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RESEARCH ARTICLE

Development and optimization of an LC-MS method for the separation of nitrosamine impurities

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Objective: This research foucuses on the development of a liquid chromatographic method for the rapid and reliable separation and identification of major nitrosamine impurities, ensuring both short analysis time and adequate resolution. Given the toxicological relevance of nitrosamines, their occurrence in pharmaceutical formulations has raised substantial concerns, leading to the reassessment of multiple drug products. In response, reverse-phase HPLC with UV detection and LC-MS techniques have been widely applied as powerful analytical tools for their detection and control.

Methods: The following impurities of the N-nitrosamine class are separated and identified by the LC-MS technique: NDMA (N-nitrosodimethylamine), NDEA (N-nitrosodiethylamine), NMEA (N-nitrosomethylethylamine) NDIPA (N-nitrosodiisopropylamine), NDBA (N-nitrosodibutylamine) NPIP (N-nitrosopiperidine). A standard solution of nitrosamines mix was prepared and subsequently diluted in methanol to achieve a final concentration of 20 µg/mL for each compound. The analysis was performed using a UHPLC chromatography system Flexar FX10 (Perkin Elmer) with MS QTOF (AB Sciex TripleTOF4600), Phenomenex Luna Omega 3 C18 (150x4.6mm, 3µm) column, column temperature 450C, mobile phase methanol and formic acid 0.1% in ultrapure water, gradient elution, flow 0.45 mL/min., injected volume 5 µl. The proposed LC-MS conditions are significantly improved compared to the European Pharmacopoeia recommendations for N-Nitrosamines impurities in active substances analysis.

Results: Based on the mass fragmentation profiles of the six investigated nitrosamines, chromatographic separation was successfully accomplished in less than 25 minutes, with the elution sequence established as follows: NDMA, NMEA, NDEA, NPIP, NDIP, NDBA.

Conclusions: The development of optimal chromatographic conditions allows further separation and identification of nitrosamines impurities in pharmaceutical products.

Keywords: itrosamines, impurities, chromatography, development

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Introduction

Nitrosamines are unwanted side products present in many substances and are suspected to have toxic and carcinogenic properties. In pharmaceutical raw materials and finished drug products, nitrosamines may also be formed as side-products from synthesis, during storage, or from packaging.

The recent discovery of nitrosamine impurities in several pharmaceuticals has increased interest in their mutagenic and carcinogenic potential. Nitrosamines generally produce systemic tumors removed from the application site, while N-nitrosamines produce both local as well as systemic tumors [1]. The list of nitrosamine impurities manufactured from drug substances using specific synthetic routes has grown after extensive synthetic route assessments.

N-nitrosamines constitute a class of compounds to which individuals are generally exposed in low doses through drinking water, tobacco consumption, and certain food products (e.g., processed meats). Regulatory authorities have required marketing authorization holders to assess both manufacturing processes and finished products containing synthesized active pharmaceutical ingredients, with the aim of identifying the potential presence of nitrosamine impurities [2,3].

Where such a risk is established, confirmatory testing and subsequent modification of the manufacturing process are necessary to eliminate the source of contamination. The ICH M7 (R1) guideline classifies nitrosamines within the "cohort of concern," alongside other highly potent carcinogens such as aflatoxin-like substances and alkyl-azoxy derivatives. As a result, acceptable levels of exposure to nitrosamines are expected to be considerably lower than the threshold of toxicological concern (TTC) defined by the guideline [4,5].

The principal analytical approaches for the detection of nitrosamines are predominantly based on ion chromatography, which employs ion-exchange separation followed by detection techniques such as conductivity, electrochemistry, ultraviolet (UV) absorption, or mass spectrometry [6]. As an alternative, high-performance liquid chromatography (HPLC) utilizing reverse-phase columns in combination with UV detection or mass spectrometry is widely applied, representing a more cost-effective option [6,7].

In addition, the identification of nitrosamine impurities can be achieved through the integration of microextraction with gas chromatography–mass spectrometry (GC–MS), a

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method that enables the separation, detection, and quantification of multiple compounds within the nitrosamine class [8,9].

For liquid pharmaceutical forms (injections, infusions, eye drops) the presence of mineral salts will interfere with the mass spectrometer signal, which implies preliminary separations using appropriate organic solvents again.

