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A Validated GC-MS Method for the Trace Level Determination of Genotoxic Impurities in Anticancer Drug, Alectinib Hydrochloride Using Liquid-Liquid Extraction Technique



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ABSTRACT

4-Fluoro-3-nitrobenzotrile (4-F-3-NBN) compound used as key starting material (KSM) in the of synthesis Alectinib Hydrochloride (ALP) drug substance. On basis of structure-activity relationship (SAR), 4-Fluoro-3-nitrobenzotrile and its isomers are 3-Fluoro-4-nitrobenzotrile (3-F-4-NBN), 2-Fluoro-5-nitrobenzotrile (2-F-5-NBN), 2-Fluoro-3-nitrobenzotrile (2-F-3-NBN) and 4-Chloro-3-nitrobenzotrile (4-Cl-3-NBN). A sensitive and specific GC-MS method was developed and validated for the trace level determination of both these impurities in the active pharmaceutical ingredient Alectinib Hydrochloride. Chromatographic separation of these five genotoxic impurities and lower level of detection was achieved on Capillary GC column (DB-624, Fused silica capillary column; 30 m length; 0.32mm internal diameter, coated with 6% Cyanopropylphenyl and 94% dimethyl polysiloxane stationary phase of 1.8 μm film thickness). Helium gas used as carrier gas with Electron Impact ionization (EI) in Selective Ion Monitoring (SIM) mode by using liquid-liquid extraction sample preparation technique. The mass fragments (m/z) were selected for the quantification of 3-F-4-NBN (m/z-166), 2-F-5-NBN (m/z-166), 4-F-3-NBN (m/z-166), 2-F-3-NBN (m/z-166) and 4-Cl-3-NBN (m/z-182). The developed GC-MS method was validated in terms of Specificity, Linearity, Sensitivity, Precision (Repeatability and Reproducibility and Accuracy, Robustness, Limit of quantitation (LOQ) and Limit of detection (LOD) experiments. The obtained limit of detection values for the 2-F-5-NBN and 4-Cl-3-NBN is 0.007 $\mu\text{g mL}^{-1}$ and for 3-F-4-NBN, 4-F-3-NBN and 2-F-3-NBN is 0.008 $\mu\text{g mL}^{-1}$. The obtained limit of quantitation values for the 2-F-5-NBN and 4-Cl-3-NBN is 0.021 $\mu\text{g mL}^{-1}$ and for 3-F-4-NBN, 4-F-3-NBN and 2-F-3-NBN is 0.024 $\mu\text{g mL}^{-1}$. The correlation coefficient values of the linearity experiment were in the range of 0.9975–0.9985. The average recoveries for the accuracy were in the range of 93.0–102.0%. The results proved that the method is suitable for the determination of 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN contents in Alectinib Hydrochloride.

INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide, generally presenting at diagnosis with locally advanced or metastatic diseases. Non-small cell lung cancer (NSCLC), accounts for 85% of the lung cancer cases and has usually reached an advance stage by the time of diagnosis [1,2].

Anaplastic lymphoma kinase (ALK) is one of the receptor tyrosine kinase (RTK) belonging to the insulin receptor family [3]. Genetic alterations in ALK are implicated in the pathogenesis of several human cancers. ALK can be aberrantly activated by mutation, gene amplification or chromosomal rearrangement, leading to the expression of a potent oncogenic driver [4]. Today, several structurally distinct small molecules have been developed as second generation ALK inhibitors, including Ceritinib and Alectinib drugs [5]. Alectinib is an orally active drug that blocks the activity of ALK and is used to treat non-small cell lung cancer [6]. It is approved in Japan in July 2014 for the treatment of ALK fusion gene Positive, unrespectable advance or recurrent non-small cell lung cancer [7].

Alectinib (ALECENSA) is a potent and highly selective anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitor. Oral Alectinib monotherapy is approved in the EU as first-line treatment for adults with advanced ALK-positive non-small cell lung cancer (NSCLC) and for the treatment of adults with advanced ALK-positive NSCLC previously treated with crizotinib[8-19]. In the USA, alectinib is indicated for the treatment of adults with ALK-positive metastatic NSCLC. The recommended dosage for alectinib in the EU and USA is 600 mg twice daily. Alectinib is an important treatment option for patients with advanced ALK-positive NSCLC who are previously untreated or those previously treated with crizotinib. Given its efficacy and tolerability, current guidelines include alectinib as a treatment option in these settings, with the NCCN guidelines recommending it as a preferred option for first-line therapy.

Alectinib is white powder or white to yellow powder with lumps in physical state. It is freely soluble in dehydrated alcohols, and in propylene glycol. Alectinib is insoluble in aqueous buffer at pH 7.5. The chemical name of Alectinib hydrochloride (ALP) is 9-ethyl-6,6-dimethyl-8-[4-(morpholin-4-yl)piperidin-1-yl]-11-oxo-6,11-dihydro-5H-benzo[b]carbazole-3carbonitrile hydrochloride corresponding to the molecular formula $C_{30}H_{34}N_4O_2 \cdot HCl$. The molecular weight is 519.08.

