DEGRADATION KINETIC STUDIES OF NON-PHARMACOPEIAL DRUG PRODUCTS AND DETERMINATION OF THEIR FORCED DEGRADANTS AND IMPURITIES

Thesis submitted for the fulfillment of the requirements for the Degree of

Doctor of Philosophy

By

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Reg. No: 089

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Bangladesh

DECLARATION

I do hereby declare that this thesis "Degradation Kinetic Studies of Non-Pharmacopeial Drug Products and Determination of their Forced Degradants and Impurities" is submitted as a requirement for the fulfillment of the Doctor of Philosophy (PhD) degree in department of Pharmaceutical Technology, University of Dhaka, is an original research works of mine and have not been previously submitted elsewhere for the award of any Degree or Diploma.

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CERTIFICATION

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Supervisor

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Abstract

Stability of a drug is assessed to ensure the chemical and physical integrity of the drug product and its capacity to remain protected against exposure to environment, such as air, light, and heat throughout its shelf-life. Development and use of stability-indicating methods are critical parameters in drug regulation to prevent counterfeit medicines. Forced degradation or stress testing according to ICH Q3B (R2) is a part of this process, used to predict the stability of drug substance or drug product with effects on purity, potency, and safety.

Three dipeptidyl peptidase IV (DPP-IV) inhibitors, sitagliptin, vildagliptin and linagliptin used to treat type 2 diabetes mellitus (T2DM) were studied, which are not yet included in the official book, i.e. USP, BP. The collected samples from pharmaceutical companies of Bangladesh were evaluated by comparing with innovator products. It is required to establish specificity of a stability indicating method, which also provide a perception into degradation pathways as well as degradation products of the drug molecules and helps in structure elucidation of the degradants by spectral analysis.

The aims of the studies were to evaluate the quality of these three DPP-IV inhibitors. The present investigation also deals with method development and optimization by applying quality by design (QbD) approaches and validation of the selective stability-indicating RP-UHPLC method according to ICH Q2 (R1) guideline. From degradation kinetics studies half-lives ($t_{1/2}$) and shelf -lives ($t_{0.9}$) of these three drug molecules were determined at room temperature by applying Arrhenius equation. Major degradation products of linagliptin were isolated and characterized by IR, ^{1}H-NMR and ^{13}C-NMR spectroscopic method and described plausible degradation pathways.

All brands which were used in these studies were similar with their innovator products in terms of weight variation, hardness, disintegration and potency. For the comparison of dissolution profile with the reference product, the difference factor (f1) and similarity factor (f2) were calculated in four different dissolution media. Seven brands of sitagliptin, seven brands of vildagliptin and five brands of linagliptin among nine are similar and

bioequivalent to innovator brand in respect to drug release pattern where the f1 value less than 15 and f2 value more than 50.

The optimized chromatographic condition for separation and quantitation of sitagliptin, vildagliptin and linagliptin was reverse phase ultra high performance liquid chromatography (RP-UHPLC) equipped with X-bridge C_{18} column (4.6 i.d. × 150 mm, 5 µm) having flow rate 1 ml/min using phosphate buffer (pH 6) and acetonitrile (70:30, v/v) as mobile phase at 246nm, 228nm and 267nm for vildagliptin, linagliptin and sitagliptin, respectively using photodiode diode array plus (PDA+) detector. The column oven temperature was ambient for analysis of all samples. The retention time for vildagliptin, linagliptin and sitagliptin were 2.423±0.04min, 3.203±0.06 min and 4.189±0.12 min respectively.

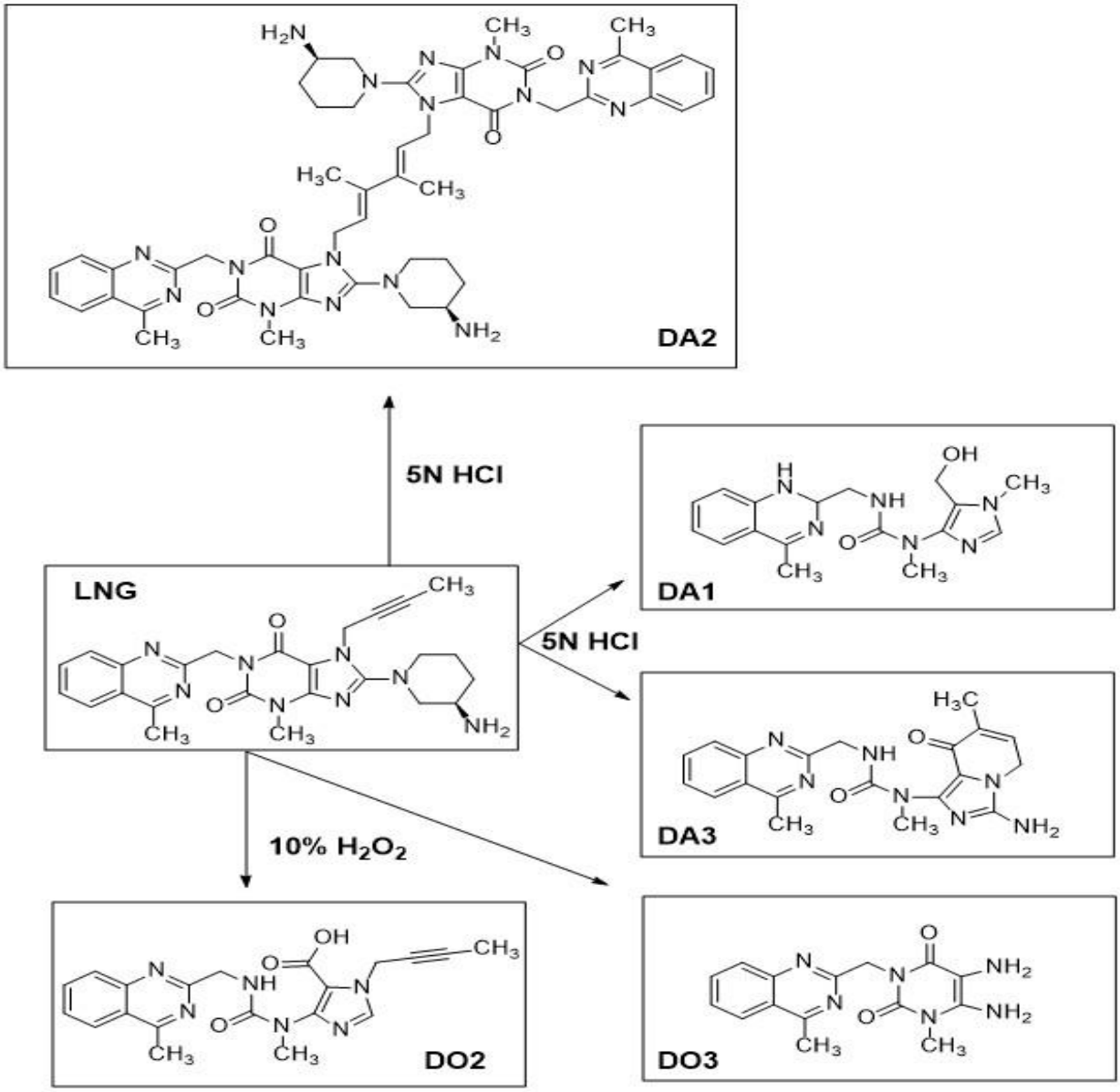
For routine analysis of these three products in pharmaceutical companies single, simple, precise, sensitive, accurate and robust method was developed and optimized by applying Quality by design (QbD) approaches using design of experiments (DoE) where 3^3 full factorial Box -Behnken Design (BBD) model were used. Three factors were utilized for the experimental design of the method as independent variables which comprise percentages of organic modifiers, pH of buffer in mobile phase and flow rate. The co-variates or responses included the retention time, resolution between peak 1 and 2(Rs1) and resolution between peak 2 and 3 (Rs2). This design was statistically analyzed by ANOVA, normal plot of residual, box-cox plot for power transform, perturbation, counter plot and 3D response surfaces plots. The quadratic effect of different variables like percentages of acetonirile in mobile phase($p< 0.0001$), flow rate ($p< 0.0001$) and pH of buffer ($p< 0.003$) separately as well as in interaction was most significant on retention time(RT), resolution between peak 1 and 2(Rs1) and resolution between peak 2 and 3 (Rs2).

