Original Article

TANDEM MASS SPECTROMETRIC METHOD FOR THE TRACE LEVEL DETERMINATION OF 2-AMINOPYRIDINE: A POTENTIAL GENOTOXIC IMPURITY IN TENOXICAM API

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ABSTRACT

Objective: This study aimed to develop a highly sensitive method for the determination of the genotoxic impurity 2-amino pyridine in Tenoxicam, employing hyphenated techniques.

Methods: The determination of 2-amino pyridine was carried out using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method in Selected Ion Monitoring mode (SIM). A LiChrospher RP-18 (100×4.6 mm) 5.0 µm column was utilized for the separation. A gradient elution technique was employed with acetonitrile (mobile phase A) and 0.01M ammonium acetate buffer (mobile phase B) in varying ratios. The gradient program (T/%B) was set as 0/5, 2.50/15, 5.00/30, 10.00/50, 15.00/95, 20.00/95. The developed method was validated according to the International Conference on Harmonization guidelines.

Results: The limits of detection (LOD) and quantification (LOQ) for 2-amino pyridine were found to be 0.09 ppm and 0.3 ppm, respectively. The method demonstrated accuracy within the range of 89.1% to 106.6% for the analyte. The method's linearity was confirmed through a six-point calibration graph spanning 6 ppm to 75 ppm, corresponding to a concentration of 20 mg/ml of Tenoxicam.

Conclusion: Developed hyphenated LC-MS/MS method presented in this study offers a highly sensitive and accurate means for the determination of the genotoxic impurity 2-amino pyridine in Tenoxicam. With validated LOD and LOQ values, as well as demonstrated accuracy, this method proves to be a robust quality control tool suitable for the quantitation of 2-amino pyridine at very low concentrations in the pharmaceutical compound Tenoxicam.

Keywords: Tenoxicam, 2-amino pyridine, Genotoxic impurities, Selected Ion Monitoring (SIM), ICH guidelines

INTRODUCTION

Tenoxicam (fig. 1) is an enolic acid derivative that inhibits high levels of COX-2 at the sites of inflammation and thus has anti-inflammatory, analgesic, and antipyretic activity. This nonselective COX inhibitor is extensively used in the treatment of rheumatoid arthritis and osteoarthritis. Chemically, tenoxicam is 4-hydroxy-2-methyl-*N*-2-pyridinyl-2*H*-thieno(2,3-e)-1,2-thiazine-3-carboxamide 1,1-dioxide with pKa 4.50 and 3.73 [1-6].
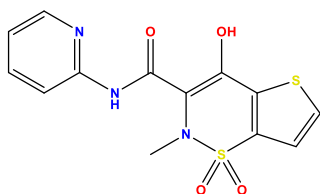

Fig. 1: Tenoxicam structure

Impurities, especially genotoxic impurities, have been at the centre of increasing regulatory and industry attention in the past decade. Active pharmaceutical Ingredients prone to contain different impurities that may arise from starting materials, reagents employed for the synthesis and by products in the synthetic process [7]. During the chemical synthesis reactants are carefully selected owing to their appropriate reactivity in order to achieve the end product with sufficient yield. However, this same reactivity of the reactants could result in genotoxicity if any unreacted material left with the final product as an impurity, which makes these impurities to consider critically eliminating them from the final drug product [8]. Often different synthetic process related modifications are employed to remove these impurities, yet it became impossible to completely eliminate the impurities from the final drug substances. According to the guidance of drug regulatory authorities it is crucial to regulate the level of genotoxic impurities in the drug substances based on the daily dose [9].

Aromatic amines are generally employed in the synthetic process as building blocks and are categorized as potentially genotoxic impurities (PGIs) in pharmaceuticals. Inherently aromatic amines genotoxicity is not owing to their reactivity but due to the generation of nitrenium ion (Ar-N⁺H) by the oxidative metabolic reactions, which is considered to be the active genotoxin that binds to DNA [10]. PGIs are known to induce genetic mutations or chromosomal aberrations and are reported as known carcinogens in rats and mice. Aryl amines possess the regulatory limit of intake at 1.5µg/d [11]. A few synthetic processes have been reported for the Tenoxicam which utilize the genotoxic substances as starting materials as well as intermediates. The reaction of 2-amino pyridine which is genotoxic in nature, with nitrogen mustard gives an aryl piperazine derivative, a key intermediate during the synthesis. The side-chain connector is then incorporated by alkylation of the second nitrogen of the piperazine ring with the genotoxic reagent 4-chloro-1-bromobutane [12].

