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HIGHLY SENSITIVE AND ROBUST DETERMINATION OF 12 NITROSAMINE IMPURITIES IN VALSARTAN DRUG PRODUCT

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ABSTRACT

Nitrosamine impurities belong to a class of probable carcinogens that are present in various pharmaceutical products and are also called as pharmaceutical contaminants. In this study 12 out of the most significant nitrosamine impurities were taken for quantitative determination in Valsartan tablet formulation that are commercially available in multiple strengths of 40mg, 80mg and 320mg etc. For acceptable intake and other relevant information, EMA and FDA guidelines were referred. The experiments in this work were performed as per current requirements in analytical chemistry where highly sophisticated analytical techniques are required such as LC-MS/MS to quantify trace levels of nitrosamine impurities in drug products. The results show that an appropriate method is required to be developed and validated for an accurate and reliable detection of nitrosamine impurities and that is what is performed in this study. Depending upon the Acceptable intake of 26.5 ng/day and 320mg of Maximum daily dose for Valsartan, 0.083 ppm (parts per million) was the specification limit. This kind of methodology supports routine product testing to ensure product safety and regulatory compliance.

KEYWORDS: Nitrosamines (NSA), Nitroso drug substance related impurities (NDSRIs), analytical chemistry, LC-MS/MS, pharmaceutical contaminants, carcinogens, acceptable intake (AI), maximum daily dose (MDD).

1. INTRODUCTION

The detection of nitrosamine impurities in pharmaceutical products has emerged as a critical concern in global drug safety, prompting significant regulatory and scientific scrutiny. Nitrosamines, particularly N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA), are classified as probable human carcinogens by the International Agency for Research on Cancer (IARC) due to their genotoxic and carcinogenic potential at trace levels[1] The issue gained international prominence in June 2018, when NDMA was first identified in valsartan active pharmaceutical ingredients (APIs) manufactured by Zhejiang Huahai Pharmaceuticals, China.[2] This discovery led to widespread recalls and initiated a comprehensive investigation into the sartan class of angiotensin II receptor blockers (ARBs), including irbesartan, losartan, and candesartan, which share a common synthetic pathway involving the tetrazole ring—a known precursor site for nitrosamine formation.

The root causes of nitrosamine contamination were traced to specific manufacturing conditions, including the use of sodium nitrite in the presence of secondary or tertiary amines under acidic conditions, as well as the reuse of contaminated solvents and reagents.[3] In response, regulatory authorities such as the European Medicines Agency (EMA), U.S. Food and Drug Administration (FDA), and World Health Organization (WHO) issued a series of risk-based guidelines and mandated the implementation of stringent control strategies, including the establishment of compound-specific acceptable intake (AI) limits and the requirement for confirmatory testing of both APIs and finished dosage forms.[4,6]

Amid these developments, Liquid Chromatography coupled with Mass Spectrometry (LC-MS) has emerged as the analytical method of choice for nitrosamine detection, owing to its high sensitivity, specificity, and robustness in complex pharmaceutical matrices. Since 2019, LC-MS/MS and high-resolution mass spectrometry (HRMS) platforms have been extensively employed to detect nitrosamines at sub-nanogram per milliliter concentrations, aligning with regulatory thresholds often set below 30 ng/day. Methodological advancements, including isotope dilution techniques, solid-phase extraction (SPE), and matrix-matched calibration, have further enhanced the reliability and reproducibility of nitrosamine quantification.

By 2025, the integration of LC-MS-based nitrosamine testing has become a regulatory and industry standard, with validated methods forming a critical component of pharmaceutical quality control and risk mitigation frameworks. This research presents a comprehensive evaluation of nitrosamine impurities in Valsartan tablet formulation of 80mg strength

arranged from market, employing state-of-the-art LC-MS/MS methodologies. The work contextualizes as to what are the basic needs to have a reliable and suitable method for analytical determination of nitrosamine impurities in samples that would ultimately bring analytical findings within the broader historical and regulatory landscape, offering insights into the evolution of nitrosamine surveillance and its implications for pharmaceutical manufacturing and public health.

