Overcome N-Nitrosamine Analysis Challenges with Chromatography and Mass Spectrometry Techniques

A case study exploring enhanced approaches to N-nitrosodimethylamine analysis in metformin drug products

Rolf Kern
Technical Marketing Manager Pharma
SCIEX

OVERVIEW

Analysis of N-nitrosamines has been a hot topic in the pharmaceutical industry since 2018 when these potential carcinogens were found in several angiotensin II receptor blockers (sartans). Their subsequent discovery in ranitidine and some slow-release metformins prompted widespread product recalls. Manufacturing requirements have now become more stringent and the industry has taken steps to evaluate root causes of N-nitrosamine contamination, apply appropriate risk assessments and implement controls. This article presents work to overcome some of the challenges in developing chromatography and mass spectrometry (MS) methods that meet increasingly demanding regulatory requirements, specifically focusing on N-nitrosodimethylamine (NDMA) analysis in metformin drug products.

NITROSAMINE CONTAMINATION IN PHARMACEUTICALS

Since the 2018 recall of a single lot of valsartan, the regulatory authorities have issued many further recalls and warnings for various sartans because of N-nitrosamine contamination. The majority of these have been due to the presence of NDMA. In late 2019, the FDA issued warnings for NDMA contamination of ranitidine, and in April 2020 requested that all products be removed from sale. In February 2020, some US lots of metformin were found to contain NDMA, which prompted the recall of several extended-release formulations. This raises questions of where else NDMA might be found, and places its analysis firmly in the spotlight.

NITROSAMINE ASSAY REQUIREMENTS

The three main components of a N-nitrosamine assay are: sample preparation, chromatographic separation, and MS

measurement. The goal of sample preparation is to solubilize all of the target analyte and produce a sample that is suitable for high-performance liquid chromatography (HPLC)-based analysis. The volatility and small molecular size of nitrosamines, NDMA in particular, means that extra care is needed. Exposure to the evaporating conditions of standard sample preparation techniques, such as solid-phase extraction or evaporation and reconstitution, can lead to problems with recovery.

In the chromatography step, nitrosamines perform reasonably well. However, the levels that must be determined are nanograms per mL of contaminant in milligrams per mL of active pharmaceutical ingredient (API). The risk is that the API will overlap chromatographically with the target analyte, which results in ionization effects that complicate analyte quantification. Therefore, the chromatography method must be able to provide acceptable peak shapes for reliable integration while clearly separating the analyte from the API.

MS is then used to detect and quantify the analyte of interest, and the method must be sensitive enough to provide accurate measurement at low levels. The method must also be sufficiently selective to avoid any issues with background. Pharmaceutical samples tend to be complex mixtures and it is generally not possible to matrix-match the standards used in their analysis. A robust ionization step is critical, for which atmospheric pressure chemical ionization (APCI) is the preferred approach because it is less susceptible than other techniques to matrix-based ionization effects.

ADDRESSING THE CHALLENGES OF ANALYZING NDMA IN METFORMIN

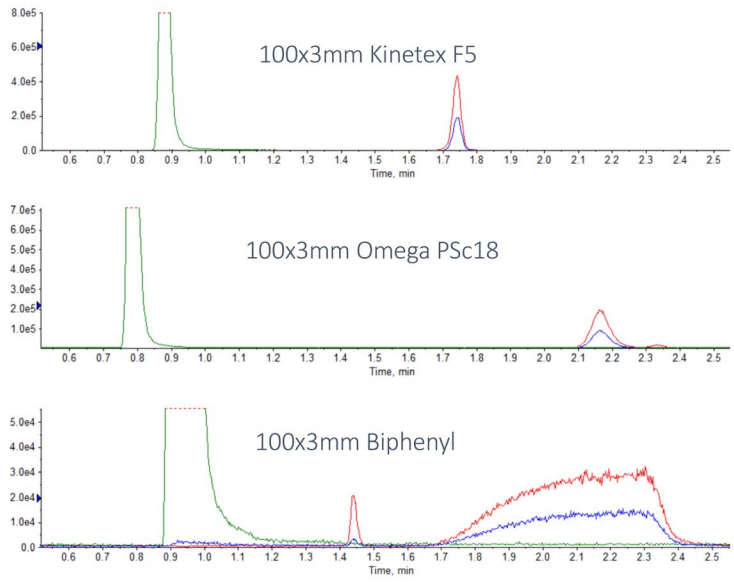
In addition to the assay requirements previously mentioned, there are complicating issues when assaying NDMA in metformin. Both are small, highly polar molecules that can be difficult to separate chromatographically. In general, small molecules such as these tend to be more prone than larger molecules to high baseline signals. Furthermore, metformin extended-release formulations are challenging to process. Unlike immediate release formulations that normally yield a dissolvable powder when using standard crushing and reconstitution techniques, extended-release formulations frequently give rise to a thick slurry or gel that is unsuitable for liquid chromatography–mass spectrometry (LC–MS) analysis.

