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Original Article

Evaluation of New Degradation Products Formed Under Stress Conditions of Betrixaban by LCMS/MS: Establishment of HPLC Method for Quantification of Genotoxic Impurities of Betrixaban

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Abstract

This study focused to optimize an accurate HPLC method for evaluation of genotoxic impurities of Betrixaban and further structural identification of forced degradation products (DPs) of betrixaban. The analytes were resolved on Zorbax SB C18 (4.6×250mm, 5µm, Agilent) column at 35°C temperature using 75 % aqueous ammonium formate (5 mM) and 50 % ammonium formate in acetonitrile in 60:40 (v/v) in isocratic elution at 1.0 mL/min and 245 nm as detection wavelength. In the optimized experimental conditions, the retention times of the analytes were precisely determined, resulting in retention times of 10.30 min for betrixaban, 3.77 min for the 2-amino impurity, 7.79 min for the 2-nitro impurity, and 13.55 min for the 4-cyanobenzamido impurity, all exhibiting acceptable system suitability and specificity. The method demonstrated excellent sensitivity, capable of detecting impurities up to 0.009 µg/mL, with 25-150 µg/mL calibration range for betrixaban and 0.025-0.15 µg/mL for impurities. Notably, all parameters studied during the validation process exhibited results within permissible limits for both betrixaban and its impurities, affirming the robustness and reliability of the analytical method. The drug underwent exposure to various stress-inducing conditions following the guidelines outlined in ICH Q1A (R2).The stress-induced DPs were identified using LCMS/MS in ESI positive mode. By comparing their collision-induced dissociation mass spectral data with that of betrixaban, potential structures for four of DPs were proposed. The results of other validation studies were also agreeable, confirming their adequacy for the routine analysis of betrixaban and its genotoxic impurities in both bulk drug and formulations. Additionally, these validated methods can be utilized to investigate the mechanisms underlying the stress-induced degradation of betrixaban.

Keywords: *Betrixaban, characterization through LCMS, genotoxic impurities, stress degradation products*

Introduction

The assurance of a pharmaceutical product's safety extends beyond solely assessing the toxicological characteristics of its active pharmaceutical ingredient (API) to encompass the impurities

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present within it (Vergote *et al*., 2009). Consequently, there is a burgeoning interest in scrutinizing impurities found in both APIs and finished pharmaceutical products (FPPs) (De Spiegeleer *et al.,* 2008). This surge in interest has propelled impurity profiling into the spotlight, a practice focused on identifying and quantifying impurities within pharmaceutical drugs, garnering significant attention from regulatory bodies. Notably, various Pharmacopoeias are now integrating specific thresholds for acceptable impurity levels in API or FPP formulations. These thresholds are established based on observed levels in approved market samples, underscoring the importance of rigorous impurity evaluation in pharmaceutical quality control (Bari *et al.,* 2007).

The origin of impurities includes various sources, such as API related impurities that can originate due to functional groups, crystallization, and stereochemistry of API, process related impurities can originate through the process of synthesis of API such as reactants, reagents, intermediate compounds, catalysts, etc., whereas the stability-related impurities can originate through degradation of API and API-excipient mutual interaction in formulation (Davis and Gidding 1983).

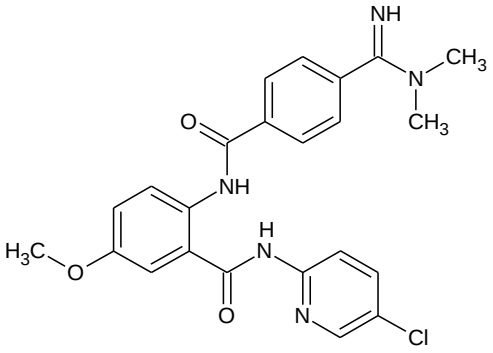
When conducting the production of pharmaceutical formulations, it is essential to conduct impurity analysis on the raw materials employed for production. These impurities have the potential to disrupt the solubility of active pharmaceutical ingredients (Chen *et al.,* 2010; Feng *et al., 2001*). The existence of these undesirable substances or extraneous chemicals can also impact the safety aspects of the drug, leading to adverse drug reactions or toxicities in the body. Such effects can compromise both the safety and effectiveness of the APIs (Bhupatiraju *et al.,* 2024).

Degradation products (DPs) are substances that are formed when a pharmaceutical or chemical compound undergoes stress conditions during testing. These stress conditions typically involve subjecting the compound to elevated temperatures, exposure to acid, base, light, and other factors that accelerate degradation (Alsante *et al.,* 2001: Bhupatiraju *et al.,* 2024). The purpose of conducting stress testing is to identify potential degradation pathways and assess stability of compound under extreme conditions. Stress degradation studies can help pharmaceutical researchers and manufacturers understand how a drug or chemical compound may degrade over time and under various environmental conditions, which is crucial for ensuring product quality, safety, and efficacy (Varma *et al.,* 2023).

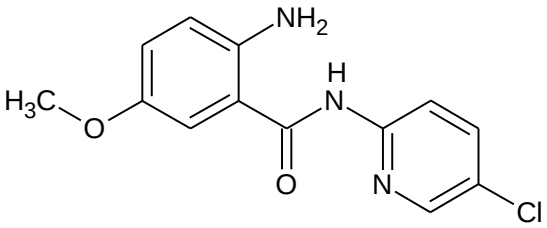
Betrixaban (depicted in Figure 1A) is categorized as an anticoagulant medication belonging to the class of drugs referred to as direct factor Xa inhibitors. Its primary purpose is to mitigate the risk of venous thromboembolism (VTE) among adults hospitalized for an acute medical condition who are at an elevated VTE risk (Eriksson *et al.,* 2009). Its mechanism of action involves the inhibition of factor Xa activity, a pivotal component in the blood clotting cascade (Cohen *et al.,* 2013: Varma *et al.,* 2023). By performing this function, it effectively hinders the formation of blood clots. While common side effects may encompass bleeding, bruising, and anemia, it's worth noting that serious bleeding events, although relatively uncommon, can occur (Turpie *et al.,*2009).

