Received: 31 December 2022 | Revised: 3 March 2023 | Accepted: 3 April 2023

DOI: 10.1002/jssc.202201067

JOURNAL OF SEPARATION SCIENCE

RESEARCH ARTICLE

# Simultaneous quantification of (*E*) and (*Z*) isomers of rilpivirine and four degradation products in bulk and tablets by reversed-phase ultra-high-performance liquid chromatography and confirmation of all by molecular weight

**Deepak Krishna Mhaske** | **Arjun Shankar Kumbhar**

Department of Chemistry, Vivekanand College, Shivaji University, Kolhapur, India

**Correspondence**
Arjun Shankar Kumbhar, Department of Chemistry, Vivekanand College, Shivaji University, Kolhapur, 416 003, India.
Email: arjun22win@rediffmail.com

The (*E*)-isomer of rilpivirine is an approved antiretroviral drug used to treat human immunodeficiency virus. A simple, fast, accurate, and precise analytical method is required to confirm the quality, purity, efficacy, and safety of drug substances and drug products containing rilpivirine. This research article offers a comprehensive ultra-high performance liquid chromatography method for the simultaneous separation and quantification of (*E*) and (*Z*) isomers of rilpivirine, including two amide impurities, one nitrile impurity, and one dimer impurity, in both bulk and tablet forms. After complete validation, the proposed reversed-phase ultra-high-performance liquid chromatography method has proven to be simple, fast, linear, accurate, and precise, with a lower limit of quantification and detection of 0.05 and 0.03 μg/ml, respectively, for all six analytes. Separation was achieved on a Waters Acquity ethylene bridged hybrid Shield RP18 (150 × 2.1 mm, 1.7 μm) column maintained at 35.0°C using a gradient elution of acetonitrile and 0.05% formic acid in 10 mM ammonium formate at a flow rate of 0.30 ml/min. A systematic forced degradation study on the undissolved rilpivirine revealed the formation of acid-base hydrolyzed amide impurities (Impurity-A and Impurity-B), oxidative nitrile impurities (Impurity-C), and *Z*-isomer and dimer impurities of rilpivirine (Impurity-D and Impurity-E) due to alkaline hydrolysis and photodegradation. The proposed method is primarily appropriate for applications requiring the precise determination of desired and undesired isomers of rilpivirine and its degradation products, such as those involving the safety, efficacy, and quality roles of rilpivirine in bulk and tablet forms. Additionally, the proposed ultra-high-performance liquid chromatography method in combination with a mass spectrometer and photo-diode array detector is helpful for the confirmation and correct identification of all analytes.

**Article Related Abbreviations:** BEH, ethylene bridged hybrid; ICH, International Conference on Harmonization; PDA, photodiode array; QC, quality control; RRF, relative response factor; RRT, relative retention time; USP, United States Pharmacopoeia.


© 2023 Wiley-VCH GmbH.

https://doi.org/10.1002/jssc.202201067

**KEYWORDS**
antiretroviral drug, degradation products, dimer impurity, rilpivirine isomer (E), rilpivirine isomer (Z)

## 1 | INTRODUCTION

According to the world health organization and summary of the global HIV epidemic 2021 report, approximately 38.4 million people worldwide were living with HIV in 2021, with an estimated 650 000 people dying as a result of HIV-related illness, demonstrating why HIV remains a significant global public health issue. There is no medicine to cure HIV infection. However, with prevention, diagnosis, inhibition, and proper nursing, it has become manageable to start long and healthy lives [1].

Rilpivirine is a highly potent second-generation non-nucleoside reverse transcriptase inhibitor used in the treatment of HIV with minimal side effects. Rilpivirine HCl is available as a white, solid powder and belongs to the biopharmaceutics classification system class II (low solubility and high permeability). It shows solubility in organic solvents (dimethyl sulfoxide, dimethylformamide, methanol, dichloromethane, and ACN) and is insoluble in water. Based on published work, there are two isomers of rilpivirine HCl, which are available as isomer (*E*) and isomer (*Z*). Rilpivirine isomer (*E*) is a more desired drug molecule in the treatment than its isomer (*Z*), and hence, the presence of the *Z* isomer in the pure drug substance of rilpivirine is treated as a specified known impurity [2]. Additionally, the literature addressed the rilpivirine HCl degradation under moderately acidic, basic, and oxidative conditions, as well as the generation of unexplained degradation products. The possibility of the formation of degradation products during different processes like the synthesis of drug substances, the manufacturing of tablets, and the storage of bulk and tablets are high.

Globally, all regulatory agencies are asking for data on impurities, their toxicity, and their control to verify any medicine's purity, quality, safety, and efficacy. Analytical data related to the number of impurities, their control, their presence, and their increment over the storage period in drug substances and drug products is important for approval for their administration in treatment [3]. Hence, a simple, fast, and stability-indicating analytical method is, therefore, necessary to ensure: (i) simultaneous quantification of assay and related substances, (ii) detection and quantification of all degradants at lower LOD and LOQs, (iii) satisfactory separation of both (*E* and *Z*) isomer, (iv) absence of isomerization during the formulation and stability period of drug products, and (v) vigilant control of new drug formulation and new packaging for the drug product.

Several different techniques have been described in the literature for the estimation of rilpivirine in pharmaceutical and biological samples. A thorough literature review study demonstrated that different analytical techniques were used for the estimation of rilpivirine as a single content using spectrophotometric [4, 5], HPTLC [6], HPLC [7–10], UPLC [11], LC-MS [12], and LC-MS/MS [13–15] in different matrixes (bulk, tablets, nanoparticles, saliva, serum, and human plasma). The literature also revealed the number of methods including RP-HPLC [16–26], HPLC-MS/MS [27–31], RP-UPLC [32–33], and UPLC-MS/MS [34–36] for the determination of rilpivirine in the presence of other antiretroviral drug molecules. To the best of our knowledge, there are only three reported works for the determination of rilpivirine with limited impurities and or in presence of degradation products in pharmaceutical samples by HPLC. Those reported methods were, however, limited to the separation, speed, and sensitivity, as well as not having more information on degradants formed in the degradation study. The HPLC method with phosphate buffer mobile phase was described by Marineni et al. with a 45-min run time for the determination of rilpivirine in the presence of potential impurities with higher LOD and LOQ values [37]. To explain the rilpivirine HCl degradation process, Chilukuri et al. employed a 10-min run-time liquid chromatographic method for rilpivirine and six impurities with limited separation [38]. The HPLC method with a 5-min run time was developed by Vejendla et al. for the determination of cabotegravir and rilpivirine with a LOD of 0.375 μg/ml and a LOQ of 1.238 μg/ml for rilpivirine, and their total four degradation products were characterized [39]. However, none of the above-described or reported methods focused or explained on the dimer impurity of rilpivirine (a photodegradation impurity) or the Rilpivirine amide-2 impurity (a base hydrolysis impurity).

The goal of the presented research work was to develop an improved, rapid, and validated UHPLC methodology for the simultaneous separation and quantification of (*E*) and (*Z*) isomers of rilpivirine, including two amide impurities, one nitrile impurity, and one dimer impurity, in both bulk and tablet forms on an achiral column. The column with the description of Waters Acquity ethylene bridged hybrid (BEH) Shield RP18, 150 mm length, 2.1 mm internal diameter, and 1.7 μm particle size was selected because of

its confirmed improved separation of (*E*) and (*Z*) isomers of rilpivirine, including four more degradation products. In this article (by explaining the development, validation, robustness, and applicability of the proposed method and force degradation study) we targeted to achieve the UHPLC method for simultaneous determination of all analytes in a single injection and a single wavelength. We also discussed the impact of the slight change in relevant chromatographic parameters and their accompanying outcomes, such as separation, LODs, LOQs, accuracy, and precision, often absent or limited in the reported work despite their importance for assessing the quality and applicability. Finally, we reported and explained here for the first time the acid-base hydrolysis, oxidation, and photodegradation of rilpivirine HCl and the formation of a series of respective degradation products (Impurity-A–E). For the first time, we described the development and validation of a UHPLC method for simultaneous qualitative and quantitative analysis of both isomers of rilpivirine (*E* and *Z*), acid-base hydrolyzed amide impurities (Impurity-A and Impurity-B), oxidative nitrile impurity (Impurity-C), and dimer degradation product (Impurity-E) with improved separation and sensitivity. Additionally, explained the significance of coupling of proposed UHPLC-PDA instrument with a mass spectrophotometer detector for correct identification and confirmation of all mentioned analytes based on their molecular weight and spectra. The structural formulas of Rilpivirine isomer (*E*), Aryl amide impurity (Impurity-A), Rilpivirine amide-1 impurity (Impurity-B), oxidative nitrile impurity (Impurity-C), *Z*-isomer of Rilpivirine (Impurity-D), and dimer impurity (Impurity-E) are displayed in Figure 1.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals and reagents

The ultrapure milli-Q water was obtained by a Milli-Q system (Merck Millipore), and ACN and methanol (LC/MS grades of Thermo Fisher Scientific and HPLC grades of Merck), dimethyl formamide (HPLC grades of Qualigens; Thermo Fisher Scientific), and formic acid (Analytical grade, Merck) were used to prepare solutions. The ammonium formate (Honeywell and Sigma-Aldrich) was used to prepare mobile phase-A, and the buffer was filtered through Millipore 0.20 μm, White Nylon, and GNWP 47 mm (Merck). The in-house reference standards for all impurities, degradation products, and rilpivirine HCl were used. The excipients and placebo in tablet formulations were of standard grade, and samples of rilpivirine HCl drug substance and tablets (25 mg) were used wherever applicable.

### 2.2 | Equipment and software

All activities related to method development, method validation, force degradation study, robustness study, and applicability of the method were performed on Waters (Milford, MA, USA) Acquity UHPLC H-class systems equipped with a quaternary solvent manager delivery pump, an autosampler with sample manager with a flow-through needle, a column manager, a photodiode array (PDA) detector, a tunable UV detector, and a Quadrupole Dalton mass spectrometer detector with an electrospray ionization source (Waters Corporation). The data acquisition, monitoring, instrument control, and data processing were performed using Empower 3 software (Waters).

### 2.3 | Preparation of solutions

A mixture of ACN and Milli-Q water (8/2, v/v) was used as the dilution solvent. The standard solution and drug substance sample solution of rilpivirine HCl (0.1 mg/ml) was prepared by dissolving content in 5% of dimethylformamide and diluted to the volume with dilution solvent. The resolution solution contained rilpivirine HCl at 100% and each impurity at a 0.5% level. Tablet sample solution (0.1 mg/ml) was prepared by using a crush of 20 tablets and in which suitable content was mixed mechanically for 10 min with 5% of dimethylformamide and diluted to the volume with dilution solvent. The detailed procedures of preparations are summarized in Section S2.3.

