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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

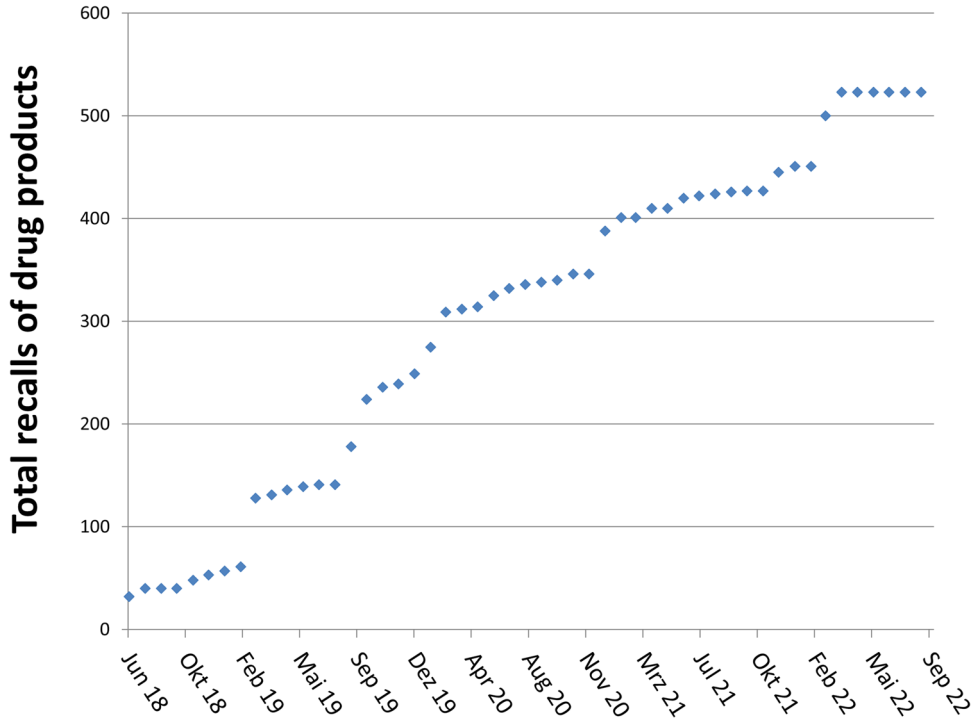


FIGURE 1 Total cumulative nitrosamine findings since July 2018 in the USA and Canada.[24,25] A flattening of the occurrence frequency cannot be observed.

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

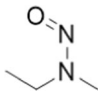
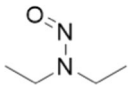
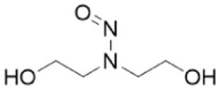
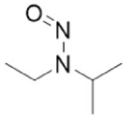
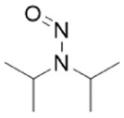
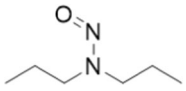
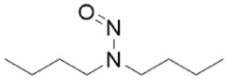
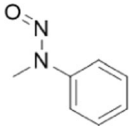
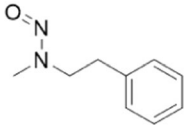
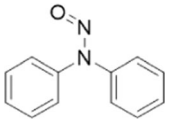
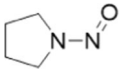
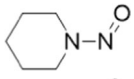
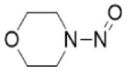
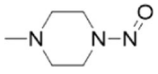
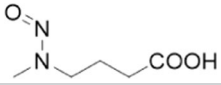
Abbreviations: API, active pharmaceutical ingredient; XR, extended-release.

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.

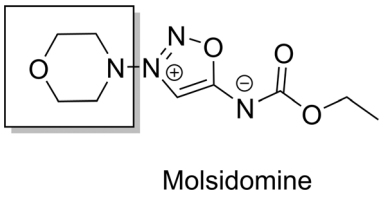


FIGURE 2 Structure of the nitric oxide donor molsidomine. Molsidomine contains a morpholine in the substructure, which can react in trace amounts to form *N*-nitrosomorpholine (NMor).

