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# Unique Quality by Design Approach for Developing HPLC and LC-MS Method for Estimation of Process and Degradation Impurities in Pibrentasvir, Antiviral Agent for Hepatitis C

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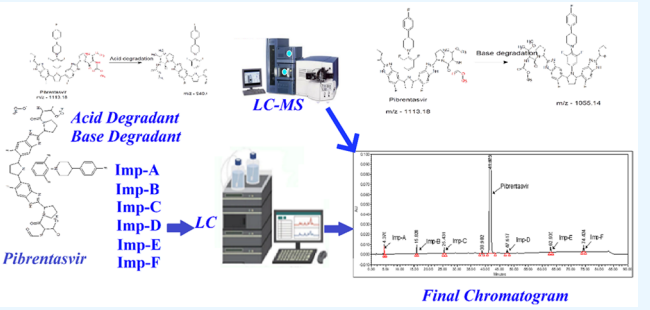
**ABSTRACT:** Pibrentasvir (PIB) was approved for treating hepatitis C patients. A specific, accurate, linear, robust, and stability-indicating method was developed and validated for determining degradation impurities present in the PIB drug substance by studying the quality by design (QbD) principles. All identified degradation impurities were separated with the stationary phase HALO C18, 150 mm × 4.6 mm, 2.7 μm. Mobile phase A contains pH 2.5 phosphate buffer and acetonitrile in the ratio of (70:30, v/v), and mobile phase B contains water and acetonitrile in the ratio of (30:70, v/v), respectively. The chromatographic conditions were optimized, such as flow rate of 0.8 mL/min, UV detection at 252 nm, injection volume of 20 μL, and column temperature of 40 °C. The proposed method was validated per the current ICH Q2 (R1) guidelines. The recovery study and linearity ranges were established from limit of quantification (LOQ) to 300% optimal concentrations. The method validation results were between 98.6% and 106.2% for recovery, and linearity $r^2$ was more than 0.999 for all identified impurities. The method precision results achieved below 5% relative standard deviation (RSD). The forced degradation results demonstrated that the drug was sensitive to chemical stress conditions. During the stress study, degrading impurities were identified by the LC-MS technique and the mechanism pathway. A QbD-based experimental design (DoE) approach was used to establish the robustness of the method.




## 1. INTRODUCTION

Hepatitis C is a viral infection that causes inflammation of the liver, leading to severe liver damage. The hepatitis C virus is spread through contaminated blood. Most diseases are caused by exposure to blood from unsafe injection methods, unsafe health care, uncontrolled blood transfusions, and sexual practices that lead to blood exposure. Worldwide, approximately 58 million people are infected with the hepatitis C virus. Currently, there is no effective vaccine against the hepatitis C virus. However, antiviral drugs can cure more than 95% of people infected with hepatitis C. Pibrentasvir (PIB) is a direct-acting antiviral and a star-targeted inhibitor of the hepatitis C virus NS5A, which is involved in viral RNA replication. PIB is available as an oral combination therapy under the brand name Mavyret. Mavyret is also indicated for the treatment of adult patients with HCV genotype 1 infection. Mavyret was approved by the USFDA on August 3, 2017. The PIB chemical is called methyl *N*-[(2*S*,3*R*)-1-[(2*S*)-2-[6-[(2*R*,5*R*)-1-[3,5-difluoro-4-[4-]4)fluorophenyl)piperidin-1-yl]-phenyl]-5-[6-fluorine-2-[(2*S*)-1-[(2*S*,3*R*)-3-methoxy-2-(methoxycarbonylamino)butanoyl]pyrrolidin-2-yl]-3*H*-benzimidazol-5-yl]pyrrolidin-2-yl]-5-fluoro-1*H*-benzimidazol-2-yl]-pyrrolidin-1-yl]-3-methoxy-1-oxobutan-carbamate 2-yl], its molecular formula is $C_{57}H_{65}F_5N_{10}O_8$, and its mass value is 1113.2 amu. Process development for the production of PIB is done by identifying possible impurities, such as impurities A, B, C, D, E, and F. Impurities A−F are those that can form during the production of pibrentasvir. The chemical structures of pibrentasvir and known impurities are shown in Figure 1.

A literature survey revealed that there is no stability-indicating method yet reported for the quantitative determination of PIB impurities. There is only one review article available for the impurities method, but there was a lack of research about stability indicating and degradants.[1] To the best

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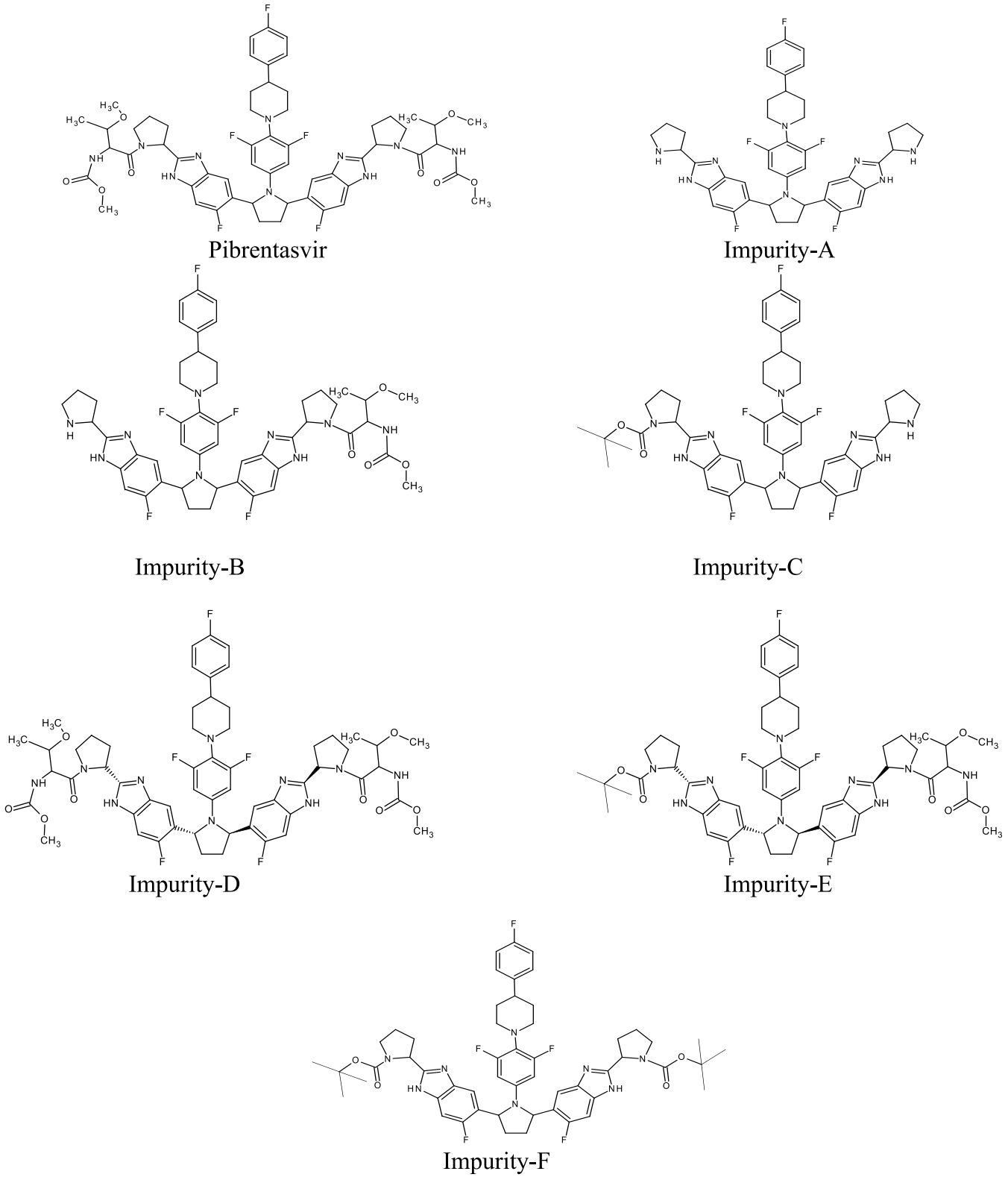


**Figure 1.** Chemical structures of PIB and impurities.

of our knowledge, a suitable analytical method for the determination of related substances and degradation products of PIB has not been reported yet. Several analytical methods were found in the literature for determination of pibrentasvir along with glecaprevir, like a stability indicating RP-HPLC method for estimation of glecaprevir and pibrentasvir in tablet dosage form[2−10] and a HPLC method for estimation of pibrentasvir and glecaprevir for in vitro dissolution studies,[11] but these methods are limited to determining the content of analytes. The literature methods did not discuss the impurities of PIB and did not prove the stability indicating nature. Therefore, it is necessary to develop a stability-indicating method to determine and estimate PIB and its related impurities. The mass of major degradation impurities was



