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Trace Level Quantification of Genotoxic Impurity "3-Chloro-N-(2-((2-Dimethylamino) Ethyl) (Methyl) Amino)-4-Methoxy-5-((4-(1-Methyl-1h-Indol-3-Yl) Pyrimidin-2-Yl) Amino) Phenyl) Propanamide" in Osimertinib Mesylate by UPLC-QDa

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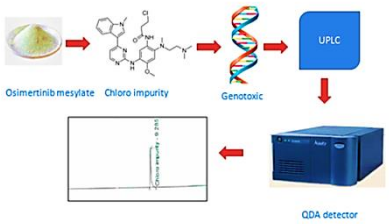
ABSTRACT

A Novel Ultra performance liquid chromatographic method with QDa detection technique was developed for trace level quantification of potential genotoxic impurity "3-Chloro-N-(2-((2-dimethylamino) ethyl) (methyl) amino)-4-methoxy-5-((4-(1-methyl-1H-indol-3-yl) pyrimidin-2-yl) amino) phenyl) propanamide" in Osimertinib mesylate an Antineoplastic agent. The chromatographic separation was achieved on Kinetics 2.6 µm Polar C18 100Å (100 mm x 4.6 mm) column at a flow rate of 0.3 mL min^{-1} and column temperature 40°C. The mobile phase consisted of 10mM Ammonium acetate at pH 5.5 and acetonitrile under programmed gradient conditions. Mass spectroscopic quantification was carried out in positive ion mode at probe temperature 600°C.

The method was validated according to ICH guidelines and achieved LOD and LOQ values as 0.3 ppm *and 1.0* ppm *respectively. The proposed method was found to be linear (r^2; ≥0.99), accurate (% Recovery ≥ 99%) and can be used for routine testing at quality control.*



Keywords: UPLC; QDa; pH; Validation; ICH; Osimertinib mesylate

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INTRODUCTION

Osimertinib mesylate [1-3] is an active pharmaceutical ingredient which is approved by FDA in November 2015 to treat non-small cell lung cancer. By blocking epidermal growth factor receptor (EGFR), Osimertinib helps to reduce the growth and spread of the cancer. The chemical name is N-(2-{2-dimethyl amino ethyl-methyl amino}-4-methoxy-5-{[4-(1-methylindol-3-yl) pyrimidin-2-yl] amino} phenyl) prop-2-enamide mesylate salt (Figure 1).

During the manufacturing process of Osimertinib mesylate, a potential genotoxic impurity 3-Chloro-N-(2-((2-dimethylamino) ethyl) (methyl) amino)-4-methoxy-5-((4-(1-methyl-1H-indol-3-yl) pyrimidin-2-yl) amino) phenyl) propanamide (chloro impurity) will generate at n-1 stage (Figure 2). According to ICHM7 impurity classification, chloro impurity comes under class-3. The acceptable limit for any genotoxic impurity [4-8] present in the Osimertinib mesylate is 18.75 ppm by considering 80 mg as maximum daily dosage and 1.5 µg as daily intake. This impurity can cause serious effects on human health if present in the drug substance at any level, so it is mandatory to control this impurity in the manufacturing process. For those genotoxic impurities that arise in starting materials or Intermediates, there is possibility to show the absence in further stages instead of including the specific test in the specification. It is essential to control the toxic impurities that arise at n-1 stage by having specific test as part of specification.

Till date there are no literature reference methodologies for the quantification of chloro impurity in Osimertinib mesylate however a few references available of Osimertinib assay determination in human plasma [9-13] .By considering the importance of chloro impurity monitoring in the manufacturing process authors decided to develop the suitable quantification method. The separation and quantification of this impurity at lower levels is critical task to the analytical scientist, as both the chloro impurity and Osimertinib have similar structures and same polarity. Chloro impurity will form at precursor step of Osimertinib mesylate synthetic scheme; hence control of this impurity during the reaction monitoring itself is a very crucial step in the manufacturing process.

Initial attempts were made to develop an HPLC method with UV detector but ended up with higher LOD, LOQ values which is not sufficient for quantification of chloro impurity. Desired responses not achieved even on the UPLC. Finally, our research emerges with most advanced and sensitive technique for the quantification of chloro impurity in Osimertinib mesylate at below TTC limit. Waters Acquity UPLC with QDa-detection technique was developed for quantification of chloro impurity at trace levels. The proposed method was validated successfully and can be used for routine analysis.