The potential presence of these genotoxins has attracted the attention of regulatory authorities. European Medicines Agency's (EMEA) Committee for Medicinal products for Human use (CHMP) has published guidelines regarding limits of genotoxic impurities [13]. In 2008, regarding the genotoxic and carcinogenic impurities in drug substances, a draft of guidelines also outlined by US FDA. It consists of the different various routes to mitigate the potential lifetime cancer risk in patients with exposure to genotoxic and carcinogenic impurities. Based on the current regulatory guidance for genotoxic impurities, analytical methods should be developed to meet the required limit of 1.5µg/d daily intake of individual impurity [14].

In accordance with the amplifying concerns of regulatory authorities regarding the control of genotoxic impurities in pharmaceuticals, an

attempt was made to develop the sensitive LC-MS/MS method to determine the 2-amino pyridine in very low levels in Tenoxicam.

MATERIALS AND METHODS

Materials

2-amino pyridine was procured from Sigma Aldrich, Bangalore, India. Analytical grade ammonium acetate and acetonitrile were purchased from Merck, India. Tenoxicam sample was procured from Remedy Labs, India.

Chromatographic conditions

The chromatographic system used was Shimadzu LCMS 8040. The analytical column was LiChrospher RP-18 (100×4.6 mm) 5.0 µm. Isocratic elution mode was applied for the operation and the mobile phase composed 50% acetonitrile and 0.01M ammonium acetate buffer (pH-4.0). The flow rate of the mobile phase was kept at 1.0 ml/min. Column oven temperature and auto sampler temperature were set as 30 °C and 25 °C, respectively and injection volume was 10 µl. Instrument operation, data collection and processing were done by LCMS Lab Solutions.

Mass spectrometer conditions

Following typical mass spectrometer conditions were applied: source temperature, 120 °C; de-solvation temperature 300 °C; sample cone, 30V; capillary voltage, 3.0KV; cone de-solvation gas (N2) flow rate 1000 L/H; gas flow rate 50 L/H; Argon as CID gas for MS/MS experiments. The selective ion monitoring (SIM) was selected for quantification of analyte. Venting was done using valco valve (Valco Instruments Co. Inc., VICI AG International). Venting was given from 8 min to 12 min.

Validation study

Ich guideline were followed for the validation of the developed LC-MS/MS method for the determination of 2-aminopyridine in Tenoxicam. By analysing the six concentrations of analyte from 0.3 ppm-7.5 ppm, linearity of the method was established. Slope, intercept and regression coefficient were determined from the least square linear regression analysis. Six repetitive injections of Standard solution were employed to establish the system precision of the mass spectrometric response. LOQ and LOD were determined via exhibiting precision, by running six replicate injections of analyte in lower concentrations. The LOQ and LOD were calculated on the basis of the lowest concentration of compound that gives %RSD<10 (for LOQ) and %RSD<15 (for LOD). The method precision was evaluated by spiking each analyte and determining the %RSD. Accuracy was determined by spiking the known amount of 2-aminopyridine with known amount of sample in six different volumetric flasks and it was calculated after making corrections for the amount pre-existed in the sample. Stability of analytes in sample solution was done by analysing spiked sample solution at different time intervals at room temperature.

Standard solution preparation

Stock solution having concentration of 7.5 mg/ml of 2-aminopyridine was prepared by dissolving it in acetonitrile. From the stock solution, diluted stock solution of 0.075 mg/ml concentration was prepared by the dilution of 1 ml of the 7.5 mg/ml solution to 100 ml with acetonitrile. From this diluted stock solution, working standard solution of 37.5 ppm strength with respect to the sample concentration of 2 mg/ml of Tenoxicam was prepared by the serial dilution in acetonitrile before injection into the chromatographic system. At all times the working standard solutions were prepared prior to the injection into chromatographic instrument [15].

Sample preparation

Tenoxicam sample solution of 2 mg/ml concentration was prepared before injection into system by dissolving about 2 mg of the drug substance with solvent in a HPLC vial.

RESULTS

Optimization of sample preparation

In trace level analysis of GTI in a drug substance, sample preparation affects the analytical sensitivity, stability, recovery, and matrix effect.

In order to achieve efficient extraction and analyte response different diluents such as methanol and acetonitrile were studied. Both the solvents produced satisfactory solubilization capacity for the analyte and drug substances. But acetonitrile was chosen finally as it provides better analyte response, proper peak shapes and good recovery levels.

