EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 11
Issue: 5
200-206
Year: 2024

DETERMINATION AND QUANTIFICATION OF A N-NITROSO-N-METHYLCYCLOHEXYLAMINE IN THE BROMHEXINE HYDROCHLORIDE ACTIVE PHARMACEUTICAL INGREDIENT BY GC-MS/MS

Vinayak T. Vele*, Kishor More, Mohan A. Chandavarkar and Krupali Shah

R & D (API), FDC Ltd., 142-148, S.V. Road, Jogeshwari (W), Mumbai-400102, Maharashtra, India.

***Corresponding Author: Vinayak T. Vele**

R & D (API), FDC Ltd., 142-148, S.V. Road, Jogeshwari (W), Mumbai-400102, Maharashtra, India.

Article Received on 15/03/2024 **Article Revised on 05/04/2024** **Article Accepted on 25/04/2024**

ABSTRACT

A gas chromatography-tandem mass spectrometry (GC-MS/MS) method was developed for the quantification of N-nitroso-N-methylcyclohexylamine in the Bromhexine Hydrochloride active pharmaceutical ingredient. Chromatographic separation was achieved using a DB-1MS column, with Helium as carrier gas at a 2.5 ml/ min flow rate. Quantification of impurity 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 0.037-0.562 ppm for N-nitroso-N-methylcyclohexylamine. The correlation coefficient obtained in each case was >0.9960. The recoveries were found to be satisfactory over the range between 80.0 and 120.0 % for N-nitroso-N-methylcyclohexylamine. The developed method was able to quantitate N-nitroso-N-methylcyclohexylamine at a concentration level of 3.75 ng mL^{-1} (0.00375 ppm with respect to 10 mg mL^{-1} Bromhexine Hydrochloride).

KEYWORDS: Nitrosamine, N-nitroso-N-methylcyclohexylamine, GCMS/MS, Validation, Bromhexine Hydrochloride.

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. As per the initial assessment nitrosamine impurities are basically formed due to reagents, solvents and catalysts used in the manufacturing process. In the recent years regulatory agencies has shifted the focused from common nitrosamine impurities to nitrosamine impurities which are possible in the drug substances due to the key starting material, intermediate and active pharmaceutical ingredient. Thus n-Nitrosamine risk assessment of pharmaceuticals moved from small molecules N-nitrosoamine to Nitrosamine drug substances related impurities (NDSRI).

Bromhexine Hydrochloride[5] (trade name Bisolvon) is a mucolytic drug used in the treatment of respiratory disorders associated with viscid and excessive mucus. It is an active pharmaceutical ingredient of an approved drug by EMA.[6] It is usually used in finished formulation as tablet and syrups.

As per the route of synthesis of Bromhexine Hydrochloride (Fig.1), N-Methyl Cyclohexylamine (Fig.1) was used as one of the key starting material in the manufacturing process. As a secondary amine, there is a possibility of formation of the N-nitrosamine impurity due to the key starting material N-Methyl Cyclohexylamine itself i.e. N-Nitroso-N-Methyl Cyclohexylamine (Fig.1). As per the latest nitrosamine guidelines" of EMA[7] and FDA[8] 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. Thus based on the maximum daily dose, N-Nitroso-N-Methyl Cyclohexylamine need to control at 0.375 ppm in Bromhexine Hydrochloride.

We have developed a GC-MS/MS method for the quantification of the nitrosamine impurity in Bromhexine Hydrochloride 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.[9]

2.0 Experimental

2.1 Reagents and Chemicals

GLCMS grade Iso-octane procured from Fischer Scientific, USA. Sample of Bromhexine Hydrochloride and standard of N-Nitroso-N-Methyl Cyclohexylamine were synthesized and analysed at FDC LTD Pharm., India.

2.2 Preparation of Sample and Standard solutions

The stock solution of the N-Nitroso-N-Methyl Cyclohexylamine and Bromhexine Hydrochloride were prepared individually by dissolving an appropriate amount of the substances in diluent. For quantitation of N-Nitroso-N-Methyl Cyclohexylamine in Bromhexine Hydrochloride a solution of 3.75 ng mL^{-1} (0.00375 ppm) concentration was used. The target analyte concentration was fixed as 10 mg mL^{-1}.