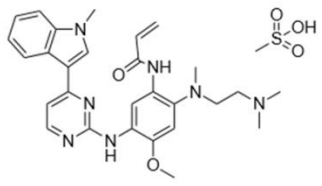

Figure 1. Chemical structure of Osimertinib mesylate

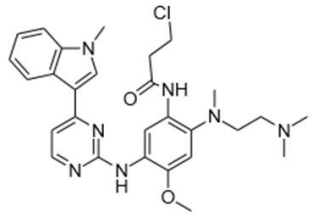

Figure 2. Chemical structure of Chloro impurity

MATERIALS AND METHODS

Chemicals and Reagents

Osimertinib mesylate samples and chloro impurity are received from Dr.Reddys laboratories (CTO-1, Hyderabad, India). Ammonium acetate and acetonitrile are purchased from Merck limited (Mumbai, India). Water was purified by using in house Milli-Q water purification system (Millipore, Bedford, MA, USA). The Kinetics 2.6 µm Polar C18 100Å, 100*2.1 mm column purchased from Phenomenex, India. The 0.22 µm membrane filter paper and 0.22 µm syringe filters were obtained from Millipore (Bangalore, India).

Instrumentation

An ACQUITY UPLC H-class system equipped with QDa detector, Quaternary solvent manager, Sample manager-flow throw needle and column heater manufacture from Waters Corporation (Waters Co., Milford, MA, USA). The data were collected and processed by using Empower™ software (Version-3).

Chromatographic System

Kinetics Polar C18 100Å column with 100 mm length, 2.1 mm internal diameter and 2.6 µm particle size was used as stationary phase. 10 mM ammonium acetate at pH 5.5 adjusted with 10% formic acid used as mobile phase-A and mixture of acetonitrile and water in the ratio of 9:1 used as mobile phase-B. The gradient program was set as Time (min)/%B=0/30, 10/50, 15/90. A flow rate of 0.3 mL min^{-1} with an injection volume of 1 µL was used and column temperature was maintained at 40°C. QDa detection parameters set as Function mode: SIR, Ionization: Positive, Probe temperature: 600°C, Cone voltage: 15 V, Capillary voltage: 0.8 kV, Sampling rate: 10 points/sec, SIR mode M.Wt of chloro impurity: 536.13 amu. A homogeneous mixture of 0.1 N HCl and Acetonitrile in the ratio of 8:2 was used as diluent to prepare standard and test sample preparation.

Preparation of Standard and Sample Solution

The impurity standard solution was prepared by dissolving about 10 mg of chloro impurity in 100 ml of diluent and further diluted to get a concentration of 0.076 µg mL^{-1} (15 ppm with respect to test concentration of 5 mg mL^{-1}) and the test solution was prepared by dissolving about 50 mg of test sample in 10 of diluent (5000 µg m^{-1}). The Limit of detection and Limit of Quantification solutions were prepared by diluting the standard solution to get a concentration of 0.005 µg mL^{-1} and 0.0016 µg mL^{-1} respectively (1 ppm and 0.3 ppm with respect to test concentration of 5 mg mL^{-1}). Spiked test solutions at LOQ level and standard level were prepared by spiking impurity stock solution to the test solution at 1 ppm and 15 ppm respectively. Linearity solutions were prepared by diluting the standard stock solution to get a concentration of 0.038, 0.061, 0.067, 0.076, 0.084, 0.091, 0.114 µg mL^{-1} which is 7.5, 12.0, 13.5, 15.0, 16.5, 18.0 and 22.5 ppm respectively with respect to test concentration.