The developed method was validated as per the requirements of ICH-Q2B guidelines for specificity, system suitability, linearity, sensitivity, precision, accuracy, and robustness. The linear regression analysis data for the linearity plot showed correlation coefficient values in case of sitagliptin of 0.999 with LOD value of 0.06 µg/mL and LOQ of 0.225µg/mL, in case of vildagliptin of 0.998 with LOD value of 0.01 µg/mL and LOQ of 0.05µg/mL and in case of linagliptin of 1.0 with LOD value of 0.005 µg/ml and LOQ of 0.015µg/ml. The relative

standard deviation (%RSD) for inter-day and intra-day precision was not more than 2.0%. The method was found to be accurate with percentages recovery of 100±2% and the % RSD was less than 2%. The results showed that the proposed method is simple, sensitive and highly robust for routine analysis.

Forced degradation or stress testing is performed according to ICH Q1A and ICH Q1B guideline to meet the stability testing of a drug substance or a drug product with effects on purity, potency, and safety. This study was carried out to ensure stability indicating assay method. The stressed condition were hydrolytic (acid and base), oxidative, thermal and photolytic. The degradation kinetics was estimated in acidic, alkaline, oxidative and thermal forced degradation condition. The half-lives ($t_{1/2}$) and shelf -lives ($t_{0.9}$) of the drugs were calculated by using an Arhenius plot. The calculated half-life of sitagliptin was maximum (2310h) in thermal and minimum (138.5h) in acid hydrolysis condition, for vildagliptin maximum (990h) in thermal and minimum (115.5h) in acid hydrolysis condition and for linagliptin maximum (1732.5h) in thermal and minimum (385h) in acid hydrolysis conditions. The proposed stability indicating method revealed that these three gliptins were stable in various heat and photolytic condition; however, protection is recommended during storage and handling in strong acidic, alkaline and oxidative condition. Five major degradants of linagliptin in acidic (3) and oxidative (2) forced degradation condition were isolated and characterized by IR, ^{1}H-NMR and ^{13}C-NMR spectroscopic methods. After acidic degradation novel compounds are 1-(2-amino-5-(hydroxylmethyl) – 1 - methyl – 1 H – imidazol – 4 – yl) – 1 – methyl – 3 - ((4 – methyl – 1 , 2 dihydroquinazolin -2-yl) methyl)urea **(DA1)**; 7,7'-((2E,4E)-3 , 4 – dimethylhexa – 2 , 4 - diene-1, 6 – diyl) bis (8 - ((R) – 3 – aminopiperidin – 1 – yl) – 3 – methyl – 1 - ((4 - methyl quinazolin-2-yl)methyl)-3,7-dihydro-1H-purine-2,6-dione) **(DA2)** and 1-(3-amino-7-methyl-8-oxo-5,8-dihydroimidazo[1,5-a]pyridin-1-yl)-1-methyl-3-((4-methylquinazolin-2- yl) methyl) urea **(DA3)**. The two novel oxidative degradants are 1 -(but – 2 – yn – 1 –yl)–4-(1-methyl – 3 - ((4 – methylquinazolin – 2 – yl) methyl) ureido) - 1H – imidazole – 5– carboxylic acid **(DO2)** and 5 , 6 – diamino – 1 – methyl – 3 - ((4 – methylquinazolin – 2 –yl) methyl)pyrimidine-2,4(1H,3H)-dione **(DO3)**.

From this study it can be concluded that the quality of antidiabetic DPP-IV inhibitors manufactured by Bangladeshi pharmaceutical companies fulfill the world class requirement based on the comparison with innovator products which are effectively worked on T2DM to reduce the global burden on diabetes.



Degradation Pathways of LNG

LNG= Linagliptin
DO2= Oxidative degradant-2 of LNG
DO3= Oxidative degradant-3 of LNG
DA1= Acidic degradant-1 of LNG
DA2= Acidic degradant-2 of LNG
DA3= Acidic degradant-3 of LNG

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List of Abbreviations

µL	Micro Liter
µm	Micro Meter
µM	Micro Mole
ACN	Acetonitrile
ANOVA	Analysis of Variance
API	Active Pharmaceutical Ingredients
AQbD	Analytical Quality by Design
ATP	Analytical target profile
AUC	area under the curve
BBD	Box-Behnken Design
BCS	Biopharmaceutics Classification System
BP	British Pharmacopoeia
cm	Centimeter
CMM	Continuous Method monitoring
CPMP	Committee for proprietary medicinal products
CQA	Critical quality attributes
DMSO	Dimethyl Sulfoxide
DoE	Design of Experiment
DPP-IV	Dipeptidyl Peptidase-IV
DT	Disintegration time
ELSD	Evaporative Light Scattering Detector
EU	European Eunion
FDA	Food and Drug Administration
FPG	Fasting plasma glucose
FT-IR	Fourier Transform Infrared
g	Gram
GC	Gas Chromatography
GIP	Gastric Inhibitory Polypeptide
GLP	Glucagon-Like Peptide
HPLC	High Performance Liquid Chromatography
HPTLC	High-Performance Thin-Layer Chromatography
ICH	International Conference on Harmonization

INN	International Nonproprietary Name
LC-MS	Liquid Chromatography–Mass Spectrometry
LNG	Linagliptin
LOD	Limit of Detection
LOQ	Llimit of Quantification
min	Minute
mL	Mili Liter
mL/min	Mili liter Per Minute
MODR	Method Operable Design Region
MP	Mobile Phase
NLT	Not Less Than
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NMT	Not More Than
ºC	Degree Celsius
PB	Phosphate Buffer
PBS(AUSTRALIAN)	Pharmaceutical Benefits Scheme
PDA	Photodiode Array
PPAR-γ	Peroxisome Proliferator-Activated Receptor Gamma
PPM	Parts Per Million
QbD	Quality by design
QC	Quality Control
RH	Relative Humidity
RID	Refractive Index Detector
RP-UHPLC	Reversed Phase- Ultra High Performance Liquid Chromatography
RRF	Relative Response Factor
RRT	Relative Retention Time
RSD	Relative Standard Deviation
SIM	Stability Indicating Method
STG	Sitagliptin
T2DM	Type 2 Diabetes Mellitus
TDI	Total Daily Intake
TLC	Thin layer chromatography
TPSA	topological polar surface area

USP	United States Pharmacopeia
USP-NF	United States Pharmacopeia - National Formulary
UV	Ultra Violet
VLG	Vildagliptin
WHO	World Health Organization
µg/mL	Microgram Per Mililiter

Introduction

Medicines are perhaps as old as mankind and the perception how their quality to be ensured has changed progressively over the time [1]. The regulation of modern medicines started only after immense progress in the field of life sciences in 19th century which laid a solid ground to work for the modern drug research and development. This process got paced up after the second world war started. In 1937 more than 100 people in 15 states of the United States were died due to diethylene glycol poisoning in sulfanilamide elixir, where the chemical was used as a solvent without any safety testing [2,3]. However, in countries with poor regulatory environment, medicines are still contaminated with diethylene glycol that have killed patients [4]. In 2009, 25 Bangladeshi children died after taking paracetamol syrup because it contained poisonous diethylene glycol according to report [5].

