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# LC-MS/MS approach for the quantification of five genotoxic nitrosoimpurities in varenicline

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**ABSTRACT**: Using an LC-MS/MS (liquid chromatography-tandem mass spectrometry) method, four probable genotoxic impurities (PGIs) in the varenicline were quantified. With 0.7 mL/min of flow rate, the separation was performed using a Phenomenex kinetex F5 100 column (150 x 4.6 mm I.D., 2.6 µm) in gradient elution mode with formic acid of 0.1 percent in water as mobile phase A and formic acid of 0.1 percent in methanol as mobile phase B. MMR mode (Multiple reaction monitoring) is utilized to measure impurities with triple quadrupole mass detection using electrospray ionization. For all five PGIs, the approach was thoroughly verified as per ICH guidelines. In each case, this correlation coefficient was greater than 0.998. The recoveries for all chosen impurities were determined to be good, ranging between 83.7 to 107.3 percent. At a concentration level of 0.521-0.549 ppm, the proposed approach was sensitive enough to quantify all five PGIs. As a result, the proposed method for identifying and quantifying PGIs in varenicline is both practical and effective.

**KEYWORDS**: Varenicline; genotoxic impurities; liquid chromatography-tandem mass spectrometry; sensitive

## 1. INTRODUCTION

Varenicline (VRC), 7,8,9,10-tetrahydro-6,10-methano-6H-azepino[4,5-g]quinoxaline (2R,3R), is a new drug that acts as a relatively selective partial agonist for the nicotinic acetylcholine receptor in the central nervous system [1]. Therapeutically, VRC reduces nicotine withdrawal effects and cigarette craving by exhibiting both antagonistic and agonistic properties while inhibiting the reinforcing effects of nicotine on relapsing smokers [2-4]. The U.S. Food and Drug Administration has approved VRC tartrate (Champix® and Chantix®; Pfizer) as an aid to smoking cessation [5]. VRC at a dosage of 1 mg is prescribed for twelve weeks, with a one-week titration period at the beginning [6].

Five potentially genotoxic impurities (PGIs) as shown in figure 1, namely, 3-Nitroso-2,3,4,5-tetrahydro-1H-1,5-methanobenzo (d) azepine (Impurity A), 7,8-dinitro-3-nitroso-2,3,4,5-tetrahydro-1H-1,5 methanobenzo (d) azepine (Impurity B), 3-nitroso-2,3,4,5-tetrahydro-1H-1,5-methanobenzo(d)azepine-7,8-diamine (Impurity C), 8-nitroso-7,8,9,10-tetrahydro-6H-6,10-methanoazepine (4,5-g) quinoxaline (Impurity D) and N-Nitroso-diethyl amine (NDEA) are involved in the synthesis of varenicline. It is extremely challenging to detect and quantify such impurities during drug manufacturing. PGIs can cause chromosomal breakage in mammalian cell systems, genetic changes, or rearrangements [7,8]. The purity of the raw material is greatly affected by the presence of impurities whereas in certain cases, the finished drug product. PGIs are difficult to eliminate from a pharmaceutical product. As a result, contaminants in starting materials and APIs ("Active Pharmaceutical Ingredients") must be kept to a bare minimum [9]. For the detection and measurement of trace contaminants, a technique that is both reliable, as well as sensitive, must be developed. Pfizer, a pharmaceutical company, has announced a recall for Chantix®, which has gotten a lot of attention. This was due to N-nitroso-varenicline (the Nitroso-Drug Substance-Related impurity (NDSRI) detected above the Pfizer established Acceptable Daily Intake (ADI) level. Literature survey reveals only a few stabilty indicating LC methods [10,12] available for estimation of varenicline. Reports on process related impurities are available in literature by HPLC method [13] but the analysis time was greater than 25 min. However, there is no single LC-MS/MS based approach available for quantifying process-related

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nitroso genotoxic impurities is varenicline. Consedering the need of LC-MS/MS method for quantification of nitroso genotoxic impurities is varenicline, we aimed to develop and validate a new sensitive LC-MS/MS technique with suitable values of LOQ ("Limit of quantification") for quantification of PGIs. The technique described here has been verified under ICH [14]("International Council for Harmonization") guidelines for specificity, LOQ, accuracy, linearity, as well as precision . Because of its great sensitivity as well as selectivity, LC/MS/MS technique was chosen in this investigation to quantify five PGIs in varenicline.

