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Original Article

A Validated LC-MS/MS Method for Determination at Trace Level of Nitrosamine Impurities in Doxofylline API

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ABSTRACT

Background: The FDA looked at popular amine-based API synthesis processes and found that these processes might additionally introduce nitrosamine-hazardous substances. The current method describes trace-level quantification of Nitrosamine impurities (NDEA, NDIPA, NDIPA, NEIPA, NMPA, NDBA, NDMA and NMBA) in the Doxofylline API. **Materials and Methods:** With the help of Waters® ACQUITY® UPLC BEH C18 (150x4.6 mm, 5 µm) and a 0.8 mL/min flow, a gradient programme (0.1%v/v formic acid and methanol) continuing for 14 min was used to achieve separation. Using Multiple Reaction Monitoring (MRM), all nitrosamine impurities were ionized and measured in the positive polarity mode of APCI. **Results:** Protonated molecular ions (M+H)+ were acquainted at: *m/z* 75 (parent), *m/z* 58 (product), *m/z* 103 (parent), *m/z* 47 (product), *m/z* 131 (parent), *m/z* 89 (product), *m/z* 117.1 (parent), *m/z* 74.8 (product), *m/z* 147.1 (parent), *m/z* 117 (product), *m/z* 137 (parent), *m/z* 66 (product), *m/z* 159 (parent), *m/z* 103 (product), *m/z* 267.2 (parent), *m/z* 181.1 (product), respectively, for NDMA, NDEA, NDIPA, NEIPA, NMBA, NMPA, NDBA and Doxofylline, with their retention times observed as 4.04, 6.83, 8.97, 7.98, 4.92, 9.25, 11.06 and 7.15 min, respectively. **Conclusion:** The coefficient of determination (r^2) for individual impurities was found to be between 0.996 and 1.000. S/N method was used to establish limits of detection and quantification, which turned out as 0.0040 µg/mL-0.0174 µg/mL and 0.0060 µg/mL-0.0262 µg/mL, respectively. Each of the validation parameters falls within the allowed ranges as per USP<1225>.

Keywords: NDMA, Doxofylline, NDEA, Nitrosamine impurities, APCI.

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INTRODUCTION

Doxofylline is a newer generation methyl xanthine derivative which has been developed to overcome the complications raised due to theophylline. Studies have demonstrated the similar's good safety and efficacy in treating COPD and asthma.[1] The structure of Doxofylline, represented by the chemical formula $C_{11}H_{14}N_4O_4$, is shown in Figure 1. Doxofylline works by limiting phosphodiesterase activity, which raises $cAMP_2$ levels and encourages smooth muscle relaxation.[2] In the year 2018, FDA was informed the presence of genotoxic impurities in ranitidine, nizatidine as well as metformin to an unacceptable level.[3-5] Further in the proceeding with the investigation, FDA and other regulatory authorities have bought into the light for thorough detection of these nitrosamine impurities utilizing effective analytical tools and to find solution to ensure drug quality thereby achieving patient safety. The FDA and other external regulators undertook the analysis of the same in various impacted APIs and associated drug products as a result of the detection of nitrosamines[6] in certain categories of drug product. Nitroso group bonded to an amine (nitrosoamine), are a class of compounds formed by a reaction nitrosating between an amine (may be 2, 3 or 4) and nitrous acid under acidic conditions is shown Figure 2. The prescribed products contain each of the seven nitrosamine impurities: N-Nitroso-di-n-butylamine, N-Nitroso-diisopropylamine, N-Nitroso-N-methyl-4-aminobutyric acid, N-Nitroso-ethylisopropylamine, N-Nitroso-methylphenylamine, N-Nitroso-dimethylamine, N-Nitroso-diethylamine whose structures are illustrated in Figure 3. The preparation of Doxofylline API involves reacting ethylene glycol and 7-(2,2-diethoxyethyl)theophylline in Dimethylformamide (DMF) as a solvent with tosic acid as a catalyst. Since the reaction involves secondary amines and nitrite esters, there are chances of nitrosamine formation as an impurity. To address this concern, a sample from manufactured Doxofylline API batch is chosen to undergo testing for the presence of nitrosamine impurities. Tagialo F *et al.*,[7] disclosed HPLC method for determination of the same in biological samples devoid of extraction, whereas

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work reported by Sivasubramanian and Patil NG revealed quantification of Doxofylline using HPTLC.[8,9] Most of the LC-MS/MS tandem methods[10-16] has shown quantification of Doxofylline using various ionization techniques with application to pharmacokinetic study. In the article published by Kamila MM it was reported molar absorptivity and sandell's sensitivity as $6.2x10^3$ $L.mol^{-1}$ $.cm^{-1}$. Few spectroscopic methods[17-20] were reported for estimating Doxofylline in combined dosage form. Methods indicating Stability and its validation using RP-HPLC[21-31] were in common. From the literature, it has been known that nitrosamines in Doxofylline API were yet to be reported. So, this article reports a LC method using MS/MS detection of the same in Doxofylline API and validating the developed method according to USP <1225> where the purpose of the study was aimed.

