



DETERMINATION AND QUANTIFICATION OF A 1-BENZHYDRYL-4-NITROSOPIPERAZINE IN THE CINNARIZINE ACTIVE PHARMACEUTICAL INGREDIENT BY LC-MS/MS

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ABSTRACT

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the quantification of 1-benzhydryl-4-nitrosopiperazine in the Cinnarizine active pharmaceutical ingredient. Chromatographic separation was achieved using a Waters Acquity HSS T3 C18 column, with 0.1% formic acid in water as mobile phase A and acetonitrile as mobile phase B in gradient elution mode at a 0.25 ml/min flow rate. Quantification of impurities was carried out using triple quadrupole mass detection with electrospray ionization in the multiple reaction monitoring mode. The method was validated with good linearity over the concentration range of 0.02-0.30 ppm of the Cinnarizine test concentration for 1-benzhydryl-4-nitrosopiperazine. The correlation coefficient obtained in each case was >0.9990. The recoveries were found to be satisfactory over the range between 80.0 and 120.0 % for 1-benzhydryl-4-nitrosopiperazine. The developed method was able to quantitate 1-benzhydryl-4-nitrosopiperazine at a concentration level of 0.6 ng mL⁻¹ (0.02ppm with respect to 30 mg mL⁻¹ Cinnarizine).

KEYWORDS: Nitrosamine, 1-benzhydryl-4-nitrosopiperazine, LCMS/MS, Validation, Cinnarizine.

1.0 INTRODUCTION

N-nitroso compounds have been listed as one of the cohorts of concern as per the ICH M7 guidance^[1] and are internationally considered as a class of strong carcinogens as per the International Agency for Research on Cancer.^[2] The issue of Nitrosamine impurities first reported in the industry in 2018 by FDA and EMA in certain class of drugs specifically sartan^[3,4] series of active pharmaceutical ingredients. From the initial phase of nitrosamine impurities assessment formed due to reagent and solvents, in recent years regulatory agencies has shifted the focused from common nitrosamine impurities to nitrosamine impurities of drug products. Thus n-Nitrosamine risk assessment of pharmaceuticals moved from small molecules N-nitrosoamine to Nitrosamine drug substances related impurities (NDSRI).

Cinnarizine^[5] (trade name Stugeron) is an antihistamine and calcium channel blocker of the diphenylmethylpiperazine group. It is an active pharmaceutical ingredient of an approved drug by EMA.^[6] It is prescribed for nausea and vomiting due to motion sickness or other sources such as chemotherapy, vertigo or Meniere's disease. It is usually used in finished formulation as tablet.

As per the route of synthesis of Cinnarizine (Fig.1), 1-benzhydrylpiperazine (Fig.1) was used as one of the key intermediate in the manufacturing process. As a secondary amine, there is a possibility of formation of the N-nitrosamine impurity due to the intermediate 1-benzhydrylpiperazine itself i.e. 1-benzhydryl-4-nitrosopiperazine (Fig.1). As per the latest nitrosamine guidelines" of EMA, if any N-nitrosamines are identified without sufficient substance specific data, to derive a substance specific limit for lifetime exposure as recommended in ICH M7 (R1) guideline, a class specific TTC for nitrosamines of 18 ng/day (derived from the Lhasa carcinogenic potency database) can be used as default option.^[7] Thus based on the maximum daily dose, 1-benzhydryl-4-nitrosopiperazine need to control at 0.20 ppm in Cinnarizine.

We have developed a LC-MS/MS method for the quantification of the 1-benzhydryl-4-nitrosopiperazine as nitrosamine impurity in Cinnarizine active pharmaceutical ingredient. The method was further validated with respect to the specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, repeatability, accuracy and robustness, in accordance with ICH guidance.^[8]

2.0 EXPERIMENTAL

2.1 Reagents and chemicals

LCMS grade Formic acid, Acetonitrile and Methanol procured from Fischer Scientific and J.T. Baker, USA respectively. LCMS grade water used was from J.T. Baker. Sample of Cinnarizine and standard of 1-benzhydryl-4-nitrosopiperazine were synthesized and analysed at FDC LTD Pharm., India.

2.2 Preparation of sample and standard solutions

The stock solution of the 1-benzhydryl-4-nitrosopiperazine and Cinnarizine were prepared individually by dissolving an appropriate amount of the substances in diluent. For quantitation of 1-benzhydryl-4-nitrosopiperazine in Cinnarizine a solution of 6.0 ng mL⁻¹ (0.20 ppm) concentration was used. The target analyte concentration was fixed as 30 mg mL⁻¹.