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identified using the LC-MS technique. An analytical method was developed using the quality by design (QbD) approach. Method development for analytical procedure was performed using a systematic approach. The steps of the QbD process are (i) definition of the analytical target profile (ATP), (ii) selection of critical method attributes (CMAs), (iii) risk assessment, (iv) identification of critical method parameters (CMPs), (v) screening and optimization using design of experiments (DoE), (vi) definition of the method operable design region (MODR), and (vii) establishment of a method control strategy. The method robustness was tested using the design expert software version 13. Design expert software is a statistical tool to evaluate the critical quality attributes (CQAs) and study the interaction between the responses, like development and validation of liquid chromatography methods using a quality by design approach[12,13] and development of green chromatography methods using a quality by design approach.[14−16] The CQAs were identified, factorial design was performed, and the robustness of the method was evaluated. Although there are many previously published stability indicating methods for pibrentasvir (PIB), many with the drug glecaprevir, it does appear that the degradation products were not previously identified for pibrentasvir; hence, a reproducible, stability-indicating HPLC/LC-MS method was developed using the quality by design approach to determine pibrentasvir and its impurities.

## 2. RESULTS AND DISCUSSION

**2.1. Method Development and Optimization.** Before starting the development of the LC method for impurity estimation in PIB, important information about the compound was collected from the literature, such as p$K_a$ ≈ 10.66, solubility (in water <0.1 mg/mL), polarity (nonpolar), melting point (187−193 °C), and hygroscopicity (nonhygroscopic) of the molecule. Based on the nature of the compound, reverse phase chromatography was chosen. The mobile phase and the stationary phase play very important roles in the impurity separation process. The p$K_a$ value of pibrentasvir is 10.6, so pH 2.5 for the mobile phase has been optimized, where all process impurities and degraded compounds are well separated, and a stationary phase is suitable. Compound C18 was selected to remove impurities. In general, the polarity of a compound is an essential property in the stationary phase; based on the polarity of the selected compound, the appropriate stationary phase was C18. Based on the solubility of the compound, a mixture of methanol and water (90:10 v/v) as the diluent was selected. Based on the UV spectrum of the compound, impurities, and decaying impurities, the UV max was determined at a wavelength of 252 nm. All trials were reviewed, and the results are shown in Table 1.

According to the final experimental results and conclusions, an appropriate method was selected for validation. In method validation studies, forced attenuation experiments were established by LC with a PDA detector. Impurities formed in forced degradation studies were determined by the LC-MS technique. The proposed method has been validated against current ICH guidelines. Therefore, based on validation results and attenuation studies, this LC method is considered a stability indicator. This method has been used to determine known impurities and degradation in PIB drug substances.

*2.1.1. Analytical Method Development.* The development and control of analytical methods across the product life cycle is a key element of the overall product control strategy. The method developed must be robust because it is intended to be used by analysts in a wide range of laboratories. Hence risk management principles were utilized for the evaluation of analytical methods. Quality risk management tools provide a framework for identifying, studying, and understanding the risks to method performance and the results generated by the method.

**Table 1. Failure Mode Effect Analysis (FMEA) and Risk Analysis of the Analytical Method**

| Failure Mode Effect Analysis | | | | |
|---|---|---|---|---|
| s.no. | variable | probability of variation (1 low, 5 high) | severity (1 low, 5 high) | risk score |
| 1 | organic ratio in mobile phase | 1 | 1 | 1 |
| 2 | pH of mobile phase | 2 | 2 | 4 |
| 3 | flow rate | 1 | 2 | 2 |
| 4 | column oven temperature | 3 | 2 | 6 |
| Risk Analysis of the Analytical Method | | | | |
| s.no. | variable | USP resolution[a] | USP tailing[b] | |
| 1 | high temperature (44 °C) | 4.69 | 1.10 | |
| 2 | low temperature (36 °C) | 4.64 | 1.01 | |
| 3 | low flow (0.7 mL/min) | 4.67 | 1.05 | |
| 4 | high flow (0.9 mL/min) | 4.62 | 1.05 | |
| 5 | existing method (40 °C, 0.8 mL/min) | 4.88 | 1.11 | |

[a]Between pibrentasvir and nearby peak. [b]Pibrentasvir peak.

*2.1.1.1. Risk Analysis.* Risk analysis involves estimating the risk associated with each of the variables identified above. It considers both the probability of occurrence and the impact on the reported results (severity). A commonly used tool for performing a quantitative analysis is failure mode effect analysis (FMEA) (Table 1). Experiments were performed based on FMEA analysis (Figure 2) to evaluate the effect on United States Pharmacopeia (USP) tailing, and resolution results showed that the variables have a negligible effect on the resolution of impurities.

*2.1.1.2. Selection of Critical Method Attributes.* From the risk analysis, it is clear that the PIB Imp-D, Imp-A, near peak, and PIB, impurity at RRT ≈ 0.93 peaks elute closely, and the three dissociations are sensitive and considered critical quality attributes (AQC). The study showed that the flow rate, buffer pH, and column temperature were considered as CMPs (critical method parameters). Method development begins using a sample of raw PIB from the aggregation process. Drug impurity profiling is the most important issue in modern pharmaceutical analysis for the development of high-purity drug production technology. Drug impurity profiling, identification, structural elucidation, and quantitative determination of impurities and degradation products in bulk drugs is one of the most important business areas of pharmaceutical analysis. The main objective of chromatography is to separate the drug substance from all impurities and degradation products involved in the process. At the beginning of method development, it is important to know various physicochemical parameters such as p$K_a$, log $P$, solubility, absorbance, and peak wavelength of the drug. Knowledge of p$K_a$ is important because most changes related to pH retention occur at pH values within ±1.5 units of the p$K_a$ value. Knowing the log $P$ of the drug and the breakdown products identified helps to better


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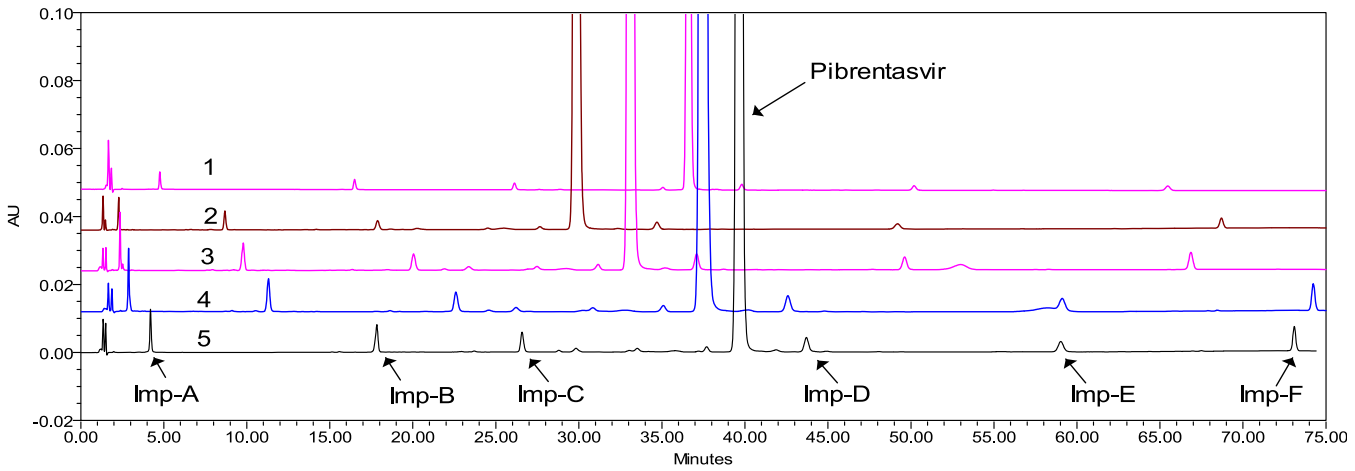


**Figure 2.** Failure Mode Effect Analysis overlaid chromatogram. (1) Finalized method, (2) high flow (0.9 mL/min), (3) high temperature (44 °C), (4) low temperature (36 °C), and (5) low flow (0.7 mL/min).

