

Degradation Pathway for Rilpivirine Hydrochloride by Validated Stability Indicating UP-LC Method

Review Article

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Abstract

Degradation pathway for Rilpivirine Hydrochloride is established as per ICH recommendations by validated and stability indicating reverse phase liquid chromatographic method. Rilpivirine Hydrochloride is subjected to stress conditions of acid, base, oxidation, thermal and photolysis. Significant degradation is observed in acid and base stress conditions. Six impurities are studied and the major degradant (RRT about 0.52) is identified by LC-MS and spectral analysis. The stress samples are assayed against a qualified reference standard and the mass balance is found close to 99.5 %. Efficient chromatographic separation is achieved on a Shimpack XR –ODS-II stationary phase with simple mobile phase combination delivered in gradient mode and quantification is carried at 295 nm at a flow rate of 1.0 mL min⁻¹. In the developed LC method the resolution between Rilpivirine Hydrochloride and six potential impurities (imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6) is found to be greater than 2.0. Regression analysis shows an r value (correlation coefficient) of greater than 0.995 for Rilpivirine Hydrochloride and six potential impurities. This method is capable to detect the impurities of Rilpivirine Hydrochloride at a level of 0.006 % with respect to test concentration of 1.0 mg mL⁻¹ for a 4- μ L injection volume. The developed LC method is validated with respect to specificity, linearity & range, accuracy, precision and robustness for impurities and degradant determination.

Keywords: Rilpivirine Hydrochloride; UP-LC; LC-MS; Forced Degradation; Validation; Stability-Indicating.

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Introduction

Rilpivirine Hydrochloride, 4-{{4-((E)-2-cyanovinyl)-2,6-dimethylphenyl}amino}pyrimidin-2-yl}amino}benzotrile, is a pharmaceutical drug, developed for the treatment of HIV infection in combination with other antiretroviral agents. Inhibits HIV-1 replication by noncompetitive inhibition of HIV-1 reverse transcriptase (RT). Trade name for Rilpivirine is edurant. Rilpivirine is a nonnucleoside reverse transcriptase inhibitor (NNRTI). It works by blocking the growth of HIV. It works by slowing the spread of HIV in the body. Rilpivirine does not cure HIV infection and may not prevent you from developing HIV-related illnesses. Rilpivirine does not prevent you from spreading HIV to other people. Rilpivirine was approved by the U.S. Food and Drug Administration (FDA) on May 20, 2011, for use in combination with other antiretroviral agents for the treatment of HIV-1 infection in treatment of HIV-1 infection in adult patients. A few chromatographic methods have appeared in the literature for the quantification of Rilpivirine hydrochloride in 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 [1-7].

To the best of our knowledge, no stability-indicating LC method for the quantitative estimation of Rilpivirine hydrochloride in bulk drug substance samples in the presence of degradation products along with its potential impurities has been reported. The purpose of the present research work is to develop a single stability-indicating UP LC method for the determination of Rilpivirine hydrochloride and its related impurities and to establish the degradation pathway for Rilpivirine hydrochloride along with its six potential impurities. The developed LC method is validated with respect to specificity, LOD, LOQ, linearity, precision, accuracy and robustness. Accordingly the aim of the present study is to establish degradation pathway of Rilpivirine hydrochloride through stress studies under a variety of ICH recommended test conditions. [8-10, 17].

Experimental

Chemicals

Samples of Rilpivirine hydrochloride and its related impurities are received from Hetero labs limited a research foundation of the firm Hetero Labs Ltd, Hyderabad, India (Fig. 1). All impurities and the Rilpivirine hydrochloride standard are of > 99% purity and as follows: Rilpivirine hydrochloride (99.1%), imp-1 (99.5%), imp-2 (95.6%), imp-3 (99.7%), imp-4 (99.5%) imp-5 (99.3%) and imp-6 (99.0%). In addition, HPLC grade acetonitrile and methanol are purchased from Merck (Darmstadt, Germany). Analytical reagent grade sodium dihydrogen phosphate monohydrate, phosphoric acid and acetic acid are purchased from Merck. Highly pure water was prepared with the Millipore Milli-Q Plus water purification system.

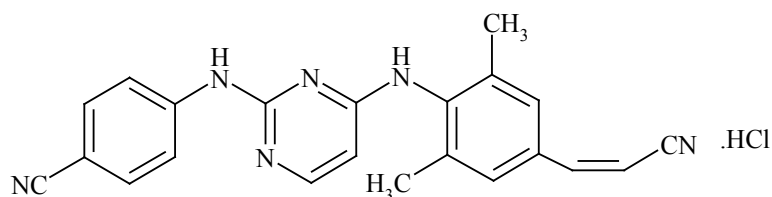
Equipment

The LC system used for method development, forced degradation studies and method validation consisted of a Waters 2695 binary pump with an auto sampler and a 2996 photo diode array detector (PDA). The output signal is monitored and processed using Empower software on a

Table 1: System suitability report

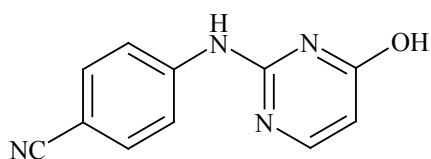
Compound	USP Resolution (RS)	Tailing factor	No. of theoretical plates (USP tangent method)
Impurity-1	--	1.198	4418
Impurity-5	6.407	1.004	7588
Impurity-2	3.370	1.023	3699
Impurity-3	10.374	0.978	10310
Impurity-6	2.099	0.998	12590
Impurity-4	2.101	1.150	15959
Rilpivirine hydrochloride	2.459	1.128	16547

