Tackling the Nitrosamine Crisis - Supercritical Fluid Chromatography Coupled with Mass Spectrometry for the Determination of Unexpected Drug Impurities

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by
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Research of the present study was conducted from 2016 till 2022 under supervision of Prof. Dr. Maria Kristina Parr at the Institute of Pharmacy of the Freie Universität Berlin, Germany.
Work was performed externally in the GMP analytical contract lab of Chromicent GmbH in Berlin, Germany.

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Independence Declaration

I hereby affirm that I have completed the presented cumulative dissertation independently and without unauthorized assistance. No aids other than these listed in the text were used in the writing of the dissertation.

The doctoral procedure has never been completed at any other university or applied to another department.

Sebastian Schmidtsdorff

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Abbreviations

AI	Allowable Intake
API	Active Pharmaceutical Ingredient
ATP	Analytical Target Profile
CDER	Center for Drug Evaluation and Research
CLD	Chemiluminescence Detector
CoC	Cohort of Concern
CYP	Cytochrome
DMF	Dimethylformamide
DNA	Deoxyribonucleic Acid
EMA	European Medicines Agency
ESI	Electrospray Ionization
FDA	U.S. Food and Drug Administration
FP	Finished Product
GC	Gas Chromatography
GMP	Good Manufacturing Practice
HRMS	High Resolution Mass Spectrometry
IARC	International Agency for Research on Cancer
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
IL	Interim Limit
LC	Liquid Chromatography
LCM	Lifecycle Management
MA	Marketing Authorization
MAH	Marketing Authorization Holder
MGMT	O^6-Methylguanine-DNA Methyltransferase
MNPaz	1-Methyl-4-nitrosopiperazine
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NA	*N*-Nitrosamine
NAP	Nitrosation Assay Procedure

NDBA	*N*-Nitrosodi-*n*-butylamine
NDEA	*N*-Nitrosodiethylamine
NDELA	*N*-Nitrosodiethanolamine
NDiPA	*N*-Nitrosodiisopropylamine
NDMA	*N*-Nitrosodimethylamine
NDPA	*N*-Nitrosodi-*n*-propylamine
NDPhA	*N*-Nitrosodiphenylamine
NDSRI	Nitrosamine Drug Substance Related Impurities
NEiPA	*N*-Nitrosoethylisopropylamine
NMBA	*N*-Nitroso-*N*-methyl-4-aminobutyric acid
NMor	*N*-Nitrosomorpholine
NMP	*N*-methylpyrrolidone
NMPhA	*N*-Nitrosomethylphenylamine
NOC	*N*-Nitroso Compound
NOX	Nitrosating Agent
OMCL	Official Medicines Control Laboratory
PGC	Porous Graphitic Carbon
Ph. Eur.	European Pharmacopeia
QbD	Quality-by-Design
RMS	Reference Member State
SAR	Structure-Activity Relationships
SFC	Supercritical Fluid Chromatography
TD_{50}	Median Toxic Dose
TEA	Thermal Energy Analyzer
TFA	Trifluoroacetic Acid
TOF	Time-of-Flight (Mass Spectrometry)
TTC	Threshold of Toxicological Concern
USP	United States Pharmacopeia
WHO	World Health Organization

Summary

The present work addresses the systematic development, enhancement and application of highly sensitive analytical methods for the investigation of nitrosamine impurities in drug substances and drug products. It is demonstrated how these can be effectively brought to the point of application in the interaction of science and regulatory compliance in order to meet the highest standards of scientific and technical knowledge.

As a first step, quality-by-design (QbD) principles are used to rapidly and systematically develop a robust and sensitive method. Due to the unique properties of supercritical fluid chromatography (SFC), this method is not only capable of separating ten pertinent nitrosamines and achieving detectability at trace levels, but also enables simultaneous analysis of the drug substances losartan, valsartan and their monographed related substances. Based on this work, it is demonstrated which advantages result from the high adaptability of the QbD development approach for the lifecycle of an analytical method. Thus analytical progression within the existing framework and design space is possible without a complete new development. For this purpose, accepted lifecycle management (LCM) principles are used to evolve the existing compound-specific method into a universally applicable method for nitrosamine determination. The published method is the only validated method to date that can simultaneously separate and detect at least 16 nitrosamines, regardless of the sample matrix. Based on this method, analytical results are subsequently presented from hundreds of marketed drug substance and drug product samples collected over a period of four years as part of the "nitrosamine crisis".

In addition, a more sophisticated and standardized testing methodology is presented. It can be used not only to test for the presence of known nitrosamines, but also to investigate the formation of novel, drug-substance-related nitrosamines. These have recently become an increasing concern of the regulatory risk assessment process and hereby the dominant issue for pharmaceutical manufacturers and authorization holders.

The present work essentially contributes to the detection and elucidation of known and novel potentially carcinogenic nitrosamines in drugs. It thus protects the health of patients, by enabling contaminated drugs to be withdrawn from the market and prevents

novel nitrosamines to occur without knowledge of the responsible manufacturer. It therefore enhances the quality and safety of existing and new drugs. However, the outlined principles of method development, analytical lifecycle management and method application can also be applied to areas outside the trace analysis of nitrosamines or pharmaceutical quality control (e.g. in forensics, bio- and environmental analysis) and are generally applicable.

Zusammenfassung

Die vorliegende Arbeit befasst sich mit der systematischen Entwicklung, Optimierung und Anwendung von hochsensitiven Analysenmethoden zur Untersuchung von Nitrosaminverunreinigungen in Arzneistoffen und Arzneimitteln. Es wird aufgezeigt, wie diese effektiv im Zusammenspiel von Wissenschaft und regulatorischen Vorschriften zur Anwendung gebracht werden können, um so dem höchsten Stand von Wissenschaft und Technik zu entsprechen.

Als erster Schritt wird demonstriert, wie mittels Quality-by-Design (QbD) Prinzipien schnell und systematisch eine robuste und sensitive Analysenethode entwickelt werden kann. Aufgrund der einzigartigen Eigenschaften der superkritischen Fluidchromatographie (SFC) ist diese nicht nur in der Lage, zehn relevante Nitrosamine zu trennen und im Spurenbereich detektierbar zu machen, sondern auch simultan die Reinheitsanalytik der Wirkstoffe Losartan, Valsartan und ihrer monographierten verwandten Substanzen zu ermöglichen. Darauf aufbauend wird gezeigt, welche Vorteile sich durch das hohe Anpassungsvermögen des QbD-Entwicklungsansatzes für den Lebenszyklus einer analytischen Methode ergeben, sodass analytische Weiterentwicklung innerhalb des bestehenden Rahmens ohne eine vollständige Neuentwicklung möglich ist. Dazu werden anerkannte Lifecycle Management (LCM) Prinzipien genutzt, um die bestehende wirkstoffspezifische Methode zu einer universell einsetzbaren Methode für die Nitrosaminbestimmung weiterzuentwickeln. Die publizierte Methode ist die bisher einzige validierte Analysenethode, die mindestens 16 Nitrosamine unabhängig von der eingesetzten Probenmatrix gleichzeitig trennen und detektieren kann. Basierend auf dieser Methode werden Analysenergebnisse von hunderten auf dem Markt befindlicher Arzneistoff- und Arzneimittelmuster präsentiert, die im Rahmen der „Nitrosaminkrise“ über einen Zeitraum von vier Jahren gesammelt wurden.

Zusätzlich wird eine weiterentwickelte und standardisierte Untersuchungsmethodik präsentiert. Mit dieser kann nicht nur auf das Vorhandensein bekannter Nitrosamine geprüft werden, sondern auch die Bildung neuartiger, wirkstoffverwandter Nitrosamine untersucht werden. Diese sind zuletzt immer mehr in den Fokus des behördlich

angeordneten Risikoanalyseverfahrens gerückt und zu der derzeit dominierenden Herausforderung für pharmazeutische Hersteller und Zulassungsinhaber geworden.

Mit der vorliegenden Arbeit wird ein essentieller Beitrag dazu geleistet, bekannte und neuartige, potentiell krebserregende Nitrosamine in Medikamenten zu entdecken und aufzuklären. Sie schützt damit die Gesundheit von Patienten, da kontaminierte Arzneimittel so vom Markt genommen werden können und das Auftreten neuartiger Nitrosamine rechtzeitig aufgedeckt werden kann. Sie steigert somit die Qualität und Unbedenklichkeit vorhandener und neuer Arzneimittel. Die dargelegten Prinzipien der Methodenentwicklung, des analytischen Lifecycle Managements und der Methodenapplikation lassen sich auch auf Bereiche außerhalb der Spurenanalytik von Nitrosaminen oder der pharmazeutischen Qualitätskontrolle anwenden (z.B. in der Forensik, Bio- und Umweltanalytik) und sind universell anwendbar.

1 Introduction

The use of pharmaceuticals has a decisive positive impact on the lifespan and, in the vast majority of cases, the quality of life of mankind. Since pharmaceutical drugs are typically highly potent agents, controlling their quality is of enormous importance and constantly poses great challenges to their manufacturers. At the time the "nitrosamine crisis" became apparent in summer of 2018, it was perceived as little more than a side note. Initially it was dismissed as one of the many problems that regularly arise from outsourcing of drug manufacturing to South and East Asia [1]. Quickly it became apparent that the extent of the problem was much greater than assumed and that this was not just a typical GMP (Good Manufacturing Practice) violation or an import alert. After more and more drugs were recalled from the market, medial interest increased rapidly and consequently also spread to the mass media (**Figure 1**). Many European manufacturers were suddenly unable to supply their patients with cheap standard medications, so health experts had to switch to alternative drugs. Thus, concern about the safety of medicines increased enormously.

In the meantime, the causes of the initial recalls have been well elaborated [2] and there has been an enormous amount of improvements at crucial regulatory points. However, or probably because of these developments, nitrosamine-related recalls continue to occur. New nitrosamines are repeatedly discovered, so it can be assumed that this issue was unattended for too long. The damage to the reputation of safe medicines sustains, since it was not avoided or at least limited.

This work was initiated at the beginning of the "nitrosamine crisis". Its aim is to provide and improve state-of-the-art analytical methods for the detection of nitrosamines in drug substances and medicinal products. It is intended to provide manufacturers and marketing authorization holders with suitable tools to investigate the presence and origin of critical nitrosamines. For this purpose, analytical methods have been developed and validated as well as prospectively aligned to the ever-increasing requirements and beyond. Furthermore, a screening assay was developed to test for possible novel nitrosamines in the context of registration studies and risk assessment procedures, as these have increasingly come into focus. With the premature finalization of the first risk assessment procedure in the EU, analytical data from hundreds of

representative drug samples have also been published, highlighting the need for further nitrosamine testing.

With this work a small contribution has been made, ensuring that drug products become safe again and to help laying the foundation for finding such potentially carcinogenic contaminants in the future, before patients, who depend tremendously on the health-preserving effects of their medicines, come into contact with them.

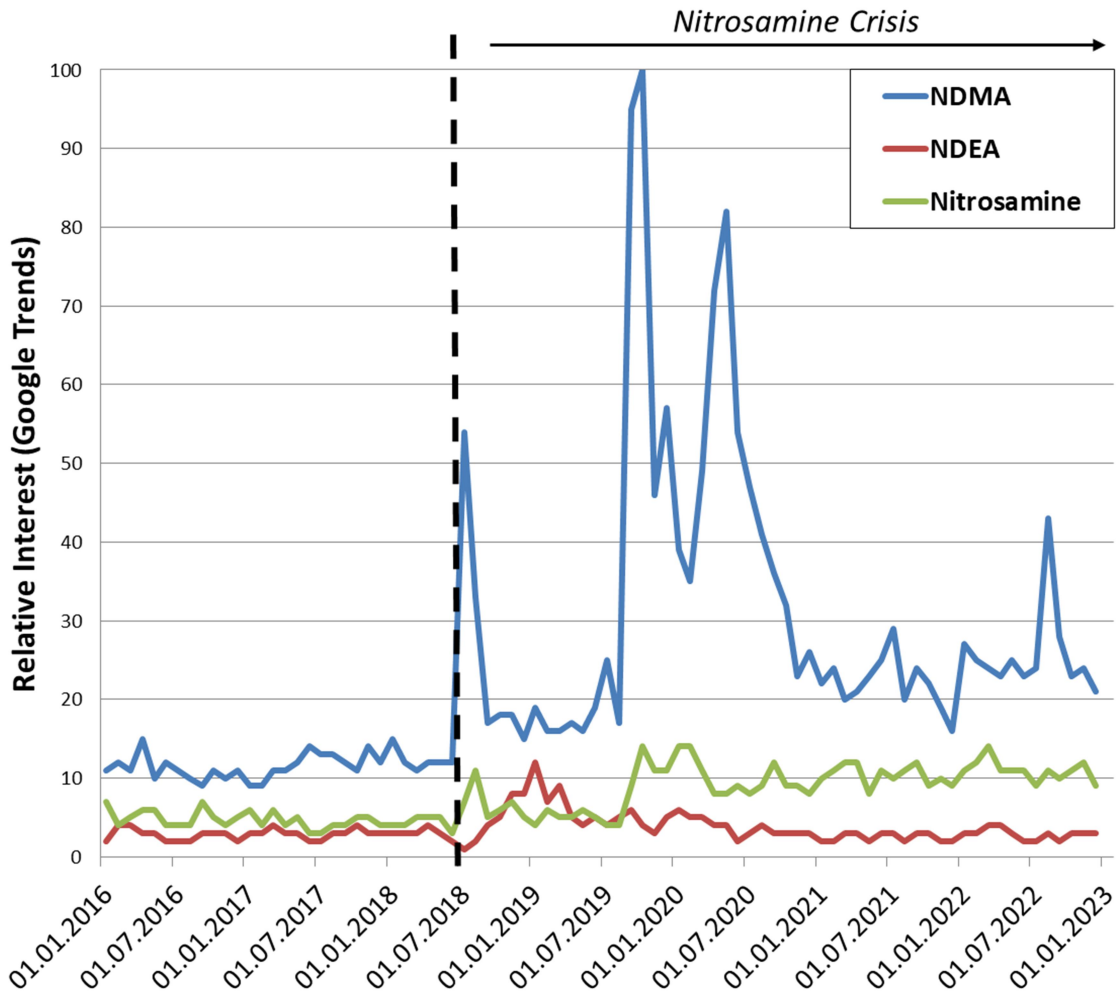

Figure 1: *Interest over time on Google Trends (Source: https://trends.google.de) for the search terms "NDMA", "NDEA" and "Nitrosamine" since January 2016.*

2 Theoretical Background

2.1 Formation and occurrence of nitrosamines

N-Nitrosamines (**Figure 2**) are a class of compounds, long-known and carcinogenic for the most part [3, 4]. They result from the reaction of secondary alkyl, aryl or cyclic amines with a "nitrosating agent" (NOX). These NOXs include in particular: nitrite salts (e.g. $NaNO_2$), nitrous acid (HNO_2), alkyl nitrites, nitrogen oxides, nitrosyl halides (e.g. NOCl), nitrosylthiocyanate and organic nitro compounds [2, 5].

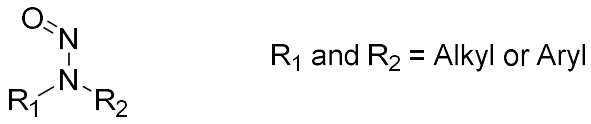

Figure 2: *General structure of N-nitrosamines*

Primary amines do not react to *N*-nitrosamines (NAs), but form diazonium compounds that further decompose under deamination [5]. Tertiary and quaternary amines are not directly able to form *N*-nitrosamines, but it is known that they can form NAs by intermediary desalkylation to a secondary amine [5, 6]. This has also been practically demonstrated for active pharmaceutical ingredients (APIs) containing tertiary amines in a broadly based assay [7]. Nevertheless, tertiary and quaternary amines are less reactive, compared to secondary amines [8].

N-Nitrosamines are ubiquitous environmental contaminants and ingested by every human being on a daily basis in small doses, mainly through food [9-11] and smoking [12], but can also enter the body through drinking water [13], rubber and latex products [14] or personal care products [15]. It is estimated that through consumption of processed or cured meat alone, each person in the western hemisphere consumes approximately 4 µg of *N*-nitrosodimethylamine (NDMA) per kilogram meat. This amount further increases approximately tenfold when the meat is fried [9, 16, 17]. Commercial available cigarettes contain about 20 – 110 ng of NAs per cigarette [18]. *N*-Nitrosamines in drinking water are limited to approximately 1 – 10 ng/L in the EU, Canada and the USA [13, 17]. Taken together, the total daily NA exposure is estimated at 1,900 – 25,000 ng/day, mainly driven by smoking and followed by food [19].

2.2 Toxicological relevance

The International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) has classified numerous NAs as “carcinogenic” or “probably carcinogenic” and categorized them in four groups based on their available scientific evidence [20]. *N*-Nitrosamines are relatively stable organic substances that are not toxic themselves (so called “pro-mutagens”). Nevertheless, they are activated by hydroxylation at the α-carbon atom mediated by cytochrome (CYP) P450. CYP isoforms 2E1 and 2A6 seem to play a significant role in this process [21-23]. The intermediate α-hydroxy-alkyl-*N*-nitrosamines (*N*-nitrosocarbinolamines) are unstable and decompose rapidly into an aldehyde and a highly active, electrophilic alkyl or aryl diazonium species (**Figure 3**) [24, 25] This is able to alkylate the DNA (deoxyribonucleic acid), especially to stable O^6-alkylguanine adducts [26, 27]. The alkylated guanine adducts can then result in DNA misreading, leading to GC→AT translation mutations.

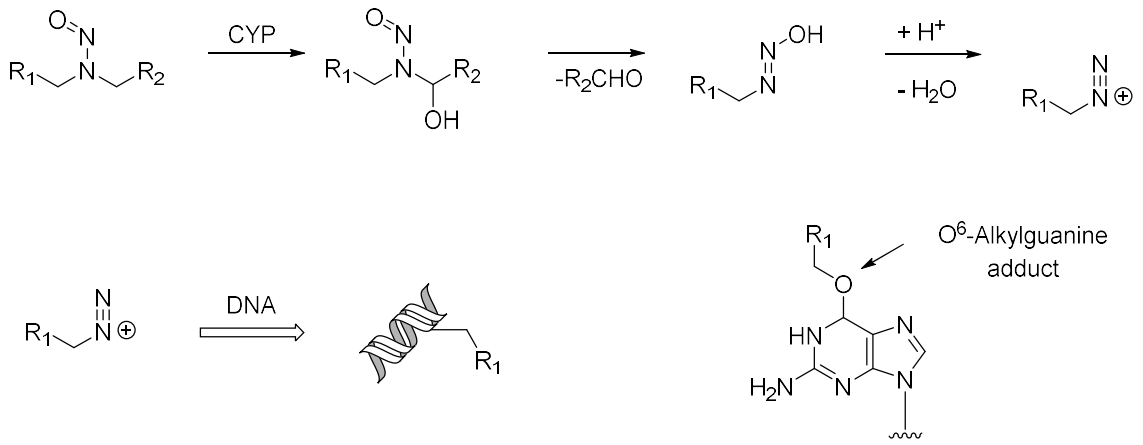

Figure 3: *Enzymatic activation of N-nitrosamines by cytochrome P450 and the resulting* O^6*-alkylguanine adduct*

Due to DNA-misreading, long-term chronic or short-term accelerated exposure can lead to tumors in a wide variety of organs. Not only in the liver, the organ with the highest metabolic competence, carcinogenic effects are observed, but also in the esophagus, urinary bladder, kidney and lung in particular [24, 28]. The type of alkyl or aryl substituents has a significant influence on the extent of the carcinogenic effect, but little is known about whether bulky, more complex molecular structures have a similar

DNA-alkylating effect [29-31]. *N*-Nitrosamines have such a strong alkylating effect [32, 33] that their daily intake must be limited. Cellular repair mechanisms, on the other hand, are only available to the organism to a very limited extent. Alkyl transferases (especially O^6-methylguanine-DNA methyltransferase, MGMT) that are able to regenerate alkylated DNA can be exhausted very quickly by the occurrence of NAs. This causes non-linear dose responses and a “cut-off” point or "practical threshold" for mutations even at very low exposure [34].

Retrospective analyses by Li et al. [35] have shown that 40 to 126 additional cancer cases from NDMA and 12 to 48 additional cancer cases from NDEA (*N*-nitrosodiethylamine) per 100,000 individuals could have occurred in the United States due to the use of NA-contaminated valsartan alone since 2012. This estimation is consistent with independent studies from other countries in the EU [2, 36, 37]. Due to the contaminated valsartan, patients could have ingested an average dose of 24.1 µg/day NDMA or 3.7 µg/day NDEA [38]. This is the 2- to 10-fold of the regular daily intake of a health-conscious person [19], although taking this medication to treat high blood pressure or heart insufficiency should actually protect the patient's life. The FDA published an exemplary list of 17 valsartan-containing products for which NDMA content up to 20 µg/tablet (Valsartan 320 mg / HCTZ 25 mg; Prinston Pharmaceutical Inc., USA) and NDEA content up to 1.3 µg/tablet (Valsartan 160 mg; Torrent Pharmaceuticals Ltd, India) [39] were found. Some of the tested batches even contained NDMA and NDEA concurrently.

2.3 Regulatory aspects to limit mutagenic substances in pharmaceuticals

The manufacturing and processing of drug substances and drug products is a highly regulated area, aiming to bring only high-quality products to the market, as well as preventing harm from patients. For this reason, there are numerous guidelines and regulations that must be adhered to (**Table 1**). Particular attention is paid to limiting impurities that significantly affect quality, effectiveness and safety.

Table 1: *Guidelines for controlling impurities in drug substances and drug products in the EU and USA (adapted from [2])*

Guideline name
European Pharmacopeia (Ph. Eur.) and U.S. Pharmacopeia (USP)
EMA Guideline - Guideline on the Chemistry of Active Substances
EMA Guideline on Summary of Requirements for Active Substances in the Quality Part of the Dossier
FDA Guidance - ANDAs: Impurities in Drug Substances
FDA Guidance - ANDAs: Impurities in Drug Products
ICH M7 - Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk
ICH Q3A - Impurities in new drug substances
ICH Q3B - Impurities in new drug products
ICH Q3C - Residual solvents
ICH Q3D - Elemental impurities
ICH Q7 - Good manufacturing practice for active pharmaceutical ingredients
ICH Q9 - Quality risk management
ICH Q10 - Pharmaceutical quality system
ICH Q11 - Development and manufacture of drug substances (chemical entities and biotechnological/biological entities)

The guideline Q3A of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) specifies: „The applicant should summarize the actual and potential impurities most likely to arise during the synthesis, purification, and storage of the new drug substance. This summary should be based on sound scientific appraisal of the chemical reactions involved in the synthesis, impurities associated with raw materials that could contribute to the impurity profile of the new drug substance, and possible degradation products“ [40]. Unfortunately, genotoxic substances are only referred to in this guideline with a marginal note: „Identification of impurities present at an apparent level of not more than (≤) the identification threshold is generally not considered necessary. However, analytical procedures should be developed for those potential impurities that are expected to be unusually potent, producing toxic or pharmacological effects at a level not more than (≤) the identification threshold“ [40].

Carcinogens and mutagens are further addressed in ICH guideline M7, which focuses on the "Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk" [41]. It provides the framework and defines how to deal with potentially mutagenic impurities to strictly limit their presence. For that purpose it introduces the threshold of toxicological concern (TTC), which establishes general lifetime acceptable intake levels. The overall goal of this guideline is, to prevent more than one additional cancer case in 100,000 treated patients. The TTC approach originates from the field of food safety [42] and has been set to 1.5 µg/day in drug substances and drug products. Below this TTC, the risk for potential mutagenic or carcinogenic effects is considered negligible, even if no carcinogenicity data are available. If sufficient data are available, allowable intake levels can be deduced on a substance- or class-specific basis.

Explicitly excluded from this TTC concept are “some structural groups […] to be of such high potency that intakes even below the TTC would theoretically be associated with a potential for a significant carcinogenic risk. This group of high potency mutagenic carcinogens is referred to as the ‘cohort of concern’ (CoC)” [41]. For this CoC class, which includes *N*-nitroso compounds (and also aflatoxin-like- and alkyl-azoxy compounds), substance-specific absolute limits must be maintained on a case-by-case basis to avoid depleting cellular repair capacities through excessive occurrence of e.g. NAs [32, 33].

These substance-specific limits are typically linearly extrapolated to low dose thresholds from animal experimental carcinogenic dose-response data, like the TD_{50} (median toxic dose), or derived from closely related compounds, to estimate lifetime acceptable intake levels for each substance by limiting the excess in the cancer incidence to 1 in 100,000 patients [41]. In contrast, the TTC value is set at a general level that is considered safe for people, without incorporating a substance-specific risk assessment every time, to abbreviate the evaluation procedures during registration.

2.4 Nitrosamine crisis

As knowledge about the manufacturing of drug substances and drug products increases over their lifecycle, the ICH encourages and calls for innovation and continuous improvement in its guideline Q12 on "Technical and regulatory considerations for pharmaceutical product lifecycle management" (derived from previous guidelines Q8, Q9, and Q10) [43]. Driven by this approach, the Chinese manufacturer Zhejiang Huahai Pharmaceuticals wanted to improve the synthesis of the tetrazole ring in one of its sartans (angiotensin II receptor blockers), viz. valsartan, by a post-approval change [44]. To increase the yield and to accelerate the synthesis, high-boiling solvents such as dimethylformamide (DMF) or *N*-methylpyrrolidone (NMP) were introduced for this purpose [45, 46]. What remained unnoticed by the drug substance manufacturer and the authorities that accepted the change in 2012, was that azides such as sodium azide (NaN_3) are used in the final step of the synthesis. These azides must be quenched (**Figure 4**) under acid conditions by sodium nitrite ($NaNO_2$), because of their extremely high toxicity [47]. During quenching, the reaction of nitrite with partially present or hydrolytically and thermally liberated secondary amines from the solvents occurred unnoticed (**Figure 5**). Since risk assessments during that change did not take this into account, the formation was unexpected and no routine screening procedures were implemented at that time.

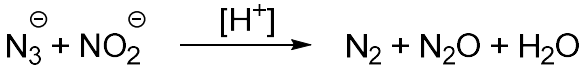

Figure 4: *Decomposition of toxic azides with sodium nitrite ("quenching") [47]*

The first time the initially unknown contamination in valsartan was noticed, was on 06 June 2018 by a potential European customer of Zhejiang Huahai Pharmaceuticals, who detected an unknown impurity while performing a residual solvent analysis. The manufacturer then conducted follow-up investigations and reported exactly two weeks later, surprisingly fast [48], that the affected batches had to be suspended immediately due to „a previously unknown impurity that may have genotoxic potential" [2]. Six days later, the result of the tests confirmed that the unknown contamination was NDMA and the responsible authorities in the EU were informed.

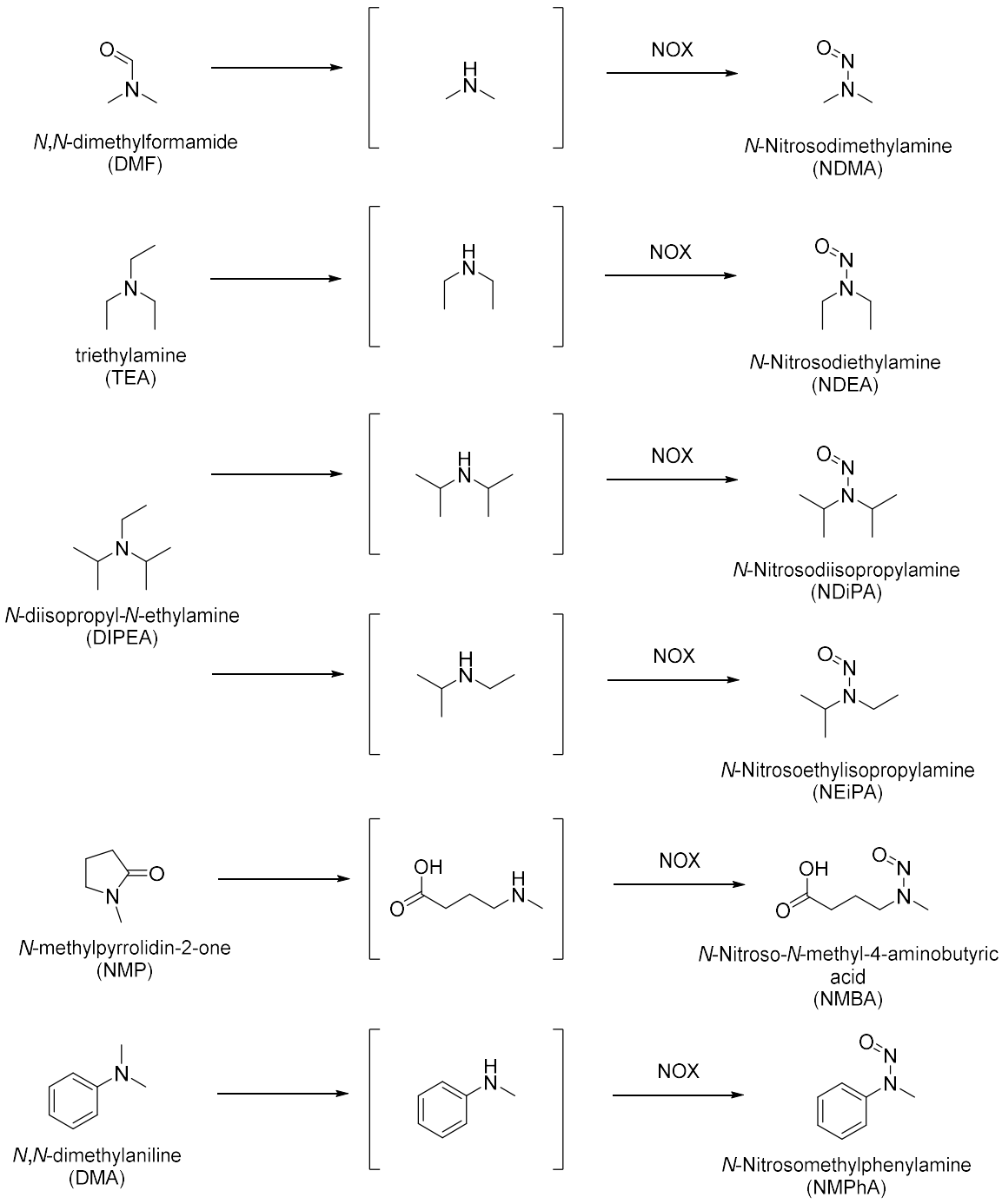

Figure 5: *Formation of different N-nitrosamines from common organic solvents used during synthesis or manufacturing of APIs*

Triggered by this unexpected finding, samples from other sartan manufacturers were examined and further findings were reported within a very short time. Shortly afterwards, NDMA and NDEA were also detected for the first time in finished products containing other sartans (e.g. losartan and irbesartan). This led to an immediate recall of

numerous medicinal products containing sartans [49] and a global review was launched to determine the extent of possible NA contamination in drug substances and drug products [50]. This review was one of the largest risk assessments ever conducted in the pharmaceutical world.

