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A case study on N-nitrosamine investigation in a marketed drug: from analytical procedure validation to risk mitigation decision

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Introduction: N-nitrosamines, a class of organic compounds recognized for their mutagenic and carcinogenic properties, have been raising major public health concerns following their detection in pharmaceuticals since 2018. These impurities can form during manufacturing processes, particularly through the interaction of amines with nitrosating agents. Regulatory bodies have launched procedures mandating a systematic investigation of medicinal products for N-nitrosamine contamination. This process involves risk evaluation, confirmatory testing, and the implementation of risk mitigation measures. Hyphenated analytical techniques, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), have become the standard for N-nitrosamine detection thanks to their high sensitivity and specificity.
Methods: In this work, based on a previously developed Quantitative Structure Retention Relationship approach for the determination of N-nitrosamines, sensitive LC-MS/MS methods were proposed and validated. These methods were subsequently applied to confirmatory testing of a marketed drug product.
Results: The methods were successfully validated in accordance with the ICH Q2 (R2) guidelines as limit tests for trace-level detection of N-nitrosamines. The confirmatory testing demonstrated the absence of most N-nitrosamines throughout the product's shelf life, except for one potentially problematic impurity that nevertheless remained below the established acceptable limit, thus requiring routine quantitative analysis.
Discussion: These findings highlight the need for continued monitoring through routine analysis to ensure the safety and quality of pharmaceutical products.

KEYWORDS
analytical procedure validation, drug safety, impurity, LC-MS/MS, N-nitrosamine, pharmaceuticals, quality control

1 Introduction

N-nitrosamines are a class of organic compounds characterized by the presence of a nitroso functional group. The majority of N-nitrosamines have been identified as mutagenic and carcinogenic in animal studies and are considered potentially carcinogenic to humans. However, Thresher *et al.* reported that approximately 18% of N-nitrosamines are considered

non-carcinogenic (Thresher et al., 2020). These compounds can occur naturally or form in trace amounts in the environment, food, drinking water, and cosmetics (Sgroi et al., 2018; Nudelman et al., 2023; Schettino et al., 2023). Since mid-July 2018, the detection of N-nitrosamines in pharmaceuticals intended for human use has raised substantial public health concerns, prompting action from international regulatory bodies (Parr and Joseph, 2019). N-nitrosamine formation may arise from the use of contaminated solvents, reagents, or raw materials, as well as from the application of nitrosating agents in the presence of certain amines during chemical synthesis or manufacturing processes (European Medicines Agency, 2025a; European Medicines Agency, 2020). Notably, excipients may also serve as a minor yet significant source of nitrite (Boetzel et al., 2023), a well-established nitrosating agent that can contribute to the formation of N-nitrosamines. Therefore, interactions between active pharmaceutical ingredients (APIs) or their related substances and excipients can lead to the formation of a distinct subclass of N-nitrosamines, known as N-nitrosamine drug substance-related impurities (NDSRIs). To date, approximately 90% of reported N-nitrosamines fall into this category (European Medicines Agency, 2025a; Zhang et al., 2025a). Within this subclass, N-nitroso-varenicline was the first NDSRI to be identified, detected in Chantix® or Champix® (European Medicines Agency, 2025a; U.S. Food and Drug Administration, 2024) at levels exceeding the established safety threshold, ultimately leading to the product's recall and market withdrawal in 2021 (U.S. Food and Drug Administration, 2022; European Medicines Agency, 2025b). N-nitroso-varenicline is absent in the varenicline raw material but forms in the finished product due to nitrosation of varenicline in the presence of nitrite originating from excipients. This nitrosation specifically occurs at the secondary amine functional group.

In response to the potential safety risks, the European Medicines Agency (EMA) launched a "Call for review". This initiative mandates that marketing authorization holders (MAHs) or applicants review their manufacturing processes, from raw materials through to finished products. The review applies to all medicinal products, encompassing both investigational and marketed drugs, as well as chemically synthesized APIs and biologicals (European Medicines Agency, 2025a; European Medicines Agency, 2020).

The "Call for review" involves a systematic investigation procedure to evaluate and detect the presence of N-nitrosamine impurities in pharmaceuticals intended for human use. This process consists of three key steps:

- Step 1: Conduct a comprehensive documentary risk evaluation for each API or finished product to identify or exclude the risk of N-nitrosamine impurities.
- Step 2: If any risk is identified, perform confirmatory testing to confirm the presence or absence of N-nitrosamines.
- Step 3: If the presence of N-nitrosamine(s) is confirmed, implement risk mitigation measures and submit a variation to the marketing authorization, if necessary (European Medicines Agency, 2025a).

The drug product selected for this case study is an oral solid dosage form formulated as uncoated tablets. Based on the documentary risk analysis (Boetzel et al., 2023), among excipients, cellulose derivatives, lactose, povidone, and magnesium stearate were identified as potential sources of nitrite. Nitrite is a well-known nitrosating precursor capable of interacting with APIs or related byproducts bearing amine functional groups. Since a risk was identified during Step 1, confirmatory testing (Step 2) was deemed necessary. This work focuses exclusively on small-molecule N-nitrosamines, as the API does not appear to form NDSRIs due to the absence of secondary amine functional groups in its chemical structure. As currently understood, secondary amines are the most reactive and susceptible functional groups for N-nitrosamine formation when exposed to nitrosating agents (Moser et al., 2023).

For confirmatory testing, analytical methods should be developed with adequate sensitivity to determine trace amounts of N-nitrosamine impurities. According to the EMA, these methods must be validated prior to testing to ensure they provide reliable, high-quality results (European Medicines Agency, 2025a). The required sensitivity is determined by the calculated acceptable limit for each N-nitrosamine in a specific product. This limit, expressed in ppm, is calculated by dividing the acceptable intake (AI) of the N-nitrosamine (in ng/day) by the maximum daily dose of the product (in mg/day) (European Medicines Agency, 2025a). AI limits are fixed values established by the EMA, while the maximum daily dose is outlined in the summary of product characteristics. In this case study, the maximum daily dose for the product under investigation is 16 mg/day. It is important to note that (recommended) AI values and relevant N-nitrosamines are listed and periodically reviewed by EMA in Appendix 1 of the document "*Questions and Answers for Marketing Authorization Holders/Applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 Referral on Nitrosamine Impurities in Human Medicinal Products*" (European Medicines Agency, 2025a), as well as on the website of the United States Food and Drug Administration (FDA) through the Center for Drug Evaluation and Research (U.S. Food and Drug Administration, 2025). Both the EMA and the FDA have stated that AI values should be established using a carcinogenic potency categorization approach (CPCA) based on the chemical structure of N-nitrosamines, which classifies N-nitrosamines into 5 categories, each associated with a specific AI value (European Medicines Agency, 2025a; Zhang et al., 2025a; Kruhlak et al., 2024). To facilitate this process, Novartis has developed an automated tool that assigns the CPCA category and corresponding AI value for NDSRIs based solely on SMILES-encoded structural information (Zhu et al., 2024). Exceptionally, the agencies have proposed interim measures to mitigate unnecessary supply disruptions when N-nitrosamine levels exceed the recommended AI limits (European Medicines Agency, 2025a; Zhang et al., 2025a; U.S. Food and Drug Administration, 2024; U.S. Food and Drug Administration, 2025). The EMA has adopted a less-than-lifetime approach, using conservative adjustment factors to derive interim limits for N-nitrosamine impurities superior to established AI values. This approach is intended as a temporary measure while corrective and preventive actions are implemented to reduce contaminant levels to or below the defined AI limits (European Medicines Agency, 2025a). In contrast, the FDA's approach consists of updated interim recommended AI limits