MATERIALS AND METHODS

Chemicals and Reagents

Highest-purity LCMS grade solvents were employed. Merck provided the methanol and Fluka provided the formic acid (96%). Doxofylline API and the pure form of nitrosamine impurities were obtained from Suven Pharmaceuticals Pvt. Ltd., and Advent Chembio Pvt. Ltd., respectively.

Instruments and Equipment

Class A glassware was used throughout the analysis where LC-MS/MS employed was of make Shimadzu Nexera X2, 6500+ (SCIEX LC-MS/MS). Weights were taken on Sartorius cubis, ASB-60-220-C2 with sensitivity of 0.01 mg and 0.0001 mg.

Preparation method for Mobile Phase (MP)

1 mL of formic acid was combined with 1,000 mL of Milli-Q water to create MP-A and 100% methyl alcohol was used as MP-B. 500 mL of formic acid and 500 mL of formic acid diluted to 0.1% v/v were combined to create the diluent. To get rid of dissolved gases, all the solvents were degassed.

Preparation X

Weight of 2.0 (in mg) of NDEA, NDBA, NDIPA, NMPA and NEIPA was taken into individual flask with capacity of 20 mL, dissolved using small quantity of methanol, sonicated to dissolve, filled the volume using the same and mixed thoroughly. 1 mL of this preparation is taken into flask with capacity of 100 mL, filled with methanol until lower meniscus is reached.

Preparation Y

Weight of 2.0 (in mg) of NDMA and NMBA was taken into individual flask with capacity of 20 mL, dissolved using small quantity of methanol, sonicated to dissolve, filled up the volume using the same and mixed thoroughly. 1 mL of this preparation is taken into flask with capacity of 50 mL, filled with methanol until lower meniscus is reached.

Preparation Z

1 mL from individual flask of preparation X and 2 mL from individual flask of preparation Y were taken into a flask with 25 mL capacity, filled up the volume with methanol then mixed thoroughly.

Standard solution

1 mL from preparation Z is taken into a flask with capacity of 10 mL, filled with methanol until lower meniscus is reached.

Test solution

2000 mg of Doxofylline API was taken into a flask with capacity of 10 mL, added small quantity of methanol and dissolved using a sonicator. Filled up the volume with the same then mixed thoroughly.

Procedure

One injection of diluent, six standard injections and one test solution were injected into LC MS/MS system. Peak responses were recorded.

Operating conditions for LC MS/MS

UHPLC with a binary pump, auto sampler and APCI interface was used to achieve chromatographic separation of standard impurities as well as Doxofylline. APCI minimizes effluent waste by handling full flow rates directly. For separation, a Waters® ACQUITY® UPLC BEH C18 (150X4.6 mm, 5 µm) column was employed with a gradient flow at the rate of 0.8 mL/min (time in min/%MP-A: 0.3/98, 10.0/10, 10.10/98, 14.0/98). 1200 µL of rinse volume was used before and after aspiration with 5 sec of dip time. All the nitrosamine impurities were quantified and ionized in positive polarity mode of APCI as ion source using Multiple Reactions Monitoring (MRM). Protonated Molecular Ions $(M+H)^+$ were acquainted at: *m/z* 75 (parent), *m/z* 58 (Product), *m/z* 103 (parent), *m/z* 47 (Product), *m/z* 131 (parent), *m/z* 89 (Product), *m/z* 117.1 (parent), *m/z* 74.8 (Product), *m/z* 147.1 (parent), *m/z* 117 (Product), *m/z* 137 (parent), *m/z* 66 (Product), *m/z* 159 (parent), *m/z* 103 (Product), *m/z* 267.2 (parent), *m/z* 181.1 (Product) respectively for NDMA, NDEA, NDIPA, NEIPA, NMBA, NMPA, NDBA and Doxofylline. 4.04, 6.83, 8.97, 7.98, 4.92, 9.25, 11.06 and 7.15 min, are respective Retention Times. Positive ion source parameters like collision gas and curtain gas pressure were maintained at 30 psi and 9 psi respectively, while drying gas temperature was 350°C and nebulizer current was 4 µA.

