

Quantitation of N-Nitroso Nebivolol in Nebivolol Tablet Formulation

Using an Agilent 6495D triple quadrupole LC/MS system

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Abstract

N-Nitroso Nebivolol, a nitroso drug substance-related impurity (NDSRI) of the drug Nebivolol, is classified by the U.S. Food and Drug Administration (FDA) as a Class 4 impurity. The maximum allowable intake of this nitrosamine impurity is 1,500 ng/day. For a 20 mg Nebivolol formulation, the specification limit for N-Nitroso Nebivolol is 22.5 ng/mL at a test concentration of 0.6 mg/mL. This study presents a highly specific, sensitive, and reproducible method for quantifying N-Nitroso Nebivolol in 20 mg Nebivolol tablets using an Agilent 1290 Infinity II LC and Agilent 6495D triple quadrupole LC/TQ. The method, optimized with the Optimizer tool in Agilent MassHunter software, achieved an LOD of 2 pg/mL and an LOQ of 5 pg/mL, with linearity from 5 pg/mL to 50 ng/mL ($R^2 > 0.997$). An approximately 10-minute separation was achieved between the API and the impurity, with the API peak diverted to the waste line during impurity quantitation.

Introduction

N-Nitroso Nebivolol is a NDSRI of Nebivolol, a beta-1 adrenergic receptor antagonist commonly used in the treatment of hypertension and heart failure. In the carcinogenic potency categorization published by the FDA, N-Nitroso Nebivolol has been categorized as Class 4, indicating the maximum allowable intake limit for the impurity to be 1,500 ng/day. Nebivolol has maximum daily dosage limit of 40 mg for adults.

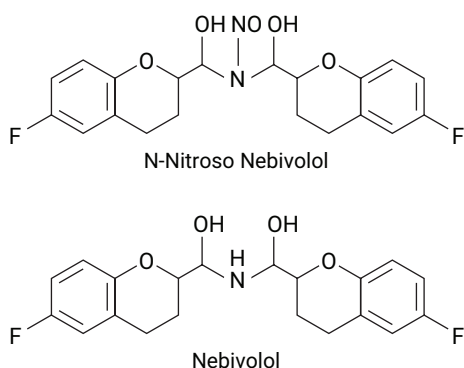


Figure 1. Chemical structures of N-Nitroso Nebivolol impurity and Nebivolol API.

Considering a maximum daily dose of 40 mg and allowable intake of 1,500 ng/day for the nitrosamine impurity in the formulation under study, the specification limit of N-Nitroso Nebivolol has been calculated as 22.5 ng/mL for the 0.6 mg/mL test concentration used during this study.

This application note demonstrates a highly specific, sensitive, and reproducible method for the quantitation of N-Nitroso Nebivolol in a 20 mg Nebivolol tablet formulation. The method uses a 1290 Infinity II LC coupled to a 6495D triple quadrupole LC/MS/MS. The multiple reaction monitoring (MRM) conditions and ion source parameters were automatically optimized using the

optimizer tool available with MassHunter Acquisition software to maximize the sensitivity of the target analyte. The API peaks were diverted to waste with the help of an integrated, software-controlled diverter valve during quantitation of the impurity.

Experimental

Materials

Calibration samples were prepared using N-Nitroso Nebivolol analytical standard material provided by an Indian pharmaceutical company. Formulation tablets, placebo, and the APIs for the drug were also provided by same pharmaceutical company.

The following other chemicals and reagents were used: ammonium trifluoroacetate and formic acid (MS-grade, Fluka, Honeywell, Muskegon, Michigan, USA), methanol (MS-grade, Biosolve, Dieuze, France), and water (MS-grade, Honeywell, Muskegon, Michigan, USA).

Equipment

The analysis was performed on a 1290 Infinity II LC system coupled to a 6495D triple quadrupole LC/MS/MS. The LC system was equipped with the following modules:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (G7117A)

Method

The chromatography conditions for the method can be found in Table 1.

Table 1. Chromatography conditions.