As a result, various new NAs in sartans were detected and reported (NMBA – *N*-nitroso-*N*-methyl-4-aminobutyric acid, NEiPA - *N*-nitrosoethylisopropylamine, NDiPA - *N*-nitrosodiisopropylamine, NDBA - *N*-nitrosodi-*n*-butylamine, NMPhA - *N*-nitrosomethylphenylamine). In addition, NDMA was also detected in other drugs: Pioglitazone-containing drug products were found to be contaminated with small amounts, due to the use of $NaNO_2$ followed by DMF during synthesis. Ranitidine- and nizatidine-containing drug products were contaminated with very high amounts that resulted after intramolecular rearrangement during degradation. Furthermore, a few metformin-containing extended-release tablets were also contaminated, mainly after reaction of NOX from the excipients during granulation [2, 17, 51, 52]. A graphical chronology of the “nitrosamines crisis” is displayed in **Figure 6**.

Regulatory authorities were now faced with the problem that they had to restore drug safety for patients from potentially carcinogenic substances as fast as possible. On the one hand, they had to ensure availability of vital drugs to patients on a permanent basis at the same time. As a result of these findings, the conclusion was drawn that all possible routes of contamination had to be assessed. For this purpose, guidelines with a mandatory multi-step assessment procedure were published (**Figure 7**). On that basis, marketing authorization holders (MAHs) were requested to submit risk assessments for all of their manufacturing processes and approved drug products on the market to this procedure under strict timelines [53, 54].

As step 1, MAHs were prompted to evaluate all of their manufactured APIs and finished drug products for potential NA risks. For „human medicines containing chemically synthesised APIs” this had to be done before 31 March 2021, or before 01 July 2021 “for human medicines containing biological active substances” [55]. In step 2, which had to be completed as fast as possible, all drug products that could have a potential risk (e.g. synthesis, excipients, cross-contamination, degradation) had to be tested representatively ("confirmatory testing") to assess the expected NA presence.

In case of a confirmed NA risk above acceptable interim limits (ILs), the responsible health authorities in the reference member state (RMS) had to be informed immediately so that suitable risk mitigation procedures could be established to push the amount

significantly below the ILs in the future [53, 54]. The (in-)availability of appropriate analytical techniques, of which there were few validated methods for drugs in the early months, was of particular importance during that time. These are essential for understanding the origin, risk control and effective prevention of occurrence.

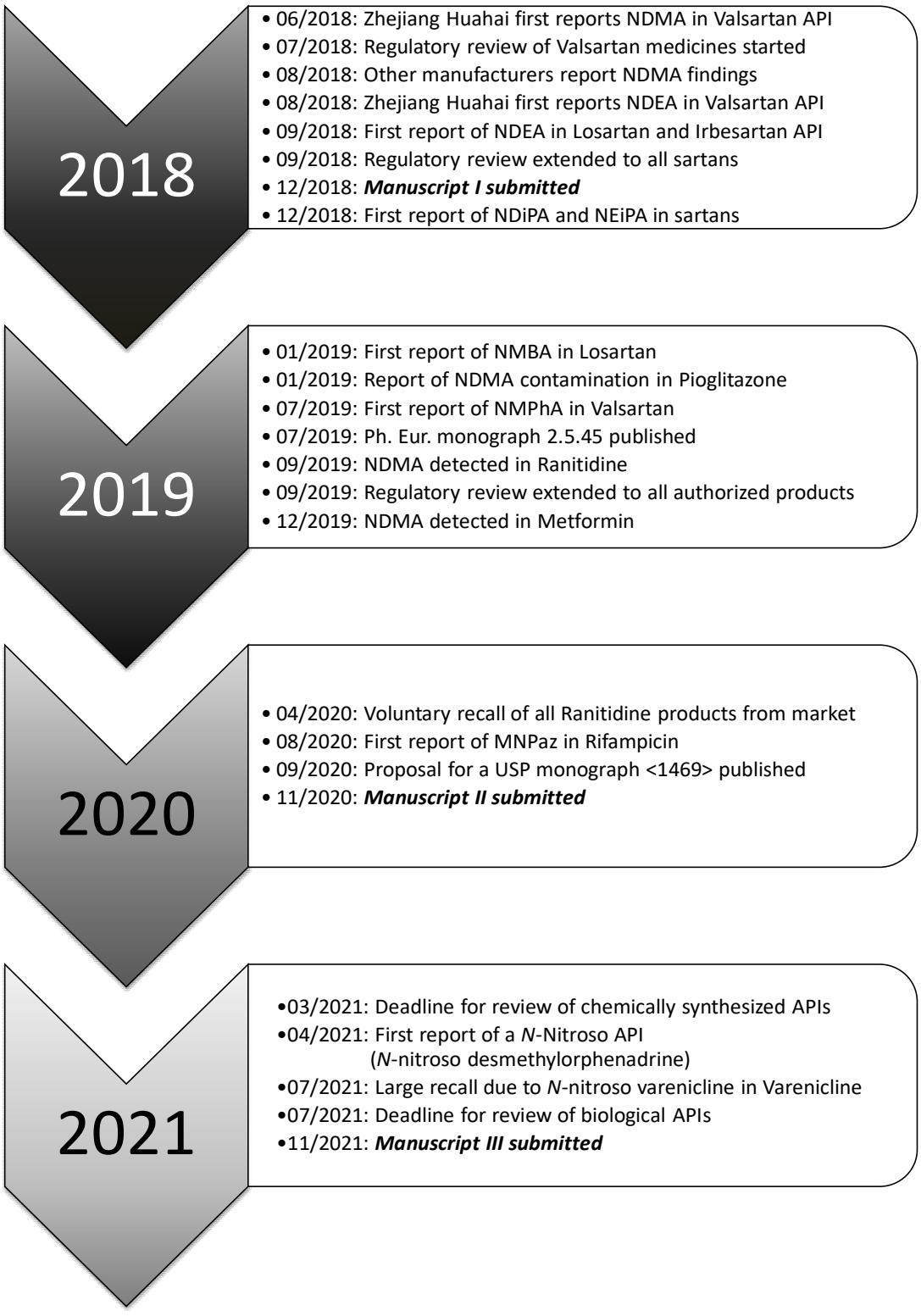

Figure 6: *Chronology of the "nitrosamine crisis" until 2021 [2, 17, 54, 56-60]*

Finally, in step 3, effective risk mitigation strategies should be implemented. This should be done the latest by 26 September 2022 (for "human medicines containing chemically [active substances]") or before 01 July 2023 (for "human medicines containing biologically active substances") in order to decrease the presence of NAs permanently below the established ILs and to develop appropriate control procedures to monitor them [55].

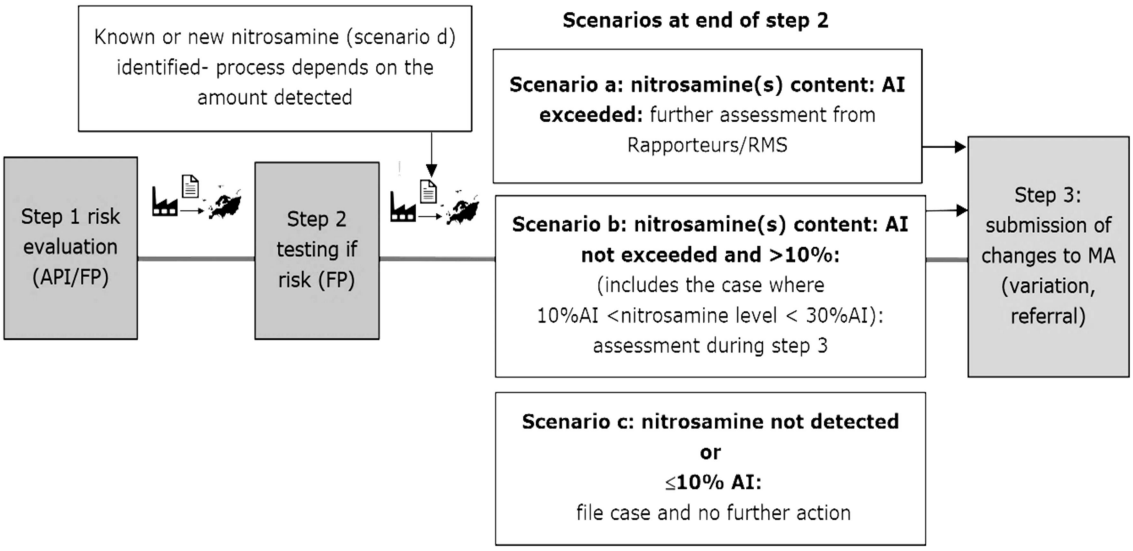

Figure 7: *Call for review to MAHs according to EMA/425645/2020 [55] - original illustration from EMA guideline. AI: Allowable Intake (ng/day); FP: Finished Product; MA: Marketing Authorization; RMS: Reference Member State.*

In the course of this investigation, further NAs were detected, which had not been considered at all before: NDSRIs (nitrosamine drug substance-related impurities). These are reaction products of a NOX with a drug substance or a related impurity. The first NDSRI found (also called "*N*-nitroso API") was *N*-nitrosodesmethylorphenadrine, which was formed by desalkylation of the tertiary amine in orphenadrine, a muscle relaxant, and subsequent nitrosation. The resulting recall [56] did not receive much attention, probably because it was only issued by the Canadian health authority “Health Canada” and because orphenadrine is a rarely used drug substance. Three months later, however, a widely-known substance, varenicline, was tested positive for another NDSRI. This drug for the treatment of nicotine dependence, which is also on the WHO's "model list of essential medicines" [61], attracted higher attention. Varenicline

tablets were contaminated with high amounts of *N*-nitrosovarenilcine, resulting in the worldwide recall of all varenicline-containing drugs, which is still valid to date [57, 62].

Since these NDSRIs became known, other nitrosated APIs (**Figure 8**) have been found and the affected drugs were recalled (*N*-nitrosopropranolol, *N*-nitrosoquinapril). It is currently assumed that "about 20% of all small-molecule drugs consist of vulnerable amines, making them susceptible to nitrosamine formation" [63]. Thus, it is likely that the "nitrosamine crisis" is far from over.

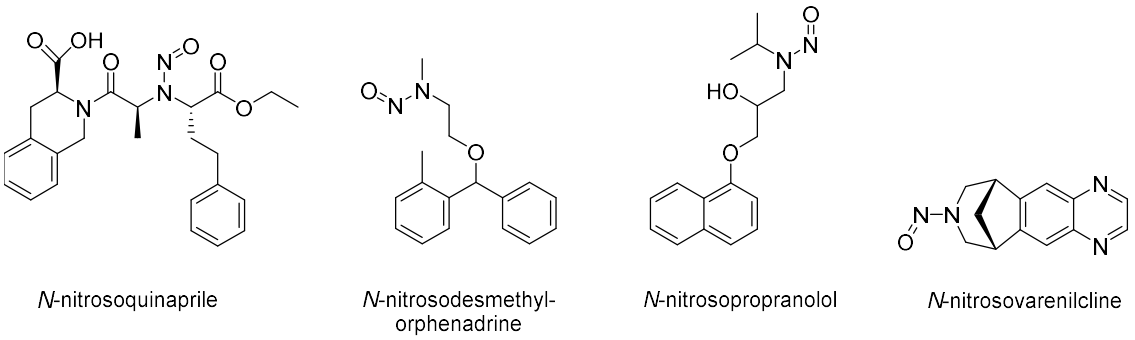

Figure 8: *Reported and recalled NDSRIs. Note that N-nitrosoirbesartan is not displayed here, since it is very likely that the initial finding might be a false-positive, caused by an irbesartan N-oxime [64].*

2.5 Analytical challenges in the detection of nitrosamines

One of the greatest challenges in the determination of NAs is that they can be toxicologically hazardous even in very small quantities. Therefore, analytical techniques must be highly sensitive to detect and quantify NAs even in trace amounts. In addition, the methods used must have high selectivity to separate matrix components, which are present to a much greater extent, so that NA analytes can be detected without interference.

The fact that drug substances are capable of forming NAs or can be contaminated by them is long known from the literature. *N*-Nitrosodimethylamine in particular has already been detected in several drug substances [65]. Nevertheless, before the "nitrosamine crisis" began, not a single monograph from the Ph. Eur. or the USP listed e.g. NDMA or NDEA as a specified impurity.

The regulatory authorities now impose strict group- and substance-specific limits, typically in the single-digit to low double-digit nanograms per day range [53, 54]. This often results in required detection limits in the parts-per-billion range (ppb; ng/g) depending on the maximum daily dose of the drug product. In order to detect these small amounts, tandem mass spectrometry (MS/MS) methods are nowadays almost exclusively used [65, 66]. Chromatographic methods such as gas chromatography (GC) [67, 68] or liquid chromatography (LC) [69, 70] are usually applied to separate the NA analyte from interfering compounds, which may originate from the drug substance or the matrix of the drug product. Alternative detection methods such as GC in combination with a thermal energy analyzer (TEA) [71] or LC combined with a chemiluminescence detector (CLD) [72] can also be utilized. Nevertheless, the latter are rarely used, especially not in GMP-controlled environments, and continue to be the subject of research.

In the early phase of the "nitrosamine crisis", GC-MS methods were widely applied. These were well established from the environmental and food chemistry field, especially for NDMA [65, 73]. The major advantage of GC is that due to the long capillary columns commonly used, very high separation efficiency can be generated. Thus GC methods are usually very selective, but sometimes time intense. In addition, GC benefits from distinct characteristic of NAs, which are very volatile and therefore easy to transfer into the gas phase. However, this is not the case for all NAs, such as

NMBA and NDELA (*N*-nitrosodiethanolamine), which are very polar compounds. Likewise, the novel NDSRIs are also difficult to vaporize and most of them cannot be analyzed by GC without considerable effort. In addition, it must be kept in mind that temperature-labile substances, such as *N*-nitrosodiphenylamine (NDPhA), can easily decompose in the injector and thus may not be accessible to GC [74]. Another problem emerged from reports that ranitidine may be contaminated with NDMA [51, 75]. Since ranitidine is subjected to temperature-induced autodegradation and decomposes in the injector, direct formation of large NDMA amounts in the GC is possible. This resulted in a number of overfindings by GC analysis [76, 77].

The problems described regarding thermos-lability or non-volatility can be circumvented by using LC methods. This also offers the advantage that modern LC methods can be faster, compared to typical GC methods. Liquid chromatography is also well suited for the analysis of NDSRIs, including alkyl, aryl and heterocyclic NAs of different polarity. Additionally, large molecules like proteins or other biopharmaceutical products are directly accessible by means of LC. Since these also have to be analyzed for NAs [54], LC can close this gap. Furthermore, LC usually only needs minimal sample preparation compared to GC, which often requires derivatization of the sample. However, during method development a strong focus must be placed on sufficient selectivity to prevent coelution of an NA analyte with the drug substance or matrix. If the remaining sample ingredients are not removed during sample preparation, they will be transferred directly to the LC column and may disturb the chromatography.

Overall, the analysis of NAs is a difficult task. In order to achieve the highest possible selectivity for a broad range of analyte polarities and their drug matrix, supercritical fluid chromatography (SFC) is increasingly being used in pharmaceutical analysis [78-81]. The exceptional properties of supercritical fluids, such as low viscosity and high diffusivity (like a gas) combined with a high solvating power and density (like a liquid), make them interesting for the use as mobile phases in chromatography [82, 83]. Therefore, SFC is combining many advantages from GC and LC [84, 85]. The most commonly used fluid in SFC is pressurized CO_2 that can make it more environmentally friendly compared to LC [86, 87], especially in preparative scale. The low viscosity and high diffusivity allows higher flow rates without the loss of separation efficiency, resulting in very fast SFC methods [88, 89]. The non-aqueous eluent can prevent on-column hydrolysis, tautomerization and degradation [90]. Moreover, the retention of highly polar substances in typically used reversed-phase LC

is a challenge. SFC on the other hand is able to unify the retention and separation of polar and non-polar substances, without the use of ion-pairing reagents or slow equilibrating normal-phase conditions [80, 86, 91].

Another benefit of SFC is the excellent compatibility with nebulizing and spraying detectors, like MS instruments [92]. As soon as the eluent-modifier mixture enters the MS source, it immediately depressurizes. The transfer of the eluent stream into the gas phase is thus considerably facilitated. This can reduce MS ion suppression compared to LC and also requires less thermal energy for evaporation, preventing in-source fragmentation [78]. Additionally, MS ionization and therefore signal intensities can benefit significantly from SFC applications [93, 94].

At the time when the "nitrosamine crisis" began, new methods were required as soon as possible. According to the quality-by-design (QbD) concept, the selection of the appropriate technology was therefore of fundamental importance. The aim was to exploit all the advantages of this technology. The initiated studies were used to verify the applicability of SFC for trace analysis of NAs and to test its feasibility. As there is no experience with the analysis of NAs by SFC so far, these studies are pioneering.

3 Manuscripts

3.1 Manuscript I: Simultaneous detection of nitrosamines and other sartan-related impurities in active pharmaceutical ingredients by supercritical fluid chromatography

Sebastian Schmidtsdorff, Alexander H. Schmidt

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Abstract: Since July 2018, the pharmacological class of "sartans" has been the subject of considerable media and analytical interest, as it became known that they are contaminated with nitrosamines such as *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA) and *N*-nitrosodiisopropylamine (NDiPA). Previous compendial methods are not able to detect these new contaminants. Using the latest and innovative Quality-by-Design (QbD) approach, it has now been possible to develop an analytical method that enables to investigate sartans, such as valsartan and losartan. Also a large class of different nitrosamines in the ppb range and sartan-related impurities can thus be determined simultaneously in a single analysis using supercritical fluid chromatography (SFC). By using SFC, a broad spectrum of nonpolar and very polar impurities can be separated and analyzed in under 20 min. The analytical method developed is validated for limit testing according to ICH Q2(R1) and fulfills default thresholds of EMA and FDA for testing of drug substances and genotoxic impurities. Additionally, it can also be adapted to other pharmaceuticals that may be contaminated with nitrosamines, since tetrazole synthesis as the underlying cause of nitrosamine contamination is important for a set of other non-sartan drug substances.

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Simultaneous detection of nitrosamines and other sartan-related impurities in active pharmaceutical ingredients by supercritical fluid chromatography

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ABSTRACT

Since July 2018, the pharmacological class of "sartans" has been the subject of considerable media and analytical interest, as it became known that they are contaminated with nitrosamines such as N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA) and N-nitrosodiisopropylamine (NDiPA). Previous compendial methods are not able to detect these new contaminants. Using the latest and innovative Quality-by-Design (QbD) approach, it has now been possible to develop an analytical method that enables to investigate sartans, such as valsartan and losartan. Also a large class of different nitrosamines in the ppb range and sartan-related impurities can thus be determined simultaneously in a single analysis using supercritical fluid chromatography (SFC). By using SFC, a broad spectrum of nonpolar and very polar impurities can be separated and analyzed in under 20 min. The analytical method developed is validated for limit testing according to ICH Q2(R1) and fulfills default thresholds of EMA and FDA for testing of drug substances and genotoxic impurities. Additionally, it can also be adapted to other pharmaceuticals that may be contaminated with nitrosamines, since tetrazole synthesis as the underlying cause of nitrosamine contamination is important for a set of other non-sartan drug substances.

1. Introduction

Since it became known in July 2018 that the drug substance (DS) valsartan from a major Chinese supplier is contaminated with N-nitrosodimethylamine (NDMA), numerous recalls have been carried out all over the world. Due to change in tetrazole synthesis of the DS, in order to increase the synthesis yield and to avoid highly toxic catalysts, nitrosamines (NAs) were formed and their occurrence not considered by the supplier and the regulatory authorities [1,2]. Valsartan is a member of the group of angiotensin II receptor antagonists, also known as ~~s~~artans, for the treatment of hypertension and heart failure. Previous compendial analytical methods for the release of the DS are not able to detect NDMA and so NAs emerged in the DS. Nitrosamines, which are not structurally related to sartans, can be formed during synthesis by the reaction of secondary amine-containing solvents (e.g. triethylamine or dimethylformamide, which can contain diethylamine or dimethylamine in trace amounts) and nitrite in an acidic environment [3]. Therefore, depending on the synthesis, different NAs can be formed. As a result, the FDA and EMA carried out further investigations in cooperation with other local organizations, which revealed that other NAs may also be present (e.g. N-nitrosodiethylamine - NDEA) and that other sartans, such as losartan and irbesartan, are also partially affected [1,4–6]. Abdel-Tawab, et al. [5] from the Central Laboratory of German Pharmacists found NDMA contents between 3.7 μg and 22.0 μg per tablet in random examinations of valsartan products.

Nitrosamines are potent carcinogens that can lead to tumors in nearly all organs. For NDMA and almost all other NAs analyzed in this article, the carcinogenic effect has already been shown in a number of animal studies in rats, mice, hamsters, guinea pigs and rabbits, irrespective of the way of exposure [7–9]. Although most the NDMA levels found in the studied valsartan products were low (in the double-digit ppm range) [2], the naturally daily intake of NAs was not negligible and the FDA and EMA assume that chronic exposure to contaminated valsartan products may lead to one additional cancer case per 5,000–8,000 threatened patients [1,10].

Based on this unsustainable situation, EMA and FDA published test methods (GC/MS) capable of detecting only NDMA in sartans

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and an additional method for the simultaneous detection of NDMA and NDEA by UHPLC-MS/MS [11,12].

Disadvantage of these methods is that they add an additional analytical test to the official pharmacopoeial purity monographs. The aim of this work is therefore to develop a new analytical method under Quality-by-Design (QbD) principles, which is capable of replacing conventional purity methods for the determination of related substances in sartans and simultaneously detecting different classes of NAs in trace amounts, so that the release of the drug substance can be implemented using a single and fast technique.

Despite continued use of the well-established liquid and gas chromatography techniques, supercritical fluid chromatography (SFC) adds a complementary strategy in the toolbox of analytical scientists and covers the application domains of different chromatographic modes as normal phase, reversed phase and hydrophobic interaction chromatography [13].

The unique physical properties of supercritical fluids such as low viscosity and high diffusivity, concurrent with a high solvating power make them very interesting for usage as mobile phases in chromatography and are leading to several advantages of SFC compared with traditional HPLC. The most common and preferred fluid used in SFC is CO_2, as it offers excellent properties. This includes a dipole moment of being zero and thus highly lipophilic properties similar to hexane or heptane and the ability to bring it easily into a supercritical state [13,14]. Analysis of non-polar compounds, such as lipids, fat-soluble vitamins and steroids are easily possible. However, for the elution of polar components, the addition of a polar organic solvent, also called modifier, is possible to prompt elution and allow substantial expansion of the polarity range [13,15].

In our study we investigated the applicability of the wide polarity range of SFC technology for the analysis of NAs and other sartan related impurities in active pharmaceutical ingredients (API) in a single run.

2. Materials and methods

2.1. Quality-by-Design development strategy and risk assessment

The term "QbD" originates from the pharmaceutical development guideline Q8(R2)-2009 of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [16]. The guideline describes the process as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management" - also known as QbD. These predefined objectives and targets were also called "Analytical Target Profile" (ATP) [17]. The ATP of the method to be developed should be that (a) all impurities are separated so that they can be detected individually and (b) the method is sufficiently sensitive for all impurities that internationally recognized pharmaceutical regulations on validation and API release are complied with. Furthermore, (c) profound knowledge of the underlying mechanisms and parameters should be acquired in order to understand and control the chromatographic and spectrometric influencing factors in depth.

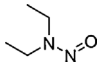
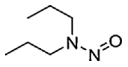
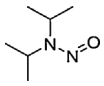
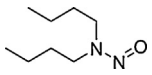
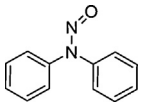
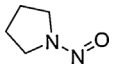
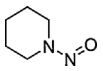
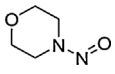
The harmonized tripartite guideline of the ICH for impurities in drug substances Q3A(R2)-2006 [16] requires a reporting threshold of $\leq$ 0.05% for process- and drug-related impurities "*for qualifying those impurities that were not present, or were present at substantially lower levels, in batches of a new drug substance used in safety*", if the daily administred dose is equal or below 2 g of the API. This defined threshold should be the level, where precise quantification is possible. Therefore, the limit of detection (LOD) for the sartan impurities should be at least 0.03% in our study, to achieve the second predefined objective of the ATP (sensitivity for sartan impurities). In addition, the ICH Q3A guideline also insists that "*lower thresholds can be appropriate if the impurity is unusually toxic*", which is the case for the genotoxic NAs [7]. For this reason, the ICH Guideline M7(R1)-2017 [16], which deals with assessment and control of mutagenic impurities in pharmaceuticals, will be applied to NAs. This guideline M7 uses the concept of the "Threshold of Toxicological Concern" (TTC), to estimate tolerable amounts with negligible risk when administered. With this TTC "*for marketed products, acceptable increased cancer risk is set at a theoretically calculated level of approximately one in one hundred thousand. These risk levels represent a small theoretical increase in risk when compared to human overall lifetime incidence of developing any type of cancer*". Therefore, the LOD for the NAs should be equal or lower than the TTC. The U.S. Environmental Protection Agency (EPA) considers "consuming up to 96 ng NDMA/day is [. . .] reasonably safe for human ingestion. It is estimated that over the course of a person's lifetime, consuming this amount of NDMA would result in less than one additional case of cancer for every 100,000 people" [1]. Related to an intake of 100 mg losartan or 320 mg valsartan, which corresponds to a usual dosage for high blood pressure and stroke prophylaxis, this amount of NDMA would be approximately equal to a TTC of 1 ppm for losartan and 0.3 ppm for valsartan. The LOD threshold of ATP point (b) for the NAs (sensitivity for nitrosamines) has to be significantly lower than for sartan related impurities and is thus set to the above mentioned values in our study. In order to demonstrate that the objective of point (b) of the ATP is achieved, the method will be validated by limit testing according to the ICH Q2(R1)-2005 guideline for impurities in DS [16].

As method development according to the QbD concept also requires process understanding & control and risk management, which is expressed by point (c) of the ATP, additional control mechanisms and structures must be established to ensure compliance. The ICH guideline Q8 therefore recommends defining "critical quality attributes" (CQAs) for the development and "critical process parameters" (CPPs), "whose variability [have] an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired quality" [16]. The following CQAs can be defined, which can be used as core parameters for development and optimization: peak resolution (Rs), peak height and symmetry, signal-to-noise and retention. In order to maximize these CQAs, for the SFC-UV-MS/MS method, eight main CPPs will be evaluated. These are the stationary phase, modifier and additives to mobile phase. Additionally, column temperature and gradient slope (and steps) of the mobile phase, type and flow rate of the make-up solvent (for MS/MS coupling) and the mass spectrometric instrument parameters were evaluated. In order to be capable of controlling and understanding all these parameters and their interaction in a multidimensional perspective, the results are evaluated and consolidated using a software-based and statistical approach. The so-called "design-of-experiments" (DoE) form the centerpiece of the development.

2.2. Chemicals and reagents

Certified reference standards of the analytes valsartan, losartan potassium, losartan impurity C, E, F, G & H and the EPA 8270/Appendix IX Nitrosamines Mix were supplied by Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). Additionally, a 5000 μg/ml reference stock solution of NDMA and a 200 μg/ml reference stock solution of NDiPA in methanol (MeOH) were purchased from LGC GmbH (Luckenwalde, Germany) – see Table 1 for the structures of the included NAs. The pharmacopeial reference substances losartan impurity D, "losartan for system suitability" (contains beside losartan also losartan impurity J, K, L and M) and "valsartan for system suitability" (contains beside valsartan also

Table 1
Nitrosamines included in the method development for impurity screening with their corresponding structures and calculated logP values.

