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Development and validation of high-precision analytical methods for monitoring nitrosamine contaminants in pharmaceutical Apis

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Abstract
The presence of nitrosamine impurities in pharmaceutical products has emerged as a significant safety concern due to their potential carcinogenic nature. In this study, a high-precision analytical method was developed and validated for the simultaneous detection and quantification of selected nitrosamines, including N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodiisopropylamine (NDIPA), N-nitrosoethylisopropylamine (NEIPA), and N-nitrosodibutylamine (NDBA), in pharmaceutical active ingredients and formulations. The method employed optimized extraction techniques combined with advanced LC–MS/MS and GC–MS/MS instrumentation to achieve high sensitivity and selectivity.
The developed method demonstrated excellent linearity over the concentration range of 0.5–100 ng/mL, with correlation coefficients (R^2) greater than 0.999 for all analytes. The limits of detection ranged from 0.15 to 0.35 ng/mL, while limits of quantification were between 0.50 and 1.20 ng/mL, indicating the capability for ultra-trace analysis. Recovery studies showed accuracy within 95.5% to 102.0%, and precision studies yielded %RSD values below 5%, confirming the reproducibility of the method.
Application of the method to pharmaceutical samples revealed the presence of NDMA in the range of 1.2–3.0 ng/g, while other nitrosamines were either not detected or present at trace levels within acceptable regulatory limits. Matrix effect evaluation showed minimal ion suppression or enhancement, with matrix factor values ranging from 0.97 to 1.02.
The method was validated in accordance with guidelines from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, demonstrating its suitability for routine quality control and regulatory compliance. Overall, the developed method provides a robust, sensitive, and reliable analytical approach for monitoring nitrosamine contaminants in pharmaceutical products.

Keywords: Nitrosamines, NDMA, LC–MS/MS, GC–MS/MS, Pharmaceutical impurities, Method validation, Trace analysis, ICH Q2, Analytical chemistry, Drug safety

Introduction

Emerging Concern of Nitrosamine Contamination

The quality and safety of pharmaceutical products have always been central to public health protection. In recent years, however, the unexpected detection of nitrosamine impurities in several widely used drug products has raised significant global concern. Nitrosamines are a class of N-nitroso compounds recognized for their strong mutagenic and carcinogenic properties. Even at extremely low levels of exposure, these compounds have been associated with an increased risk of cancer, primarily due to their ability to induce DNA alkylation and subsequent genetic mutations (Snodin, 2023) [5].
The issue gained global attention following the identification of nitrosamine contaminants in drugs such as angiotensin II receptor blockers, metformin, and ranitidine. These incidents led to widespread product recalls and intensified regulatory scrutiny. As a result, nitrosamines are now categorized under the "cohort of concern" for mutagenic impurities, requiring strict monitoring and control in pharmaceutical products.
The growing concern is not only due to their toxicity but also because of their ability to remain undetected using traditional quality control techniques. Their presence at trace levels—often in nanograms per day—poses a unique analytical challenge. Consequently, there is a pressing need for highly sensitive and reliable analytical methods capable of detecting and quantifying these impurities with high accuracy and precision.

Complexity of Formation Pathways

One of the major challenges associated with nitrosamine contamination is the complexity of their formation mechanisms. Unlike many other impurities, nitrosamines can form through multiple pathways during different stages of the pharmaceutical product lifecycle.
The most common mechanism involves the reaction between secondary or tertiary amines and nitrosating agents such as nitrites under acidic or elevated temperature conditions. These reactions are frequently encountered during API synthesis, especially when amine-containing intermediates are used. However, recent studies have shown that nitrosamine formation is not limited to synthesis alone.
Degradation processes during storage can also lead to nitrosamine formation. Environmental factors such as heat, humidity, and light exposure may accelerate chemical reactions, resulting in the generation of these impurities over time. Additionally, excipients and packaging materials have been identified as potential contributors, as they may contain trace levels of nitrites or other reactive species (Vahora Shahin 2015) [28].
Another critical aspect is the role of raw materials and solvents. The use of recycled solvents, contaminated reagents, or inadequately purified water systems can

introduce nitrosating agents into the manufacturing process. Furthermore, interactions between drug substances and excipients can lead to in situ formation of nitrosamines during formulation or storage.

The multifactorial nature of nitrosamine formation makes it difficult to predict and control their occurrence. This complexity necessitates a comprehensive understanding of chemical pathways and highlights the importance of implementing robust analytical and risk assessment strategies.

Regulatory Expectations and Risk-Based Approaches

In response to the growing concern over nitrosamine contamination, global regulatory authorities have established stringent guidelines to ensure drug safety. Agencies such as the U.S. Food and Drug Administration and the European Medicines Agency have issued detailed recommendations for the detection, evaluation, and control of nitrosamine impurities.

These regulatory frameworks emphasize a risk-based approach, which involves identifying potential sources of nitrosamine formation, assessing the likelihood of their occurrence, and implementing appropriate control measures. Manufacturers are required to conduct comprehensive risk assessments for both existing and newly developed drug products.