RESULTS

Chromatographic Method Development and Optimization of MS/MS conditions

Current work was executed to establish a highly sensitive liquid chromatographic method for separation with MS/MS detection. Initiated with various mobile phase pH conditions in both the modes of elution, 0.1%v/v Formic acid (MP-A) with 100% Methanol (MP-B) was finalized as mobile phase to obtain good peak shapes maintaining the symmetry. Efficient separation between nitrosomal impurities and Doxofylline was obtained on Waters® ACQUITY® UPLC BEH C18 150x4.6 mm, 5 µm after evaluating various columns at a flow rate of 0.8 mL/min with 10 µL of injection volume. MS/MS Tandem detection was employed in positive polarity mode in atomic pressure chemical ionization as source.

Validation of the developed method

System suitability was performed as part of compatibility of LC conditions with respect to the system. %RSD for area of each impurity standard peaks from six replicate injections was performed, and reported the R_t of each impurity peak in standard and test solution. Precision (Method and Intermediate) data was obtained by injecting the standard solutions in six replicates and obtained S/N values. Linearity was further evaluated from LOQ to 0.1 ppm. Accuracy was executed by spiking test solution with each impurity in triplicate at four different levels (LOQ, 50%, 100% and 200%) and % recovery was reported. By analysing the solutions at various points in time, the solution stability of each impurity was determined.

DISCUSSION

Specificity

Doxofylline API is spiked with all the impurities at specification level in diluent and retention time of individual impurity peaks in spiked and individual solutions were reported which were shown in Table 1. Interference of diluent was checked by injecting three solutions into LC-MS/MS system were illustrated in Figure 4. %RSD for the peak areas was calculated and found to be well within the limits.

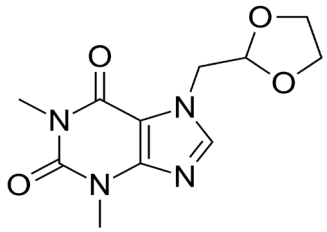


Figure 1: Chemical structure of Doxofylline.

Table 1: Results for Retention Time confirmation and Identification.

Name of the Impurity	Retention Time (min)		
	Standard	Individual	Diluent Interference[a]
NDEA	6.83	6.83	NO
NDIPA	8.97	8.96	NO
NEIPA	7.98	7.98	NO
NMPA	9.25	9.24	NO
NDBA	11.06	11.05	NO
NDMA	4.04	4.04	NO
NMBA	4.92	4.91	NO
Doxofylline	7.15	7.15	NO

[a] None of the Injected Impurity peaks were found with co-eluting peaks.

Table 2: Results for Unspiked test sample in Method Precision.

Sample name	Concentration in ppm						
	NDEA	NDIPA	NEIPA	NMPA	NDBA	NDMA	NMBA
Unspiked test solution-1	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
Unspiked test solution -2	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]	ND [a]
Average	NA [b]	NA [b]	NA [b]	NA [b]	NA [b]	NA [b]	NA [b]

[a] Not Detected; [b] Not Applicable.

Table 3: Results for Spiked test sample in Method Precision.

Sample name	Concentration in ppm						
	NDEA	NDIPA	NEIPA	NMPA	NDBA	NDMA	NMBA
Spiked solution-1	0.0197	0.0227	0.0183	0.0190	0.0206	0.0719	0.0881
Spiked solution-2	0.0213	0.0228	0.0190	0.0203	0.0214	0.0753	0.0891
Spiked solution-3	0.0190	0.0221	0.0180	0.0208	0.0213	0.0732	0.0883
Spiked solution-4	0.0181	0.0231	0.0185	0.0204	0.0207	0.0817	0.0863
Spiked solution-5	0.0192	0.0211	0.0173	0.0197	0.0210	0.0809	0.0899
Spiked solution-6	0.0181	0.0224	0.0182	0.0202	0.0206	0.0767	0.0860
Average	0.019	0.022	0.018	0.020	0.021	0.077	0.088
%RSD	6.2	3.2	3.1	3.2	1.7	5.2	1.7

Table 4: Results for LOD and LOQ Establishment.

