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RAPID AND TRACE LEVEL DETERMINATION OF POTENTIAL GENOTOXIC IMPURITY 2-AMINOPYRIDINE IN PIROXICAM API USING LC-MS/MS TECHNIQUE

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Abstract:

A sensitive genotoxic impurity detection technique Hyphenated 2-aminopyridine in Piroxicam is given. 2-aminopyridine was measured using LiChrospher RP-18 (100×4.6mm) 5.0 µm column in selected ion monitoring mode (SIM) in LC-MS/MS. Analytes were eluted using acetonitrile (mobile phase A) and 0.01M ammonium acetate buffer (mobile phase B) in varied ratios utilizing gradient method. The gradient program (T/%B) was 0/5, 2.50/15, 5.00/30, 10.00/50, 15.00/95, 20.00/95. The validated technique adhered to the guidelines set forth by the International Conference on Harmonization.It was determined that the LOD and LOQ values for 2-aminopyridine were 5.25 ppm and 75 ppm, respectively. For the analyte, the method has an accuracy that ranges from 97 to 103.4%. Quantitation of 2-aminopyridine impurity at extremely low levels in piroxicam may be accomplished with the use of this approach, which is an effective quality control tool.

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

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I. 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].

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]. A variety of alterations connected to the synthetic process are frequently utilized in order to eradicate these impurities; yet, it has become hard to totally eliminate the impurities from the drug compounds that are ultimately produced. In accordance with the recommendations made by the authorities in charge of drug regulation, it is of the utmost importance to restrict the amount of genotoxic contaminants that are present in the drug substances depending on the daily dose [8].

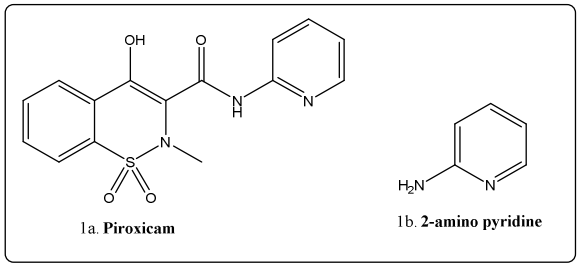

Fig. **1** a) Piroxicam b) 2-aminopyridine

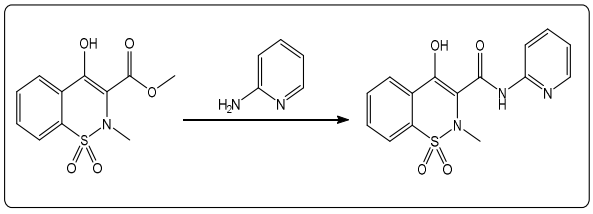

Fig. 2 Final step in the synthesis of Piroxicam with condensation of 2-aminopyridine

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-N+H) by the oxidative metabolic reactions, which is considered to be the active genotoxin that binds to DNA [10]. Polycyclic aromatic hydrocarbons (PGIs) have been shown to cause chromosomal abnormalities or genetic changes in rats and mice, and they are also known to be carcinogenic. According to reference [11], the regulatory limit of consumption for heteroaryl amines is set at 1.5µg per day. There have been a few synthetic procedures that have been described for the synthesis of piroxicam. These techniques make use of the genotoxic chemicals neither as starting materials nor as intermediates. In the end, the genotoxic compound known as 2-aminopyridine is eventually converted into piroxicam by the condensation of methyl 4-hydroxy-2-methyl-2H-benzo[e][1,2]thiazine-3-carboxylate 1,1-dioxide [12,13].

European Medicines Agency's (EMEA) Committee for Medicinal products for Human use (CHMP) has published guidelines regarding limits of genotoxic impurities13. 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.

II. MATERIAL AND METHODS

Materials

2-aminopyridine was procured from Sigma Aldrich, Bangalore, India. Analytical grade acetonitrile and ammonium acetatewere purchased from Merck, India. Piroxicam sample was procured from ShreejiPharma, 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 50.0 L/Hr; Argon gas as CID gas for MS/MS experiment. The selective ion monitoring (SIM) was selected for quantification of analyte. Venting was done using valco valve (Valco Instruments Co. Inc., VICI AG International). Six minutes to eleven minutes were allotted for venting.

