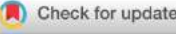

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FULL PAPER

Risk assessment for nitrosated pharmaceuticals: A future perspective in drug development

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Abstract

Since June 2018, thousands of drug products from around the world had to be recalled due to the unexpected presence of nitrosamines (NAs). Starting with the pharmaceutical group of sartans, antidiabetic drugs, antihistamines, and antibiotics also became the subject of investigation. The occurrence of NAs has shown that pharmaceutical companies and regulatory agencies did not focus on these substances in the past during drug development. In this study, we incorporated a nitrosation assay procedure into high-resolution supercritical fluid chromatography (SFC)–mass spectrometry screening to test the potential of direct nitrosation of active pharmaceutical ingredients (APIs). The forced degradation study was performed with a four-fold molar excess of sodium nitrite, relative to the drug substance, at pH 3–4 for 4 h at 37°C. Chromatographic separation was performed on a porous graphitic carbon column by SFC. The mass analysis then focused on direct N-nitrosation or *N*-nitroso compounds (NOCs) formed after dealkylation. Substances (*n* = 67) from various pharmaceutical classes were evaluated and 49.3% of them formed NOCs, of which 21.2% have not yet been reported in the literature. In addition, for two APIs, which are known to form an unidentified NOC, the structure could be identified. A few substances also showed multiple NOCs and even *N,N'*-dinitroso-species. As NAs are carcinogens, they have to be eliminated or at least limited to prevent cancer in patients, who rely on these drugs. This study contributes a procedure that can be implemented in preapproval drug development and postapproval risk assessment to prevent unexpected findings in the future.

KEYWORDS

nitrite, nitrosamines, nitrosation assay procedure (NAP), *N*-nitroso compounds (NOC), porous graphitic carbon (PGC), supercritical fluid chromatography (SFC)

1 | INTRODUCTION

The nitrosamine crisis has been a persistent problem for the past 3 years, starting on June 20, 2018, with a report that the drug substance valsartan from a Chinese manufacturer was contaminated with *N*-nitrosodimethylamine (NDMA). "Little was known about the extent of the problem or the levels of NDMA" as stated by the European Medicines Agency (EMA)[1] at this point, but an immediate regulatory response led to the initiation of a global risk assessment process. Within weeks, new nitrosamines (NAs) were detected and

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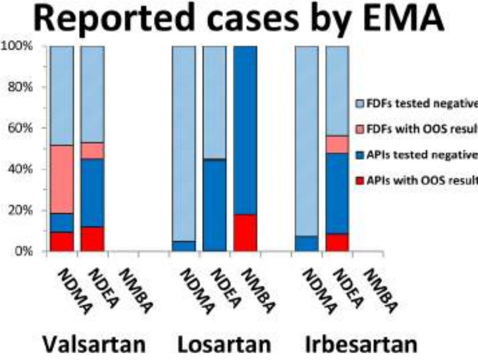
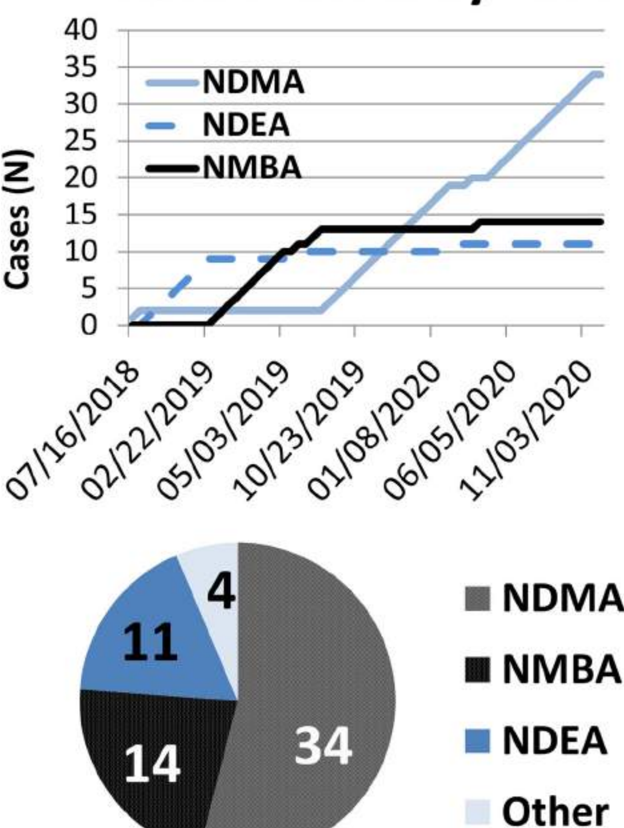