Figure. 1: Chemical structures and names of Rilpivirine hydrochloride and its impurities

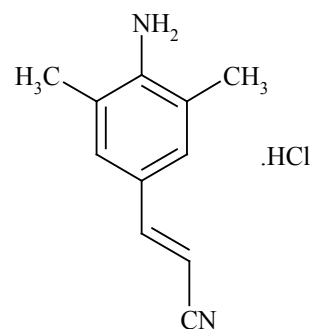


Rilpivirine hydrochloride:

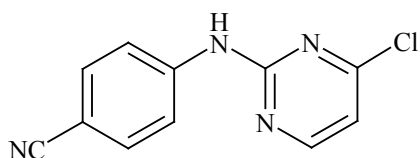
4-{{4-({4-[(E)-2-cyanovinyl]-2,6-dimethylphenyl} amino) pyrimidin-2-yl} amino} benzonitrile Hydrochloride (molecular weight 366.42) C₂₂H₁₈N₆



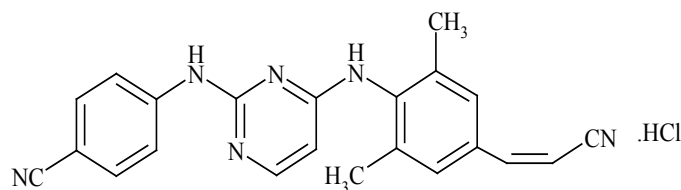
Impurity 1: 4-(4-Hydroxypyrimidin-2-ylamino) benzonitrile (molecular weight 212.21) C₁₁H₈N₄O



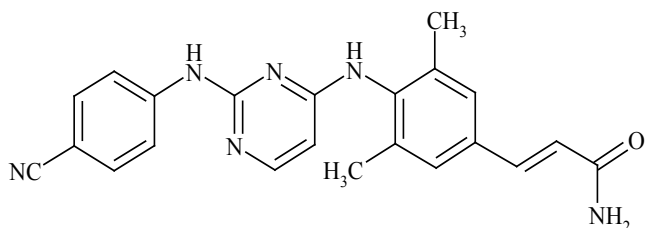
Impurity 2: (2E)-3-(4-Amino-3,5-dimethylphenyl) acrylonitrile hydrochloride (molecular weight 208.69) C₁₁H₁₂N₂ HCl



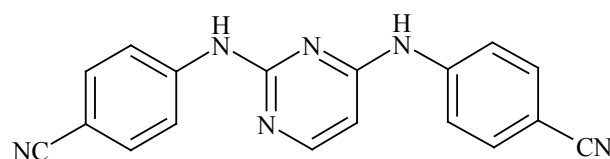
Impurity 3 : 4-[(4-Chloro-2-pyrimidinyl)amino] benzonitrile (molecular weight 230.65) C₁₁H₇ClN₄ .



Impurity 4 : 4-{{4-({4-[(Z)-2-Cyanovinyl]-2,6-dimethylphenyl} amino) pyrimidin-2-yl} amino} benzonitrile hydrochloride (molecular weight 402.88) C₂₂H₁₈N₆ HCl.



Impurity 5: (E)-3-(4-(2-(4-Cyanophenylamino) pyrimidin-4-ylamino) -3, 5-dimethylphenyl) acrylamide (molecular weight 384.43) C₂₂H₂₀N₆O.



Impurity 6: 4,4'-(Pyrimidine-2,4-diylbis(azanediyl)) dibenzonitrile (molecular weight 312.33) C₁₈H₁₂N₆.

Pentium computer (Digital equipment Co.). Photo stability studies are carried out in a photo stability chamber (Atlas Suntest CPS+). Thermal stability studies are carried out in a dry hot air oven (Cintex precision hot air oven).

Chromatographic conditions

A Shim-pack XR-ODS-II 75mm x3.0 mm column is used with a mobile phase containing a gradient of solvents A and B. The buffer is composed of 0.1 M Ammonium acetate, with its pH adjusted to 4.0 with acetic acid. Solvents A and B consisted of buffer and acetonitrile in ratios of 500:500 (v/v). The flow rate of the mobile phase is 0.5 ml/min with a gradient program of 0/50, 5/65, 6/65, 7/50, 8/50 and 10/50 (time (min) / %B). The column temperature is maintained at 45°C and the detection wavelength is set at 295 nm. The injection volume is 4 µl. The diluent consisted of buffer and acetonitrile in a ratio of 50:50 (v/v).

LC-Mass Spectrum conditions

The LC-MS system (Agilent 2100 EV series liquid chromatography system triple quadrupole mass spectrometer) was used for the identification of unknown compounds formed during forced degradation. An Inertsil ODS-3V 250 x 4.6 mm, 5-µm column is used as the stationary phase. Acetonitrile and methanol is used as mobile phase for isocratic mode. 0.01M ammonium acetate and the pH is adjusted to 4.0 using acetic acid and are used as buffer. The flow rate was 0.8 ml/min. The injection volume is 20 µl. The analysis is performed in positive and negative electrospray ionization modes. The capillary and cone voltages are 3.50 kV and 25 V, respectively. The source and dissolution temperatures are 120°C and 350°C, respectively, and the cone gas flow and dissolution gas flow are 100 Lhr⁻¹ 650 Lhr⁻¹ respectively.

