HPLC and LCMS/MS based chromatographic screening method impurities of Daprodustat with degradation kinetics, characterization and *in silico* toxicity

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Abstract

The present study reports a sensitive and stability-indicating HPLC method with LC-MS/MS confirmation for the complete impurity profiling, degradation kinetics and in silico toxicity assessment of daprodustat, a new oral hypoxia-inducible factor prolyl hydroxylase (HIF-PH) inhibitor. The earlier research describes various methods for the quantification of daprodustat in drug products and biological matrices suggest a gap for its impurity profiling, degradation kinetics and toxicity evaluation under one analytical approach. This study fills this gap by critically elaborating a reverse-phase chromatographic technique on a LiChrospher RP-18 column by isocratic elution with acetonitrile and aqueous formic acid (65:35 v/v), with baseline separation of daprodustat from its known impurities and degradation products (DPs) with resolution values of more than 2.0.

The forced degradation tests were performed according to ICH-recommended stress conditions under which significant degradation was seen upon acidic, basic and thermal stresses. The acidic stress, three different DPs were observed, one of which was determined to be a primary amide derivative of daprodustat by LC-MS/MS analysis (m/z 336). In the acidic degradation kinetics conformed to pseudo-first-order kinetics. The method was validated according to ICH Q2(R1) guidelines for specificity, linearity, sensitivity, precision, accuracy, robustness and system suitability. The technique was highly sensitive with LODs of 0.033 µg/mL for daprodustat and 0.075 µg/mL for impurities. The recovery was between 98% and 102%, with intra- and inter-day precision (% RSD) being less than 2%. The technique was very specific and did not have any interference from excipients or formulation matrices.

Overall, this study provides a comprehensive analytical framework for the quality control, safety assessment and regulatory compliance of daprodustat that makes it highly applicable for routine pharmaceutical analysis.

Keywords: Daprodustat, impurity profiling, degradation kinetics and *in silico* toxicity assessment.

Introduction

The discovery of new therapeutic drugs for chronic conditions has made the pharmaceutical industry to explore more sophisticated analytical tools to guarantee drug safety, efficacy and compliance with regulatory requirements[9]. Among these therapeutic drugs, daprodustat is a hypoxia-inducible factor prolyl hydroxylase inhibitor prescribed in the treatment for anemia in chronic kidney disease. It functions by stimulating endogenous erythropoietin production through the stabilization of HIF and offers an alternative to erythropoiesis-stimulating agents[5]. Upon consideration of its clinical significance and novel mechanism of action, the accurate quantification and characterization of daprodustat, along with its related impurities and degradation products (DPs), are critical for quality control, stability assessment and risk evaluation during drug development and post-marketing surveillance[16].

Analytical tools like HPLC and LC-MS/MS have been extensively utilized for the quantification, detection and separation of active pharmaceutical ingredients (APIs) as well as impurities[12]. They are crucial to develop stability-indicating methodologies that can identify possible DPs generated under conditions of stress such as acidic, basic, oxidative, heat and photo conditions. The occurrence of impurities, such as process-related byproducts and DPs, can affect the pharmacological properties and safety profile of the drug. Hence, a thorough study that combines chromatographic separation, structural elucidation and *in silico* toxicity prediction is needed for daprodustat to maintain regulatory compliance and patient safety[15].

The International Council for Harmonisation (ICH) guidelines like ICH Q3A (R2), Q3B (R2) and Q3C highlight identification, quantification and toxicological evaluation of drug impurities[6,7]. As per ICH Q1A (R2), forced degradation studies are of great importance to elucidate the stability profile of APIs and for establishing validated stability-indicating analytical procedures[8]. These studies assist in mimicking what a drug will experience throughout its shelf life and give information regarding its degradation pattern and kinetics. In this vein, the current research seeks to establish and prove a stable HPLC and LCMS/MS-based

quantitative method for analysing daprodustat and associated substances, along with assessing the kinetics of degradation as well as tracking possible transformation products generated under stressing conditions.

Previous reports suggest the presence of HPLC method for quantification of daprodustat in formulations[18] and stability samples. Another stability indicating method reported for characterization of DPs of daprodustat[3]. One UPLC-MS based analytical method reported for quantification of daprodustat in combination with other 8 prolyl-hydroxylase inhibitors[10]. The literature does not have extensive analytical studies that combine impurity profiling. Additionally, degradation kinetics of daprodustat under a range of ICH-recommended stress conditions has not been widely explored. Lack of a coherent analytical strategy combining quantification, degradation profiling, kinetic modeling and *in silico* safety evaluation is a major research gap.

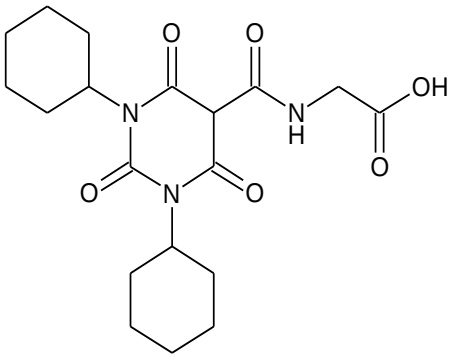
The current study focuses on the establishment of a stability-indicating HPLC and LC-MS/MS chromatographic method backed by degradation kinetics, structural identification and computer-aided toxicity evaluation for the assessment of the quality and safety of daprodustat and its impurities. The molecular structure of daprodustat and its selected impurities in the study are presented in figure 1.

