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Trace Level Determination of Potential Genotoxic Impurity (2, 4-Dichloro-5-Methoxyaniline) In Drug Substance

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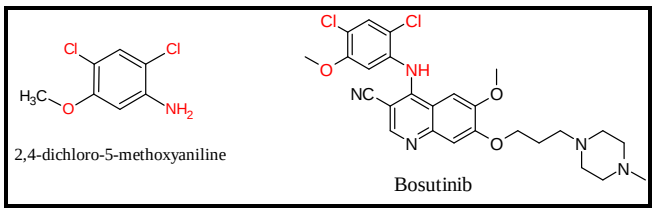
ABSTRACT

An analytical method has been developed for trace level determination of 2,4-dichloro-5-methoxyaniline (potential genotoxic impurity) in drug substances at pharmaceutical industry. The accurate quantitation of 2,4-dichloro-5-methoxyaniline was achieved on Hypersil BDS C18 column (150mm x 4.6mm, 3.0µm) with gradient elution at a flow rate of 1.0mL min^{-1}. Gradient elution containing mobile phase-A and mobile phase-B, 0.05% of Trifluoroacetic acid in water used as mobile phase-A and Acetonitrile used as mobile phase-B. The elution of 2,4-dichloro-5-methoxyaniline is monitored at 210nm, by using Ultra Visible / PDA detector at the level of 2.5 mg L^{-1}. The high correlation coefficient (R2>0.999) values indicated clear correlations between the investigated compound concentrations and their peak areas within the LOQ (limit of Quantitation) to 150% level. 2,4-dichloro-5-methoxyaniline is uses in manufacturing process of Bosutinib. Hence, 2,4-dichloro-5-methoxyaniline was major possible and potential genotoxic impurities of Bosutinib.

Graphical Abstract



Structure of 2,4-dichloro-5-methoxyaniline and Bosutinib.

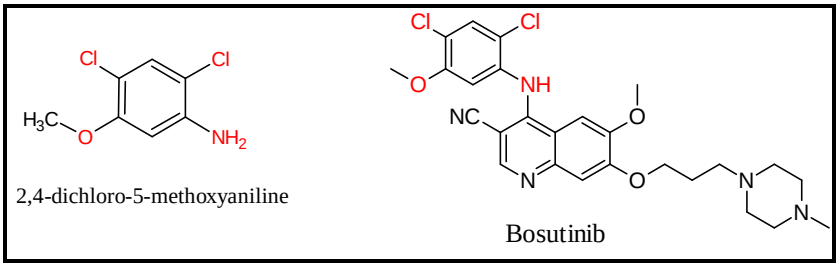
Keywords: 2,4-dichloro-5-methoxyaniline, 5-Amino-2,4-dichloroanisole, Genotoxic impurity, HPLC, Bosutinib.

INTRODUCTION

2,4-dichloro-5-methoxyaniline is the process possible genotoxic impurities of Bosutinib. This compound is using as one of the key starting material (KSM) in the manufacturing process of Bosutinib. Which is active moiety in the molecule is Bosutinib.

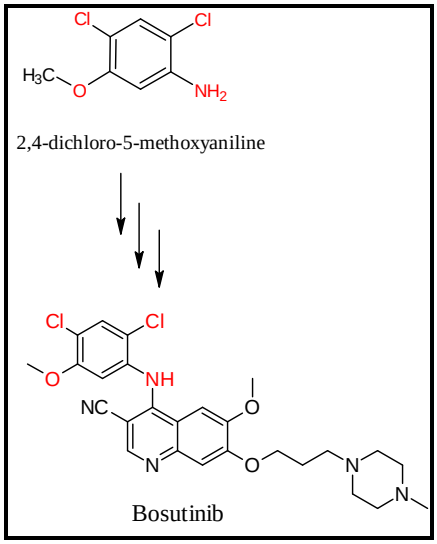
As per the daily dosage limit of Bosutinib, any genotoxic impurity shall be control below 2.5 mg L^{-1}. Moreover, Bosutinib is oncology drug/High potent (cancer drug). Hence Genotoxic impurities should be not available in the drug substances [**1-9**].

Structure of 2,4-dichloro-5-methoxyaniline and Bosutinib was given below.



Structure of 2,4-dichloro-5-methoxyaniline and Bosutinib.

Bosutinib is manufactures as per following process.