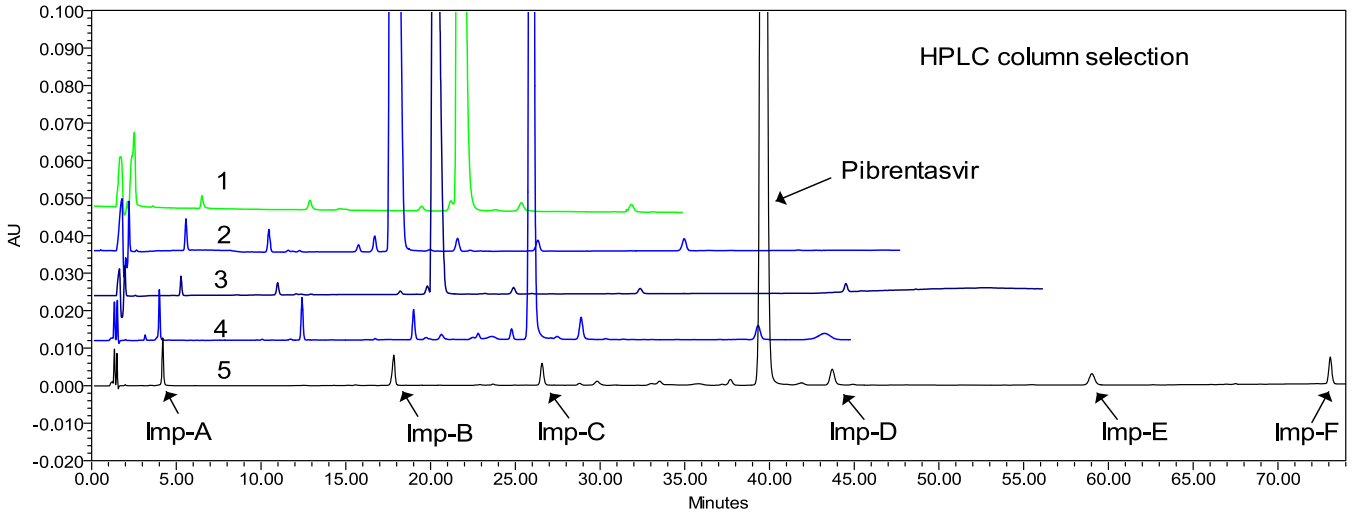


**Figure 3.** Affinity of all impurities toward different commercially available columns. (1) Sunfire C18, (2) X-Select Phenyl Hexyl, (3) X-Select CSH C18, (4) Zorbax SB C18, and (5) HALO C18.

understand the possible separation behavior on a particular stationary phase.

The ionization value ($pK_a$) helps to select the pH of the buffer used in the mobile phase. The $pK_a$ of PIB is 10.6. The effect of pH on selectivity was investigated, and it was found that a pH of 2.5 gave promising results compared to other pH. The affinity of all impurities for different commercial columns was also evaluated to obtain the best resolution. Different stationary phases have been studied such as Sunfire C18 and X-Select phenyl hexyl. Forced decomposition impurities were observed to elute close to the known impurities. Finally, the core−shell column HALO C18 at pH 2.5 was selected for good impurity separation (refer to Figure 3). Compared with other commercially available columns, this method exhibited high reproducibility and is suitable for compounds of varying polarity.

*2.1.1.3. Screening and Optimization Using Design of Experiments (DoE).* The −1 and +1 values for all three CMPs were considered for DoE experiments. Resolution between peaks PIB and impurity D, impurity-A and nearby peak, and PIB and impurity at RRT ≈ 0.93 was monitored as responses for DoE; they are denoted by R1, R2, and R3, respectively. The DoE matrix and responses are listed in Table 2.

Figure 4 provides the contour plot, 3D surface plot and 3D cube plot of CMPs on the responses R1, R2, and R3.

Pareto rank analysis showed that the effects of factors flow rate, buffer pH, and column temperature on CQAs were statistically significant ($P < 0.05$), and therefore these factors indicated were the CMPs for other optimization studies. The seminormal and Pareto plots show that column temperature has a significant effect on R2. Decreasing the column temperature increases the elution of the component and subsequently coelutes the peaks, thereby reducing the resolution. Column flow and temperature have a positive resonance effect on R2 maximization. The increased linear velocity and increased diffusion lead to narrow peaks with increased resolution. The results of the model analysis are presented in Table 3.

The $F$ value of the model implies that the model is significant. $P$ value < 0.0500 indicates that the terms of the model are significant. Unadjusted $F$-value implies that it is insignificant compared to the pure error. In addition, the feedback surface mapping was performed using the 3D feedback surface and 2D contour plots for each CQA (responses). The 2D plot for reaction 1 (R1) shows that reducing the column temperature and flow rate maximizes the



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Table 2. DoE Data

| std | run | flow rate | pH | column temperature | R1[a] | R2[b] | R3[c] |
|---|---|---|---|---|---|---|---|
| 18 | 1 | 0.8 | 2.5 | 40 | 6.2 | 2.2 | 4.1 |
| 10 | 2 | 0.7 | 2.3 | 45 | 5.8 | 1.9 | 4.5 |
| 9 | 3 | 0.7 | 2.3 | 45 | 5.5 | 1.8 | 4.6 |
| 2 | 4 | 0.7 | 2.3 | 35 | 5.1 | 1.7 | 4.0 |
| 1 | 5 | 0.7 | 2.3 | 35 | 5.0 | 1.8 | 4.1 |
| 13 | 6 | 0.7 | 2.7 | 45 | 6.5 | 2.5 | 3.5 |
| 6 | 7 | 0.7 | 2.7 | 35 | 6.0 | 2.5 | 3.2 |
| 3 | 8 | 0.9 | 2.3 | 35 | 4.8 | 1.5 | 4.2 |
| 4 | 9 | 0.9 | 2.3 | 35 | 4.6 | 1.5 | 4.1 |
| 14 | 10 | 0.7 | 2.7 | 45 | 6.1 | 2.6 | 3.7 |
| 12 | 11 | 0.9 | 2.3 | 45 | 6.2 | 1.6 | 4.0 |
| 8 | 12 | 0.9 | 2.7 | 35 | 5.7 | 2.1 | 3.2 |
| 17 | 13 | 0.8 | 2.5 | 40 | 6.3 | 2.0 | 4.2 |
| 19 | 14 | 0.8 | 2.5 | 40 | 6.0 | 2.3 | 4.0 |
| 7 | 15 | 0.9 | 2.7 | 35 | 6.3 | 2.3 | 3.5 |
| 15 | 16 | 0.9 | 2.7 | 45 | 6.0 | 2.2 | 3.4 |
| 5 | 17 | 0.7 | 2.7 | 35 | 6.5 | 2.7 | 3.6 |
| 16 | 18 | 0.9 | 2.7 | 45 | 5.8 | 2.5 | 3.3 |
| 11 | 19 | 0.9 | 2.3 | 45 | 5.2 | 1.8 | 4.0 |

[a]Resolution between pibrentasvir and imp-D. [b]Resolution between impurity A and nearby peak. [c]Resolution between pibrentasvir and impurity at RRT ≈ 0.93.

maximum resolution R1 and R2 (Figure 4). In contrast, an increase in flow rate and a decrease in column temperature and organic matter concentration increased the R3 peak resolution (Figure 5).

The desirability plot shows that the flow rate and column temperature should be kept at the minimum to obtain a peak resolution within the desired range.

The optimum conditions for the method were 0.8 mL/min flow rate, 40 °C column temperature, and pH 2.5 for the mobile phase buffer. These conditions were further considered for validation.

**2.2. Analytical Method Validation.** The described method has been validated against current ICH Q2 (R1) guidelines. The validation results have been tabulated in Table 4. Important analytical parameters, such as specificity, precision, linearity, and limits of detection and quantification (LOD and LOQ, respectively), as well as certainty, of the method were evaluated.[17−20]

*2.2.1. Specificity. 2.2.1.1. Interference Test.* The specificity parameter plays an important role in method refinement, identifying disturbances at known retention times and impurity degradation. Verification samples such as blanks, test samples, and any sample solutions with impurities were included in the current LC method. Chromatographic data showed no interference from retention times of analytes and known impurities. PIB and known impurity peaks were said to be spectrally pure.

*2.2.1.2. Forced Degradation Studies.* This study plays a significant role in identifying the stability-indicating method and could identify the molecular behavior in the different stress reagents. Any LC method must be subjected to this study before submission to US-FDA, EU, and other regulatory authorities. The forced degradation study confirms the method stability-indicating in nature. Before starting the experiment, we collected molecule solubility, polarity information, and some stress reagent concentrations and conditions from regulatory guidelines.