A numerous number of cases were related to substandard and counterfeit drugs around the world. The substandard drugs are ineffective and often dangerous to the patient because of their faulty formulation and also for low quality ingredients which do not meet the correct scientific specifications.

Products with the correct ingredients may be included in counterfeit drugs because of fake packaging, insufficient active ingredients or without active ingredients [6]. Thus health hazard effects of counterfeit drugs are greater than substandard drugs [7]. Counterfeit and substandard drugs are a prime cause of morbidity, mortality and loss of public confidence in drugs and health structures [8].

WHO has calculated approximately 10% of the global pharmaceuticals market consists of counterfeit drugs, but this percentage are to be increased 25% in developing countries, and may exceed up to 50% in certain countries [9]. FDA finds that up to 25% of the drugs consumed in poor countries are either substandard or counterfeit [10]. India and China are recognized as the most leading countries in the production of counterfeit drugs and bulk active ingredients used for counterfeiting worldwide [11]. Several studies depicted that counterfeits of pharmaceuticals sourced in China and India were noticed in 42 and 33 countries, respectively [12]. The prevalence of substandard or counterfeit medicines in Lao PDR, Tanzania, Cambodia and Uganda are 12.2–44.5%,

followed by Indonesia, Nigeria, Cameroon 18–48% and in Myanmar, Cambodia, Lao PDR, Ghana, Kenya, Tanzania, Uganda, Madagascar, Mali, Mozambique, Zimbabwe 11–44% [13].

Substandard and counterfeit drugs are also intensely noticeable in developed countries along with poor and developing countries. For example, in North America, counterfeit atorvastatin [14], erythropoietin [14], growth hormone [15], filgrastim [14,15], gemcitabine [16,17], and paclitaxel [16,17] have been reported. In 2007–2008, 149 Americans died due to the uses of adulterated blood thinner, heparin that was legally imported. In 2012, 11 people died and sickened another 100 people in the US because of contaminated steroids [18]. In another case, avastin were found to contain no active ingredients in the vials of the cancer medicine. In a study, WHO found that about 28% of antibiotic and 20–90% of antimalarial drugs were failed quality specifications [19].

Drugs are merely not ordinary consumer's products. In most cases, consumers are not in a position to make decisions about when to use drugs, which drugs to use, how to use them and to consider potential benefits against risks because no medicine is completely safe. Professional advice from either prescribers or dispensers is needed in making these types of decisions.

Pharmaceutical industries are bound to satisfy certain standards to claim it to be a quality drug. The main criteria for the quality of any drug in dosage form are its safety, potency, efficacy, stability, patient acceptability and regulatory compliance [20]. The quality of pharmaceutical products must be reliable and reproducible from batch to batch to ensure the safety and efficacy. It is required for drug manufacturers to test their products to ensure the requisite quality both during and after manufacturing at various intervals during the shelf-life of the product.

WHO supports the prescribing practice of generic medicines to minimize the expense of the health care system, but this should be carried out with sufficient and enough evidence for the replacement of one brand for another [21]. Generic drugs are not only minimizes the health care expenses [22] but also the quality of the drugs. Comparison of bioequivalence study between the generic products against the innovator product is

one of the main challenges and foremost factors for a generic marketing authorization [23]. It is very important to do bioequivalence studies for generic products on account of any significant difference in the rate and extent by which the therapeutic ingredients become available at the site of drug action, administered under identical conditions in an adequately designed study [24]. Dissolution test serves as an indicator to identify bioavailability problems [25]. Drug products which are biopharmaceutically as well as chemically equivalent must have the same quality, strength, purity, content uniformity, disintegration and dissolution rates [26]. In vitro quality control (QC) of pharmaceutical products is a fixed set of investigation started during production by in-process quality control tests and after production by finished product quality control tests according to official pharmacopoeias and different regulatory agencies. QC tests help to avoid the confusion regarding safety, potency, efficacy and stability of pharmaceuticals [27].

1.1. Stability of Drug

Stability is the ability of a drug substance or a drug product to remain stable within established or recognized specifications to make sure its identity, strength, quality and purity, all through its specified shelf lives [28].

In a rational design and evaluation of dosage forms for drugs, the stability of the active components is a major criterion to determine their suitability.

Several forms of instability can occur. Such as-

- **First,** the drug may be chemically degraded, that leads to significant reduction of the amount of the therapeutic agent in the particular dosage form. In case of drugs with narrow therapeutic index the patient needs to be carefully titrated as a result serum levels are not too high which are potentially toxic or too low that they are ineffective to give pharmacologic effect.
- **Second,** even though the degradation of the active drug may not be that extensive, a toxic degradant may be found in the decomposition process. For example, tetracycline, which is converted into more toxic compound, epianhydro tetracycline.
- **Third,** instability of a drug product can reduce its bioavailability, rather than to loss of drug or the formation of toxic degradation products. The reduction in bioavailability can hinder the therapeutic efficacy of the dosage form. This phenomenon can be caused by physical and chemical changes in the excipients in

the dosage form, which is not dependent of changes the active drug may have undergone.

- **Fourth,** there may be considerable changes in the physical state of the dosage forms since most drugs are organic molecules. The pathways of organic pharmaceutical products are must to be recognized. The major difference that has to be considered is that most pharmaceutical reactions occur in the presence of or influenced by water, oxygen, or light, rather than other active ingredients. And hence the most common routes of decomposition are: hydrolysis, oxidation, photolysis, racemization, and decarboxylation [29].

1.1.1. Stability Testing

The aim of stability testing is to endow with evidence or verification on how the quality of a drug substance or drug product varies with time due to diversity of environmental factors such as temperature, humidity, and light, and to establish a retest period for the drug substance or a shelf life for the drug product and recommended storage conditions [30].

1.1.2. Stress Testing or Forced Degradation Studies

Stress testing of the drug substance can recognize the possible degradation products and degradation pathways. Stability testing is necessary to analyze the inherent stability of the molecule and validate the stability representing power of the analytical procedures used. The nature or methodology of the stress testing will vary on each drug substance and the category of drug product involved. Stress testing is typically to be carried out on a single batch of the drug substance. It should include the effect of temperatures [in 10°C increments (e.g., 50°C, 60°C, etc.) above that for accelerated testing], humidity (e.g., 75% RH or greater) where appropriate, oxidation, and photolysis on the drug substance.

The stress testing is to be supposed to also evaluate the susceptibility of the drug substance to hydrolysis. It is done within a wide range of pH values either in solution or suspension. Photo stability testing should be done with high importance as it is considered to be an essential part of stress testing. The standard conditions or criteria for photo stability testing are described in ICH Q1B.

In stability study, degradation products are evaluated under various stress conditions is useful to establish degradation pathways and developing and validating appropriate analytical procedures. However, if any degradation product has been demonstrated that they not formed under accelerated or long term storage conditions then it is not necessary to evaluate that certain product specifically. Results from these studies will outline a vital part of the information provided to regulatory authorities [30].