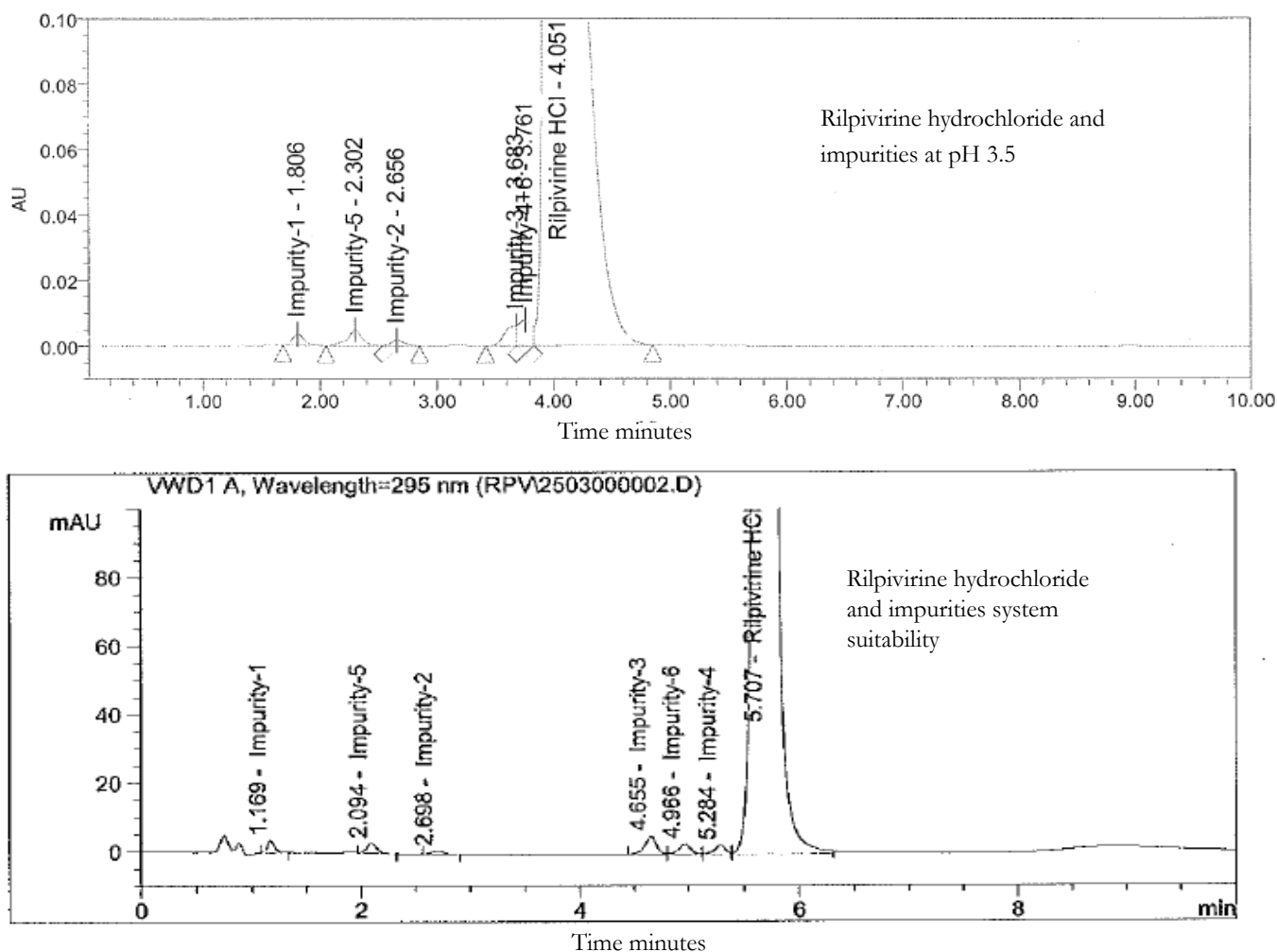
Preparation of standard solutions and sample solutions

