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Quantitative Methods for The Trace Level Determination of Potential Genotoxic Impurities in Anti-Cancer Drug, Regorafenib Using Gas Chromatography with Mass Spectrometry (GC-MS)

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ABSTRACT

Two novel gas chromatography methods coupled with selective ion monitoring mass spectrometry (GC-MS-SIM) were developed for quantitation of trace levels of five potential genotoxic impurities (PGI's) namely 2-Fluoroaniline (2-FA), 2-Fluoronitrobenzene (2-FNB), 3-Fluoronitrobenzene (3-FNB), 4-Fluoronitrobenzene (4-FNB) and 1-Chloro-2-nitrobenzene (1-Cl-2-NB) in Regorafenib (REG) drug substance. In these two methods, Chromatographic separation of potential genotoxic impurities (PGI's) were achieved on capillary GC column (Rtx-5, Fused silica capillary column; 30 m length; 0.32mm internal diameter, coated with 5% diphenyl and 94% dimethyl polysiloxane stationary phase of 0.25 µm film thickness) and passing helium as carrier gas with Electron Impact ionization (EI) in Selective Ion Monitoring (SIM) mode by using direct injection technique for 2-FA and liquid-liquid extraction sample preparation technique for remaining four impurities. The mass fragments (m/z) were selected for the quantification of 2-FA (m/z-111), 2-FNB (m/z-141), 3-FNB (m/z-141), 4-FNB (m/z-141) and 1-Cl-2-NB (m/z-157). The performance of the validation of the method was assessed by evaluating the specificity, linearity, sensitivity, precision, and accuracy experiments. For 2-FA, the limit of detection (LOD) and the limit of quantitation (LOQ) were 0.006 µg/mL and 0.002 µg/mL, respectively. For 2-FNB, 3-FNB, 4-FNB and 1-Cl-2-NB impurities, the limit of detection (LOD) and the limit of quantitation (LOQ) were 0.06µg/mL and 0.02 µg/mL, respectively. The correlation coefficient value of the linearity experiment was 0.9982 for 2-FA and the correlation coefficient value of the linearity experiment was in the range of 0.9997–0.9999 for the remaining four impurities. The average recoveries for the accuracy were in the range of 104.8–115.4% for 2-FA and average recoveries for the accuracy were in the range of 108.0–112.4% for remaining impurities. The validation results demonstrated the good linearity, precision, and accuracy of the method which can be further adopted as an adequate quality control tool for quantitation of five potential genotoxic impurities (PGI's) at trace levels in Regorafenib drug substance.

1. INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the USA and the third leading cause of cancer death in both genders. The incidence and mortality have been steadily declining over the last decade, which is probably related to the improvements in the awareness, early detection, and treatment of CRC. However, CRC-related mortality is continuing to increase in underdeveloped countries with limited resources and healthcare [1]. Combination chemotherapy (*e.g.* Fluorouracil, Capecitabine, and either Irinotecan or Oxaliplatin) with or without monoclonal antibody agents (*e.g.* Bevacizumab or Cetuximab and Panitumumab) is the backbone of treatment for metastatic CRC (mCRC) [2-5]. However, drug-related adverse events and drug resistance may limit the potential of such treatments [6]. Especially, many patients with advanced mCRC develop resistance to these agents, leaving very limited options for third-line treatment. Hence, there is a continued need to develop new effective multiple signaling pathways agents that overcome this resistance [7-8].

Regorafenib (BAY 73-4506, commercial name Stivarga) is an oral multi-kinase inhibitor [9-10] developed by Bayer which targets angiogenic, stromal, and oncogenic receptors tyrosine kinase (RTK). Regorafenib shows anti-angiogenic activity due to its dual-targeted VEGFR2-TIE2 tyrosine kinase inhibition. In 2015, it is approved for the treatment of mCRC in patients who have previously received all standard systemic anticancer treatments in the US, EU, and Canada, and in patients with unresectable, advanced, or recurrent CRC in Japan. Regorafenib is the first small-molecule tyrosine kinase inhibitor (TKI) exhibiting improvement in progression-free survival and overall survival in refractory, heavily pre-treated patients with mCRC [11-14]. The introduction of regorafenib could provide patients with CRCa new therapeutic option and help improve their survival and induce disease control.

Regorafenib is off-white to slightly pink or slightly brownish powder pink with lumps in physical state. It is freely soluble in Dimethyl sulfoxide and soluble in Dimethylformamide. The chemical name of Regorafenib (REG) is 4-[4-[[4-chloro-3-(trifluoromethyl)phenyl]carbamoylamino]-3-fluorophenoxy]-*N*-methylpyridine-2 carboxamide corresponding to the molecular formula $C_{21}H_{15}ClF_4N_4O_3$. The molecular weight is 482.82 and the chemical structure of Regorafenib is shown in **Figure 1.**

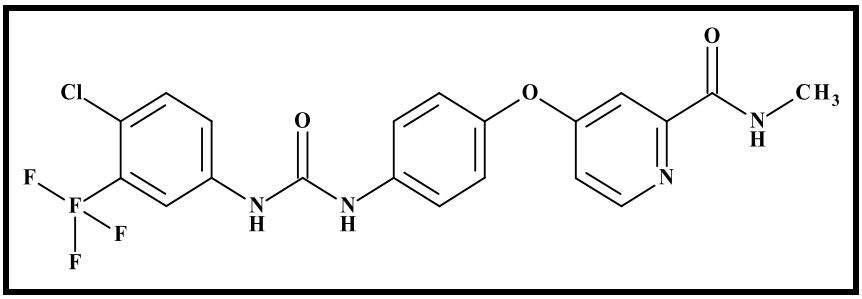

Figure No. 1. Chemical structure of Regorafenib

During the synthesis of drug substances or active pharmaceutical ingredients (API), impurities can easily arise from often used reagents, starting materials, reactive intermediates, by-product reactions and degradation during storage. Such chemically reactive impurities may have unwanted toxicities including genotoxicity and carcinogenicity and these impurities (GTI) pose a significant safety risk because they induce damage to the genetic material in the cells through interactions with the DNA sequence and structure. Certain impurities are reactive to DNA and even when present at low levels can modify the DNA and as a consequence can cause cancer. These are called as potential genotoxic impurities (PGI's). As a result, PGT's can lead to mutations or cause cancer [15-17]. The issue of potential genotoxic impurities (PGI's) in pharmaceutical products has attracted increasing attention from the industry [18–19] as well as regulatory agencies [20–25]. To ensure these undesired PGI's are reduced to an acceptable level (often at low ppm) in the final product, it is critical to monitor them closely throughout the process. However, the rapid development of analytical methods at such low levels remains a challenge for analytical chemists. For example, extremely high sensitivity, specificity and robustness are often desired. Also, complex matrix effects arising from in-process samples, API or excipients need to be overcome. On the other hand, especially for the early drug development stage, aggressive project timelines often limit the time and resources for method optimization. As a result, the analytical chemist needs to ensure the method is appropriate for its intended use. EMEA [23] and FDA [25] guidelines have established a threshold of toxicological concern (TTC) of 1.5 µg/day (1.5 ppm, assuming a daily dose of 1 g/day) for each PGI as an acceptable threshold for any marketing authorization application.

