1. INTRODUCTION:

An impure substance may be defined as any material that affects the purity of the material of interest, viz., an active pharmaceutical ingredient (API) or drug substance. Impurity control in pharmaceutical products is a primary goal of drug development. Stringent international regulatory requirements have been in place for several years as outlined in the International Conference on Harmonization (ICH) Guidelines Q3A(R), Q3B(R) and Q3C. Impurity profiling is the common name of analytical activities with the aim of detecting, identifying or elucidating the structure and quantitatively determining organic and inorganic impurities as well as residual solvents impurities in bulk drugs as well as pharmaceutical formulations.

Qualification of the impurities is the process of acquiring and evaluating data that establishes biological safety of an individual impurity thus, revealing the need and scope of impurity profiling of drugs in pharmaceutical research.

1.1 Sources of impurities:

1.1.1 Synthesis related impurities:

Impurities in a drug substance or a new chemical entity (NCE) originate mainly during the synthetic process from raw materials, solvents, intermediates, and by-products. Raw material and solvents used in the synthesis are likely to contain a number of impurities that may range from trace levels to significant amounts that can react with various chemicals used in the synthesis to produce other impurities. Although it is not possible to theorize all by products, any such impurities needs to be identified based on the whole process of synthesis and formulation.

1.1.2 Formulation related impurities:

The excipients used to formulate drug product, can originate potential impurities. In addition during the process of formulation the drug is subjected to variety of

conditions like heat, shear etc, that can attenuate undesirable reactions and form DPs.

1.1.3 Degradation related impurities:

A number of impurities can be produced because of API degradation or other interactions on storage, Eg : Nicotinamide presence in a formulation containing 4 vitamins (nicotinamide, pyridoxine, riboflavin and thiamine) causes degradation of thiamine to a substandard level within a 1-year shelf life of Vitamin Bcomplex injections. Therefore, it is very important to conduct stability studies to predict, evaluate, and ensure drug product.

1.1.4 Polymorphism related impurities:

If a substance has same elemental composition but can exist in different crystal packing arrangements, are called polymorphs and the phenomenon is called polymorphism. There is lot of focus on the physical attributes of the drug substance and drug products related to the morphology of the drugs. The mixture of multiple polymorphic forms within the single crystallization procedure was observed in several instances and in some other situations the drug converts from one polymorphic form to the pseudo polymorphic form or another crystal form when stored at different storage conditions or when the drug comes in contact with different excipients and the packaging components.

Therefore, the importance of understanding the distribution pattern and also incorporation of such type of data generated by sophisticated analytical techniques like XRPD (X-Ray Powder Diffraction chromatography), DSC (Differential Scanning calorimeter), Microscopic analysis and Raman Spectroscopy into the drug applications

1.1.5 Stereochemistry related impurities:

Stereoisomers can be considered impurity in drug substance, although ICH excludes

stereo chemical impurities, pharmacopoeias consider them as ordinary impurity. The differences in pharmacological or toxicological profiles of stereoisomers suggest that it should be monitored carefully.

1.2 Classification of Impurities:

1.2.1 Organic impurities:

Organic impurities may arise during the manufacturing process and/or storage of the drug substance. They may be identified or unidentified, volatile or non-volatile, and these include the starting material, intermediates, degradation products, by-products and reagents, ligands and catalyst used at different stages of synthesis of API and drug products.

- Starting materials or intermediates:

These are the most common impurities found in every API unless a proper care is taken in every step involved throughout the multi-step synthesis. Although the end products are always washed with solvents, there are always chances of having the residual unreacted starting materials unless the manufacturers are very careful about the impurities. In paracetamol bulk, there is a limit test for p-aminophenol, which could be a starting material for some one manufacturer or be an intermediate for the other.

- By-products:

In synthetic organic chemistry, getting a single end product with 100% yield is very rare; there is always a chance of having by-products. By-products from the side reactions are among the most common process impurities in drugs 11, 12. By-products can be formed through a variety of side reactions, such as incomplete reaction, overreaction, isomerization, dimerization, rearrangement, unwanted reactions between starting materials or intermediates with chemical

reagents or catalysts

- Degradation products:

Impurities can also be formed by degradation of the end product during manufacturing of bulk drugs. However, degradation products resulting from storage or formulation to different dosage forms or aging are also common impurities in the medicines. The degradation of penicillins and cephalosporins is a well-known example of degradation products. The presence of a ß-lactam ring as well as that of an α-amino group in the C6/C7 side chain plays a critical role in their degradation

- Reagents, ligands and catalysts:

These chemicals are less commonly found in API's; however, in some cases they may pose a problem as impurities. It has also been found that the presence of certain chemicals such as triethylamine has a degradative effect on the product. Ampicillin trihydrate samples having triethylamine content of 2000 ppm to 4000 ppm were found to be stable under accelerated stability testing. However, the product showed appreciable degradation when triethylamine content became 7000 ppm

Chemical reagents, ligands and catalysts used in the synthesis of a drug substance can be carried over to the final products as trace level impurities. For e.g. carbonic acid chloromethyltetrahydro-pyran-4-yl ester (CCMTHP), which is used as an alkylating agent in the synthesis of a β-lactam drug substance, was observed in the final product as an impurity.

1.2.2 Inorganic Impurities:

Inorganic impurities can result from the manufacturing process. They are normally

known and identified and include:

• Reagents, ligands and catalysts

• Heavy Metals or other residual metals :

The main sources of heavy metals are the reactors (if stainless steel reactors are used), where acidification or acid hydrolysis takes place and water used in the processes. These impurities of heavy metals can easily be avoided using demineralized water and glass-lined reactors.