The following study involving nine individual lots of metformin—six blinded and three with lot information—set out to address these various challenges.

Sample preparation. Sample preparation was based on a published FDA method for metformin (1) in which each tablet for analysis is crushed to a powder and dissolved in pure methanol at a rate of 1 mL of methanol to 100 mg of API, as stated on the formulation. This provides a consistent concentration that enables reporting of nanograms of contaminant per milligram of API rather than reporting as a function of the entire formulated tablet. In the study described here, each sample was then vortex mixed and sonicated to ensure exposure of the full contents of the pill to the solvent. After 15 minutes of settling at room temperature, material was pipetted from the top of each tube and clarified by centrifugation at 15000 rpm. An aliquot was then removed from the top of the supernatant and placed in an LC vial for analysis. It is important to note that these samples are in 100% methanol, a factor that must be considered in the chromatography step.

Chromatography. While the immediate aim of the chromatography method development was to analyze NDMA in metformin, the overall goal was to develop an approach that could then be applied to future drugs exhibiting

Figure 1: Chromatography.



- Generic conditions to select a stationary phase for further development
  - 0.5 mL/min
  - Mobile phase A: Water in 0.1% formic acid
  - Mobile phase B: methanol in 0.1% formic acid
  - 5-95% B over 5 mins
- Metformin shown in **green**, 2 MRMs for NDMA shown in **blue** and **red**
- Omega PS C18 was chosen for further method development due to symmetric peak shape and initial separation from API

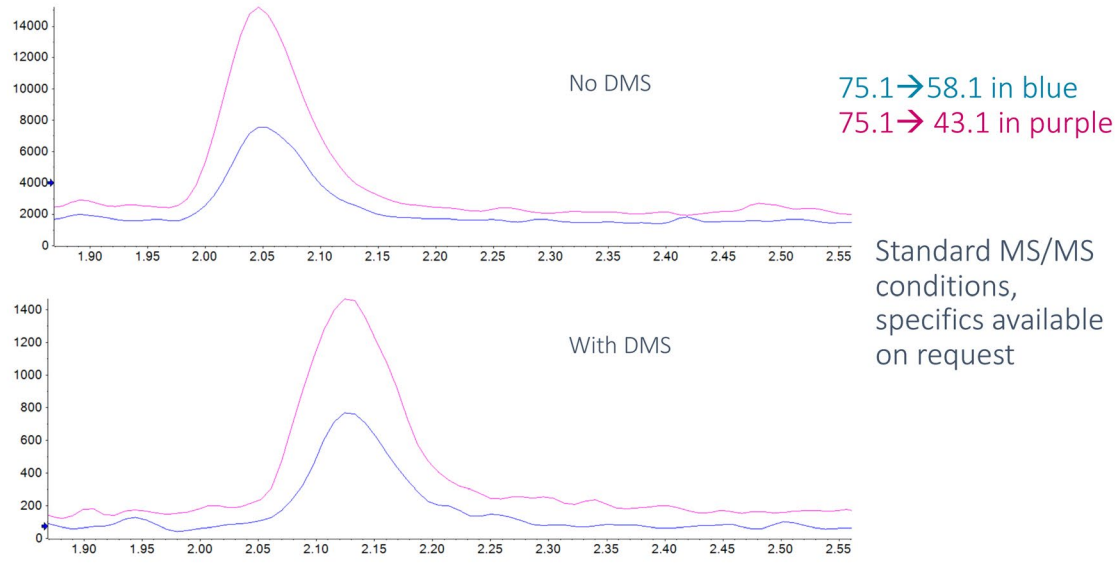
similar contamination issues. The starting point was work published by Phenomenex on the separation of a suite of nitrosamines in both ranitidine and valsartan that involved the use of three different column phases (2). This prior work meant that column phases known to be successful with nitrosamines could be evaluated using the API in this study. To determine the most appropriate column for moving forward, NDMA standard was diluted in metformin and run on multiple stationary phases of similar dimensions with a generic gradient. A relatively large column size of 100 x 3 mm was used to compensate for the expected API saturation.