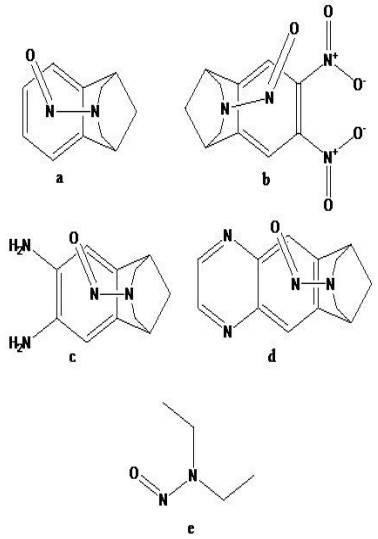


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**Figure 1.** Structure of a) Impurity A(3-Nitroso-2,3,4,5-tetrahydro-1H-1,5-methanobenzo (d) azepine) b) Impurity B (7,8-dinitro-3-nitroso-2,3,4,5-tetrahydro-1H-1,5 methanobenzo (d) azepine) c) Impurity C(3-nitroso-2,3,4,5-tetrahydro-1H-1,5-methanobenzo(d)azepine-7,8-diamine) d) Impurity D (8-nitroso-7,8,9,10-tetrahydro-6H-6,10-methanoazepine (4,5-g) quinoxaline ) e) NDEA

## 2. RESULTS & DISCUSSION

### 2.1. Method Development

The major focus of the research was to develop a selective and sensitive LC-MS/MS approach for isolating and quantifying five possible PGIs in varenicline, the API. Separating varenicline and its five PGIs is crucial due to structural and polarity similarities. As a result, the baseline separation of impurities was prioritized. A Phenomenex kinetex F5 100 Å column (150× 4.6 mm I.D., 2.6 $\mu$m) was shown to be the best suited in terms of separation and peak shape, and analyte response. With formic acid of 0.1 percent in methanol and water, the mobile phase was run in gradient mode. The temperature of column should be kept at 40 °C whereas the flow rate of mobile phase is kept constant at 0.7 mL/min. Impurities A, B, C, D, and NDEA had retention durations of 11.980, 11.075, 9.237, 10.151 and 8.158 minutes, correspondingly. The representative chromatograms were shown in the Figure 2.

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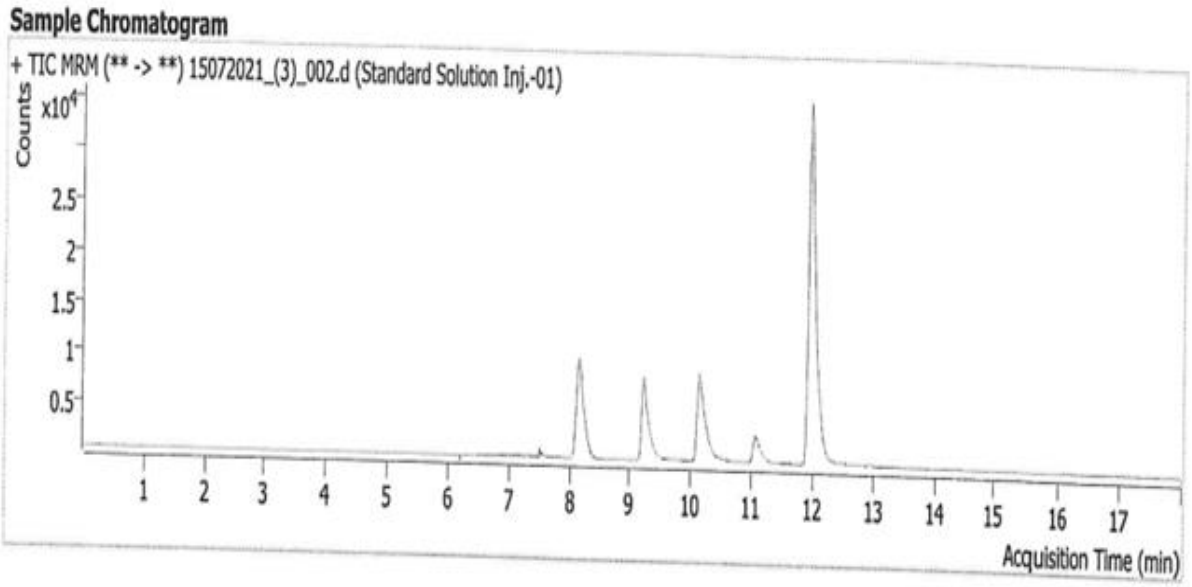


