico ADMET prediction

The ADMET PredictionTM software (version 8.1.0.11, Simulations Plus, Lancaster, CA, USA) was used for the evaluation of the physicochemical properties, absorption, distribution, Metabolism, excretion, and toxicity (ADMET) of PAN and its DPs. The ADMET Predictor is a machine learning software for ADMET modeling with an AI-driven drug design tool. It measures more than 175 properties quickly and accurately, including physicochemicals, pharmacokinetics, Metabolism, toxicity, transporters, ADMET risks, and chemical informatics.

The steps involved in evaluating the properties of the drug and its DPs in ADMET Predictor Software are as follows.

1. Initially, the chemical structures of drugs and DPs were drawn using chem draw (Chemdraw Professional 16.0) and saved into MDL SD files.
2. After being transformed into MDL SD files, the PAN and 3 DP structures were used as system input into the ADMET Software using the FILE option on the top left ribbon and clicking OPEN from the designated path.
3. The ADMET software allows canonical SMILES of PAN and DPS as system input from the Notepad or the Excel sheets.

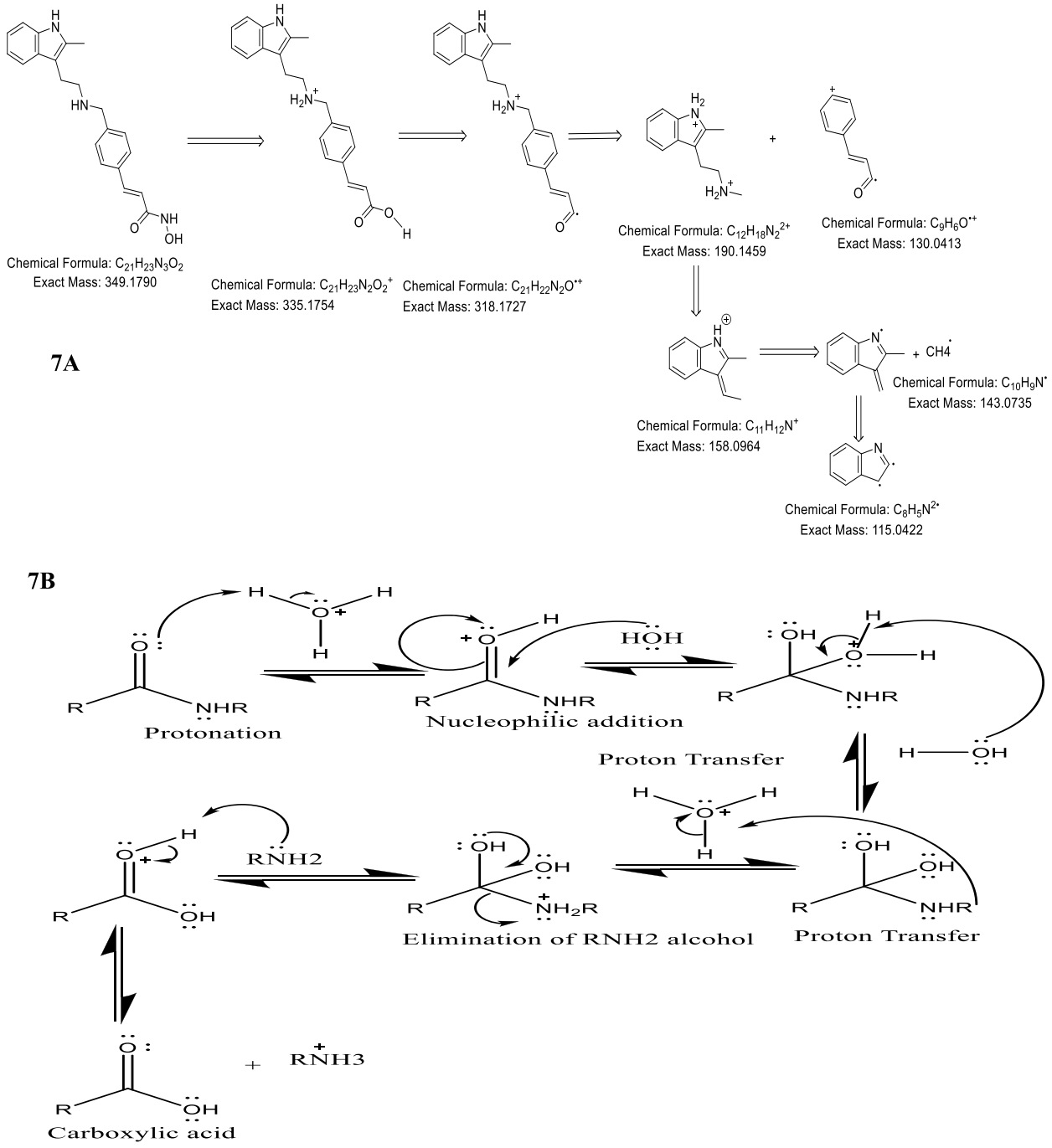

Fig. 7. Proposed Degradation pathway of DP-2 (A) and Proposed mechanism for the formation of DP-2 in alkaline condition (B).

4. The ADMET Software provides the individual identifier code and EDX to the PAN and DP structures for proper identification.
5. Using DATA from ADMET Software at the top ribbon of the software, calculate the ADMET Properties into five modules. 1. Physico-chemical, 2. Transporters, 3. Metabolism, 4. Toxicity and 5. Modeling descriptors
6. From the physico-chemical Module, the Solubility, Permeability, logP, logD, Diffusion coefficient, Air-water partition coefficient, and Pharmacokinetic parameters like Tmax (Peak Time), Cmax (Peak Concentration), Vmax (Maximum rate of Metabolism), CL (Clearance), Km (Michaelis constant).
7. The Toxicity module covers an extensive range of toxicities, including cardiac (hERG–encoded potassium channel), hepatotoxicity, endocrine, phospholipidosis, reproductive toxicity, maximum recommended therapeutic dose, acute rat toxicity, carcinogenicity, skin and respiratory sensitivity, and environmental, etc.
8. The metabolism module allows us to predict the cytochrome P450 site of Metabolism and metabolites for 9 CYP isomers for inhibition or substrate, UGT substrate.
9. The software also predicts the risk modules, i.e., MUT_Risk (Mutation Risk), TOX_Risk (Toxicity Risk), Absn_Risk (Absorption Risk), etc.
10. Customize the required attributes to evaluate the PAN and DPs properties; select FILE and go to the SAVE option to save the data into the excel sheet.
11. ADMET Predictor allows users to guide and search using the HELP option on the top right of the ribbon.