Determination of trace levels of PGI's in API is often a great analytical challenge as an extremely sensitive, selective, and robust analytical method is needed. Many traditional approaches such as HPLC-UV for non-volatile analytes and GC-FID for volatile analytes are usually not effective enough for impurity analysis at sub-ppm or trace levels [26].

Hyphenated techniques like GC-MS and LC-MS combining physical separation capabilities of chromatography (GC or HPLC) with mass spectrometry have higher sensitivity and specificity than conventional HPLC and GC methods. Their applications are oriented towards the potential identification and quantitation of trace levels of impurities in API. Several recent publications have reported systematic PGI method development and control strategies [27-29].

The following impurities 2-Fluoroaniline (2-FA), 3-Fluoronitrobenzene (3-FNB),4-Fluoronitrobenzene (4-FNB), 2-Fluoronitrobenzene (2-FNB), and 1-Chloro-2-nitrobenzene (1-Cl-2-NB) are likely present in Regorafenib (REG) drug substance. In these, 2-Fluoronitrobenzene (2-FNB) is used as a key raw material for the preparation of the Regorafenib (REG) drug substance. The other four are possible isomeric impurities. Based on literature and evaluation by Derek software, these five compounds are found to be potential genotoxic impurities. Hence, these potential genotoxic impurities are limited to a daily dose of 1.5µg/day as per ICH guidelines from the European medical agency [30]. Hence, to meet the regulatory agencies' requirements, it is essential to develop a sensitive analytical method. Hence, a gas chromatograph with a mass spectrophotometer was chosen which can detect trace level determination for the quantification of 2-FA,3-FNB,4-FNB,2-FNB, and 1-Cl-2-NB.

The European Agency for the Evaluation of Medicinal Products (EMEA), United States Food and Drug Administration (USFDA), ICH Q3A/B and ICH M7 issued the guidelines and draft guidance have established a threshold of toxicological concern (TTC) of 1.5 µg/day (1.5 ppm, assuming a daily dose of 1 g/day) for each GTI as an acceptable threshold for any marketing authorization application [31, 32]. As per the toxicological threshold concern (TTC) approach and based on the maximum daily dosage of Regorafenib (160 mg /day), these potential genotoxic impurities (2-FA,3-FNB,4-FNB,2-FNB and 1-Cl-2-NB) should be <9.0 µg g^{-1} (as per TTC concern) [17, 33-34]. To attain the best quality of REG drug, these potential genotoxic impurity levels should be monitored and controlled with appropriate analytical methods in REG drug.

Being a very novel and recently synthesized drug, there are few references for Regorafenib. Literature survey revealed that currently there is no method for the low-level quantification of these five PGI's (2-FA,3-FNB,4-FNB,2-FNB and 1-Cl-2-NB) in REG drug substances till to date. Hence, it is aimed to develop and validate a sensitive and specific method for the trace

level determination of the five PGI's in REG drug substance by GC-EI-MS with selective ion monitoring (SIM) mode. The chemical structures of these five PGI's are shown in **Figure 2 (a)** to **2 (e)** and its mass spectrums are shown in **Figure 2 (f)** to **3 (j)**.