Column selection and separation

Selection of appropriate column has a huge impact on the resolution of analyte and drug substance peak. To achieve the proper resolution in trace level analysis of the GTI, it is very crucial to select the appropriate column as the concentration of drug substance was high leading to broad peak. Various columns like Phenomenex Luna C18, Kromasil C18 and LiChrospher 100 RP-18 of different dimensions were evaluated. Luna C18 and Kromasil C18 columns were found to be not suitable, since the observance of low resolution and improper analyte response. Satisfactory response for the analyte 2-aminopyridine and good resolution between analyte and Tenoxicam were achieved on the LiChrospher 100 RP-18 column of dimensions 100 mm×4.6 mminternal diameter, 5.0 µm. Diverse composition of mobile phase using 0.1% formic acid 0.1% acetic acid, ammonium formate, and ammonium acetate with acetonitrile and methanol were studied. Decent response and separation were noticed with the combination of ammonium acetate buffer and acetonitrile in gradient elution modes. To avoid any shift in retention time the column was thermostated at 30 °C and 1.0 ml/min of mobile phase flow rate was maintained. The flow rate was reduced to 0.2 ml/min by utilizing a splitter prior to the electrospray ionization. Retention time of 2-aminopyridine was observed to be about 3.8 min.

Tenoxicam peak eluted at around 8 min. Only the analyte, 2-aminopyridine peak was permitted enter the mass detector with the help of a switching valco valve, that executed the venting of drug substance peak. This technique enables the method development process to analyse the peak of interest and also avoids the matrix effect due to high concentration of drug substance.

Optimization of mass spectrometric parameters

In Chromatographic analysis, choice of detection method is pivotal fundamental for successful method development. Comparatively, LC-MS/MS method was chosen over HPLC–UV due to the greater sensitivity and specificity offered by the mass spectrometric detection for the trace analysis of 2-aminopyridine and the analysis was executed in multiple reaction monitoring mode (MRM) which further increased the specificity of the determination. 2-aminopyridine ion mass transactions corresponding to 94>67 were recorded for the quantification. The LC-MS/MS chromatograms are shown in fig. 2 and 3.

Validation of method

The newly developed LC-MS/MS method's validation was performed according to the ICH guidelines in relation to the analytical parameters such as [14] specificity, accuracy, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, and robustness in order to demonstrate the feasibility of the method.

Specificity

Specificity of the developed LC-MS/MS method for the analyte response at specification level was indicated by the retention of the 2-aminopyridine at the time around 3.8 and the analyte response for 2-aminopyridine in MRM is about 67.15 on mass spectrum. The specificity of the developed LC-MS/MS methods was indicated by showing the m/z peak in peak as 67.15 for 2-aminopyridine.

Linearity

The linearity of method in terms of mass spectrometric response with respect to concentration of analyte was demonstrated by a six-point calibration graph between 6 ppm and 75 ppm corresponding to the concentration of 20 mg/ml of Tenoxicam. Correlation coefficients for all analytes were>0.998. Linearity results enumerated in table 1 and fig. 4 and 5 depict the linearity graph and Chromatogram respectively.
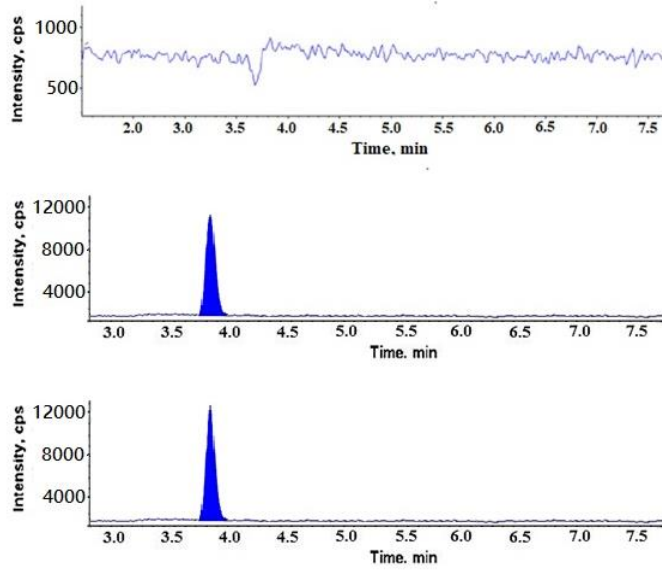
Fig. 2: Chromatograms of 2-aminopyridine using SIM scan. (A) Chromatogram of blank, (B) Chromatogram of standard containing 37.5 ppm of 2-aminopyridine, (C) Chromatogram of Tenoxicam sample spiked with 2-aminopyridine at 37.5 ppm
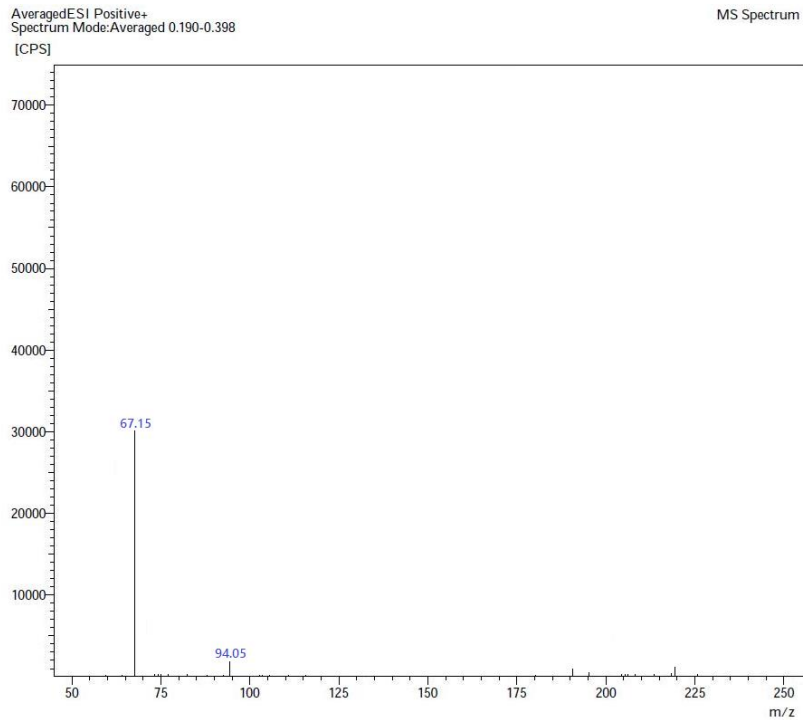