2.3 Chromatographic conditions of GC-MS/MS

Analysis was performed on Agilent GC 8890 system equipped with an auto sampler and Agilent 7010B GC-MS/MS Triple Quad with an electrospray ionization interface. The analytical column used in the study was DB-1MS, 30m x 0.32mm x0.25 µm (Agilent Co. Ltd, USA) employed in with Helium as carrier gas at a flow rate of 2.5 mL min^{-1}. The oven temperature programmed was set as initial 50°C for 1 minute hold. Further ramp rate 15°C/minute up to 320°C with hold for 2 minutes. The injector, MSD transfer line and thermal aux temperature was maintained at 220°C, 220°C and 150°C. The sample injection volume was 3.0 µL in split less mode. The positive electrospray ionization (ESI) probe was operated in MRM mode for the quantification of N-Nitroso-N-Methyl Cyclohexylamine in the form of protonated ions $(M+H)^+$ at m/z 142.2 > 125.1. The collision energy was set at 5 V along with gain that of 25. The electron energy, source temperature and quad temperature was maintained at 70 EV, 230°C and 150°C respectively.

The quench and collision gas as He and Nitrogen was maintained 4mL/min and 1.5mL/min. All parameters of GC and MS were controlled using Mass hunter work station version 10.

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 Bromhexine Hydrochloride. 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 N-Nitroso-N-Methyl Cyclohexylamine impurity in Bromhexine 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.

3.0 RESULTS AND DISCUSSION

3.1 Method development

As a part of the specific method development by GC-MS/MS for the quantitation of N-Nitroso-N-Methyl Cyclohexylamine in the Bromhexine Hydrochloride different columns were tested to obtain the most appropriate peak shape and separation. By using typical DB-Wax UI and DB-5 MS capillary column the peak shape and the separation was not desire for the N-Nitroso-N-Methyl Cyclohexylamine. A DB-1MS (30m x 0.32 mm, 0.25 µm) column was found to be the most suitable regarding both peak shape and separation, as well as the intensity of the impurity. The retention times of N-Nitroso-N-Methyl Cyclohexylamine were observed to be 8.0 min. The chromatogram of standard solution of N-Nitroso-N-Methyl Cyclohexylamine 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 N-Nitroso-N-Methyl Cyclohexylamine 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 N-Nitroso-N-Methyl Cyclohexylamine, fragmentation was carried out using different collision energy (2, 5, 10, 15 and 20 ev). The electron energy and gain gas were optimized to obtain a good response for the ions.

4.0 METHOD VALIDATION

The optimized GC-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 N-Nitroso-N-Methyl Cyclohexylamine solution was prepared at the specification level in the diluent. The spiked Bromhexine Hydrochloride solution was then subjected to GC-MS/MS analysis and the results revealed that there was no interference of the Bromhexine Hydrochloride peak with N-Nitroso-N-Methyl Cyclohexylamine 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 N-Nitroso-N-Methyl Cyclohexylamine 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 N-Nitroso-N-Methyl Cyclohexylamine and calculating their RSD% values. The chromatograms of solutions of N-Nitroso-N-Methyl Cyclohexylamine 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 N-Nitroso-N-Methyl Cyclohexylamine 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 N-Nitroso-N-Methyl Cyclohexylamine in bulk samples of Bromhexine Hydrochloride in triplicate at LOQ (0.037 ppm), 50% (0.18 ppm), 100% (0.37 ppm) and 150% (0.54 ppm) with respect to test concentration. The acceptance criterion for recovery was set at 80-120%. The percentage recoveries for N-Nitroso-N-Methyl Cyclohexylamine 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 N-Nitroso-N-Methyl Cyclohexylamine were checked at its specification level (i.e. 0.37 ppm with respect to analyte concentration, 10.0 mg mL-1). The percentage RSD of method repeatability and system repeatability for the N-Nitroso-N-Methyl Cyclohexylamine 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, oven temperature and Collision energy are altered. The data obtained confirms that these deliberately changed chromatographic conditions did not impact the chromatographic performance for N-Nitroso-N-Methyl Cyclohexylamine in spiked samples showing the robustness of the method.