2. Literature Review

2.1 Advances in Nitrosamine Detection

After initial detection of N-nitrosodimethylamine (NDMA) in valsartan in 2018[7], a surge of analytical research has focused on developing sensitive and selective methods for nitrosamine detection in pharmaceutical products. Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS) has become the method of choice due to its ability to detect nitrosamines at sub-nanogram levels. Studies have demonstrated the successful application of LC-MS/MS and high-resolution mass spectrometry (HRMS) for the quantification of multiple nitrosamines in complex matrices, including Valsartan formulations. LC-MS/MS being more useful in routine nitrosamine analysis and HRMS is best towards qualitative analysis e.g., characterization of compounds in qualtitative analysis and in investigatory and confirmatory quantitative analysis with higher mass accuracy and low ppm mass tolerance capabilities.

2.2 Methodological Gaps and Analytical Challenges

Despite these advancements, several limitations persist. One major challenge is the matrix effect, which can lead to ion suppression or enhancement, affecting quantification accuracy. Additionally, the efforts needed for developing a suitable method of analysis not only limits to minimizing matrix effect through chromatographic separation between nitrosamines and active pharmaceutical ingredient including excipients but also optimizing mobile phase additives for sensitivity and appropriate sample preparation with best sample cleanup by incorporating simple steps with sample filtration through 0.2µ PVDF or equivalent syringe filters. The co-elution of isobaric nitrosamines further necessitates advanced chromatographic separation and careful method optimization or use of HRMS technology to separate and identify isobaric masses. Therefore, the currently available methods often require labor-intensive sample preparation, limiting throughput and scalability for routine quality control.

These gaps highlight the need for more robust, standardized, and automated analytical workflows.

2.3 Regulatory Framework and Global Standards

In response to the nitrosamine crisis, regulatory agencies have established comprehensive guidelines. The U.S. Food and Drug Administration (FDA) recommends a three-step approach: risk assessment, confirmatory testing, and mitigation[8] The FDA's updated 2024 guidance emphasizes the control of NDSRIs and the use of validated LC-MS methods. The European Medicines Agency (EMA), through its Article 5(3) review, mandates risk evaluations and sets interim acceptable intake (AI) limits based on a less-than-lifetime (LTL) approach[9] The World Health Organization (WHO) has issued technical notes aligning with these standards, promoting global harmonization[10] Collectively, these frameworks underscore the importance of proactive impurity control and the integration of advanced analytical technologies in pharmaceutical quality systems.

3. METHODOLOGY

3.1 Solutions and Sample Preparation

3.1.1 Diluent Solution

60 mL of methanol mixed with 40 mL of water.

3.1.2 Nitrosamine standard Solution Preparation

Nitrosamine stock solutions were prepared in methanol at a concentration of 0.1 mg/mL for all the impurities separately.

3.1.3 Specification level standard – 100% level preparation (0.083ppm wrt sample or 2.2ng/mL) and basis of Calculation

Specification standard at 100% level was prepared at absolute concentration of 2.2 ng/mL that corresponds to 0.083 ppm with respect to the API. All other levels were prepared accordingly. To explain further, AI value of 26.5ng for all the nitrosamines (including NDMA and NMBA which have AI of 96 ng/mL) was considered to perform this study since 26.5ng is more stringent. Besides this, MDD of 320mg for Valsartan gives specification limit of 0.083 ppm {specification limit (ppm)= AI/MDD}. Therefore, since the sample preparation in this study contains API concentration of 26.5 mg/mL, 2.2 ng/mL is the absolute concentration required for specification level standard (100% level) for all the nitrosamines.

Variable volume pipettes (2-20µL, 10-100µL and 100-1000µL Finnpipette F2) were used for dilutions.

3.1.4 Linearity standards, limit of quantitation (loq), and limit of detection (lod) preparation

An intermediate mix dilution of 500ng/mL was prepared from all the 12 individual Nitrosamine stock solutions using diluent solution and then further serially diluted to prepare linearity standards using diluent solution.

Linearity standards were prepared at concentrations (ng/mL) of 0.220 (10% or LLOQ), 0.440, 2.201 (100% or specification level standard), 6.601, 14.676, 21.938, 29.250 (Highest linearity level). LOD (limit of detection) was prepared as 50% of LLOQ concentration.

3.1.5 Drug product sample preparation

To prepare samples, appropriate number of tablets were crushed into powder and homogenized. Stepwise procedure for preparing samples is given below.

Step-1: Weighed 53 mg API equivalent amount of sample and transferred to 2mL microcentrifuge tube.