Fig. 3: MRM chromatogram of 2-aminopyridine

Table 1: Linearity of 2-aminopyridine

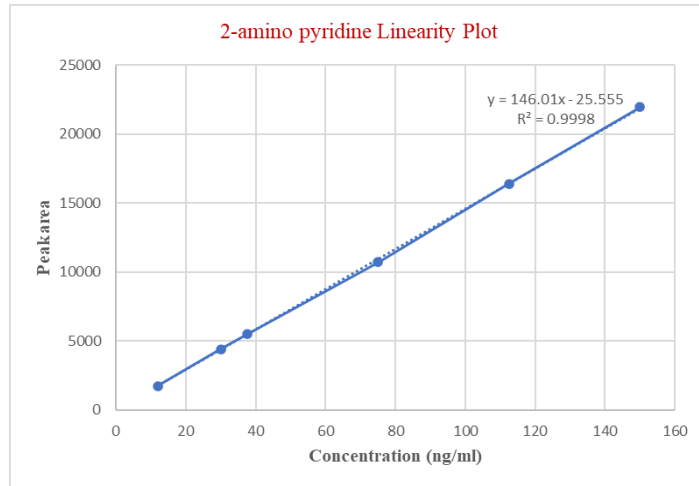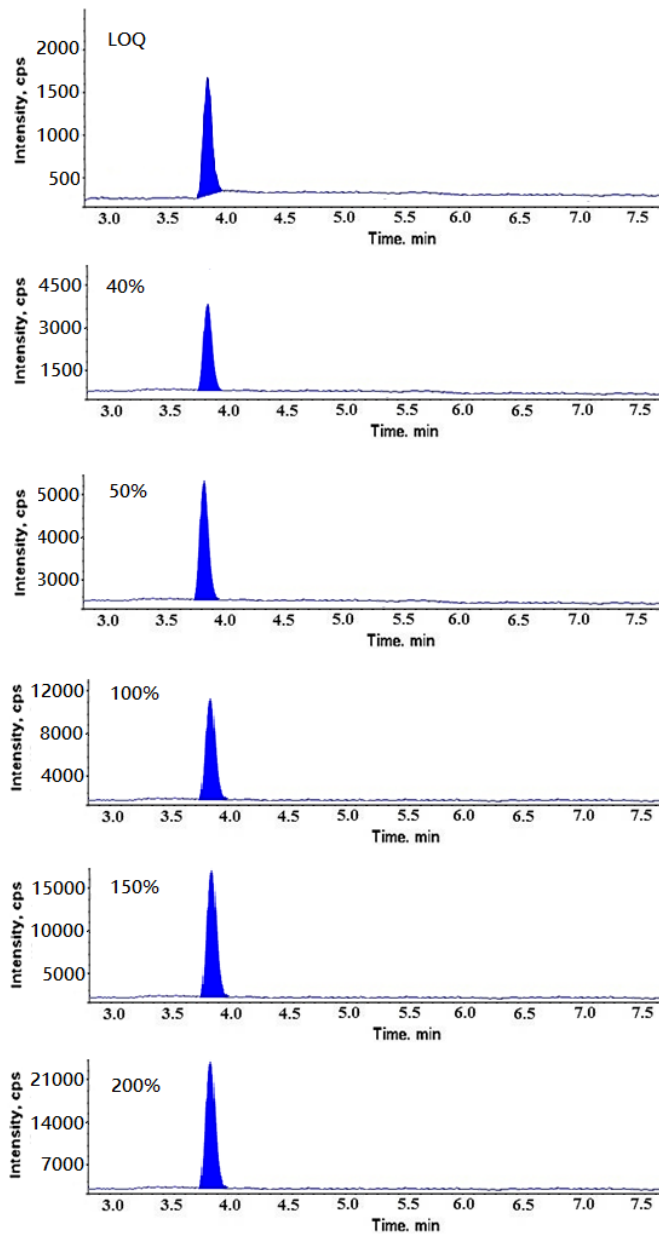| S. No.	2-aminopyridine	
	Concentration (ppm)	Area
1	6 ppm	1742
2	15 ppm	4415
3	18.75 ppm	5502
4	37.5 ppm	10723
5	56.25 ppm	16399
6	75 ppm	21952
Slope	146.01x	
Intercept	25.555	
Correlation Coefficient	0.9998	