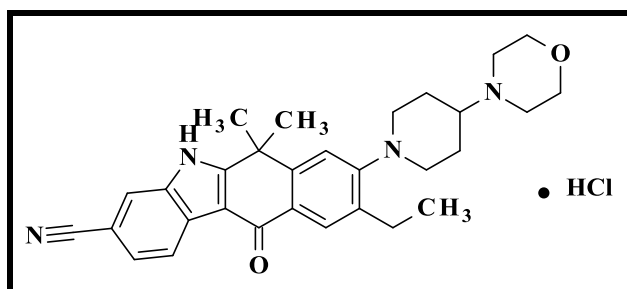


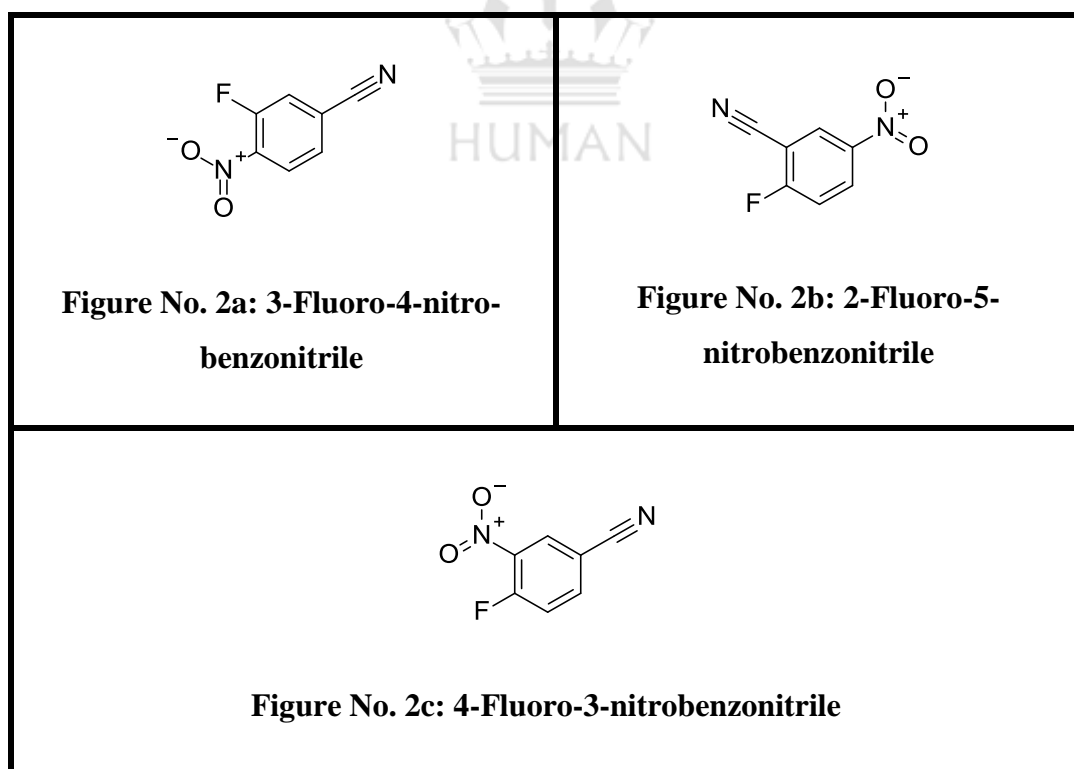
Figure No. 1: Chemical structure of Alectinib hydrochloride.

Synthesis of drug substances often involves the use of different raw materials and hence, these raw materials and its impurities may be present in the final drug substances as impurities. Such chemically reactive impurities may have unwanted toxicities including genotoxicity and carcinogenicity and are to be controlled based on the maximum daily dose [20]. These limits generally fall at low mg/mL levels and hence conventional HPLC, GC methods (or final drug substance methods) may not suitable for their determination. Hyphenated techniques like GC–MS and LC–MS combine physical separation capabilities of chromatography (GC or HPLC) with the mass analysis capabilities of mass spectrometry and have high sensitivity and specificity over conventional HPLC and GC methods. Their applications are oriented towards the potential identification and quantitation of trace level of impurities in drug substances [21].