Name	Synonym / Abbreviation	Structure	CAS-Nr.	Rel. molecular Mass [M_R]	logP
N-nitrosodimethylamine	NDMA		62-75-9	74	0.10
N-nitrosodiethylamine	NDEA		55-18-5	102	0.80
N-nitrosomethylethylamine	NMEA		10595-95-6	88	0.40
N-nitrosodi-*n*-propylamine	NDnPA		621-64-7	130	1.7
N-nitrosodiisopropylamine	NDiPA		601-77-4	130	1.4
N-nitrosodi-*n*-butylamine	NDBA		924-16-3	158	2.6
N-nitrosodiphenylamine	NDPhA		86-30-6	198	3.4
N-nitrosopyrrolidine	NPyr		930-55-2	100	0.40
N-nitrosopiperidine	NPip		100-75-4	114	0.80
N-nitrosomorpholine	NMor		59-89-2	116	−0.30

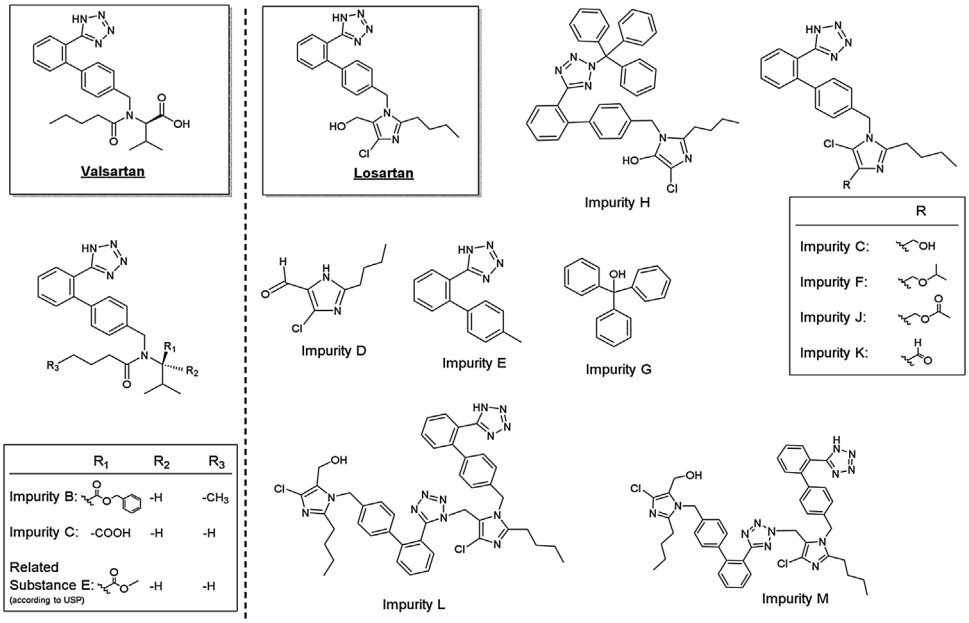


Fig. 1. Valsartan and losartan with their impurities according to Ph. Eur. (USP related Substance E for valsartan was also added).

valsartan impurity C) were acquired from the EDQM (Strasbourg, France). Valsartan impurity B and valsartan related substance E were bought from Toronto Research Chemicals (North York, ON, Canada) and losartan impurity L and M from Phast - Gesellschaft für Pharmazeutische Qualitätsstandards mbH (Homburg, Germany). The structures of all sartans and their impurities from the official Ph. Eur. monographs [18] are displayed in Fig. 1.

For screening experiments HPLC-grade solvents and reagents were used, for method optimization and performance verification these were upgraded to MS-grade purity. All were purchased from VWR International GmbH (Darmstadt, Germany). Carbon dioxide N45 (99.995%) and nitrogen N50 (99.999%) were obtained from Air Liquide Deutschland GmbH (Düsseldorf, Germany) and Argon 5.3 (99.9993%) from Linde AG (Munich, Germany).

Table 2
: Optimized SRMs with corresponding cone voltages and collision energies in ESI + mode for all NAs.

Synonym / Abbreviation	SRM 1 (Quantifier) / SRM 2 (Qualifier)			Cone voltage	Collision energy	Dwell time (sec)
NDMA	75	→	43	36	14	0.05
	75	→	58	36	10	0.05
NDEA	103	→	47	34	8	0.05
	103	→	75	34	10	0.05
NMEA	89	→	61	36	10	0.05
	89	→	47	36	8	0.05
NDPA	131	→	89	26	10	0.05
	131	→	43	26	12	0.05
NDBA	159	→	103	28	10	0.05
	159	→	57	28	14	0.05
NDPhA	199	→	66	26	26	0.05
	199	→	169	26	12	0.05
NPyr	101	→	55	34	14	0.05
	101	→	59	34	16	0.05
NPip	115	→	69	34	14	0.05
	115	→	55	34	24	0.05
NMor	117	→	45	30	14	0.05
	117	→	57	30	14	0.05

2.3. Instrumentation and software

Chromatographic analysis was performed using an Acquity UPC^2 SFC system (Waters GmbH, Eschborn, Germany) equipped with an Acquity UPC^2 column manager with active eluent preheaters for up to 4 simultaneous columns, an Acquity UPC^2 PDA detector and an Acquity TQD (Triple Quadrupole Mass Spectrometer). To interface the SFC to the MS a post-column pre-convergence manager splitter (fixed leak) was coupled with a Waters 515 HPLC pump, which was used as a make-up pump to enhance mass transfer to the MS and ionization in the source. For system control, data acquisition and data processing the Empower 3 software (Feature Release 4, Service Release 2, Hotfix 2) from Waters GmbH (Eschborn, Germany)was used.

Instrumentation was operated fully qualified according to the 4Q model of the USP <1058> [19] and under GMP-regulated laboratory environment together with the qualified and validated Empower software.

For column screening, the following columns were chosen: Viridis BEH, Viridis BEH 2-EP, Torus DIOL, Torus 2-PIC, Torus DEA, Torus 1-AA, Viridis CSH Fluorophenyl and Viridis HSS C18 SB (all 100 x 3.0 mm; 1.8 μm – Waters GmbH, Eschborn, Germany).

Fusion QbD software (Version 9.8.1.199 - S-Matrix Corporation, Eureka, California, USA) was utilized for multivariate data analysis and method screening, by full automatized DoE planning and construction. Chemical structures and logP values were generated by ChemDraw Professional (Version 16.0 - PerkinElmer Informatics, Inc., Waltham, Massachusetts, USA).

2.4. Chromatographic conditions and screening procedure

In a first attempt all columns were screened with a generic gradient of 1%B to 40%B in 20 min, followed by a 5 min reequilibration step to the initial conditions (A: CO_2; B: MeOH) at a flow rate of 1.5 ml/min at 25 °C and 50 °C column temperature. Valsartan and losartan were injected as a standard solution containing 0.5 mg/ml each and NDMA as a 10 μg/ml standard solution. Peak shape, retention behavior and peak resolution was analyzed and the four best columns were chosen. Then the nitrosamine mixture was injected as a 10 μg/ml standard solution - peak shape, resolution and retention behavior on the four chosen columns were screened again.

For modifier screening two HSS C18 SB columns were connected in series and the flow rate was adjusted to 1.2 ml/min at 1800 psi back pressure. An initial isocratic step of 2 min was added to the gradient and the gradient was reduced to 30%B in 7 min to prevent excessive back pressure. Four modifiers (B) were screened: MeOH, ethanol (EtOH), isopropanol (IpOH) and acetonitrile (ACN). These were systematically screened at 30 °C and 50 °C column temperature and 0.0, 0.5 and 1.0 initial %B of the isocratic step.

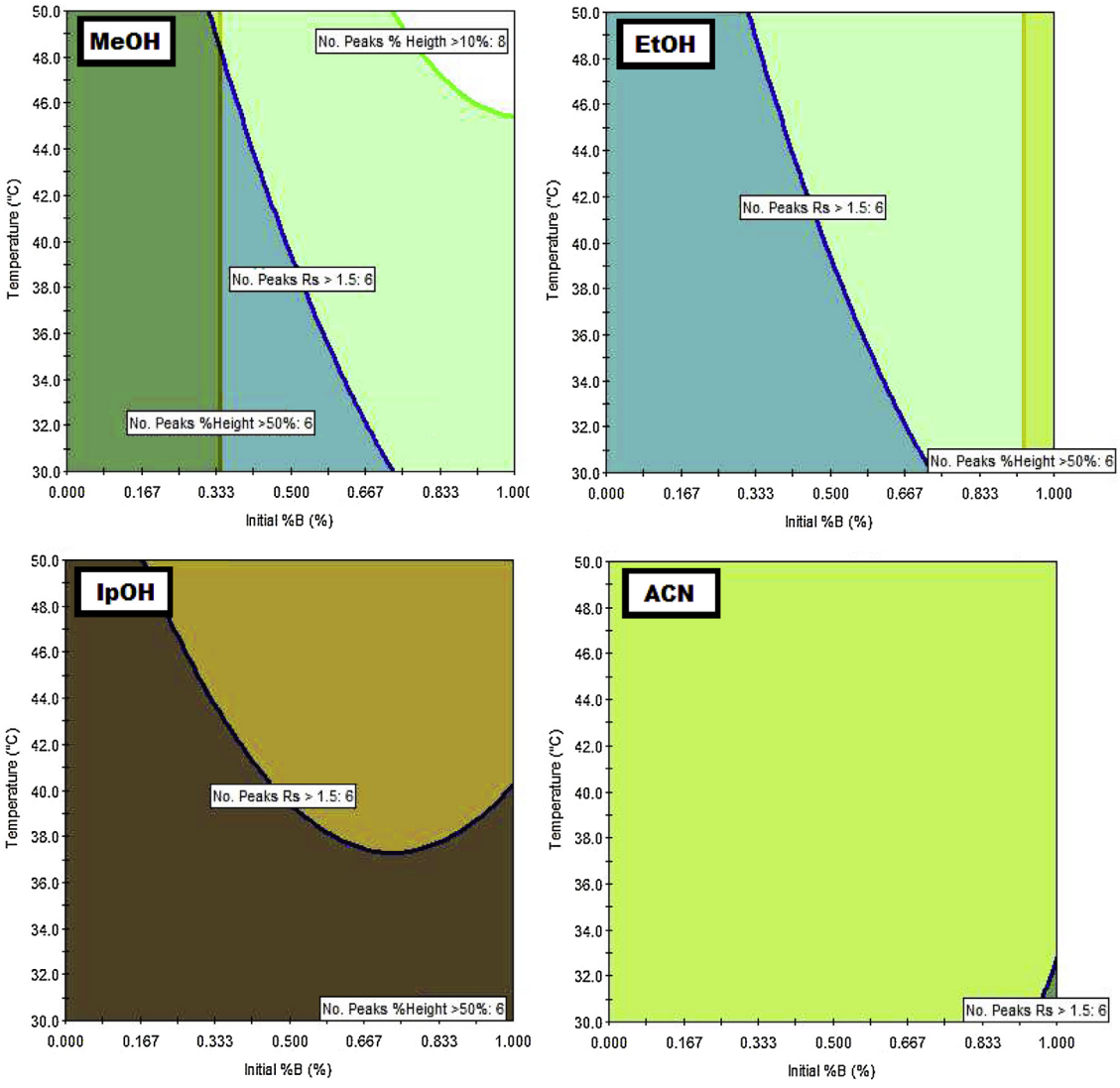
Subsequently, the influence of additives in the mobile phase and make-up solvent were investigated. The following additives were added to MeOH: formic acid (FA), acetic acid (AcOH), trifluoroacetic acid (TFA); also 30% ammonia solution (NH_3) and triethylamine (TEA) for mobile phase screening and 10 mM ammonium formate (NH_4FA) for make-up solvent screening, each 0.1% (v/v). Valsartan and losartan were spiked at the 0.5% level with all their impurities and injected each as 10 mg/ml solutions in MeOH. Samples were prepared by weighting into volumetric flasks and stirring for 5 min.

The final chromatographic method is: CO_2 as eluent A and methanol with 0.1% TFA as eluent B, starting at 2% B for 2 min, then rapidly increased linearly to 10% B within one minute with a 3 min isocratic step, followed by a slow increase to 30% B in further 4.5 min with a 1.5 min hold time and a reequilibration to 2% B for 5 min, resulting in a total run time of 17 min on two HSS C18 SB columns (each 100 x 3.0 mm; 1.8 μm) at 55 °C column temperature and a flow rate of 1.2 ml/min. The injection volume is 1 μl of a 10.0 mg/ml API sample directly dissolved in MeOH by weighting in a corresponding volumetric flask and stirring for 5 min.

2.5. Spectroscopic and spectrometric conditions

Chromatograms were recorded from 190 to 400 nm at a scan rate of 10 Hz with multiwavelength photodiode array detector and evaluated at 230 nm. For peak tracking and high sensitive detection of the NAs, the MS operated in positive electrospray ionization (ESI+) mode with selective reaction monitoring (SRM) for targeted quantitative mass analysis.

MS parameters were tuned by direct infusion of a 1 μg/ml standard solution of all NAs in MeOH (containing also 0.1% formic acid). Optimized parameters are: capillary voltage 3.80 kV, extractor voltage 3 V, source temperature 120 °C, desolvation temperature 250 °C, desolvation gas flow 500 L/h, collision gas flow 0.50 ml/min and span width 0.000. SRMs and optimal cone voltages and collision energies were displayed in Table 2.



Response Variable Goals

Name	Goal	Lower Bound	Upper Bound	Color
Peaks detected	Maximize	9.00	---	Red
No. Peaks Rs > 1.5	Maximize	6.00	---	Blue
No. Peaks %Height >10%	Maximize	8.00	---	Green
No. Peaks %Height >50%	Maximize	6.00	---	Orange

Fig. 2. Ďesign Spaceöf modifier screening for NAs generated by Fusion QbD software – white areas display a positive result for defined goals. Colored areas indicate that at least one response is not achieved – mixed colors are possible, when more than one goal is not achieved. Data generated on two connected HSS C18 SB columns with four modifiers: MeOH (top left), EtOH (top right), IpOH (bottom left) and ACN (bottom right) systematically screened at 30 °C–50 °C column temperature and 0.0, to 1.0%B (%modifier in CO_2).

3. Results and discussion

3.1. Screening

Of the eight screened SFC columns, covering a broad chemical spectrum from normal-phase (NP) to reversed-phase (RP) chromatography, only the Fluorophenyl, C18, Diol and BEH (pure silica) columns showed sufficient separation and retention behavior for losartan, valsartan and NDMA. These columns were selected and tested in order to evaluate, which column has the highest retention and separation power for the NAs, since NDMA showed only weak interactions with the stationary phase in previous screening experiments. Only the HSS C18 SB showed satisfying retention behavior for the sartans, all nine NAs (NDiPA not included at this point – see chapter 3.3 for more details) and proved to be significantly superior to all other columns, so these were immediately discarded for further experiments. This result is congruent to the LC methods usually used for separation and detection of NAs in cosmetics [20], water and biosolids [21,22], rubber [23] or for urine analysis [24], where convention RP-columns (C8 or C18) were used.

In order to further increase the separation performance for the subsequent modifier screening, two columns were connected in series. Modifiers were systemically screened with the DoE support of Fusion QbD software. This approach allows collecting knowledge about the influencing chromatographic parameters and their effects. The data was used to generate a visual “Design Space”

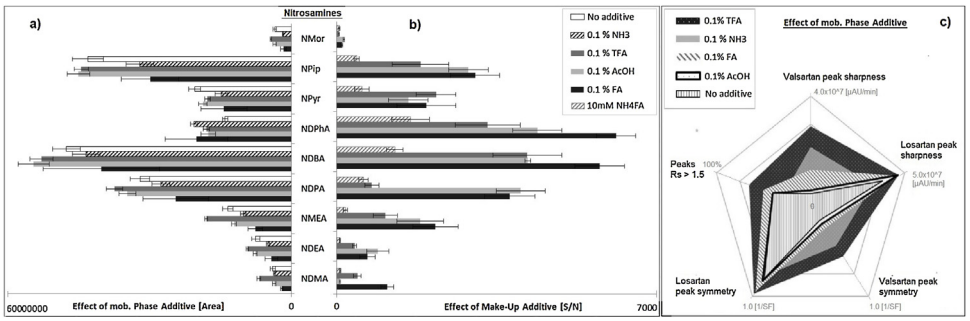


Fig. 3. [a] Effect of mobile phase additives on NA peak areas (left), [b] effect of make-up solvent additives on NA peak S/N (right) and [c] effect of mobile phase additives on sartans (peak symmetry displayed as the reciprocal of the symmetry factor and peak sharpness as peak height/peak width).

("multidimensional combination and interaction of input variables [. . .] and process parameters that have been demonstrated to provide assurance of quality" – ICH Q8), where optimal separation and peak shape is displayed. Fig. 2 shows the result of the modifier screening. The visualized design space is displayed by a white area, were the predefined CQAs are used to discriminate values of each CPP to maximize outcome and demonstrate the space with the highest efficiency for each CQA. By overlaying each CQA discrimination space (displayed by a colored area), only the design space persists uncolored.

Fusion QbD software calculates statistically, without the use of chromatographic terms and laws, the effect on the response goals in a multi-dimensional space. For modifier screening, the first step was therefore to examine only whether all NAs were detectable (response goal #1 - peaks detected), whether a good separation had already been achieved for some of the substances (response goal #2 - resolution) and whether most peaks showed a certain relative minimum intensity of their signals (response goal #3 and #4 - %Height). Response goal #3 and #4 were necessary because the weak modifiers (especially IpOH and ACN) tend to make peaks very broad and flat, so that a relative discriminating factor was necessary from the outset in order not to decrease signal intensity and loose sensitivity. The absolute peak height would not have been a suitable indicator, since all signals clearly differ in their peak height. Therefore, a relative factor was introduced to normalize all NAs.

It can be seen that only MeOH as a modifier at elevated column temperatures is able to elute all NAs from the column (peaks detected), to form them into sharp peaks (%peak height) and thus to ensure sufficient separation (Rs > 1.5). Nevertheless, the white corner section in the MeOH knowledge space of the DoE, which indicates that all response goals have been achieved, shows that the initial conditions were not ideal. Even higher temperatures and more %B (modifier) are needed, to achieve maximum separation and signal intensity. In contrast, EtOH led to a significant peak broadening and with IpOH and ACN it was partially no longer possible to detect them at all. IpOH shows an entire brown design region (mixed color of green, orange and red), which indicates that not every NA was detectable and that the detected signals were unintensive. The same is true for ACN, where all NAs were detected but highly decreased in signal intensity.

Since almost all NAs eluted during the initial isocratic step of the gradient, a very high degree of flexibility remained for the screening of the sartans. For this purpose different mobile phase additives were tested to evaluate their influence on NA separation and ionization (in order not to lose sensitivity) and sartan peak shape and retention. Figure 3a shows the influence on the peak areas for all NAs. Especially the low-responders NDMA, NDEA and NMor a major impact of the eluent additive on their sensitivity was observed.TFA in the eluent showed the best overall outcome for NAs. In parallel, only TFA was also able to decrease tailing and enhance separation for the sartans, without changes in selectivity (see Fig. 3c). AcOH showed no positive influence and formic acid only slightly on sartan separation und peak shape. Ammonia lead to an increase in peak sharpness and retention, but also caused peaks to coelute, since selectivity changed.

In contrast, the addition of TFA to the make-up solvent, as well as ammonium formate, was found to worsen ionization significantly. FA and AcOH showed comparable properties, but FA proved to be slightly superior, especially for NDMA, where the background noise increased drastically (see Fig. 3b) with AcOH.

Ammonia and TFA are known to be a strong ionization suppressor in mass spectrometry, which explains, why addition in the make-up solvent decrease the S/N values, since the entire flow enters the MS source. Addition of small amounts of TFA to the modifier of the eluent does not have a negative effect on ionization, since only small amounts of the flow are splitted to the MS, but substantially higher volumes also had a negative effect (displayed for the two low-responders NDMA and NDEA in Fig. 4). The mechanisms, which cause TFA to improve the chromatographic separation and peak sharpening, are still unclear, but it is common in SFC to add acidic additives when organic acids are present in the analyte structure - which is the case for the sartans (tetrazole and carboxylic acid derivatives). Suspected mechanisms are ion pairing, suppression of analyte charge and covering of adsorption sites on the surface of the stationary phase [13].

3.2. Optimization

The method screening showed that only MeOH as eluent with an addition of TFA was able to provide sufficient separation and of the NAs and sartans. FA showed the best results as make-up additive. Furthermore, it could be derived from the QbD screening that high temperatures are necessary for separation. Nevertheless, it turned out that the percentage of the modifier in the start gradient was not yet optimized, since only a small corner section of the knowledge space provided successful results (see Fig. 2). On this basis, the amount of the modifier in the start gradient (%B, from 1.0% to 2.5%), the column temperature (from 40 °C to 60 °C) and the concentration of TFA in the modifier (from 0.1% to 0.4% TFA) were further optimized to generate a robust and highly efficient working point for the NAs. An optimization with the goal of sufficient separation and detectability for the sartans and their impurities has not yet been carried out, because the NAs represented the major challenge. Their separation and detection in trace amounts was therefore the first step.

On the basis of Fig. 4, it can be deduced that a higher concentration of TFA does not lead to an improvement of the separation, but has a strongly negative effect on the peak height and thus on the sensitivity. Optimum separation and sensitivity was only achieved at 55 °C column temperature and 2.0%B in the start gradient with 0.1% TFA as additive. Slightly smaller concentrations of TFA prob-

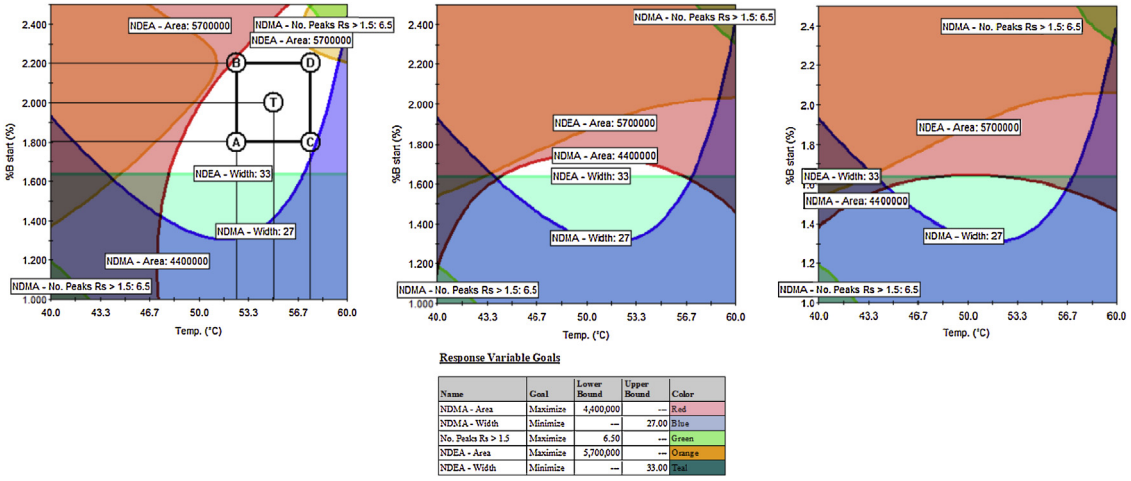


Fig. 4. Analytical method "Design Space" after QbD eluent optimization for NAs - a robust and satisfying separation is only achieved at 0.1% TFA (left); 0.25% (middle) and 0.4% (right) TFA in the mobile phase negatively impacted the peak areas of NDMA and NDEA due to ion suppression.

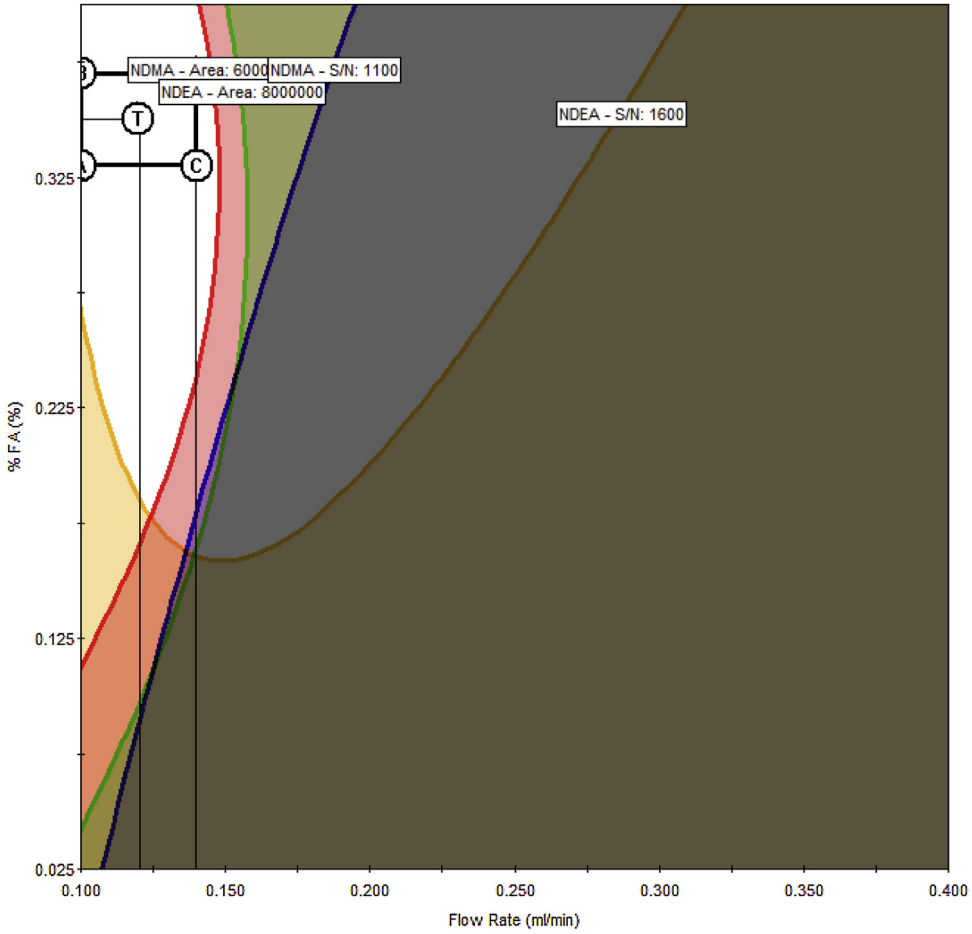


Fig. 5. Optimization of make-up flow rate and percentage of formic acid in MeOH – the best results were obtained at 0.12 ml/min and 0.35% formic acid.

ably would have improved ionization, but higher amounts were necessary to separate the sartans.

The concentration of formic acid (from 0.025% to 0.4%) in the make-up solvent and the flow rate (from 0.1 ml/min to 0.4 ml/min) of the make-up pump were also optimized. The best overall results were obtained at 0.35% formic acid at 0.12 ml/min (see Fig. 5).

After the NAs had been separated with the highest possible sensitivity and selectivity, the gradient for the elution was optimized for the separation of all sartans and their impurities and a step gradient was generated from the linear gradient. Optimum resolution was achieved at a flow of 1.2 ml/min with CO_2 as mobile phase eluent A and MeOH with 0.1% TFA as eluent B (2% B for 2 min, linear to 10% B in 1 min, 3 min hold, linear to 30% B in 4.5 min, 1.5 min hold) on two HSS C18 SB columns (2 × 100 x 3.0 mm; 1.8 μm) at 55 °C. Fig. 6 shows the resulting chromatograms for all NAs and sartans (the final method is described in detail in method chap-

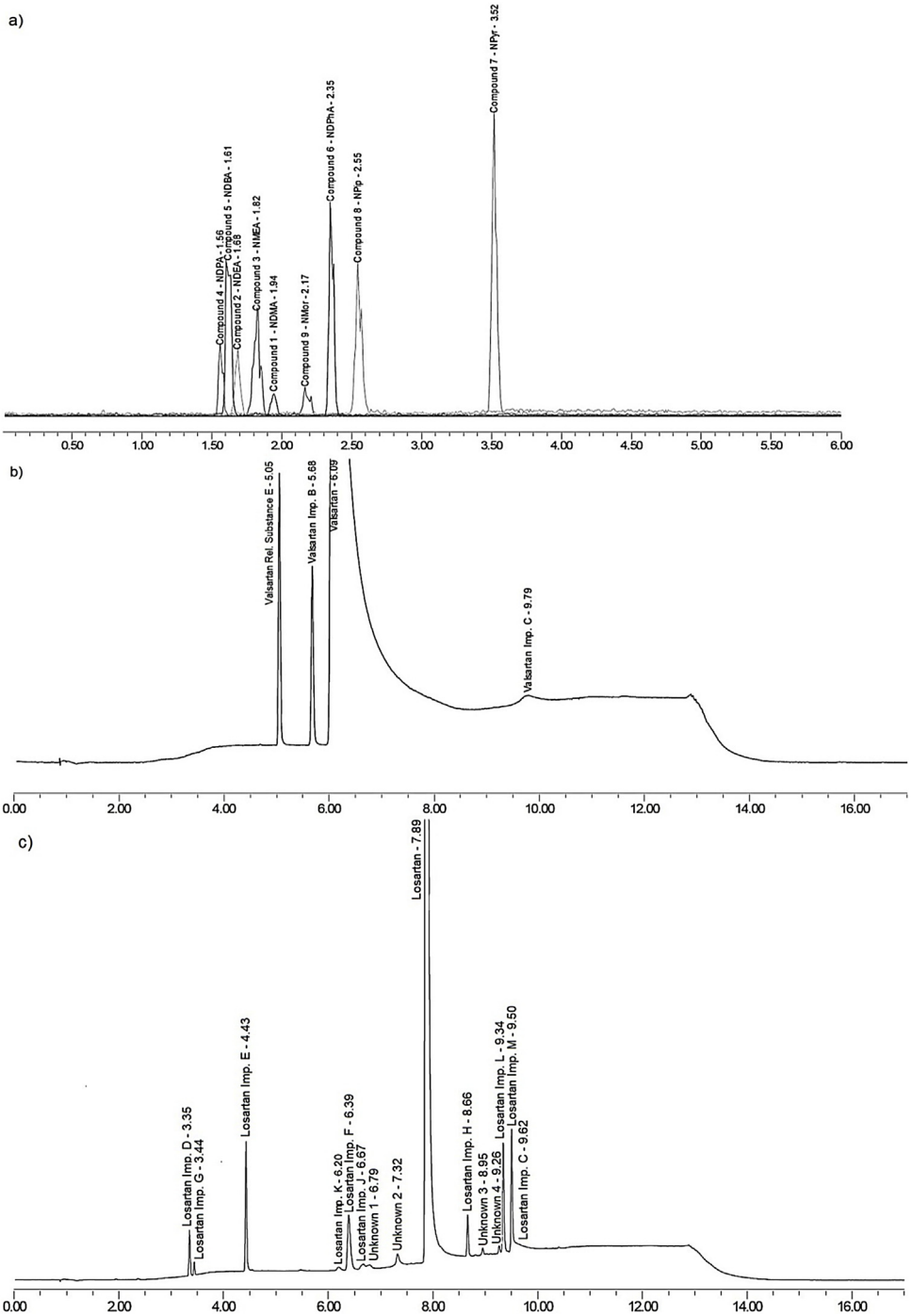


Fig. 6. Representative chromatograms of (a) the nitrosamine mixture (overlay of all 8 SRM quantifier channels – note: NDiPA and NDnPA only displayed as "NDPA", due to same retention time), (b) valsartan & (c) losartan APIs (at 230 nm detection wavelength), each spiked with their specified and unspecified sartan impurities at the 0.5% level (except for valsartan impurity C and losartan impurity C, J & K, which were spiked at the 0.05% level) - sample concentration: 10 mg/ml.

ter under section 2.4). NAs eluted in their reverse logP order (from non-polar to polar), since the elution strength is orthogonal in SFC, compared to RP liquid chromatography. This effect is inverted above four covalently bonded carbon atoms (which is the case for NDBA, NDPhA, NPip and NPyr), as from this chain length the Van der Waals force increases interaction relevance with the C18 phase of the column, resulting in a logP dependent elution order from polar to non-polar, as common in RP chromatography [25].