Fig. 4: Linearity graph



Fig. 5: Linearity chromatograms

Accuracy

The accuracy was demonstrated by the percent recovery of 2-aminopyridine from the drug substance. Results displayed in table 2 and chromatograms showing accuracy depicted in fig. 6. Satisfactory recoveries of 95.2-101.8% for 6.0, 37.5 and 56.25 ppm (six determinations, %RSDs 1.27–3.4) were obtained which are satisfactory at such low levels.

Table 2: Accuracy of method for 2-aminopyridine

LOQ level				100% level			150% level		
Amount added (ng)	12	12	12	75	75	75	112.5	112.5	112.5
Amount found (ng)	11.66	12.18	11.62	75.22	74.85	73.42	109.68	110.475	114.52
% Recovery	97.2	101.5	96.9	100.3	99.8	97.9	95.2	99.7	101.8
AVG	98.53333333			99.33333333			98.9		
SD	2.573583753			1.266227994			3.37194306		
RSD	2.611891495			1.274726169			3.409446977		
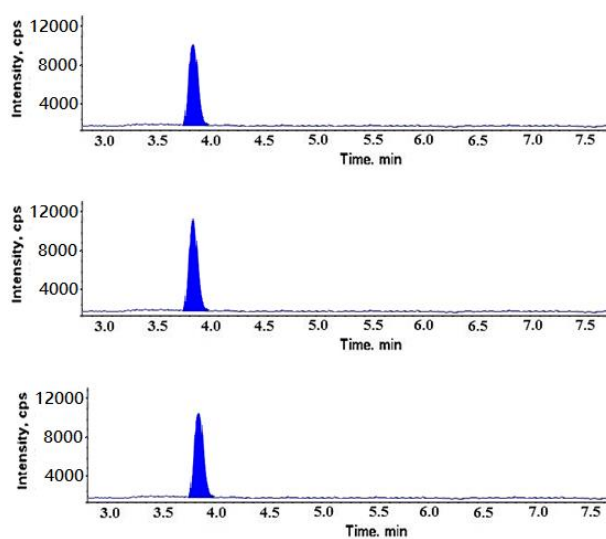
Fig. 6: Accuracy at 100% level
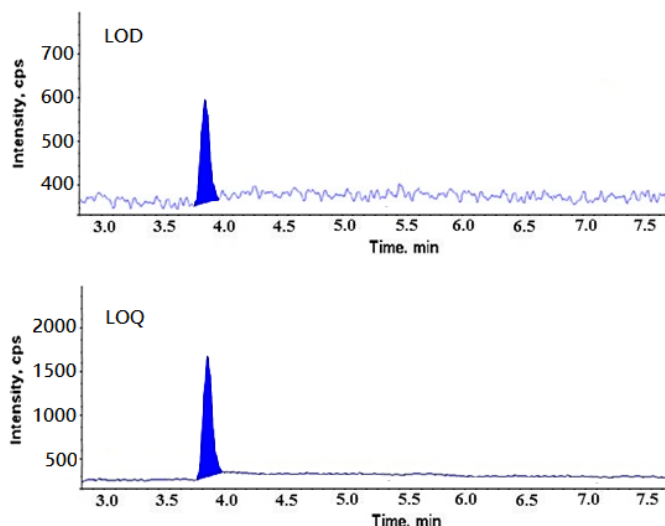
Fig. 7: Chromatograms of LOD and LOQ of 2-aminopyridine