*2.2.1.2.1. Acid Hydrolysis Degradation Sample.* About 60 mg of PIB sample was transferred into a 200 mL volumetric flask, about 20 mL of 2 N HCl solution was added, and the mixture was kept on a benchtop for about 48 h. After exposure for 48 h in acid, the solution was made up to volume with diluent, and the solutions were filtered before injection. In acid hydrolysis, one major impurity was observed at a retention time of about 15.7 min. The impurity formed in acid hydrolysis was identified by using the LC-MS technique. The impurity formed in acid hydrolysis was found to have $m/z$ 940.15. The results are shown in Figure 6.

*2.2.1.2.2. Base Hydrolysis Degradation Sample.* About 60 mg of PIB sample was transferred into a 200 mL volumetric flask, about 20 mL of 5 N NaOH solution was added, and the mixture was kept on a benchtop for about 48 h. After exposure for 48 h in base, the solution was made up to volume with diluent, and the solutions were filtered before injection. In base hydrolysis, two major degradation impurities were observed at about 3.8 and 14.9 min retention times. Impurities formed in base hydrolysis were identified by using the LC-MS technique. The major impurity formed in base hydrolysis has a $m/z$ value of 1055.37, which corresponds to impurity A. The results are shown in Figure 7.

*2.2.1.2.3. Peroxide Degradation Sample.* About 60 mg of PIB sample was transferred into a 200 mL volumetric flask, about 20 mL of 10% $H_2O_2$ solution was added, and the mixture was kept on a benchtop for about 48 h. After exposure for 48 h in peroxide, the solution was made up to volume with diluent, and solutions were filtered before injection.

Based on the degradation in solution state results, it was concluded that PIB was sensitive to acid, base, and peroxide stress conditions. In peroxide degradation, the major degradation impurity was observed at about 9.7 min retention time. Impurities formed in peroxide degradation were identified by using the LC-MS technique. The major impurity formed in peroxide degradation has a $m/z$ value of 1112.47. The results are shown in Figure 8.

*2.2.1.2.4. Solid State Degradation Studies.* Solid state degradation includes stress studies involving heat, humidity, and light. Prior to the experimental tests, the samples were kept under the respective storage conditions. In the light-stress studies, PIB was kept in a stable optical chamber and exposed to 200 W $m^2$ of UV light, 1.2 million lux hours of fluorescent light in wet conditions (85% RH) for 7 days, and heat (105 °C) for 10 days. After a period of time, samples were taken, and digested solutions were prepared and immediately injected into the LC. In photodegradation, the major degradation impurity was observed at about 60.4 min retention time. The impurity formed in photodegradation was identified by using the LC-MS technique. The major impurity formed in photodegradation has a $m/z$ value of 1128.17. The figure results are shown in Figure 9. No major degradation impurities were observed in other degradation sample chromatograms. Based on the results, the compound is stable in heat and humidity conditions. All the forced degradation results and compared chromatograms are shown in Table 5 and Figure 10.

*2.2.2. Precision.* The precision of the present method has been established through its repeatability and ruggedness. Repeatability was checked by injecting six freshly prepared drug solutions containing 0.3 μg/mL of each known impurity on the same day, and their recovery was observed. Stability was checked by injecting six freshly prepared sample solutions with the same concentration of each known impurity on different


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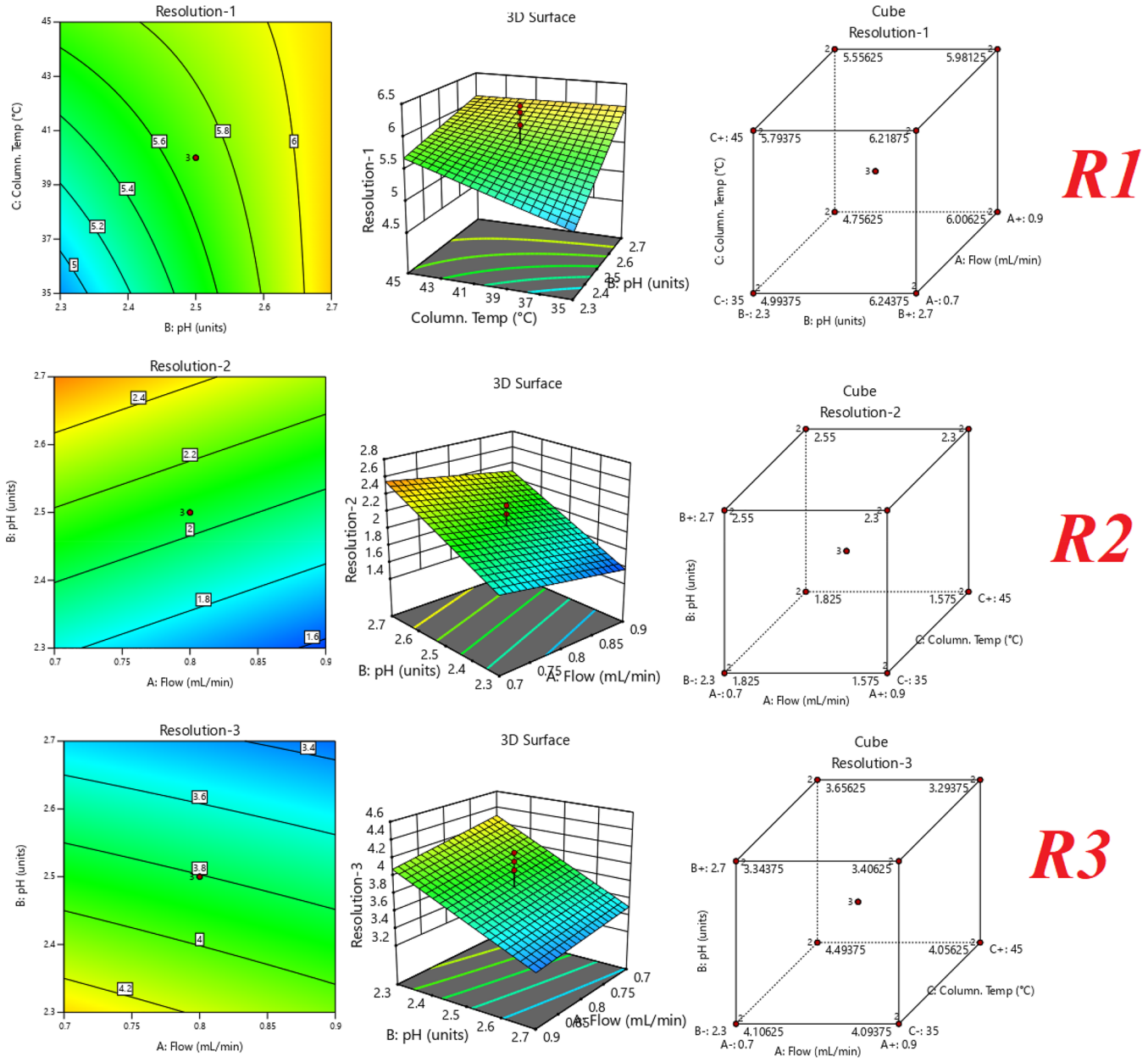


**Figure 4.** Contour plot, 3D surface plot, and 3D cube plot for R1, R2, and R3.

days and using different LC instruments with different batch columns. The overall % relative standard deviation (RSD) value for each impurity is less than <2%. This confirms that the precision of the developed test method meets the criteria and is well adapted to different laboratory conditions.

*2.2.3. Accuracy.* The accuracy of this method was assessed by marking known impurities at LOQ, 100%, and 150% of the specified concentration, 0.1, 1.5, and 2.0 $\mu$g/mL, with test concentrations, respectively. Each spike pattern determination was performed in triplicate. Contaminant recovery at three levels (LOQ, 100%, and 150%) was 98.2% to 105.1% with RSD < 3.0%, suitable for the required range from 80.0% to 120.0% and the % RSD limit of 10.0%.

*2.2.4. Linearity.* Linear studies have established the capacity of the low to high range. Thus, all known impurities (impurities A, B, C, D, E, and F) were injected at concentrations varying between LOQ (0.1 $\mu$g/mL) and 300% (4 $\mu$g/mL, 5 $\mu$g/mL) of specifications. A calibration curve was obtained by plotting peak area versus concentration of all impurities at 0.1, 0.5, 0.8, 1.2, 1.5, 3.0, and 4.5 $\mu$g/mL. The slope, intercept, and correlation values were obtained by least-squares linear regression analysis.