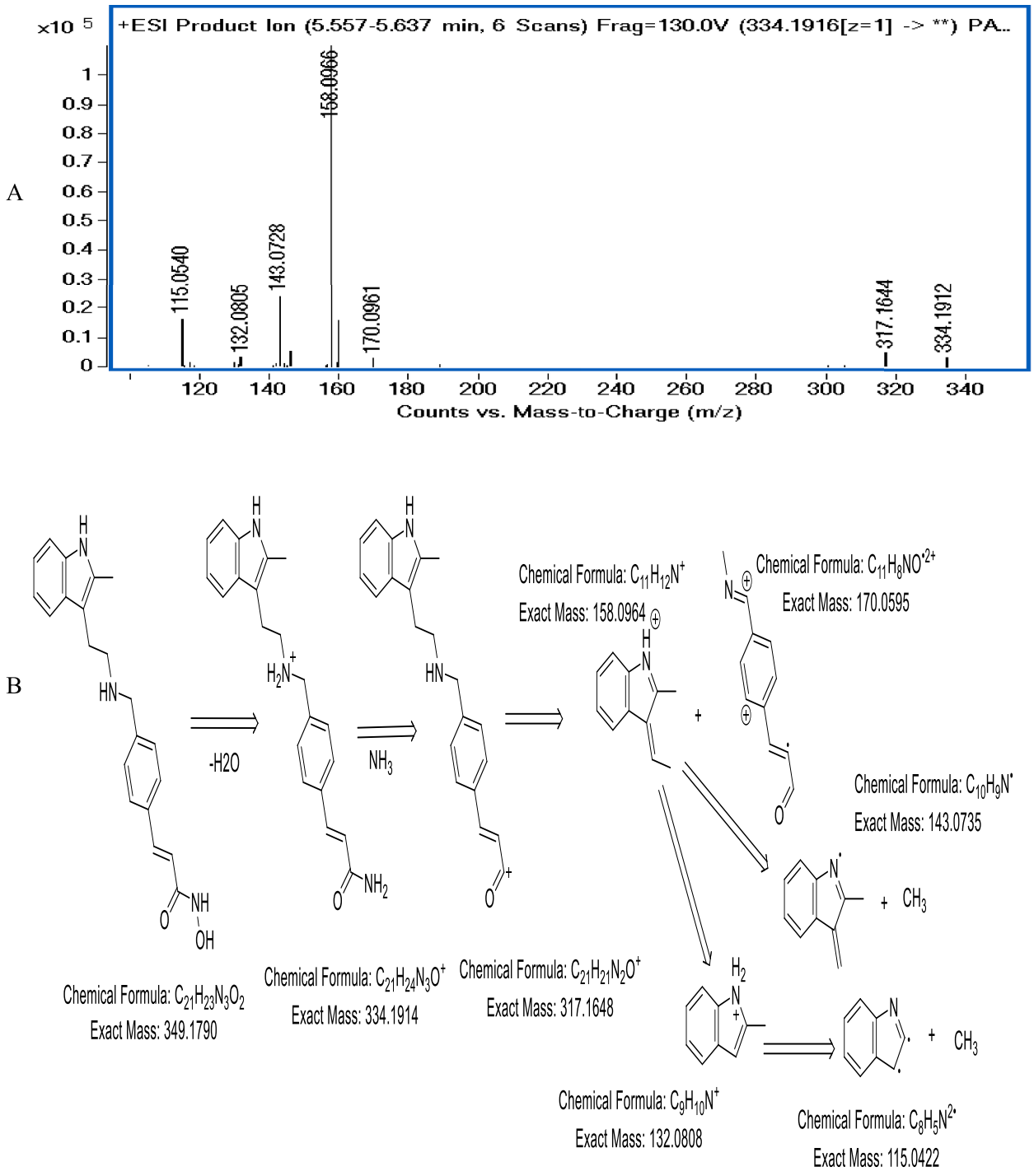

Fig. 8. Mass Spectrum for DP3 using QTOF-MS/MS (A) and Proposed Degradation pathway of DP-3.

3. Results and discussion

3.1. Development and optimization of HPLC method

Preliminary the aim on chromatographic method development for stability studies is to get the good resolution between the DPs as well as form the PAN. The HPLC method development was initiated with the Agilent Eclipse plus C18 column 5 µm (50 × 4.6 mm) with different LC-MS compatible buffers. The combination of Agilent Eclipse plus C18 column and 0.1 % HCOOH, 0.1 % CH_3COOH, and NH_4COOH with organic modifiers results in an asymmetric peak shape and tailing. The NH_4COOH and NH_4CH_3OOH buffers were tried by adjusting the pH value for the separation of the DPs, but none of the combinations found to be suitable to give optimum separation with good peak shapes. The stationary phase was changed from Agilent Eclipse plus C18 column to Waters X bridge C18 column. The symmetrical shape of the drug peak was observed when using a Waters X bridge C18 column, with a buffer solution containing 10 mM ammonium formate and a pH of 3.0, adjusted using formic acid. The degradation samples underwent analysis under similar conditions. The effective separation of PAN and DPs with high sensitivity was achieved using an X bridge C18 3 µm (50 × 4.6 mm) column in gradient elution mode. The mobile phase consisted of 10 mM

Table 3
High resolution mass spectrometry (HRMS) data of product ions of Panobinostat (PAN) and its degradation products (DP1-DP3).

