Development of a Green Stability indicating HPLC Method for Quantification of genotoxic impurities of Bexagliflozin and LC-MS/MS Profiling of its Degradation Products

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Abstract
The stability and purity of pharmaceutical compounds are crucial for ensuring drug efficacy and patient safety. Bexagliflozin, which is a sodium-glucose co-transporter 2 inhibitor, is applied to improve glycemic control in type 2 diabetes mellitus. The literature suggests a robust analytical method for its quantification and stability assessment. Hence, this study intended to propose a stability-indicating HPLC method for the separation, identification and quantification of Bexagliflozin and its impurities under various stress conditions. The developed method was validated and exhibited high selectivity and resolution (Rs > 2) between Bexagliflozin and its impurities. The calibration curve demonstrated excellent linearity within the concentration range of 8–56 µg/mL for Bexagliflozin and 0.08–0.56 µg/mL for its impurities with a correlation coefficient exceeding 0.9997. Accuracy was verified through recovery studies, yielding mean recoveries of 99.36% for Bexagliflozin and 99.20–100.36% for its impurities.

Structural elucidation of these degradation products was performed using mass spectrometry, revealing key fragmentation patterns. The proposed method successfully distinguished degradation pathways. Hence, we concluded that the developed HPLC method is a reliable and effective tool for the quantification and stability assessment of Bexagliflozin. The greenness of the method was evaluated using GAPI (Green Analytical Procedure Index) and AGREE (Analytical GREEnness) tools, confirming that the technique can significantly reduce the usage of hazardous solvents. The identification of DPs provides critical insights into Bexagliflozin's stability profile that facilitates its safe and effective therapeutic use.

Keywords: Bexagliflozin, degradation products, mass spectrometry, fragmentation.

Introduction

Bexagliflozin is a SGLT2 inhibitor prescribed for the treatment of type 2 diabetes mellitus. By selectively blocking SGLT2 in kidney, it lowers the glucose reabsorption and increases the urinary glucose excretion, decreasing the blood glucose[3]. This action not only enhances glycemic control but also has the potential for cardiovascular and renal protective effects, just like other SGLT2 inhibitors. Bexagliflozin is usually prescribed as a part of multi-faceted treatment plan that includes diet and exercise. Clinical trials have demonstrated its efficacy in lowering HbA1c, causing weight loss and decreasing blood pressure. Like other medications in this class, though, it has the potential to produce side effects such as urinary tract infections, genital infections, dehydration and increased urination[1]. Its administration should be strictly monitored in patients with impaired renal function or at risk of ketoacidosis.

Bexagliflozin is a significant step in diabetes treatment, providing a new option for patients who want to achieve better glucose control with added metabolic advantages[4]. The therapeutic significance such as efficacy and safety of Bexagliflozin necessitates a thorough understanding of its stability and degradation behaviour under various stress conditions.

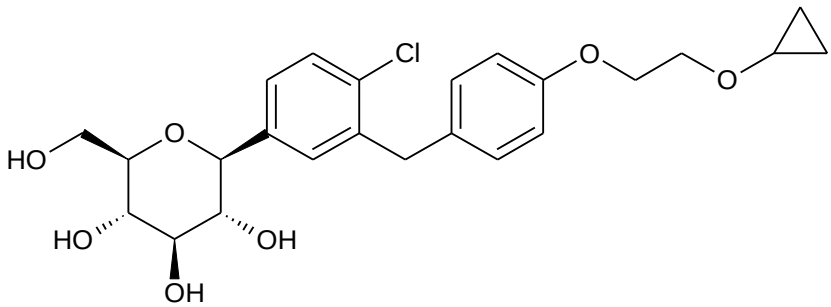


Figure 1: Molecular structure of Bexagliflozin

The forced degradation studies, which are also known as stress testing, provide significant insights into the intrinsic stability of Bexagliflozin and also identify potential DPs.

In these studies, pure Bexagliflozin was exposed to different stress conditions to simulate potential DPs and to evaluate the possible degradation pathways during manufacturing, storage and administration[2,11]. The identification/ characterization of DPs helps to optimize formulation strategies, select appropriate excipients and packaging material and establish proper storage conditions to enhance the shelf life of drug products[16]. Additionally, some DPs may be toxic or pharmacologically active and these DPs need to evaluate their toxicological risk to prevent adverse effects[8].

The green analytical methods align with green chemistry principles that minimize hazardous chemical usage, minimize waste generation and conserve resources. Furthermore, these methods can lead to cost savings and increased efficiency, making them an attractive option for the accurate and reliable quantification of impurities in pharmaceutical compounds. The HPLC coupled with mass spectrometry (LC–MS/MS) is a powerful analytical technique that was widely utilized for the identification and characterization of DPs[15]. LC–MS/MS provides structural determination of unknown compounds and helps to understand the fragmentation patterns of drugs. In addition, the optimization of a stability-indicating HPLC method is essential for routine quality control analysis to ensure accurate quantification of the drug, its impurities and DPs[12,13].

