Chromatography Solutions

Technical note #033

Addressing NDMA Over-Quantification, due to Isobaric Interference of DMF in the LC-MS/MS Analysis of Nitrosamines.

INTRODUCTION

In 2018 N-nitrosodimethylamine (NDMA) was detected in a batch of valsartan at levels exceeding acceptable intake limits for mutagenic impurities.[1,2] NDMA is an N-nitrosamine, a class of compound containing a nitroso group bonded to an amine (Figure 1). It was first reported by Barnes and Magee, who found that NDMA produced liver tumours in rats. Subsequent studies showed that, of over 300 nitrosamines evaluated, nearly 90% were carcinogenic to a wide variety of animals.[3]

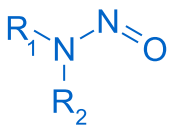


Figure 1: Chemical structure of N-Nitrosamines.

Since 2018, the analysis of nitrosamines has become an intense focus point for the pharmaceutical industry. As summarised in Figure 2, 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.[4] The FDA and EMA have highlighted several nitrosamines that could be generated during the production process and may potentially exist within drug products. These are highlighted in Table 1, with the designated daily acceptable intake (AI) limits.[4,5,6]

Due to the high potential carcinogenicity of nitrosamines, the AIs for finished drug products are in the order of ng/day. The low-level determination of nitrosamines is therefore challenging and requires the use of highly sensitive and selective detection systems. The analysis of finished drug product (i.e. drug substance and excipients) presents additional analytical challenges. 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.[7, 8]

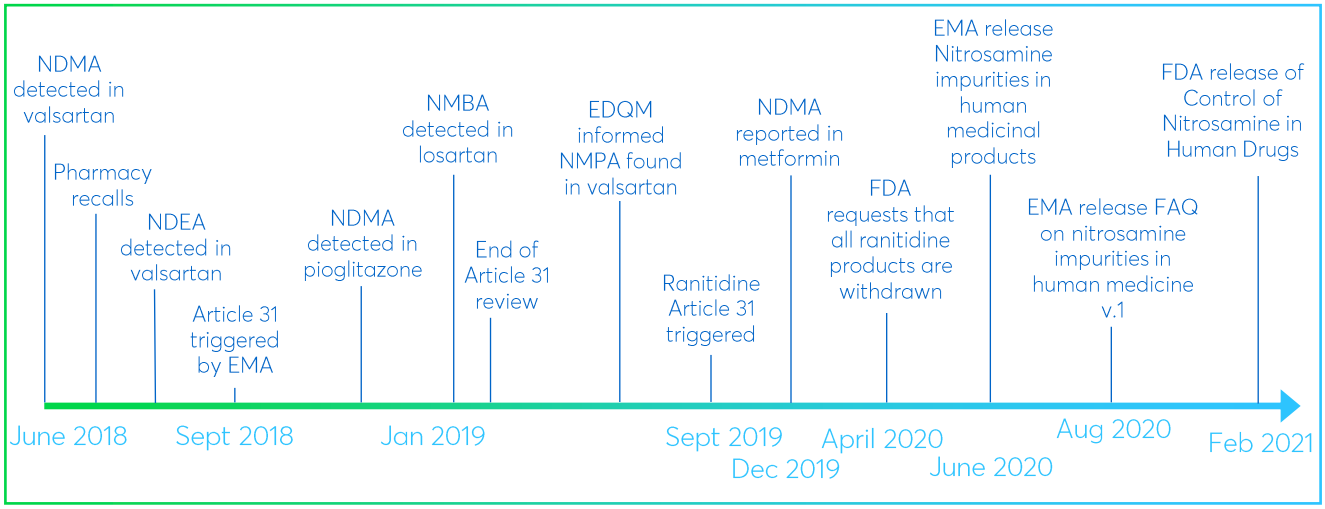


Figure 2: Timeline of main events in the evolution of regulatory requirements for nitrosamine analysis.

Table 1: List of 8 nitrosamines that have daily exposure limits defined 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.

