Nat. Volatiles & Essent. Oils, 2022; 9(1): 1809-1822

NVEO
Natural Volatiles &
Essential Oils

Rapid And Trace Level Determination Of Potential Genotoxic Imputity 2-Aminopyridine In Piroxicam Api Using Lc-Ms/Ms Technique

Manoj Kumar Rathore[1*] , T. Rama Mohan Reddy[2]

[1*]Research Scholar, Career Point University, Kota, Rajasthan-325003, India.

[2]Research Supervisor, Career Point University, Kota, Rajasthan-325003, India.

ABSTRACT

A highly sensitive method for the determination of genotoxic impurity 2-aminopyridine in Piroxicam employing hyphenated techniques have been presented. 2-aminopyridine was determined by LC-MS/MS method in selected ion monitoring mode (SIM) using LiChrospher RP-18 (100×4.6mm) 5.0 µm column. Gradient technique was applied for the elution of analytes using acetonitrile (mobile phase A) and 0.01M ammonium acetate buffer (mobile phase B) in different 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. Developed method was validated as per International Conference on Harmonization guidelines. The LOD and LOQ values found for 2-aminopyridine were 5.25 ppm and 75 ppm. Method has accuracy within 97 -103.4% for the analyte. This method is a good quality control tool for quantitation of 2-aminopyridine impurity at very low levels in Piroxicam.

Keywords: Piroxicam, 2-aminopyridine, Genotoxic impurities, Selected Ion Monitoring (SIM), ICH guidelines.

INTRODUCTION

Piroxicam (Figure 1a) is a non-steroidal anti-inflammatory drug (NSAID) widely used against pain and inflammation. Piroxicam (4-hydroxy-2-methyl-3-(pyrid-2-yl-carbamoyl)-2H-1,2-benzothiazine 1,1-dioxide) belongs to the oxicam class of NSAIDs [1–5].

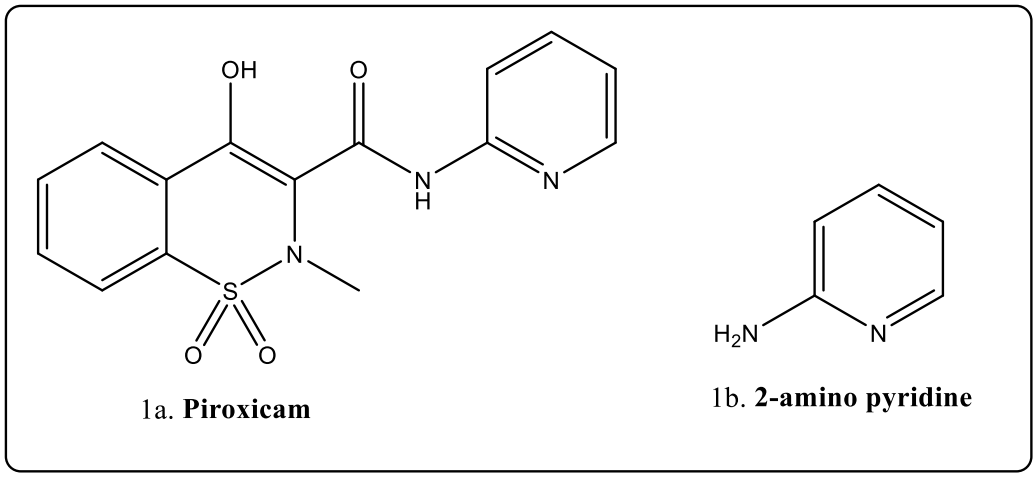

Figure 1. a) Piroxicam b) 2-aminopyridine

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 [6]. 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 to eliminate them from the final drug product [7]. 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 [8].

Hetero aromatic amines especially aminopyridines are generally employed in the synthetic process as building blocks and are categorized as potentially genotoxic impurities (PGIs) in pharmaceuticals [9]. Inherently aromatic amines genotoxicity is not owing to their reactivity but due to the generation of nitrenium ion ($Ar\text{-}N^{+}H$) 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. Heteroaryl amines possess the regulatory limit of intake at 1.5µg/day [11]. A few synthetic processes have been reported for the piroxicam which utilize the genotoxic substances as starting materials as well as intermediates. The condensation of 2-aminopyridine which is genotoxic in nature, with methyl 4-hydroxy-2-methyl-2H-benzo[e][1,2]thiazine-3-carboxylate 1,1-dioxide, finally produces the piroxicam [12,13].

