Nitrosamine Impurities – From Raw Materials to Final Drug Product

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Contents

Technology Digest: Nitrosamine impurities – from raw materials to final drug product

by Naamah Maundrell
(Editor-in-Chief, Bioanalysis Zone)

What are nitrosamines?

Nitrosamines are a class of mutagenic impurities which contain the nitroso functional group and are formed when a secondary or tertiary amine reacts with a nitrosating agent [1]. Two common nitrosamines include N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA), which have been classified as potential human carcinogens by the International Agency for Research on Cancer [1,2]. A carcinogenic response is induced when the N-nitrosamines are activated by microsomal liver enzymes and react with DNA. Low levels of these compounds can be found in food and drinks, such as roasted meats, cheese, and beer, as well as tobacco and pesticides. However, over the past few years, nitrosamines have received a great deal of global attention after being found in medicinal products [1,3].

In 2018, the US Food and Drug Administration (FDA) announced the detection of NDMA contamination in valsartan. Valsartan is an angiotensin II receptor blocker used to treat high blood pressure. Patients taking valsartan with N-nitrosamine impurities may have an increased risk of cancer, which led to lots of the drug being recalled [1–3]. In the case of valsartan, the nitrosamine impurities were only detected when a more sensitive analytical method was used many years after the initial Chemistry, Manufacturing and Controls (CMC) modification occurred (a change in the synthetic process of the Active Pharmaceutical Ingredient [API]). After impurities were detected in valsartan, global pharmaceutical and regulatory laboratories have discovered nitrosamine impurities in a growing array of pharmaceuticals (e.g., ranitidine and metformin), which have triggered numerous recalls [2,4].

The discovery of unacceptable levels of N-nitrosamines in some drugs led to companies receiving warning letters, global drug shortages and new regulatory actions requiring more stringent detection of the impurities [5]. Mi Jang, Lab Manager and Chief Researcher, Pharma & Tech (Korea) remarked on the likelihood of more rigorous regulations:

> *“Many pharmaceutical products made in Korea are exported overseas, and the regulations continue to become more stringent. When these new guidelines for nitrosamine testing came out, we knew we needed to move quickly to setup the services necessary.”*

Larger lessons have been learnt from the drug recalls, with pharmaceutical companies and manufacturers changing how they review their own quality processes for risks that could result in a similar contamination issue [6]. Scientific assessment of raw materials, critical products, process changes, and the final drug substance or drug product are necessary to ensure patient safety when the supply chain is so complex [2,3].

> *Testing should be carried out in the intermediate stage and semi-finished conditions on both raw materials and finished products, as nitrosamines can be generated at any point in the process – commented Eun-Joo Joe, Senior Researcher, Pharma & Tech (Korea).*

Regulatory response to nitrosamine impurities

Both the FDA and EMA published guidance recommendations for manufacturers of drug products and APIs to detect nitrosamine impurities in pharmaceuticals [7,8]. Key guidances also define the conditions that may introduce nitrosamine impurities, including side reactions from drug syntheses, the breakdown of unstable drug compounds and contamination from recycled solvents used in manufacturing and packaging [3]. The unexpected detection of nitrosamine impurities also highlighted the need for a risk assessment strategy for potential nitrosamine impurities. In a risk assessment all potential sources of contamination should be considered, including the excipient, drug substance, solvents, water, the manufacturing process, and packaging components [4,9]. This means that the pharmaceutical industry should examine the entire drug development and manufacturing process, which can include formulation and process development, as well as raw materials and excipients. However, this has proven to be challenging not only due to complicated manufacturing processes and a complex global supply chain, but also because of the number of different impurities and drug products of concern with many possible techniques for detection and quantification.

Much of the initial focus during the nitrosamine impurity crisis was on contaminated APIs and drugs, the final product that reaches patients. Therefore, the need to control nitrosamine impurities at lower detection limits necessitated the consideration of mass spectrometry detection to quantify these trace compounds [4,9].

> *The regulations on nitrosamine impurities in pharmaceutical products are being extended to all products including chemically synthesized drug substances. According to our developed LC–MS/MS analytical method, an LOQ of 0.025 ng/mL was achieved for eight nitrosamines. In addition, data acquisition satisfying the requirements of GMP regarding data integrity is also possible. With the use of this approach, it is possible to comply with the regulations for nitrosamines that are becoming stricter by the day, explained Hiroki Ami, Analytical Development Subgroup Leader, Production Technology Department, Shionogi Pharma (Japan).*

Nitrosamine identification & quantification

In response to the nitrosamine crisis, effort was put into developing sensitive detection methods that could meet required limits. The correct method and technology to analyze these drugs and impurities depends on the required sensitivity for each individual laboratory. This required sensitivity is based on the drug type and the stage where testing takes place during development and manufacturing. As nitrosamine impurities can occur, and be a concern, at extremely low concentration levels, sensitive and specific analytical techniques are required to enable their detection [10].

Traditional LC–UV can be used for quick method analysis of raw materials, solvents, or excipients; however, mass spectrometry has become a significant detection method for nitrosamine analysis as it enables the highly sensitive quantification of known impurities required to meet future low detection limits for final API or drug product [4]. Key mass spectrometry techniques used include GC–MS/MS, LC–MS/MS and HPLC–HRMS. These different instruments cover diverse needs, depending on what is being tested and the analytical challenges that arise [4,11]. But how do you navigate the different methods and technologies?

> *Emmanuel Desmartin, Mass Spectrometry & Bioanalysis Lab Manager, Eurofins Biopharma Product Testing (France) commented on the system his lab implemented: We have implemented in December 2020, a new LC-MS/MS system within our laboratory which is the new Xevo TQ-XS from Waters [12]. Linked to UPLC I-Class system and using MassLynx as a software this system will allow us to carry out analyses from bioanalysis to the screening of nitrosamines but also the assays of genotoxic impurities in your pharmaceutical drug products. Due to its high sensitivity, this equipment will be used for analyses for which we need to have very low limits of quantification.*

Importantly, regulators will accept any platform that can reach regulatory limits with a method that is fit-for-purpose [13]. However, each laboratory must validate the method on their own instruments to confirm it is suitable for the intended use. LC–MS is the preferred technology for high-sensitive quantification of known compounds (used for volatile and non-volatile nitrosamines), whereas HRMS is most appropriate when screening for new and unexpected impurities [1,14]. GC–MS/MS is not an appropriate technique for detecting NDMA levels in ranitidine, due to the possibility of high temperature degradation. Given the complexity and challenges of nitrosamine analysis in both API and drug products, it is important to find the best solution and method based on individual circumstances [1,10].

> *Gabriela Grijalva, Chemical Engineer, Donovan Werke (Guatemala) stated: We looked for an instrument that worked well and was appropriately suited to our needs, to detect these types of impurities at established working concentrations. We also wanted a vendor with experience in the detection of these compounds, as well as support from the brand to implement an accurate, reliable, and sensitive analytical method for the determination of nitrosamines in our raw materials, excipients and finished products.*
>
> *For these reasons, the Waters ACQUITY UPLC H-Class with QDa Mass Detector was chosen for our needs, becoming a useful and reliable tool. The UPLC-QDa is sensitive enough for our low working concentrations, so it is reliable for the identification and quantification of impurities in general. The shorter retention time, and therefore shorter run time, had a direct impact on cost and time savings by reducing the amount of solvents and reagents consumed, reducing the time of preparation and analysis, and saving work time that can be used to perform other dedicated tasks. With this instrument, the analysis of raw materials, excipients and finished product is carried out.*

Summary

Key lessons have been learnt from the presence of N-nitrosamines in medicines, most importantly how crucial it is to maintain a safe supply of medications [9,15]. Scientists and regulators must work together to ensure that analytical platforms used for nitrosamine analysis can reach regulatory limits with a method that is fit-for-purpose. Although traditional LC–UV can be used for quick method analysis of raw materials, solvents, or excipients, mass spectrometry has become the analytical platform of choice for these genotoxic impurities as it enables the highly sensitive quantification of known impurities required to meet future low detection limits for final API or drug product. Since nitrosamine contamination can have multiple possible sources, either with the API, final drug product, excipients, or solvents, it is important that the chosen method is fit for its intended purpose to accurately detect and quantify nitrosamine impurities [4].

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Application Note

Reliable HPLC/UV Quantification of Nitrosamine Impurities in Valsartan and Ranitidine Drug Substances

Margaret Maziarz, Paul D. Rainville

Waters Corporation

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This application brief demonstrates reliable quantification of six nitrosamine impurities (NDMA, NDEA, NEIPA, NDIPA, NDBA, and NMBA) in valsartan and NDMA in ranitidine by UV detection, with the added benefit of mass confirmation by mass spectral data using an ACQUITY QDa Mass Detector.

Benefits

The ACQUITY Arc System with PDA Detector, integrated with an ACQUITY QDa Mass Detector for accurate mass confirmation, enables reliable quantification of nitrosamine impurities in valsartan and ranitidine drug substances.

Introduction

Carcinogenic impurities, such as nitrosamines, can cause DNA mutations, potentially leading to cancer.[1] Several medications containing valsartan or ranitidine drug substances have been recalled due to the presence of nitrosamine impurities in the final drug products.[1,2] Due to their high toxicity, these impurities must be monitored at low levels using reliable methods to ensure safety of the pharmaceutical products.

In this work, we present an HPLC method with UV detection for the simultaneous quantification of six nitrosamine impurities in valsartan drug substance, including N-nitrosodimethylamine (NDMA), N-nitroso-N-methyl-4-aminobutyric acid (NMBA), N-nitrosodiethylamine (NDEA), N-nitrosoethylisopropylamine (NEIPA), N-nitrosodiisopropylamine (NDIPA), and N-nitrosodibutylamine (NDBA). This method also enables analysis of NDMA in ranitidine drug substance. The achievable quantitation limits for nitrosamine impurities using UV detection range from 10–20 ng/mL, with method linearity over 10–1000 ng/mL producing R^2 ≥0.999. The mass spectral data from an ACQUITY QDa Mass Detector was used for quick and accurate peak identity confirmation.

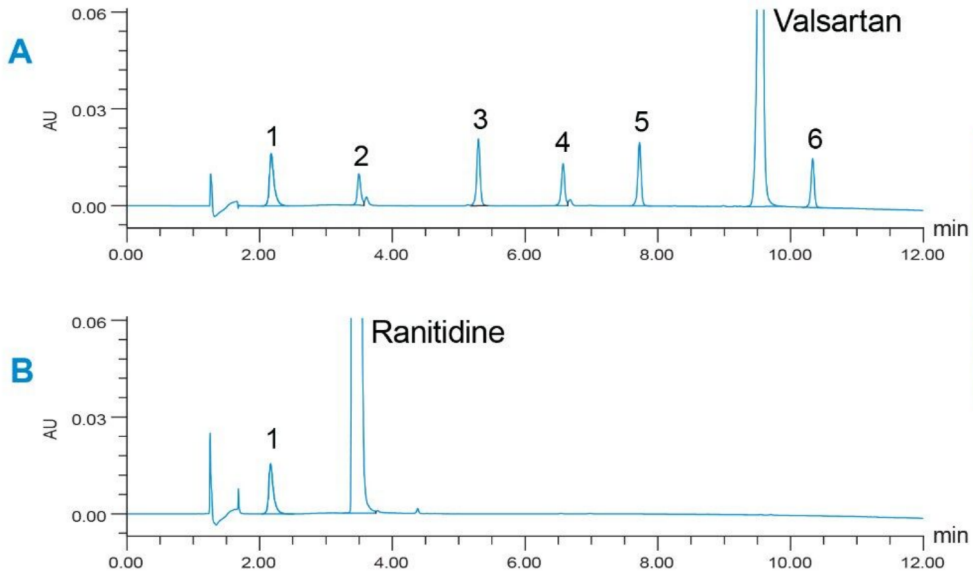
Experimental

Table 1. Instrument conditions for analysis of nitrosamine impurities

<table>
<tr><th>LC system:</th><th>ACQUITY Arc with 2998 PDA and ACQUITY QDa Detectors, passive pre-heater, and flow path 1</th></tr>
<tr><td>Column:</td><td>XSelect HSS T3 4.6 x 100 mm, 3.5 µm</td></tr>
<tr><td>Column temp.:</td><td>40 °C</td></tr>
<tr><td>Flow rate:</td><td>1.0 mL/min</td></tr>
<tr><td>Injection volume:</td><td>25.0 µL</td></tr>
<tr><td>Mobile phase:</td><td>A: water with 0.02% of formic acid
B: acetonitrile</td></tr>
<tr><td>Gradient:</td><td>
<table>
<tr><th>Step</th><th>Time (min)</th><th>%A</th><th>%B</th></tr>
<tr><td>1</td><td>Initial</td><td>95.0</td><td>5.0</td></tr>
<tr><td>2</td><td>0.50</td><td>95.0</td><td>5.0</td></tr>
<tr><td>3</td><td>12.00</td><td>5.0</td><td>95.0</td></tr>
<tr><td>4</td><td>13.00</td><td>5.0</td><td>95.0</td></tr>
<tr><td>5</td><td>13.10</td><td>95.0</td><td>5.0</td></tr>
<tr><td>6</td><td>17.00</td><td>95.0</td><td>5.0</td></tr>
</table>
</td></tr>
<tr><td>Wash solvents:</td><td>Purge: 50:50 water/acetonitrile
Sample wash: 80:20 water/methanol
Seal wash: 90:10 water/acetonitrile</td></tr>
<tr><td>PDA detection:</td><td>λ range: 210–400 nm, derived at 245 nm
Sampling rate: 10 pts/sec</td></tr>
<tr><td>Mass detection:</td><td>ACQUITY QDa
Ionization mode: ESI+
Acquisition range: 50–500 m/z</td></tr>
</table>