Impurity name	Concentration at Detection level	s/n ratio [b]	Concentration at Quantification level	s/n ratio [b]
	ppm [a]		ppm [a]	
NDEA	0.0042	5.6	0.0063	12.3
NDIPA	0.0044	6.4	0.0066	12.7
NEIPA	0.0040	7.8	0.0060	13.7
NMPA	0.0040	5.7	0.0060	12.4
NDBA	0.0042	6.6	0.0064	12.5
NDMA	0.0168	7.6	0.0252	12.9
NMBA	0.0174	7.9	0.0262	13.6

[a]concentration is expressed as parts per million. [b]calculated based on the residual standard deviation of regression line.

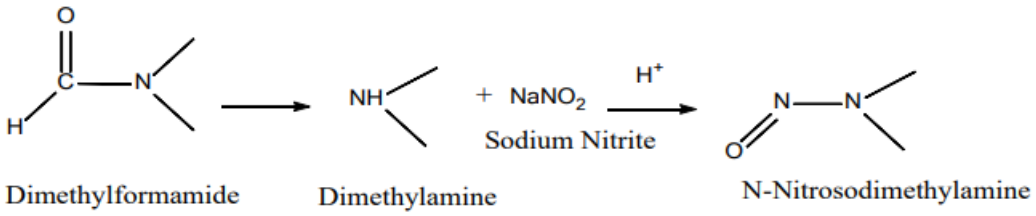


Figure 2: Common pathway for generation of nitrosamine impurities.

Precision

Method precision was performed by preparing two unspiked solutions and six spiked test solutions (at specification level). Results showing impurities not detected and Peak areas were tabulated in Tables 2 and 3 respectively. Figure 5 illustrates the m/z values of Doxofylline API. Determined the ppm of each impurity in unspiked test solution and spiked test solution where by calculating the relative standard deviation for six determinations, the method's reliability was assessed.[32,33]

Establishment of LOD and LOQ

According to the S/N ratio technique, the Limitations (LOD and LOQ) should be 3 and 10, respectively. Table 4 depicts the values. All impurities are detectable at around 0.004 µg/mL with S/N above 5 and Quantifiable at around 0.006 µg/mL with even better S/N above 12.

Linearity

LOQ to 200% of the impurity specified level was linearized. Calculated correlation coefficients for each particular impurity ranged from 0.996 to 1.00. Additionally calculated and presented were the slope, Y-intercept and residual sum of squares. Test results are tabulated in Table 5. All nitrosamine impurities in Doxofylline show excellent linearity based on correlation coefficients close to 1 and minimal impact from zero-concentration readings (Y-intercept).

Table 5: Results for Linearity.

Impurity name	Correlation coefficient	Slope	Y-intercept	Residual sum of squares
NDEA	0.996	3925593.49	4689.50	118464184
NDIPA	1.000	19714681.66	14771.53	265095185
NEIPA	0.999	25245680.03	27783.82	1091067894
NMPA	0.999	8554448.53	6158.77	150660278
NDBA	1.000	11962411.29	10928.18	55052468
NDMA	0.996	2781524.21	877.84	899202726
NMBA	1.000	6104854.49	2368.89	174887617