FIGURE 1 lists the generic conditions used to select a stationary phase, and the chromatograms indicate clear separation with acceptable peak shapes using the Omega Polar Surface C18 column (Phenomenex). This column was consequently selected. The method was further refined through the incorporation of a divert valve, which essentially collects the peaks of interest for MS whilst sending everything else to waste. This protects the mass spectrometer from contamination with the API and other materials from the original formulation.

Mass spectrometry. MS was undertaken using a QTRAP® 6500+ LC–MS/MS System (SCIEX). One of the options for the system is a bolt-on SelexION® Differential Mobility Separation Device. Since differential mobility spectrometry (DMS) is not a mass-based approach, it provides an orthogonal method of separating ions from one another and adds specificity to tandem mass spectrometry (MS/MS) results. The rationale was that while the multiple reaction monitoring (MRM) methods used in pharmaceutical analyses are already fairly selective, incorporating extra specificity into the method being developed here would be beneficial when dealing with potential unknowns in multiple lots of formulated product from different manufacturers.

Samples were prepared as described earlier, together with calibrations in methanol. No isotopically labeled internal standard (IS) was available. The sample set and additional

Figure 2: 10 ng/mL NDMA standard in methanol.



spiked samples were ran twice—once with SelexION® technology in place and once without. Running spiked samples was felt to be important given the challenges of analyzing any substance in a matrix without access to a true blank.

FIGURE 2 presents example chromatograms, with and without DMS, for a 10 ng/mL NDMA standard in methanol. Analyses were carried out using standard MS/MS conditions. The incorporation of DMS is intended to "clean up" the ions making it through to the detector and thereby lower the baseline. While there was an expected loss of signal with DMS, the results showed an improvement in signal-to-noise ratio (approximately two-fold, figures not shown).

Calibration was carried out using samples and spiked samples. With DMS applied, the lower limit of quantification (LLOQ) for NDMA was 2.5 ng/mL, compared with 10 ng/mL without DMS. While this is not a particularly low LLOQ for this molecule, these were 1 µL injections from a 100% methanol sample. In a different solvent, it might be possible to use a larger injection volume and improve the LLOQ, but

Figure 3: Sample data summary.

All values are $\frac{\text{ng NDMA}}{\text{mg Metformin}}$

Sample ID	FDA Value 1	FDA Value 2	DMS	No DMS	10 ng/mL spk % Recovery
Blind 1			0.095	BLOQ	80
Blind 2			BLOQ	BLOQ	104
Blind 3			BLOQ	BLOQ	104
Blind 4			BLOQ	BLOQ	76
Blind 5			0.039	BLOQ	86
Blind 6			BLOQ	BLOQ	92
Known 1	0.082	0.071	0.108	0.093	91
Known 2	0.314	0.292	0.352	0.296	87
Known 3	0.170	0.138	0.170	0.139	73