Material and Methods

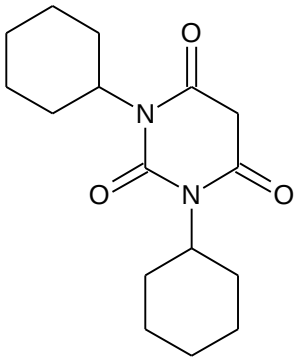
Standards and reagents: In this study, high-purity reference standards of daprodustat with a stated purity of 98.5% and its impurity 1, 2, 3 were obtained from GSK Pharmaceuticals Ltd, Mumbai. The commercially available tablets of daprodustat (Jesduvroq® - 6mg) were procured from Baidyanath Real Food Private Limited, Deoghar, Jharkhand. The HPLC grade methanol (CAS No: 67-56-1) and water (CAS No: 7732-18-5) were used. The buffer chemicals used in HPLC analysis and the reagents used for forced degradation studies included analytical grade hydrochloric acid (HCl), sodium hydroxide (NaOH) and 30% hydrogen peroxide (H_2O_2) purchased from Merck Life Science Private Limited, Mumbai.

Apparatus: The HPLC system equipped with Diode Array Detection (DAD) was utilized for the analysis of daprodustat and its impurities. An Agilent 1200 series system that includes a quaternary pump, auto-injector, vacuum degasser and DAD with G1315 C/D and G1365 C/D models, was utilized in this study. This setup was connected to a computer operating with Agilent ChemStation software (Agilent Technologies, USA) for instrument control, data acquisition and analysis. The chromatographic separation was achieved using an Agilent HC-C18 analytical column with dimensions of 250 × 4.6 mm and a particle size of 5 µm.

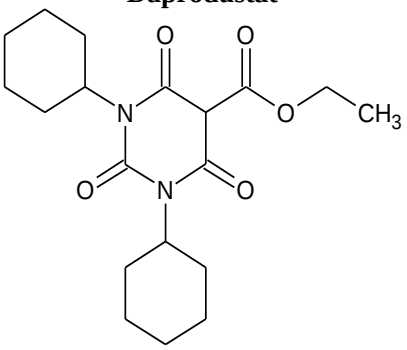
The HPLC system coupled with API 2000 triple quadrupole mass spectrometer system (Applied Biosystems Sciex, USA) with an Electrospray Ionization (ESI) source operated in positive ion mode. The ESI source parameters included ion spray voltage (+5500 V), source temperature (400°C), nebulizer gas (40–50 psi), heater gas (40–50 psi) and curtain gas (25 psi). The collision gas (nitrogen) was used at a medium pressure to aid fragmentation in the collision cell (Q2). The mass spectrometric detection was carried out in multiple reaction monitoring (MRM) mode. In this, both the precursor ion (Q1) and product ion (Q3) transitions specific to daprodustat and its impurities were monitored.



Daprodustat



Impurity 1



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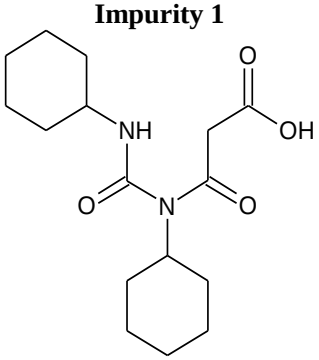


Impurity 3

Figure 1: Structure of daprodustat and its impurities

The Dwell time, collision cell exit potential, collision energy and declustering potential were individually optimized for each MRM transition to ensure maximum sensitivity and signal-to-noise ratio. The scan range was typically set between m/z 50-1000 based on the molecular weights of the analytes. Data acquisition and instrument control were managed through Analyst® software (version 1.6.2).

Preparation of solutions: The stock solutions of daprodustat and its impurities were separately prepared by dissolving each standard in HPLC-grade ethanol to reach a final concentration of 1000 µg/mL. The solutions were then kept at a freezer set at 0 °C in order to maintain their stability throughout the analysis process. The working standard solutions were prepared from these stock solutions by diluting with deionized water to obtain final concentrations between 25 µg/mL (level 1) and 150 µg/mL (level 6) for daprodustat and 0.25 µg/mL (level 1) to 1.5 µg/mL (level 6) for impurities.

The standard calibration dilutions were prepared by mixing equal volume of each linearity level solution separately and these solutions was injected into the HPLC–DAD system under optimized chromatographic conditions with an injection volume of 20 µL. Each concentration was tested in triplicate to verify consistency and accuracy. These peak responses were subsequently plotted against their known concentrations to obtain calibration curves and to determine the regression equations for quantitative analysis.

Assay sample preparation: Five tablets of Jesduvroq® (each supposed to contain 6 mg of daprodustat) were opened cautiously and the contents were weighed accurately. A quantity of powder equal to the average weight of a single tablet was transferred to a 50-mL volumetric flask. First, roughly 30 mL of ethanol was added to dissolve the powder and sonication was conducted for 5 min to facilitate dissolution. Then the solution was brought to 50 mL with ethanol. The produced solution was next filtered through a membrane filter that had a pore size of 0.22 µm in order to clear the solution of any un-dissolved material or excipients. Part of this filtered solution was again diluted to get a final test solution equivalent to 100 µg/mL of daprodustat.

In order to ensure the precision of the method, known quantities of pure daprodustat standard were spiked (added) to individual aliquots of tablet solution (standard addition technique). These spiked samples were treated and analyzed similarly as described above for reliability and recovery in the presence of tablet excipient.

Forced Degradation: The chemical stability of daprodustat was assessed and the possible DPs were detected through forced degradation studies under different stress conditions[2]. These tests assist in establishing the stability-indicating capability of analytical procedure by demonstrating the influence of extreme environments on drug stability. In every stress experiment, 1 mL of a concentrated daprodustat stock solution (1000 µg/mL) was employed and this solution was exposed to stress conditions. Then, the degraded solutions were neutralized (wherever necessary) and diluted with deionized water in 10 mL volumetric flasks to a final concentration of 100 µg/mL prior to analysis. In acid degradation, 1 mL of HCl solution (2 M) was added to daprodustat 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 daprodustat solution and the sample was left at room temperature for 12 hours. The impact of oxidative stress on daprodustat was assessed by subjecting 1 mL of standard solution to 1 mL 30% (v/v) peroxide. The stressed sample was incubated at 60 °C for 2 hours in a water bath and then cooled to room temperature. The degradation of daprodustat 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 daprodustat was put to natural sun light for 4 hours in a 10 mL volumetric flask with lid whereas in thermal degradation, the daprodustat powder was heated in an air oven for 2 hours at 100 °C.