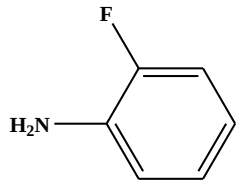

Figure. 2 (a).2-Fluoroaniline

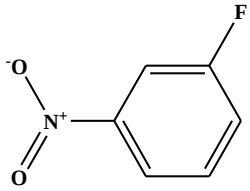

Figure. 2 (b).3-Fluoronitrobenzene

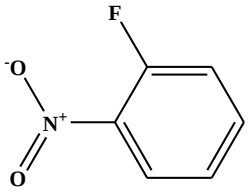

Figure. 2 (c).2-Fluoronitrobenzene

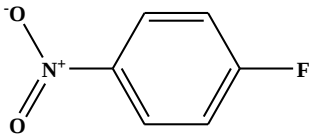

Figure. 2 (d).4-Fluoronitrobenzene

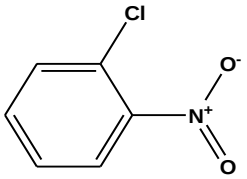

Figure. 2 (e).1-Chloro-2-nitrobenzene

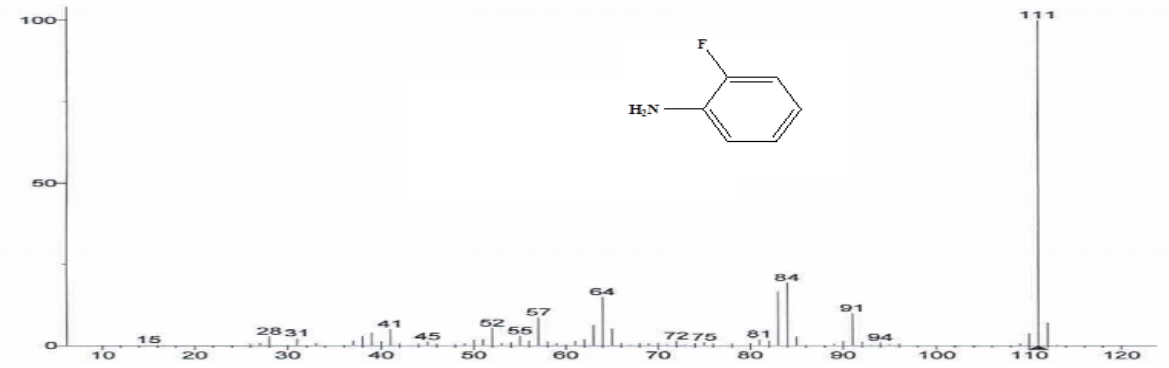

Figure. 2 (f). Mass Spectrum of 2-Fluoroaniline

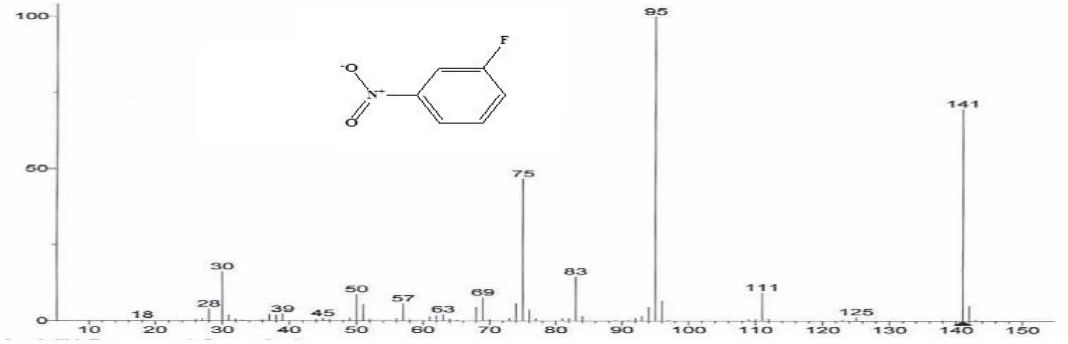

Figure. 2 (g). Mass Spectrum of 3-Fluoronitrobenzene

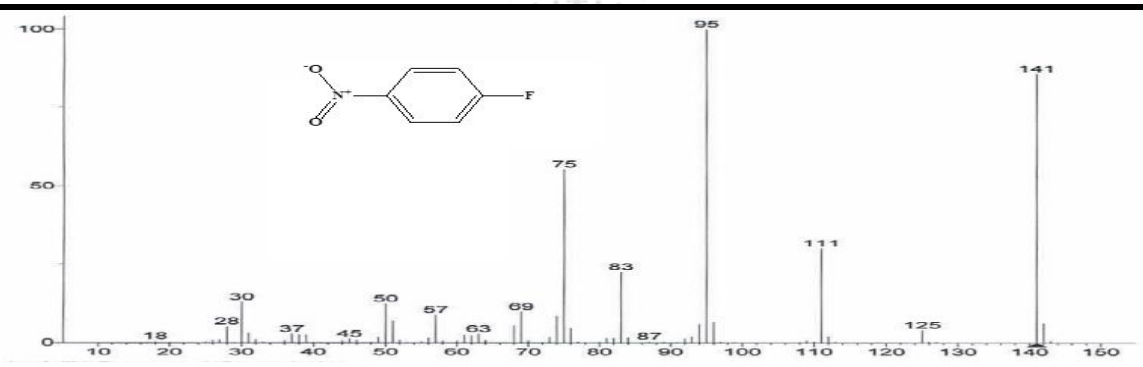

Figure. 2 (h). Mass Spectrum of 4-Fluoronitrobenzene

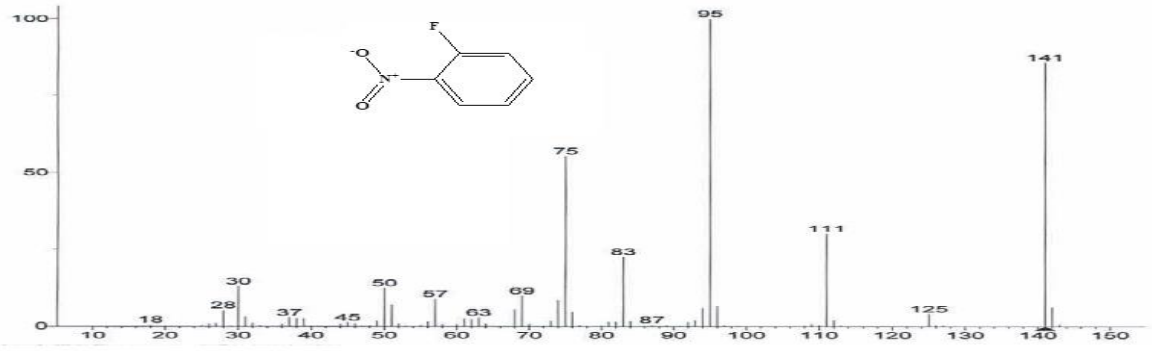

Figure. 2 (i). Mass Spectrum of 2-Fluoronitrobenzene

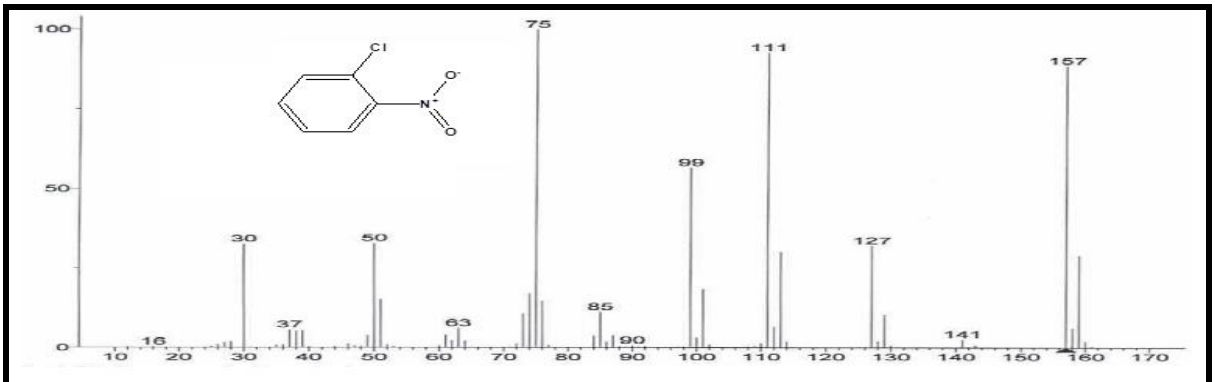

Figure. 2 (j). Mass Spectrum of 1-Chloro-2-nitrobenzene

2. EXPERIMENTAL

2.1. Chemicals and reagents:

2-FA, 3-FNB,4-FNB,2-FNB and1-Cl-2-NBand pure samples of REG were obtained from the Chemical Research Division of APL Research Centre laboratories (A division of Aurobindo Pharma Ltd., Hyderabad, India). Formic acid (Grade: EMPARTA ACS) was procured from Merck, India. Water (Grade: HPLC), Methanol and Dichloromethane (Grade: GC) were procured from Rankem, India.

2.2. Standard solutions for Method-1:

2.2.1. Preparation of Diluent

Prepare the mixture of Methanol and Dichloromethane in ratio of 1:1 (v/v).

2.2.2. Preparation of standards and test sample solutions

The standard stock solutions of 2-Fluoroaniline (2-FA) were prepared to get the concentration of 0.0452 µg/mL in the diluent. The Regorafenib test sample was typically prepared at 5mg/ml in diluent.

2.3. Standard solutions for Method-2:

A standard stock solution was prepared by weighing and diluting of 3-FNB,4-FNB, 2-FNB and 1-Cl-2-NB reference standards with Dichloromethane to get the concentration of 0.452µg/mL for each of four impurities.

2.3.1 Standard solution vial

Transfer 2.0 mL of Formic acid into a clean glass centrifuge tube followed by add 1.0 mL of water and shake the solution. To this, add 2.0 mL of the above standard solution and shake the solution for about 1 min. Allow the two phases to separate. Collect the lower layer (Dichloromethane layer) and use for analysis.

2.3.2 Blank solution vial

Transfer 2.0 mL of Formic acid into a clean glass centrifuge tube followed by add 1.0 mL of water and shake the solution. Add 2.0 mL of Dichloromethane and shake the solution about 1 min. Allow the two phases to separate. Collect the lower layer (Dichloromethane layer) and use for analysis.

