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Application Note

Quantitation of N-Nitroso Carvedilol in Drug Substance Using LC-MS/MS

Dimple D. Shah, Marian Twohig, Padmakar Wagh, Amy Bartlett, Peter Hancock

Waters Corporation

Abstract

N-nitrosamines are a class of chemical compounds known for their carcinogenic properties, posing significant health risks to humans. These compounds have garnered attention in the pharmaceutical industry due to their inadvertent presence in certain medications, raising concerns about patient safety and regulatory compliance. N-nitrosamines are not intentionally added to drugs but can form as impurities or degradation products during the manufacturing process or storage under certain conditions.[1]

In response to these concerns, regulatory agencies such as the U.S. Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the other global health authorities have implemented strict guidelines and regulations to address the presence of N-nitrosamines in drugs. This measure includes risk assessment, establish acceptable limits for N-nitrosamine impurities, testing and monitoring of pharmaceutical products, and mitigation strategies by pharmaceutical manufacturers.[4] Recently, both the EMA and the FDA provided revisions on how acceptable intake values (AI) are determined.[5,6] The revisions introduced the Carcinogenic potency categorization approach (CPCA), which aligns the relationship of impurity structure with carcinogenic potency. A CPCA category with a specific AI threshold is now determined for the majority of new Nitrosamines where in vivo toxicity of the impurity has not been determined.[7]

Carvedilol is a beta blocker that can be used alone or in combination with other medications to treat heart

conditions. This study describes the analysis of N-nitroso carvedilol in drug substance using Ultra-Performance Liquid Chromatography (UPLC™) with electrospray detection (ESI) and a Xevo™ TQ-S cronos Triple Quadrupole Mass Spectrometer. In order to avoid the need for onwards batch release testing in QC, analytical methods used for the detection of nitrosamines, including Nitrosamines drug substance related impurities (NDSRI), are required to demonstrate absence of impurity at a lower limit of quantitation (LOQ) of 10% of the limit derived from the AI and the active pharmaceutical ingredients (API) maximum daily dose (MDD). With MDD of 100 mg, the assay exceeded the regulatory requirements or N-nitroso carvedilol. Chromatographic resolution between carvedilol API and two N-nitroso carvedilol isomers was achieved for accurate quantification. Also, Nitrosamine Impurity Assay Quanpedia database provided useful starting conditions for MS or MRM analyses. The developed method demonstrated good linearity over the concentration range of 0.4–100 ppm for N-nitroso carvedilol with less than 15% concentration deviations. The R^2 was greater than 0.995. The method recovery ranged from 85–110% and the accuracy was within +/- 18% of the true value.

Benefits

- Trace level detection of N-nitroso carvedilol using the Xevo TQ-S cronos Triple Quadrupole Mass Spectrometer to the Industry requirements of 10% of the regulatory AI threshold as determined by CPCA
- Chromatographic resolution between carvedilol API and two N-nitroso carvedilol isomers for accurate quantification
- Nitrosamine Impurity Assay Quanpedia database provides useful starting conditions for MS or MRM analyses and accelerate method development

Introduction

N-nitrosamines are a class of chemical compounds known for their carcinogenic properties, posing significant health risks to humans. These compounds have garnered attention in the pharmaceutical industry due to their inadvertent presence in certain medications, raising concerns about patient safety and regulatory compliance. N-nitrosamines are not intentionally added to drugs but can form as impurities or degradation products during the manufacturing process or storage under certain conditions.[1]

The presence of N-nitrosamines in drugs gained widespread attention in the pharmaceutical industry and regulatory agencies following several high-profile recalls of medications due to contamination with these

compounds. One of the most notable cases involved the discovery of N-nitrosamine impurities, particularly in certain angiotensin receptor blockers (ARBs), including valsartan, losartan, and irbesartan.[2]

Nitrosamine drug substance related impurities (NDSRIs) are potentially potent carcinogens, with, in some cases, even low exposure levels raising concerns about long-term health risks. The contamination of pharmaceuticals with N-nitrosamines can occur through various mechanisms introducing the presence of vulnerable amines and nitrosating agents under certain conditions, including the use of contaminated raw materials, drug substance synthesis and product manufacturing processes and degradation of drug substances or excipients under certain conditions such as high temperature, humidity, or exposure to light.[3]