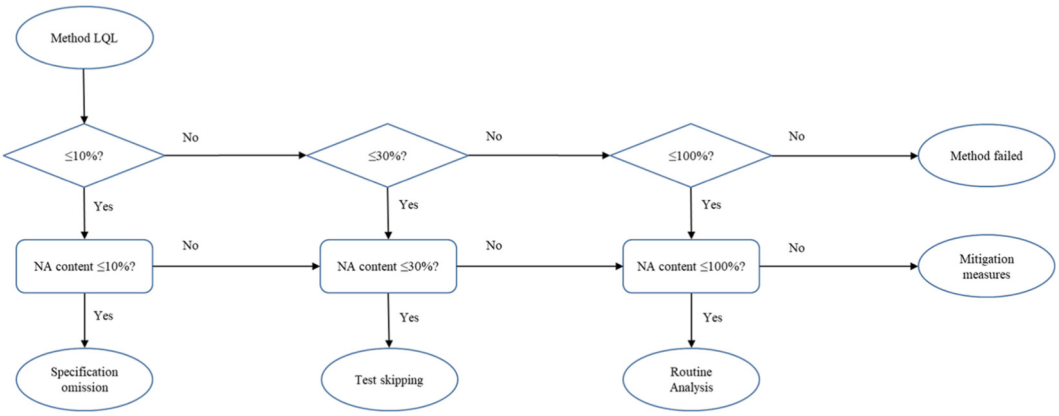

FIGURE 1
Possible testing scenarios based on the method's LQL and the N-nitrosamine content determined through confirmatory testing. LQL: Lower quantification limit; NA: N-nitrosamine.

for only certain NDSRIs, applicable for a limited duration under specific regulatory conditions (U.S. Food and Drug Administration, 2025).

Several scenarios can arise depending on the method's lower quantification limit (LQL), which may influence the outcomes of impurity testing (European Medicines Agency, 2025a). Additionally, the impurity levels detected during confirmatory testing play a crucial role in guiding final decisions regarding the necessity and frequency of further testing, as illustrated in Figure 1.

In accordance with the EMA's "Call for review", a representative number of both newly manufactured batches and retained batches that remain within their expiry date must be included in the confirmatory testing to ensure the entire shelf life of the product (European Medicines Agency, 2025a). The specification of an N-nitrosamine can be omitted if the method's LQL is below 10% of the acceptable limit and the N-nitrosamine content consistently remains below 10% of its acceptable limit across all tested batches throughout the product's shelf life. In such cases, testing for N-nitrosamines is no longer obligatory. The distinction between skip testing and routine analysis lies in the frequency of testing. Skip testing is performed at release on pre-selected batches or predetermined intervals, rather than on every batch, as is the case with routine analysis (The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, 2000).

Historically, techniques such as thin-layer chromatography, polarography, and spectrophotometry were employed for the detection of N-nitrosamines. However, these methods lacked the sensitivity required to detect trace levels of these genotoxic compounds. In contrast, modern hyphenated techniques, which combine chromatographic separation with mass spectrometric detection, offer superior sensitivity and specificity. Techniques like gas chromatography, liquid chromatography (LC), and supercritical fluid chromatography are commonly paired with mass spectrometers, ranging from simple quadrupole detectors to advanced hybrid systems such as tandem quadrupole (MS/MS) or quadrupole time-of-flight detectors (Parr and Joseph, 2019). LC is more versatile and offers a broader analytical scope compared to gas chromatography, as some N-nitrosamines are non-volatile, making gas chromatography unsuitable for their determination. Additionally, determining N-nitrosamines in drug products is more challenging than in drug substances due to the presence of excipients, which usually constitute the major portion of the formulation. In the literature, an LC-MS/MS method capable of simultaneously analyzing up to 13 N-nitrosamines has been reported for biological medicines (Luo et al., 2022). Sample preparation protocols vary significantly depending on the dosage form. For instance, protocols designed for biological formulations containing high levels of antibodies, salts, and surfactants are not suitable for oral solid dosage forms, which typically include a high proportion of insoluble excipients such as diluents, disintegrants, lubricants, and colorants.

As outlined in previous work (Zhang et al., 2025b), LC-MS/MS was selected as the analytical technique of choice due to its ability to detect analytes based on both LC retention time and structurally specific fragmentation patterns, while also reducing chemical background noise, thus improving sensitivity. This paper builds on previous work and presents three analytical methods validated under the ICH Q2 (R2) guidelines, employing a three-level limit test approach for the determination of N-nitrosamine content in a marketed product.

The three methods validated are generic, complementary, and designed to provide flexibility in routine quality control applications. Notably, the methods require no modifications to sample preparation or equipment configuration, enabling the detection of up to 17 small-molecule N-nitrosamines within a single-sequence analysis. This approach ensures i) compliance with regulatory sensitivity requirements for N-nitrosamine control, and ii) effective resolution of co-elution with the API. The case study, centered on a marketed drug product, provides a practical illustration of the structured workflow involved in conducting analytical confirmatory testing for MAHs or applicants.

2 Materials and methods

2.1 Chemicals and reagents

The same chemicals and reagents described previously (Zhang et al., 2025b) were purchased and used in this work.

500 µg/mL certified reference stock solutions of N-nitrosodimethylamine (NDMA), N-nitrosodi-n-propylamine (NDPA), N-nitrosodiisopropylamine (NDIPA), N-nitrosoethylisopropylamine (NEIPA), N-nitrosodi-n-butylamine (NDBA), N-nitroso-diethylamine (NDEA), and N-nitroso-N-methyl-4-aminobutyric acid (NMBA) were obtained from the EDQM (Strasbourg, France). Certified reference stock solutions at 1 mg/mL of 1-methyl-4-nitrosopiperazine (MeNP), and N-nitroso-1,2,3,6-tetrahydropyridine (NTHP) were supplied by the United States Pharmacopeia (Rockville, MD, United States). Reference stock solutions at 1 mg/mL of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosomethylphenylamine (NMPA) were purchased from Sigma-Aldrich/Merck (St. Louis, MO, United States). Similarly, 1000 µg/mL reference stock solutions of N-nitrosomethylethylamine (NMEA), N-nitrosopyrrolidine (NPYR), and N-nitroso-piperidine (NPIP) were purchased from Cambridge Isotopes Laboratories (Tewksbury, MA, United States). Reference standards of N-nitrosomorpholine (NMOR), N-nitrosodiphenylamine (NDPhA), and N-nitroso-diethanolamine (NDELA) were obtained from TCI (Tokyo, Japan).