Due to the genotoxic nature of this impurity (2,4-dichloro-5-methoxyaniline), a simple and accurate analytical method was developed to determination/quantify the impurity by HPLC in the Bosutinib drug substance at trace level (2.5 mg L^{-1}). Advantage of this method is trace level (2.5 mg L^{-1}) can be quantified with regular HPLC analysis.

MATERIALS AND METHODS

Chemicals, standards and impurities: Acetonitrile (HPLC grade, Merck, India), Trifluoroacetic acid (AR grade, Merck, India), High pure water is from Milli-Q water purification system from Millipore and Hydrochloric acid (LR grade, Merck, India). 2,4-dichloro-5-methoxyaniline (from Thermo fisher), Bosutinib drug substance were obtained from Process Research department of Dr. Reddy's Laboratories, Hyderabad.

Equipment: LC was carried out with Shimadzu HPLC with photodiode array detector. The output signal was monitored and processed by using LC solution software.

Chromatographic conditions: A new gradient method is developed for determination of 2,4-dichloro-5-methoxyaniline content by HPLC in Bosutinib drug substances. The chromatographic

method employs a mobile phase-A consisting 0.05% of Trifluoroacetic acid in water and a mobile phase-B consisting acetonitrile. The method employs a gradient program (Time in min / %Mobile phase B) 0.01/25, 20/25, 25/75, 30/25, 40/25. The method was developed using Hypersil BDS C18 (150mm x 4.6mm, 3.0µm) column. The flow rate of the mobile phase was 1.0 mL min^{-1}. The column temperature was maintained at 25°C, sample cooling rack temperature was maintained at 15°C and the wavelength was monitored at 210 nm. The injection volume was 40 micro liter (µL). Diluent is mixture of Acetonitrile and water in the ratio of 50:50 (v/v).

Preparations of blank solution: Accurately transferred 2.0 mL of 1N Hydrochloric acid into a 10 mL of volumetric flask and made up to the mark with diluent (Acetonitrile: water)

Preparations of impurity stock solution: Accurately weighed and transferred 12.5 mg of impurity (2,4-dichloro-5-methoxyaniline) into 100 mL of volumetric flask, added 20 mL of diluent and dissolved then made up to the mark with diluent and mixed well. Further transferred 1.0 mL of this solution in to 100 mL of volumetric flask and made up to the mark with diluent and mixed well.

Preparations of impurity standard solution: Accurately transferred 2.0 mL of 1N Hydrochloric acid 1.0 mL of impurity stock solution in to 10 mL of volumetric flask containing 5 mL of diluent, mixed well and made up to the mark with diluent.

Preparations of test sample solution: Accurately weighed and transferred 500 mg of test sample in to 10 mL of volumetric flask, added 2.0 mL of 1N Hydrochloric acid and sonicated to dissolve then made up to the mark with diluent.

Preparations of impurity spiked with test sample solution: Accurately weighed and transferred 500 mg of test sample in to 10 mL of volumetric flask, added 2.0mL of 1N Hydrochloric acid sonicated to dissolve and added 1.0 mL of impurity stock solution mixed well and made up to the mark with diluent.

RESULTS AND DISCUSSION

Linearity: Linearity test solutions for the content method are prepared from impurity stock solutions at five concentration levels from 50 to 150% of analyte concentration (50, 75, 100, 125 and 150%). The peak area versus concentration data is treated by least-squares linear regression analysis. Linearity solutions for the method impurities were prepared by diluting impurity stock solutions to the required concentrations. The solutions are prepared at different concentration levels from LOQ to 150% (3.75 mg L^{-1}). The correlation coefficients of 2,4-dichloro-5-methoxyaniline was found 0.9999. Linearity results were provided in table 1, Linearity curve for 2,4-dichloro-5-methoxyaniline was shown in figure 1.

Table 1. Linearity result of 2,4-dichloro-5-methoxyaniline

Linearity		2,4-dichloro-5-methoxyaniline
Level	Concentration(ppm)	Area
LOQ	0.30	4584
50%	1.25	19864
75%	1.88	29654
100%	2.50	39856
125%	3.13	49957
150%	3.75	60245
	Slop	16112
	Intercept	-343.6
	Correlation	**0.99998**