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. Utilizing six repeated infusions of standard solution, the precision of the mass spectrometric system was determined. In order to ascertain the LOQ and LOD with precision, six replicate injections of the analyte at lesser concentrations were performed. 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). To assess the precision of the method, the %RSD of each analyte was calculated after being spiked. 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.

III. RESULTS AND DISCUSSIONS

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. The solubilization capacities of both solvents were found to be adequate for both the analyte and drug constituents. However, acetonitrile was ultimately selected due to its superior analyte response, well-defined peaks, and high recovery levels.

Column selection and separation

Selection of appropriate column has a huge impact on the resolution of analyte and drug substance peak. In order to attain the desired resolution for trace level analysis of the GTI, it is critical to carefully choose the suitable column, given that the drug substance concentration was substantial, resulting in a broad peak. Differently sized columns, including Phenomenex Luna C18, Kromasil C18, and LiChrospher 100 RP-18, were assessed. 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. A toggling valco valve was utilized to allow entry of the analyte, specifically the 2-aminopyridine peak, into the mass detector. This valve also facilitated the expulsion of the 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. In contrast, for the trace analysis of 2-aminopyridine, the LC-MS/MS method was selected over HPLC–UV due to the enhanced sensitivity and specificity provided by mass spectrometric detection. Furthermore, the analysis was conducted in multiple reaction monitoring mode (MRM), which further augmented the determination's specificity. 2-aminopyridine ion mass transactions corresponding to 94 > 67 were recorded for the quantification. The LC-MS/MS chromatograms are shown in Figure 3 & 4.

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), precision, limit of quantitation (LOQ), and robustness in order to establish the feasibility of the technique.

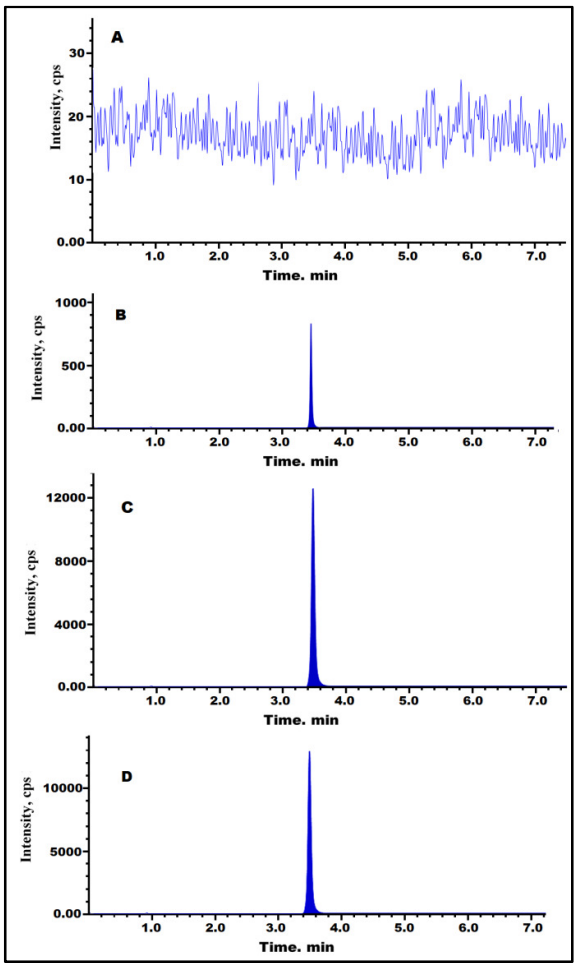

Fig. 3 Chromatograms of 2-aminopyridine using SIM scan. (A) Chromatogram of blank, (B) Chromatogram of 2-aminopyridine in Piroxicam sample, (C) Chromatogram of standard containing 37.5ppm of 2-aminopyridine, (D) Chromatogram of Piroxicam sample spiked with 2-aminopyridine at 37.5ppm.

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.