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use provides additional guidance through its M7(R1) and updated recommendations, which focus on the assessment and control of DNA-reactive (mutagenic) impurities. These guidelines establish acceptable intake limits based on toxicological data and lifetime exposure risks.

Failure to comply with these regulations can result in severe consequences, including product recalls, regulatory actions, and reputational damage. Therefore, pharmaceutical companies must adopt proactive strategies to ensure compliance, including the development of validated analytical methods and continuous monitoring systems.

Need for High-Precision Analytical Techniques

The detection of nitrosamines at ultra-trace levels presents significant analytical challenges. Conventional techniques such as high-performance liquid chromatography (HPLC) with UV detection often lack the sensitivity and selectivity required to detect these compounds at regulatory thresholds.

Recent advancements in analytical science have led to the development of highly sophisticated techniques capable of overcoming these limitations. Among these, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has emerged as the most widely used method due to its high sensitivity, specificity, and ability to analyze complex matrices. Similarly, gas chromatography–mass spectrometry (GC–MS) is particularly effective for volatile nitrosamines.

High-resolution mass spectrometry (HRMS) further enhances analytical capabilities by enabling accurate mass determination and structural elucidation of unknown impurities. These techniques allow simultaneous detection of multiple nitrosamines, improving efficiency and throughput (Akabari AH *et al* 2025) [1].

In addition to instrumental advancements, improvements in sample preparation techniques have also contributed significantly to analytical performance. Methods such as solid-phase extraction (SPE), headspace analysis, and microextraction techniques help reduce matrix interference and enhance detection sensitivity.

The integration of advanced instrumentation with optimized sample preparation protocols is essential for achieving reliable and reproducible results, particularly when dealing with complex pharmaceutical matrices.

Importance of Method Validation

While the development of advanced analytical methods is crucial, their validation is equally important to ensure reliability and regulatory acceptance. Method validation involves the systematic evaluation of analytical performance characteristics to confirm that the method is suitable for its intended purpose.

Key validation parameters include specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness. Specificity ensures that the method can accurately identify the analyte in the presence of other components, while linearity demonstrates the relationship between concentration and response.

Accuracy and precision are critical indicators of method reliability, reflecting the closeness of measured values to the true value and the reproducibility of results, respectively. Sensitivity parameters such as LOD and LOQ are particularly important for nitrosamine analysis due to their presence at trace levels.

Robustness testing evaluates the method's ability to remain unaffected by small variations in analytical conditions, ensuring consistent performance during routine use. Proper validation not only ensures compliance with regulatory guidelines but also enhances confidence in analytical results (Vikram *et al*., 2024) [3].

Aim and Significance of the Study

The increasing regulatory scrutiny and the potential health risks associated with nitrosamine impurities highlight the urgent need for reliable analytical solutions. In this context, the present study aims to develop and validate high-precision analytical methods for the detection and quantification of nitrosamine contaminants in pharmaceutical active pharmaceutical ingredients (APIs).

The primary objectives include achieving ultra-trace level detection, ensuring high accuracy and precision, and complying with international regulatory standards. The study also focuses on optimizing sample preparation techniques and minimizing matrix effects to enhance analytical performance.

The significance of this research lies in its contribution to improving pharmaceutical quality control systems and ensuring patient safety. By providing a robust and validated analytical framework, this study supports the pharmaceutical industry in meeting regulatory requirements and mitigating risks associated with nitrosamine contamination.

Furthermore, the findings of this study may serve as a foundation for future research aimed at expanding analytical capabilities, including multi-nitrosamine detection and real-time monitoring of impurity formation.

Review of Literature

Kao *et al*., (2022) [8] drug substances are at risk of contamination with N-nitrosamines (NAs), well-known carcinogenic agents, during synthesis processes and/or long-term storage. Therefore, in this study, we developed an

efficient data-based screening approach to systemically assess marketed products and investigated its scalability for benefiting both regulatory agencies and pharmaceutical industries. A substructure-based screening method employing Data Warrior, an open-source software, was established to evaluate the risks of nitrosamine (NA) impurities in drug substances. Eight nitrosamine (NA) substructures containing susceptible amino sources for N-nitrosation have been identified as screening targets: dimethylamine (DMA), diethylamine, isopropylethylamine, diisopropylamine, Nmethyl-2-pyrrolidone, dibutylamine, methylphenylamine, and tetrazoles. Our method detected 192 drug substances with a theoretical possibility of nitrosamine (NA) impurity, 141 of which had not been reported previously. In addition, the DMA moiety was significantly dominant among the eight nitrosamines (NA) substructures. The results were validated using data from the literature, and a high detection sensitivity of 0.944 was demonstrated. Furthermore, our approach has the advantage of scalability, owing to which 31 additional drugs with suspected nitrosamine (NA) contaminated substructures were identified using the substructures of 1- methyl-4-piperazine in rifampin and 1-cyclopentyl-4-piperazine in rifapentine. In conclusion, the reported substructure-based approach provides an effective and scalable method for the screening and investigation of nitrosamine impurities (NAs) in various pharmaceuticals and might be used as an ancillary technique in the field of pharmaceutical quality control for risk assessments of potential nitrosamine impurities (NAs).