2.3 Chromatographic conditions of LC-MS/MS

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 Acquity, HSS T3 C18 (50 x 2.1 mm, 1.8 µm) (Waters Co. Ltd, USA) employed in gradient mode using 0.1% formic acid in water as mobile phase A and acetonitrile as mobile phase B at a flow rate of 0.25 mL min⁻¹. The column oven temperature was maintained at 35°C. The sample injection volume was 20.0 µL. The autosampler temperature was set at 15 °C. The LC gradient program (time/% solution A) was set as follows: 0.00/50, 1.0/50, 7.0/20, 10.0/20, 12.0/50 and 15.0/50. The positive electrospray ionization (ESI) probe was operated in MRM mode for the quantification of 1-benzhydryl-4-nitrosopiperazine in the form of protonated ions (M+H)⁺ at m/z 282.10 > 167.20.

The different voltage i.e. declustering potential (DP), entrance potential and collision exit cell potential was maintained at 60 V, 11 V and 20 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 35 psi. All parameters of LC and MS were controlled using Sciex Analyst version 1.7.3.

2.4 Method validation

The developed method was successfully validated as per ICH guidance in terms of specificity, repeatability, linearity, accuracy, Limit of detection, Limit of quantification, robustness and solution stability. The repeatability at the determined Limit of detection and Limit of quantification values was verified experimentally by injecting the same solutions six times. Linearity of the method was evaluated from six concentration levels between the LOQ and 150% level. Calculate the slope, intercept, and regression coefficient values. The specificity of the developed method was assessed with Cinnarizine. Accuracy of the method was

calculated in triplicate at LOQ to 150% concentration level by the standard addition method. The recoveries and RSD values were calculated for the 1-benzhydryl-4-nitrosopiperazine impurity in Cinnarizine. 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.

3.0 RESULTS AND DISCUSSION

3.1 Method development

The aim of the study was to develop selective LC-MS/MS method that can able to quantitate 1-benzhydryl-4-nitrosopiperazine in the Cinnarizine. Columns were tested to obtain the most appropriate peak shape and separation. By using typical CSH C18 column the separation was not desired between the 1-benzhydryl-4-nitrosopiperazine and Cinnarizine. While on BEH and Kinetex peak shape was not up to the marked for the impurity. A Waters Acquity, HSS T3, C18 (50 x 2.1 mm, 1.8 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 0.1% formic acid in water as mobile phase A and acetonitrile as mobile phase B. The flow rate of the mobile phase was maintained at 0.25 mL min⁻¹, with the column temperature set at 35 °C. The autosampler temperature was set at 15 °C. The retention times of 1-benzhydryl-4-nitrosopiperazine were observed to be 3.87 min and the peak corresponding to Cinnarizine was eluted at 1.48 min. The chromatogram of standard solution of 1-benzhydryl-4-nitrosopiperazine is given in the Fig. 2.

3.2 Operating conditions of LC-MS/MS

Initial optimization of the mass parameters for the detection of the 1-benzhydryl-4-nitrosopiperazine was performed at concentration level of 1 µg mL⁻¹. The intensity obtained with electro spray ionization (ESI) in the positive mode was on higher side compare to that of negative mode for the impurity. As a part of optimization in the ESI conditions for 1-benzhydryl-4-nitrosopiperazine, fragmentation was carried out using different collision energy (10, 15, 20, 25 and 30 eV). The ion source parameters such as ion spray and collision gas were optimized to obtain a good response for the ions.

4.0 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.

4.1 Specificity

A single 1-benzhydryl-4-nitrosopiperazine solution was prepared at the specification level in the diluent. The spiked Cinnarizine solution was then subjected to LC-MS/MS analysis and the results revealed that there was

no interference of the Cinnarizine peak with 1-benzhydryl-4-nitrosopiperazine peak, and hence the specificity of the developed method was proven (Table 1).

4.2 Determination of LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) determined the sensitivity of the method. The LOD and LOQ values of 1-benzhydryl-4-nitrosopiperazine was 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 calculated by analysing six replicate injections of 1-benzhydryl-4-nitrosopiperazine and calculating their RSD% values. The chromatograms of solutions of 1-benzhydryl-4-nitrosopiperazine with concentrations of LOD and LOQ shown in Fig. 2 and table 2.