Therefore, the methods presented can be optimized for the most effective analysis of these nitrosamines, especially since in recent years a number of pharmaceutical active substances and pharmaceuticals have been reassessed in terms of the possible presence of these impurities with mutagenic or carcinogenic potential in the composition [10,11].

Concern about the harmful effects nitrosamines can have on the body has increased and with the same rapidity has increased the interest of scientists to look for ways in which the effects of these compounds toxic to be combated. In this regard, substances with the ability to capture free radicals generated by the presence of substances in the category of nitrosamines, for example ascorbic acid, anthocyanins, have been evaluated and studied, flavonoids and other potentially antioxidant substances [12,13].

The European Pharmacopoeia (Ph. Eur.) Commission has adopted a new general chapter on the analysis of *N*-nitrosamine impurities in active substances (2.5.42, previously listed as 2.4.36). The general chapter focuses mainly on the analysis of *N*-nitrosamine impurities in angiotensin-II-receptor antagonists (sartans) containing a tetrazole group, for which there are five Ph. Eur. monographs (valsartan, losartan potassium, candesartan, irbesartan and olmesartan medoxomil) [14,15].

The recent detection of carcinogenic N-nitrosamine contamination in certain pharmaceuticals has raised significant concern and drawn the focused attention of regulatory authorities. These compounds, which are commonly detectable at low concentrations in food, drinking water, cosmetics, and medicinal products, are well recognized as potent carcinogens in animal models [16,17].

Nitrosamines in pharmaceutical products can form through multiple pathways, reflecting the complexity of their occurrence and the challenges in controlling their presence. Synthetic processes represent a primary route, as certain manufacturing procedures may inadvertently generate nitrosamine impurities. Degradation pathways also contribute, with active pharmaceutical ingredients (APIs) potentially undergoing chemical transformations under specific environmental or storage conditions that lead to nitrosamine formation. Additionally, interactions between excipients, packaging materials, and APIs may further facilitate contamination. Understanding these mechanisms is critical for designing robust risk mitigation strategies, implementing effective quality control measures, and ensuring the safety of pharmaceutical products [18,19].

The occurrence of nitrosamine contamination in pharmaceuticals highlights the necessity for continuous monitoring, the application of advanced analytical methodologies, and the implementation of proactive risk management strategies. Ensuring the safety and quality of medicinal products requires coordinated efforts among researchers, manufacturers, and regulatory authorities.

Furthermore, a thorough characterization of the toxicological profiles of nitrosamines is critical for accurately assessing the risks associated with their presence in pharmaceutical formulations [18,19].

Accurate detection and quantification of nitrosamines are essential for effective risk assessment in pharmaceutical products. Nevertheless, several analytical challenges complicate this task. First, low detection limits are required, as nitrosamines frequently occur at trace concentrations, second, the complexity of pharmaceutical matrices can hinder the isolation and reliable detection of nitrosamines, given the potential for interference from excipients and other formulation components. Third, method development for diverse nitrosamines across various product types is resource-intensive, requiring extensive validation to ensure robustness and reproducibility. Overcoming these challenges is crucial for safeguarding product safety and maintaining therapeutic efficacy [20].

Their presence in certain medications has led to numerous drug recalls to ensure patient safety. Such as valsartan, irbesartan, and losartan, commonly prescribed for hypertension, have been recalled due to nitrosamine impurities. The contamination was linked to specific manufacturing processes, prompting global recalls starting in 2018. Metformin used to manage type 2 diabetes, certain batches of metformin were found to contain N-nitrosodimethylamine (NDMA), leading to recalls in 2020. Ranitidine (Zantac): This antacid was withdrawn from markets worldwide after tests revealed unacceptable levels of NDMA. Varenicline (Chantix): Pfizer recalled multiple lots of this smoking cessation aid in 2021 due to the presence of N-nitroso-varenicline above acceptable limits. Duloxetine in October 2024, over 7,000 bottles of duloxetine were recalled after detecting N-nitroso-duloxetine, a nitrosamine impurity [21].

Nitrosamines can form when compounds such as diethanolamine (DEA) or triethanolamine (TEA) react with nitrosating agents, including nitrates. These reactions may occur during manufacturing, storage, or throughout the product's shelf life, resulting in the unintended presence of nitrosamines in a range of cosmetic products, such as shampoos, lotions, and makeup formulations [22].

