Application Note

Pharmaceuticals

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Quantitation of N-Nitroso N-Desmethyl Diphenhydramine in Diphenhydramine HCl API

Using the Agilent 6475A triple quadrupole LC/MS system

Authors

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Abstract

A highly specific, sensitive, and reproducible method was developed for the quantitation of N-nitroso N-desmethyl diphenhydramine, a nitrosamine drug-substance-related impurity (NDSRI), in diphenhydramine HCl drug substance. The method uses an Agilent 1290 Infinity II LC coupled to an Agilent 6475A triple quadrupole LC/MS. Optimizing multiple reaction monitoring (MRM) parameters for the compound and optimizing the source parameters for the chromatographic conditions helped to maximize the sensitivity to the analyte. For the developed MRM method, the limit of detection (LOD) and limit of quantitation (LOQ) were found to be 0.05 ng/mL and 0.1 ng/mL, respectively. These values correspond to 0.01 parts per million (ppm) and 0.02 ppm with respect to a test concentration of 5 mg/mL. The signal-to-noise ratios at the LOD and LOQ levels were found to be more than 25:1 and 45:1, respectively. The method was found to be linear within the concentration range 0.05 ng/mL to 10 ng/mL, with a regression coefficient of more than 0.999 (linear regression and $1/x^2$ weighting).

A chromatographic separation of more than 7 minutes was achieved between the active pharmaceutical ingredient (API) and the impurity, and the analyte peak was free from any interference, demonstrating the selectivity of the method. The separated API was diverted to the waste line with the help of an integrated, software-controlled diverter valve. MRM ratios were compared between the standards and API samples; the results confirmed the presence of impurity in positive samples. This application note also suggests a triggered MRM (tMRM) confirmatory technique, as it is beneficial to avoid false-positive results. Generation of product ion spectra through tMRM can be used as an extra tool to confirm the presence of N-nitroso N-desmethyl diphenhydramine in a drug substance.

Figure 1. Agilent 1290 Infinity II LC coupled to an Agilent 6475A triple quadrupole LC/MS.

Introduction

Diphenhydramine is one of the common antihistamines and is used to treat cold or allergy symptoms. It is also used to treat motion sickness and certain symptoms of Parkinson's disease. As a drug product, it is available under different brand names.

During synthesis of amine-containing APIs such as diphenhydramine, the synthesis product can be contaminated through formation of API-derived complex nitrosamines. These compounds are called NDSRIs. Such impurities can form through various reaction pathways. Secondary amines can easily undergo nitrosation in the presence of a trace amount of acid. Tertiary amines may undergo nitrosative cleavage or nitrosative dealkylation. The presence of NDSRIs has prompted the recall of pharmaceutical drug products, drawing the attention of regulatory agencies and manufacturers.

One NDSRI of interest to various regulatory bodies is N-nitroso N-desmethyl diphenhydramine (Figure 2) as an impurity in diphenhydramine. The maximum allowable intake for this impurity is 26.5 ng/day, and the maximum daily dose of diphenhydramine is 400 mg. Therefore, the specification limit for the impurity is 0.066 ppm (maximum allowable intake/maximum daily dose).[1] A highly selective and sensitive LC/MS/MS method with MRM was developed using a 6475A triple quadrupole LC/MS (LC/TQ) to detect this impurity. The sensitivity of the 6475A LC/TQ is such that it can easily detect N-nitroso N-desmethyl diphenhydramine at the required LODs. The special design of the ion optics and the stable electronics of the system provide consistent results across multiple batches.

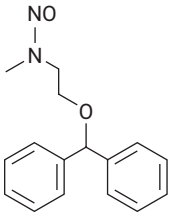

Figure 2. Structure of N-nitroso N-desmethyl diphenhydramine.

Experimental

Materials

Calibration samples were prepared using N-nitroso N-desmethyl diphenhydramine analytical standard provided by an Indian pharmaceutical company. The following other chemicals and reagents were used: ammonium trifluoro acetate (MS grade, Sigma-Aldrich, St. Louis, Missouri, USA), formic acid (MS grade, Sigma-Aldrich), 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 with a 6475A triple quadrupole LC/MS (G6475A). 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 general LC/MS chromatography conditions are given in Table 1. The parameters for dynamic MRM were optimized first (Table 2), and then the source parameters were optimized (Table 3). The MRM transitions were determined by using the MRM optimizer tool in the Agilent MassHunter Data Acquisition software (version 12.0).[2] A standard solution with an impurity concentration of 100 ng/mL was introduced into the MS by flow injection analysis, with an injection volume of 2 µL. In an automated workflow, the software selected product ions of the impurity for use in the MRM process. The tool also automatically optimized fragmentor voltages for the precursor ions (quadrupole 1) and collision energies for the product ions (quadrupole 3). Instrument MRM parameters and source parameters were optimized to maximize sensitivity while maintaining consistent performance for large batches.