The literature review of available analytical methods proved that few HPLC stability-indicating methods were reported for the quantification of betrixaban in formulations (Mastannamma *et al.,* 2018; El-Masry *et al., 2020*; Ghante *et al.,* 2021). One analytical method was reported for preparative HPLC isolation followed by spectroscopic characterization of unknown DPs of betrixaban (Surukonti *et al.,* 2023). Analytical methods were reported for the quantification of betrixabanin in combination with hexazinone (Jasemizad *et al.*, 2019) and lercanidipine (El-Masry *et al.,* 2021; El-Masry *et al.,* 2022) in biological samples.

The exhaustive examination of existing literature indicates a lack of validated HPLC or LCMS-based stability study methods, as per the ICH Q2(R1) guideline (ICH Q1B 1996; ICH Q2(R1) 1994; ICH Q1A (R2) 2003), specifically for the assessment of betrixaban and its genotoxic impurities. Taking this into account, our study aims to explore the degradation behavior of betrixaban, identify its DPs, and assess the stability of the betrixaban molecule through various stress studies. The genotoxic impurities such as 2-amino, 2-nitro, and 4-cyanobenzamido impurities of betrixaban were used in the study. The molecular structure of the genotoxic impurities of betrixaban is presented in Figure 1.



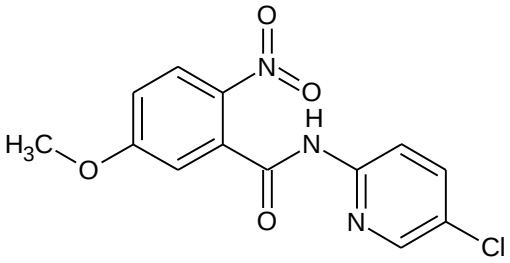
A) Betrixaban



B) 2-Amino Impurity

Molecular Formula:$C_{13}H_{12}ClN_3O_2$

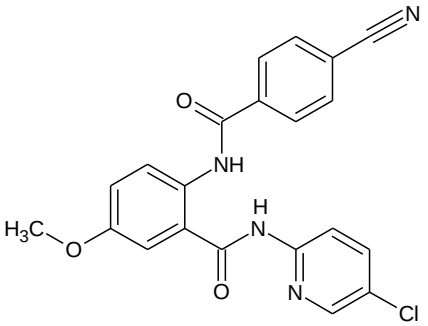
Molecular mass: 277.706 g/mol



C) 2-Nitro Impurity

Molecular Formula: $C_{13}H_{10}ClN_3O_4$

Molecular mass: 307.689 g/mol



D) 4-Cyanobenzamido Impurity

Molecular Formula:$C_{21}H_{15}ClN_4O_3$

Molecular mass: 406.821 g/mol

Figure 1: Molecular structure of betrixaban and its impurities

The process of synthesis (Li *et al.,* 2015) of betrixaban suggests the route of formation of genotoxic impurities. The synthesis of betrixaban involves a four-step mechanism, in which step one will be the reaction of *5-methoxy-2-nitrobenzoic acid* with *5-chloropyridin-2-amine* in the presence of phosphoryl chloride, pyridine, and acetonitrile, which produces *N-(5-chloropyridin-2-yl)-5-methoxy-2-nitrobenzamide,* which remains an impurity and was treated as a 2-nitro impurity. The product of the first step was reduced with Pd/C/H2 to achieve amino compounds, which were present in the final product as 2-amino impurities. This compound further reacted with 4-cyanobenzoyl chloride to produce N-(5-chloropyridin-2-yl)-2-[(4-cyanobenzoyl) amino]-*5-methoxybenzamide*. This compound was left in the final product as a 4-cyanobenzamido impurity. This compound was further processed by utilizing the Pinner reaction to produce betrixaban. The synthesis mechanism was presented in a supplementary note attached to this manuscript.

Material and Methods

Solvents and Chemicals:

The betrixaban API with a potency of 98.20%, 2-amino, 2-nitro, and 4-cyanobenzamido impurities, along with bevyxxa® brand tablet formulation with a 40 mg dosage, were procured from Tajmahal Vision Chemicals Private Limited, Mumbai, Maharastra. Merck Chemicals, Mumbai, supplied HPLC-

grade methanol, acetonitrile, Milli-Q® water, and 0.2 µ nylon membrane filters, whereas Fisher Scientific, Mumbai, provided all reagent-grade chemicals utilized in this study.

Instrumentation:

In this study, we employed cutting-edge instrumentation to conduct our analyses. Specifically, the Agilent 1100 instrument boasts an isocratic pump (G1311 A), which offers precise control over solvent delivery for our chromatographic separations. The injection of betrixaban impurities was facilitated by a temperature programmable auto sampler (G1329A), capable of accommodating volumes ranging from 0.1 to 1500 µL with exceptional accuracy and reproducibility. The instrument facilitated a programmable ultraviolet detector (G1314 A), which seamlessly integrates with the chromatographic system to provide reliable UV absorbance data. The eluents from the column were efficiently resolved, and their signals were captured and analysed using the Agilent Chem Station software. Additionally, LCMS analysis was carried out using the Waters LCMS system (Japan) with a triple quadrupole mass detector, which offers unparalleled sensitivity and specificity in identifying and quantifying analytes and was operated seamlessly with MassLynx software.

Preparation of betrixaban and impurity solutions:

The formulation sample was prepared by following the procedure described by Bhupatiraju *et al*. (2023a). The Betrixaban pure drug and its impurities, each at a concentration of 1 mg/mL (equivalent to 1000 µg/mL), were meticulously prepared in separate procedures. This involved accurately measuring 25 mg of the analyte and placing it in a 25-mL flask with 15 mL of methanol. The assistance of a sonicator was utilized to dissolve the analytes in the diluent. Following this, the analytes underwent filtration through a 0.2 µm filter and adjusted volume to achieve the specified volume using the same solvent, resulting in a concentration of 1000 µg/mL for both betrixaban and its impurities. Throughout the analysis, the necessary volume of each individual analyte concentration was combined separately as required.

