

How to Cite:

Dumbre, N. G., Chopade, V. V., & Chaudhari, P. D. (2022). Low level quantification of two potential genotoxic impurities in rilpivirine hydrochloride drug substance by HPLC technique. *International Journal of Health Sciences*, 6(S9), 3010–3019. <https://doi.org/10.53730/ijhs.v6nS9.13190>

Low level quantification of two potential genotoxic impurities in rilpivirine hydrochloride drug substance by HPLC technique

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Abstract--The goal of the research work was to develop new specific analytical method for the determination of potential genotoxic impurities 4-Iodo-2,6 Dimethyl aniline and 3-Iodo-2,6 Dimethyl aniline in Rilpivirine hydrochloride drug substances. The method was developed by using reverse phase high performance liquid chromatography technique. The comprehensive method development was done to accomplish right combination of chromatographic conditions and validated as per ICH guidelines. The method utilizes L1-octadecyl chemistry (250 millimeter (mm) x 4.6 mm ID, 5.0 micrometer (μm), HPLC column. Both impurities were detected by ultra violet detector at 210nm. The separation of both impurities was attained with 0.1% Ortho Phosphoric Acid in water and acetonitrile at 1.2 ml/min. The calibration curve exhibited good linearity over the concentration range of 0.12-0.45 μg/ml with coefficient of correlation value greater than 0.999. The accuracy in terms of % recovery of the added known amount was found in the range of 97-100 %. Based on experimental results, developed analytical method can be applied for the low level quantitation of both potential genotoxic impurities in Rilpivirine hydrochloride.

Keywords---Rilpivirine hydrochloride, 4-Iodo-2,6 Dimethyl aniline, 3-Iodo-2,6 Dimethyl aniline, potential genotoxic impurities, analytical method validation.

Introduction

In multistep synthesis process of drug substance i.e. Active Pharmaceutical ingredient (API), wide range of reactive reagents are used. Consequence of use of these reagents results in possible generation of potential genotoxic, cytotoxic, mutagenic impurities along with API. It is well known fact that the main sources of these impurities are starting materials, process related drug substance, or intermediate Impurities, catalysts, organic or inorganic reagents, enantiomer impurities, heavy metals, degradation of drug substance, organic degradation products, residual solvents. The impurities more over alter the properties of certain compounds and binds with the deoxyribonucleic acid (DNA) of human being and cause oncological disease and hence can have an impact on product risk assessment. The residual solvents induce physicochemical properties of the drug substances such as crystal nature of the bulk drug, which in turn may concern the solubility properties, odor and color changes in final products. Hence these genotoxic impurities (GTI's) also have a significant effect even in low concentration, which damages the DNA sequence and its structure ^{1,2}. The European Medicines Agency (EMA) was one of the primary controllers to build up the fundamental standards for assessing genotoxic impurities in medications³. The FDA distributed draft rules toward the finish of 2008 for drug makers and industries on genotoxic and cancer-causing impurities in a drug substances and drug products⁴. The ICH M7 guidelines, conversely with those of FDA and EMA, was material not just for the assessment of drug impurities that were accounted for registration and clinical preliminaries yet in addition for reconsideration of necessities for effectively enrolled drugs, for example, process change for a drug substance or medication generation and utilization of advanced analytical techniques⁵.

Rilpivirine (RPV) hydrochloride is a second-generation non nucleoside reverse transcriptase inhibitor (NNRTI) for the treatment of HIV. It has longer half-life and higher potency with lesser side-effect compared with older NNRTIs i.e. Efavirenz. It has demonstrated activity against NNRTI resistant viral strains due to the flexibility of interactions with the HIV RT⁶. In April 2008, RPV entered phase III clinical trial^{7,8} and it's use was approved in the United States in May 2011. A fixed-dose drug combining RPV with emtricitabine and tenofovir was approved by the U.S. FDA in August 2011 in the brand name "Complera".⁹. RPV nitrile and RPV chloro undergo reaction in presence of dimethyl acetamide and acetic acid to manufacture crude RPV. This crude converted to RPV base, purified and converted to pure RPV hydrochloride (Figure 1) form. During this process there is a possibility of generation of 4-Iodo-2,6 dimethyl aniline (GTI Imp- I) (Figure 2) and 3-Iodo-2,6 dimethyl aniline (GTI Imp-II) (Figure 3) which are potentially genotoxic.