### 2.4 | Development of an analytical method

The most important goal for this research was the development of a mass spectrometer-compatible RP-UHPLC method useful for the separation, identification, and simultaneous quantification of the assay, process-related impurities, and potential specified and unspecified degradation products in a single run and at a single wavelength. The objectives also include a timesaving validated method with a broad range of efficiencies that provides higher separation and improved detection with a quick analysis time and maximum sensitivity. Method development activity was organized and carried out to optimize the suitable RP-UHPLC chromatographic parameters (mobile phases, column, detection wavelength, injection volume, and column temperature), dilution solvent, standard and sample preparation, and suitable filter for filtration of tablet solution. In the method development activity for the optimization of different parameters (UHPLC and mass

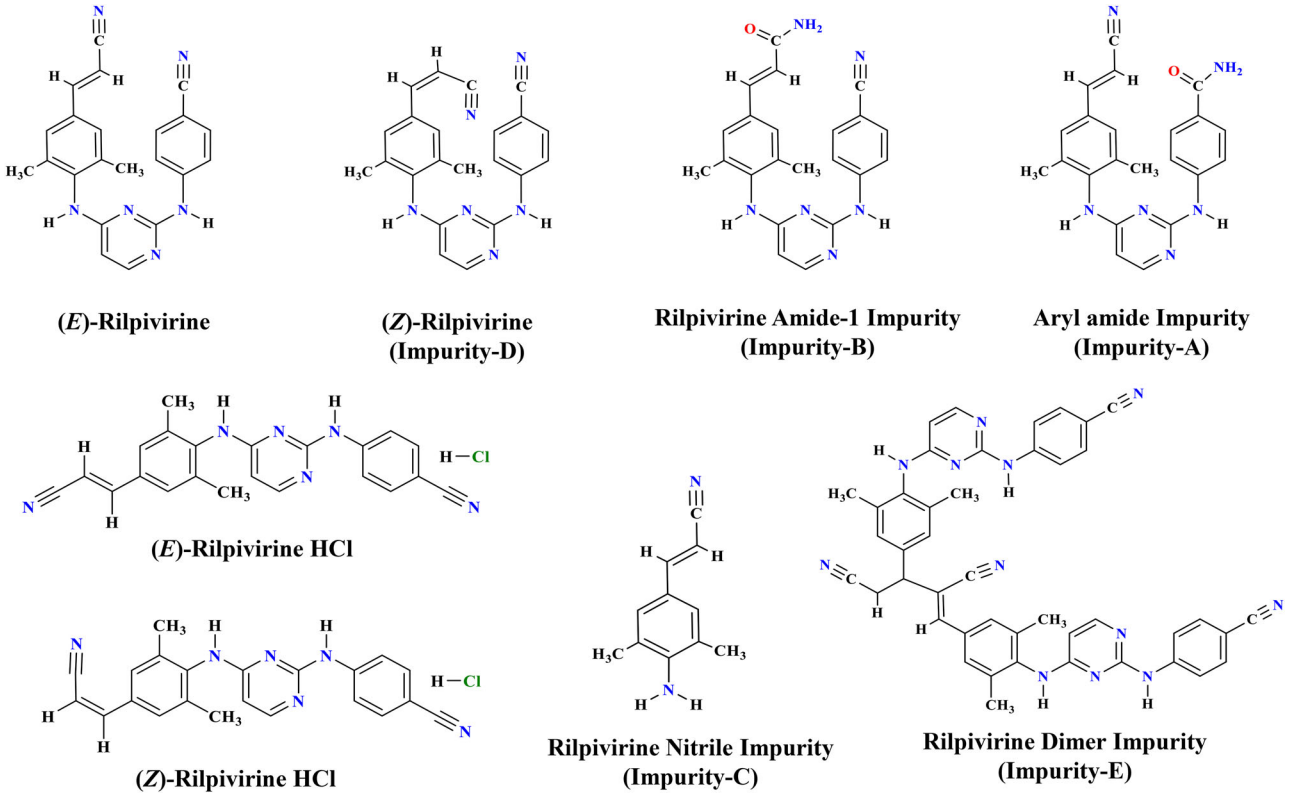


**FIGURE 1** Molecular structure of Rilpivirine (*E*) isomer, Rilpivirine (*Z*) isomer, Rilpivirine amide-1 impurity, Aryl amide impurity, Rilpivirine Nitrile Impurity, Rilpivirine dimer impurity, (*E*)-Rilpivirine HCl, and (*Z*)-Rilpivirine HCl.

spectrometer), the resolution solution and stressed drug substance sample solutions were used.

## 2.5 | Validation of the method

After optimization of all parameters in the method development activity, the proposed RP-UHPLC method was validated as per International Conference on Harmonization (ICH) guidelines on the validation of analytical procedures. Different validation parameters, including selectivity or specificity, accuracy, precision, linearity, range, LOD, LOQ, solution stability, and system suitability test, were studied [40, 41]. Additionally, the filtration study and relative response factors (RRFs) for all five degradation products were determined. The additional details, planning, and acceptance criteria of each validation parameter are summarized in Section S2.5.

## 2.6 | Robustness study

In the robustness study of the proposed experimental setup, different parameters and one parameter at a time were investigated. The robustness was examined and established with the lower and higher variation of the total of six different parameters indicated in the proposed chromatographic conditions. The chromatographic conditions parameters such as mobile phase flow (±0.05 ml/min), column temperature (±5.0°C), slope gradient (±1.0 min), the concentration of formic acid in mobile phase A (±0.05 ml), the concentration of 10 mM ammonium formate in mobile phase A (±20%), and finally change in column age with a lot to lot, different lot of chemicals and reagents were studied in the robustness study. The impact of all these purposeful changes on the retention time, United States Pharmacopoeia (USP) tailing, USP theoretical plates, S/N (at LOD and LOQ level), the precision of peak areas, and peak separation was investigated.

## 2.7 | Forced degradation study

A forced degradation study of rilpivirine HCl was performed as per ICH guidelines on stability testing of new drug substances and products. To evaluate the stability-indicating properties of the proposed UHPLC method intended to be used for the analysis of rilpivirine HCl drug substance a stressing study on solid drug substance, was initiated. These stresses were carried out to evaluate the effects of acid (HCl), alkaline (NaOH), and neutral (water) hydrolysis and an oxidizing agent ($H_2O_2$) in

combination with high temperature and room temperature on solid rilpivirine HCl. Additionally, the stressing with thermal conditions (at 80°C and 120°C), heat with humidity conditions (at 70°C and 75% relative humidity), metal ion conditions, and ICH light were carried out on solid rilpivirine HCl to evaluate stability. The objective of this forced degradation study is to generate the maximum number of degradation products from the rilpivirine HCl and separate them for qualitative and quantitative analysis. In the unbuffered aqueous hydrolysis (acid, alkaline, and neutral) stressing, 2 ml of HCl, NaOH, and water were added to a known amount of solid drug substance of rilpivirine HCl and exposed at room temperature for 4, 5, and 30 days and at 80°C for 5 h. For an oxidative stress study of rilpivirine HCl, 2 ml of $H_2O_2$ (3.0% and 30%) was added to a known amount of solid drug substance of rilpivirine HCl and exposed at room temperature for 4, 5, and 30 days and at 80°C for 5 h. To study the effect of thermal (at 80°C and 120°C) and heat with humidity (at 70°C and 75% relative humidity) stress, a solid drug substance of rilpivirine HCl was exposed in the respective oven for 30 days. At programmed time intervals of 4, 5, and 30 days, aliquots of the stressed sample were withdrawn and analyzed as per requirement. The photolytic degradation study was done by exposing the solid drug substance of rilpivirine HCl to ICH light, providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200-watt hours per square meter in the photostability chamber. Along with all stressed samples, the unspiked sample solution was prepared and kept in dark under the room temperature conditions and for the same period. To study further degradation and confirm secondary degradations in acid and basic hydrolysis, no stressed sample solution was neutralized. After each stressing, all samples were dissolved in 5.0% of dimethylformamide and then diluted with a dilution solvent to the expected concentration of 0.1 mg/ml. Then all solutions were analyzed using the proposed methods. The complete plans of forced degradation studies are summarized in tabular form in Table S1.

## 2.8 | Applicability of method in routine analysis

After achieving a successful validation outcome, the applicability of the proposed methods was confirmed by testing two batches of drug substance and two batches of tablet formulations of rilpivirine HCl. All selected samples were prepared (unspiked and spiked) as per the mentioned procedure and then analyzed and assessed on a PDA detector for simultaneous quantification and on a mass spectrometer detector to confirm their peaks and results.

# 3 | RESULTS AND DISCUSSION

## 3.1 | Development of an analytical method

Based on the main goal of this research work, optimizations of different parameters were carried out, and a simple and new RP-UHPLC method was developed for the simultaneous quantification of rilpivirine HCl and its five degradation products in a single run at a single wavelength. All these parameters were optimized and adjusted in such a way that all six analytes were successfully separated from the blank interference peak, placebo peak, and all other potential unknown degradants. Moreover, all optimized parameters were fine-tuned and evaluated based on adequate resolution, a stable baseline, a sharp peak without matrix impact, and better peak responses to make the developed method speedy, cost-effective, and simple enough to be used in the quality control (QC) laboratory of the pharmaceutical industry.

### 3.1.1 | Optimization of mobile phase and stationary phase

The optimization of chromatographic conditions, specifically the composition of the selected mobile phases and the selection of a suitable analytical column, was optimized to get sharp, symmetrical peaks and improved separation in a shorter run time. The method development work started with the selected buffers, organic phase, and stationary phase, which offer a working range in reverse phase analysis and are compatible with mass spectrometer detector analysis. In the investigation, the aqueous mobile phase contains the selected buffers such as 0.1% formic acid, ammonium formate (5, 10, and 20 mM), diverse ratios of formic acid (0.05, 0.1, and 0.2%) in ammonium formate (5, 10, and 20 mM), 0.1% acetic acid, ammonium acetate (5, 10, and 20 mM), and diverse ratios of acetic acid (0.05, 0.1, and 0.2%) in ammonium acetate (5, 10, and 20 mM). These chosen buffers were investigated in coupling with ACN, methanol, and their mixtures as an organic mobile phase. The effects of a total of four ACQUITY UPLC columns (column-1: BEH C18, column-2: BEH Shield RP18, column-3: charged surface hybrid C18, and column-4: high strength silica T3) with the same dimensions (150 mm long, 2.1 mm id, 1.7/1.8 μm particle size) were investigated as a stationary phase with the listed mobile phases.

The use of pure methanol or a mixture with ACN as an organic mobile phase with a selected buffer solution was quite useful, and the peak shape of some analytes showed a higher tailing factor when compared to pure ACN. However, in the case of ACN as an organic mobile phase, as

the composition was increased in gradient mode, the early peak elution with the suitable resolution and tailing factor in lower run time was observed with almost all aqueous buffers. However, improved separations between all analytes were accomplished with the use of a pH modifier in a formate or acetate buffer. Among all selected buffers in combination with ACN, the best sharp peak shape, and excellent separation were achieved when 0.05% formic acid was added as a pH modifier in a 10 mM ammonium formate buffer. A decreased pH value of 20 mM in the aqueous buffer with the use of 0.2% formic acid was impacted positively and increased the retention of all analytes, their resolution, and the tailing factor of the rilpivirine peak. Different flow rates of selected mobile phases were investigated in the range of 0.2–0.4 ml/min. A stable baseline, high separation in all analytes, and acceptable parameters related to peak (shape, tailing, and theoretical plates) were obtained at a flow rate of 0.30 ml/min, which eluted all desired peaks in a run time of 13 min and offered a peak resolution of more than 3.5 in any two coeluted analytes.