Formulation solution:

The process commenced with the utilization of Bevyxxa® capsules containing a dosage of 40 mg to prepare the sample solution. To begin, a precisely measured amount of 10 mg of betrixaban equivalent capsule powder was added to a 10 mL flask along with 5 mL of the chosen diluent. Utilizing the assistance of a sonicator, the analytes present in the formulation were effectively dissolved within the diluent. Subsequently, any residual undissolved particles from the capsules were meticulously removed with 0.2 µm filters. The flask was then topped up to its designated mark and further diluted to achieve the desired standard concentration. This meticulously prepared solution served as the basis for assessing the method's efficiency in evaluating the formulation assay.

Method development:

The method's development began with finding the best detection wavelength for betrixaban and its impurities using a UV-visible spectrophotometer. A standard solution with a concentration of 10 µg/mL was scanned across wavelengths from 400 to 200 nm. By comparing the UV absorption spectra, a wavelength suitable for detecting both betrixaban and its impurities was identified. Subsequently, the standard solution, maintained at a concentration of 100 µg/mL, underwent separate scanning with the spectrophotometer, and the overlaid absorption spectra validated the selection of the appropriate detection wavelength. This meticulous process ensured the accurate identification of the wavelength conducive to detecting betrixaban and its impurities, laying a solid foundation for subsequent method development and validation. To achieve the best resolution between betrixaban and its impurities, various stationary phases from different manufacturers were evaluated. Additionally, mobile phase optimization involved experimenting with various solvent compositions spanning various pH ranges, flows were also adjusted. These conditions were extensively studied to ensure the successful separation and validation of the analytes.

Method Validation:

The validation of the analytical method played a pivotal role in ensuring its reliability and accuracy in the thorough examination of betrixaban and its accompanying impurities. Rigorous scrutiny was applied to various critical parameters. This meticulous evaluation was conducted in strict adherence to ICH guidelines and the comprehensive literature (Bikshal *et al.,* 2018; Kasimala *et al.,* 2018; Mallu *et al.,* 2019; Bhupatiraju *et al.,* 2022; Bhupatiraju *et al.,* 2023b).

Assessment of method sensitivity was paramount, achieved through the determination of the signal-to-noise ratio (S/N). Here, ratios of 10:1 and 3:1 were respectively designated as LOQ (quantification limit) and LOD (detection limit). Lower values on these parameters indicated a heightened sensitivity of the method, further validated by the meticulous analysis of impurities at their lowest concentrations, confirming the method's capability to detect even minute impurity levels effectively.

The establishment of linearity was a critical step, involving the analysis of various concentration levels spanning from LOQ to maximum high concentration. Calibration range determination was meticulously achieved through least squares regression to ensure a robust correlation. Calibration curve preparation involved the meticulous formulation of standard dilutions of betrixaban with impurities, setting the impurity concentration as the minimum calibration curve concentration, and adjusting the betrixaban concentration to yield a solution containing 0.1% impurity. These solutions were then subjected to analysis using the proposed method, with resultant calibration curves plotted with peak area against analyte concentration.

Method reproducibility and repeatability were thoroughly evaluated through precision experiments, with betrixaban solutions containing 0.1% impurities analyzed intraday (n = 6), interday (n = 3), and by different analysts (n = 3) to assess method robustness. Method accuracy was meticulously assessed through spiked recovery at four independent levels within the calibration range, with triplicate analyses performed at each level to determine percentage recovery, thus establishing correlation with calibration results.

Stress studies were conducted on betrixaban capsule powder under diverse conditions, including ultraviolet, base, peroxide, acid, and thermal stress, to gauge the stability-indicating capability of the method. Samples were meticulously subjected to stress conditions, neutralized, diluted to desired concentrations, and analyzed using the optimized method. Chromatographic responses were thoroughly evaluated to ascertain the method's stability-indicating feature. Furthermore, placebo and betrixaban tablet powder underwent exposure to UV light and thermal degradation, with resultant samples analyzed using the proposed method to further corroborate method stability.

The characterization of DPs was conducted with utmost precision, requiring a thorough interpretation of mass spectral results. This involved a meticulous experimental setup, which included the utilization of a splitter to direct column eluents effectively. Furthermore, a sophisticated mass detector operating in electrospray ionization (ESI) positive ionization mode was employed to ensure optimal sensitivity and accuracy in detecting the molecular ions of interest.

To ensure reliable and reproducible results, essential instrumental parameters such as capillary, fragmentor, and skimmer voltages were meticulously configured. Each parameter was carefully optimized to maximize the signal-to-noise ratio while minimizing background noise, thereby enhancing the overall quality of the mass spectral data obtained.

Additionally, nitrogen gas was utilized for drying and nebulization purposes, ensuring the efficient conversion of liquid analytes into gas-phase ions suitable for mass spectrometric analysis. This step was critical in maintaining the integrity of the analytes and facilitating their ionization, ultimately leading to robust and reliable mass spectral data.

The practical application of the analytical HPLC method proposed in this study was successfully demonstrated in the identification and quantification of studied impurities in betrixaban injection formulations. Sample solutions prepared with Bevyxxa® formulation solution, both with and without

spiked known concentrations of studied impurities, underwent comprehensive analysis using the proposed method. The observed chromatograms and corresponding responses served as concrete evidence confirming the method's robust applicability and reliability in pharmaceutical analysis.

Results

The optimization of the mobile phase composition within a suitable pH range was a pivotal aspect of our methodology, as it directly influenced the effectiveness of our chromatographic separation. Given the presence of hydrophilic ionizable functional groups in both betrixaban and its impurities, it was imperative to utilize a mobile phase containing pH buffers to ensure optimal resolution. To achieve this, we systematically explored various solvent compositions and buffer strengths, meticulously scrutinizing their effectiveness in achieving the desired separation.