The literature reported several studies on the analytical profiling of Bexagliflozin but no literature is available on the comprehensive characterization of its DPs. This research aimed to bridge this gap by employing LC–MS/MS for structural elucidation of stress-induced DPs of Bexagliflozin. Additionally, this study intended to propose a robust stability-indicating HPLC method by following ICH guidelines to ensure the reliable quantification of Bexagliflozin and its known impurities in pharmaceutical formulations. The key objectives of this study include:

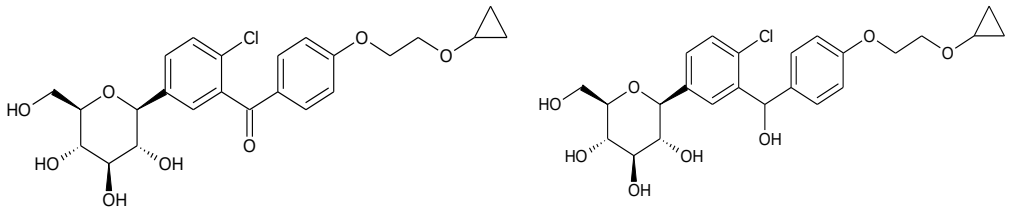
1. To establish a stable analytical method capable of distinguishing Bexagliflozin from its known impurities and DPs with high sensitivity and selectivity.
2. To validate the developed HPLC method by ICH guidelines in terms of specificity, linearity, precision, accuracy, robustness and stability.
3. To subject Bexagliflozin to different stress conditions to determine its degradation behaviour.
4. To identify and elucidate the structure of major degradation products using tandem mass spectrometry.

This study is significant form both regulatory and pharmaceutical perspectives. The comprehensive degradation profiling of Bexagliflozin will aid in better understanding its stability. The developed stability-indicating HPLC method will serve as a reliable analytical tool for analysis of Bexagliflozin and its known impurities in the study. Based on availability, the genotoxic impurities of Bexagliflozin such as keto impurity, hydroxy impurity and bromo impurity (Figure 2) were used in this study.

Material and Methods

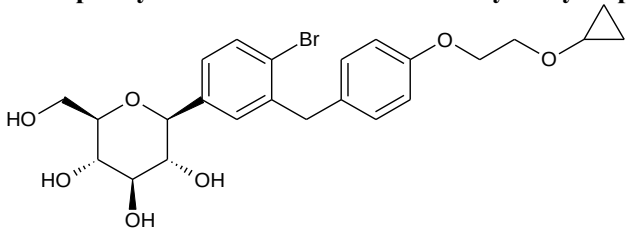
Drugs and reagents: The pure form of Bexagliflozin along with its impurities was obtained from Piramal Pharma Solutions, Ahemadabad, India as a gift sample. Ethanol, water, LR and AR grade chemicals like NaOH, HCl, H_2O_2 and buffer salts all HPLC grade chemicals were procured from Qualigens Fine Chemicals, Mumbai, Maharashtra, India.

Equipment's: The Alliance (Japan) HPLC system equipped with a binary pump (2695), online degasser, injector with fixed volume loop (20 µL) and UV detector was utilized to perform LC analysis. LC–MS/MS analysis was performed using an Alliance LC system (Waters, Japan) coupled to a MicrOTOF-Q mass spectrometer made by Bruker Daltonics, Bremen, Germany.



Keto Impurity

Hydroxy Impurity



Bromo Impurity

Figure 2: Structure of Bexagliflozin genotoxic impurities selected in this study

The LC system consisted of a binary pump with online degasser, autosampler, column oven and UV detector. Data processing was done using MassLynx software. The mass analyser was operated in positive ESI mode with an m/z range of 10–1000. In addition, a GT Sonic sonicator (India) was used for the preparation of samples.

Preparation of standard solution: The 1000 µg/mL stock solution was prepared by dissolving an equivalent weight of 50 mg of pure form in a 50 mL flask having 25 mL of ethanol and mixed thoroughly. Sonicate the solution for 3 mins for complete solubility of the drug. After the sonication, solution was filtered through 0.22µ nylon membrane filter paper and then made up the solution to the mark. Resulting solution was 1000 µg/mL, this solution was stored and used further. In the same pattern, impurities are prepared and a 1% solution of impurity was mixed with standard and used for analysis.

Stress studied samples: The stress degradation behaviour of Bexagliflozin was evaluated according to stress conditions set for ICH Q1A (R2). The stress studies conducted on the drug were exposure to hydrolysis, oxidation, dry heat and photolysis conditions. Optimizations of stress conditions were done such that the results will achieve 10-15% degradation of the drug substance. The analysis utilized HPLC with a detector at 227 nm while characterizing the same products with the LC-MS/TOF. The hydrolytic degradation was done by heating 1 mL of 1N HCl (acid), 1N NaOH (base) and water (aqueous) with 1 mL of drug solution at 80°C for 12 hours separately. After exposure, acid-treated samples were neutralized with an equivalent strength of base and vice versa. In oxidative degradation, 1 mL of the drug solution with a concentration of 1000 µg/mL was left for 24 hours with 1 mL of 10% H_2O_2 at room temperature.

In thermal degradation, the solid drug was heated in an oven at 80°C for 7 days in a sealed glass ampoule while a control sample was kept at room temperature. The photostability of both the solid and solution state drug samples was studied. A solution of Bexagliflozin at 1000 µg/mL and the solid drug was subjected to 1.25 million lux hours of fluorescent light and 200 Wh/m² of UV light with control samples protected by aluminum foil. Photolytic conditions for the solution state showed the degradation of the drug, while for the solid state, the drug was stable. Samples were subsequently diluted to reach 100 µg/mL concentration of the Bexagliflozin and 1% for the impurities with a mixture of solvent A and B in 70:30 v/v at pH 3.6 in preparation for the HPLC analysis.