3.3. Method validation and application

After the successful method development, where point (a) selectivity and (c) profound knowledge of the ATP were applied, the procedure has been validated according to the ICH Q2(R1) guideline for impurities in DS [16] by limit testing in order to also demonstrate point (b) of the ATP, the sensitivity. LODs were compared with the previously established targets and evaluated with respect to ICH Q3A and M7.

The detection limits were determined by deriving the signal-to-noise ratios from the measured linearity of a six-fold injection at

Table 3
Validation results of limit testing according to ICH Q2(R1) – note that the ICH Q3A(R2) demands a reporting threshold for impurities in drug substances to be ≤ 0.05% (for a daily intake of ≤2 g drug substance) and the ICH M7(R1) for genotoxic impurities (by adoption of the TTC concept) levels of ≤ 1 ppm.

Name	Limit of detection			Selectivity
	[ng/ml]	Per 320 mg valsartan	Per 100 mg losartan	(Separation)
NDMA	4.55	0.14 ppm	0.46 ppm	√
NDEA	1.58	0.05 ppm	0.16 ppm	√
NMEA	1.81	0.06 ppm	0.18 ppm	√
NDPA	0.24	0.01 ppm	0.02 ppm	[√]
NDBA	0.34	0.01 ppm	0.03 ppm	√
NDPhA	0.22	0.01 ppm	0.02 ppm	√
NPyr	3.71	0.12 ppm	0.37 ppm	√
NPip	2.26	0.07 ppm	0.23 ppm	√
NMor	4.20	0.13 ppm	0.42 ppm	√
valsartan impurities	≤ 0.03 %			√
losartan impurities	≤ 0.03 %			√

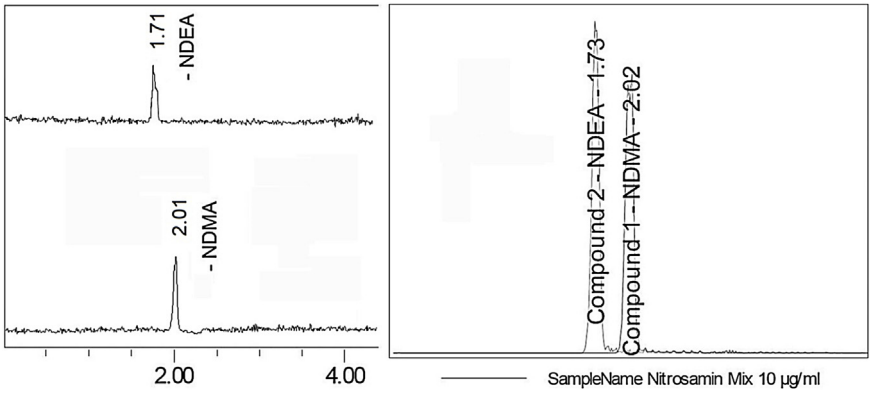


Fig. 7. Detected nitrosamines in losartan API (left) and nitrosamine standard mix for peak verification.

ten levels for each substance. The selectivity could also be shown by this experiment. Table 3 shows the LODs for all substances and displays that all criteria for successful limit testing are met and the ATP is fulfilled. The method developed far exceeds the ATP for all the studied NAs and thus achieved sufficient sensitivity, so that critical quantities can be detected in due time.

Only NDPA and NDBA are slightly coeluting, which does not interfere with its mass spectrometric determination, since this is performed by SRM acquisition, and therefore selectivity is still achieved. Since all NAs are slightly UV-active and can also be determined using PDA detection in higher quantities, this was also tested. It turned out that detection was only possible down to 100 ppm, which is clearly inferior to detection limits achieved by targeted MS/MS.

After finishing the method development it was published that another nitrosamine was detected in a API of a mexican manufacturer, which was not part of the original screening: Nnitrosodiisopropylamine (NDiPA) [26,27]. NDiPA was also subsequently integrated and validated. It turned out that a separation of NDiPA and N-nitrosodi-*n*-propylamine (NDnPA) was not possible, due to complete coelution. Individually evaluated, comparable values for the LODs were obtained for NDiPA and NDnPA. Therefore, both substances are combined in Fig. 6, Tables 2 and 3 as "NDPA" (Nnitrosodipropylamine). Based on the synthesis [3], it appears unlikely, that NDiPA and NDnPA will occur simultaneously in an API batch and the toxicological risk assessment for humans should be the same for both NAs. Therefore, and due to the fact that NDiPA was subsequently incorporated and not part of the original ATP, the coelution is acceptable.

After validation of the method for limit testing, three batches of valsartan and losartan were tested. It turned out that both NDMA and NDEA could in fact be detected in one batch of losartan. Fig. 7 clearly shows traces of the two NAs. NDEA was detected approximately at the LOD and NDMA twice above the LOD. By extrapolation of the detected amounts of NDMA and NDEA in the investigated losartan batch, a corresponding intake of about 90 ng NDMA and 15 ng NDEA per day can be estimated, but it should be noted that the developed SFC-UV-MS/MS method is not validated for quantification. Nevertheless, according to the most recent FDA interim limits, these values can be considered uncritical. FDA and EMA consider NDMA acceptable up to 96 ng per day and NDEA acceptable up to 26.5 ng per day [1]. These limits, which are slightly below the predefined acceptance criteria for LODs in the ATP, can also be achieved by the SFC method.

The values found in our study are therefore below the TTC and have to be considered uncritical. In contrast, in a random sample investigation, values of up to 20 µg NDMA per tablet (thus 200x more) were found in valsartan products by the FDA [28]. This illustrates, why there is an urgent need to develop new sensitive and effective methods, to which we want to contribute with this work.

4. Conclusion and outlook

Through our study, we were able to show that it is possible to incorporate state-of-the-art analytical techniques for the purity analysis of sartans. By using a systematic QbD development approach, contaminants from related API compounds and potentially carcinogenic NAs can be separated and detected simultaneously. This results in a highly sensitive method, which can detect NAs in the picogram to femtogram range on column. The sensitivity of the developed method is comparable to the published LC- and GC–MS/MS methods by FDA and EMA [11,12,29], but outperforms them in terms of speed and is still able to analyze the API related impurities in a single run at the same time.

Only GC headspace methods are superior due to their almost unlimited sample concentration, but are only able to detect the volatile nitrosamines and are also not able to examine sartans.

The developed method is the first approach utilizing SFC to analyze NAs and shows the high potential of this technique. New investigations by the FDA have shown that in addition to the aforementioned NAs, other previously unexpected NAs such as N-Nitroso-*n*-methyl-4-aminobutyric acid (NMBA) could also be detected in the API [1]. This shows that the current tetrazole synthesis process still involves major risks and should be closely monitored analytically. Parr and Joseph [30] have shown in their overview that not only tetrazole-containing sartans can be contaminated with NAs, but also a variety of other tetrazole drugs that might be formed by the same synthesis process than the sartans. Since the developed SFC method separates the NAs during the isocratic step at the beginning, it is also possible to adjust the gradient so that the method can be applied to other DSs, which is another great advantage.

In a next step, we will now try to investigate other non-sartans for NAs and modify another detector that far exceeds the selectivity of the mass spectrometer, to detect NAs in even smaller amounts and prove the advantages of SFC technology for pharmaceutical quality control. Additionally, further NAs will be integrated to generate a complex and comprehensive group monograph for nitrosamines.

Acknowledgments

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3.2 Manuscript II: Analytical lifecycle management for comprehensive and universal nitrosamine analysis in various pharmaceutical formulations by supercritical fluid chromatography

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Abstract: Since the detection of nitrosamines (NA) in valsartan pharmaceuticals, over two years have passed. At present, the occurrence of NAs can be limited to a few drug substances and drug products, but it is already becoming apparent that the issue appears to be much bigger than initially thought. The impact on the global pharmaceutical market has been tremendous and the problem can be attributed mainly to uncritically adopted approval changes and the lack of suitable, modern analytical methods to detect those impurities in time.

We hereby demonstrate how lifecycle management (LCM) can be used to develop and improve suitable and universal analytical methods within short time. The resulting SFC-MS/MS method is intended for a universal nitrosamine investigation in drug substances and drug products. Successful NA analysis was demonstrated for seven sartans, metformin, pioglitazone and ranitidine. Additionally, combination drug products, containing also amlodipine, hydrochlorothiazide, vildagliptin and sitagliptin, were analyzed successfully. The method achieved separation of 16 NAs in 4 min with a total run time of 11.5 min, utilizing a Supel Carbon porous graphitic carbon (PGC) column. Carbon dioxide together with 0.1% TFA in methanol as modifier were used as eluents and 0.35% formic acid in methanol as make-up solvent for mass spectrometric NA detection. By implementing LCM in this case study, development time was reduced and knowledge was implemented fast. At the same time, a high adaptability of this "vital" method was achieved, which makes it possible to implement the constantly changing regulatory requirements within the shortest possible time. Supplemental development data, according to the ICH guidelines Q8, Q12 and the proposed Q14 are

disclosed, demonstrating the scientific Quality-by-Design (QbD) development approach, the “fitness for use” and the robustness of the analytical procedure.

This method contributes to the still ongoing risk assessment process of the pharmaceutical industry and the regulatory agencies, in order to understand root causes of NA formation, maintain the drug supply and prevent drug shortage.

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Analytical lifecycle management for comprehensive and universal nitrosamine analysis in various pharmaceutical formulations by supercritical fluid chromatography

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ABSTRACT

Since the detection of nitrosamines (NA) in valsartan pharmaceuticals, over two years have passed. At present, the occurrence of NAs can be limited to a few drug substances and drug products, but it is already becoming apparent that the issue appears to be much bigger than initially thought. The impact on the global pharmaceutical market has been tremendous and the problem can be attributed mainly to uncritically adopted approval changes and the lack of suitable, modern analytical methods to detect those impurities in time.

We hereby demonstrate how lifecycle management (LCM) can be used to develop and improve suitable and universal analytical methods within short time. The resulting SFC-MS/MS method is intended for a universal nitrosamine investigation in drug substances and drug products. Successful NA analysis was demonstrated for seven sartans, metformin, pioglitazone and ranitidine. Additionally, combination drug products, containing also amlodipine, hydrochlorothiazide, vildagliptin and sitagliptin, were analyzed successfully. The method achieved separation of 16 NAs in 4 min with a total run time of 11.5 min, utilizing a Supel Carbon porous graphitic carbon (PGC) column. Carbon dioxide together with 0.1% TFA in methanol as modifier were used as eluents and 0.35% formic acid in methanol as make-up solvent for mass spectrometric NA detection. By implementing LCM in this case study, development time was reduced and knowledge was implemented fast. At the same time, a high adaptability of this "vital" method was achieved, which makes it possible to implement the constantly changing regulatory requirements within the shortest possible time. Supplemental development data, according to the ICH guidelines Q8, Q12 and the proposed Q14 are disclosed, demonstrating the scientific Quality-by-Design (QbD) development approach, the "fitness for use" and the robustness of the analytical procedure.

This method contributes to the still ongoing risk assessment process of the pharmaceutical industry and the regulatory agencies, in order to understand root causes of NA formation, maintain the drug supply and prevent drug shortage.

1. Introduction

In July 2018 regulatory agencies around the world were informed that the widely distributed drug substance (DS) valsartan, a common angiotensin II receptor blocker (ARB – also called "sartan"), may be contaminated with the carcinogenic substance N-nitrosodimethylamine (NDMA). It occurred unexpectedly after a change in the synthesis route of a Chinese supplier, which was submitted and approved by the European Medicines Agency (EMA) and United States (U.S.) Food and Drug Administration (FDA) several years ago [1]. This finding caused an immediate review of all production chains for valsartan, in order to find out whether other manufacturers are also affected. Within weeks, more valsartan producing sites with contaminated drug substances were identified and due to this issue, press releases, import alerts and withdrawals or even suspensions of compliance certificates were announced [2,3].

Nitrosamines are known and well-studied environmental, mutagenic carcinogens, which were defined as a "cohort of concern" by the ICH guideline M7 for the "Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to

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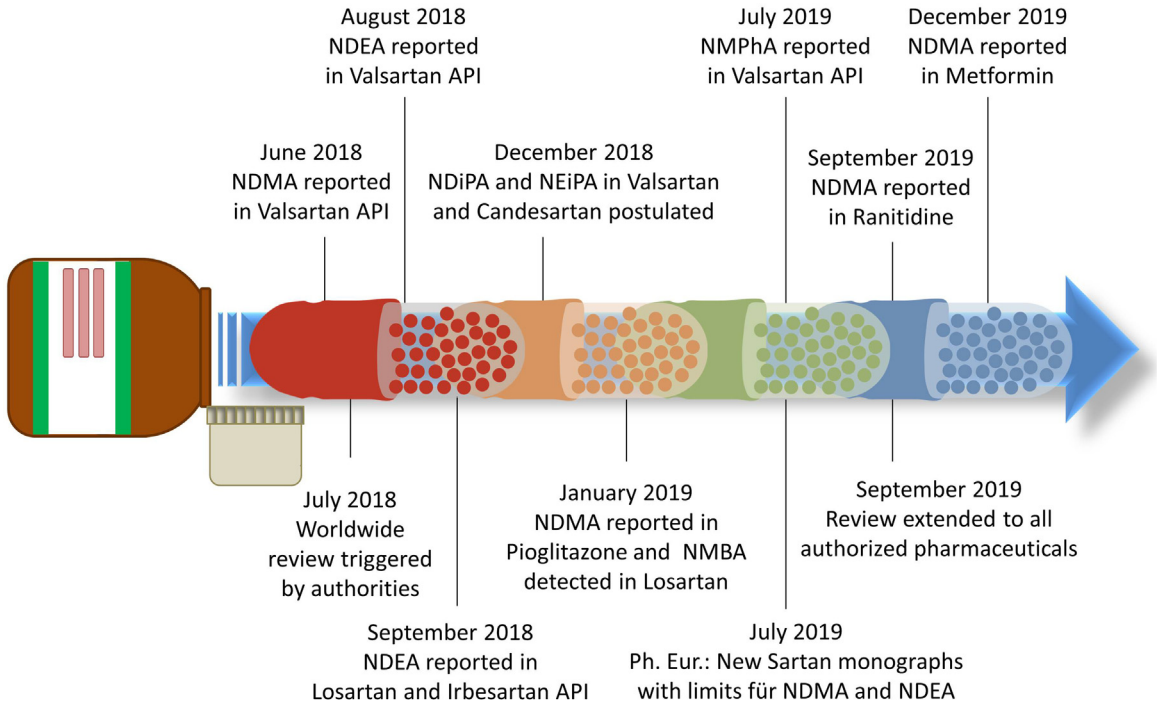


Fig. 1. Chronic of the nitrosamine crisis from June 2018 until now.

limit potential carcinogenic risk" [4]. Already in the early 1980s, pharmaceuticals like aminopyramine, disulfiram, oxytetracycline, diphenhydramine, doxylamine and methapyrilene were reported to contain contaminations of NDMA or N-nitrosodiethylamine (NDEA). Furthermore, generation of nitrosamines (NA) as disinfection by-products was reported [5–7]. Based on structure similarities and general knowledge of potential synthesis routes, several active pharmaceutical ingredients (API) out of various drug classes were hypotized as potential candidates with higher risk of NA contaminations [8]. Additionally, excipients, packing material, recycled solvents, contaminated starting materials and many more were discussed as potential root causes for NA contamination [9]. Following the findings in valsartan, these in-depth investigations by regulatory authorities in the U.S. and the EU revealed that NAs are also present in sartan drug products (DP). Furthermore, new NAs were observed - in particular: NDEA, N-nitroso-N-methyl-4-aminobutyric acid (NMBA) and N-methyl-N-nitrosoaniline (also called N-nitrosomethylphenylamine - NMPhA); N-nitrosodibuthylamine (NDBA), N-nitrosodiisopropylamine (NDiPA) and N-nitrosoethylisopropylamine (NEiPA) were further named as suspected nitrosamine impurities. Additionally, other contaminated APIs were found. Besides valsartan, also the ARBs losartan, candesartan and irbesartan as well as the anti-diabetic drugs metformin and pioglitazone and the H_2 antihistamine ranitidine were identified as contaminated with NAs [1,9,10]. The chronological progression of the crisis is shown in Fig. 1.

Due to this extensive crisis, the EMA and FDA unanimously demanded global risk assessments for each DS and DP from the marketing authorization holders (MAH) within strict deadlines. Based on these assessment reports, an evaluation was initiated for continuation or immediate withdrawal of marketing authorization, as mandated by the FDA for all ranitidine products on the U.S. market [11].

Since the absence of NAs cannot be guaranteed for all authorized formulations, interim limits (IL) with negligible risk for patients have been established, which must not be exceeded under any circumstances in case of potential contamination [12]. These ILs were based on available substance-specific toxicological data [13]. Due to the classification of NAs as carcinogenic substances, where no-effect levels are generally inappropriate, a risk-based approach was chosen [12]. In order to limit the risk of potential additional cancer cases to 1 in 100,000 patients or below over a lifetime exposure, acceptable intake (AI) levels of 26.5–96 ng/day for an individual NA were published [14]. Nevertheless, appropriate strategies for monitoring of the manufacturing processes and the finished pharmaceutical drug products to prevent formation or contamination with NAs were also requested, in order to understand and control the root-cause.

During investigation of the nitrosamine crisis, many facilities, institutes and agencies around the world published methods that were designed for quantification of NDMA in specific drug substances or drug products by LC- and GC—MS/MS or -HRMS. Later NDEA and other NAs were integrated stepwise and the list of analytical methods grew largely over time [1,11,15–17]. For none of these methods, development or complete validation data were disclosed or elucidated yet. A different approach was chosen by our working group utilizing the unique advantages of supercritical fluid chromatography (SFC) coupled with tandem mass spectrometry (MS/MS) [18]. This method was the first incorporating a large set of ten different NAs and combining the analysis of these simultaneously with the purity testing of the officially monographed impurities and related substances of the United States Pharmacopeia (USP) and the European Pharmacopeia (Ph. Eur.). The method was developed under a holistic Quality-by-Design (QbD) concept of the ICH Q8, validated for limit testing according to ICH Q2 [4] and remarked as a "rather new approach [. . .] with advantages for routine analysis" in the provisional final statement on nitrosamine impurities of the EMA in June 2020 [12]. Nevertheless, the method was only designed as a case study for the two ARBs valsartan and losartan to show the great potential of SFC in routine analysis. Additionally, only API testing was addressed and the actual list of NAs, which were suggested by health authorities, grew ever since [12].

In this work, we adapted the product "lifecycle management" (LCM) approach according to the ICH guideline Q12 [4] to the

analytical method development process, as described by Parr & Schmidt [19] to optimize an already pre-developed method. LCM excellently supports this process by adjusting, controlling and improving the existing technique. Additionally, LCM will ensure that the optimized analytical method can be brought in line with regulatory guidelines in the shortest possible time. Thus, it can be used for the comprehensive and fast analysis of nitrosamines in drug substances and drug products.

2. Experimental

2.1. Materials

Reference standards of valsartan, losartan-Na, metformin HCl, ranitidine HCl, pioglitazone, N-nitrosodiethanolamine (NDELA) and the EPA 8270/Appendix IX Nitrosamines Mix (2000 μg/mL in methanol) were supplied by Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). 1-Methyl-4-nitrosopiperazine (MNPaz) was acquired from Toronto Research Chemicals (North York, ON, Canada). Additionally, a 200 μg/mL reference stock solution of N-nitrosodiisopropylamine (NDiPA) in methanol was purchased from LGC GmbH (Luckenwalde, Germany). N-Nitrosoethylisopropylamine (NEiPA), N-Nitrosomethy(2-phenylethyl)amine (NMEPhA), N-nitroso-N-methyl-4-aminobutyric acid (NMBA) and N-nitrosomethylphenylamine (NMPhA) were bought from Enamine Ltd. (Kiev, Ukraine – supplied via Sigma Aldrich). Table 1 displays the structures of the included NAs and Fig. 2 the chemical structures of the screened APIs. Corresponding drug products were directly obtained from the suppliers or obtained on the German market.

For verification of the limit of detection a combined stock solution of all nitrosamines in methanol (MeOH) was prepared, containing 2000 ng/mL of each NA.

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

2.2. Instrumentation and software

Chromatographic analysis was performed using an Acquity UPC^2 SFC system (Waters GmbH, Eschborn, Germany) equipped with an Acquity UPC^2 column manager with active eluent pre-heaters, an Acquity UPC^2 PDA (photodiode array) detector and an Acquity TQD (triple quadrupole mass spectrometer, equipped with multimode ESI source). A fixed-leak interface from the SFC to the MS was coupled with a Waters 515 make-up pump (post-column pre-convergence manager split), to enhance mass transfer to the MS and to improve ionization. For system control, data acquisition and processing the Empower 3 software (Feature Release 5, Service Release 4 - Waters GmbH) was used.

Instrumentation operated fully qualified according to the 4Q model of the U.S. Pharmacopeia (USP) <1058> [20] in a GMP-regulated laboratory environment via the qualified and validated Empower software.

Within method development, the following columns were screened: Viridis HSS C18 SB (100 × 3.0 mm; 1.8 μm – Waters GmbH), Trefoil AMY1, CEL1 and CEL2 (all 150 × 3.0 mm; 2.5 μm – Waters GmbH), Hypercarb Porous Graphitic Carbon LC column (100 × 2.1 mm; 5 μm – Thermo Fisher Scientific GmbH, Dreieich Germany) and the Supel Carbon graphite column (100 × 3.0 mm; 2.7 μm – Merck KGaA, Darmstadt, Germany).

Sample preparation was performed with a 5415D lab centrifuge (Eppendorf AG, Hamburg, Germany - centrifugal force: 16.110 rcf; kinetic energy: 3.100 Nm) in 2 mL Safe-Lock tubes (Eppendorf). For analysis 2 mL amber glass TruView LCMS vials (Waters GmbH) were used.

Fusion QbD software (Version 9.8.1.199 - S-Matrix Corporation, Eureka, California, USA) was utilized for multivariate data analysis and chromatographic and spectrometric method optimization. Chemical structures were drawn by ChemDraw Professional (Version 19.1 - PerkinElmer Informatics, Inc., Waltham, Massachusetts, USA), which was also used for logP calculation.

2.3. Final instrumental conditions and sample preparation

Chromatograms were recorded from 200–800 nm at a scan rate of 5 Hz with a multiwavelength PDA. For dynamic peak tracking and high sensitive detection of the NAs, the MS operated in positive electrospray ionization (ESI+) mode with selected reaction monitoring (SRM) for simultaneous targeted mass analysis.

Optimized MS parameters are: capillary voltage 3.50 kV, extractor voltage 3 V, source temperature 120 °C, desolvation temperature 250 °C, desolvation gas flow 500 L/h, collision gas flow 0.30 mL/min and mass span width 0.000. No extra cone gas was used. As make-up solvent and ionization enhancer a 0.35% solution of formic acid in methanol was used with constant flow of 0.12 mL/min. SRMs, optimal cone voltages and collision energies for all analytes are displayed in Table 2.

The final chromatographic separation was performed on a Supel Carbon column (100 × 3.0 mm; 2.7 μm) at 60 °C column temperature and a flow rate of 1.5 mL/min. The back pressure regulator 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 2.92 min at 75% B, resulting in a total run time of 7 min and a next injection hold of 4.5 min for reequilibration at 2% B. The injection volume was 2.5 μL.

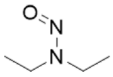
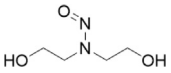
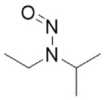
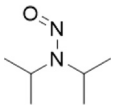
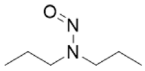
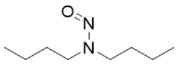
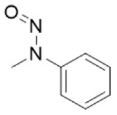
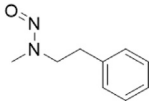
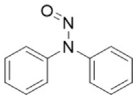
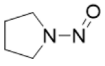
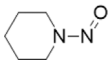
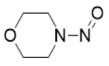
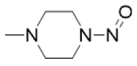
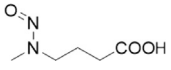
The sample concentration (mg/mL) was set to a tenth of the maximum daily dose for the individual DS, as indicated by the MAH (for specific amounts see Table 3). Samples were prepared by grounding the drug formulation (where necessary), dissolving the corresponding amount of DS or grounded DP in 50.0 mL pure methanol and by stirring for 4 min at 500 rpm. When dissolving was not possible, due to solubility issues, 2 mL of the homogenous sample suspension were centrifuged for 5 min at 13.200 rpm (16.110 rcf) and 1 mL of the particle-free supernatant was directly filled into LC vials. Spiked samples were prepared by addition of the corresponding volume of the 2000 ng/mL NA stock solution to the 50 mL volumetric flasks before extraction.

3. Results and discussion

3.1. Adaption of lifecycle concept

Analytical methods are not rigid, but are exposed to constantly changing environmental conditions and requirements and must be benchmarked continually against their suitability for the intended use. For this reason, there has been an ongoing consideration regarding pharmaceuticals and their analytical procedures as ṽitalẅithin a continuous “lifecycle”, in which requirements and specifications can change. These discussions culminated in the ICH guideline Q12 about “Technical and regulatory considerations for pharmaceutical product lifecycle management” [4], which became effective in 2020, and in the presentation of a corresponding new USP Chapter <1220> for “Analytical Procedure Life Cycle” [21]. The objective of this concept is to demonstrate the “fitness” of an analytical method over its entire lifecycle, including design, development,

Table 1
Names, structures and properties of all included nitrosamines within this study with their published interim limits (IL) according to [28] (note: not for all substances official ILs were published, substances without limits were marked "NA"). LogP values calculated by ChemDraw.

Name	Abbreviation	Structure	CAS-Nr.	Rel. mass [M_R]	logP	IL [ng/day]
N-nitrosodimethylamine	NDMA		62–75–9	74	0.08	96
N-nitrosomethylethylamine	NMEA		10595–95–6	88	0.41	*NA*
N-nitrosodiethylamine	NDEA		55–18–5	102	0.75	26.5
N-nitrosodiethanolamine	NDELA		1116–54–7	134	−0.96	*NA*
N-nitrosoethylisopropylamine	NEiPA		16339–04–1	116	1.07	26.5
N-nitrosodiisopropylamine	NDiPA		601–77–4	130	1.39	26.5
N-nitrosodi-*n*-propylamine	NDPA		621–64–7	130	1.72	*NA*
N-nitrosodi-*n*-butylamine	NDBA		924–16–3	158	2.56	26.5
N-methyl-N-nitrosoaniline (*N-nitrosomethylphenylamine*)	NMPhA		614–00–6	136	1.74	34.3
N-nitrosomethyl(2-phenylethyl)amine	NMEPhA		13256–11–6	164	2.09	*NA*
N-nitrosodiphenylamine	NDPhA		86–30–6	198	3.40	*NA*
N-nitrosopyrrolidine	NPyr		930–55–2	100	0.39	*NA*
N-nitrosopiperidine	NPip		100–75–4	114	0.81	*NA*
N-nitrosomorpholine	NMor		59–89–2	116	−0.32	*NA*
1-Methyl-4-nitrosopiperazine	MNPaz		16339–07–4	129	−0.17	26.5
N-nitroso-N-methyl-4-aminobutyric acid	NMBA		61445–55–4	146	−0.09	96

validation, continuous verification and improvement, since all previous concepts and guidelines only address single parts of the lifecycle, but do not consider it holistically. These long overdue changes have several potential benefits: They can reduce unnecessary costs and time expenditure, will improve flexibility, efficiency and productivity, can mitigate quality issues and support continuous improvement. The most promising expectation, however, is that LCM will promote the introduction of innovations, since changes are permitted and desired.