The following impurities 3-Fluoro-4-nitrobenzonitrile (3-F-4-NBN), 2-Fluoro-5-nitrobenzonitrile (2-F-5-NBN), 4-Fluoro-3-nitrobenzonitrile (4-F-3-NBN), 2-Fluoro-3-nitrobenzonitrile (2-F-3-NBN) and 4-Chloro-3-nitrobenzonitrile (4-Cl-3-NBN) are likely present in Alectinib Hydrochloride (ALP) drug substance. In these, 4-Fluoro-3-nitrobenzonitrile (4-F-3-NBN) is used as a key raw material for the preparation of Alectinib Hydrochloride (ALP) drug substance. The other four are possible isomeric impurities. Based on literature and evaluation by Derek software, these five compounds are found to be mutagenic and carcinogenic. Hence, these genotoxic impurities are limited to a daily dose of 1.5µg/day as per ICH guidelines from the European medical agency [22]. Hence, in order to meet the regulatory agencies requirements, it is essential to develop a sensitive analytical method. Hence, a gas chromatograph with mass spectrophotometer was chosen which can detect low level determinations for the quantification of 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN.

The European Agency for the Evaluation of Medicinal products (EMA), United States Food and Drug Administration (USFDA) and ICH Q3A/B issued the guidelines and draft guidance on the limitation of genotoxic impurities in pharmaceutical ingredients [23, 24]. Based on these current regulatory guidance's for genotoxic impurities, analytical methods should be developed to meet the required limit of 1.5mg/day daily intake of individual impurity. These impurities limit is considered as $1.25 \mu\text{g g}^{-1}$ with respect to Alectinib Hydrochloride maximum daily dose 1200mg/day [25-27]. Appropriate analytical methods were required for the monitored and controlled this genotoxic impurity for the best quality of ALP drug.

Being a very novel and recently synthesized drug, there are few references for Alectinib. Through the review of the literature, none of the method was described for the quantification of the five genotoxic impurities (3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN) in ALP drug substance. For the determination of all the five genotoxic impurities in ALP drug substance, a single, sensitive and specific GC-EI-MS with selective ion monitoring (SIM) mode method was developed and validated. The chemical structures of these five impurities are shown in **Figure 2a to 2e**.



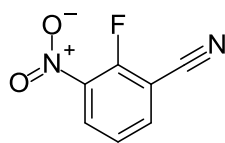


Figure No. 2d: 2-Fluoro-3-nitrobenzonitrile

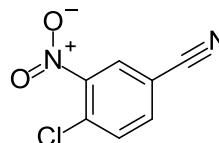


Figure No. 2e: 4-Chloro-3-nitrobenzonitrile

MATERIALS AND METHODS

Chemicals and reagents

3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN and pure samples of ALP were obtained from Chemical research division of NATCO Research Centre (A division of NATCO Pharma Ltd.), Hyderabad, India. Formic acid (Grade: EMPARTA ACS) was procured from Merck, India. Water (Grade: HPLC) and Dichloromethane (Grade: GC) were procured from Rankem, India. Methanol, Dimethyl ether, Methyl acetate, Methyl ethyl ketone, N, N-Dimethylformamide, N, N-Diisopropylethylamine, Trifluoroethanol and Benzene were procured from Sigma Aldrich, Steinheim, Germany.

Preparation of standard stock solution

A standard stock solution was prepared by weighing and diluting of 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN reference standards with Dichloromethane to get the concentration of $0.125 \mu\text{g mL}^{-1}$ for each of five impurities.

Standard solution vial

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

Blank solution vial

Transfer 2.0 mL of Formic acid into a clean glass centrifuge tube followed by 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.

Sample solution vial

Weigh and transfer about 200 mg of test sample into a clean glass centrifuge tube and add 2.0 mL of Formic acid and dissolve. Followed by add 1.0 mL of water and shake the solution 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 for analysis.

GC-MS Conditions

The complete experiments were performed 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. DB-624, (30m × 0.32 mm I.D, 1.8 μm film thickness, Agilent Technologies, USA) column consists of 6% Cyanopropylphenyl and 94% dimethyl polysiloxane as a stationary phase. Chromatographic method conditions used were as follows (Tables 1-3).

Table No. 1: Gas chromatograph conditions for GTI analysis

Instrument	Agilent 7890B
Column	DB-624, 30 m × 0.32 mm I.D. × 1.8μm Film thickness
Carrier gas	Helium
Injector temperature (°C)	240°C
Injection type	HS (headspace) control
Column oven program	10° C/min 160°C (2min) → 220°C (17min).
Flow rate (mL/min)	2.0
Injection volume (μL)	2.0
Split ratio	1 : 3
Run time (min)	25

Table No. 2: Gas chromatography mass spectrometer conditions for GTI analysis.

Instrument	Agilent GCMS-5977A and GCMS-5977B Single Quad MS
MS transfer line temperature (°C)	250
MS source temperature (°C)	230
Function type	SIM (selective ion monitoring)
Gain factor	7

Table No. 3: SIM Time segments:

Solvent delay time	Group name	Resolution	Mass(m/z)	Dwell time(ms)
2.0	3-Fluoro-4-nitrobenzotrile	Low	166*	100
	2-Fluoro-5-nitrobenzotrile			
	4-Fluoro-3-nitrobenzotrile			
	2-Fluoro-3-nitrobenzotrile			
	4-Chloro-3-nitrobenzotrile	Low	182*	100
*Quantification ion				
<u>Timed MS Detector:</u> The MS must be “ Detector Off ” after 11 min.				