Kinetics Investigation: The degradation kinetics experiment was conducted to investigate the degradation of daprodustat over time in acidic stress study[14]. In this, 1 mL aliquots of daprodustat stock solution in linearity range concentration were individually exposed to acid stress study. These stress solutions were stored at room temperature and aliquots were removed at regular time intervals to track the rate of degradation.

At every selected time interval, the reaction mixture was neutralized instantly to prevent further breakdown and subsequently, the solutions were diluted using deionized water to a final volume of 10 mL to prepare an appropriate concentration for HPLC analysis. All of these time-dependent samples were then analyzed in the optimized HPLC–DAD method. The amount of daprodustat at every time point was calculated by using the regression equation of the calibration curve. The drug concentration vs time graph was plotted and the degradation was assessed to identify the order of the reaction and the rate constant. These studies provide the detailed information about the stability of daprodustat and to predict shelf life under various storage or processing conditions.

***In silico* Toxicity Studies**: The possible toxicity of DPs was assessed by applying an *in silico* toxicity prediction strategy. The predictions were conducted with assistance of internet-based tool namely ProTox-II which is a highly accepted web server that predicts toxicological properties of chemical compounds by analyzing their molecular structures. The ProTox-II provides LD_{50} value of chemical compounds is the quantity of a substance (in milligrams) needed per kilogram

of body weight to kill 50% of a test animal group. The LD_{50} value provides the acute toxicity quantitative estimate of DPs. Along with LD_{50}, the software also provides a toxicity class from 1 (most toxic) to 6 (least toxic), which aids in the classification of the possible health hazard due to exposure to the substance. The findings provide the key information related to the safety of daprodustat, particularly in situations where it may degrade under environmental conditions or during storage mishaps[11,13].

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.

Results and Discussion

In order to conduct a comprehensive kinetic degradation study, it is very crucial to employ a stability-indicating chromatographic method that can effectively separate the daprodustat from its DPs. The method should not only ensure the accurate quantification of daprodustat but also enable the simultaneous determination of its impurities and DPs. This dual detection capability enhances the applicability of method for use in biological matrices and supports its potential utility in future pharmacokinetic and clinical investigations.

Furthermore, the DPs identified through LC MS/MS analysis must be subjected to structural characterization which is a critical step for understanding their chemical behavior and biological safety profiles. Furthermore, the *in silico* toxicity assessment was conducted to predict the potential toxicity DPs characterized in the study.

Optimization of Chromatographic Conditions: A series of chromatographic parameters were systematically evaluated and optimized to achieve effective and reliable separation of daprodustat with its known impurities and DPs. Initially, there were two types of reversed-phase columns such as LiChrospher RP-18 and Inertsil ODS-HL columns having dimensions of 250 mm and 150 mm × 4.6 mm with 5 µm particle size. The columns were assessed using equal volume compositions of methanol, acetonitrile and deionized water to achieve clear and well-resolved peaks for daprodustat and impurities with minimal tailing. These preliminary experimental trials demonstrate that both the columns produce comparable retention behaviors for daprodustat and impurities. However, the LiChrospher RP-18 column produced superior results in terms of peak shape and symmetry (tailing factor) and signal response (peak area). Hence, LiChrospher RP-18 column was selected for further method development.

The influence of pH adjustment in the aqueous phase was also explored by adjusting the mobile phase in the pH values of 3, 5, 7 and 9. The results suggest that the pH values significantly affect the retention time, peak area, or tailing factor of daprodustat and impurities. The overlapping peaks and poor separation were noticed in acidic range due to the fully protonation of daprodustat and impurities in acid pH. This may reduce their interaction with the stationary phase and leads to shorter retention times and poor peak shapes. The basic pH ranges also exhibit its poor resolution and symmetry of peaks corresponding to daprodustat and impurities and the results proved that the moderately acidic to neutral pH range was suitable for resolution of daprodustat and impurities.

Further, the choice of organic modifier such as methanol and acetonitrile was evaluated to produce sharper, more symmetrical peaks, with very minimal tailing factors. Methanol was proved as the ideal organic component to produce best resolution of daprodustat and impurities. An isocratic elution mode was found to be sufficient for the stability-indicating method, even under forced degradation conditions. The method demonstrates a resolution (Rs) greater than 2 between analytes, which is essential to confirm purity and stability of the analytes.

Based on these findings, the final optimized chromatographic conditions were established as LiChrospher RP-18 analytical column (250 × 4.6 mm, 5 µm) with an isocratic mobile phase consisting of acetonitrile and aqueous formic acid in a 65:35 (v/v) ratio at 1.0 mL/min. The detection was carried out at a wavelength of 241 nm, which corresponds to the maximum absorbance (λmax) for daprodustat and impurities. The total run time was 10 minutes, with retention times (t_R) of 5.5 minutes for daprodustat, 2.5 min for impurity 1, 7.3 min for impurity 2 and 3.8 min for impurity 3 (Figure 2).

The system suitability parameters confirm the reliability of the method. The daprodustat and impurities eluted within a time frame and the chromatograms showed a high number of theoretical plates, indicating excellent column efficiency, as well as a good resolution (Rs > 2) between the analytes (Table 1). These results were in compliance with the FDA's acceptance criteria, demonstrating that the method is suitable for routine analytical use.

Stress degradation study: The comprehensive forced degradation of daprodustat was conducted according to ICH guidelines to determine its stability under various stress conditions. The proposed HPLC–DAD method was employed to track the degradation trend of daprodustat under each condition. Initially, the mild conditions like sun light, peroxide and neutral water produced only partial degradation with no DPs in the chromatogram whereas acid, base and thermal conditions proved to be highly significant.