Results and Discussion

The HPLC separation was performed using an XSelect HSS T3 Column, based on a previously described method.[4] The conditions of the method were modified to achieve optimal UV performance for nitrosamine impurities in valsartan and ranitidine drug substances. The optimized method (Table 1) provided excellent retention for nitrosamines and separation from the drug substances (Figure 1). While the UV data was used for quantitation, the mass spectral data from an ACQUITY QDa Mass Detector enabled peak identity confirmation by mass detection.



#	Impurity	QDa *m/z*
1	NDMA	75.0
2	NMBA	147.1
3	NDEA	103.1
4	NEIPA	117.1
5	NDIPA	131.1
6	NDBA	159.2

Figure 1. Representative chromatograms of six nitrosamine impurities (1 ug/mL) in valsartan (A) and NDMA in ranitidine (B) drug substances. UV at 245 nm.

The limit of quantitation (LOQ) for nitrosamine impurities achievable with UV was determined using the signal-to-noise criteria of 10:1. The LOQ solutions were prepared by spiking ~100 µg/mL of drug substance sample in 80:20 water/methanol diluent with the nitrosamines' standards. The LOQ was found to be 10 ng/mL for NDMA, NDEA, and NDIPA, and 20 ng/mL for NMBA, NEIPA, and NDBA, respectively. The LOQ solution at 20 ng/mL of nitrosamines in ~100 µg/mL valsartan is shown in Figure 2. Data from six replicate injections was evaluated to demonstrate performance at the LOQ level (Table 2). The %RSD of the peak areas for six replicate injections of the LOQ solutions was ≤7.51%. The method exhibited a linear response over the 10–1000 ng/mL range with correlation coefficients (R^2) of >0.999 (Table 2). Data was analyzed using Empower 3 Chromatography Data System (CDS) Software.

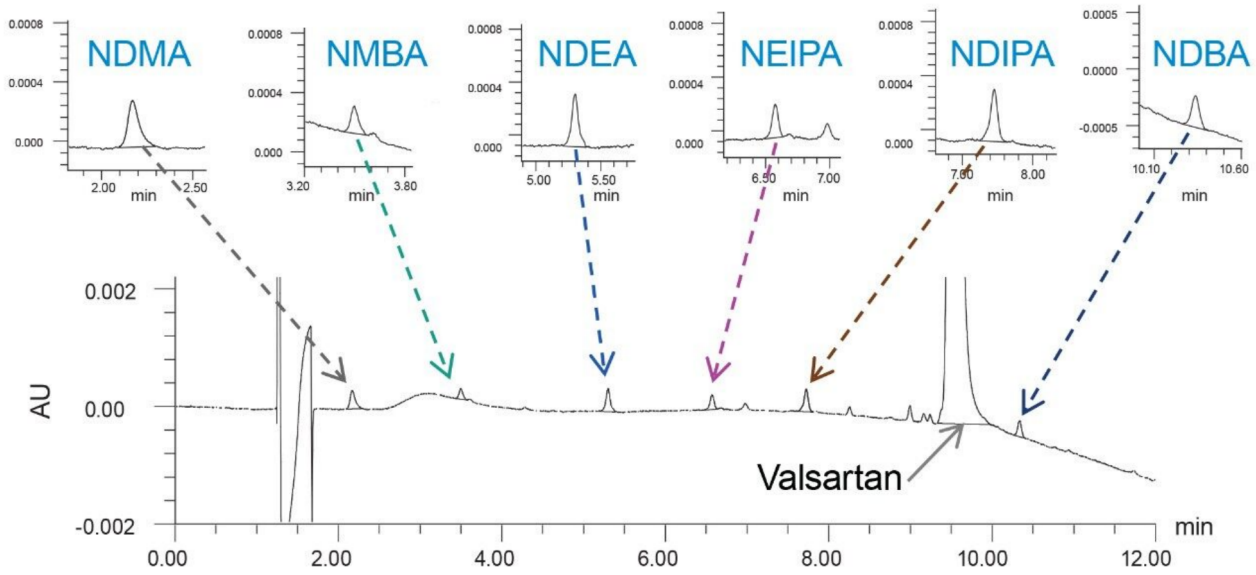


Figure 2. Limit of quantitation (LOQ) solution with six nitrosamine impurities at 20 ng/mL in valsartan drug substance sample. UV at 245 nm.

Table 2. Limits of quantitation (LOQ) and method linearity for nitrosamine impurities with UV detection, 245 nm.

Impurity	Limit of Quantitation (LOQ)			Method linearity
	LOQ Conc. (ng/mL)	S/N	% RSD of peak areas	R^2 (10–1000 ng/mL)
NDMA	10	20	7.51	0.99978
NMBA	20	25	5.25	0.99982
NDEA	10	22	5.32	0.99985
NEIPA	20	24	6.09	0.99987
NDIPA	10	20	8.16	0.99986
NDBA	20	24	5.25	0.99991

Conclusion

A single HPLC/UV method was successfully developed for the reliable quantification of six nitrosamine impurities (NDMA, NMBA, NDEA, NEIPA, NDIPA, and NDBA) in valsartan and NDMA in ranitidine drug substances, with quantitation limits ranging from 10–20 ng/mL. The analysis was performed on the ACQUITY Arc System with 2998 PDA Detector, integrated with an ACQUITY QDa Mass Detector for quick and accurate peak identity confirmation. Additionally, the XSelect HSS T3 Column, a proprietary reversed-phase column, provided retentivity and specificity for all analytes. This HPLC-UV method offers a starting point for the robust quantification of nitrosamines or similar compounds.

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Featured Products

ACQUITY Arc System <https://www.waters.com/134844390>

2998 Photodiode Array (PDA) Detector <https://www.waters.com/1001362>

ACQUITY QDa Mass Detector <https://www.waters.com/134761404>

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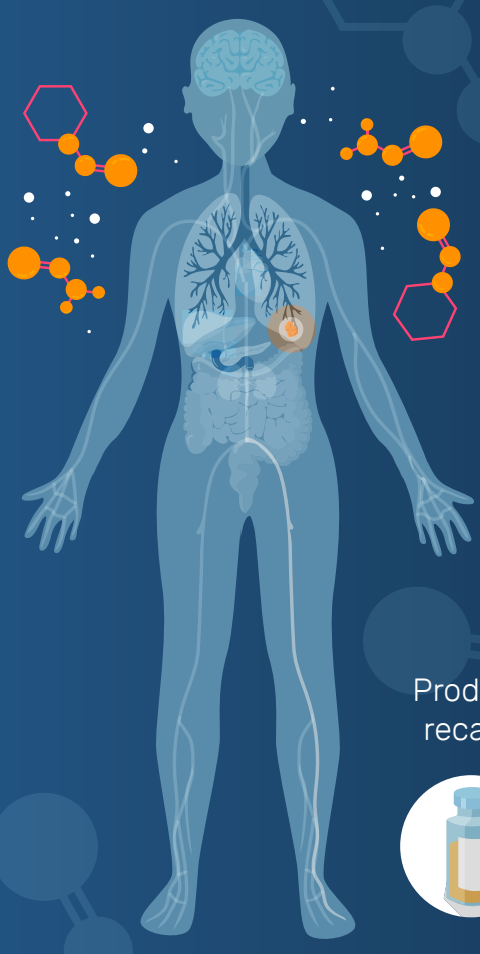
Nitrosamine analysis: techniques and instrumentation

5 steps to effectively detect and quantify nitrosamines



What are nitrosamines?

Nitrosamines are a type of impurity which may **increase the risk of cancer** if people are exposed to them above acceptable levels over long periods of time.

Since 2018, in what is known as the nitrosamine crisis, nitrosamines have been detected in different types of pharmaceutical drugs. This includes, but is not limited to, sartans (a group of hypertension drugs), ranitidine (an antacid), and metformin (a diabetes drug).

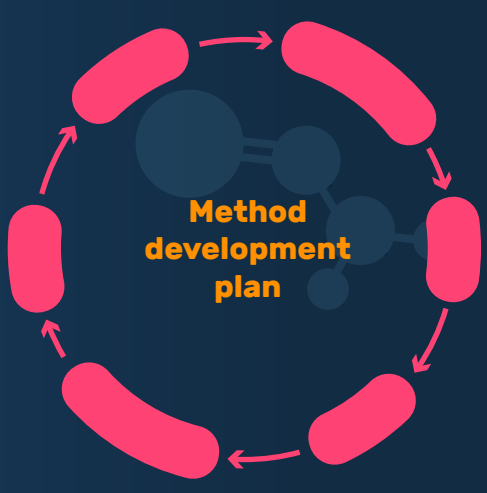
This nitrosamine crisis called into question the quality of many APIs (active pharmaceutical ingredients) produced and sourced, resulting in:

The drug manufacturing process has become increasingly outsourced and globalized, while proposals for stringent impurity regulations are imminent. Companies that manufacture and supply APIs or other raw materials must have the analytical capacity to identify and monitor impurities.

Nitrosamine detection and analysis

Method development

Variables such as ionization source and mobile phase can impact analytical performance of a method. **This highlights the importance of a good method development, transfer, optimization, and validation plan.** In general, atmospheric pressure chemical ionization (APCI) sources provide more sensitivity when observing nitrosamine impurities than compared to ESI sources. Additionally, using ammonium formate buffer mobile phase improves sensitivity and reduces baseline noise, achieving better signal to noise (S/N).



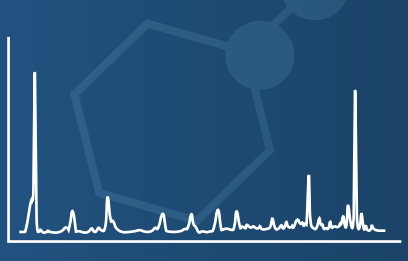
Sample preparation

One challenge with nitrosamine analysis is the wide variety of potential compounds, each with different physiochemical properties. The first step to overcoming this challenge is comprehensive sample preparation. **The more work and focus done up front in these earlier stages, the easier the later stages will be.** To maximize sensitivity without affecting peak shape, it is important to select the appropriate solvent and injection volume. When analyzing samples for low level impurities, automated sample preparation techniques can reduce possible contamination from external sources, while increasing reproducibility and, therefore, the precision of an assay.