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

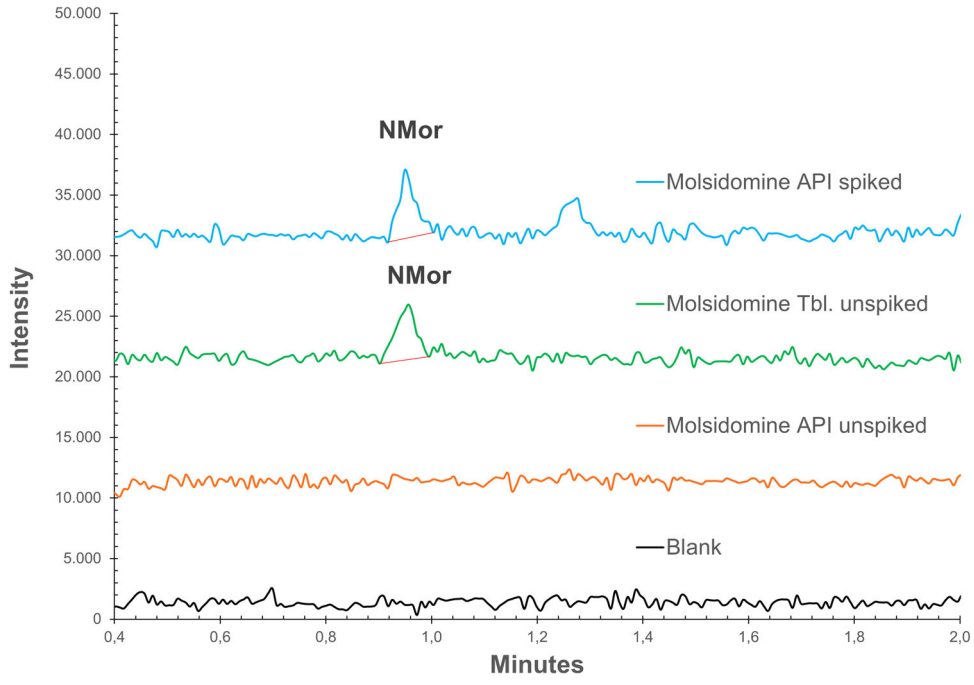


FIGURE 3 Overlay of unspiked molsidomine active-pharmaceutical ingredient (API) and drug product sample, together with a spiked API sample at the toxicological threshold corresponding to 127 ng/day *N*-nitrosomorpholine. Principal SFC-MS/MS transition of NMor displayed (*m/z* 117 > 45).

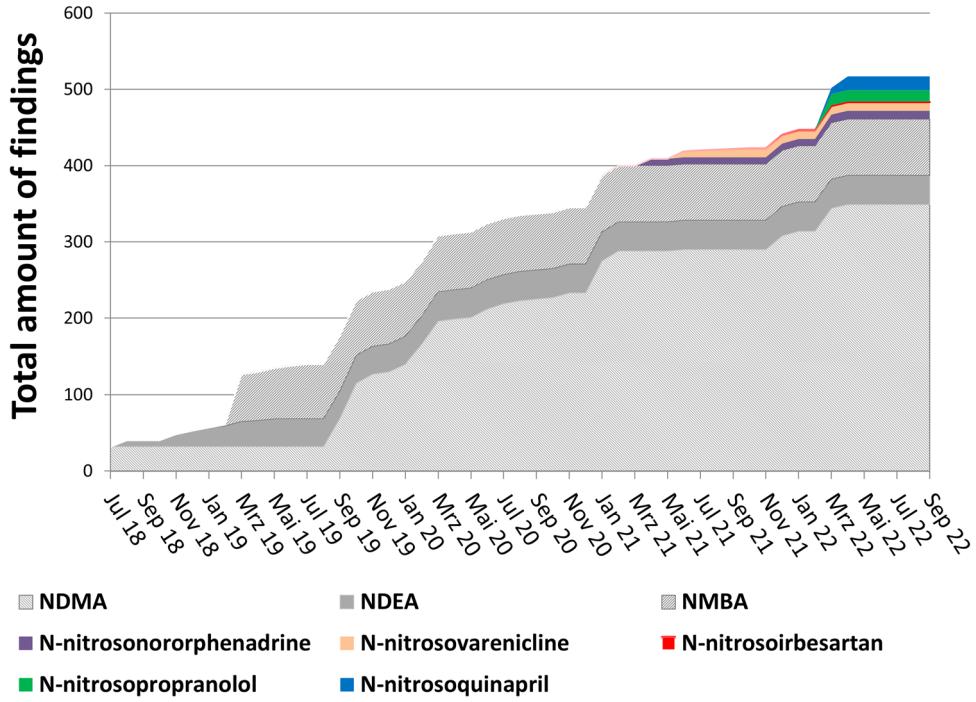


FIGURE 4 Distribution of reported nitrosamine (NA) (in total $N = 523$) findings since July 2018 in the USA and Canada. Since 2021 NDSRIs ($N = 56$) occur with increased frequency and currently account for about 10% of all cases.[24,25]

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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