Potential genotoxic impurities can be determined on the basis to the literature available in public domain, results of gene mutation in bacteria, in vitro and

in vivo tests of chromosomal damage in mammalian cells or rodent hematopoietic cells and comparative structural analysis to identify chemical functional moieties correlated with mutagenicity^{10,11}. The GTI Imp-I and GTI Imp-II impurities has been considered as a genotoxic carcinogen as per structural alert. As per the ICH M7 guideline for genotoxic impurities, a threshold of toxicological concern (TTC) based acceptable intake of a mutagenic impurity of 1.5µg/g is related with a minor risk and can in general be used for most pharmaceuticals as a default to derive an acceptable limit for intake. The maximum daily dosage of RPV is about 50 mg. Thus, a calculation based on TTC, the limit of both genotoxic impurities for intake is 30 ppm. A detailed literature survey for RPV hydrochloride revealed few analytical methods using spectrophotometry¹², high performance liquid chromatography (HPLC)¹³ and high-performance thin layer chromatography (HPTLC)¹⁴ were reported individually. Though, an intensive literature search revealed to the best of our knowledge that methods are not available for the quantitative determination of 4-Iodo-2,6 Dimethyl aniline (GTI Imp-I) and 3-Iodo-2,6 Dimethyl aniline (GTI Imp-II) with desire limit in RPV drug substances as well as drug products.

The purpose of the current research work is to develop and validate a simple and sensitive reverse phase high performance liquid chromatographic (RP-HPLC) novel method for the quantitative determination of 4-Iodo-2,6 Dimethyl aniline (Fig.2) and 3-Iodo-2,6 Dimethyl aniline in RPV hydrochloride. Many sophisticated advanced analytical techniques like HPLC coupled with mass spectrometer (MS) can be used to determine such GTI's. As analysis is carried out using HPLC, the process becomes cost effective and less tedious as compared to LCMS leading rare advantage to proposed RP-HPLC method over LCMS.

Methods and Materials

Chemicals and Reagents

The investigated sample of RPV hydrochloride and both GTI's were attained as gift sample. HPLC grade orthophosphoric acid was procured from Rankem. Acetonitrile and Methanol were procured from Qualigens thermo Fischer scientific India. Milli -Q water was used with the help of Millipore water purification system (Make Merck mili-Q integral 10).

Instrumentation

The analysis was carried out on a Shimadzu prominence- i LC-2030 3D liquid chromatography system attached with photo diode array detector (PDA). Chromatographic data were recorded and processed using thermo scientific 6.80 SR15b data handling system.

Chromatographic Conditions

Separation was performed on stainless steel column 250 mm long, 4.6 mm internal diameter filled with Octadecylsilane chemical bonded to porous silica particles of 5.0 µm (Make: GL Sciences). 0.1% orthophosphoric acid in water was used as a buffer (mobile phase A). Acetonitrile (ACN) was used as an organic

modifier (Mobile phase B). ACN in combination with methanol (MEOH) in the ratio 30:70% V/V was used as a diluent. Auto sampler was used to inject 20 μ l of sample. Analysis was carried out in gradient mode using different composition of buffer and ACN at different point of time. Initial gradient of Mobile phase B starts with 40% and at up to 25 minutes (mins) it was increased to 70%, up to 35 mins it was kept at 70% and was brought back to 40% at 40min. It was hold for 10 more mins i.e. up to 50 mins with a flow rate of 1.2ml/min. Column eluent was monitored at 210nm.

Preparation of Standard solution (GTI Imp-I and II):

Standard solution was prepared by dissolving 6mg of working standard of both the impurities in diluents in 20 ml of volumetric flask. 1 ml of each impurity standard stock solution was diluted up to 50 ml in the volumetric flask. Further 1 ml of it was diluted to 20 ml with diluent to achieve desired concentration.

Test preparation:

Sample solution was prepared by dissolving appropriate amount of RPV (10000 μ g/ml) in diluent.