Separation

It is important to select the appropriate column chemistry when setting up a method with certain impurities and APIs in mind. Having more resolution from the API enables effective use of a diverter valve to divert the largely concentrated API peak to waste, minimizing suppression and reducing source contamination. One specific column might not work when looking at different drug substances or drug products, even different formulations. **One size does not fit all.**

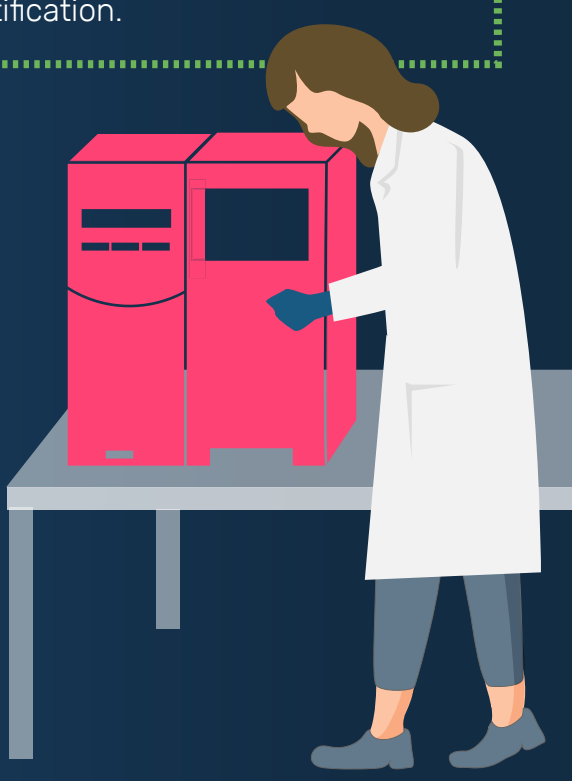
Raw material or excipient



Traditional LC–UV techniques can achieve historical regulatory limits and can still be used for quick method analysis in the lab or for raw material, solvent, or excipient testing. **Key for this technology is a platform that enables robust method development**, generating reproducible results that are transferable across labs, sites, and geographies. A single quadrupole mass spectrometer can be used for mass confirmation and identification.

Final API or drug product

To meet the low detection requirements for final API or drug products, tandem/triple quadrupole (TQ) mass spectrometry is the gold standard for robust, routine high sensitivity quantification of known impurities. Use of MS/MS is critical to detect and quantify these trace ions without interference. Extremely important to note here is the use of qualifier and quantifier ions, which might change based on the matrix, and speaks to the importance of evaluating samples using multiple MRM transitions. Different published methods might use different transitions for qualification or quantification, so **each individual lab must validate their own method** depending on their instrument and conditions before submitting data to regulatory authorities.



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The Current Concerns About Genotoxic Impurities Found in Commonly Used Prescription and Over The Counter Pharmaceutical Drugs

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INTRODUCTION

This white paper describes the current status and causes of contamination of pharmaceutical products by the genotoxic impurities known as nitrosamines. Both commonly prescribed heart protection drugs (known as angiotensin II receptor blockers – ARBs – or 'sartans) and the widely used H2 (histamine-2) blocker drugs, ranitidine (Zantac) and nizatidine (Axid), both used for prevention of acid in the stomach, have been found to contain nitrosamine compounds.

Health authorities worldwide have rescinded GMP certification, sent Warning Letters, and imposed import alerts or recalls on manufacturers of both the active pharmaceutical ingredients (API), the packaged drug products, and even manufacturing intermediates like solvents.

Dozens of drug manufacturers and pharmacies have voluntarily recalled and removed product from shelves, leaving many consumers unsure whether to continue taking their medicine.

In both cases, the culprit is the same potentially mutagenic compound, NDMA (N-Nitrosodimethylamine). While the root cause in the original 'sartans contamination is believed to have been identified, in the case of ranitidine and nizatidine, the source appears much more difficult to track down and characterize, meaning that it might not be easily solvable.

WHAT ARE NITROSAMINES?

N-nitrosamines are a class of compounds that have been shown to exhibit carcinogenic and mutagenic (or genotoxic) effects in animal models at several different tissue sites and by several different routes of exposure. Nitrosamines can potentially be formed in consumer products, cosmetics and personal care formulations, either during manufacture or product storage. N-Nitrosamine formation occurs when secondary amines are present in addition to a nitrating agent such as nitrous acid, nitrites, or nitrogen oxides, generally under acidic conditions, Figure 1 and 2.

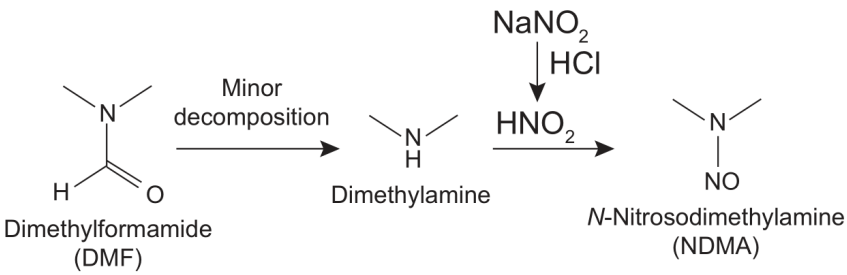

Figure 1. Formation of NDMA from Dimethylformamide.

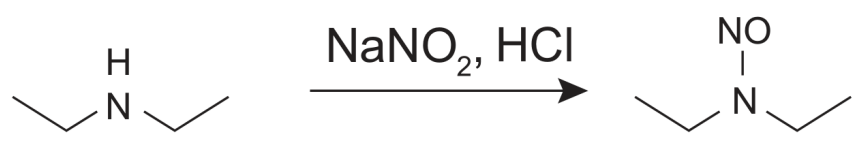

Figure 2. General reaction scheme for formation of NDEA from diethylamine.

Nitrosamine contaminants in Valsartan and related angiotensin II receptor blockers

In May 2017, an FDA 483 report[1] of an API manufacturer in Linhai Zhejiang, China, detailed concerns about the number of Out of Specification (OOS) assay tests, differential results between replicates, and a large amount of retesting of OOS samples between September 2016 and March 2017. The investigator questioned the suitability of the analytical method, given its variability.

The report goes on to detail other concerns about impurity testing:

- *"Testing of [b4] content of [b4] batch [b] by Liquid Chromatography-Mass Spectrometry yielded an unidentified peak at an approximate retention time of [b4] minute. Your firm explained this unknown peak as a 'ghost peak' that appears from time to time in chromatograms for undetermined reasons. This peak was substantially larger than that of [b4] the subject of the testing. No investigation was conducted".*
- *"Impurity testing of [b4] batches [b4] yielded a prominent coalescing[sic] peak with that of the primary [b4] peak. Nevertheless, the impurity was quantitated along with the [b4] peak as desired API and no investigation was initiated.*

However, it was observed later, following a July 2018[2] inspection, that similar lapses in investigating an OOS peak for an unnamed genotoxic impurity were reported on the company's FDA 483 observation form.

- The invalidation of 17 OOS investigations, due to either lab errors, production errors, or a combination of lab and production errors was followed by reprocessing the entire batches prior to release.
- The same form noted a significant mismanagement of a major change in the manufacturing process, which was not properly documented or overseen by the Quality unit, with no *"adequate change control system requiring scientific judgement to determine what additional testing and validation studies are appropriate to justify changes to a validation manufacturing process".*
- Other observations call into question the analytical *"in house test methods, used for Assay and Related Substances", and whether they are "at least equivalent to USP monographic test methods".*
- The report goes on to question the cleaning procedures, their validation and lack of data to show that cleaning was effective.

This section concluded with the failures of the Quality Unit: ***"you released finished API manufactured from crude intermediates with OOS levels of genotoxic impurities without conducting a thorough investigation"*** *because the QA Director stated "(these batches) met the product release specification for Related Substances".*

A later observation hints at how the FDA knew to investigate this specific OOS: they were following a 2016 complaint about the impurity levels from a customer who had responsibly tested an incoming API.

"Your VP of Analytical Operations stated,

- *A Single Quadrupole LC-MS is not as sensitive as a Triple Quad LC-MS and sometimes it gives false positive results.*
- *Your customer tested [b4] batches [b4] and [b4] using a Triple Quadrupole LC-MS.*
- *You sent samples [b4] to an outside laboratory to test using a Triple Quadrupole LC-MS.*
- *Your customer provided you with their LC-MS method.*
- *The outside laboratory used a Triple Quadrupole LC-MS but did not follow the test method provided by your customer."*

In addition, while there were forced degradation tests performed using HPLC for Related Substances, Assay, and [b4] impurity, the FDA notes "not all potential product degradations can be identified by HPLC test methods. Product release tests for [b4] include tests for identification of Residual Solvents by GC-FID. You did not test forced degradation samples for Residual Solvents by GC-FID."

By the time the follow-up Warning Letter was sent at the end of November 2018,[3] the FDA had conducted multiple tests looking for NDMA in both the company's API and drug products formulated and sold by other companies using this valsartan API. The Warning Letter also described that the company had isolated the change to the manufacturing convergence of three process related factors; one factor was the use of the solvent, DMF (Dimethylformamide), and that only product manufacturing with this specific process would be affected. However, the testing by FDA had found NDMA in other batches, too. So how had that happened? At the time of the Warning Letter, it was assumed to be contamination between batches in the company facilities, likely because of their poor cleaning procedures with inadequate cleaning validation tests.

Recalls of valsartan products sold by multiple generic companies had been demanded by both the EMA and FDA as early as July 2018. Throughout August and September 2018, further recalls were announced, and the FDA began documenting all valsartan products which were under recall as well as those that were still available and considered safe to use. The Italian Health agency issued a non-compliance report following a 'for cause' inspection at the original Chinese manufacturer in September 2018. Around the same time, the original manufacturer of the API was put on import alert to prevent any product originating there from legally entering the USA, although the corporation also had a US subsidiary which was critical to facilitating their approvals by FDA.

The FDA released their own recommended GC-MS headspace method[4] for determination of NDMA in valsartan API and finished drug products, which laboratories still needed to validate if the resulting data was used to support a required quality assessment or used in any regulatory submission.

Drug shortages for ARBs

If this was an issue confined to one API and one manufacturer, recall and correction of the manufacturing issue should have solved the quality problems in the supply chain. However, there were multiple API manufacturers involved and an unfortunate cross-contamination problem, coupled with lax testing of incoming materials, which resulted in problems with many other drugs.

By May 2019 the effects of valsartan recalls and alerts led the FDA to serious action. By this time, it was not only valsartan, but other 'sartan drug products that were affected. And the impurity was not only NDMA; another genotoxic impurity, NDEA (N-Nitrosodiethylamine), was also found to be contaminating ARBs. Just a few months before, a third nitrosamine impurity, NMBA (N-Nitroso-N-methyl-4-aminobutyric acid), was detected in losartan tablets originating in India and sold to the US.[5]

Lists of manufacturers whose products were NOT contaminated were being posted, and the FDA was quickly approving new alternative sources of these critical drugs. Notices for patients and care providers were publicized on social media, and new "interim acceptable levels" were proposed so that, for drug products in critical short supply, a very low level of NDMA or NDEA would be acceptable, if the FDA thought it was medically necessary to continue supply.[6]

The NDMA contamination affects other generic drugs

ARB drug shortages continued, as several generic companies who had purchased or manufactured API using the new manufacturing process, were now finding NDMA in other products as well.

In July 2019, a company in India received a Warning Letter[7] from the FDA following a February inspection, saying that NDMA was found in their products, and they had not tested their incoming raw materials properly. It concluded that a solvent as an incoming raw material was contaminated. This solvent had typically been sent to a contract manufacturer, who recycled or "recovered" the solvent then sold it back to pharmaceutical manufacturers and had likely been contaminated due to poor cleaning procedures at that contract supplier.

Shortly after, a contract manufacturer (providing to industry both APIs and recovered solvents), was handed a Warning Letter[8] following an inspection in March 2019. By now, all redaction of products and components were removed, and this letter was clearly related to NDMA contamination. The letter describes how a customer had reported the NDEA contamination in the delivered solvent, and the CMO (Contract Manufacturing Organization) had opened an investigation, during which they noted the NDMA contamination. However, poor record keeping around vessel cleaning in the facility failed to alert the firm that *"there is a potential for all products manufactured at your facility to contain nitrosamine through mix-ups or cross contamination."* This firm, as well as processing solvents, also manufactured similar ARBs and intermediates for non-US supply chains, which might be another source of nitrosamine contamination when equipment was also used for intermediates bound for the US supply chain. When discussing both the poor cleaning processes, and the solvent recovery process, the FDA warned that their overall risk assessment on this issue was not adequate. This included failure to perform adequate testing, and *"a procedure for investigating unknown peaks in recovered solvent chromatograms observed during analytical testing. Unknown peaks observed in chromatograms may represent unanticipated impurities and should be thoroughly investigated".*

Additionally, the firm failed to configure their software associated with the GC instruments with proper 21 CFR Part 11 expectations such as segregated use of the Administrator user role and deletion of data older than 3 months without any backup, relying instead on hard copies of chromatograms which are static and cannot be rescaled as the original electronic dynamic data could.