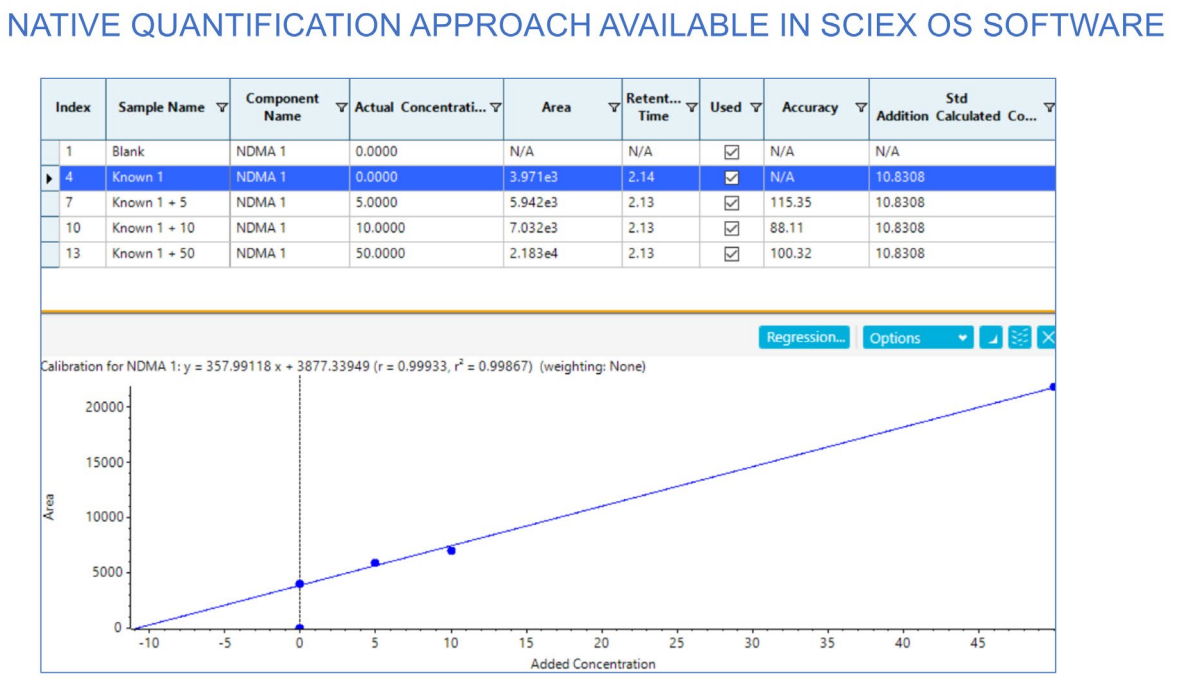
To calculate ng/mg of NDMA in metformin: $\frac{\text{ng NDMA}}{\text{mL MeOH}} \times \frac{\text{1mL MeOH}}{\text{100mg Metformin}}$

for the purposes of this work, the method was sufficient and achieved good reproducibility and linearity across the ranges.

Since the regulatory requirements around NDMA relate to total intake per day, it is important to know how much is in a given product. This requires a simple calculation to convert the calibration curve values of ng of NDMA per mL of prepared sample to ng of NDMA per mg of metformin.

Three of the metformin lots studied had previously been reported on by the FDA (1). This enabled a direct comparison of the results obtained, shown as part of FIGURE 3, which

Figure 4: Standard addition.



also gives the equation used to convert from calibration curve values. Something of note here is that the published and experimental figures for the known samples 1 to 3 lined up well. While matrix spike recovery is not typically carried out in pharmaceutical analyses, the 10 ng/mL spike recoveries obtained here were felt to be acceptable. They were particularly important in indicating no significant suppressive or enhancement effects in individual matrices of the samples being studied.

QUANTITATIVE CONSIDERATIONS

The lack of a true blank matrix and the absence of isotopically labeled standards make it essential to have some way of evaluating ionization effects. Matrix spikes are a useful tool. If spike recoveries are unacceptably high or low, then other tools are available. In the set-up for this experimental work, the system software (SCIEX OS) offers a quantification technique (Standard Addition Quant – SAQ) that is widely used in environmental and food analysis where no true blank matrix is available. The technique works well for ionization effects and elevated baseline signal, but is not intended for observable interferences that affect peak integration.