In acidic conditions, the drug shows 86.05% recovery indicating 13.95% degradation and the chromatogram display well resolved three new DPs. The known impurity 1 in the study was also detected in acid stress chromatogram (Figure 3A). In the presence of a basic environment, the % degradation of 9.81% was noticed with a recovery % of

90.19% and the formation of a single DP was noticed (Figure 3B). The dry heat stress led to 88.91% recovery of daprodustat with 11.09% degradation and this stress also produces one new DP (Figure 3C).

In contrast, daprodustat exhibits greater stability under peroxide, photolytic and neutral hydrolysis conditions, with recoveries of 98.35%, 97.54% and 98.15% respectively. No new degradation products were detected under these three conditions indicating that daprodustat is more stable in thermal, photolytic and neutral environments compared to acidic, basic and oxidative stresses. Table 2 displays the stress study results of daprodustat noticed in this study.

Degradation kinetics: The acid degradation kinetics of daprodustat was assessed to understand the impact of acid stress exposer time on the degradation of daprodustat. In this, the drug was exposed to acid stress and samples were collected at different time points (t) from the stress reaction mixture. The remaining amount of daprodustat in each sample (Ct) was measured in correlation with initial concentration (Co) using the proposed HPLC method. The achieved results were utilized to calculate its half-life ($t_{1/2}$), which helps to estimate the stability of drug stable under storage conditions. The result shows a consistent decline in daprodustat concentration over time in acidic conditions.

A straight-line relationship was observed by plotting the logarithm of the remaining concentration (log Ct) against time and results confirm that the degradation follows pseudo first-order kinetics (Figure 4). The pseudo first-order kinetics and $f_{1/2}$ were assessed using the formula[4]:

$$\log (Ct) = \log (Co) - \frac{kt}{2.303}$$

$$f_{1/2} = 0.693/k_{obs}$$

The rate of degradation of daprodustat is directly proportional to its concentration and from this linear plot, the half-life of daprodustat in acidic medium was found to be approximately 27.69 hours in the studied concentration. The corresponding degradation rate constants were 0.0120 h^{-1} indicating that daprodustat degrades slowly in the studied acid stress concentration.

Table 1
System suitability results of daprodustat and impurities in the optimized method

Parameter	Daprodustat	Impurity 1	Impurity 2	Impurity 3
t_R in min	5.5	2.5	7.3	3.8
Resolution	12.9	--	9.6	8.5
Theoretical plates	12589	5749	16058	8401
Tailing factor	1.01	0.98	1.05	1.02

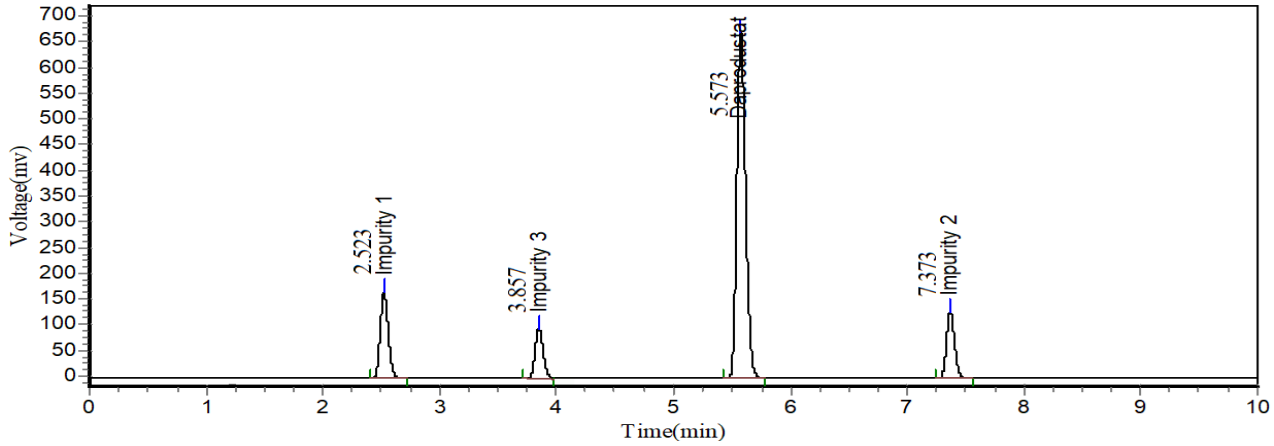


Figure 2: System suitability chromatogram of daprodustat and impurities in the optimized method

Table 2
Stress degradation study results of daprodustat in the optimized method

S.N.	Condition	Degradation conditions	% Recovery	% degradation	No of new DPs formed
1	Acid	2 M HCl, RT, 12 hours	86.05	13.95	3
2	Base	0.01 M NaOH, RT, 12 hours	90.19	9.81	1
3	Oxidative	30% H_2O_2, 60 °C for 2 hours	98.35	1.65	-
4	Dry heat	Dry heat 100°C, 2h	88.91	11.09	1
5	Photolytic	Sun light, 4h (summer)	97.54	2.46	-
6	Neutral	H_2O, 80 °C, 30 min	98.15	1.85	-