Optimization of HPLC method for Bexagliflozin and impurities: To optimize a simple and economical LC method, several attempts were made with solvents A and B in 60:40 v/v, solvent A is ethanol and solvent B is 0.01M ammonium acetate buffer (pH 5.1) as mobile phase solvents with X-bridge (250 × 4.6 mm; 5 µm particle size) and 227 nm detector wavelength. The optimization was first carried out on the individual samples of Bexagliflozin and impurities and then on their mixture. The specificity of the method was checked by peak purity and resolution for impurities and Bexagliflozin[5]. The linearity was established using solutions prepared from a stock solution at six concentration levels (8–56 µg/mL) for Bexagliflozin and 0.08-0.56 µg/mL for the studied impurities.

The triplicate of linearity solutions was analysed and the peak area versus concentration was processed using least squares linear regression analysis to determine the correlation coefficient to test accuracy. The sample mixture of Bexagliflozin and its impurities spiked with three known concentrations, each of 32, 40 and 48 µg/mL for Bexagliflozin and 0.32, 0.4, 0.48 µg/mL for impurities were analyzed in three replicates and the % recovery results were calculated to evaluate method accuracy. The precision and reproducibility within a single day and in multiple days were proved by analysing Bexagliflozin and its impurities at a 100 % recovery level concentration solution.

Evaluation of method greenness: The proposed quantitative method was green-evaluated through GAPI and AGREE on metric tools[9]. The green evaluation specifically helps to estimate different parameters for ensuring the safety of the drug by reducing the health and environmental impact. This estimation includes aspects such as sample collection, the method used, solvents and reagents used in the method, energy expenditure, steps of disposal of wastes and other pertinent considerations. Among them, GAPI is highly effective in examining environmental sustainability factors. In this evaluation, the figure was prepared with different color combinations like green, red and yellow. The whole figure is divided into six different parts, where each part signifies different factors, such as the origin of the sample, the process type that it undergoes, sample preparation, the chemicals and reagents used, the instruments and most importantly, the type of quantitative analysis, which is signified by the letter "O".

The AGREE method was used to determine the extent of environmental sustainability. The method by metric is based on the twelve principles of analytical green chemistry. This current study aims to develop AGREE, GAPI and environmental sustainability methods for the achievement of safe and effective qualitative and quantitative analysis of Bexagliflozin.

LC-MS/TOF Fragmentation Analysis of Bexagliflozin: The breakdown pattern of Bexagliflozin was analysed through LC-MS/TOF. The mass spectra were recorded within 10–1000 m/z range using positive ESI mode. The ethanolic solution of Bexagliflozin at 100 µg/mL having 1% known impurities and its stress degradation sample solutions were directly injected through a syringe pump into the MS/TOF system. The mass analyser parameters were adjusted to achieve the molecular ion peak and further

optimization of mass fragment parameters brought characteristic fragment ions[7,10]. All the mass peaks recorded were given as fourth-decimal accuracy. The stressed sample mixture was further analysed for LC-MS/TOF using the same optimized conditions as that for Bexagliflozin[17]. An analytical gradient elution method was applied during analysis and the fragmentation pattern of each degradant was obtained from the mass spectra and accurate m/z values.

Results and Discussion

The chromatographic separation was accomplished by using an X-bridge C18 column (250 mm × 4.6 mm; 5 µm), equipped with the linear gradient elution system set at 0.9 mL/min, UV detection at 227 nm. The mobile phase used was a combination of solvent A and B in 60:40 v/v, solvent A is ethanol and solvent B is 0.01M ammonium acetate buffer (pH 5.1) and the same concentration was used for the dilution process. The gradient program comprises of times/%B as 0-2 mins (5%), then 2.1 min - 5 mins (15%), 5.1 min-7 mins (40%), 7.1-8 min (15%) and 8.1-11 mins (5%). The specificity, selectivity, linearity, accuracy and precision of the proposed method were validated. The standard chromatogram is given in figure 3.

The standard and stress degradation solution in the optimized method shows high selectivity and stability-indicating capability because all degradant peaks appear to be adequately resolved. The resolution (Rs) of the peaks was greater than 2 with permissible symmetry and system suitability. The response of Bexagliflozin was linear in 8 to 56 µg/mL range for Bexagliflozin and 0.08 to 0.56 µg/mL for its impurities with correlation coefficient of higher than 0.9997 (Table 1). The precision studies were carried in terms of intra, intra-day study using 100 % level solution of Bexagliflozin and its impurities.

The achieved results are summarized in table 1 with RSD% values of below 2% providing the high precision of the method. The method accuracy was assessed via recovery studies using the standard addition method, where formulation samples were spiked with known concentrations of Bexagliflozin at three levels. The mean recovery was found to be 99.36% for Bexagliflozin, 100.25% for keto impurity, 100.36% for hydroxy impurity and 99.20% for Bromo impurity demonstrating the method's accuracy (Table 2).

Stress degradation study: The degradation through acidic and base stress was apparent for Bexagliflozin whereas its stability was achieved in oxidative, peroxide and thermal stress. The DPs produced under stress conditions were labelled as DP 1, DP 2 and DP 3 according to their elution order and formed stress conditions in the HPLC chromatogram as shown in figure 4.