Among these selected four columns with 2.1 internal diameter and 1.7 μm particle size and 150 mm longer columns, better separation, a sharp peak shape, and a lower run time were obtained with column-2 (BEH Shield RP18). The Acquity UPLC BEH Shield RP18, 150 mm long, 2.1 mm id, and 1.7 μm particle size columns are based on an embedded carbamate group into the bonded phase ligand with a wide working pH range. Compared to the other three columns of the same particle size, same internal diameter, and same 150 mm length, the BEH Shield RP18 column produces excellent efficiency and high-resolution separations. It supports enhanced sharp peak shapes and more precise and accurate results due to the embedded carbamate group into the bonded phase ligand chemistries. This optimized BEH Shield RP18 column is also known for its quite high backpressure, reproducibility with different lot numbers, and certainty in chromatographic separations. Additionally, based on some trials, it was concluded that the use of columns with a shorter length (50 and 100 mm) was helpful to decrease the run time, and all mentioned analytes were eluted within 10 min. However, degradants formed in base hydrolysis were impacting the peaks of impurity A and B, and a decreased resolution was observed between all analytes.

### 3.1.2 | Optimization of other chromatographic conditions

Further optimization of chromatographic condition parameters was done with the help of optimized mobile phases and stationary phases (columns). The influence of diverse column temperatures (in the range of 20–45°C) on peak responses, peak resolution, peak shape, tailing, and theoretical plates was also investigated. At the higher side of the column temperature (45°C), all peaks were eluted early, and the separations between all analytes were quite lower than 1.5. However, at the lower side of the column temperature (20°C), all analytes were eluted after 10 min with moderate peak separation and a quite broader peak shape. The most excellent resolution was achieved at 35°C with a sharp peak, precise elution, better peak responses, and results. The recorded PDA spectra were investigated at different wavelengths to find a common single wavelength for the quantification of all analytes. Based on the lambda max values of all analytes, we also investigated responses at 280, 282, 293, 305, and 341 nm. Maximum detection for all six analytes was observed at a 305 nm wavelength with a stable baseline.

The responses of all six analytes were investigated with diverse injection volumes in solutions containing different concentrations of analytes (0.05, 0.10, and 0.20 mg/ml). The various injection volumes in the range of 1.0–4.0 μl of spiked standard solution with different concentrations were injected to optimize the final concentration with a suitable injection volume. Finally, based on the outcome of the responses at LOD and considering the load of sample concentration on the column and the sharp peak shape, 1.0 μl of the 0.10 mg/ml standard solution concentration was assessed as a suitable injection volume and concentration for the test solution. All analytes show limited solubility in ACN and methanol and hence to increase the solubility of all analytes, dimethyl formamide was included to dissolve the content in the first place. It was investigated and concluded that the addition of 5% dimethylformamide to dissolve the content and then final dilution with a mixture of ACN and water (8/2, v/v) were providing a stable and higher response. The addition of dimethyl formamide in the first place during solution preparation was helpful to decrease the shaking time for solid content to get dissolved. It also improved the solubility and reproducibility of the peak responses for all analytes, as demonstrated by the adequate, accurate, and precise outcomes. However, the standard and drug substance sample solutions need a minimum of 30 min of mechanical shaking to dissolve all content in the mentioned dilution solvent without the use of dimethylformamide or sonication, and this was investigated. Importantly, although this method uses a simple 5% dimethyl formamide extraction step, it can easily be applied to the analysis that quantifies all analytes in more complex matrices by using the LC-MS instrument.

Additionally, different tablet placebos (with and without sodium stearyl fumarate) were studied and investigated in detail for the elution of placebo peaks. One of the major challenges when developing analytical methods is to elute

out and detect the placebo peaks in the tablet sample solution. The solution of tablet formulations manufactured with the use of sodium stearyl fumarate shows a placebo peak after the elution of all six analytes. A higher amount of ACN for a certain time needs to pass through the column to elute out the placebo peak from the column. Otherwise, the respective placebo peak will elute out in the next injection and can be misinterpreted by the user. None of the published methods investigated and explained such challenges.

In the end, the mass spectrometer parameters were evaluated with the optimized mobile phases, column, and other UHPLC chromatographic parameters for correct identification and confirmation of all peaks. The diverse values of probe temperature, cone voltage, and capillary voltage for both modes were investigated to identify suitable mass spectrometer parameters. After a detailed study, the parameters of the mass spectrometer were finalized as follows: The *m/z* was extracted in positive and negative ESI modes at 600°C as the probe temperature, with a capillary voltage for the positive mode of 1.2 kV and a negative mode of 0.8 kV. The MS scan in positive and negative modes was set to scan the *m/z* from 100.00 to 1000.00. The sampling rate was 10 points/second, and the gain value was 1.0, with 10 V as the cone voltage. All optimized UHPLC chromatographic parameters and mass spectrometer parameters are summarized in Table S2. The representative chromatogram of a spiked 25 mg tablet sample solution of rilpivirine HCl by the proposed UHPLC method is represented in Figure 2, and the chromatogram of a spiked sample solution of rilpivirine HCl drug substance is in Figure S1.

## 3.2 | Validation of the method

According to ICH guidelines [40, 41], the performance of the proposed UHPLC method was evaluated in terms of specificity, accuracy, precision, linearity, range, LOD, LOQ, solution stability, system suitability test, RRFs, and filtration study. The representative outcomes of all these parameters are reported in Table 1.

### 3.2.1 | Selectivity/Specificity

The ability of the proposed UHPLC method to separate, identify and measure all mentioned analytes in the presence of a sample matrix without interference was confirmed in the specificity/selectivity parameters. It was confirmed based on the evaluation of chromatograms of the blank, placebo solutions of the tablet, resolution solution, standard solution, individual solution of all degradation products, spiked sample solution, and unspiked sample solution of drug substance and tablet formulation. All peaks of mentioned degradation products were separated from each other and the peak of the rilpivirine. There were no interfering peaks and no coelution of peaks with blank, dilution solvent, and different placebo solutions, each other, and the peak of the rilpivirine HCl. The peak purity for rilpivirine HCl was confirmed based on an observed lower value of purity angle than the observed value of purity threshold in all injected solutions including solutions of forced degradation study. Moreover, the very negligible variations between retention times, λmax values observed in online PDA spectra, and molecular weight of each analyte in the standard and sample solutions in the mass spectrum explain the accurate and precise analyte peak identification. Based on the comprehensive outcomes, concluded that the proposed RP-UHPLC method is specific or selective for the six selected analytes in bulk and tablet forms. The overlay chromatogram of the blank, tablet placebos, individual impurity solution, and spiked sample solution of tablet and drug substance are shown in Figures S2 and S3.

### 3.2.2 | Accuracy

The accuracy parameter expresses the closeness of agreement between the true value (added amount) and the value found (observed amount). The individual recoveries for rilpivirine HCl at seven lower concentration levels (0.05%–5.0% w/w) were observed to be in the accepted range of 97.5%–112.5%, and at seven higher concentration levels (25%–150% w/w), recoveries were within 99.2%–100.7%. The accuracy for all degradation products was established at seven concentration levels in the range of 0.05%–2.0% w/w, and the individual and mean recoveries for all degradation products were found to be in the range of 91.6%–109.1% and 96.3%–107.6%, respectively. The values of the determined RRF were used in the calculation of the accuracy of all degradation products. The outcome of the accuracy parameter concerning all recoveries is presented in Tables S3 and S4.

### 3.2.3 | Precision

The precision of an analytical method is nothing but a closeness of agreement (also known as the degree of scatter) between the series of determinations acquired from several testing of the same sample under the specified conditions. The precision was expressed as system repeatability, analysis repeatability, and intermediate precision. The system repeatability parameter was estimated and

**TABLE 1** Outcome of method validation of UHPLC method (for quantification) and UHPLC-PDA-ESI-MS method (for confirmation).

| Parameter | Impurity-A | Impurity-B | Impurity-C | Impurity-D | Rilpivirine | Impurity-E |
|---|---|---|---|---|---|---|
| Peak number | 1 | 2 | 3 | 4 | 5 | 6 |
| Retention time (min) | 7.7 | 8.1 | 10.3 | 11.0 | 11.4 | 12.5 |
| Relative retention time | 0.68 | 0.71 | 0.90 | 0.96 | 1.00 | 1.10 |
| LOD (μg/ml) | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 |
| Lower LOQ (μg/ml) | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| Upper LOQ (μg/ml) | 2.0 | 2.0 | 2.0 | 2.0 | 150.0 | 2.0 |
| S/N in LOD solution | 8.0 | 6.2 | 3.8 | 5.5 | 8.6 | 3.6 |
| S/N in lower LOQ solution | 15.4 | 12.2 | 11.1 | 11.9 | 15.9 | 10.8 |
| Regression equation | y = 17143.5x − 60.5 | y = 15633.1x + 27.4 | y = 9146.3x + 20.6 | y = 16475.6x − 10.8 | y = 21380.1x + 12427.6 | y = 15956.9x − 25.7 |
| Coefficient of determination ($R^2$) | 0.9997 | 0.9997 | 0.9998 | 0.9999 | 0.9999 | 0.9998 |
| %RSD for response factor | 2.3 | 3.6 | 2.7 | 2.0 | 3.5 | 2.1 |
| Linearity range (μg/ml) | 0.050–2.0 | 0.050–2.0 | 0.050–2.0 | 0.050–2.0 | 0.050–150.0 | 0.050–2.0 |
| Stability of solutions (at RT) | 7 days | 7 days | 7 days | 7 days | 7 days | 7 days |
| Filter for sample filtration[a] | 0.45 μm | 0.45 μm | 0.45 μm | 0.45 μm | 0.45 μm | 0.45 μm |
| Relative response factor | 0.802 | 0.731 | 0.428 | 0.771 | 1.000 | 0.746 |
| Purity angle | 11.641 | 12.158 | 0.513 | 9.045 | 0.160 | 9.546 |
| Purity threshold | 16.806 | 24.292 | 1.655 | 19.330 | 0.428 | 26.451 |
| Molecular formula | $C_{22}H_{20}N_6O$ | $C_{22}H_{20}N_6O$ | $C_{11}H_{12}N_2$ | $C_{22}H_{18}N_6$ | $C_{22}H_{18}N_6$ | $C_{44}H_{36}N_{12}$ |
| Minimum USP resolution[b] | NA | 4.2 | 25.7 | 6.0 | 4.1 | 12.7 |
| Maximum USP tailing[b] | 1.2 | 1.1 | 1.1 | 1.1 | 1.1 | 1.2 |
| Minimum USP plate Count[b] | 187 536 | 192 028 | 253 857 | 278 371 | 286 348 | 306 410 |
| Molecular weight (g/mol) | 384.44 | 384.44 | 172.23 | 366.43 | 366.43 | 732.86 |
| Exact mass [M] (g/mol) | 384.17 | 384.17 | 172.10 | 366.16 | 366.16 | 732.32 |
| Molecular ion [M ± H] | $[M + H]^+$ | $[M + H]^+$ | $[M + H]^+$ | $[M—H]^-$ | $[M—H]^-$ | $[M—H]^-$ |
| *m/z* value in standard | 385.5 | 385.5 | 173.3 | 365.3 | 365.3 | 731.6 |
| *m/z* value in the sample | 385.5 | 385.5 | 173.3 | 365.3 | 365.3 | 731.6 |

NA: Not applicable.

