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Method Development And Validation Of Lc-Ms/Ms For Analyzing Potential Genotoxic Impurities In Canagliflozin

B. Akhila[*1], B.V. Ramana[2]

[1]Research Scholar, Jawaharlal Technological University Anantapur (JNTUA), Anantapuramu 515 002, Andhra Pradesh, India

[2] Professor and Principal, Dr K V subba reddy Institute of Pharmacy, Dupadu, Andhra Pradesh 518218, Andhra Pradesh, India

***Corresponding author:**

B. Akhila,

Research Scholar, Jawaharlal Technological University Anantapur (JNTUA), Anantapuramu 515 002, Andhra Pradesh, India

Email ID: akhila33friend@gmail.com

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ABSTRACT

The assessment of toxicological concentrations of possible genotoxic contaminants in drug substances was regarded as an important and challenging discipline . The International Conference and Harmonization (ICH) recommended that most pharmaceutical products be allowed to include 1.5 µg/day of a genotoxic contaminant. The goal study was to develop a quick and accurate method for measuring potential genotoxic impurities (PGIs) in canaglifoxin drug substance. The chromatographic conditions were appropriately optimized with the columns to achieve a decent separation and response of each impurities peak with the canaglifoxin. According to International conference of Harmonization (ICH) criteria for the quantification of each impurity, method validation for LC-MS was carried out regarding specificity, the limit of detection (LOD), the limit of quantification (LOQ), linearity, accuracy (recovery), precision, and solution stability. Correlation observed in the accuracy during method validation hence method can be considered linear and accurate and can be used for testing of genotoxic impurity in canaglifoxin drug substances.

Keywords: *Genotoxic impurities, LC-MS analysis, canaglifoxin, method development, method validation*

1. INTRODUCTION

The method development 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 Canagliflozin hemihydrate is 18.75 *ppm* based on TTC calculation by considering the maximum dailydosage of 100 mg for Canagliflozin hemihydrate [1]. The main challenge 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 C18 (150mm × 4.6mm, 3 µ) column. 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 Canagliflozin peak. The same method conditions were tested 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. [2]

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. [3]

The use of volatile buffers for QDa detection is mandatory to ionize the components, hence 10mM 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 100A° column with 100mm length, 2.1mm internal diameter and 2.6µm 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 withprogrammed 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 Canagliflozin samples. Tried basic diluent 0.1N NaoH with acetonitrile and methanol combination and found the compound is unstable and observed degradation in basic diluent. Checked the acidic diluent 0.1N HCl with combination of Acetonitrile in the ratio of 1:1 and found the samples are freely soluble and also 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 & 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 & 600 °c and found the good response of chloro impurity at 600 °c. Cone voltage and Capillary voltage was set as 15 & 0.8 V respectively. [4]

Table- 1:Different trails and optimization of chromatographic conditions.

Experiment	Mobile phase composition	**Elution**	**Flow rate (ml/min)**	**Column**	Detection technique	Results	**Conclusions**
1[5]	Mobile phase -0.1% Orthophosphoricacid: Acetonitrile	**Isocratic**	**1.0**	**ZorbaxRXC18 (250 x 4.6 mm, 5.0µ)**	UV Visible detectorat 240 nm	Chloro impurity mergedwithmain peak	**Rejected**
2[6]	Mobile phase -10mM Monobasicpotassium phosphate at pH-3.0 : Acetonitrile	**Gradient**	**1.0**	**ZorbaxRXC18 (250 x 4.6 mm, 5.0µ)**	UV Visible detectorat 240 nm	Peak splitting	**Rejected**
3[7]	Mobilephase-10mM Mono basic sodium phosphateatpH-5.5: Acetonitrile	**Gradient**	**1.0**	**Kromasil C8 (150x4.6mm, 5.0µ)**	UV Visible detectorat 240 nm	Chloro impurity merged with main peak	**Rejected**
4[8]	Mobilephase-10mM Mono basic sodium phosphateatpH-5.5: Acetonitrile	**Gradient**	**1.0**	**Cosmicsil PFP (150x4.6mm, 5.0µ)**	UV Visible detectorat 210 nm	Chloro impurity peak eluted in tailing of main peak	**Rejected**
5[9]	Mobilephase-10mM Mono basic sodium phosphateatpH-5.5: Acetonitrile	Gradient	1.0	Zorbax CN (250x4.6mm, 5.0µ)	UV Visible detectorat 210 nm	Separation achieved but unabletoquantify atlowlevelsdue to poorsensitivity	Rejected
6[10]	Mobilephase-10mM Ammoniumacetateat pH-5.5 : Acetonitrile	Gradient	1.0	XBridge C18 (150x4.6mm, 3.0µ)	QDA	Desiredsensitivity achieved	Accepted

2. METHODOLOGY

Optimised chromatographic conditions for quantification of chloro impurity in Canagliflozin hemihydrate[11]

Kinetics Polar C18 100 A° column with 100 mm length, 2.1 mm internal diameterand2.6µmparticlesizewasusedasstationaryphase.10mMammonium 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 gradientprogramwassetasTime(min)/%B=0/30,10/50,15/90.Aflowrateof

0.3mL min^{-1} with an injection volume of 1µL 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,Samplingrate:10 points/sec,SIR mode.Ahomogeneous mixture of 0.1N HCl and Acetonitrile in the ratio of 8:2 was used as diluent to prepare standard and test sample preparation.