RESULTS AND DISCUSSION

Characterization of Chloro Impurity

The structure of the Chloro impurity was confirmed by IR, Mass and NMR spectroscopy (Figures 3-5). The impurity obtained as off white solid. RP-UHPLC, tR=4.8 min (94.9% purity). MS (ESI, 70 eV): $[M+H^+]$ m/z 536.4. FT-IR (KBr), ν, cm^{-1}: 3415, 3234, 1667, 1583, 1533, 1417, 1371, 1262, 1231, 1205, 1127, 1101, 1025, 1264, 882, 834, 808, 748, 678. 1H NMR (400 MHz, DMSO-*d6*+D2O, TMS): δ 7.2 (s, 2H, H-7,8), 3.1-3.4 (m, 6H, H-11,13,14 and 16),2.3 (m, 1H, H-12), 2.0 (d, 1H, 11.2 Hz, H-12).13C NMR (100 MHz, DMSO-*d6*, TMS): δ 173.8 (C-2,3), 155.1 (C-5,6), 137.6 (C-9,10), 125.1 (C-7,8), 38.8 (C-11), 37.9 (C-12), 38.8 (C-13), 45.8 (C-14), 48.6 (C-16).

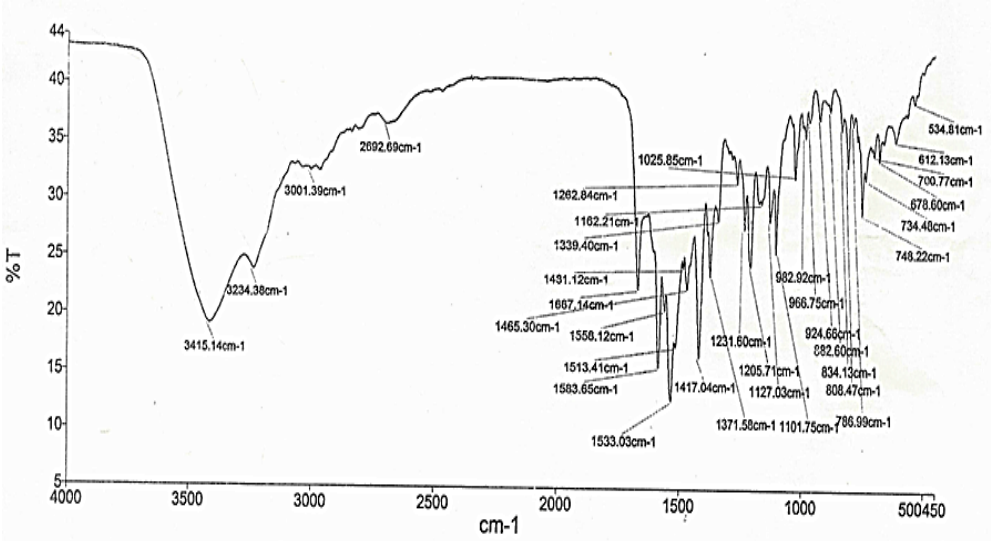

Figure 3. Infrared spectrum of Chloro impurity

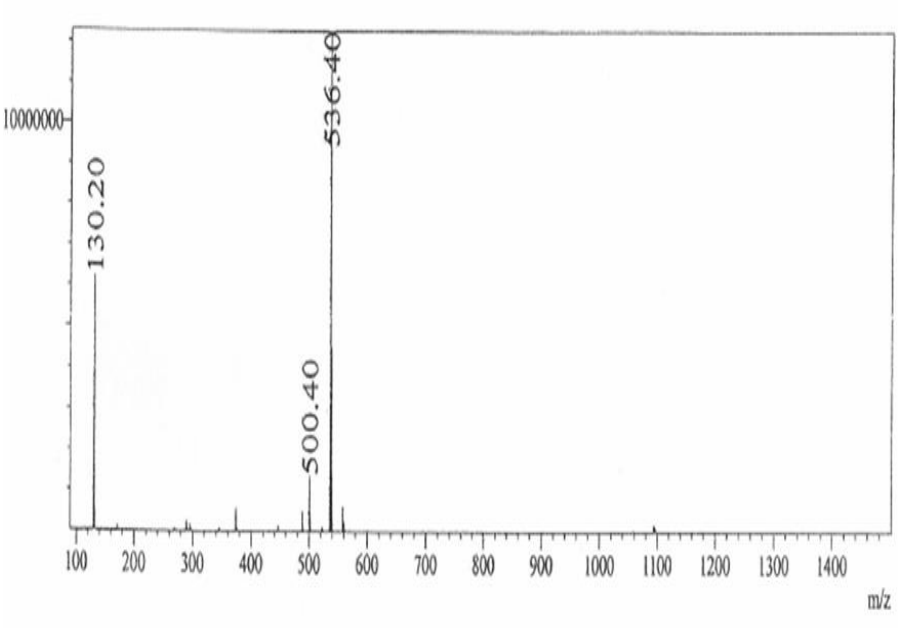

Figure 4. Mass spectrum of Chloro impurity

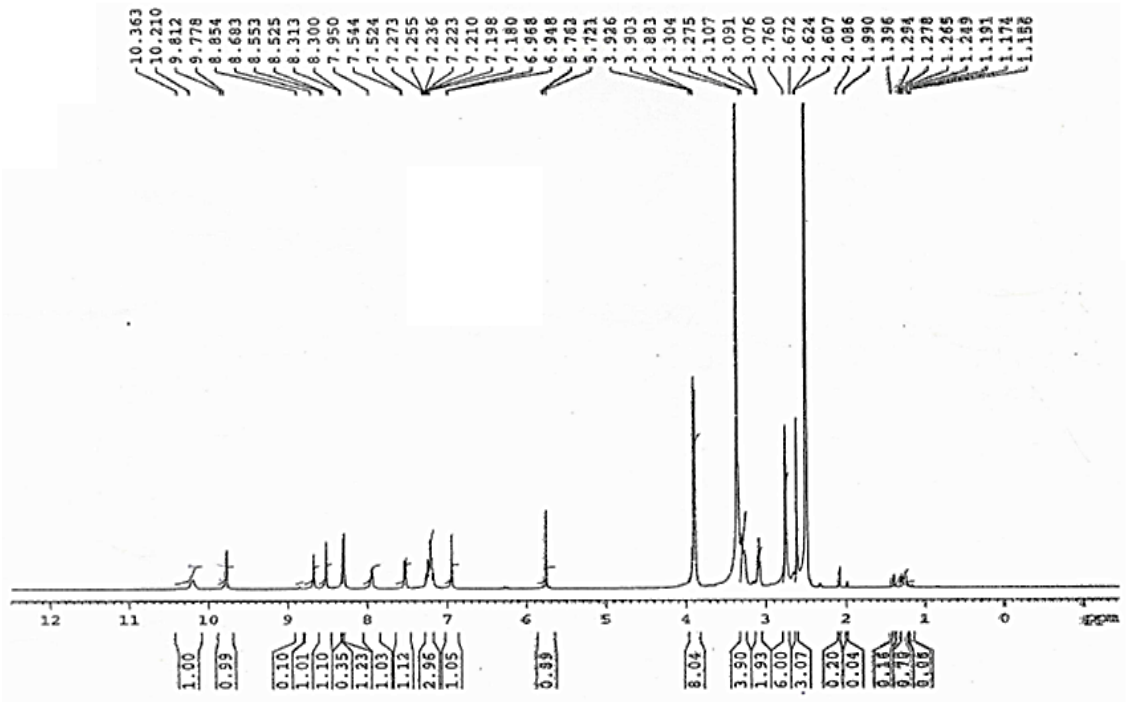

Figure 5. NMR spectrum Chloro impurity

Method development and Optimisation

The method development [14] was always has been a challenge for genotoxic impurities quantification in pharmaceutical industry because we need to control these impurities at trace levels. The acceptable limit for chloro impurity in Osimertinib mesylate is 18.75 ppm based on TTC calculation (by considering the maximum daily dosage of 80 mg for Osimertinib mesylate). The main challenge [15] for quantification of impurities at this level was not only separating the target impurity from known impurities but also from sample matrix because we need to use higher concentrations of test samples to increase the detection levels. During the method development, initial trials were done on HPLC with PDA detector and integrator but due to very close polarity of the API and the targeted chloro impurity the desired separation was not achieved. Continued the development by using different stationary phase columns (C18, C8, PFP, CN etc) and different mobile phases to improve the resolution between the API and chloro impurity. Finally, achieved the separation with a resolution of 2.5 by using gradient elution of monobasic sodium phosphate at pH 5.5 as mobile phase-A and acetonitrile as mobile phase-B in XBridge C_{18} (150 mm × 4.6 mm, 3 µ) column (Figure 6). Injected the test solutions and spiked test solution with chloro impurity and found the difficulty in the quantification at low levels (at 18.75 ppm). The difficulty in the quantification is due to the elution of chloro impurity at the tailing of the Osimertinib peak. Tested the same method condition on UPLC-PDA by using ACQUITY UPLC™ *BEH C18* 2.1 x 50 mm, 1.7 µm column. Checked the recovery and found the failure in the recovery at standard level. Based on the above trials it is concluded that the quantification of chloro impurity is not possible by HPLC with UV detection.