RESULTS AND DISCUSSION

Method development

The objective of the present work is, to establish a simple GC-MS method for the determination of 3-F-4-NBN, 2-F-5-NBN, 2-F-3-NBN and 4-Cl-3-NBN contents in Alectinib Hydrochloride (ALP) drug substance. In the synthesis process of ALP drug substance, 4-F-3-NBN was used as a key raw material. The positional isomers, *i.e.* 3-F-4-NBN, 2-F-5-NBN, 2-F-3-NBN and 4-Cl-3-NBN of 4-F-3-NBN may give corresponding potential impurities in ALP drug substance. Method development activity was initiated based on solubility studies of ALP drug, 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN. ALP drug and 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN analytes having chromophore for UV or Fluorescence detection. Initially, these analytes tried in HPLC, LCMS and LCMS/MS techniques. But in this techniques not able to achieve required levels. 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-EI-MS) technique in SIM mode for good separation and desired sensitivity. No analytical methods available in literature to quantifying these impurities in by GC-MS till date.

A well resolved, satisfactory chromatographic GC-MS-EI method was developed by using DB-624, 30m long with 0.32mm i.d., 1.8 μ m particle diameter column consists of 6% Cyanopropylphenyl and 94%-dimethylpolysiloxane as stationary phase and passing helium as carrier gas. Dichloromethane used as diluent and the temperature of column oven is used initially 160°C is maintained for 2 min and then increased to 220°C at a rate of 10°C/min, followed by holding at 220°C for 17 min. The developed method was used for validation study to evaluate its performance characteristics. The present investigation was initiated for the quantification of 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN by GC-MS-EI technique in ALP drug substance.

Method validation

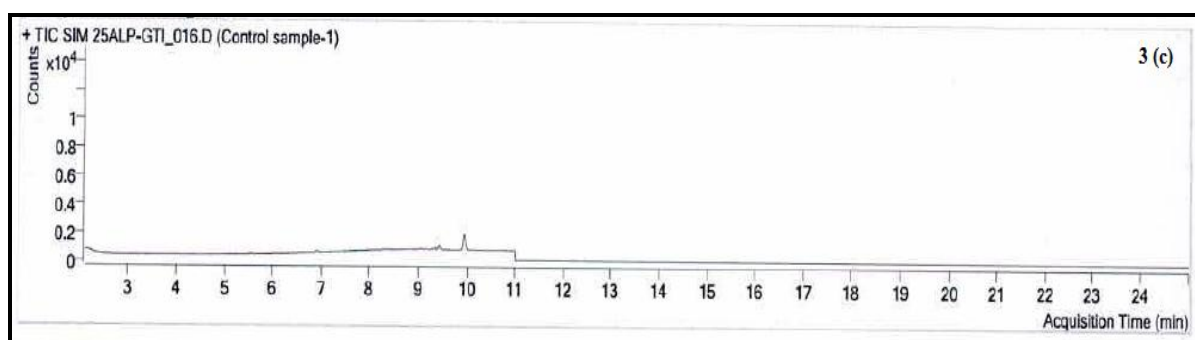
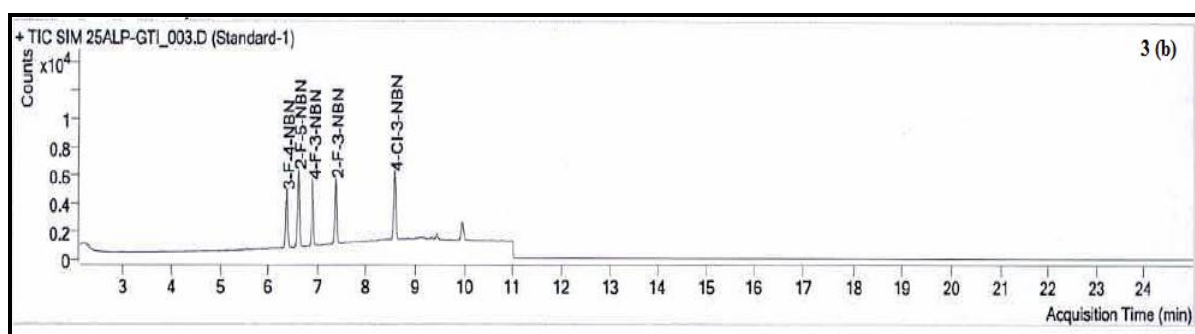
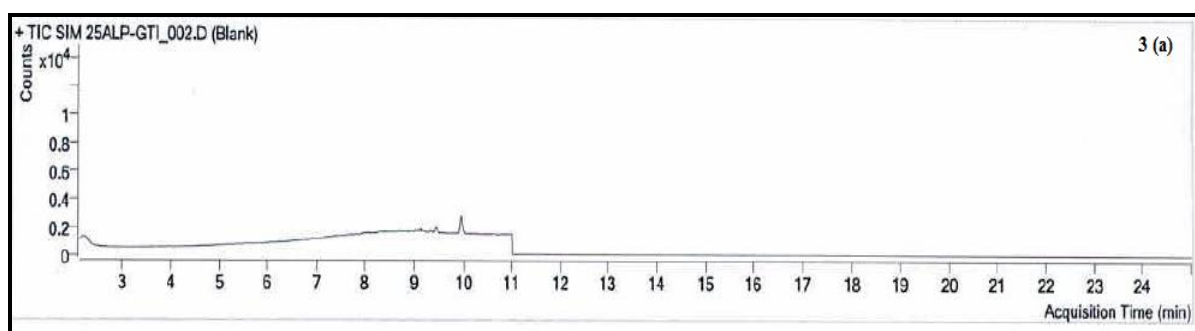
The developed method was validated as per the ICH guidelines [28] for the determination of the contents of 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN in ALP drug substance. Individually in terms of specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy and precision (system precision, method precision and intermediate precision) and robustness and system suitability.