FIGURE 1 Number of out-of-specification (OOS) results for analyzed active pharmaceutical ingredients (APIs) and finished dosage forms (FDFs) as of April 15, 2019, according to the European Medicines Agency (EMA).[1] From 758 tested APIs, 165 were positive (21.8%), and 320 of 1802 tested FDFs (17.8%) were above the acceptable daily dose of the specific nitrosamine (NA). For *N*-nitrosodimethylamine (NDMA), 39.5% of the APIs (70 of 177) and 21.2% of the FDFs (253 of 1193) were contaminated; for *N*-nitrosodiethylamine (NDEA), 16.1% of the APIs (82 of 509) and 11.0% of the FDFs (67 of 609) were contaminated; for *N*-nitroso-*N*-methyl-4-aminobutyric acid (NMBA), 18.1% of the losartan APIs were contaminated (13 of 72)—note that other sartans and sartan drug products were not analyzed at the time of data acquisition[1]

other active pharmaceutical ingredients (APIs) were reported to be contaminated with NAs. Ten months after the first report of a detected NA, about 22% of all tested API batches and 18% of all drug product batches containing valsartan, losartan, and irbesartan showed levels of NDMA, *N*-nitrosodiethylamine (NDEA), and/or *N*-nitroso-*N*-methyl-4-aminobutyric acid (NMBA) above the acceptable limits in the European Union (Figure 1).[1] To date, more than 1800 drug product batches (sartans, antidiabetic drugs, antihistamines, and antibiotics) have been recalled in the United States (Figure 2) due to NA detection.[2] The U.S. Food and Drug Administration (FDA) and the EMA additionally initiated a total withdrawal of all ranitidine[3,4] and varenicline drug products[5,6] from the market, as the occurrence of NAs could not be eliminated.

This triggered a worldwide scientific evaluation of nitrosamine contamination in all drug substances and medicinal products.[7–9] Marketing authorization holders have been requested to review all their supply chains and medicinal products for the possibility of NA formation and their root cause. A major part of this referral is the consensus that analytical measurements are necessary to detect and control *N*-nitrosamines and to mitigate their occurrence in medicinal products. Lists of nine potential known NAs have been established with interim limits for acceptable daily intake on a toxicological basis,[3,8] as NAs are mutagenic and carcinogenic substances from a "cohort of concern" as defined by the ICH guideline M7.[10] This unexpected finding of *N*-nitrosovarenicline shows that nitrosation of APIs or other drug product ingredients is possible and has not been addressed yet.[5,6]

For the scientific investigation, the EMA published a multilevel approach with a strict time frame (Figure 3) for the implementation of



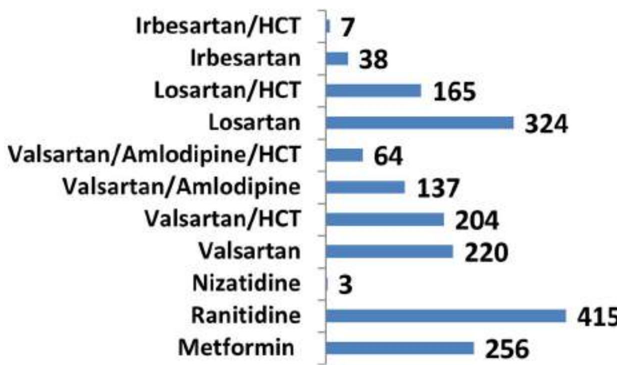


FIGURE 2 Cumulative cases of recalled or withdrawn drug products after Food and Drug Administration (FDA) safety alerts since 2018[2,3] —note that rifampicin and rifapentine were also subject to safety alerts, but not recalled, to prevent drug shortage. No specified nitrosamines were reported after January 4, 2021. HCT, hydrochlorothiazide; NDEA, *N*-nitrosodiethylamine; NDMA, *N*-nitrosodimethylamine; NMBA, *N*-nitroso-*N*-methyl-4-aminobutyric acid

a regulatory referral process. This approach includes a request for evaluation (step 1), additional confirmatory testing (step 2), and follow-up risk mitigation (step 3), if NAs are detected or likely to occur. In this EMA publication, a rather new scenario has been introduced within step 2, which has not been discussed yet: "one or

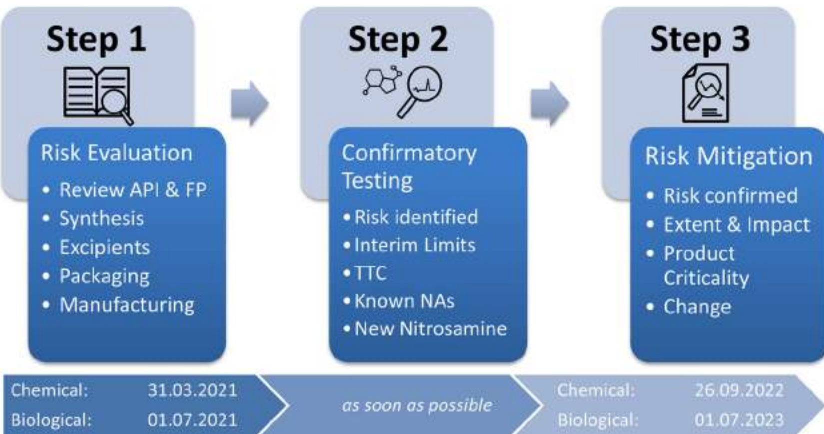


FIGURE 3 Time frame and expectation for nitrosamine referral according to EMA/425645/2020[11,12] for active pharmaceutical ingredients (APIs) and finished products (FPs). EMA, European Medicines Agency; NA, nitrosamine; TTC, threshold of toxicological concern

more new *N*-nitrosamines have been detected in a medicinal product which has not yet been assessed."[11] This was the first time that more than the nine[12] common nitrosamines were directly addressed, without further details being provided.