Limit of quantification (LOD) and detection (LOQ)

The LOD and LOQ were calculated from S/N ratio data generated from six injections of 2-aminopyridine with respect to sample concentration of 2 mg/ml. Chromatograms of sensitivity study were displayed in fig. 7. The LOD and LOQ values observed for 2-aminopyridine were 3.75 ng/ml and 12 ng/ml.

Precision

The precision of the methods was checked by injecting 0.075 µg/ml solution for six times. The values of RSDs for areas of each 2-aminopyridine were calculated. The % relative standard deviation (%RSD) was found to be below 4% for both the analytes in system precision and the data were enumerated in

table 3 and chromatograms of precision study were presented in fig. 8.

Robustness

The robustness of the method was ensured by getting the resolution between analyte and drug substance to be greater than 2.0, when mobile phase flow rate (±0.2 ml/min), organic solvent ratio in both mobile phases A and B (±2%) and column temperature (±5 °C) were deliberately varied.

Solution stability

Stability studies were performed using primary standard solution (75 ng/ml) of 2-amino pyridine and spiked samples of piroxicam (2 mg/ml) with the analyte at 100% concentration levels up to 48 h at ambient laboratory temperature (25±5 °C) and refrigerated condition (2-8 °C). The percent recoveries of primary standard solution of 2-amino pyridine and spiked samples subjected to

stability studies were calculated by comparing against the freshly prepared primary standard solutions (75 ng/ml) of 2-amino pyridine.

Table 3: Precision results

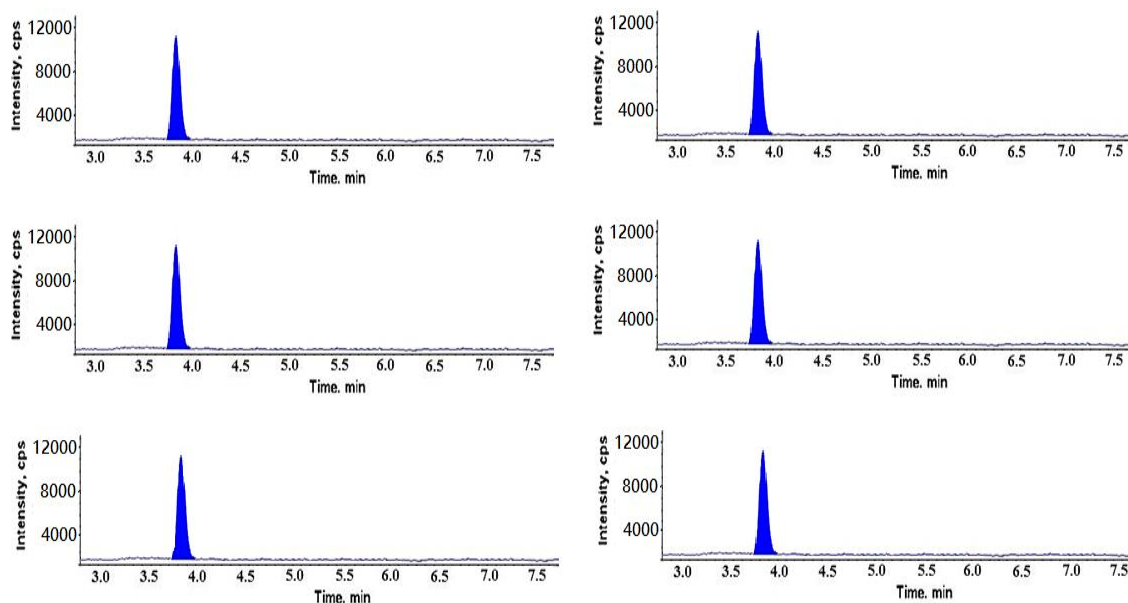| Injection ID	2-aminopyridine (0.075 µg/ml)
1	11129
2	11201
3	10862
4	10963
5	10567
6	11408
Mean	11021.66667
SD	292.7768206
% RSD	2.656375206
95 % Confidence interval	±101.3

