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Quantification of Potential Genotoxic Impurities at Trace Level in Gefitinib Drug Substance by LCMS Method

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Abstract: ***A sensitive and novel reversed-phase liquid chromatography (UPLC) coupled with triple quad (TQS-micro) mass detector (LC-MS system) method was developed and validated [13] for the trace analysis of 4-(3-chloropropyl) Morpholine (KSM-02), 3-Chloro-4-fluoroaniline and 4-Chloroanilinegenotoxic impurities in Gefitinib drug substance with the shorter run time. These impurities of Gefitinib were identified as genotoxic impurities using SAR / QSAR models. The method developed on waters Xevo TQS micro mass system attached with Acquity H class UPLCand Acquity UPLC BEH C18, 50x2.1mm, 1.7µmcolumn with electro spray ionization (ESI) in SIR (Single ion reaction) detection mode. The gradient mode of elution for the impurities was carried out with the aid of the mobile phase-A (0.1% solution of formic acid in water)and mobile phase-B(Acetonitrile).The flow rate was 0.5mL/min, column oven temperature 30ºC and elution was monitored by mass spectrometer. The method was validated as per International Conference on Harmonization (ICH) guidelines. The method quantitates up to 0.01ppm with respect to sample concentration of 4-(3-chloropropyl) Morpholine (KSM-02), 3-Chloro-4-fluoroanilineand 4-Chloroaniline***

Keywords: Gefitinib, UPLC-MS, Genotoxic impurities, SIR (Single ion reaction and SAR / QSAR models.

1. Introduction

Impurities in drug substances or products typically belong to the category of starting materials, intermediates, or by-products [1]. Some impurities in the drug substance or pharmaceutical products containing structural alters are called potential genotoxic impurities (PGIs) [12]. These impurities may react with and damage DNA, induce genetic mutation, and potentially cause cancer. PGIs may be generated at any phases of the drug substance synthesis, drug product manufacture, or during storage of drug substance or drug product. In many instances, these impurities can be controlled and effectively purged out or removed during the drug substance development process through an alternate synthesis route. If changing the synthesis process is not possible, control of genotoxic impurities to an acceptable level that follows the regulatory standards is required. Regulatory guideline recommends that the genotoxic impurity should be controlled to a level based on a threshold of toxicological concern (TTC), which is 1.5 µg/day over lifetime of exposure. The analytical procedures with "as low as reasonably practicable" (ALARP) detection limits will enable accurate monitoring of the fate and purging levels of the impurities during the drug development process. Ultimately, accurate identification and control of these toxic impurities at various stages of pharmaceutical development is a critical element to ensure product quality and minimum risk to the patient safety.

In the last several years, growing importance has been given to the quantification of potential genotoxic impurities [2–6], i.e. those which could cause DNA damage involving genetic mutations[7]. In order to make a preventive evaluation of the potential genotoxic activity of a given impurity, lists of alerting functions have been compiled on the basis of the structure of known genotoxic compounds and their mechanism of action [8, 9]: impurities bearing one or more alert functions have to be considered as potential genotoxic compounds if no toxicological data are available, and their limit has to be calculated according to specific guidelines [10].

Gefitinib is a targeting drug of epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs). It is mainly used in the treatment of progressive-stage non-small cell lung cancer (NSCLC) patients who received platinum as basic chemotherapy, and it was sensitive to adenocarcinoma in non-smoking Asian women. Gefitinib may be considered a first-line therapy regimen [11].

The chemical name for Gefitinib is 4-(3'-chloro-4'-Fluoroanilino)-7-methoxy-6-(3-morpholinopropoxy)quinazoline. The molecular formula is $C_{22}H_{24}ClFN_4O_3$, which corresponds to a formula weight of 446.91g/mol. The drug substance is very slightly soluble in methanol, sparingly soluble in dimethylsulphoxide and practically insoluble in water. Gefitinib is marketed by AstraZeneca under the trade name IRESSAavailable in tablet and maximum daily dose is 250mg.

1.1 Description of Brief Synthetic process of Gefitinib

The Gefitinib has been synthesized by reaction of 4-((3-chloro-4-fluorophenyl) amino)-7-methoxyquinazolin-6-olate (GEF-01) with 4-(3-chloropropyl) Morpholine (KSM-02), produced crude Gefitinib on purification yield pure Gefitinib. Synthetic scheme of Gefitinib is represented in fig.-01. Chemical structure of4-(3-chloropropyl) Morpholine (KSM-02), 3-Chloro-4-fluoroaniline and 4-Chloroaniline are represented in fig.02, 03 & 04 respectively.

1.2 Genotoxicity and carcinogenicity study

The above impurities (Fig. 2, 3&4) are considered as a genotoxic carcinogen as per structural alert. KSM02 is key starting material of Gefitinib, it is potentially genotoxic hence need to control at acceptable level.3-Chloro-4-fluoroaniline is degradation product of GEF-01 and may be generated in the reaction and 4-Chloroaniline is genotoxic impurity of 3-Chloro-4-fluoroaniline. The alerts have been taken from the ViTAL toxicity alert through software program of VLife science a division of Novalead pharma pvt. ltd (Estimation of toxic hazard–A decision tree approach) version 2.4. The maximum daily dosage of Gefitinib is about 250 mg. The genotoxic impurities limit for each impurity is 6ppm, calculated as per the calculation provided in ICH guideline M7, considering 250 mg maximum daily dose of Gefitinib and based acceptable intake of 1.5µg/day the threshold of toxicological concern (TTC) was protective for a lifetime of daily exposure.