Table 1. Chromatography conditions.

Parameter	Value
Column	Agilent InfinityLab Poroshell HPH-C18, 4.6 × 150 mm, 2.7 µm (p/n 693975-702T)
Mobile Phase A	1 mM Ammonium trifluoroacetate with 40 µL of formic acid in 1 L water (buffer solution)
Mobile Phase B	Methanol
Flow Rate	0.5 mL/min
Injection Volume	10 µL
Sample Cooler Temperature	10 °C
Column Temperature	50 °C
Needle Wash	Methanol:water (60:40)
Diluent	Methanol:water (60:40)
Elution	Gradient
Acquisition Time	18 minutes
Gradient Program	Time (min) %B 0 60 14 60 16 95 Postrun 2.0

Table 2. Parameters for the dynamic MRM method for N-nitroso N-desmethyl diphenhydramine.

Precursor	Product Ion (*m/z*)	MRM ID	Fragmentor Voltage (V)	Collision Energy (V)	Polarity
271.3	167	Quantifier	88	10	Positive
271.3	152	Qualifier	88	45	Positive

Table 3. MS source parameters.

Parameter	Value
Ionization Source	Agilent Jet Stream Technology Ion Source (AJS) (p/n G1958B)
Gas Temperature	300 °C
Gas Flow	13 L/min
Nebulizer	45 psi
Sheath Gas	200 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	5,000 V
Nozzle Voltage	500 V
Gain	8.0

From these MRM parameters, a retention-based dynamic MRM method was set up, with a retention time (RT) window of 1.0 minute, to increase the selectivity of the method. Both *m/z* 167 and *m/z* 152 were selected as fragments, and their ion ratios were recorded throughout the quantitation batch.

Calibration curves were plotted from 0.05 ng/mL to 10 ng/mL, using working and calibration standards containing N-nitroso N-desmethyl diphenhydramine prepared as described in Table 4. The calibration established the LOQ as 0.1 ng/mL with a signal-to-noise ratio more than 45:1, where noise was calculated using the root mean square (rms) method.

Sample preparation

The samples for the quantitation batch were prepared as follows. Twenty milligrams of diphenhydramine API were weighed and transferred to a 15 mL centrifuge tube. Four milliliters of diluent were added, and the tube was vortexed for 1 minute, then subjected to rotational shaking for 20 minutes using a Rotospin (Tarsons, Kolkata, India). Even though the API is completely soluble, the solution was centrifuged at 9,000 rpm for 10 minutes. The supernatant was transferred to an HPLC vial for analysis.

Table 4. Preparation of working standards to generate the calibration curve.

Stock ID	Volume of Stock	Volume of Diluent	Final Concentration	Standard Name
Stock*	100 µL	9.9 mL	10 mg/L	Working Standard 1
Working Standard 1	100 µL	9.9 mL	100 ng/mL	Working Standard 2
Working Standard 2	200 µL	1.8 mL	10 ng/mL	Calibration Level 8
Calibration Level 8	1,000 µL	1.0 mL	5 ng/mL	Calibration Level 7
Calibration Level 7	800 µL	1.2 mL	2 ng/mL	Calibration Level 6
Calibration Level 6	1,000 µL	1.0 mL	1 ng/mL	Calibration Level 5
Calibration Level 5	1,000 µL	1.0 mL	0.5 ng/mL	Calibration Level 4
Calibration Level 4	800 µL	1.2 mL	0.2 ng/mL	Calibration Level 3
Calibration Level 3	1,000 µL	1.0 mL	0.1 ng/mL	Calibration Level 2
Calibration Level 2	1,000 µL	1.0 mL	0.05 ng/mL	Calibration Level 1

* The initial stock solution was prepared as follows: 10.0 mg of the certified reference material was accurately weighed and dissolved in 10 mL of methanol to obtain a stock solution containing 1 mg/mL of the impurity N-nitroso N-desmethyl diphenhydramine.

Data acquisition and data analysis

All samples were acquired using Agilent MassHunter Data Acquisition software (version 12.0). Chromatograms were viewed through MassHunter Qualitative Analysis software (version 10.0). Quantitation of each batch was carried out using MassHunter Quantitative Analysis software (version 12.0). 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%.

Results and discussion

The chromatogram of the blank run is given in Figure 3.

The developed chromatographic method provided excellent separation between the impurity and the API to avoid any possible interference (Figure 4). To avoid contamination of the MS, an integrated diverter valve program was included to divert high-concentration API as it elutes.