Internal standards (stable isotope-labeled analogs) were sourced from various suppliers. Deuterated N-nitrosamine standards, including NMBA-d3, NMPA-d5, NDPhA-d6, NDPA-d14, and MeNP-d4 were purchased from BOC Sciences (New York, NY, United States), while NMOR-d8 was obtained from J.H. Ritmeester B.V. (Nieuwegein, Netherlands). A 10 µg/mL reference stock solution of ^{13}C6-NNK, and 1000 µg/mL reference stock solutions of NDMA-d6, NDBA-d18, NPYR-d8, NMEA-d3, NDEA-d10, and NPIP-d10 were acquired from Cambridge Isotope Laboratories. NDELA-d8 was obtained from Sigma-Aldrich/Merck.

17α-hydroxyprogesterone was obtained from TCI. ULC-MS grade methanol, water, formic acid, and ammonium acetate were purchased from Biosolve (Dieuze, France). The pharmaceutical formulation used in the validation and case studies was sourced by a pharmaceutical industry partner. Due to a confidentiality agreement, the nature and dosage of the API cannot be disclosed.

Prior to the validation studies and confirmatory testing, individual stock solutions of internal standards NDPA-d14, MeNP-d4, and NDELA-d8 were prepared at a concentration of 1 mg/mL. Both reference stock solutions and prepared stock solutions were aliquoted and stored at −20 °C to prevent instability of the compounds due to repeated freeze-thaw cycles.

2.2 Instrumentation and software

Validation studies and confirmatory testing were conducted using an Acquity® Premier UPLC® system (Waters, Milford, MA, United States), equipped with an Acquity® UPLC® HSS T3 VanGuard pre-column (1.8 µm, 2.1 mm × 5 mm) connected to either an Acquity® Premier HSS T3 column (1.8 µm, 2.1 mm × 100 mm) or an Acquity® UPLC® HSS T3 column (1.8 µm, 2.1 mm × 100 mm) from Waters. The chromatographic system was coupled with a Xevo® TQ-Absolute tandem quadrupole spectrometer (Waters), featuring an atmospheric pressure chemical ionization (APCI) source.

Instrument control, data acquisition, and data processing were managed using MassLynx® Security software (Waters).

2.3 Analytical methods

2.3.1 Sample preparation

An extraction protocol was employed to isolate the analytes from the drug product. Uncoated tablets were crushed into a fine powder. Following the addition of internal standards (see Section 2.4.1 for preparation details) to 425 mg of powdered material, 5.0 mL of methanol was added to solubilize and extract N-nitrosamines while simultaneously removing insoluble excipients such as cellulose derivatives, lactose, and magnesium stearate. The mixture was ultrasonicated for 25 min and then centrifuged at 11,000 rpm for 15 min. To ensure the removal of residual water-soluble excipients, 1.0 mL of the resulting supernatant was collected and mixed with 4.0 mL of 1% formic acid solution, followed by a second centrifugation under the same conditions. The final supernatant was transferred into an analytical vial for injection.

2.3.2 Chromatographic conditions

The chromatographic conditions used for the two validation studies and confirmatory testing are summarized in Table 1. As shown, the only difference between the mobile phase conditions 1 and 2 is their composition. An aqueous mobile phase adjusted to pH 2.7 was used for the validation of Method 2, whereas a pH of 5.0 was applied for the validation of Method 3. As demonstrated in previous work, Method 1 was also validated under pH 2.7 conditions. This decision was made during the development of the three methods to optimize sensitivity and mitigate matrix effects. Working at pH 2.7 yielded greater signal intensities for most N-nitrosamines. However, under these experimental conditions, MeNP, NDEA, NTHP, NPIP, and NDELA co-eluted with the API. Therefore, an alternative pH of 5.0 was selected for the analysis of these compounds to prevent such co-elution observed at pH 2.7. In addition, the N-nitrosamines were divided into two source temperature groups, 150 °C and 250 °C, to further enhance signal intensities (Zhang et al., 2025b). The effect of source or probe temperature must be carefully considered, as elevated temperatures can cause in-source fragmentation by promoting analyte dissociation. Therefore, thermolabile compounds required a lower probe temperature and were analyzed at 150 °C to prevent undesirable thermal degradation, while the source temperature was consistently maintained at 120 °C.

Based on these parameters, the N-nitrosamines were categorized into three groups, each assigned to one of the flexible methods for validation, as follows:

- Method 1 (pH 2.7/250 °C): NMOR, NMBA, NDPhA, NNK, and NMPA,
- Method 2 (pH 2.7/150 °C): NDMA, NDPA, NDIPA, NEIPA, NDBA, NPYR, and NMEA,
- Method 3 (pH 5.0/150 °C): MeNP, NDEA, NTHP, NPIP, and NDELA (Zhang et al., 2025b).

These methods only differ in the mobile phase composition or the temperature applied to the APCI probe, enabling i) compliance with regulatory sensitivity requirements for N-nitrosamine control, and ii) effective resolution of co-elution with the API. Following

TABLE 1 Chromatographic conditions.

Stationary phase	Pre-column: - Acquity® UPLC® HSS T3 VanGuard pre-column (1.8 µm, 2.1 mm × 5 mm) Columns: - Acquity® Premier HSS T3 column (1.8 µm, 2.1 mm × 100 mm) - Acquity® UPLC® HSS T3 column (1.8 µm, 2.1 mm × 100 mm)
Mobile phase	pH condition 1: - Solvent A: 0.1% FA in water, pH 2.7 - Solvent B: 0.1% FA in methanol pH condition 2: - Solvent A: 10 mM AcAm, pH 5.0 - Solvent B: Methanol
Flow rate	0.4 mL/min
Gradient	Time (min) / Solvent A (%) / Solvent B (%) Initial / 100.0 / 0.0 1.00 / 100.0 / 0.0 10.90 / 5.0 / 95.0 13.50 / 5.0 / 95.0 13.60 / 100.0 / 0.0 17.00 / 100.0 / 0.0
Temperature	Column: 45 °C Autosampler: 10 °C
Injection volume	10 µL
Needle wash	Water/Methanol (10%/90%, v/v)
Purge solvent	Water/Methanol (90%/10%, v/v)

FA: formic acid; AcAm: Acetate ammonium.

successful validation, the same chromatographic conditions were used for confirmatory testing.

2.3.3 Mass spectrometric parameters

The ion source was configured in positive mode with a corona current of 1.5 µA and a source temperature of 120 °C. The APCI probe temperature was set to 150 °C (Methods 2 and 3) or 250 °C (Method 1). Desolvation, nebulization, cone, and collision gas flows were adjusted to 950 L/h, 250 L/h, 250 L/h, and 0.15 mL/min, respectively. Data acquisition was performed in multiple reaction monitoring (MRM) mode, targeting specific precursor ions and product ions. The autodwell option was enabled for all N-nitrosamines, while the soft transmission was activated only for fragile analytes: NMBA, NEIPA, NDPA, NDELA, and their deuterated analogs. The MRM parameters are detailed in Table 2.

2.4 Validation protocol

Method 1 (pH 2.7/250 °C) was successfully validated in previous work for the determination of NMOR, NMBA, NDPhA, NNK, and NMPA, both as a quantitative method and as a limit test. This approach was justified by the identification of one of these five N-nitrosamines as being "at risk" of occurring in the finished product due to the nature of the API. Validation results for Method 1 are detailed in the previous publication (Zhang et al., 2025b).

