Application Note

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Quantitation of N-Nitroso Moxifloxacin in 400 mg Moxifloxacin Tablet Formulation

Using the Agilent 6495D triple quadrupole LC/MS/MS system

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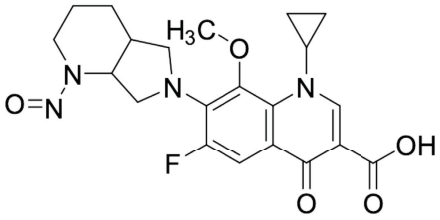
Abstract

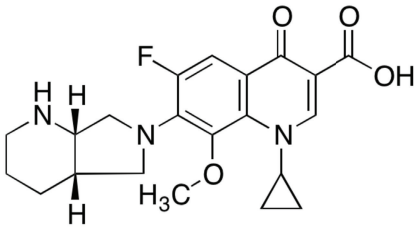
N-Nitroso moxifloxacin, a nitrosamine drug substance related impurity (NDSRI) of the fluoroquinolone antibiotic moxifloxacin, is categorized as Class-4 by the U.S. Federal Drug Administration (FDA), with a maximum allowable intake of 1,500 ng/day. This application note presents a sensitive and reproducible method for quantifying N-Nitroso moxifloxacin in 400 mg moxifloxacin tablets using an Agilent 1290 Infinity II LC and 6495D triple quadrupole LC/MS/MS system. The method achieved a limit of detection (LOD) of 1 pg/mL and a limit of quantification (LOQ) of 2.5 pg/mL, with linearity from 2.5 pg/mL to 10 ng/mL ($R^2 > 0.991$). Chromatographic separation exceeded 1.5 minutes between the active pharmaceutical ingredients (APIs) and the impurity, with API peaks diverted during impurity quantitation. The method showed a signal-to-noise ratio (S/N) of 232:1 at the LOQ level and an average recovery of 86.33% in placebo. Reproducibility at the LOQ level was confirmed with a percent relative standard deviation (%RSD) of approximately 4.7% for seven replicate injections.

Introduction

N-nitroso moxifloxacin is a NDSRI of moxifloxacin, which is a fluoroquinolone antibiotic widely used to treat various bacterial infections. N-nitroso moxifloxacin is characterized as an N-nitroso derivative of the secondary amine present in moxifloxacin. In the carcinogenic potency categorization published by the FDA, N-nitroso moxifloxacin has been categorized as Class-4, which implies a maximum allowable intake limit for the impurity of 1,500 ng/day. Moxifloxacin has a usual daily dosage limit of 400 mg for oral formulations.



N-Nitroso moxifloxacin



Moxifloxacin

Figure 1. Chemical structures of moxifloxacin and the impurity N-nitroso moxifloxacin.

Considering 400 mg moxifloxacin active pharmaceutical ingredient API in the formulation under study, the specification limit of N-nitroso moxifloxacin has been calculated as 5.625 ng/mL for a 1.5 mg/mL test concentration, which was used during this study.

This application note demonstrates a highly specific, sensitive, and reproducible method for the quantitation of N-nitroso moxifloxacin in the 400 mg moxifloxacin tablet formulation. The method uses an Agilent 1290 Infinity II LC coupled to an Agilent 6495D triple quadrupole LC/MS/MS.

Experimental

Materials

Calibration samples were prepared using N-nitroso moxifloxacin analytical standard material provided by an Indian pharmaceutical company. Formulation tablets, placebo, and the API for the drug was also provided by same pharmaceutical company.

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

Equipment

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

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

Method

The LC/MS chromatography conditions for the method are listed in Tables 1, 2, and 3.

Table 1. Chromatography conditions.

Parameter	Value				
Column	Agilent Pursuit XRS3 Diphenyl, 150 × 3 mm (p/n A6021150X030)				
Mobile Phase A	4.5 mM Ammonium formate, 0.5 mM ammonium fluoride, and 0.1% formic acid in water				
Mobile Phase B	Methanol				
Flow Rate	0.4 mL/min				
Injection Volume	20 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	16 minutes				
Gradient Program	Time (min)	%A	%B	Flow (mL/min)	Max. pressure limit (bar)
	0.0	75	25	0.400	1,300
	0.5	75	25	–	–
	6.0	25	75	–	–
	10.0	2	98	–	–
	13.0	2	98	–	–
	31.1	75	25	–	–
	16.0	75	25	–	–

Table 2. MS acquisition parameters.