PAN and DPs	Molecular formula	Calculated *m/z*	Observed *m/z*
PAN	$C_{21}H_{24}N_3O_2^+$	350.1863	350.1860
	$C_{21}H_{24}N_3O^{+\cdot}$	333.1836	333.1596
	$C_{10}H_{12}N_2O^{\cdot 2+}$	176.0939	176.0703
	$C_{11}H_{12}N^{\cdot}$	158.0970	**158.0964** [b]
	$C_{10}H_9N^{\cdot +}$	143.0730	143.0729
	$C_9H_8N^{\cdot}$	130.0657	130.0649
	$C_8H_5N^{2\cdot}$	115.0422	115.0540
DP1	$C_{21}H_{24}N_3O_3^+$	366.1812	366.1806
	$C_{21}H_{22}N_3O_2^+$	348.1707	348.1693
	$C_{21}H_{23}N_2O^+$	319.1805	319.1419
	$C_{12}H_{17}N_2O^{\cdot 2+}$	205.1330	205.0966
	$C_{10}H_{10}NO_2^+$	176.0706	176.0700
	$C_{11}H_{12}N^+$	158.0964	158.0597
	$C_{10}H_{10}N^+$	144.0808	**144.0804** [b]
	$C_9H_8N^+$	130.0651	130.0651
DP2	$C_{21}H_{23}N_2O_2^+$	335.1754	335.1754
	$C_{21}H_{22}N_2O^{\cdot +}$	318.1727	318.1485
	$C_{12}H_{18}N_2^{2+}$	190.1459	190.0858
	$C_{11}H_{12}N^+$	158.0964	**158.0965** [b]
	$C_{10}H_9N^{\cdot}$	143.0735	143.0729
	$C_9H_6O^{\cdot +}$	130.0413	130.0646
	$C_8H_5N^{2\cdot}$	115.0422	115.0540
DP3	$C_{21}H_{24}N_3O^+$	334.1914	334.1912
	$C_{21}H_{21}N_2O^+$	317.1648	317.1644
	$C_{11}H_8NO^{\cdot 2+}$	170.0595	170.0961
	$C_{11}H_{12}N^+$	158.0964	**158.0966** [b]
	$C_{10}H_9N^{\cdot}$	143.0735	143.0728
	$C_9H_{10}N^+$	132.0808	132.0805
	$C_8H_5N^{2\cdot}$	115.0422	115.0540

[b] Bold indicates base peak.

NH_4COOH buffer, pH 3.0 adjusted with HCOOH, and Ethanol as mobile phase A and B, respectively. The gradient program consisted of the following values: 0/15, 2/15, 6/25, 8/25, 10/70, 12/70, 14/90, 16/90, 18/15, and 20/15 (T min/%B). The flow rate of the mobile phase has been set at 0.5 mL/min, while the injection volume is 3 μL. The sample and column temperatures are 25 °C and 40 °C respectively. Using PDA detector, the degradation samples were analyzed in a scan mode (190–400 nm). At a wavelength of 277 nm, the drug and DPs exhibited superior detection sensitivity. The transfer of the HPLC method to LC-MS/MS studies was accomplished successfully. Furthermore, the MS conditions were optimized to attain maximum sensitivity in detecting all the DPs.

3.2. Degradation study of PAN

The drug's behavior has been investigated using HPLC under various stressor conditions. The PAN was observed to be prone to deterioration when subjected to hydrolytic and oxidative stress conditions. The chromatogram overlay of the degradation samples, as shown in Fig. 2, illustrates the successful separation of the PAN from the DPs using HPLC. The three DPs were produced under conditions characterized by the presence of hydrolytic and oxidative stress. The structures have been discovered and described using ESI/Q-TOF/MS/MS. The illustrated structure of PAN and its DPs are presented in Fig. 1. The final optimal

Table 5
Accuracy data for PAN.

Amount (μg mL^{-1})	Amount added (μg mL^{-1})	Final concentration (μg mL^{-1})	% Recovery	% RSD
100	100	200 (80 %)	100.36	0.55
100	150	250(100 %)	99.28	0.34
100	200	300 (120 %)	100.05	0.27

Table 6
Precision Study by Intra-day and Inter-day Assay of PAN.

Obtained concentration (μg mL^{-1})	Method Precision (μg mL^{-1})	Intermediate Precision (μg mL^{-1})		
		Day 1	Day 2	Day 3
250.2	252.3	251.9	250.7	252.5
249.8	249.4	254.3	251.9	251.4
250.1	251.3	248.9	250.4	252.3
250.1	253.1	251.5	250.1	252.5
250.3	250.1	253.4	251.9	250.6
250.2	252.8	250.1	252.1	249.8
Average	251.5	251.6	251.8	251.5
SD	1.50	2.01	0.88	1.13
% RSD	0.60	0.80	0.35	0.45

Table 7
Robustness study of PAN on system suitability criteria.

Robustness conditions	Resolution between PAN and DP2	PAN peak asymmetry	PAN peak plate count	% Assay
Temperature As Such (40 °C)	1.82	1.12	10,564	99.5
Temperature Minus (35 °C)	1.75	1.18	9566	99.3
Temperature Plus (45 °C)	1.86	1.05	12,689	99.3
Buffer pH as Such (pH 3.0)	1.82	1.12	10,564	99.5
Buffer pH Minus (pH 2.8)	1.79	1.1	10,136	99.3
Buffer pH Plus (pH 3.2)	1.80	1.12	11,587	99.3
Flow Rate as Such (0.5 mL/Min)	1.82	1.12	10,564	99.5
Flow Minus (0.4 mL/Min)	1.89	1.16	9816	99.3
Flow Plus (0.6 mL/Min)	1.72	1.1	11,687	99.5

Table 8
Sample and Standard Solution stability.