Fig. 8: Chromatograms of precision study

DISCUSSION

In ensuring the safety and quality of pharmaceutical formulations, a thorough examination of genotoxic impurities (GTIs) is essential. The identification of 2-aminopyridine, a genotoxic impurity present in tenoxicam-a frequently utilized nonsteroidal anti-inflammatory drug-emphasizes the imperative for the development of a meticulous and highly sensitive analytical methodology. This investigative effort is focused on the refinement and validation of a liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach, aiming to detect and quantify the presence of 2-aminopyridine in Tenoxicam. The optimization and validation of this analytical method are pivotal steps towards ensuring the integrity and safety of pharmaceutical formulations containing Tenoxicam, thereby addressing the potential risks associated with genotoxic impurities.

Efficient sample preparation is crucial for trace-level analysis, impacting sensitivity, stability, recovery, and matrix effects. Acetonitrile was chosen over methanol for its superior analyte response, peak shapes, and recovery levels, enhancing the overall efficacy of the extraction process. A similar choice of acetonitrile is utilized for the sample preparation techniques by Gerd Vanhoenacker *et al.* [16] who reported superior analyte response, peak shape and extraction efficiency for the two analytes aryl amine and aminopyridine. The choice of column significantly influences resolution, especially in the context of high drug substance concentrations leading to broad peaks. LiChrospher 100 RP-18 column exhibited satisfactory response and separation, overcoming

limitations observed with other columns. The optimized mobile phase composition and gradient elution modes further contributed to achieving desirable resolution, with the retention time of 2-aminopyridine at approximately 3.8 min. In research conducted by Gyoergy Szekely and colleagues, they presented a method for developing LC-MS/MS for the trace analysis of the potentially genotoxic impurity, 4-dimethylaminopyridine, in glucocorticoids [17]. A crucial aspect of this method involved utilizing gradient elution as a critical process parameter. The application of gradient elution was found to be instrumental in achieving robust quantification of the analyte, particularly in trace levels.

LC-MS/MS was selected over HPLC-UV owing to its heightened sensitivity and specificity, deemed essential for meticulous trace analysis. The employment of the multiple reaction monitoring mode (MRM) augmented specificity, notably utilizing 2-aminopyridine ion mass transactions for dependable quantification. A parallel LC-MS/MS methodology for quantifying Pyridine, 3-aminopyridine, 4-dimethylaminopyridine, and N, N-dimethylamine in Alogliptin was previously elucidated by Bashar Al-Sabti *et al.* [18]. Additionally, Al-Sabti *et al.* also reported LC-MS/MS methods for quantifying pyridine, 4-dimethylaminopyridine, and N, N-dimethylaniline impurities in vildagliptin [19]. Chromatograms displayed in fig. 2 and 3 validate the effectiveness of the chosen LC-MS/MS method.

The validation of the newly developed LC-MS/MS method followed ICH guidelines, covering specificity, accuracy, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, and

robustness. Specificity was confirmed by the retention time and analyte response for 2-aminopyridine. The method demonstrated excellent linearity over a concentration range of 6 ppm to 75 ppm, with correlation coefficients exceeding 0.998. Accuracy, precision, and robustness were well-established, as indicated by recovery percentages, %RSD values, and deliberate variations in method parameters, respectively.

CONCLUSION

In conclusion the developed method is a direct tandem mass spectrometric method for screening and quantification of 2-aminopyridine in the Tenoxicam drug substances. Multiple reaction monitoring (MRM) mode relatively provided better selectivity and sensitivity for the screening and quantitation of the analyte. The described analytical method is cost-effective, direct, accurate and convenient quality control tool for determination of 2-aminopyridine in Tenoxicam. The method is advantageous owing to its improved sensitivity and simpler sample preparation technique to those formerly reported methods. Multiple reaction monitoring (MRM) mode consents radically discounts or eliminates the matrix effects that restricts the accuracy and LOD and LOQ levels. This method can be further studied for its application to other drug substances.