The LCM approach defines three main stages that an analytical method traverses: The development phase (stage 1), which also includes robustness testing for the final chosen method operable design region (MODR) based on the collected data and knowledge. Subsequently the performance qualification of the established method (stage 2), which will demonstrate the "fitness for use" and the capability of "consistently generating a reportable value that meets the intended purpose" [21]. Once this is demonstrated, the method is released for routine application in an ongoing controlled

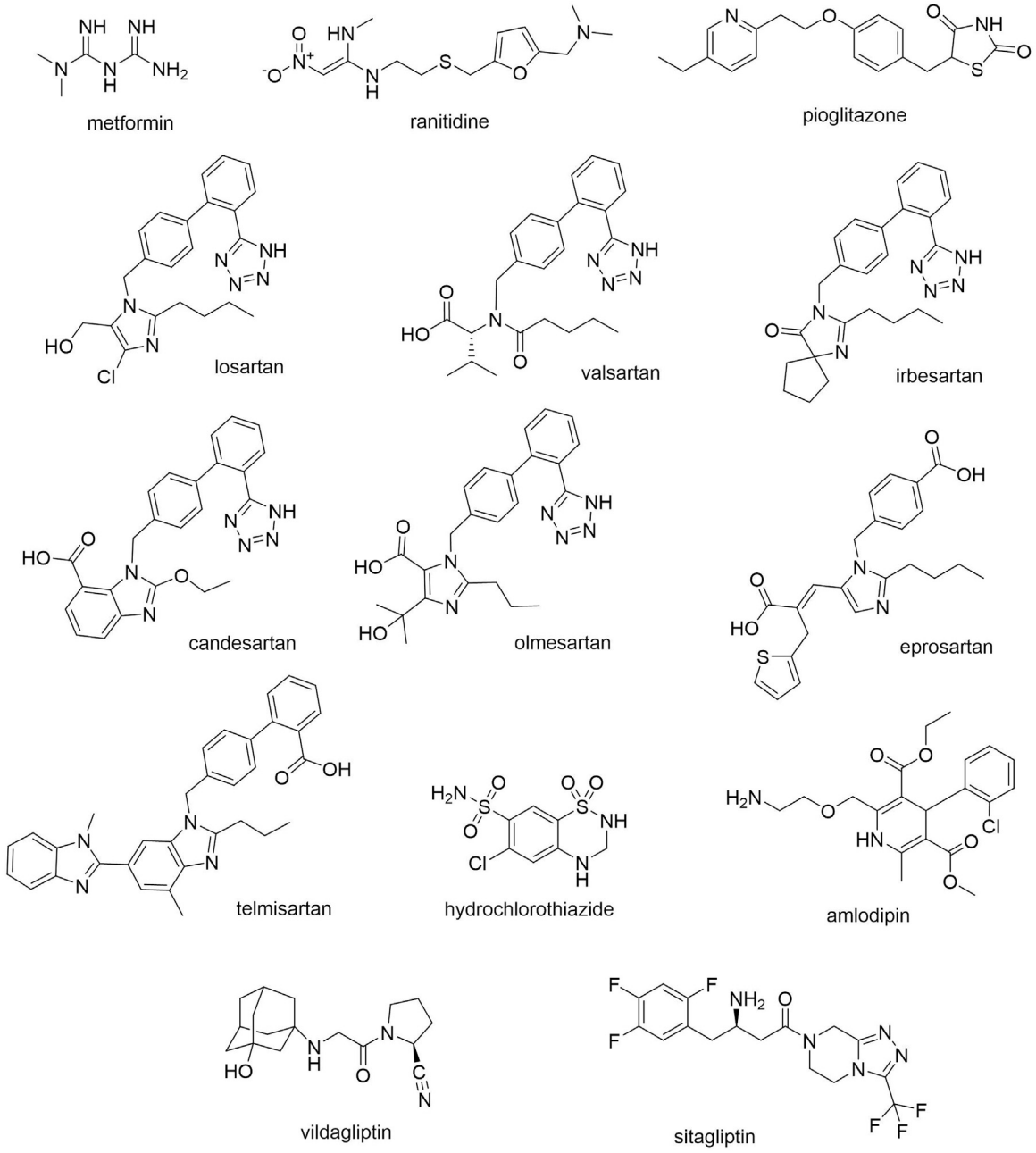


Fig. 2. Chemical structures of all APIs investigated within this study.

environment (stage 3). The LCM concept for analytical methods is display in Fig. 3 according to Parr & Schmidt [19].

Within the three stages, failures to achieve the predefined objectives may be revealed or an unexpected or trending effect can occur that makes it necessary to implement changes to maintain the "fitness for purpose" status of the method. Therefore, these defined objectives are of superior importance and guide the entire LCM concept – they are called "Analytical Target Profile" (ATP) [22]. The ATP prospectively describes the required performance of an analytical method as quality characteristics.

In this work the initial ATP of the previously published SFC method [18] was adjusted, in order to provide full capability of sensitive and universal analytical testing, since new analytes were requested and additional NAs had to be integrated. Profound chromatographic data were already obtained and deep physico-chemical knowledge was generated in the earlier investigation. Therefore, multivariate and interactional relationships of instrumental and chemical parameters could be integrated, which significantly accelerated development work, since large parts of the methodology could be retained.

As ATP the following points were considered as prerequisites:

A) Enable analysis of NAs in DS as well as DP (ARBs, metformin, ranitidine and pioglitazone) and preferably more,
B) meet requirements of ICH Q2 for limit test,
C) perform method re-development under QbD principles utilizing SFC separation,
D) integrate all published and expected NAs (Table 1) for simultaneous detection,
E) allow rapid turn-around time (as fast as the initial method or preferably faster), and
F) improve separation (no coelution of isomers or matrix artefacts).

Table 2
Optimized MS parameter for all 16 included nitrosamines.

Synonym / Abbreviation	SRM 1 (Quantifier)		SRM 2 (Qualifier)	Cone voltage	Collision energy	Dwell time (sec)
NDMA	75	→	43	38	18	0.05
		→	58		12	
NMEA	89	→	61	32	14	0.05
		→	47		12	
NDEA	103	→	47	32	20	0.05
		→	57		14	
NDELA	135	→	74	16	12	0.10
		→	87		8	
NEiPA	117	→	75	24	8	0.05
		→	43		14	
NDiPA	131	→	89	26	14	0.05
		→	43		18	
NDPA	131	→	43	26	16	0.05
		→	89		12	
NDBA	159	→	103	30	16	0.25
		→	57		18	
NMPhA	137	→	66	32	20	0.30
		→	77		24	
NMEPhA	165	→	77	22	31	0.10
		→	51		43	
NDPhA	199	→	66	22	30	0.30
		→	169		30	
NPyr	101	→	55	36	18	0.05
		→	59		18	
NPip	115	→	69	36	18	0.05
		→	55		20	
NMor	117	→	45	32	20	0.05
		→	57		16	
MNPaz	130	→	58	21	17	0.10
		→	43		28	
NMBA	147	→	44	20	10	0.10
		→	117		6	

Table 3
Investigated samples with findings of nitrosamines within this study. Levels of detected NAs marked with ↓ are below the toxicological threshold (detected peak area in the unspiked sample <50% of the spiked sample) and can be considered as safe. A ↑ indicate amounts above the tolerable daily dose. Absolute amounts not displayed, since limit testing is a semi-quantitative test and not intended for quantification.

Sample name	Maximum daily dose [mg]	Available formulation	Findings
Candesartan	32	DP	–
Candesartan / Hydrochlorothiazide	32 / 25	DP	–
Eprosartan	800	DP	–
Irbesartan	300	DP	–
Losartan	100	DS, DP	NDMA ↓, NDEA ↓
Losartan / Hydrochlorothiazide	100 / 25	DP	–
Metformin	2550	DS, DP	NDMA ↓
Metformin/Sitagliptin	2000 / 100	DP	–
Metformin/Vildagliptin	2000 / 100	DP	–
Olmesartan	40	DP	–
Olmesartan / Hydrochlorothiazide	40 / 25	DP	–
Pioglitazone	45	DS	–
Ranitidine	300	DS	NDMA ↑
Telmisartan	80	DP	–
Valsartan	320	DS, DP	NDEA ↓
Valsartan / Amlodipine	320 / 10	DP	–

This ATP substitutes the former profile of the analytical method we previously developed following lifecycle management. Thus, the new method reported inhere terminates the previous method and a total rollback to "Stage 1″ (method development) was chosen [19].

3.2. Method re-development under LCM principles

As defined in the ATP, new NAs had to be integrated and an implementation of various sample formulations was obligatory. The original SFC method [18] performed separation on a Viridis HSS C18 SB column, which showed strong retention for the non-polar compounds, but the less aliphatic and more polar NAs eluted early. Therefore, NMBA (logP = -0.09) and NDELA (logP = −0.96) were hypotized to elute with the void volume, which would lead to massive matrix problems and signal suppression. Additionally, the selectivity of the C18 column was low, which led to a coelution of the isomers NDiPA and NDPA. Since method development had previously been carried out entirely according to the QbD principles, comprehensive and in-depth chromatographic information was available. This allowed deducing that all the silica-based reversed-phase (RP) or normal-phase (NP) columns previously screened would not be able to meet the requirements of the ATP. Also polysaccharide-modified phases failed to retain the majority of the NAs.

Therefore, porous graphitic carbon (PGC) based columns were investigated, which have been accessible since the 1990s [23], but have barely found their way into routine pharmaceutical or pharmacopeial applications and especially into SFC. Nevertheless, studies reported PGC columns proved to be a valuable tool to

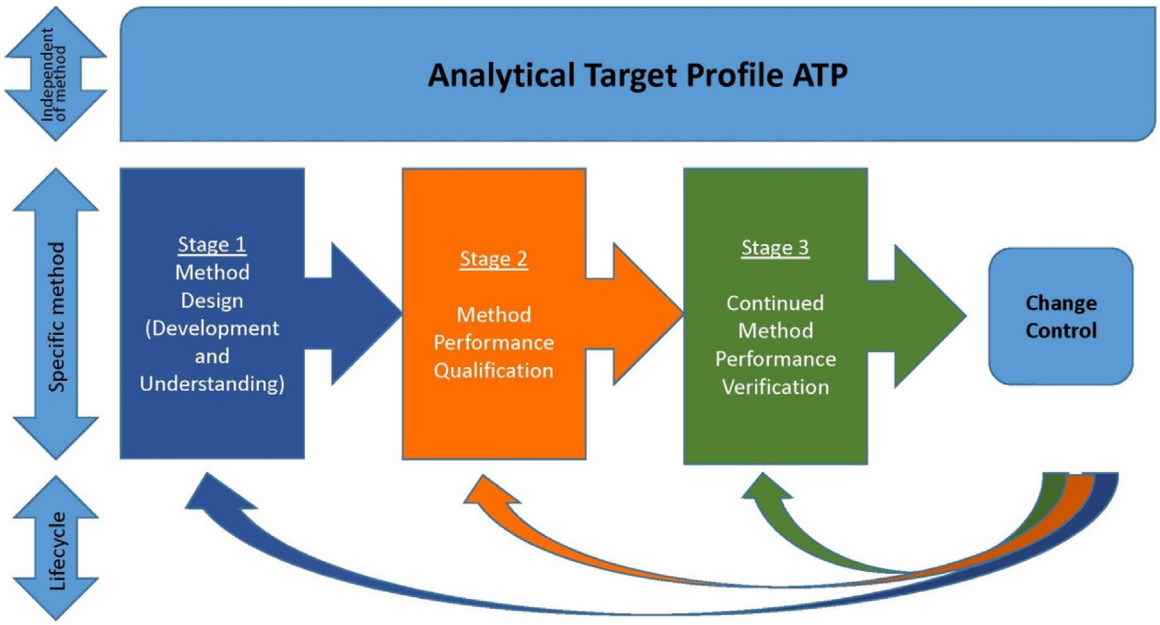


Fig. 3. Lifecycle management of analytical methods concept according to Parr & Schmidt [19], with permission of the publisher (Elsevier).

achieve separation of isomers [24] or structures showing a high similarity [25]. In addition, a broad range of polarities is accessible on PGCs, enabling separation of aliphatic, aromatic as well as isomeric substituted compounds [26]. A highly varying selectivity, compared to the earlier studied silica-based stationary phases, was expected due to the completely different chemistry. Since it was also expected that the list of NAs could expand further in the future, a stationary phase allowing the separation of a wide range of potential similar compounds will be even more advantageous. Thus, PGC stationary phases were chosen due to their high potential for separation of these NAs.

Two commercially available graphite columns were selected and appeared to have a promising and comparable retention effect on all NAs in screening experiments. The initial modifier (methanol with .1% TFA), which was already known from the previous study [18] to be the best for separation efficiency, peak sharpening and especially MS ionization enhancement, was used for multivariate QbD gradient optimization. Other acidic or even basic additives or organic modifiers were not screened again, since they showed significant chromatographic disadvantages or ion suppression in the MS. The make-up solvent (methanol with 0.35% formic acid) was also transferred unchanged, because it showed the best overall results for NA ionization and detection. Thus, only the eluent gradient profile, backpressure of the SFC system, column temperature and the percentage of TFA in the modifier were optimized, which reduced the method development time drastically, demonstrating the advantages of analytical LCM.

Representative chromatograms of all investigated samples and an exemplary metformin sample, which was spiked with all 16 NAs, are displayed in Figs. 4 and 5. The resulting chromatographic gradient method (as mentioned in chapter 2.3) was evaluated for robustness and reproducibility of NA separation. These multivariate design spaces together with the robust MODR are displayed in Fig. 6.

As displayed, an elevated temperature of about 60 °C was necessary for sufficient peak separation of the isomers NDiPA and NDPA, as well as the isobars NEiPA and NMor, since the reduced density of the mobile phase and therefore the increased permeability and mass transfer resulted in sharper and better retained peaks.

Since most of the NAs are separated during the initial isocratic hold of the method, they are affected by the percentage of modifier (methanol with TFA) during this step. Amounts of more than 2% modifier showed a decrease in resolution of the critical peak pairs NDiPA & NDPA and NEiPA & NMor, due to a decreased retention on column. Additionally, the low responders NDMA, NDEA, NPip, NPyr and NDPhA were negatively affected by higher amounts of the modifier, which resulted in signal suppression caused by the higher amount of TFA. Amounts of less than 2% modifier were also tested and showed satisfactory separation results. At the same time, a higher pressure ripple of the binary high-pressure SFC pump was observed, which could be related to reduced mixing of CO_2 and modifier or a modifier-clustering. Modifier-clustering can form larger solvated spheres with polar analytes, which diffuse less, due to the increased cross-section, and leads to a peak broadening. Peak broadening at the lower percentages of modifier is also related to weak solubility of the analyte in the mobile phase (CO_2) and strong adsorption to the PGC.

An increased pressure of the backpressure regulator should theoretically result in a higher split-ratio to the MS, which was not observed, but a significant decrease in the resolution of the isomers (NDiPA & NDPA) and the isobars (NEiPA & NMor) was ascertained above 1800 psi (about 120 bar). This effect can be explained, since an increased backpressure results in an increased density and viscosity of the mobile phase. This decreases the diffusion coefficients or mass transfer with the stationary phase and therefore the retention of the analytes.

As recently studied [18], small amounts of TFA are necessary to elute the APIs from the column and to form narrow and sharper peaks. TFA improves the chromatographic behavior and peak shape by ion pairing and suppression of analyte charge. Covering of silanol-adsorption sites on the stationary phase surface is not occurring on PGC columns, but the flat layers of hexagonal arranged carbon atoms (sp^2 hybrids) with delocalized electrons [26] can be polarized, thus altering the analyte interactions with the graphite stationary phase.

By varying the amount of TFA in the modifier, about 0.1% of TFA was found as the optimum in terms of chromatographic performance and ionization enhancement. Amounts of more than 0.1% TFA showed major impact on ionization, resulting in a significant mass suppression in the MS. Less TFA showed slightly better MS results, but led also to peak broadening of the APIs, which might result in carryover and increase the

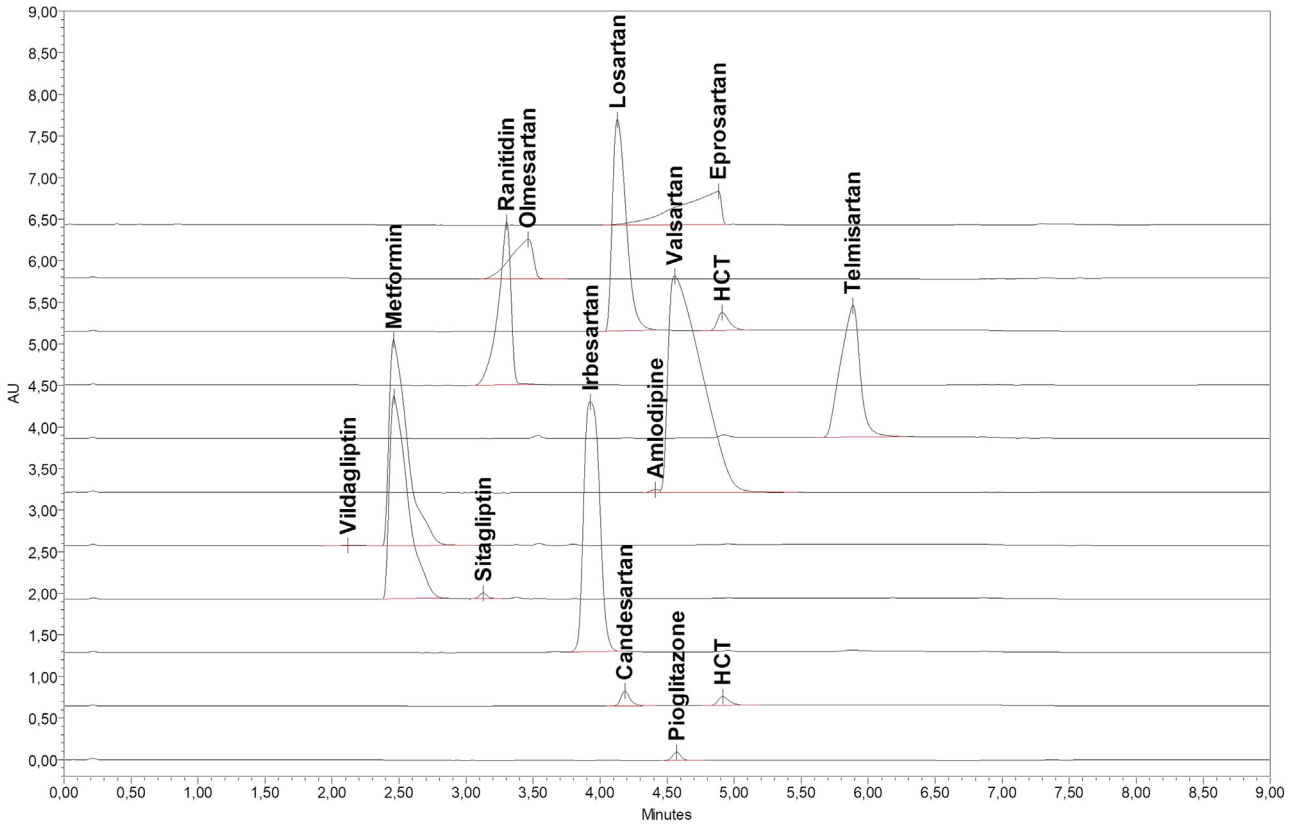


Fig. 4. Representative UV chromatograms of the investigated drug substance and drug product samples within this study, obtained with the final method at 250 nm detection wavelength (HCT = Hydrochlorothiazide).

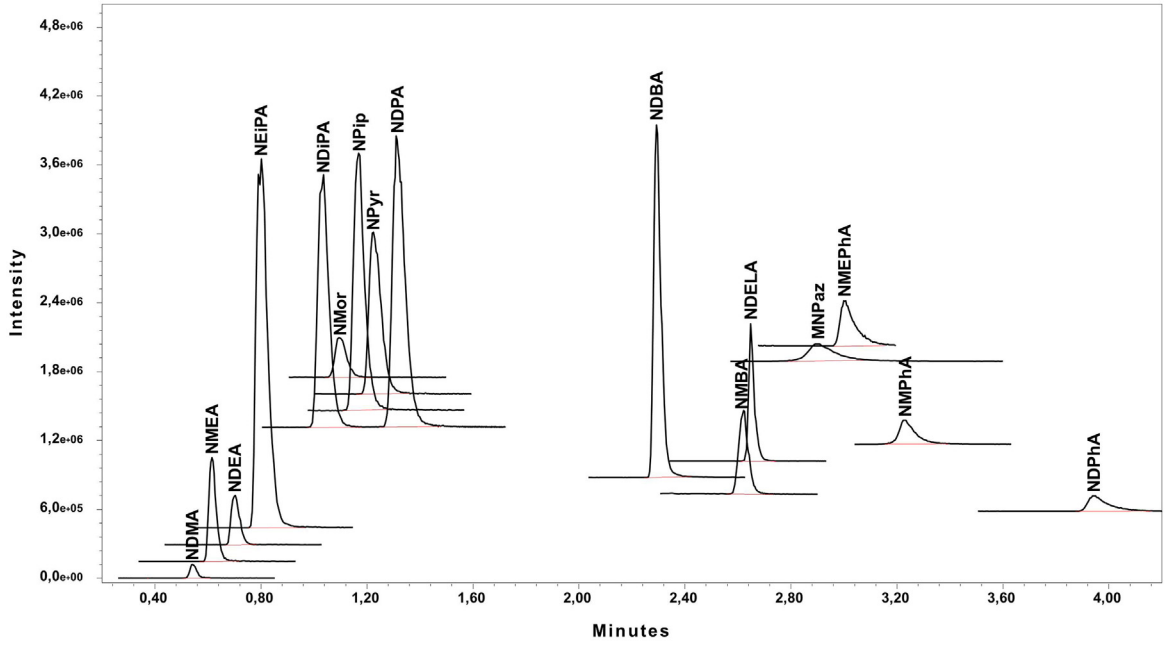


Fig. 5. Representative MS/MS chromatogram of a metformin DP sample spiked with all 16 nitrosamines (20 ng/mL) and their retention times (min), obtained on the Supel Carbon column with the final method.

possibility of matrix artefacts over a broad retention time window.

Both investigated PGC columns showed a very good separation capacity, which was fully capable to achieve the ATP (fast run time, no coelution of isomers or isobaric substances, integration of all NAs) under the optimized chromatographic parameters. Due to the chosen QbD approach, the working point and the MODR were visualized within the knowledge space (Fig. 6) and LCM implementation helped to accelerate the development process, by integrating a scientific concept.

The FDA reported in July 2020 that coelution of NDMA and ^{15}N-DMF or ^{13}CDMF (dimethylformamide) may result in a

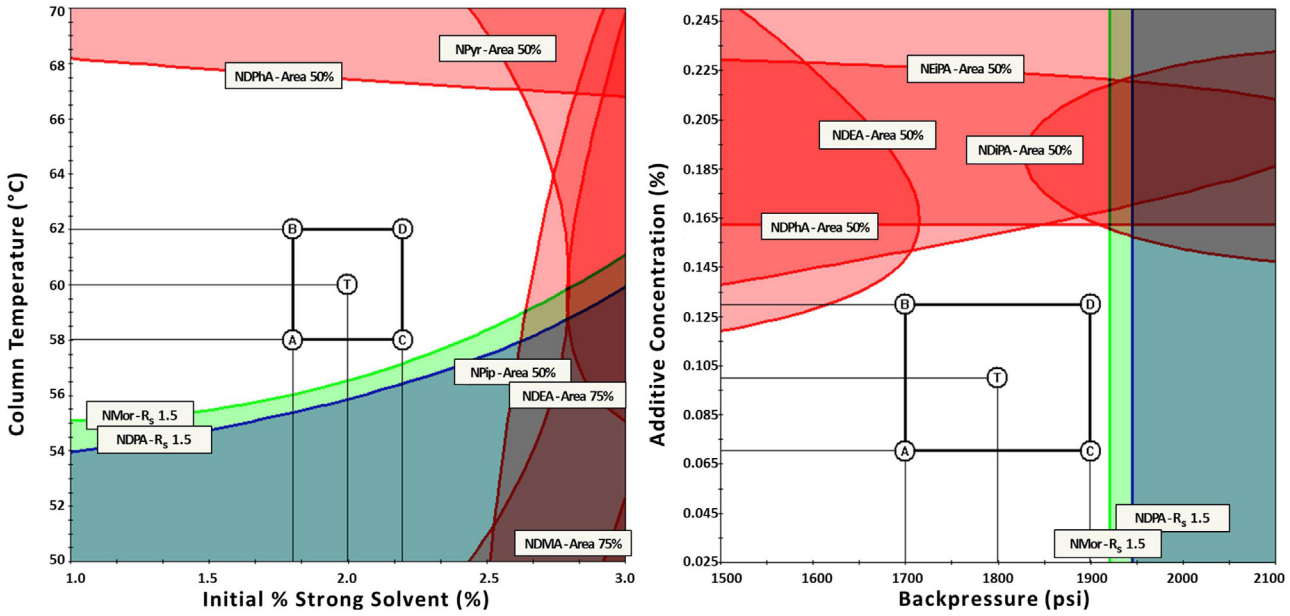


Fig. 6. Robustness evaluation for the optimized SFC method's design space. Left: Column temperature and the initial percentage of modifier ("strong solvent") during the first isocratic step of the gradient method. Right: Concentration of TFA (additive) in the modifier and backpressure. Displayed in green the resolution of NMor to NEiPA (isobar peak pair) and in blue NDPA to NDiPA (isomers). In red relative peak areas for each single NA, compared to the most intense peak within this full-factorial experiment, are displayed. Colored areas indicate where the predefined and listed objective is not achieved (note that mixed-colors are possible) and therefore white areas indicate positive results. Final working point (T) with MODR shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

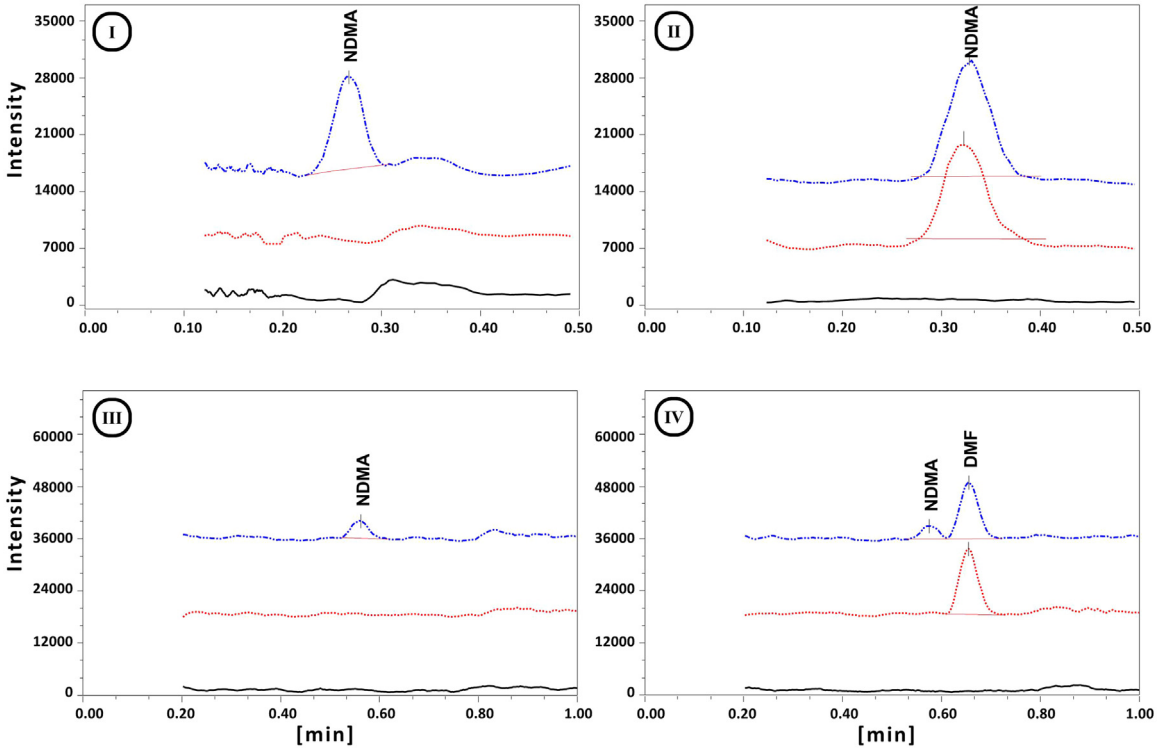


Fig. 7. Overlay of a blank (MeOH – black solid), unspiked Metformin sample (red dotted) and a Metformin sample spiked at 96 ng/day level (blue dash-dotted) – Overlay I: Metformin API without DMF or NDMA contamination on Hypercarb column; Overlay II: DMF-contaminated and NA-free tested Metformin tablet on Hypercarb column; Overlay III: identical Metformin API sample without contamination on Supel Carbon column; Overlay IV: identical DMF-contaminated Metformin tablet on Supel Carbon column – note that due to DMF coelution in Overlay II the sample would be claimed false NDMA-positive. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

(false-positive) overestimation of NDMA [27], due to comparable fragmentation patterns and almost no mass difference of both structures. Therefore, NDMA and DMF have to be separated chromatographically or must be evaluated with sufficient mass resolution (mass tolerance not more than ±15 ppm). In order to demonstrate the high selectivity of SFC, the separation approach was chosen, since targeted MS/MS analysis was also intended. For this purpose, both PGC columns were challenged with a non-

contaminated metformin API and a drug product sample, which was known to contain trace amounts of DMF and no NDMA. In order to simulate a contaminated sample, the API and the drug product were spiked with NDMA at the level corresponding to an intake of 96 ng/day, which is the interim limit for an allowable daily intake (based on a daily dose of 2550 mg metformin) [11,28]. On the Supel Carbon column a full separation of DMF and NDMA was observed, demonstrating a NA-free sample, but on the Hypercarb column a complete coelution occurred. The chromatographic results are displayed in Fig. 7. This coelution causes false-positive findings in routine analysis. Therefore, the Hypercarb column was excluded from the development program and only the Supel Carbon column was selected for a later routine use, at the end of stage 1.