RT: Room temperature.

[a]Pall, Acrodisc CR 25 mm syringe filter with 0.45 μm PTFE membrane, part No. 4219T filter.

[b]In method validation, robustness, and forced degradation study.

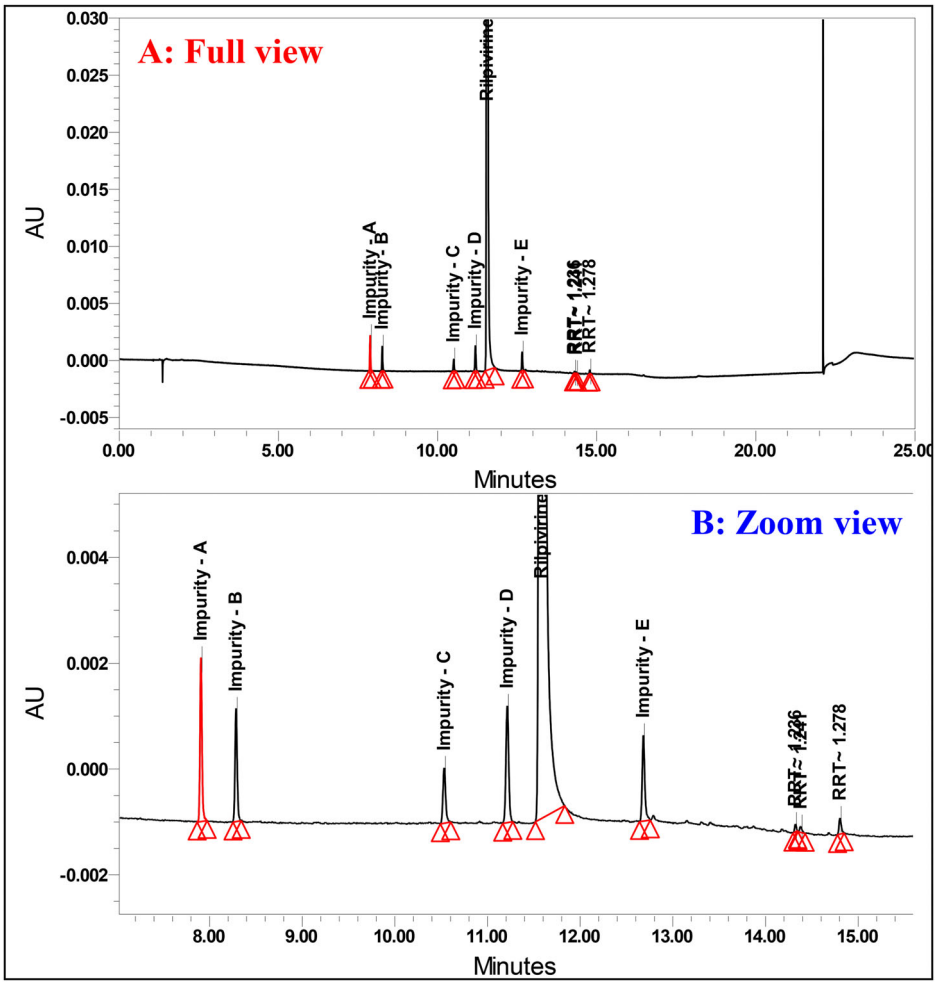


**FIGURE 2** Full view and zoom view chromatogram of spiked 25 mg tablet sample solution of rilpivirine HCl.

confirmed by injecting six replicates of 100% standard solution of the rilpivirine HCl with acceptance criteria for a %RSD of ≤ 0.73% for peak area and retention time in each sample set of complete method validation. The analysis repeatability was determined as a %RSD on three determinations at all seven different concentration levels, which concluded as an individual recovery in the accuracy parameter. The %RSD was found to be below 4.7% for all five degradation products in the range of LOQ to 2.0%. The %RSD was found to be below 0.2% for the assay range of rilpivirine HCl, and at a lower range (0.05%–5.0%), it was within 4.4%.

The intermediate precision was proved by investigating two separate series of six spiked samples on different UHPLCs by using different columns on separate days with separate preparations. The selected drug substance of rilpivirine HCl did not contain all the mentioned degradation products, and samples of tablets of rilpivirine HCl contained only two degradation products. Hence, all degradation products were spiked into the six separate sample solutions of rilpivirine HCl drug substance and tablet samples in the range of 0.2%–0.6%. For both analysts, the %RSD values obtained for the six replicates of spiked sample solution and the average of the 12 replicates yielded for each degradation product was less than 3.8%, and the %RSD for the rilpivirine HCl assay values was less than 0.6%. The total results of all three parameters were confirmed by the precision of the method. The symbolic outcomes of these three parameters are presented in Tables S3–S7.

### 3.2.4 | Linearity and range

The linearity of the proposed UHPLC method was examined by preparing and injecting a series of reference standard solutions in triplicate preparation in the range of the LOQ (0.05%) to 150.0% of the concentration of rilpivirine HCl in the test solution. For all five degradation products, the linearity was tested by preparing and injecting a series of standard solutions in triplicate preparations in the range of LOQ (0.05%) to 2.0% of the concentration of the test solution. Three separate stock standard

solutions of all degradation products were prepared at a concentration of 0.1 mg/ml and were further diluted to obtain seven different concentrations in the range of 0.05–2.0 μg/ml (0.05%–2.0%). Three separate stock standard solutions (0.1 mg/ml) were prepared for all seven lower-concentration solutions of rilpivirine HCl, ranging from 0.05 to 5.0 μg/ml (0.05%–5.0%). At a higher concentration of rilpivirine HCl, seven levels of solutions were prepared in the range of 25–150 μg/ml (25%–150%) in triplicate preparations. An observed value of the correlation coefficient ($R^2$) of ≥ 0.9997 for rilpivirine HCl and for all five degradation products confirmed that the proposed UHPLC method is linear in the tested concentration range. The %RSD of response factors was less than 3.6% for all five degradation products, and it was less than 3.5% for rilpivirine HCl (in the range of 0.05–150 μg/ml), and less than 0.4% for rilpivirine HCl in the assay range (25–150 μg/ml). Based on the total outcome observed in linearity, the range for rilpivirine HCl was identified as between the verified lower concentration of 0.05% (0.05 μg/ml) and a higher concentration of 150% (150 μg/ml). However, the range for all five degradation products was between 0.05% (0.05 μg/ml) and 2.0% (2.0 μg/ml). The linearity graphs for all six validated analytes are presented in Figure S4, and the observed regression equation with the value of the correlation coefficient is presented in Table 1.

### 3.2.5 | LOD and LOQ

The limit of detection was proven by three injections of a solution containing rilpivirine HCl and all five degradation products at a LOD level of 0.03 μg/ml (0.03%). The S/N (USP s/n) obtained in the solution of the LOD for all six analytes was in the range of 3.6–8.0 on two UHPLC instruments and by using separate columns of different ages.

The limit of quantitation was determined by injecting three injections of a solution containing rilpivirine HCl and all five degradation products at a LOQ level of 0.05 μg/ml (0.05%). The S/N (USP s/n) obtained in the solution of the LOQ for all six analytes was in the range of 10.8–15.9 on two UHPLC instruments and by using separate columns of different ages. The overlay chromatogram of LOD and LOQ solutions for all six analytes is presented in Figure S5, and the individual value of the S/N is in Table 1.

### 3.2.6 | Solution stability

The stability of the solutions was studied against freshly prepared standard solutions. The total outcome in solution stability was confirmed that all standard solutions (unspiked and spiked solutions at LOD, LOQ, and 100% level) and sample solutions of drug substance and tablets (unspiked and spiked) were found to be stable for 7 days at room temperature (e.g., 22°C) and at 5°C in the refrigerator. The mobile phases, blank solution, and dilution solvent stability are also investigated, and it is found that all are stable for 7 days when stored at room temperature.

### 3.2.7 | System suitability test

A system suitability test is an essential part of each analytical method, and hence this test was evaluated as part of the method validation design. The system suitability test parameters (resolution, %recovery, %RSD, S/N at LOD level, %recovery at LOQ level, 5% retention time shift window, and interference-free blank) were checked and demonstrated during the whole method validation activity. In the evaluation of each validation parameter, it was confirmed that %RSD of retention time and peak area in replicate injections of standard solution for the rilpivirine peak was less than 0.73%. The USP tailing was in the range of 1.0–1.2 for all six analytes in all injections of standard and sample solutions. The USP theoretical plates (efficiency) for all the analytes were above 150 000 and separation in between any mentioned coeluted two peaks was more than 4.0. By this assessment, it was ensured that all chromatographic conditions were suitable for their intended purpose before sample injection, intermittently after 12 injections of the sample, and after the last injection of the sample. The different parameters listed in Table S8 were ensured, and each time, all parameters met the acceptance criteria.

### 3.2.8 | Relative response factors

The RRFs for each degradation product were calculated by using the respective slope values obtained in the linearity test. The RRFs are a ratio of the slope of the respective impurity to the slope of rilpivirine HCl in the assay range. The calculated RRFs in three decimals are presented in Table 1.

### 3.2.9 | Filtration study

The filtration study was conducted on the blank, unspiked, and spiked 25 mg tablet sample solutions of rilpivirine HCl by using a Pall Acrodisc CR 25 mm syringe filter with 0.45 μm polytetrafluoroethylene membrane, part number 4219T filter. It was confirmed that there were no additional

interfering peaks in the extracts of blank and sample solutions collected from the filter. Based on the comparison of results between unfiltered (centrifuged) and filtered sample solutions, it was assured that there is no significant adsorption of any analytes under investigation onto the filter bed. Based on the validation outcomes, concluded that the developed RP-UHPLC method is specific or selective for the six selected analytes in bulk and tablet forms.

In summary of the validation outcome, based on all acceptable outcomes it was concluded that the developed UHPLC method defined here has improved separation, speed, and sensitivity to determine concentrations of rilpivirine and its five specified related substances in drug substances and drug products. It also meets the accuracy, precision, and linearity criteria for method validation, providing improved quality checks, and accurate and precise quantification in different pharmaceutical matrices.

