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LC-MS/MS METHOD FOR THE DETERMINATION AND QUANTIFICATION OF A N-NITROSO-DORZOLAMIDE IN THE DORZOLAMIDE HYDROCHLORIDE ACTIVE PHARMACEUTICAL INGREDIENT

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ABSTRACT

Background: Aim of the present study to developed and validate an UPLC-MS/MS method for the quantification of N-nitroso-Dorzolamide in Dorzolamide Hydrochloride drug substance. **Results:** This technique involves chromatographic separation with a Phenyl Hexyl column with 0.05 % ammonia solution in water as mobile phase A and equal acetonitrile as mobile phase B in gradient elution mode at a 0.40 ml/ min flow rate. Quantification of Nitrosamine impurity was carried out using triple quadrupole mass detection with electrospray ionization in the multiple reaction monitoring mode. The validation experiments involve the demonstration of system suitability, specificity, limit of detection, quantitation, linearity, precision and accuracy experiments. The results obtained were found to be within the acceptable limit. **Conclusions:** The proposed method is simple quality control tool for the identification and quantification of N-nitroso-Dorzolamide at low level in Dorzolamide Hydrochloride drug substance.

KEYWORDS: Nitrosamine, N-nitroso-Dorzolamide, LCMS/MS, Method Validation, Dorzolamide Hydrochloride.

INTRODUCTION

Over the past almost six years issue related to Nitrosamine impurities[1] found to be challenging task for the overall pharmaceutical industry and regulatory agencies. The issue first arose in the industry when US Food and drug Administatation (FDA) and European Medicines agency (EMA) evaluated the nitrosamine impurities in certain "sartan" family active pharmaceuticals ingredient.[2,3] The initial assessment in 2018 was limited to the formation of Nitrosamine impurities mainly due to the reagent and solvents uses in the manufacturing process containing the secondary amine in presence of sodium nitrite/nitrate in acidic condition as the key ingredients to form the concern Nitrosamine impurities.

In next phase of the guidance (FDA & EMA), N-nitrosamine precursors of secondary or tertiary amines from starting materials, intermediates and Active pharmaceutical ingredients itself should also be considered comprehensively in the risk assessment for the possible formation of nitrosamine impurities. FDA and EMA first communicated the possible presence of Nitrosamine drug substances related impurities (NDSRI) in November 2021.

Dorzolamide hydrochloride[4,5] (trade name Trusopt), (4S,6S)-4-ethylamino-5,6-dihydro-6-methyl-4H-thieno(2,3-b)thiopyran-2-sulfonamide 7,7-dioxide monohydrochloride (Figure 1), is a carbonic anhydrase inhibitor used in eye drops to treat increased pressure in the eye caused by open-angle glaucoma and to treat a condition called hypertension of the eye. It is an active pharmaceutical ingredient of an approved drug by EMA[6] and USP.[7]

As per the latest nitrosamine guidelines of EMA[8] and USFDA[9] N-nitroso-Dorzolamide (Fig. 1) was reported with the acceptable intakes established for N-nitrosamines under the Carcinogenic Potency Categorization Approach (CPCA) with maximum allowable acceptable intake (AI) of 1500ng/day and 100ng/day. As per the literature the Dorzolamide Hydrochloride used as an ophthalmic solution in finished dosage form with maximum daily dose ranging from 2mg/day to 7mg/day. Based on the maximum daily dose, N-nitroso-Dorzolamide needs to control at 214.29 ppm in Dorzolamide Hydrochloride. In the present study the quantification of the N-nitroso- Dorzolamide was performed at stringent limit i.e. 2.57 ppm considering the stringent acceptable intake (AI) of 18 ng/day.

Herein, we would like to present the development of an LC-MS/MS method for the determination of N-nitroso-Dorzolamide in Dorzolamide Hydrochloride followed by the validation of the method with respect to the specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, repeatability, accuracy and robustness, in accordance with ICH guidance.

METHODS

Chemicals and reagents

Reagents used were of LCMS grade with the highest purity. Acetonitrile was purchased from J.T Baker (USA). The ammonium solution and acetic acid as eluent additive for LC-MS was purchased from SIGMA-ALDRICH. LCMS grade water was used throughout the analysis from J.T baker. Dorzolamide Hydrochloride and N-nitroso-Dorzolamide were synthesized and analyzed at FDC LTD Pharm., India.