3.3. Implementation and performance verification

After successful completion of method development, evaluation of robustness and performance demonstration (end of "Stage 1″), suitability of the method was verified for limit testing according to ICH Q2 "Validation of Analytical Procedures: Text and Methodology" [4], in order to prove the fitness for purpose ("Stage 2″) as the last step within the LCM before routine use ("Stage 3″). Since QbD method development provided detailed information and reliability on the separation performance of the method, selectivity has already been demonstrated and is assured by both chromatographic separation and MS/MS detection using SRM. Limit of detection (LOD) is usually evaluated as the "lowest amount of analyte in a sample which can be detected but not necessarily quantitated" [4]. Since EMA and FDA published specific threshold ILs for each NA [11,28], based on their toxicological profile, these amounts were spiked onto each DS or DP sample solution (Table 1 for the individual ILs of each NA). A total of 25 different samples were prepared, covering 7 ARBs, metformin, ranitidine and pioglitazone. In addition, combinatory products of the aforementioned APIs were also integrated, as the new method is intended for universal NA analysis. The list of investigated drug substances and drug products is displayed in Table 3. All NAs were detected with the necessary intensity in each spiked sample (signal-to-noise-ratio ≥ 3), thus verifying the performance for semi-quantitative limit testing (system suitability test).

Subsequently, unspiked samples were analyzed and possible occurring signals evaluated against the spiked samples. For limit testing, the detected peak area of the unspiked samples should not exceed 50% of the spiked sample. It turned out that one Losartan sample was contaminated with NDMA and NDEA simultaneously and one Valsartan sample with NDMA, but both below the toxicological threshold. Furthermore, a Ranitidine DS showed large amounts of NDMA, more than 10-times above the tolerable limit. NDMA was also detected in one Metformin drug product, but below the toxicological threshold. All other investigated samples did not contain NAs.

4. Conclusions

The above displayed LCM process was able to provide a universal analysis method, resulting in a rapid, sensitive and versatile analytical SFC-MS/MS method for screening and investigation of nitrosamine impurities in various pharmaceuticals. Not only drug substances, but also complex drug products and even combination drug product formulations were successfully analyzed, which indicates the broad applicability. The data reflect the method's compliance in line with international guidelines, regulatory expectations and requirements and therefore the contemporary approach. Additionally, the highly flexible and beneficial advantages of supercritical fluid chromatography were demonstrated, indicating that SFC separation can be used as state-of-the-art technique in pharmaceutical analysis. We highlighted that many different pharmaceutical substances can be analyzed by a single analytical method in a very short run time under full GMP conditions. Through the intensive preliminary QbD study [18], the gathered knowledge could be rapidly implemented, demonstrating the superiority of LCM.

As the FDA's "Center for Drug Evaluation and Research" (CDER) stated: "Today, we have better testing methods than ever before, and we know what to look for [. . .]. As our investigations and testing continues, along with the investigations done by other drug regulatory agencies, we may find low levels of nitrosamines in additional drugs. [. . .] These investigations take time. [. . .] The FDA will communicate any information we have scientifically confirmed to ensure the public knows as much as possible as soon as possible." [29]. Embedded in this process we intend to provide this method to MAHs and the scientific community in order to sustain the process of risk assessment and to reduce the patient health hazards. The method is capable of meeting the requirements of the newly proposed "nitrosamine impurities" monograph of the USP [30] and the fully disclosed development data prove conformity with the ICH guidelines M7, Q8, Q9, Q12 and the proposed Q14 [4].

As our current laboratory data indicate, other drug products besides the published samples (Fig. 2) may also be contaminated without the knowledge of the MAH or the responsible regulatory authorities. Based on this assumption, analytical departments around the world should focus on universal testing methods to ensure that any positive finding is detected before market release and to prevent imported contamination.

CRediT authorship contribution statement

Sebastian Schmidtsdorff: Conceptualization, Methodology, Investigation, Visualization, Writing - original draft. **Jonas Neumann:** Investigation, Writing - original draft. **Alexander H. Schmidt:** Conceptualization, Methodology, Supervision, Writing - review & editing, Resources. **Maria K. Parr:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest./p>

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3.3 Manuscript III: 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.

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FULL PAPER

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

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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

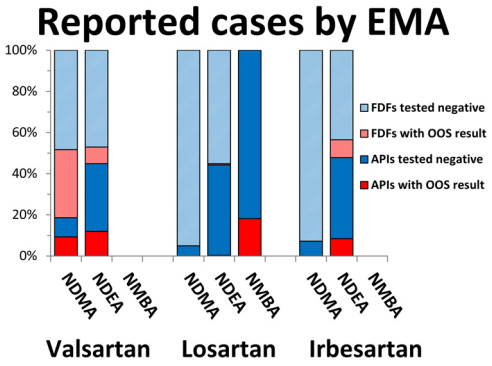

FIGURE 1 Number of out-of-specification (OOS) results for analyzed active pharmaceutical ingredients (APIs) and finished dosage forms (FDFs) as of April 15, 2019, according to the European Medicines Agency (EMA).[1] From 758 tested APIs, 165 were positive (21.8%), and 320 of 1802 tested FDFs (17.8%) 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

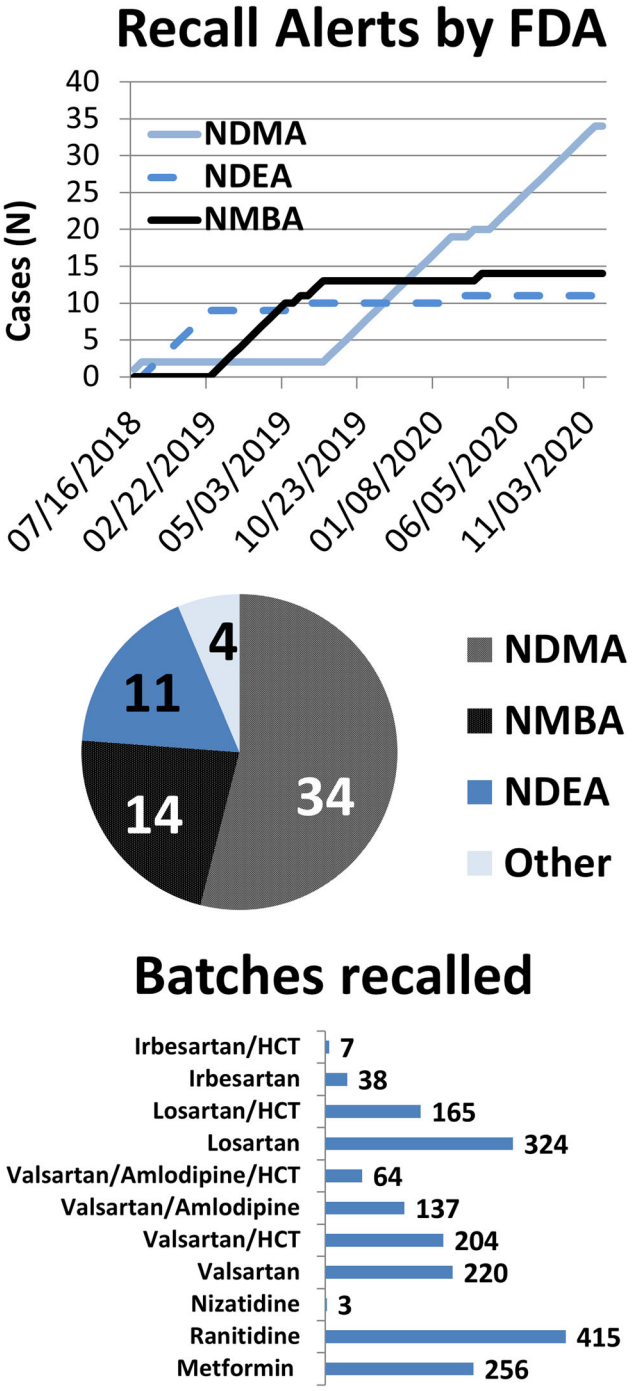

FIGURE 2 Cumulative cases of recalled or withdrawn drug products after Food and Drug Administration (FDA) safety alerts since 2018[2,3]—note that rifampicin and rifapentine were also subject to safety alerts, but not recalled, to prevent drug shortage. No specified nitrosamines were reported after January 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

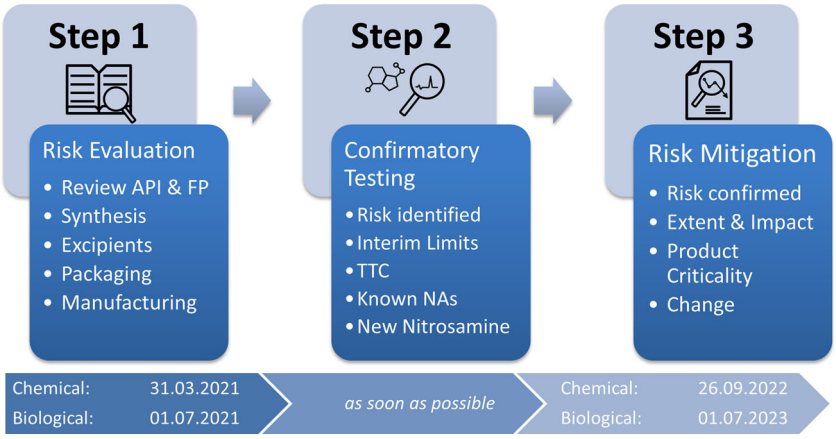

FIGURE 3 Time frame and expectation for nitrosamine referral according to EMA/425645/2020[11,12] for active pharmaceutical ingredients (APIs) and finished products (FPs). EMA, European Medicines Agency; NA, nitrosamine; TTC, threshold of toxicological concern

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

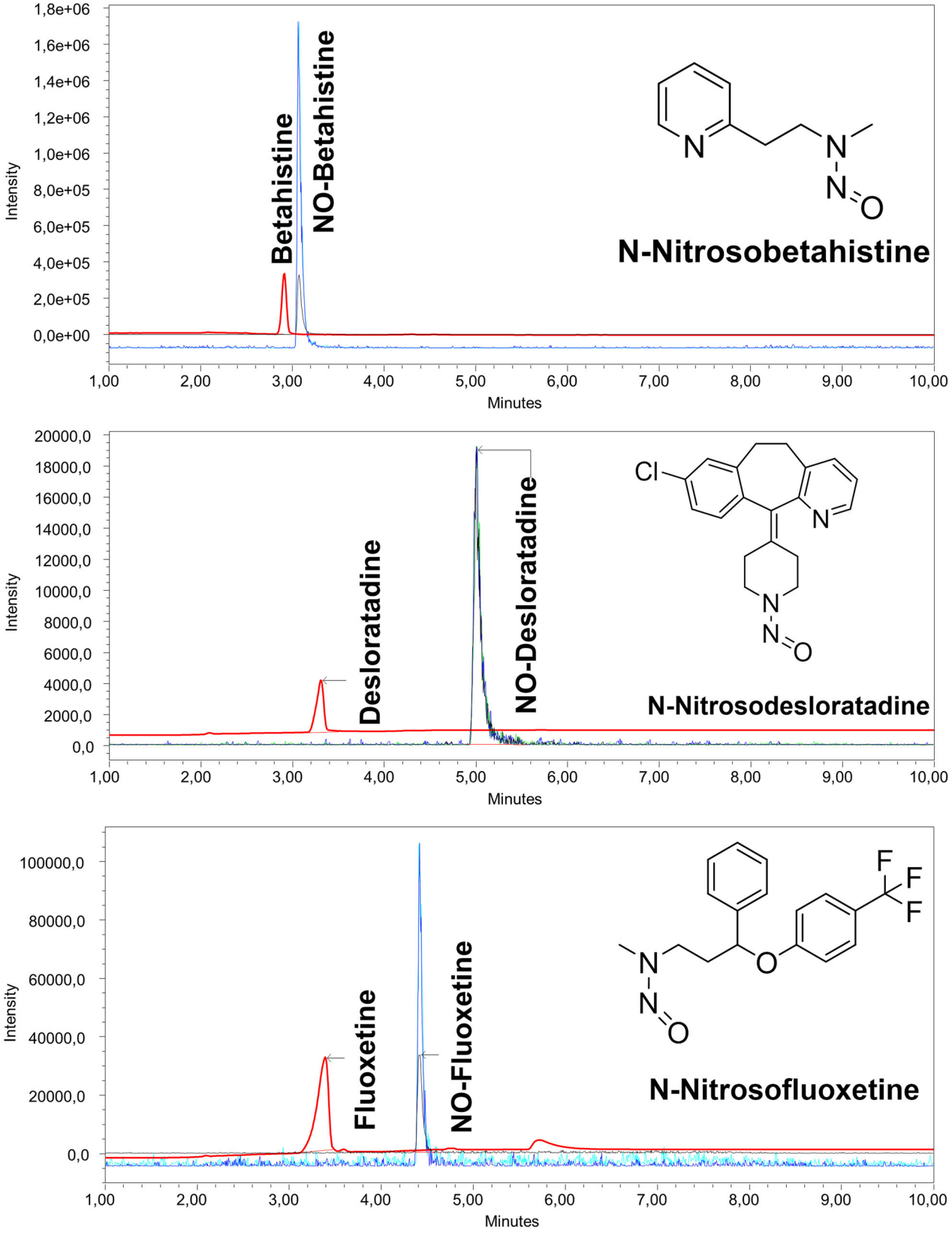

FIGURE 4 Commercially available and investigated *N*-nitroso derivatives of active pharmaceutical ingredients (APIs) for method implementation (original APIs displayed with the red UV channel only, to prevent oversaturation of the mass detector, and three mass transitions of each *N*-nitroso compound 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.

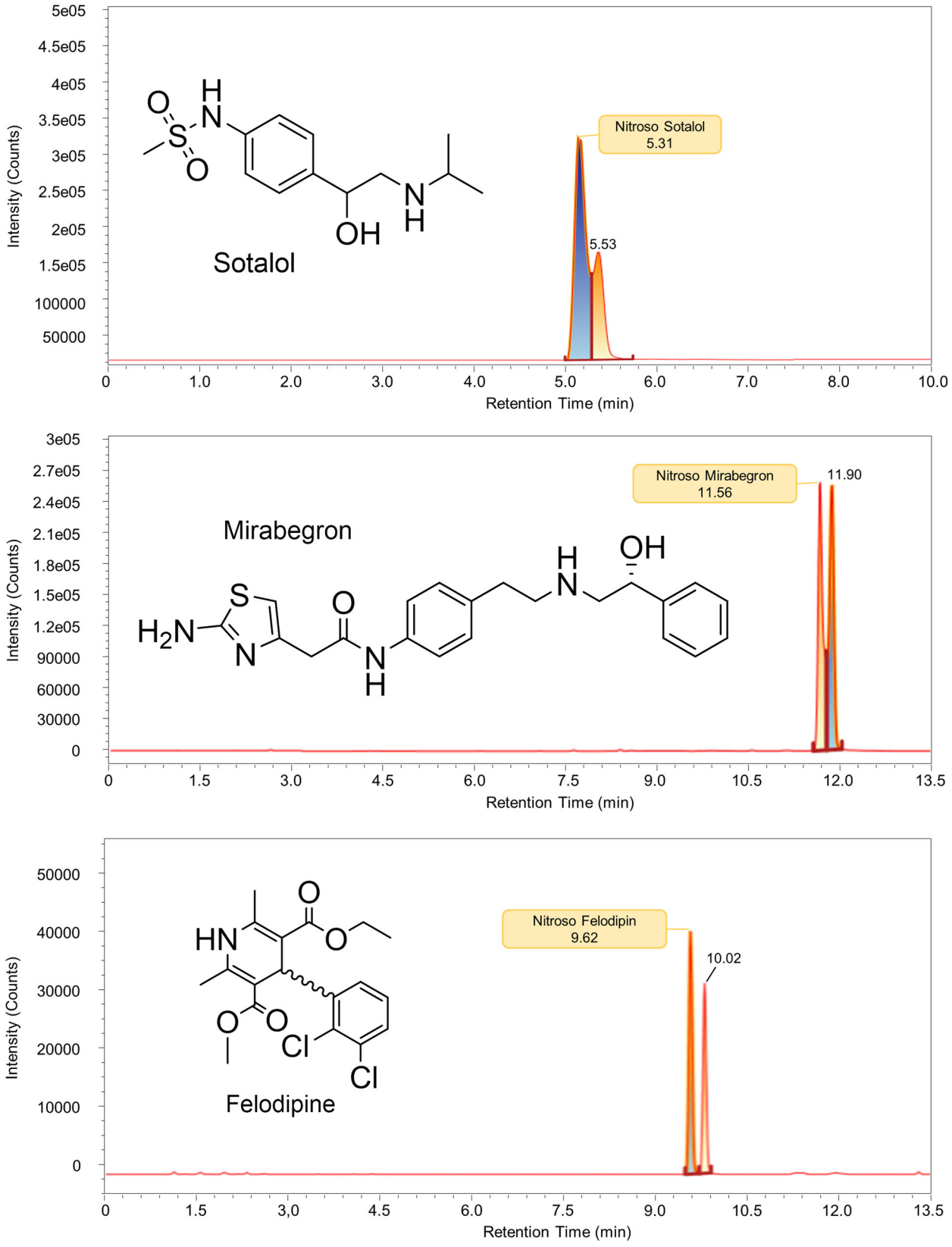

FIGURE 5 Extracted ion SFC-MS chromatogram (15.0 ppm mass accuracy window) of sotalol (EIC = 301.1096 Da), felodipine (EIC = 412.0593 Da), and mirabegron (EIC = 425.1522 Da) samples after the NAP test—two separated peaks of *N*-nitroso compounds with mass errors of <5 ppm detected. Extracted ion SFC-MS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

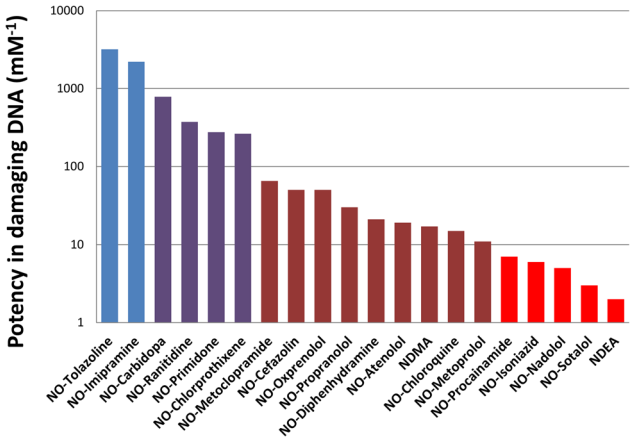

FIGURE 6 Genotoxic potential of selected nitrosamines and nitrosated pharmaceuticals according to Brambilla and Martelli[22] by inducing DNA fragmentation in cell culture. High values indicate a lower potential

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 re-equilibration 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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3.4 Manuscript IV: Prevalence of nitrosamine contaminants in drug samples: Has the crisis been overcome?

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Abstract: Various drug samples (N = 249; drug substances, tablets, capsules, solutions, crèmes, and more) from the European pharmaceutical market were collected since 2019 and analyzed for 16 nitrosamines (NAs). In 2.0% of the cases, NAs were detected. These findings included four active pharmaceutical ingredients already known for potential NA contamination: losartan (*N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine, simultaneously), valsartan (NDMA), metformin (NDMA) and ranitidine (NDMA). The fifth new finding, which has not been reported yet, discovered a contamination of a molsidomine tablet sample with *N*-nitrosomorpholine (NMor). The tablet contained 144% of the toxicological allowable intake for NMor. NMor was included in our screening from the beginning and is currently in the focus of regulatory authorities, but was added to the guidelines only last year. Thus, it may not have been the focus of regulatory investigations for too long. Our results indicate that the majority of drug products on the market are non-hazardous in terms of patient safety and drug purity. Unfortunately, the list of individual affected products keeps growing constantly and new NA cases, such as molsidomine or nitrosated drug substances (nitrosamine drug substance-related impurities, NDSRI) continue to emerge. We therefore expect nitrosamine screenings to remain a high priority.

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FULL PAPER

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Prevalence of nitrosamine contaminants in drug samples: Has the crisis been overcome?

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Abstract

Various drug samples ($N = 249$; drug substances, tablets, capsules, solutions, crèmes, and more) from the European pharmaceutical market were collected since 2019 and analyzed for 16 nitrosamines (NAs). In 2.0% of the cases, NAs were detected. These findings included four active pharmaceutical ingredients already known for potential NA contamination: losartan (*N*-nitrosodimethylamine [NDMA] and *N*-nitrosodiethylamine, simultaneously), valsartan (NDMA), metformin (NDMA) and ranitidine (NDMA). The fifth new finding, which has not been reported yet, discovered contamination of a molsidomine tablet sample with *N*-nitrosomorpholine (NMor). The tablet contained 144% of the toxicological allowable intake for NMor. NMor was included in our screening from the beginning and is currently the focus of regulatory authorities, but was added to the guidelines only last year. Thus, it may not have been the focus of regulatory investigations for too long. Our results indicate that the majority of drug products in the market are nonhazardous in terms of patient safety and drug purity. Unfortunately, the list of individual affected products keeps growing constantly and new NA cases, such as molsidomine or nitrosated drug substances (nitrosamine drug substance-related impurities [NDSRI]), continue to emerge. We therefore expect nitrosamine screenings to remain a high priority.

KEYWORDS

nitrosamine drug substance-related impurities, nitrosamines, *N*-nitroso compounds, porous graphitic carbon, supercritical fluid chromatography

1 | INTRODUCTION

The numerous nitrosamine findings that have followed the first *N*-nitrosodimethylamine (NDMA) detection in valsartan since 2018 are now concerning manufacturers, regulatory authorities, and marketing authorization holders (MAH) for 4 years. The list of recalled products is growing constantly and there is no evidence that the number of recalls is declining (Figure 1).

Nitrosamines (NA) are high-potent carcinogens that can cause tumors in nearly all organs, due to their alkylating potential after

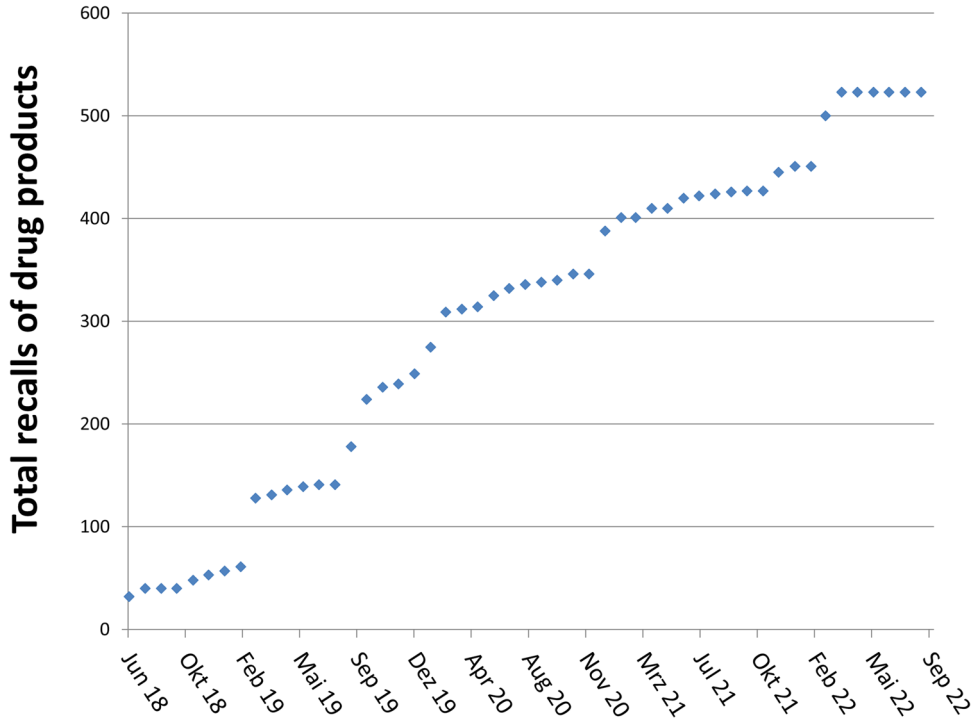


FIGURE 1 Total cumulative nitrosamine findings since July 2018 in the USA and Canada.[24,25] A flattening of the occurrence frequency cannot be observed.

enzymatic activation to alkyl diazonium species. Affected organs are in particular the liver, esophagus, urinary bladder, kidney, and lung.[1,2]

Since it became apparent that not only sartans were affected, the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) started a worldwide call for review of each active pharmaceutical ingredient (API) and drug product. Both have mandated harmonized guidelines that manufacturers and MAHs have to review (step 1—risk assessment) and, if necessary, test registered drug products (step 2—confirmatory investigation) for potentially occurring NAs.[3,4]

Not only synthesis has to be considered as hypothesized for various APIs,[5] which was the main reason for NA findings in sartans (e.g., valsartan, losartan),[6,7] but also storage instabilities, as in the case of ranitidine,[8] as well as incompatibilities with excipients.[9,10] The latter is increasingly coming into focus due to residual amounts of nitrite and nitrate from drug excipients or water (wet granulation) that may promote formation of NAs over the shelf-life of finished products.[11,12] Especially the common excipients hypromellose (HPMC), magnesium stearate, microcrystalline cellulose, crospovidone, and povidone often contain high amounts of nitrite [13] and can have a significant impact on nitrosamine formation.

As most MAHs cannot exclude the occurrence of NAs for every product in the market only by risk assessments, analytical testing is demanded as step 2 of the investigation.[14] The results of these confirmatory tests have to be submitted by September 26, 2022 for products containing chemically synthesized APIs or until July 01, 2023 for biological APIs.[3]

Our group started developing and validating universal and comprehensive analytical methods targeting nitrosamines and nitrosated drugs based on supercritical-fluid chromatography (SFC) early on,[15–17] so we were able to investigate APIs and drug products over the entire 4 years of the NA crisis. We are now sharing the results to assess, from our (analytical) perspective, how pervasive the NA crisis problem was and whether it was resolved in a sustainable manner.

The study presented here is the first to address the topic of nitrosamine analysis comprehensively and delivers results not only for individual drug substances (e.g., sartans) or their specific formulation. The aim is to investigate all conceivable drug formulations in a diversified study, since the focus to date has been primarily on APIs and tablets. Comparable studies have not yet been published in the literature indicating the necessity of the undertaken research. Based on the obtained analytical results and available literature, the question is furthermore discussed whether an end to the nitrosamine crisis can really be expected with the ending of the investigation deadlines set by EMA and FDA.