Parameter	Value				
Column	Agilent Pursuit XRs 3 Diphenyl, 150 mm × 3 mm (p/n A6021150X030)				
Mobile Phase A	1 mM Ammonium trifluoroacetate and 0.004% formic acid in water				
Mobile Phase B	Methanol				
Flow Rate	0.5 mL/min				
Injection Volume	10 µL				
Sample Cooler Temperature	8 °C				
Column Temperature	40 °C				
Needle Wash	Acetonitrile:water (90:10)				
Diluent	Acetonitrile:water (1:1)				
Elution	Gradient				
Acquisition Time	25 min				
Gradient Program	Time (min)	%A	%B	Flow (mL/min)	Max. pressure limit (bar)
	0.00	55.00	45.00	0.500	1,300.00
	3.00	55.00	45.00	–	–
	6.00	40.00	60.00	–	–
	13.00	40.00	60.00	–	–
	16.00	15.00	85.00	–	–
	21.00	15.00	85.00	–	–
	21.10	0.00	100.00	–	–
	25.00	0.00	100.00	–	–

Preparation of standard and samples

Preparation of concentration linearity:

The standard stock solution of 100 µg/mL was prepared by dissolving 5 mg of analytical standard using diluent and making up the volume in a 50 mL volumetric flask. A 1 mL portion of the stock solution was then diluted to 100 mL to prepare a 1 ppm working standard solution. A 13-concentration level linearity was prepared by serial dilution of the working standard solution.

Preparation of test samples:

Five formulation tablets were weighed accurately, and the average weight was determined. The tablets were crushed to powder and mixed well using mortar and pestle. A 0.6 mg/mL Nebivolol content equivalent of the tablet powder was transferred into a 15 mL centrifuge tube and 10 mL diluent was added. The tube was then vortexed for 1 minute and subjected to rotational shaking for 20 minutes using a Rotospin (Tarsons, Kolkata, India). The solution was centrifuged at 9,000 rpm for 10 minutes. The supernatant was transferred to an HPLC vial for analysis. Spiked API, placebo, and tablet samples were also prepared using the same method, spiking 200 pg/mL of N-Nitroso Nebivolol in all. The API solution was also prepared at a concentration of 0.6 mg/mL.

Data acquisition and data analysis

All samples were acquired using Agilent MassHunter Data Acquisition software (version 12.0). Chromatograms were viewed in MassHunter Qualitative Analysis software (version 12.0). Quantitation of each batch was carried out using MassHunter Quantitative Analysis software (version 12.1).

Validation parameters (linearity, reproducibility, recovery, specificity, and sensitivity in terms of LOQ) were characterized to ensure good method performance. Accuracies for calibration points were within ± 20%.

Table 2. MS Acquisition parameters.

Compound	Precursor (m/z)	Product Ion (m/z)	Collision Energy (CE; V)	Polarity
N-Nitroso Nebivolol	435.2	254.0	12	Positive

Table 3. MS source parameters.

Parameter	Value
Ionization Source	Jet Stream Technology ion source (AJS) (G1958B)
Gas Temperature	250 °C
Gas Flow	12 L/min
Nebulizer	30 psi
Sheath Gas	400 °C
Sheath Gas Flow	10 L/min
Capillary Voltage	4,500 V
Nozzle Voltage	500 V
Gain	10.0

Timetable		
Start time (min)	Type	Value
0	Diverter	To waste
12	Diverter	To MS
21	Diverter	To waste

Figure 2. Diverter valve settings.

Results and discussion

The separation between the impurity and both the APIs is shown in Figure 3.

The chromatogram shows an approximately 10-minute separation between the impurity and the API. To avoid further interference of the APIs on impurity response, the diverter program was used to automatically send the LC flow to waste during the elution of high concentration APIs, then divert it back to MS for the impurity.

Figure 4 shows the responses and signal-to-noise at LOD and LOQ levels (2 and 5 pg/mL, respectively), where the noise was calculated by the Peak-to-Peak algorithm.

