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A HIGHLY SENSITIVE LC-MS/MS METHOD FOR THE DETERMINATION AND QUANTIFICATION OF A N-NITROSAMINE IMPURITY IN THE TIMOLOL MALEATE ACTIVE PHARMACEUTICAL INGREDIENT

Vinayak T. Vele*, Kishor More, Vishal Telvekar, Shivaji Kadam, Mohan A. Chandavarkar

India.

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

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ABSTRACT
A liquid chromatography-tandem mass spectrometry (L C-MS/MS) method was developed for the quantification of n-Nitrosamine Timolol maleate impurity (NTML) in the Timolol maleate active pharmaceutical ingredient. Chromatographic separation was achieved using a Waters Acquity HSS T3 C18 column, with 0.01 mol L^{-1} ammonium formate in water as mobile phase A and 0.1% formic acid in methanol 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 fully validated with good linearity over the concentration range of 0.0061-0.0303 ppm of the Timolol Maleate test concentration for NTML. The correlation coefficient obtained in each case was >0.9994. The recoveries were found to be satisfactory over the range between 80 .0 and 120.0 % for NTML. The developed method was able to quantitate NTML at a concentration level of 200 ng mL^{-1} (0.02 ppm with respect to 100 mg mL^{-1} Timolol Maleate).

INTRODUCTION
N-Nitrosamines (NAs) are internationally recognized as a class of strong carcinogens according to the International Agency for Research on Cancer (IARC). N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) are classified as the most potent carcinogens of the N-nitrosamines, and N-nitrosodibutylamine (NDBA), N-nitrosopiperidine (NPIP) and N-nitrosopyrrolidine (NPYR) are listed as potential carcinogens.[1-2] The Environmental Protection Agency (EPA) of the United States considers that NDMA at extremely low concentrations (0.7 ng L^{-1}) can cause cancer, and the risk index of NAs for human health damage is grade B2.[3]

Given the great harm to human health that can be caused by NAs, the risk of NAs in active pharmaceutical ingredients (API) has been strictly controlled by regulatory agencies and pharmaceutical industries. The ICH M7 (R1) guideline[4] defines N-nitrosamines as substances of the "cohort of concern" for which limits in medicinal products refer to the so-called substance specific acceptable intake (AI) (the Threshold of Toxicological Concern, TIC, of 1.5 µg per day cannot be applied), which is associated with a negligible risk (theoretical excess cancer risk of <1 in 100 000 over a lifetime of exposure). In addition to the conventional genotoxic impurities (GTIs) described in the ICH M7 guideline, the European Medicines Agency (EMA)[5,6] and the United States Food and Drug Administration (FDA)[7,8] have released specific documents regarding regulatory issues related to the risk assessment and control strategy of NAs in new drug applications as well as approved drugs. According to the latest updated documents, N-nitrosamine precursors of secondary or tertiary amines from starting materials, intermediates and API should also be considered comprehensively in the risk assessment.

Timolol maleate[9] is a non-selective beta-adrenergic receptor blocking agent that lowers the ocular pressure in open angle glaucoma and ocular hypertension.[10] It is chemically described as (S)-1-(tert-butylamino)-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]-propan-2-ol Maleate which is official in IP, U.S.P, B.P and E.P.[11] Ever since its introduction for clinical usage in the management of glaucoma in 1978, none of the new generation beta blocker have been found more effective than Timolol Maleate.

As per the structure of Timolol maleate with secondary amine present there is a possibility of formation of the N-nitrosamine impurity of the Timolol Maleate drug substances itself. An N-nitrosamine impurity (Fig.1) named (S)-N-(tert-butyl)-N-(2-hydroxy-3-((4-morpholino-1,2,5-thiadiazol-3-yl)oxy)propyl)nitrous amide (NTML) was possible in Timolol maleate the active pharmaceutical ingredient of an approved drug by

EMA and FDA for the treatment of patients with lowers the ocular pressure in open angle glaucoma and ocular hypertension of eyes. As per the latest nitrosamine guidelines" of EMA, if 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.[12] Based on this information, the NTML concentration must be controlled in Timolol Maleate at concentrations lower than 0.22 ppm with respect to the maximum daily dose.