Step-2: Added 2mL of diluent solution and vortexed briefly.

Step-3: Samples were sonicated in an ultrasonic bath for 15 minutes.

Step-4: Vortexed for 1 minutes to mix again.

Step-5: Centrifuged at 12000rpm and 5°C for 25 minutes.

Step-6: The supernatant liquid sample was filtered through a 0.22µm PVDF syringe filter

Step-7: Clear solution was transferred to HPLC glass vials for analysis on LC-MS/MS.

3.2 Analytical technique and method of analysis (LC-MS/MS)

LC-MS/MS is a highly sensitive analytical technique as a hyphenation of HPLC and triple quadrupole MS that is capable of quantitative analysis required for a reliable molecular determination. The tandem MS process involves fragmentation of selected precursor ions producing fragment ions that serve as molecular fingerprints. This enhances both qualitative identification and quantitative accuracy.

To perform analysis on LC-MS/MS first step is to develop a method and validate as per the required area of analysis. For nitrosamine analysis, the primary requirement is to chromatographically separate the retention time of API and nitrosamine impurities where the

ultimate goal is to setup time segmentation to allow the sample flow into mass spectrometer for detection of nitrosamine impurities and avoid the sample flow going into MS when API elutes, since API is present in very high concentration that can contaminate MS. In this method, LC and MS both the parameters were optimized for best sensitivity of nitrosamine impurities and good chromatographic separation from retention time of Valsartan. LC parameters are presented in Table 1.

Table 1: HPLC conditions.

Sr.no.	Particular	Description
1	Mobile phase A	0.1% Formic acid in water
2	Mobile phase B	0.1% Formic acid in methanol
3	Needle wash	Methanol: water::80:20
4	Flow rate	0.5 mL/minute
5	Column oven temperature	45°C
6	Column	Acclaim 120, C18, 250x4.6mm, 5µ
7	Autosampler temperature	20°C
8	Injection volume	40µL
9	Flow mode	Gradient; %B = 25% to 95% in 30 minutes followed by re-equilibration with 25%
10	Total runtime	40 minutes

The LC flow needs to be sent to MS through Ion source module. The ion source converts incoming LC flow from liquid state into gaseous phase by using a combination of high temperature and gases with optimized values as given in Table 2.

Table 2: Ion source parameters.

Sr.no.	Particular	Description
1	Ion source type	Atmospheric pressure chemical ionization
2	Polarity	Positive
3	Sheath gas	50
4	Auxillary gas	10
5	Sweep gas	1
6	Corona discharge voltage	6µA
7	Ion transfer tube temperature	325°C
8	Vaporizer temperature	325°C

Further for setting up the time segmented flow to either send it into MS or avoid when API which is in high concentration elutes, divert program is established which is given in Table 3.

Table 3: Divert valve setup.

Sr.no.	Time (minutes)	Divert position (2 – Into MS, 6 – Diverted)
1	0	2
2	15.7	6
3	16.3	2
4	21.8	6
5	28.4	2
6	31.25	6
7	39.5	2

For detection of nitrosamine impurities in MS, compound-based parameters were optimized to give best sensitivity which are given in table 4.

Table 4: SRM settings on MS.

Nitrosamine impurity Name	Q1/Q3 ions (m/z)	Collision energy (V)	RF Lens (V)	Source Fragmentation (V)	Resolution width (Q1/Q3)
NDMA	75/58	14	38	25	0.7/1.2
NMEA	89/61	13	45	20	0.4/1.2
NPYR	101/41	29	48	18	0.4/1.2
NDEA	103/29	18	44	25	0.4/1.2
NPIP	115/69	16	54	30	0.4/1.2
NMOR	117/87	12	55	20	0.4/1.2
NEIPA	117/43	19	34	16	0.4/1.2
NDIPA	131/89	10	35	5	0.7/1.2
NDPA	131/89	10	43	10	0.4/1.2
NMPA	137/66	22	52	25	0.4/1.2
NMBA	147/117	7	33	0	0.7/1.2
NDBA	159/103	12	50	20	0.4/0.7

4. RESULTS

The method of analysis for determination of 12 nitrosamines in Valsartan drug product has been found to be promising with high sensitivity, good chromatographic peak separation between nitrosamines and Valsartan. The MS chromatograms of nitrosamine impurities were found to be good in terms of appropriate distribution throughout the runtime as given in figure 1, and good separation from chromatographic elution of Valsartan as per UV chromatogram in figure 2.