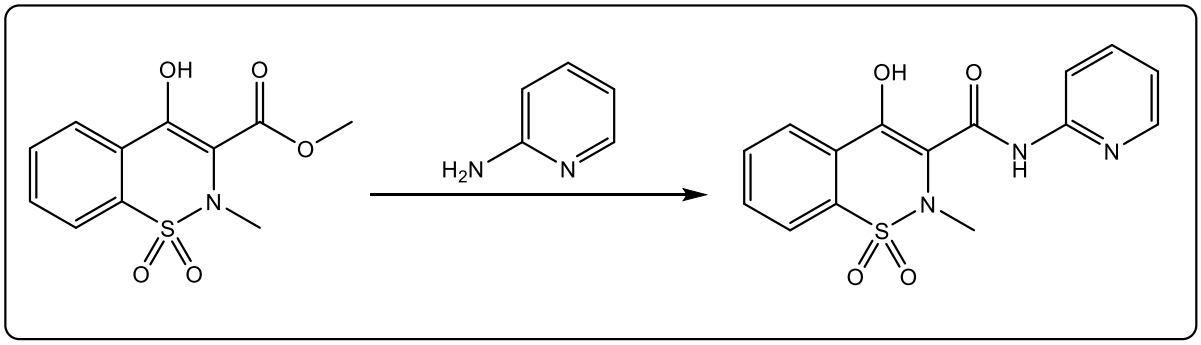

Figure 2: Final step in the synthesis of Piroxicam with condensation of 2-aminopyridine

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/day 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-aminopyridine in very low levels in Piroxicam.

EXPERIMENTAL

Materials

2-aminopyridine was procured from Sigma Aldrich, Bangalore, India. Analytical grade ammonium acetate and acetonitrile were purchased from Merck, India. Piroxicam sample was procured from Shreeji Pharma, India.

Chromatographic conditions

The chromatographic system used was Shimadzu LCMS 8040. The analytical column was LiChrospher RP-18 (100×4.6mm) 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 1.0mL/min. Column oven temperature and auto sampler temperature were set as 30^0C and 25^0C, 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/Hr, gas flow rate 50 L/Hr; 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 6 mins to 11 mins.

Validation study

Ich guideline were followed for the validation of the developed LC-MS/MS method for the determination of 2-aminopyridine in Piroxicam. By analysing the six concentrations of analyte from 0.3ppm -7.5ppm, 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 EMS 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 1mL of the 7.5 mg/mL solution to 100mL with acetonitrile. From this diluted stock solution, working standard solution of 37.5ppm strength with respect to the sample concentration of 2mg/mL of Piroxicam 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.

Sample preparation

Piroxicam sample solution of 10mg/ml concentration was prepared before injection into system by dissolving about 10mg of the drug substance with solvent in a HPLC vial.

RESULTS AND DISCUSSION

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 Piroxicam were achieved on the LiChrospher 100 RP-18 column of dimensions 100mm×4.6mminternal diameter, 5.0µm. 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 30^0C and 1.0mL/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 3.5 mins.

Piroxicam peak eluted at around 8 mins. 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 Figure 2 & 3.