Ultimately, we successfully achieved efficient resolution of betrixaban and its impurities using a Zorbax SB C18 column (4.6×250 mm, 5µm). Our chromatographic conditions, including a flow rate of 1.0 mL/min and a mobile phase consisting of 75% aqueous ammonium formate (5 mM) and 50% ammonium formate in acetonitrile at a ratio of 60:40 (v/v), were meticulously optimized. Throughout our analyses, we maintained strict control over the column temperature (35°C), sample volume (20 µL), and detector wavelength (245 nm).

Under these optimized conditions (Figure 2), the resulting chromatographic peaks exhibited symmetrical shapes, with satisfactory resolution between neighboring peaks exceeding 2. To further confirm the identities of individual analytes, we conducted injections of individual analyte solutions and correlated their retention times (tR) with standards. Our analyses revealed retention times of 10.30 min for betrixaban, 3.77 min for the 2-amino impurity, 7.79 min for the 2-nitro impurity, and 13.55 min for the 4-cyanobenzamido impurity.

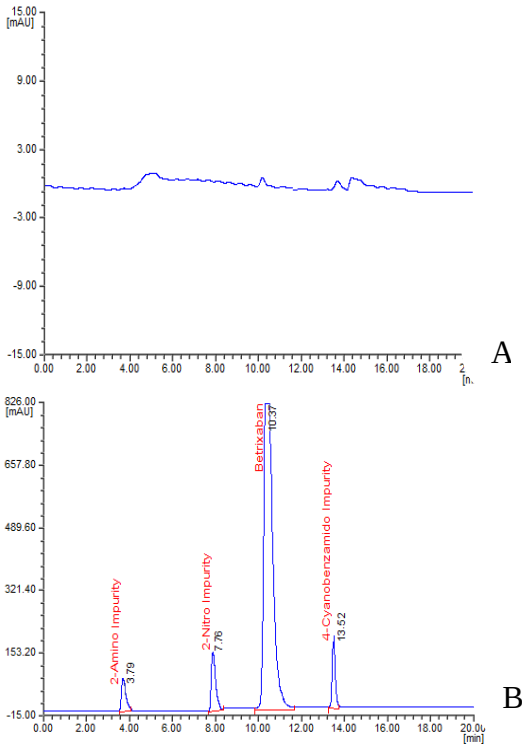


Figure 2: Chromatographic profiles observed during the specificity investigation

A) The chromatogram obtained from the analysis of the placebo solution does not exhibit any chromatographic detections; B) The standard chromatogram, obtained during the analysis of a known strength of betrixaban spiked with 0.1% impurities, clearly reveals symmetric peaks corresponding to the analytes.

System suitability tests were conducted to validate the adequacy of our method, with analyte peaks meeting established guidelines, including theoretical plates (N) of > 2000, resolution (RS) of > 2, and an asymmetric factor (AS) of ≤ 2.0. Additionally, sensitivity evaluation yielded impressive limits of detection (LOD) and quantification (LOQ) values of 0.007 µg/mL and 0.05 µg/mL for impurities, respectively, highlighting the method's high sensitivity at very low concentrations. Method validation results are given in Table 1.

Table 1: Summary results noticed in method validation

Parameter	Results			
	Betrixaban	**2-Amino impurity**	**2-Nitro impurity**	**4-Cyanobenzamido Impurity**
System suitability[$]				
t_R(min)	10.36	3.77	7.79	13.55
Relative retention time	--	0.37	0.75	1.30
Relative response factor	--	0.045	0.070	0.082
R_S	7.13	--	11.25	6.58
A_S	1.05	0.98	1.07	0.97
N	9311	4960	7412	12149
Linearity(µg/mL)	25-150	0.025-0.15	0.025-0.15	0.025-0.15
Slope	11449	557372	802219	937871
Intercept	38639	- 550.95	4546.8	1933.5
Correlation coefficient	0.9995	0.9991	0.9999	0.9991
Intraday precision[$$]	0.44	0.44	0.46	0.54
Precision (day 1)[$]	0.28	0.23	0.85	0.51
Precision (day 2)[$]	0.45	0.22	0.46	0.39
LOQ level	--	0.57	0.43	0.98
50 % level accuracy[$]				
Prepared(µg/mL)	75	0.075	0.075	0.075
Estimated(µg/mL)	74.45	0.075	0.073	0.075
% accuracy	99.26	99.61	97.64	99.57
% RSD	1.73	0.30	1.53	0.85
100 % level accuracy[$]				
Prepared(µg/mL)	100	0.100	0.100	0.100
Estimated(µg/mL)	98.50	0.100	0.099	0.100
% accuracy	98.50	100.08	99.10	100.04
% RSD	1.32	0.45	0.79	0.68
150 % level accuracy[$]				
Prepared(µg/mL)	125	0.125	0.125	0.125
Estimated(µg/mL)	124.17	0.124	0.124	0.125
% accuracy	99.34	99.40	99.51	99.62
% RSD	0.92	0.91	0.76	0.81
Sensitivity				
Detection limit (µg/mL)	--	0.025	0.025	0.025
Quantification limit (µg/mL)	--	0.007	0.007	0.007

Average results of n=3[$] and 6[$$]

Method accuracy was rigorously assessed through recovery (%R) studies, with observed % recovery falling within the acceptable range of 98–102% across different spiked concentrations. Moreover, the robustness of our method was confirmed through minor parameter modifications, which resulted in negligible changes in our analyses. Robustness results are given in Table 2.

Table 2: Robustness study results.