Owing to the extremely limited time of the impurity identification, there was not a specific and sensitive method for the determination and quantification of NTML in the Timolol Maleate reported in the literature except for related substance methods. Herein, we would like to present the development of an LC-MS/MS method for the determination of NTML in Timolol Maleate, as well as validation of the method with respect to the specificity, limit of detection (LOD), the limit of quantification (LOQ), linearity, repeatability, accuracy and robustness, in accordance with ICH recommended conditions.

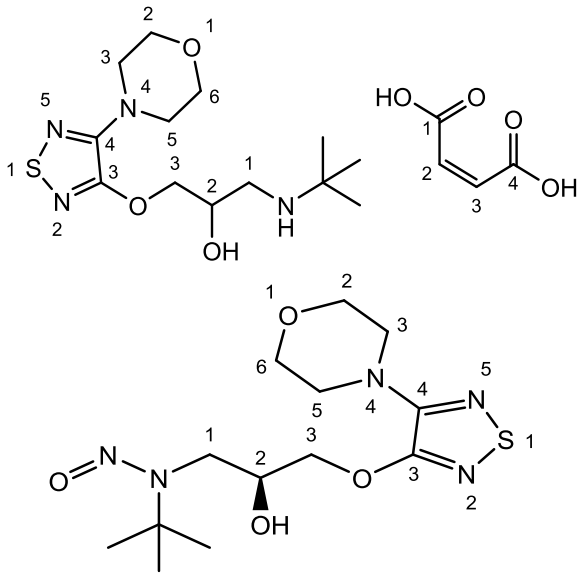

Fig. 1: Structures of Timolol maleate and N-Nitrosamine Timolol maleate (NTML).

EXPERIMENTAL

Reagents and chemicals

All reagents used were of LCMS grade with the highest purity of >99.8%. Methanol were purchased from J.T Baker (USA). The ammonium formate and formic acid eluent additive for LC-MS was purchased from SIGMA-ALDRICH. LCMS grade water was used throughout the analysis from J.T baker. Timolol Maleate and n-Nitrosamine Timolol maleate impurity (NTML) were synthesized and analysed at FDC LTD Pharm., India.

Mobile phase preparation

Mobile phase solution A was prepared by adding 0.01 mol of ammonium formate and 1.0 mL of Formic acid to 1000 mL of water. 0.1% formic acid in Methanol was used as mobile phase solution B. Both mobile phase solutions were degassed and stored for further use at ambient temperature. Solvents were prepared before each series of analyses.

Preparation of sample and standard solutions

A stock mixture of NTML (0.2 mg mL^{-1}) was prepared by dissolving suitable amounts of NTML in 100% methanol as the diluent. From this solution, the diluted stock solution of 2000 ng mL^{-1} was prepared by dilution with Water: Acetonitrile (1:1 v/v) as diluent. A series of calibration standards were prepared by diluting a 2000 ng mL^{-1} solution to obtain the following final concentrations of 6.1, 10.1, 16.2, 20.2, 24.2 and 30.3 ng mL^{-1}. Solutions for recovery determination were prepared by accurately weighing 500 mg of Timolol maleate in 5 mL volumetric flasks (in triplicate). By adding an appropriate amount of NTML to the Timolol maleate solutions, 6.1 ng mL^{-1} (0.060 ppm), 20.2 ng m-L 1 (0.202 ppm) and 30.3 ng mL-l (0.303 ppm) solutions of NTML with respect to 100 mg mL^{-1} Timolol maleate were prepared. All solutions prepared for analytical recovery were prepared in triplicate to ensure repeatability.