Table 1b: Optimised linear gradient programme

Time (min)	Flow(ml/min)	Mobile phase-A	Mobilephase- B
0.0	0.3	65	35
10.0	0.3	40	60
15.0	0.3	20	80
15.5	0.3	65	35
20.0	0.3	65	35

Table- 1c:Optimized chromatographic conditions

Parameters	**Conditions**
Stationary phase(column)	Kinetics PolarC18100A°,100×2.1mm
Mobile phase	10mM Ammonium acetate and Acetonitrile
Flow rate(mL/min)	0.3
Column temperature	27° C
Volume of injection loop (µL)	1
Detection	QDA(+ve)
Run time(min)	20

Characterization of Chloro impurity

The structure of the Chloroimpurity was confirmed by IR,Mass and NMR spectroscopy.

The impurity obtained as off white solid. RP-UHPLC, tR = 4.8 min(94.9% purity). MS (ESI, 70 eV): $[M + H]^+$: 536.4. FT-IR (KBr), ν, cm−1: 3415, 3234,1667,1583,1533,1417,1371,1262,1231,1205,1127,1101,1025,1264,

882,834,808,748,678.1HNMR(400MHz,DMSO-*d6*+D2O,TMS):δ7.2(s,

2H, H-7,8), 3.1-3.4 (m, 6H, H-11,13,14 &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).