4.7 Solution stability

The solution stability of N-Nitroso-N-Methyl Cyclohexylamine was carried out by leaving spiked and unspiked sample solutions in firmly capped LC vials at room temperature for about 24 h in an autosampler. The concentration of N-Nitroso-N-Methyl Cyclohexylamine was determined against freshly prepared standard solutions and no significant changes were observed in the concentration for N-Nitroso-N-Methyl Cyclohexylamine. The data confirmed the stability of impurity in the sample solution for 24 hrs.

5.0 CONCLUSIONS

In this study, we have developed a GC-MS/MS method that is capable of quantifying N-Nitroso-N-Methyl Cyclohexylamine in Bromhexine Hydrochloride 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 N-Nitroso-N-Methyl Cyclohexylamine were set very low and well below that of acceptable limit. The sample prepared in the analytical solution was found to be stable for the 24 hrs. The method was fully validated and presents good linearity, accuracy, repeatability, and robustness. This method could be very useful for the determination of N-Nitroso-N-Methyl Cyclohexylamine in Bromhexine Hydrochloride during its manufacture and product release.

Table 1: System suitability criteria

Component	Retention time (min)	Relative retention time (min)
N-Nitroso-N-Methyl Cyclohexylamine	8.00	2.13
Iso-octane	3.75	1.00

Table 2: LOD, LOQ and Linearity results.

Validation parameter	Results
LOD-LOQ	
LOD (ng mL^{-1})	0.2
LOQ (ng mL^{-1})	0.4
Precision at LOQ (%RSD)	5.10
Linearity	

Regression (r)	0.9956
Calibration range (ng/mL^{-1})	0.40-5.62
Slope	39738919.85
Intercept	-12316.79
% Intercept	-4.19

Table 3: Accuracy (Recovery) results of N-Nitroso-N-Methyl Cyclohexylamine in bulk sample.

Accuracy Level	Mean Recovery (%)	% RSD
LOQ%	93.11	5.05
50 %	95.49	3.79
100 %	98.48	2.23
150 %	108.33	4.21

Table 4: Precision results of N-Nitroso-N-Methyl Cyclohexylamine.

Precision	% RSD
System precision	1.79
Method precision	2.94
Intermediate precision	4.67

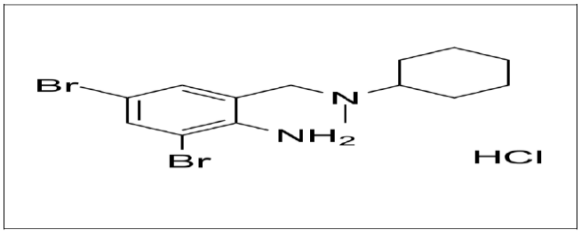

Fig. 1: Chemical structure of bromhexine hydrochloride.

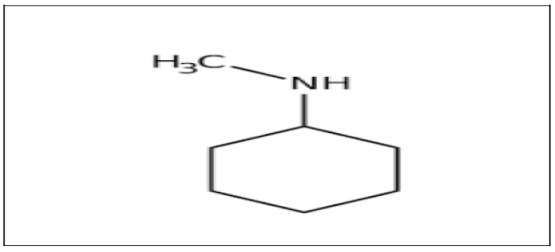

Fig. 1: Chemical structure of N-Methyl Cyclohexylamine.

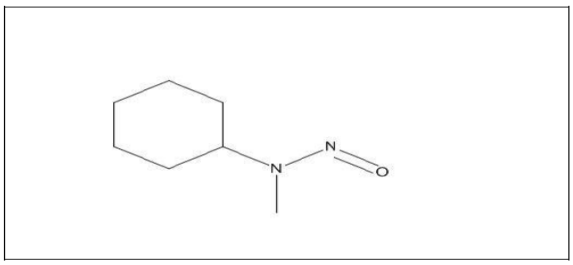

Fig. 1: Chemical structure of N-Nitroso-N-Methyl Cyclohexylamine (NMCA).