In this work, we develop and validate a sensitive and fast UPLC method coupled with a triple quadrupole mass spectrometer for the quantitative determination of three genotoxic impurities of the Gefitinib drug substance. There is no method available in literature for quantification of these three impurities in gefitinib. We demonstrated sensitivity, specificity, linearity and accuracy of the method.

2. Materials and Methods

2.1 Apparatus

The analysis was carried out on aWaters Xevo TQS Micro MS system equipped with Acquity H class UPLCand mass hunter data handling system.Acquity UPLC BEH C18, 50x2.1mm, 1.7µm UPLC column was used.

2.2 Chemicals and Solvents

Millipore generated water used forUPLC, acetonitrile (Fischer LCMSgrade, formic acid (LCMS grade)were obtained from Fischer scientific. 4-(3-chloropropyl) Morpholine (KSM-02) is a starting material of Gefitinib and purchased from China.3-Chloro-4-fluoroaniline is potential genotoxic impurity generated in the reaction and isolated by research center of Oncogen pharma (Malaysia) Sdn. Bhd and 4-Chloroaniline was purchase from Sigma Aldrich. Gefitinibdrug substance sample is synthesized at research center of Oncogenpharma (Malaysia) Sdn. Bhd.

2.3 Method development

The initial trials were carried out with HPLC using different buffers. During the trials it was observed that there was a lag in attaining the sensitive & specificity of the method to reach the targeted level and hence finally the method development trials were carried out using the LCMS technique for better sensitivity. The final chromatographic condition was achieved on a acquity UPLC BEH C18, 50x2.1mm, 1.7µm UPLC column in gradient mode of elution using the mobile phase-A (0.1% formic acid in water) and mobile phase-B (acetonitrile). The flow rate was 0.5mL/min, MS parameters were set to get maximum sensitivity for the impurities of 4-(3-chloropropyl) Morpholine, 3-Chloro-4-fluoroaniline and 4-Chloroaniline. Before obtaining the final method, the method was scrutinized with different stationary phase columns which includes C18, C8,

2.4 Optimized LCMS condition

The UPLC column used was Acquity UPLC BEH C18, 50x2.1mm, 1.7µm procured from waters technologies (Malaysia). A gradient elution was used. The mobile phase-A was 0.1% formic acid in water and mobile phase-B was acetonitrile. The gradient elution program was represented in Table-1. The flow rate was 0.5mL/min. The column oven temperature was maintained at 30^0C and sample cooler temperature was 20^0C. The injection volume was 2µl. Positive ion electrospray ionization probe &single ion reaction (SIR) detection mode were used for LC-MS method for quantification of 4-(3-chloropropyl) Morpholine, 3-Chloro-4-fluoroaniline and 4-Chloroaniline genotoxic impurities in Gefitinib drug substance. Mass spectrometer conditions was represented in Table-2.

2.5 Sample and standard preparation

The test concentration of Gefitinib was 5mg/mL. The diluent was optimized as acetonitrile and water in the ratio of 50:50 (v/v). The standard solution 4-(3-chloropropyl) Morpholine, 3-Chloro-4-fluoroaniline and 4-Chloroaniline were prepared 6.0 ppm with respect to the test concentration.

2.6 Procedure

LCMS system was equilibrated.Injected blank and standard solutions into the chromatograph by followed the test method conditions. Monitored these impurities with its molecular ion [M+1] (protonated) as mentioned below by SIRmode. (Note: M = Molecular weight) as shown in Table-3.

Evaluated system suitability by the relative standard deviation of replicate injections of all impurities should not be more than 5 %. The injected sample solution and monitored content of these impurities with its molecular ion [M+H] as per above.Calculated the content of each impurities in sample chromatogram (TIC) by external standard method.

The SIR chromatograms of blank (diluent), individual injection of 4-(3-chloropropyl) Morpholine,4-Chloroaniline and 3-Chloro-4-fluoroaniline,genotoxic impurity standard (individual), Gefitinib sample and Gefitinib drug substance spiked with all impurity mixture at specification level using the proposed method is shown in figure 5,6, 7, 8,9 10 &11 respectively.

3. Method Validation

The developed method was validated as per ICH guidelines. The specificity of the developed LCMS method for Gefitinib was determined in the presence of its process impurities. All the analysis was carried out by Waters Xevo TQS Micro MS system equipped with Acquity H class UPLC and mass hunter data handling system. Acquity UPLC BEH C18,

50x2.1mm, 1.7µm UPLC column was used. Using the SIR acquisition mode, linearity test solutions for six concentration levels from LOQ to 200 % of the specification level. Peak area versus concentration data was performed by least-squares linear regression analysis with the correlation coefficients of ≥0.999 for all genotoxic impurities.