*2.2.5. Limit of Detection and Limit of Quantitation.* This study helps us to determine the sensitivity of the analytical method. The LOD and LOQ were calculated using the signal-to-noise ratio method. The LOQ and LOD solutions were prepared from the standard impurity stock solutions. In the study, a series of dilute solutions of all impurities were prepared to obtain the LOQ, and the signal-to-noise ratio was 12 for impurity A, 16 for impurity B, 18 for impurity C, 15 for impurity D, 13 for impurity E, and 16 for impurity F. LOD was calculated using the formula LOD = LOQ/3.3, and the LOQ solution was diluted three times, and the LOD solution was injected into the LC. The experimental and theoretical LOD values appear to be comparable and meet the criteria. The



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Table 3. ANOVA Results

| source | sum of squares | df | mean square | *F*-value | *p*-value | |
|---|---|---|---|---|---|---|
| **model** | 4.31 | 4 | 1.08 | 12.26 | 0.0002 | significant |
| flow | 0.2256 | 1 | 0.2256 | 2.56 | 0.1333 | |
| pH | 2.81 | 1 | 2.81 | 31.89 | <0.0001 | |
| column temp | 0.6006 | 1 | 0.6006 | 6.83 | 0.0215 | |
| BC | 0.6806 | 1 | 0.6806 | 7.74 | 0.0156 | |
| curvature | 0.565 | 1 | 0.565 | 6.42 | 0.0249 | |
| **residual** | 1.14 | 13 | 0.088 | | | |
| lack of fit | 0.1219 | 3 | 0.0406 | 0.3976 | 0.7577 | not significant |
| pure error | 1.02 | 10 | 0.1022 | | | |
| **cor total** | 6.02 | 18 | | | | |
| **model** | 2.35 | 2 | 1.18 | 70.11 | <0.0001 | significant |
| flow | 0.25 | 1 | 0.25 | 14.9 | 0.0015 | |
| pH | 2.1 | 1 | 2.1 | 125.31 | <0.0001 | |
| curvature | 0.0274 | 1 | 0.0274 | 1.63 | 0.2206 | |
| **residual** | 0.2517 | 15 | 0.0168 | | | |
| lack of fit | 0.085 | 5 | 0.017 | 1.02 | 0.4553 | not significant |
| pure error | 0.1667 | 10 | 0.0167 | | | |
| **cor total** | 2.63 | 18 | | | | |
| **model** | 2.73 | 6 | 0.4556 | 21.27 | <0.0001 | significant |
| flow | 0.1406 | 1 | 0.1406 | 6.56 | 0.0264 | |
| pH | 2.33 | 1 | 2.33 | 108.57 | <0.0001 | |
| column temp | 0.0756 | 1 | 0.0756 | 3.53 | 0.087 | |
| AB | 0.0056 | 1 | 0.0056 | 0.2626 | 0.6185 | |
| AC | 0.1806 | 1 | 0.1806 | 8.43 | 0.0143 | |
| BC | 0.0056 | 1 | 0.0056 | 0.2626 | 0.6185 | |
| curvature | 0.218 | 1 | 0.218 | 10.18 | 0.0086 | |
| **residual** | 0.2356 | 11 | 0.0214 | | | |
| lack of fit | 0.0506 | 1 | 0.0506 | 2.74 | 0.1291 | not significant |
| pure error | 0.185 | 10 | 0.0185 | | | |
| **cor total** | 3.19 | 18 | | | | |

LOQ and LOD for all known impurities were 0.12 and 0.02 $\mu$g/mL, respectively.

*2.2.6. Solution Stability.* This study helps to understand the stability of cooled standard and sample solutions under refrigerated conditions and at room temperature. Stability study was evaluated by injecting standard and enriched sample solutions at 2 °C, 8 °C, and room temperature (25 °C). The results show that the solution is stable for 4 days under refrigerated and room temperature conditions.

*2.2.7. Robustness.* The optimized test method was examined by statical tool design expert software. QbD based DoE design was planned. Critical method parameters (CMPs) were identified based on the development. The selected CMPs were flow rate (0.8 ± 0.1 mL/min), pH (2.5 ± 0.2 units), and column temperature (40 ± 5 °C). The parameters were changed per the guidelines, and the three factorial design with 3 center points, zero blocks, and two replicates was designed. A total of 19 runs of spiked samples were injected in the LC instrument, and three responses were recorded. The DoE data is recorded in Table 2. All responses were analyzed by considering the major effects. The ANOVA data is shown in Table 3. In the ANOVA table lack fit was found not significant for three responses, and the model was found significant for all three responses, *P* value was obtained below 0.05. Column temperature and pH show clear impact on R1, resolution between PIB and impurity D. Flow and pH show clear impacts on R2 (resolution between impurity A and nearby peak) and R3 (resolution between PIB any impurity at RRT ≈ 0.93). All of the parameters mentioned above changes did not show any significant differences between identified impurities.

## 3. CONCLUSIONS

A highly sensitive, accurate, linear, specific, and robust analytical method has been developed and validated for the determination of PIB degradation and the determination of impurities in tablet dosage forms using the stability indicator method. The LC-MS technique determines the mass values of degraded impurities. The proposed method has been validated against current ICH guidelines. During the forced decomposition study, we identified three impurities that are not known to degrade under acidic, basic, and oxidizing conditions. Therefore, it is sensitive to chemical stress conditions. No significant degradation was observed in the solid-state degradation study. The molecule is stable under conditions of heat, humidity, and photodegradation. This method can estimate the impurity of PIB in the drug substance based on the validation results. Therefore, this method can be used to assess product quality. The current method is cost-effective and simple to handle for quality control analysis.

## 4. EXPERIMENTAL SECTION

**4.1. Chemicals and Reagents.** The PIB standard (99.9% purity) and its related known impurities (Impurities A, B, C, D, E, and F) were provided by Natco pharma ltd (Pharmaceutical company), Hyderabad, Telangana, India. A PIB sample was supplied by GITAM University, Hyderabad, Telangana, India. For this research, we selected a more compatible buffer,



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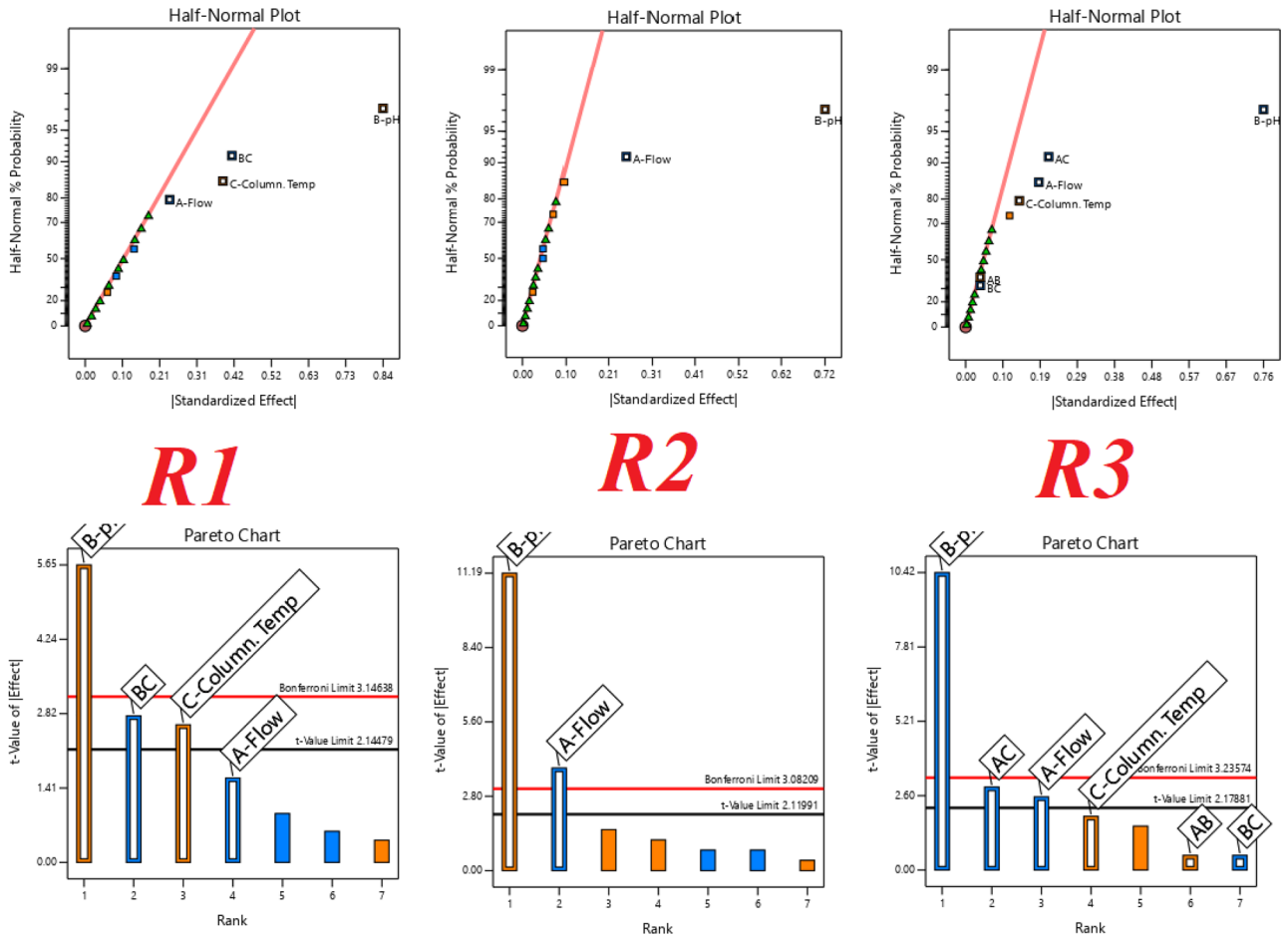