Compound	Precursor	Product Ion (*m/z*)	Fragmentor Voltage (V)	Collision Energy (CE; V)	Polarity
N-nitroso Moxifloxacin	431.2	232.0	166	50	Positive

Table 3. MS source parameters.

Parameter	Value
Ionization Source	Agilent Jet Stream Technology Ion Source (AJS) (p/n G1958B)
Gas Temperature	290 °C
Gas Flow	11 L/min
Nebulizer	35 psi
Sheath Gas	375 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	2,500 V
Nozzle Voltage	0 V
Gain	10.0

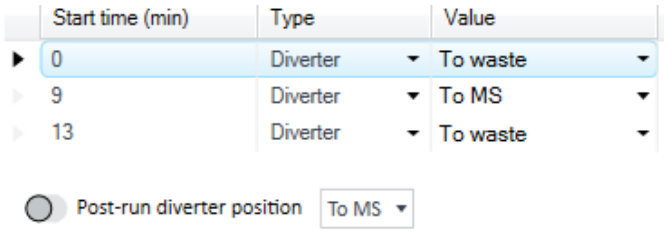


Figure 2. Diverter valve settings.

Preparation of standards and samples

Preparation of concentration linearity standards: The standard stock solution of 100 µg/mL was prepared by dissolving 5 mg of analytical standard using diluent and making up the volume of a 50 mL volumetric flask. Then, 1 mL of the stock solution was diluted to 100 mL to prepare a 1 ppm working standard solution.

A 12-point set of concentration linearity standards was prepared by serial dilution of the working standard solution.

Preparation of test samples: Five formulation tablets were accurately weighed, and the average weight was determined. The tablets were then crushed to powder and mixed well using pestle and mortar. A 1.5 mg/mL moxifloxacin content equivalent of the tablet powder was placed in a 15 mL centrifuge tube, and 10 mL of diluent were added. The tube was then vortexed for 1 minute, then subjected to rotational shaking for 20 minutes using a Rotospin (Tarsons, Kolkata, India). Then, the solution was centrifuged at 9,000 rpm for 10 minutes. The supernatant was transferred to an HPLC vial for analysis.

The same process was followed to prepare a placebo solution.

A spiked placebo sample was also prepared using the same method by spiking the LOQ level (2.5 pg/mL) of N-nitroso moxifloxacin into the placebo.

API solution was also prepared at a concentration 1.5 mg/mL.

Data acquisition and data analysis

All data were acquired using Agilent MassHunter Data Acquisition software (version 12.0). Chromatograms were viewed through Agilent MassHunter Qualitative Analysis software (version 12.0). Quantitation of each batch was carried out using Agilent 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%. No manual integration was needed.

Results and discussion

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

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

Figure 4 shows the responses and S/N at LOD and LOQ levels, which were 1 and 2.5 pg/mL, respectively.