Yamamoto *et al*., (2022) [9] N-Nitrosodimethylamine (NDMA) has been detected in some drug substances and pharmaceutical products containing sartans, ranitidine and metformin, and a potential risk of NDMA contamination exists in other drug substances and their pharmaceutical products. To quantitate NDMA in various drugs having diverse physicochemical properties, a specific, sensitive, and reliable analytical method is required, in addition to methods that can be applied to a class of nitrosamines. We aimed to develop an off-line isolation method for NDMA in drug substances using SPE for quantification with LC–APCI–MS/MS. Impediments to accurate quantitation of NDMA in drug substances using LC–MS/MS and insufficient durability of the system are attributed to the extremely large amounts of active pharmaceutical ingredients (APIs) in sample solutions in comparison to the trace amount of NDMA. A reduced retention of NDMA and/or decreased separation from other substances in LC, matrix effect in MS detection, and undesirable contamination of instruments with API and other substances may be occasionally encountered, all of which consequently result in deterioration of system performance and generation of unreliable data, even in the cases where a divert valve is configured between the column and ion source of the MS instrument. To address these problems, an off-line NDMA isolation methodology from APIs exhibiting diverse physicochemical properties, namely ranitidine hydrochloride (ranitidine), metformin hydrochloride (metformin), nizatidine, valsartan, and telmisartan, was developed. The applicability of the method was confirmed by batch analysis of metformin and ranitidine. Furthermore, contrary to previous reports, NDMA was found to be stable over a wide pH range. The proposed methodology and data from this study would contribute to the control of NDMA contamination in various drugs to realize the safe delivery of pharmaceuticals to patients.

Zheng *et al*., (2022) [10] the recent detection of potent carcinogenic nitrosamine impurities in several human medicines has triggered product recalls and interrupted the supply of critical medications for hundreds of millions of patients, illuminating the need for increased testing of nitrosamines in pharmaceutical products. However, the development of analytical methods for nitrosamine detection is challenging due to high sensitivity requirements, complex matrices, and the large number and variety of samples requiring testing. Herein, we report an analytical method for the analysis of a common nitrosamine, N-nitrosodimethylamine (NDMA), in pharmaceutical products using full evaporation static headspace gas chromatography with nitrogen phosphorous detection (GC-HS-NPD). This method is sensitive, specific, accurate, and precise and has the potential to serve as a universal method for testing all semi-volatile nitrosamines across different drug products. Through elimination of the detrimental headspaceliquid partition, a quantitation limit of 0.25 ppb is achieved for NDMA, a significant improvement upon traditional LC-MS methods. The extraction of nitrosamines directly from solid sample not only simplifies the sample preparation procedure but also enables the method to be used for different products as is or with minor modifications, as demonstrated by the analysis of NDMA in 10 plus pharmaceutical products. The in situ nitrosation that is commonly observed in GC methods for nitrosamine analysis was completely inhibited by the addition of a small volume solvent containing pyrogallol, phosphoric acid, and isopropanol. Employing simple procedures and low-cost instrumentation, this method can be implemented in any analytical laboratory for routine nitrosamine analysis, ensuring patient safety and uninterrupted supply of critical medications.

Hu *et al*., (2021) Since July 2018 several drugs have been recalled due to contamination with N-nitrosodimethylamine (NDMA), a probable human carcinogen. Dimethylamine (DMA) and nitrite are precursors in the formation of NDMA. In this study, Ion chromatography (IC) methods were developed for the determination of these two precursors in drug substances and drug products. Two methods were developed to determine DMA in two drug products using a cation exchange separation coupled to suppressed conductivity detection. The limit of detection of DMA is < 1 μg/g of active pharmaceutical ingredient (API) for both methods. Nitrite was determined using an anion exchange separation coupled with UV absorbance detection. The limit of detection of nitrite was 0.918 μg/g API. The developed methods were successfully applied to DMA and nitrite determinations in five drug products including metformin, losartan, ranitidine, Nytol, and Benadyrl, and two drug substances (APIs), losartan potassium and metformin hydrochloride. Some samples contained nitrite and DMA at detectable levels. Dimethylamine and nitrite recovery from pharmaceutical samples ranged from 96.0 - 104 %. The developed methods should be useful for the rapid screening and quantification of nitrite and DMA in pharmaceuticals and in-process samples to assess the likelihood of NDMA formation. The methods for DMA should be applicable to other amines to assess the likelihood of the formation of other nitrosamines in pharmaceutical products.