The main source for NAs previously has been the use or carryover of sodium nitrite ($NaNO_2$) within API synthesis and drug product manufacturing. Additionally, the use of recycled and/or contaminated raw and starting materials, carryover or cross-contamination of NA intermediates, degradation processes generating, for example, nitrosyl or oxime functionalities, and the use of certain packaging materials (e.g., nitrocellulose lidding foil) or any other nitrosating agents in the presence of secondary or tertiary amines were discussed as a source of NAs.[12–15]

Recently, mainly organic solvents and short-chain aliphatic amines (e.g., dimethylamine, diethylamine, or *N*-methyl-2-pyrrolidone) have been considered to be the precursors of nitrosamines, but as the varenicline case shows, nitrosation should also be considered for APIs.

In 2003, Adachi et al.[16] reported a case of 12 liver injuries with severe health consequences (one patient required liver transplantation and another patient died) due to the ingestion of a Chinese weight-loss dietary supplement. This supplement, which was labeled as herbal medicine, contained *N*-nitrosofenfluramine. In addition, it was already shown in the 1970s that in vivo and in vitro nitrosation of APIs[17–20] is possible. It is, therefore, obvious that the potential for NA formation has not yet been scrutinized to the end, as nitrosation studies are not mandatory during drug development and registration or forced degradation studies.

2 | RESULTS AND DISCUSSION

2.1 | Method implementation

As our workgroup has already developed a universal and selective supercritical fluid chromatography-tandem mass spectrometry SFC-MS/MS method for 16 aliphatic, cyclic, and aromatic nitrosamines using Quality-by-Design principles,[21] analysis was easily extended to nitrosated APIs. Three commercially available nitrosated APIs (Figure 4) were spiked at the 2-ppm level to their corresponding drug products to evaluate method suitability for this new group of nitrosamine species. Selectivity and detectability were evaluated at a minimum of two drug product batches from different manufacturers by tuned selected reaction monitors. All *N*-nitroso derivatives were fully separated from their respective APIs.

The implementation experiments demonstrated that SFC-MS/MS is able to separate and detect nitrosated APIs very efficiently and sensitively (resolution factor > 1.5 and signal-to-noise ratio > 1000 at the 2-ppm level), which is necessary during drug and process development.

2.2 | Nitrosation experiments

After the SFC method was successfully implemented for advanced nitrosamine screening, samples of 67 drug products were incubated according to the nitrosation assay procedure (NAP test) and investigated by SFC-high-resolution mass spectrometry (HRMS) time-of-flight (TOF). The NAP test is an in vitro forced degradation test with fourfold excess nitrite in acidic solution. It was originally designed in 1980 by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) to simulate in vivo formation of nitrosamines in the stomach, but it was never included in registration dossiers, as selective and sensitive analytical techniques were not commonly available at that time.

Out of the investigated model compound samples (Table 1), 33 (49.3%) showed intense peaks in the extracted ion chromatograms that were associated with drug–nitrite interaction products and identified by the exact mass. The experiments were then repeated with the same samples using a liquid chromatography (LC)-HRMS (TOF) system. All results were verified and confirmed using this orthogonal technique.

Of the 33 drug–nitrite interaction products, 24 (72.7%) are already commercially available or reported as known *N*-nitroso compounds (NOC).[22–24] Seven drugs (21.2%) have not yet been reported to form *N*-nitroso derivatives and two additional drugs (opipramol and amoxicillin) were already reported[22–24] to form