Operating conditions of LC-MS/MS

Chromatographic analysis was performed on Waters Acquity H class UPLC system equipped with a binary pump and an autosampler and Waters LC-MS/MS 2020 Triple Quad with an atmospheric pressure chemical 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 0.01 mol L^{-1} ammonium formate and 1.0 mL Formic acid in water as mobile phase A and 0.1% formic acid in methanol as mobile phase B at a flow rate of 0.25 mL min^{-1}. The column oven temperature was maintained at 40 °C. The sample injection volume was 20.0 µL. The LC gradient program (time/% solution A) was set as follows: 0.00/90, 0.5/90, 2.00/10, 3.80/10, 4.20/90 and 6.00/90. The positive Atmospheric pressure chemical ionization (APCI) probe was operated in MRM mode for the quantification of NTML in the form of protonated ions $(M+H)^+$ m/z 345.97 > 127.34. Source and Desolvation temperature set was 150 °C and 400 °C respectively. Cone voltage set was 15 V while collision energy (ev) at 10.0. The corona current of 1.0 µA was applied on NTML. All parameters of LC and MS were controlled using Waters Mass link version 4.1.

Method validation

The developed method was successfully validated in terms of specificity, repeatability, linearity, accuracy, LOD, LOQ, robustness and solution stability. Method validation for NTML in Timolol Maleate was conducted

following ICH guidelines. Initially, individual solutions of NTML were prepared (NTML impurity) = 20.2 ng mL^{-1}, 0.20 ppm with respect to 100 mg mL^{-1} Timolol Maleate) 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 30.3 ng mL^{-1} impurity concentration. Least squares linear regression analysis was employed to calculate the slope, intercept, and regression coefficient values. The specificity of the developed method was assessed with Timolol Maleate. 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 NTML impurity in Timolol Maleate. 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 a sensitive and selective LC-MS/MS method that can separate and quantify NTML in the Timolol Maleate active pharmaceutical ingredient. A few columns were tested to obtain the most appropriate peak shape and separation. There was a greater overlap between NTML and API peaks when using the Hypersil C18 column, and poor peak shapes were observed when using an Inertsil and PFP column. 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 0.01 mol L^{-1} ammonium formate and 1.0 mL Formic acid in water as mobile phase A and 0.1% formic acid in methanol as mobile phase B (see the subsection on operating conditions of LC-MS/MS). Methanol was chosen for the good resolution purpose. The flow rate of the mobile phase was maintained at 0.25 mL min^{-1}, with the column temperature set at 40 °C. The autosampler temperature was set at 25 °C. The retention times of NTML were observed to be 2.70 min and the peak corresponding to Timolol Maleate was eluted at 1.93 min. The chromatogram of standard solution of NTML is given in the APCI. Fig. 1