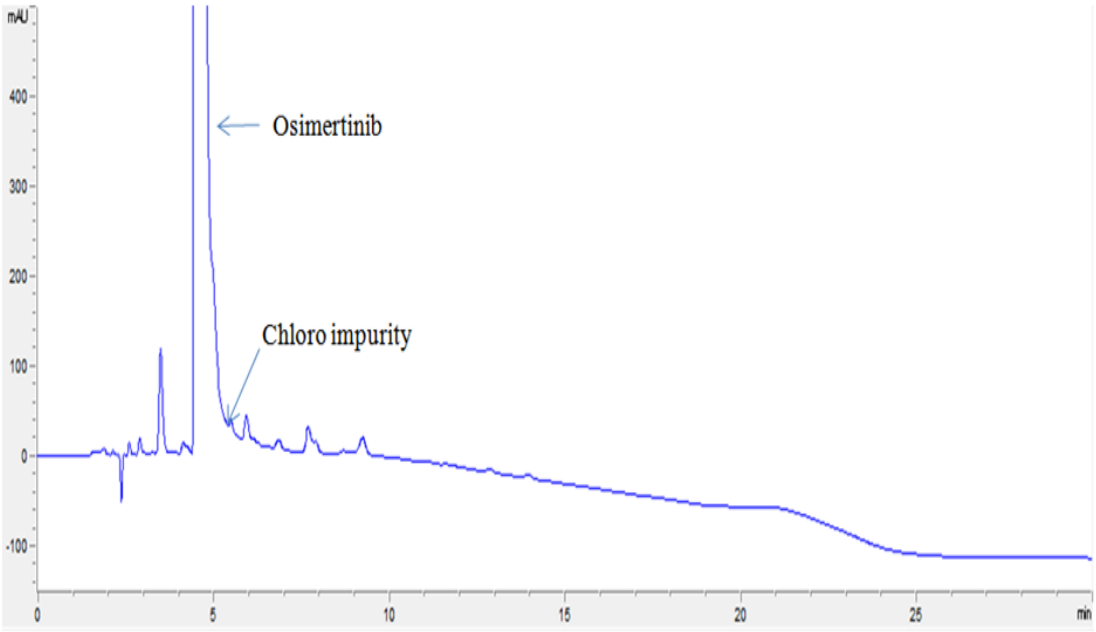

Figure-6: Representative chromatogram of Osimertinib mesylate spiked with chloro impurity by HPLC in XBridge C_{18} (150 mm × 4.6 mm, 3 µ)

The method development was continued by using the advanced detection technique ACQUITY QDa® Detector. The ACQUITY QDa® Detector is a non-complex, sensitive and robust mass detector used in the chromatographic analysis especially for the quantification of trace level impurities.

The use of volatile buffers for QDa detection is mandatory to ionize the components, hence 10 mM ammonium acetate adjusted to pH-5.5 with diluted formic acid used as buffer. The pH of the buffer was selected based on the separation achieved in the HPLC chromatographic conditions. A symmetrical peak shape was achieved on Kinetics Polar C18 100**Å** column with 100 mm length, 2.1 mm internal diameter and 2.6 µm particle size. The gradient programme was optimized by changing the ratios of %A (100% Buffer) and %B (Acetonitrile and water in the ratio of 9:1) and achieved the desired separation with programmed linear gradient as Time (min)/%B: 0/30, 10/50, 15/90, 20/90, 20.5/30, 25/30. During the development different diluents were used like water, acetonitrile, methanol at different compositions but all the attempts were failed to dissolve the Osimertinib samples. Tried basic diluent 0.1 N NaoH with acetonitrile and methanol combination and found the compound is unstable and observed degradation in basic diluent. Checked the acidic diluent 0.1 N HCl with combination of Acetonitrile in the ratio of 1:1 and found the samples are freely soluble and stable, so the same diluent is finalized for test and standard preparations. Tested the peak shape at different column thermostat temperatures like 25**°C**, 30**°C**, 35**°C** and 40**°C** and found the excellent peak shape at 40**°C**. Auto sampler temperature was set as 10**°C** to increase the solution stability during the analysis. To fix the QDa parameters initially tested the impurity standard at scan mode with both positive and negative modes and noted that chloro impurity was ionized in positive mode. The detected mass number for chloro impurity in positive mode was $[M+H^+]$ m/z 536.40. So the function mode was set as SIR (Selective ion recording) in positive ionization mode. Optimized the probe temperature at 300°C, 500**°C** and 600**°C** and found the good response of chloro impurity at 600**°C**. Cone voltage and Capillary voltage was set as 15 and 0.8 V respectively.