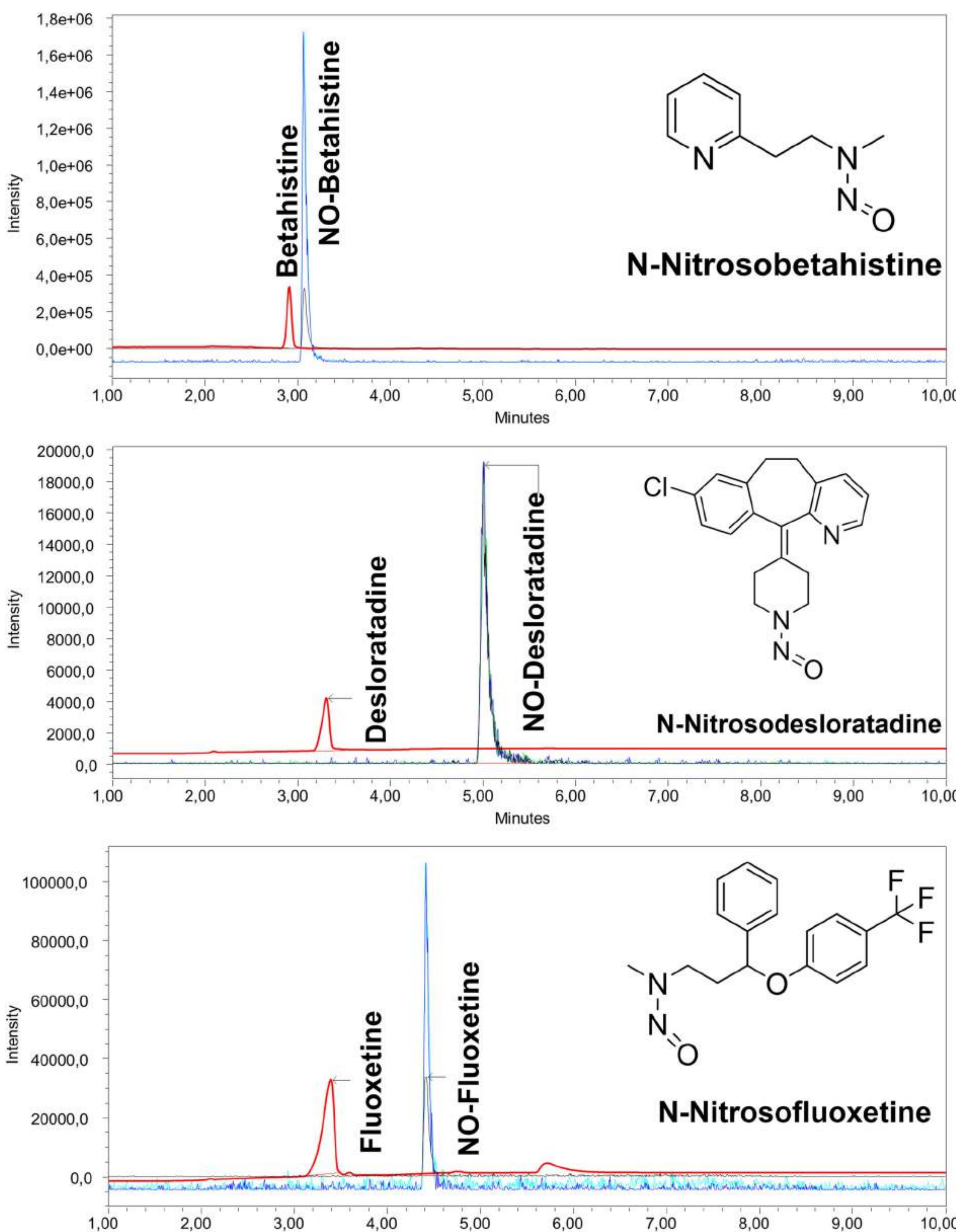


FIGURE 4 Commercially available and investigated *N*-nitroso derivatives of active pharmaceutical ingredients (APIs) for method implementation (original APIs displayed with the red UV channel only, to prevent oversaturation of the mass detector, and three mass transitions of each *N*-nitroso compound species by electrospray ionization tandem mass spectrometry). *N*-Nitrosobetahistine: *m/z* 166.0 > 44.0, 93.0, 136.0; *N*-nitrosodesloratadine: *m/z* 340.1 > 266.1, 280.5, 310.2; *N*-nitrosofluoxetine: *m/z* 339.2 > 73.0; 117.1; 177.0

TABLE 1 Positive and negative findings by SFC-TOF-MS of the investigated APIs after nitrosation with the NAP test and corresponding *N*-nitroso derivatives with mass error (only findings with a mass error of ≤5 ppm are reported) and NOC yield (intensity ratio of MS signals in extracted ion chromatograms between an *N*-nitroso derivative and API)