Fragmentation pathway of DP 1 and DP 3: In acid degradation, two DPs are formed at 2.123 min and 3.223 mins, they are named as DP 1 and DP 3 based on elution of time. Bexagliflozin brokedown in two structural units, one with a molecular formula of $C_{19}H_{21}ClO_6$ named DP 1 and the second one with a molecular formula of $C_{21}H_{23}ClO_7$ which was named as DP 3 because they are formed at stress condition of acid. Formation of DP 1 is formed by the loss of C_5H_8O form Bexagliflozin. Formed DP 1 has core functional groups like polycyclic and chloro-substituted with oxygen containing functional groups are like esters, carboxyl, or hydroxyl groups. DP 1 is again formed into two fragments based on the possible splits (i) Formed by the loss of OH group with molecular formula $C_{19}H_{20}ClO_5$ (m/z 363.81) and (ii) compound with molecular formula of $C_{19}H_{18}ClO_4$ (m/z 345.79).

By all this fragmentation pattern DP 1 is identified as *(2S,3R,4R,5S,6R)-2-[4-chloro-3-(4-hydroxybenzyl)phenyl]-6-(hydroxymethyl) tetrahydro-2H-pyran-3,4,5-triol*. DP 3 is also formed in acid degradation study by the loss of simple molecule C_3H_6 from Bexagliflozin. DP 3 is split into two product ions: one is by the loss of single hydrogen and single oxygen atoms (OH) group and second fragment is formed by the loss of ethynyl carbonate (C_3HO_3). Resulted fragments are $C_{21}H_{24}ClO_6$ (m/z 407.86) and second one is $C_{18}H_{22}ClO_4$ (m/z 337.81). Fragment ion of $C_{21}H_{24}ClO_6$ forms product ion by the loss of vinyl chloride or chloroethene. Resulted fragment is $C_{19}H_{21}O_6$, m/z 345.36.

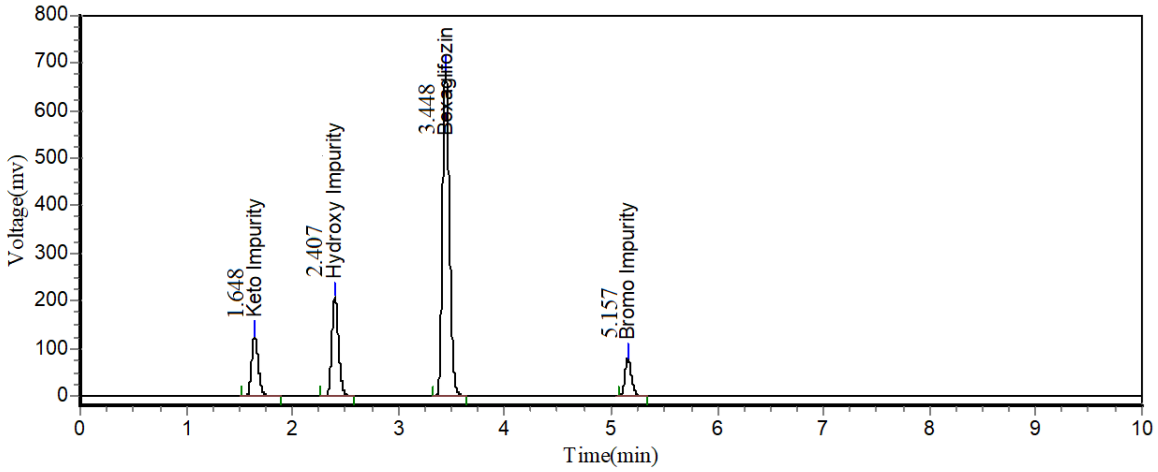


Figure 3: Standard chromatogram of Bexagliflozin and its impurities

Table 1
Precision study of Bexagliflozin and its studied impurities

Parameter	Bexagliflozin	Keto Impurity	Hydroxy Impurity	Bromo Impurity
Linearity range (µg/mL)	8-56	0.08-0.56	0.08-0.56	0.08-0.56
Regression equation	y = 153057x + 23168	y = 220926x + 1506.9	y = 317335x + 123.79	y = 148089x + 3091
Correlation coefficient	0.9997	0.9996	0.9999	0.9993
Intraday precision (n=6)				
Mean Area value	6110351.8	90715.1	124039.7	61146.9
SD	29486.4	255.3	2130.3	560.10
%RSD	0.483	0.281	1.717	0.916
Interday precision (Day 1)				
Mean Area value (n=3)	6108716.5	90547.7	125665.6	61407.0
SD	41751.8	196.0	671.8	130.8
%RSD	0.683	0.216	0.535	0.213
Interday precision (Day 2)				
Mean Area value (n=3)	6115493.8	91139.9	125971.1	61652.2
SD	90082.6	209.8	769.2	224.8
%RSD	0.982	0.230	0.611	0.365
Ruggedness (Analyst 1)				
Mean Area value (n=3)	6137961.9	90945.0	125618.0	61431.6
SD	48132.8	629.3	858.6	290.4
%RSD	0.784	0.692	0.683	0.473
Ruggedness (Analyst 2)				
Mean Area value (n=3)	6098905.3	91407.1	125441.1	61420.3
SD	23301.7	224.0	23890.0	264.3
%RSD	0.382	0.245	1.905	0.430

Table 2
Recovery results of Bexagliflozin and its studied impurities

Parameter	Injected concentration (µg/mL)	Estimated concentration (µg/mL) mean±SD	% Recovery	RSD (%)
Bexagliflozin	24	23.50±0.281	99.85	0.218
	32	31.85±0.397	99.36	0.396
	40	39.05±0.193	98.39	0.574
Keto Impurity	0.24	0.238±0.003	100.01	1.250
	0.32	0.315±0.028	99.87	0.587
	0.40	0.397±0.036	99.63	0.336
Hydroxy Impurity	0.24	0.239±0.006	100.77	1.097
	0.32	0.317±0.009	98.65	1.139
	0.40	0.396±0.002	99.05	0.463
Bromo Impurity	0.24	0.239±0.006	100.14	0.851
	0.32	0.320±0.008	100.33	0.627
	0.40	0.399±0.001	99.58	0.351