**Figure 5.** Pareto charts and half normal plots for R1, R2, and R3.

**Table 4. Analytical Method Validation Data**

| s.no. | parameter | imp-A | imp-B | imp-C | imp-D | imp-E | imp-F |
|---|---|---|---|---|---|---|---|
| 1 | specificity (peak purity) | pass | pass | pass | pass | pass | pass |
| Limit of Detection and Quantitation | | | | | | | |
| 2 | LOD ($\mu$g/mL) | 0.02 | 0.02 | 0.04 | 0.04 | 0.04 | 0.02 |
| 3 | LOQ ($\mu$g/mL) | 0.06 | 0.06 | 0.12 | 0.12 | 0.12 | 0.06 |
| Linearity | | | | | | | |
| 4 | slope | 58458 | 42047 | 39910 | 41730 | 38386 | 48016 |
| 5 | intercept | −4171.6 | −1175.3 | −1245.7 | −1291.0 | −555.8 | −857.1 |
| 6 | correlation coefficient | 0.9998 | 0.9999 | 0.9999 | 1.0000 | 0.9999 | 0.9999 |
| 7 | regression coefficient | 0.9985 | 0.9998 | 0.9998 | 0.9999 | 0.9999 | 0.9999 |
| 8 | correction factors | 0.70 | 0.98 | 1.03 | 0.98 | 1.07 | 0.85 |
| Precision | | | | | | | |
| 9 | precision (% RSD) | 1.99 | 0.62 | 1.28 | 0.58 | 0.48 | 0.26 |
| 10 | intermediate precision (% RSD) | 4.58 | 0.83 | 0.55 | 0.43 | 0.32 | 0.38 |
| Accuracy (Recovery) | | | | | | | |
| 11 | at LOQ | 108.0 | 103.6 | 99.1 | 114.8 | 76.4 | 104.9 |
| 12 | at 100% | 114.5 | 104.2 | 97.3 | 100.7 | 96.0 | 101.0 |
| 13 | at 150% | 110.7 | 103.0 | 96.5 | 100.3 | 95,9 | 100.47 |
| Solution stability | | | | | | | |
| 14 | day −2 at BT % difference < 10.0 | 0.028 | 0.002 | 0.001 | 0.001 | 0.002 | 0.002 |
| 15 | day −4 at BT % difference < 10.0 | 0.017 | 0.001 | 0.049 | 0.017 | 0.030 | 0.002 |
| 16 | day −2 at 2−8 °C % difference < 10.0 | 0.033 | 0.025 | 0.048 | 0.020 | 0.005 | 0.005 |
| 17 | day −4 at 2−8 °C % difference < 10.0 | 0.128 | 0.003 | 0.006 | 0.004 | 0.025 | 0.008 |

potassium dihydrogen phosphate, purchased from Merck KGaA, Frankfurter Str. 250, 64293 Darmstad, Germany. Orthophosphoric acid and acetonitrile (HPLC grade) were purchased from JT Baker (Avantor performance materials India limited, Gurgaon -122002, Haryana, India), and water was Milli-Q grade. The PIB sample was exposed to different



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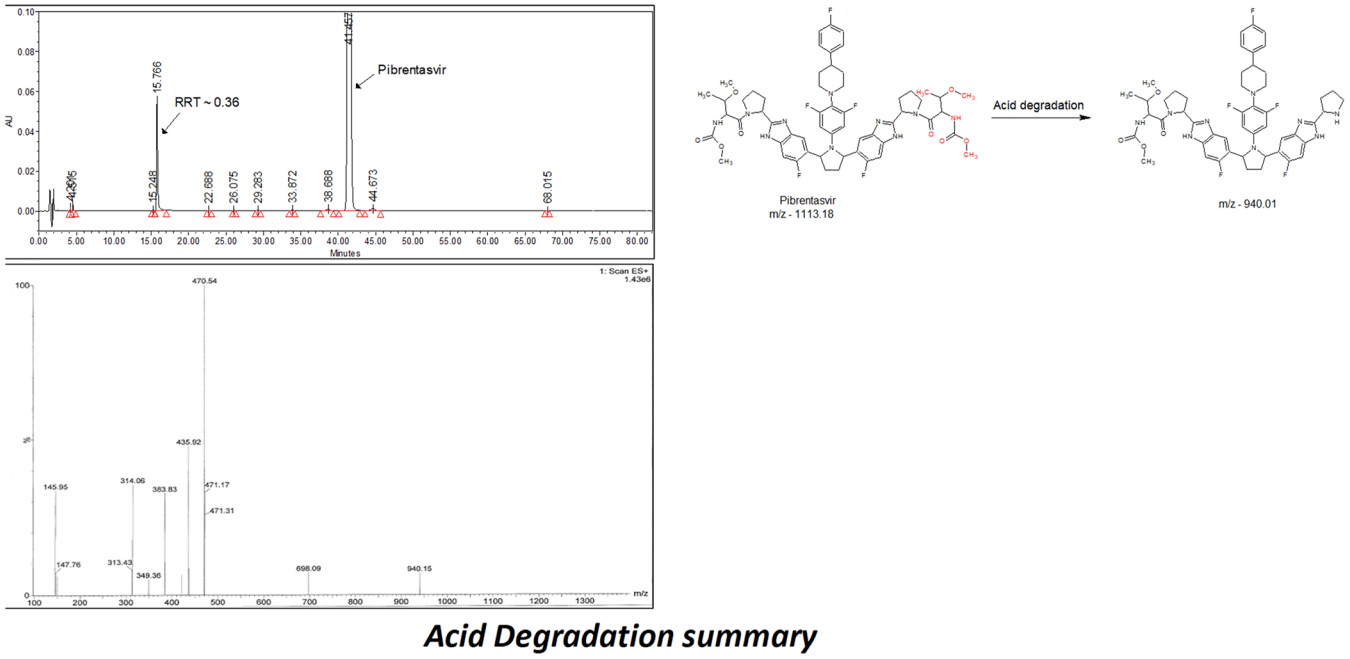