2.3.3 Sample solution vial

Weigh and transfer about 100 mg of test sample into a clean glass centrifuge tube and add 2.0 mL of Formic acid and dissolve. Add 1.0 mL of water and shake the solution for about 1 min and add 2.0 mL of Dichloromethane and shake the solution for about 1 min. Allow the two phases to separate. Collect the lower layer (Dichloromethane layer) and use it for analysis.

2.4 GC-MS Conditions

The analysis was carried out on the Agilent GCMS-5977A and GCMS-5977B gas chromatograph equipped with 7890B GC System autosampler and data handling system having Mass Hunter solution software. The instrument was run in EI mode. Rtx-5, (30m × 0.32 mm I.D, 0.25µm film thickness, Agilent Technologies, USA) column consists of 5% Diphenyl and 95% dimethyl polysiloxane as a stationary phase. Chromatographic method conditions used were as follows **(Tables 1-3).**

Table No. 1: Gas chromatograph conditions for PGI's analysis

	Method-1			Method-2		
Instrument	Agilent 7890B			Agilent 7890B		
Column	Rtx-5, 30 m × 0.32 mm I.D. × 0.25 µm Film thickness			Rtx-5, 30 m × 0.32 mm I.D. × 0.25 µm Film thickness		
Carrier gas	Helium			Helium		
Injector temperature	240°C			240°C		
Injection type	ALS mode (Auto liquid sampler)			ALS mode (Auto liquid sampler)		
Column oven program	Heating rate (°C/min)	Initial temperature (°C)	Hold time (min)	Heating rate (°C/min)	Initial temperature (°C)	Hold time (min)
		60	4		60	4
	10	100	2	10	140	3
	20	240	13	20	240	10
Flow rate (mL/min)	1.5			1.5		
Injection volume (µL)	2.0			2.0		
Split ratio	5			10		
Run time (min)	30			30		

Table No. 2: Gas chromatography mass spectrometer conditions for PGI's analysis

	Method-1	Method-2
Instrument	Agilent GCMS-5977A and GCMS-5977B Single Quad MS	Agilent GCMS-5977A and GCMS-5977B Single Quad MS
MS Transfer line temperature (°C)	250	250
MS Source temperature (°C)	230	230
MS Quad temperature (°C)	150	150
Function type	SIM (selective ion monitoring)	SIM (selective ion
Gain factor	5	3

Table 3: SIM Time segments

<table>
<tr><td colspan="5">Method-1</td><td colspan="5">Method-2</td></tr>
<tr><td>Solvent delay</td><td>Group Name</td><td>Resolution</td><td>Mass (m/z)</td><td>Dwell time (ms)</td><td>Solvent delay time</td><td>Group Name</td><td>Resolution</td><td>Mass (m/z)</td><td>Dwell Time(ms)</td></tr>
<tr><td>2.0</td><td>2-Fluoroaniline</td><td>Low</td><td>111*</td><td>100</td><td rowspan="4">2.0</td><td>3-Fluoronitrobenzene</td><td rowspan="3">Low</td><td rowspan="3">141*</td><td rowspan="3">100</td></tr>
<tr><td colspan="5">*Quantification ion</td><td>4-Fluoronitrobenzene</td></tr>
<tr><td colspan="5" rowspan="2">Timed MS Detector:

The MS must be ‘‘Detector Off’’ after 8 min.</td><td>2-Fluoronitrobenzene</td></tr>
<tr><td>1-Chloro-2-nitrobenzene</td><td>Low</td><td>157*</td><td>100</td></tr>
<tr><td colspan="5" rowspan="2"></td><td colspan="5">*Quantification ion</td></tr>
<tr><td colspan="5">Timed MS Detector:

The MS must be ‘‘Detector Off’’ after 13 min.</td></tr>
</table>

3. RESULT AND DISCUSSION

3.1 Method development

The objective of the present work is, to establish a simple GC/MS-SIM method for the determination of 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NBcontents in Regorafenib drug substance. In the synthesis process of REG drug substance, 2-FNB was used as a key raw material. The positional isomers, i.e. 3-FNB, 4-FNB, 2-FA and 1-Cl-2-NBof 2-FNB may give corresponding potential impurities in REG drug substance. Method development activity was initiated based on the solubility studies of REG drug substances and five PGI’s. REG drug substance and 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB analytes having chromophore for UV or Fluorescence detection. Initially, these analytes tried in HPLC, UPLC, LC-MS and LC-MS/MS techniques. But in these techniques, the required levels are not able to be achieved. Moreover, based on the tendency of volatility and polarity of the analytes, there is a possibility to develop a chromatography method by GC equipped with flame ionization detector (FID). We made few trials by changing different diluents and chromatographic conditions in GC with FID. Due to the lower response of these impurities by GC-FID technique, we have chosen a gas chromatography electron ionization mass spectrometry (GC-MS-EI) technique in SIM mode for good separation and desired sensitivity. No analytical

method available in the literature to quantifying thisPGI's in REG drug substances by GC-MS till date.

Due to high boiling points of 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB, the peaks were not eluted in the head-space technique. Further, development trials were initiated in the direct-liquid injection technique using the stationary phase, 6% cyanopropyl and 94% dimethyl polysiloxane (DB-624; Make: Agilent). The sample solution was prepared by dissolving the sample in diluent (i.e. Methanol and Dichloromethane mixture in the ration of 1:1 v/v) and injecting into the GC-MS. Background interference was encountered in this trial and peak shapes were also not good. After cleaning the inlet port (to avoid ghost peaks), a broad peak shape of analytes was observed, which suggests another type of sample preparation required to reduce the interference from the sample matrix and proper peak shapes. During the optimization procedure, we have tried with few of diluents i.e. chloroform, diethyl ether and ethyl acetate and different columns. Finally, Methylene chloride extraction is used for sample preparation and using the stationary phase, 5% diphenyl and 95% dimethyl polysiloxane (Rtx-5; Make: Restek). Sample dissolved in Formic acid and extracted with Methylene chloride has given satisfactory results. But in this extraction procedure, 2-FA response was very poor. 2-FA did not come to Methylene chloride layer, it presents in Formic acid layer. Due to this reason for 2-FA developed single method in direct injection technique. In this method, mixture of Methanol and Dichloromethane in the ration of 1:1 v/v used as diluent, further method optimization purpose m/z-111 ion was selected for quantification of 2-FA and no interference observed from sample matrix. For remaining analytes (3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB) extraction procedure method developed and m/z-141ion was selected for quantification of 3-FNB, 4-FNB and 2-FNB and m/z-157 ion selected for quantification of 1-Cl-2-NB.