• Inorganic salts

• Other materials (filter aids, charcoal)

The filters or filtering aids such as centrifuge bags are routinely used in the bulk drugs manufacturing plants, and in many cases, activated carbon is also used. The regular monitoring of fibers and black particles in the bulk drugs is essential to avoid these contaminations

1.2.3 Residual solvents:

These are generally inorganic/organic liquids that are used during synthesis of drug substances as a vehicle for preparation of solution or suspension. Appropriate control of residual solvent is necessary since they have known toxicity. ICH Q3C (5) guideline provides the limits of residual solvent based on existing safety and toxicity data. These were classified in three categories:

- Class 1 (The most toxic and/or environmentally hazardous):

These are highly toxic in nature and are limited to 2–8 ppm, for environmentally hazardous chemical like trichloroethane the limit of 1500 ppm is applied. During manufacturing of pharmaceuticals Class 1 solvents should be avoided. But if their presence is unavoidable, the definite concentration limit is applied, regardless of

the actual patient intake dose.

- Class 2 (Considered a lesser risk):

These should be limited in their usage. Two different approaches were described in guideline for setting limits of class 2 solvents. The first approach is used when PDE (permitted daily dose) can not be estimated; concentration limits are calculated on the basis of daily intake of theoretical product mass of 10g. The second approach is used when dose is known; the PDE and/or dose value can be used to determine the permissible concentration.

- Class 3 (The lowest risk category):

These have low toxic potential and are limited to 5000 ppm (0.5% w/w)

- In the process of developing the drug products, one has to understand the potential toxicological profiles of each of the identified impurities and its class either being genotoxic, mutagenic or in cases of organic volatile impurities (OVI) / residual solvents whether they are in the class I, class II or class III.

- The regulatory requirements for impurity profiling and forced or stress degradation study have been extended to generic drugs and products in recent years. Due to the stringent environment laid down by regulatory authority, there is steady increase in product recalls from the market. One of the reasons often cited is ―due to the presence of impurities or degradation products (DPs) beyond the prescribed limits.

- The major deficiencies in the DMF's (Drug master files) and ANDA's applications submitted to agencies like USFDA and EMEA, after the review of the application are mainly revolving around the identification, reduction in levels, characterization, justification of levels, and qualification of impurities.

- This also includes the queries on the data related to the identification of the degradation pathways, illustration of the different degradation pathways, stability studies, validation of stability indicating analytical methods, separation of the identified and unidentified impurities from the drug substance and between the impurities itself, the stress studies under different harsh conditions and the residual solvents and the justifications of their levels.

- Another most important physical property of drug which is severely scrutinized and reviewed and demanded in the drug applications is the particle size data (Analyzed by Malvern analyzer or other equivalent techniques).The extent of distribution of various sizes of the particles in Active Pharmaceutical Ingredients in combination with the polymorphic forms has a huge bearing on the bio-equivalence of the generic drugs when formulated.

- Having a thorough understanding of the impurity profiles of any drug substance and drug product helps immensely in understanding the safety and efficacy of the drug substances and also helps the pharmaceutical companies to get the approvals without any delay.

2. AIMS AND OBJECTIVES:

- To understand the potential impurity profiles and its degradation pathway of the recently approved drugs.

- The focus is fully on all the potential impurities like organic, inorganic, enantiomeric and polymorphic impurities.

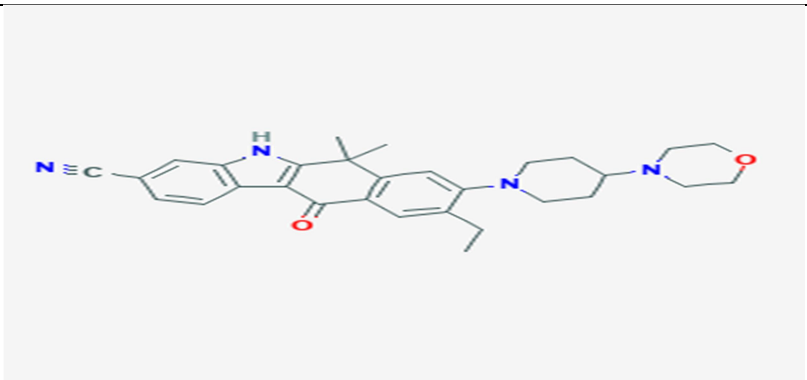
- Structure elucidation and characterization of the unknown impurities which are present more than identification threshold in all the drugs.

- The sophisticated instruments like LC-MS/MS, HRMS, NMR, XRD, FTIR, Raman, HPLC, GC, etc. shall be used.

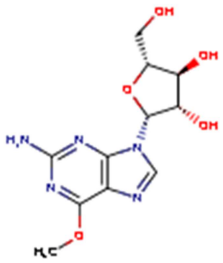
3. LITERATURE REVIEW:

- Several review articles and published papers are available on the importance of the impurity profiling and degradation pathways for the drug products
- However, there are so many recent drug products which are approved by the state governing bodies of various countries in the form of NDA's in the last couple of decades. There is very limited literature and publications on these drugs with respect to impurity profiles and degradation pathways as it is limited to only the innovator companies work on the NCE during the developing phase and is in the confidential data submitted to the regulatory agencies.
- Another factor which becomes important in order to study the impurity profiles and degradation pathways for the recent drugs is that when the drug substance becomes off-patent and is available for the rest of the world to make the generic products of the same drug, each company can synthesize the drug substance in several different ways, which might lead to new impurity profiles.
- Hence, there is a strong need for conducting research in this area to systematically study the impurity profiles of these recently approved drugs as there are new inventions in the sophisticated analytical instruments which are enabling to improve the level of understanding of the impurity profiles constantly which might not be at the innovator company's disposal during the development phase.
- **Drug specific literature review:**

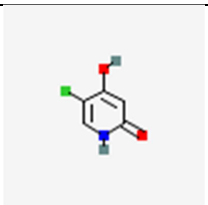
As far as drug specific review of literature is concerned, for Alectinib drug no any reported research paper was found to contain the detailed impurity profiling and degradation impurities study. Alectinib drug has been approved drug as 'Alecensa' in USFDA for the indication of Non-small cell lung cancer since 2015.