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Nil

AUTHORS CONTRIBUTIONS

All authors have contributed equally

CONFLICT OF INTERESTS

Declared none

REFERENCES

1. Madni MA, Raza A, Abbas S, Tahir N, Rehman M, Kashif PM. Determination of tenoxicam in the plasma by reverse phase HPLC method using single step extraction technique: a reliable and cost effective approach. Acta Pol Pharm. 2016 Sep;73(5):1123-8. PMID 29638052.
2. Abdel Hamid ME. LC-MS analysis of selected sulfur-containing non-steroid anti-inflammatory agents: applications to pharmaceutical products. J Liq Chromatogr Relat Technol. 2000;23(20):3095-107. doi: 10.1081/JLC-100102370.
3. AMIN AS. Spectrophotometric determination of piroxicam and tenoxicam in pharmaceutical formulations using alizarin. J Pharm Biomed Anal. 2002;29(4):729-36. doi: 10.1016/s0731-7085(02)00035-3, PMID 12093502.
4. Ji HY, Lee HW, Kim YH, Jeong DW, Lee HS. Simultaneous determination of piroxicam, meloxicam and tenoxicam in human plasma by liquid chromatography with tandem mass spectrometry. J Chromatogr B. 2005;826(1-2):214-9. doi: 10.1016/j.jchromb.2005.08.023.
5. Raghavan CV, Abimon VD. Intranasal delivery of tenoxicam in rat. Int J Pharm. 2001;221(1-2):227-9. doi: 10.1016/s0378-5173(01)00635-4, PMID 11397584.
6. Stebler T, Guentert TW. Bioavailability of intramuscularly administered tenoxicam. Biopharm Drug Dispos. 1993;14(6):483-90. doi: 10.1002/bdd.2510140604, PMID 8218966.
7. Keck PE, Orsulak PJ, Cutler AJ, Sanchez R, Torbeyns A, Marcus RN. Aripiprazole monotherapy in the treatment of acute bipolar I mania: a randomized, double-blind, placebo- and lithium-controlled study. J Affect Disord. 2009;112(1-3):36-49. doi: 10.1016/j.jad.2008.05.014, PMID 18835043.
8. Pierson DA, Olsen BA, Robbins DK, DeVries KM, Varie DL. Approaches to assessment, testing decisions, and analytical determination of genotoxic impurities in drug substances. Org Process Res Dev. 2009;13(2):285-91. doi: 10.1021/op8002129.
9. Miller JA, Miller EC. Ultimate chemical carcinogen as reactive mutagenic electrophiles. In: Origin of human cancers. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1977. p. 605-7.
10. Committee for Medicinal Products for Human Use (CHMP). Safety Working Party (SWP). Questions and answers on the 'guideline on the limits of genotoxic impurities'; EMA/CHMP/SWP/431994/2007 Rev. 3. London: European Medicines Agency; 2010.
11. Snodin DJ. Genotoxic impurities: from structural alerts to qualification. Org Process Res Dev. 2010;14(4):960-76. doi: 10.1021/op100118e.
12. Kroes R, Renwick AG, Cheeseman M, Kleiner J, Mangelsdorf I, Piersma A. Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet. Food Chem Toxicol. 2004;42(1):65-83. doi: 10.1016/j.fct.2003.08.006, PMID 14630131.
13. Oshiro Y, Sato S, Kurahashi N. Carbostyril derivatives. U.S. Patent 5,006,528; 1991.
14. Guideline on the limits of genotoxic impurities. Committee for Medicinal Products for Human Use (CHMP). London: European Medicines Agency (EMEA): (CPMP/SWP/5199/02. EMEA/CHMP/QWP/251344/2006); 2006.
15. Genotoxic and carcinogenic impurities in drug substances and products: recommended approaches. Silver Spring, MD, USA: United States Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER); 2008.
16. Vanhoenacker G, Dumont E, David F, Baker A, Sandra P. Determination of arylamines and aminopyridines in pharmaceutical products using in-situ derivatization and liquid chromatography–mass spectrometry. J Chromatogr A. 2009 Apr;1216(16):3563-70. doi: 10.1016/j.chroma.2008.08.102, PMID 18804773.
17. Szekely GY, Henriques B, Gil M, Ramos A, Alvarez C. Design of experiments as a tool for LC–MS/MS method development for the trace analysis of the potentially genotoxic 4-dimethylaminopyridine impurity in glucocorticoids. J Pharm Biomed Anal. 2012 Nov;70:251-8. doi: 10.1016/j.jpba.2012.07.006, PMID 22841555.
18. Al-Sabti B, Harbali J. HPLC-MS analysis of four potential genotoxic impurities in alogliptin pharmaceutical materials. J AOAC Int. 2022 Mar 15;105(2):362-9. doi: 10.1093/jaoacint/qsab152, PMID 34849990.
19. Al-Sabti B, Harbali J. Development and validation of an analytical method for quantitative determination of three potentially genotoxic impurities in vildagliptin drug material using HPLC-MS. J Sep Sci. 2021 Jul;44(13):2587-95. doi: 10.1002/jssc.202100136, PMID 33934507.