1.1.2.1. Reasons for Conducting Forced Degradation Studies

Forced degradation studies are carried out for the following reasons:

- To develop and validate stability indicating methodology;
- To determine the intrinsic stability of a drug molecule, and to elucidate the structure elucidation of degradation products;
- To determine the degradation pathways of drug substances and products;
- To differentiate the drug and non drug related degradation products in the formulations.

1.1.2.2. FDA Recommended Degradation Studies for a Drug Substance

The following are FDA recommended degradation studies for a drug substance (FDA 1998):

- Stressing the drug substance in solution and suspension form at acidic and alkaline pH medium and under high oxygen environment;
- Stressing the solid drug at temperature and humidity conditions in excess to accelerated conditions;
- Stressing the drug under photolytic condition in the solid state and solution;
- Manifestation of the stability indicating methods with forced degraded / spiked samples;
- Separation or complete depiction of degradation products (by NMR, MS, UV etc);
- Determination of the mechanism and kinetics of the degradation products if possible.

Thus, for degradation study of a drug substance, it should be exposed to acid /base, oxidative, exposure to light, thermal and humidity.

1.1.2.3. Regulatory Considerations

In accordance to the International Conference on Harmonization (ICH) guidelines impurities in pharmaceuticals can be defined as components that remain with the active pharmaceutical ingredients, or arise during the manufacturing process and/or storage of the drug substance [31]. The performance of the pharmaceutical products may be influenced by the presence of these impurities, even in small amounts. The ICH and FDA have published guidelines for the identification and qualification of impurities in new drug substances and drug products [32-33]. According to the guidelines, impurities can be characterized as organic or inorganic impurities and residual solvents. Organic impurities may include impurities in starting synthesis materials, synthesis by-products, degradation products and intermediates. For degradation products, the ICH Guidance Q3B (R2) provides recommendations for reporting, control, identification and qualification in drug products [32]. The critical values for reporting, identifying and qualifying impurities vary based on drug dosing regimens, and are shown in **Table 1.1** [32].

Table 1.1- Thresholds for Degradation Products in New Drug Products

Reporting Thresholds	
Maximum daily dose	Threshold
≤ 1 g	0.1%
> 1 g	0.05%
Identification Thresholds	
≤ 1 g	0.1%
> 1 g	0.05%
Identification Thresholds	
< 1 mg	1.0% or 5 µg TDI, whichever is lower
1 mg - 10 mg	0.5% or 20 µg TDI, whichever is lower
>10 mg - 2 g	0.2% or 2 mg TDI, whichever is lower
>2 g	0.10%

TDI: Total daily intake of the degradation product

The critical value for *reporting* impurities ranges from 0.05% to 0.1%, and reporting an impurity may or may not require identification.

Identification is required for any degradation product observed in stability studies present at a level greater than the identification threshold. Identification requires assignment of a specific chemical composition of the impurity. The critical value for identification is typically between 0.1% and 0.5% depending on the daily drug dose. For

low dose drugs (< 1mg per day), the identification threshold is 1% of the total daily intake (TDI) or 5 µg (whichever is lower).

Qualification is the process of evaluating safety data and establishing acceptance criteria for a degradation product. If any degradation product exceeds the limit of quantification threshold then it must be qualified. Depending on the maximum daily dose, the critical range of the qualification threshold ranges from 0.15% to 1%.

For a given degradation product, its acceptance criteria should be established no higher than its qualified level and along with safety considerations [33]. Sometimes the qualification thresholds are exceeded and adequate data are not available to qualify the degradation product. In this case, additional studies should be conducted on the drug product containing the degradant or isolated degradation products.

The guidelines of FDA and ICH provide a feasible way to control degradation products of drug. However, degradation products that exceed qualification thresholds or that are potentially toxic compounds are not under this guidance as they do not provide a rationale for them.

1.1.2.4. Purposes of Forced Degradation Studies

Forced degradation studies are carried out to achieve the following purposes:

- Establishment of the degradation pathways of drug substances and drug products.
- Differentiation of the degradation products that are related to drug products from those that are generated from non-drug product in a formulation.
- Elucidation of the structure of degradation products.
- Determination of the intrinsic stability of a drug substance in formulation.
- Depiction of the degradation mechanisms such as hydrolysis, oxidation, thermolysis or photolysis of the drug substance and drug product [30, 34].
- Establishment of stability indicating nature of a developed method.
- Evaluation of the chemical properties of drug molecules.
- Generation of stable formulations.
- Production of a degradation profile similar to that of what would be observed in a formal stability study under ICH conditions.

- Rectify the stability-related anomalies [35].

1.1.2.5. Forced Degradation Testing Time

Before stress testing, it is very important to know the appropriate time to carry out stress studies to develop new drug substance and new drug product. FDA guidance states that force degradation testing should be performed during phase III of regulatory submission process. Force degradation studies should be done using solutions of different pH, in presence of oxygen and light, and at increased temperatures and humidity levels so that the stability of the drug substance can be determined. Generally, force degradation studies are carried out on a single batch. The results are to be summarized and submitted in an annual report [36]. Starting force degradation would be very effective if it is done during early in preclinical phase or phase I of clinical trials. These tests are performed on drug substance in order to attain enough time to identify products formed after degradation and elucidate structures and also to optimize the stress conditions. An early stress study also provides appropriate recommendations for improvisation of manufacturing process and selecting suitable stability-indicating analytical procedures [37].

1.1.2.6. Limits of Forced Degradation Testing

Many discussions among pharmaceutical scientists have already been held about the question of how much degradation is sufficient. Degradations of drug substances of 5-20% are considered acceptable and reasonable in case of chromatographic assays validation [38, 39]. Some pharmaceutical scientists suggest that degradation of 10% can be favorable to use in analytical validation for small pharmaceutical molecules. So acceptable stability limits of 90% of label claim is frequent [28]. Other suggestion is like that the drug substance spiked with a combination of known degradation products can be used to challenge the methods engaged to monitor in stability of drug product [34]. No such limits for physiochemical changes, loss of activity or degradation during shelf life have been established for individual types or groups of biological products [40]. It is not always mandatory that forced degradation study would result in a degradation product. The study can be concluded if no degradation is observed after drug substance or drug product has been exposed to stress conditions than those conditions mentioned in an accelerated stability protocol [41]. Over-stressing a sample is not recommended

as this may cause the formation of a secondary degradation product that is not to be seen in formal shelf-life stability studies. On the other hand, under-stressing may not generate sufficient degradation products which can fail the whole stress study [42]. Protocols for generation of product related degradation may be different for each drug substance and drug product because of their differences in matrices and concentrations. It is recommended that maximum of 14 days for stress testing in solution (a maximum of 24h for oxidative tests) to provide stressed samples for methods development [43].