4.3 Linearity

Linearity of the method was studied by using the standard solution of 1-benzhydryl-4-nitrosopiperazine at different concentration level from the limit of quantification (LOQ) to 150% of the impurity. The slope, intercept, and correlation coefficient values were derived from the linear regression analysis of the average peak area versus the concentration of analytes. A good correlation between the peak area and concentration of analytes was obtained, as can be seen in table 2.

4.4 Accuracy (Recovery)

The standard addition and recovery experiments were conducted for the 1-benzhydryl-4-nitrosopiperazine in bulk samples of Cinnarizine in triplicate at LOQ (0.02 ppm), 30% (0.06 ppm), 50% (0.10 ppm), 100% (0.20 ppm) and 150% (0.30 ppm) with respect to test concentration. The acceptance criterion for recovery was set at 80-120%. The percentage recoveries for 1-benzhydryl-4-nitrosopiperazine are presented in Table 3.

4.5 Precision (Repeatability)

The precision of an analytical procedure expresses the closeness of agreement among a series of measurements obtained from multiple samplings of the same homogenous sample under prescribed conditions. The system and method precision for the 1-benzhydryl-4-nitrosopiperazine were checked at its specification level (i.e. 0.20 ppm with respect to analyte concentration, 30.0 mg mL⁻¹). The percentage RSD of method repeatability and system repeatability for the 1-benzhydryl-4-nitrosopiperazine were reported (Table 4) confirms good precision of the method.

4.6 Robustness

The robustness of an analytical procedure is measured by its capability to remain unaffected through small, but deliberate, variations in method parameters and provide an indication of its reliability during normal usage. The optimized flow rate of the mobile phase was 0.25 mL min⁻¹ and the column oven temperature was 35 °C. The both parameters are altered from 0.22 to 0.28 mL min⁻¹

and 32°C and 38°C receptively. The data obtained confirms that these deliberately changed chromatographic conditions did not impact the chromatographic performance for 1-benzhydryl-4-nitrosopiperazine in spiked samples showing the robustness of the method.

4.7 Solution stability

The solution stability of Cinnarizine and 1-benzhydryl-4-nitrosopiperazine was carried out by leaving spiked and unspiked sample solutions in firmly capped LC vials at 15°C for about 24 h in an autosampler. The concentration of 1-benzhydryl-4-nitrosopiperazine was determined against freshly prepared standard solutions and no significant changes were observed in the concentration for 1-benzhydryl-4-nitrosopiperazine. The data confirmed the stability of impurity in the sample solution for at least 24 h.

Table 1: System suitability criteria

Component	Retention time (min)	Relative retention time (min)
Cinnarizine	1.48	1.00
1-benzhydryl-4-nitrosopiperazine	3.87	2.61

Table 2: LOD, LOQ and Linearity results of 1-benzhydryl-4-nitrosopiperazine.

Validation parameter	Results
LOD-LOQ	
LOD (ng mL ⁻¹)	0.30
LOQ (ng mL ⁻¹)	0.60
Precision at LOQ (%RSD)	3.24
Linearity	
Regression (r)	0.9990
Calibration range (ng/mL ⁻¹)	0.60-9.00
Slope	700720547.22
Intercept	-4335.95
% Intercept	-0.10

Table 3: Accuracy (Recovery) results of 1-benzhydryl-4-nitrosopiperazine in bulk sample.

Accuracy Level	Mean Recovery (%)	% RSD
LOQ	108.7	4.95
30 %	96.3	3.11
50 %	100.5	2.98
100 %	97.8	2.62
150 %	103.5	2.34

Table 4: Precision results of 1-benzhydryl-4-nitrosopiperazine.

Precision	% RSD
System precision	1.34
Method precision	3.34
Intermediate precision	4.38

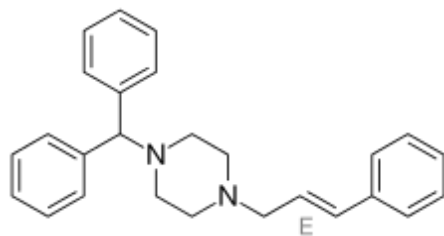


Fig. 1: Chemical structure of Cinnarizine.

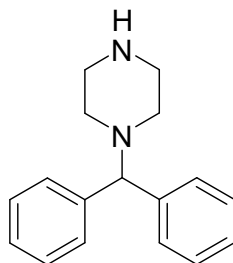


Fig. 1: Chemical structure of 1-benzhydrylpiperazine.