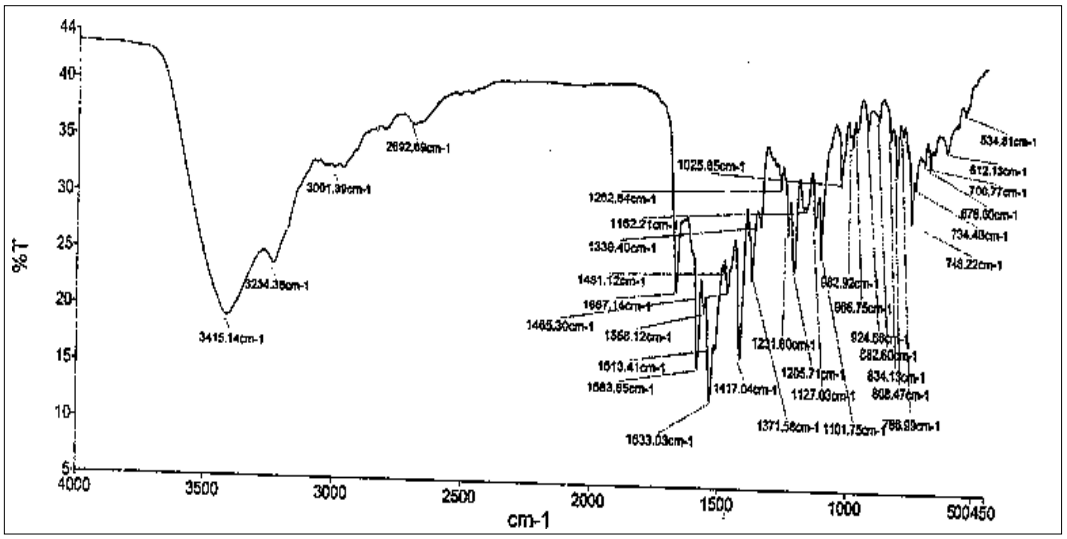

Figure-2a:Representative Infrared spectrum of Chloroimpurity

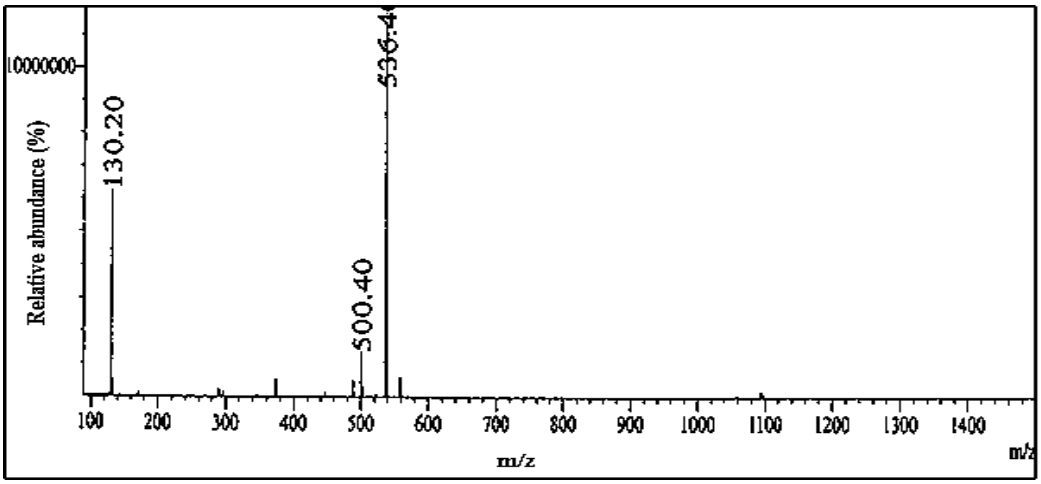

Figure- 2b:Representative LC-MS spectrum of Chloroimpurity

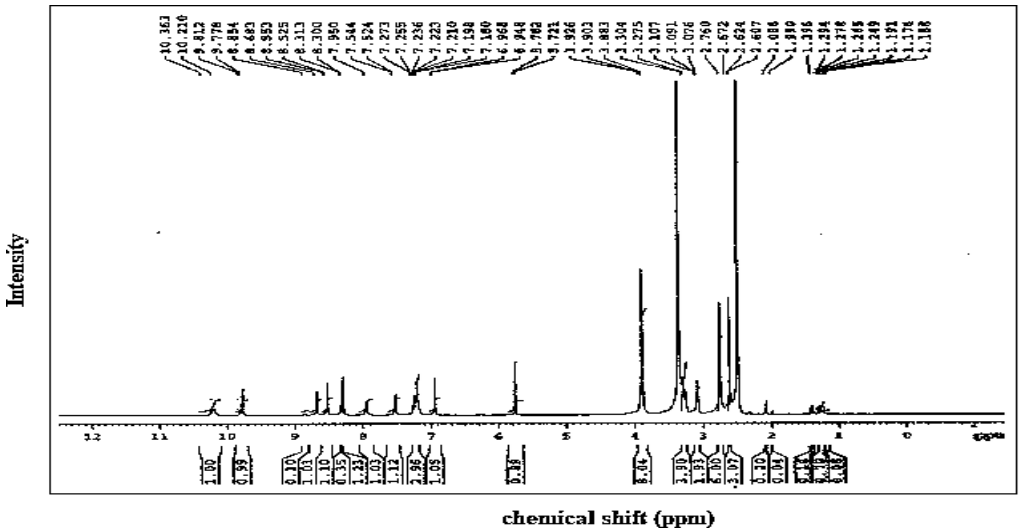

Figure- 2c:Representative NMR spectrum of Chloroimpurity

Analytical method validation

The above method was validated 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 chloroimpurity in Canagliflozin 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 1.5 %.

Preparation of solutions

Preparation of chloroimpurity stock solution:

Accurately weighed and transferred about 10 mg of chloro impurity in to 100 ml volumetric flask containing 30 ml of diluent, sonicated for 2 minutes to dissolve the contents and made up to the mark with diluent.

This solution is 20000 ppm with respect to test concentration 5mg/ml.

Preparation of Intermediate stock solution:

Transferred 1 ml of above chloro impurity stock solution in to 100 ml volumetric flask containing 30 ml of diluent, cyclomix for 2 minutes and made up to the volume with diluent.

This solution is 200 ppm with respect to testconcentration5 mg/ml.

Preparation of standard solution:

Transferred 3.8 ml of above chloro impurity intermediate stock solution in to 50 ml volumetric flask containing 30 ml of diluent, cyclomix for 2 minutes and made up to the volume with diluent.

3. RESUTS AND DISCUSISION [12]

Table-3a: System suitability/System precision results of chloroimpurity

Injection	**Area of chloroimpurity**
1	370748
2	374145
3	369774
4	367179
5	359316
6	359746
Average	366818
Standard deviation	5542.130667
(%) RSD	**1.5**