Alectinib	
Description	Alectinib is a second generation oral drug that selectively inhibits the activity of anaplastic lymphoma kinase (ALK) tyrosine kinase. It is specifically used in the treatment of non-small cell lung cancer (NSCLC) expressing the ALK-EML4 (echinoderm microtubule-associated protein-like 4) fusion protein that causes proliferation of NSCLC cells. Inhibition of ALK prevents phosphorylation and subsequent downstream activation of STAT3 and AKT resulting in reduced tumour cell viability. Approved under accelerated approval in 2015, alectinib is indicated for use in patients who have progressed on or were not tolerant of crizotinib, which is associated with the development of resistance.
IUPAC name:	9-ethyl-6,6-dimethyl-8-[4-(morpholin-4-yl)piperidin-1-yl]-11-oxo-5H,6H,11H-benzo[b]carbazole-3-carbonitrile
Molecular Structure:	

Water solubility:	0.0105 mg/mL

Nelarabine	
Description	Nelarabine is an antineoplastic agent that is typically employed to treat acute T-cell lymphoblastic leukemia. Nelarabine is a purine nucleoside analog converted to its corresponding arabinosylguanine nucleotide triphosphate (araGTP), resulting in inhibition of DNA synthesis and cytotoxicity.
IUPAC name:	2-Amino-9-beta-D-arabinofuranosyl-6-methoxy-9H-purine
Molecular Structure:	

Gimeracil	
Description	Gimeracil is an adjunct to antineoplastic therapy, used to increase the concentration and effect of the main active componets within chemotherapy regimens. Approved by the European Medicines Agency (EMA) in March 2011
IUPAC name:	5-Chloro-2,4-dihydroxypyridine

Molecular Structure:	

5.1 ALECTINIB:

Cancer is the second leading cause of death worldwide according to the World Health Organization data of the year 2018 [1]. The most common type of human cancer is lung cancer with estimated 1.76 million deaths in 2018 [1]. About 85 % cases of lung carcinoma are from non-small-cell lung cancer (NSCLC) as its dominant type [2]. Initially, for NSCLC treatment, the first generation of tyrosine kinase inhibitors were used which did not successfully inhibit kinase activity of fusion protein, generated between protein (EML4) and anaplastic lymphoma kinase (ALK). Therefore, studies have focused on the discovery and development of new alternative drugs for treatment of NSCLC including patients with EML4-ALK fusion proteins [3]. Previously crizotinib was the first-generation drug that can be used in the treatment of anaplastic lymphoma kinase (ALK) positive Non-small cell lung cancer (NSCLC). However, since more and more patients were found to be resistant to this drug. Subsequently, alectinib has been approved for the treatment with crizotinib resistant patients [3]. Alectinib (Alecensa) is a second-generation, orally active, potent, and highly selective inhibitor of anaplastic lymphoma kinase (ALK). Alectinib has been approved for the treatment of ALK fusion-gene positive, unresectable, advanced or recurrent non-small cell lung cancer (NSCLC) in Japan, where it has been given orphan drug designation [4]. International Conference on Harmonization (ICH) has recommended Q1A (R2) and Q1B guidelines to perform forced degradation studies on drug substances to generate all possible degradation products to establish its stability attributes [5][6]. ICH has also recommended Q3A(R2) and Q3B(R2) to provide requirements of characterization of impurities present at

higher than the identification threshold in a drug substance or product [7][8][9].

The literature mentions a RP-HPLC method for the estimation of alectinib in bulk and pharmaceutical dosage form [10] and the HPLC-PDA method for the determination of alectinib concentrations in the plasma of adolescent [11]. Nevertheless, no study in literature was found to be reported on the related degradation products of alectinib hydrochloride, hence present research work may help pharmaceutical industries to identify any of the state degradants in their drug substance [12].

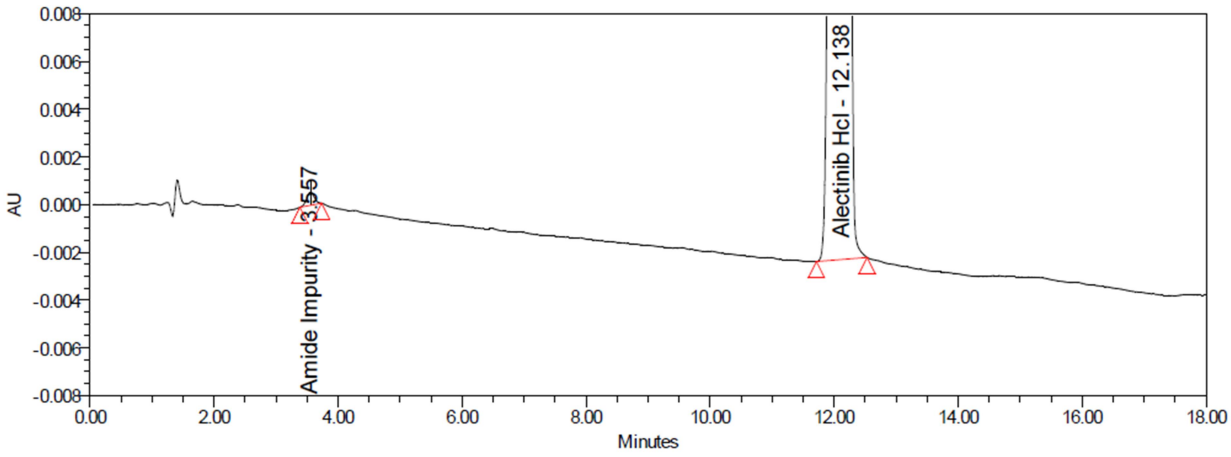
5.1.1 Experimental:

5.1.1.1 Instrumentation and analytical condition

Liquid chromatography Mass spectrometry

The HPLC system (Waters Alliance 2695) equipped with the photodiode array (PDA) detector used with the Empower 3.0 software for data acquisition. For the LC-MS study, LC-MS/MS (Waters Micro-mass, ZQ-Mass detector) with electro spray ionization (positive) mode and PDA detector was used. The pH of the buffer solution was adjusted using Eutech (PH-510) pH meter. Ultrasonic cleaner (Leelasonic-500) was used for degassing the mobile phase and other solutions. Kromasil C18 (250 X 4.6) mm, i.d., 5µ (Make: Akzonobel) column was maintained at 50 °C temperature during analysis. The mobile phase-A was composed of 10mM ammonium formate buffer (the pH was adjusted to 7.00 with 0.25 % ammonia solution in water) and acetonitrile in the ratio of 95:05. The mobile phase-B was a mixture of 10mM ammonium formate buffer (the pH was adjusted to 7.00 with 0.25 % ammonia solution in water) and acetonitrile in the ratio of 25:75. The gradient program was planned as linear gradient decrease of mobile phase-A from 50% to 0.0% for 25 minutes, maintaining mobile phase-A at 0.0 % for 5 minutes and the last 5 minutes as of initial

gradient condition for equilibration. The injection volume was 20 µL, flow rate was kept at 1.5mL/min for entire 35 minutes of run time and detection wavelength was maintained at 230nm. These chromatographic conditions were used in both HPLC and LC-MS/MS studies as the conditions were compatible with LC-MS/MS instrument.



Preparative high performance liquid chromatography (Prep-HPLC)

Preparative HPLC (Shimadzu LC-20AP) with the PDA detector was used for isolation of all identified degradants. The column used was Phenomenex C18 (250X25) mm, 5µ. The mobile phase used was similar to that was used in analytical studies. The chromatographic separation was achieved with an injection volume of 5 ml and flow rate of 25 mL/min for 35 minutes run time. The detection wavelength and gradient were similar to that used in analytical method.

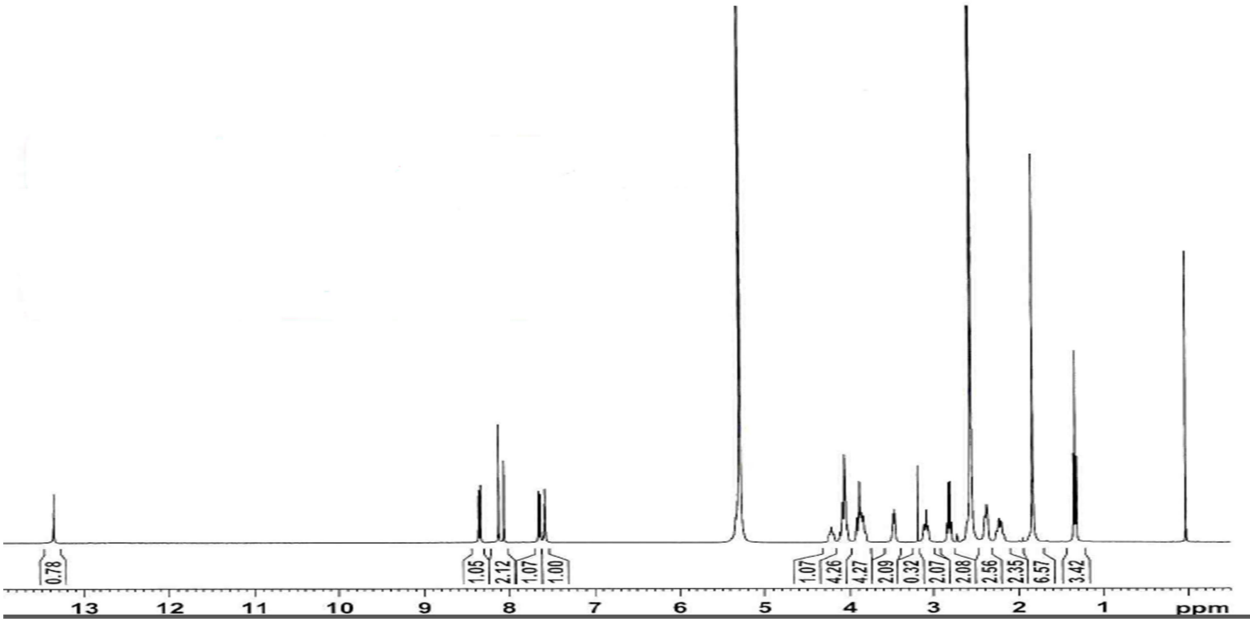
High resolution mass spectrometry

The high resolution mass spectrometry (HRMS) orbitrap Q-Exactive plus of Thermo system was used for identification of fraction masses of impurities observed in degradation studies. The instrument and method parameters are same as mentioned in LC-MS.