A well resolved, satisfactory chromatographic GC-MS-EI method were developed by using Rtx-5, 30m long with 0.32mm i.d., 0.25µm particle diameter column consists of 5% diphenyl and 95%-dimethylpolysiloxane as stationary phase and passing helium as carrier gas. Mixture of Methanol and Dichloromethane in the ration of 1:1 v/v used as diluent for 2-FA method and Dichloromethane used as diluent for another method. In the Quantification of 2-FA, the temperature of column oven is used initially 60°C is maintained for 4 min and then increased to 100°C for 2 min at a rate of 10°C/min, then increased to 240°C at a rate of 20°C/min followed by holding at 240°C for 13 min. In the Quantification of the remaining four impurities, the temperature of column oven is used initially 60°C is maintained for 4 min and

then increased to 140°C for 3 min at a rate of 10°C/min, then increased to 240°C at a rate of 20°C/min followed by holding at 240°C for 10 min. The developed methods were used for validation study to evaluate its performance characteristics. The present investigation was initiated for the quantification of 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB by GC-MS-EI technique in REG drug substance.

3.2 Method validation

To determine the contents of 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB in REG drug substance, the developed methods were validated as per the ICH guidelines [35] individually in terms of specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy and precision (system precision, method precision and intermediate precision) and robustness and system suitability.

3.2.1. Specificity

The specificity of the developed GC-MS-EI methods was indicated by showing the m/z peaks in the method as 111 for 2-FA, 141 for 3-FNB, 4-FNB, 2-FNB and 157 for 1-Cl-2-NB. Specificity is the ability of the method to measure the analyte response in presence of all impurities (2-FA, 3-FNB, 4-FNB, 2-FNBand 1-Cl-2-NB) in REG drug substance. To evaluate the specificity experiment, all impurity solutions (2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB) were prepared individually and injected into GC-MS to confirm the retention times. Further, blank, control sample (REG sample) and spiked sample solutions (REG sample spiked with 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB) were prepared as per methodology and injected into GC-MS. From the chromatograms of all individual injection solutions (2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB), blank solution, control sample solution and spiked sample solutions, it was observed that 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB peaks were well resolved from each other and there was no other interference (co-elution) from the sample matrix indicated that the method is selective and specific for the determination of 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB contents in REG drug substance. A typical representative overlaid GC-MS chromatograms of Method-(1) and Method-(2) are shown in **Figure 3** and **Figure 4.**

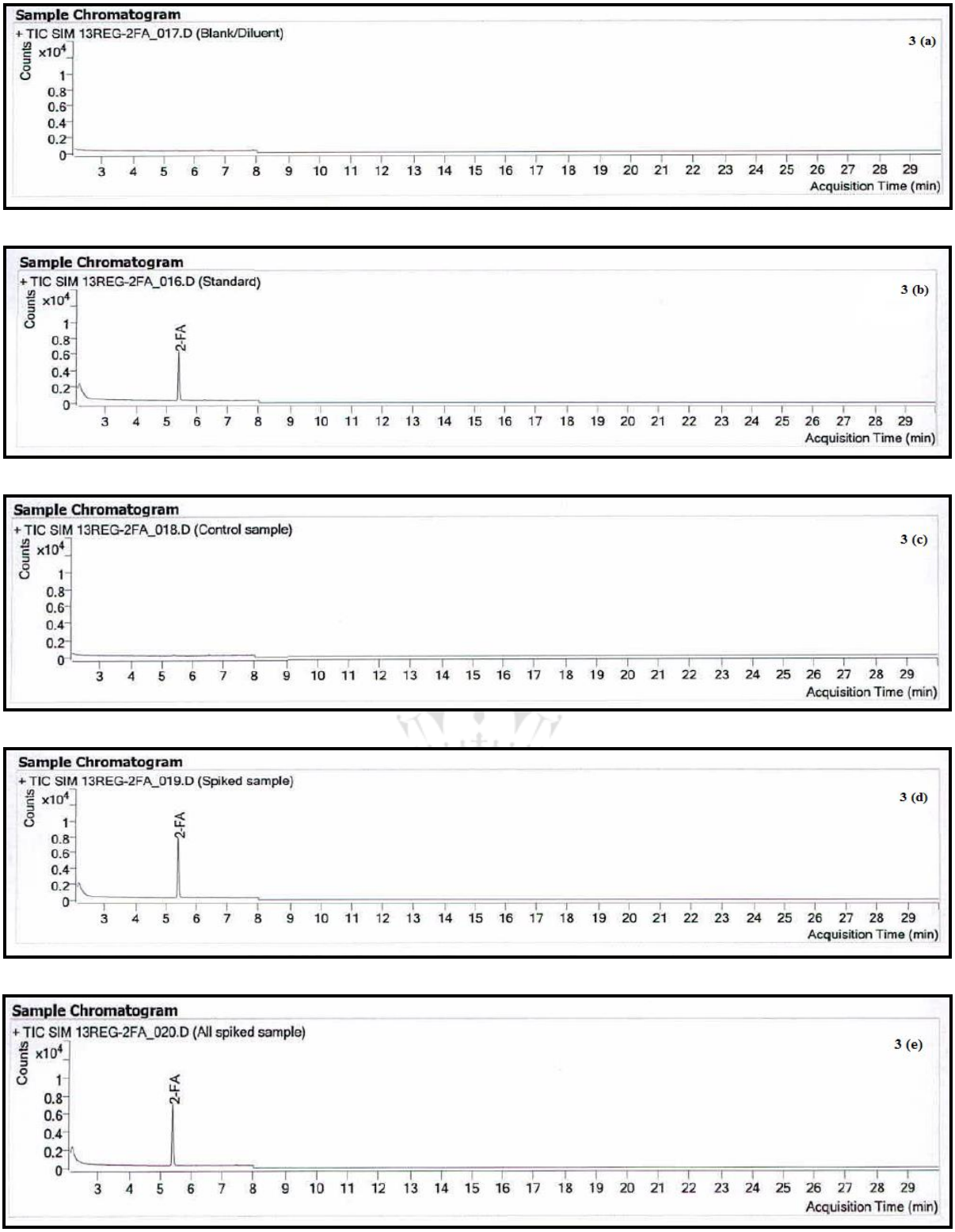

Figure No. 3: Typical GC-MS chromatograms of Method-(1) a) Blank solution, (b) Standard solution, (c) Regorafenib drug substance (as such sample), (d) Regorafenib drug substance spiked with 2-FA (spiked sample) and (e) Regorafenib drug substance spiked with 2-FAincluding all residual solvents (all spiked sample)