1.1.2.7. Approach for Forced Degradation Conditions Selection

Forced degradation is conducted to make representative samples for the development of stability-indicating methods for drug substances and drug products. The options of stress conditions are supposed to be consistent with the products breakdown under normal manufacturing process, storage, and use conditions which are specific in each individual case [44]. A common procedure of degradation conditions used for drug substance and drug product is shown in **Figure 1.1**. To conduct force degradation studies successfully a list of stress factors are recommended to take account like acid and base hydrolysis, thermal degradation, photolysis, oxidation [36,45-47] and may include freeze–thaw cycles and shear stress conditions [40]. The conditions of pH, temperature and specific oxidizing agents to be used are not specified in the guidelines. The protocol of photolytic degradation studies is left to the applicant's judgment even though it is specified in ICH Q1B that the light source is supposed to produce combination of visible and ultraviolet (320–400 nm) outputs, and exposure time should be reasonable [41]. The initial trial should be aimed to degrade the drug by approximately 10%. It is found to be practical to start with extreme conditions such as 80◦C or even higher temperatures and testing at shorter (2, 5, 8, 24h, etc.) multiple time points, so that the rate of degradation can be evaluated [48]. The primary and secondary degradants can be illustrated by testing at initial stage. Thus improved degradation pathway can be established. In another approach, the drug substance is considered labile when degradation is started. Then stress would be augmented or lessened to obtain enough degradation. As compared to more stressful environment and less time approach, this tactic is better because of some reasons. (i) If there is any modification in the mechanism of reaction during a insensitive condition , and (ii) If

there is any practical difficulty in neutralizing or diluting every sample, when it is associated with a high concentration of reactants, e.g., acid or base, before an injection can be made on the HPLC column. Both these reasons suggest as normal as possible conditions to carry out the degradation of the drug [49]. Studies should be repeated when formulations or methods change because the change may lead to the production of new degradation products.

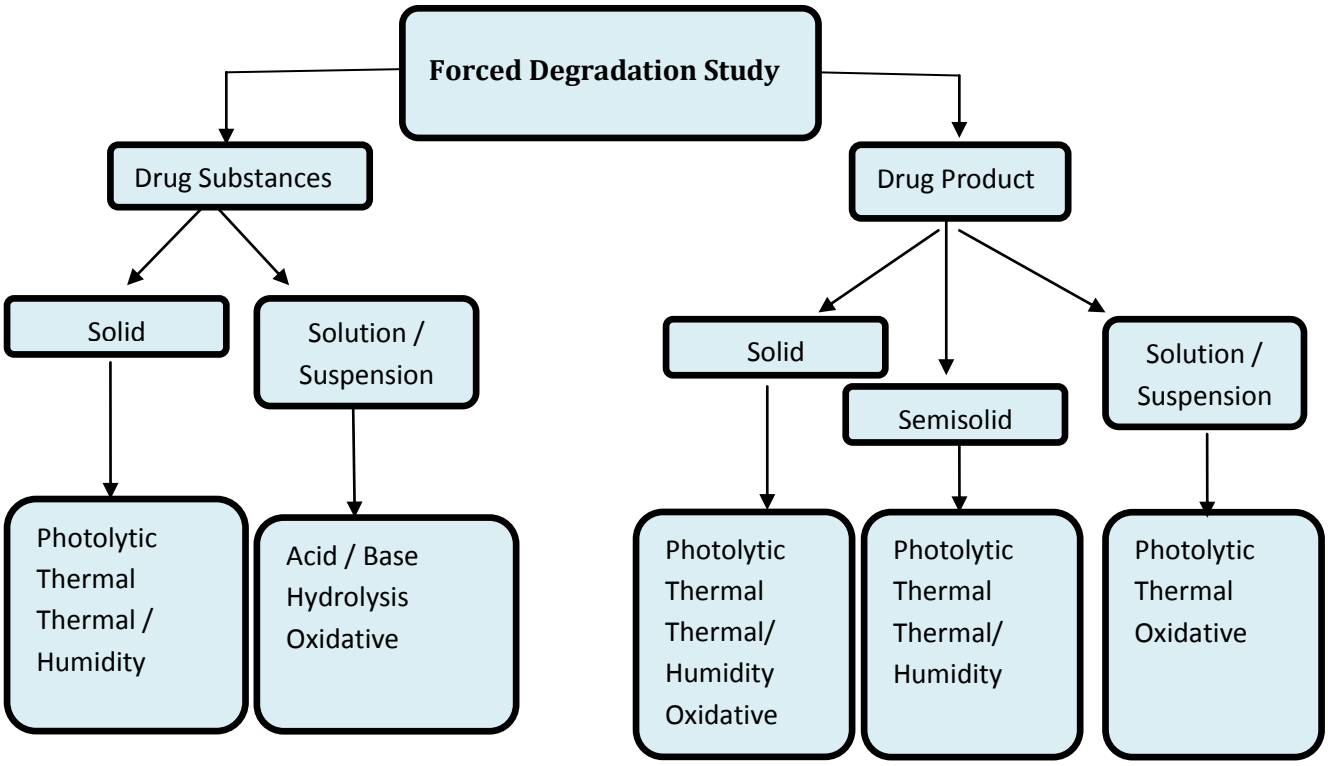


Figure1.1. Diagram of Stress Study Used for Degradation of Drug Substance and Drug Product. [50]

1.1.2.8. Conditions for Forced Degradation Study

Different conditions to conduct forced degradation studies according to ICH Q3B (R2) are discussed below.

1.1.2.8.1. Hydrolytic Conditions

Hydrolysis is one of the most common degradation chemical reactions over different pH range. Hydrolysis which is a chemical process includes decomposition of a chemical compound by reaction with water. Acidic or basic hydrolysis study involves catalysis of

ionizable functional groups that are present in the molecule. In forced degradation study, when drug substance is exposed to acidic or basic conditions, primary degradants are found in desirable range. The selection of the type and concentrations of acid or base is dependent on the stability of the drug substance. Hydrochloric acid or sulfuric acid (0.1–1M) for acid hydrolysis and sodium hydroxide or potassium hydroxide (0.1–1M) for base hydrolysis are suggested as suitable reagents for hydrolysis [51,52]. Co-solvents can be used to dissolve the drugs in HCl or NaOH if the compounds for stress testing are poorly soluble in water. Drug substance structure is used as the basis for selection of co-solvent. Stress testing assessment is usually started at room temperature and if there is no degradation, an increased temperature (50–70◦C) is applied. Stress testing should be completed within 7days. The degraded sample is then neutralized by means of appropriate acid, base or buffer to circumvent added decomposition.

1.1.2.8.2. Oxidation Conditions

Though other oxidizing agents such as metal ions, oxygen and radical initiators can also be used, hydrogen peroxide is widely used for oxidation of drug substances in forced degradation studies. Selection of an oxidizing agent, its concentration, and conditions are dependent on the drug substance. It is reported that subjecting the solutions to 0.1–3% hydrogen peroxide at neutral pH and room temperature for seven days or up to a maximum 20% degradation could potentially generate relevant degradation products [52]. An electron transfer mechanism is involved in the oxidative degradation of drug substance to form reactive anions and cations. Amines, sulfides and phenols are susceptible to electron transfer oxidation to give N-oxides, hydroxylamine, sulfones and sulfoxide [53]. The functional group with labile hydrogen like benzylic carbon, allylic carbon and tertiary carbon or α-positions with respect to hetero atom is susceptible to oxidation to form hydroperoxides, hydroxide or ketone [54, 55].

1.1.2.8.3. Photolytic Conditions

The photo stability testing of drug substances must be evaluated to demonstrate that a light exposure does not result in unacceptable change. Photo stability studies are performed by exposure to UV or fluorescent conditions to generate primary degradants of drug substance. ICH guidelines recommend some conditions for photo stability testing [41]. Samples of drug substance and solid or liquid drug product must be out in

the open to a minimum of 1.2 million lxh and 200 Wh/m^2 light. The most commonly accepted wavelength of light is in the range of 300 – 800 nm to cause the photolytic degradation [56,57]. The highest illumination suggested is 6 million lxh [55]. Light stress conditions can persuade photo oxidation through free radical reaction mechanism. Functional groups like carbonyls, nitro-aromatic, N-oxide, alkenes, aryl chlorides, weak C–H and O–H bonds, sulfides and polyenes are expected to initiate photosensitivity of drugs [58].