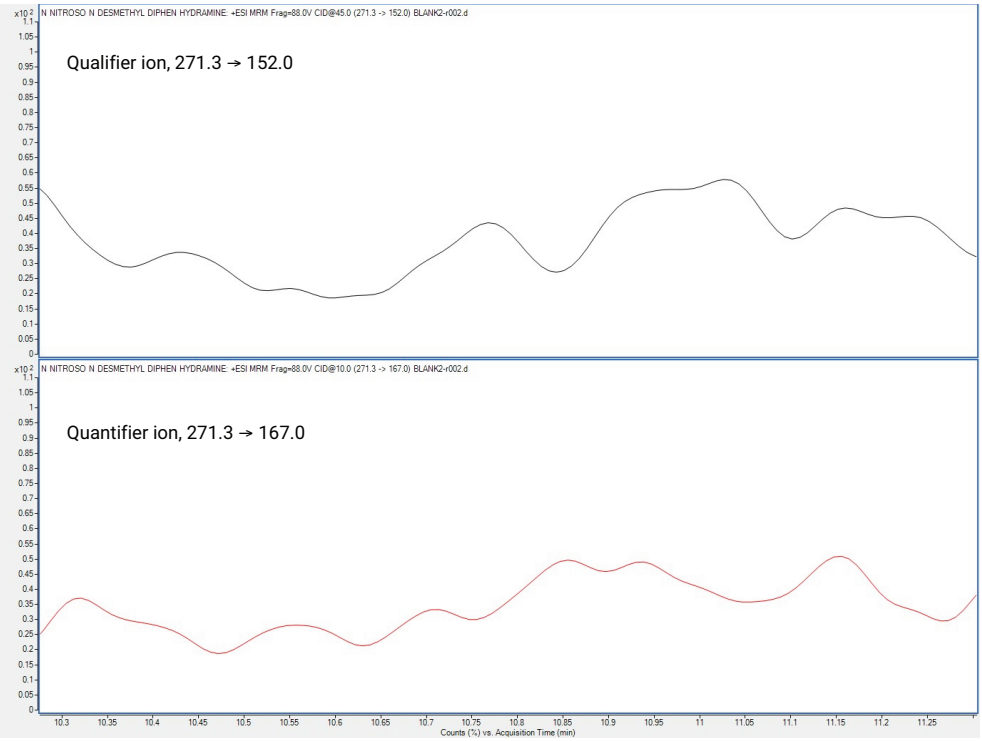

Figure 3. Blank chromatogram. RT of N-nitroso N-desmethyl diphenhydramine is 10.96 minutes.

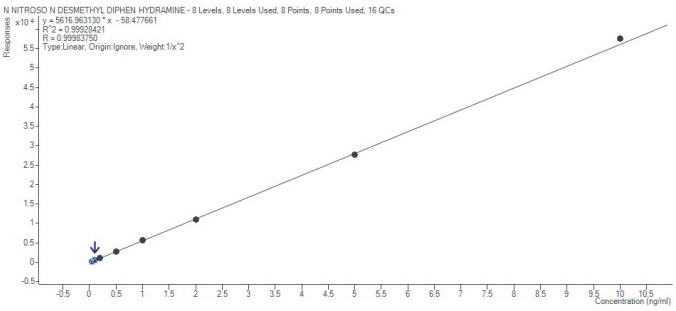

Figure 4. Calibration curve generated from 0.05 to 10 ng/mL; R^2 = 0.9992 with linear regression and $1/x^2$ weighting. The arrow designates the LOQ.

LOD and LOQ of the analyte were found to be 0.05 ng/ml and 0.1 ng/mL, respectively. These values correspond to 0.01 parts per million (ppm) and 0.02 ppm with respect to a test concentration of 5 mg/mL. The signal-to-noise ratio (S/N) was calculated for both LOD and LOQ by the rms algorithm and found to be more than 25:1 and 45:1, respectively (Figure 5).

A precision and accuracy batch was submitted to verify the method performance. A calibration curve spanning more than 2.0 orders of magnitude in concentration was generated, with a concentration range of 0.05 ng/mL to 10 ng/mL (Figure 6). The curve was found to be linear, with a regression coefficient of 0.9992 for linearity plotted between area against concentration of the analyte with a weighting factor of $1/x^2$. The accuracy of the individual calibration standards measured from the linearity curve ranged from 96% to 104%. The results of the quantitation are given in Table 5.