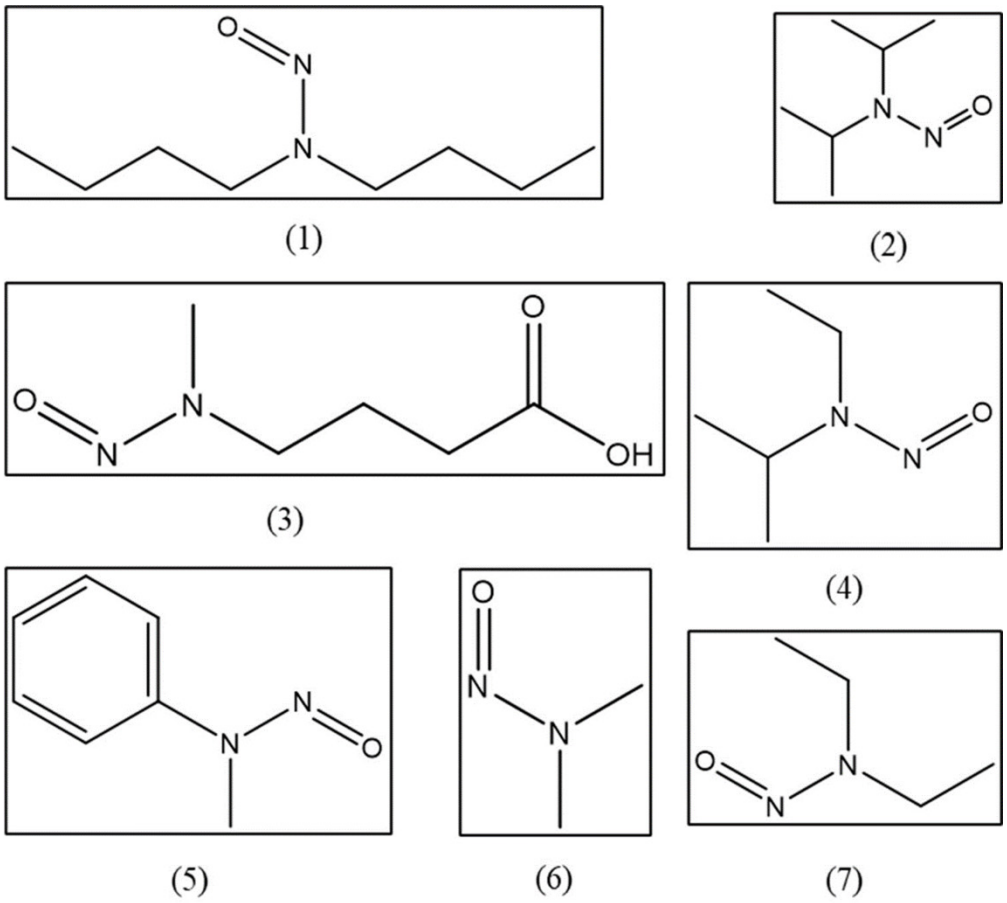


Figure 3: Five out these (NDMA, NDEA, NMBA, NIPEA, NMPA) have actually been detected in drug substances and drug product.

Accuracy and Recovery

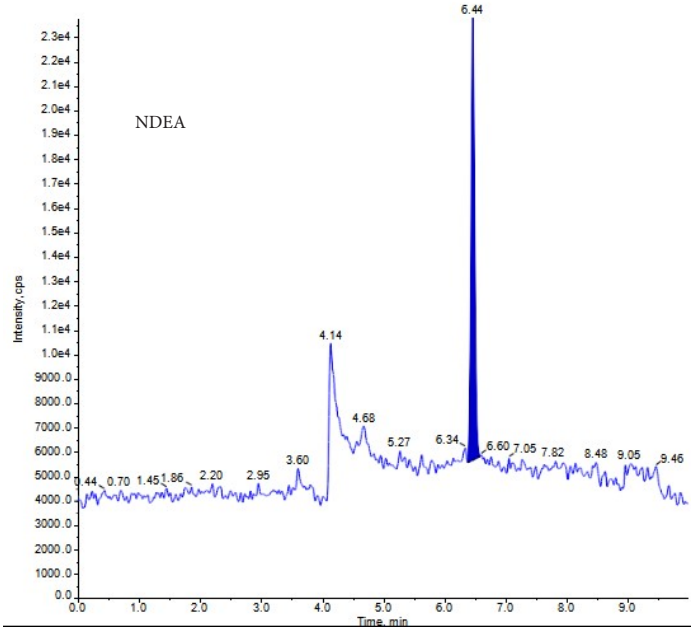
By creating sample (i.e., spiking on test solution with each contaminant) at the level of LOQ, 50%, 100% and 200% of target concentration, the test method's accuracy was examined. With the exception of the LOQ and 200% levels, the accuracy samples were made in replicate of three for each level (six preparations). Obtained recovery values were discussed in Table 6. Individual %recovery of each impurity should be from 70.0 to 130.0, indicating good accuracy of the method.

Stability of Analytical Solutions

Prepared standard solutions and spiked test solution (impurity at specification level) and kept them on bench top. Injected the solutions into the chromatographic system at time points up to day-1. Calculated the ppm of each impurity in spiked sample and similarity factor for standard preparation at initial and day-1 as per the test method against the fresh prepared standard solution. The solutions under study were found to be consistent for up to one day on the bench top, according to the data that were collected.

Table 6: Results for accuracy from LOQ to 200% level.