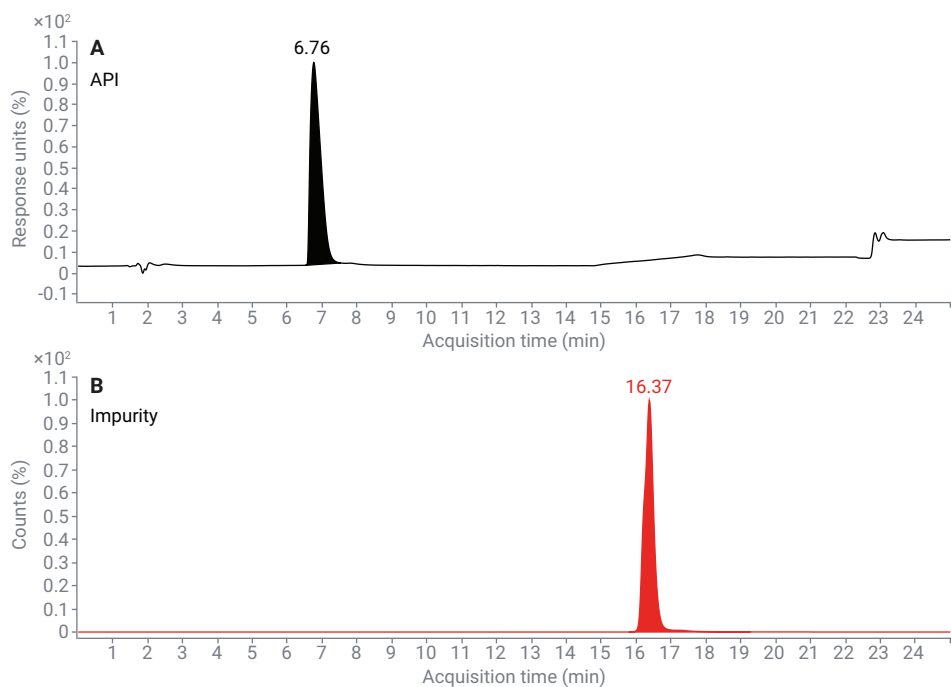


Figure 3. Chromatographic separation between the API (A) and the nitrosamine impurity (B).

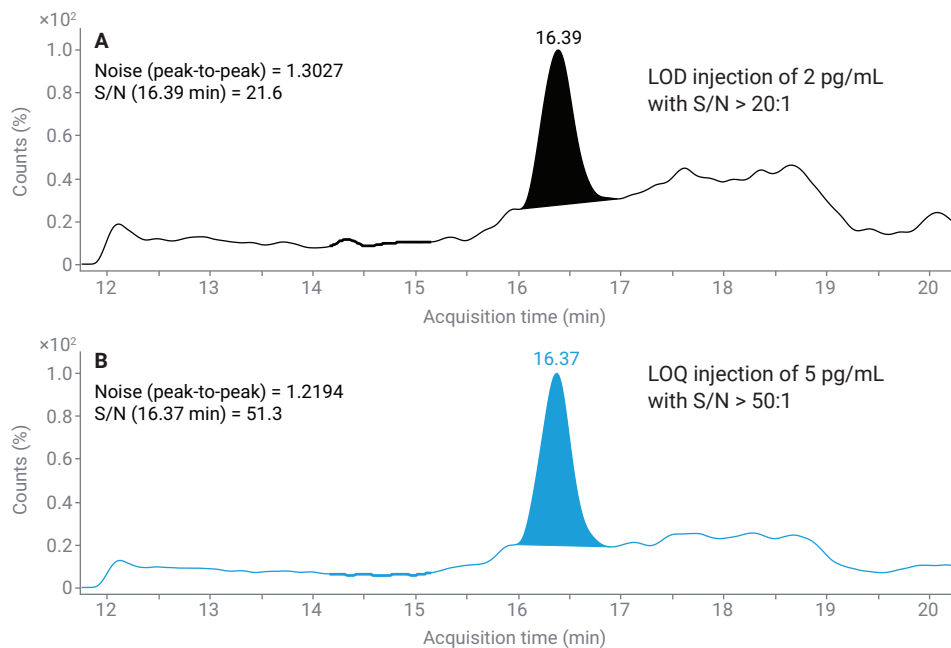


Figure 4. Chromatograms of (A) LOD (2 pg/mL) and (B) LOQ (5 pg/mL) of N Nitroso Nebivolol impurity.

A 13-point calibration curve was constructed with concentration linearity from 5 pg/mL to 50 ng/mL (Figure 5). The R² value for the calibration was found to be 0.997, well within acceptable range.