Drug	Nitroso compound	NOC neutral mass (Da)	Mass error observed (ppm)	Nitrosation yield (%)	Retention time (min)	Retention factor (k_1)
Ambroxol	-					
Amlodipine	-					
Amoxicillin	*N*-Nitrosoamoxicillin	394.09471	0.0	5.5	8.09	12.5
Aripiprazole	-					
Bendroflumethiazide	*N*-Nitrosobendroflumethiazide	450.02795	−2.1	0.3	9.33	14.6
Betahistine	*N*-Nitrosobetahistine	165.09021	1.2	99.9	3.05	4.1
Bisoprolol	*N*-Nitrosobisoprolol	354.21547	−1.1	50.7	7.62	11.7
Bromazepam	*N*-Nitrosobromazepam	343.99089	−2.2	0.4	9.32	14.5
Carvedilol	*N*-Nitrosocarvedilol	435.17942	−2.9	20.7	8.71	13.5
Cetirizine	1-[(4-Chlorophenyl)(phenyl)methyl]-4-nitrosopiperazine	315.11384	−1.7	0.06	5.21	7.7
Citalopram	-					
Clopidogrel	-					
Desloratadine	*N*-Nitrosodesloratadine	339.11384	4.6	29.2	5.03	7.4
Diclofenac	*N*-Nitrosodiclofenac	324.0073	1.4	28.9	5.20	7.7
Diphenhydramine	-					
Doxylamine	*N*-Nitrosonordoxylamine	285.14773	−4.4	2.3	9.02	14.0
Duloxetine	*N*-Nitrosoduloxetine	326.10890	0.1	85.4	5.32	7.9
Enalapril	*N*-Nitrosoenalapril	405.18999	2.9	67.8	7.22	11.0
Entacapone	-					
Ergometrine	*N*-Nitrosonorergometrine	340.15354	0.9	0.8	7.34	11.2
Felodipine	*N*-Nitrosofelodipine (1)	412.05928	−0.8	45.1	9.62	15.0
	N-Nitrosofelodipine (2)	412.05928	1.1	40.4	10.02	15.7
Fesoterodine	-					
Fexofenadine	-					
Flecainide	*N*-Nitrosoflecainide	443.12798	−0.4	17.1	6.82	10.4
Fluoxetine	*N*-Nitrosofluoxetine	338.12421	2.8	99.0	4.46	6.4
Furosemide	-					
Glibenclamide	-					
Glimepiride	-					
Haloperidol	-					
Hydrochlorothiazide (HCT)	*N*-Nitroso-HCT	325.95464	5.4	19.2	4.77	7.0
Levofloxacin	*N*-Nitrosonorlevofloxacin	376.11830	0.6	0.03	8.01	12.4
Levomepromazine	-					
Loperamide	-					
Melperone	-					
Metoclopramide	-					

(Continues)

TABLE 1 (Continued)

Drug	Nitroso compound	NOC neutral mass (Da)	Mass error observed (ppm)	Nitrosation yield (%)	Retention time (min)	Retention factor (k_1)
Metoprolol	*N*-Nitrosometoprolol	296.17361	4.5	48.9	7.35	11.3
Mirabegron	*N*-Nitrosomirabegron (1)	425.15216	−0.8	19.1	11.56	18.3
	N-Nitrosomirabegron (2)	425.15216	0.5	51.9	11.90	18.8
	N,N'-Dinitrosomirabegron (3)	454.14232	0.4	29.2	12.75	20.3
Mirtazapine	*N*-Nitrosonormirtazapine	280.13241	1.6	0.8	8.22	12.7
Moclobemide	-					
Molsidomine	-					
Moxifloxacin	*N*-Nitrosomoxifloxacin	430.16525	0.0	30.6	4.19	6.0
Mycophenolate mofetil	-					
Nebivolol	*N*-Nitrosonebivolol	434.16533	0.9	75.8	5.46	8.1
Nifedipine	-					
Olanzapine	-					
Opipramol	5-[3-(4-Nitrosopiperazin-1-yl)propyl]-5*H*-dibenzo[*b,f*]azepine	348.19501	−0.4	0.5	8.95	13.9
Perazine	-					
Pergolide	*N*-Nitrosopergolide	343.17183	4.2	52.3	5.54	8.2
Pipamperone	-					
Pramipexole	-					
Promethazine	-					
Propafenone	-					
Propranolol	*N*-Nitrosopropranolol	288.14739	2.4	15.7	6.25	9.4
Quetiapine	11-(4-Nitrosopiperazin-1-yl)dibenzo[*b,f*][1,4]thiazepine	324.10448	0.5	0.2	6.69	10.2
Ramipril	*N*-Nitrosoramipril	445.22129	−1.0	0.0	5.98	9.0
Roxithromycin	*N*-Nitrosonorroxithromycin	851.49908	1.1	17.6	5.30	7.8
Sertraline	*N*-Nitrososertraline	334.06397	−1.2	3.6	5.26	7.8
Sotalol	*N*-Nitrososotalol (1)	301.10963	−0.2	18.8	5.31	7.9
	N-Nitrososotalol (2)	301.10963	1.3	17.3	5.53	8.2
Spiramycin	-					
Sumatriptan	*N*-Nitrososumatriptan	324.12561	0.0	34.3	7.96	12.3
Terbinafine	-					
Torasemide	-					
Tramadol	-					
Varenicline	*N*-Nitrosovarenicline	240.10111	0.0	69.3	4.82	7.0
Venlafaxine	-					
Verapamil	-					
Zolpidem	-					

Note: Retention factor (k_1 value) was calculated on the experimental determined unretained hold-up time T_0 = 0.6 min.

Abbreviations: API, active pharmaceutical ingredient; NAP, nitrosation assay procedure; NOC, *N*-nitroso compound; SFC-TOF-MS, supercritical fluid chromatography–time-of-flight–mass spectrometry.