Name	% Recovery						
	NDEA	NDIPA	NEIPA	NMPA	NDBA	NDMA	NMBA
LOQ level-1	107.9	98.5	105.0	100.0	107.8	103.2	99.2
LOQ level-2	117.5	97.0	101.7	96.7	117.2	99.2	98.1
LOQ level-3	104.8	101.5	105.0	121.7	112.5	100.4	100.4
LOQ level-4	104.8	110.6	103.3	86.7	103.1	106.7	96.9
LOQ level-5	107.9	106.1	106.7	108.3	101.6	114.3	98.8
LOQ level-6	104.8	95.5	101.7	116.7	98.4	102.0	99.6
50% level-1	100.0	95.5	104.0	98.0	101.9	108.1	100.9
50% level-2	121.0	95.5	101.0	97.0	105.6	100.7	101.6
50% level-3	108.6	99.1	101.0	103.0	94.4	95.0	99.1
100% level-1	101.0	99.1	99.0	97.5	95.3	98.9	99.0
100% level-2	106.2	96.8	97.0	103.5	98.6	104.9	101.0
100% level-3	105.2	95.9	99.5	100.0	95.8	110.6	100.8
200% level-1	103.1	94.4	99.0	98.0	97.7	103.0	97.8
200% level-2	98.3	96.9	98.0	102.0	97.4	102.1	99.8
200% level-3	102.6	95.7	97.0	103.5	95.3	102.5	97.4
200% level-4	106.4	98.2	98.3	102.2	97.2	104.7	98.8
200% level-5	100.5	94.8	96.3	97.5	96.5	105.1	96.6
200% level-6	101.7	93.5	99.0	98.3	99.8	106.6	99.0



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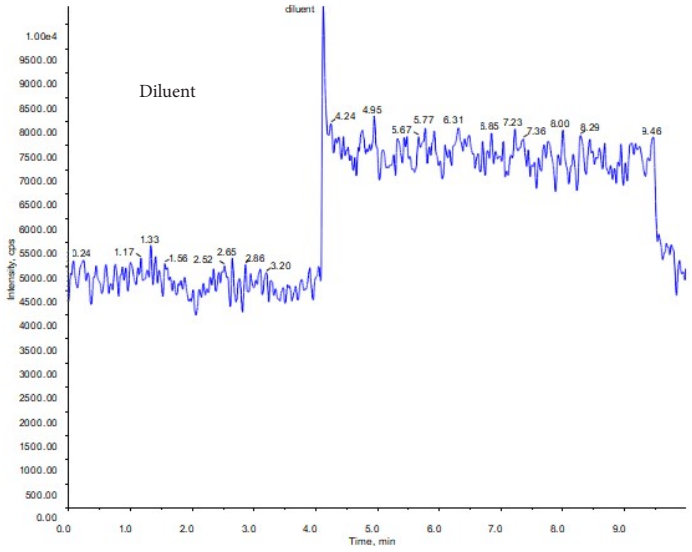