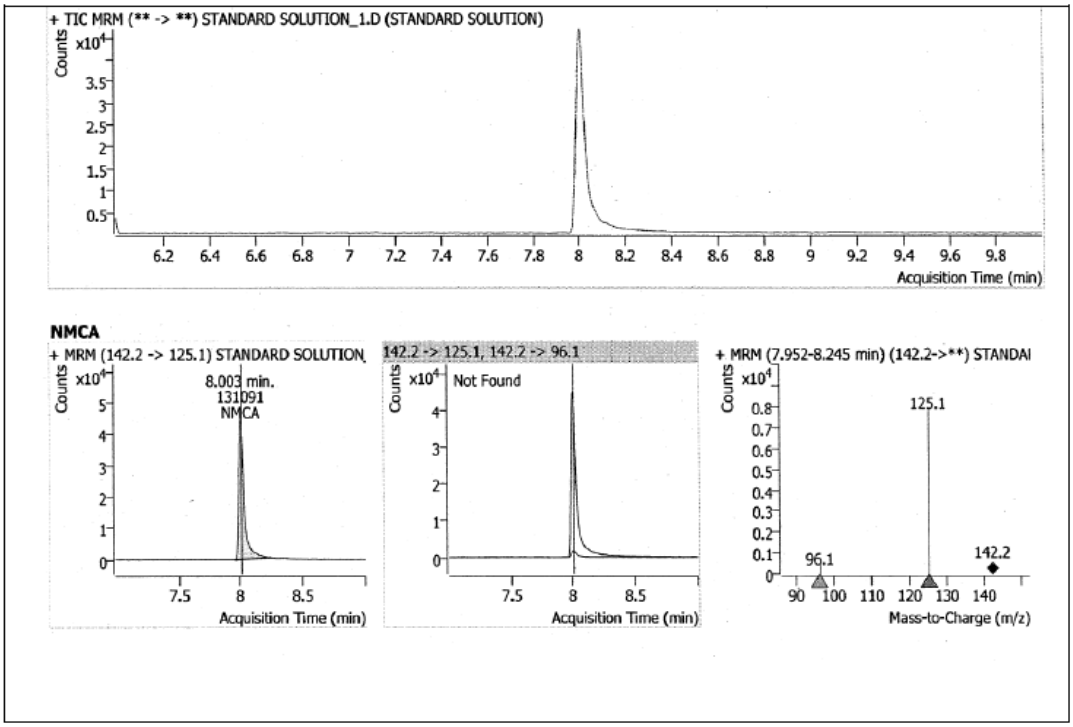

Fig. 2: A typical GCMS/MS chromatogram of Standard solution.