Time Interval	Sample	Standard
Initial	99.5	99.6
After 24 Hrs at RT	99.2	99.4
After 48 Hrs at RT	99.2	99.1
% Difference (Initial-48 hrs/Initial) *100	0.3 %	0.5 %

Table 4
The Assay, Total Degradation, Mass Balance and Peak purity of PAN at various degradation conditions.

Stress Condition	Assay (%)	Degradation (%)	Total Related Substance (%)	Mass Balance (%)	Purity Angle	Purity Threshold
As Such	98.82	NA	0.7	NA	0.028	0.222
Acid hydrolysis	96.22	2.3	3.29	98.51	0.028	0.225
Base hydrolysis	95.01	3.11	3.53	98.54	0.027	0.226
Thermal degradation	98.54	0.38	0.68	99.18	0.028	0.239
Photolytic degradation	98.32	0.30	0.51	99.02	0.026	0.224
Neutral Hydrolysis	98.45	0.07	0.82	99.27	0.026	0.223
Oxidative degradation	95.95	3.18	3.96	99.21	0.027	0.537

Table 9
Represents the GAC tool Eco scale to assess the Greenness analytical method.

Analytical Eco Scale for assessing the analytical method			
S. No	**Name**	**Proposed Method Penalty Points**	**Reported Method Penalty Points**
Chemicals or reagents			
1	Ammonium formate	4	0
2	Ethanol	4	4
3	Formic acid	6	6
4	Acetonitrile	0	7
5	Methanol	0	7
Instruments			
1	Energy- 1.5 kWh of energy per sample for HPLC and LC-MS	2	2
2	Occupational Waste- Procedure release vapours into the environment	0	3
Waste			
1	Total Amount of waste generated (Waste Generated >10 mL)	5	5
2	Management (The generated waste has a degradation process)	1	3
Total Penalty Points		**24**	**37**
Analytical Eco Scale Total Score (100-Total Penalty Points)		**76**	**63**
Green ness Evaluation		**Excellent**	**Acceptable**

Table 10
Represents the GAC tool AGREE to assess the Greenness analytical method.

AGREE tool for assessing the Analytical Method on HPLC and LC-MS/MS for Proposed and Reported sample procedure			
S. No	**Green analytical Chemistry Principles**	**Proposed Sample Procedure**	**Reported Sample Procedure**
1	Direct analytical techniques should be applied to avoid sample treatment	Off-Line Analysis	External sample pre-treatment and batch analysis
2	Minimal sample size and minimal number of samples are goals	1 g	2 g
3	If possible, measurement should be performed in-situ	At-line	At-line
4	Integration of analytical processes and operations saves energy and reduces the use of reagents	3 distinct steps involved in the sample preparation procedure	5 distinct steps involved in the sample preparation procedure
5	Automated and miniaturized methods should be selected	Semi-Automatic and miniaturized	Semi-Automatic and miniaturized
6	Derivatization should be avoided	No Derivatization	No Derivatization
7	Generation of a large volume of analytical waste should be avoided, and proper management of analytical waste should be provided	1 g	3 g
8	Multi-analyte or multi-parameter methods are preferred versus methods using one analyte at a time	4 analytes determined with single run, 3 samples analysed per hour	4 analytes determined with single run, 15 samples analysed per hour
9	**The use of energy should be minimized:** Select the most energy-intensive technique used in the method, or the closest equivalent	HPLC, LC-MS	LC-MS
	Alternatively, estimate the total power consumption of a single analysis in kWh	1	1
10	Reagents obtained from renewable sources should be preferred.	All reagents are from bio-based	None of the reagents are from bio-based
11	Toxic reagents should be eliminated or replaced	No toxic reagents or solvents used	Yes toxic reagents or solvents used
12	Operator's safety should be increased.	The threats that are not avoided are a. Bioaccumulative b. Highly flammable c. Explosive	The threats that are not avoided are a. Bioaccumulative b. Highly flammable c. Explosive d. Toxic to aquatic life e. Corrosive

stress conditions are listed in Table 1.

3.2.1. *Hydrolytic degradation*

The optimum degradation was observed to the PAN by performing acid hydrolysis at 2 N HCl (3 d at 80°C) in the reflux results in the formation of DP3, where, in base hydrolysis when refluxed with 3 N NaOH for 6 h at 80°C the excess degradation was observed, two DPs (DP2 and DP3) were formed. Neutral degradation was conducted in diluent (50:50 water: Ethanol) for 6 h when refluxed at 80°C which does not result in the formation of DP (**Refer** Fig. 2).

3.2.2. *Oxidative degradation*

Oxidative forced degradation studies using 30 % H_2O_2 for 6 h were performed at room temperature. DP1 was formed as the result of above conditions (**Refer** Fig. 2).

3.2.3. *Thermal and photolytic degradation*

The PAN was kept in a Petri dish in the form of a thin homogeneous layer in a hot air oven to examine the effect of temperature. No degradation was detected in the drug PAN even after being subjected to a temperature of 80°C for a period of 2 days. To study the effect of light, the PAN was exposed to photolytic stress conditions (UV: 200 W h/m2 and FL: 1.2 × 106 Lux. H) in solid and liquid form. PAN was not found to be degraded under UV light in the solid and liquid states.

3.3. *LC–MS/MS studies of PAN and its DPs*

The analysis of the degradation products (DP) was carried out using LC-ESI-QTOF-MS/MS under various conditions of experimentation. The accurate determination of mass depends upon the examination of high-resolution mass fragmentation patterns. The accurate prediction of DP structures was achieved by utilizing RDB measurements and adhering to the nitrogen rule. Table 2 presents the elemental composition of the protonated DPs, as well as their corresponding product ions. Figs. 3–8 presents the high-resolution mass fragmentation patterns and their corresponding mechanisms for the generation of PAN and DPs.