**Figure 2.** Typical MRM chromatogram of five PGIs

### 2.2. Optimization of LC-MS conditions

The objective of mass spectrometric ionization optimization is to design a method for identifying five PGIs in the varenicline API that was rapid, simple, stable, as well as sensitive. The five PGIs were detected and quantified using LC-MS/MS at a 1 µg/mL concentration level during development of method. During the early phases of method development, it was found that the signal intensity in positive mode was substantially stronger than in negative mode, constraining development of method to the positive ESI source. Fragmentation was performed utilizing four distinct collision energy voltages to improve the ESI conditions for PGIs (0 V, 10 V, 20 V, and 30 V). The capillary voltage has also been changed. Table 1 shows the ion source characteristics that were adjusted for a favorable response. Figure 3-7 shows the MS/MS spectra of five PGIs at varying collision energies.

**Table 1.** Multiple reactions monitoring (MRM) transitions and optimized collision energy for five PGIS

| Analyte | Retention Time (min) | Precursor→Product(m/z) | Fragmentor Voltage(V) | Collision Energy(eV ) |
|---|---|---|---|---|
| Impurity A | 11.739 | 189→128 | 100 | 20 |
| Impurity B | 10.877 | 1279.1→156.1 | 150 | 20 |
| Impurity C | 9.344 | 230.1→170 | 100 | 30 |
| Impurity D | 10.118 | 241.1→169 | 120 | 20 |
| NDEA | 8.450 | 103.1→47.1 | 80 | 19 |

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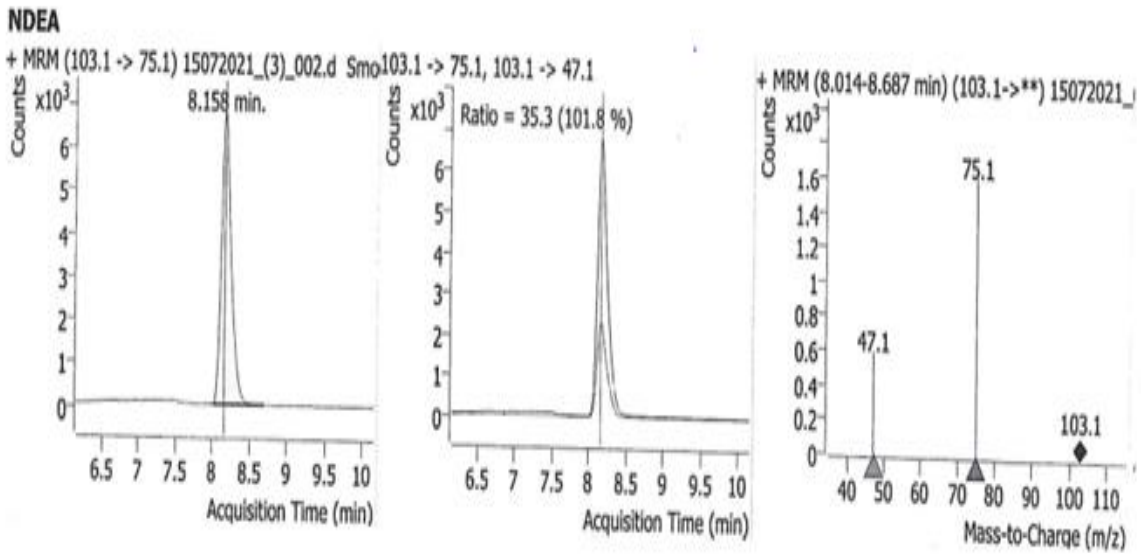