2 | RESULTS AND DISCUSSION

Using an earlier published SFC-MS/MS method,[16] which was developed using quality-by-design (QbD) principles, 249 different, randomly selected samples from 66 different manufacturers (Table 1) were tested over the last 4 years for 16 aliphatic, cyclic

TABLE 1 List of investigated samples for nitrosamine testing

Drugs	Manufacturer	Dosage strength	Formulation	Maximum daily dose (mg/day)
Acetylsalicylic acid	#1	500 mg	Tablets	3000
Aciclovir	#2	200 mg	Tablets	1000
Aciclovir	#3	800 mg	Tablets	1000
Aciclovir	#4	400 mg	Tablets	1000
Agomelatine	#5	25 mg	Tablets	50
Allopurinol	#4	300 mg	Tablets	800
Allopurinol	#6	300 mg	Tablets	800
Ambroxol	#1	75 mg	XR Capsules	75
Amiloride/Bendroflumethiazide	#7	5 mg/2.5 mg	Tablets	10/5
Amitriptyline HCl	#8	-	API	150
Amlodipine	#9	10 mg	Tablets	10
Amlodipine	#10	10 mg	Tablets	10
Amoxicillin/Clavulanic acid	#11	875 mg/125 mg	Tablets	1750/250
Amoxicillin	#3	1000 mg	Tablets	2000
Apixaban	#12	2.5 mg	Tablets	10
Aripiprazole	#11	5 mg	Tablets	30
Atorvastatin	#4	20 mg	Tablets	80
Atropine	#13	5 mg/ml	Drops	15
Baclofen	#14	10 mg	Tablets	80
Betahistine	#4	12 mg	Tablets	36
Betahistine	#15	12 mg	Tablets	36
Bisoprolol	#11	5 mg	Tablets	20
Bisoprolol	#1	5 mg	Tablets	20
Bisoprolol/Amlodipine	#16	5 mg/5 mg	Tablets	10/10
Brinzolamide	#17	10 mg/ml	Drops	30
Bromazepam	#1	6 mg	Tablets	18
Butylscopolamine-Br	#18	10 mg	Tablets	60
Candesartan	#3	16 mg	Tablets	32
Candesartan	#6	16 mg	Tablets	32
Candesartan	#16	16 mg	Tablets	32
Candesartan	#3	32 mg	Tablets	32
Candesartan/Hydrochlorothiazide	#11	8 mg/12.5 mg	Tablets	8/12.5
Candesartan/Hydrochlorothiazide	#11	16 mg/12.5 mg	Tablets	16/12.5
Candesartan/Hydrochlorothiazide	#14	16 mg/12.5 mg	Tablets	16/12.5
Carbamazepine	#2	200 mg	XR Tablets	1600
Carvedilol	#3	25 mg	Tablets	100
Cefaclor	#2	500 mg	Capsules	1000
Cefuroxime	#3	500 mg	Tablets	1000

(Continues)

TABLE 1 (Continued)

Drugs	Manufacturer	Dosage strength	Formulation	Maximum daily dose (mg/day)
Cefuroxime	#1	500 mg	Tablets	1000
Celecoxib	#19	200 mg	Capsules	400
Cetirizine	#20	10 mg	Tablets	10
Cetirizine	#21	10 mg	Tablets	10
Cetirizine	#3	10 mg	Tablets	10
Levocetirizine	#16	5 mg	Tablets	5
Chlorphenamine Maleate	#8	-	API	32
Chlorpromazine HCl	#8	-	API	150
Escitalopram	#22	20 mg	Tablets	20
Citalopram	#2	10 mg	Tablets	40
Citalopram	#2	20 mg	Tablets	40
Clindamycin	#23	600 mg	Tablets	1800
Clobetasone	#24	0.5 mg/g	Crème	2.5
Clopidogrel	#25	75 mg	Tablets	75
Codeine	#11	16 mg/g	Drops	200
Colecalciferol	#26	1000 I.U.	Tablets	0.025
Desloratadine	#25	5 mg	Tablets	5
Diclofenac	#27	75 mg	XR Capsules	150
Diclofenac	#3	10 mg	XR Tablets	150
Dienogest/Ethinylestradiol	#24	2 mg/0.03 mg	Tablets	2/0.03
Diltiazem HCl	#8	-	API	360
Dimenhydrinate	#28	20 mg	Dragee	140
Dimenhydrinate	#1	50 mg	Tablets	300
Diphenhydramine HCl	#8	-	API	200
Donezepil	#29	10 mg	Tablets	23
Dorzolamide	#30	20 mg/ml	Drops	60
Doxylamine Succinate	#8	-	API	25
Doxylamine	#1	25 mg	Tablets	25
Doxycycline	#31	40 mg	Capsules	300
Doxycycline	#1	100 mg	Tablets	300
Duloxetine	#21	60 mg	XR Capsules	120
Edoxaban	#32	60 mg	Tablets	60
Enalapril	#33	20 mg	Tablets	40
Entacapone	#22	200 mg	Tablets	1600
Epinephrine	#34	1 mg/ml	Solution	10
Eprosartan	#1	600 mg	Tablets	800
Ergometrine Maleate	#8	-	API	0.5
Erythromycin	#8	-	API	4000

TABLE 1 (Continued)

Drugs	Manufacturer	Dosage strength	Formulation	Maximum daily dose (mg/day)
Esomeprazole	#16	40 mg	XR Capsules	80
Etomidate	#8	-	API	30
Etoricoxib	#4	60 mg	Tablets	120
Ezetimibe	#35	10 mg	Tablets	10
Febuxostat	#36	80 mg	Tablets	80
Felodipine	#6	10 mg	XR Tablets	10
Fesoterodine	#37	8 mg	XR Tablets	8
Fexofenadine	#34	180 mg	Tablets	180
Flecainide	#14	100 mg	Tablets	400
Fluoxetine	#3	40 mg	Tablets	80
Furosemide	#1	40 mg	Tablets	80
Gabapentin	#39	300 mg	Capsules	3600
Glibenclamide	#1	1.75 mg	Tablets	3.5
Glimepride	#3	2 mg	Tablets	8
Glimepride	/#25	3 mg	Tablets	8
Granisetron	#21	2 mg	Tablets	2
Haloperidol	#22	1 mg	Tablets	15
Hydrochlorothiazide	#3	12.5 mg	Tablets	100
Hydrochlorothiazide	#10	12.5 mg	Tablets	100
Ibuprofen	#41	200 mg	Tablets	3200
Ibuprofen	#25	600 mg	Tablets	3200
Ibuprofen	#2	600 mg	Tablets	3200
Imiquimod	#29	50 mg/g	Crème	12.5
Imipramine HCl	#8	-	API	200
Indometacin	#1	25 mg	Capsules	200
Irbesartan	#4	75 mg	Tablets	300
Irbesartan	#3	300 mg	Tablets	300
Irbesartan	#3	300 mg	Tablets	300
Isosorbide Dinitrate	#4	20 mg	Tablets	60
Isosorbide Dinitrate	#42	120 mg	XR Capsules	120
Dexetoprofen	#43	25 mg	Tablets	75
Ketotifen	#44	0.25 mg/m	Drops	0.2
Lamotrigine	#2	200 mg	Tablets	400
Lamotrigine	#2	50 mg	Tablets	400
Lercanidipin	#45	20 mg	Tablets	30
Levetiracetame	#2	1000 mg	Tablets	3000
Levetiracetam	#46	1000 mg	Granules	3000
Levodopa/Carbidopa	#22	100 mg/25 mg	XR Tablets	700/175

(Continues)

TABLE 1 (Continued)

Drugs	Manufacturer	Dosage strength	Formulation	Maximum daily dose (mg/day)
Levofloxacin	#47	500 mg	Tablets	750
Levomepromazine	#22	25 mg	Tablets	300
Levomepromazine	#22	100 mg	Tablets	300
Letrozole	#48	2.5 mg	Tablets	2.5
Letrozole	#49	2.5 mg	Tablets	2.5
Lidocaine	#50	20 mg/ml	Solution	300
Loperamide	#4	2 mg	Capsules	16
Loratadine	#20	10 mg	Tablets	10
Lorazepam	#14	1 mg	Tablets	10
Losartan	#51	50 mg	Tablets	100
Losartan	#52	100 mg	Tablets	100
Losartan/Hydrochlorothiazide	#47	100 mg/12.5 mg	Tablets	100/12.5
Melperone	#1	25 mg	Tablets	100
Memantine	#22	10 mg	Tablets	20
Memantine	#21	20 mg	Tablets	20
Meropenem	#8	-	API	3000
Metamizole	#3	500 mg/ml	Drops	4000
Metamizole	#3	500 mg	Tablets	4000
Metamizole	#1	500 mg	Tablets	4000
Metamizole	#43	500 mg	Tablets	4000
Metformin/Vildagliptin	#53	1000 mg/50 mg	Tablets	2000/100
Metformin	#3	850 mg	Tablets	3400
Metformin	#10	1000 mg	Tablets	3000
Methotrexate	#55	10 mg	Tablets	10
Methotrexate	#55	33 mg/ml	Solution	20
Methylprednisolone	#24	1 mg/g	Crème	5
Metoclopramide	#4	4 mg/ml	Drops	40
Metoclopramide	#4	10 mg	Tablets	40
Metoprolol Succinate	#3	95 mg	XR Tablets	190
Metoprolol Succinate	#55	190 mg	XR Tablets	190
Metoprolol Succinate	#1	95 mg	XR Tablets	190
Metronidazole	#8	-	API	4000
Mirabegron	#56	50 mg	XR Tablets	50
Mirtazapine	#47	15 mg	Tablets	45
Mirtazapine	#11	30 mg	Tablets	45
Molsidomine	#8	-	API	24
Molsidomine	#4	8 mg	Tablets	24
Montelukast	#47	4 mg	Tablets	4

TABLE 1 (Continued)

Drugs	Manufacturer	Dosage strength	Formulation	Maximum daily dose (mg/day)
Montelukast	#3	10 mg	Tablets	10
Moxifloxacin	#47	400 mg	Tablets	400
Moxonidine	#11	0.2 mg	Tablets	0.6
Nebivolol	#57	5 mg	Tablets	40
Nebivolol	#58	5 mg	Tablets	40
Nifedipine	#4	20 mg	XR Tablets	120
Nifedipine	#1	20 mg	XR Tablets	120
Nitrofurantoin	#54	100 mg	Tablets	400
Ofloxacin	#60	3 mg/ml	Drops	12
Olanzapine	#61	2.5 mg	Tablets	30
Olanzapine	#29	7.5 mg	Tablets	30
Olmesartan	#32	40 mg	Tablets	40
Olmesartan/Hydrochlorothiazide	#16	40 mg/12.5 mg	Tablets	40/12.5
Olmesartan/Hydrochlorothiazide	#16	40 mg/25 mg	Tablets	40/25
Olmesartan/Amlodipine	#16	40 mg/5 mg	Tablets	40/5
Olmesartan/Amlodipine	#43	40 mg/5 mg	Tablets	40/5
Omeprazole	#21	40 mg	XR Capsules	80
Omeprazole	#6	40 mg	XR Capsules	80
Ondansetron	#51	8 mg	Tablets	16
Opipramol	#22	100 mg	Tablets	300
Osimertinib	#62	80 mg	Tablets	80
Oxytetracycline HCl	#8	-	API	2000
Pantoprazole	#2	20 mg	XR Tablets	240
Pantoprazole	#16	20 mg	XR Tablets	240
Pantoprazole	#29	40 mg	XR Tablets	240
Pantoprazole	#55	40 mg	XR Tablets	240
Pantoprazole	#14	40 mg	XR Tablets	240
Paracetamole	#1	500 mg	Tablets	4000
Perazine	#22	100 mg	Tablets	1000
Pergolide	#22	1 mg	Tablets	3
Phenylephrine	#13	100 mg/ml	Drops	200
Pipamperone	#3	40 mg	Tablets	360
Pramipexole	#1	0.35 mg	Tablets	4.5
Prednicarbate	#63	2.5 mg/g	Crème	12.5
Prednisolone	#27	5 mg	Tablets	60
Prednisolone	#27	10 mg	Tablets	60
Pregabalin	#3	50 mg	Capsules	600
Promazine HCl	#8	-	API	1000

(Continues)

TABLE 1 (Continued)

Drugs	Manufacturer	Dosage strength	Formulation	Maximum daily dose (mg/day)
Promethazine	#46	20 mg/ml	Drops	100
Promethazine	#22	20 mg/ml	Drops	100
Promethazine	#22	25 mg	Tablets	100
Propafenone	#6	150 mg	Tablets	900
Propranolol	#1	40 mg	Tablets	640
Quetiapine	#1	25 mg	Tablets	800
Quetiapine	#11	50 mg	Tablets	800
Ramipril	#11	5 mg	Tablets	20
Ramipril/Hydrochlorothiazide	#1	5 mg/12.5 mg	Tablets	10/25
Ramipril/Hydrochlorothiazide	#25	5 mg/25 mg	Tablets	5/25
Ranitidine HCl	#8	-	API	5/25
Risperidone	#10	0.5 mg	Tablets	16
Risperidone	#6	2 mg	Tablets	16
Rivaroxaban	#64	20 mg	Tablets	20
Rosuvastatin	#52	5 mg	Tablets	40
Roxithromycin	#8	-	API	300
Salbutamol	#65	0.5 mg/ml	Solution	3
Sertraline	#47	100 mg	Tablets	200
Simvastatin	#29	20 mg	Tablets	80
Simvastatin	#3	60 mg	Tablets	80
Simvastatin	#4	80 mg	Tablets	80
Sitapliptin	#35	50 mg	Tablets	100
Sotalol	#66	160 mg	Tablets	320
Spiramycin	#8	-	API	3000
Spironolactone	#1	50 mg	Tablets	400
Sulfamethoxalzole/Trimethoprim	#1	800 mg/160 mg	Tablets	2400/640
Sulpiride	#4	50 mg	Tablets	1600
Sumatriptan Succinate	#8	-	API	200
Sumatriptan	#15	50 mg	Tablets	300
Telmisartan	#3	80 mg	Tablets	80
Terbinafine	#57	250 mg	Tablets	250
Tetracycline	#8	-	API	2000
Tetracycline HCl	#8	-	API	2000
Thiamizole	#2	10 mg	Tablets	20
L-Thyroxin	#55	75 µg	Tablets	0.3
L-Thyroxin	#34	50 µg	Tablets	0.3
Ticagrelor	#62	90 mg	Tablets	180
Tilidine/Naloxone	#3	50 mg/4 mg	XR Tablets	600/48

TABLE 1 (Continued)

Drugs	Manufacturer	Dosage strength	Formulation	Maximum daily dose (mg/day)
Tilidine/Naloxone	#4	50 mg/4 mg	XR Tablets	600/48
Tilidine/Naloxone	#4	100 mg/8 mg	XR Tablets	600/48
Tofacitinib	#37	5 mg	Tablets	20
Torasemide	#55	2.5 mg	Tablets	200
Torasemide	#3	5 mg	Tablets	200
Torasemide	#3	50 mg	Tablets	200
Tramadol	#4	50 mg	Capsules	300
Trospium-Cl	#59	30 mg	Tablets	40
Urapidil	#40	30 mg	XR Capsules	180
Valaciclovir	#47	500 mg	Tablets	3000
Valsartan/Amlodipine	#4	160 mg/5 mg	Tablets	320/10
Valsartan/Hydrochlorothiazide	#3	160 mg/12.5 mg	Tablets	320/25
Valsartan/Hydrochlorothiazide	#55	160 mg/12.5 mg	Tablets	320/25
Valsartan/Hydrochlorothiazide	#16	160 mg/12.5 mg	Tablets	320/25
Valsartan/Hydrochlorothiazide	#3	160 mg/25 mg	Tablets	160/25
Valsartan/Amlodipine/ Hydrochlorothiazide	#16	160 mg/10 mg/12.5 mg	Tablets	160/10/12.5
Venlafaxine	#6	150 mg	XR Capsules	225
Verapamil	#34	40 mg	Tablets	480
Verapamil	#7	80 mg	Tablets	480
Xipamide	#38	20 mg	Tablets	80
Zolpidem	#4	10 mg	Tablets	10

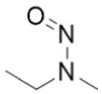
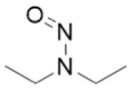
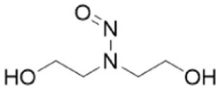
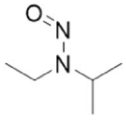
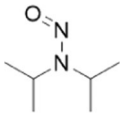
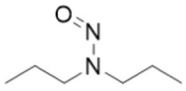
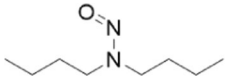
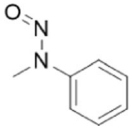
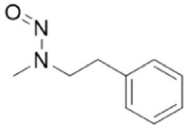
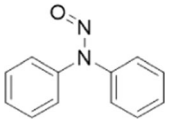
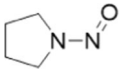
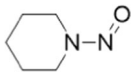
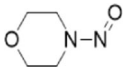
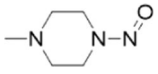
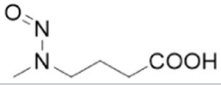
Abbreviations: API, active pharmaceutical ingredient; XR, extended-release.

and aromatic nitrosamines (Table 2). The samples were composed of APIs as well as liquid, semi-solid and solid dosage forms. The majority of the tested drug products contained chemically synthesized APIs. In addition, also seven samples of botanical origin were investigated. The latter are not listed in Table 1 for confidentiality reasons, as their composition would reveal the manufacturers.

Nitrosamines were detected in 5 of the 249 samples (2.0%). A ranitidine API showed enormous amounts of NDMA (about 10-times above the interim limit (IL) of 96 ng/day). Intramolecular degradation of ranitidine followed by intermolecular rearrangement and thus the formation of significant amounts of NDMA is a known problem,[18] which led to a total global suspension of ranitidine drug products.[19] In addition, two sartan samples showed NA contamination below the IL: one valsartan sample was contaminated with NDMA and one losartan sample contained both NDMA and NDEA (*N*-nitrosodiethylamine) simultaneously. The mentioned sartan samples were withdrawn from the market after analysis. Trace amounts of NDMA were also detected in a metformin drug product significantly below the IL.

A new NA finding, which was not reported yet, is NMor in molsidomine, a nitro-vasodilator used for long-term prophylaxis of angina pectoris. The nitric oxide donor has a morpholine-containing substructure (Figure 2) that resulted in formation of NMor. We analyzed one API and one drug product, of which the finished product showed elevated levels (about 44% above the threshold, corresponding to approx. 183 ng/day) of NMor above the IL of 127 ng/day (Figure 3). Based on the requirements for limit tests in the general chapters USP <1469> "Nitrosamine impurities"[20] and Ph. Eur. 2.5.42 on "N-Nitrosamines in active substances"[21] the peak area ratio between the unspiked and spiked sample was 0.59.

TABLE 2 List of 16 investigated nitrosamines (NA = no IL published yet)

Name	Abbreviation	Structure	CAS-No.	IL (ng/day)
N-Nitrosodimethylamine	NDMA		62-75-9	96
N-Nitrosomethylethylamine	NMEA		10595-95-6	*NA*
N-Nitrosodiethylamine	NDEA		55-18-5	26.5
N-Nitrosodiethanolamine	NDELA		1116-54-7	NA
N-Nitrosoethylisopropylamine	NEiPA		16339-04-1	26.5
N-Nitrosodiisopropylamine	NDiPA		601-77-4	26.5
N-Nitrosodi-*n*-propylamine	NDPA		621-64-7	26.5
N-Nitrosodi-*n*-butylamine	NDBA		924-16-3	26.5
N-Methyl-*N*-nitrosoaniline (*N*-nitrosomethylphenylamine)	NMPhA		614-00-6	34.3
N-Nitrosomethyl(2-phenylethyl)amine	NMEPhA		13256-11-6	8
N-Nitrosodiphenylamine	NDPhA		86-30-6	NA
N-Nitrosopyrrolidine	NPyr		930-55-2	NA
N-Nitrosopiperidine	NPip		100-75-4	1300
N-Nitrosomorpholine	NMor		59-89-2	127
1-Methyl-4-nitrosopiperazine	MNPaz		16339-07-4	26.5
N-Nitroso-*N*-methyl-4-aminobutyric acid	NMBA		61445-55-4	96

Abbreviations: IL, interim limit; NA, not applicable.

The API sample, which does not pertain to the analyzed tablets, was out of shelf life, thus formation in drug products does not seem to be linked to instability of the API, which was NMor-free. NMor is the specified impurity B in the Ph. Eur. (threshold 3 ppm in manufactured API).[21] Based on the maximum daily dose for molsidomine this corresponds to 72 ng/day NMor. Therefore, contamination from the API in the molsidomine tablet can also be excluded, since the tested product originated from the German market. The most probable source is either carryover of nitrite from synthesis, in which sodium nitrite is used,[22,23] or a nitrosating agent (e.g., nitrite) from tablet excipients.[13] Thus, it is hypotized, that reaction with traces of morpholine, another specified Ph. Eur. impurity E of molsidomine (limited to 100 ppm),[21] might have occurred in the finished product.

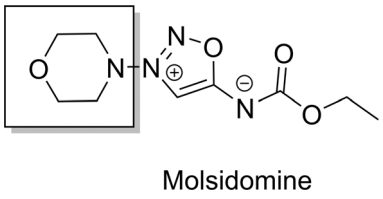


FIGURE 2 Structure of the nitric oxide donor molsidomine. Molsidomine contains a morpholine in the substructure, which can react in trace amounts to form *N*-nitrosomorpholine (NMor).

Throughout our screening, we have considered furthermore that the formation of NAs is not only possible from short-chain aliphatic amines, such as those used during synthesis but that basic amine functions of entire API molecules can also be nitrosated. We, therefore, adapted our SFC-MS/MS method in the meantime to an SFC-TOF-MS nitrosation assay. With this assay, we demonstrated that screening should not be restricted to known NAs (e.g., NDMA, NDEA, and NMBA) only, but also to "nitrosamine drug substance-related impurities" (NDSRI). We confirmed that these NDSRIs may be formed in many nitrogen-containing APIs and high amounts,[17] which repeatedly led to recalls recently (varenicline, orphenadrine, irbesartan, propranolol, and quinaprile).[24,25] EMA and FDA have therefore incorporated this general approach and updated their guidelines, now addressing the screening for NDSRIs.[3,26]

The number of recalls since 2022 is no longer dominated by NDMA only, but NDSRIs are increasingly coming to the fore (Figure 4). Buschmann and Holzgrabe [27] have stated that "Increasingly frequent press releases of newly identified contaminants cannot become the standard in Europe" (translation from the original German text), which we agree with, but we expect the opposite to happen. More NDSRI-related recalls will probably arise in the future. Therefore, NA screening should be implemented on a mandatory basis during API and drug product

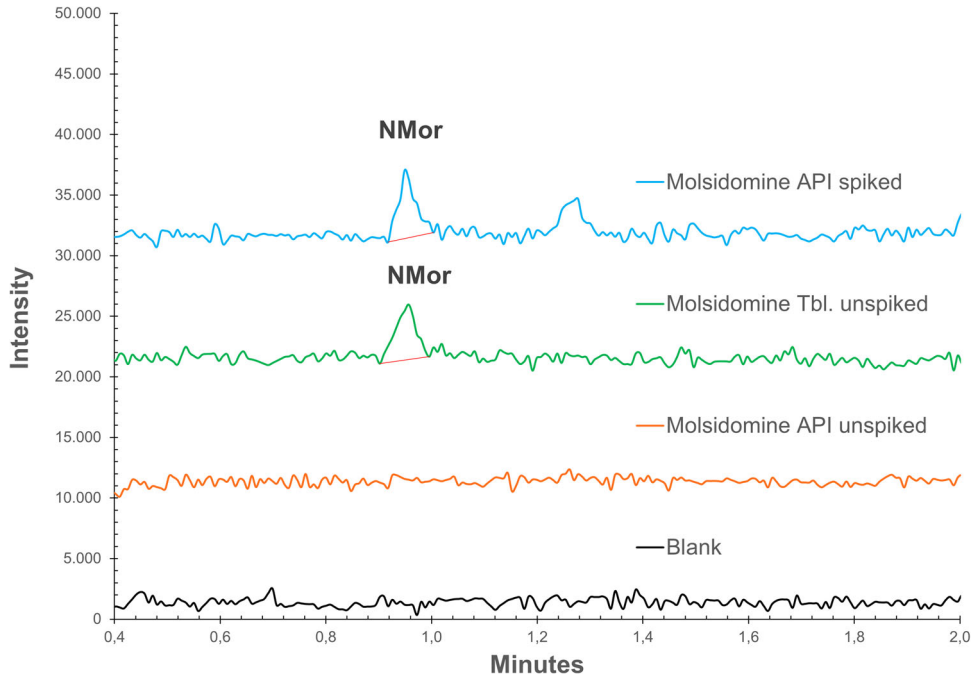


FIGURE 3 Overlay of unspiked molsidomine active-pharmaceutical ingredient (API) and drug product sample, together with a spiked API sample at the toxicological threshold corresponding to 127 ng/day *N*-nitrosomorpholine. Principal SFC-MS/MS transition of NMor displayed (*m/z* 117 > 45).

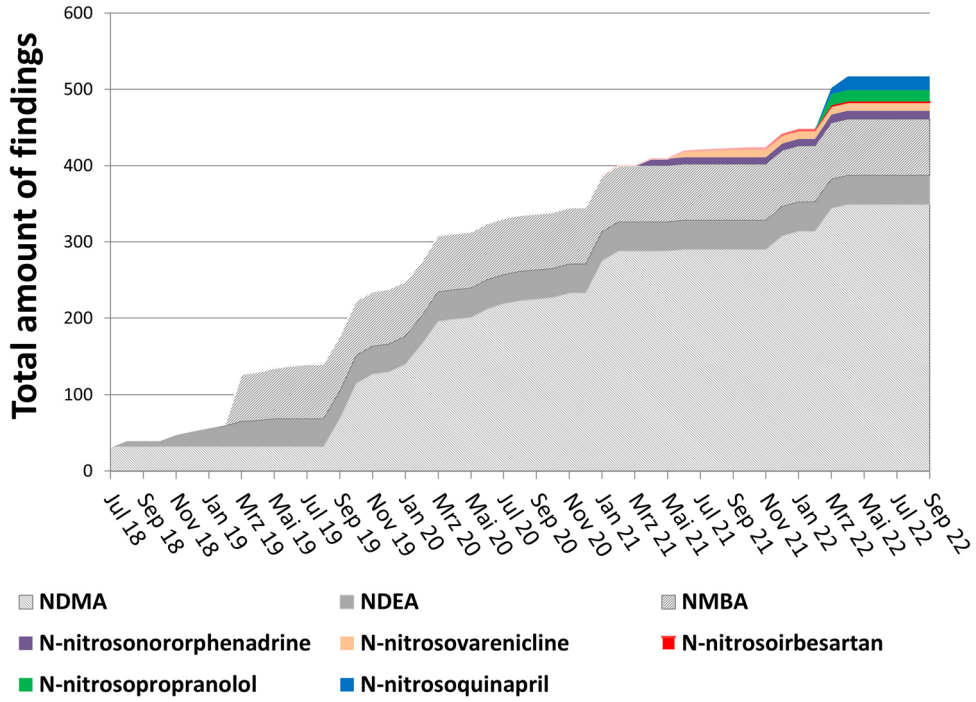


FIGURE 4 Distribution of reported nitrosamine (NA) (in total $N = 523$) findings since July 2018 in the USA and Canada. Since 2021 NDSRIs ($N = 56$) occur with increased frequency and currently account for about 10% of all cases.[24,25]

development. In addition, each new API should be screened for the possibility of NDSRI formation before marketing authorization so that their kinetics can then be investigated during drug product formulation and stability studies. A particular focus should also be placed on limiting nitrite levels, as this appears to be a key determinant of the extent of NA formation.[11]

The NA formation can be suppressed by antioxidants such as ascorbic acid (vitamin C) or alpha-tocopherol (vitamin E).[28,29] Where NA or NDSRI formation in drug product formulations cannot be ruled out, the addition of antioxidants should be considered by MAHs.

3 | CONCLUSIONS

Throughout our study, we have found five samples containing one of the investigated 16 nitrosamines (Table 2). Only two of these positive findings showed NAs above the allowable intake (AI) levels according to EMA and FDA. A new finding was NMor in a molsidomine drug product significantly above the IL. The investigated molsidomine product was still within its shelf life specification at the time of the analysis and was also not recalled. Thus, formation and analysis of NMor should be taken into account with priority. All other investigated samples did not show any NA, indicating that the majority of drug products available in the market are safe for patients in this regard.

Nevertheless, we conclude that the NA crisis is not solved yet. Although recalls no longer lead to persistent supply problems, as was initially the case with sartans, it shows that the issue has been ignored for too long. From our analytical perspective, we further advocate extending the timelines for the NDSRI screenings significantly. Since this problem has been identified for a relatively short time, analytical reference standards must first be procured or synthesized. In addition, test methods must be validated as quickly as possible, which requires even more time. To set proper limits for the NDSRIs individual toxicity testing is required as well.

4 | EXPERIMENTAL

4.1 | Materials

In this study, only MS-grade solvents and additives were used and purchased from VWR International GmbH (Darmstadt, Germany). 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.

The following standards were acquired: *N*-nitrosodiethanolamine (NDELA) and the EPA 8270/Appendix IX Nitrosamines Mix (2000 µg/ml in methanol—Sigma Aldrich Chemie GmbH); *N*-nitrosoethylisopropylamine (NEiPA—EDQM); 1-methyl-4-nitrosopiperazine (MNPaz—Toronto Research Chemicals); *N*-nitrosodiisopropylamine (NDiPA, 200 µg/ml in methanol) and

TABLE 3 Selected reaction monitoring parameters of the 16 investigated nitrosamines

Synonym/ Abbreviation	SRM 1 (Quantifier)/SRM 2 (Qualifier)			Cone voltage	Collision energy
NDMA	75	→	43	38	18
		→	58		12
NMEA	89	→	61	32	14
		→	47		12
NDEA	103	→	47	32	20
		→	57		14
NDELA	135	→	74	16	12
		→	87		8
NEiPA	117	→	75	24	8
		→	43		14
NDiPA	131	→	89	26	14
		→	43		18
NDPA	131	→	43	26	16
		→	89		12
NDBA	159	→	103	30	16
		→	57		18
NMPhA	137	→	66	32	20
		→	77		24
NMEPhA	165	→	77	22	31
		→	51		43
NDPhA	199	→	66	22	30
		→	169		30
NPyr	101	→	55	36	18
		→	59		18
NPip	115	→	69	36	18
		→	55		20
NMor	117	→	45	32	20
		→	57		16
MNPaz	130	→	58	21	17
		→	43		28
NMBA	147	→	44	20	10
		→	117		6

N-nitrosomethylphenylamine (NMPhA—LGC GmbH); *N*-nitroso-*N*-methyl-4-aminobutyric acid (NMBA—Enamine Ltd., Kyiv, Ukraine via Sigma Aldrich).