Analytical Method Validation

The above method was validated [16] by establishing Quantification, Detection limits and by determining the Accuracy, Precision, Linearity, Robustness, solution stability and Range as per International Council on Harmonization (ICH) validation guidelines Q2, (R1) guidelines.

System precision/System suitability

As the main aim of the stated method was quantification of chloro impurity in Osimertinib the system suitability parameter was set as %Relative Standard deviation to the area of Chloro impurity at standard level by injecting six replicates in to the chromatographic system. Prepared and injected the standard solution of chloro impurity into the chromatographic system. Calculated the %*RSD* for the area of Chloro impurity and the value obtained was 1.7%.

Establishment of Quantification and Detection limits

Calculated signal to noise ratio for the standard solution from the system suitability parameter and found the signal to noise ratio is ~150. LOQ, LOD solutions were prepared by diluting the standard solution up to 15 volumes for LOQ and 50 volumes for LOD respectively and injected in to the chromatographic system. Calculated the signal to noise ratios and the results were tabulated in Table 1.

Table 1. LOD, LOQ results

Parameter	Concentration (ppm)	S/N ratio
Limit of Detection	0.3	2.8
Limit of Quantification	1	9.8

Precision

Precision was established by calculating %Relative standard deviation for six replicate injections of LOQ solution and spiked test solution. Prepared the LOQ solution as mentioned in the section 3.4. Spiked test solution was prepared by weighing about 50 mg of test sample in to 10 ml volumetric flask, to it spiked the chloro impurity to get concentration of 15 ppm and finally made up to volume with diluent. Injected the above two solutions into the chromatographic system for 6 times individually. Calculated the %RSD for the area of chloro impurity and results are tabled in below Table 2.

Table 2. Method precision results

Precision	Concentration (ppm)	% RSD
LOQ level	1	2.8
100% level	15	1.7
150% level	22.5	7.9

Accuracy

Method accuracy was determined by estimating the amount of chloro impurity recovered from spiked test solutions of chloro impurity at different levels. Accuracy was performed at LOQ, 50%, 100%, 150% levels by injecting each solution in triplicate. Prepared the Accuracy solutions by spiking the 0.005 µg (LOQ level), 0.038 µg (50% level), 0.076 µg (100% level), 0.114 µg (150% level) of chloro impurity to the test sample and estimated the amount of chloro impurity recovered by injecting the above solutions in to chromatographic system. The %recovery of chloro impurity obtained was 107.8, 111.7, 104.3 and 112.7 at LOQ, 50%, 100%, 150% levels respectively. The results are tabulated in table 3.

Table 3. Accuracy/Recovery results

Accuracy	(%) Recovery	Average (%) Recovery
LOQ Spiked-1	99.6	
LOQ Spiked-2	110	
LOQ Spiked-3	113.9	107.8
50% Spiked-1	104.6	
50% Spiked-2	116	
50% Spiked-3	114.4	111.7
100% Spiked-1	104.8	
100% Spiked-2	103.3	
100% Spiked-3	104.7	104.3
150% Spiked-1	112.9	
150% Spiked-2	112.7	
150% Spiked-3	112.6	112.7

Linearity and Range

Evaluated the linearity of the method by injecting diluted solutions of chloro impurity at LOQ, 50%, 80%, 90%, 100%, 110%, 120% and 150% with respect to the test concentration. The above series of diluted impurity standard solutions were prepared by diluting the impurity stock solution with the diluent. Injected all the above linearity solutions into the chromatographic system and recorded the area of chloro impurity (Table 4). Plotted the linearity graph by taking the areas on Y-axis and impurity concentration on X-axis. Calculated the correlation coefficient and found the value ≥0.99 (Figure 7**)**.