In response to these concerns, regulatory agencies such as the U.S. Food and Drug Administration (FDA), the European Medicines Agency (EMA), and other global health authorities have implemented strict guidelines to regulate the presence of N-nitrosamines in drugs. This measure includes risk assessment, establish acceptable limits for N-nitrosamine impurities, testing, and monitoring of pharmaceutical products and mitigation strategies by pharmaceutical manufacturers.[4]

Recently, both the EMA and the FDA provided revisions on how AI are determined.[5,6] The revisions introduced the Carcinogenic potency categorization approach (CPCA), which aligns the relationship of impurity structure with carcinogenic potency. A CPCA category with a specific AI threshold is now determined for the majority of new Nitrosamines where in vivo toxicity of the impurity has not been determined.[7]

Carvedilol is a beta blocker that can be used alone or in combination with other medications to treat heart conditions. This study describes development of a robust quantification method for the analysis of N-nitroso carvedilol in drug substance using Ultra-Performance Liquid Chromatography (UPLC) with electrospray detection (ESI) and a Xevo TQ-S cronos Triple Quadrupole Mass Spectrometer.

Experimental

Sample Preparation

Standards and reagents

Authentic standards of N-nitroso carvedilol and carvedilol were purchased from Clearsynth and Sigma Aldrich, respectively. Optima LC/MS grade solvents and acetic acid were purchased from Fisher Scientific.

10 mg carvedilol ($C_{24}H_{26}N_2O4$) was dissolved in 1 mL methanol to make 10 mg/mL stock solution. A 1 mg/mL working solution of carvedilol was prepared by taking a 1 mL aliquot from the stock solution and diluting in 9 mL 5% acetic acid.

10 mg N-nitroso carvedilol ($C_{24}H_{25}N_3O_5$) was weighed and 3 mL DMSO and 7 mL methanol was added to make a 1 mg/mL stock solution. A working solution of N-nitroso carvedilol was further diluted with 5% acetic acid solution.

Recovery

Recovery was studied at three levels: threshold, 10% threshold and ten times threshold equating to 0.4 µg/mL (ppm), 4 µg/mL (ppm), and 40 µg/mL (ppm) of N-nitroso carvedilol with respect to 1 mg/mL carvedilol.

Spiking solutions of N-nitroso carvedilol were prepared at 0.2 µg/mL, 2 µg/mL, and 20 µg/mL with 5% acetic acid in 20 mL glass scintillation vials. The spiking solutions were used to prepare three concentrations for recovery calculations.

Pre-spikes:

500 µL methanol was pipetted into three tubes each containing 5.0 mg carvedilol in glass scintillation vials. To avoid subsequent impact on recovery experiences, solvent choice was critical, and all tubes were vortexed, ensuring both API and impurities were solubilized. Remove 10 µL of each spiking solution was added followed by 4.990 mL 5% acetic acid in water to bring final concentrations of 0.4 ng/mL, 4 ng/mL, and 40 ng/mL (n=3 at each concentration level). The scintillation vials were vortexed for five minutes and centrifuged at 4000 rpm for ten minutes before transfer into LCMS vials.

Matrix blanks:

Matrix blanks were prepared by adding 500 µL methanol to three vials containing 4.99 mg carvedilol. These were vortexed until dissolved. 4.5 mL 5% acetic acid in water was added and the scintillation vials were vortexed for five minutes and centrifuged at 4000 rpm for ten minutes before transfer into LCMS vials.

Standard solutions:

Standard solutions (n=3) at each test concentration level (0.4 ng/mL, 4 ng/mL, and 40 ng/mL) were prepared in 20 mL scintillation glass vials using 10 µL spiking solutions in 4.990 mL 5% acetic acid in water. The vials were vortexed and centrifuged in the same way as the matrix blanks.

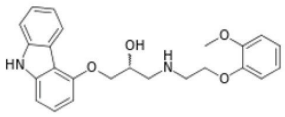
Calibration curve preparation

Authentic Standard Calibration Curve

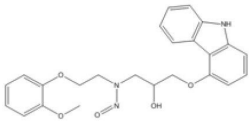
An authentic standard of N-nitroso carvedilol (1 mg/mL) in 5% acetic acid in water was sequentially diluted to create a calibration curve ranging from 0.4 ng/mL to 100 ng/mL.