Venkatesan *et al*., (2020) [17] a simple, precise and accurate GC-MS method was developed for estimation the content estimation of N-Nitrosodimethylamine (NDMA) &

NNitrosodiethylamine (NDEA) in Olmesartan medoxomil (OLM) in drug substances. The content was determined by GC-MS on DB-CAM 30.0 m X 0.32 mm, 0.5µm Capillary column and helium was used as carrier gas, using methanol as diluent at column flow rate of 2.0 mL/min and Ion source temperature & Interface temperature at 200ºC and Detector gain mode relative to tuning file with acquisition mode Q3 SIM. The method was developed and evaluated for validation parameter as per ICH guidelines for Specificity, linearity, accuracy and precision. The method shows good linearity over the range of 10% - 150% for NDMA and NDEA for olmesartan. The average percentage recoveries were found within predefined acceptance criteria (10% and 100%) for N-Nitrosodimethylamine (NDMA) & N-Nitrosodiethylamine (NDEA) in Olmesartan medoxomil, respectively. Therefore, the proposed method can be applied for routine analysis of the bulk drugs as well as combined pharmaceutical dosage forms of Olmesartan medoxomil.

Chang *et al*., (2020) [18] an incident of sartan medicine contamination was notified by Europe in June 2018. The contaminant was identified as a probable carcinogenic nitrosamine and the recalls of sartan medicines were soon made. Since then, more nitrosamine contaminants in sartan medicines were reported. To broaden the applicability and variety in nitrosamine determination, a multi-analyte method is required. In this study, a feasible and sensitive multi- analyte LC-MS/MS method for determination of 12 nitrosamines in sartans was established, where the active pharmaceutical ingredients and final products merchandised in Taiwan were also examined. Chromatographic separation was achieved on an Xselect® HSS T3 column (15 cm £ 3 mm i.d., 3.5 mm) with gradient elution using mobile phase A consisting of 0.1% formic acid in water and mobile phase B consisting of 0.1% formic acid in acetonitrile/methanol (2:8). Validation of the proposed method was also carried out. The limit of detection and limit of quantification for 12 nitrosamines were 20 ng/g and 50 ng/g, respectively. The intra-day and inter-day recoveries of nitrosamines were among 80% - 120% with precision of 20% for most of nitrosamines within sartans matrices. The method was successfully established and applied to authentic samples which a total of 98 positive samples containing 5 distinct nitrosamines, including N-nitrosodiethylamine, N-nitrosodimethylamine, N-nitroso-N-methyl-4- aminobutyric acid, N-nitrosomorpholine and N-nitrosopiperidine, were detected from 557 authentic samples

Materials and Methods

Chemicals and Solvents

All chemicals and solvents used in this study were of high analytical grade to ensure accuracy and reliability of the results. Methanol and acetonitrile of LC–MS grade were employed as primary organic solvents for extraction and chromatographic separation. Ultrapure water obtained from a Milli-Q purification system was used throughout the analysis. Dichloromethane and hexane were utilized for liquid–liquid extraction procedures due to their excellent partitioning properties for organic compounds. Formic acid was used as a mobile phase modifier to enhance ionization efficiency in mass spectrometric analysis, while ammonium acetate served as a buffering agent to improve chromatographic performance. Sodium chloride was added during extraction processes to enhance analyte partitioning, and sodium nitrite was included in controlled experiments to study the formation of nitrosamines under specific conditions. All reagents were procured from certified suppliers and used without further purification.

Nitrosamine Standards

Analytical reference standards of selected nitrosamines were used for calibration and method validation. These included N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodiisopropylamine (NDIPA), N-nitrosoethylisopropylamine (NEIPA), and N-nitrosodibutylamine (NDBA). These compounds were selected based on their regulatory relevance and frequent detection in pharmaceutical products. Standard stock solutions were prepared in methanol and stored under controlled temperature conditions to prevent degradation.

Internal Standards

Isotopically labeled internal standards were incorporated into the analytical method to improve quantification accuracy and compensate for matrix effects. NDMA-d6 and NDEA-d10 were selected as internal standards due to their structural similarity to the target analytes. These standards were added to all calibration and sample solutions prior to analysis to ensure consistent and reliable results.

Pharmaceutical Samples

A variety of pharmaceutical active ingredients and finished dosage forms were selected to evaluate the applicability of the developed analytical method. Losartan potassium was chosen as a representative API with known susceptibility to nitrosamine contamination during synthesis. Ranitidine hydrochloride was included due to its tendency to form nitrosamines through degradation pathways. Metformin hydrochloride tablets were selected as widely used formulations with reported nitrosamine concerns. Valsartan tablets were analyzed as representative angiotensin receptor blockers, while rifampicin capsules were included to study nitrosamine formation associated with specific synthetic routes. All samples were obtained from commercial sources and stored under controlled environmental conditions to prevent degradation or contamination prior to analysis.

Sample Preparation Materials

Solid-Phase Extraction (SPE) Materials

Solid-phase extraction was performed using C18 and polymeric cartridges to achieve effective cleanup and concentration of analytes. A vacuum manifold system was employed to facilitate controlled flow through the cartridges. PTFE syringe filters with a pore size of 0.22 µm were used to remove particulate matter from the extracts before analysis. All processed samples were stored in amber glass vials to minimize light-induced degradation.