The tablet, API, and placebo solutions were injected as samples, and spiked placebo solution was injected as quality control (with known spiked concentration of 200 pg/mL) along with the calibration standards. All points of the calibration and QC were found to be within an acceptable accuracy range of 80 to 120%.

The recovery of the spiked API, placebo, and tablet were calculated by comparing the area of the neat standard of LOQ level and the average area in spiked samples using Equation 1.

Equation 1.

$$\left[\frac{(\text{spiked area} - \text{unspiked area}) / \text{standard area}}{\text{standard area}} \times 100 \right]$$

The calculation table is shown in Table 4.

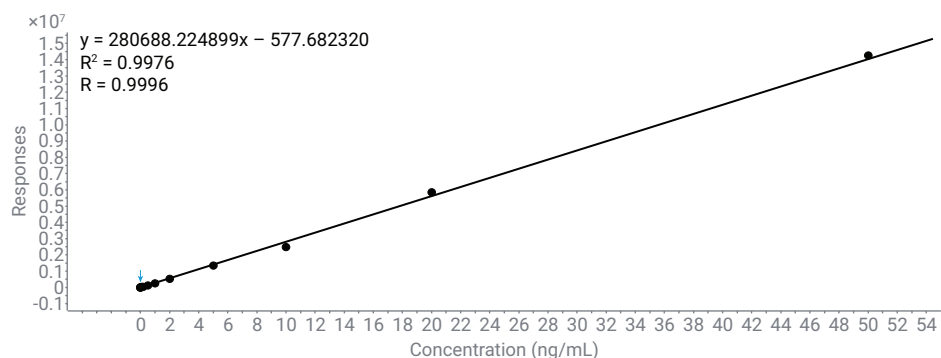


Figure 5. Calibration curve for N-Nitroso Nebivolol impurity.

Sample				NEBIVOLOL Results			
Name	Type	Level	Pos.	RT	Resp.	Final Conc.	Accuracy
STD 0.005 PPB	Cal	1	P1-A4	16.375	1383	0.0049	98.6
STD 0.01 PPB	Cal	2	P1-A5	16.375	2903	0.0108	107.7
STD 0.02 PPB	Cal	3	P1-A6	16.375	5006	0.0188	94.2
STD 0.05 PPB	Cal	4	P1-A7	16.375	12171	0.0464	92.7
STD 0.1 PPB	Cal	5	P1-A8	16.375	26243	0.1004	100.4
STD 0.2 PPB	Cal	6	P1-A9	16.375	45657	0.1749	87.5
STD 0.5 PPB	Cal	7	P1-B1	16.375	128798	0.4941	98.8
STD 1.0 PPB	Cal	8	P1-B2	16.375	256012	0.9825	98.3
STD 2.0 PPB	Cal	9	P1-B3	16.375	529394	2.0321	101.6
STD 5.0 PPB	Cal	10	P1-B4	16.375	1340147	5.1448	102.9
STD 10.0 PPB	Cal	11	P1-B5	16.375	2488584	9.5539	95.5
STD 20.0 PPB	Cal	12	P1-B6	16.375	5855750	22.4814	112.4
STD 50.0 PPB	Cal	13	P1-B7	16.375	14249415	54.7068	109.4
BLANK	Blank		P1-B9	16.408	62	0.0000	
BLANK	Blank		P1-B9	16.375	90	0.0000	
API SAMPLE 0.6MG_ML	Sample		P1-C3	16.392	65879	0.2525	
BLANK	Blank		P1-B9	16.425	54	0.0000	
API SPIKE	Sample		P1-C5	16.400	119216	0.4573	
BLANK	Blank		P1-C6	16.392	16	0.0000	
PLACEBO	Sample		P1-C7	16.425	1209	0.0043	
BLANK	Blank		P1-C8	16.475	31	0.0000	
PLACEBO SPIKE	Sample		P1-C9	16.392	50673	0.1942	
BLANK	Blank		P1-D1	16.475	31	0.0000	
TABLET SAMPLE	Sample		P1-D2	16.392	551207	2.1158	
BLANK	Blank		P1-D3	16.392	33	0.0000	
TABLET SPIKE	Sample		P1-D4	16.392	602243	2.3118	
BLANK	Blank		P1-D5				

Figure 6. Quantitation table showing analyte response, accuracy of calibration standards, MRM ratios, and percent recovery of the analyte from API sample and formulation.

