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Research Article

Determination and Quantification of a N-nitroso-Brinzolamide in the Brinzolamide 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 N-nitroso-Brinzolamide in the Brinzolamide active pharmaceutical ingredient. Chromatographic separation was achieved using a Phenyl hexyl column, with 2 mm Ammonium formate in water and Acetonitrile as mobile phase in gradient elution mode at a 0.5 ml/min flow rate. The detection and quantification was done using triple quadrupole mass detection with electrospray ionization in the multiple reaction monitoring mode. The method validation was conducted with a stringent linearity criteria and the concentration of the analyte N-Nitroso Brinzolamide to the lowest range of 1ppb to 15.5ppb. The result for the linearity test was satisfactory with the correlation coefficient >0.9990. The Accuracy parameter was found to be satisfactory over the range 80% to 120% for the analyte. The analytical method successfully quantified N-nitroso-Brinzolamide at a low concentration of 10 ppb relative to 0.2 mg/mL. Additionally, the quantification demonstrated consistent repeatability and reproducibility.

Keywords: Nitrosamine; N-nitroso-Brinzolamide; LCMS/MS; Validation; Brinzolamide

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

Introduction

With the discovery and reporting of the nitrosamine impurity in a sartan-based drug substance [1,2] in June 2018 to USFDA and EMA, N-nitroso compounds have been categorized under the cohort of concern group of compounds according to ICH M7 [3]. These compounds are recognized worldwide as strong carcinogens by the International Agency for Research on Cancer [4]. The initial phase of the nitrosamine impurity probe was confined to a search and examination of the seven nitrosamine impurities generated throughout the drug substance's synthetic process. The regulatory agencies have now extended their probe from the listed nitrosamine impurities to the nitrosamine impurities of the drug substances and drug products. Thus the search and assessment of N-Nitrosamine impurities has now extended from the small molecules N-Nitrosamine to N-Nitrosamine drug substance related impurities (NDSRI).

Brinzolamide, marketed as Azopt [5], is an ophthalmic medication that belongs to the carbonic anhydrase inhibitor class. It is prescribed to reduce elevated intraocular pressure in patients diagnosed with open-angle glaucoma or ocular hypertension.

It is an active pharmaceutical ingredient (API) in a drug approved by the USFDA [6]. Typically, it is used in finished formulations as an ophthalmic solution, administered as eye drops in the form of a suspension.

According to the latest and recent guidance from both the EMA [7] and the USFDA [8], N-nitroso-Brinzolamide (Figure 1) has been identified as a potential nitrosamine impurity. This identification is attributed to the presence of a secondary amine in the active pharmaceutical ingredient Brinzolamide (Figure 2) itself, which can lead to the formation of nitrosamine impurities.

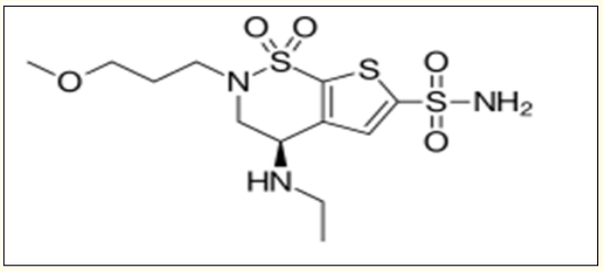

Figure 1: Chemical structure of Brinzolamide.

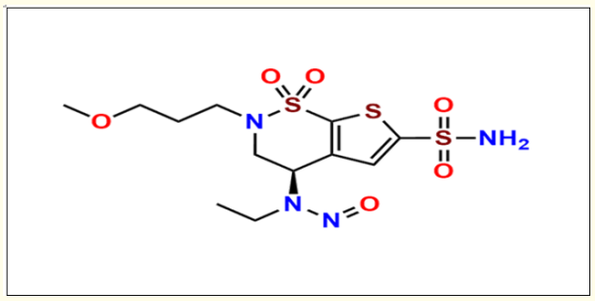

Figure 2: Chemical structure of N-nitroso-Brinzolamide.

According to the carcinogenic potency categorization approach outlined in the guidance, the N-nitroso-Brinzolamide impurity is classified as a category 2 carcinogen, with an acceptable intake (AI) limit of 100 ng/day. Given the maximum daily dose of Brinzolamide is 1.92 mg/day, it is necessary to control the level of N-nitroso-Brinzolamide to 52.0 ppm in the Brinzolamide formulation to ensure safety and compliance with regulatory standards.