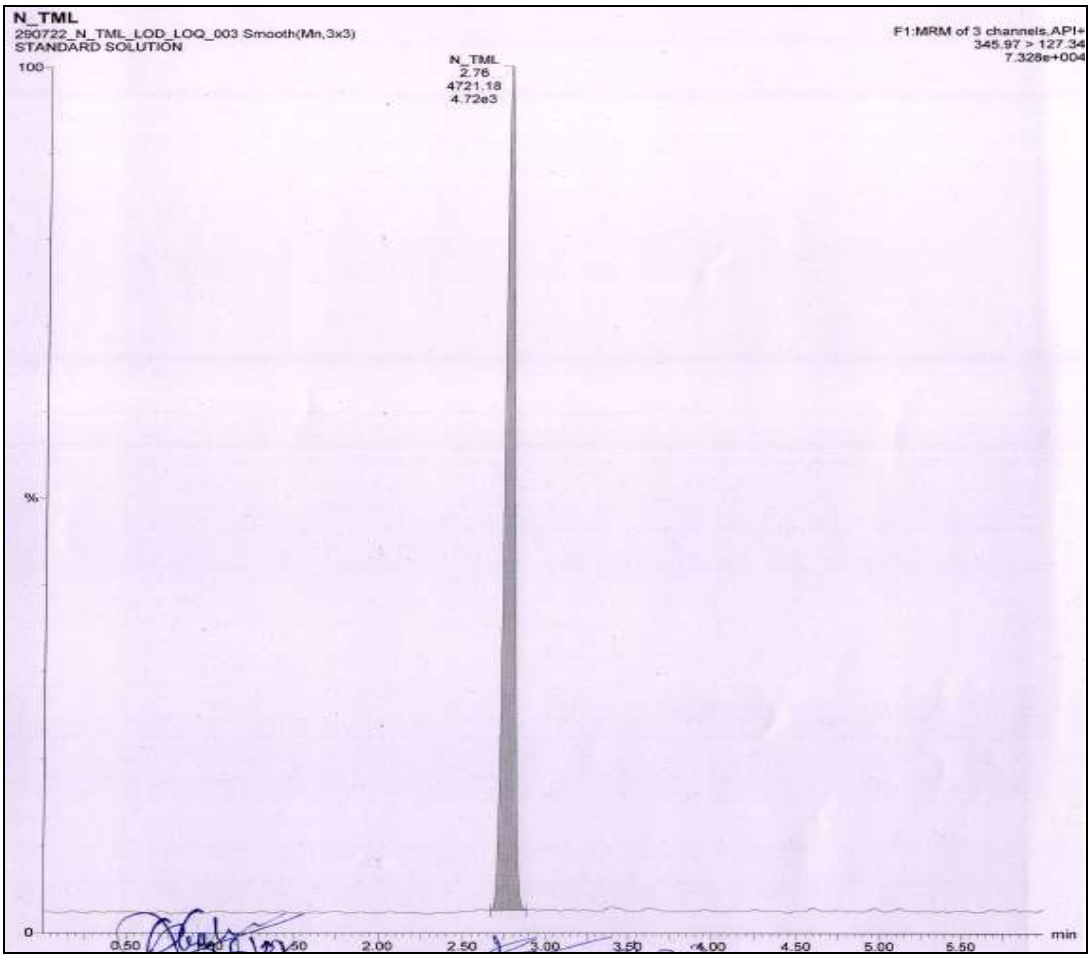

Fig. 1: LCMS-MS chromatogram of NTML (Standard solution).

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 NTML in the Timolol Maleate API. Method development was carried out using LC-MS/MS for the detection and quantification of NTML at a concentration level of 1 µg mL^{-1}. During the early stages of method development, the signal intensity in the positive mode was found to be much higher than that in the negative mode, limiting the method development to the positive APCI source. To optimize the APCI conditions for NTML, fragmentation was carried out using different collision energy (2, 5, 10, and 20 ev). The ion source parameters such as cone voltage and collision energy were optimized to obtain a good response for the 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

A single NTML solution was prepared at the specification level in the diluent. The spiked Timolol Maleate solution was then subjected to LC-MS/MS analysis and the results revealed that there was no interference of the Timolol Maleate peak with NTML peaks, and hence the specificity of the developed method was proven.

Linearity

The linearity of the method was established over the concentration range from 6.1 to 30.3 ng mL^{-1} (0.0061-0.0303 ppm) for NTML. 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 analytes. A good correlation between the peak area and concentration of analytes was obtained, as can be seen in Table 1.

