avantor™

Chromatography Solutions

Chromatography white paper

Matt James, Senior Research Scientist
Tony Edge, Site Director (Production & R&D)

Determination of nitrosamine impurities in pharmaceutical API's and addressing issues associated with DMF and NDMA co-elution.

ABSTRACT

In 2018 N-nitrosodimethylamine (NDMA) was detected in a batch of valsartan at levels exceeding ICH acceptable intake limits for mutagenic impurities. Since then, the analysis of nitrosamines has become an intense focus point for the pharmaceutical industry. The identification and low-level determination of nitrosamines in potentially affected materials is challenging and requires the application of highly sensitive analytical techniques. This white paper reviews the evolution of the regulatory landscape and discusses the development of analytical methods for the determination of nitrosamine impurities referenced by regulatory authorities. The development of a separation of these compounds from the active pharmaceutical ingredient (API) is discussed, together with application of mass spectrometry (MS) to ensure that the required detection limits can be reached. Additionally, the potential for interference, notably from N,N-dimethylformamide (DMF), is considered, along with strategies for mitigating the risks of inaccurate quantification that arises.

INTRODUCTION

In July 2018, the pharmaceutical landscape for the manufacture of small molecules changed with the detection of a small mutagenic compound in a batch of valsartan. Valsartan, is a prescription only selective angiotensin II receptor blocker (ARB) drug, used to treat high blood pressure and heart failure. During routine analysis, QC chemists identified the presence of a nitrosamine, N-nitrosodimethylamine (NDMA), and subsequently reported an average level of 66.5 parts per million in affected batches[1], which is high enough to have a detrimental impact on patient safety.[2] Mutagenic compounds damage the genetic information within a cell, causing mutations which may result in cancer. The damage to the cell is caused by interactions with the DNA sequence and the DNA structure. DNA alteration may also result in permanent heritable changes to the somatic cells of the organism or germ cells which can then be passed on to future generations. It is vital that mutagenic compounds such as nitrosamines are detected and their production avoided wherever possible.

Nitrosamines are a class of compound containing a nitroso group bonded to an amine (Figure 1) and were first reported by Barnes and Magee, who found that NDMA produced liver tumours in rats. Subsequent studies showed that of over 300 nitrosamines tested, nearly 90% were carcinogenic to a wide variety of animals.[3] Nitrosamines have since been reported in numerous sources, including environmental samples, drinking water and processed food products. Their potential formation in pharmaceutical drug products was established in the 1970's with the detection of NDMA in aminophenazone. In this case, NDMA was believed to form as an API degradation product, via hydrolysis of the API to release dimethylamine, followed by nitrosation in the presence of nitrosating agents.[4] Subsequently, it has been reported that nitrosamines can potentially form during the synthesis of several other APIs, including aminopyrimidine, amitriptyline, chloramphenicol, oxytetracycline, promazine, propoxyphene, chlorpromazine, diphenhydramine, doxylamine, trimipramine, tetracycline, erythromycin, imipramine and methapyrilene.[4,5] The detection of high-levels of NDMA in batches of valsartan drug product in 2018 prompted renewed focus on this class of potentially mutagenic impurities.

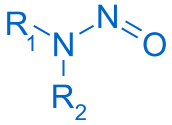


FIGURE 1: Chemical structure of N-nitrosamines.

Nitrosamines can be formed by the reaction of a secondary amine with nitrite (Figure 2), therefore, it is suspected that the NDMA identified in valsartan may originate from the $ZnCl_2$-catalysed disproportionation of DMF to dimethylamine (DMA) and CO. DMA then reacts with sodium nitrite resulting in formation of NDMA. It has also been proposed that carryover of nitrites or amines from subsequent synthetic steps may result in nitrosamine formation. Notably, contamination from external sources has been highlighted as a potential source of nitrosamines, such as the use of recycled solvents.[6] Similar reaction schemes have been proposed for the formation of other nitrosamines that have been

identified during impurity profiling. An additional theoretical source of nitrosamines that should be considered is the formation of higher molecular weight API-like nitrosamines within a drug product. In the case of valsartan, two valsartan specific nitrosamines were confirmed by a single API manufacturer to the EMA, albeit at acceptably low levels.[4]

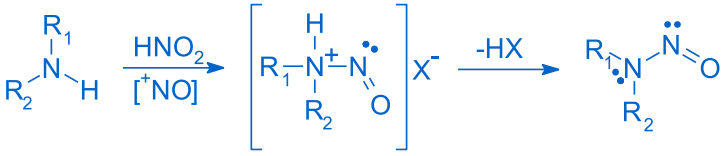


FIGURE 2: Reaction scheme for the formation of N-nitrosamines.

As summarised in Figure 3, the regulatory landscape has evolved very quickly since the first observation of NDMA in valsartan. In September 2020, the FDA released documentation related to controlling nitrosamine impurities in human drugs, which was recently updated in February 2021.[7] The World Health Organisation has used the principles outlined in the ICH M7(R1)[8] guideline to determine acceptable daily intake limits for mutagenic impurities. This information is then used to determine the allowable daily intake of mutagenic impurities for a specific medication.

Since 2018, nitrosamines other than NDMA have been detected in other tetrazole ring-containing sartan drug products, along with other API/medicinal products including ranitidine and pioglitazone. The FDA and EMA have highlighted several nitrosamines, that could be generated during the production process and therefore may potentially exist within drug products. These are highlighted in Table 1, with the designated acceptable daily intake limits.[4, 7, 9] Additionally, it should be noted that the authorities stipulate that any carcinogenic impurity should be assessed in accordance with ICH M7(R1) guidelines, indeed the EMA documentation refers to 16 different nitrosamines that are routinely monitored under EPA guidelines.[4, 10] Separation and detection of these impurities within solvents, intermediates, drug substance and drug product has therefore become of high importance within the pharmaceutical industry.

N-nitrosamine	Abbreviation	FDA limit ng/day	EMA limit ng/day
N-nitrosodimethylamine	NDMA	96,0	96,0
N-nitrosodiethylamine	NDEA	26,5	26,5
N-nitrosoethylisopropylamine	NEIPA	26,5	26,5
N-nitroso-diisopropylamine	NDIPA	26,5	26,5
N-nitroso-N-methyl-4-aminobutyric acid	NMBA	96,0	96,0
1-Nitroso-4-methyl piperazine	MeNP	N/A	26,5
N-nitrosodibutylamine	NDBA	26,5	26,5
N-nitrosomethylphenylamine	NMPA	26,5	34,3

TABLE 1: List of 8 nitrosamines that have defined daily exposure limit by the EMA and FDA. It should be noted that these limits are only applicable if the finished product contains a single N-nitrosamine. For multiple N-nitrosamines a different set of thresholds has been set.