This study aimed to develop an LC-MS chromatographic method to optimize the conditions for the efficient identification and separation of a wide range of nitrosamine impurities within a short analysis time and lower consumption of reagents considering the applied flow rate and elution gradient. In the developed method, the column temperature was adjusted from 40 °C to 45 °C, while the mobile phase flow rate was modified from 0.50 mL/min to 0.45 mL/min. Although faster GC-MS techniques are also described, not all nitrosamine impurities are suitable for this type of analysis, therefore, the improved method refers to the LC-MS technique for the analysis of nitrosamine impurities described by the European Pharmacopoeia.

Material and methods

Nitrosamine standards: N-Nitrosodimethylamine (NDMA) EuPhY0002259500 200 µg/mL (1mL), N-Nitroso methylethylamine (NMEA) 100mg, N-Nitrosodiethylamine (NDEA) EuPhY0002258 500 µg/mL (1mL) from Sigma Aldrich.

Nitrosamine MIX EPA521 were used containing 6 nitrosamines in diclomethane 2000 µg/mL each component (Sigma Aldrich) of each nitrosamine: N-Nitrosodimethylamine (NDMA), N-Nitrosomethylethylamine (NMEA), N-Nitrosodiethylamine (NDEA), N-Nitrosodipropylamine (NDIPA), N-Nitrosodibutylamine (NDBA), chromatographic purity 96-99%, was diluted 100 times to obtain a concentration of 20 µg/mL from each nitrosamine.

Formic acid LC-MS grade (Merck, Germany), methanol and water LC-MS grade, were employed for mobile phase preparation.

The chromatography mass spectrometry equipment consisted in an UHPLC Flexar FX10 system (Perkin Elmer) with a QTOF- quadrupol time of flight mass spectrometer 4600 Serie (Sciex TripleTOF4600), an APCI- ionisation source (Atmospheric pressure chemical ionization), a Phenomenex Luna Omega 3 C18 (150x4.6mm, 3µm) chromatographic column, column temperature was set at 45^{o}C, the mobile phase consisted of LC-MS-grade solvents as follows: mobile phase A, 0.1% formic acid in ultrapure water, and mobile phase B, 0.1% formic acid in methanol. Separation was achieved using a gradient elution mode (Table I), mobile phase flow of 0.45 mL/min. The injected volume was set to be 5 µl.

The SciexTriple 4600 QTOF mass spectrometer with Analyst 1.7 software and the Perkin Elmer FX10 with Chromera Software were utilized for this method development device control, data chromatographic analysis. In our case the ionization mode was APCI (Atmospheric pressure chemical ionization) positive, the positive mode of ionization is suitable for most nitrosamines, whereas the negative mode is preferred in the case of NMBA.

The heater temperature was set at 550^{0}C and the fragmentor voltage was adjusted for each nitrosamine to enable the identification of the main MRM (Multiple Reaction Monitoring) transitions (m/z), the system detects masses with an accuracy of four decimal places. The overall analysis time was 25 minutes, allowing the separation of the nine nitrosamine compounds present in the processed impurity mixture.

Table I. Gradient conditions for the mobile phase in LC-MS analysis

Time (min)	Phase A %	Phase B %	Flow	Injection volume
0	80	20	0.45mL/min	5 µl
1	80	20		
6	55	45		
15	55	45		
16	5	95		
20	5	95		
22	80	20		
25	80	20		

Standard preparation: Nitrosamine standards were dissolved in methanol (LC-MS purity grade), and serial dilutions were prepared to obtain concentrations ranging from 20 to 100 µg/mL from N-Nitrosodimethylamine (NDMA), N-Nitroso methylethylamine (NMEA) and N-Nitrosodiethylamine (NDEA).

Nitrosamine Mix preparation: Nitrosamines standard solution, a MIX EPA521 (Sigma Aldrich) were used containing 6 nitrosamines in diclomethane 2000 µg/mL each component, the solvent was evaporated with SpeedVacConcentrator Savant SPD121P, followed by resumption with methanol (LC-MS grade), was diluted 100 times to obtain a concentration of 20 µg/mL from each nitrosamine: NDMA, NDEA, NDBA, NDIPA, NMEA, NPIP.

Each nitrosamine was individual investigated, according to molecular wieght and m/z ratio. In mass spectrometry, the m/z ratio represents the mass-to-charge ratio of ions detected in the mass spectrometer, this ratio is displayed on the mass spectrum, helping determine the composition of molecules in a sample.