Mobile phase preparation

Mobile phase solution A was prepared by adding 0.05 % of ammonia solution in water. Adjust the pH 3.5 with diluted Acetic acid. Acetonitrile was used as mobile phase solution B. Both mobile phase solutions were degassed and stored for further use at ambient temperature. The mobile phase were prepared before each series of analyses.

Preparation of sample and standard solutions

A stock mixture of N-nitroso-Dorzolamide (0.1 mg mL^{-1}) was prepared by dissolving suitable amounts of N-nitroso-Dorzolamide in 0.05% of ammonia in water, adjusted pH 7.4 with Acetic acid as the diluent. From this solution, the diluted stock solution of 250 ng mL^{-1} was prepared by dilution with diluent. A series of calibration standards were prepared by diluting a 250 ng mL^{-1} solution to obtain the following final concentrations of 0.7, 1.2, 2.0, 2.5, 3.0 and 3.7 ng mL^{-1}. Solutions for recovery determination were prepared by accurately weighing 10.0 mg of Dorzolamide Hydrochloride in 10 mL volumetric flasks (in triplicate). By adding an appropriate amount of N-nitroso-Dorzolamide to the Dorzolamide Hydrochloride solutions, 0.7 ng mL^{-1} (0.0007 ppm), 1.2 ng mL^{-1} (0.0012 ppm), 2.5 ng mL^{-1} (0.0025 ppm) and 3.7 ng mL^{-1} (0.0037 ppm) solutions of N-nitroso-Dorzolamide with respect to 1 mg mL^{-1} Dorzolamide Hydrochloride were prepared. All solutions prepared for analytical recovery were prepared in triplicate to ensure repeatability.

Chromatographic conditions of LC-MS/MS

Chromatographic analysis was performed on Nexa Shimadzu UPLC system equipped with a binary pump and an autosampler and Sciex 5500+ LC-MS/MS Triple Quad with an electrospray ionization interface. The analytical column used in the LCMS/MS study was Phenyl Hexyl (100 x 4.6 mm, 2.6 µm) (Phenomenex, USA) employed in gradient mode using 0.05% ammonia in water (pH 3.5) as mobile phase A and acetonitrile as mobile phase B at a flow rate of 0.4 mL min^{-1}. The column oven temperature was maintained at 30°C. The sample injection volume was 20.0 µL. The autosampler temperature was set at 20°C. The LC gradient program (time/% solution A) was set as follows: 0.00/80, 3.0/80, 7.0/5.0, 15.0/5.0, 16.0/80.0 and 20.0/80.0. The negative electrospray ionization (ESI) probe was operated in MRM mode for the quantification of N-Nitroso Dorzolamide in the form of protonated ions $(M-H)^-$ at m/z 352.0 > 216.0.

The different voltage i.e. declustering potential (DP), entrance potential and collision exit cell potential was maintained at 100 V, 10 V and 10.5 V respectively. The ion spray voltage (V) was maintained at 2500 V. The curtain gas flow, ion source gas (GS1) and ion source gas (GS2) pressure was maintained at 50, 35 psi respectively. All parameters of LC and MS were controlled using Sciex Analyst version 1.7.3.

Method validation

The developed method was successfully validated in terms of specificity, repeatability, linearity, accuracy, LOD, LOQ, robustness and solution stability. Method validation for N-nitroso-Dorzolamide in Dorzolamide Hydrochloride was conducted following ICH guidelines. Initially, individual solutions of N-nitroso-Dorzolamide were prepared of 2.5 ng mL^{-1}, 0.0025 ppm with respect to 1.0 mg mL^{-1} Dorzolamide Hydrochloride and their S/N ratios were calculated. The repeatability at the determined LOD and LOQ values was verified experimentally by injecting the same solutions six times. Next, the linearity of the method was evaluated from six concentration levels between the LOQ and a 3.7 ng mL^{-1} impurity concentration. Linear regression analysis was employed to calculate the slope, intercept, and regression coefficient values. The specificity of the developed method was assessed with Dorzolamide Hydrochloride. Next, the accuracy of the method was calculated in triplicate at LOQ, 50%, 100% and 150% concentration level by the standard addition method. The recoveries and RSD values were calculated for the N-nitroso-Dorzolamide in Dorzolamide Hydrochloride. The robustness of the method was tested by altering the mobile phase flow rate and column temperature. Further, the analysis of the sample solution at different intervals of time was compared against fresh samples to evaluate the stability of impurity in the sample solution.