Specificity

The specificity of the developed GC-MS method was indicated by showing the m/z peaks in the method as 166 for 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 182 for 4-Cl-3-NBN. Specificity is the ability of the method to measure the analyte response in presence of all impurities (3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN) in ALP drug substance.

The specificity of the developed GC-MS method was verified in presence of residual solvents like Methanol, Dimethyl ether, Methyl acetate, Methyl ethyl ketone, N, N-Dimethylformamide, N, N-Diisopropylethylamine, Trifluoroethanol and Benzene which were used in the Alectinib Hydrochloride process. These solvents and five analytes were

injected individually to confirm retention times. ALP sample solution (Control sample), ALP drug substance spiked with spiked with 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN at specification level (Spiked Sample) and ALP drug substance spiked with 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN and all other known residual solvents at specification level (All Spiked Sample), Blank and Standard solutions were injected into GCMS to confirm any co-elution of 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN peaks with each other and with any other known residual solvents. The specificity experiment typical GCMS Chromatograms of Blank, Standard, control sample, spiked sample and all spiked sample are shown in Figure.3 (a) to (e). Based on evaluation of specificity studies, it was concluded that the 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN peaks are well separated from each other as there is no other solvent co-elution indicated that the method is selective and specific for five analytes in Alectinib Hydrochloride drug substance.