The LOQ levels for 4-(3-chloropropyl) Morpholine (KSM-02), 3-Chloro-4-fluoroaniline and 4-Chloroaniline and are at 0.009ppm, 0.006ppm and 0.008ppm respectively with respect to sample concentration. Precision study was carried at LOD &LOQ level by injecting six times and calculating the percentage of relative standard deviation (RSD) ofarea of all impurities were found well withinlimits.Standard addition and recovery experiments were conducted to determine accuracy of impuritiesquantitation in bulk drug samples. The study was carried out in triplicate at LOQ, 100% and 200% level with respectto specification limit. The percentages of recoveries for impurities were calculated.

The recoveries for all impurities from the spiked drug substance sample range from 80 to 120%. The results show that the triple quadrupole spectrometer with SIR acquisition mode is ideal for quality monitoring of genotoxic impurities in the development of drug substances. Method Validation data is represented in Table-4.

4. Conclusion

A sensitive liquid chromatography mass spectrometer (LCMS) method has been developed and validated for determination of 4-(3-chloropropyl) Morpholine (KSM-02), 3-Chloro-4-fluoroaniline and 4-Chloroaniline potential genotoxic impurities of Gefitinib at trace level. The method was found to be precise, linear and accurate with decent and constant recoveries. The validated method may be used for the regular analysis of Gefitinib drug substance.

5. Acknowledgement

Author is thankful to Oncogen pharma (Malaysia) Sdn Bhd for providing Gefitinib drug substance sample, reference standard of 4-(3-chloropropyl) Morpholine (KSM-02), 3-Chloro-4-fluoroaniline and 4-Chloroaniline impurities and other necessary facilities to conduct this development work.

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Figures and Tables

Fig.-1: Synthetic scheme of Gefitinib
Fig.-2: Chemical structure of 4-(3-chloropropyl) Morpholine (KSM-02).
Fig.-3: Chemical structure of 3-Chloro-4-fluoroaniline
Fig.-4: Chemical structure of 4-Chloroaniline

Fig.-5 to 11 Chromatograms (TIC)
Fig.-12 to 14 Linearity graphs of impurities.

Table-1: Gradient Program
Table -2: Mass spectrometer conditions
Table-3: Retention time and Molecular ion.
Table -4: Validation data

Figures

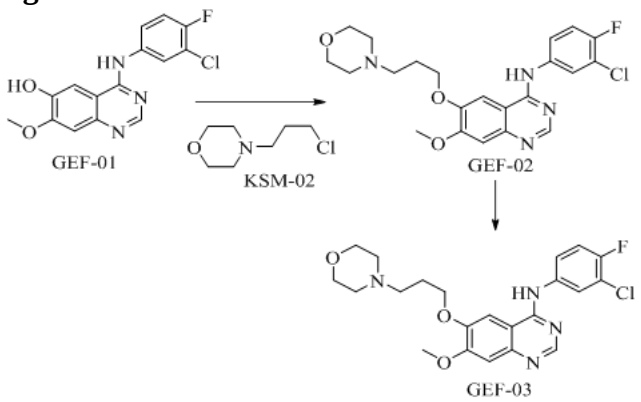


Figure 1: Synthetic scheme of Gefitinib

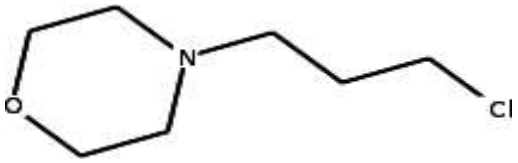


Figure 2: 4-(3-chloropropyl) Morpholine (KSM-02). $C_7H_{14}NOClM$. Wt: 163.65

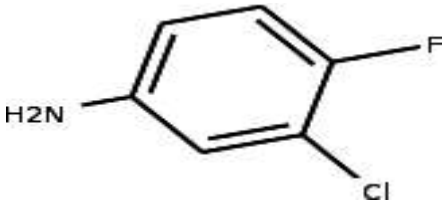


Figure 3: 3-Chloro-4-fluoroaniline C_6H_5NClFM. Wt: 145.56

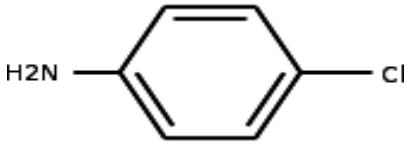


Figure 4: 4-Chloroaniline.C_6H_6NClM. Wt: 127.57

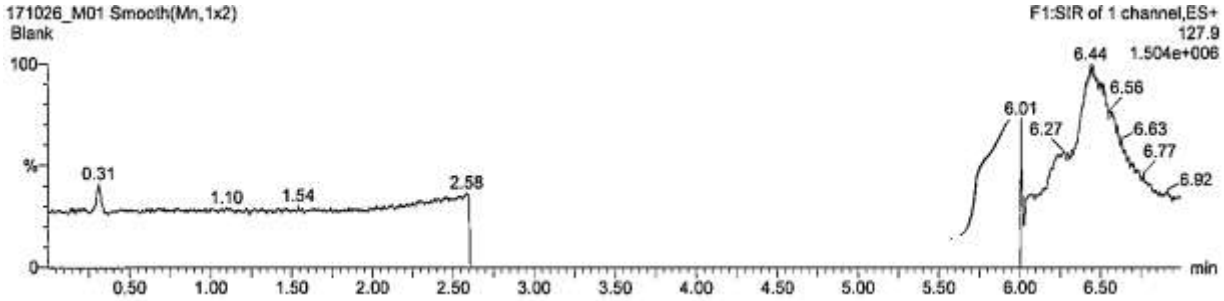


Figure 5: Blank (Diluent)