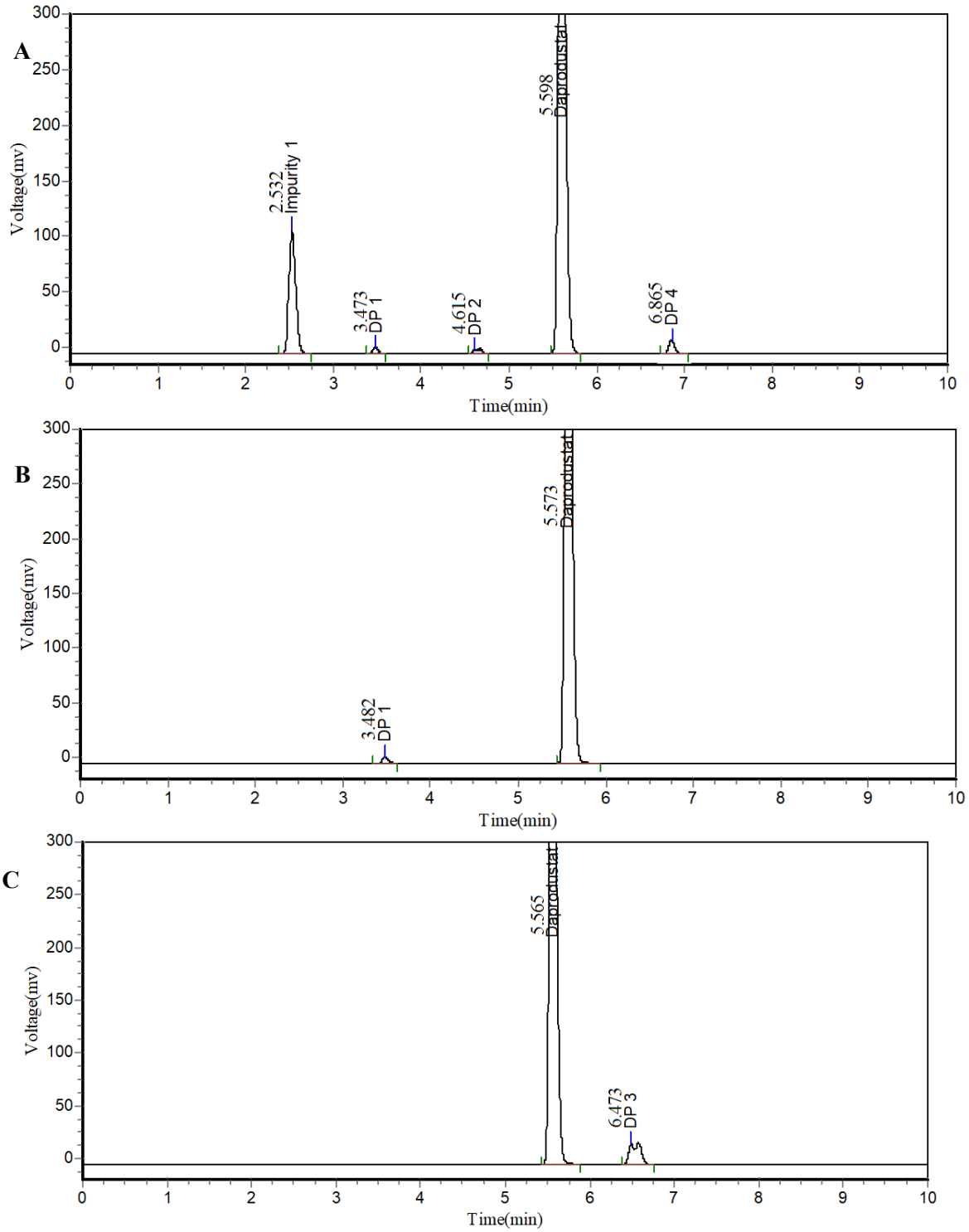


Figure 3: (A) Acid, (B) base and (C) dry heat stress study chromatograms of daprodustat in the optimized method

Method Validation: The method developed for quantification of daprodustat, its impurities and DPs was validated according to the ICH guidelines[17]. The range of standard and impurity solutions with varying concentrations that exhibit high correlate linear plot, was assessed in linearity. The method shows a strong linear response over the concentration range of 25 µg/mL to 150 µg/mL for daprodustat and 0.25 µg/mL to 1.5 µg/mL for impurities. The regression equations obtained were y = 10269x + 21029 for daprodustat, y = 90307x + 955.34 for impurity 1, y = 66572x + 562.17 for impurity 2 and y = 76215x + 610.47 for impurity 3. The correlation coefficients (r) were exceptionally high with corresponding r^2 values of more than 0.999 clearly indicating that the method exhibits excellent linearity. In addition, the % RSD of the slope was noticed to be well below the acceptable limit of 2% for daprodustat and its impurities indicate minimal fluctuation in the response factor. The sensitivity of method was assessed by determining LOD and LOQ for both daprodustat and its impurities. These values were established based on the

signal-to-noise ratio where a ratio of 3:1 was used for LOD and 10:1 for LOQ. The method proved to be highly sensitive, as evidenced by the very low concentrations that could be reliably detected and quantified. The LOD for daprodustat was noticed to be 0.033 µg/mL whereas the LOQ of impurities was noticed to be 0.075 µg/mL. These findings highlight the method's suitability for detection and quantification of trace amounts of impurities.

The intra-day and inter-day tests were conducted at three concentration levels to evaluate the accuracy and precision of method. In intra-day precision, each concentration was analyzed three times on the same day, while for inter-day precision, the tests were repeated across three consecutive days. Accuracy was evaluated by calculating the relative error (Er) whereas precision was assessed using the % RSD. The results, summarized in table 3, shows that both Er and RSD values of daprodustat and its impurities were consistent within the acceptable range of below 2%. Additionally, the recovery rates for all tested concentrations were excellent in the range of 98% and 102%, further confirming that the developed method provides reliable and reproducible results for routine quality control and stability testing of daprodustat and its impurities.

The method robustness was tested by intentionally introducing small variations in key chromatographic parameters to see if these changes would affect the method's performance. The conditions like detection wavelength, pH of the aqueous phase, ratio of solvents and mobile phase flow rate were altered. These deliberate changes did not significantly impact the results. The daprodustat and its impurities display consistent peak areas with % RSD of values below 2% indicating the excellent reproducibility. Additionally, the retention times of the peaks showed very small standard deviation values confirming that the method remains reliable and accurate even when minor operational changes are introduced, thereby proving its robustness.