3.3.1. *MS/MS of PAN*

The MS/MS data of PAN displayed a fragmentation pattern for drug molecules with the elemental composition of $C_{21}H_{23}N_3O_2$ and an accurate mass 349.42 *m/z*. The product ions $[M+H]^+$ of protonated PAN (Fig. 3) (t_R =5.5 min.) displays at *m/z* 350.1860 in ESI-MS/MS spectrum. The fragmentation at hydroxylamine bond from $(M+H)^+$ of Panobinostat resulted in a fragment at 333.1596, which would further break at NH linker to give *m/z* 176.0703 and characteristic high abundant base peak at *m/z* 158.0964. The methyl radical originating from the base peak fragment at *m/z* 158.0964 would undergo fragmentation, resulting in the formation of a radical cation with *m/z* 143.0729. Subsequent to the CH radical cation loss from the *m/z* 143.0729 fragment, *m/z* 130.0649 was generated. The dissociation of the methyl cation at *m/z* 130.0649 results in the generation of *m/z* 115.0540. The accurate

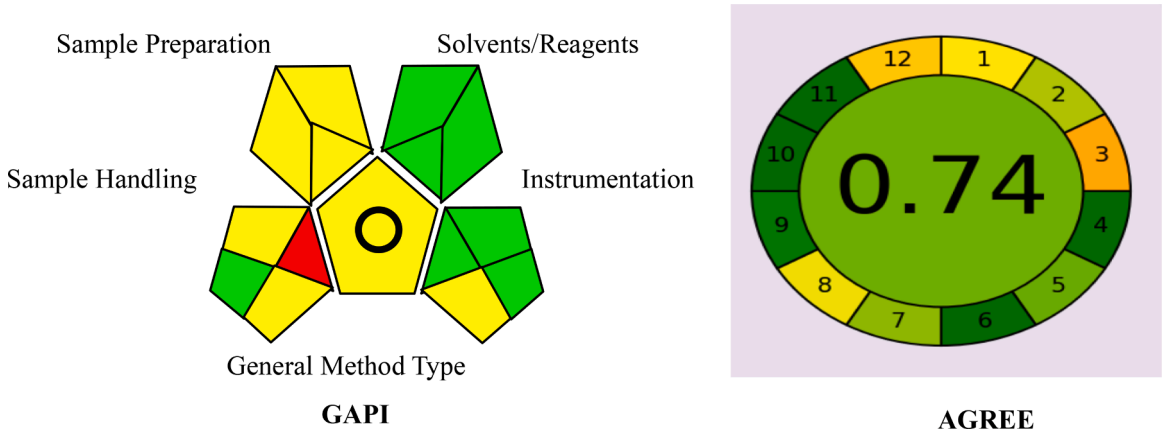

Fig. 9. Representation of GAPI and AGREE pictogram for the proposed analytical method.

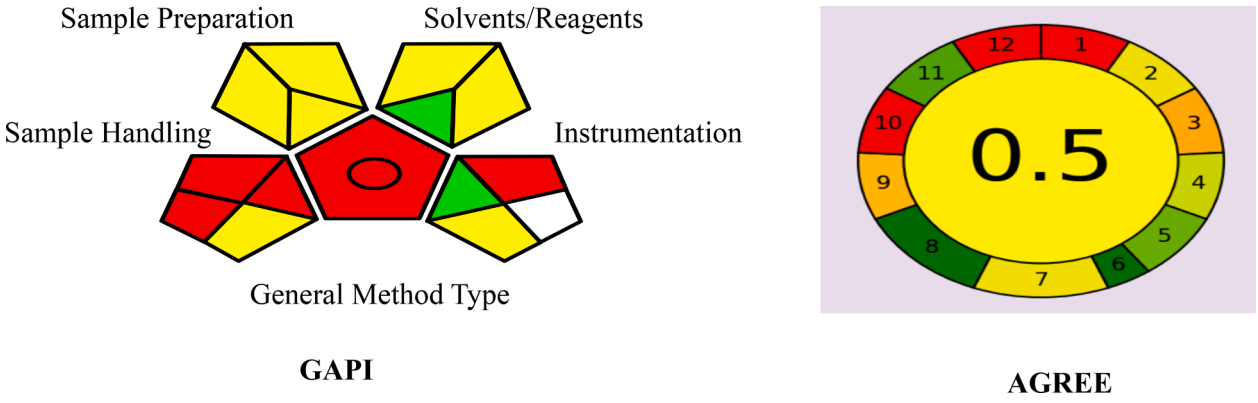

Fig. 10. Representation of GAPI and AGREE pictogram for the reported analytical method.

mass measurement studies have confirmed the elemental composition of all the fragment ions, as presented in Table 2. Table 3 represents the accurate mass, calculated mass and the predicted molecular formula for PAN and its fragmented ion. (Fig. 4)

3.3.2. *MS/MS of degradation products*

The structural analysis of all the formed DPs was carried out by comparing the mass fragmentation of the PAN with that of DPs. Additionally, an elemental composition analysis was performed using RDB values and accurate mass measurement. Fig. 5-8 depicts the mass fragmentation pattern of DPs. Table 3 represents the accurate mass, calculated mass and the predicted molecular formula for DP1 to DP3 and its fragmented ion.

3.3.2.1. Oxidative DP1 (m/z 366). The formation of protonated DP1 (*m/z* 366.1806) occurred under oxidative (major) conditions, as depicted in Fig. 5**A**. The elution of this compound took place at a retention time of 5.2 min. DP1′s molecular formula is $C_{21}H_{24}N_3O_3+$. This indicates that peroxide has been added to the drug, which has a mass-to-charge ratio of 349.18. The elimination of water from DP1 (*m/z* 366.18) results in the formation of product ions at *m/z* 348.17, which provide insight into the molecule's structure. The aforementioned product ions generate distinct fragment ions with mass-to-charge ratios of 205.10 and 158.09. The fragmentation of *m/z* 205.10 results in the formation of the tropylium ion fragment with *m/z* 176.07, subsequent to the loss of methylamine. Additionally, the cyclization of the indole structure gives rise to the formation of a quinoline structure with *m/z* 144.08. The process by which DP1 undergoes fragmentation can be elucidated through the illustration presented in Fig. 5**B**. Table 3 represents the accurate mass, calculated mass and the predicted molecular formula for DP1 and its fragmented ion.