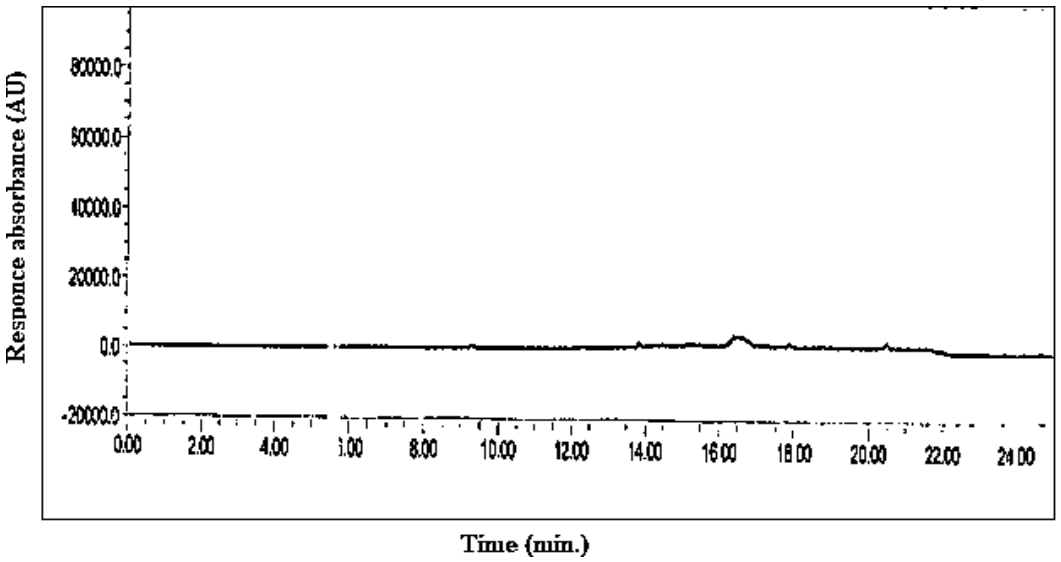

Figure- 3a:Representative chromatograms of Blank solution

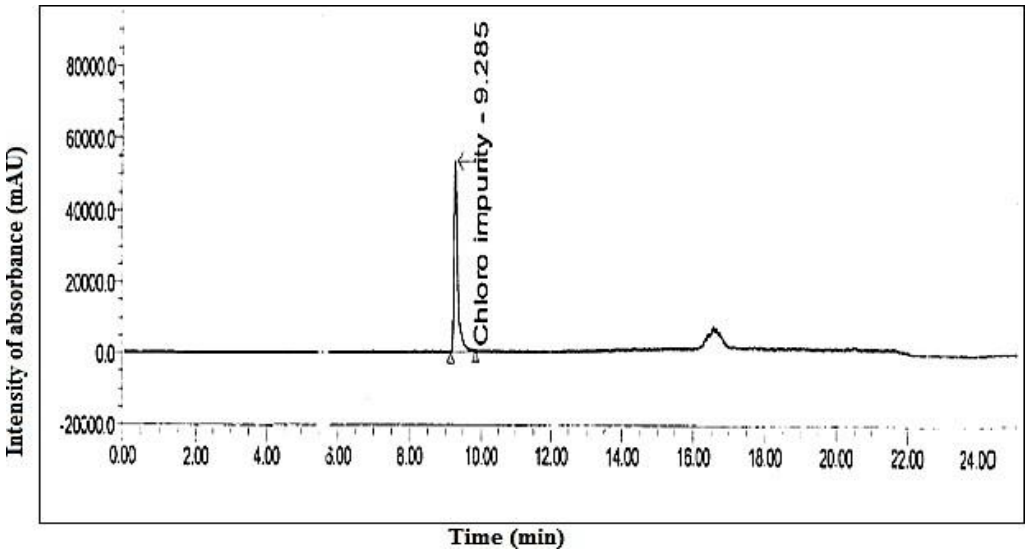

Figure- 3b: Representative chromatograms of Chloroimpurity standard solution

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 LOQand50volumesfor LOD respectively and injected into the chromatographic system. Calculated the signal to noise ratios and the results are tabulated in Table 3b.

Preparation of LOQ solution:

Transferred 0.5 ml of above Chloro impurity intermediate stock solution in to 100 ml volumetric flask containing30 ml of diluent, cyclomix for 2 minutes and made up to the volume with diluent.

This solution is 1ppm with respect to test concentration5mg/ml.

Preparation of LOD solution:

Transferred 3.3 ml of LOQ solution in to 10 ml volumetric flask containing 5 ml of diluent, cyclomix for 2 minutes and made up to the volume with diluent.

Table- 3b:Results of Chloroimpurity LODandLOQ

Parameter	**Concentration** (***ppm***)	**Signal to noise ratio**
Limit of Detection	0.3	2.8
Limit of Quantification	1	9.8

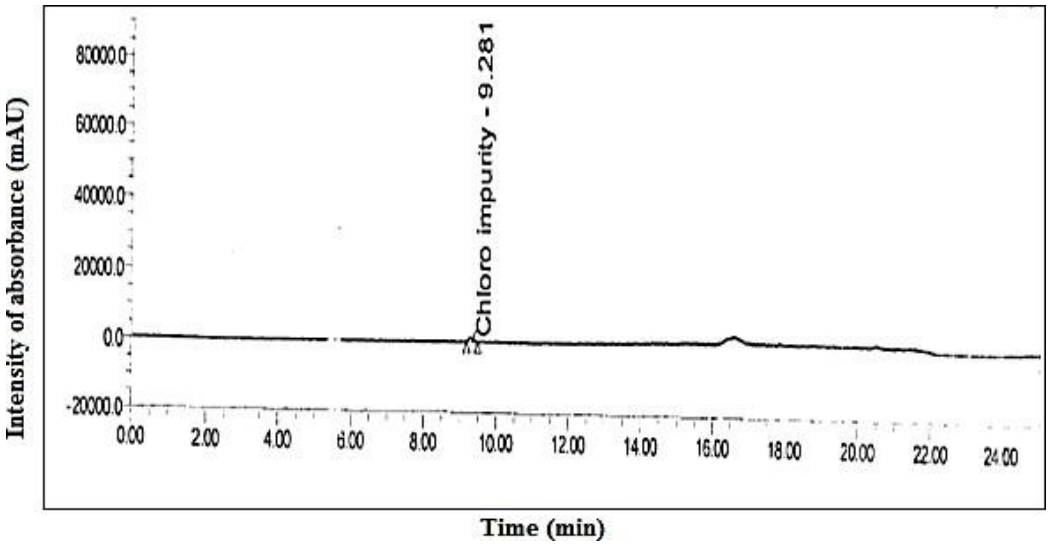

Figure-3c:Typical Representative chromatograms of Limit of detection for chloro impurity

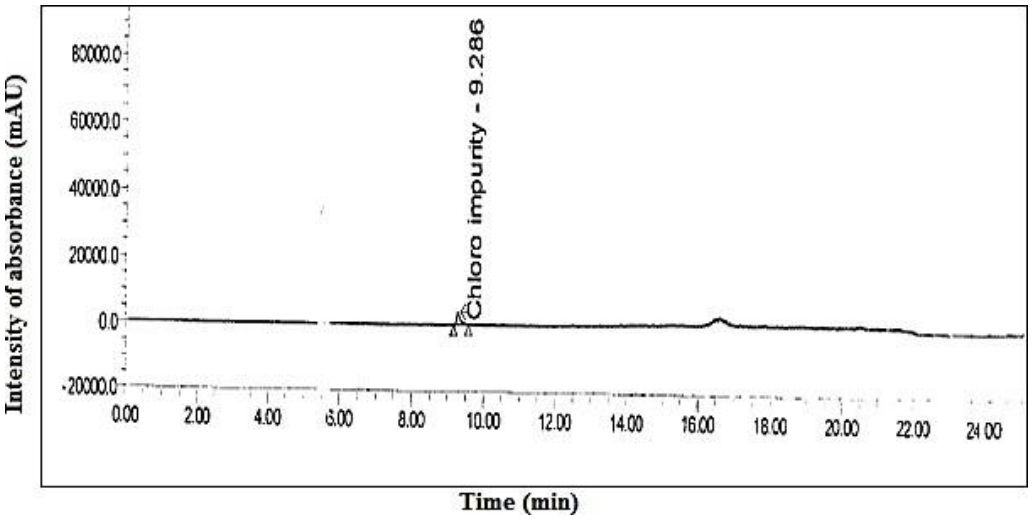

Figure- 3d:Typical Representative chromatograms of Limitof quantification for chloro impurity

Precision

Precision was established by calculating % Relative standard deviation for six replicate injections of LOQ solution, standard solution at 150 % level to the target concentration i.e. 22.5 ppm with respect to test concentration.