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

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*,[9] 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 samples determined to contain NDMA above the limit. It was postulated that interference from DMF, which co-eluted with NDMA, resulted in the over-estimation of NDMA content in the testing laboratory. Specifically, the ^{15}N DMF isotopic ion (which differs from the NDMA monoisotopic ion by just 0.0016 amu (21 ppm)) could potentially be mis-identified as NMDA, resulting in inaccurate quantification. Subsequent experiments recorded higher NDMA concentrations in samples containing DMF. It was concluded that if inappropriate mass accuracy and tolerance settings are applied, the ^{15}N DMF isotopic ion can be mis-identified as NDMA in the LC-HRMS analysis, resulting in over-quantification of NDMA.

Given the lower mass resolution of triple quadrupole MS compared to HRMS, 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 if they are not sufficiently resolved chromatographically. In this technical note, the potential for interference from N,N-dimethylformamide (DMF)

using an existing LC-MS/MS method is investigated, along with strategies for mitigating the risks of inaccurate quantification that arises.

NDMA OVER QUANTIFICATION DUE TO DMF CO-ELUTION

A previously published LC-MS/MS method for the analysis of eight nitrosamines in drug substances, developed using an Avantor® ACE® UltraCore SuperC18 solid-core column,[10] was used to investigate the potential for NDMA over-quantification. This was assessed by analysing a series of 1.0 ng/mL NDMA samples, spiked with varying concentrations of DMF (Table 2). The DMF concentrations selected are within the defined residual solvent limits specified in ICH Q3C(R8).[11] Both NDMA and DMF showed very low retention on the solid core C18, with a retention factor (k) of just 0.3 and were found to co-elute. At this low-level concentration, the presence of DMF detrimentally impacted the calculated accuracy (Table 2), leading to falsely high predicted NDMA concentrations. This could be particularly impactful in situations where multiple nitrosamines are detected, requiring lower level quantification limits.[4, 6, 12] 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, chromatographic separation of DMF and NDMA would clearly be advantageous. The hydrophilic nature of both DMF and NDMA 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.[13] Fully porous columns are typically more retentive than their solid core counterparts, due to their increased porosity, and consequently, a larger surface area. 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 provided increased aliphatic interactions between the analytes and the stationary phase. This improved NDMA retention (k = 1.1) and provided additional separation of DMF from NDMA (Figure 3B).

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 3C, 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 prove useful for addressing ion suppression effects that may arise in the analysis of drug products containing hydrophilic APIs and/or excipients. The LC gradient conditions were optimised on both columns to provide maximum NDMA retention plus separation of the seven additional nitrosamines. The separation on the Biphenyl phase is shown in Figure 4,

Table 2: Summary of spiking experiment used to assess potential interference from DMF on NDMA quantification.

				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**

for full method details on both columns, please refer to reference 14. Calibration curves and QC samples showed excellent linearity, accuracy and precision, whilst LOD and LOQ values were determined and found to be comparable to data obtained for the original method.[14]

Both LC-MS/MS methods were then assessed using the spiking approach in Table 2, to determine whether they could be utilised to reduce NDMA quantification errors in the presence of DMF. The additional chromatographic resolution of NDMA and DMF provided by both the