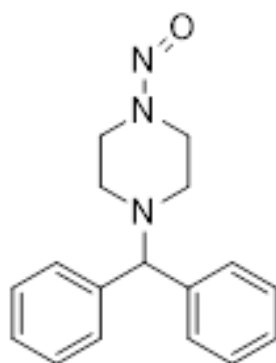


Fig. 1: Chemical structure of 1-benzhydryl-4-nitrosopiperazine.

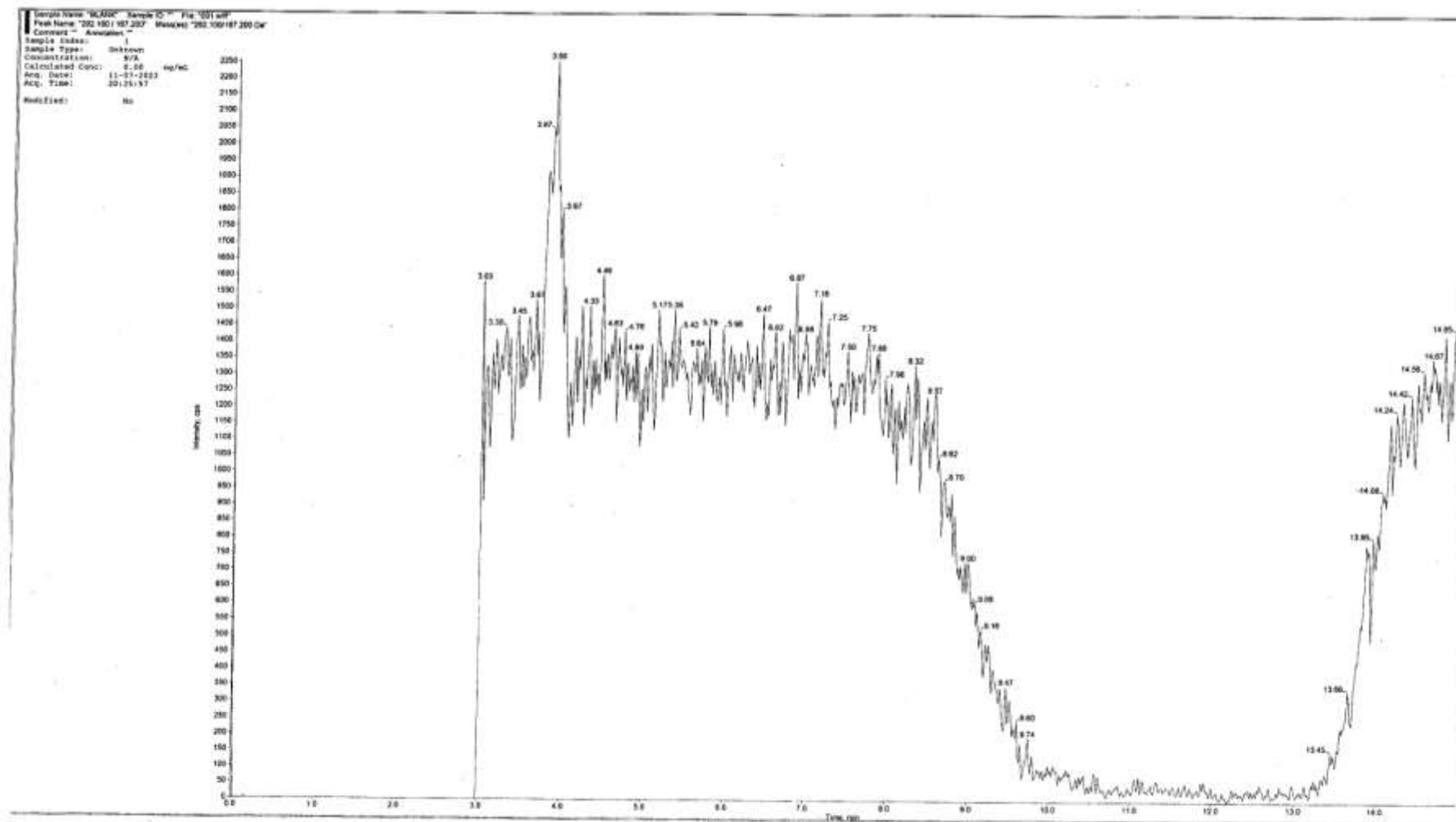


Fig. 1 A typical LCMS/MS chromatogram of Blank.