RESULTS AND DISCUSSION

Method development

This study was conducted to develop LC-MS/MS method that can separate and quantify N-nitroso-Dorzolamide in the Dorzolamide Hydrochloride active pharmaceutical ingredient. A few columns were tested to obtain the most appropriate peak shape and separation. The peaks due to N-nitroso-Dorzolamide and API found closely eluting when using the CEH C18 column, and poor peak shapes were observed when using a BEH and HSS column. A Phenomenex, Phenyl Hexyl (100 x 4.6 mm, 2.6 µm) column was found to be the most suitable

regarding both peak shape and separation, as well as the response of analytes. The mobile phase was operated in gradient mode using 0.05% ammonia, adjust the pH 3.5 with diluted Acetic acid as mobile phase A and acetonitrile as mobile phase B. The flow rate of the mobile phase was maintained at 0.40 mL min^{-1}, with the column temperature set at 30 °C. The autosampler temperature was set at 30 °C. The retention times of N-nitroso-Dorzolamide were observed to be 10.33 min and the peak corresponding to Dorzolamide Hydrochloride was eluted at 2.78 min. The chromatogram of blank and standard solution of N-nitroso-Dorzolamide is given in Fig. 2.

Operating conditions of LC-MS/MS

The optimization of mass spectrometric ionization was aimed at developing a simple, rapid, sensitive, and stable analytical method for the determination of N-nitroso-Dorzolamide in the Dorzolamide Hydrochloride API. Method development was carried out using LC-MS/MS for the detection and quantification of N-nitroso-Dorzolamide at a concentration level of 1 µg mL^{-1}. During the early stages of method development, the signal intensity in the negative mode was found to be much higher than that in the positive mode, limiting the method development to the negative (-) ESI source. Optimization of the method on the ESI mode for the determination of N-nitroso-Dorzolamide, fragmentation was carried out using different declustering potential, entrance potential and collision potential. The ion source parameters such as ion spray and collision gas were optimized to obtain a good response for the qualifier and target ions.

Method validation

The optimized LC-MS/MS method was successfully validated in accordance with the ICH guidelines. Method validation was carried out in terms of its adequate selectivity, linearity, LOD and LOQ, accuracy, repeatability, recovery, and robustness.

Specificity

N-nitroso-Dorzolamide solution was prepared at the specification level in the diluent. The spiked Dorzolamide Hydrochloride solution was then subjected to LC-MS/MS analysis and the results revealed that there was no interference of the Dorzolamide Hydrochloride peak with N-nitroso-Dorzolamide peaks, and hence the specificity (Table 1) of the developed method was proven.

Determination of LOD and LOQ values

The LOD and LOQ values (Table 2) of N-nitroso-Dorzolamide were determined based on S/N ratios of 3.0 and 10 by injecting standard solutions of known concentrations. The repeatability at the LOD and LOQ value was also calculated by analysing six replicate injections of N-nitroso-Dorzolamide impurity and calculating their RSD% values. The chromatograms of solutions of N-nitroso-Dorzolamide impurity with concentrations of LOQ and LOD shown in Fig. 2.

Linearity

The linearity of the method was established over the concentration range from 0.7 to 3.7 ng mL^{-1} (0.0007-0.0037 ppm) for N-nitroso-Dorzolamide. The slope, intercept, and correlation coefficient values were derived from the least squares linear regression analysis of the average peak area versus the concentration of analyte. A good correlation between the peak area and concentration of analytes was obtained, as can be seen in Table 2.

Accuracy and recovery

Accuracy as recovery study performed for the N-nitroso-Dorzolamide impurity from LOQ level (0.0007 ppm), 50% (0.0012 ppm), 100% (0.0025 ppm) and 150% (0.0037 ppm) with respect to test concentration of Dorzolamide Hydrochloride bulk sample. The acceptance criterion for recovery was set at 70-130% along with relative standard deviation of NMT 10% for each level. The percentage recoveries for N-nitroso-Dorzolamide are presented in Table 3.