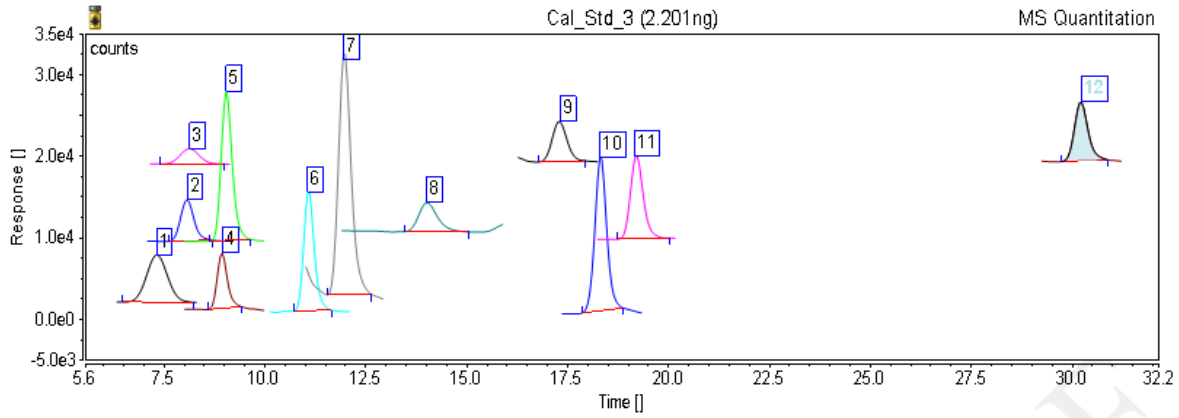

Figure 1: Chromatographic elution of 12 nitrosamines in a Specification level standard (2.201 ng/mL).

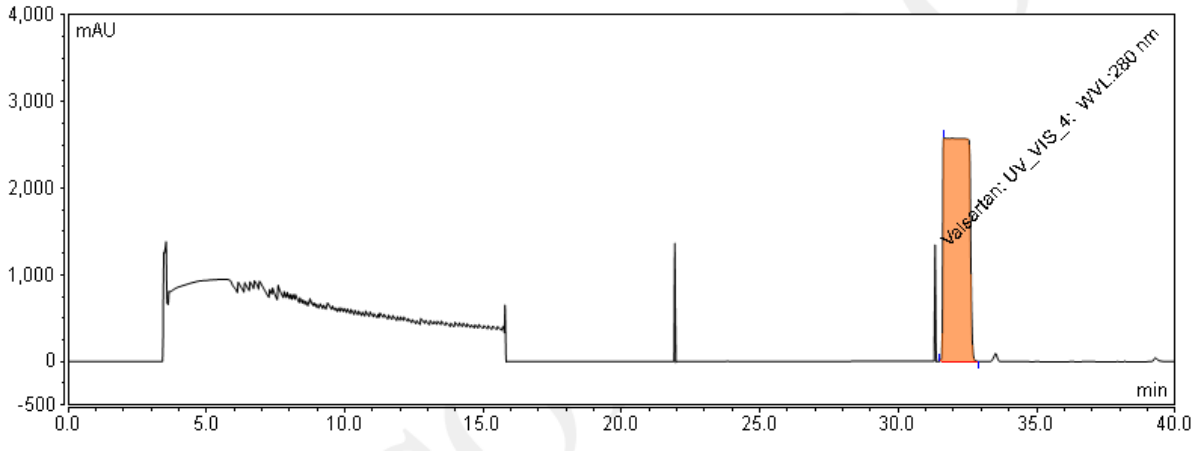

Figure 2: UV chromatogram of Valsartan from sample.

4.1 Regulatory requirements and data acceptance

From the validation activities for 12 nitrosamine impurities in Valsartan drug product, the analytical method used in this study, demonstrated robust performance across key parameters as outlined by FDA and EMA guidelines. The limit of quantitation (LOQ) exhibited a signal-to-noise ratio exceeding 10, confirming adequate sensitivity for trace-level detection. Linearity was established over the relevant concentration range with a regression coefficient (R^2) consistently greater than 0.98, indicating strong correlation between analyte concentration and detector response. Recovery assessments conducted in triplicate across spiked sample matrices yielded values within the acceptable range of 70–130%, affirming method accuracy and reliability for quantitative analysis. These results collectively satisfy the criteria for specificity, accuracy, precision, and quantitation limit as defined in the ICH

Q2(R2) guideline[11] thereby supporting the method's suitability for routine quality control and regulatory compliance.