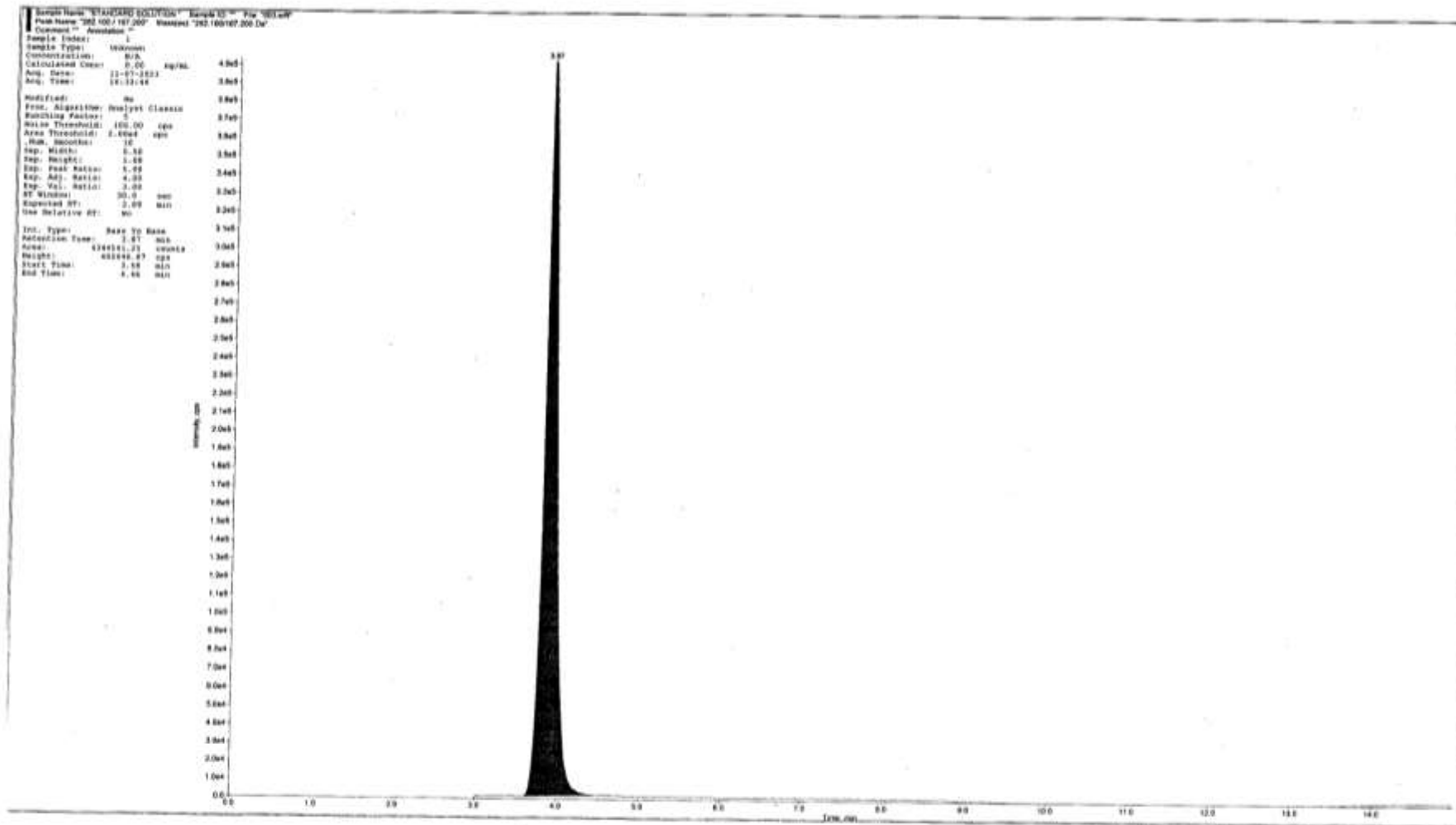


Fig. 2 A typical LCMS/MS chromatogram of Standard solution