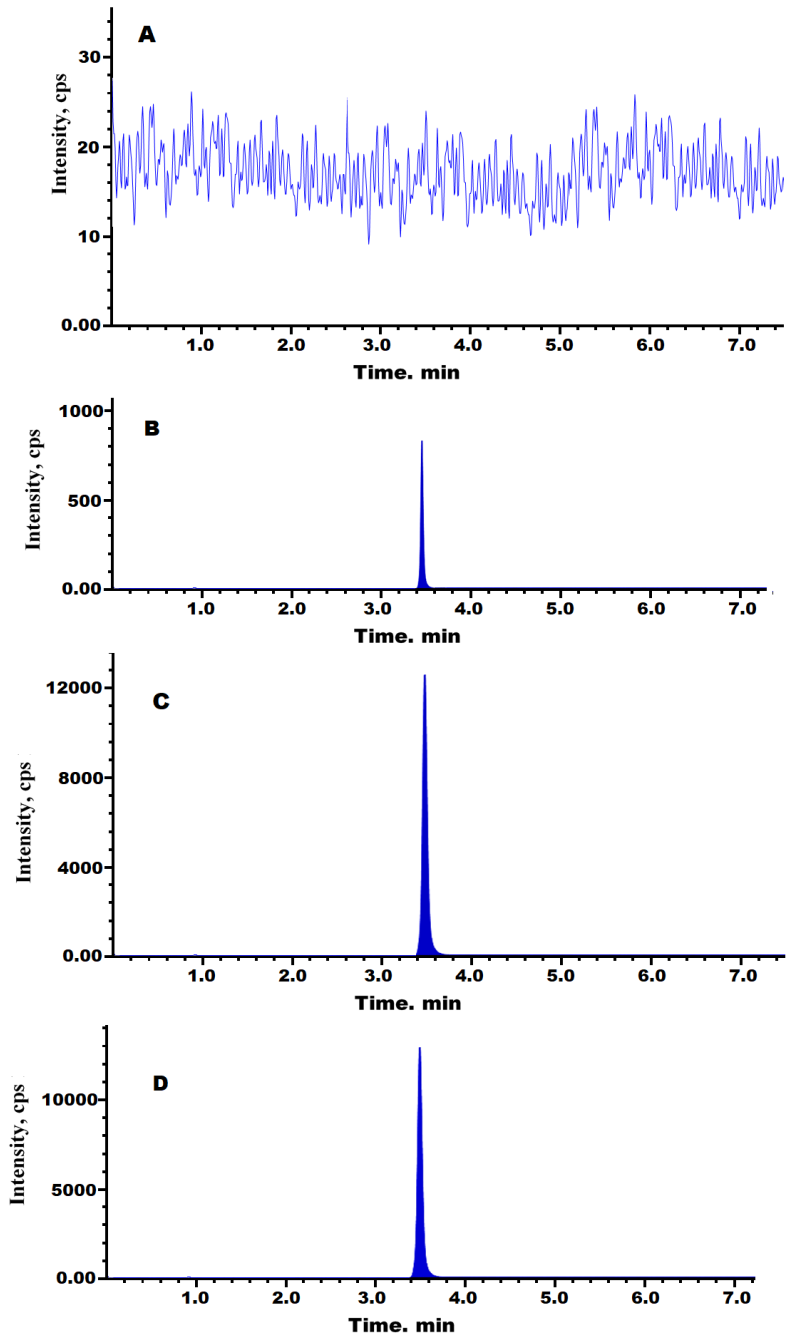

Figure 2: Chromatograms of 2-aminopyridine using SIM scan. (A) Chromatogram of blank, (B) Chromatogram of 2-aminopyridine in Piroxicam sample, (C) Chromatogram of standard containing 37.5ppm of 2-aminopyridine, (D) Chromatogram of Piroxicam sample spiked with 2-aminopyridine at 37.5ppm.