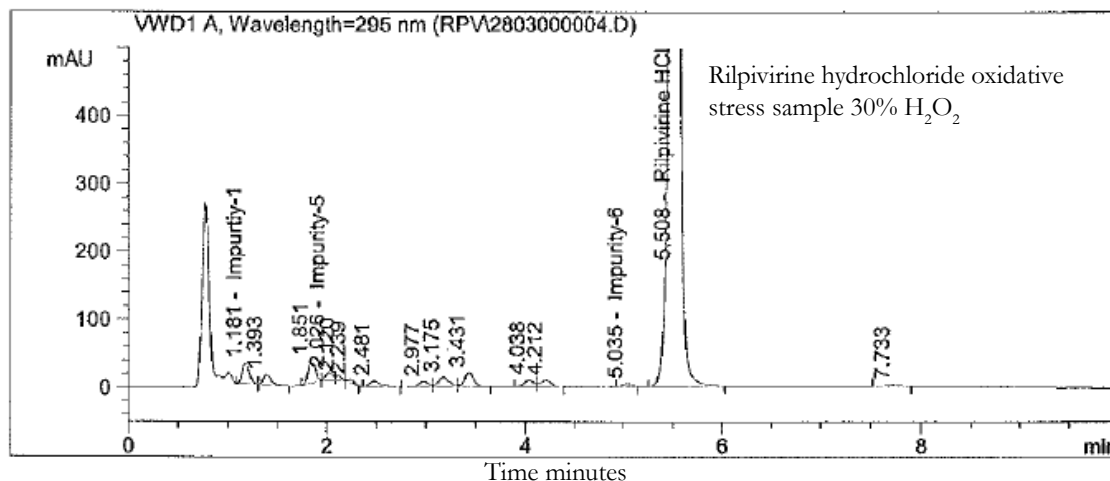
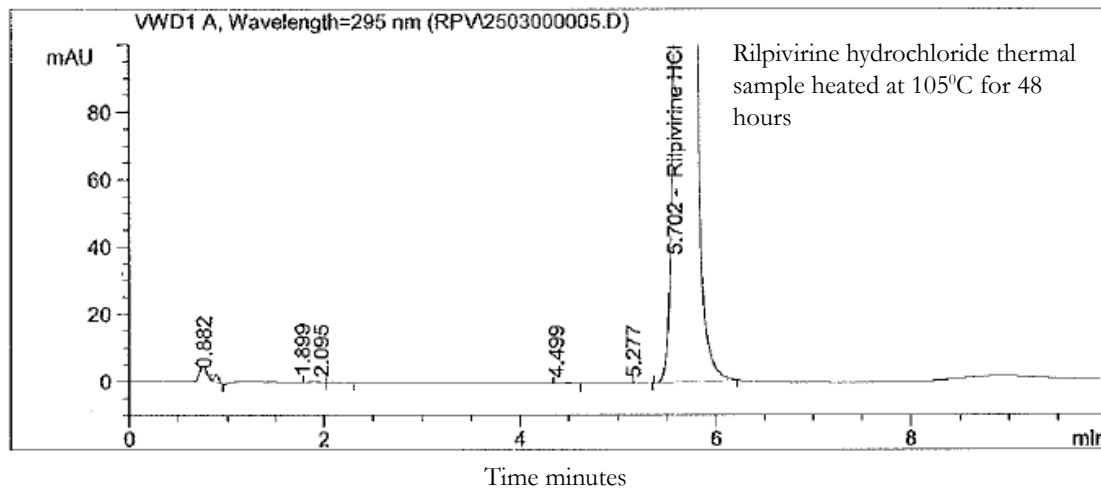
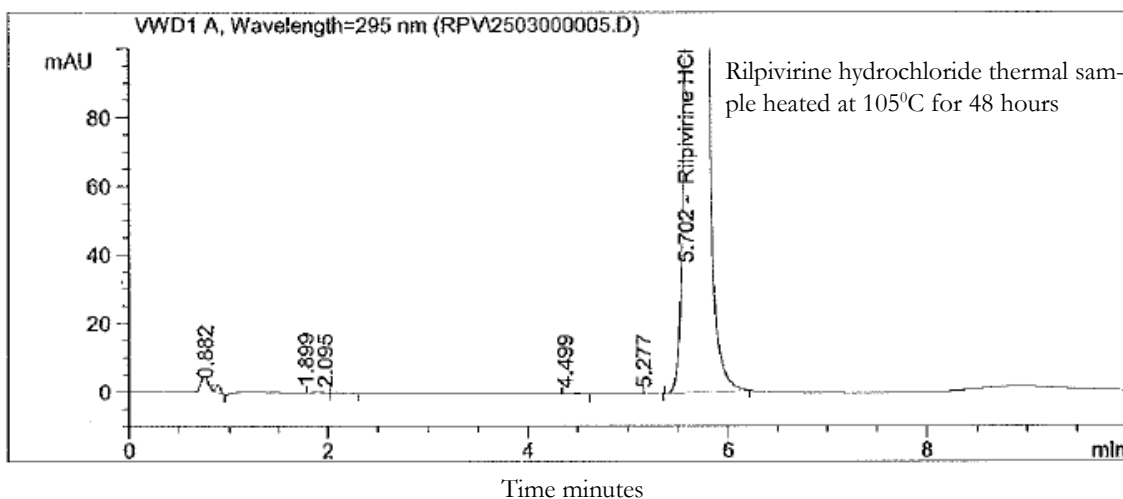
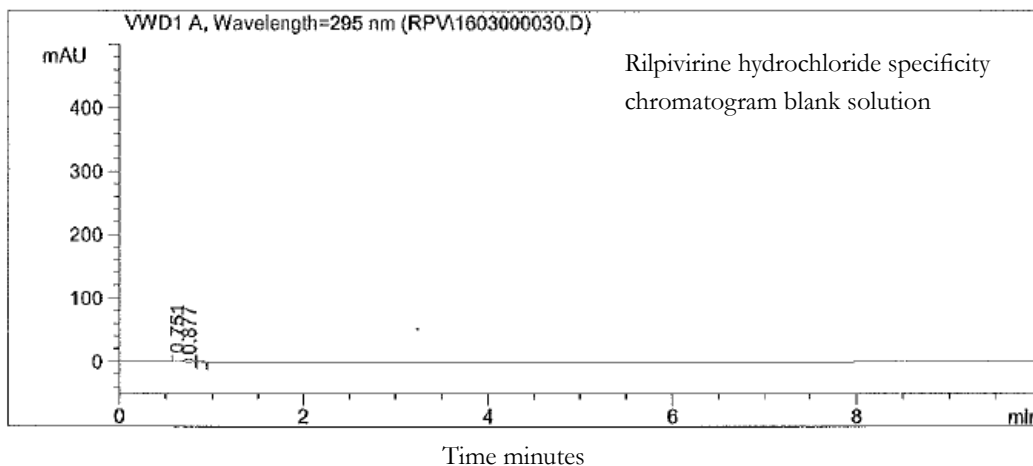
A stock solution of Rilpivirine hydrochloride (1.0 mg/ml) is prepared by dissolving the appropriate amount of Rilpivirine hydrochloride solid in the diluent. Working solutions of Rilpivirine hydrochloride 0.10 % and for impurities 0.15% are prepared from the stock solution for the determinations of related substances respectively. A stock solution of impurities (mixture of imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6) at 0.05 mg/ml is also prepared in the diluent. The drug substance powder equivalent to 100 mg of sample is transferred into a 100-ml volumetric flask, and 70 ml of diluent is added. The flask is attached to a rotary shaker and shaken for 10 min to disperse the powder completely. The mixture is sonicated for 10 min and then diluted to the appropriate volume with diluent to make a solution containing 1.0 mg/ml. The solution is then filtered through a 0.45-µ Nylon 66 membrane filter.