Nuclear magnetic resonance spectrometry

The NMR experiments for alectinib hydrochloride and its degradation products were performed on Bruker AVANCE 400 MHz NMR instrument equipped with BBO probe. The probe temperature was set as 298K throughout experiment cycle. The chemical shifts of ^{1}H and ^{13}C spectra were recorded on delta scale in ppm with reference to tetra methyl silane (TMS). The axis of the scale was calibrated as 2.56 ppm for DMSO-d6 peak in ^{1}H spectra and at 39.5 ppm for DMSO-d6 peak in ^{13}C NMR spectra (Figure-1).

Figure-1: Proton spectra of alectinib

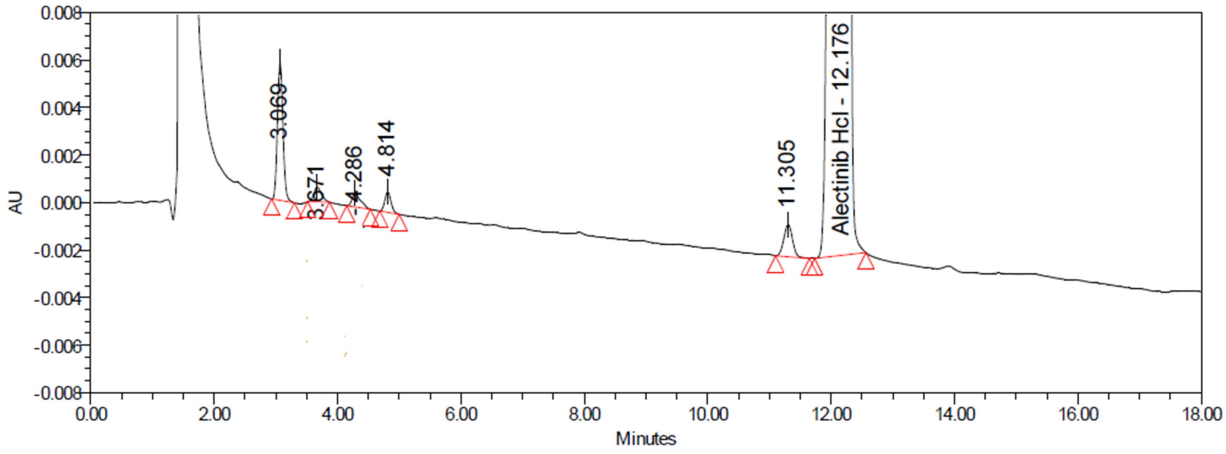


5.1.1.2 Analytical method development, method validation, forced degradation study and degradation kinetic study

The forced degradation experiments were performed according to ICH Q1B guidelines to test the stability of alectinib under the following conditions: acidic and alkaline hydrolysis, oxidation, heat and light. The sample solutions used for forced degradation study were having concentration of 0.3mg/mL in diluent. The diluent used was a mixture of 0.05 % formic acid (98-100%) in water and acetonitrile in the ratio of 80:20. Degradation kinetics study was performed degradation of the sample during varied duration of time and varied

condition of degradation. Comparison of these results through statistical values proves some degradation kinetics results which were summarized in detail.

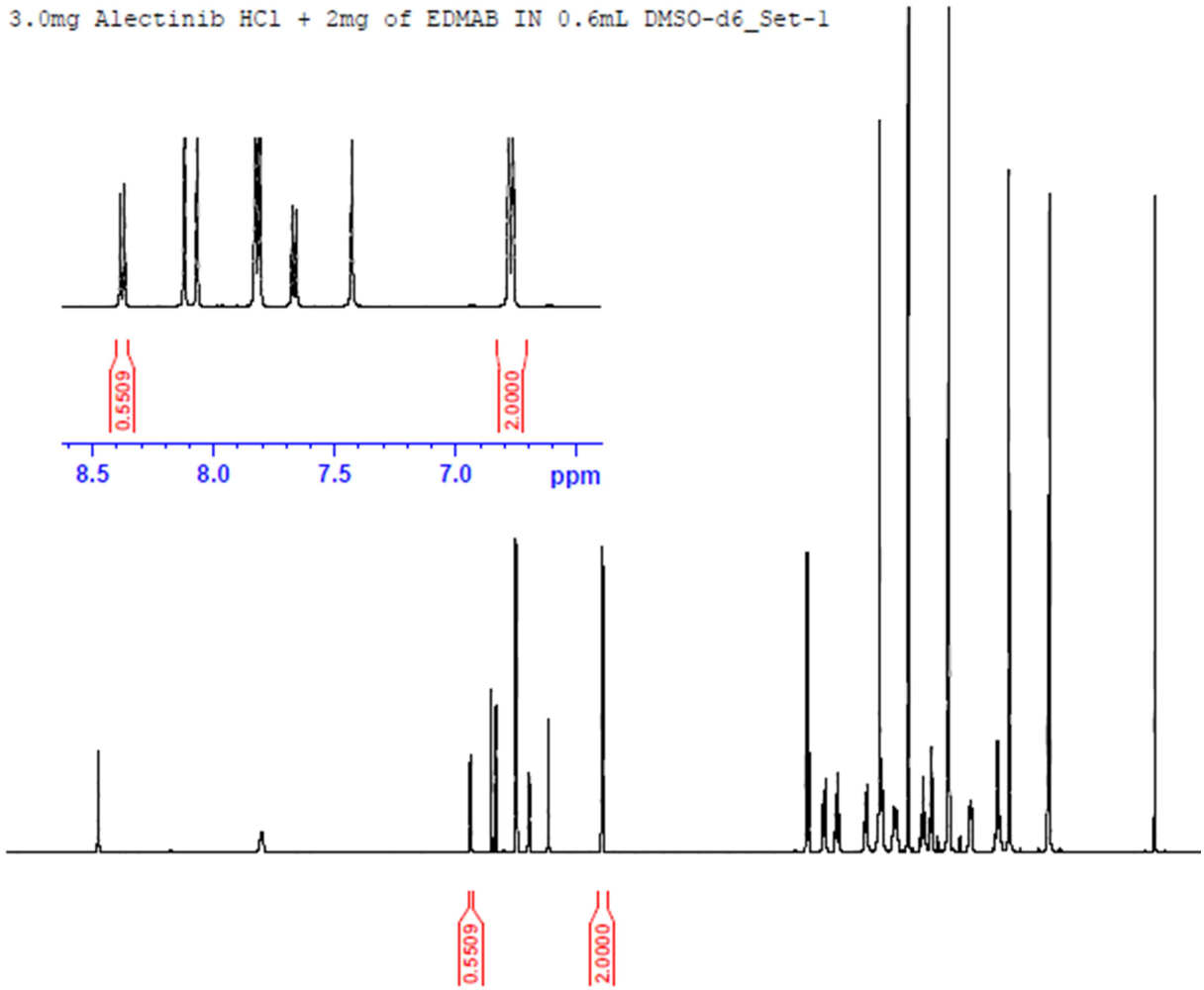
Figure-2: Forced degradation chromatogram oxidative stress condition.