Matrix Calibration Curve

Carvedilol (20 mg) was weighed into a 20 mL glass scintillation vial. A stock solution of 10 mg/mL carvedilol was prepared by adding 2 mL methanol and further diluted with 5% of acetic acid to create a 1 mg/mL carvedilol solution. This solution was used as the diluent for the N-nitroso carvedilol matrix calibration curve. An authentic standard of N-nitroso carvedilol was sequentially diluted using the 1 mg/mL carvedilol solution to a calibration ranging from 0.4 ppm to 100 ppm.



Carvedilol
CAS # 72956-09-3
Molar mass – 406.4 g/mol
Formula- $C_{24}H_{26}N_2O_4$



N-nitroso carvedilol
CAS # 2248746-67-8
Molar mass – 435.5 g/mol
Formula- $C_{24}H_{25}N_3O_5$

Figure 1. Structures of carvedilol and N-nitroso carvedilol along with their CAS number, molar mass, and formula.

Method Conditions

LC system:	ACQUITY™ Arc™ Premier System
Detection:	ACQUITY™ PDA (UV absorbance 220 nm)
Solvent manager:	Quaternary Solvent Manager – R
Sample manager:	Sample Manager FTN-R
Mobile phase A:	0.1% Acetic Acid in Water

Mobile phase B:	Methanol
Purge solution:	Methanol:H2O (1:1)
Wash solution:	Methanol:water (6:4)
Seal wash:	Methanol:water (1:9)
Injection volume:	30 µL
Sample temperature:	10 °C
Column temperature:	25 °C

Table 1. LC Conditions

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0.00	0.40	50	50	6
0.50	0.40	50	50	6
11.00	0.40	10	90	6
11.10	0.40	50	50	6
20.00	0.40	50	50	6

Table 2. LC Gradient Program

MS conditions	Parameters
Ionisation mode:	Electrospray negative
Capillary voltage (kV):	2
Cone voltage (V):-	Table 4
Collision energy (eV):	Table 4
Source temp. (° C)	130
Desolvation temp. (° C)	600
Desolvation gas flow (L/hr):	1000
Cone gas flow (L/hr):	150

Method events

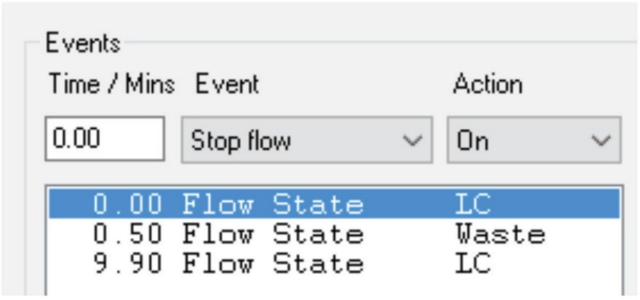


Table 3. Optimised MS conditions and method events from MS method to divert the flow.

Compound name	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Carvedilol	405.3	181.04 (Quantifier)	48	30
		196.06 (Confirmation)	48	12
N-nitroso carvedilol	434.08	181.05 (Quantifier)	50	44

Table 4. MRM transitions for carvedilol and N-nitroso carvedilol. Quantification and confirmatory transitions along with cone voltage and collision energy settings. These MRM transitions were obtained from the Waters MassLynx™ Nitrosamine Impurity Assay Quanpedia database.

Results and Discussion

Standards and samples were analyzed using ACQUITY Arc UPLC System coupled with two detectors, ACQUITY PDA and Xevo TQ-S Cronos. The separation between the carvedilol (API) and two isomers of N-nitroso carvedilol is vital. As carvedilol concentrations are considerably higher and to avoid saturation of the MS the first 9.9 minutes of the chromatographic run was diverted to waste using the integrated solvent divert valve (see Table 3). Chromatographic resolution of the API from the trace level impurities is important to avoid any potential matrix effects that could occur due to the proximity of the high levels of API present in the samples.

The MRM transitions were obtained from the Nitrosamine Impurity Assay Quanpedia Database in the MassLynx™ Software. The Quanpedia database is an extensible and searchable database for UPLC-MS/MS analytical methods. The database is a comprehensive repository of information related to a large number of nitrosamines MRM, or Multiple Reaction Monitoring (MRM), analysis assays for a given compound. By simply selecting compounds from the database, Quanpedia automatically creates both the data acquisition methods and the associated data processing methods required to perform the analysis. Quanpedia enables efficient, rapid, and easy information management of both methods and compounds, allowing new methods to be rapidly generated for any of the compounds contained within the database. Compound and method information can easily be shared between multiple workstations or laboratories, thus reducing the risk of transcription errors. This database includes multiple MRM transitions of over 60 nitrosamines which includes small alkyl nitrosamines, Nitrosamine Drug Substance Related Impurities (NDSRI), and some corresponding APIs and internal standards in one place. To achieve optimum sensitivity, the MRM transitions in the Quanpedia database can be further optimized. Figure 2 shows a screenshot of the Quanpedia library for Nitrosamines.