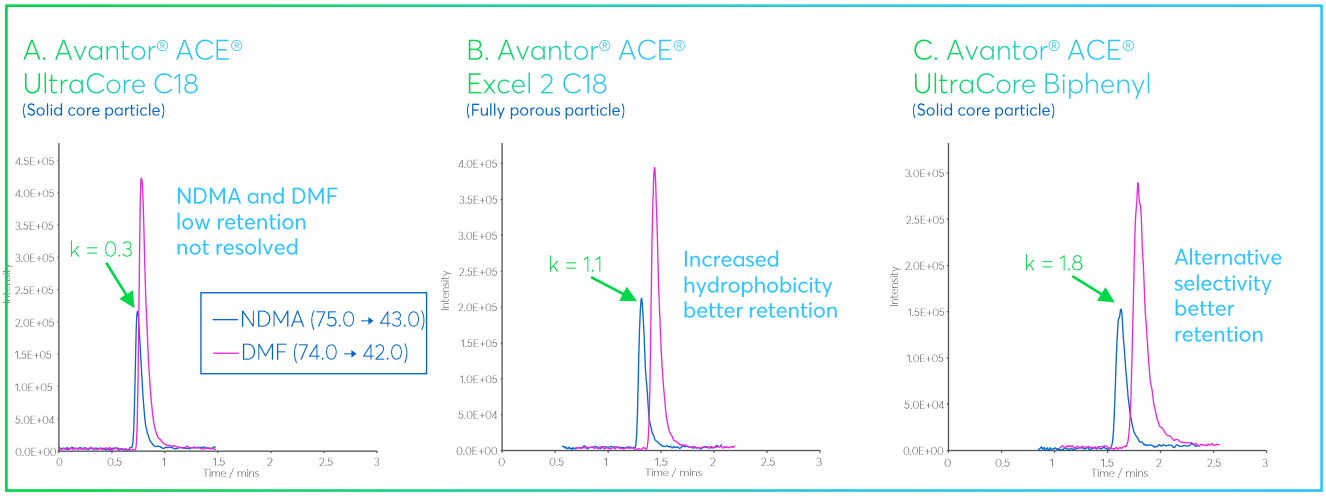
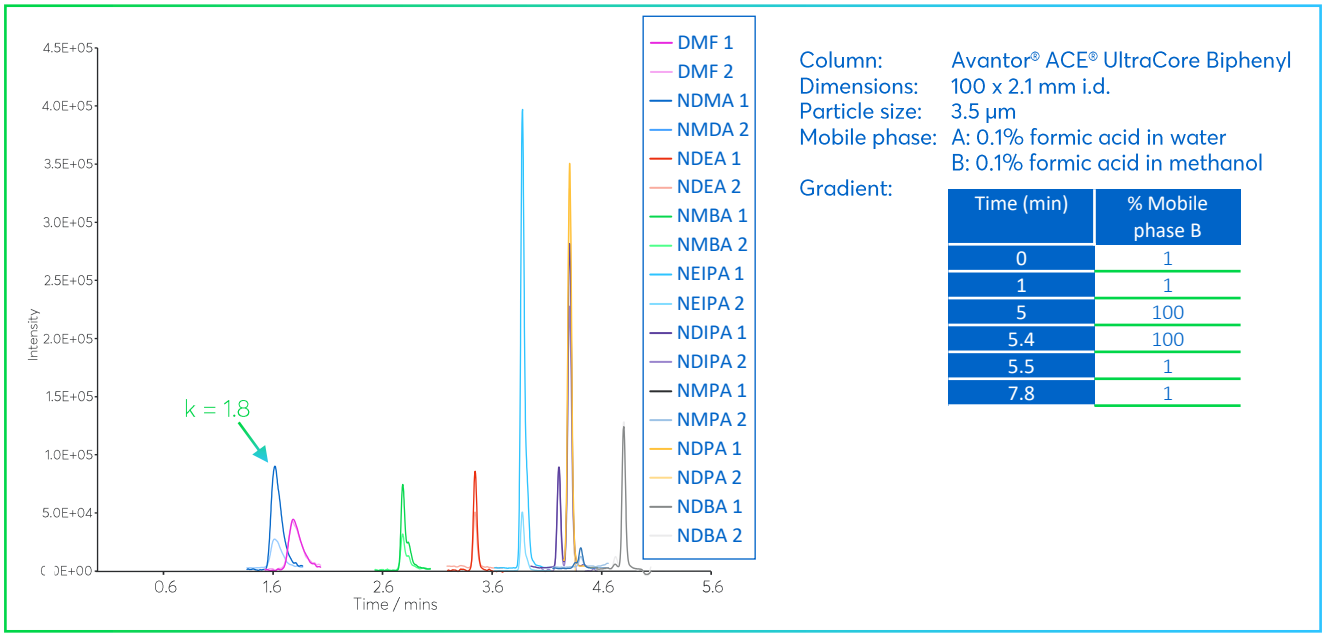


Figure 3: The chromatographic separation between NDMA and DMF on the three stationary phases tested.



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

Figure 4: Example LC-MS/MS separation of nitrosamines spiked into valsartan drug substance at 0.1 ng/mL on an Avantor® ACE® UltraCore Biphenyl column. Overlayed traces represent the quantifier and qualifier transitions for each nitrosamine and DMF. Please refer to reference 14 for full MS conditions and details of the MRM transitions.