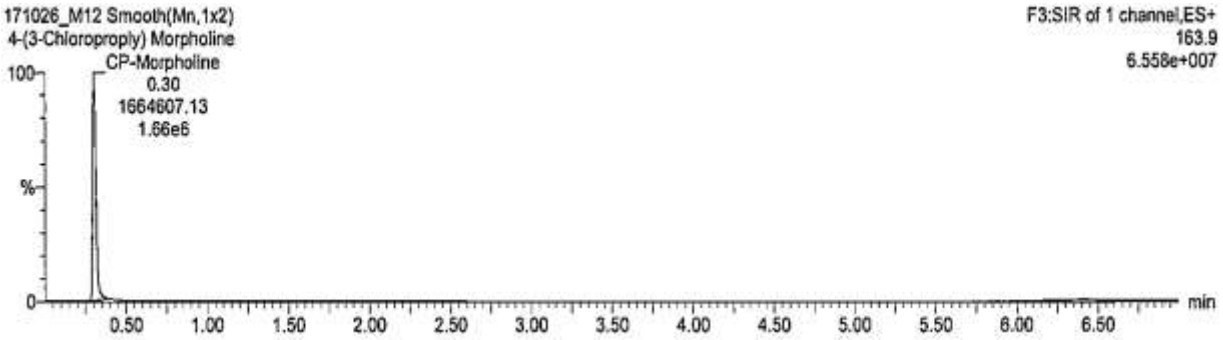


Figure 6: 4-(3-chloropropyl) Morpholine**.**

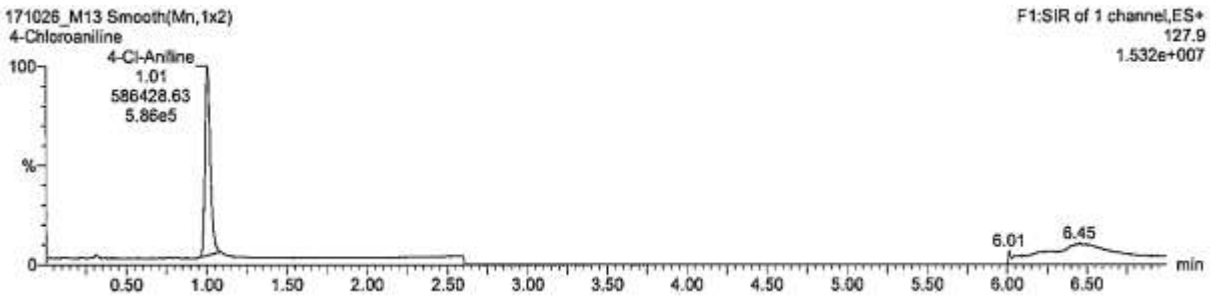