NDMA contamination goes global

While the source of the contamination was being investigated, regulatory agencies around the world began recalls and public notifications about the actual medical risk of this contamination. The European Medicines Agency (EMA) was already working with the FDA back in July 2018 over the recall of products from the original Chinese firm and had sent in their own inspectors. On January 31, 2019 the EMA announced that any company making sartan medicines review their manufacturing processes for nitrosamine impurities. The EMA published their own information website,[9] including Q&As for medical professionals, their own interim acceptable levels (of not more than ONE nitrosamine impurity) with a plan to revise those downwards in 2 years (March 2021) to allow companies to make necessary changes to their manufacturing processes and suppliers. In addition, the Council of Europe published a list of possible analytical methods and links to methods from other agencies.[10]

Valisure, an online digital pharmacy

Following concerns about both counterfeit and poor quality or inconsistent drugs slipping through the oversight of FDA, and the insights provided by healthcare professionals to Katherine Eban as she researched her book, "A Bottle of Lies",[11] a new online pharmacy called Valisure was founded in 2015 and played a role in finding nitrosamine contaminants. The company tests each batch of commonly prescribed drugs before they are shipped to the consumer, rejecting about 10% before they are sent. A batch-specific Certificate of Analysis is included with each cleared shipment. In March 2019, Valisure began looking for the nitrosamine impurities, as well as DMF, the industrial solvent implicated in creating and carrying these contaminants, which is itself probably carcinogenic. In June 2019 they issued a Citizen Petition about the levels of DMF they were finding in common drugs, even when NDMA was not observed.[12]

Concerns in the US Congress about drug pricing and drug supply chain

Currently in the US, a number of official hearings are taking place at governmental levels discussing the globalization of the pharmaceutical supply chain. While the press may be reporting these with the threat of national security due to the entire population being reliant on a very few sources of API or excipients, questions of both quality and cost are also a concern. If relatively few manufacturers can make a mistake while attempting to drive down costs and in doing so contaminate drugs across the globe, how can we secure alternative suppliers? How much lower should prices be, for consumers and patients, to ensure access to lifesaving medicines, and what compromises to quality, safety and efficacy, or even to environmental concerns, can we accept to get the prices we want?

NDMA impurity found in ranitidine products

As well as testing ARBs, Valisure looked at other products, and using the FDA's GC method, also found very high NDMA levels in ranitidine products and notified the FDA in June 2019 in another Citizen Petition.[13]

While many ranitidine products are prescribed by physicians, there are also many over-the-counter dosage forms available and relied on by consumers for controlling acid in the digestive system. Regulators across the world began examining ranitidine products, issuing recalls, and US pharmacies began to voluntarily pull over-the-counter ranitidine products from their shelves during September 2019. The Valisure website has a list of actions taken by global Health Authorities in relation to ranitidine.[14]

However, two facts are different when considering the presence of NDMA in ranitidine products compared with in ARBs.

First, since the 1980's it was known that, under certain oxidizing conditions, the ranitidine molecule itself can break down or degrade into NDMA, conditions which could occur in storage, but also conditions which could occur in a patient's stomach. Critically, this implies that NDMA might show up in dissolution tests performed by pharmaceutical firms.

Dissolution tests are only validated to perform the assay of the drug over time, as tablets are dissolved in an acidified media replicating typical stomach environments in a controlled experiment. If a new peak appears, which might be an impurity, a contaminant or a degradation product, is there a prescribed procedure as to how it would be investigated and

documented? Especially if the NDMA is not seen in a typical impurity or related substance test performed on tablets not subjected to the acidified conditions?

Secondly, because it appears that the formation of NDMA may be a degradation of the active molecule, could it be formed during the test itself, specifically in a high temperature test like GC? The FDA updated their ranitidine information page[15] to ask all ranitidine manufacturers to assess NDMA in their products, but also noted that the GC method used by "a third-party laboratory uses higher temperatures (which) generate very high levels of NDMA because of the test method". The FDA's test methods for ranitidine used LC-HRMS at lower temperatures, and then shortly after, released an LC-MS/MS method, which does detect NDMA, but at lower levels than those detected by the GC-MS methods.

In its latest post dated November 1, 2019,[16] the FDA announced they are investigating similar NDMA degradation products in another acid-reducing product, nizatidine. Further, they claim that, using these prescribed methods to detect NDMA during tests in a simulated gastric fluid (SGF) model to estimate the biological significance of *in vitro* findings, as well as tests to simulate the environment of the small intestines (simulated intestinal fluid or SIF), LC-MS/MS analysis reveals no additional NDMA formation during the tests.

However, in both the Valisure Citizen Petition and an ongoing court action in California, [17] Valisure claims that they: a) developed a new GC test that only subjects the sample to 37 °C, which should not increase the NDMA formation and, b) agree that in industry standard dissolution tests, using both SGF and SIF media, they saw no NDMA formation. However, when significant sodium nitrite is introduced to the dissolution media (as might be induced by eating pizza or tacos for instance), NDMA formation occurs at detectable levels.

The FDA website also mentions that low levels of NDMA have been found, not only in grilled food, but also in drinking water. Surely, widespread use of ranitidine, in people who might have the right combination chemical environments inside their digestive systems, could in turn be contaminating water supplies. If ranitidine degradation, rather than contamination, is identified as the source of NDMA in dissolution tests, is this a problem which has been occurring over many years, and only now that companies are looking specifically for NDMA, has it come to light?

CONCLUSION

The source of the 'sartan contamination appears to be an understudied and unvalidated production change, resulting in the creation of excess quantities of nitrosamine contaminants, exacerbated by three other factors:

a) A complex and poorly understood supply chain.

b) Lax QC testing on incoming raw material, including solvents.

c) Ineffective cleaning or cleaning validation testing.

It is not yet agreed upon whether the NDMA found in ranitidine and nizatidine is a contamination problem similar to that of the 'sartans, or if it is a degradation issue (either in packaging or in the body).

In the meantime, regulatory agencies have tasked manufacturers in all stages of production, including pharmacies, to take action immediately to understand if these or other harmful impurities are contained in pharmaceutical drugs. On November 14, 2019, the FDA issued a warning letter to a convenience store about the source of their drug products from previous indicted suppliers and some questionable testing of products they packaged to sell.[18]

As impurity testing increases, there will be more impurities found, and as method sensitivity increases, they will be found at lower and lower levels. For instance, the regulatory Health Authority in Singapore, found trace levels of NDMA in 3 out of 46 marketed metformin medicines[19] in December 2019. Ensuring that pharmaceutical scientists are using appropriate analytical methods and accurately reporting unexpected or unusual test results is key to product safety. Analytical methods that are not optimized for such low-level detection can give a false sense of product safety. Simply following the prescribed monograph method is clearly a potential cause for why these impurities went undetected, as the original analytical method might no longer be fit for purpose. But equally, there is the possibility that the nitrosamine impurities were detected but not reported, which is a Data Integrity concern.

Are quality concerns like this the result of driving drug prices down? Whether impurities are benign or harmful, we need to ask if the risks of these low-level impurities are outweighed by the benefits that the drugs provide. The question becomes how, with globalization of the drug supply, do we balance the need to maintain high standards and a good culture of quality with the need for safe, affordable medicines.

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Analysis of nitrosamine impurities: an interview with Javier Jimenez-Villarin

About the speaker

Javier Jimenez-Villarin
Support & Applications Specialist
Waters Corporation

Javier Jimenez-Villarin received his PhD in analytical chemistry from the University of Barcelona (Barcelona, Spain). For his thesis, Javier utilized state-of-the-art LC–HRMS/MS and ion mobility mass spectrometry to identify and characterize pharmaceutical transformation products and their metabolites. Javier then joined a CDMO where he applied his knowledge of analytical instrumentation to the resolution of complex contract manufacturing analysis projects and the characterization of API impurities utilizing HRMS/MS. He joined Waters Corporation in 2019 as a Support & Applications Specialist, focusing on delivering innovative and fit-for-purpose solutions to customers in a wide variety of scientific fields, such as pharma, biopharma, clinical, food and environment and chemical materials. This has included the development of novel analytical methods for the detection and quantification of nitrosamine impurities in raw materials, active pharmaceutical ingredients and final product formulations utilizing LC–MS/MS.

Questions

Nitrosamines are genotoxic impurities that have made their presence known in the pharma industry. To what levels do you believe nitrosamines need to be determined?

Nitrosamines are genotoxic impurities that appeared in 2018 when the European Medicines Regulatory Network alerted us about their presence in certain APIs. This was due to the formation of nitrosamines, and it is believed that contamination from solvents, reagents and equipment also made an impact and led to the presence of nitrosamines in the APIs and the drug products. That's why not only the APIs are affected but also excipients and final drug products are analyzed in order to make sure that no nitrosamines are found in the final drug product. Bearing this in mind, the levels of detection are dependent on the acceptable intake of the impurity in nanograms per day and the maximum daily dose of the drugs in milligrams per day. Therefore, for each API or drug product, the level of detection needs to be calculated for each drug impurity.

What considerations do you take into account when creating a new method for nitrosamine analysis?

That is the key question when I, or we at Waters, start talking about these analyses with customers. The most important thing, I believe, is the limits of detection and quantitation that the customer requests or that the lab requests. This has a close link, or a direct link, to the instrumentation available in the lab or that we will need to implement in the customer's lab. Solubility of the API or the final drug product is another consideration to take into account, as it will have a strong impact on the later development of the method. Finally, the last consideration we usually take into account is all the information that we can gather from the API. as this will help us fully understand the complexity of the separation that is needed to develop for the customer in order to work on these analyses.

What are the main technical challenges you usually face when setting up a new method for nitrosamine analysis? How do you overcome them?

The limit of detection and quantitation requested by the customer is the main thing that we have in mind during the overall method development. The solubility or the physical/chemical properties of the API are also something that is important to have on hand. Waters believe that each customer and API should have their own method. We create fit-for-purpose method development or we implement this kind of method for each customer as the physical/chemical properties of the API, the purity substances and the impurities may differ from one API to another. The main technical challenge about this analysis is to separate nitrosamines from the API, other impurities or residual solvents in the matrix. This is of great importance because when we couple liquid chromatography to mass spectrometry, we have to separate everything as we need to divert the API or the whole matrix goes to waste. In this way we prevent matrix effects coming from the sample. A useful tool in order to overcome this analysis is to use online UV detection with MS to make sure that the API or other impurities at higher concentrations than nitrosamines are diverted to waste. We make sure, in real-time during the analysis and during the chromatographic runs, that the API or the matrix doesn't go into the mass spectrometer. As we are talking about separation all the time, we at Waters are very lucky and that's why we have been successful at overcoming these analyses because we have the complete portfolio of chemistries, in all formats possible and a wide variety of instruments, so we cover sample prep to the final result.

What are the different technologies that might be needed and when would each of them be necessary for an individual lab?

The traditional technique was liquid chromatography coupled to UV detection. This was when the lead of detection and quantification were high enough for the quantitation of nitrosamines, but since 2018 newer limits of detection and quantification were needed. So mass spectrometry started to play a role here. When we need more detection or more power to data detection and quantitation, then we start talking about single-quad mass spectrometry especially in those cases where the concentration of nitrosamines are expected to be high or high enough to be detected by this technique or by this kind of instrumentation. But the tool of reference here in nitrosamine analysis is triple quadrupole as they have the ability to achieve the limits of detection and quantification commonly required by the pharma companies. It is also the tool of reference because we double check each chromatographic run for the presence of nitrosamines, so this technique is less prone to provide false positives. On the other side, we have also high-resolution mass spectrometry for those labs that may expect new nitrosamines or new genotoxic impurities. And finally, on the list, gas chromatography can be an appropriate instrument for the detection and quantitation of nitrosamines, but it may lead to false positive for certain APIs.