We have developed a new, sensitive, a high signal to noise ratio and reproducible LC-MS/MS method for evaluating the nitrosamine impurity in the Brinzolamide drug substance, capable of detecting it at a limit of 10 ppb.

The developed analytical method was screened, tested and validated for the ICH guidelines [9] validation parameters of specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, repeatability, accuracy, robustness and solution stability. The method successfully met all the required parameters.

Materials and Methods

Reagents and chemicals

LCMS grade Ammonium Formate, Formic Acid Make- Fisher Scientific, Acetonitrile and Water Make – J T Baker were used. A synthesized and well characterized standard of N-Nitroso Brinzolamide was used in the analysis of N-nitrosamine in the synthesized Brinzolamide at FDC LTD Pharm., India.

Preparation of sample and standard solutions

The stock solutions of N-Nitroso Brinzolamide and Brinzolamide were prepared separately by dissolving their appropriate quantities in a suitable diluent. For quantitation of N-Nitroso-Brinzolamide in Brinzolamide the standard concentration of 10.0 ng mL^{-1} and the test concentration of 0.2 mg mL^{-1} were used.

Chromatographic conditions of LC-MS/MS

The analysis was performed on Nexa Shimadzu UPLC system which is equipped with a binary pump, autosampler and Sciex 5500+ LC-MS/MS Triple Quad with an electrospray ionization interface. The analytical column used in this analysis was Phenyl Hexyl, (100 x 4.6 mm, 2.6 µm) (Phenomenex, USA). A gradient mode mobile phase was employed, consisting of 2 mM ammonium formate adjusted to pH 3.0 with formic acid in water as mobile phase A, and acetonitrile as mobile phase B, with a flow rate of 0.5 mL/min. The LC gradient program (time/% mobile phase A) was set as follows: 0.00/80, 2.0/80, 8.0/10, 15.0/10, 16/80 and 20.0/80.

The column oven temperature was maintained at 30°C. The sample injection volume was 10.0 µL and the autosampler temperature was set at 15°C.

The negative electrospray ionization (ESI) probe was operated in MRM mode for the quantification of N-nitroso Brinzolamide in the form of protonated ions $(M-H)^-$ at m/z 411.00 > 216.00.

The different voltage i.e. declustering potential (DP), entrance potential and collision exit cell potential was maintained at 110 V, 10 V and 15 V respectively. The ion spray voltage (V) was maintained at 4500 V. The curtain gas flow, ion source gas (GS1) and ion source gas (GS2) pressure was maintained at 55 psi, 60 psi and 50 psi. All parameters of LC and MS were controlled using Sciex Analyst version 1.7.3 software.

Method validation

The developed analytical method was successfully validated for the ICH guidelines validation parameters of specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, repeatability, accuracy, robustness and solution stability. The linearity parameter was evaluated over a range from the limit of quantification (LOQ) to 150% of the target level. The correlation coefficient, slope, and intercept values were subsequently calculated. The specificity of the analytical method was tested for N-Nitrosamine in presence of Brinzolamide. The recovery and %RSD were calculated for the N-nitroso-Brinzolamide impurity in Brinzolamide at each level. 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

The aim of the study was to develop a specific, sensitive LC-MS/MS analytical method which is capable of detecting and quantifying N-Nitroso Brinzolamide in Brinzolamide. A comprehensive evaluation of various chromatographic columns was conducted to optimize peak shape and resolution. Utilizing standard CSH and BEH C18 columns resulted in inadequate retention of both N-nitroso-Brinzolamide and Brinzolamide, leading to their premature elution and unsatisfactory peak separation. On HSS T3 there was an improvement in the retention but the separation and the peak shapes were unsatisfactory for N-Nitroso Brinzolamide.

The Phenomenex, Phenyl Hexyl (100 x 4.6 mm, 2.6 µm) column was found to be the most suitable regarding both peak retention, shape and separation, as well as the response of analytes.

A gradient elution method was employed using a mobile phase consisting of 2 mM ammonium formate (pH adjusted to 3.0 with formic acid) in water (mobile phase A) and acetonitrile (mobile phase B). The chromatographic separation was conducted at a flow rate of 0.5 mL per minute and a column temperature of 30 °C.

