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Research Article

Eco-Friendly and AQbD Approach for the estimation of trace level of NDMA and NMBA impurity in Rilpivirine formulation by UHPLC-MS/MS

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Abstract
Rilpivirine, a second-generation non-nucleoside reverse transcriptase inhibitor, used for the treatment of HIV-1 in antiretroviral-naive individuals. Rilpivirine tablets may form impurities NDMA (N-Nitroso dimethylamine) and NMBA (N-Nitroso methylbenzylamine) under various conditions in the presence of secondary or tertiary amines particularly in acidic environments. The quantification of NDMA and NMBA was carried out in the Rilpivarine tablet sample using a Potoshell 120 HPH-Octadecyl column of (150 X 4.6) mm ID with 2.7µm as particle size, at a flow rate of 0.5 mL/min using gradient programme with 0.1% formic acid and ethanol as mobile phase. The eluent was directed to the Mass Spectrum and ionized via APCI in positive ionization mode. Quantification was achieved at the 6 ppb utilizing MRM transitions at m/z 75.0 to 43.1 for NDMA and m/z 147.1 to 117.4 for NMBA. The optimised method was validation in accordance with ICH Q2 (R1) guidelines, with the results were meeting the accepting criteria for parameters like specificity, linearity (R2 greater than 0.990 from QL to 200%), accuracy, robustness, and method precision. The accuracy for NDMA and NMBA was achieved from 88% to 120%. The Design Expert statistical tool was employed to evaluate the robustness study using AQbD and shown to be significant for the parameters <0.05. The green analytical chemistry tools like GAPI, AGREE, and Analytical eco-scale study confirmed the method's favorable attributes in terms of environmental sustainability.

Keywords: Rilpivirine, NDMA, NMBA, AQbD, Green Analytical Chemistry, UHPLC-MS/MS.

1. Introduction:

Rilpivirine (RPE) is a second-generation NNRTI that has been authorized for administration as a once-daily oral medication in combination therapy for treatment-naive HIV-1 patients. It is available in a 25 mg tablet form, which should be taken once daily, ideally with food. Consequently, RPE tends to be better tolerated, exhibiting fewer neuropsychiatric side effects such as rashes and lipid alterations compared to efavirenz. RPE undergoes metabolism primarily through the cytochrome P450 3A4 isoenzyme. It is advised not to use it alongside medications that either induce or inhibit this metabolic pathway, or with drugs that reduce stomach acidity, as these can diminish its effectiveness. [1–3]. RPE exhibits low water solubility at 0.0166 mg/mL and a moderate bioavailability of only 32%; consequently, it is classified as a BCS II drug, with the dissolution rate serving as the rate-limiting factor. RPE is noted for its favourable pharmacokinetics, including a prolonged half-life in the body, allowing for a once-daily dosage. The molecular formula of RPE is

$C_{22}H_{18}N_6$, and its IUPAC designation is 4-[(4-[(E)-2-cyanovinyl]-2,6-dimethylphenyl) amino] pyrimidin-2-ylamine (Refer: Figure 1 for chemical structure of RPE). The molecular weight of RPE is approximately 366.44 g/mol. [4–7].

The N-Nitrosamine-related impurities in pharmaceutical products have garnered significant regulatory attention due to the fact that nitrosamines are extremely potent carcinogens and mutagens. The FDA and EMA, overseeing numerous known impurities, have issued specific guidelines to align with risk assessments and employ sensitive strategies for detecting such impurities. Various nitrosamines, including N-nitrodimethylamine (NDMA), N-Nitroso-n-butylamine (NMBA), and N-Nitrosodiethylamine (NDEA), have been identified in the drugs of valsartan, losartan, ranitidine, and metformin, leading to a series of product recalls (Refer Figure 1 for chemical structures of NDMA and NMBA). LC-MS/MS and GC–MS/MS are employed due to their superior sensitivity in detecting extremely low concentrations of nitrosamines to comply with regulatory standards. Additionally, limits on nitrosamines have been established, with the FDA, for instance, permitting only up to 26.5 ng/day, thus highlighting the necessity for a low-level methodology capable of detecting and characterizing distinct and complex nitrosamine structures [8–13]. NDMA exists in extremely low concentrations, it can only be identified and measured using hyphenated techniques such as LC-MS/MS and GC-MS/MS. This encompasses instances involving the compound NDMA, which has been discovered in drugs like metformin and valsartan, leading to regulatory actions aimed at safeguarding patient health [14–16]. Regulatory bodies, including the FDA and EMA, have issued guidelines on managing these impurities and have determined that the methods employed for their detection must be highly sensitive and selective, with techniques such as LC-MS/MS used to identify trace levels of nitrosamines [17–21].

In method development, integrating chemometric tools with Design of Experiments (DoE) can help minimize solvent use while enhancing the robustness of the methodology. In summary, the ecological consequences of these methodologies can be analyzed through tools such as the Analytical Eco-Scale, GAPI, and NEMI [22–25]. AQbD is a structured methodology for improving the quality, sensitivity, specificity, and reliability of analytical procedures using statistical analysis and risk assessment. The Design of Experiments (DoE) can be performed with the help of Design Expert software which helps in optimizing the method parameters (CMPs) and analytical attributes (CAAs). Further, the software helps in identifying the correlation between the variables to maintain the uniformity of the drug products. The Design Expert software enhanced the analytical method by conducting an analysis of the buffer pH, column temperature, and mobile phase composition. This led to a validated method which was highly precise with regard to the accuracy aspect. Statistical designs like fractional factorial, Plackett-Burman, central composite designs provided by Design Expert help in risk analysis [26–28].