Supel Carbon LC graphitic carbon columns (100 × 3.0 mm; 2.7 µm—Merck KGaA) were used for analysis.

Drug products and APIs investigated in this study were directly obtained from the European market, with the main origin in Germany.

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 pre-heaters and an Acquity UPC^2 PDA (photodiode array) detector. A fixed-leak interface from the SFC to a Waters Acquity TQD (triple quadrupole mass spectrometer) was coupled with a Waters 515 make-up pump (post-column split) to enhance mass transfer to the MS and to improve ionization. For system control, the Empower 3 software (Feature Release 5, Service Release 4— Waters) was used.

Instrumentation operated fully qualified according to the 4Q model of the U.S. Pharmacopeia (USP) general chapter <1058>[20] in a GMP-regulated laboratory environment.

Chemical structures and exact molecular masses were drawn and calculated by ChemDraw Professional (Version 20.1—PerkinElmer Informatics, Inc.).

4.3 | Instrumental conditions

For highly sensitive targeted detection of nitrosamines, our published SFC-MS/MS method was used.[16] Chromatographic separation was performed on a Supel Carbon column (100 × 3.0 mm; 2.7 µm) at 60°C column temperature and a flow rate of 1.5 ml/min. The back pressure was set to 1800 psi. The gradient method is: CO_2 (carbon dioxide) 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 2.92 min at 75% B, resulting in a total run time with reequilibration of 11.5 min. The injection volume was 2.5 µl. Make-up solvent was a 0.35% solution of formic acid in MeOH at 0.12 ml/min constant flow to transfer the SFC-split to the MS.

The MS/MS operated in positive electrospray ionization (ESI+) mode with timed selected reaction monitoring (SRM). Optimized MS/MS parameters are: 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. Nitrosamine transitions and SRM parameters are listed in Table 3.

Sample preparation was performed with a 5415D lab centrifuge (Eppendorf AG, Hamburg, Germany - centrifugal force: 16.110 rcf; kinetic energy: 3.100 Nm) in 2 ml Safe-Lock tubes (Eppendorf).

4.4 | Sample preparation and analysis

The concentration (mg/ml) for sample preparation was set to one-twentieth of the maximum daily dose (MDD in [mg]—Table 1) per milliliter for the individual API or drug product, as published by the MAHs. Samples were prepared by grinding the solid drug formulation (tablets, capsules, dragees, and granules) with a blade mill. APIs and crèmes were directly prepared without grinding. Solutions (injections,

drops) were diluted in the sample solvent or analyzed directly where the concentration related to the MDD was already in the necessary range. The corresponding amount of samples was transferred to 50 ml amber glass volumetric flasks and dispersed in the sample solvent methanol for 15 min. 2 ml of the homogenous sample suspension were then centrifuged for 5 min at 13.200 rpm (16.110 rcf) and 1 ml of the particle-free supernatant was filled into amber glass vials. In parallel also spiked samples were prepared by addition of a NA stock solution, containing all 16 NAs (Table 2), before extraction with methanol. For this purpose, all samples were spiked at the EMA-published ILs.[3] Unspiked and spiked samples were then analyzed by limit testing and the peak area ratio was calculated according to USP <1469> und Ph. Eur. 2.5.42.[20,21]

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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4 Declaration of Own Contribution

The following list displays the author's contribution to the publications listed under "Manuscripts".

Manuscript I

- Conceptualization in cooperation with co-authors
- Methodology in cooperation with co-authors
- Method development and optimization
- Data curation and visualization
- Preparation of manuscript (original draft & revision)

Manuscript II

- Conceptualization in cooperation with co-authors
- Methodology in cooperation with co-authors
- Method development and optimization
- Data curation and visualization
- Preparation of manuscript (original draft & revision)

Manuscript III

- Conceptualization in cooperation with co-authors
- Methodology in cooperation with co-authors
- Method development and investigation
- Data curation and visualization
- Preparation of manuscript (original draft & revision)

Manuscript IV

- Conceptualization in cooperation with co-authors
- Methodology in cooperation with co-authors
- Investigation
- Data curation and visualization
- Preparation of manuscript (original draft & revision)

5 Discussion and Outlook

This work provides insights into method development, application and continuous improvement of analytical methods to investigate potential NA contaminations. It covers the entire lifecycle of an analytical method [95] and demonstrates science and compliance in terms of state-of-the-art analytical chemistry. Therefore, the methodology and the results obtained are essential for research in NA analysis and drug safety, but will also have impact on future authorization of drugs. The findings and knowledge from the publications in this thesis are discussed concerning their benefits and limitations in NA analysis and formation.

The first step in detecting and identifying NAs is the use of suitable and appropriate analytical techniques respectively the development of those. With the onset of the "nitrosamine crisis", it was rapidly realized that no suitable analytical methods were available for the examination of potentially contaminated drug substances or drug products, as formation of NAs in sartans was never considered. For this reason, in 2018 the “Official Medicines Control Laboratories” (OMCLs), a network of public institutes in the EU responsible for controlling the quality of medicines, and the U.S. “Center for Drug Evaluation and Research” (CDER) quickly started to set up ad-hoc projects to develop such methods [96-99]. Since initially only NDMA- and NDEA-contaminated valsartan was in focus, laboratories limited themselves to this for the time being. Another disadvantage of the published methods of the OMCLs and the CDER was that they introduced an additional testing method to the existing pharmacopeial tests from Ph. Eur. and USP. These first had to be established as a supplementary method in a cumbersome manner in the MAHs or their contract manufacturing laboratories.

In parallel, work on Manuscript I „Simultaneous detection of nitrosamines and other sartan-related impurities in active pharmaceutical ingredients by supercritical fluid chromatography“ [100] began, since holistic and efficient methods were needed rapidly (section 3.1). For this purpose a total of ten NAs were selected, instead of just the two NAs monitored at that time (NDMA and NDEA). This proved immensely beneficial at a later stage, as the list of elucidated NAs expanded swiftly (**Figure 6**), which made the previously published methods of the OMCLs and the CDER insufficient. The reason for directly incorporating more NAs from the beginning was that these are well-known

contaminants from the environmental, food and chemical industries. For example, the U.S. Environmental Protection Agency (EPA) lists seven additional NAs in their established testing routine instead of only NDMA and NDEA [73]. So it seemed evident that the “nitrosamine crisis” might not be limited to these two NAs only, which later proved correct. In order not to provide the laboratories of the MAHs with an additional method, the "Analytical Target Profile" (ATP) [101] of the new method to be developed was selected in such a manner that the analysis of ten NAs could be performed simultaneously with the analysis of the related substances (purity analysis). Thus, only one analytical run had to be carried out, instead of two separate runs.

For this purpose, sub-/supercritical fluid chromatography (SFC) was chosen as the analytical technique, because of its capability to separate a wide spectrum of hydrophobic as well as polar compounds [78, 86, 91]. Method development was conducted according to ICH Guidelines Q8 and Q14 [102, 103] using the quality-by-design (QbD) concept, so that a multi-variate method optimization (chromatography as well as spectrometry) was performed and a broad spectrum of non-polar and very polar valsartan- and losartan-related substances were separated in less than 20 minutes. In addition, all ten NAs were detected in the parts-per-billion (ppb; ng/g) range simultaneously.

At the same time, from a regulatory point of view the many scientific advantages of this method were also compliance disadvantages, since it would have led to a post-approval change process to the registration dossier, in which the release-testing method for the related substances is explicitly described. Submitting this change and having it approved by all responsible authorities would have taken months and was too cumbersome in the first acute phase of the “nitrosamine crisis”. Nevertheless, the published method shows that state-of-the-art development in the pharmaceutical field, which is highly reluctant to embrace development and improvements, is possible in a very short time and benefits for the users are enormous. Applying this type of method development prior to regulatory approval, allows its advantages to be exploited even more effectively. Furthermore, during the early phase of the recalls and the uncertainty towards API suppliers offering potentially contaminated valsartan or losartan, the method was able to provide important analytical results in a GMP contract lab that prevented the processing of contaminated APIs several times during purity assessment. Additionally, the method was the only holistic and comprehensive method published at the time, which made it possible to analyze the discovered NAs without constantly

revalidating the adapted method. For this reason the EMA acknowledged this research as an outstanding approach with “advantages for routine control of *N*-nitrosamine[s]” in their preliminary assessment report on “Nitrosamine impurities in human medicinal products” in June 2020 [17].

Due to regulatory requirements and the ever-growing list of potential NAs (**Figure 5**) [54], which were repeatedly found not only in drug substances but also in drug products, it was inevitable to further develop the existing SFC-MS/MS method. Since the previous development was performed under a QbD approach, an adaptation could be made without a complete new development in the context of lifecycles management (LCM) of analytical methods [43, 95, 104, 105]. Instead, the original ATP of the method was adapted: A simultaneous determination of related substances was discarded, as this was no longer desired. The list of NAs to be investigated was increased from 10 to 16. In addition, the new aim now was to re-develop a method that can be used universally (not only for valsartan and losartan) and that can also analyze drug products in addition to APIs. The steps taken to achieve this were finally published in Manuscript II "Analytical lifecycle management for comprehensive and universal nitrosamine analysis in various pharmaceutical formulations by supercritical fluid chromatography" (section 3.2) [106].

Particular importance was assigned to the choice of the stationary phase. From the development described in Manuscript I [100] it was known that the polar, short-chain, aliphatic NAs eluted almost with the dead volume. Furthermore, the isomers NDiPA and NDPA (*N*-nitrosodi-*n*-propylamine) could not be separated by the initial method. Since more polar NAs such as NMBA and NDELA now had to be integrated, PGC (Porous Graphitic Carbon) [107] was chosen as the stationary phase chemistry, which is able to strongly retain polar as well as non-polar substances [108-110] and is also capable of separating isomers [111-113] in contrast to conventional normal- or reversed-phase columns.

The resulting method is able to separate all 16 acidic, basic, neutral, aliphatic or aromatic NAs in under 4 minutes in a single run. All APIs and drug products studied, including seven sartans, metformin, pioglitazone and ranitidine and also combinatory drug products, additionally containing amlodipine, hydrochlorothiazide, vildagliptin or sitagliptin were successfully separated from the NAs, so that no matrix effects and suppressions occur. The published method [106] is still in routine use in a GMP contract lab today and has been applied to over 400 different APIs and drug products, so

that the proclaimed universality has been practically proven by Manuscript IV once again [114].

Another advantage of the PGC chemistry was demonstrated when the FDA published a rapid communication in July 2020, warning analytical divisions about discrepancies of elevated NDMA levels during metformin analysis [115]. The private testing laboratory "Valisure" had previously raised a "Citizen Petition" to the FDA, informing them that levels of NDMA above the IL were detected in 16 of 38 investigated metformin tablet batches available on the market and advocated the recall of these [116]. After confirmatory tests of the FDA it was shown that the analytical results of Valisure were over- or false-positive findings due to interference with co-eluting dimethylformamide (DMF). The ^{13}C- and especially the ^{15}N-isotopologue of DMF have the same nominal mass as NDMA (**Figure 9**). Thus, they need to be distinguished by the use of high-resolution mass spectrometers (HRMS) or by sufficient chromatographic separation of NDMA and DMF. The method published in Manuscript II is the first method capable of achieving this chromatographically. The FDA in turn was only able to address that issue by the use of TOF (Time-of-Flight) or Orbitrap HRMS instruments, which are rarely used in routine pharmaceutical laboratories, since this method was not able to chromatographically separate NDMA and DMF.

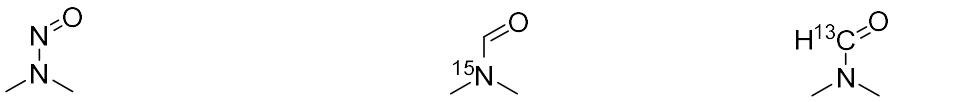

Figure 9: *Mass difference between N-nitrosodimethylamine (NDMA) and dimethylformamide (DMF)*

In a follow-up study, based on the developed SFC-MS/MS method, more than 400 APIs and drug products were tested for NAs in a period of over 2 years until the premature conclusion of the MAHs' investigation period (**Figure 7**). For this purpose, random samples from the European market, with a special focus on Germany, were

collected and analyzed under GMP conditions. From those analyzed samples, which were neither protected by non-disclosure agreements nor clearly traceable to a manufacturer by their ingredients, 249 results were finally published in the Manuscript IV "Prevalence of nitrosamine contaminants in drug samples: Has the crisis been overcome?" (section 3.4) [114].

In 2.0% of the tested samples (N=5) one of the 16 monitored NAs was detected. These included NDMA and NDEA simultaneously in a losartan API, NDMA in a valsartan tablet, NDMA in a metformin tablet and large amounts of NDMA in a ranitidine API. The vast majority of the analyzed samples were free of contamination or at least below acceptable thresholds. This impression is consistent with the published findings of the EMA's "Assessment report on Nitrosamine impurities in human medicinal products", which stated that "it has to be noted that the vast majority [...] were not affected, indicating high probability of process-specific route causes" [17].

For the findings published in Manuscript IV, the root-causes are now understood to a high degree. For example, after the initial findings of high levels of NDMA in products containing ranitidine, a global suspension of ranitidine drug products was triggered [75, 117]. The API itself tends to undergo intramolecular degradation in which NDMA is then formed by rearrangement, resulting in increasingly higher NDMA amounts after long-term storage [60, 118]. In metformin samples minor amounts of NDMA were also found. It seems plausible that the reaction of dimethylamine, a synthesis educt and degradation product, with a NOX source (mainly nitrite) from the excipients during wet granulation and coating by heat and mechanical stress occurred [52, 119]. For the sartans, the synthesis- and process-related reasons have already been adequately described [2, 17, 60, 120].

Nevertheless, an additional new finding was reported in Manuscript IV that is not reported yet elsewhere: a contamination of molsidomine tablets with *N*-nitrosomorpholine (NMor). The tablet contained significantly more NMor than the tolerable IL of 127 ng/day [54]. *N*-Nitrosomorpholine is not a typical NA in the pharmaceutical field unlike NDMA and NDEA, but it is a known hepato-cancerogenic NA [28, 121, 122]. It can be found in drinking water [123], tobacco [124] or artificial snow spray [125]. Besides the fact that NMor was found above the tolerable IL, it is particularly problematic that NMor was already mentioned in the "Assessment report on Nitrosamine impurities in human medicinal products" of the EMA in June 2020 13-times in 90 pages [17], but the incorporation into the "Questions and answers for

marketing authorisation holders" guideline with a specific IL [54] inexplicably took over a year. Thus, it was most likely not on the focus of most regulatory agencies and contract analytical laboratories. Therefore, it may have been overlooked in step 2 of the risk assessment procedure, as the MAH are very much aligned with these guidelines. Nevertheless, in the Manuscripts I, II and IV it was part of the published methods and was included in the targeted screening at all times.

As the origin for NMor occurrence, a reaction of morpholine, a degradation product of molsidomine, with a NOX from the excipients is suspected (similar to the occurrence of NDMA in metformin). In a parallel tested molsidomine drug substance, which was more than 10 years older and out of shelf life, no NMor was detected, so that simple degradation can be excluded.

In fact, excipients are increasingly coming into focus as a cause and starting point for nitrosation. In particular, trace levels of nitrates and nitrites are found in many excipients like polyvinylpolypyrrolidone (crospovidone), magnesium stearate, polyvinylpyrrolidone (PVP, povidone), hypromellose (HPMC), microcrystalline cellulose, pre-gelatinized starch and lactose [17, 126, 127], which are some of the most commonly used excipients for solid dosage forms in the recently approved new drug product formulations [128]. Other causes, such as reactions of packaging materials (e.g. nitrocellulose lidding foils) with amine-containing printing ink, can also lead to leachable NAs that can migrate into the drug product [119, 129].

So the legitimate question remains whether the NAs reported so far in drug substances and drug products are not just the tip of the iceberg. These have formed during synthesis, processing or storage from the reaction of a NOX with a short-chain, aliphatic or aromatic amine and are therefore very process-specific. However, in addition to solvents or starting materials in the finished product, there is an even more substantial amine source that can neither be avoided nor replaced: The API itself.

Since 2021, dozens of batches have already been recalled due to findings of *N*-nitrosodesmethylorphenadrine, *N*-nitrosovarenilcine, *N*-nitrosopropranolol and *N*-nitrosoquinapril (**Figure 8**) and currently account for 10% of all recalls affected by NAs in Canada and the USA [7], where freely accessible lists of all recalls are available.

For this reason, in November 2021, in parallel with the ongoing work on Manuscript IV, a nitrosation assay was developed, which finally culminated in the publication of Manuscript III: „Risk assessment for nitrosated pharmaceuticals: A future perspective in drug development“ (section 3.3) [7]. For this purpose, the WHO

Nitrosation Assay Procedure (NAP) test [130] was modified to establish a standardizable procedure to study the possibility of the occurrence of NDSRIs (so-called "NO-APIs") in the context of the risk assessments in steps 1 and 2 of the NA referral. The original WHO NAP test was designed to simulate formation of NAs from drugs after joint ingestion of potentially nitrite-containing foods in the stomach. On this basis, an *in vitro* assay was developed to examine the formation of possible NDSRIs under comparable conditions by non-targeted HRMS. In the subsequent study, 67 different drug substances and drug products were subjected to the modified NAP test and tested for possible NDSRIs. Almost half (N=33; 49.3%) of all samples tested showed at least one reaction product, some even multiples simultaneously. It is therefore apparent that, in addition to the very process-specific NAs (such as NDMA and NDEA), a much greater focus has to be directed at NDSRIs in the future, which, in addition to APIs, could also arise from amine-containing excipients such as colorants.

Unfortunately, since there is no universal NA assay that is selective and also highly sensitive to NAs only, the availability of specific nitrosation assays is crucial. The latter can close another knowledge gap about NA formation in order to further reduce the risk for public health. Thus, in combination with the NA referral procedure by EMA and FDA now in progress, the modified NAP test can also be applied during early drug substance and drug product development to increase knowledge about NAs that may occur within shelf life. For example, the previous NDSRI recalls (*N*-nitrosodesmethylorphenadrine, *N*-nitrosovarenilcine, *N*-nitrosopropranolol and *N*-nitrosoquinapril) could have been avoided if appropriate screening had been done prior to approval.

It is well known that NDSRIs can also cause cancer and some of them have a high genotoxic potential [131, 132]. Nevertheless, toxicological data are generally not available for NDSRIs. Therefore, the limit for NAs with unknown genotoxic potential was set even below NDEA (26.5 ng/day), one of the most potent mutagenic NAs. These are limited to a default class limit of 18.0 ng/day, due to lack of experimental carcinogenicity data, although structure-activity relationships (SARs) suggest that most NDSRIs may be significantly less reactive and mutagenic [29, 30, 133]. This highly conservative approach was chosen without incorporating dose-response relationships, which are not well-studied for the entire NA CoC, to establish general ILs applicable to other NAs. It should be noted that it is very likely that this massively overestimates the oncogenic effect of larger bulky NAs, such as the novel NDSRIs. Therefore, it is

questionable whether their complex molecular architecture is equally accessible and susceptible to CYP-induced oxidative metabolism. In addition, SAR analyses of low-molecular weight NAs already demonstrated that some structural features significantly diminish or result in a total loss of the genotoxic effect. Available toxicological data further indicates that about one in five studied NAs (18%) was non-carcinogenic in rodent bioassays [31, 134, 135]. Despite this, the occurrence and prevention of NDSRIs must be given a greater attention, since these are not process-specific and can be formed from a large variety of drug substances [63].

In the meantime, the EMA and FDA have adapted their guidelines accordingly to include these "novel nitrosamines" and now explicitly demand testing [54, 136]. Manuscript III is an early contribution to this procedure. With the respective positive or negative findings, a simple decision can be made as to whether further confirmatory tests are necessary (**Figure 10**):

a) If a tested substance is not prone to the formation of NDSRIs (e.g. in the adapted NAP test), it can be assumed that they will not occur at a later stage of the drug product. The NAP test is a very strong forced degradation test and utilizes a fourfold excess of nitrite in an aqueous-acidic environment, which is the optimal nitrosation condition [8]. This represents a "worst case" scenario that does not prevail in drug products. Thus, in the presence of a negative result, the occurrence of NDSRIs in the drug product can be excluded with the highest probability.
b) In case of a positive result (by e.g. NAP testing), it must be assumed that nitrosation may occur in the drug product. This does not necessarily have to be the case, since the NAP test involves more drastic conditions than those actually present in the finished drug product formulation. Nevertheless, with this knowledge, it is necessary to establish suitable NDSRI reference standards in order to carry out corresponding follow-up tests with confidence and to ensure unambiguous detection and identification of the potential reaction product.

As it has been shown by the NAP test in Manuscript III, a large number of drugs are capable of forming NDSRIs. Therefore, more extensive studies should be initiated at least for these, but also for other NAP-positive substances, in order to ensure that no NAs are present in the drug products on the market.

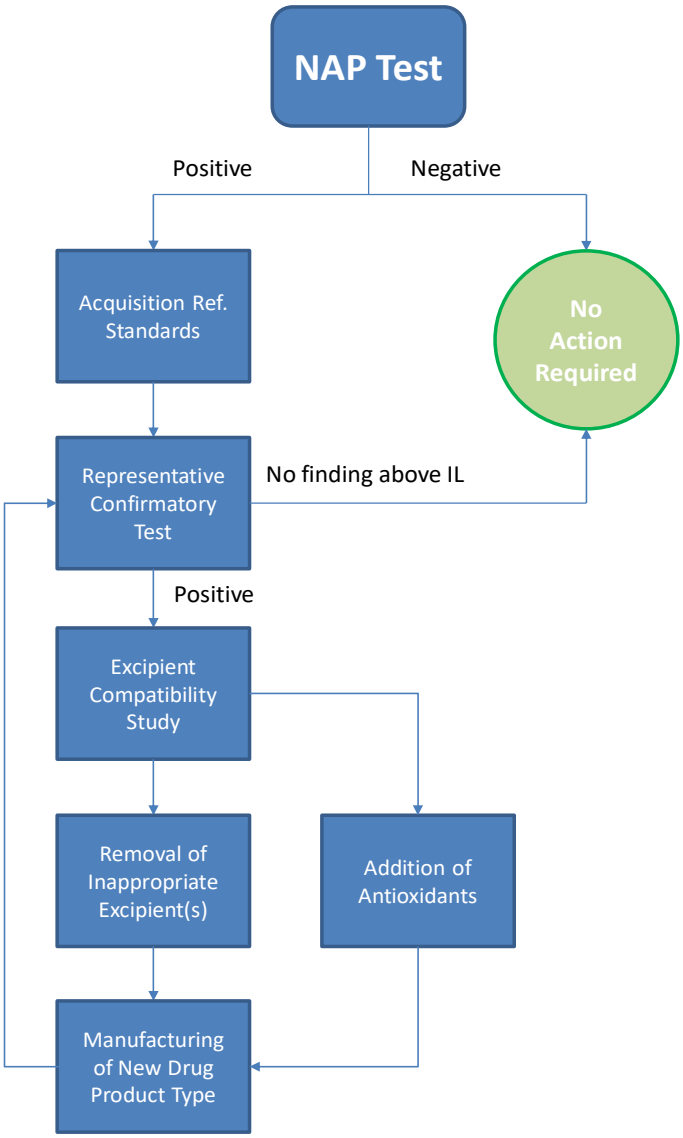

Figure 10: *Decision tree for NAP test application during drug development and post-approval risk assessment*

In the first step, this includes confirmatory tests on representative drug product batches over the entire shelf life. If a finding above the acceptable ILs is discovered, appropriate risk mitigation strategies must be established. If these actions are unsuccessful, the drug product must be withdrawn from the market. To find the root cause of nitrosation, excipient compatibility tests should be carried out with the API and one excipient at a time. If the root cause is found, the involved excipient can be replaced by another or a differently manufactured excipient of the same type with less NOX potential. Alternatively, antioxidants or other inhibitors (e.g. ascorbic acid, cysteine or propyl gallate) can be added, as some studies show that these NOX scavengers can

significantly lower nitrosation [132, 137-140]. This, however, requires additional stability, dissolution and bioequivalence studies to maintain the regulatory compliance status.

In addition, it should be considered whether a nitrosation assay, such as the one presented in Manuscript III, could be established as a mandatory part of the forced degradation and stability tests required by ICH Guideline Q1A "Stability Testing of new Drug Substances and Products" [141] prior to registration. It is now apparent that more and more potential drug-related nitrosation products are mentioned in the guidelines [54], even though they have not yet led to major recalls (**Table 2**).

Table 2: *Excerpt from the current EMA guideline EMA/409815/2020 [54] with known NDSRIs and their source*

***N*-Nitrosamine**	**Source**
N-Nitrosodabigatran	Dabigatran
N-Nitrosoduloxetine	Duloxetine
N-Nitrosofluoxetine	Fluoxetine
N-Nitrosomefenamic acid	Mefenamic acid
N-Nitrosomethylphenidate	Methylphenidate
N-Nitrosonortriptyline	Amitriptyline, Nortriptyline
N-Nitrosoparoxetine	Paroxetine
N-Nitrosorasagiline	Rasagiline
7-Nitroso-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-*a*]pyrazine	Sitagliptine
N-Nitrosovarenicline	Varenicline

Furthermore, once NAs become known, the root cause must be investigated at a far more systematic level and focus must be placed on ensuring that the process leading to the formation is not only identified but also controlled continuously (e.g., by QbD in drug manufacturing).

An excellent example of this has been presented by Schlingemann et al. [119], who systematically investigated the formation of NDMA in different metformin drug products. The investigations included synthesis and crystallization of the API as well as blending, granulation, drying, sieving, tableting and coating of the final formulation. Additionally, primary and secondary packaging was investigated to incorporate formation of NDMA during long-term storage. Similarly, the quality of excipients must receive greater attention when it comes to the formation of NAs. In this regard, the preliminary work of Boetzel et al. [127] is an outstanding achievement. They have started a data sharing initiative for common excipients and built up a ready-to-use database that systematically compares nitrite values of hundreds of excipient batches.

On the basis of Manuscripts III and IV, which were built on the preceding Manuscripts I and II, the question as to whether the "nitrosamine crisis" has now been overcome must be answered in the negative. It is probable that no acutely toxic amounts of NAs are present in drug products for the most part, but their general prevalence is very likely, higher than originally suspected and has been ignored for far too long. One reason for this could be that suitable analytical methods and screening procedures were not available and the problem was therefore overseen, or perhaps there was an absence of hazard awareness, so that suitable methods were not developed as a result. Unfortunately, this is probably accompanied by a not inconsiderable degree of negligence and possibly criminal activity of the Chinese manufacturer of valsartan. As excerpts from a whistleblower reveal, the analytical department was aware of the presence of NDMA long beforehand. However, warnings to management were ignored and downplayed internally [48]. This could explain why the manufacturer's initial investigation, after a European customer became aware of a possible contamination, proceeded so fast [2].

The increased awareness that has now arisen as a result of the "nitrosamine crisis" must therefore be used so that MAHs, regulatory agencies and especially patients can benefit from it in a long-term and sustainable way. It is imperative that the lost confidence of patients in the innocuousness of their prescribed medicines must be restored. In addition, the public disgrace caused by the “nitrosamine crisis” must also be eradicated. Confidence in the safety and quality of public health care is one of the highest goods we have, and it is invaluable.

As Tuesuwan and Vongsutilers [59] have stated: “[…] current recommendations from regulatory authorities serve as useful guidance for pharma industries on how to

deal with nitrosamine contamination, risk identification remains one of the biggest challenges in the risk assessment towards this global concern." With the published manuscripts, a substantial contribution was made to the broadening of the horizon of identification and surveillance, so that manufacturers and their service providers can reduce and avoid NAs in the long term. It is not possible to screen for everything, but we should be careful and have to see the red flags.

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7 List of Figures

8 List of Tables

9 List of Publications

9.1 Publications in scientific peer reviewed journals

Parr, M.K., Schmidtsdorff, S. & Kollmeier, A.S. Nahrungsergänzungsmittel im Sport – Sinn, Unsinn oder Gefahr? *Bundesgesundheitsblatt* **60**, 314-322 (2017). https://doi.org/10.1007/s00103-016-2498-1

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9.2 Presentations at international conferences and forums

Schmidtsdorff, S., Stanic, M. & Schmidt, A.H. Simultaneous detection of nitrosamines and other sartan-related impurities in active pharmaceutical ingredients by supercritical fluid chromatography. LC/MS and Hyphenation – HPLC 2019 Milan (2019) Poster P383

Schmidtsdorff, S., Stanic, M., Schmidt, A.H. & Parr, M.K. Structure assisted impurity profiling for rapid method development in liquid chromatography. Retention and Selectivity – HPLC 2019 Milan (2019) Poster P483

Schmidtsdorff, S. UPLC-TOF Platform for *N*-Nitrosamine Investigation in Pharmaceuticals – Waters' Genotoxic Impurities: Nitrosamines webinar (07.12.2021)

Schmidtsdorff, S. State-of-the-art Untersuchung von (neuen) Nitrosaminen in Arzneistoffen und Fertigprodukten mittels SFC-MS – Avantor Chrom Forum (29.09.2022)

Schmidtsdorff, S. & Schmidt, A.H. ICH Q14 - Entwicklung analytischer Methoden - Von QbD zum Life-Cycle-Konzept – Vortragsreihe für Concept Heidelberg (14.-16.02.2023)

10 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.