3.3.2.2. Base DP2 (m/z 335). The major alkaline hydrolytic DP2 (*m/z* 335.1754, $C_{21}H_{23}N_2O_2+$) was formed by the hydrolysis of CO—NH—OH to COOH in the studied stress conditions and eluted at a RT of 5.8 min and ESI-MS/MS spectrum for DP2 in alkaline hydrolysis (Fig. 6). When compared with the PAN, the molecular formula of DP2 shows the elimination of 1 N (Nitrogen) which indicates the loss of *m/z* 14. The loss of OH from COOH presented characteristic product ions at *m/z* 318.1485. The Fragmentation between alpha-carbon with respect to the NH linker and phenyl ring would give *m/z* 190.0858 and along with *m/z* 130.0646 due to fragmentation between phenyl and the alpha-carbon with respect to NH linker. The peaks *m/z* 158.0965, *m/z* 143.0729 and *m/z* 115.0540 are similar to the original fragmentation pattern of Panobinostat. The presence of all these characteristic product ions and elemental composition were compatible with the proposed structure of DP2. The proposed mass fragmentation for DP2 is explained in Fig. 7**A**. The mechanism for fragmentation of DP2 can be explained as shown in Fig. 7**B**. It involves amide hydrolysis in the conversion of amide to COOH in alkaline hydrolysis. Table 3 represents the accurate mass, calculated mass and the predicted molecular formula for DP2 and its fragmented ion.

3.3.2.3. Base DP3 and acid DP3 (m/z 334). The formation of the protonated DP3 (with a retention time of 5.6 min) was seen under both acidic and basic forced degradation conditions, as shown in Fig. 2. The protonated DP3 showed its parent ion $[M + H]+$ with a mass-to-charge ratio of 334.19. The obtained mass spectrum reveals a mass difference of 15 Da between PAN and DP3. The provided diagram, labeled as Fig. 8, is presented for reference. The spectrum indicates that the substance PAN

Table 11
Represents the GAC tool GAPI to assess the Greenness analytical method for Proposed and reported method.

GAPI tool for assesing the Analytical Method on HPLC and LC-MS/MS			
Sample Sourcing		**Proposed Method**	**Reported Method**
1	Collection	Off-line	Off-line
2	Preservation	Physical or Chemical	Physical or Chemical
3	Transport	None	None
4	Storage	None	Under special conditions
Method Type and Sample Preparation			
5	Type of Method	Simple Procedure	Extraction required
6	Scale of extraction	Micro-Extraction	Micro-Extraction
7	Solvent or reagent used	Green Solvents/ Reagents used	Non-Green solvents/ reagents used
8	Additional Treatment	Simple Treatment	Advanced Treatments
Reagents and Solvents			
9	Amount	<10 g used	<10 g used
10	Health hazard	Slightly toxic, slight irritant; NFPA health hazard score of 0 to 1. No special hazards.	Moderately toxic; could cause temporary incapacitation; NFPA = 2 or 3.
11	Safety hazard	Highest NFPA flammability or instability score of 0 or 1. No Special hazards.	Highest NFPA flammability or instability score = 2 or 3, or a special hazard is used.
Instrumentation			
12	Energy	<=0.1 kWh per sample	<=0.1 kWh per sample
13	Occupational hazard	Hermetic sealing of the analytical process	Emission of vapours to the atmosphere
14	Waste	< 1 g	1–10 mL (1–10 g)
15	Waste treatment	Degradation, Passivation	Degradation, Passivation
Quantification/Qualification			
1	Symbol O	Procedure for qualification and quantification	Procedure for qualification and quantification

undergoes dehydration, resulting in the loss of a water molecule. The mass spectrum of PAN exhibited similarities in the product ions at *m/z* 158, *m/z* 143, and *m/z* 115. Additional fragment ions were detected at mass-to-charge ratios of 317, 170, 158, 143, 132, and 115. The structure of DP3 is confirmed by the validation of its structural integrity through the analysis of fragment ions and accurate estimation of its mass. The process of degradation for the generation of DP3 under both acidic and basic hydrolytic conditions is illustrated in Fig. 8. The mechanism for the formation of DP3 is mainly due to the loss of water molecules from the drug PAN. Table 3 represents the accurate mass, calculated mass and the predicted molecular formula for DP3 and its fragmented ion.

3.4. HPLC analytical method validation

After optimization, the HPLC method for PAN was validated following ICH Q2 (R1) guidelines.

3.4.1. Specificity and selectivity

The evaluation of the method's specificity and selectivity included assessing peak purity for PAN and its degradation products using a PDA detector, and determining purity angle and threshold values. The assessment of peak purity was conducted by verifying that the purity angle of PAN remained below the predetermined purity threshold in the presence of all DPs. Refer Table 4 for peak purity at various degradation conditions. From the blank, no interference is seen at the Retention time (RT) of PAN and DPs.

3.4.2. LOD and LOQ

The term LOD refers to the minimum concentration level of a PAN (analyte of interest) at which the detection limit is determined, utilizing a signal-to-noise (S/N) ratio of 3. In contrast, LOQ refers to the minimum quantifiable limit determined by utilizing a S/N ratio of 10. The estimation of the concentration of LOD and LOQ was carried out utilizing the S/N Ratio approach. The results of the research show that the study has established the LOD and LOQ for PAN as 5 and 12 μg/mL, respectively.