**Figure 3.** MRM chromatogram and mass spectra of NDEA

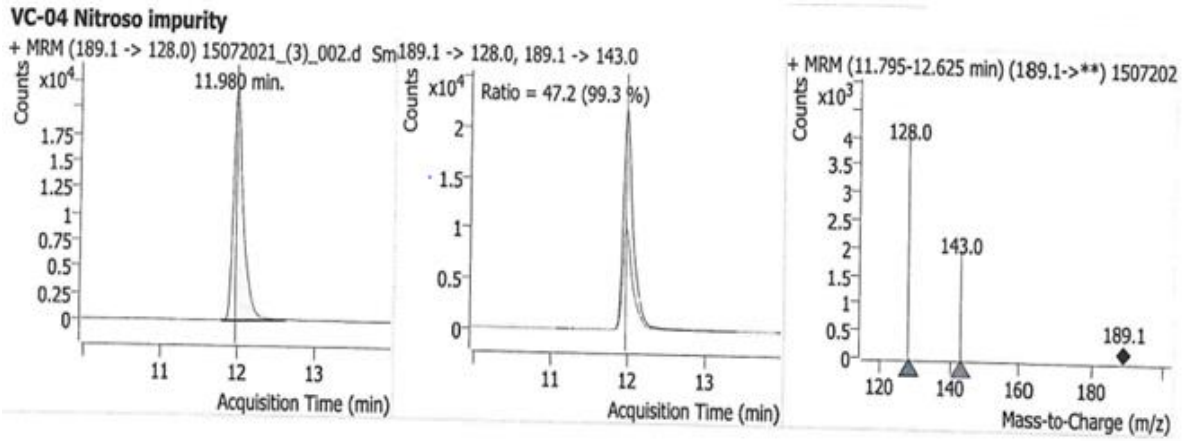


**Figure 4.** MRM chromatogram and mass spectra of Impurity A

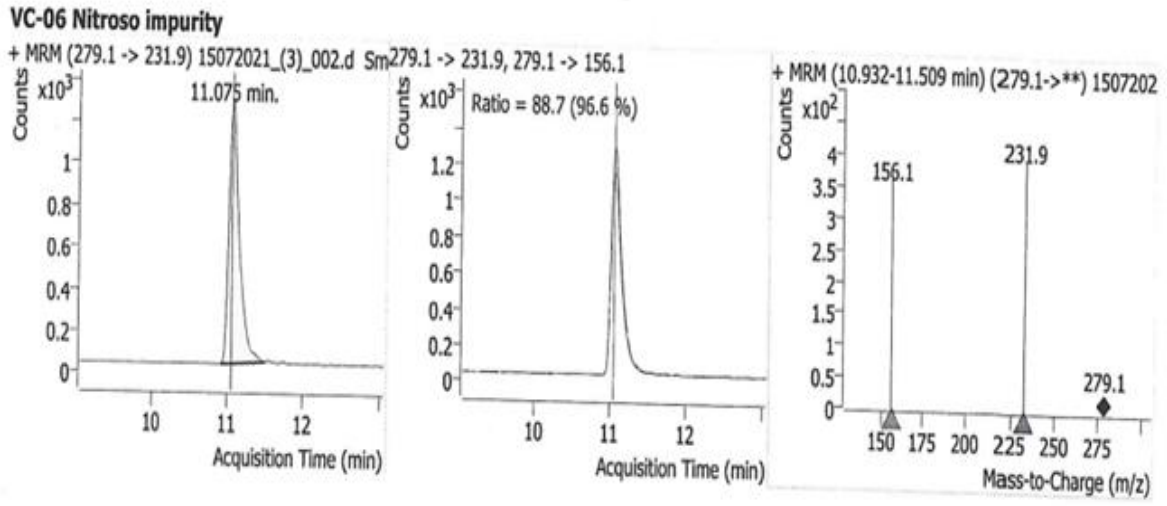


**Figure 5.** MRM chromatogram and mass spectra of Impurity B

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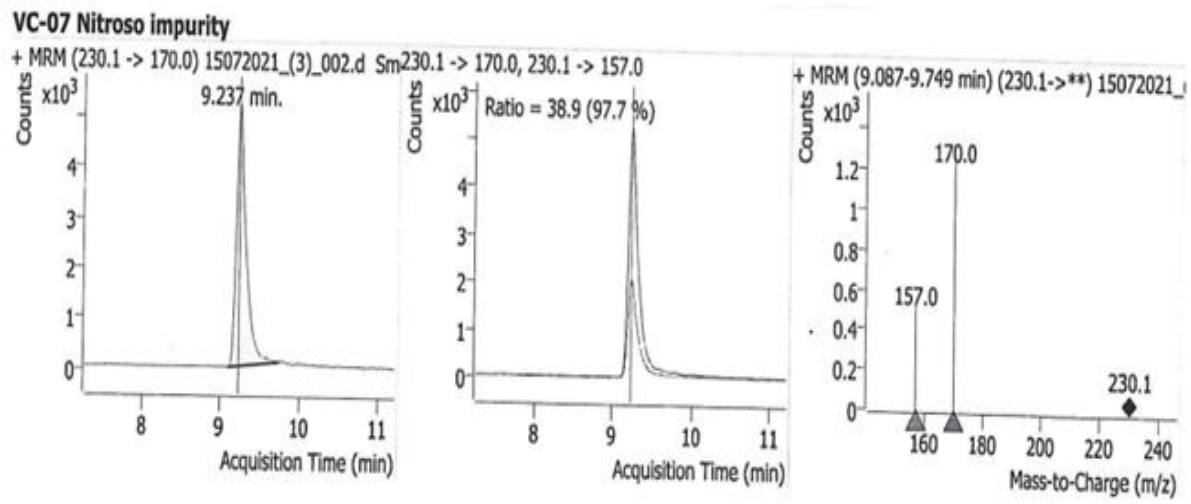


**Figure 6.** MRM chromatogram and mass spectra of Impurity C

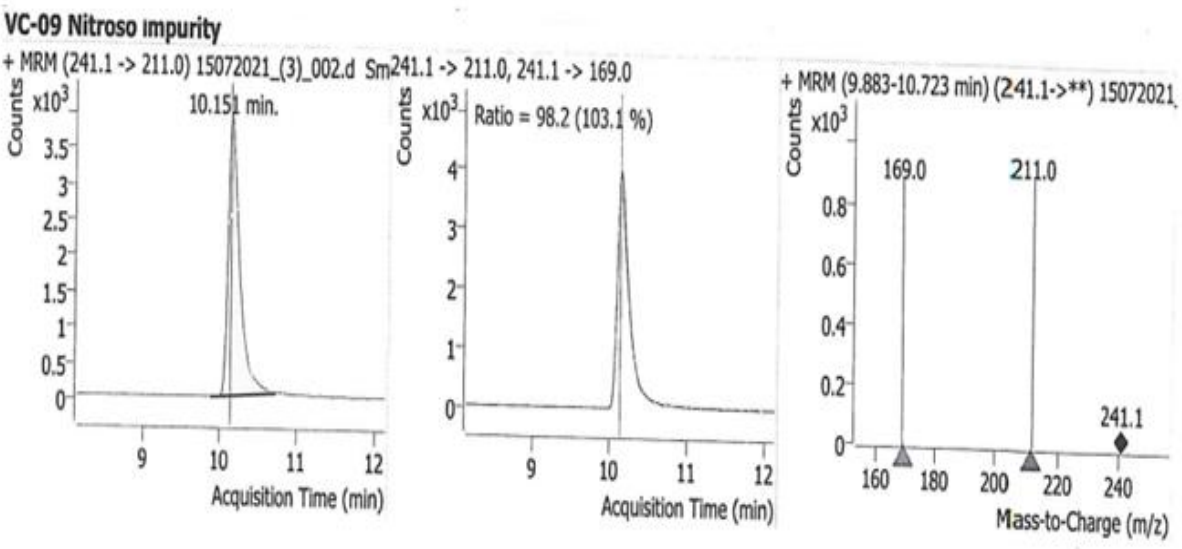


**Figure 7.** MRM chromatogram and mass spectra of Impurity D

## 2.3. Method validation

*2.3.1. Specificity*

In diluent, a single varenicline placebo solution was made at the specified concentration. The LC-MS/MS analysis of the spiked varenicline placebo solution was then performed and the findings demonstrated that the varenicline placebo peak did not interfere with Impurities A, B, C, D, or NDEA peaks, demonstrating the specificity of the proposed approach.