How has the transition and/or technology transfer been from HPLC–UV/Vis to UPLC–MS/MS in QC labs?

This transition has been quite smooth as our benchtop single-quad MS, our Acquity PDA, has been a big intermediate step to start acquiring some training, as it is important to gain a lot of training and knowledge in MS analysis for most QC labs. But I still have the feeling when I am in front of the customer that they have a kind of fear. They are worried about using mass spectrometry specifically if they are not fully trained for running this kind of a routine analysis with this instrumentation. They are also worried about being compliant. That's why we at Waters believe that we deliver benefit to customers, not by only providing the instrumentation, columns or the solutions, but also providing training, knowledge and what's most important for me is accompaniment over time.

As a final thought, do you see nitrosamines going away at any point and do you see any new impurities in the near future?

That's for sure. Nitrosamines have been the first milestone in genotoxic impurity analysis and for sure the search and investigation of these impurities will be part of future drug development. Pharma companies from now on will be cautious during synthesis from raw material to final product in analyzing each critical step, looking for nitrosamines in this case or new genotoxic impurities. In the end, nitrosamines won't go away anytime soon and they will remain a hot topic in the field. That's because the regulators like the FDA or the EMA are being more strict in terms of regulation, especially in the content of these kinds of impurities in the final products or the APIs and that's why, when we talk to customers, we try to emphasize that it is important to be prepared for today but also for the future. The key advice is to acquire or to be trained in this kind of more advanced technology for the detection and quantitation at these compounds. I am sure Waters will continue to play a key role in supporting our customers and for making them successful during the synthesis or manufacturing of medicines.

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Application Note

High Sensitivity Quantitation of Nitrosamine Genotoxic Impurities: LC-MS Analysis of Ranitidine Drug Product using the Waters ACQUITY UPLC I-Class/Xevo TQ-XS Tandem Quadrupole Mass Spectrometer

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Abstract

This application note presented herein, provides practical considerations for optimization of LC-MS conditions to achieve sensitive and robust simultaneous quantification of several nitrosamine GTIs (NDMA, NDEA, NEIPA, NDIPA, NDBA, and NMBA).

Benefits

- A simple and reproducible method for detection and quantification of multiple nitrosamine impurities
- Use of the UPLC HSS T3 Column for excellent reversed-phase chromatographic retentivity of NDMA, and resolution from the drug product ranitidine
- Highly sensitive and accurate quantification using the ACQUITY UPLC I-Class PLUS for separation and Xevo TQ-XS Mass Spectrometer for nitrosamine detection
- Nitrosamine quantification achieving LLOQs of 0.025–0.1 ng/mL

Introduction

Ranitidine is a histamine-2 blocker, which decreases the amount of acid created by the stomach and is approved for multiple indications, including treatment and prevention of stomach and intestinal ulcers, as well as treatment of gastroesophageal reflux disease.[1] Ranitidine is manufactured by many pharmaceutical and generic companies and is available over the counter (OTC) and by prescription. In 2019, reports appeared that the N-nitrosamine impurity, N-nitrosdimethylamine (NDMA) was found to be present in ranitidine drug products and resulted in recalls of this product.[2,3]

N-nitrosamines, as a class, are known environmental contaminants with suspected carcinogenic/genotoxic effects in animals and humans.[4,5] In response to public concern, regulatory agencies have issued guidance for allowable limits of these genotoxic impurities (GTIs) with an acceptable daily intake limit of 96 ng/day (0.32 ppm) for NDMA in ranitidine and a proposed limit in the future of 0.03 ppm. Information on how to assess and control these impurities can be found in the ICH M7 (R1) guideline.[7]

Due to the regulatory guidance's low safety threshold levels for these compounds, there exists a strong need for LC-MS methods that can accurately quantify them at low ppm levels. Developing such methods is challenging due to the chemical diversity of nitrosamines, poor chromatographic retention, MS ionization, and fragmentation, often limiting sensitivity and selectivity. This work presented herein, provides practical considerations for optimization of LC-MS conditions to achieve sensitive and robust simultaneous quantification of several nitrosamine GTIs (NDMA, NDEA, NEIPA, NDIPA, NDBA, and NMBA). A list of these impurities, including their chemical information, is shown in Table 1. The developed analytical method employs ultra performance liquid chromatography (UPLC) and tandem quadrupole MS-MS detection. Using the low dispersion ACQUITY UPLC I-Class PLUS and reversed-phase (UPLC-RP) separation with a sub-2-µm C_{18} column designed specifically for retention and separation of polar compounds coupled to a high sensitivity tandem quadrupole MS, lower limits of quantification (LLOQ) between 0.025–0.1 ng/mL (<1 pg on column) in ranitidine drug substance and product were achieved. This method was used to analyze a ranitidine drug product tablet, achieving an LLOQ of 0.1 ng/mL (0.0025 ppm based on a 30 mg/mL dose) and determining the concentration of NDMA in the tablet to be 29.0 ng/mL, or 1 ppm relative to the ranitidine API.

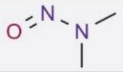
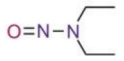
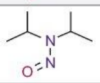
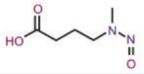
Impurity	Common name	Empirical formula	Chemical structure
NDMA	N-nitrosodimethylamine	$C_2H_6N_2O$ MM: 74.05	
NDEA	N-nitrosodiethylamine	$C_4H_{10}N_2O$ MM: 102.8	
NEIPA	N-nitrosoethyl isopropylamine	$C_5H_{12}N_2O$ MM: 116.09	
NDIPA	N-nitrosodiisopropylamine	$C_6H_{14}N_2O$ MM: 130.11	
NDBA	N-nitrosodibutylamine	$C_8H_{18}N_2O$ MM: 158.14	
NMBA	N-nitroso-N-methyl-4-aminobutyric acid	$C_5H_{10}N_2O_3$ MM: 146.07	

Table 1. List of nitrosamine impurities with common names and chemical information.

Experimental

Preparation of samples and calibration standards

NDMA, NDEA, and NMBA were obtained from Sigma-Aldrich (St. Louis, MO, USA). NDBA, NEIPA, and NDIPA were obtained from Toronto Research Chemicals (Ontario, Canada). Individual stock solutions (5.0 mg/mL) of the nitrosamines were prepared in methanol. Using the 5 mg/mL individual nitrosamines stock solutions, a combined working stock solution (250 µg/mL) of the 6 nitrosamines was prepared in methanol. Stock solutions containing 30 mg/mL ranitidine drug substance (DS) or drug product (DP) were prepared in water. The age, expiry, and storage conditions of the ranitidine drug tablet were unknown. Calibration curve standards (0.025–100 ng/mL) were prepared by spiking the working solution of the nitrosamine impurities into the prepared ranitidine DS and DP solutions. The prepared samples (30 µL) were then analyzed using the described LC-MS method in a previously published application (Application Note 720006751EN) using the ACQUITY UPLC I-Class PLUS (A) and Xevo TQ-XS Tandem Quadrupole Mass Spectrometer.

Results and Discussion

Mass Spectrometry

Detection and quantification of the nitrosamine impurities was performed using atmospheric pressure chemical ionization (APCI) MS operating in the positive ion mode using the Xevo TQ-XS Tandem Quadrupole Mass Spectrometer. A previous proof of concept application (Application Note 720006751EN) for MS nitrosamine impurity quantification provides detailed MS conditions, including the nitrosamine impurity MRM transitions. The multiple reaction monitoring (MRM) transitions and conditions chosen for nitrosamine analysis were optimized using MassLynx IntelliStart Software and confirmed with manual infusion. Representative MS spectra of the MH+ precursors for the 6 nitrosamines (10 µg/mL), using a combined infusion, is highlighted in Figure 1a, while an example of product ion spectra generated for NDMA, using optimal collision energies (CEs) for the 43.1 and 58.1 NDMA fragments is demonstrated in Figure 1b.

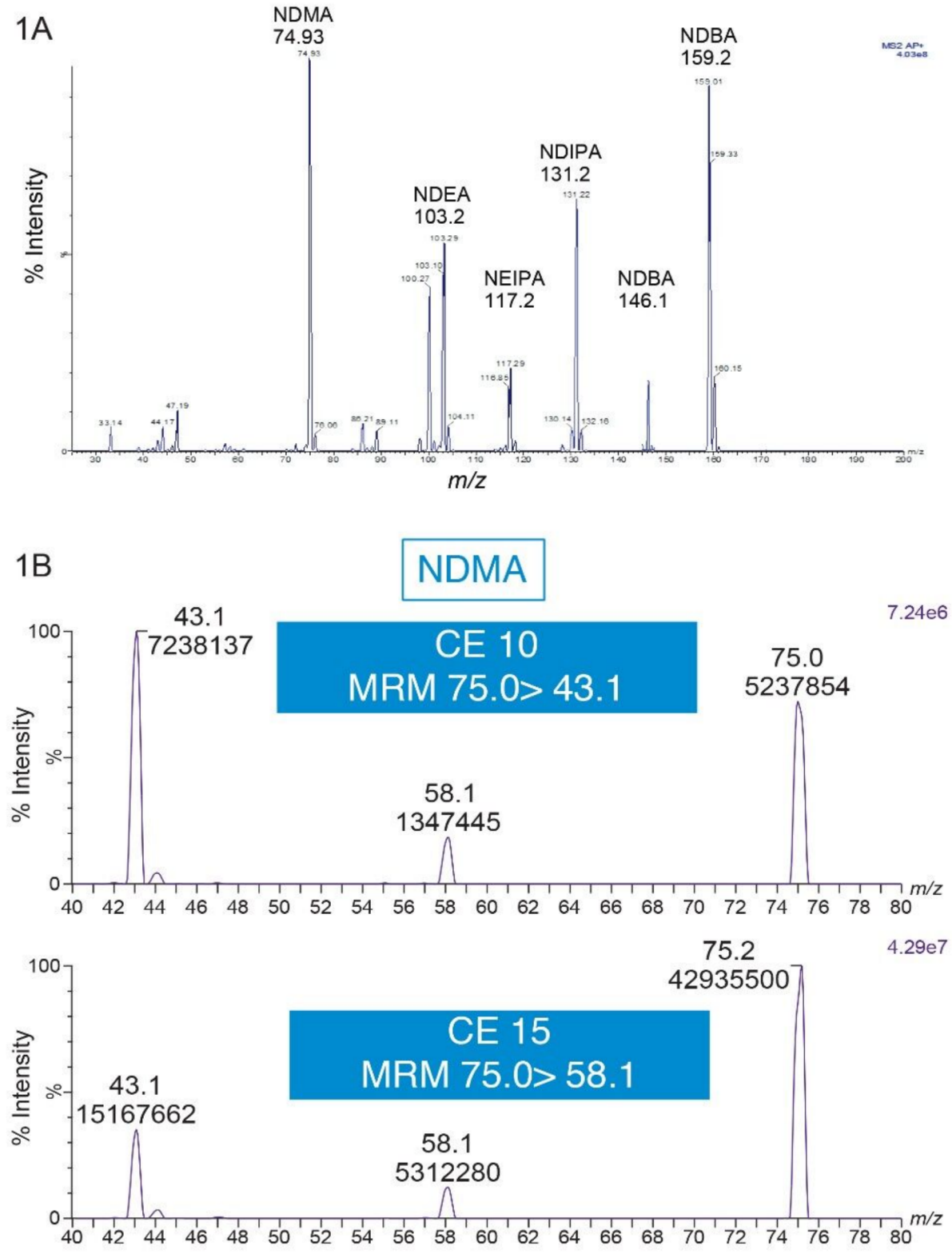


Figure 1. Representative MS precursor MH+ spectra for the nitrosamine impurities (A) and representative

product ion spectra for the NDMA nitrosamine (B), identifying the primary fragments of 43.1 and 58.1 with optimal collision energies of 10 and 15, respectively.