Figure 7: 4-Chloroaniline**.**

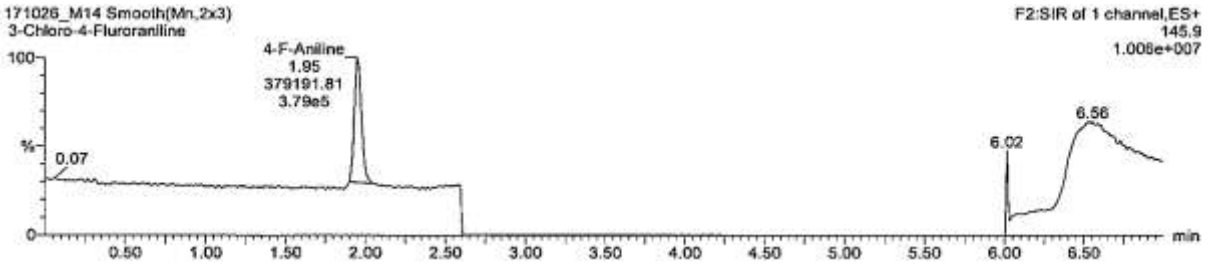
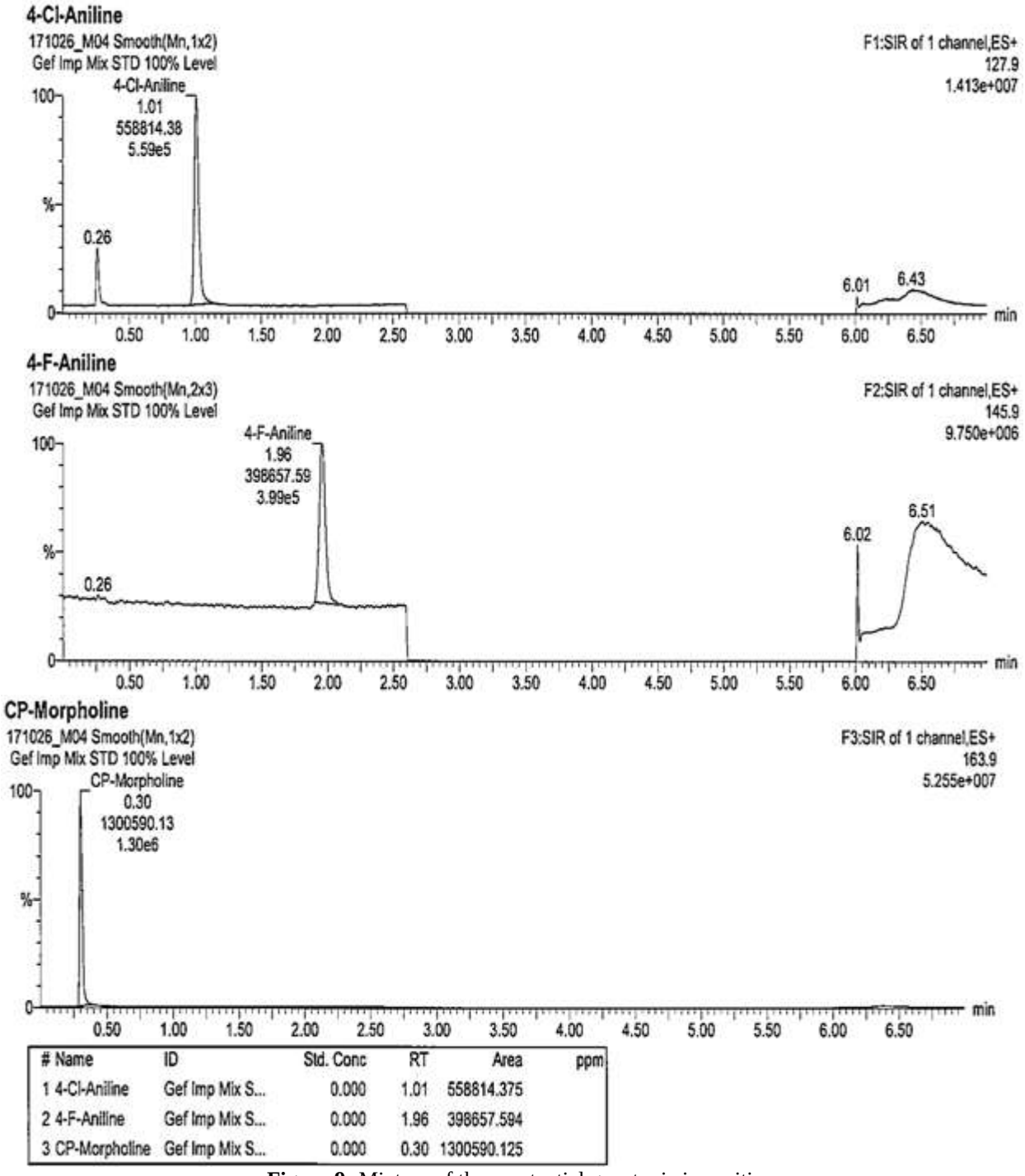
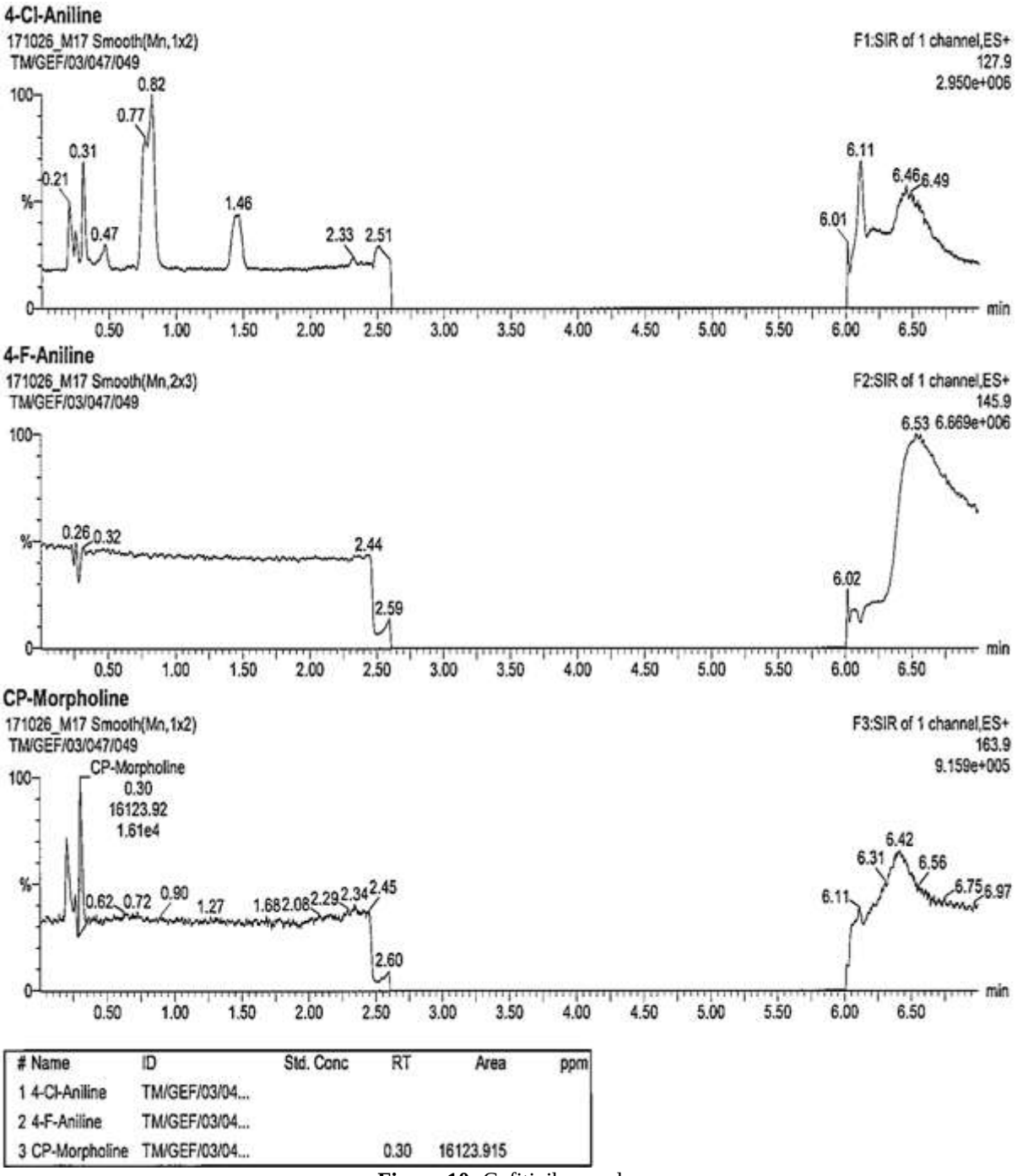