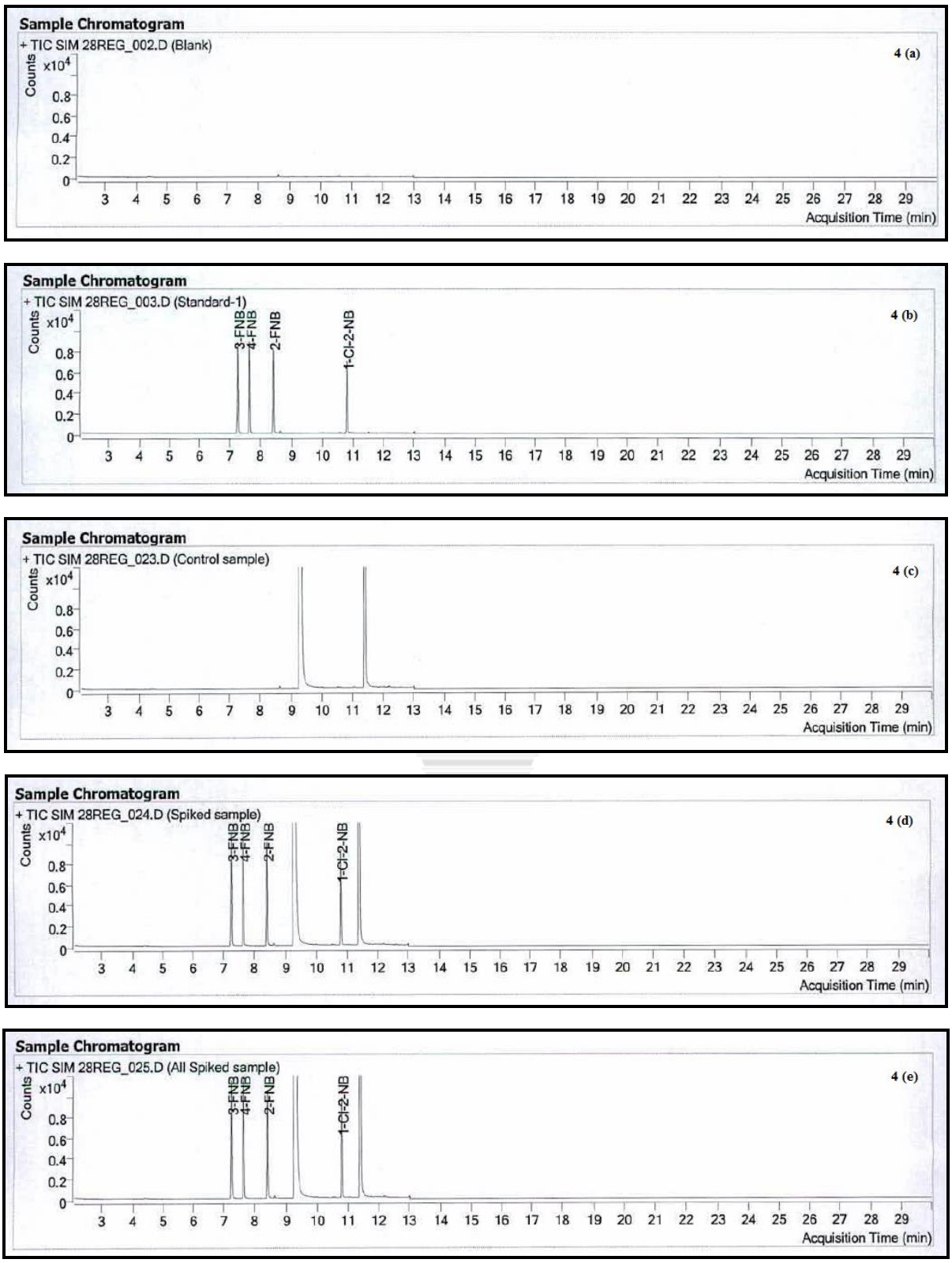

Figure No. 4: Typical GC-MS chromatograms of Method-(2) (a) Blank solution, (b) Standard solution, (c) Regorafenib drug substance (as such sample), (d) Regorafenib drug substance spiked with four PGI's (spiked sample) and (e) Regorafenib drug substance spiked with four PGI's including all residual solvents (all spiked sample)

3.2.2. Limit of detection and limit of quantification

In these both methods, Specification level standard solution was injected in to GC-MS and S/N ratios for all analytes were recorded. Based on these values, the LOD and LOQ values of 2-FA, 3-FNB, 4-FNB, 2-FNBand 1-Cl-2-NBwere predicted. At LOQ level S/N ratio was > 10 and LOD level S/N ratio was > 3 for all analytes. Each predicted concentration was verified for precision by preparing the solutions containing 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB about its detection limit and quantification limit concentrations. The LOD and LOQ solutions were injected six replicates into GC-MS. The relative standard deviation [% RSD (n = 6)] for LOD precision of 2-FA, 3-FNB, 4-FNB, 2-FNBand 1-Cl-2-NB were 3.2, 1.1, 2.6, 2.4 and 2.5; for LOQ precision 1.8, 2.2, 2.2, 2.0 and 1.9 respectively. The details of the précised LOD and LOQ values are shown in Table 5. The overlaid GC-MS chromatograms of Method-(1) LOD solution and LOQ solution are shown in **Figure 5(a)** and **(b)**.The overlaid GC-MS chromatograms of Method-(2) LOD solution and LOQ solution are shown in **Figure 6 (a)** and **(b)**.

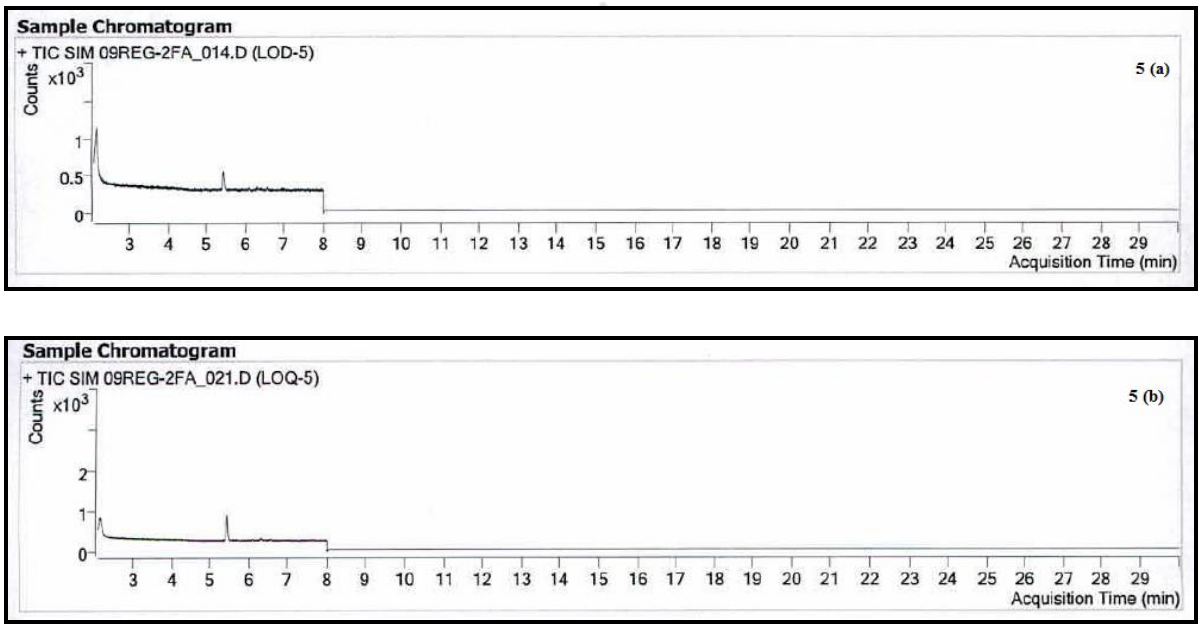

Figure No. 5: Typical GC-MS chromatograms of Method-(1) (a) LOD solution and (b) LOQ solution