**Figure 6.** Acid forced degradation LC chromatogram, LC-MS chromatogram, and unknown identification and fragmentation.

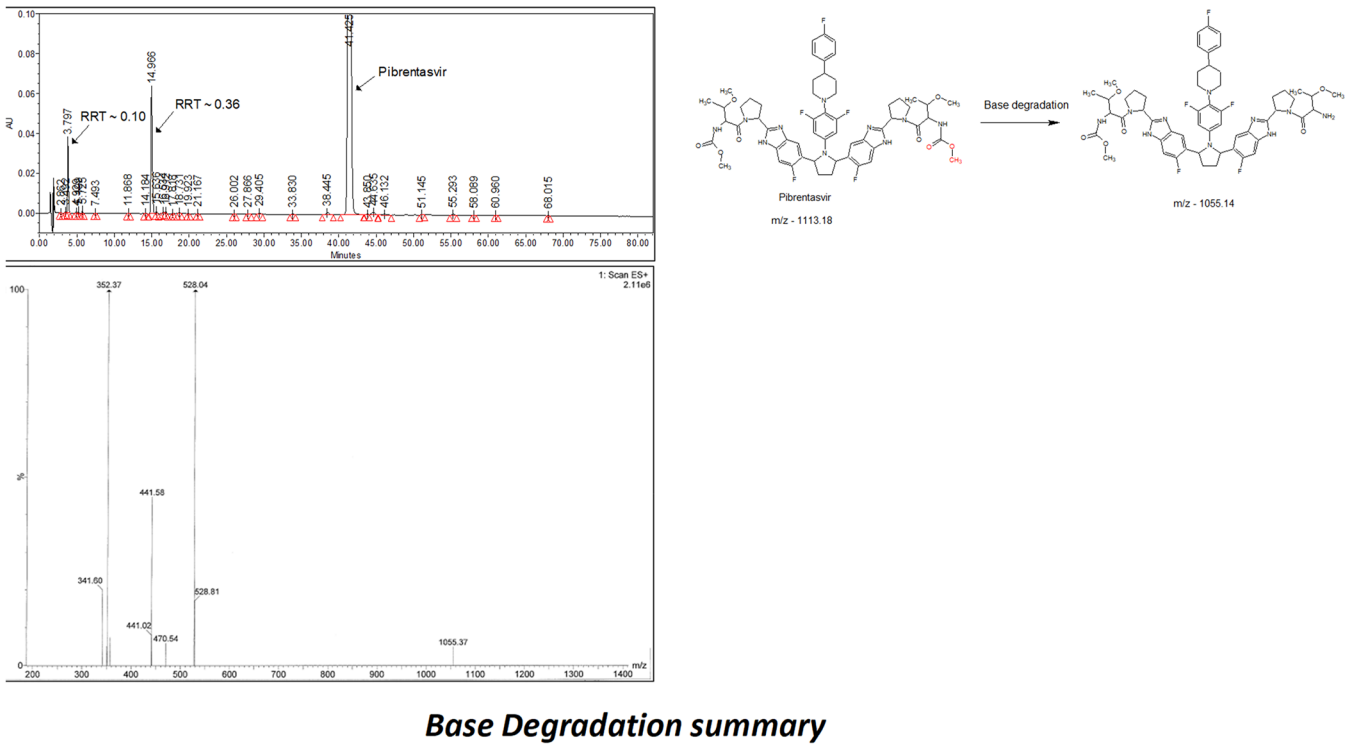


**Figure 7.** Base forced degradation LC chromatogram, LC-MS chromatogram, and unknown identification and fragmentation.

stress reagents such as acid, base, and peroxide solution, and forced degradation study was performed. For these studies, we purchased concentrated hydrochloric acid solution, sodium hydroxide pellets, and peroxide solution (AR grade) from Rankem chemicals, Mumbai, Maharashtra, India.

**4.2. Instruments, Column, and Software.** A quaternary pump and photodiode array detector (PDA) were used to develop and analyze liquid chromatographic methods (Waters Alliance e2695 HPLC System, 34 Maple St, Milford, MA 01757, USA). Waters Corporations USA's Empower 3 software was used to collect and process the data. Weighing was conducted on an XP4002S precision balance, an XP205 Delta Range analytical balance, an AX205 Delta Range analytical balance, or an MX5 microbalance (Mettler Toledo, Columbus, OH, USA). Mettler Toledo Columbus, OH, USA, Seven Multi pH meter was used to measure pH. A Branson 8510 ultrasonic bath (Emerson Electric, St. Louis, MO, USA) was used. The photostability of the material was measured using the Suntest XLS+xenon test instrument (Atlas Material



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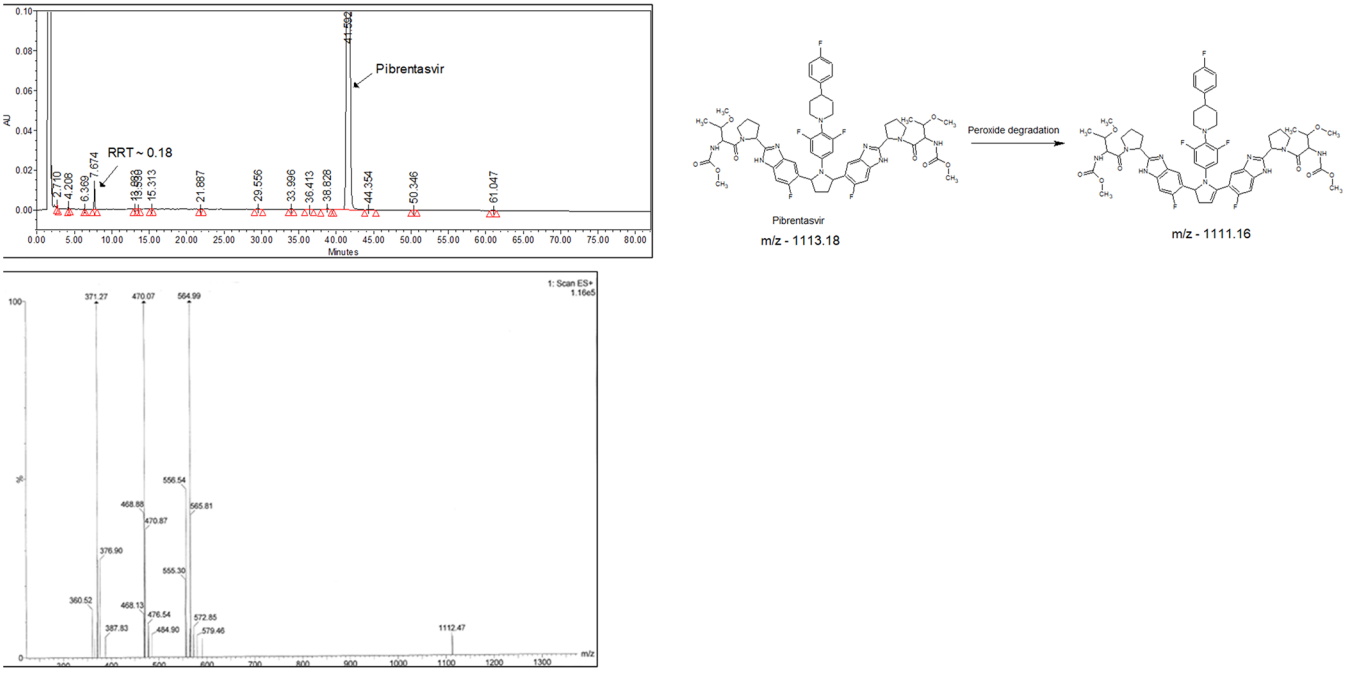


**Figure 8.** Peroxide forced degradation LC chromatogram, LC-MS chromatogram, and unknown identification and fragmentation.

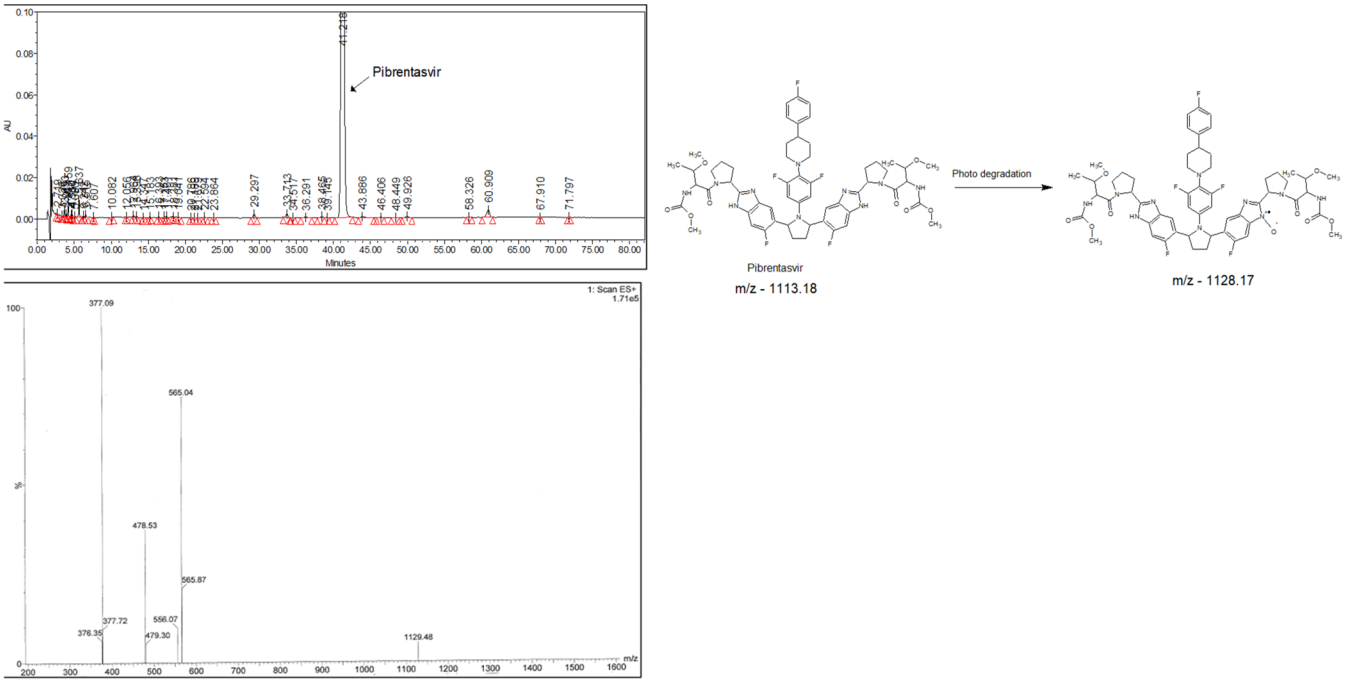


**Figure 9.** Photodegradation LC chromatogram, LC-MS chromatogram, and unknown identification and fragmentation.

Testing Technology, a division of Ametek, Mount Prospect, IL).