Standard addition begins with the unspiked samples as the zero point. Successively higher levels of analyte are then added and analyzed, giving the typical regression shown in FIGURE 4. The absolute value of where the regression line crosses the x-axis is used to calculate the concentration in the sample, a calculation that is native in the software. Results for the samples studied here lined up closely with the values from the standard calibration curve, which provides added confidence in the developed method.

ASSAY INTERFERENCES

FDA and others using high-resolution quantification methods have observed interference of dimethyl formamide (DMF) with

Figure 5: Notes on assay interferences HR-MS/MS.

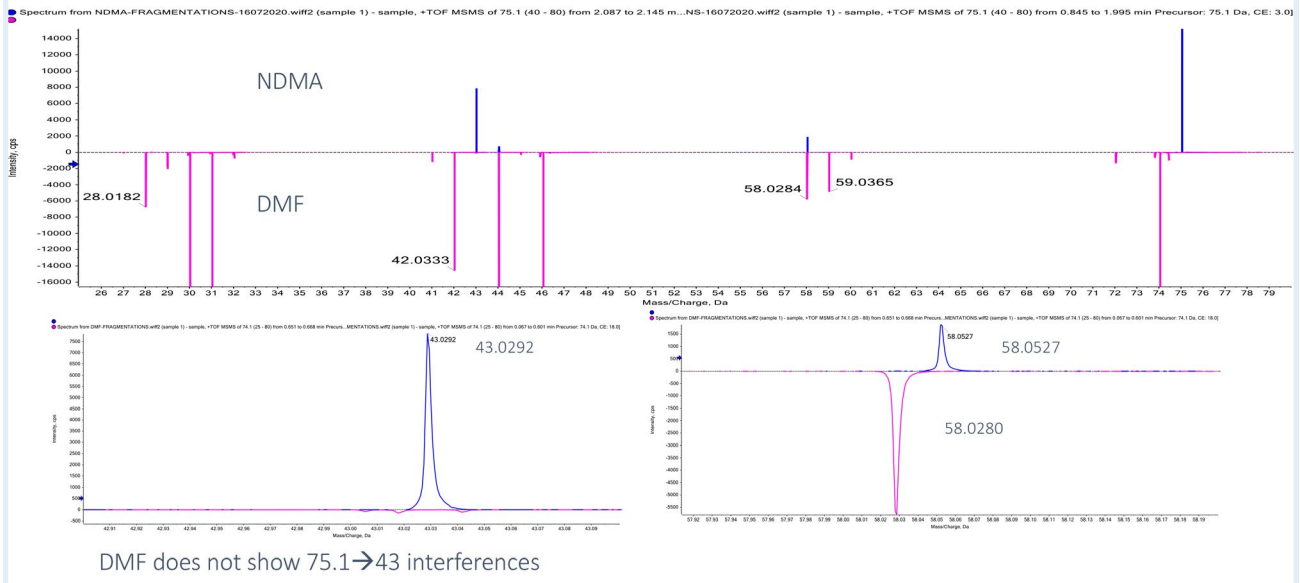
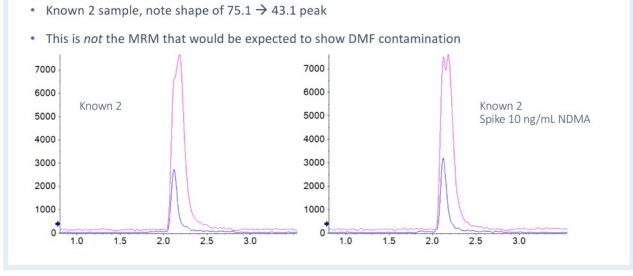


Figure 6: Notes on assay interferences.