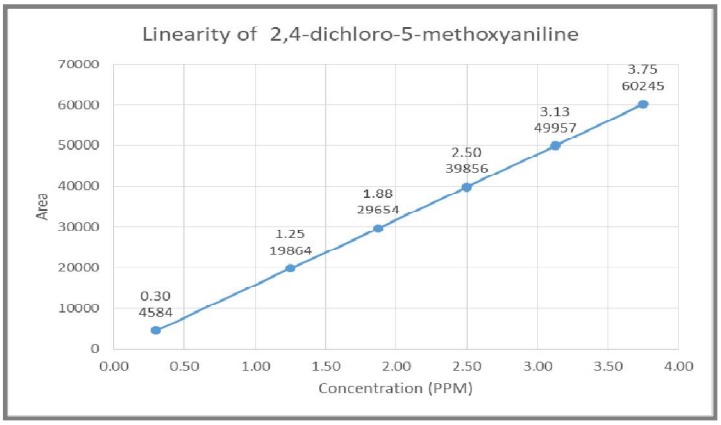


Figure 1. Linearity curve for 2,4-dichloro-5-methoxyaniline

Limits of detection (LOD) and Limit of quantitation (LOQ): The LOD and LOQ for 2,4-dichloro-5-methoxyaniline was estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of diluted solutions with known concentration. Precision study was also carried at the LOQ level by injecting six individual preparations of 2,4-dichloro-5-methoxyaniline and calculating the % R.S.D. of the area. Accuracy at LOQ level was evaluated in triplicate for the 2,4-dichloro-5-methoxyaniline by spiking at the estimated LOQ level to test solution. Limit of Quantitation (LOQ) was found 0.3 mg L^{-1} and Limit of Detection (LOD) was found 0.1 mg L^{-1} for 2,4-dichloro-5-methoxyaniline with respect to test concentration. Relative standard deviation for Limit of Quantitation (LOQ) found 1.54% for 2,4-dichloro-5-methoxyaniline. The limit of detection, limit of Quantitation and precision at LOQ values for 2,4-dichloro-5-methoxyaniline are shown in table 2 and 3.

Table 2. Concentration of LOD and LOQ for 2,4-dichloro-5-methoxyaniline

Level	2,4-dichloro-5-methoxyaniline
LOD	**0.10ppn**
LOQ	**0.30ppm**
Specification	2.5ppm

LOD and LOQ values with respect to test concentration.

Table 3. Precision at Limit of Quantitation results for 2,4-dichloro-5-methoxyaniline

LOQ	2,4-dichloro-5-methoxyaniline
Preparation	**Area**
Preparation-1	4586
Preparation-2	4513
Preparation-3	4605
Preparation-4	4518
Preparation-5	4706
Preparation-6	4577
Mean	4584
SD	70.39
% RSD	**1.54**

Accuracy: The accuracy of the 2,4-dichloro-5-methoxyaniline content method is evaluated in triplicate at three concentration levels, i.e. 50, 100 and 150% of the specification concentration along with Limit of Quantitation level. The recovery is calculated against 50 mg mL^{-1} of test concentration. Recovery study of 2,4-dichloro-5-methoxyaniline was performed at 1.25 mg L^{-1}, 2.5 mg L^{-1} and 3.75 mg L^{-1} levels and found that accuracy of the method falls in the range of 97.8% to 101.7%. Accuracy

data is shown in the table 4. The Accuracy study was performed with API samples of Bosutinib. Accuracy result for 2,4-dichloro-5-methoxyaniline was given in the table 4.

Table 4. Accuracy result for 2,4-dichloro-5-methoxyaniline

2,4-dichloro-5-methoxyaniline						
Levels	**Preparation**	**Area**	**Added**	**Obtained**	**Recovery**	**Avg**
	Preparation-1	4596	0.30	0.29	98.3	
At LOQ	Preparation-2	4521	0.30	0.29	96.7	**97.8**
	Preparation-3	4612	0.30	0.30	98.6	
	Preparation-1	19154	1.25	1.23	98.3	
At 50%	Preparation-2	20985	1.25	1.34	107.7	**101.7**
	Preparation-3	19316	1.25	1.24	99.1	
	Preparation-1	39765	2.50	2.55	102.0	
At 100%	Preparation-2	39567	2.50	2.53	101.5	**101.2**
	Preparation-3	38987	2.50	2.50	100.0	
	Preparation-1	59515	3.74	3.81	101.8	
At 150%	Preparation-2	59562	3.74	3.81	101.9	**101.1**
	Preparation-3	58254	3.74	3.73	99.6	

Precision: The precision of the method is evaluated by analyzing six test samples of spiked with 2,4-dichloro-5-methoxyaniline at 2.5 ppm level. The Relative standard deviation is found to be 0.98% for 2,4-dichloro-5-methoxyaniline. Precision data is shown in table 5, (Figure 2-5).