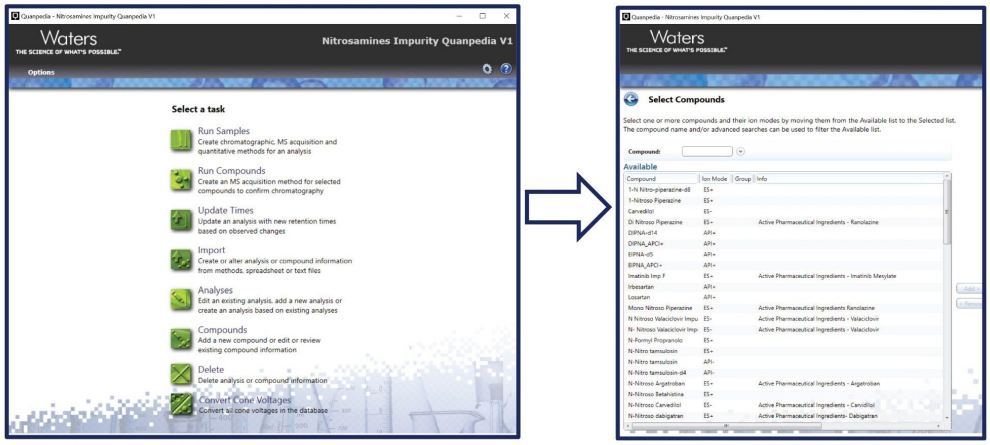


Figure 2. Nitrosamines Impurity Quanpedia database in MassLynx.

Using the Quanpedia MRM starting point, further fine tuning was achieved within a combined flow of the mobile phase used in the assay, using the MassLynx auto tune tool Intellistart.

Figure 3 shows the chromatogram of carvedilol (PDA retention time 2.38 min) and separation of two isomers of N-nitroso carvedilol (MS isomer 1 retention time 10.17 min, isomer 2 retention time 10.52 min) in mass spectrometer and PDA. The CORTECS™ C_{18}+ column provides sufficient separation between carvedilol (API) and N-nitroso carvedilol (impurity) and also resolving the two isomers of N-nitroso carvedilol.