Nowadays, UHPLC-MS/MS is the appropriate technique for quantification of nitroso amine impurities and provides the valuable contribution on process development. The literature review showed that the quantification of related impurities is published only limited to the HPLC techniques in Rilpivirine formulation. **Abhijit A. Date et.al.** using HPLC, reported the publication for the quantification of Rilpivirine in biological matrices. **S. Venkatesan et. al.** using HPLC, article reported for the quantification of Tenofovir, Emtricitabine and Rilpivirine simultaneously in tablet dosage form. **Deepak Krishna Mhaske et.al.** using HPLC, reported the article on quantification of 4 related impurities and two isomers in Rilpivirine API. **J. Kalpana et.al.** published the HPLC for simultaneous quantification of related impurities of antiretroviral drugs. However, these methods have not provided their method is environmentally friendly and robust using AQbD technique. To the best of knowledge there is only two papers reported a quantification of process related impurities in Rilpivirine tablets. Further, the estimation of Nitrosamine impurities in Rilpivirine formulation is not yet published in any of the pharmacopoeia. As a result from the above literature review, the requirement on developing the sensitive, accurate and precise method for the quantification of Nitrosamine impurities (NDMA and NMBA) in formulated drug product on UHPLC-MS/MS [29–34].

The objective of the present study is to identify and quantify the nitrosoamine impurities NDMA and NMBA on UHPLC-MS/MS in Rilpivarine Tablets, validating the proposed method following the ICH

Q2 guidelines and to study the green analytical chemistry (GAC) principles and analytical quality by design (AQbD) to study the robustness of the proposed method.

2. Experimental Work

2.1. Chemical and Reagents

The nitroso amine impurities NDMA, NMBA and Rilpivarine API were provided as a gift sample from Hetero Pharma Limited. The sample Rilpivarine Tablets 25 mg tablets was purchased from the local pharmacy. Analytical reagent grade HCOOH and CH_3CH_2OH was procured from Honeywell, India. Milli-Q Water obtained from the Lab Q Pvt.Ltd.

2.2. Analytical Instruments, Columns and Software's

The analytical experimental work was performed on a Waters ACQUITY UHPLC-MS/MS installed with Empower 3 Software, equipped with auto injector, column heater, binary pumps, and a column heater. MassLynx software was employed for integrating the MS spectral data. The separation of analytes was achieved using Potoshell 120 HPH-Octadecyl column with 4.6 mm x 150 mm ID and 2.7µm particle size. The weighing was performed using an Analytical balance and a Micro Balance manufactured from Sartorius. 0.22µm PVDF filters was used for filtering the mobile phase Microfiltration unit with from Millipore was utilized for mobile phase. The Power Sonic 410 Ultra Sonicator was used for sonicating the sample and standard. Micropipette supplied by Eppendorf was used for pipetting.

2.3. Optimised UHPLC-MS/MS conditions

The UHPLC-MS/MS optimised method is composed of mobile phase with aqueous phase includes 0.1% HCOOH and organic phase includes 0.1% formic acid in CH_3CH_2OH, operated in gradient elution mode with 20 minutes total run time. The optimised gradient program is as follows: time (min)/%B (0/10, 2/10, 7/70, 10/75, 11/90, 16.5/90, 16.6/10, 20/10). The separation and selectivity of the analytes were achieved in Poroshell 120 HPH-C18 column, (4.6 x 150) mm ID, with 2.7 µm as particle size. The optimised flow rate of the mobile phase is operated at 0.5 mL/min, with the sample cooler temperature maintained at 5°C, while the column compartment was maintained at 40°C. The optimised injection volume is operated at 50 µL into the system. The diluent utilized for this procedure was Milli-Q Water. The Optimised Analytical methods involving mass spectrometry were executed using Q-TOF mass analyzer, which featured APCI as the ionization source in the positive scan mode. Data acquisition was facilitated by the Mass Lynx software via MRM during the optimization phase for analyte identification and quantification. The operational parameters at the mass source included a gas flow rate of 7 L/min, a gas temperature of 250°C, a nebulizer pressure of 25 psi, a vaporizer temperature of 300°C, a capillary voltage of 4000V, a corona current of 4+, delta EMV of 500+, a dwell time of 50 milliseconds, and a fragmentor voltage of 80V for NDMA. In the collision cell, energies were calibrated at 13 eV for NDMA and 4 eV for NMBA, with a cell acceleration set at 3 eV. The mass-to-charge ratio of the precursor ion for NDMA was 75, while the product ion had a mass-to-charge ratio of 43; for NMBA, the respective peaks were recorded at 147 m/z and 117.4 m/z.

2.4. Analytical Solutions

I. Preparation of standard Solution

Individually prepared the standard stock solution-1 (SS-1) for NDMA and NMBA in diluent to 1200 µg/mL concertation. Further transfer 0.30 mL of SS-1 into a 25 mL flask, and made upto the mark with diluent and mixed well (SS-2). Further transfer 1.00 mL of SS-2 into a 25 mL flask, subsequently made up to the mark with the diluent and mixed well (SS-3). Finally transfer 0.50 mL of SS-3 into a 50 mL flask and made up the mark with diluent and mixed well. (concertation: 0.00576µg/mL)

II. Preparation of Quantitation Limit (QL) solution

Accurately transfer 0.05 mL of SS-3 in to a 50 mL flask, subsequently made up to the mark with diluent and mixed well. (Concentration: 0.000576 µg/mL).

III. Preparation of Detection Limit (DL) solution

Accurately transfer 0.02 mL of SS-3 in to a 50 mL flask, subsequently made up to the mark with the diluent and mixed well. (Concentration: 0.0002304 µg/mL).

IV. Preparation of Sample Solution

Weigh and calculate the average weight of 10 tablets and pulverizing them into a fine powder using a mortar and pestle. Weigh accurately and transfer equivalent to 2.5 mg of Rilpivirine tablet powder into a 15 mL centrifuge tube, add about 5 mL of diluent, and vertex for about 5 minutes, followed by sonication for 2 minutes, filtration was performed using PVDF syringe filter with a pore size of 0.22 µm to attain the cconcentration of 2.5 mg/mL

2.5. Validation

2.5.1 System suitability

System suitability of the optimised method was performed by injecting 6 replicate injections of standard solution of analytes (NDMA and NMBA impurities). The peak asymmetry and the theoretical plate count for the analytes should meet the acceptable limit of NMT 2.0 and NLT 2000 respectively. %RSD pertaining to the Ion count for the analytes from the 6 replicate injections of the standard solution is not more than 15.0.