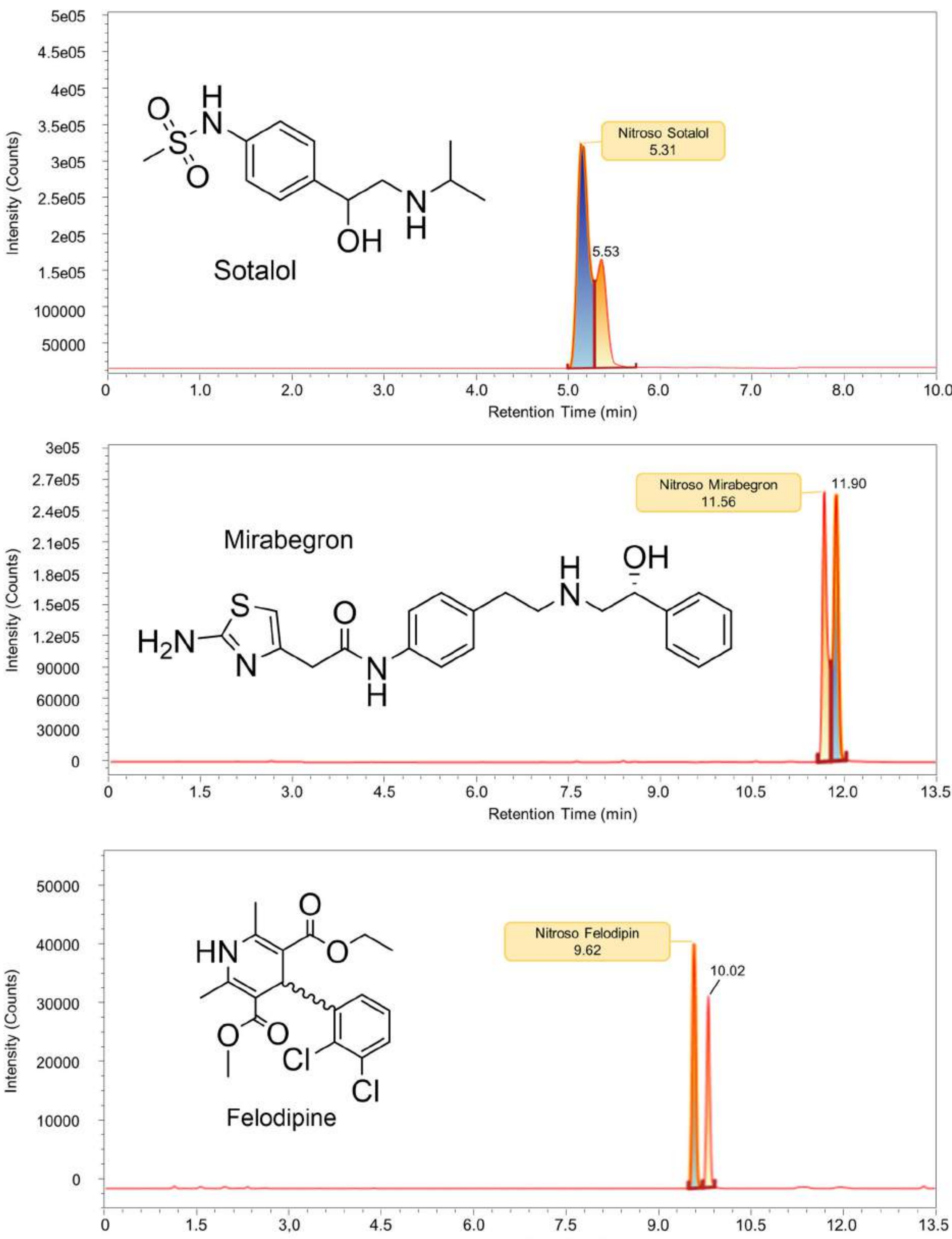


FIGURE 5 Extracted ion SFC-MS chromatogram (15.0 ppm mass accuracy window) of sotalol (EIC = 301.1096 Da), felodipine (EIC = 412.0593 Da), and mirabegron (EIC = 425.1522 Da) samples after the NAP test—two separated peaks of *N*-nitroso compounds with mass errors of <5 ppm detected. Extracted ion SFC-MS chromatogram (15.0 ppm mass accuracy window) of sotalol (EIC = 301.1096 Da), felodipine (EIC = 412.0593 Da), and mirabegron (EIC = 425.1522 Da) samples after the NAP test—two separated peaks of *N*-nitroso compounds with mass errors of <5 ppm detected. NAP, nitrosation assay procedure; SFC-MS, supercritical fluid chromatography-mass spectrometry

drug–nitrite interaction products but without knowledge of their chemical structure. They were now elucidated by HRMS analysis.