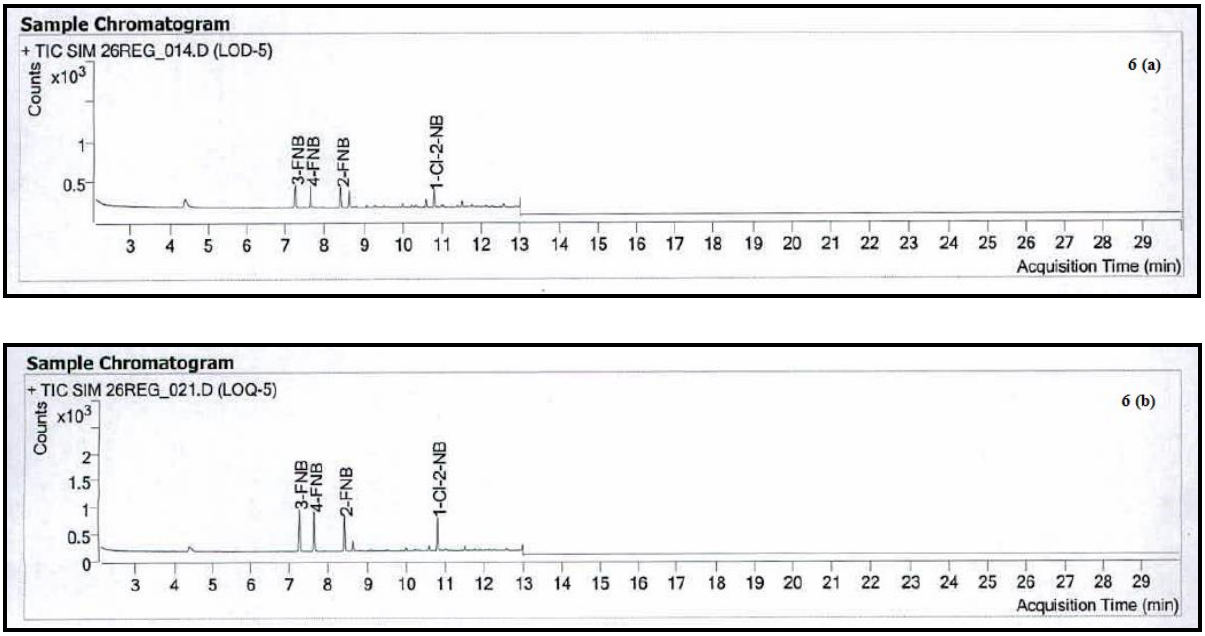

Figure No. 6: Typical GC-MS chromatograms of Method-(2) (a) LOD solution and (b) LOQ solution

3.2.3. Linearity

The linearity was evaluated by measuring the response of 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB seven different concentrations were prepared across the range concentrations were studied in the range of LOQ to 150% of the specification level (~0.006–0.066µg/mL for 2-FA and (~0.03–0.702µg/mL for remaining). The linearity level solutions of 2-FA (~0.006–0.066 µg/mL), 3-FNB (~0.03–0.702 µg/mL), 4-FNB (~0.03–0.682 µg/mL), 2-FNB (~0.03–0.693 µg/mL) and 1-Cl-2-NB (~0.03–0.695µg/mL) were prepared and injected each in duplicate injections into GC-MS. The data were subjected to statistical analysis using a linear regression model. The statistical parameters slope, intercept, residual standard on deviation and correlation coefficient values were calculated. The derived correlation coefficients were in the range of 0.9982–0.9999 indicating the best fitness of the linearity curves of the developed methods. The calculated statistical results are shown in **Table 4.**

Table No. 4: LOD, LOQ and Linearity experiments results

Statistical parameters	Results				
	2-FA	3-FNB	4-FNB	2-FNB	1-Cl-2-NB
Correlation coefficient	0.9982	0.9999	0.9998	0.9997	0.9997
Concentration range (µg/mL)	0.006 – 0.066	0.030 – 0.702	0.030 – 0.682	0.030 – 0.693	0.030 – 0.695
Calibration points	7	7	7	7	7
Intercept	525.6700	237.6762	5.0823	34.1583	20.6541
Slope(S)	222447.9714	34729.9022	36056.9202	31498.4141	21578.9687
STEYX	328.1917	122.7704	207.6836	218.1738	132.5247
LOD (µg/mL)	0.002	0.010	0.010	0.010	0.010
LOQ (µg/mL)	0.006	0.030	0.030	0.030	0.030
Precision at LOD level (%R.S.D)	3.2	1.1	2.6	2.4	2.5
Precision at LOQ level (%R.S.D)	1.8	2.2	2.2	2.0	1.9

3.2.4. Accuracy

Accuracy experiment was performed by spiking the known amounts of 2-FA, 3-FNB, 4-FNB, 2-FNBand 1-Cl-2-NB at LOQ level, 50%, 100% and 150% levels (for 9 µg/g limit) into REG drug substance. In the accuracy experiment, REG sample solutions (control sample) were prepared without spiking any impurity in triplicate and injected into GC-MS. Further, REG sample solutions (spiked sample) were prepared in triplicate by spiking with the all the impurities (2-FA, 3-FNB, 4-FNB, 2-FNBand 1-Cl-2-NB) at LOQ level, 50% level (4.5µg/g), 100% level (9.0µg/g) and 150% level (13.5µg/g) and injected into GC-MS. Control samples, Spiked samples were analyzed and the percentage recoveries were calculated. The average % recovery values of four levels (LOQ, 50%, 100% and 150% levels) for twelve determinations for 108.5 (2-FA), 108.6(3-FNB), 108.6(4-FNB), 108.0 (2-FNB) and 112.4 (1-Cl-2-NB). The complete validated accuracy results are shown in **Table 5.**

Table 5: Accuracy experiment results

Identification	2-Fluoroaniline (2-FA)				3-Fluoronitrobenzene (3-FNB)				4-Fluoronitrobenzene (4-FNB)			
Control sample	ND				ND				ND			
	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)
*Added (µg/g)	1.207	4.44	8.88	13.32	0.597	4.68	9.35	14.03	0.600	4.55	9.09	13.64
*Found (µg/g)	1.295	4.65	10.24	14.20	0.720	4.59	9.92	15.39	0.728	4.42	9.61	15.04
Recovery (%)	107.3	104.7	115.3	106.6	120.6	98.1	106.1	109.7	121.3	97.1	105.7	110.3
% RSD	3.6	4.6	5.9	3.6	6.9	0.8	1.1	2.6	6.4	1.1	1.0	2.4

Identification	2-Fluoronitrobenzene (2-FNB)				1-Chloro-2-nitrobenzene (1-Cl-2-NB)			
Control sample	ND				ND			
	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)
*Added (µg/g)	0.610	4.62	9.24	13.86	0.604	4.62	9.24	13.86
*Found (µg/g)	0.736	4.47	9.67	15.24	0.804	4.54	9.90	15.38
Recovery (%)	120.6	96.8	104.7	110.0	133.1	98.3	107.1	111.0
% RSD	7.3	1.2	0.7	2.1	5.2	0.5	1.2	2.6

*Average of three replicates.