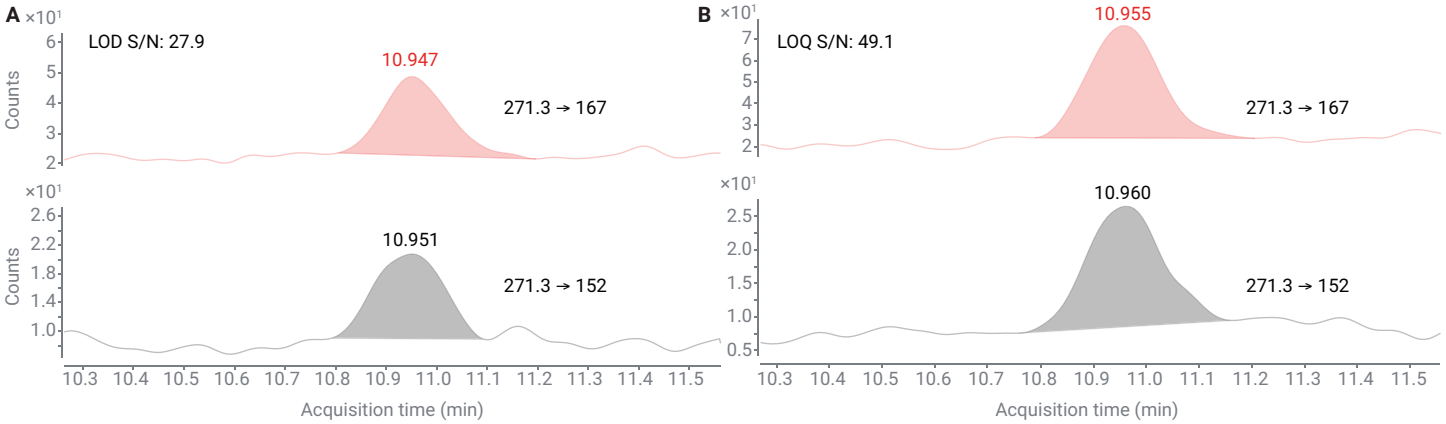

Figure 5. LOD and LOQ chromatograms with S/N (acquisition time corresponding to ratio given above peak).

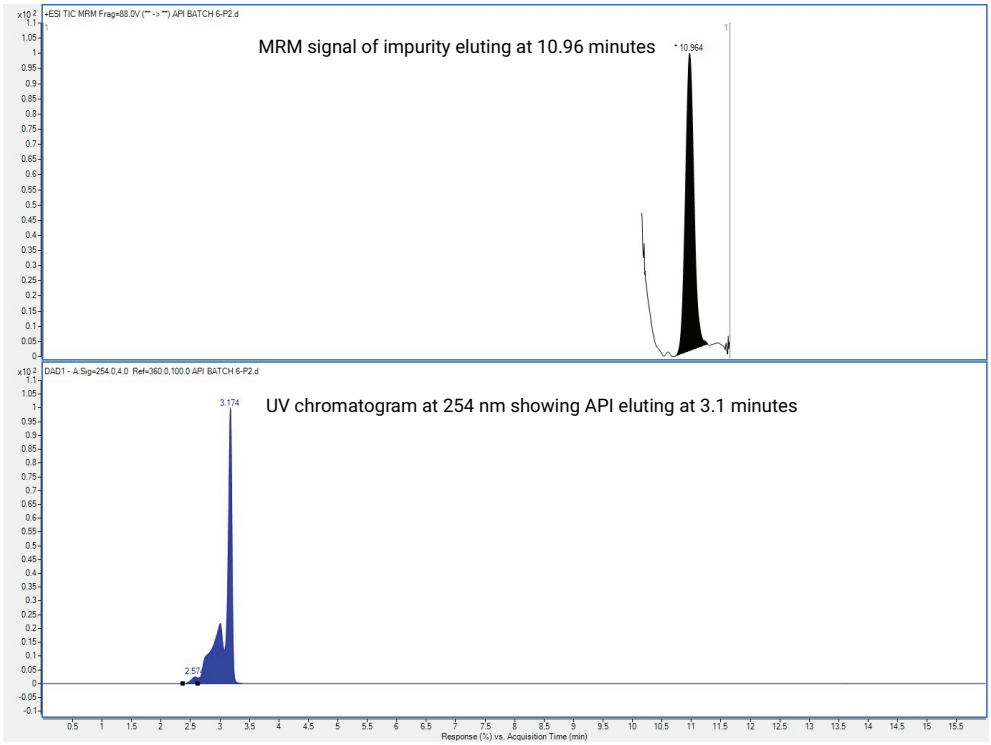

Figure 6. Chromatographic separation between the impurity and the API (more than 7 minutes separation).

Table 5. Quantitation table showing the response of analyte, accuracy of calibration standards, MRM ratios, and percent recovery of the API samples. Samples were spiked with the impurity standard at LOQ (0.1 ng/mL). Calculated concentration is in ng/mL, and the final concentration is in ppm (with respect to a test concentration of 5 mg/mL).