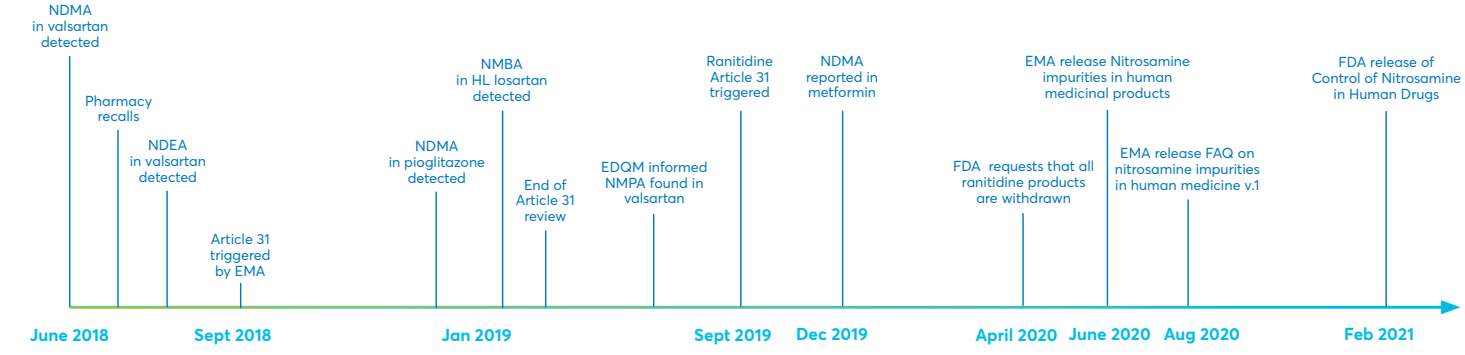


FIGURE 3: Timeline of main nitrosamine events.

Drug	Maximum daily dose (mg/day)	Acceptable intake NDMA (ng/day)	Acceptable intake NDMA (ppm)	Acceptable intake NDEA (ng/day)	Acceptable intake NDEA (ppm)	Acceptable intake NMBA (ng/day)	Acceptable intake NMBA (ppm)
Valsartan	320	96	0,3	26,5	0,083	96	0,3
Losartan	100	96	0,96	26,5	0,27	96	0,96
Irbesartan	300	96	0,32	26,5	0,088	96	0,32
Azilsartan	80	96	1,2	26,5	0,33	96	1,2
Olmesartan	40	96	2,4	26,5	0,66	96	2,4
Eprosartan	800	96	0,12	26,5	0,033	96	0,12
Candesartan	32	96	3,0	26,5	0,83	96	3,0
Telmisartan	80	96	1,2	26,5	0,33	96	1,2

TABLE 2: List of sartan APIs with acceptable daily intakes of nitrosamine impurities, based on the maximum daily dose.

The acceptable daily intake of nitrosamines is related to the maximum daily dose (MDD) of the drug substance, therefore different concentrations of nitrosamines are acceptable for different drugs (Table 2). However, these limits are applicable only if a drug product contains a single nitrosamine. For scenarios where multiple nitrosamines are detected, the maximum daily dose levels can vary,[7, 8, 9] however it should be stated that in all cases the levels are in the order of ng/day which necessitates the need for highly sensitive and selective detection systems to be employed. This has resulted in the routine use of mass spectrometry in the analysis of this class of mutagenic compounds. Although mass spectrometers are used routinely within the pharmaceutical market, they are not typically used for routine quantitative analysis within final product testing. This has resulted in a range of challenges that need to be addressed, that are not present with other detectors, such as UV. This will be discussed in detail later in this article.

This white paper discusses the development of an LC-MS/MS application for the determination of a range of nitrosamines referenced by regulatory authorities, initially showing how to perform a separation of these compounds from the API. It then examines how mass spectrometry can be applied to the analysis to allow the required detection limits to be achieved. Finally, the presence of residual DMF, which has been reported to potentially lead to errors in the quantification of NDMA in drug products, is examined and potential strategies to alleviate the arising issues discussed.

MATERIALS AND METHODS

LC-MS applications were run on a Sciex QTRAP® 6500+ LC-MS/MS system equipped with an ExionLC AD UHPLC system (Sciex, Framingham). LC separations were performed on Avantor® ACE® UltraCore C18 and Biphenyl 3.5 µm superficially porous particle and Avantor® ACE® Excel C18 2 µm fully porous particle columns in 100 x 2.1 mm dimensions (VWR, Lutterworth). Solvents and reagents were purchased from VWR, Lutterworth. NDMA, NDEA, NPIDA, NDPA, NDBA, NMPA, NDMA-d6 and valsartan standards were purchased from VWR, Lutterworth, NMBA and NEIPA from Enamine Ltd, Kiev and NMBA-d3, NDEA-d10 and NDBA-d18 from LGC Ltd, Teddington.

Samples and standards were prepared according to a method described in the proposed USP general chapter on the analysis of nitrosamine impurities.[11] A standard containing 200 ng/mL of NDMA, NMBA, NEIPA, NDIPA, NDPA, NMPA and NDBA was prepared, along with a 132 ng/mL solution of NDEA. The internal standard solution contained 10 µg/mL NDMA-d6 and NMBA-d3 and 1 µg/mL NDEA-d10 and NDBA-d18. L1-L7 calibration solutions were prepared as per Table 3. The spiked valsartan sample was prepared by spiking 80 mg of drug substance with 6 and 9 µL of the respective standard solutions and 12 µL of internal standard solution, followed by addition of 1173 µL 1% formic acid (aq) to extract nitrosamines, whilst precipitating the valsartan. The sample was vortexed at 2500 rpm for 20 minutes and then centrifuged at 6,000 rpm for 20 minutes, then filtered using a Whatman Mini-UniPrep 0.45 µm PVDF syringeless filter.

Concentration level	Concentration of NDMA, NMBA, NDBA, NEIPA, NDIPA, NMPA, NDPA		Concentration of NDEA	
	ng/mL	ppb*	ng/mL	ppb*
L1	1,33	19,95	0,66	10
L2	2	30	0,88	13,5
L3	5	75	3,3	49,5
L4	7,5	112,5	4,95	74,25
L5	10	150	6,6	99
L6	15	225	9,9	148,5
L7	30	450	19,8	297

* with respect to 66.67 mg/mL drug substance

TABLE 3: Concentrations of nitrosamines in the calibration solutions used to construct the calibration curves.