The present work focuses on the validation of Methods 2 and 3, which target groups 2 and 3 of N-nitrosamine impurities, respectively. These methods were developed under the experimental conditions outlined in the earlier study. Specifically, Method 2 (pH 2.7/150 °C) was used for the determination of NDMA, NDPA, NDIPA, NEIPA, NDBA, NPYR, and NMEA; whereas Method 3 (pH 5.0/150 °C) was applied for the determination of MeNP, NDEA, NPIP, NTHP, and NDELA (Zhang et al., 2025b).

Validation of Methods 2 and 3 was conducted in accordance with ICH Q2 (R2) guidelines, using a limit test approach. This decision was based on the absence of any N-nitrosamine from groups 2 and 3 being identified as "at risk" for presence in the final product. Four independent validation series were performed, involving two operators to account for variability. The Acquity® Premier HSS T3 column and the Acquity® UPLC® HSS T3 column were used to evaluate the feasibility of employing them interchangeably in routine analyses. Due to the photosensitivity of N-nitroso compounds, all preparations were carried out under light-protected conditions to prevent photodegradation.

2.4.1 Internal standards

On each validation day, aliquots of stock solutions were thawed and used once they reached room temperature. Individual stock solutions of internal standards were diluted to 100 µg/mL with

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TABLE 2 MRM acquisition parameters of N-nitrosamines and internal standards.

N-nitrosamine	MRM transition (m/z)	Retention window (min)	Cone voltage (V)	Collision energy (eV)	Soft transmission
NMOR	Q: 117.10 → 86.93 q: 117.10 → 45.09	2.20–3.80	27	13 13	No
NMPA	Q: 137.05 → 66.10 q: 137.05 → 107.15	6.20–7.50	28	12 11	No
NMBA	Q: 147.10 → 117.10 q: 147.10 → 44.10	2.80–3.90	17	7 10	Activated
NDPhA	Q: 199.10 → 66.10 q: 199.10 → 169.10	8.30–10.20	23	22 12	No
NNK	Q: 208.10 → 122.10 q: 208.10 → 148.00	4.20–5.30	32	14 10	No
NDMA	Q: 75.05 → 58.05 q: 75.05 → 43.00	1.65–2.90	32	9 13	No
NDBA	Q: 159.20 → 57.10 q: 159.20 → 103.23	8.70–9.70	30	12 10	No
NMEA	Q: 89.11 → 61.00 q: 89.11 → 43.00	3.00–4.20	24	9 9	No
NEIPA	Q: 117.20 → 74.99 q: 117.20 → 47.10	5.40–6.50	25	8 14	Activated
NDIPA	Q: 131.20 → 89.10 q: 131.20 → 43.10	6.30–7.50	17	10 9	No
NDPA	Q: 131.16 → 89.16 q: 131.16 → 43.14	6.90–7.90	30	11 11	Activated
NPYR	Q: 101.12 → 55.00 q: 101.12 → 41.00	3.10–4.30	22	15 18	No
MeNP	Q: 130.00 → 58.00 q: 130.00 → 100.00	2.00–3.00	27	13 5	No
NDEA	Q: 103.20 → 74.90 q: 103.20 → 46.90	4.30–5.30	33	9 12	No
NPIP	Q: 115.15 → 69.00 q: 115.15 → 41.00	4.80–5.70	13	14 17	No
NTHP	Q: 113.10 → 67.10 q: 113.10 → 83.10	4.40–5.40	17	10 13	No
NDELA	Q: 135.10 → 74.00 q:135.10 → 104.00	0.70–2.20	8	10 6	Activated
NMOR-d8	Q: 125.17 → 95.00	2.40–3.80	20	15	No
NMPA-d5	Q: 142.05 → 112.01	6.20–7.50	20	12	No
NMBA-d3	Q: 150.10 → 120.00	2.80–3.90	20	5	Activated
NDPhA-d6	Q: 205.10 → 175.10	8.30–9.80	20	11	No
NNK-^{13}C6	Q: 214.14 → 128.00	4.20–5.30	32	12	No
NDMA-d6	Q: 81.12 → 46.00	1.60–2.80	32	13	No
NDBA-d18	Q: 177.35 → 66.00	8.60–9.60	25	13	No
NDPA-d14	Q: 145.28 → 50.00	6.80–7.80	9	12	Activated
NPYR-d8	Q: 109.10 → 62.10	3.10–4.30	22	15	No
NMEA-d3	Q: 92.06 → 64.00	3.00–4.20	25	10	No
MeNP-d4	Q: 134.18 → 104.00	2.00–3.00	17	7	No

(*Continued*)

TABLE 2 Continued

N-nitrosamine	MRM transition (m/z)	Retention window (min)	Cone voltage (V)	Collision energy (eV)	Soft transmission
NDEA-d10	Q: 113.20 → 34.00	4.30–5.30	15	11	No
NPIP-d10	Q: 125.21 → 78.00	4.60–5.60	22	16	No
NDELA-d8	Q: 143.05 → 80.01	0.70–2.20	8	10	Activated

Q: Quantifier ion; q: Qualifier ion.

methanol. Two distinct concentrations of a mixture containing NDMA-d6, NDPA-d14, NDBA-d14, NPYR-d8, and NMEA-d3 were prepared: one (IS 1) was used for spiking the validation standards, while the other (IS 2) was used to supplement the calibration standards. In both validation and calibration standards, the injected concentrations of NDMA-d6, NDPA-d14, NDBA-d14, NPYR-d8, and NMEA-d3 were 5, 1.25, 1.25, 50, and 5 ng/mL, respectively.

Similarly, two mixtures of MeNP-d4, NDEA-d10, NPIP-d10, and NDELA-d8 were prepared: one (IS 3) was used to spike the validation standards, while the other (IS 4) was used to supplement the calibration standards. In both validation and calibration standards, the injected concentrations of MeNP-d4, NDEA-d10, NPIP-d10, and NDELA-d8 were 20, 1.5, 50, and 10 ng/mL, respectively.

To normalize the signal, a known quantity of internal standards was added to calibration and validation standards to ensure consistent internal standard concentrations across all standards. This practice is recommended to account for variability in sample preparation and to compensate for variability in the ionization process inherent to the MS(/MS) technique. Although stable isotope-labeled internal standards are expensive, they are generally preferred over structural analogs. These internal standards share identical physicochemical properties and theoretically co-elute with the analytes in LC-MS/MS analyses. Their use can mitigate the enhancement or suppression of ionization in the ion source caused by matrix effects (Stokvis et al., 2005).

2.4.2 Calibration standards

In alignment with the three-level limit test approach proposed based on the acceptable limit, calibration standards were prepared at three concentration levels: 10, 30% and 100% of the respective acceptable limit. The calculated three levels of acceptable limits, derived from the AI limits (European Medicines Agency, 2025a) and maximum daily dose for each N-nitrosamine, are presented in Table 3.

For each validation series, a 1 mg/mL stock solution of NDELA was freshly prepared in methanol. Aliquots of the stock solutions were thawed and used after reaching ambient temperature. Each stock solution was individually diluted to 10 µg/mL with diluent. The diluent consisted of a mixture of water/methanol (80%/20%, v/v) containing 0.1% formic acid.

Two mixtures of NDMA, NDPA, NDIPA, NEIPA, NDBA, NPYR, and NMEA were prepared at concentrations corresponding to the acceptable limits for each N-nitrosamine: one with internal standards (solution IS 2) and one without internal standards. The mixture without internal standards was then diluted and supplemented with solution IS 2 to reach 10% and 30% of the respective acceptable limits.