Batch Table

Sample: STD 0.1 PPB | Sample Type: <All> | Compound: N NITROSO N DESMETHYL DIPHI | ISTD:

Sample								N NITROSO N D...		N NITROSO N DESMETHYL DIPHEN HYDRAMINE Results						Qualifier...
		Data File	Type	Level	Amt.	Dil.	Tot. Amt.	Exp. Conc.	Units	RT	Resp.	MI	Calc. Conc.	Final Conc.	Accuracy	Ratio
		BLANK1-r001.d	Blank			1.0			ng/ml	11.097	10	☐	0.0122	0.0122		70.0
		BLANK1-r002.d	Blank			1.0			ng/ml	10.922	7	☐	0.0117	0.0117		42.9
		STD 0.05 PPB.d	Cal	1		1.0		0.0500	ng/ml	10.968	223	☐	0.0502	0.0502	100.3	43.5
		STD 0.1 PPB.d	Cal	2		1.0		0.1000	ng/ml	10.955	493	☐	0.0982	0.0982	98.2	36.9
		STD 0.2 PPB.d	Cal	3		1.0		0.2000	ng/ml	10.955	1104	☐	0.2070	0.2070	103.5	38.9
		STD 0.5 PPB.d	Cal	4		1.0		0.5000	ng/ml	10.955	2663	☐	0.4845	0.4845	96.9	36.8
		STD 1.0 PPB.d	Cal	5		1.0		1.0000	ng/ml	10.964	5627	☐	1.0122	1.0122	101.2	34.5
		STD 2.0 PPB.d	Cal	6		1.0		2.0000	ng/ml	10.964	11007	☐	1.9700	1.9700	98.5	36.7
		STD 5.0 PPB.d	Cal	7		1.0		5.0000	ng/ml	10.964	27691	☐	4.9403	4.9403	98.8	36.2
		STD 10.0 PPB.d	Cal	8		1.0		10.0000	ng/ml	10.955	57564	☐	10.2587	10.2587	102.6	34.7
		BLANK2-r001.d	Blank			1.0			ng/ml	10.880	8	☐	0.0118	0.0118		125.0
		BLANK2-r002.d	Blank			1.0			ng/ml	10.855	6	☐	0.0115	0.0115		
		API BATCH 1-P1.d	Sample		20	1.0	4		ng/ml	10.972	789	☐	0.1509	0.0302		38.1
		API BATCH 1-P2.d	Sample		20	1.0	4		ng/ml	11.006	772	☐	0.1478	0.0296		31.6
		BLANK3_1.d	Blank			1.0			ng/ml	10.922	7	☐	0.0117	0.0117		128.6
		API BATCH 2-P1.d	Sample		20	1.0	4		ng/ml	10.964	1171	☐	0.2189	0.0438		36.4
		API BATCH 2-P2.d	Sample		20	1.0	4		ng/ml	11.001	1129	☐	0.2114	0.0423		44.7
		BLANK3_2.d	Blank			1.0			ng/ml	10.905	3	☐	0.0109	0.0109		66.7
		API BATCH 4-P1.d	Sample		20	1.0	4		ng/ml	10.955	2788	☐	0.5068	0.1014		38.3
		API BATCH 4-P2.d	Sample		20	1.0	4		ng/ml	10.955	2760	☐	0.5018	0.1004		38.1
		BLANK3_3.d	Blank			1.0			ng/ml	11.006	19	☐	0.0138	0.0138		47.4
		API BATCH 5-P1.d	Sample		20	1.0	4		ng/ml	10.964	2614	☐	0.4758	0.0952		38.1
		API BATCH 5-P2.d	Sample		20	1.0	4		ng/ml	10.964	2749	☐	0.4998	0.1000		36.3
		BLANK3_4.d	Blank			1.0			ng/ml	11.006	6	☐	0.0115	0.0115		
		API BATCH 6-P1.d	Sample		20	1.0	4		ng/ml	10.964	2327	☐	0.4247	0.0849		35.9
		API BATCH 3-P1.d	Sample		20	1.0	4		ng/ml	10.955	1047	☐	0.1968	0.0394		33.1
		BLANK3_5.d	Blank			1.0			ng/ml	10.922	10	☐	0.0122	0.0122		140.0
		API BATCH 3-P2.d	Sample		20	1.0	4		ng/ml	10.972	1055	☐	0.1982	0.0396		34.1
		API BATCH 6-P2.d	Sample		20	1.0	4		ng/ml	10.972	2462	☐	0.4487	0.0897		34.2
		BLANK3_6.d	Blank			1.0			ng/ml	10.914	6	☐	0.0115	0.0115		33.3
		API SPIKE1_1.d	Sample		20	1.0	4		ng/ml	10.972	1650	☐	0.3042	0.0608		33.4
		API SPIKE1_2.d	Sample		20	1.0	4		ng/ml	10.964	1603	☐	0.2958	0.0592		33.0
		BLANK3_7.d	Blank			1.0			ng/ml	10.989	4	☐	0.0111	0.0111		
		BRACKETING STD-r001.d	QC	2		1.0		0.1000	ng/ml	10.964	528	☐	0.1044	0.1044	104.4	40.9
		BRACKETING STD-r002.d	QC	2		1.0		0.1000	ng/ml	10.972	588	☐	0.1151	0.1151	115.1	39.8

The results given in Table 6, Figure 7, and Figure 8 show consistent performance throughout the sample batch. Reproducibility was checked by injecting 0.05 ng/mL (LOD) and 0.1 ng/mL (LOQ) of standards seven times. The relative standard deviation (%RSD) of the seven injections of LOD and LOQ was calculated as 5.3 and 6.6 %, respectively. A metrics plot of area response and RT of all seven injections of LOD and LOQ standards is shown in Figure 8, demonstrating the consistency in the results.