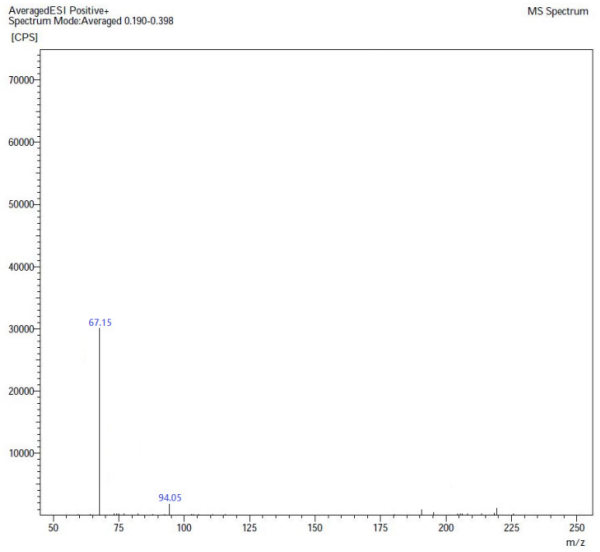

Fig. 4 MRM Chromatogram of 2-aminopyridine

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 5 & 6 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	

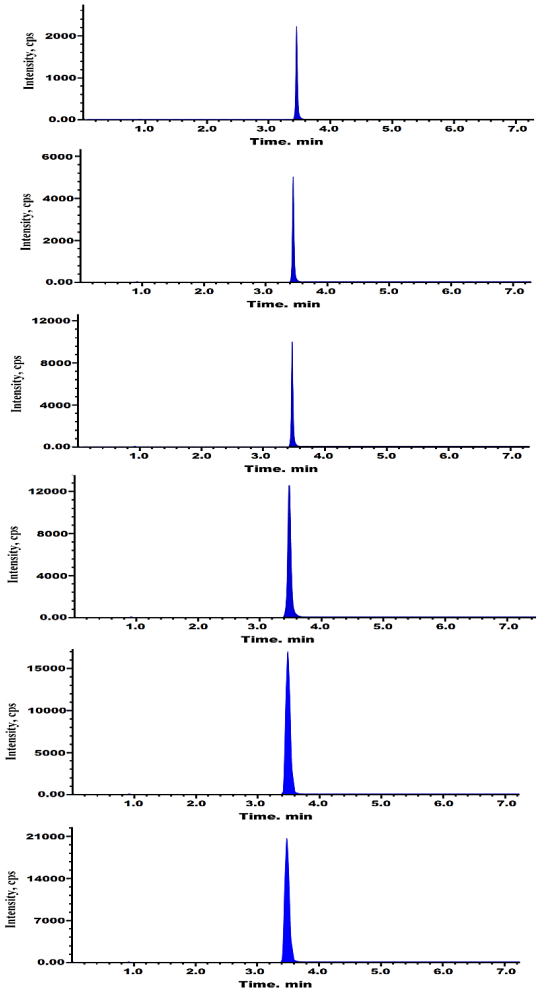

Fig. 5 Linearity Chromatograms

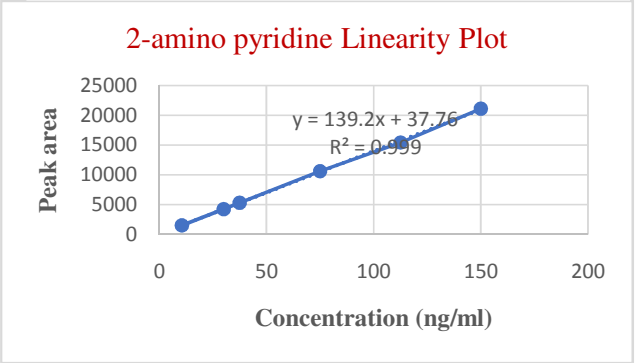

Fig. 6Linearity graph

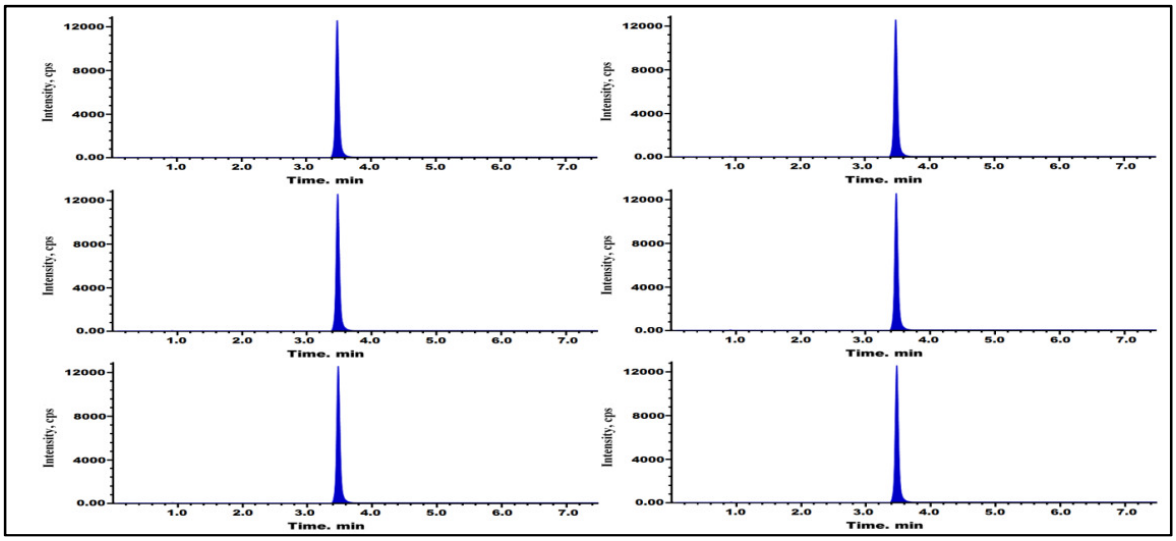

Fig. 7Accuracy at 100% level

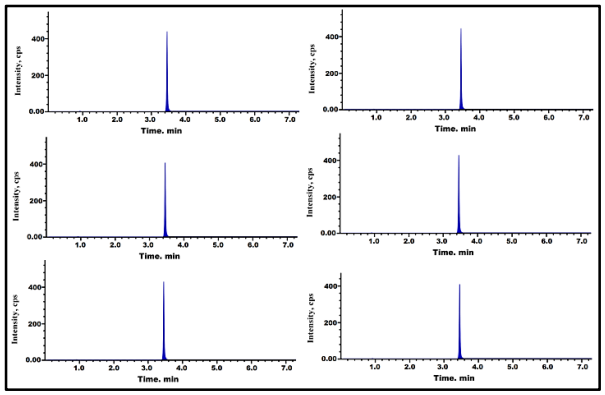

Fig. 8 LOD Precision

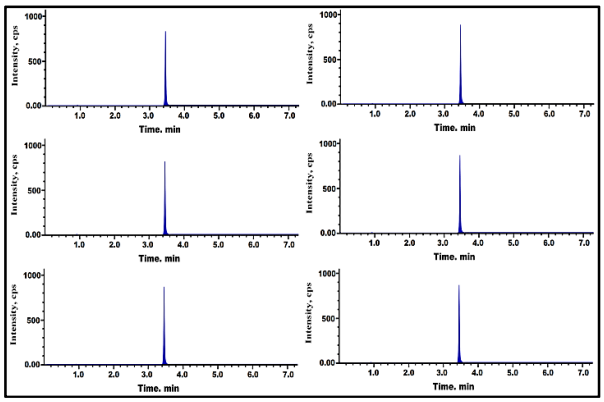

Fig. 8 LOQ precision

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 7. 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.

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 8 & 9. The LOD and LOQ values observed for 2-aminopyridine were 0.003 µg/mL and 0.0105 µg/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 4.

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		

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

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.

IV. CONCLUSION

In conclusion the developed method is a direct tandem mass spectrometric method for screening and quantification of 2,6dichloroaniline 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 eliminate or drastically reduce the matrix effects that impede precision and LOQ and LOD levels. Additional research can be conducted to explore the potential of this method on other pharmaceutical substances.

Conflicts of interest:

Declared None.

Acknowledgments:

None

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