Preparation of standard solution at 150%:

Transferred 5.6 ml of above chloro impurity intermediate stock solution in to 50 ml volumetric flask containing 30 ml of diluent, cyclomix for 2 minutes and made up to the volume with diluent.

Injected the above two solutions into the chromatographic system for 6 times individually and calculated the *% RSD.*

Table- 3c: Resultsof Method precision

Injection	Area of chloroimpurity	
	LOQ level	**100 %level**
1	24924	534882
2	24472	533071
3	26361	556682
4	21040	567923
5	22546	535368
6	24801	530974
Average	24024	543150
Standard deviation	1740.0	13997.5
(%) RSD	**7.2**	**2.6**

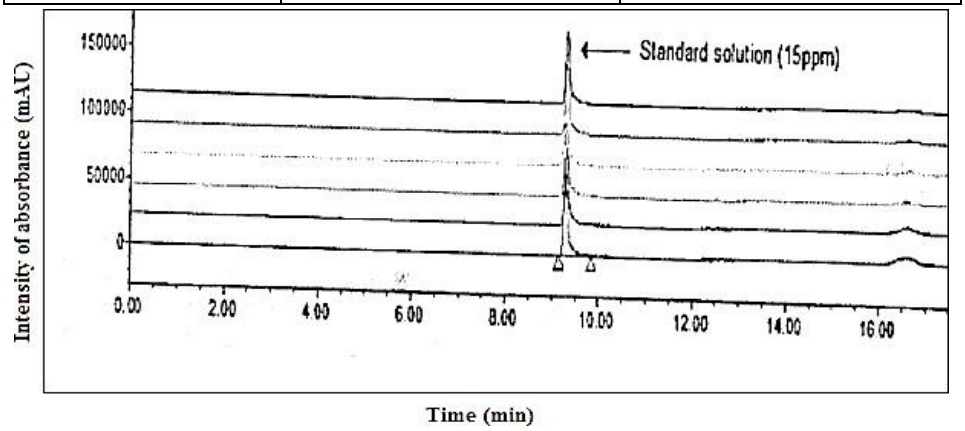

Figure- 3e:Representative chromatograms of Standard precision at 100% level for chloro impurity

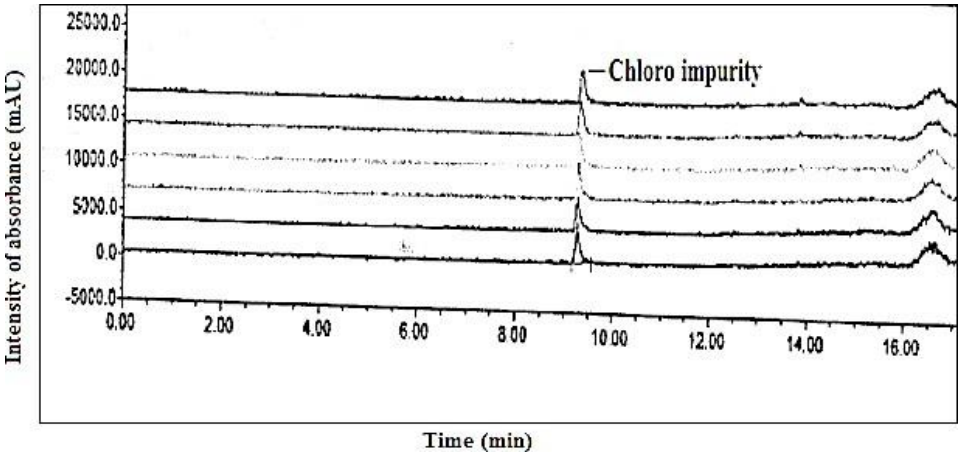

Figure-3f: Representative chromatograms of Standard precision at LOQ level for chloro impurity

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 solutionintriplicate.Preparedtheaccuracysolutionsbyspikingthe0.005µg (LOQ level), 0.038 µg (50% level), 0.076 µg (100% level), and 0.114 µg (150% level) of chloroimpurity 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 108.7, 101.3, 104.2 and 110.1 at LOQ, 50%, 100%, 150% levels respectively.

Accuracy solutions preparation:

Preparation of test solution spiked at LOQ level:

Accurately weighed about 50 mg of test sample in 10 ml volumetric flask containing 5 ml of diluent, sonicated for few minutes to dissolve, spiked 50 µl of chloro impurity stock solution and made up to the mark with diluent.

The above solution was prepared three times individually. Preparation of test solution spiked at 50 % level:

Accurately weighed about 50 mg of test sample in 10 ml volumetric flask containing 5 ml of diluent, sonicated for few minutes to dissolve, spiked 400 µl of chloro impurity stock solution and made up to the mark with diluent.

The above solution was prepared three times individually. Preparation of test solution spiked at 100% level:

Accurately weighed about 50 mg of test sample in 10 ml volumetric flask containing 5 ml of diluent, sonicated for few minutes to dissolve, spiked 750 µl of chloro impurity stock solution and made up to the mark with diluent.

The above solution was prepared three times individually. Preparation of test solution spiked at 150 % level:

Accurately weighed about 50 mg of test sample in 10 ml volumetric flask containing 5 ml of diluent, sonicated for few minutes to dissolve, spiked 1150 µl of chloro impurity stock solution and made up to the mark with diluent.

The above solution was prepared three times individually.