2.5.2 Specificity

The optimised method was validated using the parameter specificity using the diluent (blank), standard solution, sample solution, and impurity-spiked sample solution. The evaluation criteria for the specificity is to no interference should be observed at the retention time of analytes (NDMA and NMBA impurities) from the blank chromatogram and sample chromatogram.

2.5.3 QL and (DL

The optimised method was evaluated for the lower quantification and detection limit. As per ICH Q2 guidelines the QL and DL was identified using visual examination method where the signal to noise ratio (S/N) is consider. The 10% standard concentration for NDMA and NMBA was considered as QL concentration, whereas the 5% standard concentration for NDMA and NMBA was considered as DL concertration. The S/N ratio for QL should be more than 10 and for DL is more than 3.0. The precision at the QL has been evaluated by injecting the six-replicate injection of QL solution. The %RSD for the six-replicate injection should not exceed 15.0.

2.5.4 Linearity

The linearity of the proposed method was evaluated with five different concentration levels of QL, 50%, 100%, 150%, and 200% in accordance with the defined parameters. The concentration for QL, 50%, 100%, 150%, 200% as 0.000576 µg/mL, 0.00288 µg/mL, 0.0576 µg/mL, 0.00864 µg/mL, 0.01152 µg/mL respectively. Plot the linear graph between the concentration with that of Ion count. The correlation coefficient for the analytes NDMA and NMBA should not be less than 0.990.

2.5.5 Method precision

The optimised method was determined using the method precision parameter by injecting the six individual spiked sample preparations which is spiked with the analytes at 100% specification level. The %RSD for each analyte obtained from the six individually prepared sample preparations, should not exceed 15.0.

2.5.6 Accuracy

Accuracy of the proposed method was determined by injecting a sample solution spiked with the analytes with the concentration at LOQ, 50, 100%, and 200% levels with respect to the specified threshold. The % recovery for NDMA and NMBA at each level should be in the range of 70.0% and 130.0%. The %RSD for the recoveries of NDMA and NMBA at each level should be NMT 15.0.

I. Preparation of accuracy sample solution

Weight and calculate the average weight of 10 tablets, pulverise the tablets into fine powder using mortar and pestle. Accurately weight and transfer equivalent to 2.5 mg of the Rilpivirine tablet powder into a 15 mL centrifuge tube, add about 5 mL of standard solutions (Similarly with LOQ, 50%, 100%, and 200% concentrations). Further, vertex the spiked sample solution for 5 minutes, followed by the sonication for two minutes. Filtered the resultant solution using 0.22µm PVDF syringe filter. Prepared triplicate at every accuracy level.

2.5.7 Robustness study using AQbD (Design Expert using Surface response)

The robustness of the proposed analytical method was analyzed utilizing the Design Expert 13 AQbD software. The critical quality parameters of the proposed method were identified as the flow rate and the column temperature. The column temperature was modified to remain within ±5°C of the proposed column temperature of 40°C. The flow rate was adjusted by ±10% compared to the proposed flow rate of 0.5mL/min. The developed Response Surface Randomized Central Composite design (RSR-CCD), encompassed with two factors and included three central points within a single block. The software facilitated the execution of 13 experimental runs on UHPLC-MS/MS. The main response variables are calculated for the retention time for NDMA and NMBA. The response variables were analyzed through the software, revealing any of the significant effects illustrated in the parent and contour plots. Conduct an Analysis of Variance (ANOVA) to assess the significance of the design and validate the model's accuracy.

2.6. Green Analytical Principles

The environmental sustainability of the enhanced methodology was evaluated using the twelve principles of green chemistry tools, including the Analytical Eco-Scale, AGREE, and GAPI. The suggested methodology employed the mobile phase with a 0.1% HCOOH solution along with ethanol, necessitating lesser volume of ethanol for a single injection as mobile phase and diluent.

AGREE (Analytical GREEnness metric): This tool serves as a crucial assessment of the friendliness to the environment of an analytical proposed method, considering into various green factors, such as the toxicity of solvents employed, the energy consumed, and the waste generated. It yields a score that reflects the method's impact concerning the environmental burden it imposes.

GAPI (Green Analytical Procedure Index): In assessing the environmental sustainability of an analytical procedure, GAPI considers the overall analytical procedure cycle, encompassing the preparation of sample solutions and waste disposal. It utilizes a color-coded system that directs the program's development in areas requiring improvement.

Analytical Eco-Scale: This instrument assesses the combustibility of an analytical approach regarding its ecological impact, assigning penalty points for the use of environmentally harmful materials and energy-intensive processes. A higher Eco-Scale score indicates that the method in question is recognized as being more environmentally friendly.

3. Results and Discussion

3.1 Validation

The validation of the proposed analytical method on UHPLC-MS/MS technique was performed in accordance with the ICH Q2 (R1) guidelines concerning the specified parameters, including system precision, specificity, accuracy, linearity, method precision, robustness, DL, and QL.

3.1.1 System Precision

The proposed optimised method was validated for the System precision by injecting the 6 replicate injections of the NMDA and NMBA standard solution with the concentration range of 0.006 μg/mL. The system precision or system suitability criteria for the analytes are meeting the acceptance standards as the %RSD for NDMA and NMBA are 7.3 and 6.5 with mean of 85678 and 16983 respectively. The Plate count for NDMA and NMBA are greater than 2000 and Asymmetry factor is less than 2.0.

3.1.2 Specificity

The efficacy of the proposed analytical method was further evaluated for its specificity by injecting the blank (diluent), standard, sample and spiked sample solutions. The method's specificity was confirmed, as there was no interferences detected in the blank at the retention times for NDMA and NMBA. Figure 2, 3, 4, and 5 depicts the representative ion chromatogram of the blank, standard, sample and spiked sample. The retention times for the identified impurity NDMA in Standard and Spiked samples shows 4.71+ and 4.712 minutes where as for NMBA the retention time is 7.249 and 7.242 minutes for both the standard and spiked samples.