Avantor® ACE® Excel® C18 and Avantor® ACE® UltraCore Biphenyl methods permitted accurate integration of NMDA in the presence of DMF and significantly improved accuracy compared to the original method (Figure 5). Given that the *m/z* 75.0 → 58.0 NDMA transition was found to provide improved accuracy in the presence of DMF in the previous experiments (Table 2), it is recommended that this transition be assigned as the quantifier transition for NDMA.

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. MRM transitions were therefore established and optimised for selective monitoring of DMF (Figure 6). The transitions were found to be highly selective in the presence of NDMA. Consequently, these DMF transitions can be used in any LC-MS/MS approach to monitor the DMF content of real-life samples, to screen for samples that may be prone to NDMA quantification issues. Figure 5 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.

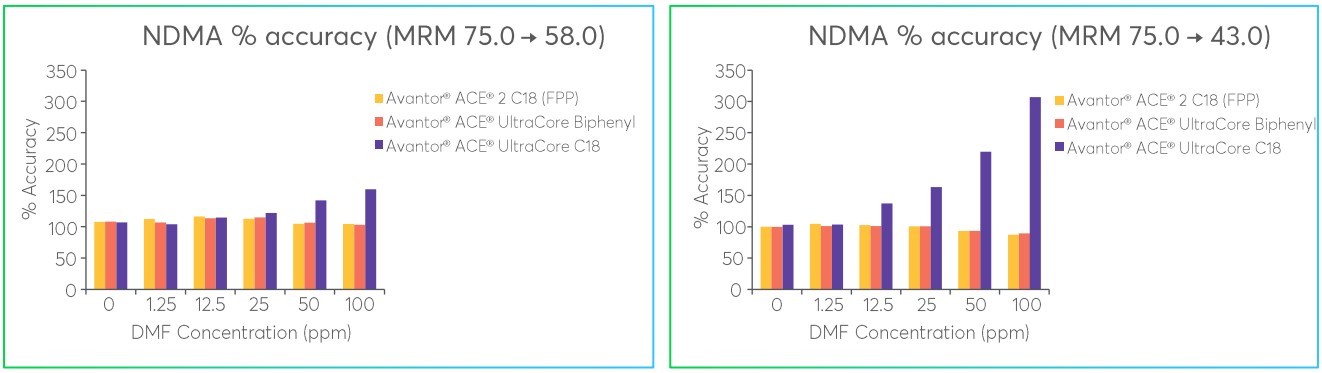


Figure 5: Percentage accuracy data for NDMA quantification in the DMF spiking experiment using original method (purple) and the alternative approaches on the Avantor® ACE® Excel® 2 C18 and Avantor® ACE® UltraCore biphenyl phases.

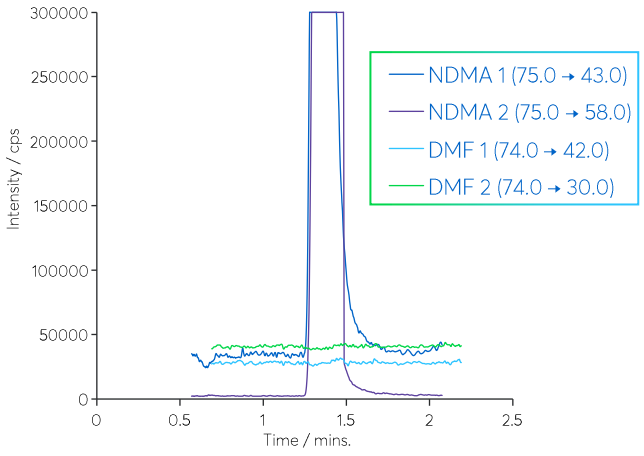


Figure 6: NDMA and DMF MRM transitions in a 30 ng/mL solution of NDMA, demonstrating high selectivity of the DMF transition in the presence of NDMA.

CONCLUSION

The combined approach of monitoring samples, using appropriate MRM transitions to identify residual DMF, and the 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. The chromatographic resolution provided reduces the risk of isobaric interference and 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.

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