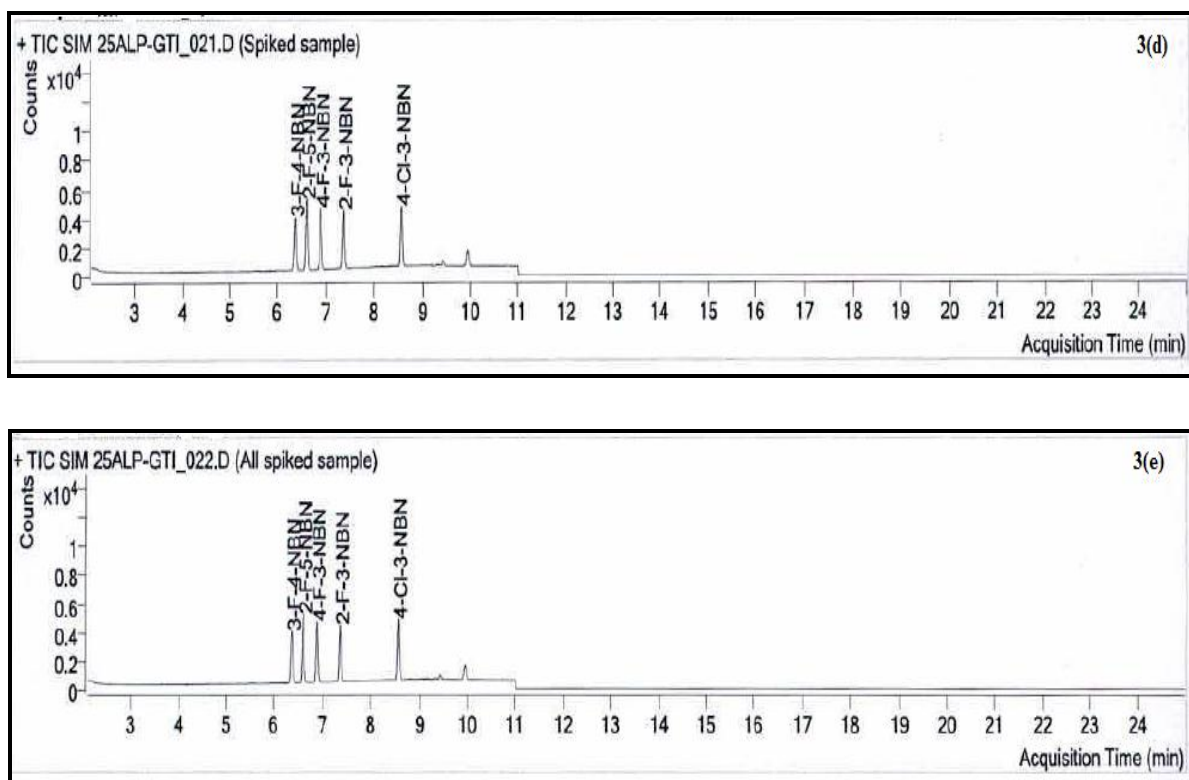


Figure No. 3: Typical GC-MS chromatograms of a) Blank solution, (b) Standard solution, (c) Alectinib Hydrochloride drug substance (as such sample), (d) Alectinib Hydrochloride drug substance spiked with GTI's (spiked sample) and (e) Alectinib Hydrochloride drug substance spiked with GTI's including all residual solvents (all spiked sample)

Limit of detection and limit of quantification

In this method, 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 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN were 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 at about these predicted concentrations and injected each solution six times into the GCMS. The relative standard deviation [% RSD (n = 6)] for LOD precision of 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN were 4.1, 4.4, 3.7, 4.5 and 8.3; for LOQ precision 1.9, 1.9, 1.6, 1.7 and 2.5 respectively. The details of the précised LOD and LOQ values are shown in Table 4. The overlaid GC-MS chromatograms of LOD solution and LOQ solution are shown in Figure. 4 (a) and (b).

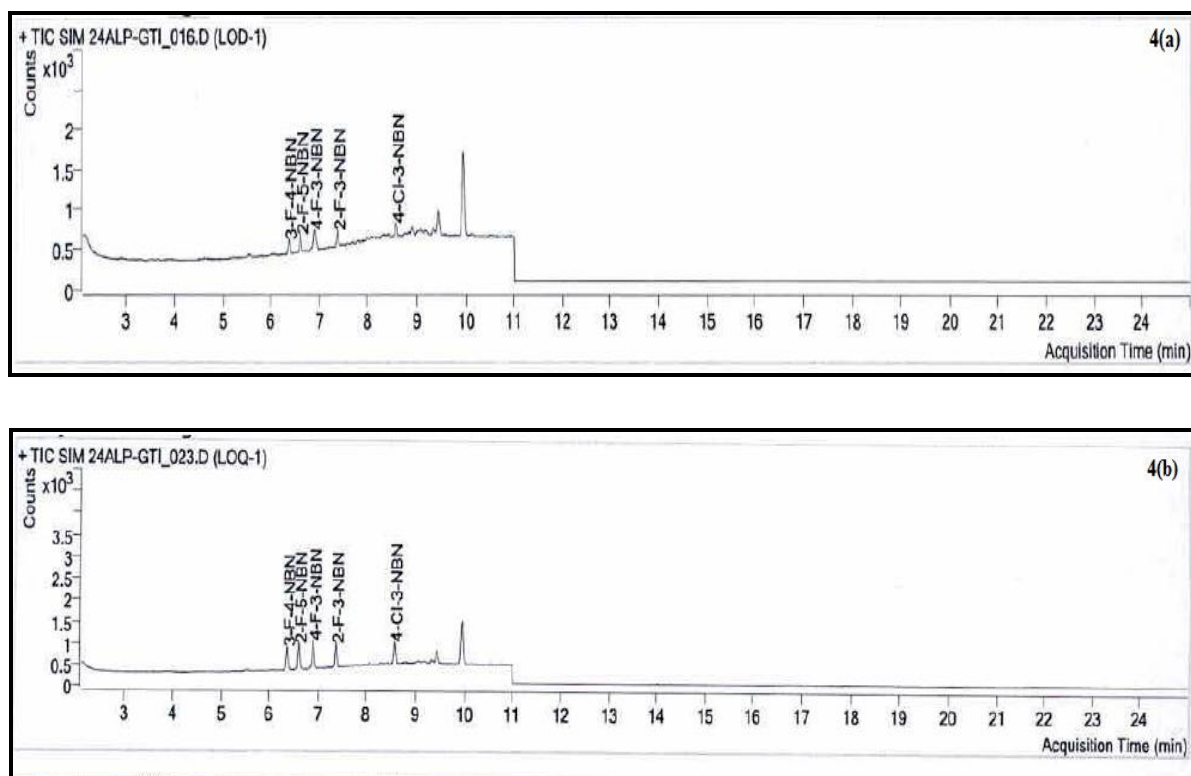


Figure No. 4: Typical GC-MS chromatograms of a) LOD solution and (b) LOQ solution

Linearity

The linearity of 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN was satisfactorily done. A series of solutions were prepared across the range concentrations were studied in the range of LOQ to 150% of the specification level (~0.021–0.196 µg/mL). The level linearity solutions of 3-F-4-NBN (~0.024–0.192 µg/mL), 2-F-5-NBN (~0.021–0.196 µg/mL), 4-F-3-NBN (~0.024–0.193 µg/mL), 2-F-3-NBN (~0.024–0.196 µg/mL) and 4-Cl-3-NBN (~0.021–0.196 µg/mL) were prepared and injected each in duplicate injections into GC-MS. Statistical data like slope, intercept, STEYX and correlation coefficient were established by using the peak area response versus concentration data. The derived correlation coefficients were in the range of 0.9975–0.9985 indicating the best fitness of the linearity curves of the developed method. The calculated statistical results are shown in Table 4.