3.1.3 Linearity

The optimised proposed analytical method was validated using linearity by plotting the calibration curves between the ion counts of the both NDMA and NMBA and the respective concentrations from QL to 200% with minimum five distinct solutions prepared separately. The resulting calibration curve exhibited a correlation coefficient of 0.998 for NDMA and 0.996 for NMBA which is exceeding the acceptable limit of 0.990. The results pertaining to NDMA and NMBA have been meticulously compiled and are presented in the Table 1 and Table 2.

3.1.4 Method Precision The optimised proposed analytical method was validated using method precision parameter by preparing 6 different sample solutions spiked with the NDMA and NMBA impurities at 0.005 µg/mL concentration level. The results demonstrate that the method shows repeatability and meeting the acceptance threshold of not more than 15.0%. A summary of these results is provided in Table 3.

3.1.5 Accuracy

The accuracy (recovery) of the proposed analytical method was evaluated by the standard spiking technique. The investigation was performed with triplicate preparations at four varying concentrations, at QL, 50%, 100%, and 200%, following which the percentage recovery was determined. The data for these percentage recoveries and their RSDs for NDMA and NMBA were compiled and presented in Table 4 and Table 5 respectively. All recovery percentages reported in the results fall within the range of 70.0% to 130.0% for all samples analysed at this concentration level, with the %RSD remaining below 15.0%.

3.1.6 DL and QL

The Detection Limit (DL) is defined as 5% of the specification limit, whereas the Quantification Limit (QL) is determined at 10% of the specification limit, where the concentration for DL stands at 0.0002 µg/mL and for QL at 0.0005 µg/mL. At the DL level, the ion count chromatogram can detect signals, and the signal to noise ratio exceeds 3.0, whereas at the QL level, the ion count chromatogram can quantify signals, with the signal to noise ratio surpassing 10. The representative ion counts chromatograms for both DL and QL levels are presented in Figures 6 and 7 respectively.

3.1.7 Robustness Study using SCC

The two main Critical Quality Attributes (CQAs) identified through the AQbD investigation and optimisation of method development on UHPLC-MS/MS were

1. The column compartment temperature maintained from 35 to 45°C
2. The mobile phase flow rate ranging from 0.4 to 0.6 mL/min.

The responses measures for the above CQAs are the

1. Retention time of the NDMA and NMBA

It is significant that the research did not reveal any flow rate or column temperature retention time that notably influenced the critical analytical attributes (CQA). The research design employed a three-factor approach utilizing a three-centre point without a block design. More specifically, 13 experimental trials were conducted systematically, and their records are detailed in Table 6. This study was carried out through all 13 trials, and the results obtained were analysed as well. The ANOVA analysis results presented in Table 7 also indicate a P value that is significantly below 0.05. This significance reinforces the validation of the model for the two response variables. The pictorial diagrams for 2D contour diagrams, 3D contour diagrams, all-factor composite response surface plots, and cube graphs are illustrated in Figures 8, while those for the second response are displayed in Figures 9. From the statistical results it is concludes there is no sensitivity analysis for any of the responses. Among all the fit statistics, Predicted R^2 and Adjusted R^2 for Response 1 and 2 are the most suitable for this analysis, as they demonstrate a close relationship. This well-established model serves as a valuable reference framework for exploring the design domain.

3.1.8 Green Analytical Chemistry assessment tools

The suggested method employed CH_3CH_2OH and HCOOH in the mobile phase as well as during the solution preparation procedure. The findings indicated that the use of CH_3CH_2OH and H_2O as the mobile phase and diluent is considerably safer than the toxic chemicals like CH_3OH or CH_3CN. A usage of shorter column enabling a total analysis time of 20 minutes for the evaluation of two compounds (NDMA and NMBA), while using less than 5 mL of ethanol for each sample preparation. The environmental assessment for the proposed analytical method was evaluated using green analytical metric tools like GAPI, Analytical Eco-Scale and AGREE. Table 8 shows the calculated results for the Analytical Eco-Scale tool provides assessments on chemicals or reagents used, instrument used for the energy consumption, generation of waste, and management in the proposed method compared to other methodologies. The results showed that the suggested analytical method is having a total penalty point of 18, for which the total greenness score is 82. Figure 10 showed the AGREE and GAPI metrics calculations, where the AGREE tool provides a greenness level of 0.65 for the proposed analytical method, exceeding the acceptance threshold of 0.50. The application of the proposed method in alignment with green analytical principles is illustrated in Table 9, utilizing the AGREE tool. Furthermore, Figure 10 indicates that the AGREE representation affirms the method's eco-friendliness, with the only drawback being the total energy consumption of the instrument (UHPLC-MS/MS, marked in red). Table 10 shows the results of the GAPI tool for evaluating sources of the sample, type of the method, preparation of sample technique, usage of reagents and solvents in the optimised method, instruments, and quantification of analytes in the proposed analytical method. The GAPI test result confirmed that the proposed analytical method is environmentally sustainable, with the exception of the sample handling process noted in red.

4. Discussion

This paper highlights the necessity of minimizing the environmental footprint of analytical techniques, particularly in LC-MS. This can be achieved by either reducing or entirely removing the reliance on toxic and highly flammable organic solvents, substituting them with safer alternatives like ethanol and buffers under subcritical conditions. It also effectively eliminates hazardous waste in practice while enhancing the safety of analysts handling chemicals. The environmental relevance of the method has been assessed using GAPI, AGREE, and Analytical Eco-scale tools. Through the analysis of these results, it has been demonstrated that the proposed method possesses an Analytical Eco-Scale score exceeding 82, establishing it as an effective means of promoting eco-friendly practices in Quality Control (QC) Laboratories. This paper discusses the miniaturization of chromatographic and online sample preparation systems, which holds the potential to significantly reduce solvent usage and waste generation. UHPLC-MS/MS methods are favored for their speed and lower solvent consumption. ANOVA statistical analysis was employed to evaluate the results and detect any systematic differences among the analytical methods; the p-value obtained was <0.05, signifying high reliability. The developed method was validated in accordance with the specified ICH Q2 (R2) criteria encompassing specificity, linearity, accuracy, robustness, and precision. The proposed analytical method is deemed highly advantageous for QC laboratories, particularly in the detection and quantification of nitrosamine impurities like NDMA and NMBA-related impurities in RPE formulations. This proposed analytical method ensures the safety and efficacy of for the identification of nitrosamine impurities available in the market and is recommended for implementation.