During MS method development, use of an APCI probe over the more common electrospray ionization (ESI) probe provided 10X better sensitivity. This is illustrated in Figure 2 for the NDMA (A) and NDEA (B) nitrosamines. APCI is a soft ionization method well-suited for polar and relatively less polar thermally stable compounds with small molecular weights. Additionally, use of soft transmission/ionization mode within the experimental method further minimized in-source fragmentation, aiding in improved MS signal performance (peak area/height) for NEIPA, NDIPA, and NMBA (data not shown).

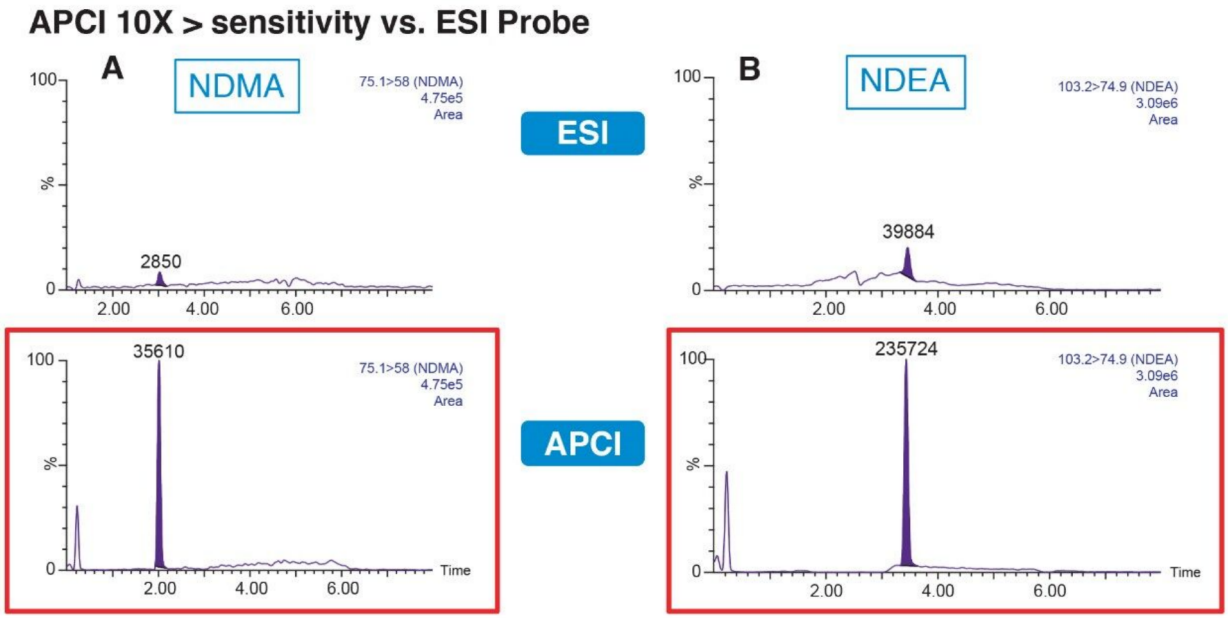


Figure 2. Comparison of APCI and ESI MS performance for the nitrosamines, NDMA (A) and NDEA (B). Use of the IonSABRE APCI probe provided a 10X improvement in analyte response vs. the ESI probe.

An additional improvement in MS signal (peak area/height) for the nitrosamines was achieved by decreasing probe and source temperatures (250/130 °C). This improvement is highlighted in Figure 3. Use of short dwell times (<30 msec.) for each MRM transition and fast scan time of the Xevo TQ-XS Mass Spectrometry system allowed for the simultaneous acquisition of all compounds with >/= 10 data points for each nitrosamine. Final MS conditions are provided in Figure 4.

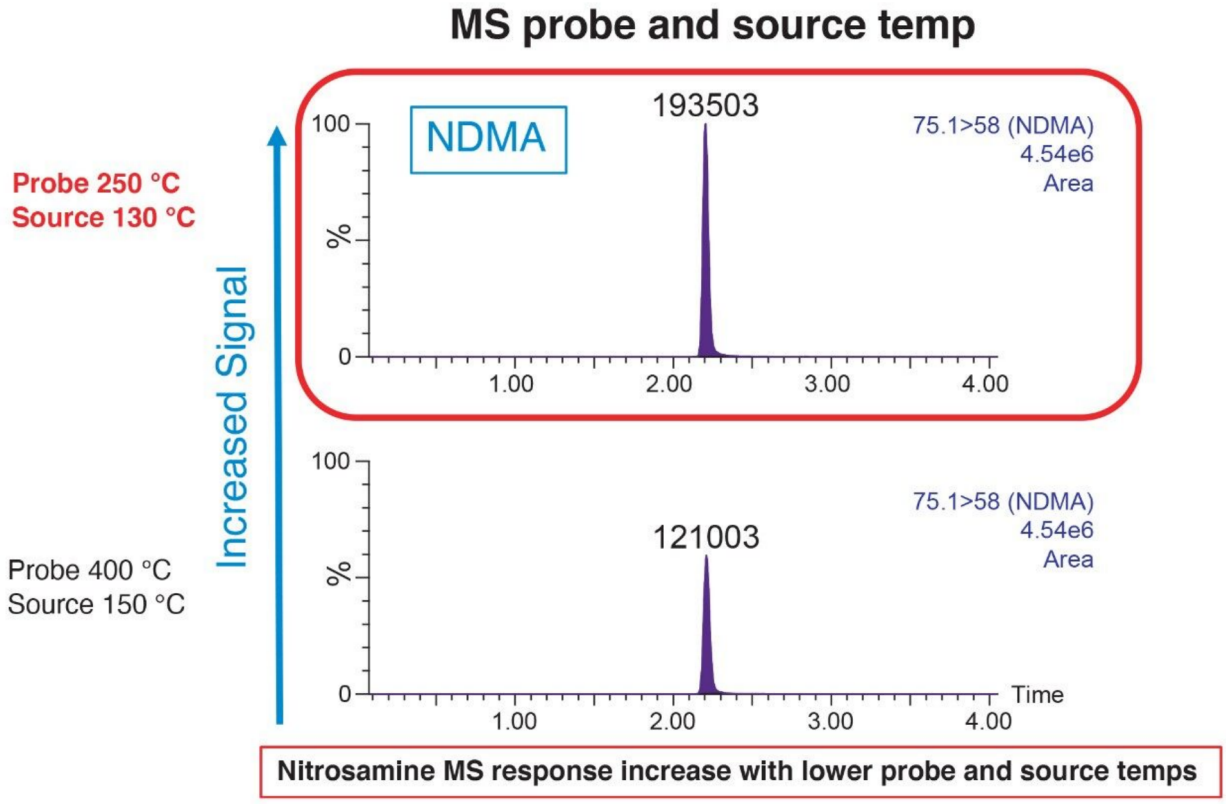


Figure 3. Demonstration of improved nitrosamine impurity analyte MS response with probe and source temperatures.

MS system	Xevo TQ-XS Tandem Quadrupole
Source	APCI +
Corona	1.3 µA
Nebuliser	3.5 bar
APCI probe temp.	250 °C
Desolvation flow	1000 L/Hr
Cone gas flow	150 L/Hr
Cone voltage	30 V
Source temp.	130 °C
Data management:	Instrument control software: MassLynx (v4.2) Quantification software: TargetLynx

Figure 4. Xevo TQ-XS Mass Spectrometer final optimized instrument conditions detection, and quantification of nitrosamine impurities from ranitidine drug substance and product.

UPLC Chromatography

During method development, both reversedphase (RP) and reversed-phase/anion exchange columns (RP-AX) were evaluated for overall chromatographic performance (e.g., assessment of retention, peak shape, influence of diluent composition, area counts, and signal to noise). While the ACQUITY CSH Phenyl Hexyl and Atlantis PREMIER BEH C_{18} AX columns provided adequate retention for the six nitrosamines and ranitidine API during method development, best overall chromatographic performance for the most polar nitrosamine, NDMA, and ranitidine was achieved using the ACQUITY HSS T3 column (Figure 5). The HSS T3 column not only provided significantly better retention for NDMA and ranitidine, but also facilitated resolution from the closely eluting NMBA nitrosamine impurity.

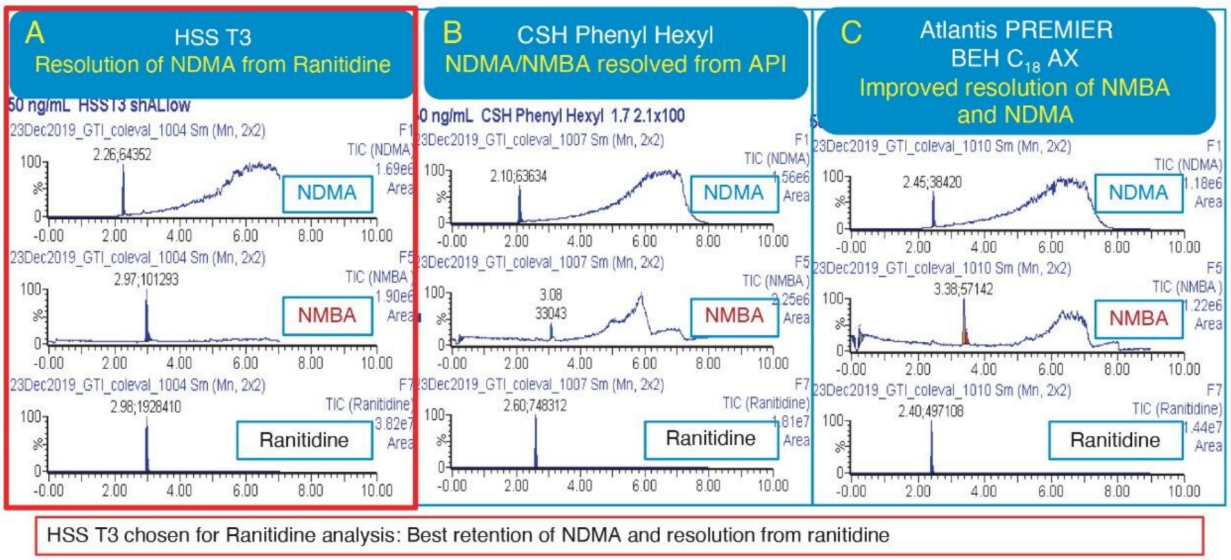


Figure 5. Comparison of chromatographic performance for the NDMA and NMBA nitrosamine impurities and ranitidine API in water: methanol neat solution (80:20) using the ACQUITY UPLC HSS T3 (A), CSH Phenyl Hexyl (B), and Atlantis PREMIER BEH C_{18} AX (C), 2.1 mm × 100 mm columns. The HSS T3 provided best retention for NDMA and facilitated resolution from ranitidine and the closely eluting NMBA nitrosamine impurity.

Full UPLC chromatographic separation for all nitrosamines and ranitidine is illustrated in application note 720006751EN. Separation of the API from the impurities is critical, as it allows use of the divert valve to send the API to waste during analysis, minimizing impact of large quantities of the API (mg/mL) from interfering in the trace analysis (pg/mL) of the nitrosamine impurities. Use of ammonium formate buffer improved analyte performance (peak area/height) and minimized baseline noise, further improving levels of detection of the nitrosamine impurities in this assay (data not shown). While one of the benefits of using a low dispersion LC system and sub-2-µm chemistry is use of high flow rates for fast analysis, in this assay it was determined that a lower flow rate of 0.35 mL/min further improved analyte intensity (peak area/intensity).

Quantitative Performance

The quantitative performance using the optimized LC-MS method was excellent, achieving LLOQs between 0.025–0.1 ng/mL for the nitrosamine impurities in drug substance (DS) with S/N ratios ≥10. Relative to load on column with a 30 uL injection, this would be equivalent to 0.75–3 pg/mL for a 0.1 ng/mL LLOQ. Calibration curves were linear (R^2 ≥0.99) with accuracies between 85–115% for all points on the curve (Table 2), meeting recommended method validation guidelines for LC-MS quantitative analysis. Representative

chromatographic performance of the 0.025, 0.05, and 0.1 ng/mL over-spiked DS samples as compared to the blank is illustrated in Figure 6. Relative to the ranitidine API concentration (30 mg/mL), these LLOQs (<0.003 ppm) exceed the recommended regulatory limits of nitrosamine impurity detection of 0.3 ppm.