Similarly, two mixtures of MeNP, NDEA, NPIP, NTHP, and NDELA were prepared at concentrations corresponding to the acceptable limits of each N-nitrosamine: one with internal standards (solution IS 4) and one without. The mixture without internal standards was diluted and supplemented with solution IS 4 to reach 10% and 30% of the respective acceptable limits.

2.4.3 Validation standards

The pharmaceutical product under investigation, in tablet form, was crushed into a powder and used as the drug matrix to prepare validation standards, simulating real samples. Validation standards were freshly prepared in independent triplicate on each validation day, and separately from the calibration standards. For NDELA, a 1 mg/mL stock solution was prepared. Each stock solution was then diluted to the appropriate concentration before being used to prepare mixtures for supplementing validation standards by adding known quantities of N-nitrosamines.

Two N-nitrosamine validation mixtures were prepared: one containing NDMA, NDPA, NDIPA, NEIPA, NDBA, NPYR, and NMEA (solution M1), and the other containing MeNP, NDEA, NPIP, NTHP, and NDELA (solution M2).

Solutions M1 and IS 1 were added to the drug matrix, as were solutions M2 and IS 3. Validation standards were prepared to match the concentrations of the calibration standards, corresponding to 10, 30, and 100% of the acceptable limits for each N-nitrosamine. The respective concentrations are presented in Table 3.

Following the addition of N-nitrosamine standards and internal standards, each validation standard was prepared according to the procedure described in Section 2.3.1. For NDELA, a 10-fold dilution was applied prior to injection.

2.4.4 Unspiked matrices

Unspiked matrix solutions were prepared independently in triplicate without the addition of N-nitrosamine standards or internal standards. These solutions were processed following the same extraction protocol as the samples detailed in Section 2.3.1 to assess method selectivity.

2.5 Equipment performance verification

17α-hydroxyprogesterone is recommended by the instrument manufacturer for verifying system performance when operating in

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TABLE 3 Recommended AI limits published by the EMA and respective acceptable limits for N-nitrosamines in the product under investigation.

	AI limit (ng/day)	MDD (mg/day)	Acceptable limit (ppm)		
			100%	30%	10%
Method 1 – pH 2.7/250 °C					
NMOR	127	16	7.94	2.38	0.79
NMBA	1500		93.75	28.13	9.38
NDPhA	78,000		4875.00	1462.50	487.50
NNK	100		6.25	1.88	0.63
NMPA	100		6.25	1.88	0.63
Method 2 – pH 2.7/150 °C					
NDMA	96	16	6.00	1.80	0.60
NDPA	26.5		1.66	0.50	0.17
NDIPA	1500		93.75	28.13	9.38
NEIPA	400		25.00	7.50	2.50
NDBA	26.5		1.66	0.50	0.17
NPYR	1700		106.25	31.88	10.63
NMEA	AI limit undefined by the EMA				
Method 3 – pH 5.0/150 °C					
MeNP	400	16	25.00	7.50	2.50
NDEA	26.5		1.66	0.50	0.17
NTHP	37		2.31	0.69	0.23
NPIP	1300		81.25	24.38	8.13
NDELA	1900		118.75	35.63	11.88

This work was conducted using data published by the EMA, as updated in September 2024. AI, acceptable intake; MDD, maximum daily dose.

APCI positive mode. Accordingly, a 50 ng/mL solution of 17α-hydroxyprogesterone was freshly prepared each day and injected in triplicate prior to the N-nitrosamine analysis sequence. This verification step was performed independently of the formal N-nitrosamine analyses to confirm instrument sensitivity and repeatability. The equipment performance verification is considered compliant if the mean intensity calculated from the three injections is equal to or greater than 8,000,000 counts, and the relative standard deviation (RSD%) of the intensities obtained is not more than 5%, as established from data obtained during method development.

2.6 System suitability test solutions

System suitability tests (SSTs) are commonly used by laboratories to ensure that the analytical system is suitable for its intended use on the day of analysis. In agreement with the ICH Q14 (R2) guidelines, a set of SSTs was validated alongside the validation studies. SSTs are an integral and critical part of an analytical procedure to verify its performance (The International Council for Harmoni sation of Technical Requirements for Pharmaceuticals for Human Use, 2024).

2.6.1 System sensitivity verification for trace N-nitrosamine analysis

Two N-nitrosamine standard solutions at 5% of the acceptable limits were independently prepared: one containing NDMA, NDPA, NDIPA, NEIPA, NDBA, NPYR, and NMEA, and the other containing MeNP, NDEA, NPIP, NTHP, and NDELA. The signal-to-noise (S/N) ratio for each analyte was calculated to verify the system's sensitivity. There were no predefined specifications for this SST, instead, specifications were established based on data generated throughout the validation studies.

2.6.2 Ion ratio

The ion ratio was calculated by dividing the area under the curve (AUC) of the less intense by that of the most intense ion. The standard solution containing the highest concentration of N-nitrosamines at the acceptable limit was used to establish the reference value. The ion ratio in all calibration and validation standards was then compared to this reference value to determine conformity.

Considering that the ion ratio can vary slightly from series to series, there were no predefined specifications for this SST, instead, specifications were established based on data generated throughout the validation studies.

2.6.3 NDBA carry-over verification

An NDBA-related carry-over has been observed since the method's development. To monitor this carry-over, multiple blank injections were performed at the beginning of the analysis sequence. The mean AUC of the carry-over-related peaks observed at the quantifier transition and retention time of NDBA was calculated using the last three blank injections before injecting calibration standards. The SST is considered compliant if the mean AUC of the carry-over-related peaks, corresponding to the retention time of NDBA, does not exceed 10% of the AUC of NDBA at the quantifier transition obtained from the calibration solution at 10% of the acceptable limit.

2.7 Confirmatory testing

Confirmatory testing is part of Step 2 of the "Call for review" procedure, and it was performed on the finished product in tablet form. According to the investigation procedure, the number of batches to be tested should correspond to the risk and be representative of annual production. Since fewer than 10 batches were manufactured each year, three batches were randomly selected from each production year between 2021 and 2024, resulting in a total of 12 batches. This included three newly produced batches and nine retained samples from batches still within their expiry date. The retained samples were stored under controlled stability conditions of 25 °C and 60% relative humidity (RH). Testing was performed using Method 1, previously validated (Zhang et al., 2025b), along with Methods 2 and 3, validated in the present study. All three LC-MS/MS methods were successfully validated in accordance with the ICH Q2 (R2) guidelines.

From an experimental perspective, confirmatory testing was carried out using a three-level limit test approach, corresponding to 10, 30, and 100% of the acceptable limit for each N-nitrosamine. To maintain consistency with the validation studies, calibration standards were prepared at these same three concentration levels (refer to Table 3). A known amount of internal standards was added to both the calibration standards and the samples, mirroring the procedure applied during validation.

Sample preparation adhered to the protocol described in Section 2.3.1, employing an extraction procedure to isolate N-nitrosamines from the samples, if present. 12 batches were analyzed over two separate days. On the first day, batches 1 to 6 were tested, while on the second day, batches 7 to 12 were analyzed.