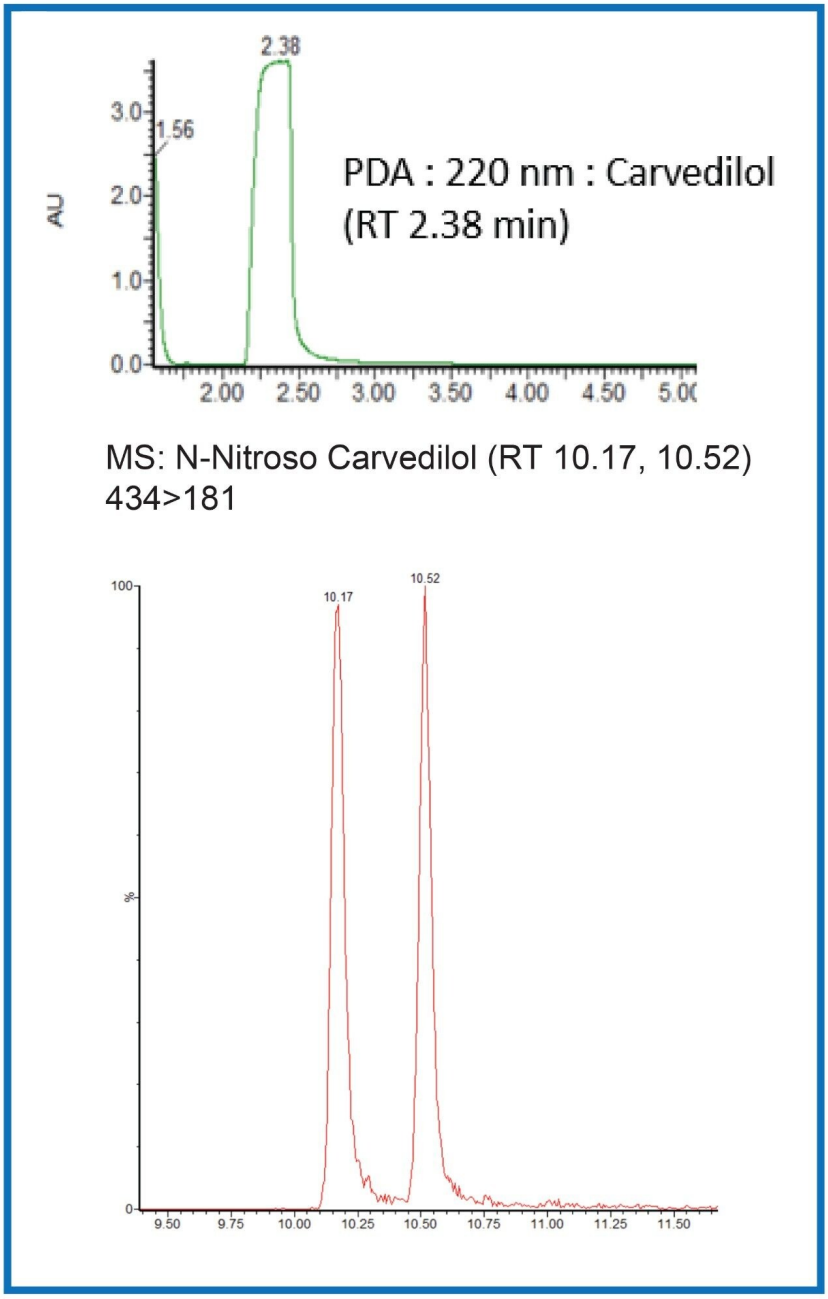


Figure 3. Chromatograms of carvedilol and N-nitroso carvedilol (both isomers) with PDA chromatogram (220 nm) in solvent standard.

Limit of Quantitation (LOQ) and Linear Dynamic Range

The quantitative limits of the assay were initially established using authentic standards of N-nitroso carvedilol with the LOQ based on a 10:1 signal-to-noise (S/N) ratio using the peak-to-peak algorithm (PtP). The threshold level for detection of N-nitroso carvedilol was 4 ppm with respect to 1 mg/mL carvedilol (API). To illustrate the ability of the method to exceed the sensitivity required at the regulatory threshold levels, the method was assessed at the 10% level, a common requirement to avoid need for onward batch testing, which is 0.4 ppm. Figure 4 shows chromatograms at threshold and 10% threshold. Signal to noise ratio for both isomers for N-nitroso carvedilol was 42 and (PtP) respectively at 0.4 ppm.

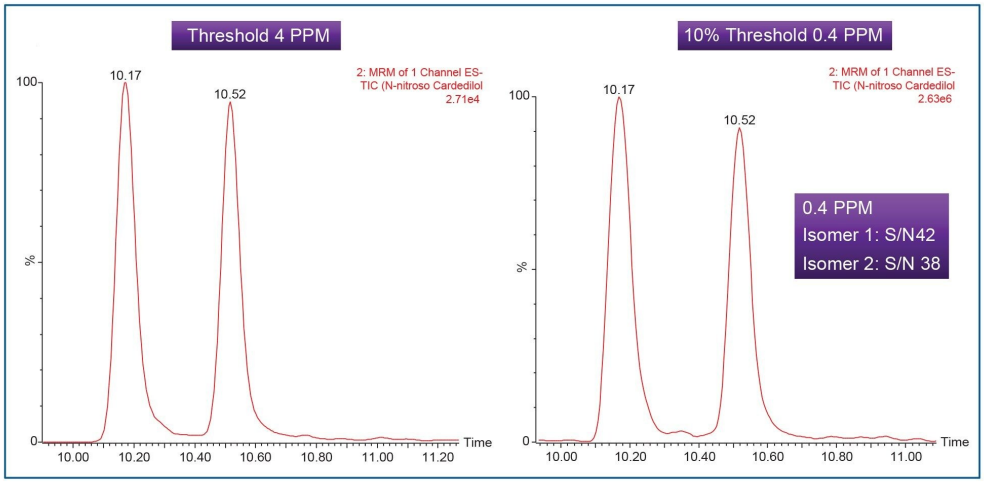


Figure 4. The S/N was measured using the Peak-to-peak (PtP) algorithm at 0.4 PPM which is 10% threshold in an authentic standard of N-nitroso carvedilol.