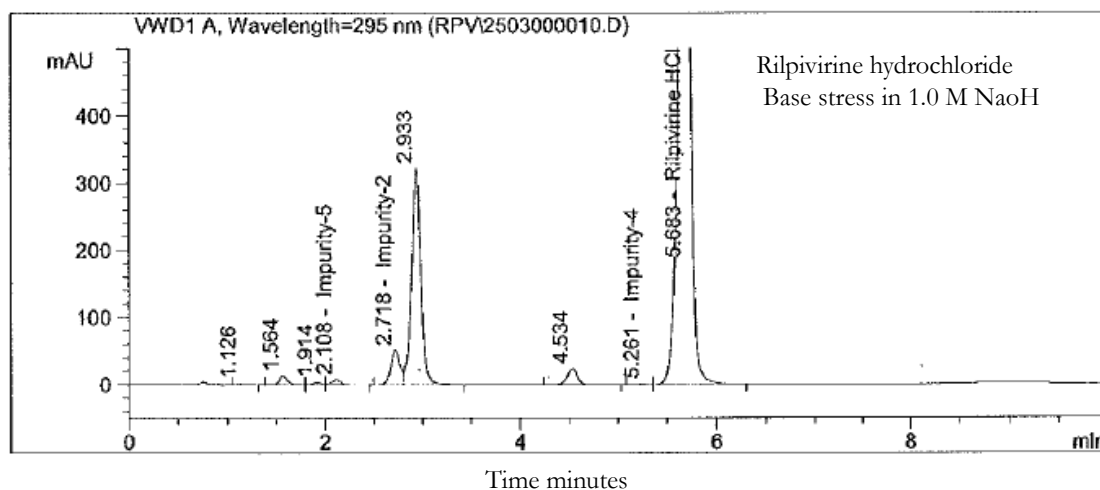
Stress studies / Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities.[9-10] The specificity of the developed UPLC method for Rilpivirine hydrochloride is determined in the presence of its six related impurities (namely imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6) and degradation products. Forced degradation studies are also performed on Rilpivirine hydrochloride to provide an indication of the stability-indicating property and specificity of the proposed method [9-16]. The stress conditions employed for the degradation study included light (carried out as per ICH Q1B), heat (105°C), acid hydrolysis (2 M HCl), base hydrolysis (1.0 M NaOH) and oxidation (30% H₂O₂). For heat and light studies, the samples are exposed for 48 hrs, whereas the samples are treated for 2 h for acid, base hydrolysis and for oxidation.

Figure 2: Typical chromatogram from the method development trials and subsequent optimized conditions and stressed Rilpivirine hydrochloride samples.







The peak purity of the Rilpivirine hydrochloride stressed samples is also checked by using a waters 2996 photo diode array detector (PDA). The purity angle is within the purity threshold limit in all of the stressed samples, demonstrating the homogeneity of the analyte peak. The contents of impurities were calculated for the stress samples against a qualified reference standard. The mass balance (% assay + % of impurities + % of degradation products) is calculated for all of the samples.

Analytical Method validation

The proposed method was validated per ICH guidelines. [8]

Precision

The precision of the related substance method is investigated by injecting six individual preparations of (100% sample) Rilpivirine hydrochloride spiked with 0.15% each of imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6. The %RSD of the areas of imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 is calculated. The intermediate precision of the method is evaluated using a different analyst and instrument located within the same laboratory.

The precision of the method is evaluated by carrying out six independent preparations of a test sample of Rilpivirine hydrochloride against a qualified reference standard. The %RSD of six obtained values are calculated.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for Rilpivirine hydrochloride, imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 are estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. The precision study is also carried out at the LOQ level by injecting six individual preparations of Rilpivirine hydrochloride, imp-1, imp-2, imp-3, imp-4, imp-5, imp-6 and calculated the %RSD of the areas.