Recovery experiments were conducted in duplicate by spiking the API sample with the impurity standard at LOQ level. Average recovery of analyte in the sample corresponding to the LOQ was 102.5% (calibration level 2, absolute concentration 0.1 ng/mL, or 0.02 ppm with respect to the test concentration of 5 mg/mL), showing the accuracy of the method. A summary of key parameters for the recovery study of N-nitroso N-desmethyl diphenhydramine in the API is given in Table 7.

Table 6. %RSD of seven injections at LOD and LOQ levels.*

Batch Table

Sample: STD 0.05 PPB | Sample Type: <All> | Compound: N NITROSO N DESMETHYL DIPHI | ISTD:

Sample								N NITROSO N D...		N NITROSO N DESMETHYL DIPHEN HYDRAMINE Results					Qualifier...
		Name	Data File	Type	Level	Amt.	Dil.	Tot. Amt.	Exp. Conc.	Units	RT	Resp.	MI	Calc. Conc.	Final Conc.	Accuracy	Ratio
		BLANK	BLANK5.d	Blank			1.0			ng/ml	10.964	11	☐	0.0124	0.0124		63.6
▸		STD 0.05 PPB	STD 0.05 PPB-r001.d	QC	1		1.0		0.0500	ng/ml	10.964	247	☐	0.0544	0.0544	108.8	48.8
		STD 0.05 PPB	STD 0.05 PPB-r002.d	QC	1		1.0		0.0500	ng/ml	11.014	268	☐	0.0582	0.0582	116.4	39.8
		STD 0.05 PPB	STD 0.05 PPB-r003.d	QC	1		1.0		0.0500	ng/ml	10.968	269	☐	0.0583	0.0583	116.7	37.0
		STD 0.05 PPB	STD 0.05 PPB-r004.d	QC	1		1.0		0.0500	ng/ml	11.006	259	☐	0.0566	0.0566	113.2	43.2
		STD 0.05 PPB	STD 0.05 PPB-r005.d	QC	1		1.0		0.0500	ng/ml	10.980	234	☐	0.0520	0.0520	104.0	44.9
		STD 0.05 PPB	STD 0.05 PPB-r006.d	QC	1		1.0		0.0500	ng/ml	10.955	271	☐	0.0587	0.0587	117.3	41.8
		STD 0.05 PPB	STD 0.05 PPB-r007.d	QC	1		1.0		0.0500	ng/ml	11.001	258	☐	0.0564	0.0564	112.9	30.1
		STD 0.1 PPB	STD 0.1 PPB_1-r001.d	QC	2		1.0		0.1000	ng/ml	10.964	537	☐	0.1060	0.1060	106.0	41.2
		STD 0.1 PPB	STD 0.1 PPB_1-r002.d	QC	2		1.0		0.1000	ng/ml	11.010	539	☐	0.1063	0.1063	106.3	38.4
		STD 0.1 PPB	STD 0.1 PPB_1-r003.d	QC	2		1.0		0.1000	ng/ml	10.964	556	☐	0.1094	0.1094	109.4	46.0
		STD 0.1 PPB	STD 0.1 PPB_1-r004.d	QC	2		1.0		0.1000	ng/ml	10.972	529	☐	0.1046	0.1046	104.6	42.2
		STD 0.1 PPB	STD 0.1 PPB_1-r005.d	QC	2		1.0		0.1000	ng/ml	10.972	609	☐	0.1188	0.1188	118.8	37.8
		STD 0.1 PPB	STD 0.1 PPB_1-r006.d	QC	2		1.0		0.1000	ng/ml	10.955	510	☐	0.1012	0.1012	101.2	42.0
		STD 0.1 PPB	STD 0.1 PPB_1-r007.d	QC	2		1.0		0.1000	ng/ml	10.972	598	☐	0.1169	0.1169	116.9	47.3
		BLANK	BLANK6-r001.d	Sample			1.0			ng/ml	10.964	22	☐	0.0143	0.0143		36.4
		BLANK	BLANK6-r002.d	Sample			1.0			ng/ml	10.930	10	☐	0.0122	0.0122		

* %RSD of area response at LOD (0.05 ng/mL) and LOQ (0.1 ng/mL) were 5.3% and 6.6%, respectively (n = 7).