Table-3d:Results of Accuracy at LOQ level

S.No	Area of chloroimpurity		Average area of standard (15ppm)	Amount of chloroimpurity spiked (ppm)	Chloroimpurity content (ppm)		(%) Recovery	(%) Average recovery
	Test sample (asis)	Spiked at LOQ			Test sample (as is)	Spiked at LOQ		
Replicate-1	16487	43942	366818	1	0.67	1.8	107.3	**108.7**
Replicate-2	21078	50019			0.86	2.05	109.9	
Replicate-3	23081	51809			0.94	2.12	109	

Table-3e:Results of Accuracy at 50% level

S.No	Area of chloroimpurity		Average area of standard (15ppm)	Amount of chloroimpurity spiked (ppm)	Chloroimpurity content (ppm)		(%) Recovery	(%) Average recovery
	Test sample (asis)	Spiked at 50 %level			Test sample (as is)	Spiked at 50 % level		
Replicate-1	16487	205021	366818	8	0.67	8.38	96.7	**101.3**
Replicate-2	21078	227035			0.86	9.28	104.8	
Replicate-3	23081	223915			0.94	9.16	102.4	

Table-3f:Results of Accuracy at 100% level

S.No	Area of chloroimpurity		Average area of standard (15ppm)	Amount of chloroimpurity	Chloroimpurity content (ppm)		(%) Recovery	(%) Average
	Tests ample	Spiked at			Test	Spikedat		

	(asis)	100%level		spiked (ppm)	sample (as is)	100% level		recovery
Replicate-1	16487	410765	366818	15.2	0.67	16.8	105.8	**104.2**
Replicate-2	21078	404653			0.86	16.55	103	
Replicate-3	23081	410273			0.94	16.78	103.9	

Table-3g:Results of Accuracy at 150% level

S.No	Area of chloroimpurity		Average area of standard (15ppm)	Amount of chloroimpurity spiked (ppm)	Chloroimpurity content (ppm)		(%) Recovery	(%) Average recovery
	Test sample (asis)	Spiked at 100%level			Test sample (as is)	Spikedat 100% level		
Replicate-1	16487	646790	366818	23	0.67	26.45	111.7	**110.1**
Replicate-2	21078	638802			0.86	26.12	109.5	
Replicate-3	23081	638144			0.94	26.1	109	

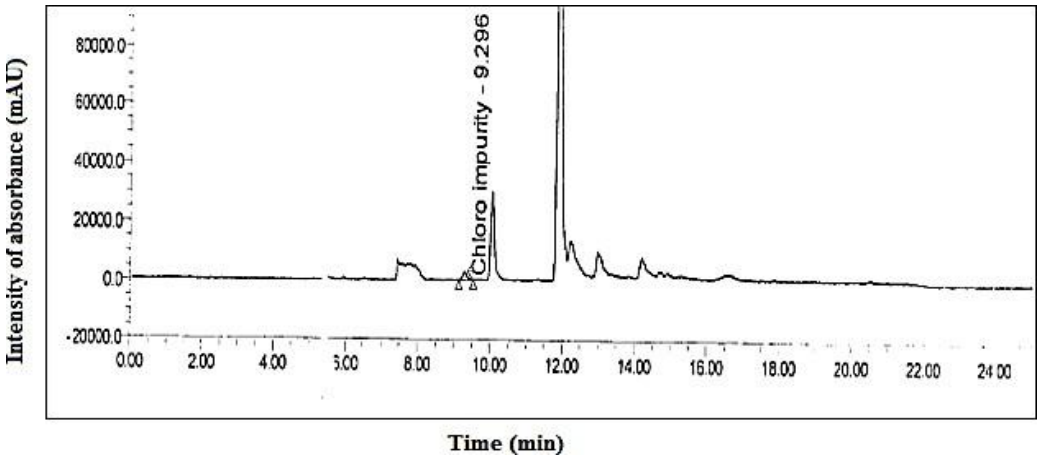

Figure- 3g:Representative chromatograms of Accuracy experiment–ControlTest sample as is

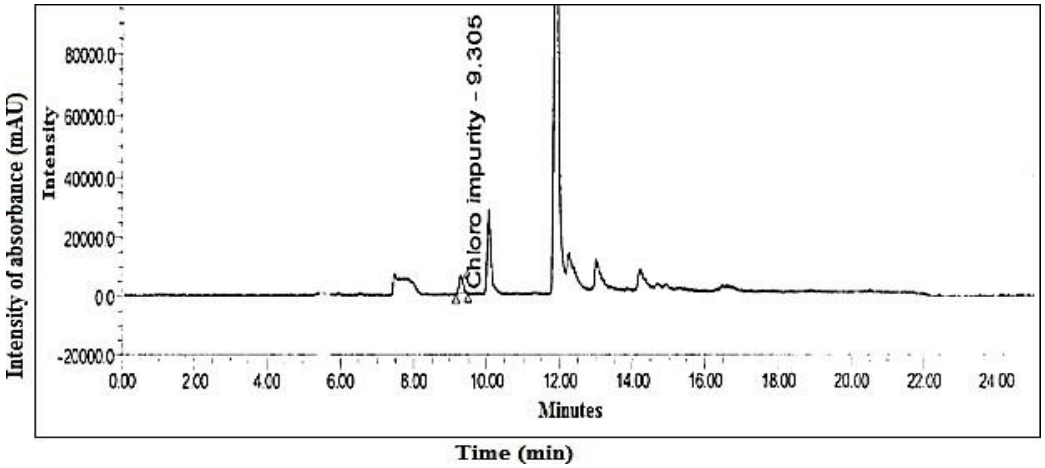

Figure- 3h:Representative chromatograms of Accuracy experiment– Spiked test solution at LOQ level