Resulted fragment forms another product ion by the loss of $C_{13}H_{10}O_2$. Resulted product is m/z 147.14 with formula of $C_6H_{11}O_4$. Product ion of m/z 337.81 forms fragment ion by the loss of methane group (CH_4). Resulted fragment ion is $C_{17}H_{18}ClO_4$. This product ion loses $C_2H_4O_2$ and forms fragment ion of $C_{15}H_{14}ClO_2$ (m/z 261.72), this compound again loses oxirane or epoxyethane group to form $C_{13}H_{10}ClO$ (m/z 217.67). Already formed product ion of $C_{17}H_{18}ClO_4$ is due to loss hydroxytyrosol group ($C_8H_{10}O_3$) and forms another product ion of C_9H_8ClO m/z 167.61. This product ion loss ketene group i.e. C_2H_2O. Resulted fragment is m/z 125.57 (C_7H_6Cl). By all the degradation pathway, DP 3 is identified as *(2S,3R,4R,5S,6R)-2-(4-chloro-3-(4-(2-hydroxyethoxy)benzyl)phenyl)-6-(hydroxymethyl)tetrahydro -2H-pyran-3,4,5-triol*. Available successive fragments of DP 1 and DP 3 are given in figure 5 and mass spectra was given in figure 7.

Fragmentation pathway of DP 2: In the base degradation of Bexagliflozin, single DP is formed and it is named as DP 2 based on elution time; DP 2 was eluted to 2.823 min. In base degradation, loss of simple molecule of $C_6H_6O_4$ resulted to form DP2.

Molecular formula of DP 2 is $C_{18}H_{19}ClO_3$ with m/z 318.79 mass value. The fragmentation pathway of DP 2 forms three product ions by the loss of hydroxypropyl group (C_3H_5O), another one is by loss of phenylpentanoate group ($C_{11}H_{13}O_2$) and third fragment by the loss of C_2H_2 resulted in fragments with m/z of 261.72 ($C_{15}H_{14}ClO_2$), m/z 141.57 (C_7H_6ClO) and m/z 292.75 ($C_{16}H_{17}ClO_3$). First formed product ion was further formed another fragment was by the loss of two carbon atom and two hydrogen atom i.e. C_2H_2. Resulted fragment is with 235.68 m/z and with $C_{13}H_{12}ClO_2$ molecular formula. Resulted fragment is loss of water molecule (H_2O) and resulted to form $C_{13}H_{10}ClO$ with 217.67 m/z. This fragment ion loses C_6H_3Cl and forms fragment ion of C_7H_7O with m/z value of 107.12. Resulted fragment loses single oxygen atom and forms C_7H_7 (m/z 91.12). Already formed product ion of $C_{16}H_{17}ClO_3$ is by loss of chloromethyl group (CH_2Cl) and resulted to form $C_{15}H_{15}O_3$. m/z value is 243.27, this fragment again loses two carbon atoms, four hydrogens and single oxygen atom to form another product ion with mass of 199.22 with $C_{13}H_{11}O_2$ molecular formula. By observing all the fragmentation pathway, DP 2 is identified as 4-chloro-3-{4-[2-(cyclopropyloxy)ethoxy]benzyl} phenol. Fragmentation pathway of DP 2 is given in figure 6. Masses of the all DPs are given in figure 7.

Assessment of Green Analytical Chemistry: The current work was conducted with a focus on the green aspects of developing a method for evaluating Bexagliflozin and its impurities. Unlike previous methods that used hazardous solvents like acetonitrile and methanol, this study employed green solvents such as ethanol and water. These two solvents were exclusively chosen for preparing the mobile phase and solutions to minimize environmental impact. Additionally, 100 mm column was utilized that facilitates shorter runtime which reduces both solvent consumption as well as energy usage.