5.1.1.3 Quantitative NMR:

Analytical method for Q-NMR estimation of Alectinib by NMR updated to make necessary modifications. Method validation of the same method was also performed. (Figure-3)

Figure-3: Quantitative NMR spectrum for alectinib



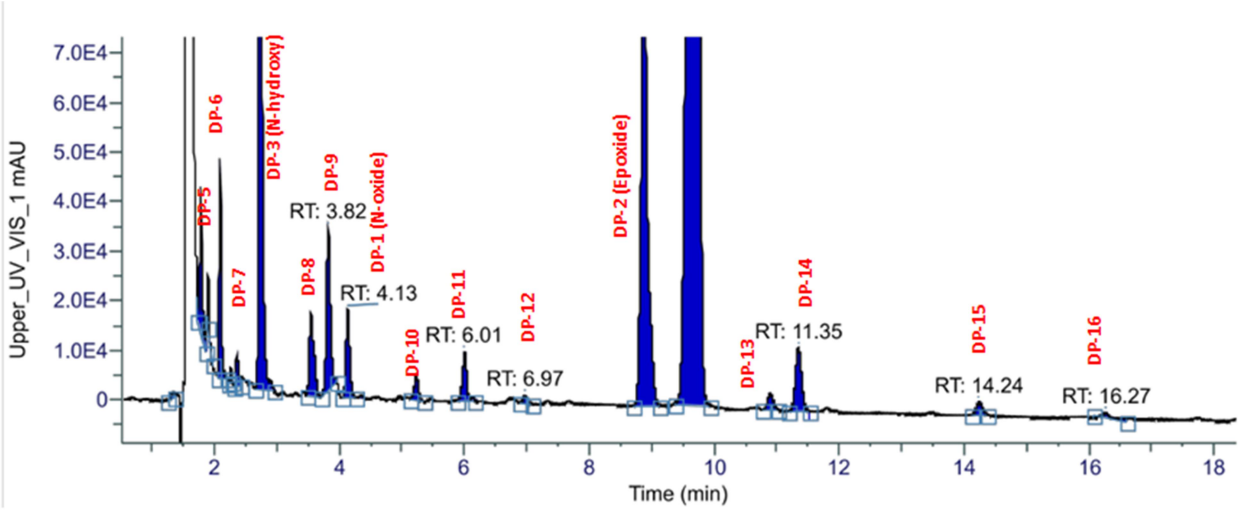
5.1.1.4 Results and discussion

Alectinib hydrochloride was found stable in all the degradation conditions except oxidation. Oxidative stress degradation has generated four degradation products which were isolated by preparative-HPLC and then efforts were made to elucidate their structure by studying various spectroscopic data as mentioned in each individual DP section. Percentage of degraded drug was calculated using a stability indicating HPLC method the developed and validated in our laboratory.

Alectinib hydrochloride was found to generate four degradation products in oxidative stress condition. Later on, during HRMS study, other twelve degradation products were identified. The characteristic differences in their structures were confirmed through LC-MS, HRMS,

NMR, and FTIR spectra. (Figure-4)

Figure-4: UV chromatogram of oxidative degradation sample of alectinib in HRMS:



5.2 NELARABINE:

5.2.1 Introduction:

Antimetabolites specifically nucleobase and nucleoside analogues are the anticancer agents that have revolutionised the clinical oncology and made the cancer into a curable disease[14]. Benchmark drugs in this category are cytarabine for acute myeloid leukemia and gemcitabine for pancreatic and lung cancer [15]. Nucleobase and nucleoside analogues act by mimicking endogenous nucleosides and thereby following phosphorylation and nucleotides. This can be mediated either by enzyme inhibition or by substitution of endogenous nucleosides leading to DNA and RNA damage and interference with DNA methylation [1]. Nelarabine is a guanosine nucleoside analogue, arabinosylguanine (ara-G) which is prodrug of arag-G and based on its activity was approved for the treatment of T-cell acute lymphoblastic leukemia (ALL) and lymphoma (LBL) [16].