S No	Changed condition	Parameter	Results observed			
			Betrixaban	2-Amino impurity	2-Nitro impurity	4-Cyanobenzamido Impurity
1	MP 1	% change	0.34	0.54	0.33	0.77
		t_R	10.35	3.77	7.79	13.54
		N	9272	5084	7401	12098
2	MP 2	% change	0.08	1.36	0.33	0.13
		t_R	10.32	3.71	7.72	13.52
		N	9277	5119	7394	12179
3	pH 1	% change	0.65	0.15	0.62	1.75
		t_R	10.39	3.75	7.77	13.59
		N	9241	5089	7388	12189
4	pH 2	% change	0.25	1.69	1.15	0.55
		t_R	10.33	3.72	7.79	13.56
		N	9262	4939	7403	12219
5	WL 1	% change	1.44	0.63	0.68	0.03
		t_R	10.34	3.76	7.71	13.58
		N	9278	4942	7371	12085
6	WL 2	% change	0.83	0.35	0.75	-0.78
		t_R	10.31	3.78	7.75	13.54
		N	9281	4923	7409	12096
7	CT 1 (33°C)	% change	0.52	0.91	0.43	0.58
		t_R	10.33	3.75	7.76	13.52
		N	9544	4934	7362	12109
8	CT 2 (38°C)	% change	0.32	0.37	0.58	0.39
		t_R	10.32	3.71	7.75	13.58
		N	9609	4943	7381	12131

Forced degradation studies (table 3) constitute a critical component of pharmaceutical analysis, aimed at unraveling potential degradation pathways and shedding light on the stability of molecules under various stress conditions. The ability of an analytical method to effectively indicate stability is paramount in ensuring the confirmation of product shelf life. Therefore, in this study, meticulous forced degradation experiments were conducted to elucidate potential DPs and gain insights into the degradation pathways and stability of the molecule under investigation. Establishing the stability-indicating capability of the analytical method was imperative, and the resulting DPs were meticulously characterized through LCMS/MS analysis. The percentage of degradation was comprehensively evaluated under diverse stress conditions, revealing intriguing insights into the molecule's behavior. Specifically, the percentage of degradation was quantified as follows: 9.97% under acidic conditions, 8.31% under basic conditions, 6.27% under peroxide-induced stress, 5.88% under thermal stress, and 3.47% under UV light-induced stress.

Table3: Stress degradation results of betrixaban

Condition	Betrixaban % degradation[$]	Betrixaban % assay[$]	% Mass balance[$]	Remark
Acid	9.97	90.03	99.32	DP 2 was identified
Base	8.31	91.69	99.14	DP 1 and 3 were identified
Peroxide	6.27	93.73	98.75	DP 4 was identified
Thermal	5.88	94.12	99.58	No degradation was identified
UV light	3.47	96.53	99.63	No degradation was identified

[$]sum of all detected compounds in the chromatogram (n=3)

Interestingly, thermal degradation studies yielded no significant degradation, with no detectable DPs observed. However, under acidic degradation conditions, a notably high percentage of degradation, reaching 9.97%, was observed. Chromatographic analysis of the acid-degraded sample revealed the presence of DP 2 at a retention time (tR) of 5.80 min, providing valuable insights into the specific degradation pathway under acidic conditions. Similarly, base degradation unveiled the presence of

two distinct DPs, labeled DP 1 and DP 3, with retention times of 3.79 min and 6.85 min, respectively, highlighting the complexity of degradation pathways under basic conditions. Peroxide-induced degradation exhibited peaks corresponding to DP 4, retained at 13.25 min, along with peaks corresponding to known impurities, further emphasizing the necessity of understanding degradation pathways to ensure product stability and safety. The stress degradation behavior of betrixaban is given in Figure 3.

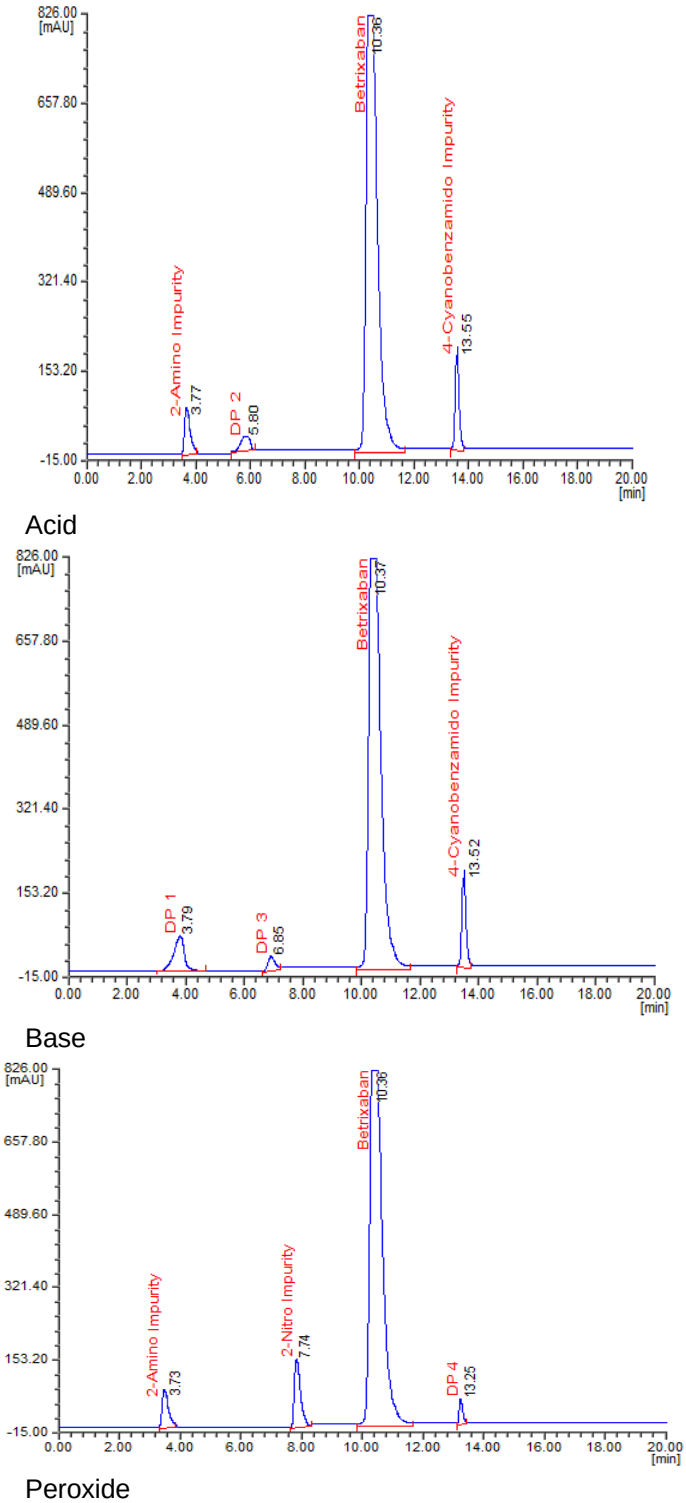


Figure 3: Chromatographic profiles observed during the investigation of stress degradation behaviour of betrixaban

Overall, these forced degradation studies underscored the robustness and reliability of the analytical method in identifying and characterizing DPs, thereby providing valuable insights into the molecule's stability profile under diverse stress conditions. This comprehensive understanding is indispensable for ensuring the efficacy, safety, and shelf life of the pharmaceutical product.