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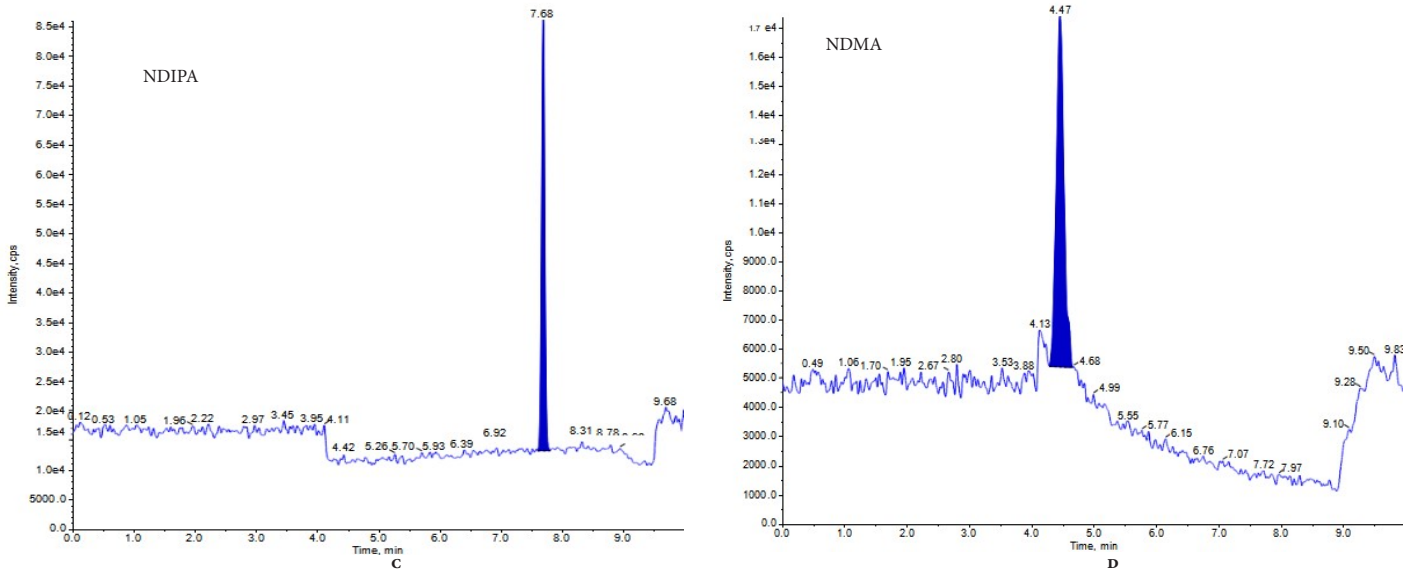
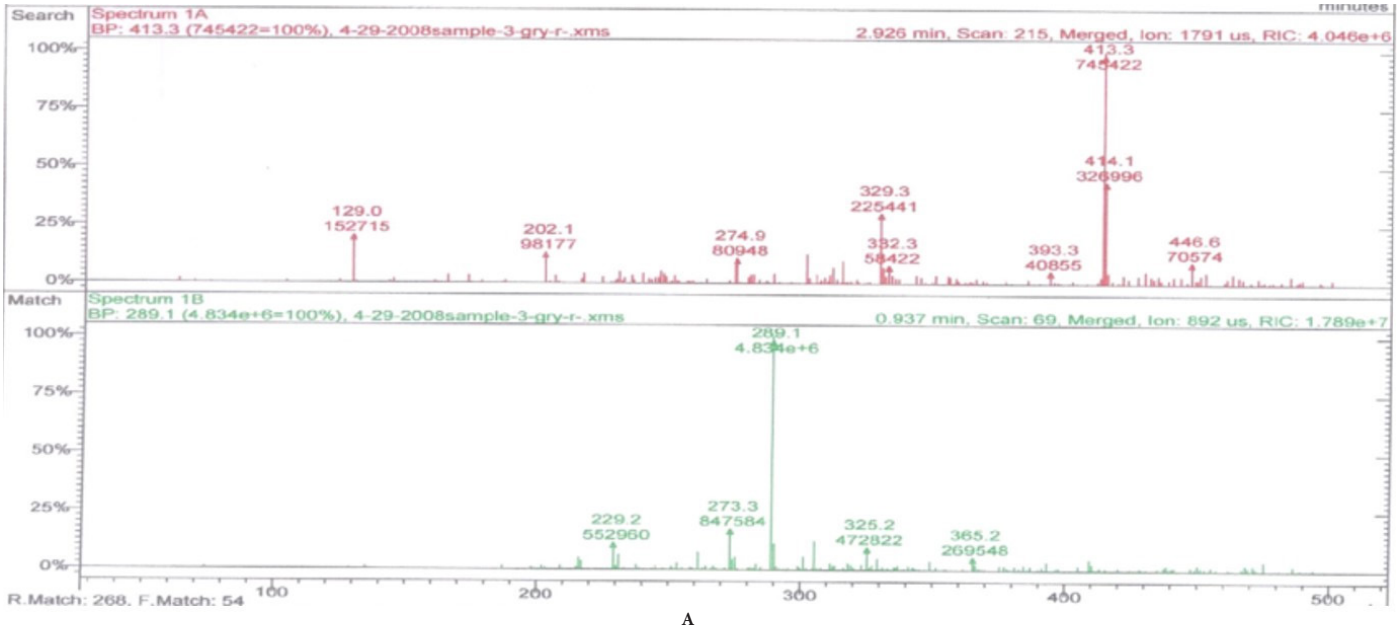
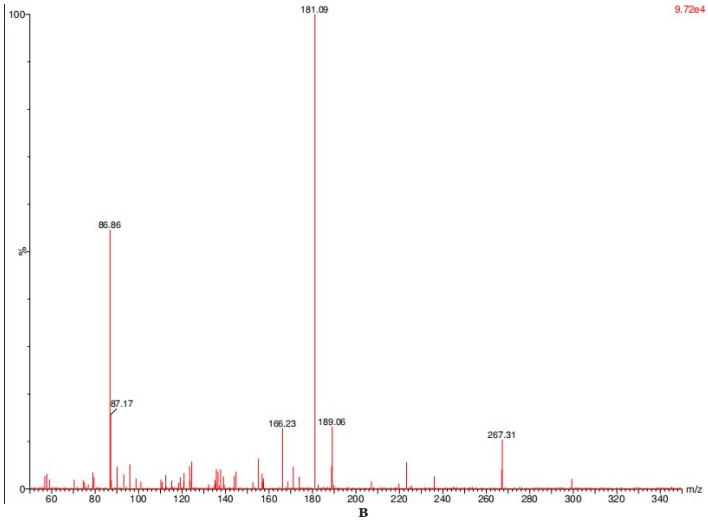


Figure 4: Chromatograms of individual nitrosamine impurity and no interference in diluent.