## 3.3 | Robustness study

A comprehensive robustness study was carried out and measured that the capacity of the proposed UHPLC method remained unaffected by slight but intentional variations in method parameters related to the instrument, gradient, and mobile phase. Outcomes of robustness revealed that the proposed UHPLC method was controlled in terms of column temperature, flow rate, gradient slope, the concentration of buffer and formic acid in mobile phase A, and changes in a lot number of columns, chemicals, and reagents, and proved its reliability for the intended purposes of qualitative and quantitative analysis. The sequence of minimum injections, including blanks, LOD solution, LOQ solution, impurity spiked standard solution-1 (six replicates), and impurity spiked standard solution-2, was injected in the evaluation of each robustness parameter. The injected solutions, such as the blank, LOD solution, and LOQ solution, confirmed that there was no impact of varied parameters on the acceptable blank pattern, S/N values, or accuracy at lower LOQ levels for all six analytes. The recoveries for all six analytes (rilpivirine at 100% level and all five impurities at 0.5% level) were obtained in the range of 98%–102% with impurity-spiked standard solution-2, confirming the consistency of accuracy in all intentionally changed parameters. The obtained %RSD values of six replicate measurements for retention time and peak area responses below 0.73% (for rilpivirine at 100% level) and below 2.0% (for all five impurities at 0.5% level) endorsed the scattering of a homogeneous solution (precision) in all purposefully modified parameters. The slight variations in column temperature (35.0 ± 5.0°C), flow rate (0.30 ± 0.05 ml/min), gradient slope (10.0 ± 1.0 min), the concentration of buffer in mobile phase-A (10 ± 2 mM), the concentration of formic acid in mobile phase-A (0.05% ± 0.01%), and change in a lot number of column, chemicals, and reagents on separate UHPLC had no significant effect on the chromatographic outcome and results. The impact on the repeatability of retention time, peak responses, USP tailing factor, USP theoretical plate, and USP resolution was not significantly affected under these six varied parameters at lower and higher than nominal conditions. The detailed information (overall impact, chromatograms, and major system suitability parameters) of the robustness study is presented in Figures S6–S11 and Tables S9–S14.

## 3.4 | Forced degradation study

These forced degradation studies were executed to examine and prove the specificity, selectivity, and stability-indicating nature of the proposed UHPLC method for the identification, assay, and chromatographic purity of rilpivirine. All prepared stressed sample solutions of rilpivirine HCl under various stress conditions were analyzed as per the proposed UHPLC instrument equipped with a PDA detector and a mass spectrometer detector to identify degradation products.

### 3.4.1 | Acidic conditions

Based on the total outcome (results and chromatograms) with acid stressing conditions, it can be concluded that a solid drug substance sample of rilpivirine HCl is prone to minor degradation under acidic conditions (5 M HCl at 80°C for 5 h). About 0.60% degradation was observed as active is degrading slowly in mentioned respective acidic conditions. Both nitrile groups in the rilpivirine molecule were hydrolyzed and formed two separate amide impurities. Both formed amide impurities were confirmed with PDA and mass spectral data. However, stressing at room temperature with 5 M HCl does not show such degradation and degradants, so it was concluded that rilpivirine HCl was not susceptible to degradation under acidic conditions (0.1 and 5 M HCl) at room temperature for 30 days. No secondary degradation was observed, and all generated degradants were confirmed based on PDA and mass spectral data. Mass balance is considered to be met since stressed assay (%) and total degradation (%) values are near the initial unstressed assay and impurity values.

### 3.4.2 | Alkaline conditions

Stressing a solid drug substance sample of rilpivirine HCl with NaOH (0.1 and 5 M) has shown that rilpivirine HCl is susceptible to minor degradation under alkaline

conditions. About 1.9% degradation was achieved, as active is slightly reactive towards an alkaline condition. Within 5 h of stressing with 5 M NaOH at 80°C, rilpivirine HCl was degraded and formed an increment of 0.1% of impurity A, 0.7% of impurity B, and 0.1% of impurity D, plus five more degradants in the range of 0.1%–0.4% of total impurities. However, after stressing with NaOH (0.1 and 5 M) at room temperature for 30 days, rilpivirine HCl was degraded and formed only an increment of 0.1% in the peak of impurity-B, 0.2% in the peak of Impurity-D, two more degradants up to 0.1% and slight increment in Impurity-A. The increases in total impurities were seen from 0.25% (unstressed sample) to 0.7%, including all minor degradants, at room temperature for 30 days and up to 1.9%, including all minor degradants, at 80°C for hours when a solid drug substance of rilpivirine HCl was stressed with 5 M NaOH. No further degradation was observed, and all generated degradants were confirmed based on PDA and mass spectral data. Mass balance is considered as met since stressed assay (%) and total degradation (%) are close to initial assay and impurity values.

### 3.4.3 | Neutral conditions

Based on the observed results and chromatograms, it can be concluded that rilpivirine HCl is not prone to degradation under neutral conditions when treated with water at room temperature for 30 days and at 80°C for 5 h. The observed results and chromatograms under these conditions were exactly in line with the results and chromatograms of the unstressed sample. Ideally, it was expected that (no degradation) based on published information that practically rilpivirine HCl is insoluble in water across a broad pH range.

### 3.4.4 | Oxidation conditions

Based on the total outcome (results and chromatograms) with oxidation conditions, it can be concluded that a solid drug substance sample of rilpivirine HCl is slightly susceptible to minor degradation under oxidation conditions with 3.0 and 30% $H_2O_2$ at room temperature. About 1.9% degradation was observed as active is degrading slowly at room temperature in 30% $H_2O_2$ for 30 days oxidation condition. However, it is prone to degradation with 3.0 and 30% $H_2O_2$ at 80°C for 5 h and forms total degradants of 7.5% and 22.7%, respectively. It was also identified that dissolved rilpivirine (in solution form) was prone to major degradation and responsible for forming the degradation product named nitrile impurity of rilpivirine (Impurity-C). Mass balance is considered as met since stressed assay (%) and total degradation (%) are near the values of the unstressed sample.

### 3.4.5 | Thermal conditions and heat with humidity conditions

Based on the observed results and chromatograms, it can be concluded that rilpivirine HCl is not susceptible to degradation under thermal conditions when exposed at 80 and 120°C for 30 days. As well, rilpivirine HCl is not prone to degradation under 70°C heat with 75% relative humidity conditions for 30 days. The observed results (assay, total impurities, and mass balance) and chromatograms under these conditions were exactly in line with the results and chromatograms of the unstressed sample.

### 3.4.6 | Metal ion conditions

Based on the observed results and chromatograms, it can be concluded that rilpivirine HCl is not susceptible to degradation under metal ion conditions with 0.3 mM $CuCl_2$ and 0.2 mM $FeCl_2.4H_2O$ solution for 30 days. The observed chromatograms and results under these conditions were exactly in line with the results and chromatograms of the unstressed sample.

### 3.4.7 | Photolytic conditions

Based on the observed results and chromatograms, it can be concluded that rilpivirine HCl is slightly susceptible to degradation under photolytic (ICH Light) conditions for three cycles. Photodegradation of the rilpivirine HCl drug substance was observed upon exposure to ICH light with isomerization of rilpivirine HCl, which yielded the *Z*-isomer of rilpivirine (Impurity-D). Besides the *Z*-isomer of rilpivirine, another degradation product named dimer impurity (Impurity-E) was detected. Mass balance is considered as met since stressed assay (%) and total degradation (%) are near to values of unstressed sample solution.

### 3.4.8 | Total summary of the forced degradation study

All major degradation compounds were separated, and no degradation compounds were found to co-elute with the active peak for rilpivirine. This proves that the proposed UHPLC method is the stability-indicating method. Not all degradation compounds observed in the force

degradation study experiment were structurally identified, since the observed degradation compounds have never been detected under accelerated or long-term storage conditions. The major outcome of force degradation studies on the solid drug substance of Rilpivirine HCl in reaction representation forms is presented in Figure 3. The detailed information (outcomes, chromatograms, and total degradations) of complete forced degradation studies is presented in Section 3.4, Figures S12–S30, and Table S15.

## 3.5 | Applicability of method in routine analysis

The applicability of the proposed validated methods in routine quality control testing was confirmed by conducting simultaneous analysis of chromatographic purity and assay determinations on two batches of bulk samples and tablet formulation samples. In addition to it, the same selected sample solutions were spiked with all five degradation products and analyzed on a PDA detector and a mass spectrometer detector. The observed content of all mentioned analytes in unspiked and spiked sample solutions was determined using known amounts of the reference standard rilpivirine HCl and determined values of RRFs for impurities. The generated results indicate the closeness of the observed values and the true values of respective selected batches of drug substance and tablet formulation. Based on the total outcome, it was confirmed that the proposed UHPLC method can be applied to any type of pharmaceutical formulation without any further conversion or after simple method verification. Additionally, it was confirmed that the proposed UHPLC-PDA-ESI-MS method is very useful for the correct identification and confirmation of all peaks based on observed molecular weight.

## 3.6 | Correct identification and confirmation by UHPLC-PDA-ESI-MS method

The correct identification and confirmation of all desired peaks and their results are important parameters in their quantifications. Sometimes, due to the use of different excipients in the drug products, such as tablet formulations, the complex sample matrix may have an impact on the analyte peak and the results. All standard and sample solutions were analyzed by the UHPLC-PDA-ESI-MS method, and the observed results were confirmed for all desired peaks in the sample chromatogram. Additionally, all prepared solutions in force degradation studies were also analyzed by this proposed method to confirm the peak of formed degradants. All analytes' peaks were pure, and no coelution was observed. The consistency of observed results in all sample solutions was demonstrated by comparing the values of molecular weights observed with those in a standard solution of all analytes. The consistency of observed *m/z* values with UHPLC-PDA-ESI-MS of all analytes was also confirmed with expected *m/z* values with ESI-MS, extracted from ChemDraw Software. Both isomers of rilpivirine (*E* and *Z*) and the dimer impurity of rilpivirine were identified in the negative mode as $(M\text{-}H)^-$ and the positive mode as $[M+H]^+$. While all three remaining degradation products were identified only in the positive mode as $[M+H]^+$ and confirmed as impurity-A, impurity-B, and impurity-C. The generated comprehensive data of mass spectral analysis and PDA data were very useful for the correct identification and confirmation of all desired peaks.

### 3.6.1 | Confirmation of rilpivirine

In the UHPLC chromatogram, the main peak eluted at a retention time of 11.4 min. was for the rilpivirine analyte. In mass spectral data, the molecular ion peak of rilpivirine was identified in positive and negative modes. However, in the negative mode, the peak intensity was higher than in the positive mode. In the negative mode of the mass spectra, the molecular ion peak $[M\text{-}H]^-$ of rilpivirine as a base peak appeared at *m/z* 365.3, having the molecular formula $C_{22}H_{18}N_6$. The exact molecular weight of rilpivirine is 366.4, and ideally, in mass spectra with ESI, it was expected to appear at *m/z* 365.4 in negative mode. The peak purity for rilpivirine was passed in PDA spectral data, and it was confirmed by mass spectral data in all injected solutions. The mass spectral data and observed online UV spectra for rilpivirine observed in an unspiked tablet sample solution are presented in Figure 4A,B.