Characterization of DPs by LCMS/MS:

The acid induced stress degradation study chromatogram shows one unknown peak considered as DP and based on time of elution, this DP was marked as DP 2. In positive ionization mode, the exact masses of DPs were identified based on protonated molecule ion and nitrogen rule. The molecular formula of $C_{18}H_{19}N_3O_4$was confirmed with molecular formula of 341.36 g/mol, DP 2. DP 1 was characterized by observing fragment ion at 178, 134, 108, 78 in positive ionization mode corresponds to $C_9H_9N_2O_2$ (formed by lose of CH_3 from parent ion), $C_8H_9N_2$(lose of $C_2H_3O_2$ from 192 *m/z*, C_7H_7O (formed by lose of $C_3H_5N_2O$ from 192 *m/z*) and C_6H_5 (formed by lose of $C_4H_7N_2O_2$ from 192 *m/z*). The parent ion fragment at 263 (m+1) was noticed in positive ionization mode suggest the compound mass of DP 3 as 262.69 g/mol. The parent ions noticed at 218 (m+1), 198 (m+1), 140 (m+1) and 108 (m+1) represents fragment with molecular formula $C_{12}H_{10}ClN_2$ (due to lose of CHO from parent ion), $C_9H_{10}ClN_2O$ (due to lose of C_4HO from 263 *m/z*), $C_6H_4ClN_2$ (due to lose of $C_7H_7O_2$ from 263 *m/z*) and C_7H_7O(due to lose of $C_6H_4ClN_2O$ from 263 *m/z*). The mass pattern of DP 4 confirms fragments ions at 354 (m+1), 228 (m+1), 150 (m+1) and 123 (m+1) with parent ion at 407 (m+1) confirms its molecular mass as 406.82 g/mol. The formula of parent ion confirmed as $C_{21}H_{15}ClN_4O_3$ derived from fragment ion formula of $C_{18}H_{14}ClN_4O_2$ (generated due to lose of C_3HO from parent ion), $C_{10}H_{12}ClN_4O_2$ (due to lose of $C_{11}H_3N_2O$ from 406 *m/z*), $C_8H_7NO_2$ (due to lose of $C_{13}H_8ClN_3O$ from 406 *m/z*) and C_7H_8NO (due to lose of $C_{14}H_7ClN_3O_2$ from 406 *m/z*). The collision-induced dissociation analysis suggests that DP 1 and 4 were originate from betrixaban. The possible fragmentation mechanism of DP 1, 2, 3 and 4 was presented in figure 4-7. Mass spectra are given in Figure 8.