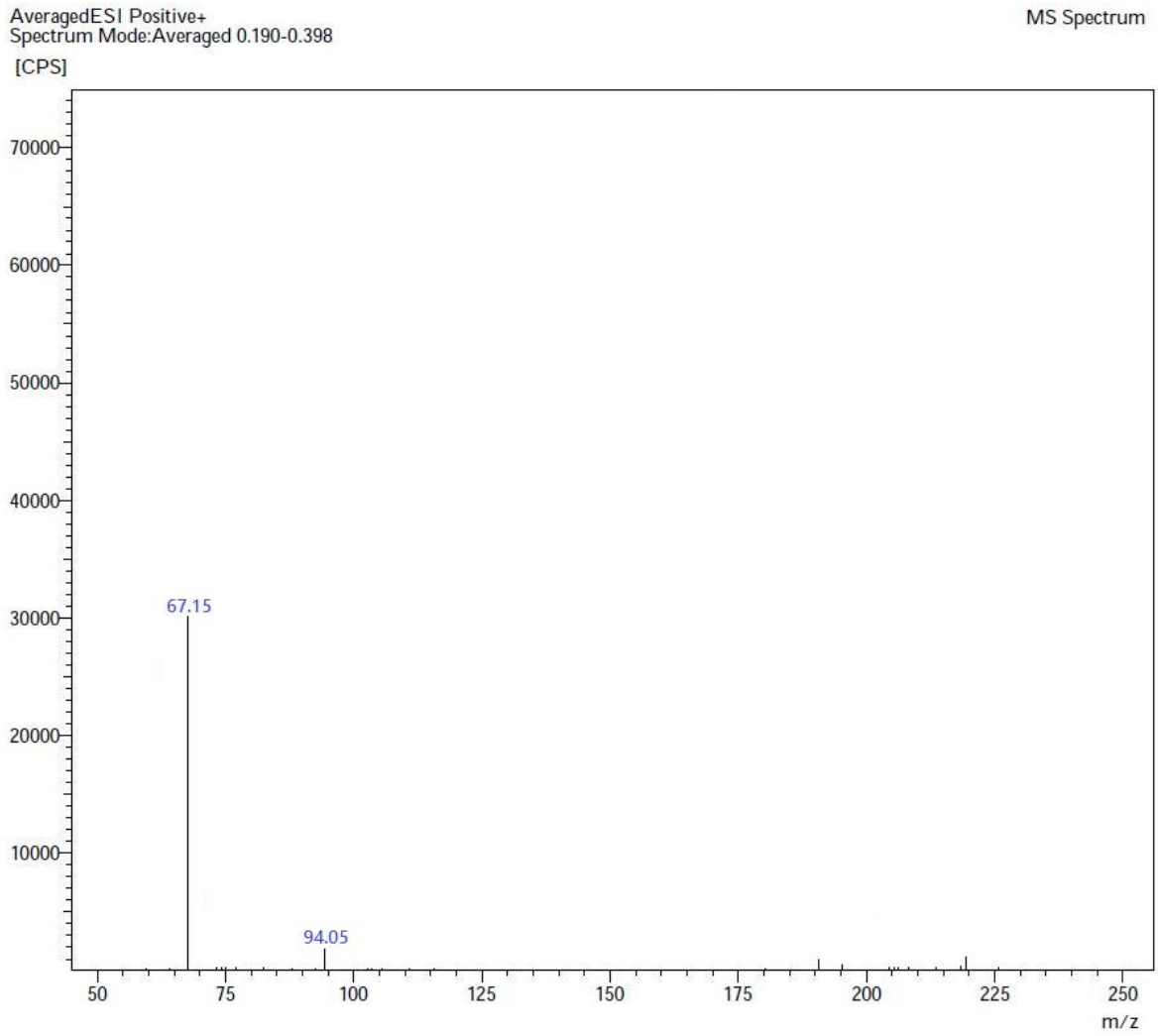

Figure 3: MRM Chromatogram of 2-aminopyridine

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 3.5 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 5.25ppm and 75ppm corresponding to the concentration of 20 mg/mL of Piroxicam. Correlation coefficients for all

analytes were >0.998. Linearity results enumerated in Table 1 and Figure 4 & 5 depict the linearity graph and Chromatogram respectively.

Table 1: Linearity of 2-aminopyridine

S. No	2-aminopyridine	
	Concentration (ppm)	**Area**
1	5.25ppm	1492
2	15ppm	4227
3	18.75ppm	5281
4	37.5ppm	10589
5	56.25ppm	15399
6	75ppm	21083
Slope	139.22x	
Intercept	37.766	
Correlation Coefficient	0.9995	