Figure 8: 3-Chloro-4-fluoroaniline



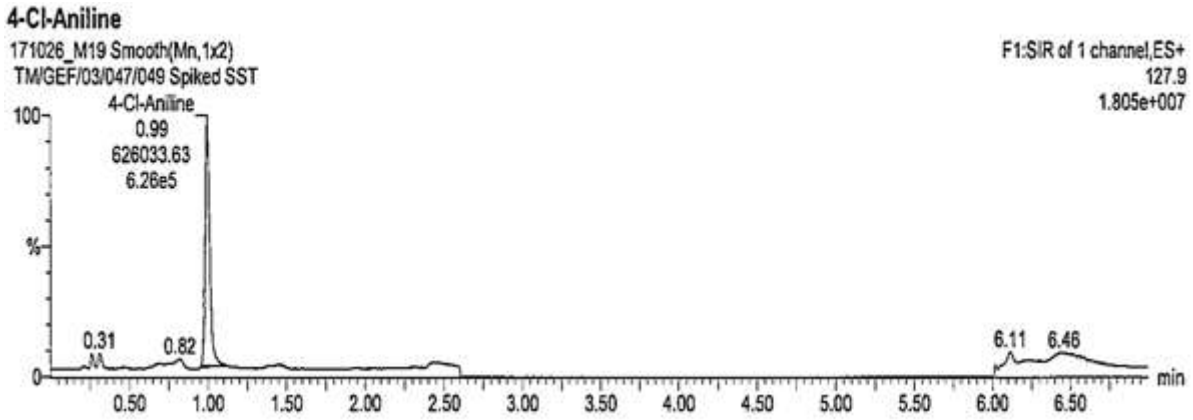
#	Name	ID	Std. Conc	RT	Area	ppm
1	4-Cl-Aniline	Gef Imp Mix S...	0.000	1.01	558814.375	
2	4-F-Aniline	Gef Imp Mix S...	0.000	1.96	398657.594	
3	CP-Morpholine	Gef Imp Mix S...	0.000	0.30	1300590.125	

Figure 9: Mixture of three potential genotoxic impurities**.**



#	Name	ID	Std. Conc	RT	Area	ppm
1	4-Cl-Aniline	TM/GEF/03/04...				
2	4-F-Aniline	TM/GEF/03/04...				
3	CP-Morpholine	TM/GEF/03/04...		0.30	16123.915	