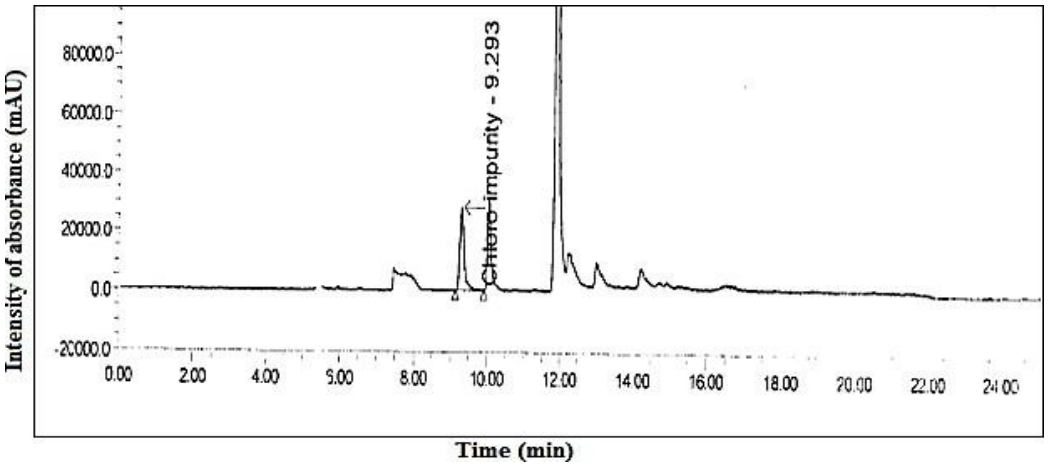

Figure- 3i:Representative chromatograms of Accuracy experiment–Spiked test solution at 50% level

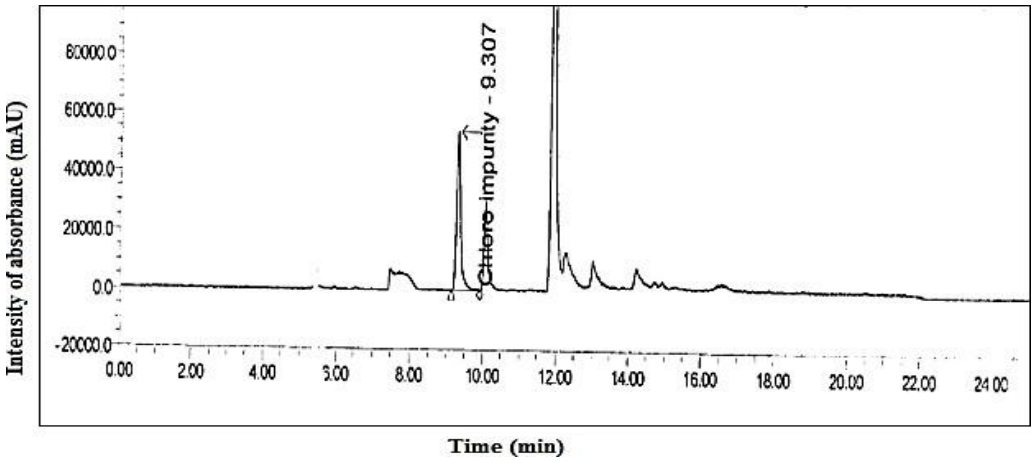

Figure- 3j:Representative chromatograms of Accuracy experiment–Spikedtest solution at 100% level

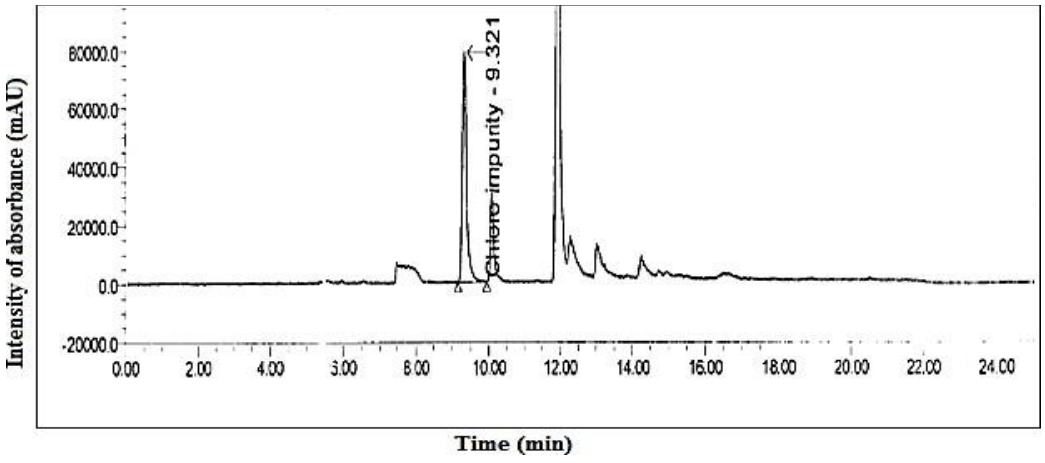

Figure- 3k:Representative chromatograms of Accuracy experiment–Spiked test solution at 150% level

Linearity and Range

The linearity of the method was evaluated by injecting diluted solutions of chloro impurity at 50 %, 80 %, 100 %, 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. The linearity graph was plotted by taking the areas on Y-axis and impurity concentration on X-axis. Calculated the correlation coefficient and found the value ≥0.99.

Preparation of linearity solutions:

50 %solution:

Transferred 1.9 ml of above chloro impurity intermediate stock solution in to 50 ml volumetric flask containing30 ml of diluent, cyclomix for 2 minutes and made up to the volume with diluent.