5.2.2 Experimental:

Analytical method was developed for LC as well as LC-MS compatible mobile phase.

The forced degradation experiments were performed according to ICH Q1B guidelines to test the stability of nelarabine under the following conditions: acidic and alkaline hydrolysis, oxidation, heat and light. The sample solutions used for forced degradation study were having concentration of 0.1mg/mL in milli-Q water as a diluent.

Degradation kinetics study in oxidative stress condition was performed and summarized in detail.

Method validation experiments were conducted as per ICH guideline Q2 (R1)

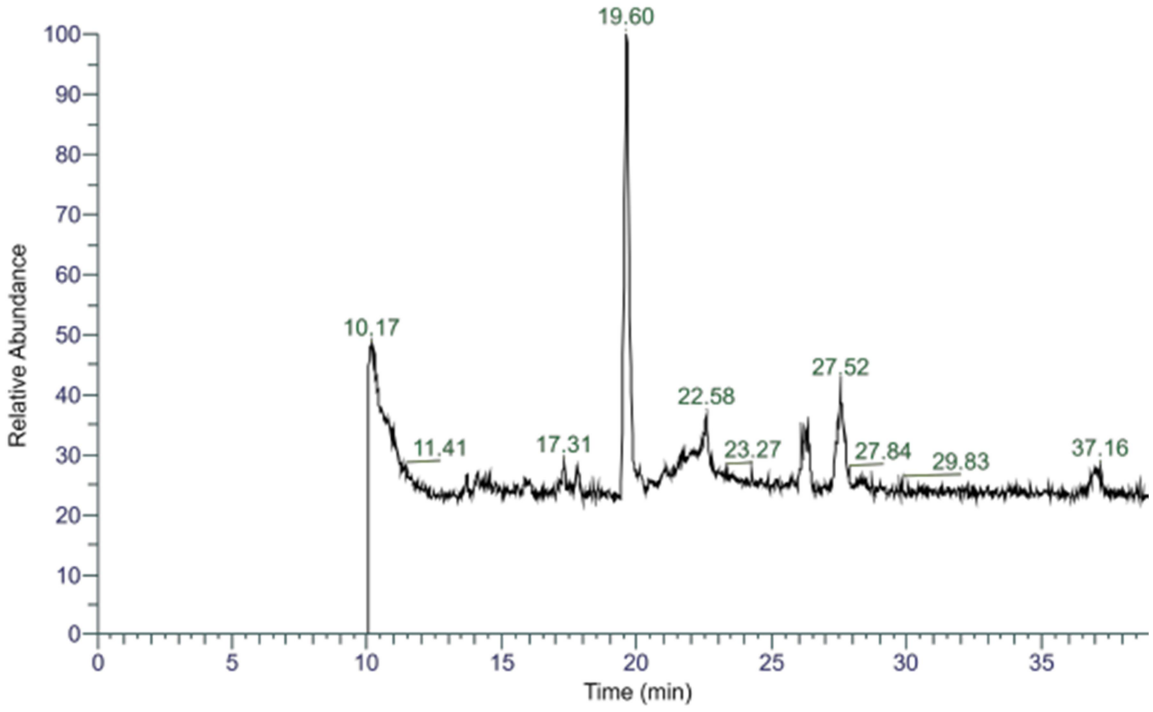
5.2.3 Results and discussion:

Analytical method was developed with proper peak shape and all other chromatographic parameters satisfactory.

The method was validated for the parameters of linearity, sensitivity, LOD, LOQ, Accuracy, Precision, and Robustness and all the results of parameters were found satisfactory.

The drug nelarabine was found to be very susceptible to acidic degradation condition and found to follow zero order degradation kinetics. In section-C, total 9 degradation products were characterized based on their LC, LC-MS, HRMS, and NMR data. Out of this, DP-1 was observed in acidic and peroxide degradation condition, DP-2 was observed in alkaline degradation condition and remaining all the degradation products were observed in HRMS TIC spectra in oxidative degradation condition (Figure-5). All the nine degradation products were satisfactorily characterized and their probable structures with chemical names were presented in individual sections.

Figure-5: TIC spectra of degradation sample in oxidative condition by HRMS



5.3 GIMERACIL:

5.3.1 Introduction

Out of all types of cancer, gastric cancer standard second most frequent type of cancer which is responsible for the death of the patients [17]. There are various first and second line treatment strategies have been developed however recently oral fluoropyrimidines have been developed as inactive prodrugs of 5-fluorouracils which get absorbed intact through gastrointestinal mucosa and then converted to 5-fluorouracil by one or more enzymatic system [17]. One such development was combination of tegafur, gimeracil and oteracil which was approved by European Medicines Agency (EMA) in March-2011 as commercially available product as “Tysuno” [18]. The main active drug in this combination is tegafur which is prodrug of 5-fluorouracil and rapidly acts on cancer cells halting their growth by inserting itself into the DNA and RNA strands and inhibiting their replication process [18]. The main role of gimeracil is to prevent the breakdown of 5-fluorouracil so that

the high enough concentration of 5-fluorouracil can be maintained for sustained effect on cancer cells [19]. Gimeracil acts by selectively blocking the dihydropyrimidine dehydrogenase enzyme which is responsible for degradation of 5-fluorouracil [20]. The main action of oteracil in the combination is to block the conversion of tegafur prodrug into 5-fluorouracin in the gastrointestinal tract and thereby preventing the toxic effects of 5-fluorouracil in the gastrointestinal tract. Oteracil blocks the orotate phosphoribosyltransferase enzyme which is responsible for generation of 5-fluorouracil in the gastrointestinal tract [19]. Hence to enhance the efficacy and to reduce the unwanted toxicity, all the three components are vital in the combination therapy.