Table 4. Linearity statistics

Conc. (µg/ml)	Area	Correlation	Slope	Intercept
0.0375	184573			
0.06	303051			
0.075	345187	0.997	4609851	13784.01
0.09	429921			
0.1125	534882			

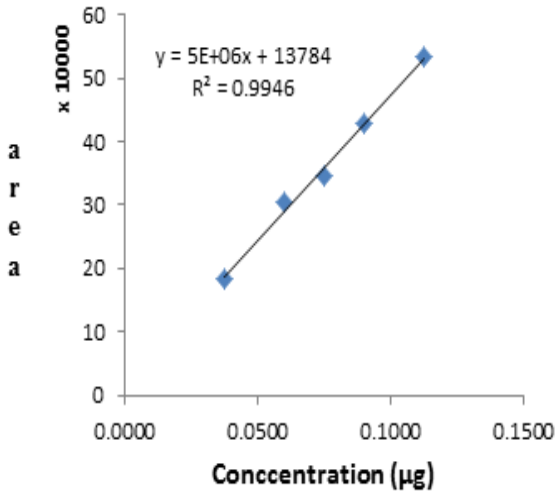

Figure 7. Linearity plot of chloro impurity

Robustness

Method robustness was proved by studying the variation in system suitability results verses the changes in flow rate ±0.1 mL min^{-1} to the actual flow rate of 0.3 mL min^{-1}, mobile phase pH ±0.2 units to the actual pH of 5.5, and column temperature ±5°**C** to the actual temperature of 40°**C**. Calculated the %*RSD* to the area of chloro impurity by injecting the impurity standard in to the chromatographic system at all the above changed chromatographic conditions and found the method was robust (Table 5).

Table 5. Robustness results

Robustness condition	Average area of chloro imp	Standard deviation	% RSD
Optimal	370748	10380.94	2.8
f=0.27	410253	13128.1	3.2
f=0.33	365287	9132.175	2.5
T=35°C	371587	7803.327	2.1
T=45°C	365972	12809.02	3.5
pH 5.3	368058	4416.696	1.2
pH 5.7	345631	10714.56	3.1

Stability of Standard, Test solution and Mobile Phase

Standard and test solutions were injected into the chromatographic system after stored separately at room temperature, 2-8°C to establish solution stability (Table 6). Stored the mobile phase on bench top at room temperature for 24 h, injected freshly prepared standard and test sample solutions by using stored mobile phase to know the mobile phase stability (Figures 8-11).

Table 6. Solution stability results

Solution stability	Chloro impurity content			
Stored at	0 h	6 h	12 h	24 h
Room temperature	15.2	14.6	12.3	12
2-8°C	15.2	14.9	15.4	15.1

From the above study, it is found that the standard, test sample solutions were stable up to 24 h at both RT and 2-8 °C and mobile phase is found to be stable for 24 h at RT.