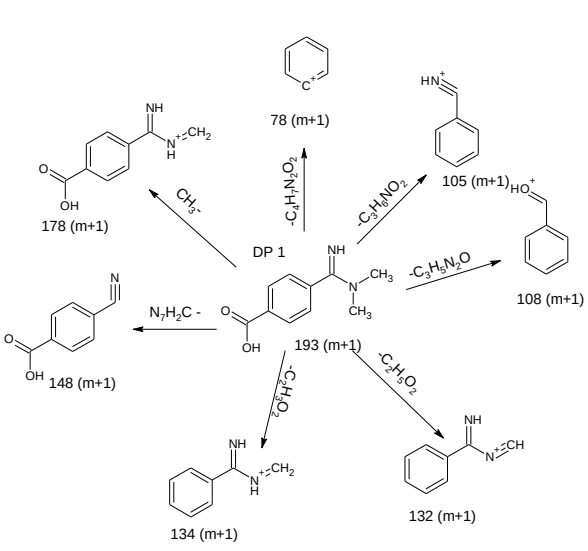


Figure 4: Proposed fragmentation mechanism of DP 1

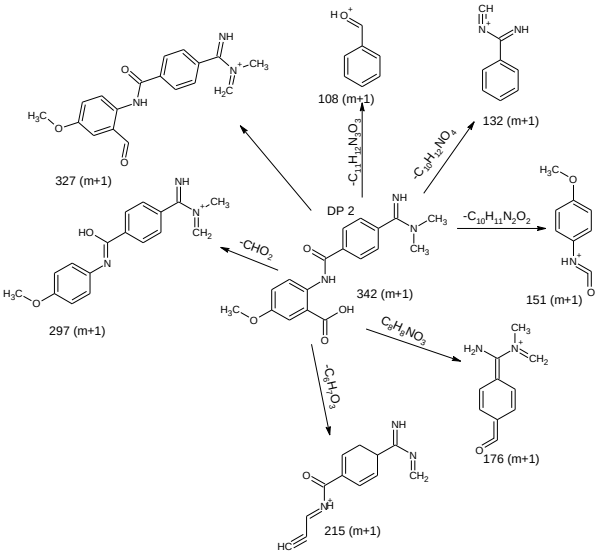


Figure 5: Proposed fragmentation mechanism of DP 2

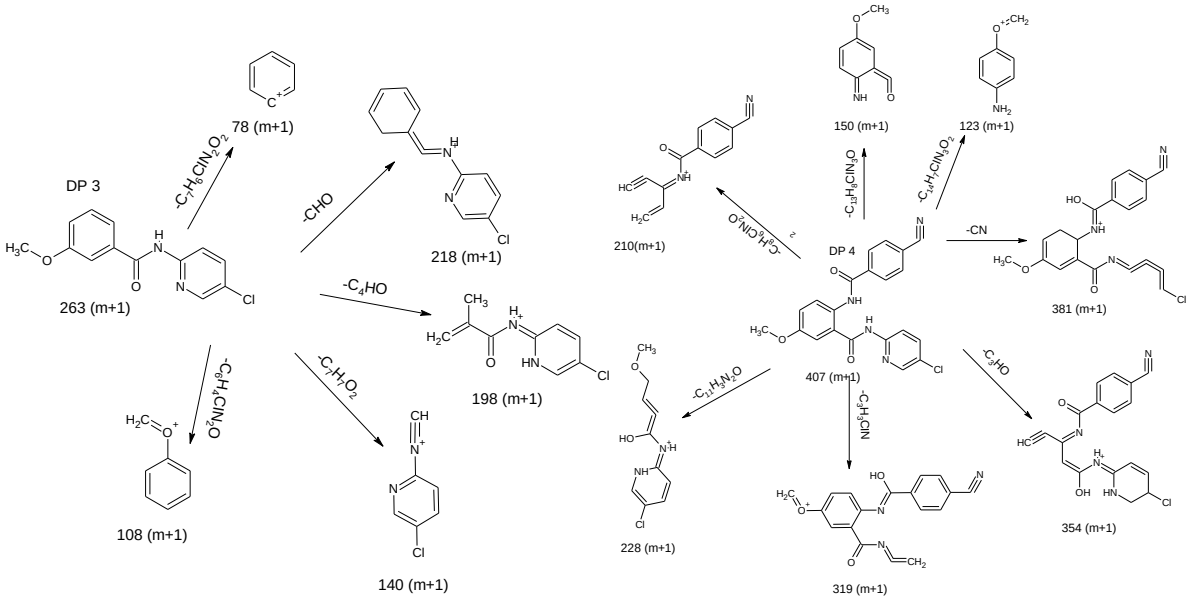


Figure 6: Proposed fragmentation mechanism of DP 3 **Figure 7:** Proposed fragmentation mechanism of DP 4

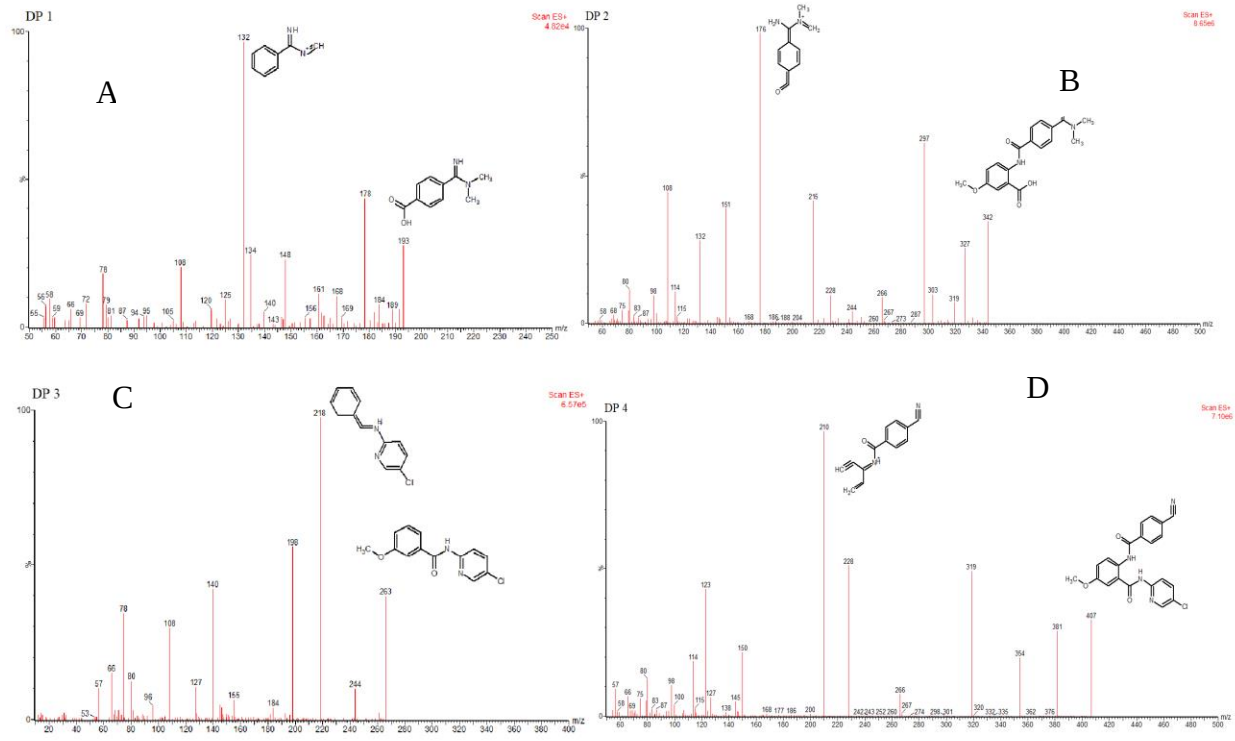
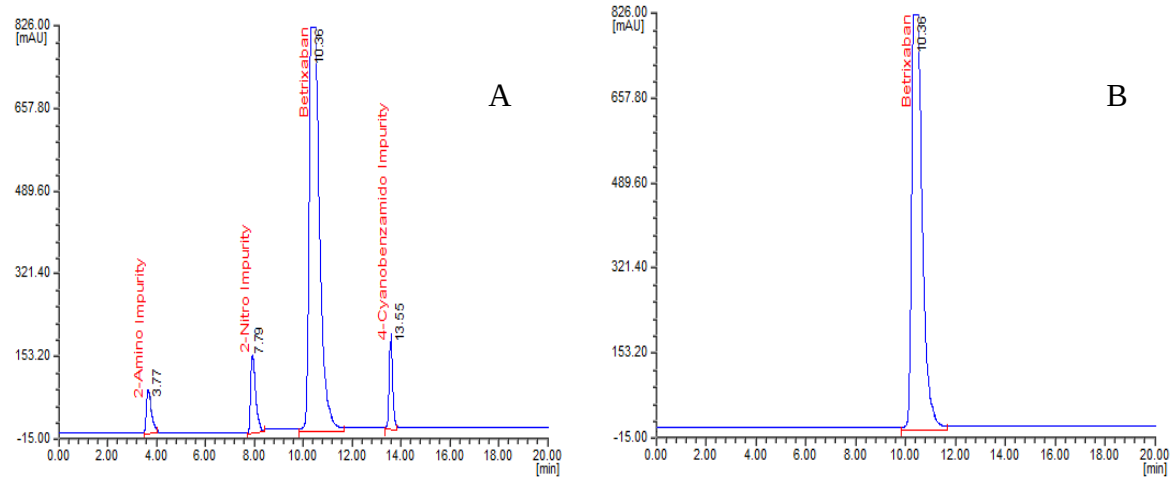