5. Conclusion

The enhanced experimental and verified outcomes endorse the conclusions that the analytical method outlined by UHPLC-MS/MS is capable of measuring NDMA and NMBA concentrations in RPE, facilitated by appropriate stationary phases, chromatographic conditions, and MS/MS parameters. The suggested analytical approach underwent validation in accordance with the procedures specified in ICH Q2. The established methodology demonstrated attributes such as specificity, linearity, accuracy, robustness, and precision, fulfilling the required analytical detection limit (DL) and quantification limit (QL). This technique enables the assessment and linear generation of the NDMA and NMBA impurity within a concentration range from QL to 200% concerning the defined limit. By employing the AQbD strategy, the method's applicability was analyzed through ANOVA statistical evaluation, yielding p-values less than 0.05, thus confirming the method's robustness. The method's environmental suitability was assessed using GAP evaluation tools like AGREE, GAPI, and the Analytical Eco-Scale. The findings suggest that the proposed technique aligns with green practices, as indicated by Analytical Eco-Scale values exceeding 82. This methodology proves to be particularly beneficial for Quality Control Laboratories in accurately identifying and quantifying the levels of NDMA and NMBA found in RPE.

6. Abbreviations: RPE: Rilpivirine, NDMA : N-Nitrosodimethylamine, NDBA : N-Nitrosodi-n-butylamine, UHPLC : Ultra High-Performance Liquid Chromatography, MS: Mass Spectrometry, APCI : Atmospheric Pressure Chemical Ionization, MRM : Multiple Reaction Monitoring, ICH: International

Conference on Harmonization, DE: Design Expert, GAPI : Green Analytical Procedure Index, AGREE : Analytical Greenness, NNRTI : Non-Nucleoside Reverse Transcriptase Inhibitor, HIV-1: Human Immune deficiency Virus-1, CYP : Cytochrome P450 , BCS : Biopharmaceutics Classification System, FDA : Food and Drug Administration, EMA : European Medical Agency, LC-MS/MS : Liquid Chromatography-tandem Mass Spectrometry, GAC : Green Analytical Chemistry, DoE : Design of Experiments, NEMI : National Environmental Methods Index, AQbD : Analytical Quality by Design, CMPs : Critical Method Parameters, CAAs : Critical Analytical Attributes, MODR : Method Operable Design Region, QL : Quantitation Limit, DL : Detection Limit, %RSD : Percentage Relative Standard Deviation, QC : Quality Control.

7. Data Availability: Any material and data required will be available for the journal on request.

8. Ethical Approval: This experimentation has not harmed any animal or human

9. References

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10. Figure Legends:

Figure 1: Represents the chemical structure of Rilpivirine (RPE), NDMA and NMBA,

Figure 2: Typical selected Ion Chromatogram of Blank solution.

Figure 3: Typical selected Ion Chromatogram of NDMA and NMBA Standard solution

Figure 4: Typical selected Ion Chromatogram of Rilpivirine Tablets 25 mg

Figure 5: Typical Selected Ion Chromatogram of NDMA and NMBA at Rilpivirine Tablets 25mg Spiked solution.

Figure 6: Typical Ion Chromatogram of DL level

Figure 7: Typical Ion Chromatogram of QL level

Figure 8: All factor coding plot, Contour plot, 2D Plot, and 3D plot for Response Factor 1 (NDMA Retention time)

Figure 9: All factor coding plot, Contour plot, 2D, Plot and 3D plot for Response Factor 2 (NMBA Retention Time)

Figure 10: Represents GAP tools GAPI and AGREE pictograms for the proposed method.

Table 1: Represents the Linearity for NDMA.

Name	Level	Volume transfer (mL)	Volumetric flask (mL)	Concertation (µg/mL)	Area
QL Solution	Linearity Level-1	0.05 mL of SS-3	50	0.0005	14417
50% level solution	Linearity Level-2	0.250 mL of SS-3	50	0.0027	45010
100% level solution	Linearity Level-3	0.500 mL of SS-3	50	0.0055	89370
150% level solution	Linearity Level-4	0.750 mL of SS-3	50	0.0082	121468
200% level solution	Linearity Level-5	1.000 mL of SS-3	50	0.0109	165379

Table 2: Represents the Linearity for NMBA.

Name	Level	Volume transfer (mL)	Volumetric flask (mL)	Concertation µg/mL	Area
QL Solution	Linearity Level-1	0.05 mL of SS-3	50	0.0006	2288
50% level solution	Linearity Level-2	0.250 mL of SS-3	50	0.0029	10510
100% level solution	Linearity Level-3	0.500 mL of SS-3	50	0.0058	16593
150% level solution	Linearity Level-4	0.750 mL of SS-3	50	0.0087	30711
200% level solution	Linearity Level-5	1.000 mL of SS-3	50	0.0115	39617

Table 3: The table represents the method precision results for NDMA and NMBA

Method Precision	Results in %	
	NDMA	NMBA
Preparation-1	96.023	115.144
Preparation-2	94.459	107.086
Preparation-3	93.143	102.789
Preparation-4	95.154	103.587
Preparation-5	94.368	102.861
Preparation-6	93.586	104.287
Average	94.456	105.959
STD.DEV	1.04	4.77
% RSD	1.1	4.5

Table 4: Represents the preparation and results for NDMA accuracy spiked sample

Name	Volume transfer in mL	Linearity solution spiked from	Sample Concentration in ppm		%Recovery	% RSD
			Added	Found		
QL Spiked Sample	5	QL Level	0.218	0.263	120.69	6.45
50% Spiked Sample	5	50% Level	1.092	1.040	95.26	4.26
100% Spiked Sample	5	100% Level	2.183	2.064	94.54	1.52
200% Spiked Sample	5	200% Level	4.366	3.972	90.97	0.69