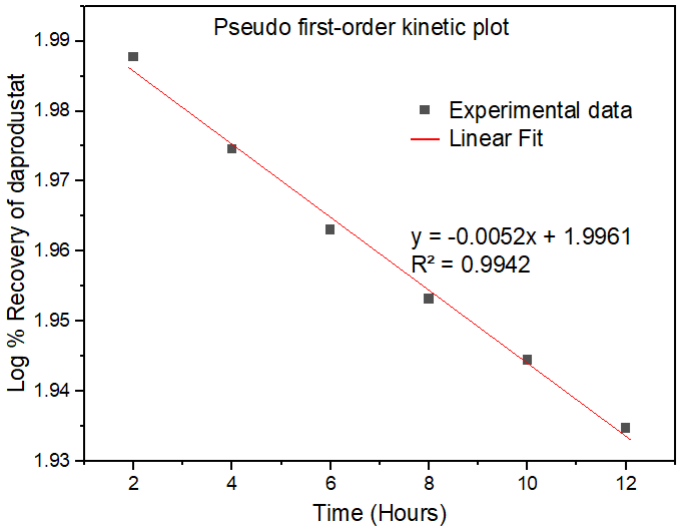


Figure 4: Pseudo first-order kinetic plot observed of daprodustat under acid stress conditions

Table 3
Precision and accuracy results of daprodustat and its impurities in the proposed method

Analyte	Test	Intra-day			Inter-day		
Standard	% Recovery	99.25±0.582	98.69±0.663	99.01±0.245	98.63±0.332	99.58±0.295	98.73±0.748
	% RSD	0.59	0.67	0.25	0.34	0.30	0.76
	% error	0.75	1.31	0.99	1.37	0.42	1.27
Impurity 1	% Recovery	98.69±0.515	99.21±0.774	100.25±0.326	98.79±0.858	100.91±1.004	100.58±1.026
	% RSD	0.52	0.78	0.33	0.87	0.99	1.02
	% error	1.31	0.79	0.25	1.21	0.91	0.58
Impurity 2	% Recovery	99.45±0.552	98.58±0.639	99.52±0.441	100.58±0.928	100.73±0.782	98.73±0.614
	% RSD	0.56	0.65	0.44	0.92	0.78	0.62
	% error	0.55	1.42	0.48	0.58	0.73	1.27
Impurity 3	% Recovery	99.36±0.679	99.12±0.841	100.88±1.025	101.32±1.036	100.55±0.552	98.24±0.582
	% RSD	0.68	0.85	1.02	1.02	0.55	0.59
	% error	0.64	0.88	0.88	1.32	0.55	1.76

We assessed whether any commonly used excipients or inactive ingredients in the pharmaceutical formulation interfered with the detection of the active compounds in specificity study. The result does not show any such interference indicating that the method is highly specific to the analytes of interest. This was further confirmed by analyzing the pharmaceutical formulation solution of daprodustat. The formulation solution displayed daprodustat peak at its specific retention time and no additional or overlapping peaks were detected confirming that none of the excipients or inactive ingredients present in the formulation interfered with the analysis.

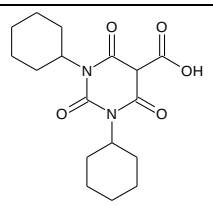
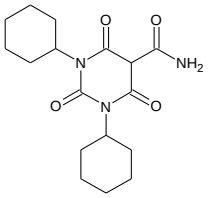
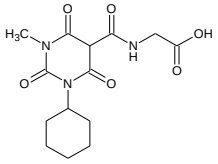
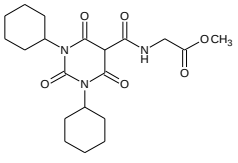
That the method is highly selective for the analysis of daprodustat in complex sample matrices. The reliability of the method was strengthened by conducting standard addition test. A known quantity of impurities was added to the tablet solution. The resulting chromatographic analysis confirms the successful recovery of the added impurities with no interference from the formulation matrix demonstrating that the method is not only accurate but also robust enough for routine quality control analysis.

LCMS/MS study of DPs: The acid, base and dry heat stress samples were further analyzed through LCMS/MS and results provide the adequate information to understand the breakdown of daprodustat under the studied stress conditions. This approach allows for both the separation and identification of the DPs formed during stress testing. The stress degraded samples were injected into the system and the mass-to-charge ratios (m/z) of the resultant peaks were recorded using scan mode.

The acid stress chromatogram shows DP 2 at an RRT of 0.824 and was formed by the conversion secondary amide (R–CO–NHR) skeleton of daprodustat into the primary amide structure (R–CO–NH_2). The degradation begins with protonation of the carbonyl oxygen that enhances the electrophilicity of the carbonyl carbon which facilitates nucleophilic attack by water. This leads to the formation of a tetrahedral intermediate that subsequently breaks down, releasing the amine (R'NH_2) and yields the primary amide. This chemical transformation reflects in the mass spectral data, where a prominent ion at m/z 336 appears that represents the protonated primary amide product of daprodustat. These DPs show the total mass loss of 59 Da compared to daprodustat suggesting that DP was formed by the loss of 59 Da from the parent ion suggesting the formation of primary amide.

The disappearance of the molecular ion corresponds to carboxylic acid also providing strong support of the proposed degradation mechanism to confirm the identity of the primary amide as a DP.