1.1.2.8.4. Thermal Conditions

Thermal degradation (e.g. dry heat and wet heat) should be carried out at more strenuous conditions than recommended ICHQ1A accelerated testing conditions. Solid-state drug substances samples and drug products are to be exposed to dry heat, moist heat and liquid drug products are to be exposed to dry heat. Studies may be conducted at higher temperatures for a shorter period [52]. Effect of temperature on thermal degradation of a substance is studied through the Arrhenius equation [55, 59, 60]. Thermal degradation study is carried out at 40–105◦C.

1.2. Stability Indicating Method (SIM)

A stability indicating method (SIM) is an analytical process which is used to enumerate the decline in the sum of the active pharmaceutical ingredient (API) in drug product due to degradation. According to an FDA guidance document, a stability-indicating method is a validated quantitative analytical process used to identify the stability of the drug substances and drug products changes with time. A stability-indicating method measures the changes in active ingredients concentration avoiding intrusion from other degradation products, impurities and excipients precisely [45]. Force degradation is carried out to demonstrate specificity of the developed method to measure the changes in concentration of drug substance when little information is available about potential degradation product. The development of a suitable stability indicating method provides a background for the pre-formulation studies, stability studies and the development of proper storage requirements. Bakshi and Singh [49] discussed some critical issues about developing stability indicating methods. Comments and suggestions on stability indicating assays are also made by Dolan [61]. Smela [62] discussed from a regulatory point of view about stability indicating analytical methods. The RP-HPLC is a

most widely used analytical tool for separation and quantifying the impurities and it is most frequently coupled with a UV detector [59]. The following are the steps involved for development of SIM on HPLC which meets the regulatory requirements.

1.2.1. Sample Generation

The API is tested stressfully at conditions which are more acute than accelerated degradation conditions to generate samples for SIM. Decomposition of drug at hydrolytic, oxidative, photolytic and thermal conditions are involved as discussed earlier. The stress study of API both in solid and solution form is performed with an aim to produce degradation products which are likely to be formed in realistic storage conditions [63]. This sample is then used to develop a stability indicating method.

1.2.2. Method Development

For method development, various physiochemical properties like pKa value, log P, solubility and absorption maximum of the drug must be recognized, as it lays a foundation for HPLC method development. Solubility and logP aids in selecting mobile phase and sample solvent and pKa value aids in determining the pH of the mobile phase [49]. Reverse phase column is better option to begin the separation of sample components because the degradation is carried out in aqueous solution. Methanol, water and acetonitrile can be used in mobile phase in various amounts at the initial stages of separation. Solubility of analyte is the key factor to select between methanol and acetonitrile for organic phase. Initially the water and organic phase ratio starts with 50:50 and suitable modifications is done as trials proceed to obtain a good separation of peaks. Buffer is also added to get better peak separation and peak symmetry if necessary. Sometimes the method is extended to liquid chromatography–mass spectrometry (LC–MS). In this case mobile phase buffer should be MS compatible like triflouro acetic acid and ammonium formate. The selectivity of the method is affected by variation in column temperature as analytes respond differently to temperature changes. 30–40◦C temperature range is optimum to generate good reproducibility [64]. Pushing the drug peak further in chromatogram results in separation of all degradation products. Also a sufficient run time after the drug peak is to be allowed to obtain the degradants peak eluting after the drug peak [49]. It can be possible that the drug peak may hide an impurity or degradants peak. These types of

peaks may co-elute with the drug during the method development. This leads to the requirement of peak purity analysis to determine the method specificity. Direct analysis can be done online by using photodiode array (PDA) detector. PDA gives information of the homogeneity about the spectral peak. But it is not appropriate for the degradants having the similar ultraviolet spectrum to the drug. Indirect method involves change in the chromatographic conditions like mobile phase ratio and column which will affect the peak separation. The spectrum of altered chromatographic condition is then compared with the original spectra. If the degradant peaks and percentage of the drug peak remain same, then it can be confirmed that the drug peak is homogeneous [65]. The degradant that co-elutes with the drug would be acceptable if it is not found during accelerated and long term storage conditions [30]. The method is then optimized for separating closely eluting peaks by changing flow rate, injection volume, column type and mobile phase ratio.

1.3. Quality by Design (Qbd) Approach in Method Development and Optimization:

Joseph M. Juran was first defined the term Quality by Design (QbD) [66] and applied with great success. The underlying concept of QbD is that quality must be designed in to a product through the systematic implementation of an approach to establish a absolute understanding of the product and the processes utilized to develop and manufacture it. To improve quality control strategies are developed and used to verify continuously. Recently the FDA has begun to approve the QbD approach for the pharmaceutical sector [67]. But there are a lots of claim of the concept. For example the requirement for modeling the influence of variables on quality, methodical experimental design strategies and to make sure the traceability of information from the stages of design during validation.

Now a days analytical chemists have started to apply QbD approaches for chromatographic methods development, prompting a revisit of method development strategies. Modern technology allows to investigate the strategies for chromatographers to develop new methods and optimization by applying Quality by Design.

Pharmaceutical industry has paying attention on product quality, safety, and efficacy. By applying scientific tools, QbD (Quality by Design) product quality has been improved.

These QbD tools will minimize the threat by increasing the quality and productivity. The implementation of ICH quality guidelines Q8 to Q11 are always recommended by regulatory authorities [68-70].

Analytical Quality by Design (AQbD)

According to ICH, is defined as "A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management."

The outcome of AQbD is well establised and suitable for intended purpose with robustness throughout the lifecycle. Different tools of AQbD life cycle are ATP (Analytical Target Profile), CQA(Critical Quality Attributes), risk assessment, method optimization and development with DoE (design of experiment), MODR (method operable design region), control strategy and risk assessment, AQbD method validation, and continuous method monitoring. **Figure 1.2** represents the AQbD life cycle with each tool.

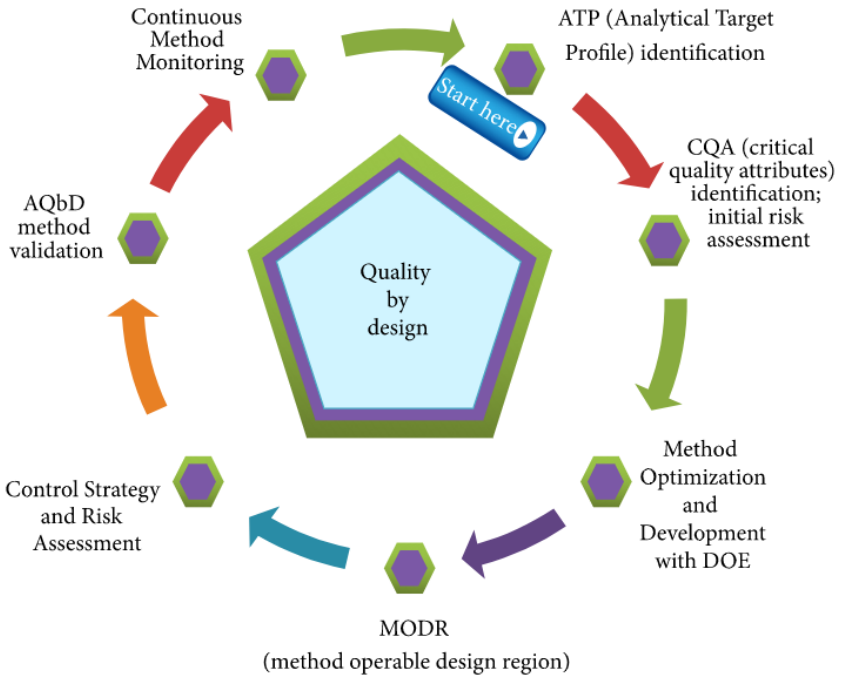


Figure1.2. Lifecycle of Qbd [71]

1.3.1. Analytical Target Profile (ATP)

General ATP (**Table 1.2**) for analytical procedures is as follows:

(a) Selection of target analytes (API and impurities),

(b) Selection of technique to be used (HPLC, GC, HPTLC, ion chromatography, chiral HPLC)

(c) Selection of method requirements (assay or impurity profile or residual solvents).