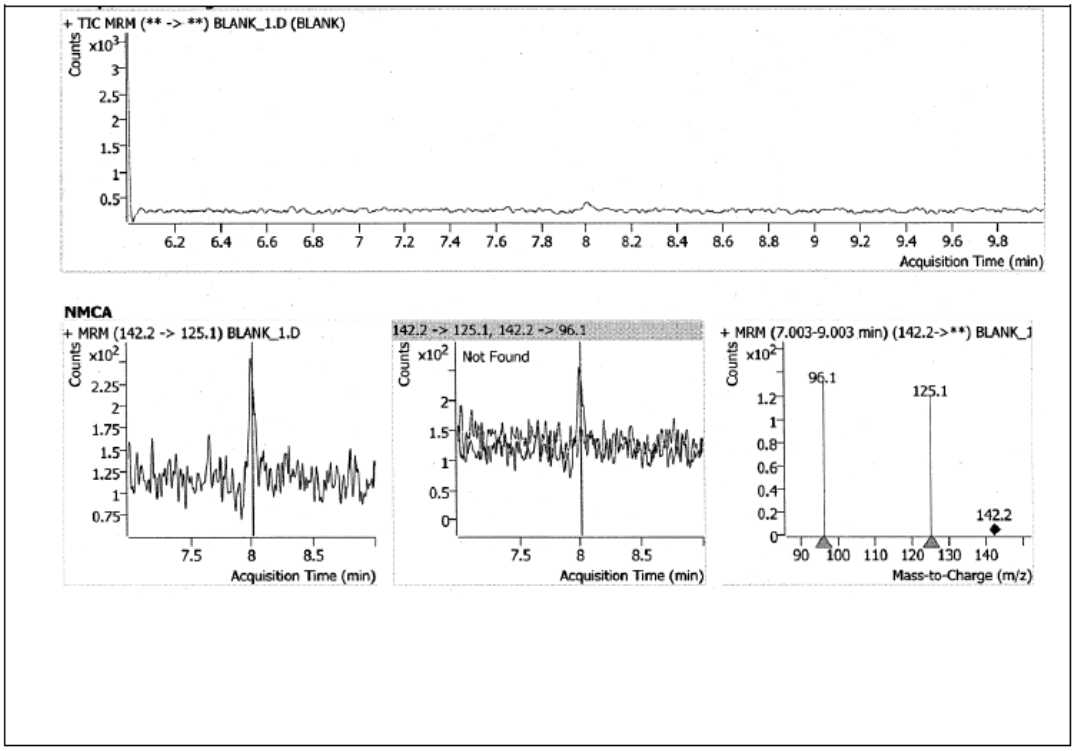

Fig. 2: A typical GCMS/MS chromatogram of Blank.

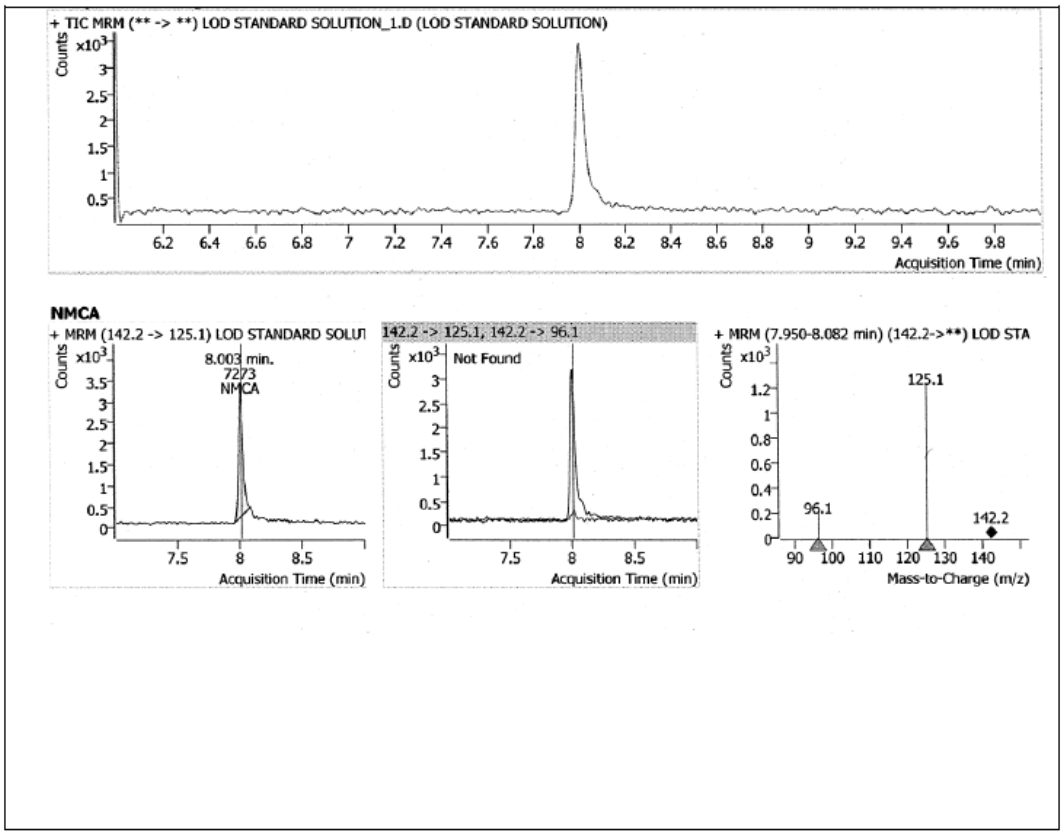

Fig. 2: A typical GCMS/MS chromatogram of Limit of Detection.