**4.3. Analytical Solutions.** Standard solution was prepared at 1.5 $\mu$g/mL with diluent, and sample solution was prepared at 300 $\mu$g/mL. Impurity stock solutions were prepared at concentration of 3.75 $\mu$g/mL. The impurity spiked test sample was prepared with PIB concentration of 300 $\mu$g/mL and impurities concentration of 0.11 $\mu$g/mL using the diluent.

**4.4. Chromatographic Conditions.** A HALO C18, 150 mm × 2.7 mm, 5 $\mu$m particle, was used as the stationary phase in the chromatographic separation. The mobile phases A and B consisted of phosphate buffer, pH 2.5, and acetonitrile in proportions of 70:30, v/v, and acetonitrile and water in proportions of 70:30, v/v, respectively. A 20 $\mu$L injection volume, 0.8 mL/min flow rate, and column temperature of 40 °C were used for the measurements. UV detection was performed at 252 nm with a flow rate of 0.8 mL/min. Water



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**Table 5. Forced Degradation Data**

| s.no. | stress conditions | assay (%) | single maximum impurity (%) | total impurities (%) | mass balance | peak purity |
|---|---|---|---|---|---|---|
| 1 | as such sample | 99.6 | 0.17 | 0.71 | *a* | pass |
| 2 | acid hydrolysis | 88.8 | 12.6 | 14.0 | 102.4 | pass |
| 3 | base hydrolysis | 87.2 | 6.9 | 10.9 | 97.8 | pass |
| 4 | peroxide degradation | 95.9 | 0.67 | 1.6 | 97.2 | pass |
| 5 | photodegradation | 80.9 | 0.81 | 4.0 | 84.8 | pass |
| 6 | thermal degradation | 96.9 | 0.20 | 1.9 | 98.9 | pass |
| 7 | humidity degradation | 97.6 | 0.18 | 0.85 | 98.5 | pass |

[a]Not applicable.

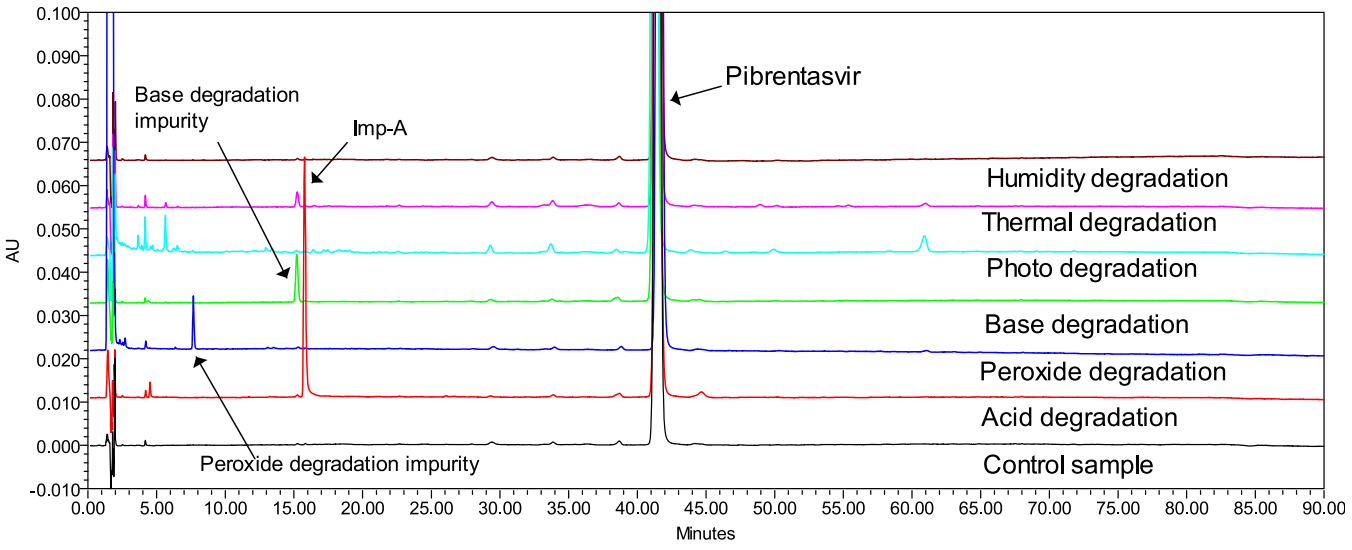


**Figure 10.** Overlay all forced degradation conditions chromatograms.

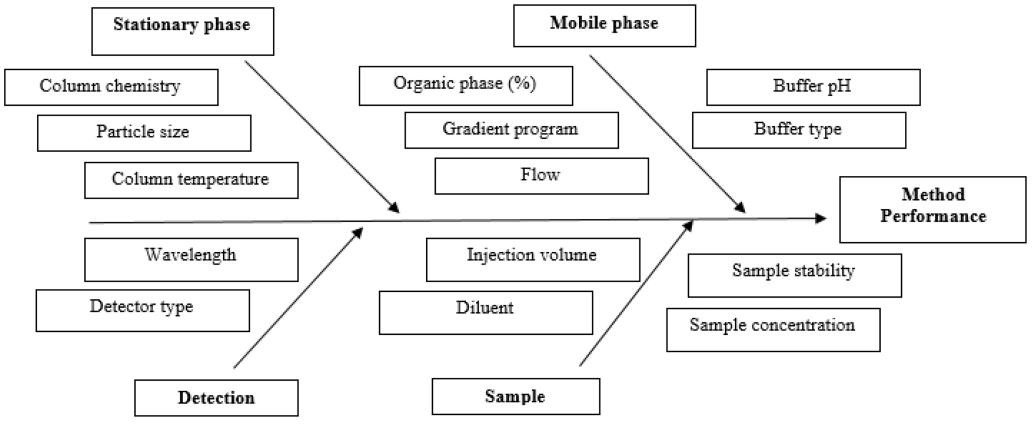


**Figure 11.** Ishikawa or fish bone diagram.

and methanol were mixed in a proportion of 10:90, v/v, to prepare diluent. Time (in minutes)/% mobile phase B of 0/10, 20/30, 50/37, 70/80, 80/80, 82/10, 90/10 was the gradient program.

**4.5. LC-MS Conditions.** Waters TQD triple quadrupole mass spectrometer was used in the LC-MS studies. The source voltage used was 5000 V, and 400 °C was used as the capillary temperature. Positive ionization mode was selected to cover the mass range of 90−2000. Mobile phase A consisted of 0.01 M ammonium acetate (pH 2.5) and acetonitrile in a ratio of 70:30 (v/v), and mobile phase B consisted of acetonitrile and water in a ratio of 70:30 (v/v).

**4.6. Analytical Target Profile.** The analytical method must be able to separate PIB and related substances and its degradation products with a resolution of >2.0. This method should be able to quantify degradation products and related substances within the range of the reporting threshold, that is, 0.05% to 120% of the working concentration (i.e., 0.5 mg/mL) with an accuracy 70−130% recovery and RSD repeatability <5%.

**4.7. Risk Identification.** The risk process begins by systematically identifying the potential variables associated with the analytical procedure. Tools such as Ishikawa or fish bone diagram are used to identify the potential variables that can affect the analytical method (Figure 11).



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### Notes

The authors declare no competing financial interest.

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