(a) Target Analytes Selection.

Common ATP for HPLC methods during impurities profile are described in **Table 1.2**.

Table1.2. Common ATPs for Impurity Profile by HPLC Method

Sl#	Method requirements for impurity profile
1	Number of analytes (API and impurities)
2	Separation of all analytes
3	Mobile Phase (buffer and organic modifier)
4	Elution method (gradient or isocratic)
5	Sample concentration
6	Sample diluents
7	Sample solution stability
8	Sample preparation process (dilution process and sonication time, etc.)
9	Filter or centrifuge
10	Impurity specification limits
11	Column type (stationary phase and dimensions)
12	Detection (UV/RID/ELSD)
13	Flow rate
14	Injection volume
15	Column oven temperature
16	Run time
17	System suitability parameters selection with limits
18	LOD and LOQ concentrations establishment
19	Impurities/ Degradants calculation method
20	Recovery establishment

(b) Technique Selection.

Every analytical technique has its own principle. So based on the analytes characteristics it can be chosen. Analytical test items and analytical techniques are the following:

(1) Identification by IR: FT-IR spectrophotometer,

(2) Impurity profile (Chromophore): HPLC with UV detector,

(3) Impurity profile (non-Chromophore): HPLC with RID/ELSD and so forth,

(4) Assay by HPLC (Chromophore): HPLC with UV detector,

(5) Assay by HPLC (non-Chromophore): HPLC with RID/ELSD and so forth.

(c) Method Requirements Selection. Method requirements can vary from one method to another. The common ATPs for impurity profile by HPLC method are listed in **Table 1.6**.

1.3.2. Critical Quality Attributes (CQA)

Critical quality attributes for analytical methods describes method attributes and parameters. Different CQA is required for each analytical technique.

For HPLC analysis CQA are

1. Buffer of mobile phase
2. pH of mobile phase
3. Diluents
4. Column selection
5. Organic modifier and
6. Elution method

For GC analysis CQA are

1. Gas flow
2. Oven temperature and program
3. Injection temperature
4. Sample diluents and
5. Concentration

For HPTLC method CQA are

1. TLC plate
2. Mobile phase

3. Injection concentration and volume
4. Plate development time
5. Color development reagent and
6. Detection method

The CQA for analytical method development based on the nature of impurities and DS can such as solubility, pH value, polarity, charged functional groups, boiling point, and solution stability.

1.3.3. Risk Assessment.

Risk Assessment is a science-based process can be performed from initial stage of method development to continuous method monitoring. AQbD come up to involve the risk detection at early stages of progress followed by appropriate improvement plans with control strategies that will be recognized. Ishikawa fishbone diagram is generally used for risk identification and assessment. The following **Figure1.3** shows fishbone risk identification approach for typical analytical test procedure according Raman et al [71].

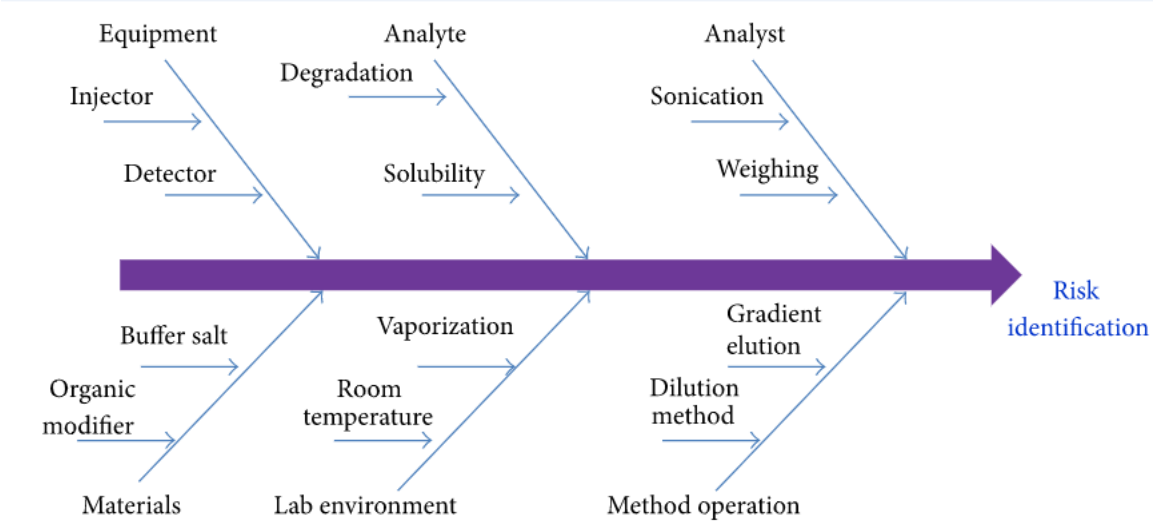


Figure1.3. Ishikawa Fishbone Diagram for Risk Identification

1.3.4. Design of Experiment (DoE) for Method Optimization and Development

Sometimes the potential and critical analytical method variables are defined with initial risk assessment. At that time, DoE is performed to verify and purify critical method

variables. And this process is based on statistical significance. It is determined as per unit operation or combination of selected multiple method variables and their interactions and responses (critical method attributes). This tactic provides a brilliant prospect to screen a number of conditions generated from a limited number of experiments. Data evaluations are very vital to determine critical method variables and this is done by using statistical tools. The proper optimal ranges for method variables where a robust region for the critical method attributes could be obtained.

According to ICH Q8 guideline, process robustness is defined as "the ability of a process to tolerate variability of materials and changes of the process and equipment without negative impact on quality." Starting materials properties can affect the drug substance synthetic process robustness, impurity profile, physicochemical properties, process capability, and stability. Process understanding endow with the adequate information to establish robustness parameters after evaluation of different operating conditions, difference scales, and different equipments.

1.3.5. Method Operable Design Region (MODR)

Method operable design region (MODR) is used for set up of a multidimensional space based on method factors and settings. MODR can present appropriate method act. It is also used to establish significant method controls like system suitability, relative retention time (RRT), and relative response factor (RRF). Additional method verification exercises are applied to establish ATP conformance and ultimately define the MODR.

1.3.6. Control Strategy and Risk Assessment

Control strategy [72-73] is a planned set of controls, derived from analyte nature and MODR understanding. Complete statistical data is used to establish method control strategy which is collected during the DoE and MODR stages discussed earlier. Correlations can be drawn between method and analyte attributes using this statistical experimental data for the ability to meet ATP criteria. Control strategy will decide the method parameters irregularity for example reagent grade, instrument brand or type, and type of column. Method control strategy is not appeared as noticeably dissimilar under the AQbD approach while compared to the traditional or conventional approach. However, method controls are established based on CQA, DoE. MODR experimental data is used to make sure a stronger connection between the method purpose and performance.