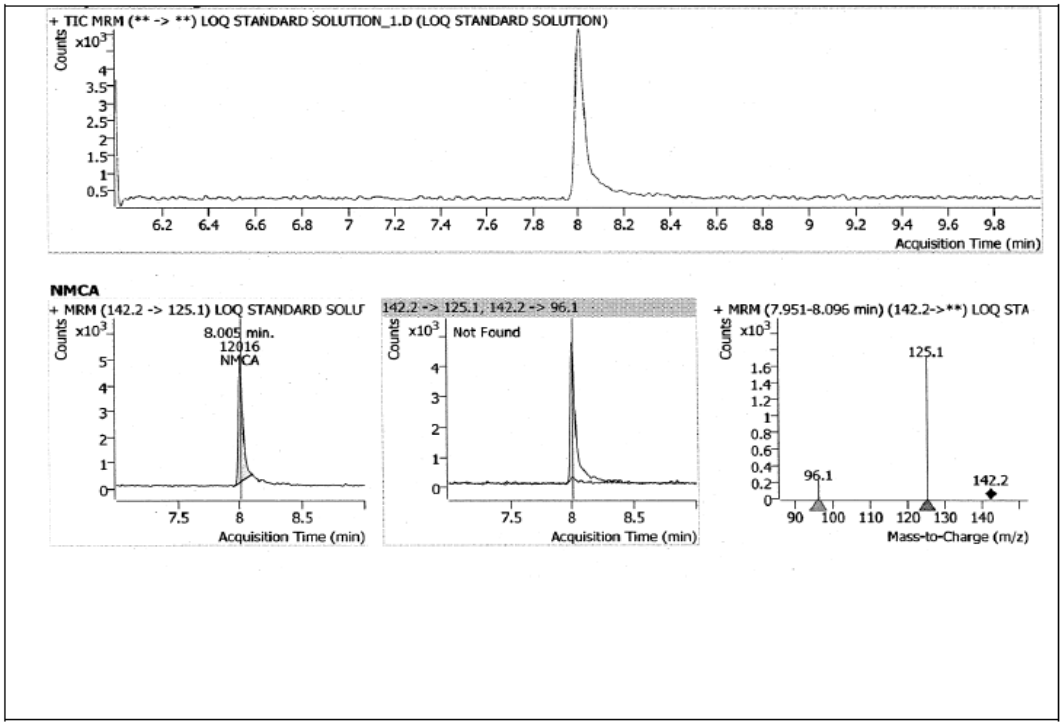

Fig. 2: A typical GCMS/MS chromatogram of Limit of Quantification.

6.0 Abbreviations

GC-MS: Gas chromatography mass spectrometry
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

7.0 Conflicts of interest

There are no conflicts to declare.

8.0 ACKNOWLEDGEMENTS

We acknowledge FDC Ltd. Management for support during the entire work of method development and method validation.

9.0 REFERENCES

1. International Council for Harmonization, Assessment and control of DNA reactive (Mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk M7 (R2).
2. International Agency for Research on Cancer, IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Lyon, 1978; 17.
3. Angiotensin-II-receptor antagonists (sartans) Article 31referralCHMP assessment report, https://www.ema.europa.eu/en/documents/variation-report/angiotensin-ii-receptor antagonists- sartans-article-31-referral-chmp-assessment report_en.pdf.
4. Pharmaceutical analysis and characterization of nitrosamine impurities within angiotensin ii receptor blocker drug products, https://www.fda.gov/media/148748/download.
5. www.wikipedia.com.
6. European pharmacopeia, Page no, 2000; 10.0.
7. Questions and answers for marketing authorisation holders/ applicants on the CHMP Opinion for the Article 5{3} of Regulation {EC} No 726/2004 referral on nitrosamine impurities in human medicinal products, 2024; 15 EMA/409815/2020 Rev.20.
8. Nitrosamine Drug Substances-Related impurities (NDSRIs) Guidance for industry, 2023. https://www.fda.gov/drugs/guidance-compliance-regulatory-information/guidances- drugs.
9. International Council for Harmonization, Validation of analytical procedures: Text and methodology Q2 (R2).