DEVELOPING AN LC-MS METHOD FOR THE ANALYSIS OF NITROSAMINES

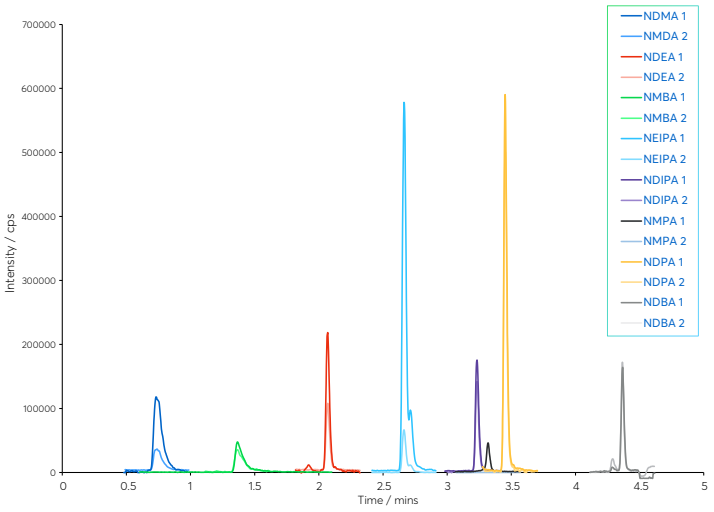
The separation of nitrosamines can be achieved by reversed-phase LC (RPLC), although given their hydrophilic, polar nature, retention of some nitrosamines can be challenging.[12, 13] Various stationary phases have been utilised to obtain satisfactory retention, with C18 or pentafluorophenyl (PFP) phases often specified. The proposed USP general chapter on nitrosamine impurities, currently under review (September 2021), provides monograph methods for the use of both these LC phases.[11] The FDA additionally specifies the use of an Avantor® ACE® C18-AR phase with enhanced aromatic selectivity for the determination of NDMA in ranitidine.[14, 15]

As previously stated, the low detection limits required necessitates the use of highly sensitive and highly specific detectors, such as mass spectrometry, although the low molecular weight of nitrosamines and their relatively low hydrophobicity can make developing a sensitive and robust method somewhat challenging. The use of LC-MS/MS has become increasingly common, with nitrosamine detection, quantification and confirmation achieved through monitoring analyte specific MRM (multiple reaction monitoring) transitions. Alternatively, LC-high resolution MS (LC-HRMS) has also been employed to provide highly specific detection and quantification.

To demonstrate the applicability for the high sensitivity quantification of nitrosamines in drug substances, an LC-MS/MS method for the determination of six nitrosamines (NDMA, NDEA, NMBA, NEIPA, NDIPA, NDBA) in selected sartans, specified in the proposed USP general chapter on the analysis of nitrosamine impurities, was used as a starting point.[11] The monograph method specified the use of a C18 column and uses a 12.2 minute gradient profile, giving a cycle time of 18-24 minutes (assuming 5-10 column volumes of mobile phase for post-gradient re-equilibration). A previously developed LC-UV separation, using non-MS compatible mobile phases, demonstrated that the Avantor® ACE® UltraCore C18 solid core column provides excellent resolution of nitrosamine impurities.[16] This column was therefore selected to develop the LC-MS/MS method, targeting a >50% reduction in overall cycle time compared to the USP method. In addition to the six nitrosamines specified in the USP method, NMPA (identified as a nitrosamine of concern by EMA and FDA) and NDPA (isobaric to NDIPA) were also included.

The LC separation was refined on a 100 x 2.1 mm Avantor® ACE® UltraCore 3.5 µm C18 column.[13] Formic acid is widely used in the mobile phase for nitrosamine analysis by LC-MS. Ngongang et al. reported an increase in MS signal intensity for five out of nine nitrosamines tested over non-buffered mobile phase, with 0.1% formic acid proving optimal for lower response nitrosamines.[17] Under acidic mobile phase conditions, the analytes have log D values ranging from -0.17 to 5.30, therefore gradient elution is required. An initial isocratic hold of 0.2 mins was used to maximise retention of NDMA and NMBA, whilst the remaining analytes were eluted on a 4.0 minute gradient (full LC conditions are detailed in Figure 4). It is worth noting that the peaks for asymmetric NMBA and NEIPA may be observed as doublet peaks of the syn- and anti-conformers in LC analysis, due to hindered rotation around the N-N bond.[18] Resolution of the syn- and anti-conformers for NMBA and NEIPA was not deemed to be necessary in this case, as these would be integrated as a single peak.

Electrospray ionisation (ESI) has been used for LC-MS analysis of nitrosamines, however, it has been reported that atmospheric pressure chemical ionisation (APCI) is preferential for nitrosamine



Column:	Avantor® ACE® UltraCore C18
Dimensions:	100 x 2.1 mm i.d.
Particle size:	3.5 µm
Mobile phase:	A: 0.1% formic acid in water B: 0.1% formic acid in methanol
Gradient:	**Time (min) / % Mobile phase B** 0 / 2.5 0.2 / 2.5 4.2 / 80 4.5 / 80 4.6 / 2.5 7 / 2.5
Flow rate:	0.5 mL/min
Temperature:	40 °C
Inj. volume:	40 µL
MS source parameters:	**Parameters / Optimised value** Ionisation mode / APCI, positive mode Source temperature / 300 °C Curtain gas / 33 psig Ionspray™ source voltage / 5500 V Ion Source Gas 1 / 30 psig Ion Source Gas 2 / - Needle current / 2 µA

FIGURE 4: Example LC-MS/MS separation of nitrosamines spiked into valsartan drug substance at 0.1 ng/mL. Overlayed traces represent the quantifier and qualifier transitions for each nitrosamine (see Table 4).[13]

analysis, providing much improved sensitivity,[4, 19] due to reduced impact from ion suppression from matrix effects compared to ESI. As per the USP methodology, four deuterated internal standards were included for quantification; NDMA-d6, NMBA-d3, NDEA-d10 and NDBA-d18 (NEIPA, NDIPA, NDPA and NMPA are quantitated against NDEA-d10).

The individual transitions specified in the USP method for quantification and confirmation were assessed by direct infusion of reference standard solutions of each compound and were either confirmed or replaced by higher performing transitions. The individual transitions were optimised by infusion (Table 4), whilst MS source parameters were optimised by flow injection analysis, targeting maximisation of the responses for NDMA and NMBA.

Seven-point calibration curves were constructed (Figure 5) by injection of L1-L7 calibration solutions, outlined in Table 3. Excellent data was obtained across the calibration range. The

Nitrosamine Impurity	Optimised MS Parameters			
	MRM	Declustering potential (V)	Collision energy (V)	Cell exit potential (V)
NDMA	+75.0 amu → +43.0 amu	11	19	10
	+75.0 amu → +58.0 amu	11	17	28
NDMA-d6	+81.2 amu → +46.0 amu	40	22	11
	+81.2 amu → +64.1 amu	40	17	12
NMBA	+147.1 amu → +117.1 amu	11	11	12
	+147.1 amu → +87.1 amu	11	17	10
NMBA-d3	+150.1 amu → +120.2 amu	16	11	8
	+150.1 amu → +47.1 amu	21	17	8
NDEA	+103.1 amu → +75.1 amu	16	21	10
	+103.1 amu → +47.1 amu	16	23	22
NDEA-d10	+113.2 amu → +34.2 amu	21	33	6
	+113.2 amu → +49.1 amu	6	23	6
NEIPA	+117.1 amu → +75.1 amu	26	17	10
	+117.1 amu → +47.1 amu	21	23	10
NDIPA	+131.1 amu → +89.1 amu	76	15	10
	+131.1 amu → +47.1 amu	71	23	10
NMPA	+137.1 amu →+66.0 amu	21	23	8
	+137.1 amu →+107.1 amu	16	21	12
NDPA	+131.1 amu → +89.1 amu	16	17	10
	+131.1 amu → +43.1 amu	16	21	10
NDBA	+159.2 amu → +57.1 amu	46	17	10
	+159.2 amu → +103.2 amu	51	15	10
NDBA-d18	+177.3 amu → +66.2 amu	46	23	8
	+177.3 amu → +46.2 amu	41	37	22

TABLE 4: MRM transitions monitored and optimised MS parameters.

accuracy and precision for the method was very good, as detailed in Table 5, demonstrating reproducibility across the calibrated range. In all cases the calibration used was a linear or a quadratic relationship between the ratio of analyte to internal standard and the concentration of the analyte. It should also be noted that a weighting of 1/x was also used for all calibration curves.