Sotalol, which is already known to form *N*-nitrososotalol, showed two NOC species (Figure 5) with the same mass spectrum that were chromatographically separated by SFC on the graphite column, due to its high isomeric separation performance,[25] but only partially with LC. As the first *N*-nitrososotalol peak in the SFC, the chromatogram shows a more abundant $[M+H]^+$ adduct and the second peak shows a more abundant $[M+Na]^+$ adduct in the HRMS spectra; we, therefore, suggest that it was nitrosated at two different amine entities (secondary amine and methanesulfonamide). Multiple NOC species were also observed in felodipine and mirabegron (Figure 5). For mirabegron, an *N*,*N′*-dinitroso derivate was additionally detected (most likely due to nitrosation at the secondary amine and phenylacetamide structure). Felodipine is manufactured as a racemic formulation and has a planar, symmetrical 1,4-dihydropyridine structure. By NO binding to the amine moiety, felodipine deracemizes and forms two isomers that were separated by SFC.

Additional MS/MS experiments (e.g., QTOF) might be relevant in the future to support the thesis that has been made in this paper, as NOC standards are not available on the market. Fragmentation experiments would then support the postulated structures. If these experiments support nitrosation, synthesis of the elucidated NAs should be performed to allow for structural substantiation. With these NOC standards, definite verification of NA occurrence by chromatographic and spectrometric behavior would then be possible (level 1 structure confirmation).[26]

Furthermore, MS/MS analysis is able to detect NAs in very low quantities, due to the targeted approach. This allows precise and sensitive root-cause analysis, as requested by health authorities.

On the contrary, the NAP test results are able to exclude theoretically proposed nitrosamines, derived from the chemical structure of the API or excipient. If the predicted structure cannot be detected after incubation, it is highly unlikely that it will form in the product.

3 | CONCLUSIONS

Although evidence to support major risk concerns for patients' health is missing or at least very low,[1,27] it has become apparent that the analytical focus of drug analysis was too narrow in the past. Not all nitrosamines are inevitably mutagenic or carcinogenic (e.g., *N*-nitrosodiphenylamine), as Elder et al.[28] have shown in their review, but without the knowledge of NA formation, it is already evident that this public health problem will persist.

Nevertheless, a lot of NAs are known for their high potential to cause cancer in almost all organs[29] and case studies have shown that not only small aliphatic or aromatic NAs but also nitrosated APIs can cause cancer.[16] Additionally, in vitro data of nitrosated APIs, which were summarized by Brambilla and Martelli,[22] suggest a high genotoxic potential of some drug–nitrite interaction products (Figure 6), some of them even higher than NDMA.

After completion of this study, the Canadian health authority published a short notice on the recall of orphenadrine tablets, used as

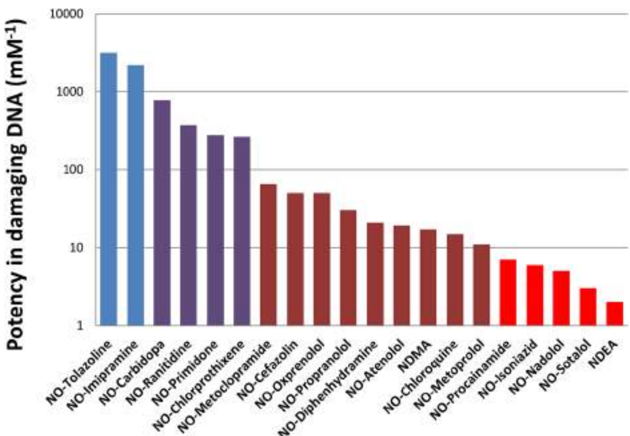


FIGURE 6 Genotoxic potential of selected nitrosamines and nitrosated pharmaceuticals according to Brambilla and Martelli[22] by inducing DNA fragmentation in cell culture. High values indicate a lower potential

a muscle relaxant, as certain batches showed elevated levels of *N*-methyl-*N*-nitroso-2-[(2-methylphenyl)phenylmethoxy]ethanamine (*N*-nitrosonororphenadrine),[30] another unexpected NOC species, formed from the API. Also, irbesartan tablets were recently recalled in the United States due to the potential presence of *N*-nitrosoirbesartan.[31] It is, therefore, apparent that the security of drug supply and possibly also the health of patients may be affected, as this group of nitrosamines has not been addressed to date. Such findings could have been avoided if appropriate nitrosation assays had been performed before registration.

In the present study, it was demonstrated how direct risk assessment of pharmaceuticals can be performed using a state-of-the-art SFC-HRMS screening method to integrate nitrosated API species into drug development and forced degradation studies. Thus, the potential formation of NOCs can be detected, controlled, and limited. If this had been performed earlier, varenicline, which was recently elevated to global "essential medicines" status by the WHO,[32] could have been prevented from having to be recalled.

The "nitrosamine crisis," with all its negative effects on patient health and the supply assurance of drug products, should be used to draw advantages for the future. All health participants will benefit from the increased awareness. The lifecycle of drug products will also profit from this in the long term if relevant conclusions are drawn and consequences are taken into consideration. It is crucial to broaden the horizon of surveillance and to investigate drug harmlessness before registration with appropriate analytical techniques, which are now available.