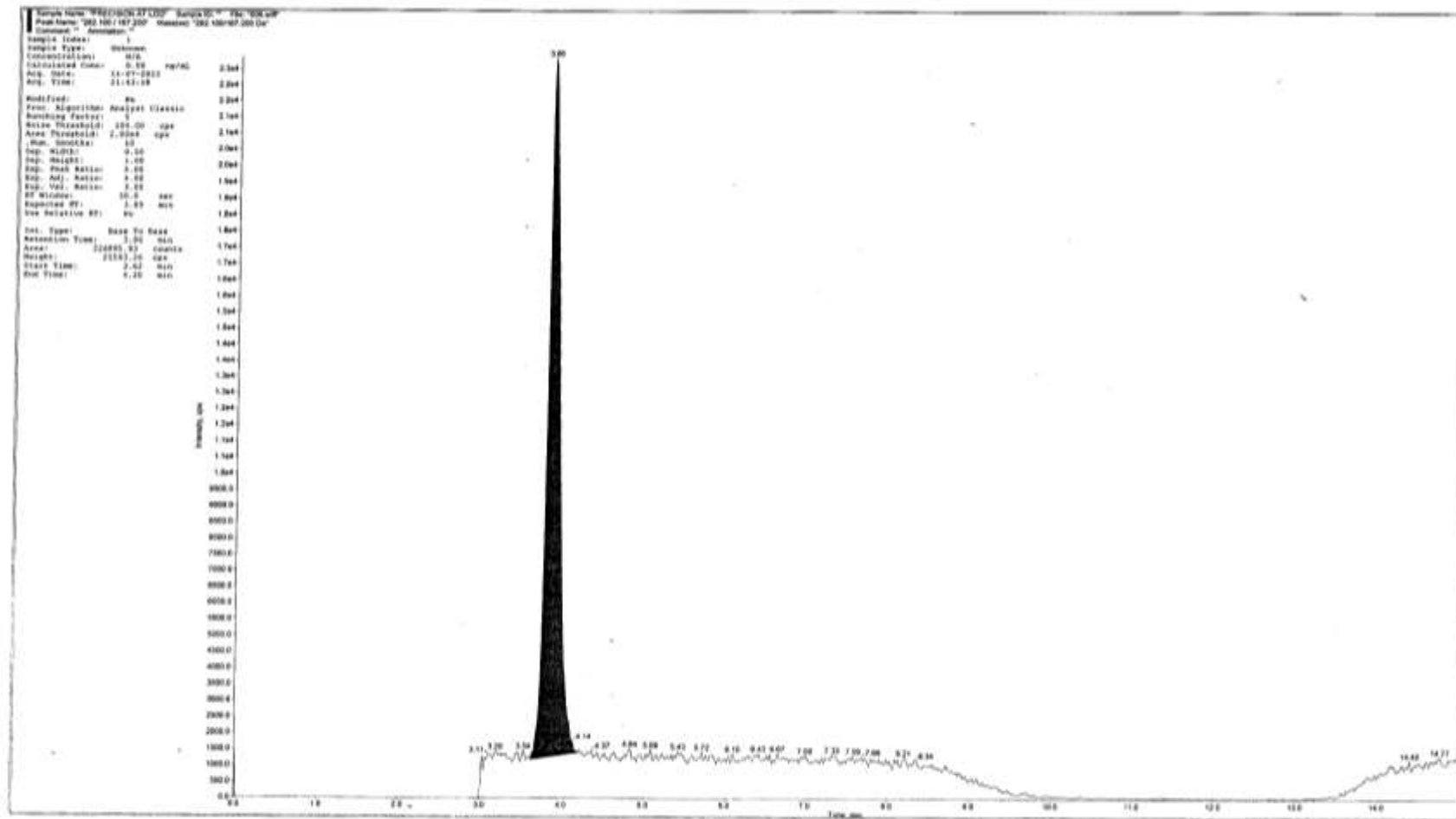


Fig.2 A typical LCMS/MS chromatogram of Limit of detection (LOD)