Nitrosamine quantification performance (neat solution)				
Nitrosamine	**Std curve range (ng/mL)**	**Weighting**	**Linear fit (R^2)**	**MRM transition**
NDMA	0.1-100	1/x	0.99	75.1>58.0 75.1>43.1
NDEA				103.2>74.9 103.2>46.9
NDBA				159.2>103.2 159.2>57.1
NMBA				147.1>117.1 147.1>44
NEIPA				117.2>74.9 117.2>43.1
NDIPA				131.2>89.1 131.2>47.1

Table 2. Calibration curve performance of the nitrosamine impurities spiked in a neat solution of water:methanol (80:20) using a 30 µL injection of prepared sample.

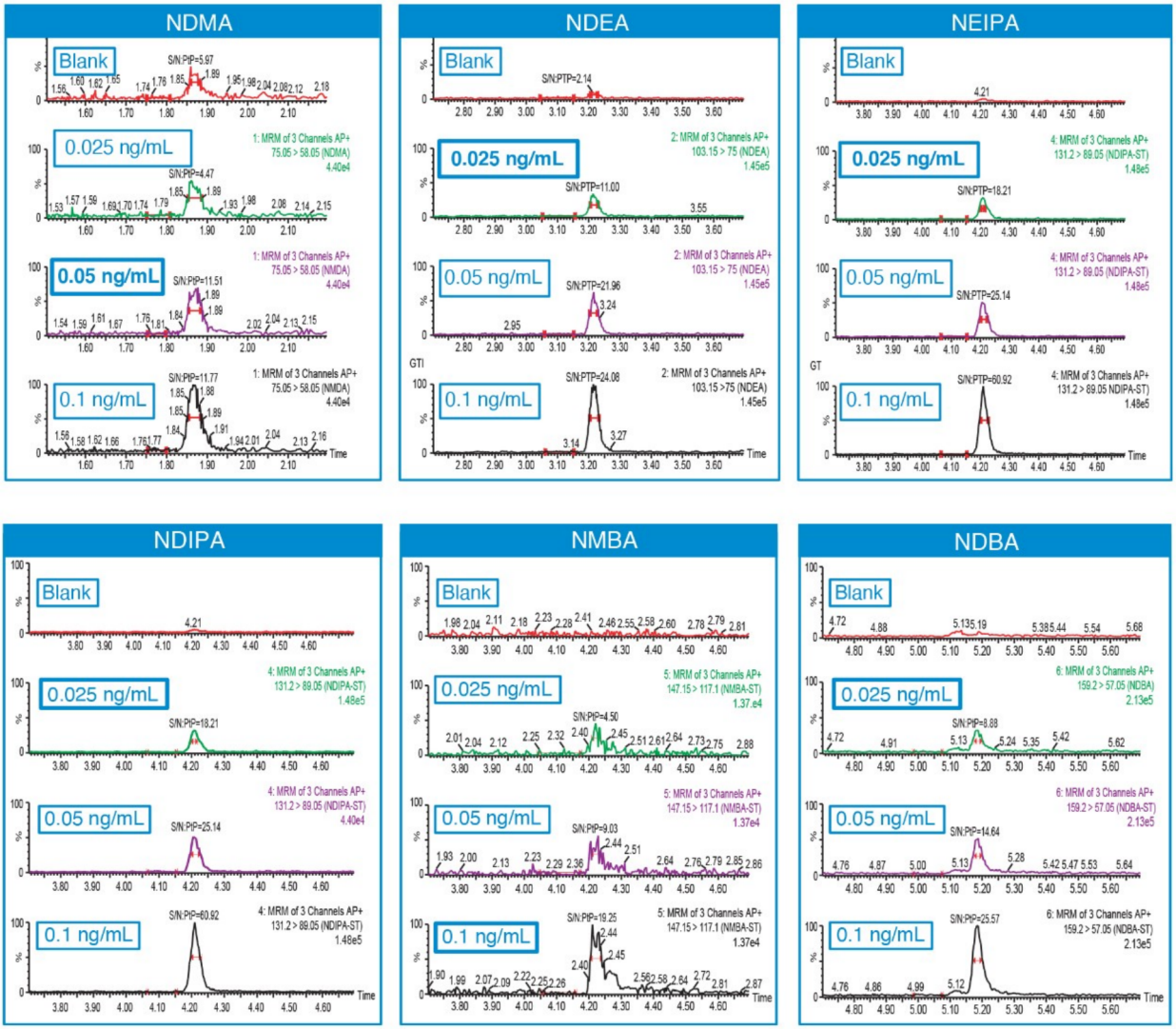


Figure 6. Representative chromatographic performance of the nitrosamine impurities spiked in a neat solution of water:methanol (80:20) at concentrations of 0.025, 0.05, and 0.1 ng/mL.

Analysis of the nitrosamine impurities in drug product (DP) also resulted in excellent quantitative performance with linear dynamic range of the calibration curves from 0.1–100 ng/mL. Representative chromatograms for NDMA (A), NDEA (B), NDBA (C), and NDIPA (D), over-spiked in drug product (0.5 ng/mL) as compared to the blank DP sample are illustrated in Figure 7, while the representative calibration curves are highlighted in Figure 8. It is important to highlight the use of multiple MRMs for each nitrosamine impurity, to ensure adequate sensitivity and selectivity. During MS optimization, the 75.1>58.1 MRM fragment of NDMA provided the best overall peak intensity and reduced baseline. However, during analysis of ranitidine DP and DS over-spiked with the nitrosamine impurities, it was found that the NDMA MRM

75.1>43.1 transition was more intense than the 75.1>58.1 MRM transition. This is illustrated in Figure 9 for ranitidine DP (A) and DS (B).

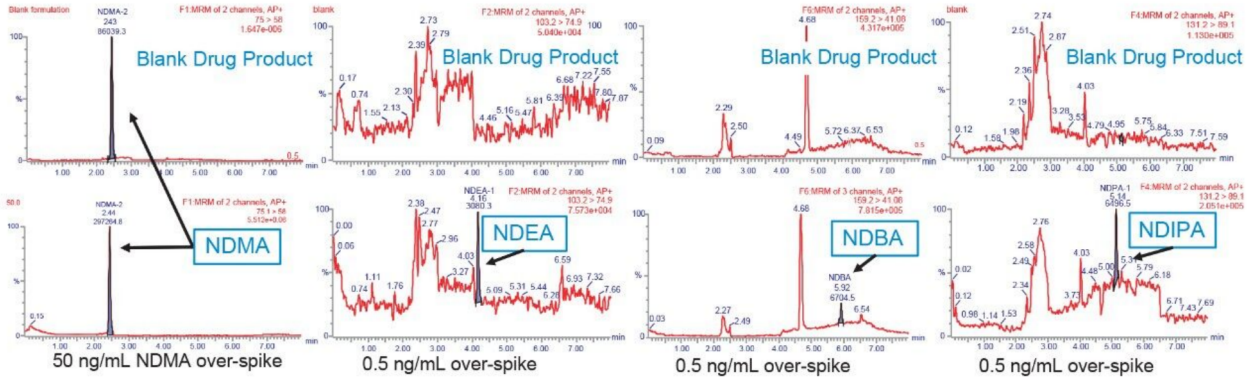


Figure 7. Representative chromatographic performance of prepared ranitidine drug product samples, comparing blank to a 0.5 ng/mL nitrosamine impurity over-spike sample for NDMA, NDEA, NDBA, and NDIPA.

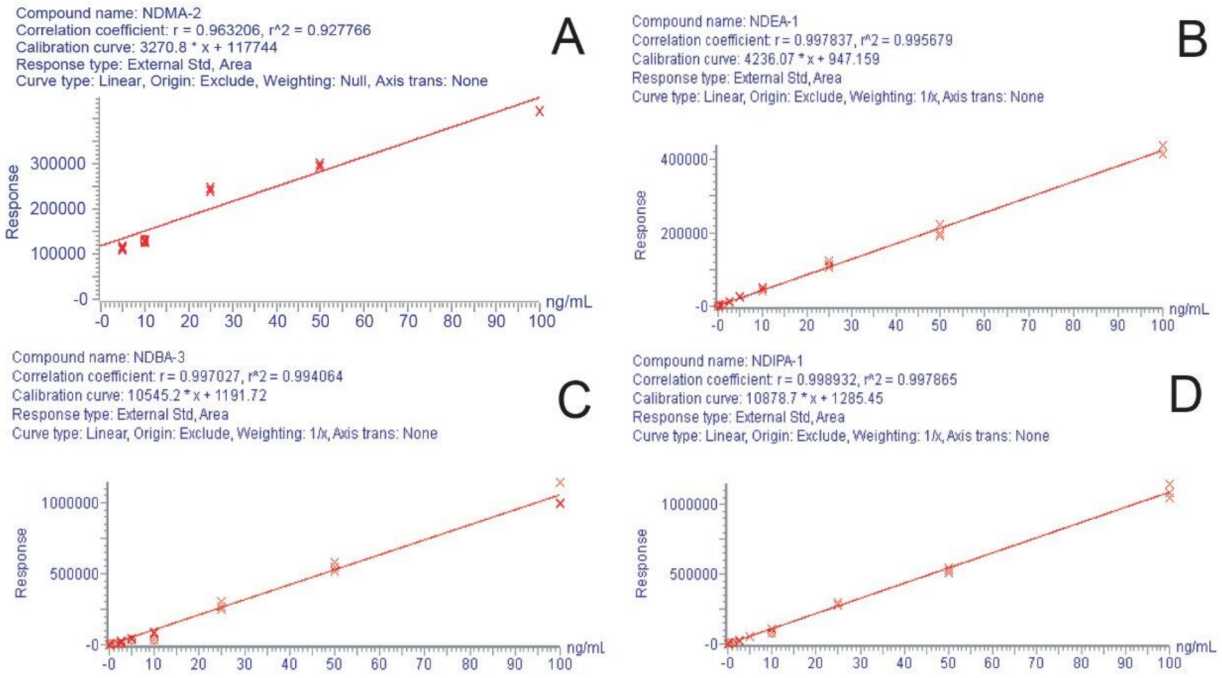


Figure 8. Representative ranitidine drug product calibration curves (0.1–100 ng/mL) with linearity ≥0.99 for the NDMA (A), NDEA (B), NDBA (C), and NDIPA (D) nitrosamine impurities. Note: Due to endogenous NDMA levels found in drug product, the NDMA intercept does not pass through zero.