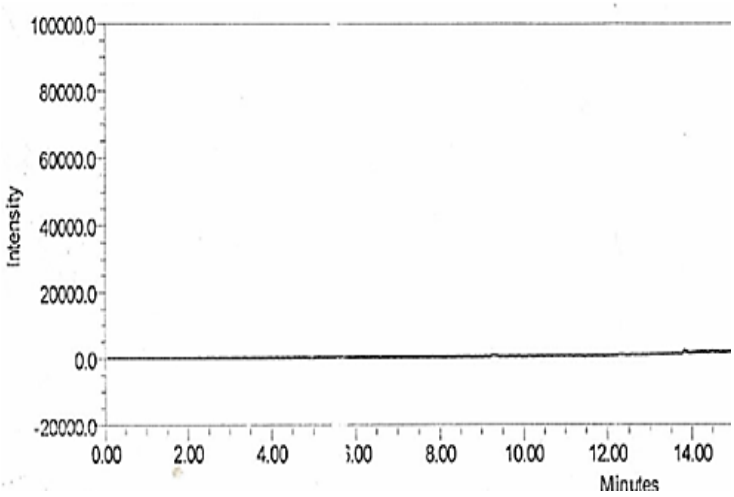

Figure 8. Blank chromatogram

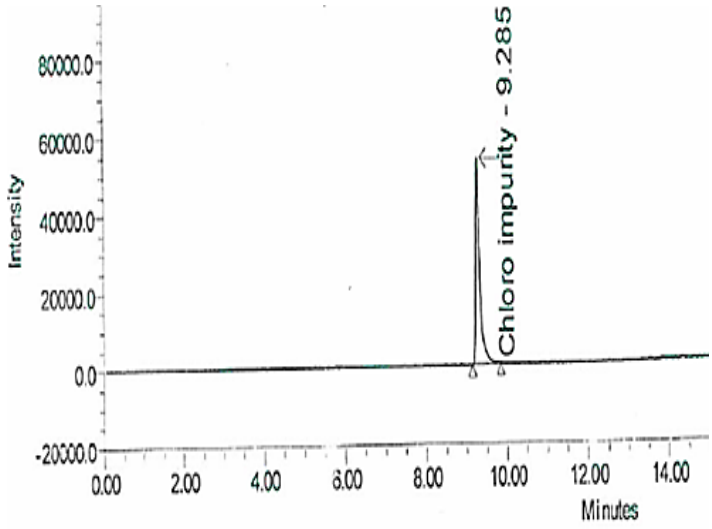

Figure 9. Standard chromatogram

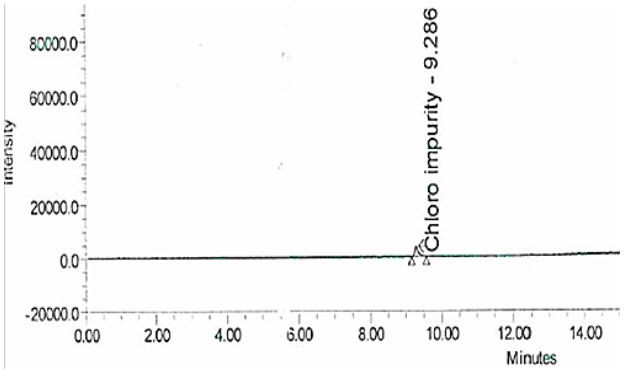

Figure 10. LOQ chromatogram

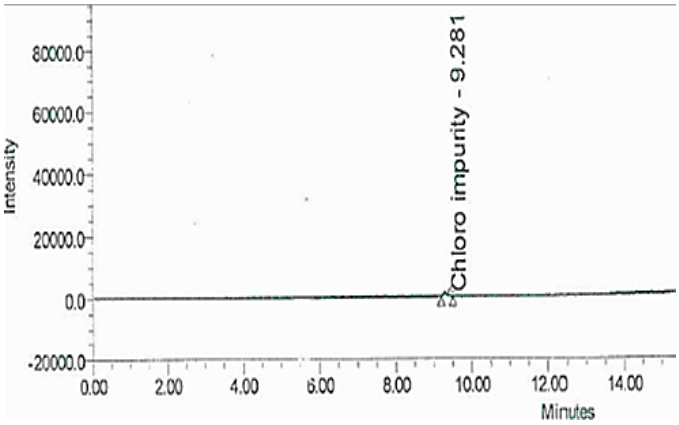

Figure 11. LOD chromatogram

CONCLUSION

The present research study emerges with a simple and novel analytical method for trace level quantification of genotoxic impurity 3-Chloro-N-(2-((2-dimethylamino) ethyl) (methyl) amino)-4-methoxy-5-((4-(1-methyl-1H-indol-3-yl) pyrimidin-2-yl) amino) phenyl) propanamide (chloro impurity) in Osimertinib mesylate. The described method is proven to be highly sensitive, accurate and precise for quantification of Genotoxic impurity in Osimertinib mesylate and can be used for routine testing.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest

The authors Krishna Katta, T. Siva rao, J. Mosesbabu and D. Venugopala Rao declare that there is no conflict of interest and this article does not contain any studies with human participants or animals performed by any of the authors.

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