Robustness

The robustness of the method evaluated with deliberated change in the optimized parameters such as flow rate, mobile phase pH and column oven temperature. The optimized parameters such as flow rate (0.40 mL min^{-1}), pH (3.5) and column oven temperature (30 °C). The optimized flow rate, pH and column oven temperature was 0.40 mL min-1, 3.5 and 30 °C. The parameters are altered in the range of change 0.37 to 0.43 mL min.-1, 3.0 to 4.0, and 27 °C and 33 °C receptively. The data obtained confirms that these deliberately changed chromatographic conditions did not impact the quantification of N-nitroso-Dorzolamide impurity in spiked samples showing the robustness of the method.

Repeatability and Solution stability

Repeatability of the method confirmed by the spiked study with N-nitroso-Dorzolamide impurity at standard concentration level in six individual preparation in Dorzolamide Hydrochloride. The results obtained are well within the acceptance criterion (Table 4). The study of the solution stability of Dorzolamide Hydrochloride and N-nitroso-Dorzolamide was carried out by leaving spiked and unspiked sample solutions in firmly capped LC vials at 20 °C for about 24 h in an autosampler. The concentration of N-nitroso-Dorzolamide was determined against freshly prepared standard solutions and no significant changes were observed in the concentration for N-nitroso-Dorzolamide. Data obtained confirmed the stability of impurity in the sample solution for 24 h.

Table 1: System suitability criteria.

Component	Retention time (min)	Relative retention time (min)
Dorzolamide Hydrochloride	1.48	1.00
N-nitroso-Dorzolamide (NDRZ)	3.87	2.61

Table 2: LOD, LOQ and Linearity results.

Validation parameter	Results
LOD-LOQ	
LOD (ng mL^{-1})	0.35
LOQ (ng mL^{-1})	0.70
Precision at LOQ (%RSD)	0.61
Linearity	
Regression (r)	0.9990
Calibration range (ng/mL^{-1})	0.70-3.70
Slope	663150312
Intercept	+68059.15
% Intercept	3.88

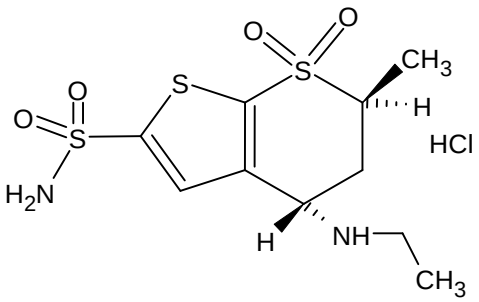

Fig. 1: Chemical structure of Dorzolamide Hydrochloride.

Table 3: Accuracy (Recovery) results of N-nitroso-Dorzolamide in bulk sample

Accuracy Level	Mean Recovery (%)	% RSD
LOQ	80.05	4.72
50 %	83.22	3.86
100 %	82.15	0.63
150 %	82.54	0.97

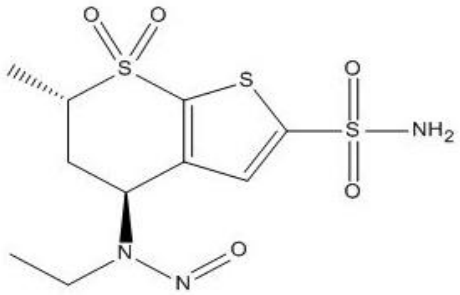

Fig. 1: Chemical structure of N-nitroso-Dorzolamide.

Table 4: Precision results of N-nitroso-Dorzolamide.

Precision	% RSD
System precision	1.77
Method precision	2.15
Intermediate precision	2.33