Table 4
HRMS data observed for DPs of daprodustat characterized in this study

Name	RRT	Experi-mental m/z	Proposed Molecular formula of $[M+H]^+$	Error (ppm)	m/z of major Fragments	Proposed $[M+H]^+$ Formula	Proposed structure
DP 1	0.620	337.3828	$C_{17}H_{24}N_2O_5$	0.89	280.2680	$C_{13}H_{15}N_2O_5$	
					252.2579	$C_{12}H_{15}N_2O_4$	
					215.2808	$C_{11}H_{20}NO_3$	
					142.1903	$C_7H_{13}N_2O$	
					113.1922	$C_7H_{14}N$	
DP2	0.824	336.3981	$C_{17}H_{25}N_3O_4$	0.59	279.2832	$C_{13}H_{16}N_3O_4$	
					252.2579	$C_{12}H_{15}N_2O_4$	
					214.2960	$C_{11}H_{21}N_2O_2$	
					142.1903	$C_7H_{13}N_2O$	
					113.1922	$C_7H_{14}N$	
DP3	1.156	326.3171	$C_{14}H_{19}N_3O_6$	0.31	269.2884	$C_{12}H_{18}N_3O_4$	
					252.2579	$C_{12}H_{15}N_2O_4$	
					244.2790	$C_{11}H_{19}N_2O_4$	
					157.2447	$C_8H_{16}NO$	
					143.2181	$C_8H_{16}NO$	
					113.1922	$C_7H_{14}N$	
DP4	1.226	408.4607	$C_{20}H_{29}N_3O_6$	0.49	327.3245	$C_{14}H_{20}N_3O_6$	
					286.3587	$C_{14}H_{25}N_2O_4$	
					245.1809	$C_8H_{10}N_3O_6$	
					204.2151	$C_8H_{15}N_2O_4$	
					173.1182	$C_5H_6N_3O_4$	

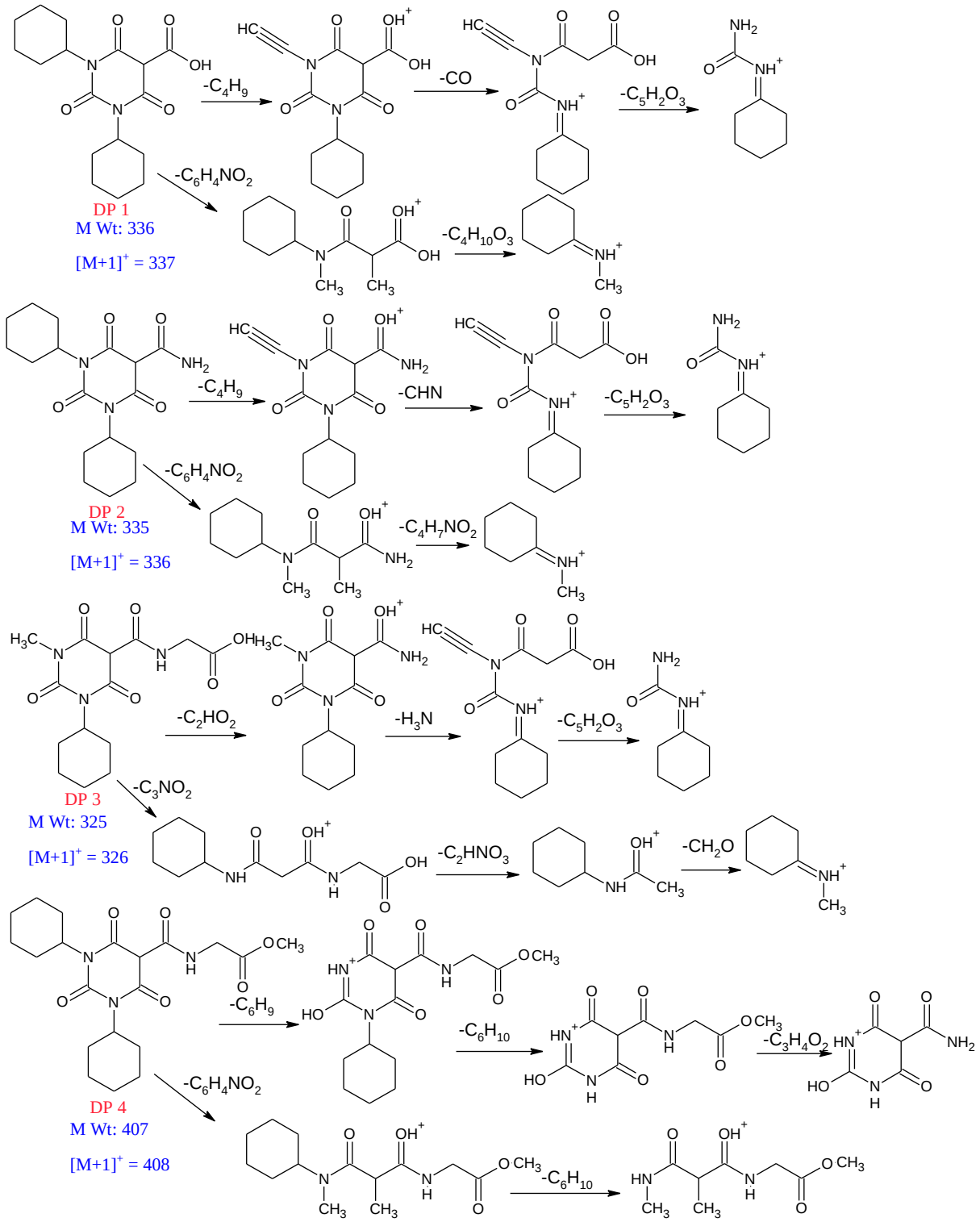


Figure 5: Fragmentation mechanism proposed for DPs of daprodustat characterized in this study

The DP 2 was characterized as 1,3-dicyclohexyl-2,4,6-trioxohexahydropyrimidine-5-carboxamide with mass of 335 g/mol. The acid hydrolysis of a primary amide group (R–CO–NH_2) in DP 2 was further continued and then convert the amine into carboxylic acid (R–COOH) through a protonation-driven mechanism. This process results in the formation of carboxylic acid compound as DP 1 and was identified at an RRT of 0.620. The conversion of amide into carboxylic acid was further confirmed by observing a mass difference of 1 Da in the mass spectrum. The DP 1 shows parent ion at m/z of 337 [m+1] and its fragments were almost similar to DP 2 suggesting that the DP 1 was originate from DP 2.