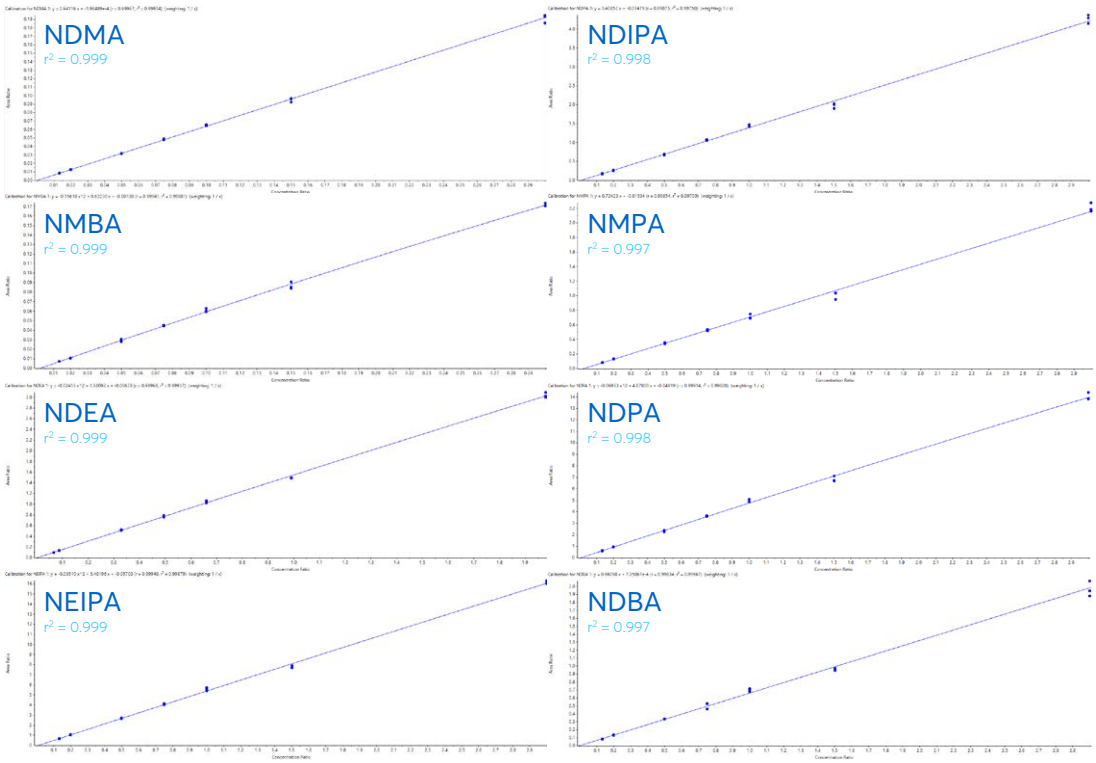


FIGURE 5: Seven-point calibration curves for each nitrosamine, curves used a 1/x weighting factor and either linear or quadratic fit.

Concentration level	NDMA		NMBA		NDEA		NEIPA		NDIPA		NMPA		NDPA		NDBA	
	Accuracy %	% CV	Accuracy %	% CV	Accuracy %	% CV	Accuracy %	% CV	Accuracy %	% CV	Accuracy %	% CV	Accuracy %	% CV	Accuracy %	% CV
L1	99,35	1,91	103,25	0,55	99,53	1,55	99,09	2,92	101,41	4,83	99,74	2,68	99,35	4,93	94,88	2,42
L2	99,56	1,79	95,97	1,71	98,95	2,38	99,24	4,44	98,74	4,30	101,93	1,66	100,00	1,75	99,99	2,87
L3	98,92	1,00	99,18	4,46	101,00	0,84	100,18	2,54	98,88	1,91	100,59	2,23	98,44	3,00	101,33	1,05
L4	101,47	1,72	100,92	1,07	100,87	2,37	101,54	1,36	101,83	1,68	100,65	2,14	101,50	1,10	101,88	7,57
L5	101,84	1,02	102,50	2,92	102,14	2,16	103,37	2,74	103,45	2,01	100,53	4,66	104,05	2,55	105,92	2,78
L6	99,21	2,39	97,98	4,29	97,10	0,38	96,07	1,28	94,18	3,47	94,22	5,11	96,22	3,64	96,86	1,56
L7	99,65	2,52	100,22	1,03	100,42	1,59	100,52	0,97	101,51	2,65	102,33	2,75	100,46	2,38	99,13	4,83

TABLE 5: Accuracy and precision data for the quantifier transitions for each calibration level used to generate the calibration curves.

To further assess accuracy and precision, six injections of a QC sample containing the six nitrosamines specified in the USP method were performed (Table 6). The QC standard was prepared at a concentration of 20 ng/mL, which corresponded to the AI limit for NDMA and NMBA in valsartan. Determined accuracies were within 10% of the expected range, with %CV values all being below 3.1%, demonstrating excellent accuracy and reproducibility.

	Determined concentration (ng/mL)						% Accuracy	%CV
	Inj #1	Inj #2	Inj #3	Inj #4	Inj #5	Inj #6		
NDMA	18,34	18,95	18,26	18,69	18,87	18,49	93,00	1,53
NMBA	19,56	19,97	19,30	19,52	18,87	18,96	96,81	2,12
NDEA	19,03	18,27	17,98	17,87	17,89	18,72	91,37	2,65
NEIPA	19,15	19,75	20,08	18,78	18,54	19,45	96,46	3,02
NDIPA	19,92	20,09	18,94	20,11	19,96	18,97	98,33	2,82
NDBA	19,40	19,00	20,11	19,09	19,20	19,72	97,09	2,19

TABLE 6: Accuracy and precision data for six replicate injections of the QC sample.