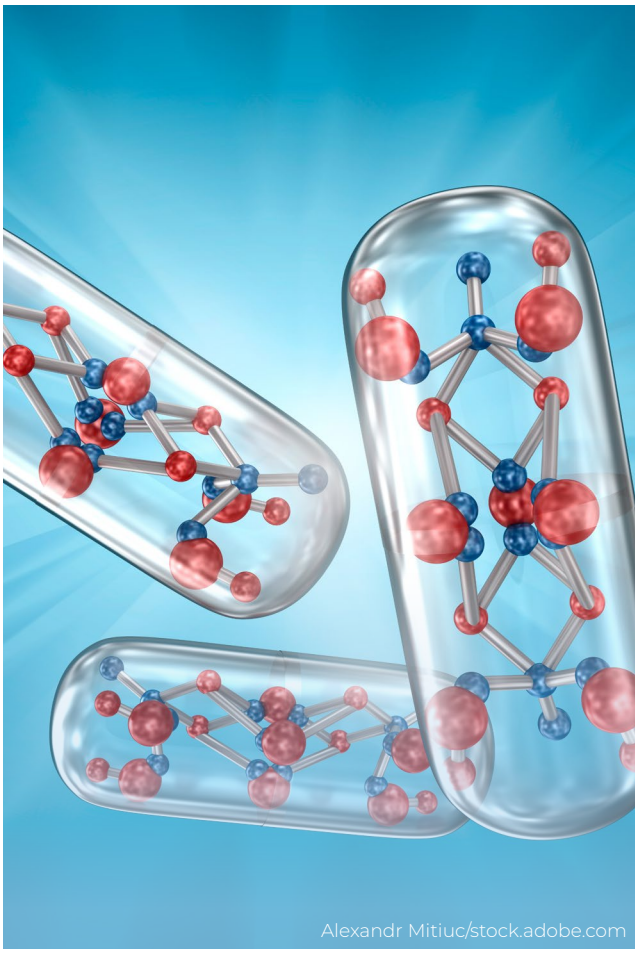
NDMA, and it is clear that there are isotopes that could potentially overlap. If these are not chromatographically well separated for high-resolution quantification, and if fragmentation is not being taking into account, then interference is a possibility. However, there are ways of working around this.

FIGURE 5 shows fragmentation patterns for NDMA (parent and two fragments) and also DMF. It can be seen that there is a DMF fragment very close to the NDMA fragment at 58, but nothing in the range of the NDMA 43 fragment. DMF therefore does not appear to show interferences at the 75 to 43 transition, so monitoring this trace is important, as illustrated by the example from the current study shown in FIGURE 6.

FIGURE 6 shows the MRMs for known sample 2. The 43 peak is the higher of the two with the other being the 58 peak. The peak shape is not quite right, and it appears that something is co-eluting with one of the fragments. In the spiked sample, the 43 peak is split indicating something else is present, but it is not the MRM that would be expected from DMF contamination. This effect was seen even with SelexION® technology installed. Tuning voltages for the SelexION® device to selectively filter individual m/z can be done in one of two ways: to optimize transmission of a particular ion (used in these experiments) or ramped up to affect the separation of two known compounds. The latter works only where there are two known compounds whose voltages can be dialed in for specific separation. However, this approach might have some value in a generic separation.

CONCLUSION

There are many aspects to consider when developing a new LC–MS method for NDMA, or nitrosamines in general. Sample preparation is key and can be challenging for extended-release formulations. In this metformin case study, methanol was used, but an aqueous phase would enable higher injection volumes and improved LLOQ. Chromatography column selection can leverage known separation methods with orthogonal column phases. These make it easy to quickly test which is the most effective phase for a given sample type. The difficulties of working with complex matrices of unknown composition make spiking experiments essential for assessing data quality.



Alexandr Mitiuc/stock.adobe.com

REFERENCES

1. J. Yang et al. *The AAPS Journal* online, DOI:10.1208/s12248-020-00473-w (Jul. 1, 2020).
2. R. Eggleston-Rangel et al., "LC-MRM-MS Method for the Detection and Quantification of Six Nitrosamine Impurities in Sartan (ARBs) Drugs," Phenomenex (ASMS 2020 Poster Presentation).