4 | EXPERIMENTAL

4.1 | Materials

Reference standards of *N*-nitrosobetahistine, *N*-nitrosodesloratadine, and *N*-nitrosofluoxetine were acquired from Toronto Research

Chemicals. The drug products investigated in this study were directly obtained from the German market.

In this study, only MS-grade solvents and additives were used and purchased from VWR International GmbH. Carbon dioxide N45 (99.995%) and nitrogen N50 (99.999%) were obtained from Air Liquide Deutschland GmbH and Argon 5.3 (99.9993%) from Linde AG.

A Supel Carbon graphite column (100 × 3.0 mm, 2.7 µm; Merck KGaA) was used for analysis.

4.2 | Instrumentation and software

Chromatographic analysis was performed using an Acquity UPC^2 SFC system (Waters GmbH) equipped with an Acquity UPC^2 column manager with active eluent preheaters and an Acquity UPC^2 PDA (photodiode array) detector. A fixed-leak interface from the SFC to the MS was coupled with a Waters 515 make-up pump (post-column split) to enhance mass transfer to the MS and to improve ionization. For method implementation, a Waters Acquity TQD (triple quadrupole mass spectrometer) was used for targeted analysis (Section 2.1). For untargeted nitrosation experiments (Section 2.2), a Waters Acquity RDa (TOF and HRMS) was hyphenated to the SFC system.

For system control, Empower 3 software (Feature Release 5, Service Release 4; Waters) was used. Nitrosation experiments were further verified on the orthogonal Waters BioAccord LC-TOF system, to validate the SFC-MS results. Acquisition and processing of high-resolution mass data were performed on the UNIFI Scientific Information System (Waters).

Instrumentation operated fully qualified according to the 4Q model of the U.S. Pharmacopeia general chapter <1058>[33] in a GMP-regulated laboratory environment.

Incubation (chapter 4.4) was performed on a 5436 thermomixer at 1200 rpm (Eppendorf AG) in 2 -ml Safe-Lock tubes (Eppendorf). Afterward, samples were prepared on a 5415D lab centrifuge (centrifugal force: 16.110 rcf; kinetic energy: 3.100 Nm; Eppendorf). For injection, 2 -ml amber glass TruView LCMS vials (Waters) were used.

Chemical structures and exact molecular masses were drawn and calculated using ChemDraw Professional (Version 20.1; PerkinElmer Informatics, Inc.). Graphical abstract was created with free images from Servier Medical Art (SMArt) and Wikimedia commons.

4.3 | Instrumental conditions

For high-sensitivity detection of the three nitrosated API standards, the MS/MS was operated in positive electrospray ionization (ESI+) mode with timed selected reaction monitoring for method implementation. Optimized MS/MS parameters are as follows: capillary voltage 3.50 kV, source temperature 120°C, desolvation temperature 250°C, desolvation gas flow 500 l/h, and collision gas flow 0.30 ml/min. No extra cone gas was used.

For peak identification after the nitrosation assay procedure (NAP test), the TOF-MS was operated in ESI+ mode with a capillary voltage of 1.50 kV, a source temperature of 120°C, a desolvation temperature of 550°C, a desolvation gas flow rate of 940 l/h, cone gas flow of 36 l/h, and nebulizer gas flow of 133 l/h.

As a make-up solvent, a 0.35% solution of formic acid in MeOH was used at a 0.12 ml/min constant flow to transfer the SFC-split to the MS. Chromatograms were also recorded from 195 to 400 nm using a PDA detector.

Chromatographic separation was performed on a Supel Carbon column (100 × 3.0 mm; 2.7 µm) at a column temperature of 60°C and a flow rate of 1.5 ml/min. The back pressure was set to 1800 psi. The gradient method is: CO_2 as eluent A and a 0.1% solution of trifluoroacetic acid in methanol as eluent B, starting isocratic at 2% B for 1 min. The gradient profile was then rapidly increased linearly to 60% B within 2 min with an additional 0.5 min isocratic step, followed by a second rapid, linear increase to 75% B in 0.58 min and a final hold for 7.92 min at 75% B, resulting in a total run time with reequilibration of 16.5 min. The injection volume was 2.5 µl.

4.4 | NAP test and sample preparation

According to the 1980 IARC monograph from the WHO,[34] a standardized procedure was applied with an excess of nitrite. Before NAP testing, all drug products were ground and dispersed (for solid dosage forms) or directly dissolved (for liquid and semisolid dosage forms) in the incubation medium.

All samples were incubated at 37°C for 4 h with an API concentration of 10 mmol/l in a 40-mol/l sodium nitrite solution at pH 3.5 (with 1 mol/l hydrochloric acid). The pH was adjusted in the sodium nitrite solution, measured (pH 3.5 ± 0.5), and corrected if necessary after the addition of sample material. After incubation, samples were centrifuged and the particle-free supernatant was analyzed by SFC-HRMS. Only peaks with a mass error of ≤5 ppm were investigated and reported.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests.

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