3.4.3. Linearity

The linearity of the method was assessed by constructing a calibration curve utilizing a standard solution of PAN. The solution was prepared at six separate concentrations, ranging from 12 to 300 μg/mL, and was subjected to triple analysis. The calibration curve was constructed by plotting the mean peak area, obtained from three replicate measurements, at different concentrations covering the limit of quantification (LOQ), as well as levels corresponding to 25 %, 50 %, 80 %, 100 %, and 120 % of the PAN test concentrations. The data pertaining to linearity was analyzed utilizing a linear regression model through statistical analysis. The linear regression equation has been calculated as $y = 11355x + 54{,}857$, while the coefficient of determination (r^2) has been determined to be 0.998. Based on the data, it appears that the method exhibits a linear correlation.

3.4.4. Accuracy

The accuracy of the HPLC method that was developed has been evaluated by employing the standard addition technique. The methodology involved the preparation of drug PAN at three discrete concentration levels, specifically 80 %, 100 %, and 120 %, which were subsequently incorporated into the sample solutions. The specimens were examined in triplicate, with a sample size of $n = 3$. The% recovery of PAN was determined through a comparison of the peak area values between the spiked sample and the standard PAN solutions. Based on the results outlined in Table 5, the% recovery and% RSD values were observed to fall within the range of 99.28–100.36 and 0.60, respectively.

3.4.5. Precision

The HPLC method that was developed underwent an evaluation of precision parameters, specifically repeatability and inter-day precision. The determination of the method's repeatability involves the injection of the sample or standard solution multiple times within a short time frame, commonly referred to as intraday precision. The precision of PAN was assessed by performing six measurements at 100 % of the sample concentration (250 μg/mL). The Table 6 represents the recovered value expressed as a percentage of the relative standard deviation, denoted as % RSD and found to be less than 0.60, indicating that the method is reproducible within the specified conditions and concentration.

3.4.6. Intermediate precision

Intermediate precision, also known as inter-day precision, was evaluated on multiple occasions using distinct instruments and analysts. The experiment was conducted over a period of 3 consecutive days using the assay concentration of 250 μg/mL. The study's outcomes were presented in the form of% RSD values. The Table 6 represents the recovered values at different days were consistently below 1.0 %, indicating that the HPLC method exhibits excellent precision.

3.4.7. Robustness

The HPLC method that was developed and optimized underwent an evaluation of its robustness parameter. The study evaluated the robustness of the method by intentionally varying specific parameters such as the flow rate (0.5 ± 0.1 mL/min), pH of the mobile phase (3.0 ± 0.2), and column temperature (40±5 °C). The method's robustness was assessed by intentionally altering the method parameters. It was subsequently validated using system suitability criteria, including resolution, peak asymmetry, peak plate count and assay results. From the Table 7 represents the analytical method was determined to be robust, as no significant changes were observed in the system suitability criteria and the assay value of the PAN.

Table 12
Insilco toxicity prediction of PAN and its DPs using ADMET prediction software.

ADMET Properties	**Identifier**	**PAN**	**DP-1**	**DP-2**	**DP-3**
ADMET Code	Predicts carcinogenicity in rats and mutations and likely Ames Positive	Carcinogenicity; Mutations	Carcinogenicity	Carcinogenicity	Carcinogenicity
pKa	***S*+Acidic_pKa**	13.59	9.61	13.93	13.87
	***S*+Basic_pKa**	8.37	6.28	8.72	8.54
Lipophilicity	***S*+logP**	2.234	1.401	1.637	3.084
	***S*+logD**	1.243	1.356	1.616	1.917
Solubility	***S*+Sw** Water solubility (mg/mL)	0.106	0.096	0.018	0.079
pH	***S*+pH_Satd** Native pH calculated at *S*+Sw solubility.	8.668	7.571	6.573	9.418
Pharmacokinetics	Cmax_hum-1.0 (ng/mL).	4.27	8.45	15.44	5.36
	Tmax_hum-1.0 Time (hr)	3.92	2.73	2.98	3.25
	AUC_hum-1.0 (ng-hr/mL)	65.15	116.24	135.71	75.71
	CL_hum-1.0 Total clearance (L/hr)	12.36	7.92	6.73	11.74
	T Half_hum-1.0 Half-life (hr)	7.05	7.81	3.27	7.38
Permeability	***S*+Peff** (cm/s x 10^4).	0.71	2.058	0.827	1.313
	Perm_Cornea (cm/s x 10^7).	108.827	137.185	35.711	367.385
	Perm_Skin (cm/s x 10^7)	1.147	0.16	0.132	4.523
Clearance mechanism	**S+CL_Metab**	Yes	Yes	No	Yes
	S+CL_Mech	Renal	Renal	Renal	Renal
CYP Inhibition	CYP1A2_Inh	Yes	No	No	Yes
	CYP2C9_Inh	No	No	No	No
	CYP2C19_Inh	No	No	No	Yes
	CYP2D6_Inh	Yes	Yes	Yes	Yes
	CYP3A4_Inh	Yes	Yes	Yes	Yes
CYP Substrate	CYP1A2_Substr	Yes	No	Yes	Yes
	CYP2A6_Substr	No	No	No	No
	CYP2B6_Substr	No	No	No	No
	CYP2C8_Substr	Yes	Yes	Yes	Yes
	CYP2C9_Substr	No	No	No	No
	CYP2C19_Substr	No	No	No	No
	CYP2D6_Substr	Yes	Yes	Yes	Yes
	CYP2E1_Substr	No	No	No	No
	CYP3A4_Substr	Yes	Yes	Yes	Yes
Sensitization	**Sens_Skin**	Sensitizer	Nonsensit.	Sensitizer	Sensitizer
	Sens_Resp	Nonsensit.	Sensitizer	Sensitizer	Nonsensit.
Cardiac Toxicity	**hERG_Filter**	No	No	No	No
	hERG_pIC50	4.82	4.707	4.776	5.074
Lethal dose	**Rat_Acute** LD50 (mg/kg)	704.505	442.027	993.57	592.311
	Rat_TD50 TD50 (mg/kg/day in oral dose)	1.194	1.306	1.766	1.235
	Mouse_TD50 TD50 (mg/kg/day in oral dose)	490.378	201.43	226.679	194.617
Phospolipidosis	Qualitative estimation of causing phospholipidosis.	Toxic	Nontoxic	Nontoxic	Toxic
Reproductive toxicity	Qualitative estimation of reproductive / developmental toxicity.	Toxic	Nontoxic	Toxic	Toxic
Risk	**ADMET_Risk**	3.074	1.157	3.545	2.037
	Absn_Risk	0.074	0.157	0.946	0.037
	MUT_Risk	1.5	0.6	0	0.6
	TOX_Risk	2	1	1	1
Blood brain barrier	Predicts Blood Brain Barrier	Low	Low	Low	High