5.3.2 Experimental:

Analytical method was developed for LC as well as LC-MS compatible mobile phase.

The forced degradation experiments were performed according to ICH Q1B guidelines to test the stability of gimeracil under the following conditions: acidic and alkaline hydrolysis, oxidation, heat and light. The sample solutions used for forced degradation study were having concentration of 0.1mg/mL in milli-Q water as a diluent.

Method validation experiments were conducted as per ICH guideline Q2 (R1)

5.3.3 Results and discussion:

The developed method was found to be validated as per ICH guideline and found satisfactory as per good chromatographic response.(Figure-6)

The degradation products generated from gimeracil in oxidative degradation condition only (Figure-7). These degradation products were only identified in HRMS study which is characterized in detail by MS and MS-MS spectra of the degradation products.

Figure-6: Chromatogram of gimeracil sample as per method

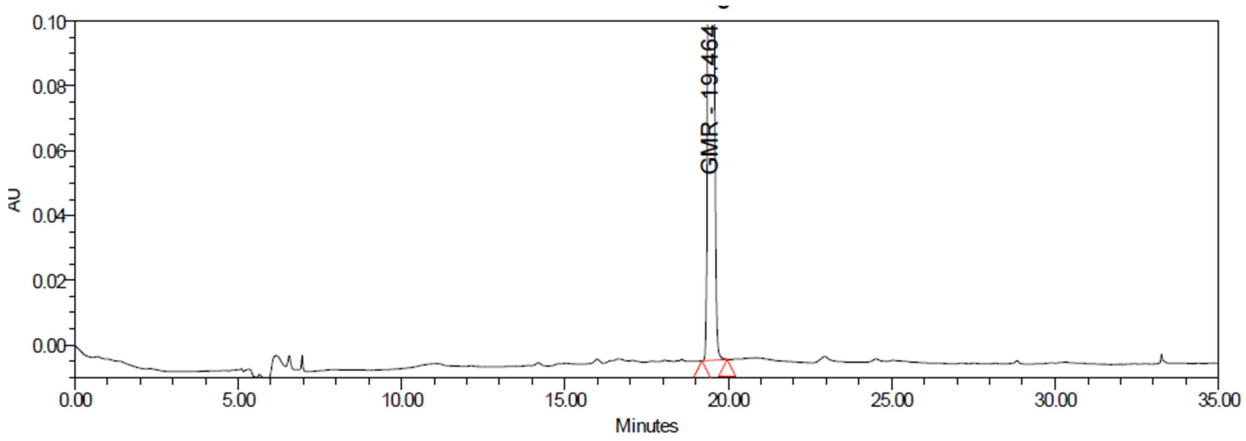
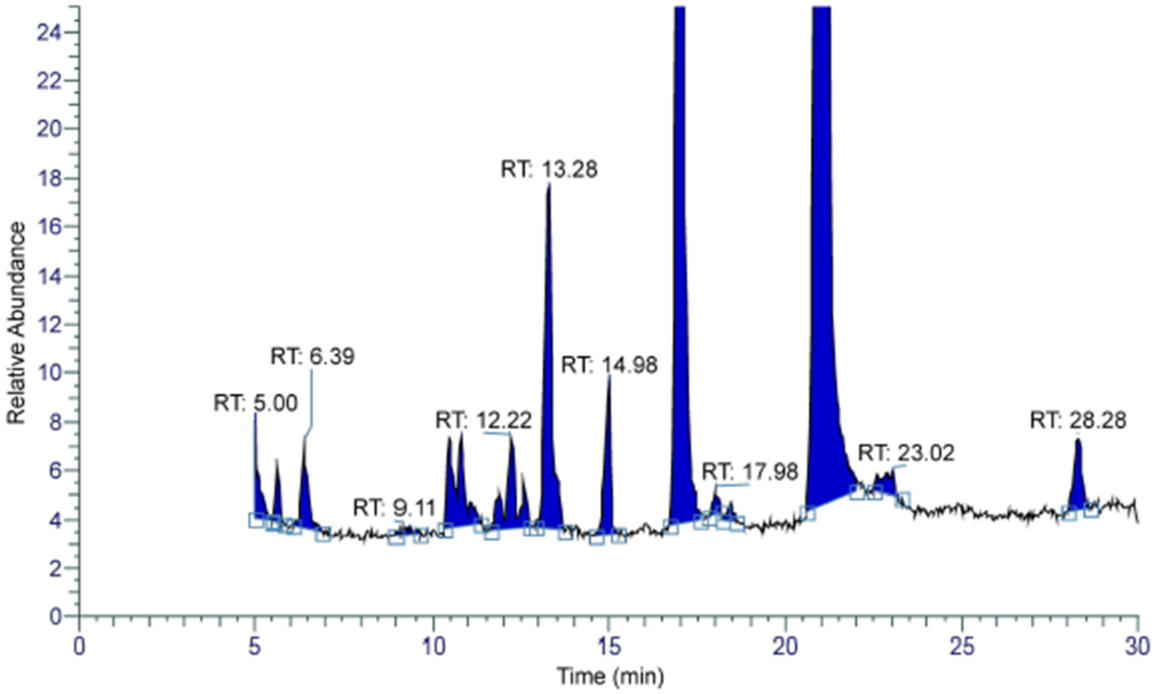


Figure-7: TIC Chromatogram of gimeracil in oxidative stress condtion.



6. FUTURE WORK:

- Data compilation and submission.
- Publication of research work
- Thesis writing.

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