*2.3.2. Linearity*

The method's linearity was confirmed across a range of concentrations between 0.525-52.54 ppm for Impurity A, 0.536-53.61 ppm for Impurity B, 0.521-52.11 ppm for Impurity C, 0.532-53.2 ppm for Impurity D, and 0.549-5.93 ppm NDEA). The intercept, correlation coefficient, and slope data are calculated using the least-squares linear regression analysis of the average peak area vs analyte concentration. Table 2 shows a satisfactory correlation between analyte concentration and peak area. The calibration curve shown in figure 8.

**Table 2.** Calibration curves, LODs, and LOQs for five PGIS

| Analyte | Linearity Range(ppm ) | Regression Equation | $R^2$ | LOD(ppm ) | LOQ(ppm ) |
|---|---|---|---|---|---|
| Impurity A | 0.525-52.54 | Y=21577X-16907 | 0.9983 | 0.263 | 0.526 |
| Impurity B | 0.536-53.61 | Y=3031.3X-144.6 | 0.9986 | 0.263 | 0.526 |
| Impurity C | 0.521-52.11 | Y=9582.1X-12326 | 0.9970 | 0.263 | 0.526 |
| Impurity D | 0.532-53.22 | Y=18789X-11650 | 0.9978 | 0.263 | 0.526 |
| NDEA | 0.549-54.93 | Y=12794X-5616.7 | 0.9997 | 0.263 | 0.526 |

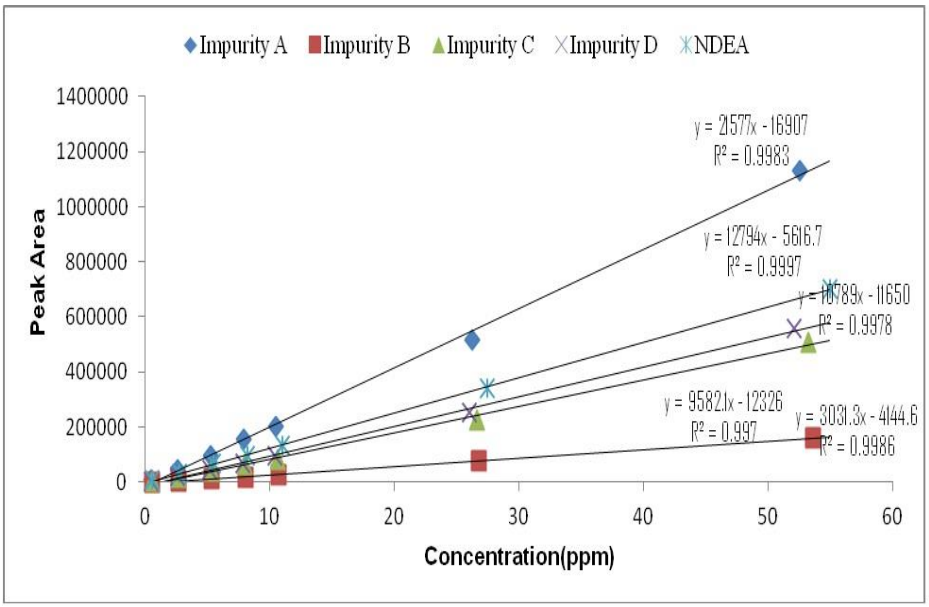


**Figure 8.** Calibration curves of PGIs

*2.3.3. Sensitivity*

By injecting standard solutions of known concentrations, the LOD and LOQ values (Table 2) of the five impurities were obtained based on S/N ratios of 3.0 and 10, respectively . PGIs with the least response factors were used to calculate the values. At LOQ value, the repeatability is determined by analyzing and calculating the % RSD values of six injections of each of the five impurities. It was found that impurity A had the weakest response and was thus less sensitive, whereas impurity D had the largest response and was, therefore, more sensitive. Such low values of LOQ are deemed acceptable as well as satisfactory for the precise analysis.