Table 5. Precision result for 2,4-dichloro-5-methoxyaniline

Precision	2,4-dichloro-5-methoxyaniline
Preparation	**Area**
Preparation-1	38318
Preparation-2	38941
Preparation-3	38813
Preparation-4	39136
Preparation-5	39403
Preparation-6	39232
Mean	38974
SD	383
% RSD	**0.98**

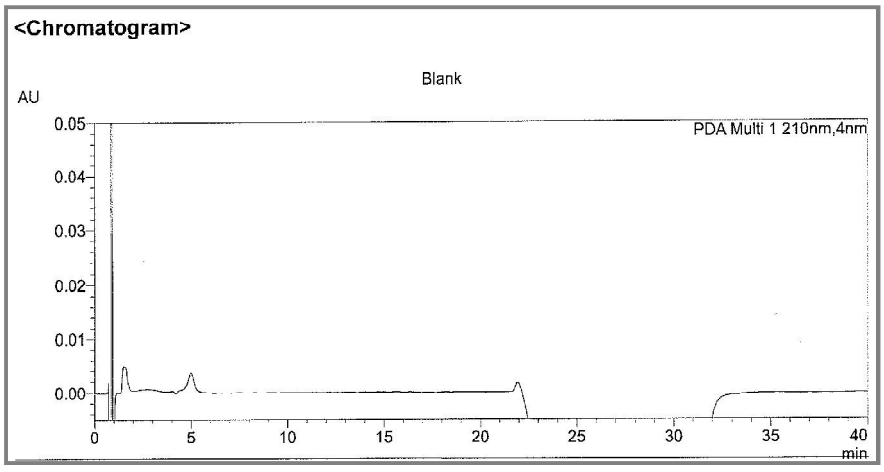


Figure 2. Blank run chromatogram at 210nm

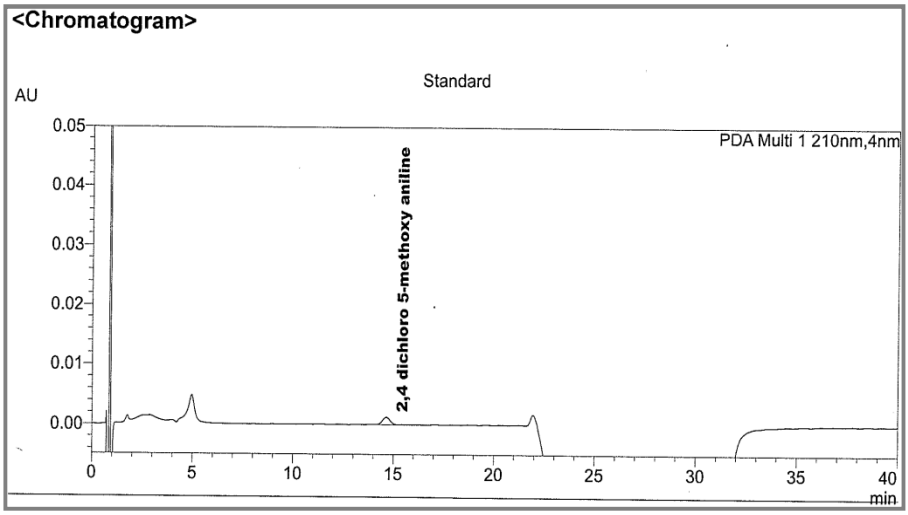


Figure 3. Impurity standard chromatogram at 210 nm

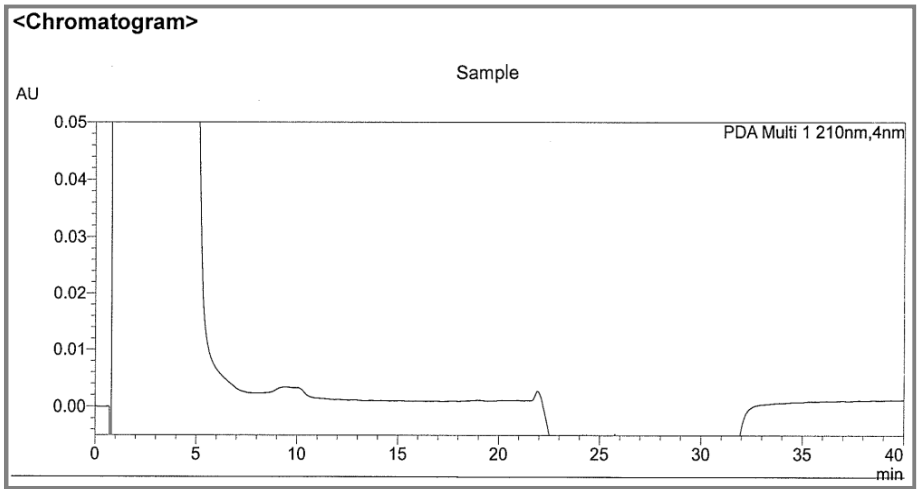


Figure 4. Bosutinib test sample chromatogram at 210 nm.