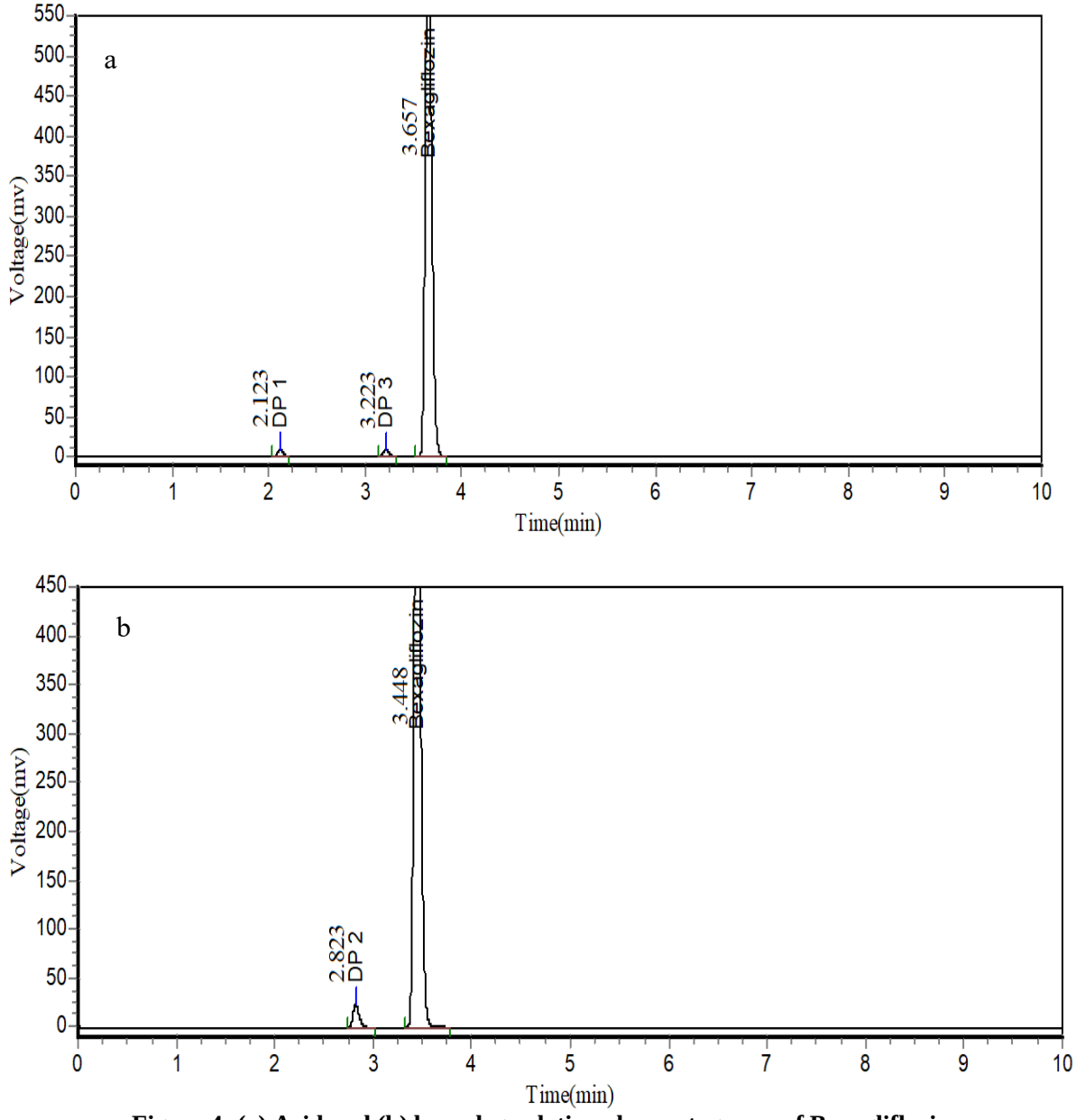


Figure 4: (a) Acid and (b) base degradation chromatograms of Bexagliflozin

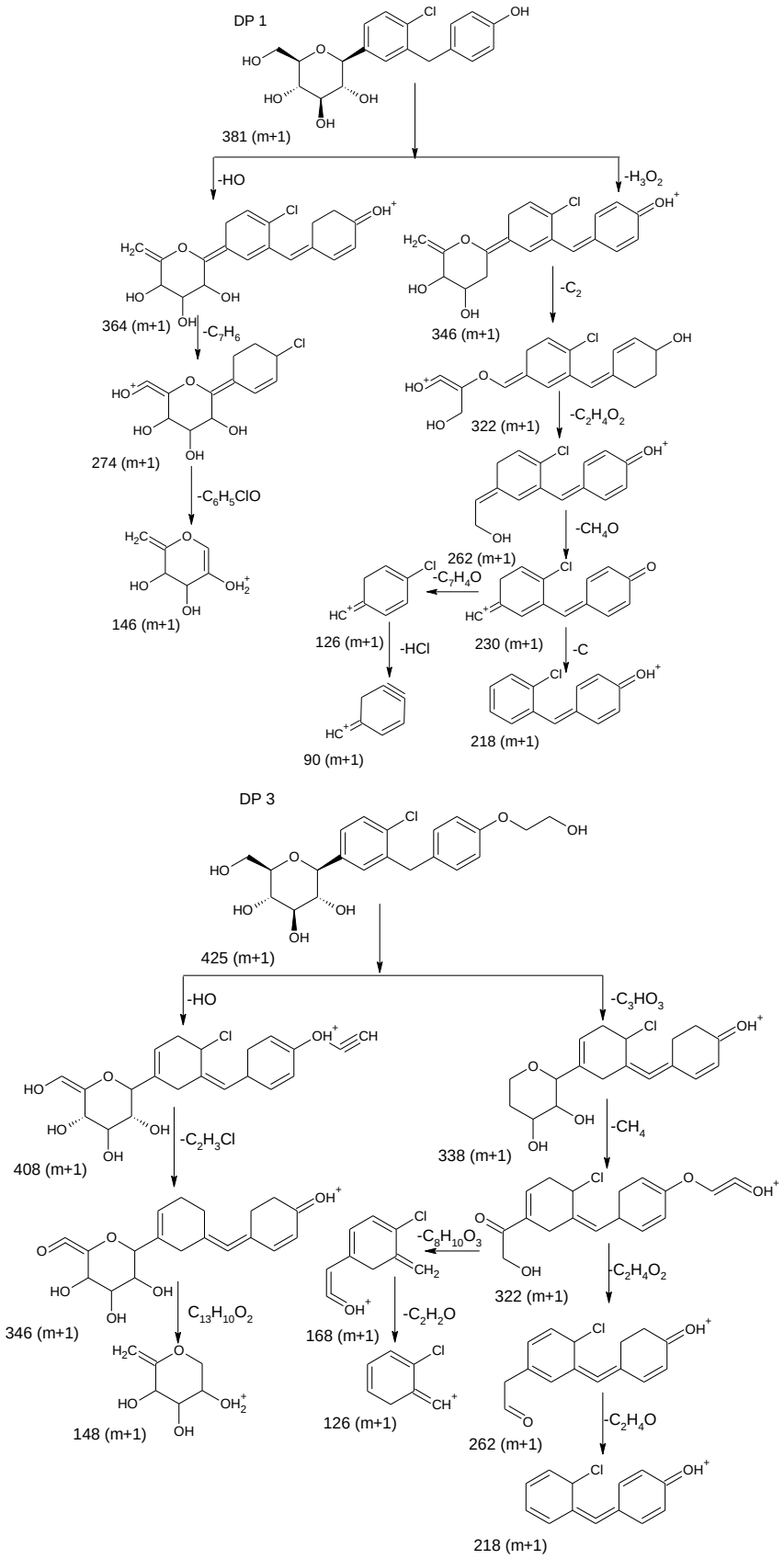


Figure 5: Fragmentation pathway of DP 1 and DP 3

The environmental assessment of the proposed analytical method was conducted through two green evaluation tools viz., AGREE and GAPI. AGREE software assessed 12 green analytical principles of the method with 0.1 to 1.0 scores scale. A total AGREE score of 0.79 (Figure 8A) indicates that this method was highly eco-friendly and had minimal

environmental impact. The GAPI tool also provided a detailed visual representation of the method's environmental impact. This tool produces pictograms and pentagrams that will be helpful for assessing the eco-friendliness of the proposed method. The analysis revealed very minor red pictograms and all others are green, yellow suggesting no major environmental issues in the proposed method (Figure 8B). The GAPI rating of 1.4E+00 produced confirmed that these methods have low environmental pressure. The yellow pictograms were noted corresponding to sample handling.

Conclusion

In this study, a precise and reliable chromatographic method was successfully developed and validated for the analysis of Bexagliflozin and its impurities. The separation was achieved using an X-bridge C18 column with a linear gradient elution system, demonstrates high specificity, selectivity and stability-indicating capability. The developed method effectively resolved all degradant peaks with resolution (Rs) values greater than 2, ensures adequate separation and quantification of analytes. The stress degradation study reveals that Bexagliflozin exhibited notable degradation under acidic and base conditions whereas it remained stable under oxidative, peroxide and thermal stress conditions. The DPs identified in acid and photolytic stress conditions were thoroughly characterized based on their elution order and fragmentation pathways.

The major acidic degradation products (DP 1 and DP 3) were formed due to chloro-substituted aromatic or polycyclic system and subsequent cleavage reactions, leading to the formation of stable intermediates. The base degradation product (DP 2) resulted from the loss of Isosorbide and other functional groups, forming distinct degradation compounds.

The proposed degradation pathways, supported by mass spectral analysis, provided comprehensive insights into the breakdown mechanisms of Bexagliflozin under stress conditions. The fragmentation studies further confirmed the structural integrity and transformation of degradation products, with mass spectrometric evidence highlighting key product ions and their successive fragmentation patterns. The observed degradation behavior and formation of stable degradation products suggest that Bexagliflozin's structural stability is influenced by specific stress conditions, emphasizes the necessity of controlled storage conditions to maintain drug integrity.

The green assessment tools such as GAPI and AGREE verified that this method significantly reduces the use of hazardous solvents with excellent chromatographic performance. This study contributes valuable information regarding the degradation behavior of Bexagliflozin, which can aid in formulation development, stability studies and regulatory submissions. Further research may explore additional stability parameters and the potential impact of DPs on pharmacological efficacy and safety.