5. DISCUSSION

5.1 RESULTS AND REGULATORY EXPECTATIONS

The method has been experimented with validation activities of Linearity, reproducibility and recovery which were found to meet all significant regulatory recommended criteria. This indicates suitability of this method to analyze the Valsartan tablets for 12 nitrosamines in single run while in this method 80mg tablets were evaluated. Although it has been 7 years since the first finding of nitrosamines in valsartan still there is no such method incorporating multiple nitrosamines other than the conventional 6 nitrosamines in one LC-MS/MS method for routine quantitation. The methods that are available have either less nitrosamines or they were developed for API samples to verify the purity of drug substances. This method should stand one step ahead of the approach to have high quality and high throughput data from routine LC-MS/MS analysis of a drug product for multiple nitrosamines in single run.

5.2 Significance of study

To explain the significance of such study, we consider futuristic approach wherein many of the consumable products would be tested in short period of time. One method will suffice the need of testing multiple impurities or traces of many unwanted molecular species belonging to various categories in a few methods only.

Besides this, such approach or study supports public health and well-being and the pharmaceutical industries also to modify the approach and switch to high throughput and at same time enhance the quality of drug products they manufacture.

5.3 limitations and potential sources of error

While the LC-MS/MS method for determination of 12 nitrosamines in Valsartan drug product demonstrated strong performance in terms of sensitivity, selectivity and reproducibility certain limitations must be acknowledged. Matrix effects arising from the complex composition of the valsartan drug product may influence ionization efficiency, potentially leading to signal suppression or enhancement. Although internal standards can be used to mitigate this, variability across different batches or formulations could still introduce bias. Additionally, the method’s reliance on electrospray ionization (ESI) may limit its applicability to nitrosamines with poor ionization efficiency under the selected conditions,

necessitating further optimization or alternative ionization techniques for broader applicability. Therefore, in this method we specifically focused on mitigation of matrix effect by enhancing percentage recovery and used APCI which gives best ionization efficiency for robust analysis of nitrosamines.

Potential sources of error include sample preparation inconsistencies, particularly during extraction and dilution steps, which can affect recovery and quantitation. Instrumental factors such as source contamination, column degradation, or fluctuations in mobile phase composition may also impact method robustness over time. Moreover, the co-elution of structurally similar nitrosamines poses a challenge for accurate quantification, especially when retention times are closely spaced. Continuous monitoring, system suitability testing, and periodic revalidation are essential to ensure long-term reliability and regulatory compliance of the method.

6. CONCLUSION

6.1 Key findings

The LC-MS/MS method developed for the simultaneous quantification of twelve nitrosamine impurities in valsartan drug product demonstrated high analytical performance across all validation parameters as provided in Table 5. The method achieved a signal-to-noise ratio exceeding 10 at the limit of quantitation (LOQ), ensuring reliable detection of trace-level contaminants. Linearity was confirmed with regression coefficients (R^2) consistently above 0.98 as shown in Table 5 and Figure 3, indicating strong correlation between concentration and response. Recovery rates across three replicates at three levels, from low to high concentration levels, remained within the regulatory acceptance range of 70–130%, affirming the method's accuracy and precision. These findings support the method's suitability for routine quality control and regulatory compliance, contributing to enhanced safety monitoring of pharmaceutical products in accordance with FDA and EMA guidelines.

Chromatographically, the method shows good peak shapes fo all 12 nitrosamines that are appropriately separated from peak of Valsartan. The chromatography of diluent blank (Figure 4), when compared with LLOQ (Figure 5) and specification level (Figure 6), show no or insignificant interference at the RT of corresponding nitrosamine.

Table 5: Validation results for method's accuracy and precision determination.