Table 5: Represents the preparation and results for NMBA accuracy spiked sample

Name	Volume transfer in mL	Linearity solution spiked from	Sample Concentration in ppm		%Recovery	% RSD
			Added	Found		
QL Spiked Sample	5	QL Level	0.231	0.228	88.73	2.19
50% Spiked Sample	5	50% Level	1.1549	1.242	107.54	2.76
100% Spiked Sample	5	100% Level	2.3098	2.502	108.34	5.79
200% Spiked Sample	5	200% Level	4.6195	5.303	114.80	1.15

Table 6: The table represents the robustness study with factors influencing the NMDA and NMBA retention time

		Factor 1	Factor 2	Response 1	Response 2
Std	**Run**	**A: Column Temperature**	**B: Flow Rate**	**NMDA Retention Time**	**NMBA Retention Time**
		°C	mL	Minutes	Minutes
8	1	40	0.641421	4.558	7.096
5	2	32.9289	0.5	4.706	7.219
3	3	35	0.6	4.463	7.112
11	4	40	0.5	4.712	7.242
13	5	40	0.5	4.719	7.248
4	6	45	0.6	4.563	7.118
1	7	35	0.4	4.906	7.368
6	8	47.0711	0.5	4.712	7.221
9	9	40	0.5	4.735	7.243
10	10	40	0.5	4.716	7.251
2	11	45	0.4	4.915	7.352
7	12	40	0.358579	4.986	7.406

12	13	40	0.5	4.711	7.246

Table 7. The table represents the ANOVA Table for response factor 1 and response factor 2

Response	Source	Sequential p-value	Lack of Fit P-value	Adjusted R^2	Predicted R^2
Factor:1 NDMA Retention Time	**Linear**	< 0.0001	0.0042	0.9356	0.8834
	2FI	0.243	0.0042	0.939	0.8568
	Quadratic	0.2248	0.0043	0.9488	0.7956
	Cubic	0.0347	0.0138	0.9813	0.5921
Factor:2 NMBA Retention Time	**Linear**	< 0.0001	0.0053	0.9806	0.9663
	2FI	0.4381	0.0045	0.9799	0.9625
	Quadratic	0.0079	0.0303	0.9935	0.9758
	Cubic	0.0086	0.4844	0.9986	0.9946

Table 8: The table represents the Analytical Eco-Scale for assessing the GAC method

Analytical Eco Scale for assessing the analytical method		
S.No	**Name**	**Penalty Points**
Chemicals or reagents		
1	Formic acid	6
2	Ethanol	4
Instruments		
1	Energy- 1.5kWh of energy per sample for UHPLC-MS/MS	2
2	Occupational Waste- The procedure releases vapors into the environment	0
Waste		
1	Total Amount of waste generated (Waste Generated >10mL)	5
2	Management (The generated waste has a degradation process)	1
Total Penalty Points		18
Analytical Eco Scale Total Score (100-Total Penalty Points)		82
Green ness Evaluation		Excellent

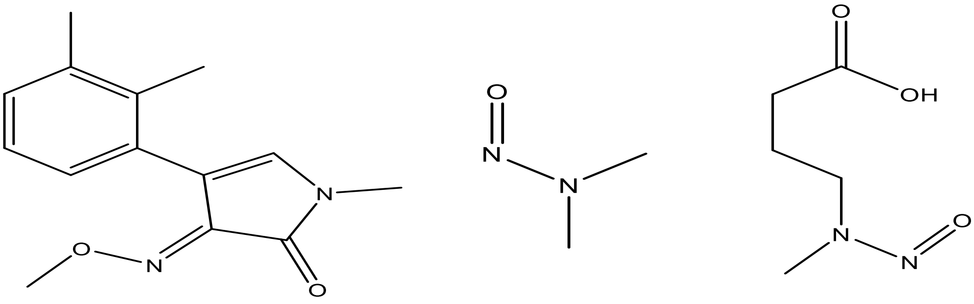
Table 9: The table represents the AGREE tool for assessing the Green analytical method.

AGREE tool for assessing the Analytical Method on UHPLC-MS/MS for the Proposed sample procedure		
S.No	**Green Analytical Chemistry Principles**	**Proposed Sample Procedure**
1	Direct analytical techniques have to be used to eliminate the treatment of samples	Off-Line Analysis
2	Minimum sample size and minimum number of samples are objectives	1 g
3	It is preferred if measurements are made directly on the existing environment.	At-line
4	Combined operations of analytical processes and operations help in reducing energy use and cutting down on reagents.	Four distinct steps are involved in the sample preparation procedure.
5	Automated and miniaturized methods should be selected	Semi-Automatic and miniaturized

6	Derivatization should be avoided	No Derivatization
7	There must be a minimal generation of analytical waste and adequate management of the waste generated.	1 g
8	There is a preference for using multi-analyte or multi-parameter rather than the use of the single analyte.	Two analytes were determined with a single run, and three samples were analyzed per hour
9	**The use of energy should be minimized:**	
	Choose the highest energy consumption technique applied in the method or the one that is closest to it.	UHPLC-MS
	Alternatively, rounded to the nearest kilowatt-hour, determine an approximate value of the total power consumption of a single analysis in kilowatt-hours.	1.5
10	The reagents used should be sourced from renewable sources in case they can be obtained from them.	All reagents are from bio-based
11	Hazardous reagents must be minimized or substituted	No toxic reagents or solvents used
12	The safety of the operator should be enhanced.	The threats that are not avoided are a. Bioaccumulative b. Highly flammable c. Explosive

Table 10: The table represents the GAPI tool for assessing the Green analytical method.