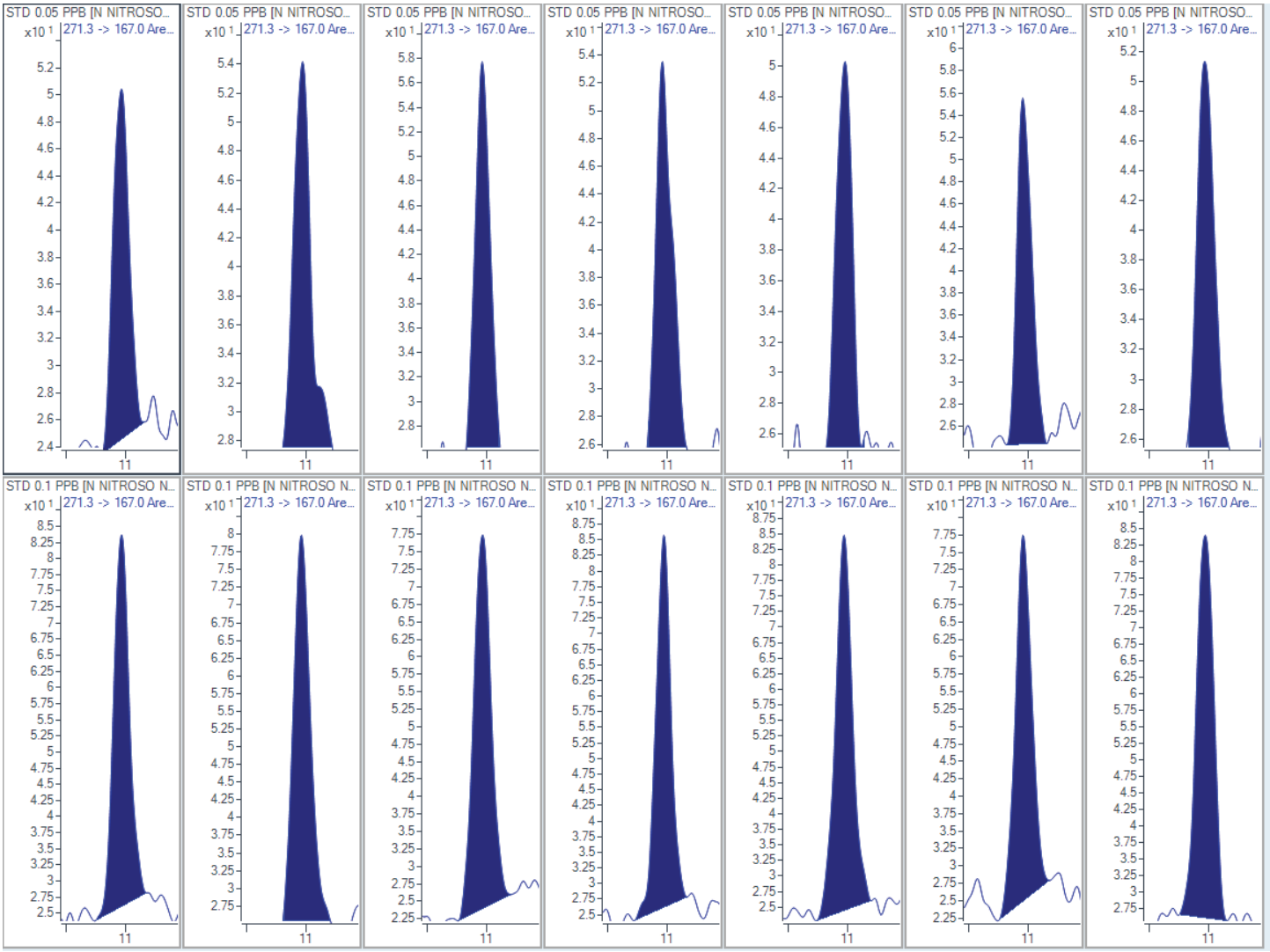

Figure 7. Peak review of the seven replicates of N-nitroso N-desmethyl diphenhydramine impurity at LOD and LOQ levels.