Linearity & Range

Linearity test solutions for the method are prepared from a stock solution at six concentration levels from 50 to 150% of the impurities concentration (50(0.075%), 75(0.12%), 100(0.15%), 125(0.18%) and 150(0.225%). The peak area versus concentration data is analyzed with least-squares linear regression. Linearity test solutions for the related substance method are prepared by diluting the impurity stock solution (2.5) to the required concentrations. The solutions are prepared at six concentration levels from the LOQ to 150%. The solution concentrations used for Rilpivirine hydrochloride is LOQ, 0.05%, 0.08% 0.10%, 0.12%, 0.15%. The peak area versus concentration data is analyzed with least-squares linear regression. Linearity test solutions for the related substance method are prepared by diluting the impurity stock solution (2.5) to the required concentrations. The solutions are prepared

Table 2: Summary of forced degradation results

Stress condition	%Total Impurities	Study Time	% Assay of active substance	Mass balance (% assay + % impurities + % degradation products)	Remarks
Acid hydrolysis (2 M HCl)	0.12%	2 h	99.4	99.5	Prominent Degradation observed
Base hydrolysis (1.0 M NaOH)	24.4%	2 h	75.2	99.6	major degradation product was formed RRT about 0.52 and identified as (E)-methyl4-(4-(4-(2-cynovinyl)-2,6-dimethylphenylamino) pyrimidine-2-ylamino benzoate
Oxidation (30% H2O2)	8.54%	2 h	91.2	99.7	Prominent Degradation observed
Thermal (100°C)	0.07%	2 days	99.5	99.6	No degradation products formed
Light (photolytic degradation)	0.09%	1200 KLUX/ Hr	99.4	99.5	No degradation products formed