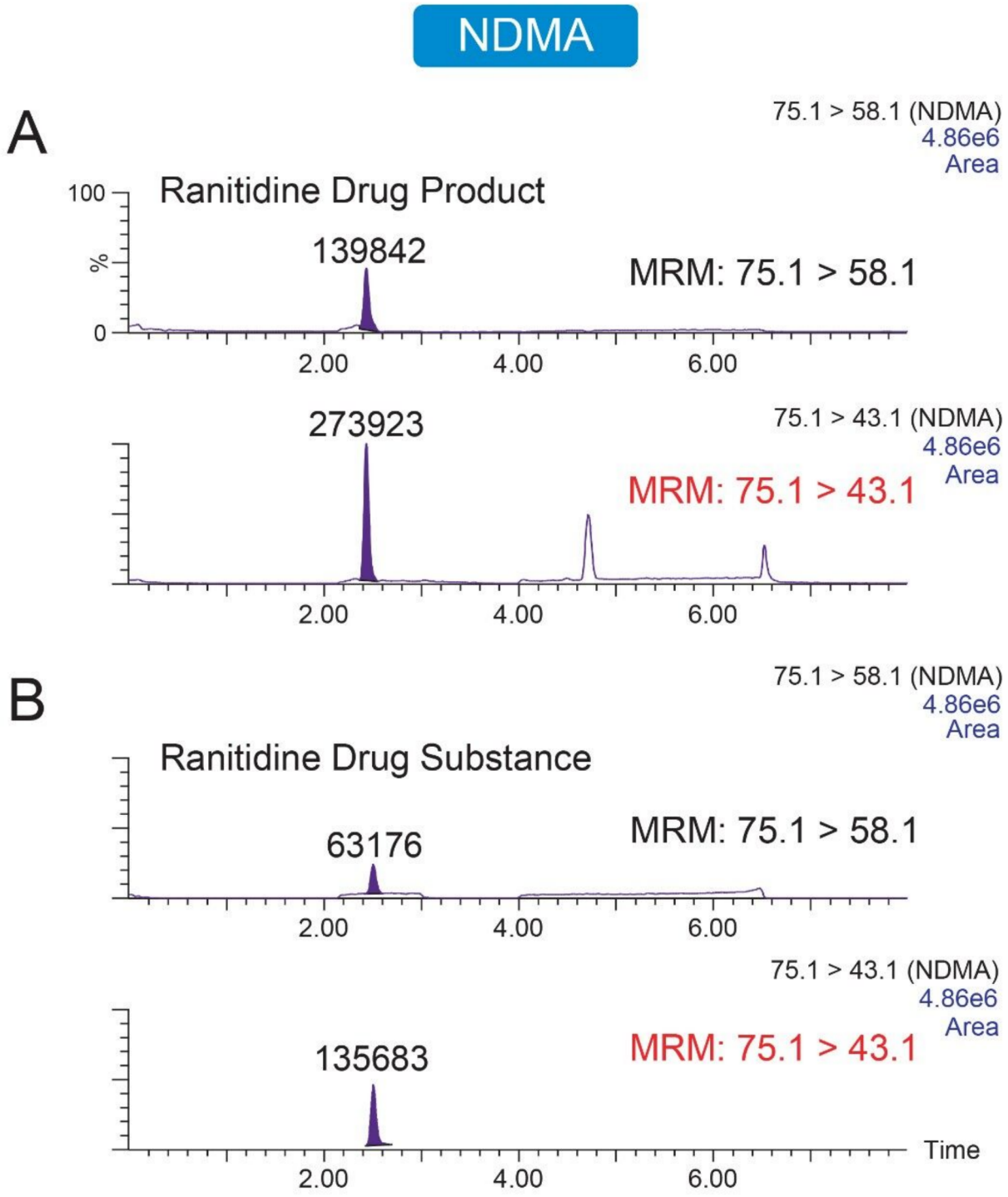


Figure 9. Chromatography illustration highlighting better MS sensitivity of the NDMA nitrosamine in ranitidine drug product (A) and drug substance (B) using the 75.1 > 43.1 MRM transition, shown by increased peak area.

Due to the detection of a large NDMA impurity peak for the prepared ranitidine drug product with unknown age, expiry, and storage conditions, highlighted in Figure 7, the analysis was repeated with re-prepared DP samples. For the repeated assay, the gradient was slowed to 0.3 mL/min and the divert valve was not employed. Figure 10 shows confirmation of a large NDMA peak in DP, using both MRM transitions, which is well separated from ranitidine (Panel A). Peak area of both MRM transitions increases with NDMA over-spike of 10 ng/mL (Panel B). Green dashed lines indicate where the divert valve would be used to switch to waste during normal analysis, shuttling ranitidine API to waste.

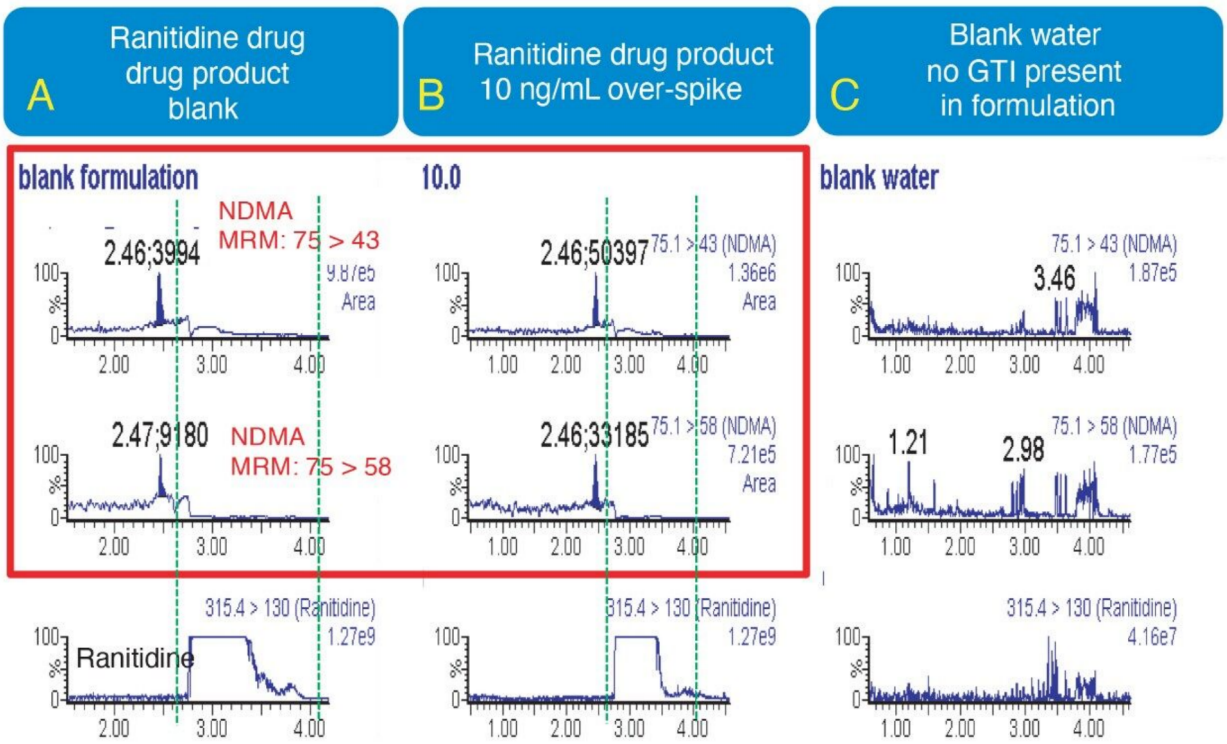


Figure 10. Confirmation of endogenous NDMA nitrosamine impurity (2 MRM transitions) in ranitidine DP (A), increase in NDMA peak response with 10 ng/mL NDMA over-spike in DP (B), and absence of the NDMA peak in blank neat solutions before and after injection of DP samples (C). Green dashed line indicates time divert valve switched to shuttle rantidine API to waste during analysis.

Finally, there was no detection of the NDMA GTI in blank water, which was injected before and after the blank, un-spiked, and DP samples (Panel C). This confirmed that the NDMA was real and not a result of contamination from other samples or carry over due to the LC-MS method.

Due to the presence of endogenous NDMA in the DP sample, the standard addition method was used to

determine NDMA concentration. Using linear least squares analysis, the slope (3270.8), and intercept (117744) of the calibration line were used to estimate NDMA levels. Performing this regression analysis, NDMA levels in DP sample were estimated to be 36 ng/mL (>1 ppm) and was confirmed with both MRM transitions. Following NDMA level estimation, all calibration points were corrected, by adding the estimated 36 ng/mL to each spiked NDMA impurity concentration and a corrected calibration curve was regenerated. The dynamic range of the corrected calibration curve was 1–100 ng/mL ($R^2 \geq 0.99$ using 1/x weighting) with recoveries between 85–115%. This performance is highlighted in Table 3. Mean calculated NDMA concentration in the DP samples (N=4) was determined to be 28.18 ng/mL (~1 ppm), as shown in Table 4.

Corrected standard curve performance of NDMA in DP with recoveries between 85–115%

	Name	Sample Text	Vial	Type	Std. C...	Area	Response	S/N	ng/ml	%Rec
18	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1015	0.25	1:F,9			3.17e4	31700	168.607	11.01	
19	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1016	0.5	1:F,8			1.08e5	108000	225.363	20.36	
20	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1017	0.5	1:F,8			7.61e4	76100	326.839	16.47	
21	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1018	1.0	1:F,7	Analyte	36.000	1.07e5	107000	345.026	20.31	56.4
22	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1019	1.0	1:F,7	Standard	36.000	2.34e5	234000	663.546	35.89	99.7
23	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1020	1.0	1:F,7	Standard	36.000	2.24e5	224000	381.029	34.72	96.4
24	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1021	2.5	1:F,6	Standard	37.500	2.49e5	249000	500.458	37.69	100.5
25	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1022	2.5	1:F,6	Standard	37.500	2.46e5	246000	616.546	37.33	99.5
26	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1023	5.0	1:F,5	Standard	41.000	2.66e5	266000	764.654	39.79	97.1
27	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1024	5.0	1:F,5	Standard	41.000	2.68e5	268000	698.564	40.06	97.7
28	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1025	5.0	1:F,5	Standard	41.000	2.73e5	273000	342.203	40.63	99.1
29	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1026	10.0	1:F,4	Standard	46.000	3.04e5	304000	1031.6...	44.50	96.8
30	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1027	10.0	1:F,4	Standard	46.000	3.05e5	305000	917.715	44.65	97.1
31	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1028	10.0	1:F,4	Standard	46.000	3.08e5	308000	999.476	44.93	97.7
32	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1029	25.0	1:E,3	Standard	61.000	4.93e5	493000	2650.8...	67.76	111.1
33	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1030	25.0	1:E,3	Standard	61.000	4.85e5	485000	1976.1...	66.81	109.5
34	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1031	50.0	1:E,2	Standard	86.000	6.57e5	657000	2071.5...	87.87	102.2
35	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1032	50.0	1:E,2	Standard	86.000	6.51e5	651000	2624.0...	87.15	101.3
36	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1033	100.0	1:E,1	Standard	136.000	9.84e5	984000	3843.5...	128.16	94.2
37	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1034	blank water	1:H,3	Blank						
38	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1035	blank water	1:H,2	Blank		1.35e3	1350	2.160	7.27	

Table 3. Corrected NDMA standard curve performance in prepared ranitidine DP samples (1.0–100 ng/mL) with NDMA recoveries between 94.2–111.1%.

Mean (N=4) Calculated NDMA concentrations in DP = 28.18 ng/mL (~1 ppm)

	Name	Sample Text	Vial	Type	Std. C...	Area	Response	S/N	ng/mL	%Rec
1	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1001	blank water	1:H,3	Blank						
2	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1002a	blank water	1:H,2	Blank						
3	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1002b	blank water	1:H,2	Blank						
4	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1002	blank water	1:H,2	Blank						
5	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1003	blank formul...	1:H,7	Analyte		1.20e5	120000	189.655	21.915	
6	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1004	blank formul...	1:H,8	Analyte		1.79e5	179000	421.920	29.133	
7	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1004_2	blank formul...	1:H,8	Analyte		1.88e5	188000	376.751	30.193	
8	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1005	blank formul...	1:H,9	Analyte		1.98e5	198000	694.523	31.487	
9	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1006	blank water	1:H,1	Blank						
10	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1007	blank water	1:H,2	Blank						
11	24Mar2020_Zantac Drug Formulation30mgmL_6mixGTI_1008	blank water	1:H,3	Blank						

Table 4. Mean (N=4) calculated NDMA concentration (29.0 ng/mL) in prepared ranitidine DP samples.

Conclusion

Use of the reversed-phase HSS T3 Column provided excellent retentivity for nitrosamine impurities, particularly the most polar nitrosamine, NDMA, while also providing separation from ranitidine API. Detection using a tandem quadrupole MS system with MRM analysis using atmospheric pressure chemical ionization (APCI), provided a 10X fold sensitivity improvement compared to electrospray ionization (ESI) for the nitrosamines. With this developed assay, LLOQs 0.025–0.1 ng/mL (<3 pg/mL on-column), for the various nitrosamine impurities were achieved for neat standard solutions, DS, and DP, with recoveries between 85–115% for the calibration points. The specificity, sensitivity, and broad linear dynamic range of this developed assay easily detected 0.1 ng/mL (0.0033 ppm, relative to 30 mg/mL DP or DS) of the nitrosamines in ranitidine drug product. Using this method, endogenous levels of NDMA from a prepared ranitidine drug tablet were detected and calculated to be 28 ng/mL (~1 ppm). The performance of this developed assay demonstrates a highly sensitive, accurate, and robust method for simultaneous nitrosamine impurity detection and quantitation, easily achieving regulatory guidance threshold values for these nitrosamine impurities in drug substance and drug product.

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