### 3.6.2 | Confirmation of impurity-A

The peak for impurity-A in the UHPLC chromatogram was eluted at a retention time of 7.8 min. The molecular ion peak $[M+H]^+$ of impurity-A appeared at *m/z* 385.5, having molecular formula $C_{22}H_{20}N_6O$. The exact molecular weight of impurity-A is 384.4, and ideally, in a mass spectral analysis with ESI, it should appear at *m/z* 385.4. In the force degradation study, impurity-A was generated as a degradant due to hydrolysis (acidic and basic) and oxidative degradation of rilpivirine HCl. The increment in impurity-A was confirmed based on the increased peak area, and peak purity was confirmed by PDA spectral and mass spectral data for the respective peak. The mass

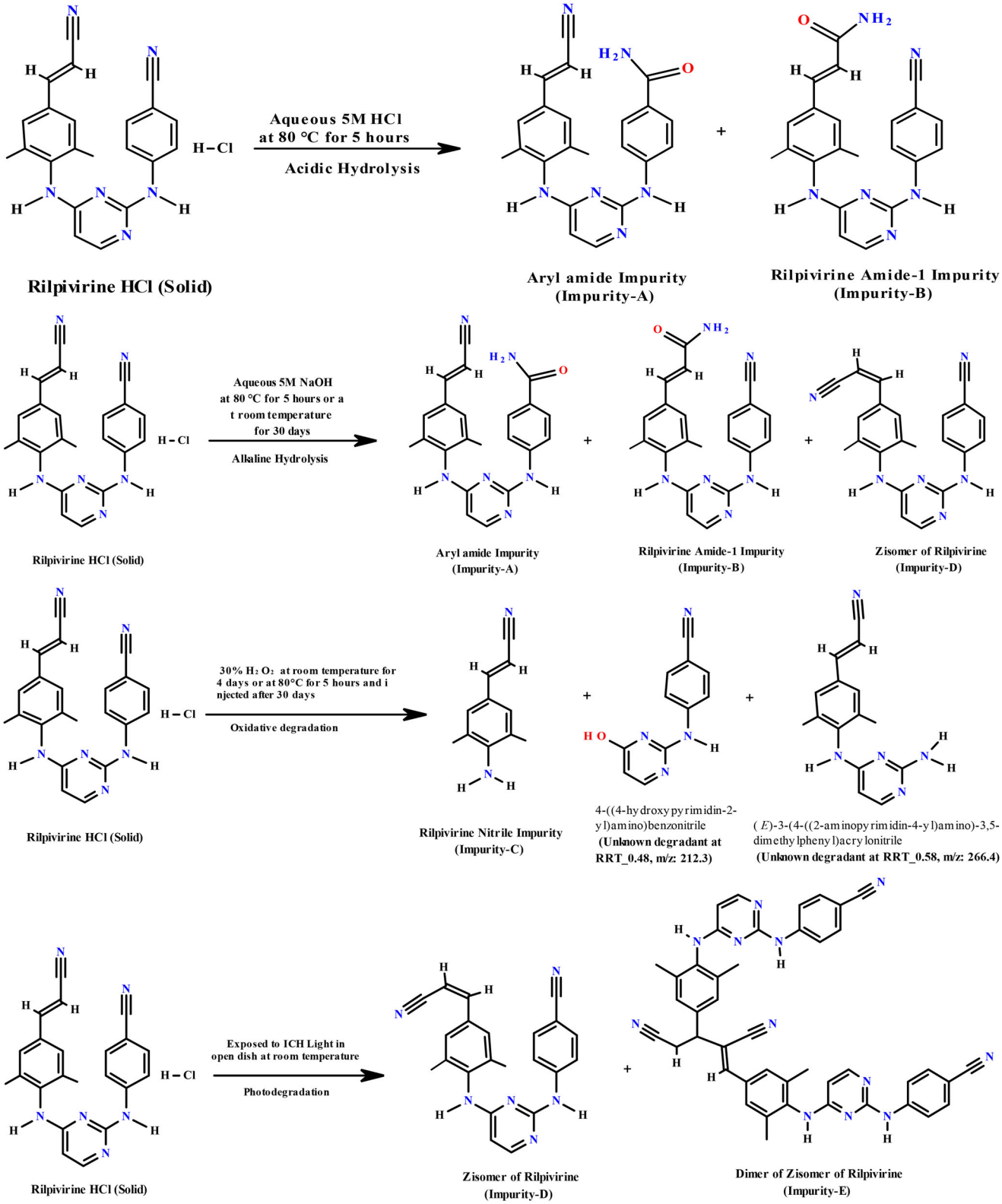


**FIGURE 3** Representation of forced degradation studies (hydrolysis, oxidation, and photodegradation) on the solid drug substance of rilpivirine HCl.

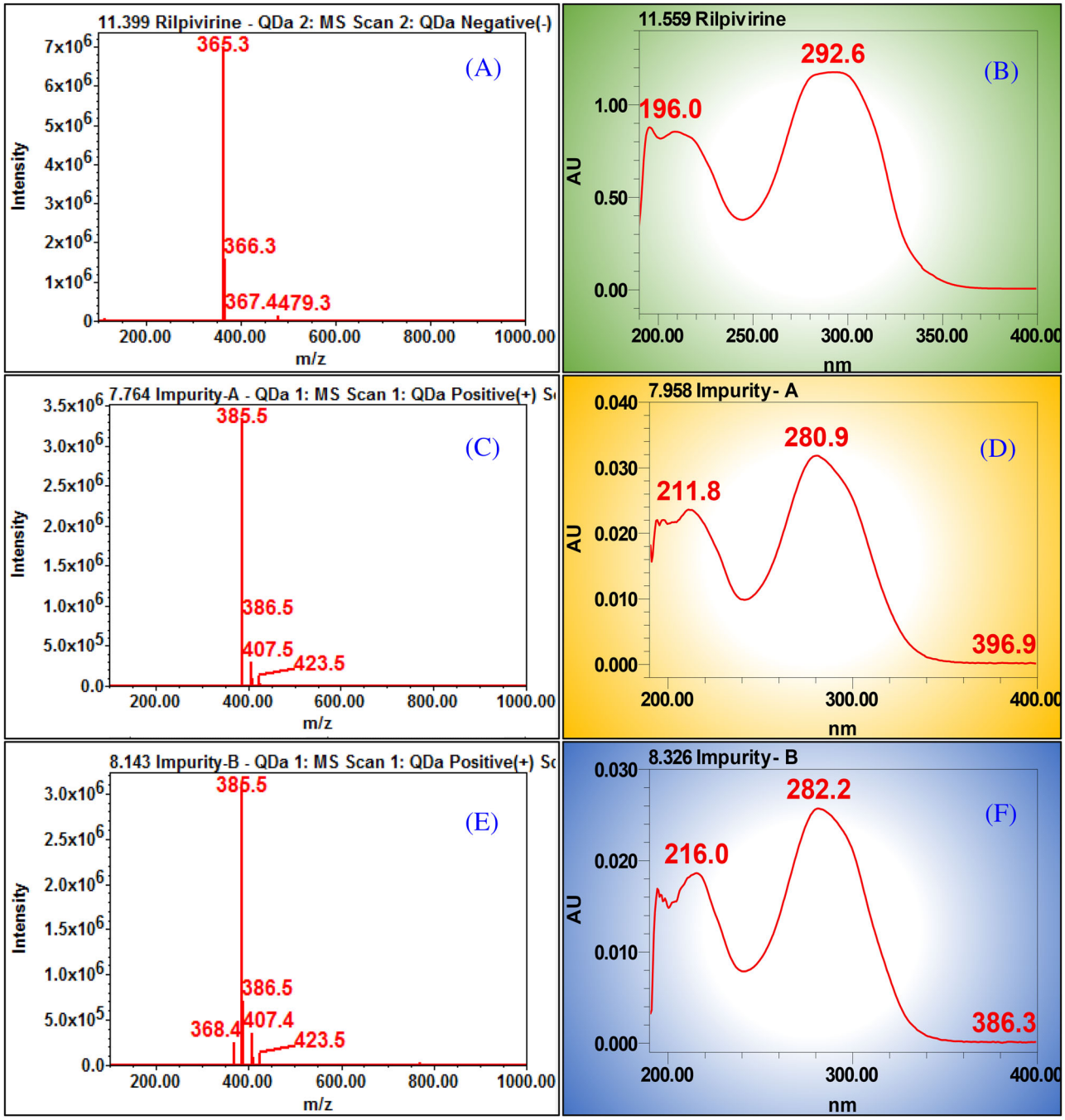


**FIGURE 4** Mass spectra and online UV absorption spectra recorded by photodiode array (PDA) detector for Rilpivirine and two degradation products (Impurity-A and Impurity-B).

spectral data and observed online UV spectra for impurity-A observed in HCl stressed sample solution are presented in Figure 4C,D.

### 3.6.3 | Confirmation of impurity-B

The peak for impurity-B in the UHPLC chromatogram was eluted at a retention time of 8.1 min. The molecular ion peak $[M+H]^+$ of impurity-B appeared at *m/z* 385.5, having molecular formula $C_{22}H_{20}N_6O$. The exact molecular weight of impurity-B is 384.4, and ideally, in a mass spectral analysis with ESI, it should appear at *m/z* 385.4. Impurity-B was generated due to the hydrolysis of one of the nitrile functional groups of rilpivirine HCl. The increment of impurity-B was identified in acidic and basic conditions degradation and confirmed the same based on the increased peak area, and peak purity was confirmed by

PDA spectral and mass spectrum data for respective peaks. In the stressing of rilpivirine HCl under these basic conditions, the rate of formation of impurity-B was greater than the rate observed in acidic conditions at higher temperatures. The mass spectral data and observed online UV spectra for impurity-B observed in NaOH-stressed sample solutions are presented in Figure 4E,F.

### 3.6.4 | Confirmation of impurity–C

The peak for impurity-C in the UHPLC chromatogram was eluted at a peak retention time of 10.3 min. The molecular ion peak $[M+H]^+$ of impurity-C appeared at *m/z* 173.3 and had molecular formula $C_{11}H_{12}N_2$. The exact molecular weight of impurity-C is 172.2, and ideally, in mass spectra with ESI, it was expected to appear at *m/z* 173.2. The traces of impurity-C were identified only in the oxidative degradation of dissolved rilpivirine with 30% $H_2O_2$ and exposed for 30 days. The formation of impurity-C was confirmed only by PDA spectral data, and due to the higher LOD for a mass spectrophotometer, it was not identified in the mass spectrum. The mass spectral data and observed online UV spectra for impurity-C observed in $H_2O_2$ stressed spiked sample solution is presented in Figure 5A,B.