Table 4. Recoveries of nitrosamine impurity from API, placebo, and tablet formulation.

Sample ID	Area	Spiked Amount (ng/mL)	Area for Neat Standard of Spike Concentration	Recovery %
API	65,879	–	–	116.8
API Spike	119,216	0.2	45,657	
Placebo	1,209	–	–	108.3
Placebo Spike	50,673	0.2	45,657	
Tablet	551,207	–	–	111.8
Tablet Spike	602,243	0.2	45,657	

Conclusion

A highly sensitive and robust MRM method was developed for the quantitation of N-Nitroso Nebivolol in 20 mg Nebivolol tablet formulation using an Agilent 6495D triple quadrupole LC/MS system. The chromatographic method developed provided good separation between the analyte and the API. The method showed LOD and LOQ of 2 and 5 pg/mL, respectively. The minimum signal-to-noise ratio at the LOQ level was found to be more than 50:1 (Peak to Peak). The calibration curve from 5 pg/mL to 50 ng/mL was found to be linear, with 1/x weighting. R and R² values were 0.999 and 0.997, respectively. Spike recovery analysis showed the efficiency of sample extraction with a recovery percentage of 116.8% in API, 108.3% in placebo, and 111.8% in tablet formulation at the spiking level of 200 pg/mL. The method was found to be highly reproducible at the LOQ level, while the %RSD value for the area response of seven replicate injections was approximately 3.1%.

Sample		NEBIVOLOL Method		
Name	Type	Exp. Conc.	RT	Resp.
STD 0.005 PPB	Cal	0.0050	16.375	1383
STD 0.005 PPB	Cal	0.0050	16.375	1328
STD 0.005 PPB	Cal	0.0050	16.375	1387
STD 0.005 PPB	Cal	0.0050	16.392	1286
STD 0.005 PPB	Cal	0.0050	16.375	1369
STD 0.005 PPB	Cal	0.0050	16.375	1313

Figure 7. Reproducibility at LOQ, % RSD is 3.1% (N = 6).

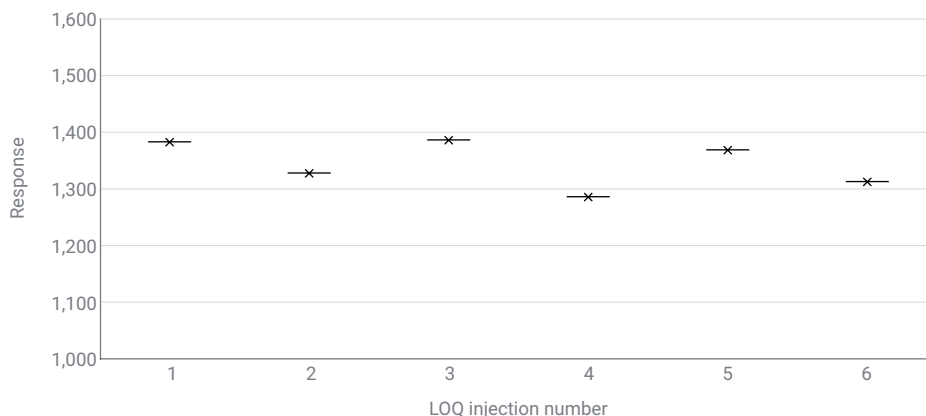


Figure 8. Box and whisker chart representing the reproducibility at LOQ.

References

1. Prasanth, J.; Banerjee, S.; Vyas, S. Determination of Genotoxic Nitrosamine Impurity in Bumetanide API and Tablets Using the Agilent 6470 Triple Quadrupole LC/MS. *Agilent Technologies application note*, publication number 5994-2967EN, **2020**.
2. Wu, L.; Bertram, L.; Chan, S.-A. Determination of Nitrosamine Impurities Using the Agilent 6475 Triple Quadrupole LC/MS System. *Agilent Technologies application note*, publication number 5994-5919EN, **2023**.