Establishing the LOQ of N-nitroso carvedilol in the carvedilol API was complicated by the presence of endogenous levels of the impurity in the carvedilol API sample tested. The neat standard of carvedilol (API) revealed 4 peaks in the MRM of N-nitroso carvedilol. Hence, RADAR acquisition was utilized enabling a full scan MS acquisition simultaneously with the MRM transitions.

Figure 5 shows MRM, RADAR full scan, and PDA for carvedilol at 1 mg/mL. Four peaks were observed and 2 were confirmed to be N-Nitroso carvedilol using authentic standards (RT 10.15 and 10.52 minutes), with two still to be identified but the resolution achieves in the chromatographic method meant that these unidentified peaks did not interfere with the analysis.

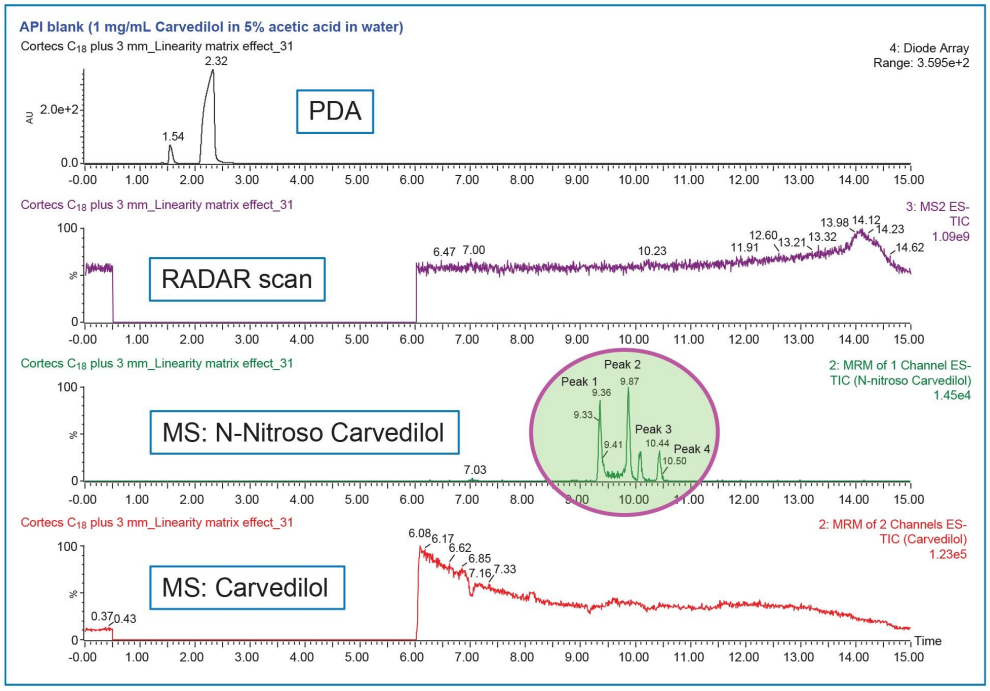


Figure 5. The MRM and full scan RADAR data showing the presence of four peaks for a standard of carvedilol at 1 mg/mL.

Spectrum underneath all four peaks is shown in Figure 6. Suggesting they could be other isomers of N-nitroso carvedilols as there are multiple nitroso impurities possibilities in carvedilol.[8]