3 Results

3.1 Validation studies

3.1.1 Selectivity

Both methods were validated as limit tests for impurity, in compliance with the ICH Q2 (R2) recommendations for screening purposes. At the onset of the validation studies, the selectivity and specificity of the methods were independently demonstrated for each N-nitrosamine by injecting the following solutions: i) blank; ii) N-nitrosamine calibration standard at the lowest concentration (10% of the respective acceptable limit); iii) unspiked matrix solutions in independent triplicate. The selectivity of the methods is illustrated in Supplementary Figure S1, demonstrating chromatographic separation of all N-nitrosamines from the API.

For the N-nitrosamines and their paired internal standards (where only the quantifier transition was monitored), no interfering peaks were observed at their respective retention times. However, only one exception occurred with NDBA, for which a consistent carry-over was detected across the validation series. This carry-over-related peak appeared at the same retention time as NDBA in both the quantifier and qualifier transitions, even in blank injections or when no injection was performed, as demonstrated in Figure 2.

Carry-over refers to the unintended appearance of residual analyte signals, often observed as undesirable small peaks in blank injections. Understanding and controlling carry-over is essential because the residual signals may interfere with analyte detection and potentially compromise the accuracy of the method. Therefore, identifying and eliminating the sources of carry-over during the method development phase is crucial (Hansen et al., 2013).

The source of this unexpected carry-over related to NDBA was investigated during method development. Through testing, which included the assessment of different needle wash solution compositions, use of two different columns, involvement of two operators, and evaluation of various batches of solvents from multiple suppliers, the carry-over was confirmed to be related to the equipment itself, rather than external factors like solvents or isolated contamination events.

To validate the selectivity of NDBA, throughout all validation series, the mean percentage of the NDBA-related carry-over AUC in matrices A, B, and C consistently remained below 10% of the AUC of NDBA at the quantifier transition at the lowest concentration level of the calibration solution (as shown in Supplementary Table S1). This approach ensures that the AUC of the carry-over-related peak will not exceed 1% of the AUC at the acceptable limit (or the highest concentration level). Therefore, the interference due to carry-over is considered acceptable and does not compromise the method's selectivity for NDBA determination concerning the intended purpose.

3.1.2 Detection limit

The detection limit is the lowest amount of an analyte that can be detected in a sample but cannot be precisely and accurately quantified (The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, 2024a; The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, 2024b). According to the ICH Q2 (R2) recommendations, an S/N ratio of 3 is generally considered acceptable for estimating the detection limit. In the studies, the detection limit was estimated by extrapolating the average S/N ratio obtained at the lowest concentration level across all

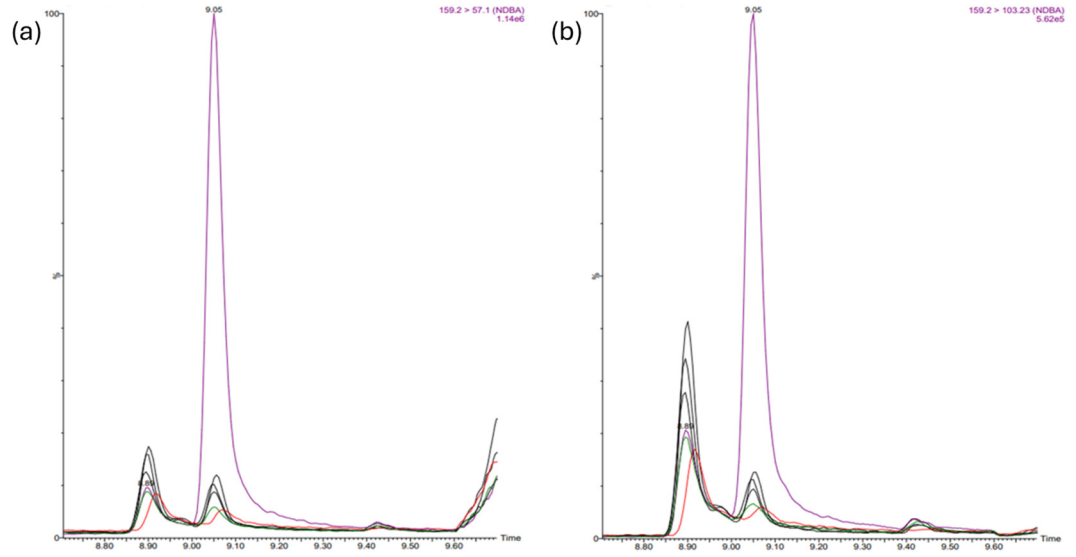

FIGURE 2
Overlayed extracted ion chromatograms. **(a)** left: NDBA at quantifier ion transition; **(b)** right: NDBA at qualifier ion transition. (red) No injection; (green) Blank; (black) Unspiked matrices in triplicate; (purple): Calibration standard at the lowest concentration level (10% of the acceptable limit).

TABLE 4 **Estimated detection limit for each N-nitrosamine.**

	Estimated detection limit *(p = 4)*						
Method 2 pH 2.7/150 °C	NDMA	NDPA	NDIPA	NEIPA	NDBA	NPYR	NMEA
Detection limit (ppb)	40	9	1	2	7	22	3 pg/mL[a]
Method 3 pH 5.0/150 °C	MeNP	NDEA	NPIP	NTHP	NDELA		
Detection limit (ppb)	52	23	135	23	89		

[a]The detection limit is expressed in pg/mL, as the EMA has not established an AI limit for NMEA. For the purpose of confirmatory testing, threshold concentration of 30 ng/mL was exceptionally and arbitrarily selected for NMEA.
p: Number of series of validation.

validation series to a threshold of 3. The main advantage of this extrapolation-based approach lies in its ability to reasonably lower the method detection limit based on experimental data. However, its principal limitation is that the estimated detection limit was not experimentally demonstrated. At the extrapolated concentration level, there remains a risk that the analyte may not be detectable. Nevertheless, as the experimentally confirmed detection limits (corresponding to 10% of the acceptable limit) exhibited overall S/N ratios significantly greater than 3, the risk of non-detectability is thus lower than 0.3% and is therefore considered negligible in the context of the present case study.

As summarized in Table 4, the detection limit estimated for each N-nitrosamine was found to be below 10% of the acceptable limit in the product, meeting the required method sensitivity criteria for omitting the specification. Since NMEA is not listed in Appendix 1 of the document "*Questions and Answers for Marketing Authorization Holders/Applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 Referral on Nitrosamine Impurities in Human Medicinal Products*", no AI limit is available for NMEA. Based on the concentration range chosen in the previous validation study, the highest concentration level of 30 ng/mL was arbitrarily and exceptionally selected as the threshold for NMEA.

3.1.3 Recovery

Matrix effects may compromise the sensitivity, accuracy, and precision of analytical procedures. They arise from various factors, including the components present in the sample matrix, the sample preparation technique, and the ionization type used in MS analyses (Panuwet et al., 2016). Matrix effects were evaluated by assessing the recovery of responses calculated for both the calibration and validation standards at three levels of acceptable limits: 10, 30, and 100%. Response is defined as the ratio of the analyte AUC to that of its corresponding internal standard. Recovery was individually calculated for each validation standard across the validation series, and determined by comparing the responses

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TABLE 5 Response recovery and RSD% for matrix effects evaluation—Method 2 (pH 2.7/150 °C) and Method 3 (pH 5.0/150 °C).