Finally, the method was applied to the analysis of valsartan drug substance spiked with nitrosamine standard solutions at a level of 1 ng/mL (equating to 15 ppb of drug substance according to the sample preparation procedure and as such below the lowest AI for a single nitrosamine). A simple extraction protocol was used to extract nitrosamines with 1 % aqueous formic acid, whilst precipitating the valsartan.[9] For the analysis of other drug substances, such as other sartans, ranitidine or metformin, alternative extraction methods may be required to achieve suitable removal of the API.[20, 21, 22]

Figure 4 shows an example MRM chromatogram obtained. Chromatographic resolution is achieved for all analytes and the isobaric analytes NDIPA and NDPA are clearly separated. The syn- and anti-conformers of NEIPA are partially resolved but are integrated as a single peak for quantification. According to Table 2, the AI limits for NDEA in a high daily dose drug substance correspond to 33 ppb, therefore the method clearly shows excellent performance in the low ppb ranges required by regulatory authorities. LOD and LOQ values were estimated based on the signal-to-noise ratio determined from individual MRM chromatograms obtained for the L1 calibration levels and are summarised in Table 7. These values clearly demonstrate the low ppb sensitivity that can be achieved using the tested methodology.

		ng/mL		ppb*	
	S/N (3σ) at L1 level	LOD	LOQ	LOD	LOQ
NDMA	99,1	0,040	0,134	0,60	2,01
NMBA	433,1	0,009	0,031	0,14	0,46
NDEA	197,7	0,010	0,033	0,15	0,50
NEIPA	3199	0,001	0,004	0,02	0,06
NDIPA	1204	0,003	0,011	0,05	0,17
NMPA	401	0,010	0,033	0,15	0,50
NDPA	3428	0,001	0,004	0,02	0,06
NDBA	629,9	0,006	0,021	0,10	0,32

* with respect to 66.67 mg/mL drug substance.

TABLE 7: Estimated LOD and LOQ values.

ASSESSING POTENTIAL INTERFERENCE FROM DMF AND SOLUTIONS TO ENABLE ACCURATE QUANTIFICATION

The analysis of finished drug product (i.e. drug substance and excipients) presents additional analytical challenges, particularly for drug products with a high MDD. The potential for interference from drug substance or excipients and the low detection limits required means that in some cases sample clean-up and concentration approaches, such as SPE, may need to be employed to mitigate the impact of the matrix.[20, 23]

Additionally, interference from other low molecular weight trace impurities could potentially result in inaccurate quantification. It has been reported that co-elution of N,N-dimethylformamide (DMF) with NDMA can result in over-quantification of NDMA. Yang et al,[24] document a case in which a private testing laboratory reported that 16 of 38 metformin drug products tested by LC-high resolution MS (LC-HRMS) contained quantities of NDMA above the AI limit of 96 ng/day. However, subsequent FDA testing of the same samples, reported overall lower values, with

only 8 of the samples determined to contain NDMA above the AI threshold. In the subsequent investigation, it was hypothesised that DMF (molecular weight = 73.09) co-eluting with NDMA (molecular weight = 74.08) caused interference, resulting in the over-estimation of NDMA content and could account for the inter-lab discrepancy in reported values. Specifically, interference from isotopic ions of DMF (^{13}C or ^{15}N) could theoretically result in inaccurate NMDA quantification if the specificity of the analytical approach is not sufficient to distinguish from NDMA. The ^{15}N DMF isotopic ion differs from the NDMA monoisotopic ion by just 0.0016 amu (21 ppm) and could therefore potentially be mistakenly identified as NDMA. The ^{13}C DMF isotopic ion differs by 104 ppm, so is less likely to interfere. Experiments using mass tolerance settings of ±15 and ±30 ppm recorded higher NDMA concentrations in samples containing DMF when the wider tolerance setting was applied. It was concluded that if inappropriate mass accuracy and tolerance settings are applied, the ^{15}N DMF isotopic ion can be miss-identified as NDMA in the LC-HRMS analysis, resulting in over-quantification of NDMA. The potential for interference only occurs when DMF and NDMA co-elute.

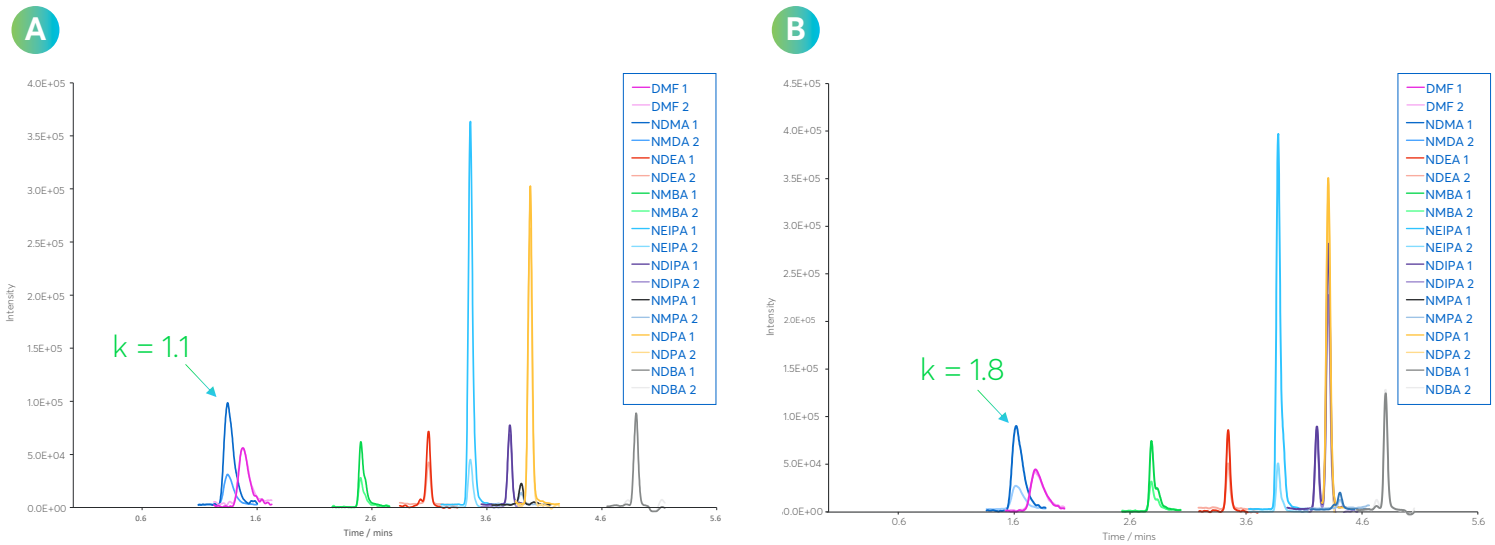
Given the lower mass resolution of triple quadrupole MS compared to HRMS, it was anticipated that if residual DMF was present in API or drug product, then transitions from ^{13}C and ^{15}N DMF isotopic ions could potentially interfere with NDMA quantification in the current method. This was assessed by analysing a series of 1.0 ng/mL NDMA samples, spiked with varying concentrations of DMF (Table 8). The DMF concentrations selected are within the defined residual solvent limits specified in ICH Q3C(R8).[25] DMF was found to co-elute with NDMA and at this low-level concentration, detrimentally impacted the quantification accuracy as shown in Table 8, leading to falsely high calculated NDMA concentrations. This could in particular be impactful in situations where multiple nitrosamines are detected, requiring lower level quantification limits.[7, 8, 9] It was also noted that the *m/z* 75.0 → 58.0 NDMA qualifier transition was affected to a lesser degree than the *m/z* 75.0 → 43.0 quantifier transition.