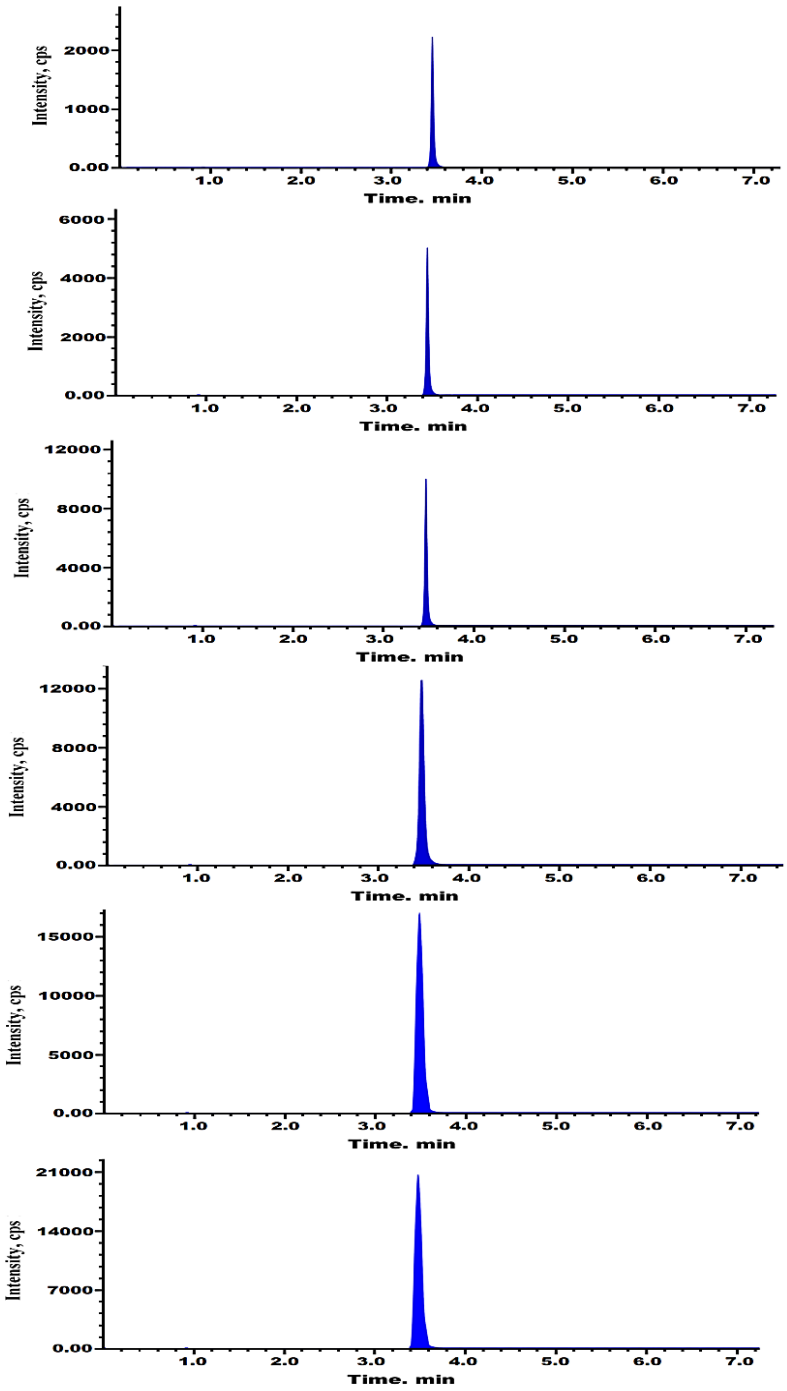

Figure 4: Linearity Chromatograms

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 Figure 6. Satisfactory recoveries of 97.9-103.8% for 5.25, 37.5 and 56.25 ppm (six determinations, %RSDs 1.2 – 2.25) 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)	10.5	10.5	10.5	75	75	75	112.5	112.5	112.5
Amount found (ng)	10.42	10.89	10.66	73.4	75.1	73.8	109.6	110.4	114.5
% Recovery	99.3	103.8	101.6	97.9	100.2	98.4	97.5	98.2	101.8
AVG	101.5666667			98.83333333			99.16666667		
SD	2.250185178			1.209683154			2.307234997		
RSD	2.215476053			1.223962719			2.326623527		