*2.3.4. Accuracy and recovery*

The method's accuracy was calculated using the recovery outcomes of five PGIs. Improvements in five PGIs were determined after blank varenicline samples were spiked with three different levels of five PGIs at 50 percent, 100 percent, and 150 percent of the limits, respectively, to assess the output of the proposed technique. The recoveries of impurities A, B, C, D, and NDEA vary between 87.68 to 122.75 %. In the perspective of the study's ultra-trace nature, PGIs recovery was determined to be within an acceptable range of 70-130 percent, showing that the suggested approach for PGIs is accurate.'The data presented in Table 3.

**Table 3.** Accuracy data of five PGIS

| Analyte | Varenicline Concentration (mg/mL) | Mean % Recovery at LOQ level ± SD | Mean % Recovery at 100 % level ± SD | Mean % Recovery at 200 % level± SD |
|---|---|---|---|---|
| Impurity A | 1.33 | 93.2 ± 0.23 | 98.2 ± 0.98 | 103.3 ± 1.89 |
| Impurity B | | 74.6 ± 1.75 | 86.0 ± 0.76 | 91.9 ± 0.49 |
| Impurity C | | 93.8 ± 0.31 | 99.4 ± 1.38 | 93.6 ± 1.66 |
| Impurity D | | 89.6 ± 1..73 | 90.2 ± 0.99 | 100.5 ± 0.26 |
| NDEA | | 121.7 ± 0.58 | 97.8 ± 0.45 | 102.4 ± 0.23 |

*Precision*

Intra and inter-day accuracy were employed to test the method's precision. Comparison of "standard deviation" of spiked specimens recovery percentage was used to estimate intra-day precision. Spiked samples were evaluated on three distinct days to find inter-day accuracy. Intermediate accuracy was evaluated using data from a different study conducted on a different day with different analysts and fresh solutions. Table 4 shows that the acceptable RSD percent values for the intra and inter-day precision are between 2.88-9.15 % and 1.45 and 6.38%, respectively, for this "LC-MS/MS" technique.

**Table 4.** Precision results of five PGIS

| Drug(API) | Analyte | Con. (ppm)* (µg/g API) | System Precision (% RSD) | Method Precision (% RSD) | | Intermediate Precision (% RSD) | |
|---|---|---|---|---|---|---|---|
| | | | | Interday | Intraday | Analyst I | Analyst II |
| Varenicline | Impurity A | 5.255 | 2.43 | 7.44 | 6.37 | 3.53 | 6.74 |
| | Impurity B | 5.361 | 5.52 | 7.46 | 6.83 | 7.28 | 5.83 |
| | Impurity C | 5.211 | 3.78 | 5.26 | 7.69 | 7.35 | 7.97 |
| | Impurity D | 5.322 | 5.26 | 3.79 | 2.93 | 5.41 | 8.27 |
| | NDEA | 5.494 | 7.21 | 9.26 | 8.39 | 7.32 | 3.33 |

*1 ppm corresponds to 0.0013 µg/mL of Impurity A , impurity B , Impurity C , Impurity D and NDEA respectively.

*2.3.5. Stabilities of PGIs*

Standard solutions of 0.007 µg/mL were made for each of the five PGIs and analyzed every 4 h to a new standard. These solutions were kept at a temperature of 25 °C in complete darkness. The recovery % of PGIs from such stock solutions ranged from 97.51 to 105.04%, and the difference in PGI recoveries at 0 h and 24 h was just 10 %, indicating that the stock solution was stable for at least 24 h.

*2.3.6. Robustness*

To evaluate the method's robustness, the column oven temperatures as well as mobile phase flow rate were purposefully adjusted. The flow rate of mobile phase was optimized at 0.7 mL/min, however, it was changed from 0.65 to 0.75 mL/min. At 37°C and 43°C (changed by 3°C), the impact of column temperature on the resolution was investigated. The findings demonstrate that changing the column temperature and flow rate did not influence chromatographic effectiveness for any contaminants in spiked specimens, demonstrating the method's robustness while the mobile phase components remained unchanged.

## 2.4. Applications in samples

Four batches of commercial varenicline-containing drugs were tested for "five PGIs" and none were found to contain any of the five "PGIs" in any of the four batches of the commercialized formulations.