From this data, it is clear that chromatographic separation of DMF and NDMA would be highly favourable to avoid this issue when using a low mass resolution detector such as a tandem mass spectrometer. Additionally, the ability to monitor drug product and substance for the presence of DMF in the same analytical run to identify samples potentially at risk of NDMA over-quantification would be beneficial.

Both NDMA and DMF showed very low retention on the solid core C18 used in Figure 4, with a retention factor (k) of just 0.3. The hydrophilic nature of both analytes and the low starting percent organic used in the gradient makes obtaining better retention challenging. Varying column stationary phase is a powerful tool by which analyte selectivity and retention can be adjusted, therefore a range of stationary phases were screened to assess whether better retention and separation was possible.[26] Fully porous columns are typically more retentive than their solid core counterparts, due to increased porosity, and therefore larger surface area, of the stationary phase material. By exchanging the solid core column with an Avantor® ACE® Excel 2 C18 fully porous column, it was found that the increased hydrophobicity of this phase improved NDMA retention (k = 1.1). This column was also found to provide additional separation of DMF from NDMA (Figure 6A), and a potential route forward.

				Quantifier *m/z* 75.0 → 43.0		Qualifier *m/z* 75.0 → 58.0	
Spike level	NDMA (ng/mL)	DMF (ng/mL)	DMF (ppm*)	Calculated NDMA Conc. (ng/mL)	% Accuracy	Calculated NDMA Conc. (ng/mL)	% Accuracy
0	1,0	0	0	1,03	102,9	1,07	106,7
1	1,0	83,3	1,25	1,03	103,3	1,04	103,6
2	1,0	833,3	12,5	1,37	137,0	1,15	114,6
3	1,0	1666,7	25	1,64	163,6	1,22	121,6
4	1,0	3333,3	50	2,20	220,0	1,42	141,9
5	1,0	6666,7	100	3,07	306,8	1,60	159,7

TABLE 8: Summary of spiking experiment used to assess potential interference from DMF on NDMA quantification.



Column:	Avantor® ACE® Excel 2 C18
Dimensions:	100 x 2.1 mm i.d.
Particle size:	2 µm
Mobile phase:	A: 0.1% formic acid in water: methanol 98:2 v/v B: 0.1% formic acid in water: methanol 2:98 v/v
Gradient:	

Time (min)	% Mobile phase B
0	0
1	0
2.5	50
5	86.5
5.5	86.5
5.6	0
8.1	0

All other conditions:	as per Figure 4

Column:	Avantor® ACE® UltraCore Biphenyl
Dimensions:	100 x 2.1 mm i.d.
Particle size:	3.5 µm
Mobile phase:	A: 0.1% formic acid in water B: 0.1% formic acid in methanol
Gradient:	

Time (min)	% Mobile phase B
0	1
1	1
5	100
5.4	100
5.5	1
7.8	1

All other conditions:	as per Figure 4

FIGURE 6: Example LC-MS/MS separation of nitrosamines spiked into valsartan drug substance at 0.1 ng/mL on A) Avantor® ACE® Excel C18 and B) Avantor® ACE® UltraCore Biphenyl columns. Overlayed traces represent the quantifier and qualifier transitions for each nitrosamine (see Table 4) and the DMF transitions specified in Table 10.

As an alternative approach, the Avantor® ACE® UltraCore Biphenyl solid core stationary phase was assessed to determine whether an alternative stationary phase selectivity could provide better retention and separation.

As shown in Figure 6B, π-π interactions with the biphenyl phase provided enhanced retention for NDMA (k = 1.8) and DMF and a similar degree of separation to the C18 fully porous phase.

The added retention offered by the biphenyl phase could also potentially prove useful for addressing ion suppression effects that may arise in the analysis of drug products containing very hydrophilic APIs and/or excipients. The gradient conditions were optimised on both columns as shown in Figure 6 and calibration curves and QC samples showed excellent linearity, accuracy and precision. LOD and LOQ values were determined and found to be comparable to data obtained for the previous method (Table 9).

	Method 1 ACE UltraCore C18	Method 2 ACE Excel 2 C18	Method 3 ACE UltraCore Biphenyl
NDMA 1	0,60	0,55	0,47
NMBA 1	0,14	0,07	0,07
NDEA 1	0,15	0,24	0,33
NEIPA 1	0,02	0,02	0,02
NDIPA 1	0,05	0,13	0,13
NMPA 1	0,15	0,24	0,20
NDPA 1	0,02	0,03	0,03
NDBA 1	0,10	0,04	0,04

TABLE 9: Calculated LOD values (ppb) for the three LC-MS/MS method.

		Optimised MS Parameters		
	MRM	Declustering potential (V)	Collision energy (V)	Cell exit potential (V)
DMF	+74.0 amu → +42.0 amu	1	46	12
	+74.0 amu → +30.0 amu	1	33	14

TABLE 10: MRM transitions for monitoring DMF content.

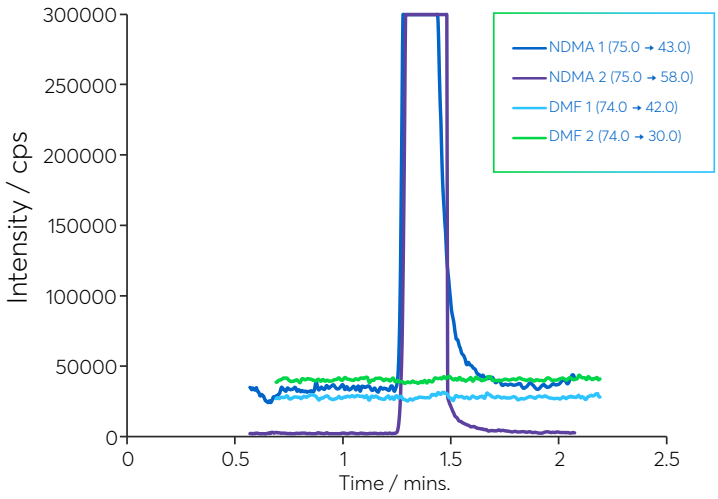


FIGURE 7: NDMA and DMF MRM transitions in a 30 ng/mL solution of NDMA, demonstrating high selectivity of the DMF transition in the presence of NDMA.

For both methods, MRM transitions were selected and optimised to allow for selective monitoring of DMF (Table 10). The transitions were found to be highly selective in the presence of NDMA and could therefore be utilised in any LC-MS/MS approach to monitor the DMF content of real-life samples and identify samples that may be prone to NDMA quantification issues. Figure 7 shows the NDMA and DMF transitions for a 30 ng/mL solution of NDMA. At this high NDMA concentration, no response is seen in either DMF transition, thereby demonstrating the applicability of these MRM transitions to monitor samples for residual DMF.

Both LC-MS/MS methods were then assessed using the spiking approach in Table 8, to determine whether they could be utilised to reduce NDMA quantification errors in the presence of DMF. Figure 8 shows the *m/z* 75.0 → 43.0 NDMA MRM transition on the Avantor® ACE® Excel C18 column and clearly shows the impact of interference from increasing DMF concentrations. Given that the *m/z* 75.0 → 58.0 NDMA transition performed better in the presence of DMF in previous experiments, this transition could therefore be assigned as the quantifier transition for NDMA.