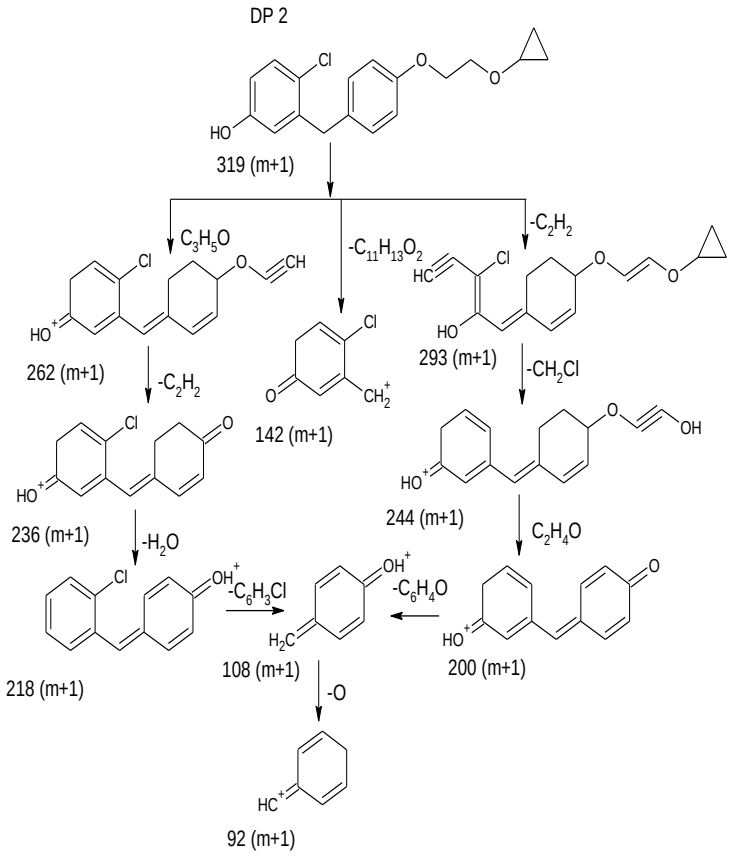


Figure 6: Fragmentation pathway of DP 2

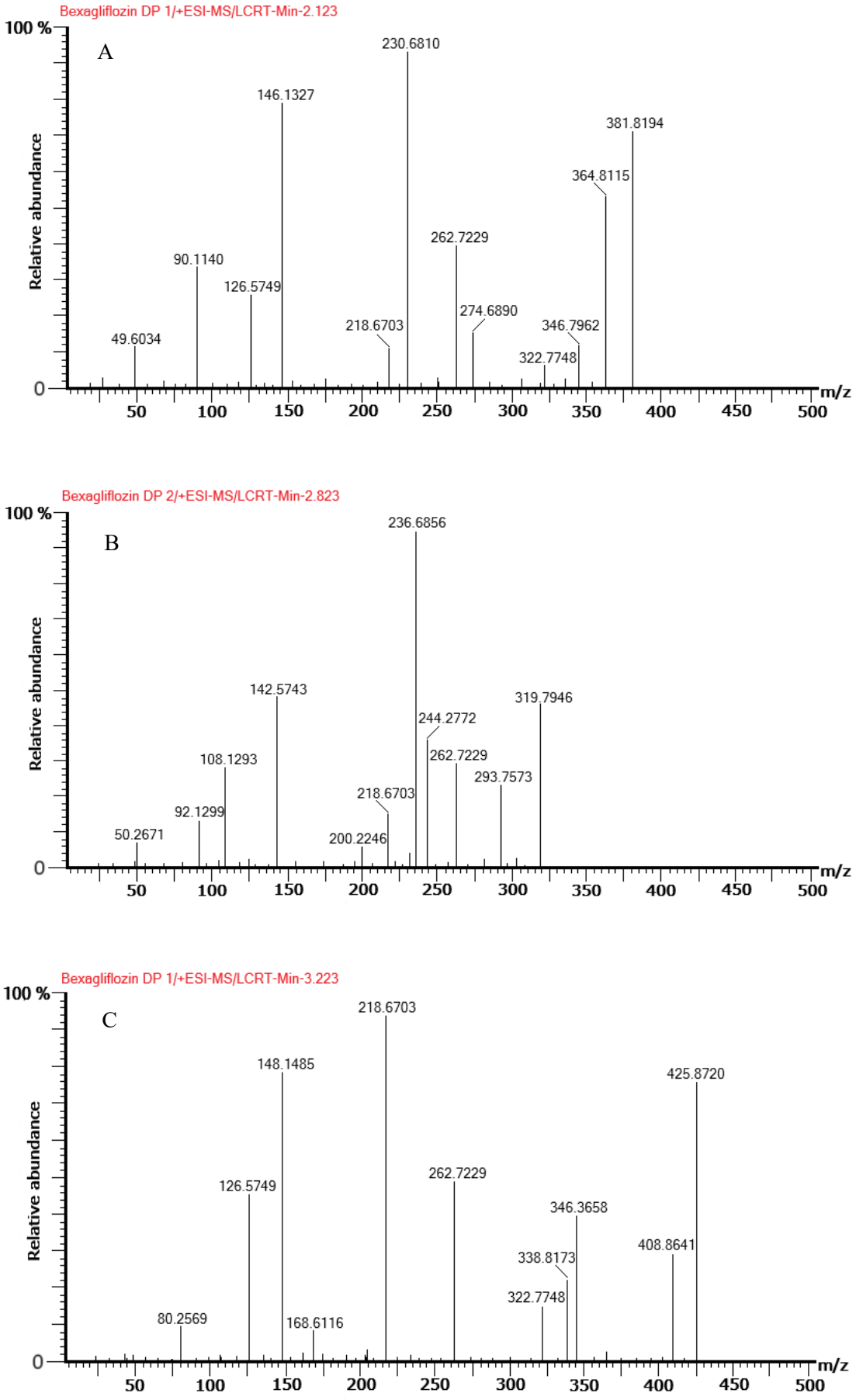
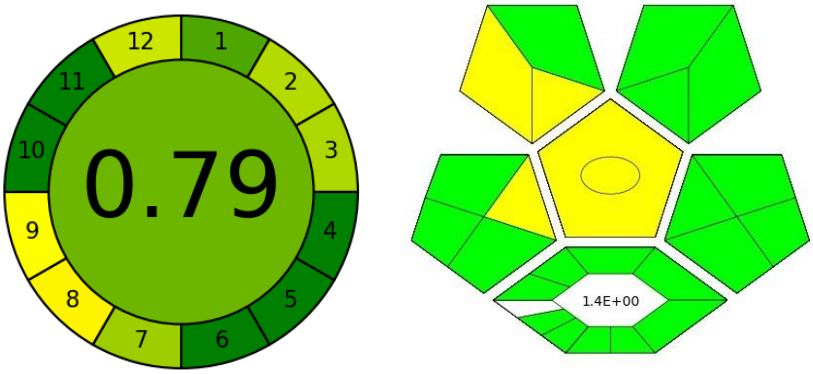


Figure 7: Mass spectrums of DP 1(A), DP 2 (B), DP 3 (C)



(A) AGREE **(B) GAPI**

Figure 8: Pictogram noticed in AGREE and GAPI green assessment tools

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