### 3.6.5 | Confirmation of impurity–D

The peak for impurity-D in the UHPLC chromatogram is eluted at a peak retention time of 11.0 min. The molecular ion peak $[M-H]^-$ of impurity-D appeared at *m/z* 365.3 and is the base peak having the molecular formula $C_{22}H_{18}N_6$. The exact molecular weight of impurity-D is 366.4, and ideally, in a mass spectral analysis with ESI, it should appear at *m/z* 365.4 in negative mode. Isomerization of rilpivirine was observed in stressing with basic conditions and on exposure to the ICH light. In this isomerization conversion, the *E* isomer of rilpivirine was converted into the *Z*-isomer of rilpivirine as an impurity-D. The peak response intensity of the *Z*-isomer of rilpivirine (Impurity-D) was higher in the negative mode than the peak response intensity in the positive mode. The mass spectral data and observed online UV spectra for impurity-D observed in an ICH light-stressed sample solution are presented in Figure 5C,D.

### 3.6.6 | Confirmation of impurity–E

The peak for impurity-E in the UHPLC chromatogram is eluted at a peak retention time of 12.5 min. The molecular ion peak $[M-H]^-$ of impurity-E appeared at *m/z* 731.6, which is the base peak having molecular formula $C_{44}H_{36}N_{12}$. The exact molecular weight of the dimer impurity of rilpivirine is 732.9 and ideally, in mass spectra with ESI it was expected to appear at *m/z* 731.9 in negative mode. Impurity-E was identified as a degradant only in the stressing of rilpivirine HCl with ICH light and hence concluded as a photodegradation impurity. Due to ICH light exposure, the *E* isomer of rilpivirine was yielded in the *Z*-isomer of rilpivirine, and then due to photodegradation of the *Z*-isomer dimer impurity of rilpivirine was formed. The mass spectral data and observed online UV spectra for impurity-D observed in ICH light-stressed sample solution are presented in Figure 5E,F.

### 3.6.7 | Degradation product eluted at a relative retention time of 1.07 in ICH light sample

After exposure of rilpivirine HCl drug substance to ICH light, only three degradants were formed and identified in chromatographic analysis. Out of three degradants, two were identified as the *Z*-isomer of rilpivirine (Impurity-D) and the dimer impurity of rilpivirine (Impurity-E). The third identified degradant was eluted at a relative retention time of 1.07, and the same peak appeared at *m/z* 731.8 in negative mode. Based on data observed with PDA spectra and mass spectra, it was concluded that it was a possible isomer of a dimer impurity of rilpivirine.

## 3.7 | Significance of the proposed research work compared to published methods

Very few methods in the literature were reported, and these methods are not able to provide adequate separation of both isomers of rilpivirine from each other and their potential degradation products. The newly proposed method reduces these concerns; it is efficient in improving the separation and simultaneous quantification of both isomers and four potential degradants in a single run on the achiral column. The significances of the proposed research work are that: (1) The first reported research work investigated and confirmed the formation of rilpivirine amide-1 impurity (Impurity-B) due to base hydrolysis. (2) The first study described the formation of rilpivirine dimer impurity (Impurity-E) due to photodegradation (ICH light). (3) The presented work explored, verified, and showed the conversion of isomer (*E*) into isomer (*Z*), that is, the isomerization of rilpivirine due to base hydrolysis and photodegradation. This information will be helpful during the new formulation and packaging of drug products. Also, provide a

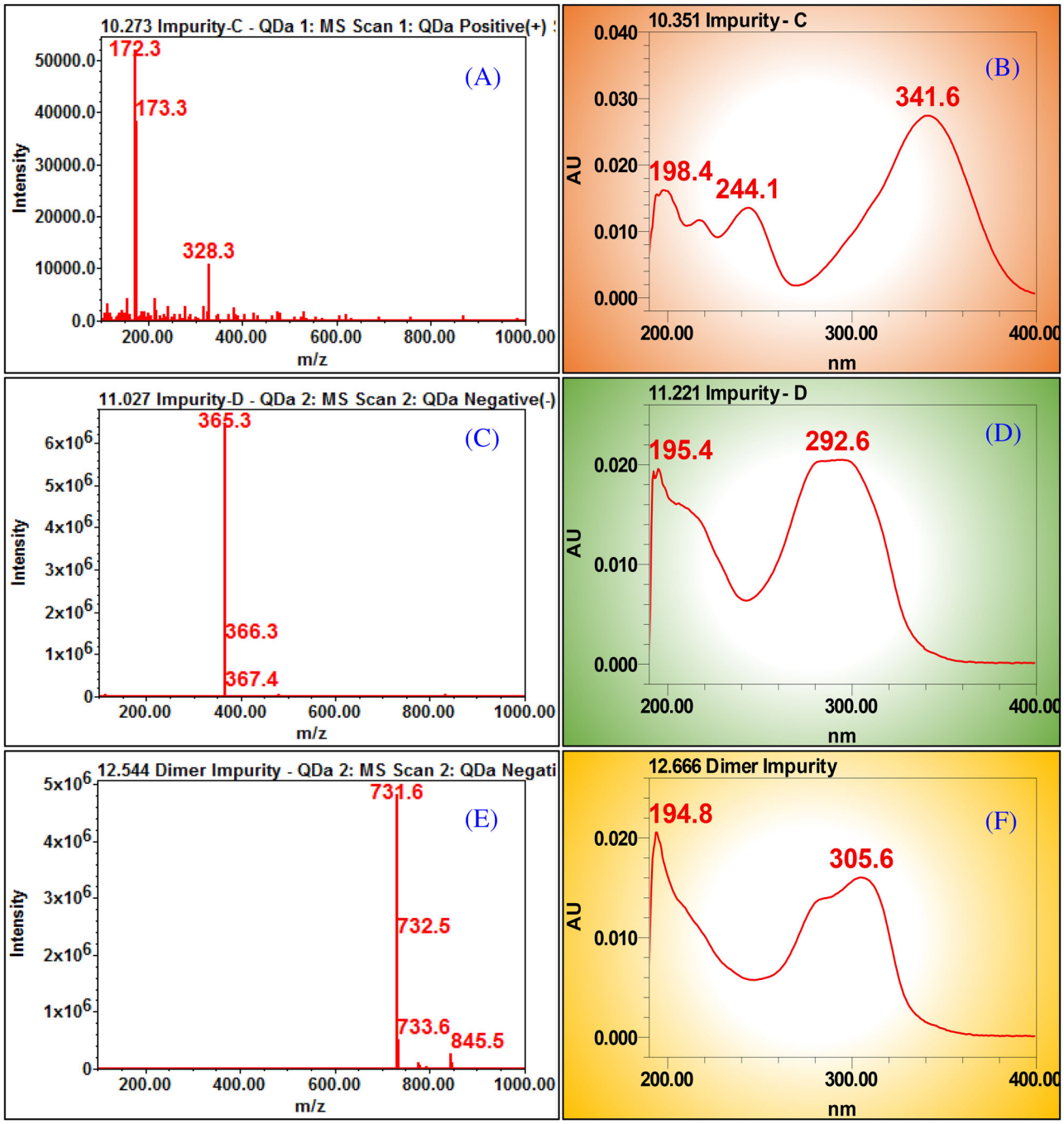


**FIGURE 5** Mass spectra and online UV absorption spectra recorded by photodiode array (PDA) detector for three degradation products (Impurity-C, Impurity-D, and Impurity-E).

method for the separation and quantification of a desired and undesired isomer of rilpivirine without the use of an achiral column. (4) The proposed method offers improved separation (minimum resolution 4.0), speed (all analytes elute within 13 min), and sensitivity (LOD: 0.03 and LOQ: 0.05 μg/ml) compared to published HPLC methods in the literature. (5) Simultaneous quantitative analysis of the assay and related substances of rilpivirine HCl. (6) Common methods for the analysis of drug substances and drug products (tablets) (7) The stability-indicating nature of the method is confirmed by forced degradation studies. (8) Capable of producing precise and accurate analytical data for a wide range of applications, including release analysis, investigational studies, stability studies, pharmacokinetic studies, toxicity studies, and bioequivalence studies. (9) Comprehensive data generated with a PDA detector and an MS detector is useful for the correct identification and confirmation of peaks based on spectra and molecular weight.

(10) The molecular weight of an unspecified degradation product or peak will be helpful to identify or predict the root cause or source of contamination in the analysis.

## 4 | CONCLUDING REMARKS

A simple, new, fast, and MS-compatible RP-UHPLC method was developed and then validated as per the ICH Q2 (R1) guideline for the simultaneous quantification of rilpivirine HCl and its five degradation products in bulk and tablet formulations for the first time. Notably, the assay of all six analytes was validated to detect concentrations at 0.03 μg/ml (LOD) and to quantify concentrations accurately and precisely at or above 0.05 μg/ml (LLOQ) in sample matrices, demonstrating improved sensitivity. The outcome of the robustness study revealed that this new method provides improved speed, separation, and sensitivity, as well as accurate and precise results over a wide operating range in a single run at a single wavelength. In the forced degradation study, it was confirmed and explained that the hydrolysis of rilpivirine HCl generated aryl amide impurity (Impurity-A) and rilpivirine amide-1 impurity (Impurity-B). Dissolved rilpivirine HCl undergoes oxidation at room temperature and generates traces of the rilpivirine nitrile impurity (Impurity-C). Photodegradation of the rilpivirine HCl drug substance and drug product was observed upon exposure to ICH light with isomerization and yielded the *Z*-isomer of rilpivirine (Impurity-D) and another degradation product named dimer impurity (Impurity-E) was detected. Moreover, the further analysis of all solutions with the UHPLC-PDA-ESI-MS method was helpful for the confirmation and correct identification of all analytes in the complex matrixes. This method offers well-separated sharp peaks in a low analysis time with fast and simple sample preparation and is inexpensive concerning solvent and reagent consumption. The accurate and precise results obtained in terms of the applicability of the method revealed the suitability and stability-indicating nature of the presented RP-UHPLC method. Hence, this method can be used by pharmaceutical quality control laboratories for release analysis, stability analysis, developmental studies, pharmacokinetics studies, and investigational studies. Based on the comprehensive outcome, we assured that the presented method has significance in the pharmaceutical sector and will provide meaningful support to ensure the efficacy, safety, and quality of rilpivirine HCl in bulk and tablet formulation.

## AUTHOR CONTRIBUTIONS

Deepak Krishna Mhaske: method development; investigation; validation; writing the original draft; review; and editing. Arjun Shankar Kumbhar: methodology; writing the original draft; reviewing; editing; supervision.

## ACKNOWLEDGMENTS

The authors would like to thank all individuals for providing the necessary extensive support in planning, execution, and the laboratory facility to achieve this milestone.

## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## FUNDING INFORMATION

This research work received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

## DATA AVAILABILITY STATEMENT

All related data are included in the manuscript as Supporting Information. Additional data are available from the authors upon request.