The additional chromatographic resolution of NDMA and DMF provided by both the Avantor® ACE® Excel C18 and Avantor® ACE® UltraCore Biphenyl methods permitted integration of NMDA in the presence of DMF and significantly improved accuracy determination compared to the original method (Figure 9).

The combined approach of monitoring samples, using appropriate MRM transitions, to identify residual DMF and use of a column stationary phase that provides at least partial resolution of NDMA and DMF is recommended. The Avantor® ACE® Excel 2 C18 and Avantor® ACE® UltraCore Biphenyl phases have both been demonstrated to achieve this separation and provide more accurate NDMA quantification at low concentrations by LC-MS/MS analysis. Additionally, these LC columns can be applied to analyses utilising HRMS. The chromatographic resolution provided reduces the risk of isobaric interference and also guards against any potential for ion suppression or enhancement in the ionisation process that may result from co-elution of these two species. The improved retention provided by these phases could also aid in reducing the possibility for interference from other low retention matrix components. Provided suitable mass accuracy and tolerance settings are used, the chromatographic separation provided by these two stationary phases can provide additional safeguards against quantification errors for NDMA.

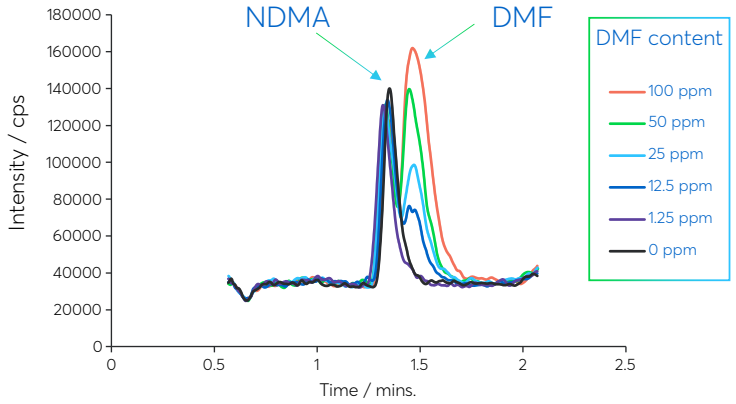


FIGURE 8: NDMA MRM transition (*m/z* 75.0 → 43.0) in 1.0 ng/mL solutions spiked with increasing concentrations of DMF showing increasing interference from DMF as the concentration is increased.

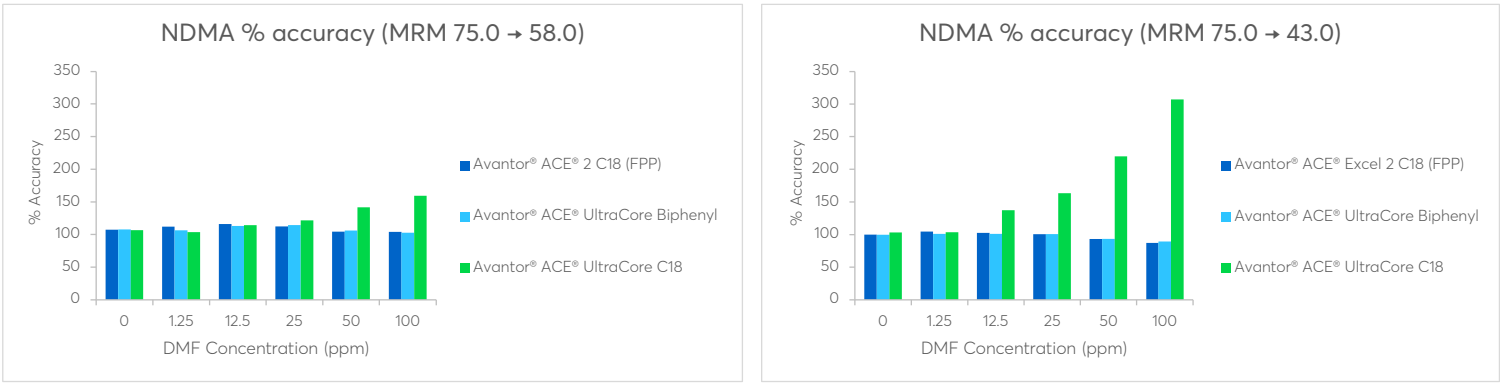


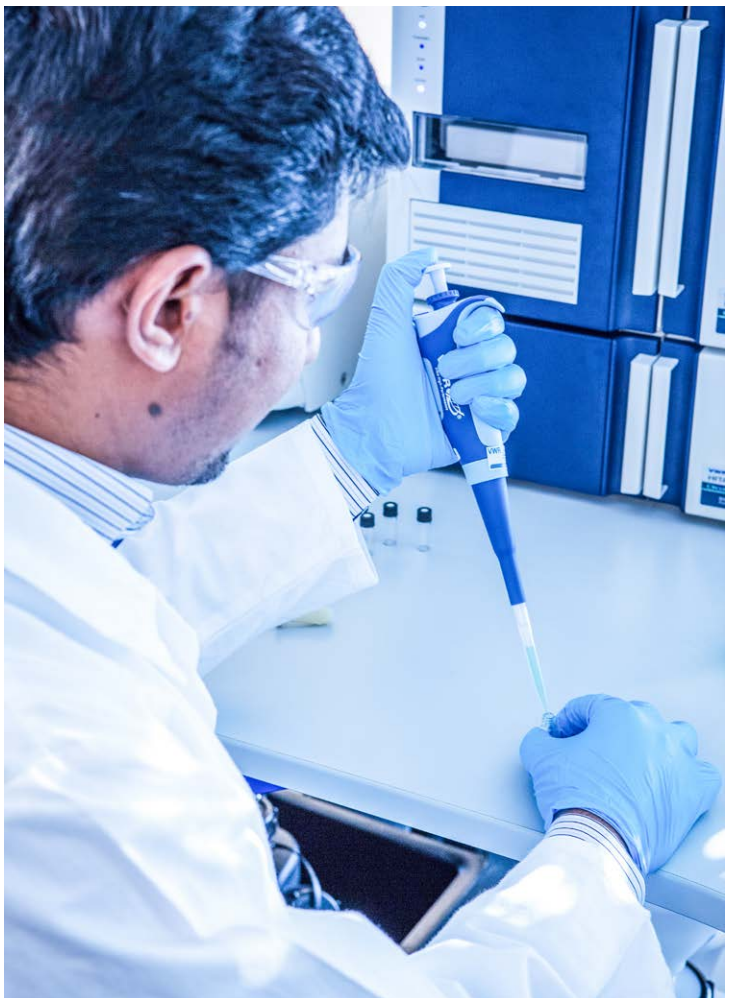
FIGURE 9: Percentage accuracy data for NDMA quantification in the DMF spiking experiment using original method (green) and the alternative approaches on the Avantor® ACE® Excel 2 C18 and Avantor® ACE® UltraCore Biphenyl phases.