Determination of LOD and LOQ values

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

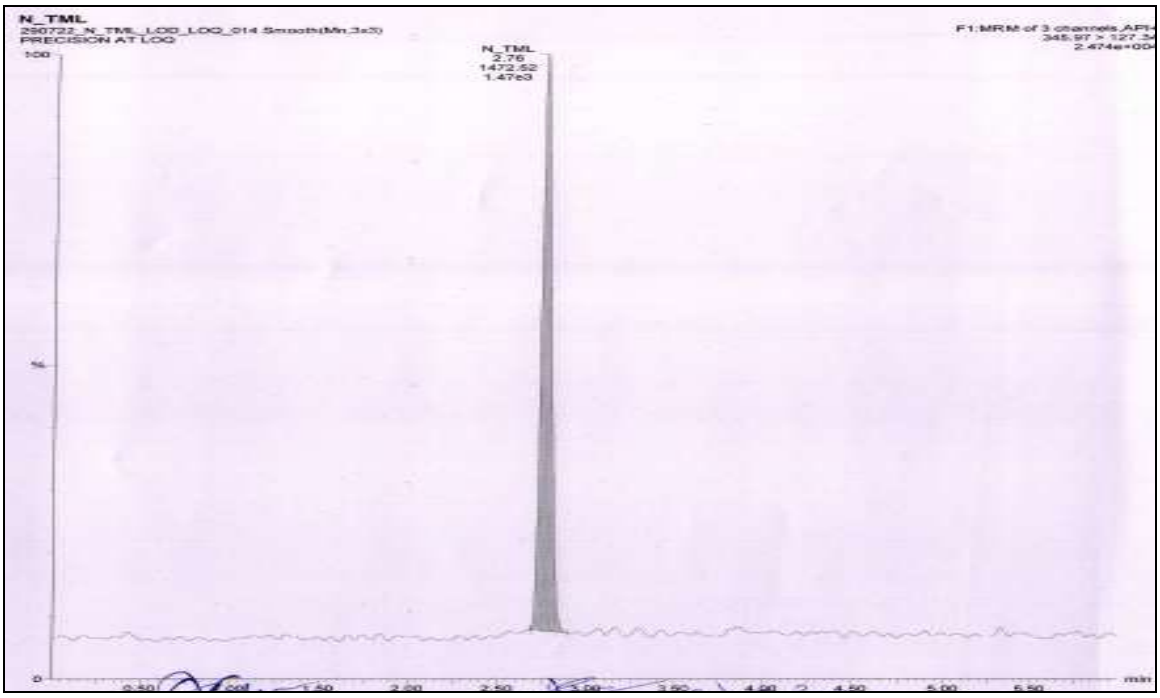

Fig. 2: LCMS-MS chromatogram of NTML (Limit of Quantification).

Accuracy and recovery

Accuracy as a deviation from linearity was evaluated by injecting a mixture of NTML at the LOQ concentration and on the specification level three times. The acceptance criterion for accuracy in such a low concentration range is 70-130%. The deviation of the peak areas for each impurity was <10.0 % at the LOQ level, which is acceptable for the developed analytical method (Table 2). The accuracy (as recovery) of the method was evaluated by the standard addition method in triplicate at the LOQ (0.060 ppm), 0.100 ppm, 0.200 and 0.303 ppm in the Timolol Maleate active ingredient. The acceptance criterion for recovery was set at 80-120%. The percentage recoveries for NTML are presented in Table 1.

Table 1: Summary of Method Validation Data.

Parameter	ng mL^{-1}	r	% Mean Recovery	% RSD
LOD	3.0	-	-	4.60
LOQ Linearity	6.0	-	-	2.89
(LOQ to 150%) Accuracy	-	0.9994	-	-
LOQ % spiking	-	-	89.2	1.94
50% spiking	-	-	85.2	0.35
100 % spiking	-	-	95.1	0.97
150 % spiking Precision	-	-	91.1	1.03
System precision	-	-	-	1.85
Method precision	-	-	-	2.35
Intermediate pre (Ruggedness)	-	-	-	4.01

Robustness

To determine the robustness of the method, experimental conditions including the flow rate of the mobile phase and column oven temperatures were deliberately changed. The optimized flow rate of the mobile phase was 0.25 mL min^{-1} and the same was altered from 0.21 to 0.28 mL min^{-1}. The effects of column oven temperature on the resolution were studied at 37 °C and 43 °C (altered by 3.0 °C). The results revealed that these deliberately changed chromatographic conditions of the flow rate and column oven temperature did not impact the chromatographic performance for NTML in spiked samples showing the robustness of the method if the mobile phase components were held constant.

Solution stability and repeatability

To confirm the repeatability of the method, the samples of Timolol Maleate spiked with NTML at three concentration levels in triplicate were evaluated. The acceptance criterion for repeatability was RSD = ±10%. The RSD was $\leq$ 5.0% (Table 4). The study of the solution stability of Timolol Maleate and NTML was carried out by leaving spiked and unspiked sample solutions in firmly capped LC vials at 25 °C for about 24 h in an autosampler. The concentration of NTML was determined against freshly prepared standard solutions and no significant changes were observed in the concentration for NTML. Therefore, we have confirmed the stability of impurities in the sample solution for at least 24 h.

CONCLUSIONS

In this study, we have developed a highly sensitive LC-MS/MS approach that is capable of quantifying NTML in Timolol Maleate 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 NTML were set very low and lie within the range of the 30% 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 NTML in Timolol Maleate during its manufacture and product release.

Conflicts of interest

There are no conflicts to declare.

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