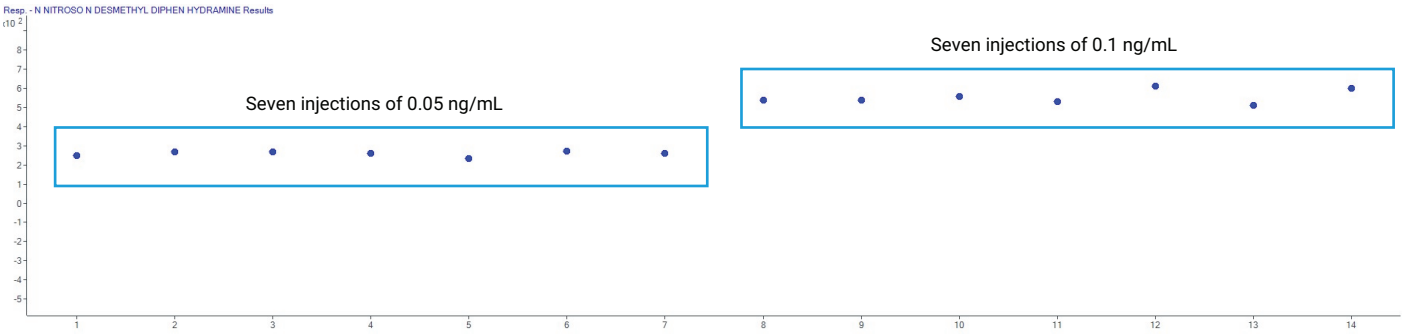

Figure 8. Metrics plot of response of seven replicate injections at the LOD (0.05 ng/mL) and LOQ (0.1 ng/mL) levels, showing the consistency in results.

Table 7. Results of the recovery study.

Parameter	Value
Maximum Allowable Intake for the Impurity	26.5 ng/day
Maximum Daily Dose of Drug	400 mg
Specification Limit for Impurity	0.066 ppm
Test Concentration	5 mg/mL
LOQ for the Impurity	0.1 ng/mL = (0.1 ng/mL)/5 mg/mL = 0.02 ppm
Impurity Concentration (Average) in API	0.0395 ppm
Spiked Concentration	0.02 ppm
Calculated Concentration (Average) of the Spiked Samples	0.06 ppm
Recovery	[(0.06–0.0395)/0.02] × 100 = 102.5%

Carryover was also evaluated by injection of the solvent blank/diluent sample after the injection of the highest standard. The peak area count obtained for the blank in this test was less than 5% of the area of the LOQ sample (calibration level 2). MRM ratios were compared between the standards and API samples; the results confirmed the presence of the impurity in positive samples.

The 6475A LC/TQ features mixed-mode acquisition, which enables simultaneous acquisition of both quantitative information (MRM) and qualitative information (full scan and product ion scan) from the sample. Mixed-mode acquisition works by incorporating scanning functions into the same time segment as the MRMs for the analytes of interest. This feature can be useful in monitoring the background of samples.[3] Results of a mixed-mode scan in the present study are given in Figure 9.

Figure 9. Mixed-mode scan results extracted at analyte RT, showing total ion chromatogram (TIC), extracted ion chromatogram (EIC), and product ion spectra at different collision energies.

The fragmentation pattern generated by a product ion scan can be susceptible to a matrix effect. In contrast, the spectra generated by the tMRM feature available in the 6475A LC/TQ are less susceptable to matrix effects because they are based on the MRM scan. The fragments selected during MRM can be stored as tMRM spectra for future comparison with sample fragments. In a tMRM method using Agilent 6400 Series triple quadrupole LC/MS systems, up to 10 MS/MS transitions can be acquired for each analyte and combined into a product ion spectrum (at optimum collision energies for each product ion). This product ion spectrum is used for library matching and provides increased confidence in identifications. When using the tMRM function, some of the transitions (primary transitions) are acquired during the entire analyte acquisition window. The acquisition of additional transitions is triggered (and performed for a defined number of scans) when one of the primary transitions exceeds the set abundance threshold.[4] The tMRM spectra obtained for the analyte generated from a spiked sample are given in Figure 10.

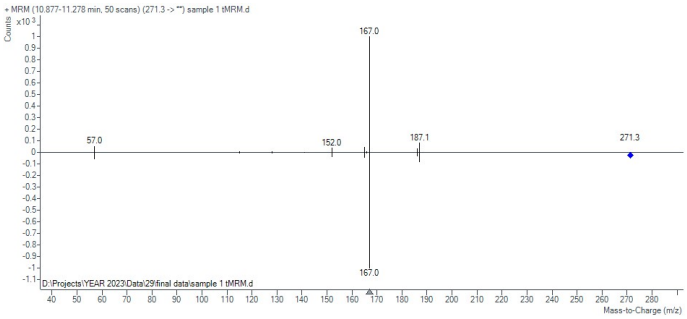

Figure 10. tMRM spectrum obtained for the analyte generated from the spiked sample.

Conclusion

A highly sensitive and robust MRM method was developed for quantitation of N-nitroso N-desmethyl diphenhydramine in diphenhydramine drug substance using the Agilent 6475A 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 0.05 ng/mL and 0.1 ng/mL, respectively. The minimum S/N at the LOQ level was found to be more than 45:1 (rms). The calibration curve from 0.05 ng/mL to 10 ng/mL was found to be linear, with $1/x^2$ weighting. R and R^2 values were 0.9998 and 0.9992, respectively. Spike recovery analysis showed the efficiency of sample extraction with an average recovery percentage of 102.5% at the LOQ spiking level. The method was found to be highly reproducible at the LOQ level: the %RSD value for the area response of seven replicate injections was 6.6%. The method was found to be suitable for the routine quality control of diphenhydramine drug substance.

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