Results and Discussion

Optimization of chromatographic conditions

In order to achieve desired separation different chromatographic conditions were tried. Based on hydrophobic nature of RPV hydrochloride and both impurities, reverse phase mode was selected for separation. Trials were done with different chemistry columns like C4, octylsilane, octadecylsilane with different carbon loading capacity. Different particle size like 2.5 μ m, 2.7 μ m, 3.0 μ m and 5.0 μ m were tried. The stationary phase was selected finally on the basis of its efficiency in separating both impurities with good peak shape, tailing factor, number of theoretical plates and acceptable retention time. To achieve better separation, acidic, basic and neutral buffers with different concentration were tried..The retention time of GTI Imp-I and II was determined by injecting 20 μ L of six replicates of standard solution at 1.2 ml/min flow rate into. The 1.2 ml/min was found to be optimal for better retention time and good peak shape. To avoid carryover, diluents was used as a needle wash. The final optimized method was considered for the validation.

Analytical Method Validation

To confirm the performance characteristics of the developed method, the validation was performed using parameters as per the ICH guidelines¹⁵.The parameter with which analytical method is validated is system suitability Specificity, system precision, method precision Limit of detection, Limit of quantitation, Linearity and range, recovery/Accuracy, Precision, Robustness, Solution stability.

Specificity

As specificity test performed for detection of potential impurities in the sample . GTI Imp-I and GTI Imp-II were spiked in test sample at its limit level and analyzed. Other known impurities of Rilpivirine hydrochloride solutions were prepared at its limit level concentration and injected into chromatographic system. It was observed that peaks of API and all other impurities were well resolved from each other. At the retention time of GTI Imp-I and GTI Imp-II i.e. at 16.9 mins and 17.5 mins interference due to any other impurity was not observed. (Refer Image 4, Image 5 and Image 6).

Linearity

Linearity of both genotoxic impurities was determined by preparing and injecting a sequence of solutions at concentrations ranging from limit of quantitation (LOQ) to 150% of specification level. The RSD for area response of both impurities at each concentration and plotted the linearity graph by average area vs. concentration. The linearity range, slope with correlation coefficient also calculated. Linearity ranges were obtained in concentration range of 0.12 µg/ml to 0.45 µg/ml with the correlation coefficient of more than 0.999 in both cases.

Sensitivity [LOD (Limit of detection) and LOQ (Limit of quantification)]

Sensitivity of the method was proven by establishing the LOD and LOQ for GTI Imp-I and GTI Imp-II. LOD and LOQ for both impurities were determined by using the values of signal to noise ratio (S/N). For the RP-HPLC method, LOD was found to be 0.04 µg/ml, and LOQ was found to 0.12 µg/ml for GTI Imp-I and GTI Imp-II.

Precision

Precision had to be established as % relative standard deviation (%RSD). Method precision was established by replicate analysis of RPV. As both the GTI's were not detected, to establish precision working level concentration i.e. 0.30 µg/g of GTI-Imp-I and GTI Imp-II was spiked in RPV sample of 10000 µg/g and samples were subjected for analysis. The %RSD for the analyte response of GTI-Imp-I is about 1.1 and GTI-Imp-II is about 1.5 which is well within acceptance criteria (not more than 5%) indicating that the method is precise.

Accuracy

Accuracy was performed to evaluate the closeness of the experimental values to the actual amount of samples. Accuracy of the method was performed using standard addition technique (spiking study). The recoveries were determined by spiking both GTI's at four concentration levels from about LOQ to 150% levels i.e. 0.12 µg/g, 0.15 µg/g, 0.30 µg/g and 0.45 µg/g into RPV drug substance. These accuracy samples were prepared as per respective test procedure and analyzed in duplicate for LOQ, 50 %, 150 % level. For 100% level six replicates were prepared and the percentage recoveries were calculated. Based on linear regression and

$y=mx + c$ formula, concentration of each impurity in spiked samples was determined. The accuracy was calculated using equation,

$$\text{Accuracy (\%)} = \frac{\text{Amount found}}{\text{Amount added}} \times 100$$

The recovery was found in 80% to 120% and the observed recovery for GTI Imp-I and GTI Imp-II at each level is tabulated in Table 1. Observed recoveries were well within acceptance criteria. This is indicating that method is accurate.

Robustness

The optimal chromatographic conditions set for this method were slightly modified to evaluate the method robustness. The small, but deliberate variations were made in method parameter such as flow rate, column oven temperature. Injected standard solution in replicate (six injections) and evaluate system suitability test. Significant effect was not observed on system suitability parameters such as tailing factor, and theoretical plates of individual component and retention time when small but deliberate changes were made to chromatographic conditions. Thus, the method was found to be robust with respect to variable conditions in method parameters.