Solid-Phase Microextraction (SPME) Materials

Solid-phase microextraction was carried out using fibers coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), which are suitable for extracting volatile and semi-volatile nitrosamines. Headspace vials with appropriate volume capacity were used for sample incubation, and aluminum crimp caps with septa ensured airtight sealing during extraction.

Fig 1: Sample Preparation

Liquid–Liquid Extraction (LLE) Materials
Liquid–liquid extraction procedures were conducted using separatory funnels and centrifuge tubes to facilitate phase separation. A vortex mixer was used to ensure efficient mixing of solvents and samples, while a nitrogen evaporator system was employed to concentrate extracts under controlled conditions without thermal degradation.

Soxhlet Extraction Materials
Soxhlet extraction was utilized for exhaustive extraction of analytes from solid samples. The setup included a Soxhlet extractor, a heating mantle to maintain consistent temperature, and cellulose extraction thimbles to hold the sample during extraction.

Contamination Control Measures
Strict contamination control measures were implemented to avoid artefactual formation of nitrosamines during analysis. All glassware was pre-baked at elevated temperatures to remove residual contaminants. Blank solvent runs were performed prior to sample analysis to verify system cleanliness, and carryover effects were evaluated between successive injections. Special care was taken to minimize exposure to nitrite-containing materials and environmental factors that could contribute to nitrosamine formation.

Instrumentation
LC–MS/MS System
Quantitative analysis was performed using a liquid chromatography system coupled with a triple quadrupole mass spectrometer. The system was equipped with an electrospray ionization source operating in positive ion mode, providing high sensitivity and selectivity for nitrosamine detection. Chromatographic separation was achieved using a reverse-phase column under optimized conditions.

GC–MS/MS System
A gas chromatography system coupled with tandem mass spectrometry was employed for the analysis of volatile nitrosamines. The system operated under electron ionization mode, allowing efficient fragmentation and detection of analytes with high sensitivity.

Fig 2: LC–MS/MS and GC–MS/MS Systems

Supporting Equipment
Additional laboratory equipment included an analytical balance for precise weighing, an ultrasonic bath for sample dissolution, a centrifuge for phase separation, a pH meter for solution adjustment, and a nitrogen gas supply system for sample concentration. Temperature-controlled systems were used to maintain consistent experimental conditions.

Method Development
Extraction Optimization
Various extraction techniques were evaluated to achieve maximum recovery of nitrosamines from pharmaceutical matrices. Parameters such as solvent selection, extraction time, salt addition, and sample-to-solvent ratio were systematically optimized. The objective was to minimize matrix interference while ensuring high extraction efficiency.

LC Optimization
Chromatographic conditions were optimized by adjusting mobile phase composition, flow rate, column temperature, and gradient profile. A combination of water containing formic acid and acetonitrile was found to provide optimal separation and peak shape for the target analytes.

MS Parameter Optimization
Mass spectrometric parameters were optimized to achieve maximum sensitivity and selectivity. Parameters such as ionization voltage, collision energy, and gas flow rates were adjusted. Multiple reaction monitoring transitions were selected for each analyte to ensure accurate identification and quantification.

GC Temperature Programming
Temperature programming in gas chromatography was optimized by selecting appropriate initial temperature, ramp rate, and final hold time. This ensured efficient separation of volatile nitrosamines within a reasonable analysis time.

Method Validation (ICH Q2 Guidelines)
The developed analytical method was validated in accordance with guidelines established by the International

Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use to ensure its reliability and suitability for routine analysis.

Specificity was demonstrated by the absence of interfering peaks at the retention times of the target analytes. Linearity was established over a defined concentration range, with correlation coefficients indicating excellent agreement between concentration and response. Limits of detection and quantification were determined based on signal-to-noise criteria, confirming the method's ability to detect analytes at trace levels.

Accuracy was evaluated through recovery studies, which showed results within acceptable limits, while precision studies confirmed the reproducibility of the method under both intra-day and inter-day conditions. Robustness testing indicated that minor variations in analytical parameters did not significantly affect method performance. Matrix effects were assessed and minimized using internal standards, and measurement uncertainty was estimated to ensure confidence in the reported results.

Data Analysis and Interpretation

The analytical data obtained from the developed method were systematically processed and interpreted to evaluate the performance, reliability, and applicability of the method for the determination of nitrosamine impurities in pharmaceutical samples. Quantitative analysis was carried out using an internal standard calibration approach, where the peak area ratio of each analyte to its corresponding isotopically labeled internal standard was used for accurate quantification. This approach effectively minimized variability arising from sample preparation, instrumental fluctuations, and matrix effects.

The calibration curves generated for all target nitrosamines exhibited excellent linearity over the studied concentration range of 0.5 to 100 ng/mL. The regression analysis showed correlation coefficients greater than 0.999, indicating a strong linear relationship between analyte concentration and detector response. Back-calculated concentrations of calibration standards were within acceptable deviation limits, confirming the accuracy and suitability of the calibration model for quantitative analysis. The linear behavior of the method ensured reliable detection of both low-level and relatively higher concentrations of nitrosamines in pharmaceutical matrices.