GAPI tool for assessing the Analytical Method on UHPLC-MS/MS		
Sample Sourcing		
1	Collection	Off-line
2	Preservation	Physical or Chemical
3	Transport	None
4	Storage	Under normal conditions
Method Type and Sample Preparation		
5	Type of Method	Extraction required
6	Scale of extraction	Macro-Extraction
7	Solvent or reagent used	Green Solvents/ Reagents used
8	Additional Treatment	Simple Treatment
Reagents and Solvents		
9	Amount	<10 mL used
10	Health hazard	Slightly toxic, slight irritant; NFPA health hazard score of 0 to 1. No special hazards.
11	Safety hazard	Highest NFPA flammability or instability score of 0 or 1. No Special hazards.
Instrumentation		
12	Energy	<=1.5 kWh per sample
13	Occupational hazard	Hermetic sealing of the analytical process
14	Waste	1-10 mL (1-10g)
15	Waste treatment	Degradation, Passivation
Quantification/Qualification		
1	Symbol O	Procedure for qualification and quantification



A B C

Figure 1: Represents the chemical structures of Rilpivirine (A), NDMA (B), NMBA (C)

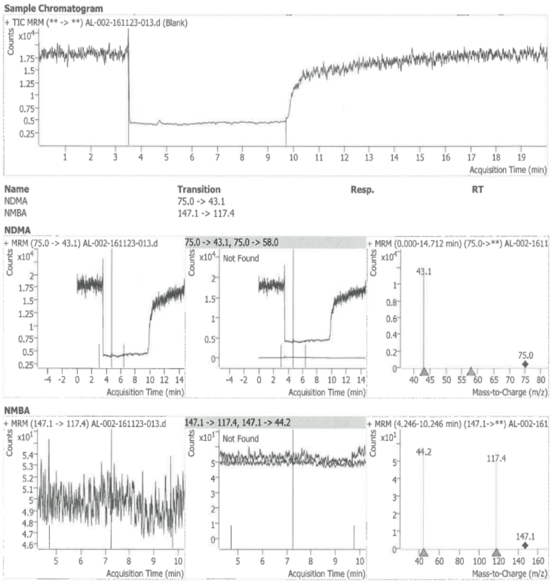


Figure 2: Represents the Ion Chromatogram of Blank solution.

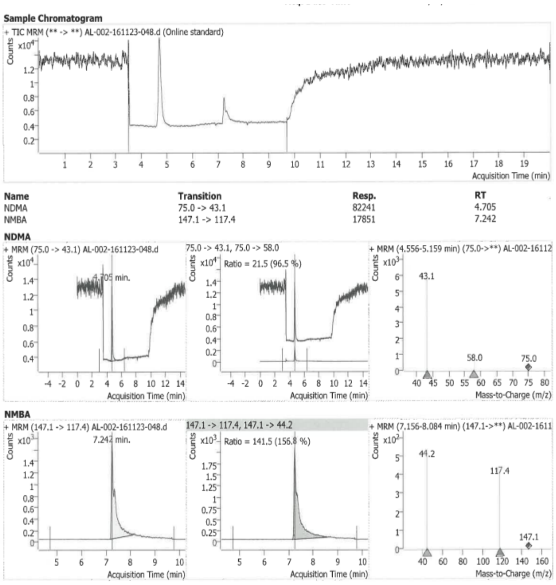


Figure 3: Illustrates the Ion Chromatogram of NDMA and NMBA Standard solution

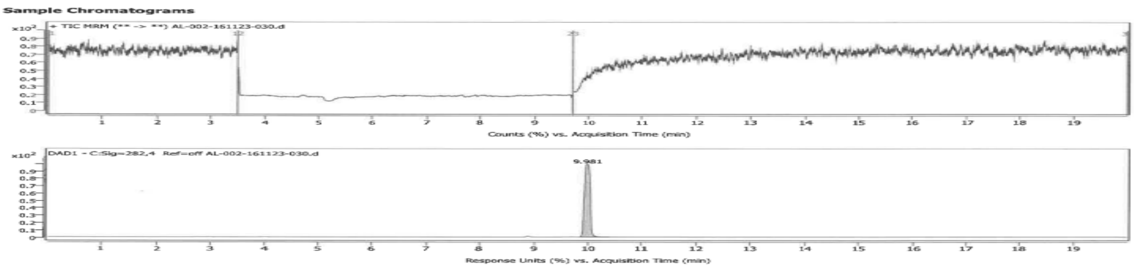
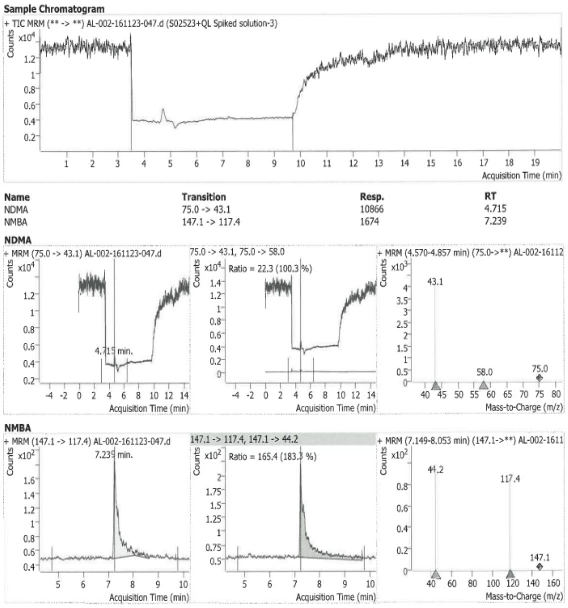
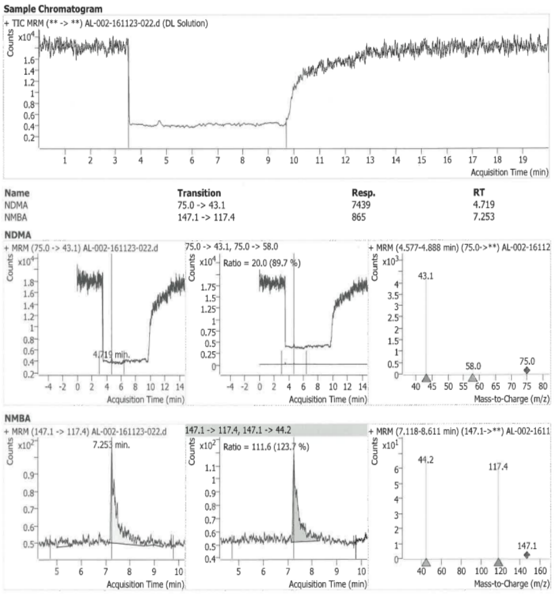