Figure 10: Gefitinib sample



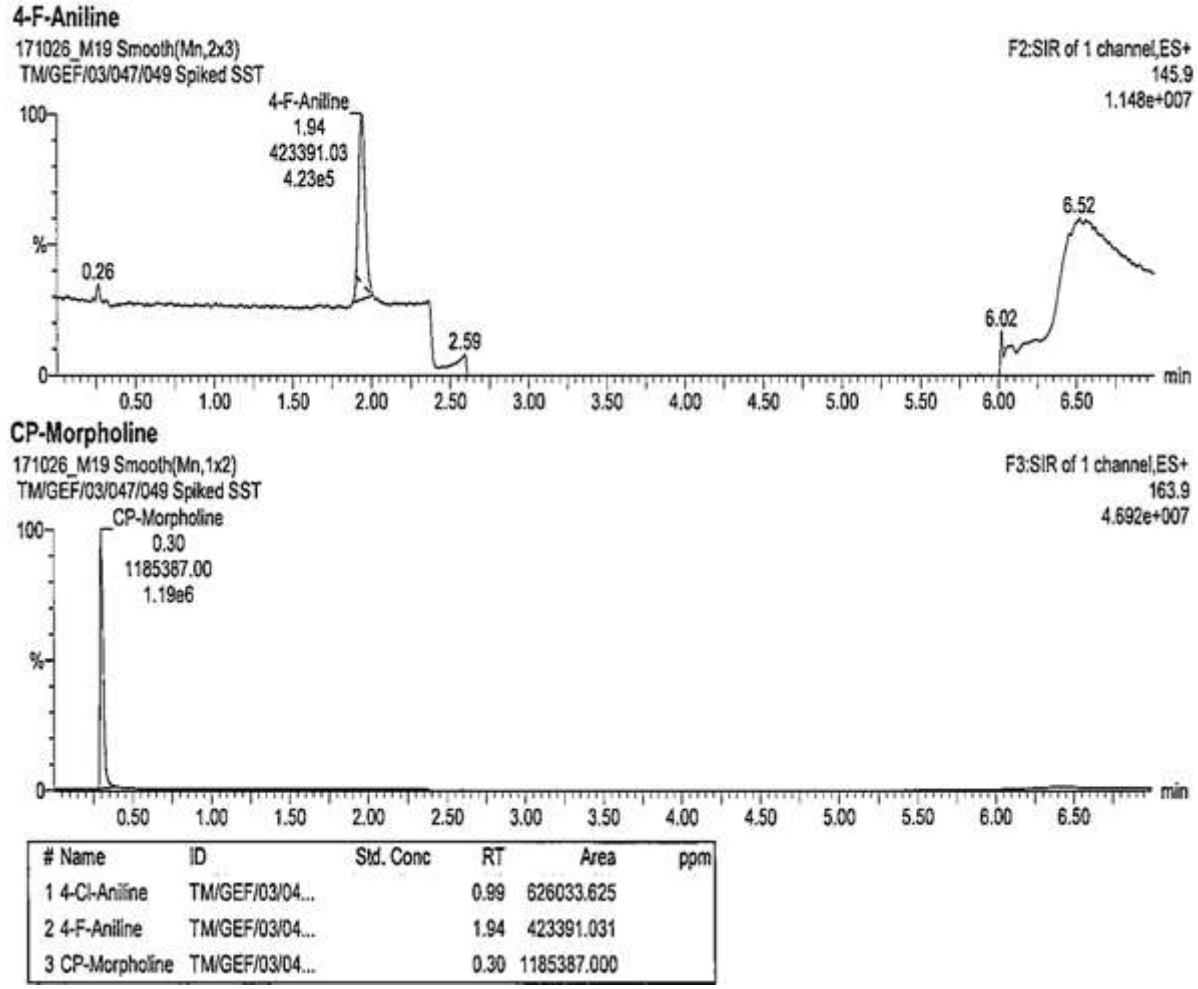


Figure 11: Gefitinib sample spiked with all impurity mixture at specification level

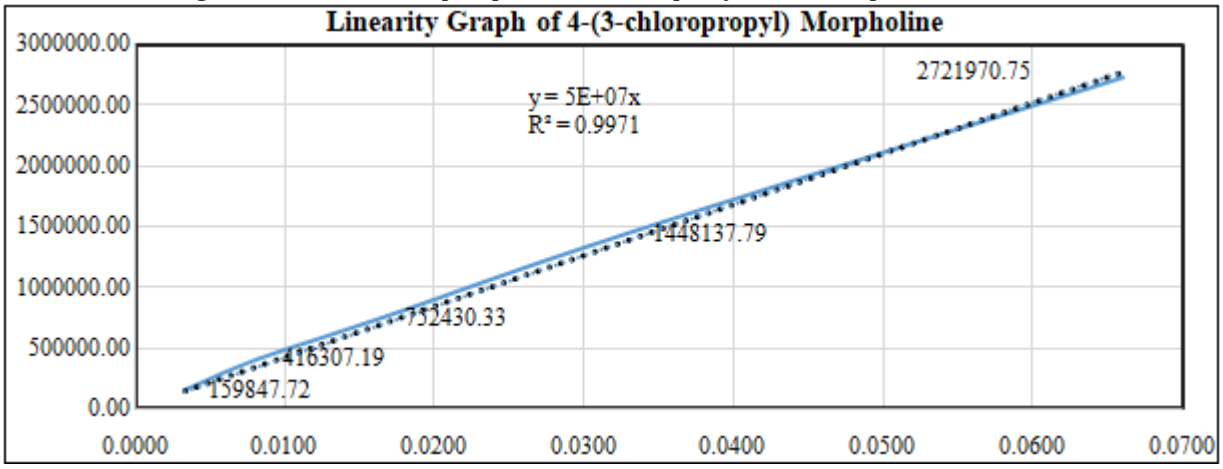


Figure 12: Linearity graph of 4-(3-chloropropyl) Morpholine (KSM-02)