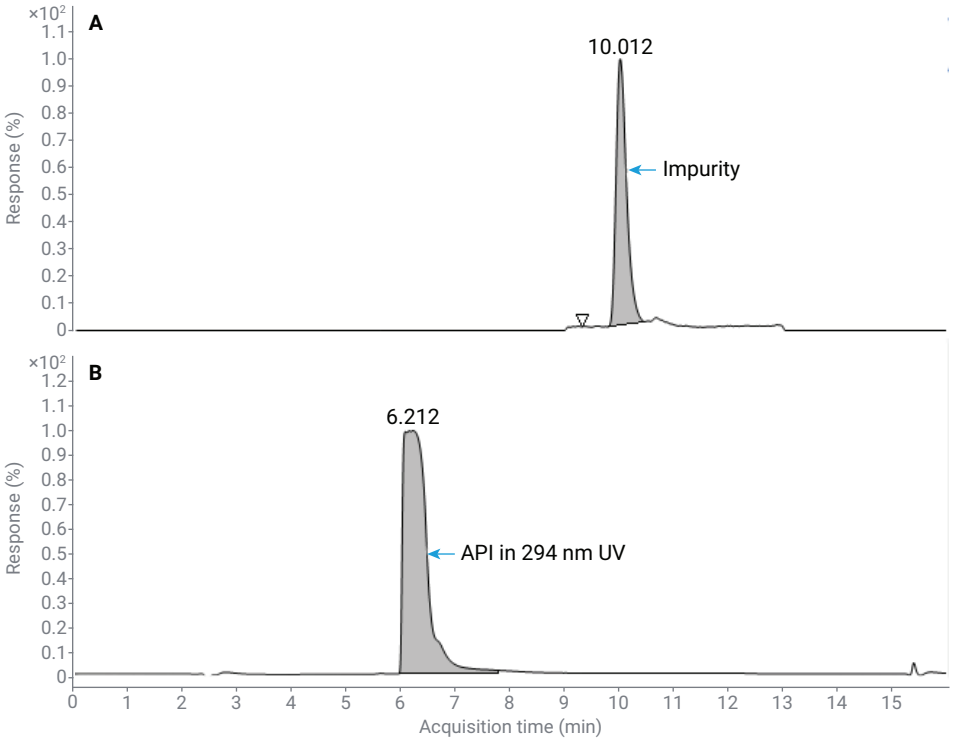


Figure 3. Graphs of % response versus acquisition time, showing clear separation between impurity (A) and API (B).

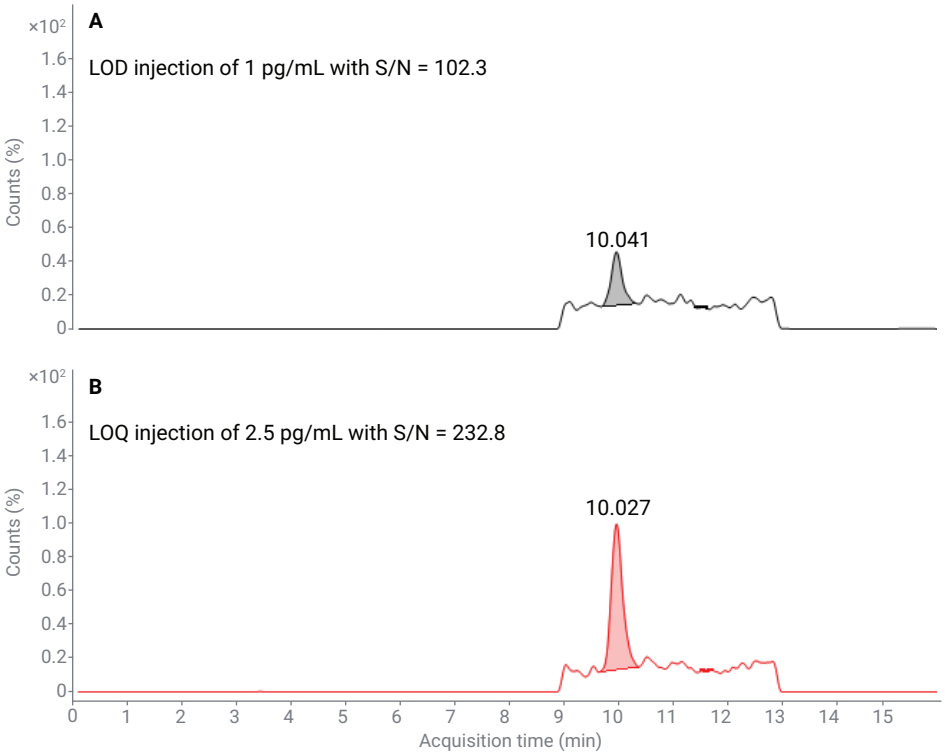


Figure 4. Chromatograms showing signal-to-noise ratios at (A) LOD and (B) LOQ levels.

A 12-point calibration curve was drawn with concentration linearity from 2.5 pg/mL to 10 ng/mL (Figure 5). The R^2 value for the calibration was found to be 0.991, which is well within the acceptable range.

The tablet, API, and placebo solutions were injected as samples, and spiked placebo solution was injected as a QC (with a known spiked concentration of 2.5 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 quant batch shows that the impurity content in the placebo was below the LOQ level (Figure 6).

The recovery of the spiked placebo was calculated by comparing the area of the neat standard at the LOQ level and the average area in the spiked placebo using Equation 1.

Equation 1.

$$\frac{(\text{spiked area} - \text{unspiked area})}{\text{standard area}} \times 100$$

Table 4 shows the calculations.