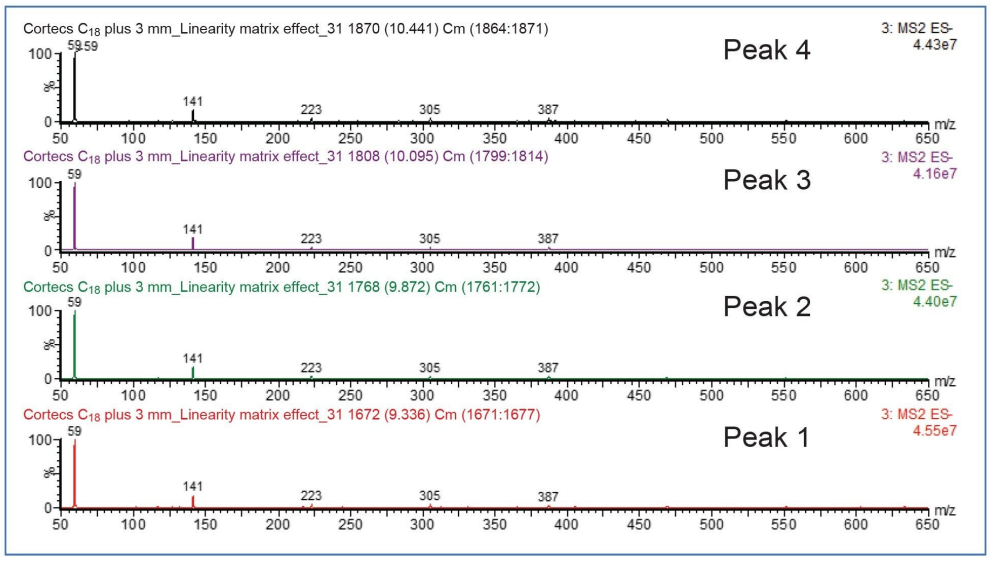


Figure 6. Full scan RADAR spectrum underneath peak 1, peak 2, peak 3, and peak 4.

The linear dynamic range was determined by evaluating an authentic standard of N-nitroso carvedilol and N-nitroso carvedilol in the presence of the API. Both the curves, when injected in triplicate were linear across the analyzed range, 0.4–100 ng/mL) with R^2 greater than 0.995 and <15% concentration deviations using 1/X weighting across the range for both isomers in both curves. Figure 7 shows the calibration curves of both isomers in the authentic standard and in the presence of the API.

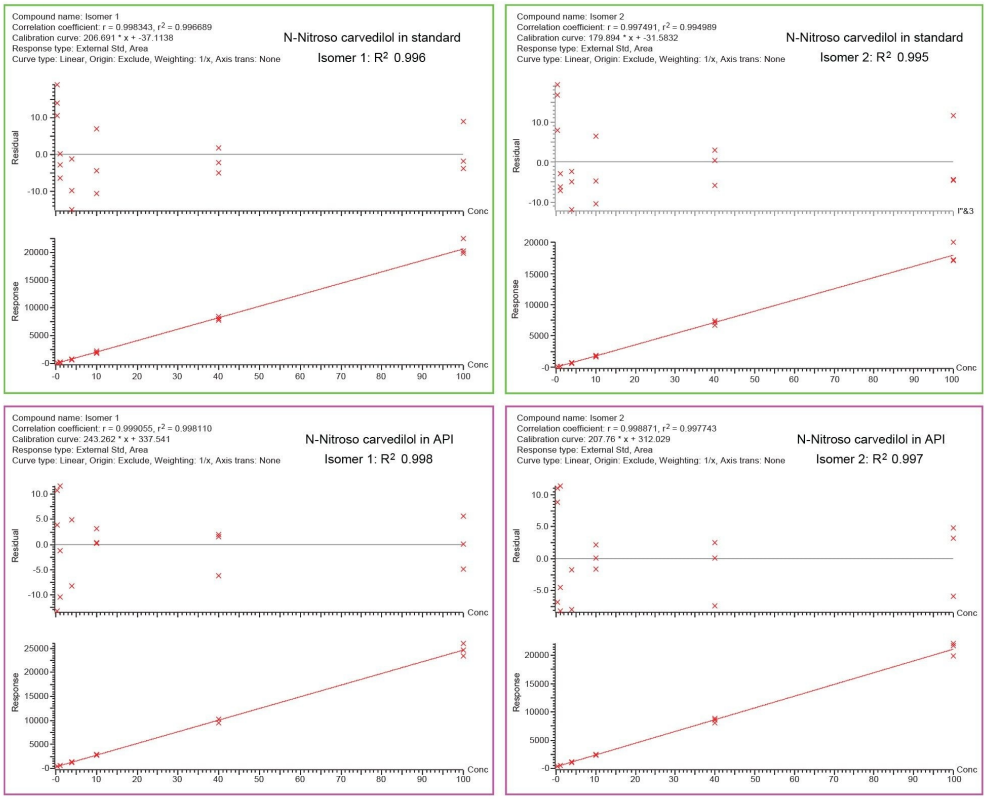


Figure 7. Linear range for N-nitroso carvedilol in the authentic standard and in the presence of the API (1 mg/mL carvedilol) from 0.04–100 ng/mL with R^2> 0.995 and residuals <15% across the calibration range tested, injected in triplicate.