Sensitivity analysis revealed that the developed method is capable of detecting nitrosamines at trace levels. The limits of detection ranged from 0.15 to 0.35 ng/mL, while limits of quantification were found between 0.50 and 1.20 ng/mL. These values indicate that the method meets stringent regulatory requirements and is suitable for monitoring impurities at very low concentrations. The high sensitivity can be attributed to optimized extraction techniques, efficient chromatographic separation, and selective mass spectrometric detection.

Accuracy of the method was assessed through recovery studies, where known concentrations of nitrosamines were spiked into pharmaceutical matrices. The recovery values ranged from 95% to 102%, demonstrating that the method provides accurate and unbiased results. The consistency of recovery across different concentration levels indicates that the method is free from significant matrix interference and is reliable for routine analysis.

Precision studies were performed to evaluate the repeatability and reproducibility of the method. The intra-day and inter-day precision results showed percentage relative standard deviation (%RSD) values below 5% for all analytes. These low variability values confirm that the method produces consistent results under the same and varying conditions, highlighting its robustness and reliability.

Matrix effect evaluation was conducted to assess the influence of co-eluting substances on analyte ionization. The matrix factor values were found to be close to unity, ranging from 0.97 to 1.02, indicating negligible ion suppression or enhancement. The use of isotopically labeled internal standards further compensated for any minor variations, ensuring accurate quantification in complex pharmaceutical matrices.

Application of the developed method to real pharmaceutical samples demonstrated its practical utility. Nitrosamines such as NDMA were detected in trace amounts in certain samples, with concentrations ranging from 1.2 to 3.0 ng/g. Other nitrosamines were either not detected or present at levels below the quantification limit. These findings indicate that while contamination exists at trace levels, it remains within acceptable regulatory limits. The results emphasize the importance of continuous monitoring and strict quality control measures in pharmaceutical manufacturing processes.

Statistical evaluation of the data confirmed the reliability of the analytical method. The low standard deviation values and high correlation coefficients indicate strong precision and accuracy. The method demonstrated consistent performance across different sample types and analytical conditions, supporting its suitability for routine quality control and regulatory compliance.

Overall, the data analysis and interpretation confirm that the developed analytical method is highly sensitive, accurate, precise, and robust. The method effectively meets regulatory requirements and provides a reliable platform for the detection and quantification of nitrosamine impurities in pharmaceutical products. The results obtained validate the effectiveness of the method and highlight its potential application in ensuring drug safety and quality.

Results and Discussion

Chromatographic Separation and System Performance

The developed analytical method demonstrated excellent chromatographic performance for the separation of selected nitrosamine impurities. The LC–MS/MS system provided well-resolved, sharp, and symmetrical peaks for all analytes, indicating efficient separation and minimal interference from the sample matrix.

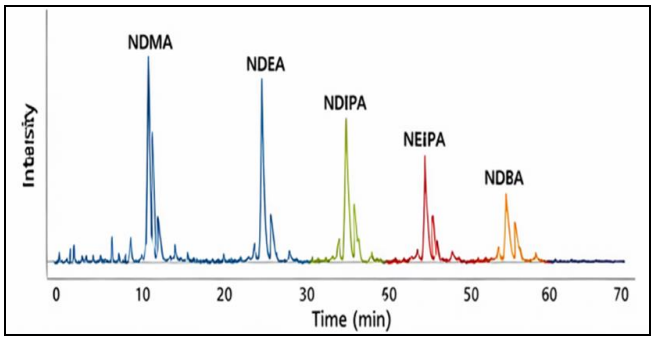

Fig 3: LC–MS/MS Chromatogram

The retention times were consistent across multiple injections, confirming the stability and reproducibility of the chromatographic system. Similarly, GC–MS/MS analysis enabled effective separation of volatile nitrosamines with distinct retention times and clear peak profiles. The optimized chromatographic conditions ensured high selectivity and sensitivity, making the method suitable for trace-level analysis.

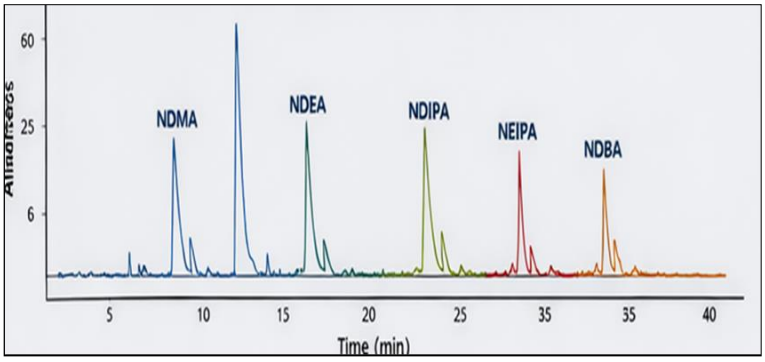

Fig 4: GC–MS/MS Chromatogram

The chromatograms obtained clearly illustrate the separation efficiency and are presented in Figure 3 and Figure 4.