Results

LC-MS and GC-MS techniques are widely employed in nitrosamine detection, as they offer good sensitivity with detection limits at the nanogram level. LC-MS is a preferable analytical tool for the identification and quantification of polar, nonpolar, and non-volatile analytes. This method describes the use of liquid chromatography coupled with tandem mass spectrometric detection for the analysis of six nitrosamines.

Initially, standard solutions of nitrosamines at a concentration of 20 µg/mL were analyzed individually. The chromatograms obtained under the specified conditions for the standard solutions of nitrosodimethylamine (NDMA) and nitrosomethylethylamine (NMEA) are presented in Figure 1 and Figure 2.

Nitrosamines often produce a characteristic fragmentation patterns, for example: NDMA (N-Nitrosodimethylamine): m/z 75 (common fragment m/z 43), NMEA (N -Nitrosomethylethylamine) m/z 89 (common fragment m/z 61) NDEA (N-Nitrosodiethylamine): m/z 103 (common fragment m/z 75).

Table II provides a comprehensive overview of the retention times acquired under optimized chromatographic conditions, molecular mass and MRM transitions m/z, identified for the nitrosamines under investigation.

Discussions

The elution order using the mentioned chromatographic conditions were: NDMA with a retention time 5.03 min-

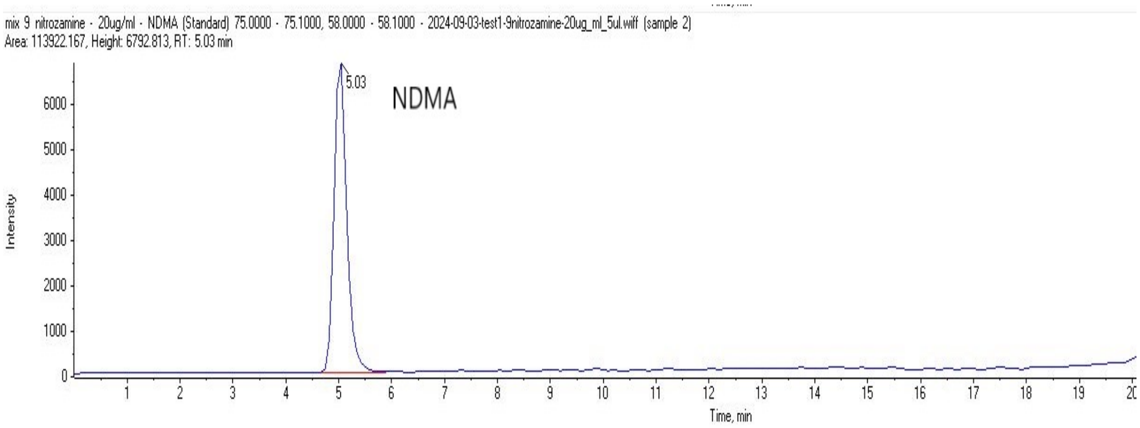

Fig. 1. Chromatogram of 20 µg/mL N-Nitrosodimethylamine standard solution

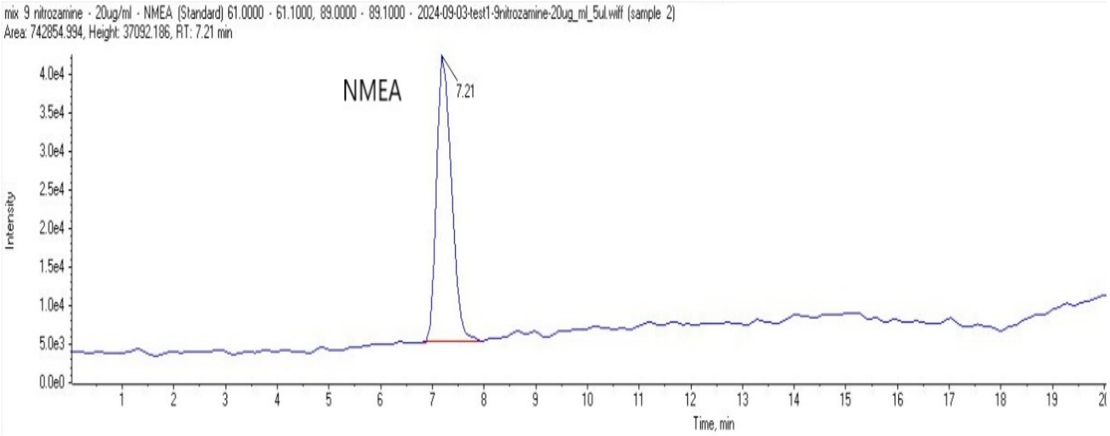

Fig. 2. Chromatogram of 20 µg/mL N-Nitrosomethylethylamine standard solution

Table II. The retention times and MRM transitions (m/z) principal (qualifier) for the studied nitrosamines