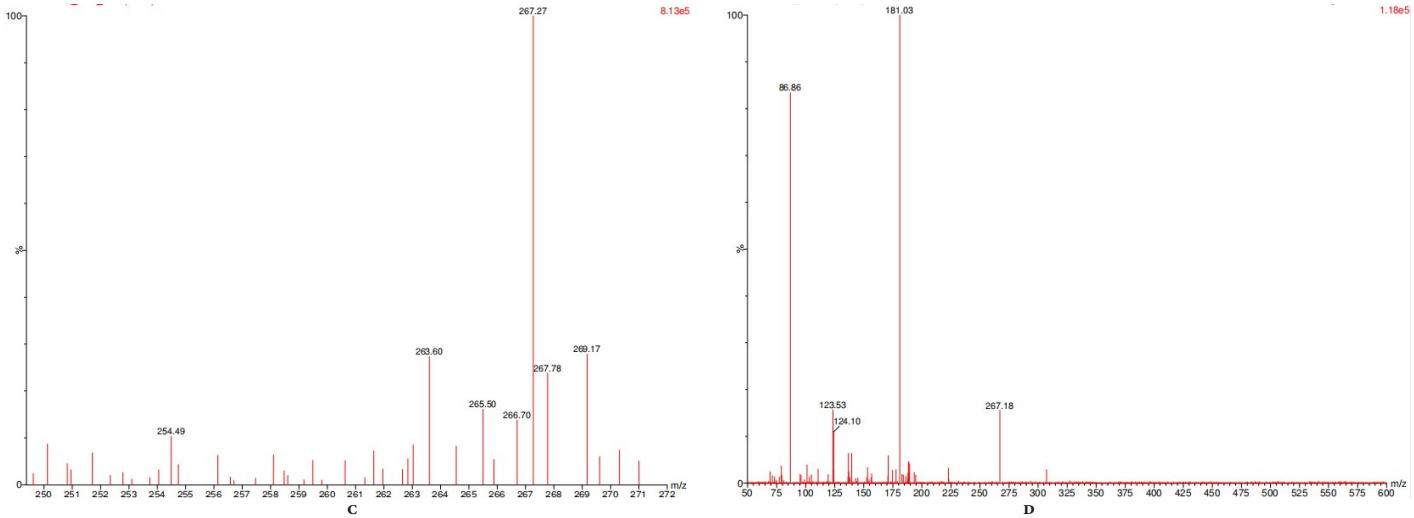


Figure 5: Tandem Mass spectrum of Doxofylline API with no detected *m/z* of nitrosamines.

CONCLUSION

According to ICH Q2 (R1) criteria, the analytical method validation for LC-MS/MS measurement of Nitrosamine impurities in Doxofylline was completed. The approach is specific, exact, linear and accurate for quantifying Nitrosamine impurities in Doxofylline, according to the data obtained. The aforementioned information proved that certain batches of Doxofylline API did not include the nitrosamine impurities (NDEA, NDIPA, NEIPA, NMPA, NDBA, NDMA, and NMBA). The current LC-MS/MS method provides a strong foundation for extending the analysis to encompass the detection of leachables and extractables during Doxofylline storage.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

mL: Millilitre; **g:** Gram; **mg:** Milligram; **µg:** Microgram; **NMT:** Not more than; **RSD:** Relative standard deviation; **VF:** Volumetric flask; **LC-MS/MS:** Liquid chromatography tandem mass spectrometry; **NDMA:** N-Nitroso dimethyl amine; **NDEA:** N-Nitroso diethyl amine; **NDIPA:** N-Nitrosodiisopropylamine; **NEIPA:** N-Nitrosoethylisopropylamine; **NMBA:** N-Nitroso-N-methy l-4-aminobutyric acid; **NMPA:** N-Nitrosomethylphenylamine; **NDBA:** N-Nitrosodi-n-butylamine; **APCI:** Atomspheric Pressure Chemical Ionization; **FDA:** Food and Drug Administration; **API:** Active Pharmaceutical Ingredient; **S/N:** Signal to Noise ratio; **COPD:** Chronic Obstructive Pulmonary Disease.

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