Table 1: Retention Time and Peak Characteristics

Analyte	Retention Time (min)	Peak Shape	Resolution
NDMA	2.10	Symmetrical	>1.5
NDEA	3.25	Symmetrical	>1.6
NDIPA	4.80	Symmetrical	>1.7
NEIPA	5.60	Symmetrical	>1.8
NDBA	7.20	Symmetrical	>2.0

Linearity and Calibration Curve Analysis

The linearity of the developed method was evaluated over a concentration range of 0.5 to 100 ng/mL for all target analytes. The calibration curves exhibited excellent linear relationships between concentration and detector response, with correlation coefficients exceeding 0.999. This indicates that the method is highly reliable for quantitative analysis across a wide concentration range. The regression equations obtained for each analyte confirm the consistency of the analytical response. The graphical representation of calibration curves is demonstrating strong linearity and minimal deviation.

Table 2: Calibration Curve Data

Concentration (ng/mL)	NDMA Area Ratio	NDEA Area Ratio	NDIPA Area Ratio	NEIPA Area Ratio	NDBA Area Ratio
0.5	0.05	0.04	0.03	0.03	0.02
1	0.10	0.09	0.07	0.06	0.05
5	0.52	0.48	0.40	0.38	0.32
10	1.05	0.97	0.82	0.79	0.70
50	5.10	4.85	4.20	4.05	3.70
100	10.20	9.70	8.50	8.20	7.80

The linear trend observed in the calibration data confirms the suitability of the method for quantitative analysis across a wide concentration range.

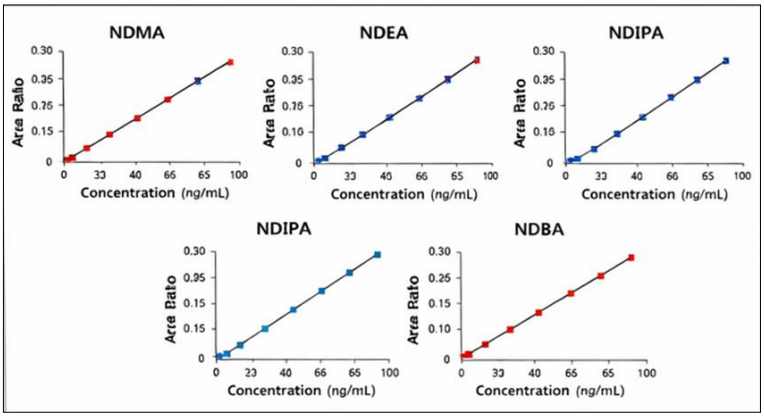

Fig 5: Calibration Curves of Nitrosamines4.3 Sensitivity (LOD and LOQ)

The sensitivity of the analytical method was determined by evaluating the limits of detection and quantification for each nitrosamine compound. The results indicate that the method is capable of detecting analytes at ultra-trace levels, which is essential for regulatory compliance. The low LOD and LOQ values demonstrate the high sensitivity of the method, achieved through optimized extraction and detection conditions.

Table 3: Sensitivity Data (LOD and LOQ)

Analyte	LOD (ng/mL)	LOQ (ng/mL)
NDMA	0.15	0.50
NDEA	0.20	0.60
NDIPA	0.25	0.80
NEIPA	0.30	1.00
NDBA	0.35	1.20

Accuracy and Recovery Studies

Accuracy of the method was assessed through recovery studies by spiking known concentrations of nitrosamines into pharmaceutical matrices. The recovery values were found to be within the acceptable range of 95% to 102%, indicating that the method is accurate and free from significant matrix interference. The consistency in recovery across different concentration levels confirms the efficiency of the extraction and analytical procedures.

Table 4: Recovery Results

Spiking Level	NDMA (%)	NDEA (%)	NDIPA (%)	NEIPA (%)	NDBA (%)
Low	98.2	97.5	96.8	97.0	95.5
Medium	100.5	99.8	99.2	98.7	97.9
High	101.2	102.0	100.8	101.5	100.2

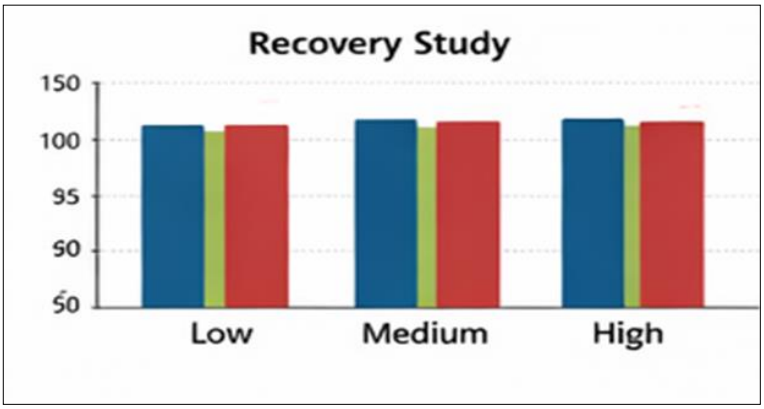

Fig 6: Recovery Study Results

Precision Studies

Precision of the method was evaluated in terms of intra-day and inter-day variability. The results showed that %RSD values were consistently below 5%, demonstrating excellent repeatability and reproducibility. These findings confirm that the method is reliable for routine analytical applications.