Substance	Retention time (min)	Molecular mass (g/mol)	MRM Transitions (m/z) principal (qualifier)
N-Nitrosodimethylamine (NDMA)	5.03	75.04	58, 43
N-Nitrosomethylethylamine (NMEA)	7.21	88.11	61
N-Nitrosodiethylamine (NDEA)	9.80	103.07	75, 47
N-Nitrosopiperidine (NPIP)	10.56	114.15	97, 69, 55
N-Nitrosodipropylamine (NDIPA)	18.14	130.19	89, 85, 99, 113
N-Nitrosodibutylamine (NDBA)	20.48	159	41, 57, 107

utes, NMEA at 7.21 minutes, followed by NDEA at 9.80 minutes, NPIP at 10.56 minutes, NDIPA at 18.14 minutes, and NDBA at 20.48

For the nitrosamine mixture comprising six compounds, which are among the most relevant impurities, the developed LC-MS method achieves efficient separation with good resolution in a relatively short analysis time of less than 25 minutes (Figure 3). The signal intensity for NPIP (N-Nitrosopiperidine) is stronger than NDEA (N- Nitrosodiethylamine) at the similar retention time around 10 minutes suggesting higher ionisation yield.

The elution order was established according to the main fragments derived from unique precursor ions for each nitrosamine, initially each nitrosamine was idividually assessed in relation to the resulting masses after fragmentation.

Figure 4 presents the retention times of the nitrosamines NPIP (NNitrosopiperidine) and NDEA (NNitrosodiethylamine) obtained from individual analyses, facilitating the determination of the elution sequence for the overlapping signals observed in the mixture.

Different signal intensities at the same concentration (20 µg/mL) for NDMA, NMEA, NDEA, NPIP, NDIPA, and NDBA require an adjustment of collision energies to facilitate molecular fragmentation and signal alignment. It is highly relevant the fact that all six nitrosamines exhibits different ionisation yield on one hand and on the other the fragmentation is advisable to be utterly controlled in order to obtain high intennsities of fragment ions that can be used for the establishment of ions transitions. However nitrosamines are molecules with very low molecular weight meaning that commonly, after fragmentation, the smaller fragment ions are generated with a significant loss in intensity which may further impact the limits of detection and quantification. Ionisation process moreover is relatively efficient with APCI due to nitrosamines chemical structures.

Nitrosamine separation in LC-MS analysis is a crucial step for accurately identifying and quantifying individual nitrosamine impurities. The separation process can be challenging due to the structural similarities of nitrosamines,