Recovery

The recovery experiments were performed at 0.4 ppm, 4 ppm, and 40 ppm (n=3, injected three times). The results are summarized in Table 5. Due to the presence of the endogenous level of N-nitroso carvedilol in the API, the recovery calculations were based on the corrected responses for the pre-spiked API samples using the average response for the N-nitroso carvedilol peak present in the API blank (n=6). The average response for the peak in the API was subtracted from each of the responses in the pre-spiked samples. The recovery was calculated using the following formula:

% Recovery = (Response Pre-spike corrected/Standard Response) *100%

The calculated recoveries were between 85–110%. The %RSD for the recoveries at each concentration level were

less than 18%.

Isomer 1 concentration (PPM)	Average recovery (n=9)	STD dev	%RSD	Isomer 2 concentration (PPM)	Average recovery (n=9)	STD dev	%RSD
0.4	87.0	15.4	17.8	0.4	85.8	14.4	16.8
4	102.0	12.9	12.7	4	102.8	13.1	12.8
40	109.7	12.0	11.0	40	109.4	12.2	11.1

Table 5. Summary of the method recovery for both isomers at 0.4 ppm, 4 ppm, and 40 ppm in 1 mg/mL API (n=3, injected three times, total n=9).

Matrix effects were assessed by comparing the response obtained in neat standards to the corrected response for the matrix across the calibration curve ranged from 0.4 to 100 ppm. The matrix effects were found to be <5% for both isomers.

Carryover

The highest standard of N-nitroso carvedilol was injected at 100 ng/mL and carvedilol at 1 mg/mL. After running 1 mg/mL, with no carryover observed in the first blank sample.

Totals

N-Nitroso carvedilol has two isomers. TargetLynx™ offers the Totals functionality where both the isomers can be summed to be reported as one. Figure 8 shows example data of totals in MassLynx.

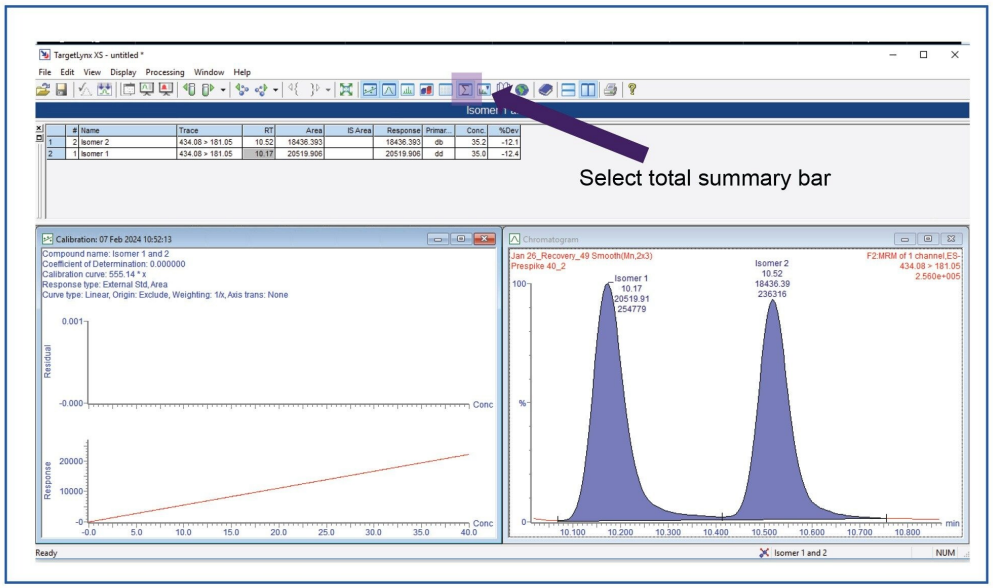


Figure 8. TargetLynx showing totals functionality for N-nitroso carvedilol isomers 1 and 2.

Conclusion

In the presented study, a UPLC-MS/MS method was developed for the quantitation of N-nitroso carvedilol in drug substance. The method is capable exceeding the regulatory guidelines as determined by CPCA, recovery, carry over, linearity, and reproducibility is demonstrated to meet the expected quantitative assay performance expected of a validated impurity analysis. In addition, both the isomers of N-nitroso carvedilol can be chromatographically resolved from carvedilol and from each other. Summed impurity isomer concentrations were afforded through use of the totals function in the MassLynx software. Method development efficiency was aided through the use of a Nitrosmaines Impurity Quanpedia database, simplifying the optimization workflows by providing an MRM starting point.

References

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