Figure 4: Illustrates the sample Chromatogram of Rilpivirine Tablets 25 mg



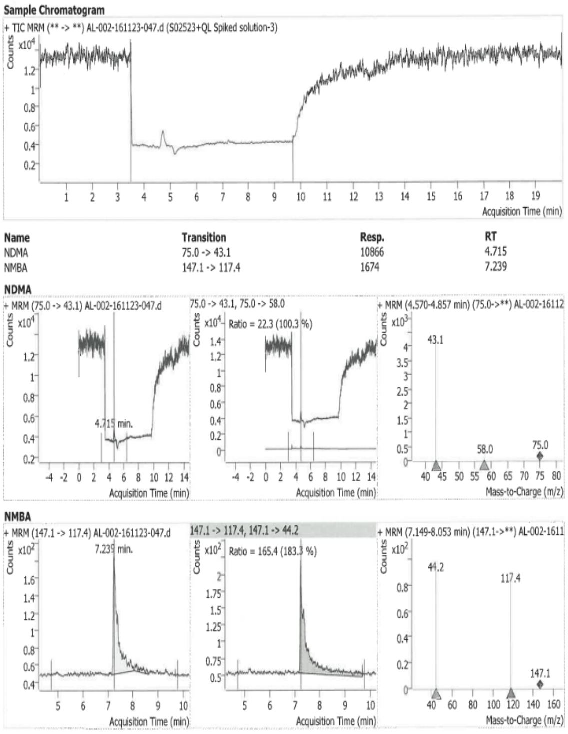
Name	Transition	Resp.	RT
NDMA	75.0 -> 43.1	10866	4.715
NMBA	147.1 -> 117.4	1674	7.239

5: Illustrates the Ion Chromatogram of Spiked NDMA and NMBA in Rilpivirine Tablets.



Name	Transition	Resp.	RT
NDMA	75.0 -> 43.1	7439	4.719
NMBA	147.1 -> 117.4	865	7.253

Figure 6: Illustrates the Ion Chromatogram of standard NDMA and NMBA at DL level



Name	Transition	Resp.	RT
NDMA	75.0 -> 43.1	10866	4.715
NMBA	147.1 -> 117.4	1674	7.239

Figure 7: Typical Ion Chromatogram of standard NDMA and NMBA at QL level

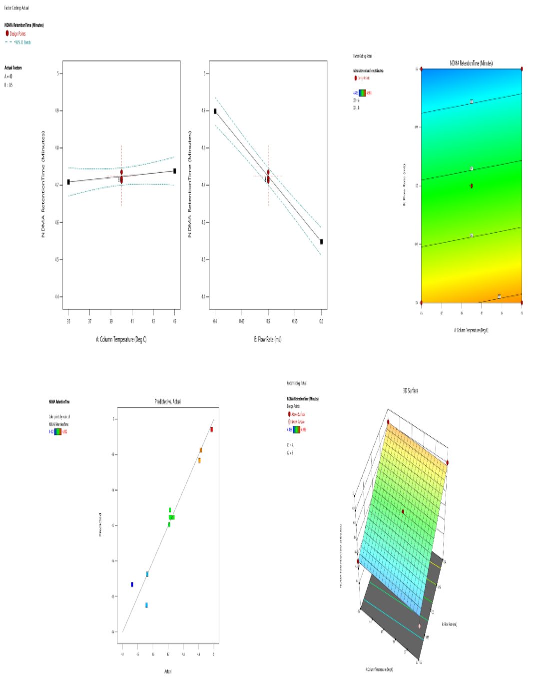
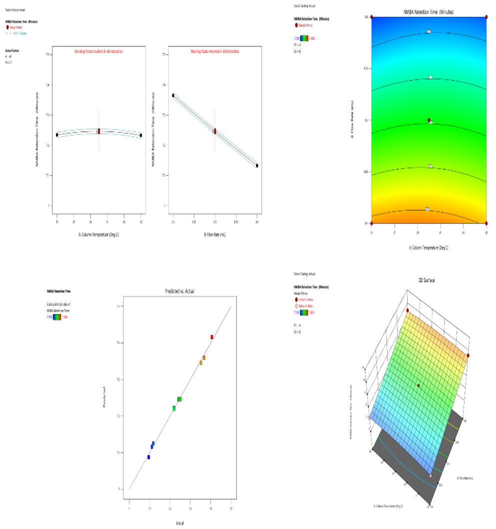


Figure 8: All factor coding plot, Contour plot, 2D Plot, and 3D plot for Response Factor 1 (NDMA Retention time)



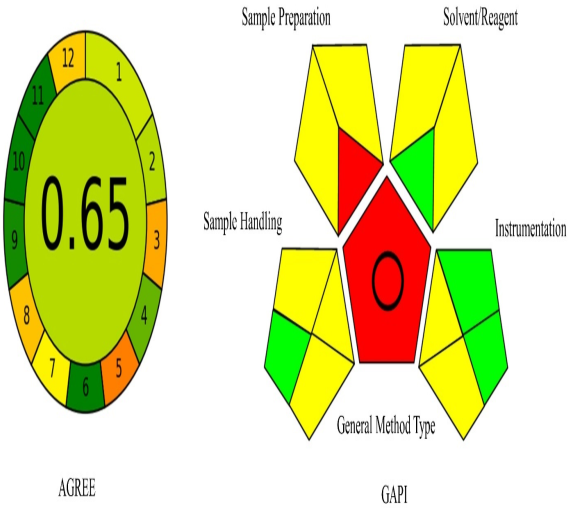


Figure 9: All factor coding plot, Contour plot, 2D, Plot and 3D plot for Response Factor 2 (NMBA Retention Time)

Figure 10: Represents GAP tools GAPI and AGREE pictograms for the proposed method.

Citation: G Raghuvaran and Shobha Rani Satla 2026. “Eco-Friendly and AQbD Approach for the estimation of trace level of NDMA and NMBA impurity in Rilpivirine formulation by UHPLC-MS/MS”. International Journal of Academic Research, 13(1): 74-90.