Figure 3: LC-Mass Chromatograms

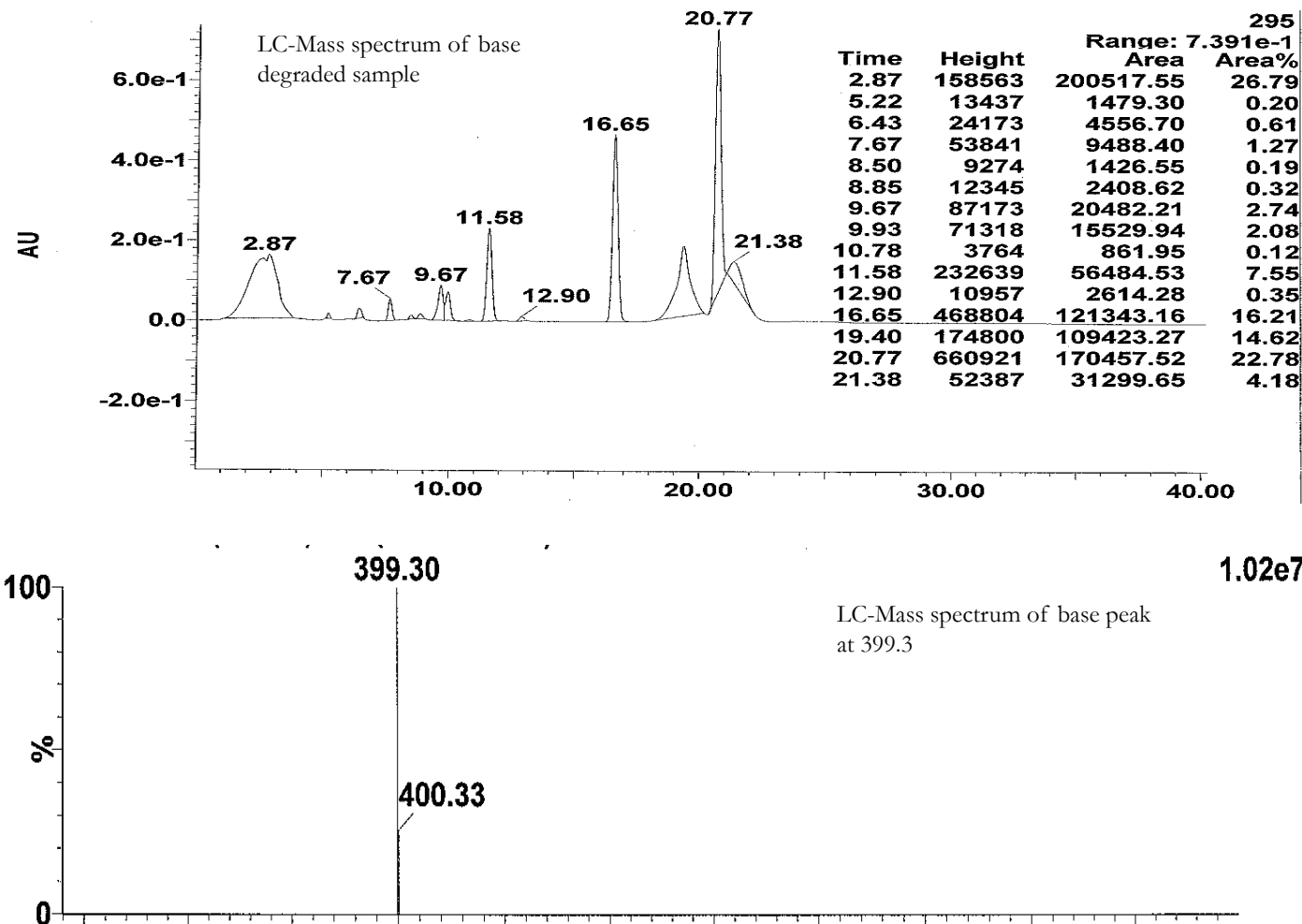


Table 3: LOD, LOQ, Regression and precision data

Parameter	Rilpivirine hydrochloride	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6
LOD (%)	0.007	0.010	0.009	0.008	0.006	0.006	0.006
LOQ (%)	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Slope (m)	125.9925	46.834	229.02	111.51	113.48	146.71	154.55
Intercept(C)	-0.01437	0.00092	-0.05067	-0.02311	0.00121	0.02469	-0.06247
Correlation coefficient	0.9999	0.9999	0.9998	1.00000	0.9999	0.9999	0.9999
Precision (%RSD) ^a	4.0	3.0	3.8	2.5	3.2	3.2	3.2

Linearity range was LOQ-150% w r t 1.0 mg/ml Rilpivirine hydrochloride for impurities; Linearity range was 50-150% of Rilpivirine hydrochloride. a Six determinations using LOQ solutions for impurities and Rilpivirine hydrochloride.

at six concentration levels from the LOQ to 150%. The slope and y-intercept of the calibration curve is reported.

Accuracy

Accuracy of the method is evaluated in triplicate at three concentration levels 50(0.075%), 100(0.15%) and 150(0.225%), and the percentage recoveries are also calculated. Rilpivirine hydrochloride did show the presence of imp-2 imp-4 at minor concentrations, and also contained 0.02% of unknown. Standard addition and recovery experiments are conducted to determine the accuracy of the related substance method for the quantification of all six impurities (imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6) in the drug substance. The study is carried out in triplicate at LOQ level, 0.075%, 0.15% and 0.225% of the analyte concentration

(1.0 mg/ml). The percentage of recoveries for imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 are calculated.

Robustness

To determine the robustness of the developed method, the experimental conditions are altered and the resolution between Rilpivirine hydrochloride and imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 is evaluated. The flow rate of the mobile phase is 0.5 ml/min. To study the effect of the flow rate on the resolution, the flow rate is changed by 0.1 units (to 0.4 and 0.6 ml/min). The effect of pH on the resolution of the impurities is studied by varying the pH by ± 0.2 units (buffer pH of 4.2 and 3.8). The effect of the column temperature on the resolution is studied at 40°C and 50°C instead of 45°C. In all these varied conditions, the components of

the mobile phase remained constant, as outlined in Section 2.3.

Solution stability and mobile phase stability

The solution stability of Rilpivirine hydrochloride in the impurities method was carried out by leaving both the sample and reference standard solutions in tightly capped volumetric flasks at room temperature for 48 h. The same sample solutions are assayed for in 6-h intervals over the study period. The mobile phase stability was also examined by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions for 6 h intervals up to 48 h. The prepared mobile phase remained constant during the study period. The %RSD of the Rilpivirine hydrochloride assay is calculated for the mobile phase and solution stability experiments. The solution stability of Rilpivirine hydrochloride and its impurities in the related substance method is carried out by leaving a spiked sample solution in a tightly capped volumetric flask at room temperature for 48 h. The content of imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 is determined at 6 h intervals up to the study period. The mobile phase stability is also investigated for 48 h by injecting the freshly prepared sample solutions for every 6 h interval. The content of imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 is determined in the test solutions. The prepared mobile phase remained constant during the study period.

Results and Discussion

Method development and optimization

The main objective of the chromatographic method was to separate imp-1, imp-2, imp-3, imp-4, imp-5, imp-6 and Rilpivirine hydrochloride and the generated degradation products from the analyte peak during stress studies. Impurities and degradation products are co-eluted by using different stationary phases, such as C18 with various mobile phases with buffers, such as phosphate, sulphate and acetate with different pH values (2-5), and organic modifiers, including acetonitrile and methanol, in the mobile phase. 0.1M ammonium acetate buffer with a pH value of 4.0 and methanol (30:70, v/v) at a flow rate of 1.0 ml/min is chosen for the initial trial with a Hypersil BDS C18 250 stationary phase. When an impurity-spiked solution is injected, the resolution between the impurities and two peaks are merging. Impurities are almost co-eluted with the analyte. To improve the resolution between the impurities and analyte, mobile phase ratio is slightly changed (60:40, v/v) and injected into the impurity-spiked solution. The resolution between the impurities and analyte is very poor (0.210 and 0.198 between impurity 6 and Rilpivirine hydrochloride). In this case peak tailing, no separation between impurities and compound (imp-3, imp-4, Rilpivirine hydrochloride and imp-6). To optimize the resolution between the impurities and the retention time of the impurities, trials are carried out with different mobile phase ratios using buffer and acetonitrile (buffer: acetonitrile: 90:10, 80:20, 70:30 v/v). Isocratic trials are not successful in achieving a favorable resolution between impurity and analyte peaks and in the elution process of impurities. Therefore, a gradient method is selected using buffer and acetonitrile in a ratio of 50:50 (v/v) as solution A, buffer and acetonitrile in a ratio of 50:50 (v/v) as

solution B. Different gradient programs are investigated and satisfactory results are obtained when a gradient program with a flow rate of the mobile phase at 0.5 ml/min and a gradient program of 0/50, 5/65, 6/65, 7/50, 8/50 and 10/50 (time (min) / %B) is used. The column used with the said satisfactory conditions is Shim-pack XR-ODS-II 75mm x3.0 mm column.