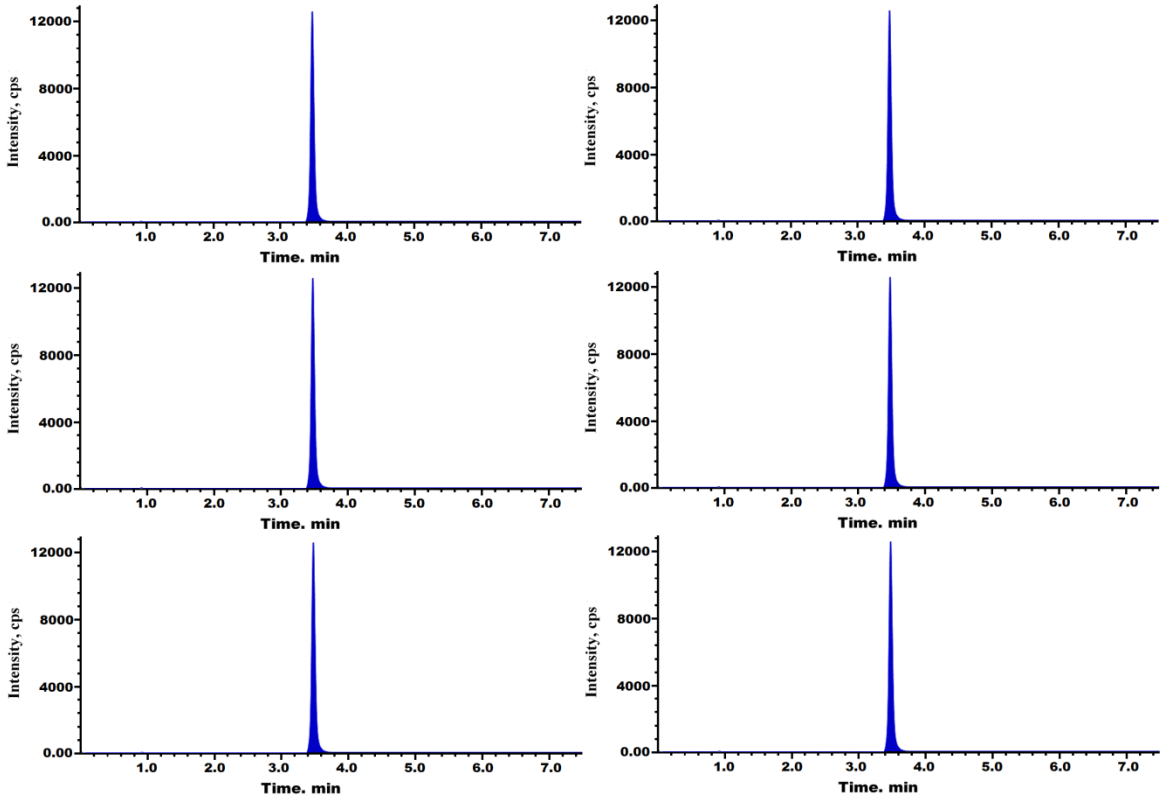

Figure 6: Accuracy at 100% level

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 10 mg/mL. Results disclosed in Table 3 and chromatograms displayed in Figure 7 & 8. The LOD and LOQ values observed for 2-aminopyridine were 0.003 µg/mL and 0.0105 µg/mL.

Table 3: LOD, LOQ Precision

Injection ID	**2-aminopyridine**	
	LOD (0.003 µg/mL) area	**LOQ (0. 0105 µg /mL) area**

1	451	827
2	460	890
3	409	810
4	431	874
5	439	880
6	416	886
Mean	434.3333333	861.1666667
SD	18.01542549	30.96458551
% RSD	4.147833958	3.595655371