Figure 8: Mass spectra of degradation products formed due to stress expose
Mass spectra identified at t_R of 3.78 min for DP 1 (A), 5.80 min for DP 2 (B), 6.85 min for DP 3 (C), 13.25 min for DP 4

In this study, the evaluation of process-related impurities in betrixaban was conducted utilizing a method tailored specifically for the Bevyxxa® brand formulation of betrixaban. Both impurity-spiked and un-spiked capsule solutions of betrixaban were meticulously analyzed using the developed method. The resulting chromatograms obtained from the analysis of these samples clearly depicted the presence of impurities alongside the standard betrixaban peaks.The clear visualization of these impurities in both the spiked and un-spiked samples suggests that these impurities were present in concentrations above the detection limit within the formulation. Furthermore, noteworthy was the absence of any visualization of formulation excipients or unidentified compounds in either the spiked

or un-spiked samples. This observation underscores the reliability and adequacy of the method for routine quality evaluation of process-related impurities in betrixaban. The chromatograms obtained from the spiked and un-spiked formulation samples, as depicted in Figure 9, provide tangible evidence of the method's effectiveness in detecting and quantifying impurities, thereby ensuring the integrity and quality of the betrixaban formulation.



Impurities spiked (A) no impurities spiked (B)

Figure 9: Formulation analysis chromatogram of betrixaban

Discussion

Upon conducting a thorough literature review, it became evident that there were no previously published analytical methods tailored specifically for quantifying genotoxic impurities in betrixaban. Consequently, the primary objective of this study was to optimize a robust HPLC method capable of accurately quantifying three genotoxic impurities—namely, 2-amino, 2-nitro, and 4-cyanobenzamido impurities of betrixaban. Given the polar nature of both the impurities and betrixaban, the optimization process focused on testing various column configurations to achieve optimal resolution of the analytes.

Optimizing the mobile phase composition within a suitable pH range was crucial for our chromatographic separation, particularly due to the hydrophilic ionizable functional groups in both betrixaban and its impurities. The use of pH buffers in the mobile phase was essential to achieving optimal resolution. We systematically evaluated various solvent compositions and buffer strengths to determine the most effective conditions for separation. Forced degradation studies were essential to understanding the stability of betrixaban under various stress conditions. The DPs formed during the stress study were characterized based on mass spectral fragmentation data.

The findings achieved in this study were correlated with reports published in the literature. In methods reported by Mastannamma *et al.,* 2018, El-Masry *et al., 2020* and Ghante *et al.,* 2021 were available for quantification of betrixaban in formulations. The method reported by Surukonti *et al.,* 2023 adopted preparative HPLC technic for isolation of stress DPs and further characterized the structure of three DPs using spectroscopic techniques. The method reported here doesn't require preparative HPLC for structural characterization of DPs. Whereas the methods reported by Jasemizad *et al.*, 2019, El-Masry *et al.,* 2021 and El-Masry *et al.,* 2022 were suitable for quantification of betrixabanin in combination with other drugs such as hexazinone and lercanidipine in biological samples. Hence, based on literature it was was confirmed that no method was reported for the characterization of DPs of betrixaban, and no method resolved the impurities of betrixaban. Hence, the method reported in this study will be the best choice for resolution, quantification, and identification of both impurities and DPs of betrixaban.

Conclusion

This study presents a highly selective and sensitive HPLC method designed for the resolution and quantification of genotoxic impurities in betrixaban formulations. Utilizing a Zorbax SB C18 column (4.6 × 250 mm, 5 µm, Agilent) maintained at 35°C, the method employed a mobile phase consisting of 75% aqueous ammonium formate (5 mM) and 50% ammonium formate in acetonitrile, in a 60:40 (v/v) ratio, with isocratic elution at a flow rate of 1.0 mL/min and detection at 245 nm. A highly sensitive calibration curve ranging from LOQ (0.025 µg/mL) to 0.15 µg/mL was successfully generated for the studied impurities. Betrixaban underwent rigorous forced degradation studies following ICH guidelines, demonstrating remarkable stability under thermal and UV light degradation conditions but showing susceptibility to acidic, basic, and peroxide degradation. A total of four DPs, namely N-(5-chloropyridin-2-yl), -3-methoxybenzamide (DP 1), 2-{[4-(N,N-dimethylcarbamimidoyl)benzoyl]amino} -5-methoxybenzoic acid (DP 2), N-(5-chloropyridin-2-yl) -3-methoxybenzamide (DP 3) and N-(5-chloropyridin-2-yl) -2-[(4-cyanobenzoyl)amino] -5-methoxybenzamide (DP 4) was characterized using LCMS/MS analysis in ESI positive mode. The study also explored the mechanisms and pathways leading from betrixaban to these DPs, providing valuable insights for identifying trace levels of genotoxic impurities and potential DPs in bulk drug samples.Moreover, this analytical method offers practical utility for estimating the quality control samples, serving both routine analysis and stability studies of betrixaban and its genotoxic impurities.

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Conflict of Interest:

Authors confirm that there is no conflict of interest to declare.

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