ND: Not Detected.

3.2.5 Precision

The precision was the study of the method using repeatability (Method precision). The performance of the method was evaluated with replicate injections of standard and sample solutions. The standard solution was analyzed six times for checking the performance of the GC-MS system under test method conditions on the day tested (System Precision). The relative standard deviation results obtained for the system precision experiment were 1.5 (2-FA), 2.2 (3-FNB), 2.2 (4-FNB), 2.2 (2-FNB) and 2.5 (1-Cl-2-NB) respectively. Repeatability (Method Precision) experiment was performed by prepared six sample solutions were using single batch of REG drug substance spiked with 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB about known concentration (9.0 µg/ g) level and injected into GC-MS. The relative standard deviation for the content results of the Method precision experiment 6.3 (2-FA), 3.2 (3-FNB), 2.9 (4-FNB), 3.1 (2-FNB) and 2.9 (1-Cl-2-NB). The intermediate precision was the inter-day variation (ruggedness) defined as the degree of reproducibility obtained by following the same procedure as mentioned for Method precision experiment. The ruggedness of the method was evaluated by preparing six individual sample preparations (same sample which was used in Method precision experiment) by spiking 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB to REG drug substance and injected into different column, different instruments and different analyst on different days. The achieved precision (System precision, Method precision and Intermediate precision) experiment results are shown in **Table 6.**

Table No. 6: Statistical Data of Precision experiment

Repeatability (System precision)	2-FA	3-FNB	4-FNB	2-FNB	1-Cl-2-NB
1	13130	21685	21192	19205	13829
2	12939	21269	20680	18795	13572
3	12600	21524	20984	18975	13603
4	13034	21037	20513	18630	13383
5	12768	20803	20337	18389	13154
6	12839	20444	19962	18036	12892
Average	12885	21127	20611	18672	13406
STDEV	191	462	444	419	339
% RSD	**1.5**	**2.2**	**2.2**	**2.2**	**2.5**
Reproducibility (Method precision) (µg/g)					
1	10.66	9.97	9.58	9.63	9.84
2	10.50	9.98	9.69	9.75	9.88
3	9.55	10.17	9.77	9.73	10.06
4	9.33	9.93	9.62	9.68	9.72
5	10.31	10.69	10.28	10.37	10.51
6	9.26	10.56	10.14	10.21	10.24
Average	9.94	10.22	9.85	9.90	10.04
STDEV	0.63	0.33	0.29	0.31	0.29
% RSD	**6.3**	**3.2**	**2.9**	**3.1**	**2.9**
Reproducibility (Intermediate Precision) (µg/g)					
1	8.79	9.74	9.61	9.65	10.65
2	8.87	10.94	10.79	10.85	11.72
3	9.71	11.30	11.14	11.25	12.15
4	9.24	11.57	11.45	11.52	12.44
5	10.01	12.14	12.00	12.09	13.10
6	9.71	12.19	12.00	12.18	13.20
Average	9.39	11.31	11.17	11.26	12.21
STDEV	0.50	0.91	0.90	0.93	0.95
% RSD	**5.3**	**8.0**	**8.1**	**8.3**	**7.8**
Overall statistical data (n=12)					
Average	9.66	10.77	10.51	10.58	11.13
STDEV	0.61	0.87	0.94	0.97	1.32
% RSD	**6.3**	**8.1**	**8.9**	**9.2**	**11.9**

3.2.6 Robustness

Robustness of the method was evaluated by deliberately altering the method conditions from original method parameters and verifying compliance to the system suitability parameters. The impact of variation of column oven temperature and flow rate of carrier gas on system suitability was conducted. In robustness verification of test method, one parameter changed while keeping the other unchanged from actual parameter. The study was carried out for column flow variation of carrier gas initial flow rate ±10% and column oven initial temperature ± 2°C as follow mentioned in **Table 7 (a)** and **Table 7 (b)**. Results of peak areas for 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB are summarized in **Table 8**.

Table No. 7 (a): Flow variations: -

Column Flow (ml/min)		
	Method-1	**Method-2**
As per Methodology	1.5	1.5
-10% Flow variation	1.35	1.35
10% Flow variation	1.65	1.65

Table No. 7 (b): Column Oven Temperature variations: -

Column Oven Temperature for Method-1	
As per Methodology	60°C (4min) $\xrightarrow{10°\ C/min}$ 100°C (2min) $\xrightarrow{20°\ C/min}$ 240°C (13min)
-2°C Column Oven Temperature variation	58°C (4min) $\xrightarrow{10°\ C/min}$ 100°C (2min) $\xrightarrow{20°\ C/min}$ 240°C (13min)
+2°C Column Oven Temperature variation	62°C (4min) $\xrightarrow{10°\ C/min}$ 100°C (2min) $\xrightarrow{20°\ C/min}$ 240°C (13min)
Column Oven Temperature for Method-2	
As per Methodology	60°C (4min) $\xrightarrow{10°\ C/min}$ 140°C (3min) $\xrightarrow{20°\ C/min}$ 240°C (10min)
-2°C Column Oven Temperature variation	58°C (4min) $\xrightarrow{10°\ C/min}$ 140°C (3min) $\xrightarrow{20°\ C/min}$ 240°C (10min)
+2°C Column Oven Temperature variation	62°C (4min) $\xrightarrow{10°\ C/min}$ 140°C (3min) $\xrightarrow{20°\ C/min}$ 240°C (10min)

Table No. 8: Robustness experiment results

Robustness condition	System suitability criteria (% RSD)				
	2-FA	3-FNB	4-FNB	2-FNB	1-Cl-2-NB
As per methodology	0.8	2.1	2.3	2.2	2.1
Flow variation					
-10%	1.0	2.9	2.6	3.2	3.6
+10%	0.5	2.9	2.7	2.9	3.1
Temperature variation -Initial Oven					
-2°C	0.7	4.9	4.9	5.0	5.4
+2°C	0.4	3.4	3.3	3.4	2.8

4. CONCLUSION:

The present study established a well-resolved analytical method for the determination of five genotoxic impurities by GC-EI-MS with SIM mode at a very low level. Method validation data demonstrated that the developed method is simple, sensitive, specific, precise, linear, accurate, user-friendly and cost-effective for the estimation of 2-FA, 3-FNB, 4-FNB, 2-FNB and 1-Cl-2-NB contents in Regorafenib drug substance.

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