Conclusion

A novel selective, precise, robust and accurate RP-HPLC method was successfully developed and validated for determination of 4-Iodo-2, 6 Dimethyl aniline and 3-Iodo-2, 6 Dimethyl aniline impurity from Rilpivirine hydrochloride drug substance. Within the analysis both drug and another known impurities of Rilpivirine hydrochloride were well resolved from both genotoxic impurities. Method validation parameters have proven that the developed method is selective, precise, accurate, linear, rugged, and robust with low LOD, LOQ values. The proposed method was successfully applied for quantization of 4-Iodo-2, 6 Dimethyl aniline and 3-Iodo-2, 6 Dimethyl aniline impurity from Rilpivirine hydrochloride drug substance in order to control both GTI's below TTC level.

Acknowledgment

The authors would like to thank management of M/s SmartChem plus Ltd. for supporting this work. The authors are also thankful to the colleagues of analytical departments for their cooperation.

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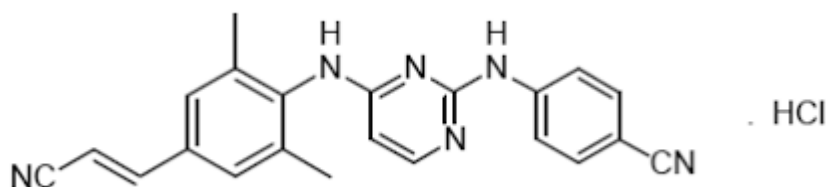


Figure 1: Chemical structure of Rilpivirine hydrochloride

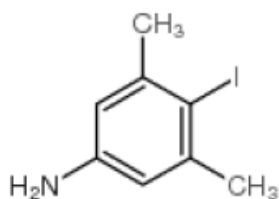


Figure 2: Chemical structure of 4-Iodo-2,6 Dimethyl aniline (GTI Imp- I)

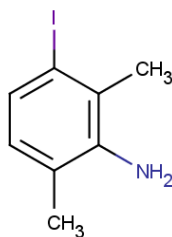


Figure 3: Chemical structure of 3-Iodo-2,6 Dimethyl aniline (GTI Imp-II)

Table 1: Recovery results for GTI Impurity I and II

% Accuracy	GTI Imp-I	GTI Imp-II
Level 1 (LOQ)	98%	98%
Level 2 (50%)	100%	99%
Level 3 (Working Level)	98%	97%
Level 4 (150%)	99%	99%

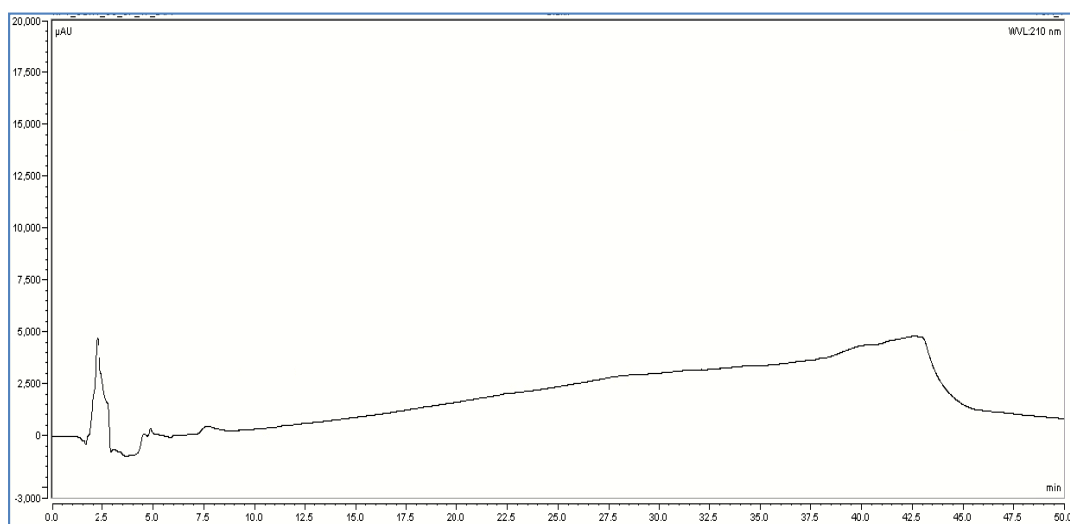


Image 4: Representative typical chromatogram of Blank (Diluent)