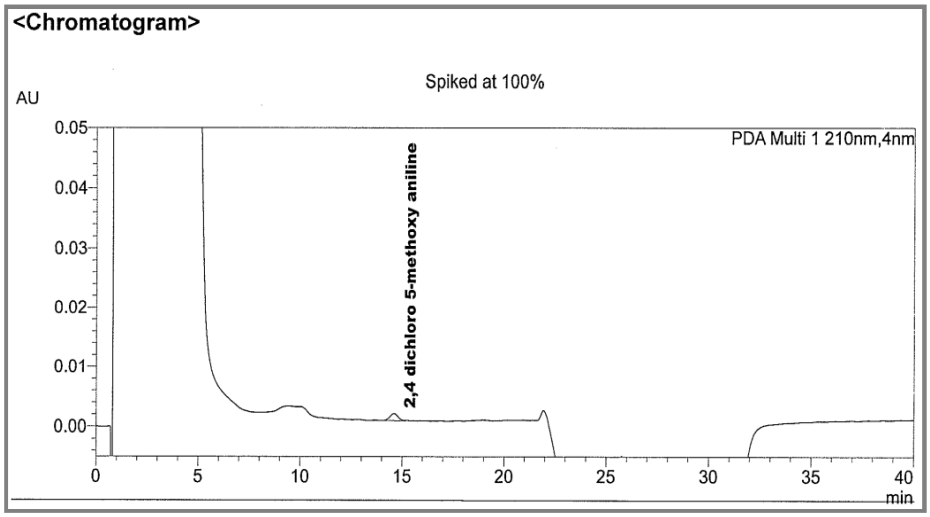


Figure 5. Impurity spiked to Bosutinib test sample chromatogram at 210 nm.

Summary: All the results were summarized in the following table 6.

Table 6. Validation results for the parameters

S. No	Validation parameter	Description	Result
1	LOD	Impurity concentration (ppm)	0.1
2	LOQ	Impurity concentration (ppm)	0.3
3	Specification	Impurity concentration (ppm)	2.5
4	Precision(n=6)	% RSD for area	0.98
5	LOQ Precision(n=6)	% RSD for area	1.54
6	Accuracy at LOQ	% Recovery	97.8
7	Accuracy	% Recovery at 50%	101.7
		% Recovery at 100%	101.2
		% Recovery at 150%	101.1
8	Linearity	Correlation coefficient	0.99998

APPLICATION

This method was developed at the wave length of 210 nm to get high response for 2,4-dichloro-5-methoxyaniline. Hypersil BDS C18 column (150 mm x 4.6 mm, 3.0 µm) was used for the separation and retain purpose of the impurity (2,4-dichloro-5-methoxyaniline) in the column and good peak (Gaussian peak). This study demonstrates the analytical method was linear accurate and precise at specification level (2.5 mg L^{-1}) and quantitation level (0.3 mg L-1).

CONCLUSION

Simple, sensitive and accurate analytical method was developed for the trace level determination and quantitation of 2,4-dichloro-5-methoxyaniline in pharmaceutical drug substance (Bosutinib) using regular high performed liquid chromatography. Selection of diluent (1N Hydrochloric acid) is the key step for the analytical approach which dissolves the Bosutinib compounds at such a high concentration (50mg mL^{-1}) and meets the specific requirement of analytical strategy. Since the trace level (2.5 mg L^{-1}) determination is the major task with regular HPLC. Method was developed with the wave length of 210 nm is get high response for 2,4-dichloro-5-methoxyaniline. Hypersil BDS C18 column (150mm x 4.6mm, 3.0µm) was used for the separation and retain purpose of the impurity (2,4-dichloro-5-methoxyaniline) in the column and good peak (Gaussian peak). This study was demonstrated the analytical method was linear accurate and precise at specification level (2.5ppm) and Quantitation level (0.3ppm).

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