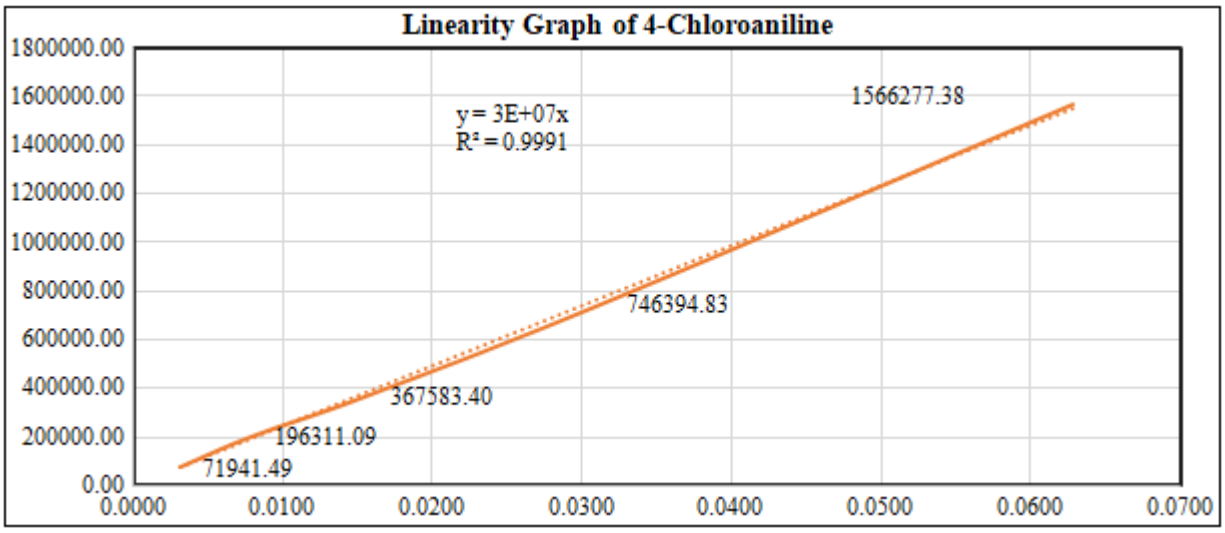


Figure 13: Linearity graph of 4-Chloroaniline

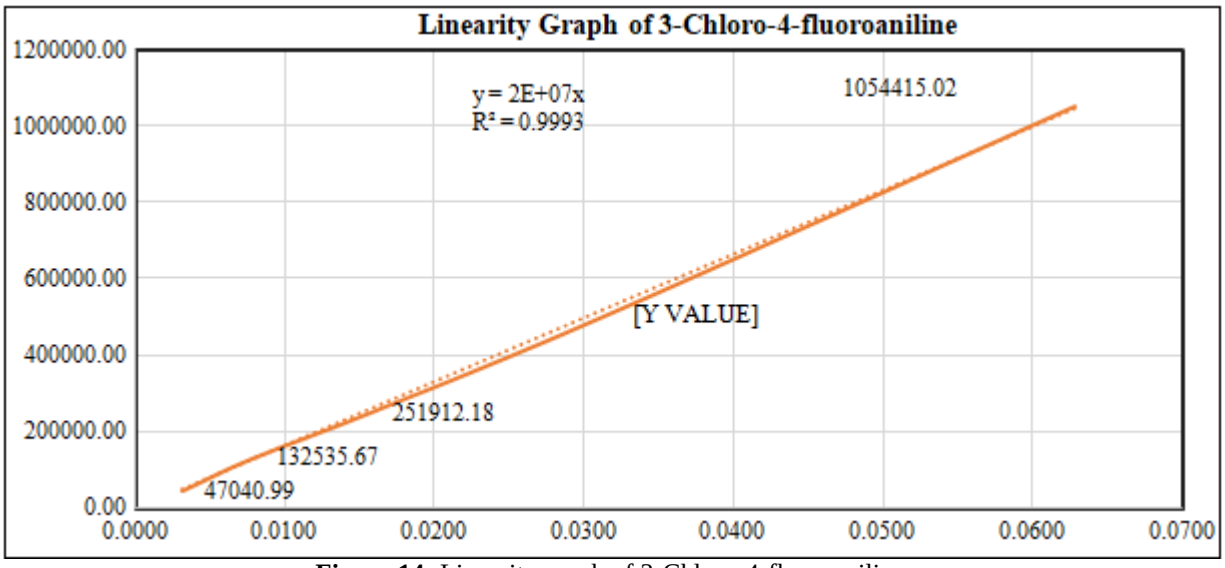


Figure 14: Linearity graph of 3-Chloro-4-fluoroaniline

Tables

Table 1: Gradient Program

Time (min)	Mobile Phase-A (%)	Mobile Phase-A (%)
0.0	90	10
1.0	90	10
4.0	50	50
4.1	20	80
5.5	20	80
5.6	90	10
7.0	90	10

Table 2: Mass spectrometer conditions

Ionization mode / Polarity electrospray ionization probe)	:	+ESI, (Positive ion
Data Type	:	SIR / SIM
Calibration	:	Static 2
Capillary	:	3.00 kv
Cone	:	38.00V
Source Temperature	:	150°C
Desolvation Temperature	:	350°C
Cone Gas Flow	:	0 L/Hrs.
Desolvation Gas Flow	:	1000 L/Hrs.
Dwell	:	0.025secs
Delay	:	Auto

Table 3: Retention time and Molecular ion

Components	Retention time (min	[M+1]
4-(3-chloropropyl) Morpholine	0.3	163.9
4-Chloroaniline	1.1	127.9
3-Chloro-4-fluoroaniline	2.1	145.9

Table 4: Validation data

Parameters	KSM-02	3-Cl 4-fluoroaniline	4-Chloroaniline
Linearity (CORRL)	0.997	0.999	0.999
LOD (ppm)	0.003	0.002	0.0025
LOQ (ppm)	0.009	0.006	0.0075
Method Precision (% RSD)	2.43	4.75	1.43
% Recovery at LOQ level	80.8	116.8	97.8
% Recovery at 100% level	82.0	98.8	96.8
% Recovery at 200% level	94.0	101.1	102.5