Acceptance limit	Series 1		Series 2		Series 3		Series 4	
	Recovery (%) Min. – Max.	RSD (%)	Recovery (%) Min. – Max.	RSD (%)	Recovery (%) Min. – Max.	RSD (%)	Recovery (%) Min. – Max.	RSD (%)
NDMA (*p = 4; n = 3*)								
Level 10%	99–101	1	102–103	1	93–99	3	97–100	1
Level 30%	99–103	2	99–103	2	90–92	1	95–97	1
Level 100%	101–106	2	106–108	1	93–94	1	97–99	1
NDPA (*p = 4; n = 3*)								
Level 10%	101–104	2	97–102	2	98–101	1	92–98	3
Level 30%	105–106	1	99–100	1	91–95	3	90–93	1
Level 100%	105–108	1	99–101	1	93–96	2	94–96	1
NDIPA (*p = 4; n = 3*)								
Level 10%	101–106	2	98–102	2	94–101	4	96–100	2
Level 30%	101–107	3	95–97	1	89–95	4	90–95	2
Level 100%	101–105	2	96–99	2	90–92	1	92–96	2
NEIPA (*p = 4; n = 3*)								
Level 10%	92–94	1	99–103	2	93–94	1	90–95	3
Level 30%	98	1	98–100	1	86–89	2	89–92	1
Level 100%	99	0	98	0	90–93	2	93–96	1
NDBA (*p = 4; n = 3*)								
Level 10%	101–103	1	101–105	2	101–102	1	99–102	2
Level 30%	103–106	1	98–101	2	96–98	1	94	0
Level 100%	103–105	1	102–104	1	100–102	1	103–104	1
NPYR (*p = 4; n = 3*)								
Level 10%	90–95	3	95–96	1	99–102	2	100–103	1
Level 30%	96–98	1	93–94	1	92–94	1	95–97	1
Level 100%	93–95	1	96–98	1	97–98	1	96–97	1
NMEA (*p = 4; n = 3*)								
30 ng/mL	99–102	1	103–105	1	99–101	1	99–101	1
MeNP (*p = 4; n = 3*)								
Level 10%	96–100	2	93–100	4	101–102	1	99–105	3
Level 30%	97–104	3	98–100	2	98–100	1	100–103	1
Level 100%	102–110	4	99–102	1	102–104	1	104–105	1
NDEA (*p = 4; n = 3*)								
Level 10%	98–109	5	92–96	2	102–107	2	96–98	1
Level 30%	109–112	1	93–95	1	101–102	1	93–100	4
Level 100%	112–113	0	95–97	1	100–102	1	101–107	3
NPIP (*p = 4; n = 3*)								
Level 10%	103–110	3	95–96	0	103	0	100–101	1

(*Continued*)

TABLE 5 Continued

Acceptance limit	Series 1		Series 2		Series 3		Series 4	
	Recovery (%) Min. – Max.	RSD (%)	Recovery (%) Min. – Max.	RSD (%)	Recovery (%) Min. – Max.	RSD (%)	Recovery (%) Min. – Max.	RSD (%)
Level 30%	112–114	1	93–94	1	95–101	3	100–102	1
Level 100%	112–113	1	95–96	1	99–101	1	101–103	1
NTHP *(p = 4; n = 3)*								
Level 10%	101–110	4	92–96	2	97–100	1	96–100	2
Level 30%	111–113	1	91–92	0	100–104	2	101–103	1
Level 100%	112–113	0	93–95	2	102–103	1	102–104	1
NDELA *(p = 4; n = 3)*								
Level 10%	97–100	1	99–100	0	93–98	3	100–105	2
Level 30%	98–102	2	97–99	1	97–99	1	97–99	1
Level 100%	95–97	1	100–101	1	101–103	1	101–103	1

p: Number of series of validation; *n*: Number of replicates per series and per level.

obtained in the validation standard to those in the calibration standard at the same concentration level.

To ensure that the matrix effect is acceptable, the individual recovery must fall within the range of 85%–115% for each validation standard across all validation series. Additionally, the repeatability of recovery was assessed based on three replicates at the same concentration level for each validation series. Except for NMEA, recovery and repeatability were assessed only at an arbitrarily chosen concentration of 30 ng/mL. Within a single validation series, the repeatability, expressed as the RSD% of recovery for the three replicates, must not exceed 15%.

Detailed data for recovery and repeatability are presented in Table 5. Overall, the recovery and RSD% values meet the pre-established specifications, indicating that the matrix effects can be considered acceptable for all N-nitrosamines.

3.1.4 Equipment performance verification

In the first instance, the equipment's sensitivity and repeatability were verified daily before the principal validation analysis sequence. The mean intensity and RSD (%) values obtained from the three injections of 17α-hydroxyprogesterone solution are reported in Supplementary Tables S2, S3. All averaged intensities and RSD (%) values fulfill the specifications pre-established, *i.e.*, mean intensity not less than 8,000,000 counts, and RSD (%) not more than 5%.

3.1.5 System suitability tests

The ICH Q14 (R2) guidelines emphasize that SSTs are established and implemented to ensure that the measurement system and the associated analytical operations fit the intended applications, thereby enhancing the detectability of undesirable and unacceptable performance (The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, 2024).

3.1.5.1 System sensitivity verification for trace N-nitrosamine analysis

Analytical system sensitivity is crucial for trace analyses such as the determination of N-nitrosamine impurities. According to the ICH Q2 (R2) guidelines, an S/N ratio of 3 is typically considered acceptable for estimating the detection limit. Therefore, the S/N ratio should not be less than 3 for each N-nitrosamine in the standard solution prepared at 5% of the acceptable limit. All values are summarized in Supplementary Tables S2, S3, and conform to the specifications.

3.1.5.2 Ion ratio

The ion ratio is commonly used in MS/MS analyses, particularly when using MRM acquisition mode. This SST confirms the identity of analytes and was verified for all calibration and validation standards. All ion ratio values fell within the maximum permitted tolerance for the LC-MS/MS technique (European Parliament and the Council of the European Union, 2002). Detailed data are provided in Supplementary Tables S2, S3.

3.1.5.3 NDBA carry-over verification

Throughout all validation series, the mean percentage of the NDBA-related carry-over AUC consistently remained below 10% of the AUC of NDBA at the quantifier transition in the calibration solution at 10% of the acceptable limit. Therefore, the verification of the carry-over conforms to the pre-established specifications (refer to Supplementary Tables S2).

3.2 Confirmatory testing

Alongside the validation studies, equipment performance verification and SSTs were also validated to support the routine

TABLE 6 Summary of confirmatory testing results.

	N-nitrosamine content											
	Batch 1 T0	Batch 2 T0	Batch 3 T0	Batch 4 T12M	Batch 5 T12M	Batch 6 T12M	Batch 7 T24M	Batch 8 T24M	Batch 9 T24M	Batch 10 T30M	Batch 11 T36M	Batch 12 T36M
Manufacturing year	2024	2024	2024	2023	2023	2023	2022	2022	2022	2021	2021	2021
NA 1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 6	≤10%	≤10%	≤10%	≤30%	≤30%	≤30%	≤100%	≤100%	≤100%	≤100%	≤100%	≤100%
NA 7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 9	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 10	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 11	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 12	≤10%	≤10%	≤10%	N.D.	N.D.	N.D.	≤10%	≤10%	≤10%	N.D.	N.D.	N.D.
NA 13	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 14	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 15	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 16	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
NA 17	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

The retained samples were stored under controlled stability conditions of 25 °C/60% RH. NA: N-nitrosamine; N.D.: not detected.