Sr. no.	Name	RT	LLOQ (10%) conc. (ng/mL)	LLOQ S/N ratio	Spec Level conc. (100%) conc. (ng/mL)	RSD Spec level (n=6)	Linearity R^2	% Recovery (3 Levels)		
								Low	Mid	High
1	NDMA	7.30	0.22	12.3	2.201	3.74%	0.9997	92.85	93.51	96.80
2	NMOR	8.07	0.22	20.6	2.201	1.68%	0.9998	87.09	95.82	96.96
3	NMBA	8.14	0.22	12.3	2.201	4.30%	0.9996	80.30	101.20	95.29
4	NPYR	8.97	0.22	11.6	2.201	2.37%	0.9994	83.45	92.48	94.59
5	NMEA	9.00	0.22	62.7	2.201	0.93%	0.9990	102.32	95.60	95.55
6	NDEA	11.10	0.22	27.9	2.201	2.17%	0.9993	104.83	92.07	95.88
7	NPIP	11.97	0.22	25.4	2.201	0.50%	0.9995	81.77	89.31	91.33
8	NEIPA	13.90	0.22	18.7	2.201	4.35%	0.9997	94.34	76.27	94.78
9	NDIPA	17.27	0.22	23.7	2.201	1.78%	0.9993	87.27	89.16	90.51
10	NMPA	18.35	0.22	20.0	2.201	1.21%	0.9998	81.51	85.32	83.72
11	NDPA	19.20	0.22	19.2	2.201	0.85%	0.9997	86.20	97.48	97.84
12	NDBA	30.21	0.22	15.7	2.201	2.18%	0.9998	96.38	87.90	90.22

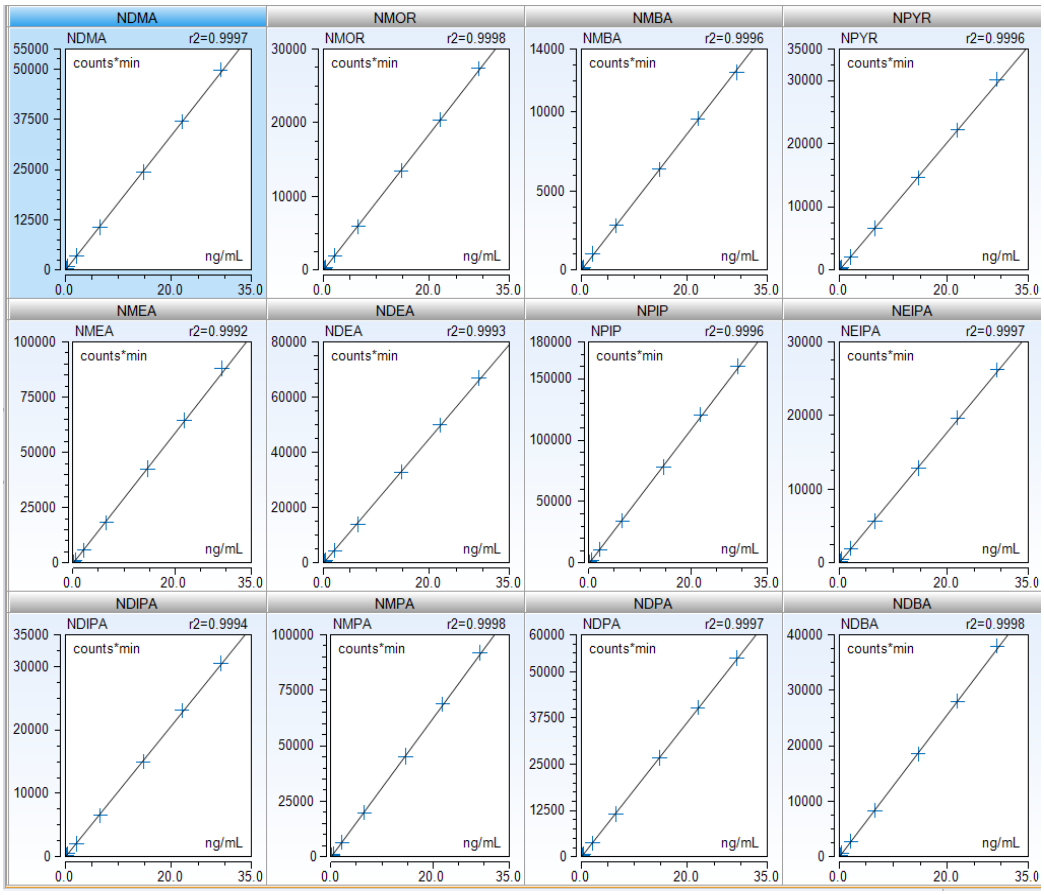

Figure 3: Linearity curves for 12 nitrosamines.