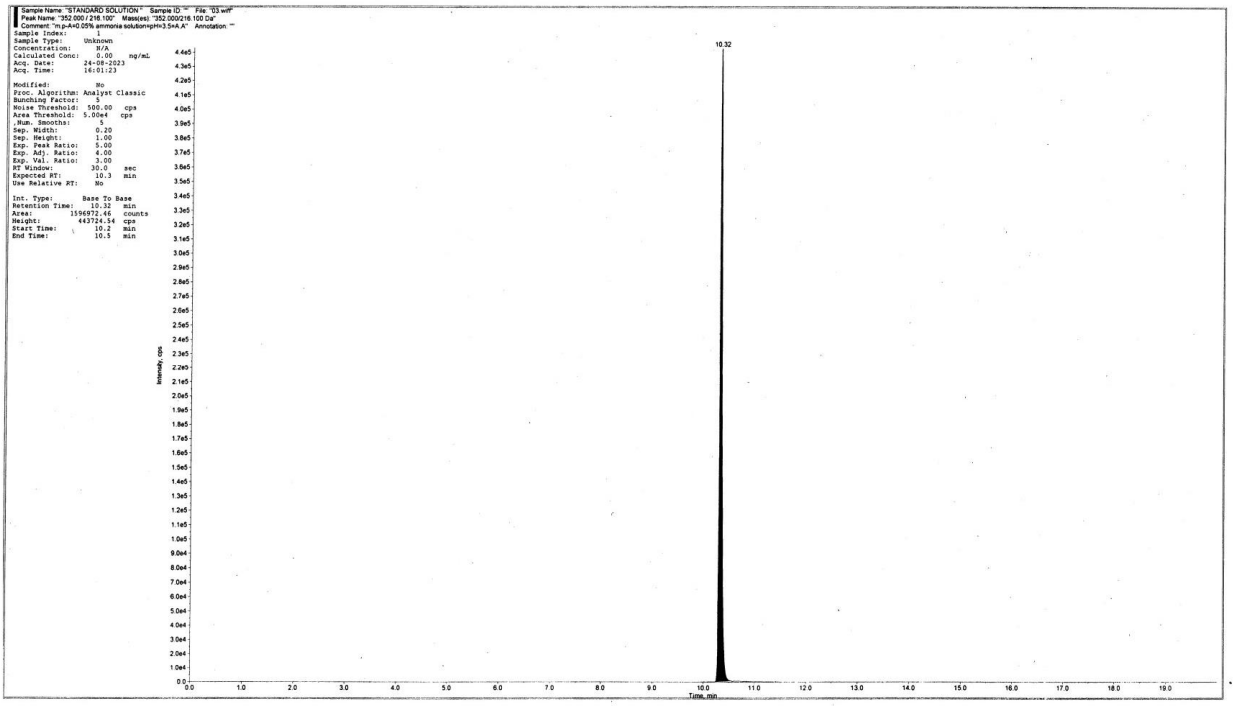

Fig. 2 A typical LCMS/MS chromatogram of Standard solution

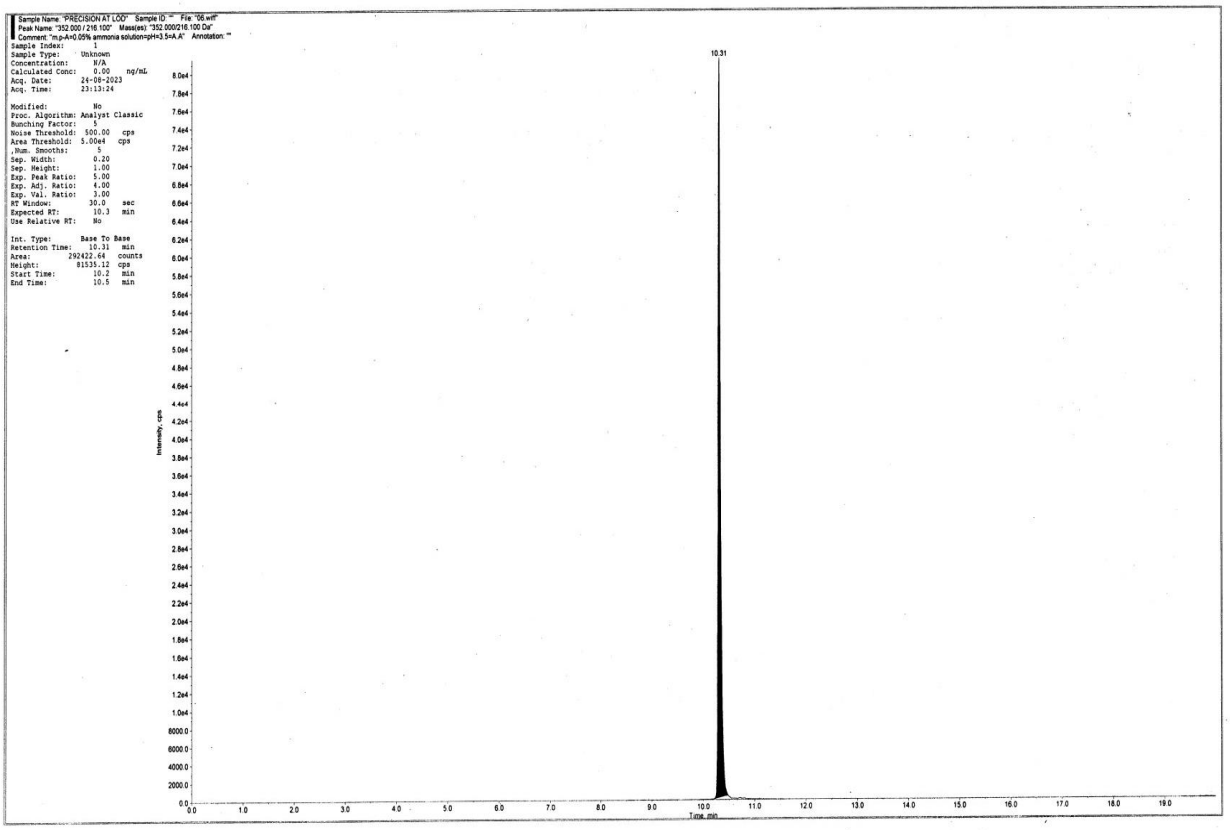

Fig.2 A typical LCMS/MS chromatogram of Limit of detection (LOD)