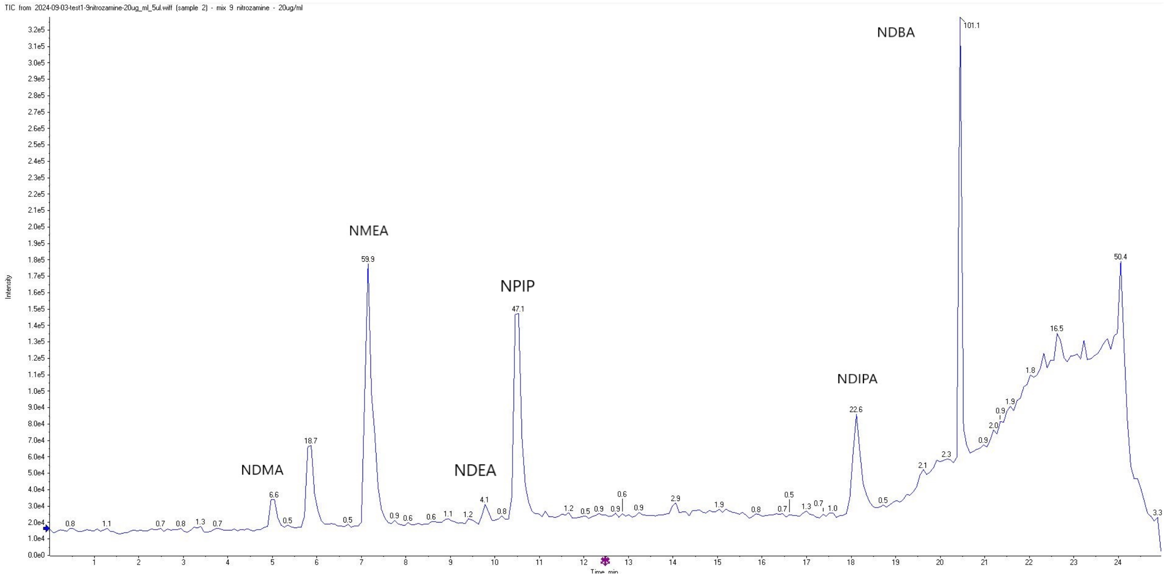

Fig. 3. Chromatogram of nitrosamine mixture analysis with the elution order: NDMA, NMEA, NDEA, NPIP, NDIPA, NDBA

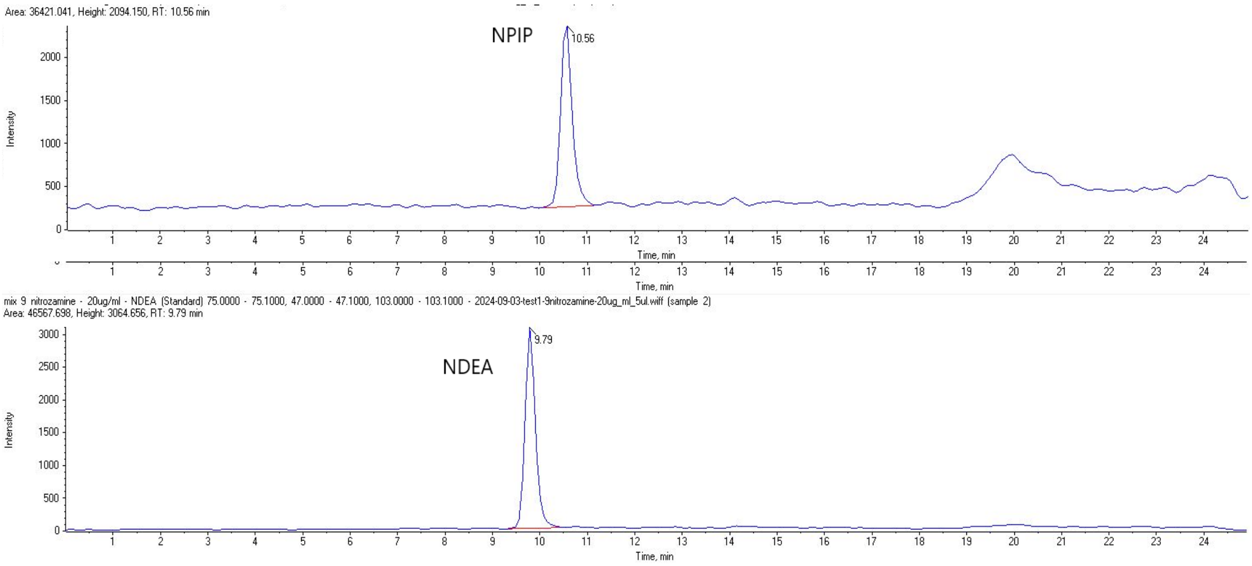

Fig. 4. Chromatograms of standard solutions of NPIP (N-nitrosopiperidine) and NDEA (N-nitrosodiethylamine) at a concentration of 20 µg/mL

low concentrations in pharmaceutical products, and potential matrix effects.

Conclusion

The proposed LC–MS conditions represent a improvement over the recommendations of the European Pharmacopoeia, leading to a significant reduction in analysis time and reagent consumption, resulting from the optimized flow rate and elution gradient. These conditions allow the effective analysis of six nitrosamine impurities while ensuring efficient separation with good resolution

Abbreviations

NDMA – N-nitrosodimethylamine
NDEA- N-nitrosodiethylamine
NDBA- N-nitrosodibutylamine
NDIPA- N-nitrosodiisopropylamine
NMEA- N-nitrosomethylethylamine
NPIP- N-nitrosopiperidine

Author´s contributions

AB (Conceptualization, data curation, methodology, project administration, writing original draft, funding acquisition)
LF (Data curation, formal analysis, resources, Software)
ACG (Conceptualization, supervision, validation)
VI (Investigation, formal analysis)
All authors have read and agreed to the published version of the manuscript.

Conflict of interest

None to declare.

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