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

Statistical parameters	Results				
	3-F-4-NBN	2-F-5-NBN	4-F-3-NBN	2-F-3-NBN	4-Cl-3-NBN
Correlation coefficient	0.9985	0.9975	0.9982	0.9976	0.9984
Concentration range (µg/mL)	0.024 – 0.192	0.021 – 0.196	0.024 – 0.193	0.024 – 0.196	0.021 – 0.196
Calibration points	7	7	7	7	7
Intercept	462.5318	721.5925	132.2059	545.1633	626.1328
Slope(S)	12000.4630	16376.9331	14637.0083	12485.8453	14060.7246
STEYX	461.8345	834.4853	626.5933	613.5213	576.7494
LOD (µg/mL)	0.008	0.007	0.008	0.008	0.007
LOQ (µg/mL)	0.024	0.021	0.024	0.024	0.021
Precision at LOD level (% R.S.D)	4.1	4.4	3.7	4.5	8.3
Precision at LOQ level (% R.S.D)	1.9	1.9	1.6	1.7	2.5

Accuracy

Standard addition experiments were conducted in triplicate preparations (*i.e.* ALP drug substance sample solutions were prepared in triplicate by spiking with 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN) to determine accuracy of the methods at LOQ level, 50% level (0.625 µg/g), 100% level (1.25 µg/g) and 150% level (1.875 µg/g). In the accuracy experiment, ALP sample solutions (control sample) were prepared without spiking any impurity in triplicate and injected into GC-MS. Further, ALP sample solutions (spiked sample) were prepared in triplicate by spiking with all the impurities and injected into GC-MS. Control samples, Spiked samples were analysed and the percentage recoveries were calculated. The average % recovery values of four levels (LOQ, 50%, 100% and 150% levels) for twelve determination for 100.5 (3-F-4-NBN), 100.9 (2-F-5-NBN), 102.0 (4-F-3-NBN), 93.0 (2-F-3-NBN) and 101.7 (4-Cl-3-NBN). The accuracy experiment results are reported in Table 5.

Table No. 5: Accuracy experiment results

Identification	3-Fluoro-4-nitrobenzonitrile (3-F-4-NBN)				2-Fluoro-5-nitrobenzonitrile (2-F-5-NBN)				4-Fluoro-3-nitrobenzonitrile (2-F-5-NBN)			
Control	ND				ND				ND			
	LOQ Level	Level-I	Level-II	Level-III	LOQ Level	Level-I	Level-II	Level-III	LOQ Level	Level-I	Level-II	Level-III
*Added	0.242	0.65	1.31	1.96	0.215	0.65	1.30	1.95	0.239	0.63	1.27	1.90
*Found	0.230	0.62	1.38	2.08	0.205	0.61	1.37	2.13	0.229	0.63	1.33	2.04
Recovery	95.0	95.4	105.3	106.1	95.3	93.8	105.4	109.2	95.8	100.0	104.7	107.4
% RSD	1.5	0.9	0.7	0.3	1.4	0.3	0.4	0.8	1.1	0.9	0.2	0.5
Identification	2-Fluoro-3-nitrobenzonitrile (3-F-4-NBN)				4-Chloro-3-nitrobenzonitrile (4-Cl-3-NBN)							
Control	ND				ND							
	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)	LOQ Level	Level-I	Level-II (100%)	Level-III (150%)				
*Added	0.242	0.64	1.27	1.91	0.210	0.66	1.33	1.99				
*Found	0.226	0.52	1.21	1.95	0.198	0.67	1.38	2.13				
Recovery	93.4	81.3	95.3	102.1	94.3	101.5	103.8	107.0				
% RSD	2.9	0.4	0.8	0.7	0.1	0.9	0.7	0.7				

*Average of three replicates.

ND: Not detected.