In acidic conditions, daprodustat undergoes a transformation of its carboxylic acid group (R–COOH) into a methyl ester (R–$COOCH_3$). This reaction was facilitated in the presence of methanol which was used as diluent and acts as a

nucleophile. The mechanism begins with protonation of carbonyl oxygen that enhances the electrophilicity of the carbonyl carbon. Methanol then attacks this carbon and facilitates the formation of the methyl ester (R–$COOCH_3$). This structural change is supported by mass spectral data. The methyl ester product shows an increase in the molecular ion peak (m/z) by +14 Da corresponding to the addition of a CH_2 group. This change in mass aligns with the conversion of the –COOH group into a –$COOCH_3$ ester and confirms the proposed degradation mechanism.

It was confirmed that the DP 4 is an ester impurity of daprodustat with molecular mass of 407 g/mol. The DP 3 was identified at an RRT of 1.1 in dry heat degradation study and was formed by the breakdown cyclohexane moiety of daprodustat. The total mass deference of 38 Da was noticed in the mass spectrum of DP 3 than daprodustat. The mass spectrum displays 326 [m+1] with product fragments at m+1 of m/z 269, 252, 244, 157, 143 and 113. By The correlation and interpretation of these data, the compound was confirmed as [(1-cyclohexyl-3-methyl-2,4,6-trioxohexahydr opyrimidin-5-yl)carbonyl]amino}acetic acid with molecular mass of 325 g/mol and formula of $C_{14}H_{19}N_3O_6$. The mechanism of formation of DPs was visualized in figure 5 and MSMS results are summarized in table 4.

***In silico* toxicity assessment of DPs**: The evaluation of the toxicity profile of DPs is essential to ensure the overall safety and efficacy of pharmaceutical compounds. In this context, *in silico* toxicity studies were conducted using the ProTox-II webserver which is a predictive tool based on machine learning models that estimated various toxicological endpoints including LD_{50} values, hepatotoxicity, carcinogenicity, immunotoxicity, mutagenicity and cytotoxicity. These studies are particularly valuable because they offer a cost-effective, rapid and ethical alternative to conventional animal-based toxicity testing[1] and hence the toxicity of DPs characterized in the study was performed through ProTox-II. The *in silico* toxicity assessment of the four major (1-4) of daprodustat shows that the DPs possess relatively high LD_{50} values in the range of 3000 to 4000 mg/kg. These DPs were classified under toxicity class 5 indicating low level of acute toxicity.

All the DPs do not exhibit hepatotoxicity, cardiotoxicity, carcinogenicity, immunotoxicity, mutagenicity, or cytotoxicity, suggesting that these DPs have favorable safety profile. However, neurotoxicity, respiratory toxicities and clinical toxicity were consistently predicted as active for all four DPs. Additionally, nephrotoxicity was found to be active for DP1, DP3 and DP4, whereas DP2 remains inactive and indicates some variability in kidney toxicity potential.

Notably, blood-brain barrier (BBB) permeability was predicted for DP1, DP2 and DP4, suggesting a likelihood of CNS exposure. Despite the absence of major toxic alerts in several categories, the active predictions for neurotoxicity and respiratory toxicity were noticed for the DPs and these predictions highlight the need for further experimental validation to fully assess the safety of these DPs.

Conclusion

The present study aimed to develop and validate a comprehensive analytical strategy for the stability evaluation of daprodustat through a robust, stability-indicating HPLC method in conjunction with LC-MS/MS characterization. Unlike previous analytical approaches that focused solely on quantification or degradation studies, this work integrates impurity profiling, forced degradation under ICH-recommended conditions, degradation kinetics modeling and *in silico* toxicity assessment that fulfill the critical gap in the current literature. The developed method successfully separated daprodustat from its known impurities and DPs with high resolution (Rs > 2) and demonstrated excellent linearity, accuracy, precision, specificity, sensitivity and robustness in line with ICH guidelines.

Among the stress conditions tested, acidic, basic and thermal conditions result in the most significant degradation, with four DPs identified and structurally characterized using LC-MS/MS. In acidic conditions, DP 2 was identified at an RRT of 0.824 which was formed via the conversion of the secondary amide to a primary amide and was evidenced by a mass loss of 59 Da and m/z 336. Further acid hydrolysis of DP 2 led to DP 1 (RRT 0.620) which is a carboxylic acid derivative and was confirmed by a +1 Da shift and similar fragmentation patterns. Additionally, methanol-induced esterification under acidic conditions produced DP 4 which is a methyl ester impurity of daprodustat with a molecular mass of 407 g/mol which was supported by a +14 Da shift in m/z. In dry heat stress.

DP 3 emerges at RRT 1.1 due to cyclohexane ring cleavage that shows 38 Da mass loss and characteristic fragments and was identified as a compound with the formula $C_{14}H_{19}N_3O_6$ and molecular weight 325 g/mol. The *in silico* toxicity evaluation was limited to theoretical assessments and requires experimental validation through *in vitro* or *in vivo* toxicity assays. Moreover, the developed method proved to be efficient for degradation, impurity profiling in a laboratory setup and its application to biological matrices (e.g. plasma or tissue samples) was not explored in this work.

The future study was planned to focus on expanding this analytical framework to include real-time stability studies, matrix-based pharmacokinetic profiling and bioanalytical validations. In conclusion, the present study successfully developed and validated a robust, stability-indicating HPLC and LC-MS/MS method for the comprehensive analysis of daprodustat and its impurities that integrated degradation kinetics, structural elucidation of DPs and *in silico* toxicity evaluation, thereby offering a holistic analytical approach to ensure the drug's quality, stability and safety.

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