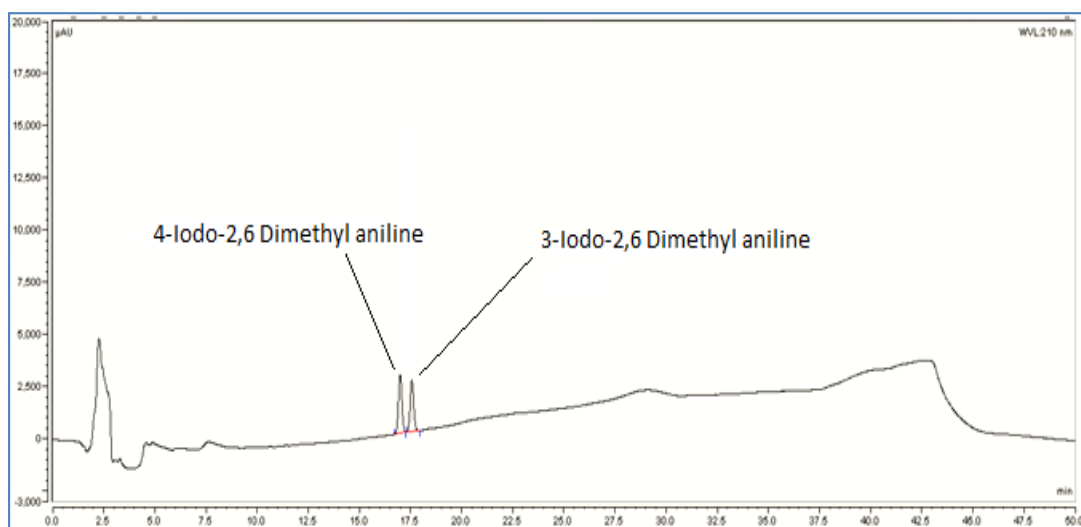


Image 5: Typical HPLC chromatogram of potential genotoxic 4-Iodo-2, 6 dimethyl aniline and 3-Iodo-2, 6 dimethyl aniline impurity of working level.

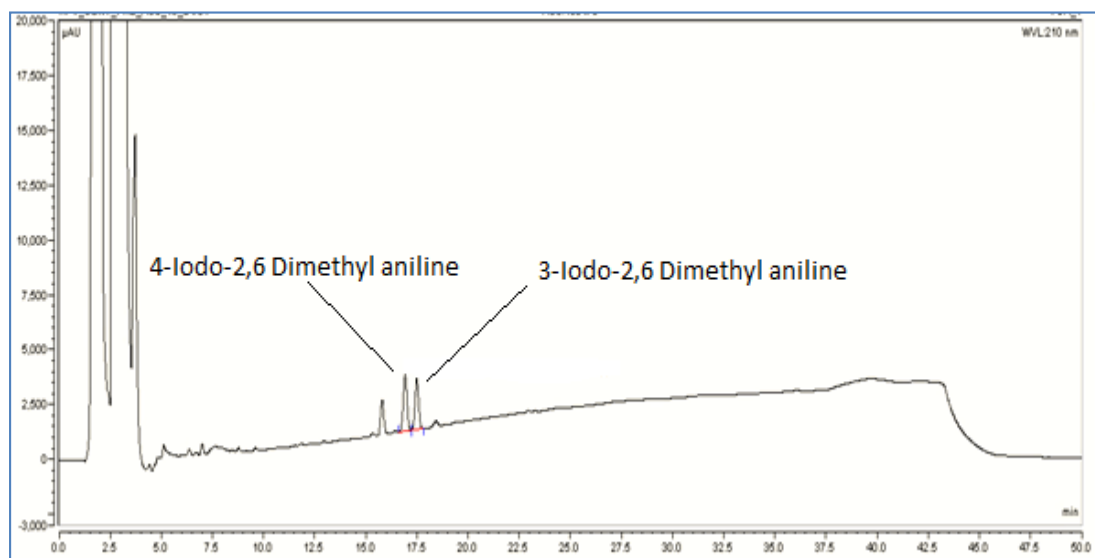


Image 6: Typical HPLC chromatogram of Rilpivirine Hydrochloride drug substance spiked with potential genotoxic 4-Iodo-2, 6 Dimethyl aniline and 3-Iodo-2, 6 Dimethyl aniline impurity (spiked chromatogram).