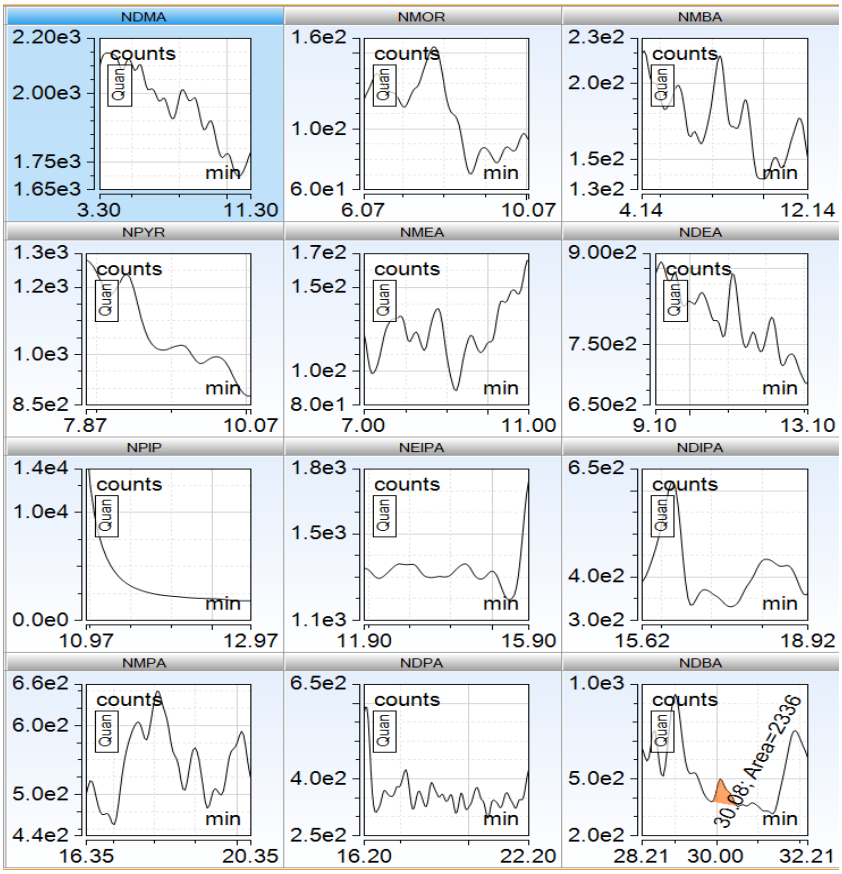

Figure 4: MS chromatograms of Diluent blank.