Precision

The precision was the study of the method using repeatability (Method precision) and reproducibility (Ruggedness). The performance of the method was evaluated with replicate injections of standard and sample solutions. Standard solution was analyzed by injecting six times for checking the performance of the GC-MS system under the test conditions on the day tested (System Precision). The relative standard deviation results achieved for the system precision experiment were 0.5 (3-F-4-NBN), 0.5 (2-F-5-NBN), 0.5 (4-F-3-NBN), 0.3 (2-F-3-NBN) and 0.7 (4-Cl-3-NBN) respectively. Repeatability (Method Precision) experiment was performed by prepared six sample solutions were using single batch of ALP drug substance spiked with 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN about known concentration (1.25 µg/ g) level and injected into GCMS. The relative standard deviation for the content results of the Method precision experiment 0.7 (3-F-4-NBN), 1.4 (2-F-5-NBN), 0.7 (4-F-3-NBN), 1.6 (2-F-3-NBN) and 0.7 (4-Cl-3-NBN).

The intermediate precision was the inter-day variation (ruggedness) was defined as the degree of reproducibility obtained by following the same procedure as mentioned for method

precision experiment. Ruggedness of the method was evaluated by preparing six individual sample preparations (same sample which was used in Method precision experiment) by spiking 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN to ALP drug substance and injected into different column, different instrument and different analyst on different days. The obtained 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) Area	3-F-4-NBN	2-F-5-NBN	4-F-3-NBN	2-F-3-NBN	4-Cl-3-NBN
1	15429	20915	17882	14977	16475
2	15570	21149	18015	15039	16702
3	15567	21169	18072	14981	16566
4	15523	21183	17908	14993	16706
5	15560	21172	18069	14956	16534
6	15420	21083	17892	14921	16405
Average	15512	21112	17973	14978	16565
STDEV	70	103	89	39	121
%RSD	0.5	0.5	0.5	0.3	0.7
Reproducibility (Method precision) (µg/g)					
1	1.39	1.37	1.34	1.23	1.39
2	1.38	1.37	1.34	1.22	1.40
3	1.38	1.38	1.35	1.23	1.41
4	1.37	1.39	1.35	1.23	1.41
5	1.39	1.41	1.37	1.26	1.42
6	1.40	1.41	1.35	1.27	1.41
Average	1.39	1.39	1.35	1.24	1.41
STDEV	0.01	0.02	0.01	0.02	0.01
%RSD	0.7	1.4	0.7	1.6	0.7
Reproducibility (Intermediate Precision) (µg/g)					
1	1.33	1.33	1.29	1.29	1.28
2	1.32	1.30	1.26	1.26	1.25
3	1.31	1.31	1.26	1.26	1.25
4	1.24	1.23	1.19	1.19	1.18
5	1.21	1.21	1.17	1.16	1.14
6	1.22	1.22	1.17	1.18	1.15
Average	1.27	1.27	1.22	1.22	1.21
STDEV	0.05	0.05	0.05	0.05	0.06

%RSD	3.9	3.9	4.1	4.1	5.0
Overall statistical data(n=12)					
Average	1.33	1.33	1.29	1.23	1.31
STDEV	0.07	0.07	0.08	0.04	0.11
%RSD	5.3	5.3	6.2	3.3	8.4

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 with respect to column flow variation of carrier gas initial flow rate $\pm 10\%$ and column oven initial temperature $\pm 2^\circ\text{C}$ as follow listed in Table 7a and Table 7b. Results of peak areas for 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN are summarized in Table 8. In each robustness conditions remaining GCMS conditions are same as per test method.

Table No. 7a: Flow variations:-

Column Flow (ml/min)	
As per Methodology	2.0
-10% Flow variation	1.8
10% Flow variation	2.2

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

Column Oven Temperature	
As per Methodology	160°C (2min) $\xrightarrow{10^\circ\text{ C/min}}$ 220°C (17min).
-2°C Column Oven Temperature variation	158°C (2min) $\xrightarrow{10^\circ\text{ C/min}}$ 220°C (17min).
+2°C Column Oven Temperature variation	160°C (2min) $\xrightarrow{10^\circ\text{ C/min}}$ 220°C (17min).

Table No. 8: Robustness experiment results

Robustness condition	System suitability criteria (% RSD)				
	3-F-4-NBN	2-F-5-NBN	4-F-3-NBN	2-F-3-NBN	4-Cl-3-NBN
As per methodology	0.8	0.6	0.9	0.7	1.1
Flow variation					
-10%	1.0	0.9	1.0	1.3	1.2
+10%	0.5	0.4	0.8	0.7	1.4
Temperature variation -Initial Oven					
-2°C	0.7	0.7	0.9	0.7	0.8
+2°C	0.4	0.6	0.7	0.8	0.8

CONCLUSIONS:

The present study established a well resolved analytical method for the determination of five genotoxic impurities at very low level by GC-EI-MS with SIM mode. The validated method is Specific, Sensitive, Linear, Precise, Accurate and Suitable for the determination of 3-F-4-NBN, 2-F-5-NBN, 4-F-3-NBN, 2-F-3-NBN and 4-Cl-3-NBN contents in Alectinib Hydrochloride drug substance. Hence, the validated GC-MS method can be employed in to the routine analysis.

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