## ORCID

*Deepak Krishna Mhaske* https://orcid.org/0000-0001-8563-9185

*Arjun Shankar Kumbhar* https://orcid.org/0000-0001-5927-3482

## REFERENCES

1. https://www.who.int/images/default-source/departments/hiv/summary-of-the-global-hiv-epidemic-2021.png?sfvrsn=73ac5b6a_9. Accessed November 23, 2022.
2. https://newdrugapprovals.org/tag/rilpivirine/. Accessed November 23, 2022.
3. Mhaske DK, Kumbhar AS. Development and validation of rapid, timesaving, and cost-effective UHPLC method for simultaneous quantification of cinnarizine, its five specified impurities, two degradation products, and two antioxidants. Anal Chem Lett. 2022;12(4):488–504.
4. Bhavar GB, Pekamwar SS, Aher KB, Chaudhari SR. Development and validation of UV spectrophotometric method for estimation of rilpivirine hydrochloride in bulk and pharmaceutical formulations. Am J PharmTech Res. 2013;3(1):450–8.
5. Vijaya KS, Jain GV, Madhuri M. UV-visible spectrophotometric method for the estimation of rilpivirine hydrochloride in pharmaceutical dosage form by using multivariate technique. Chem Sci Trans. 2015;4(3):799–805.
6. Sudha T, Shanmugasundram P. Reverse phase high performance and HPTLC methods for the determination of rilpivirine bulk and in the tablet dosage form. World J Pharm Res. 2012;1(4):1183–96.
7. Somsubhra G, Sowjanya B, Laxmi PV, Vidyadhar S, David B, Subhadip R. Method development and validation of rilpivirine in bulk and tablet doses form by RP-HPLC method. Res. J Pharm Technol. 2013;6(3):240–3.
8. Alekhya CH, Vijay Shree K. RP-HPLC method development, and validation of rilpivirine hydrochloride quantification of saliva samples. SunText Rev Pharm Sci. 2020;1(1):104.

9. Date A, Shibata P, Destache Bruck CJ. Development and validation of a simple and isocratic reversed-phase HPLC method for the determination of rilpivirine from tablets, nanoparticles, and HeLa cell lysates. Biomed Chromatogr. 2015;29:709–15.
10. Patel S, Nagappan K, Santhosh GR. A new quantitative reverse-phase high-performance liquid chromatographic method for the quantification of Rilpivirine hydrochloride in bulk and dosage form. J Appl Pharm Sci. 2018;8(11):157–62.
11. Krishnaphanisri P, Sunitha K. Analytical method development and validation of cabotegravir and rilpivirine by RP-UPLC method. High Technol Lett. 2021;27(12):173–8.
12. Shibata M, Takahashi M, Yoshino M, Kuwahara T, Nomura T, Yokomaku Y, et al. Development and application of a simple LC-MS method for the determination of plasma rilpivirine (TMC-278) concentrations. J Med Investig. 2013;60(1-2):35–40.
13. Raju AV, Nemala AR. Development and validation of an LC-MS/MS method for the determination of rilpivirine in Sprague Dawley rat serum and its application to pharmacokinetic study. Asian J Biomed Pharm Sci. 2013;3:23–9.
14. Gupta A, Guttikar S, Patel Y, Shrivastav PS, Sanyal M. Reliable LC-MS/MS assay for the estimation of rilpivirine in human plasma: application to a bioequivalence study and incurred sample reanalysis. Drug Test Anal. 2015;7(4):290–9.
15. Burugula L, Pilli NR, Makula A, Lodagala DS, Kandhagatla R. Liquid chromatography-tandem mass spectrometric assay for the non-nucleoside reverse transcriptase inhibitor rilpivirine in human plasma. Biomed Chromatogr. 2013;(2):172–8.
16. Pranitha D, Vanitha C, Francies P. Simultaneous estimation of Emtricitabine, Tenofovir disoproxil fumarate, and Rilpivirine in bulk form by RP-HPLC method. J Pharm Res. 2012;5(8):4600–2.
17. Karunakranth D, Midha AK, Babu RS, Kishore D. Development and validation of HPLC method for simultaneous estimation of emtricitabine, rilpivirine, and tenofovir disoproxil fumarate tablet dosage form. Indian, J Res Pharm Biotechnol. 2018;6:8–15.
18. Venkatesan S, Kannappan N, Mannemala SS. Stability-indicating HPLC method for the simultaneous determination of HIV tablet containing emtricitabine, tenofovir disoproxil fumarate, and rilpivirine hydrochloride in pharmaceutical dosage forms. Int Sch Res Notices. 2014;2014:1–9.
19. Babu MN, Chandrasekar R. Development and validation of stability indicating RP-HPLC method for the simultaneous estimation of dolutegravir and rilpivirine by forced degradation studies. Int J Pharm Sci Res. 2021;12(9):4954–63.
20. Reddy KS, Shirisha SS, Kumar KP. Validated stability indicating RP-HPLC method for the simultaneous estimation of rilpivirine and dolutegravir in bulk form. Int J Pharm Sci & Res. 2020;11(10):4991–7.
21. Ismail Y, Prasad MVV, Shaheedha SM, Habeeb M. A new stability indicating RP-HPLC method development and validation for the simultaneous estimation of dolutegravir and rilpivirine in bulk and its dosage forms. Iran. J Pharm Sci. 2019;15(4):53–72.
22. Veeraswami B, Naveen VMK. Development and validation of RP-HPLC method for the estimation of dolutegravir and rilpivirine in bulk and pharmaceutical dosage form and its application to rat plasma. Asian J Pharm Clin Res. 2019;12(2):267–71.
23. Tempestilli M, Ammassari A, D'Avolio A. Development and validation of an HPLC-UV method for quantification of elvitegravir and two other new antiretrovirals, dolutegravir and rilpivirine, in the plasma of HIV-positive patients. Biomed Chromatogr. 2018;e4274.
24. Vijaya SK, Madhuri M, Anusha V, Siresha VRK. Development, Validation of RP-HPLC method for the simultaneous estimation of Tenofovir disproxil fumarate, emtricitabine, and Rilpivirine hydrochloride in the bulk formulation and used in nanosuspension. Indian Drugs. 2019;56(09):43–54
25. Panigrahy UP, Reddy ASK. A novel validated RP-HPLC method for the simultaneous estimation of Emtricitabine, Tenofovir Disoproxil Fumarate, and Rilpivirine in bulk and pharmaceutical tablet dosage forms. Der Pharm Lett. 2015;7(1):303–14.
26. Cozzi V, Charbe N, Baldelli S, Castoldi S, Atzori C, Cattaneo D, et al. Development and Validation of a Chromatographic Ultraviolet Method for the Simultaneous Quantification of Dolutegravir and Rilpivirine in Human Plasma. Ther Drug Monit. 2016;38(3):407–13.
27. Grégoire M, Deslandes G, Renaud C. A liquid chromatography-tandem mass spectrometry assay for quantification of rilpivirine and dolutegravir in human plasma. J Chromatogr B Anal Technol Biomed Life Sci. 2014;971:1–9.
28. Parsons TL, Marzinke MA. Development and validation of a liquid chromatographic-tandem mass spectrometric method for the multiplexed quantification of etravirine, maraviroc, raltegravir, and rilpivirine in human plasma and tissue. J Pharm Biomed Anal. 2016;30(131):333–44.
29. Else L, Watson V, Tjia J, Hughes A, Siccardi M, Khoo S, et al. Validation of a rapid and sensitive high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) assay for the simultaneous determination of existing and new antiretroviral compounds. J. Chromatogr B: Anal Technol Biomed Life Sci. 2010;878(19):1455–65.
30. Zheng Y, Aboura R, Boujaafar S, Lui G, Hirt D, Bouazza N, et al. HPLC-MS/MS method for the simultaneous quantification of dolutegravir, elvitegravir, rilpivirine, darunavir, ritonavir, raltegravir and raltegravir-β-D-glucuronide in human plasma. J Pharm Biomed Anal. 2020;182:113–9.
31. Prathipati PK, Mandal S, Destache CJ. Simultaneous quantification of tenofovir, emtricitabine, rilpivirine, elvitegravir and dolutegravir in mouse biological matrices by LC–MS/MS and its application to a pharmacokinetic study. J Pharm Biomed Anal. 2016;129:473–81.
32. Khaleel N, Abdul Rahaman SK. Stability-indicating RP-UPLC method for the simultaneous determination of dolutegravir and rilpivirine in Bulk and Pharmaceutical Dosage Form. Der Pharmacia Lettre. 2019;11(2):29–39.
33. Kavitha KY, Geetha G, Hariprasad R, Venkatnarayanan R, Kaviarasu M. Development and validation of RP-UPLC analytical method for simultaneous estimation of the emtricitabine, tenofovir disoproxil fumarate and rilpivirine, and its pharmaceutical dosage form. Int Res J Pharm. 2013;4(1):150–5.
34. Gouget H, Noé G, Barrail-Tran A, Furlan V. UPLC–MS/MS method for the simultaneous quantification of bictegravir and 13 others antiretroviral drugs plus cobicistat and ritonavir boosters in human plasma. J Pharm Biomed Anal. 2020;181:113057.
35. Courlet P, Alves Saldanha S, Cavassini M, Marzolini C, Choong E, Csajka C, et al. Development and validation of a multiplex UHPLC-MS/MS assay with stable isotopic internal standards for the monitoring of the plasma concentrations of the antiretroviral

drugs bictegravir, cabotegravir, doravirine, and rilpivirine in people living with HIV. J Mass Spectrom. 2020;55(6):e4506.
36. Simiele M, Ariaudo A, De Nicolò A, Favata F, Ferrante M, Carcieri C, et al. UPLC-MS/MS method for the simultaneous quantification of three new antiretroviral drugs, dolutegravir, elvitegravir, and rilpivirine, and other thirteen antiretroviral agents plus cobicistat and ritonavir boosters in human plasma. J Pharm Biomed Anal. 2017;138:223–30.
37. Marineni B, Krishna V, Sreenivasulu Reddy T. A validated stability indicating HPLC assay method for rilpivirine HCl in bulk drug. Int J Pharm Biol Sci. 2017;3(4):278–87.
38. Chilukuri M, Hussain KR, Narayanareddy P, Venkataramana M. Degradation pathway for rilpinavir hydrochloride by validated stability indicating UP-LC method. Int J Clin Pharmacol Ther Toxicol. 2012;1(1):1–8.
39. Vejendla1 A, Talari S, Moturu R, Murthy Boddapati SN, Emmanuel Kola A. Method development and validation for Cabotegravir and Rilpivirine by using HPLC and its degradants are characterized by LCMS and FTIR. Future J Pharm Sci. 2021;7:226.
40. ICH, Q2 (R1). Validation of analytical procedures: text and methodology, ICH Harmonized Tripartite Guideline. 2005
41. ICH of technical requirements for registration of pharmaceuticals for human use. ICH Q2(R2)/Q14 EWG analytical procedure development and revision of Q2(R1) analytical validation (business plan, concept paper, work plan). 2018. Available from: https://www.ich.org/page/quality-guidelines Accessed November 23, 2022

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Mhaske DK, Kumbhar AS. Simultaneous quantification of (*E*) and (*Z*) isomers of rilpivirine and four degradation products in bulk and tablets by reversed-phase ultra-high-performance liquid chromatography and confirmation of all by molecular weight. J Sep Sci. 2023;2201067.
https://doi.org/10.1002/jssc.202201067