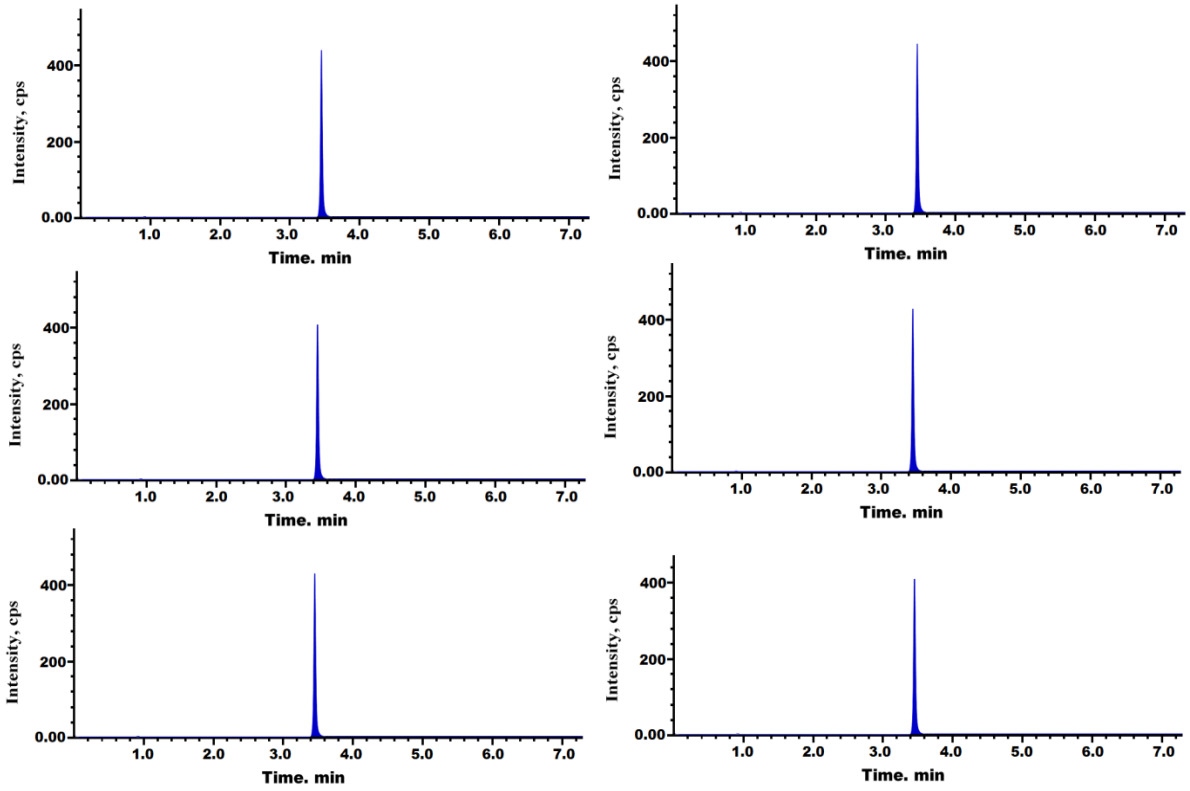

Figure 7: LOD Precision

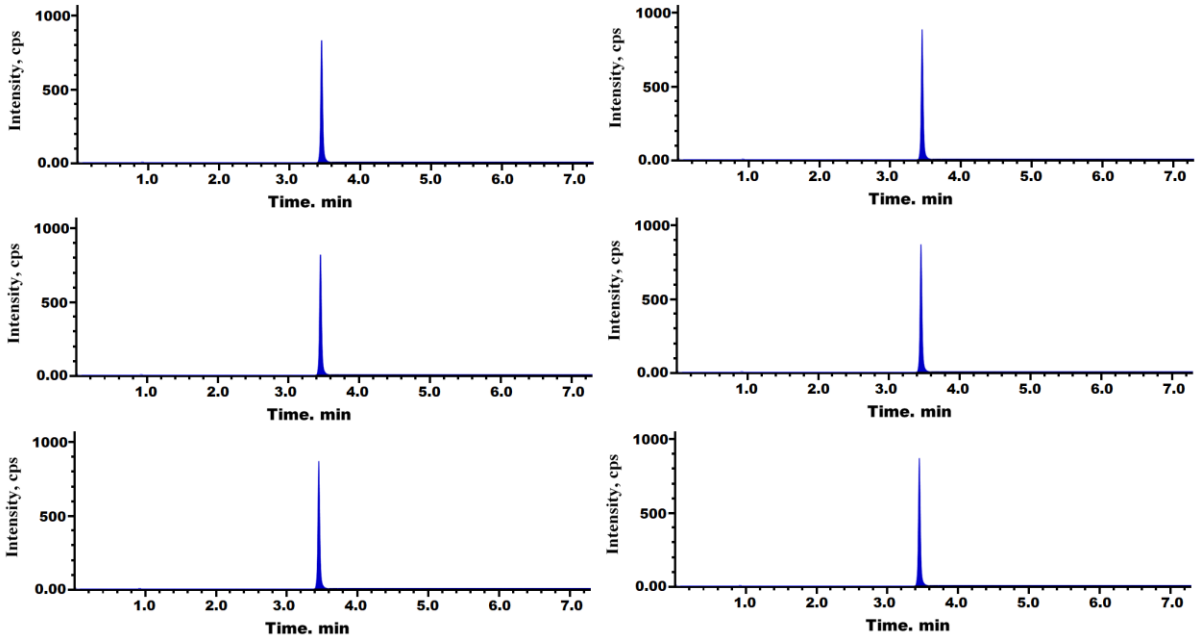

Figure 8: LOQ Precision

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 4.