3.4.8. Solution stability

The solution stability was assessed for the sample and standard at 250 μg/g concentration kept at room temperature (RT). It is evaluated by injecting sample and standard solutions at the regular intervals and calculated the relative difference in percentage from the initial assay results to the time intervals. The Table 8 represents the results and found below 2 % difference from the initial results for both sample and standard and stable up to 48 h

3.5. Green analytical chemistry assessment

The proposed green analytical method was developed using greener and more economical solvents like ethanol and water was used instead of traditional hazardous solvents like methanol or acetonitrile. A shorter column with dimensions (50 mm x 4.6 mm) 3.0 μm was used to have a shorter run time and rapid analysis, so the energy utilization for each run was reduced. The reported bioanalytical method on HPLC for the simultaneous detection of four HDAC inhibitors includes PAN in mice plasma consisting the mobile phase at a 25: 75 % ratio (0.2 % Formic acid: Acetonitrile), and diluent at 80:20% ratio (Methanol: water)

All the above features promoted greener method for proposed method than the reported method. The comparison between the proposed and reported methods was evaluated using the green metric tools like GAPI, AGREE and Analytical Eco-scale [31–37].

The Analytical Eco-scale, a metric tool, is employed to measure penalty points. The proposed method shows total penalty points amount to 24 whereas the reported method shows the total penalty points amounts to 37, thus the proposed analytical method of analysis obtains a score of 76, indicating excellent performance and the reported analytical method obtains a score of 63 indicating acceptable [39]. For detailed results; please refer to Table 9 The AGREE software was developed by The Gdansk University of Technology in Poland, using a structure that consists of 12 green analytical principles. Each principle is assigned a score ranging from 0.1 to 1.0, denoting its level of adherence to sustainability criteria. The proposed method's overall AGREE score of 0.74 where as reported methods overall AGREE score is 0.50 is depicted in Table 10, Fig. 9 and Fig. 10. The GAPI tool displays a collection of 15 pictograms and 5 pentagrams. For proposed method, among the several pictograms utilized, it is significant that the pictogram denoted by the number 8 is depicted in the color red, symbolizing the specific sample treatment used. Conversely, the pictograms about sample preparation and sample handling are illustrated in yellow. The reagents and

instruments utilized in the study are indicated by a green pictogram. The reported method shows significant red color for sample treatment, instrumentation, and general method type. The sample preparation and solvents or reagents used are shown yellow color. The results obtained from the Green Analytical Process Index (GAPI) are presented in Table 11, Fig. 9 and Fig. 10.

3.6. In-silico ADMET prediction

The in silico ADMET prediction of the PAN, DP1, DP2 & DP3 was generated using in silico ADMET Prediction™ software. Parameters such as the ADMET code, Physico-chemical properties, Pharmacokinetic properties, Permeability, Clearance mechanism, CYP (Cytochrome P) enzymes predictions, Sensitization, Cardiac toxicity, Lethal dose, Phospolipidosis, reproductive toxicity, ADMET risk, MUT risk (Mutation), TOX risk (Toxicity) and Blood-brain barrier and the results are summarized in Table 12. PAN, DP-1, DP-2 and DP-3 possess carcinogenicity whereas PAN also possesses Mutagenicity from ADMET Code. PAN shows slightly higher solubility in water than the DPs. The PAN and DPs are showing basic pH. The PAN and DPs are shown more permeability through the cornea than the jejunal and skin. The PAN and DPs show both metabolic and renal clearance except DP-2 in metabolic clearance. PAN and DPs are shown to have more affinity to the hERG (Human ether-a-go-go-related gene) potassium channels results in cardiac toxicity. PAN and DP-3 are shown to cause Phospolipidosis. PAN, DP-2 and DP-3 are shown to cause reproductive toxicity. The DP-3 has shown high prediction to penetrate the Blood Brain Barrier.

4. Conclusion

A HPLC method was developed to determine the stability of PAN under different conditions such as hydrolytic, oxidative, thermal, and photolytic degradation. This method is considered stability-indicating. A stress degradation study was conducted by examining the factors recommended by ICH guidelines. The pharmaceutical compound experienced degradation when exposed to acidic, peroxide, and basic conditions, resulting in the creation of three distinct degradation byproducts. The identified and characterized DPs were analyzed using LC-QTOF-MS/MS and molecular formula generation, which relied on accurate mass measurement. The HPLC method that was developed has undergone validation for specificity and selectivity, linearity, accuracy, precision, LOD, LOQ and robustness. The developed HPLC method holds potential for application in quality control analysis and identification of degradation impurities. The developed analytical method was assessed with green analytical metric tools GAPI, AGREE and Eco-scale and found that the method is green. Based on the findings generated by the ADMET prediction software, it has been ascertained that both PAN and DPs possess carcinogenic attributes. Studies have shown that PAN, DP-2, and DP-3 exhibit teratogenic properties.

Ethical approval

This experimentation has not harmed any animal or human.

Consent to publish

We affirm that the article has been studied, accepted and approved by all listed authors.

CRediT authorship contribution statement

Vijay Nayak Bhukya: Formal analysis, Investigation, Resources, Validation, Writing – original draft. **Durga Prasad Beda:** Conceptualization, Methodology, Project administration, Supervision, Visualization, Writing – review & editing.

Declaration of Competing Interest

No Conflict of Interest.

Data availability

Data will be made available on request.

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