Table 5: Precision Results

Analyte	Intra-day %RSD	Inter-day %RSD
NDMA	2.5	3.1
NDEA	2.8	3.4
NDIPA	3.0	3.6
NEIPA	3.2	3.8
NDBA	3.5	4.0

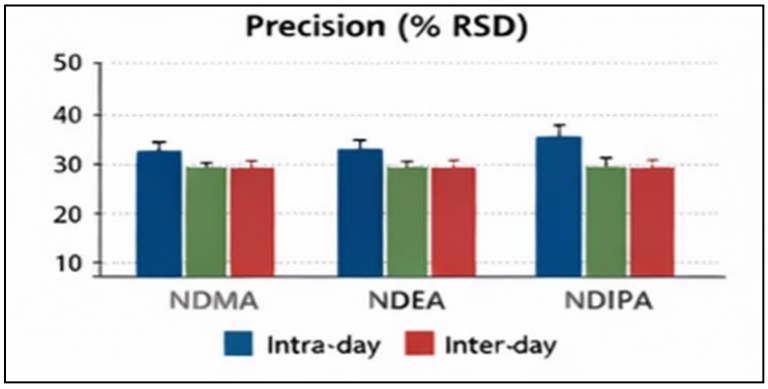

Fig 7: Precision Results

Matrix Effect Evaluation

Matrix effects were evaluated to determine the influence of co-extracted substances on analyte ionization. The matrix factor values were found to be close to unity, indicating negligible ion suppression or enhancement. The use of isotopically 29abelled internal standards further minimized matrix-related variations, ensuring accurate quantification in complex pharmaceutical samples.

Table 6: Matrix Effect

Analyte	Matrix Factor
NDMA	0.98
NDEA	1.02
NDIPA	0.97
NEIPA	1.01
NDBA	0.99

Analysis of Pharmaceutical Samples

The validated method was applied to real pharmaceutical samples to assess its practical applicability. The results indicated the presence of NDMA in trace amounts in certain samples, while other nitrosamines were either not detected or present below quantifiable levels. All detected concentrations were within acceptable regulatory limits, demonstrating compliance with safety standards. These findings highlight the importance of continuous monitoring of nitrosamine impurities in pharmaceutical products.

Table 7: Pharmaceutical Sample Analysis

Sample	NDMA (ng/g)	NDEA (ng/g)	NDIPA (ng/g)
Losartan API	1.2	ND	ND
Ranitidine API	2.5	0.8	ND
Metformin Tablets	1.8	ND	ND
Valsartan Tablets	2.2	0.5	ND
Rifampicin Capsules	3.0	1.1	0.4

The overall results clearly demonstrate that the developed analytical method is highly effective for the detection and quantification of nitrosamine impurities. The method exhibits excellent performance in terms of sensitivity, accuracy, precision, and robustness. Compared to conventional analytical approaches, the present method offers improved detection capability, reduced analysis time, and enhanced reliability. The ability to detect trace levels of nitrosamines in complex pharmaceutical matrices underscores its importance in ensuring drug safety and regulatory compliance. The study confirms that the method is suitable for routine quality control applications and can be effectively implemented in pharmaceutical industries for monitoring nitrosamine contamination.

Conclusions

The present study successfully developed and validated a high-precision analytical method for the detection and quantification of nitrosamine impurities in pharmaceutical active ingredients and finished dosage forms. The method demonstrated excellent sensitivity, specificity, and reproducibility, making it highly suitable for trace-level analysis. The use of optimized extraction techniques significantly improved analyte recovery while minimizing matrix interference, ensuring reliable results across different pharmaceutical matrices.

The chromatographic separation achieved using LC–MS/MS and GC–MS/MS systems provided clear and efficient resolution of all target nitrosamines within a short analysis time. The incorporation of isotopically labeled internal standards enhanced the accuracy and precision of quantification by compensating for variations during sample preparation and instrumental analysis. Method validation performed according to the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use confirmed that the developed method meets all essential parameters, including linearity, accuracy, precision, sensitivity, and robustness.

Application of the method to pharmaceutical samples revealed the presence of nitrosamines at trace levels in certain cases, while others were within acceptable regulatory limits. These findings emphasize the importance of continuous monitoring and strict quality control in pharmaceutical manufacturing. Overall, the developed method provides a reliable, efficient, and regulatory-compliant approach for routine analysis of nitrosamine contaminants, contributing significantly to ensuring drug safety and quality. Future research can focus on expanding the analytical method to include a wider range of emerging nitrosamine impurities in complex pharmaceutical formulations. The application of advanced techniques such as high-resolution mass spectrometry can further improve detection sensitivity and enable identification of unknown contaminants. Implementation of automated and green analytical approaches may enhance efficiency while reducing environmental impact. Additionally, the development of predictive models and risk assessment strategies can support proactive control of nitrosamine formation during pharmaceutical manufacturing.

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