CONCLUSIONS

Since the initial discovery of NDMA in valsartan in 2018, detection and quantification of nitrosamine impurities in drug substances has become critical, ensuring the safety of pharmaceutical products. Assessment of nitrosamine contamination risks has been widely implemented to identify and mitigate potential sources of nitrosamine contamination in pharmaceutical products. Where risks are present, analytical determination of nitrosamines is essential to ensure the ongoing safety of drug products. The low acceptable daily intake limits established for nitrosamines, which must be considered against the maximum API daily dose, requires the application of highly sensitive and selective MS detection, capable of quantification at the low ppb level.

This white paper has discussed the application of LC-MS/MS for low-level nitrosamine quantification. A separation of eight nitrosamines was demonstrated on using an Avantor® ACE® UltraCore C18 column. The excellent separation and peak shape provided by the LC column and separation conditions employed readily provides the sensitivity to quantify nitrosamine impurities at the low levels required by regulatory authorities. The method was demonstrated to show excellent accuracy and precision across the calibration concentration range assessed.

The low molecular weight of nitrosamines means increased potential for interference from other co-eluting sample components. Co-elution of DMF with NDMA has been highlighted in the literature as having potential to cause inaccurate quantification of NDMA by LC-HRMS, due to isobaric interference. The potential impact when using a lower resolution triple quadrupole instrument was therefore investigated. Although the Avantor® ACE® UltraCore C18 method was found to be affected by this issue, successful strategies to mitigate the risk of over-quantification of NDMA have been demonstrated using modified chromatographic methodology based on both Avantor® ACE® UltraCore Biphenyl and Avantor® ACE® Excel C18 stationary phases. Additionally, MRM transitions have been established for the selective detection of DMF, which can be used to monitor samples for residual DMF content and flag potentially affected samples.



REFERENCES

1. European Medicines Agency, "Lessons learnt from presence of N-nitrosamine impurities in sartan medicines" June 2020. (Online). Available: https://www.ema.europa.eu/en/documents/report/lessons-learnt-presence-n-nitrosamine-impurities-sartan-medicines_en.pdf (Accessed June 2021).

2. European Medicines Agency, "Update on review of recalled valsartan medicines. Preliminary assessment of possible risk to patients" August 2018. (Online). Available: https://www.ema.europa.eu/en/documents/press-release/update-review-recalled-valsartan-medicines_en.pdf (Accessed September 2021).

3. J. Barnes and P. Magee, Brit. J. Ind. Med. 11(3) (1954) 167-174

4. European Medicines Agency, "Nitrosamine impurities in human medicinal products, Procedure number: EMEA/H/A-5(3)/1490," 25 June 2020. (Online). Available: https://www.ema.europa.eu/en/documents/referral/nitrosamines-emea-h-a53-1490-assessment-report_en.pdf (Accessed March 2021).

5. M. K. Parr and J. F. Joseph, J. Pharm. Biomed. Anal. 164 (2019) 536-549

6. World Health Organization, "Update on Nitrosamine Impurities", April 2020. (Online) Available: https://cdn.who.int/media/docs/default-source/substandard-and-falsified/informationnote-nitrosamine-impurities-april2020.pdf?sfvrsn=4f06ed1b_4&download=true (Accessed September 2021).

7. US Food and Drug Administration, "Control of nitrosamine impurities in human drugs," February 2021. (Online). Available: https://www.fda.gov/media/141720/download

8. FDA/ICH, "M7(R1) Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk," (Online). Available: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/m7r1-assessment-and-control-dna-reactive-mutagenic-impurities-pharmaceuticals-limit-potential

9. European Medicines Agency, "Questions and answers for marketing authorisation holders/applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 referral on nitrosamine impurities in human medicinal products," 23 April 2021. (Online). Available: https://www.ema.europa.eu/en/documents/referral/nitrosamines-emea-h-a53-1490-questions-answers-marketing-authorisation-holders/applicants-chmp-opinion-article-53-regulation-ec-no-726/2004-referral-nitrosamine-impurities-human-medicinal-products_en.pdf (Accessed June 2021).

10. United States Environmental Protection Agency website: https://comptox.epa.gov/dashboard

11. United States Pharmacopeia, "Proposed General Chapter: (1469) Nitrosamine Impurities," September 2020. (Online). (Accessed 2021 February).

12. Avantor® ACE® webinar, "Devising Solutions for Nitrosamine Analysis" (Online). Available: https://uk.vwr.com/cms/webinar_method_development (Accessed September 2021).

13. M. James and T. Edge, LCGC Europe 34(7) (2021) 267-276

14. US Food and Drug Administration, "Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) Method for the Determination of NDMA in Ranitidine Drug Substance and Drug Product," September 2019. (Online). Available: https://www.fda.gov/media/130801/download (Accessed March 2021).

15. US Food and Drug Administration, "Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of NDMA in Ranitidine Drug Substance and Solid Dosage Drug Product," October 2019. (Online). Available: https://www.fda.gov/media/131868/download (Accessed March 2021).

16. Avantor® ACE® Application Note #7250 "Nitrosamine Contaminants in Valsartan API" (Online). Available: https://uk.vwr.com/cms/chromatography_chrom_library (Accessed September 2021).

17. A. D. Ngongang, S. V. Duy and S. Sauve, Anal. Methods 7 (2015 5748-5759)

18. T. W. Iwaoka, T. Hansen, S. T. Hsieh and M. C. Archer, J. Chrom. A 103 (1975) 349-354

19. J.-H. Lee, S.-U. Lee and J. E. Oh, Int. J. Environ. Anal. Chem. 93(12) (2013) 1261-1273

20. H.-H. Lim, Y.-S. Oh and H.-S. Shin, J. Pharm. Biomed. Anal. 189 (2020) 113460

21. O. Scherf-Clavel, M. Kinzig, A. Besa, A. Schreiber, C. Bidmon, M. Abdel-Tawab, J. Wohlfart, F. Sörgel, U. Holzgrabe, J. Pharm. Biomed. Anal. 171 (2019) 278-284

22. F. Sörgel, M. Kinzig, M. Abdel-Tawab, C. Bidmon, A. Schreiber, S. Ermel, J. Wohlfart, A. Besa, O. Scherf-Clavel, U. Holzgrabe, J. Pharm. Biomed. Anal. 172 (2019) 395-405

23. K. M. Shaik, B. Sarmah, G. S. Wadekar, P. Kumar, Crit. Rev. Anal. Chem. (2020) 1-19

24. J. Yang, T. Andres Marzan, W. Ye, C. D. Sommers, J. D. Rodriguez, D. A. Keire, AAPS J. 22 (2020) 89

25. International Council for Harmonisation, "Impurities: Guidline for Residual Solvents" April 2021. (Online). Available: https://database.ich.org/sites/default/files/ICH_Q3C-R8_Guideline_Step4_2021_0422_1.pdf (Accessed September 2021).

26. Avantor® ACE® Knowledge Note 0013 "The Power of Stationary Phase Selectivity" https://uk.vwr.com/cms/ace_knowledge_notes

avantor™ delivered by VWR™

VWR.COM

1021-BB-KS

ZPROAVTR35444-EN