Table 4. System precision

Injection ID	2-aminopyridine (0.075 µg/mL)
1	12849
2	12598
3	12745
4	11986
5	11890
6	12082
Mean	12358.33
SD	383.413
% RSD	3.102466
95 % Confidence interval	±102.5

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^{0}C) were deliberately varied.

CONCLUSION

In conclusion the developed method is a direct tandem mass spectrometric method for screening and quantification of 2,6 dichloroaniline in the Piroxicam drug substances. Selected ion monitoring (SIM) 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 Piroxicam. The method is advantageous owing to its improved sensitivity and simpler sample preparation technique to those formerly reported methods. Selected ion monitoring (SIM) 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.

REFERENCES

1. Sharif ZA, Lieberman RA, Schatzberg AF, Nemeroff CB. Textbook of Psychopharmacology. The American Psychiatric Publishing American Psychiatric Pub. Inc. Washington, DC 2009; 4: 613–625.
2. Kwon JS, Jang JH, Kang DH et al. Long-term efficacy and safety of piroxicam in patients with schizophrenia, schizophreniform disorder, or schizoaffective disorder: 26-week prospective study. Psychiatry and Clinical Neurosciences2009; 63: 73–81.
3. Keck PE, Marcus R, Tourkodimitris S, Ali M, Liebeskind A, Saha A, et al. A placebo-controlled, double-blind study of the efficacy and safety of piroxicam in patients with acute bipolar mania. American Journal of Psychiatry 2003; 160:1651-8.
4. Sachs G, Sanchez R, Marcus R, Stock E, McQuade R, Carson W, et al. Piroxicam in the treatment of acute manic or mixed episodes in patients with bipolar I disorder: A 3-week placebo-controlled study. Journal of Psycho-pharmacology (Oxford) 2006; 20:536-46.
5. Vieta E, Tjoen C, McQuade RD, Carson WH, Marcus RN, Sanchez R et al.Efficacy of adjunctive piroxicam to either valproate or lithium in bipolar mania patients partially nonresponsive to valproate/ lithium monotherapy: A placebo-controlled study. American Journal of Psychiatry 2008; 165:1316-25.
6. Keck PE, Orsulak PJ, Cutler AJ, Sanchez R, Torbeyns A, Marcus RN et al. Piroxicam monotherapy in the treatment of acute bipolar I mania: A randomized, double-blind, placebo- and lithium-controlled study. Journal of Affective Disorders 2009; 112:36-49.
7. Pierson DA, Olsen BA, Robbins DK, DeVries KM, Varie DL. Approaches to Assessment, Testing Decisions, and Analytical Determination of Genotoxic Impurities in Drug Substances. Organic Process Research Development 2009; 13: 285−291.
8. Miller JA, Miller EC. Ultimate Chemical Carcinogen as Reactive Mutagenic Electrophiles. In Origin of Human Cancers. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1977; 605-607.

9. 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; European Medicines Agency: London, 2010.
10. Snodin DJ. Genotoxic Impurities: From Structural Alerts to Qualification. Organic Process Research Development 2010; 14: 960−976.
11. Kroes R, Renwick AG, Cheeseman M, Kleiner J, Mangelsdorf I et al. Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet. Food and Chemical Toxicology2004; 42: 65–83.
12. OshiroY, Sato S, Kurahashi N. Carbostyril derivatives. U.S. Patent 5,006,528, April 09, 1991.
13. Guideline on the Limits of Genotoxic Impurities, Committee for Medicinal Products for Human Use (CHMP), European Medicines Agency (EMEA), London, 28 June, 2006 (CPMP/SWP/5199/02, EMEA/CHMP/QWP/251344/2006).
14. Genotoxic and carcinogenic impurities in drug substances and products: recommended approaches. U.S. Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Silver Spring, MD, USA, December 2008.