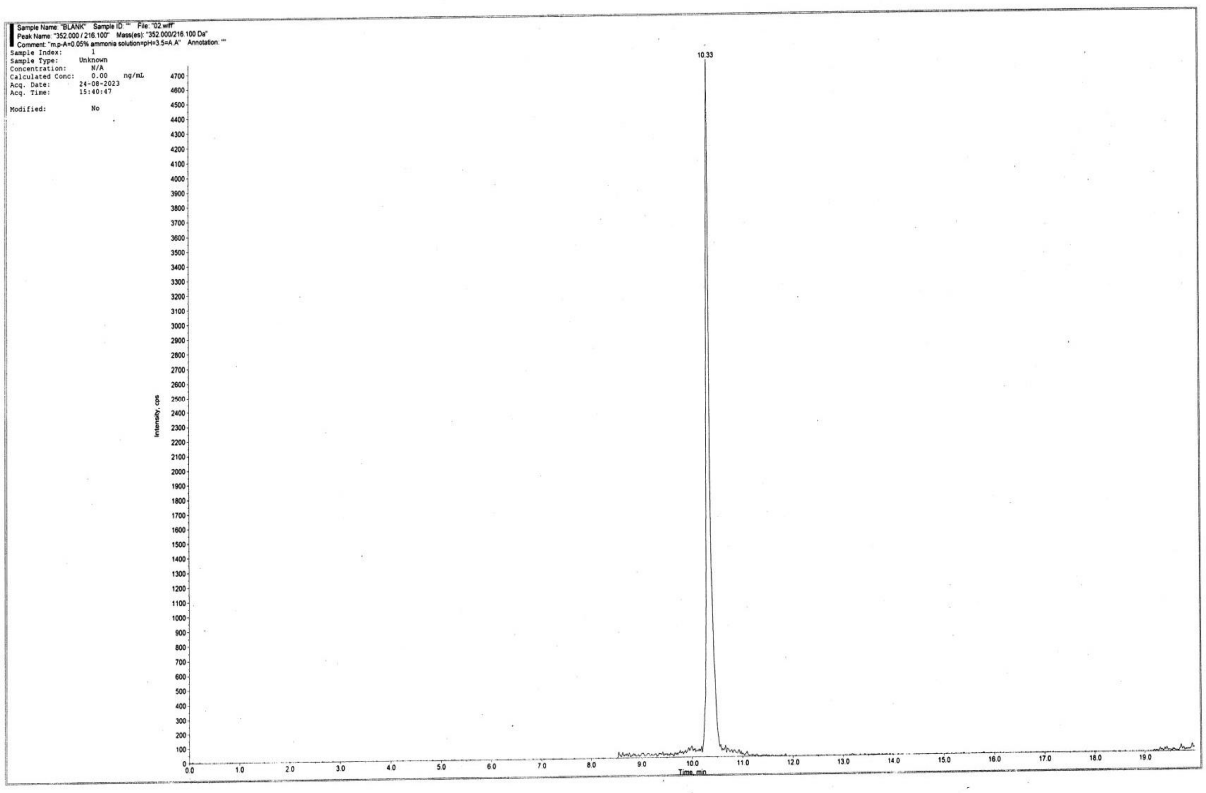

Fig.2 A typical LCMS/MS chromatogram of Blank

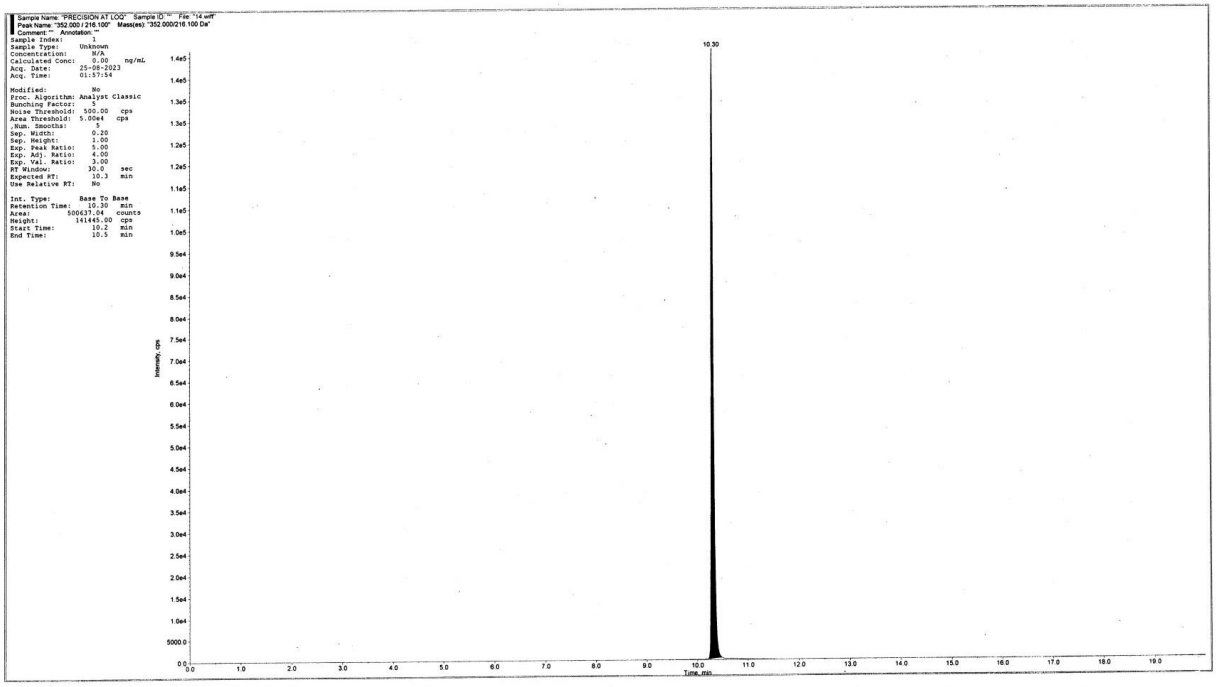

Fig.2 A typical LCMS/MS chromatogram of Limit of detection (LOD)

CONCLUSIONS

The study defined the LC-MS/MS method for the quantification of N-Nitroso-Dorzolamide impurity in Dorzolamide Hydrochloride in negative ionization mode with multiple reaction monitoring (MRM). The method was validated as per ICH guidance and found to be specific and linear over the specified concentration range. The determined LOD and LOQ values for N-nitroso-Dorzolamide were set very low and lie within the acceptable range. The method was fully validated and presents good linearity, accuracy, repeatability, and robustness. The sample prepared in the analytical solution was found to be stable for at least 24 h. The method could be very useful for the determination of N-nitroso-Dorzolamide in Dorzolamide Hydrochloride during its manufacture and product release for the bulk i.e. API and in finished formulation ophthalmic solutions.

Conflicts of interest

There are no conflicts to declare.

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