80 %solution:

Transferred 3.0 ml of above chloro impurity intermediate stock solution in to 50 ml volumetric flask containing30 ml of diluent, cyclomix for 2 minutes and made up to the volume with diluent.

100 %solution:

Transferred 3.8 ml of above chloro impurity intermediate stock solution in to 50 ml volumetric flask containing30 ml of diluent, cyclomix for 2 minutes and made up to the volume with diluent.

120 %solution:

Transferred 4.5 ml of above chloro impurity intermediate stock solution in to 50 ml volumetric flask containing30 ml of diluent, cyclomix for 2 minutes and made up to the volume with diluent.

150 %solution:

Transferred 5.6 ml of above chloro impurity intermediate stock solution in to 50 ml volumetric flask containing30 ml of diluent, cyclomix for 2 minutes and made up to the volume with diluent.

Table- 3h:Linearity results of Chloroimpurity

Level	Conc. (µg/ml)	area	Correlation	Slope	Intercept
50%	0.0375	184573	0.9946	4609851	13784.01
80%	0.0600	303051			
100%	0.0750	345187			
120%	0.0900	429921			
150%	0.1125	534882			

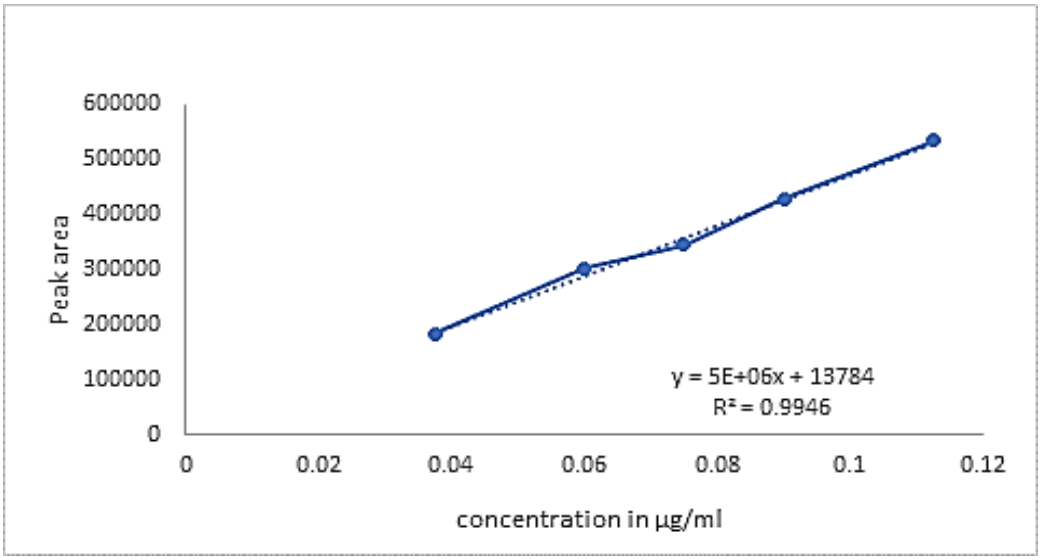

Figure- 3l:Linearity graph of chloroimpurity

Robustness

Method robustness was proved by studying the variation in system suitability results verses the changes in flow rate± 0.1 $mLmin^{-1}$ 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 chromatographicsystematalltheabovechangedchromatographicconditions and found the method was robust.

Moified chromatographic conditions for Robustness

Table-3i:Results of Robustness-flow variations

Replicate	**Area of chloroimpurity**		
	f=*0.3*	**f=*0.2***	**f=0.4**
1	370748	415698	352560
2	374145	424458	346692
3	369774	422569	354892
4	367179	409658	355879
5	359316	411361	335897
6	359746	412587	365152
Average	366818	416055.1667	351845.33
Standard deviation	6071.1	6134.4	9839.1
(%) RSD	1.7	1.5	2.8

Table- 3j:Results of Robustness-Temperature variations

Replicate	**Area of chloroimpurity**		
	t=40°c	**t=35°c**	**t=45°c**
1	370748	385645	378528
2	374145	376891	376588
3	369774	378954	365500
4	367179	381369	364221
5	359316	378745	371258
6	359746	376985	382564
Average	366818	379764.8333	373109.83
Standard deviation	6071.099966	3311.604229	7364.59
(%) RSD	1.7	0.9	2.0

Table- 3k:Results of Robustness-pHvariations

Replicate	**Area of chloroimpurity**		
	pH=5.5	**pH=5.3**	**pH=5.7**
1	370748	365520	359125
2	374145	335248	362310

3	369774	345669	369580
4	367179	371259	352897
5	359316	374561	366542
6	359746	362589	345896
Average	366818	359141	359391.67
Standard deviation	6071.099966	15424.47991	8807.17
(%) RSD	1.7	4.3	2.5

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. 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

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.

Table- 3l:Results of Solution stability

Injection	**Area of chloroimpurity**	
1	356558	
2	365287	
3	345980	
4	378522	
5	355412	
6	362585	
Average	360724	
Standard deviation	10991	
(%) RSD	3.00	
Duration	Area of chloroimpurity	Chloro impurity(ppm)
SS/RT/0h	395689	16.5
SS/RT/24h	405965	16.9
Difference		0.4

Table3.3m:Results of Mobile phase stability

Conditions	**Chloroimpurity area**	**Concentration**	**Similarity factor**
Asis	377852	0.0752	--

MS/0h	380628	0.0755	0.99
MS/24h	376525	0.0755	1.01

4. CONCLUSION

The method was found to be specific, selective, precise, and robust. The developed method can successfully be applied for the determination of chloro impurity in canagliflozin up to very low trace level concentration.

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