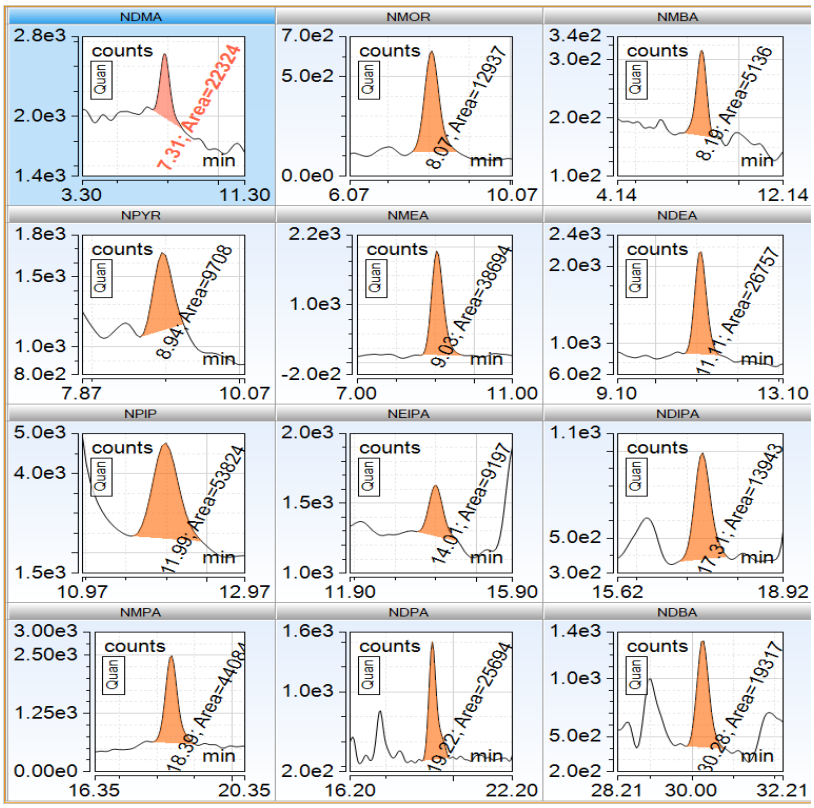

Figure 5: MS chromatograms of LLOQ.

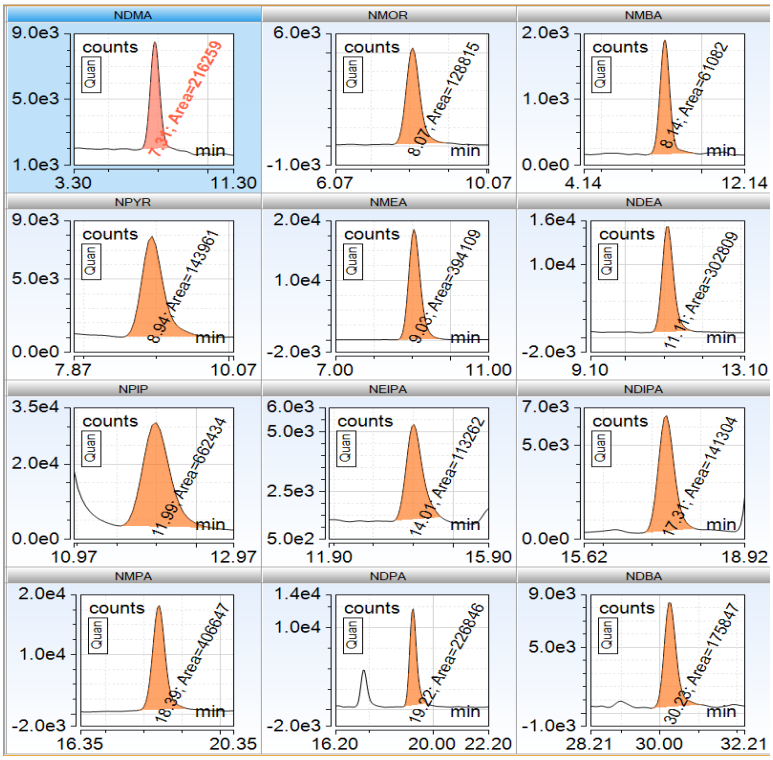

Figure 6. MS chromatograms of Specification level standard.

6.2 Future research directions

Building on the successful quantification of twelve nitrosamines in valsartan formulations, future research could focus on extending the method's applicability to other high-risk drug classes such as ranitidine, metformin, and other sartans, which have shown susceptibility to nitrosamine contamination (EMA, 2020). Investigating the influence of excipient variability and packaging materials on nitrosamine formation could enhance risk assessment models and improve formulation strategies (EMA, 2025). Additionally, integrating high-resolution mass spectrometry or alternative ionization techniques may improve detection of thermally labile or low-volatile nitrosamines (EMA, 2020). Long-term studies evaluating nitrosamine formation under stress and storage conditions would provide valuable insights into impurity pathways, supporting proactive mitigation and lifecycle management of pharmaceutical products (Shomali et al., 2025).[12]

6.3 Importance of continued monitoring

Continuous monitoring of nitrosamine impurities in drug products is critical to maintaining product safety and regulatory compliance over time. Given the dynamic nature of manufacturing processes, excipient variability, and storage conditions, nitrosamines may form post-production, making routine surveillance essential. Regulatory bodies such as the

European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) have emphasized the need for ongoing risk evaluation, confirmatory testing, and lifecycle management to detect and mitigate nitrosamine contamination (EMA, 2020; EMA, 2025). EMA's Article 5(3) review outlines a structured approach requiring marketing authorization holders to implement control strategies and continuously assess their products for nitrosamine risks (EMA, 2020). This proactive monitoring not only ensures patient safety but also supports regulatory transparency and rapid response to emerging impurity profiles.

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