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implementation of the methods. During confirmatory testing, these verifications were carried out to ensure analytical performance on the day of analysis.

3.2.1 Equipment performance verification and system suitability tests

Confirmatory testing was conducted over two separate days of analysis. On each day, equipment performance verification and SSTs, based on validation studies, were verified to ensure the equipment's sensitivity and repeatability, system sensitivity (S/N ratio), ion ratio compliance, and to monitor the carry-over related to NDBA. The equipment performance verification and all SSTs met the required specifications, validating the analysis sequences. Supplementary Table S4 summarizes the results.

3.2.2 Testing results

Due to confidentiality considerations, the results of the confirmatory testing are presented in Table 6 without identifying the specific N-nitrosamines detected. This case study is only intended to provide a practical example of the workflow involved in conducting analytical confirmatory testing.

Confirmatory testing was conducted under the "Call for review" procedure. Appropriate future testing scenarios were determined based on the N-nitrosamine levels detected during the study, as summarized in Figure 1. The results demonstrated that most N-nitrosamines were below the detection limit, indicating that their levels consistently remained under 10% of the respective acceptable limits throughout the product's shelf life. This observation supports the omission of their specifications and eliminates the need for further testing. For N-nitrosamine 12, no intra-year batch-to-batch variation was observed, however, inter-year variation was noted. This impurity was detected in 6 out of 12 batches, with its content consistently remaining below 10% of the acceptable limit. As a result, N-nitrosamine 12 also does not require further testing, and its specification can be omitted. In contrast, one N-nitrosamine impurity, N-nitrosamine 6, was consistently detected across all batches, including both release and ongoing stability samples stored under 25 °C/60% RH conditions. At release (T_0), its content remained consistently below 10% of the acceptable limit. At 12 months, the amount exceeded 10% but stayed below 30% of the acceptable limit. By 24 months, its content consistently surpassed 30%, though it remained strictly below the acceptable limit. These findings indicate that the product remains compliant with the specification at the end of its shelf life. Risk mitigation measures are therefore not required. However, quantitative routine analysis remains necessary for N-nitrosamine 6, in alignment with the "Call for review" procedure (Figure 1).

Given the evolving profile of N-nitrosamine 6 over storage, this impurity is presumed to originate from API degradation. If sufficient financial and human resources are available, efforts may be undertaken to establish conditions enabling skip testing. To achieve this objective, the level of N-nitrosamine 6 would need to be reduced to, and consistently maintained below 30% of its acceptable limit. Potential risk mitigation strategies include the use of low-nitrite excipients, although this approach may increase manufacturing costs. Lowering the storage temperature to 5 °C instead of 25 °C may slow API degradation and, consequently, the kinetics of N-nitrosamine formation. An alternative measure involves redesigning or modifying the packaging, for example, by replacing the primary packaging material with a nitrocellulose-free lidding foil for blister packs in direct contact with tablets (Zhang et al., 2025a).

4 Discussion

The control of N-nitrosamine impurities in pharmaceuticals has become a critical focus due to their mutagenic and carcinogenic potential, necessitating rigorous regulatory oversight. Based on a previously developed Quantitative Structure Retention Relationship approach to the determination of N-nitrosamines, this study successfully proposed specific and sensitive LC-MS/MS methods for detecting trace levels of small-molecule N-nitrosamines in a marketed pharmaceutical product. These methods have been successfully validated in accordance with the ICH Q2 (R2) guidelines, meeting the requirements for limit tests as specified therein. The methods demonstrated high specificity, adequate sensitivity, and acceptable recovery with detection limits below 10% of the acceptable limit, ensuring reliable screening capabilities. The proposed methods provide a solid basis for further development, particularly for the determination of newly identified small-molecule N-nitrosamines, and may also serve as a starting point for the investigation of NDSRIs in both drug substances and drug products. For NDSRI investigations, it is necessary to verify and eliminate potential interference from small-molecule N-nitrosamines and to demonstrate method specificity to ensure method validity for the intended use.

This case study serves as a practical example illustrating the structured workflow of analytical confirmatory testing for MAHs or applicants. The results showed that most N-nitrosamines were undetected, eliminating the need for further testing. However, one impurity was consistently present and increased over time, though remaining below the acceptable limit, necessitating routine quantitative analysis. This finding highlights the importance of ongoing surveillance, particularly for impurities resulting from API degradation. Future efforts could focus on optimizing storage conditions or packaging materials to mitigate impurity formation risk, ensuring continued product safety. The methods proposed in this work provide a useful tool for routine quality control of 17 small-molecule N-nitrosamines in pharmaceutical products, supporting regulatory compliance and safeguarding public health.

From a broader perspective, the development of platform methods for small-molecule N-nitrosamines could help centralize analytical efforts, thereby supporting wider applications and facilitating testing across products with diverse dosage forms, APIs, and excipients. To achieve this goal, the main challenges lie in adapting sample preparation protocols and managing matrix effects in mass spectrometry, which may lead to ion suppression or enhancement.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

YZ: Conceptualization, Formal Analysis, Methodology, Software, Writing – original draft, Writing – review and editing. SH: Formal Analysis, Writing – review and editing. TV: Methodology, Software, Writing – review and editing. PK: Methodology, Software, Writing – review and editing. EZ: Writing – review and editing. PH: Conceptualization, Project administration, Supervision, Writing – review and editing. CH: Conceptualization, Project administration, Supervision, Writing – review and editing.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

LC-MS/MS	liquid chromatography-tandem mass spectrometry
APIs	active pharmaceutical ingredients
NDSRIs	N-nitrosamine drug substance-related impurities
EMA	European Medicines Agency
MAHs	marketing authorization holders
AI	acceptable intake
FDA	Food and Drug Administration
CPCA	carcinogenic potency categorization approach
LQL	lower quantification limit
NDMA	N-nitrosodimethylamine
NDPA	N-nitrosodi-n-propylamine
NDIPA	N-nitrosodiisopropylamine
NEIPA	N-nitrosoethylisopropylamine
NDBA	N-nitrosodi-n-butylamine
NDEA	N-nitroso-diethylamine
NMBA	N-nitroso-N-methyl-4-aminobutyric acid
MeNP	1-methyl-4-nitrosopiperazine
NTHP	N-nitroso-1,2,3,6-tetrahydropyridine
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NMPA	N-nitrosomethylphenylamine
NMEA	N-nitrosomethylethylamine
NPYR	N-nitrosopyrrolidine
NPIP	N-nitroso-piperidine
NMOR	N-nitrosomorpholine
NDPhA	N-nitrosodiphenylamine
NDELA	N-nitroso-diethanolamine
APCI	atmospheric pressure chemical ionization
MRM	multiple reaction monitoring
RSD	relative standard deviation
SSTs	system suitability tests
S/N	signal-to-noise
AUC	area under the curve
RH	relative humidity.