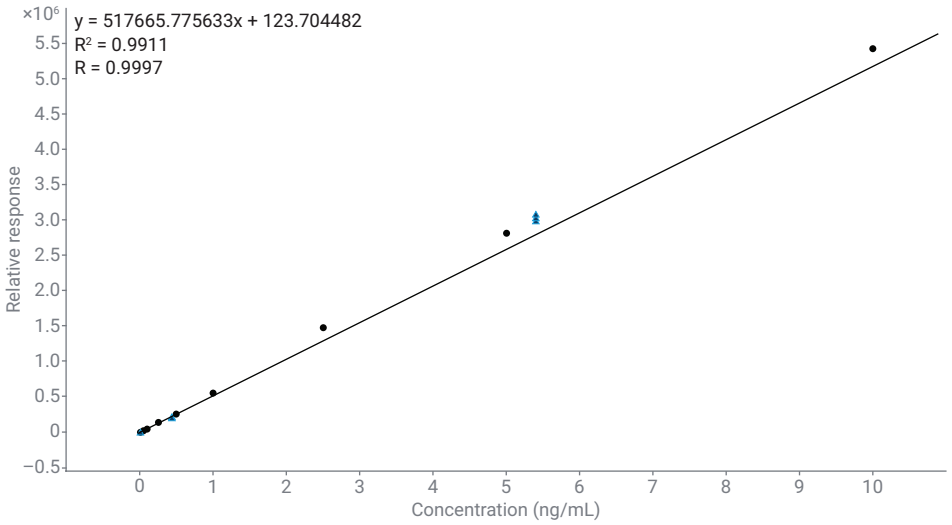


Figure 5. Calibration curve demonstrating linearity of the method over the range 2.5 pg/mL to 10 ng/mL.

Sample				NN MOXIFLOXACIN Results			
Name	Type	Level	Pos.	RT	Resp.	Final Conc.	Accuracy
BLANK1	Blank		P1-A1	9.945	4	0.0000	
0.0025PPB	Cal	1	P1-A3	10.038	1507	0.0027	106.9
0.005PPB	Cal	2	P1-A4	10.027	2594	0.0048	95.5
0.01PPB	Cal	3	P1-A6	10.033	4666	0.0088	87.7
0.025PPB	Cal	4	P1-A7	10.022	11820	0.0226	90.4
0.05PPB	Cal	5	P1-A8	10.027	23983	0.0461	92.2
0.1PPB	Cal	6	P1-B1	10.016	47288	0.0911	91.1
0.25PPB	Cal	7	P1-B2	10.016	133705	0.2580	103.2
0.5PPB	Cal	8	P1-B3	10.016	253713	0.4899	98.0
1PPB	Cal	9	P1-B5	10.016	556454	1.0747	107.5
2.5PPB	Cal	10	P1-B6	10.016	1476623	2.8522	114.1
5PPB	Cal	11	P1-B7	10.016	2815064	5.4378	108.8
10PPB	Cal	12	P1-B9	10.016	5423936	10.4774	104.8
BLANK2	Blank		P1-C1	10.027	145	0.0000	
BLANK4	Blank		P1-C5	10.032	156	0.0001	
TAB	Sample		P1-C6	10.016	1004568	1.9403	
BLANK5	Blank		P1-C7	10.027	136	0.0000	
API	Sample		P1-D4	10.016	2253187	4.3524	
BLANK6	Blank		P1-C1	10.060	58	0.0000	
PLACEBO	Sample		P1-C8	10.120	96	0.0000	
BLANK7	Blank		P1-C1	10.005	10	0.0000	
PLACEBO LOQ SPIKE	QC	15	P1-D1	10.016	1397	0.0025	98.4
BLANK7	Blank		P1-C1	10.027	21	0.0000	

Figure 6. Quant batch data. The concentration of N-nitroso moxifloxacin impurity in the placebo was below LOQ.

Table 4. Recovery calculations.

Sample ID	Average Area	Spiked Amount (ng/mL)	Area for the Neat Standard of Spike Concentration	Recovery (%)
Placebo	96	0.0025	1,507	86.33
Placebo Spike	1,397			

Conclusion

A highly sensitive and robust MRM method was developed for the quantitation of N-nitroso moxifloxacin in a 400 mg moxifloxacin 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 an LOD and LOQ of 1 pg/mL (0.0006 ppm with respect to test) and 2.5 pg/mL (0.0017 ppm with respect to test), respectively. The minimum signal-to-noise ratio at the LOQ level was found to be 232:1 (AUTO RMS). The calibration curve from 2.5 pg/mL to 10 ng/mL was found to be linear, with $1/x^2$ weighting. R and R^2 values were 0.996 and 0.991, respectively. Spike recovery analysis showed the efficiency of sample extraction with an average recovery percentage of 86.33% in placebo at the LOQ spiking level. The method was found to be highly reproducible at the LOQ level with the %RSD value for the area response of seven replicate injections of approximately 4.7%.

References

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DE002442

Printed in the USA, March 11, 2025
5994-7933EN

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