Analytical Method validation

Precision

The %RSD of Rilpivirine hydrochloride during the method precision study is within 3.0% and the %RSD values of the area of imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 in the related substance method precision study are within 10.0%. The %RSD of the results obtained in the intermediate precision study was within 3.0% and the %RSD of the areas of

imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 are well within 5%, revealing the high precision of the method (Table 3).

Limit of detection and limit of quantification

The limits of detection and quantification of Rilpivirine hydrochloride, imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 for a 4- μ l injection volume are given in Table 3. The precision at the LOQ concentration for imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 is below 10.0%.

Linearity & Range

The linear calibration plot for the method is obtained over the tested calibration range (50%-150% level) and the obtained correlation coefficient is greater than 0.995. The results revealed an excellent correlation between the peak area and analyte concentration. The linear calibration plot for the related substance method is determined over the calibration range (LOQ to 0.225% w/r to analyte concentration) for imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6, a correlation coefficient of greater than 0.995 is obtained. The linearity is checked for the related substance method over the same concentration range for three consecutive days. The %RSD values of the slope and y-intercept of the calibration curves are within 15.0%. These results showed an excellent correlation between the peak areas and concentrations of imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 (Table 3). Residuals are within $\pm 10\%$ scattered with respect to 100% concentration response. Sensitivities are scattered within $\pm 10\%$ with respect to 100% concentration sensitivity.

Accuracy

The percentage recovery of Rilpivirine hydrochloride impurities in the drug substances i.e. imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 ranged from 99.48 to 101.29; 98.95 to 100.05; 99.00 to 100.05; 98.11 to 100.88; 97.85 to 101.10 and from 98.65 to 101.02 respectively. The UPLC chromatograms of spiked samples at the 0.15% level of all six impurities in the Rilpivirine hydrochloride drug substance sample are shown in Fig. 2.

Robustness

In all of the deliberately varied chromatographic conditions carried out as described in Section 2.7.5 (flow rate, pH and column temperature), the resolution between the closely eluting impurities, namely imp-5 and imp-2 and imp-4 and Rilpivirine hydrochloride is greater than 5.0, illustrating the robustness of the method. The variability of Rilpivirine hydrochloride and the impurities area response is within $\pm 2\%$ and within $\pm 3\%$, respectively.

Solution stability and mobile phase stability

The %RSD of assaying Rilpivirine hydrochloride during the solution stability and mobile phase stability experiments is within 1%. No significant changes are observed in the content of imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 during the solution stability and mobile phase stability experiments when performed using the related substances method. The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the related substance determinations are stable up to 48 h. Mobile phase is proved to be stable up to two days.

Results of forced degradation studies

Degradation is not observed in Rilpivirine hydrochloride stressed samples subjected to light and heat. Significant degradation of the drug substance and product is detected under base hydrolysis, leading to the formation of one major unknown degradation product at 0.52 RRT (Fig. 2). Peak purity test results derived from the PDA detector confirmed that the Rilpivirine hydrochloride peak and the degraded peaks are homogeneous and pure in all of the analyzed stress samples. Degradation studies are carried out for the stress samples (mg/ml) against a qualified reference standard of Rilpivirine hydrochloride.

The mass balance of the stressed samples is close to 99.5%. The assay of Rilpivirine hydrochloride is unaffected by the presence of imp-1, imp-2, imp-3, imp-4, imp-5 and imp-6 and its degradation products, confirming the stability-indicating power of the developed LC method.

Identification of major degradation product (RRT 0.52) formed in base hydrolysis (Stress conditions)

A LCMS study was carried to determine the m/z value of the major degradation product formed under acid and base hydrolysis using an Agilent 2010 EV series liquid chromatography system coupled with triple quadrupole mass spectrometer. Acetonitrile is used as mobile phase for isocratic mode. 0.01 M ammonium acetate and the pH is adjusted to 4.0 using acetic acid and is used as buffer and the conditions are described in section 2.4. The m/z value obtained for the degradation product resolving at 0.52 RRT in ESI positive mode was 399.32(M+1). The impurity is isolated using preparative LC-MS. Based on the mass number the identified degradant is (E)-methyl 4-(4-(4-(2-cynovinyl)-2,6-dimethylphenylamino) pyrimidine-2-ylamino benzoate with molecular weight of 399.45

Conclusions

The degradation pathway of Rilpivirine hydrochloride is established as per ICH recommendations. The gradient LC method developed and used for stress studies also fit for quantitative, related substance and assay determination of Rilpivirine hydrochloride. The behavior of Rilpivirine hydrochloride under various stress conditions is studied, and the hydrolysis (base) degradant is identified as (E)-methyl 4-(4-(4-(2-cynovinyl)-2,6-dimethylphenylamino) pyrimidine-2-ylamino benzoate by LCMS and presented. All of the degradation products and process impurities are well separated from the drug substance demonstrates the stability-indicating power of the method. The method is validated as per ICH recommendations. The developed method is stability indicating which can be used for the impurity testing in routine analysis of production samples and also to analyze stability determining samples.

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