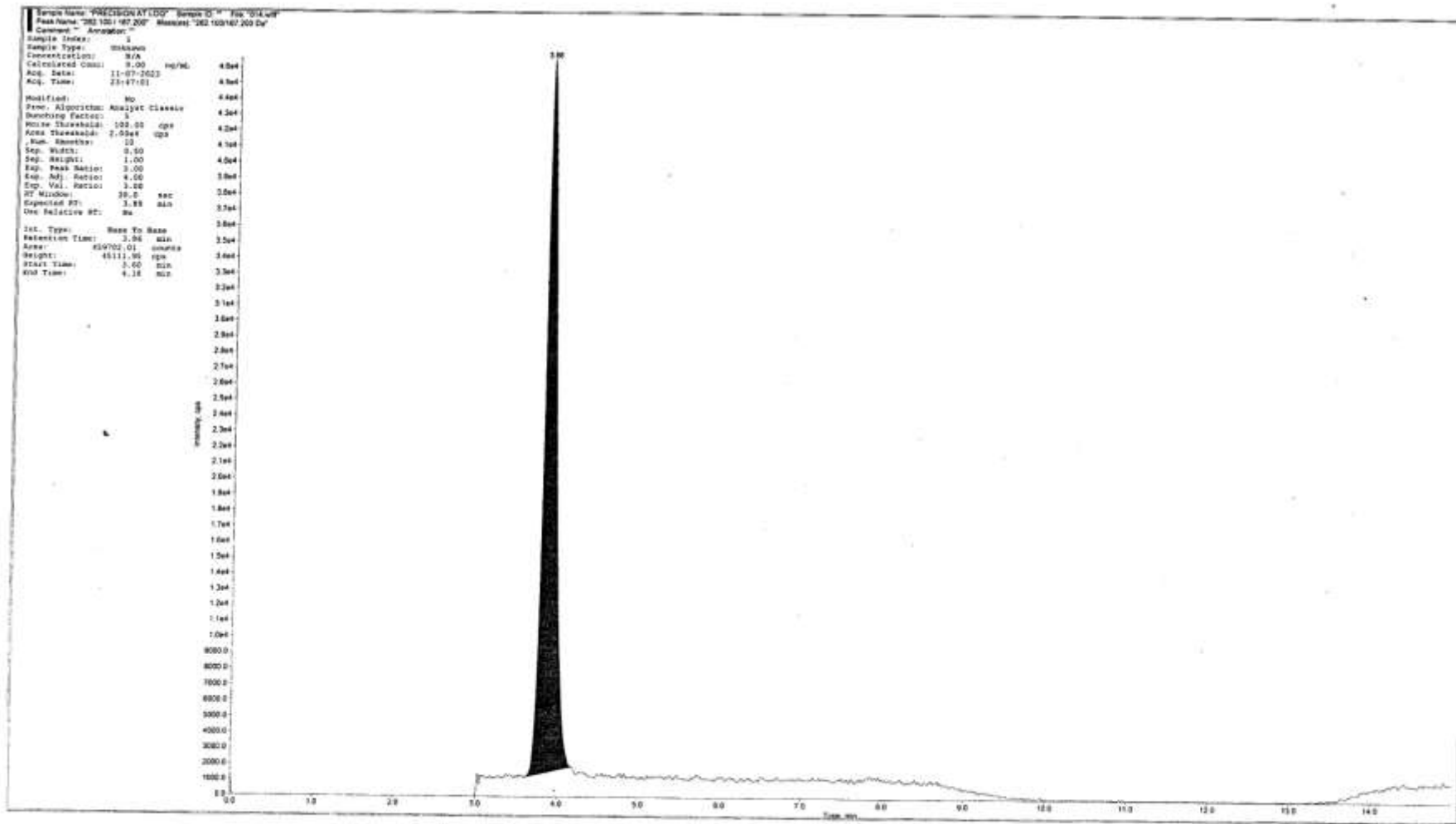


Fig. 2 A typical LCMS/MS chromatogram of Limit of quantitation (LOQ)

5.0 CONCLUSIONS

In this study, we have developed a LC-MS/MS method that is capable of quantifying 1-benzhydryl-4-nitrosopiperazine in Cinnarizine using the positive ionization mode with multiple reaction monitoring (MRM). The method was validated as per ICH recommendations and it was found to be specific and linear over the specified concentration range. The determined LOD and LOQ values for 1-benzhydryl-4-nitrosopiperazine were set very low and well below that of acceptable limit. The sample prepared in the analytical solution was found to be stable for at least 24 h. The method was fully validated and presents good linearity, accuracy, repeatability, and robustness. This method could be very useful for the determination of 1-benzhydryl-4-nitrosopiperazine in Cinnarizine during its manufacture and product release.

6.0 Abbreviations

LC-MS: Liquid chromatography mass spectrometry

UPLC: Ultra performance liquid chromatography

NDSRI: Nitrosamine drug substances related impurities

LOD: Limit of detection

LOQ: Limit of quantification

ESI: Electrospray ionization

MRM: Multiple reaction monitoring

RSD: Relative standard deviation

ICH: The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use

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