The mobile phase was operated in gradient mode using 2 mM Ammonium formate, pH adjusted to 3.0 with formic acid in water as mobile phase A and Acetonitrile as mobile phase B. The flow rate of the mobile phase was maintained at 0.5 mL min^{-1}, with the column temperature set at 30 °C. The autosampler temperature was set at 15 °C. The retention time of N-nitroso-Brinzolamide was observed to be 10.2 min and the peak corresponding to Brinzolamide eluted at 4.2 min.

Operating conditions of LC-MS/MS

Initial optimization of the mass parameters for the detection of the N-nitroso-Brinzolamide 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-Brinzolamide, fragmentation was carried out using different collision energy (15, 20, 25, 30 and 35 ev). The ion source parameters such as ion spray and collision gas 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 N-Nitroso-Brinzolamide solution was prepared at the specification level in the diluent.

The spiked Brinzolamide solution was analyzed using LC-MS/MS, and the results demonstrated no interference between the Brinzolamide peak and the N-nitroso-Brinzolamide peak, confirming the specificity of the developed method (Table 1).

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-Brinzolamide was determined based on S/N ratios of 3.0 and 10 by injecting standard solutions of known concentra-

Table 1: System suitability criteria.

Component	Retention time (min)	Relative retention time (min)
Brinzolamide	4.20	1.00
N-nitroso Brinzolamide	10.20	2.43

tions. The repeatability at the LOD and LOQ value was calculated by analysing six replicate injections of N-nitroso-Brinzolamide and calculating their RSD% values. The LOD and LOQ data shown in table 2.

Table 2: LOD, LOQ and Linearity results.

Validation parameter	Results
LOD-LOQ	
LOD (ng mL^{-1})	0.5
LOQ (ng mL^{-1})	1.0
Precision at LOQ (% RSD)	1.78
Linearity	
Regression (r)	0.9990
Calibration range (ng/mL^{-1})	1.00-15.0
Slope	222277973
Intercept	+91536.94
% Intercept	3.91

Linearity

Linearity of the method was studied by using the standard solution of N-nitroso-Brinzolamide 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.

Accuracy and recovery

The standard addition and recovery experiments were conducted for the N-Nitroso-Brinzolamide in test samples of Brinzolamide in triplicate at LOQ (0.0010 ppm), 50% (0.005 ppm), 100% (0.010 ppm) and 150% (0.015 ppm) with respect to test concentration. The acceptance criterion for recovery was set at 80-120%. The percentage recoveries for N-nitroso-Brinzolamide are presented in Table 3.

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

Accuracy Level	Mean Recovery (%)	% RSD
LOQ%	113.34	3.23
50 %	95.32	2.15
100 %	103.11	1.24
150 %	107.37	0.74

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-Brinzolamide were checked at its specification level (i.e. 0.010 ppm with respect to analyte concentration, 0.20 mg mL^{-1}). The percentage RSD of Method Precision and System Precision for N-nitroso-Brinzolamide were reported (Table 4) confirms good precision of the method.

Table 4: Precision results of N-nitroso Brinzolamide.

Precision	% RSD
System precision	1.17
Method precision	1.32
Intermediate precision	2.88

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 0.5 mL min^{-1} pH 3.0, and the column oven temperature was 30 °C. The parameters were altered from flow rate 0.47 to 0.53 mL min^{-1}, pH 2.90 to 3.10 and column oven temperature 27 °C and 33 °C receptively. The data obtained confirms that these deliberately changed chromatographic conditions did not impact the chromatographic performance for N-nitroso-Brinzolamide in spiked samples showing the robustness of the method.

Solution stability

The stability of Brinzolamide and N-nitroso-Brinzolamide was assessed by storing both spiked and unspiked sample solutions in

tightly capped LC vials at 15 °C for approximately 24 hours in an autosampler. The concentration of N-nitroso-Brinzolamide was determined against freshly prepared standard solution and no significant changes were observed in the concentration for N-nitroso-Brinzolamide. The data confirmed the stability of impurity in the sample solution for at least 24 h.

Conclusion

In this study, we have developed a LC-MS/MS method which is capable of quantifying N-nitroso-Brinzolamide in Brinzolamide using the negative 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-Brinzolamide 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 N-Nitroso-Brinzolamide in Brinzolamide during its manufacture and product release.

Conflicts of Interest

There are no conflicts to declare.

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