## 3. CONCLUSION

In this study, positive ionization mode with MRM was used to develop a simplified LC-MS/MS technique to evaluate five different PGIs in varenicline simultaneously. The method was validated according to ICH guidelines, and it was confirmed to be both accurate and linear over the entire concentration range. The calculated values of LOQ and LOD for all five impurities are modest and within the acceptable range. The analytical sample solution was found to be stable for at least 48 hours. The approach has been thoroughly tested and exhibits excellent accuracy, linearity, robustness, as well as repeatability. Moreover, the method proposed here could be of great use for the determination of impurities A, B, C, D, and NDEA in varenicline during manufacturing.

## 4. MATERIALS AND METHODS

### 4.1. Chemicals and materials

The drug varenicline has been acquired as a free sample by the local pharmaceutical company. Sigma Aldrich acquired NDEA (purity ≥ 99.82%), Impurity A (purity ≥ 99.70%), Impurity B (purity≥ 99.82%), Impurity C (purity ≥ 99.85%), and Impurity D (purity≥99.89%) standards. Methanol, formic acid, as well as water were all HPLC-grade solvents bought from Merck Ltd India, Mumbai.

### 4.2. Instrumentation

The sample analysis was performed using an Agilent 6470 series HPLC system and a 6470B triple quadrupole MS (Agilent Technologies, Inc.; Santa Clara, CA, United States) coupled using an electrospray ionization mode. A Phenomenex kinetex F5 100 Å column (150×4.6 mm I.D., 2.6 $\mu$m) has been bought from W.R. Grace & Co. (Columbia, MD, United States). Column temperature was maintained at 40°C using thermostat column compartment.. Mobile phase A is formic acid of 0.1 percent in water, while mobile phase B is formic acid of 0.1 percent in methanol. Both mobile phases run in a gradient elution mode. The injection volume and flow rate of mobile phase are optimized at 40 $\mu$L and 0.7 mL/min, correspondingly. In MRM mode, a triple quadrupole MS was used in conjunction with a positive electrospray ionization source. For nebulizer pressure, drying gas flow, spray voltage, and gas temperature, the apparatus was set to 25 psi, 7 L/min, 4500 V, and 300 °C, correspondingly. The MRM conditions are tailored particularly for all five PGIs due to their distinct structures, and Table 1 specifies the MS requirements for MRM.

### 4.3. Preparation of standard and sample solutions

By dissolving adequate quantities of all impurities in 100 percent methanol as the diluent, a stock mixture of varenicline PGIs (Impurities A, B, C, D, and NDEA) (1 mg/mL) has been made. Dilution with methanol yielded a diluted stock solution of 0.007 µg/ml (5.26 ppm for API) from this solution.

### 4.4. Method validation

Precision, linearity, repeatability, accuracy, LOQ, LOD, solution stability, and robustness were all satisfactorily verified using the proposed technique. The validation method for Impurity A – Impurity D, NDEA in varenicline was carried out in compliance with the International Conference on Harmonisation (ICH) criteria. Individual solutions of all four contaminants (1 mg/mL; 1 ppm for 0.0013 µg/mL varenicline) are made first, and their S/N ratios have been determined. The repeatability of the same solutions at the defined LOD and LOQ values was tested experimentally by injecting the same solutions six times. The method's linearity was then tested using seven different concentration levels between the LOQ and a predetermined impurity concentration. The regression coefficient, slope, and intercept values are determined with least-squares linear regression analysis. The developed method's specificity was tested using varenicline tablets and a placebo. The accuracy of the procedure was then determined in triplicate using the standard addition technique at three distinct concentration levels, namely LOQ, 100 %, and 200 %. For five contaminants in three pure API batches, RSD and recovery values are computed. Changes in the column temperature and mobile phase flow rate were used to test the method's robustness. The stability of the contaminants in the specimen solution was measured by comparing the analysis of the specimen solution at varying time intervals to newly produced samples.

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**Author contributions:** Concept – K.M., S.R.; Design – S.R., K.M.; Supervision – K.M.; Resources – K.M.; Materials – S.R.; Data Collection and/or Processing – S.R.; Analysis and/or Interpretation – K.M., S.R.; Literature Search – S.R.; Writing – S.R.; Critical Reviews – K.M., S.R.

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