1.3.7. Analytical Quality by Design (Aqbd) Method Validation

AQbD [74] method validation approach is the validation of analytical method over a range of different API batches. It uses both DoE and MODR knowledge for designing method validation for all kinds of API manufacturing changes without revalidation. This approach is very important as it provides the required ICH validation elements and also information on interactions, measurement uncertainty, control strategy, and continuous improvement. It requires less resource than the traditional validation approach without any compromising quality.

1.3.8. Continuous Method Monitoring (CMM) and Continual Improvement

Life cycle management is a control strategy which is applied to implement the design space in commercial stage. CMM is final step in AQbD life cycle. It is a continuous process of sharing knowledge gained during development and implementation of design space including results of risk assessments, assumptions based on prior knowledge or information, statistical design considerations, and bridge between the design space, MODR, control strategy, CQA, and ATP. After establishment of the method validation procedure, it can be used for routine purpose and continuous method performance can be monitored investigations, and so forth. CMM allows the analyst to proactively recognize and deal with any out-of-trend performance.

AQbD is an approach that is concerned with moving away from reactive trouble shooting to proactive failure diminution. The stage of project in the development timeline is very important as the type and extent of the risk assessment depends on it. AQbD success rate depends on many variables such as exact approach, planning, tools usage, and performance of work in a appropriate time. The appropriate risk assessment tools at the right time prevent method failures and better understanding on the design space and control strategy [75-84].

1.4. Method Validation

Analytical method validation is the process which is established by laboratory studies. It fulfills the effectiveness of the method to acquire the official requirements for the intended analytical application. Validation is required for any new or modified method

to confirm that it is capable of providing precise, reproducible and robust results with a variation of equipment, operators in the same or different laboratories. The method validation procedure for analysis starts with the designed and organized approach by the applicant of the validation data to maintain analytical procedures [85]. The obtained results from method validation can be used to evaluate the quality, acceptability, reliability and stability of analytical results. The analytical methods validation is conducted as per ICH guidelines.

Validation or revalidation of analytical methods is required [86]

- Before their introduction into routine use
- Whenever any conditions are changes
- Whenever the method is changed
-

1.4.1. Parameters for Method Validation

Typical parameters recommended by FDA, USP, and ICH are as follow [86, 88]

- Specificity
- Linearity & Range
- Precision
- Method precision (Repeatability)
- Intermediate precision (Reproducibility)
- Accuracy (Recovery)
- Solution stability
- Limit of Detection (LOD)
- Limit of Quantification (LOQ)
- Robustness
- System suitability
- Forced degradation studies

1.4.2. Components Required for Validation

The common compendial requirements for the establishment of analytical methods for finished products are categorized in following ways (**Table 1.3**):

Category 1: Identification and Qualification of main components or active ingredients in its finished pharmaceutical products.

Category 2: Determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products. It includes the quantitative assay and limit tests.

Category 3: Determination of performance characteristics

Category 4: Identification tests.

Table 1.3. Parameters to be Covered in Validation

Test	Cat.1	Cat.2		Cat.3	Cat.4
		Quantitative	Limit test		
LOD	NR	NR	R	NR	NR
LOQ	NR	R	NR	*	NR
Linearity	R	R	NR	*	NR
Range	R	R	*	*	NR
Specificity	R	R	R	*	R
Precision	R	R	NR	R	NR
Accuracy	R	R	*	*	NR

R: Required

NR: Not required

*: May be required depending on the nature of the specific test

1.4.2.1. Specificity: Specificity or selectivity of an analytical method as its capacity to determine any interference with the analyte accurately, such as excipients, synthetic precursors, enantiomers, and degradation products that may be anticipated to be present in the sample matrix [87].

1.4.2.2. Linearity and Range: The linearity of an analytical method validation is its aptitude to get result which is directly proportional to the concentration (amount) of analyte in the sample. A linear relationship should be assessed across the range of the analytical procedure. It is described directly on the drug substance by diluting a

standard stock solution of the drug product with proposed method. Linearity is usually articulated as the confidence limit about the slope of the regression line [86-88]. For determining the linearity, minimum of five concentrations are recommended according to ICH guideline [89]. The range of an analytical method is the difference between the upper levels and lower levels which have been confirmed to be determined with linearity, precision and accuracy using the method [87].

1.4.2.3. Precision: The precision of an analytical procedure expresses the closeness of agreement or the degree of scatter between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility [89].The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation of series of measurements. Precision may be defined as either the degree of reproducibility or the repeatability of the analytical procedure under normal conditions. Intermediate precision (also known as ruggedness) expresses within laboratories variations, as on different days, or with different analysts or equipment within same laboratory. Precision of an analytical procedure is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation.

1.4.2.4. Accuracy (Recovery): The accuracy of an analytical procedure is defined as the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. lt is determined by applying the method to samples to which known amounts of analyte have been added. These should be analyzed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. It may often be expressed as the recovery by the assay of known, added amounts of analyte [88,89].

1.4.2.5. Limit of Detection (LOD): Limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected but may not be quantitated as an exact value of an individual procedure. In analytical procedures that exhibit baseline noise, the LOD

can be based on a signal-to-noise (S/N) ratio (3:1), which is usually expressed as the concentration of analyte in the sample.

1.4.2.6. Limit of Quantification (LOQ): The limit of Quantitation (LOQ) or Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. For analytical procedures such as HPLC that exhibit baseline noise, the LOQ is generally estimated from a determination of S/N ratio (10:1) and is usually confirmed by injecting standards which give this S/N ratio and have an acceptable percent relative standard deviation as well [89].

1.4.2.7. Robustness: is defined as the measure of the ability of an analytical method to remain unaffected by small but deliberate variations in method parameters (e.g. pH, mobile phase composition, temperature and instrumental settings) and provides an indication of its reliability during normal usage. Determination of robustness is a systematic process of varying a parameter and measuring the effect on the method by monitoring system suitability and/or the analysis of samples [88, 89].

1.4.2.8. System suitability: System suitability tests are an integral part of liquid chromatographic methods. They are used to verify that the detection sensitivity, resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Factors, such as the peak resolution, number of theoretical plates, peak tailing and capacity have been measured to determine the suitability of the used method [86-89]. The acceptance criteria of system suitability parameters are described in **Table 1.4**.

Table-1.4. Acceptance Criteria (Limits) of System Suitability Parameters

Sl#	Parameter name	Acceptance criteria
1	Number of theoretical plates or Efficiency (N)	> 2000
2	Capacity factor (K)	< 1
3	Separation or Relative retention (α)	> 1
4	Resolution (Rs)	> 1.5
5	Tailing factor or Asymmetry(T)	< 2
6	Relative Standard Deviation (RSD)	< 2

1.5. Drug Selection:

Diabetes is one of the major public health problem, one of four priority non-communicable diseases (NCDs) targeted for action over the world. It is a serious, chronic disease that occurs either when enough insulin is not produced from pancreas, or when the body cannot effectively use the insulin it produces. Both the number of cases and the prevalence of diabetes have been increasing over the past few decades. Globally, it was reported that 422 million adults were living with diabetes in 2014, compared to 108 million in 1980. Since 1980, the global prevalence of diabetes has nearly doubled rising from 4.7% to 8.5% in the adult population [90]. This reflects an increase in associated risk factors like being overweight or obese.

Diabetes prevalence has been rising faster in low- and middle-income countries than in high-income countries over the past decade. It was reported that diabetes caused 1.5 million deaths in 2012. An additional 2.2 million deaths were caused by higher-than-optimal blood glucose, by increasing the risks of cardiovascular and other diseases. 43% of these 3.7 million deaths occur before the 70 years of age [90].

The percentage of deaths caused by high blood glucose or diabetes that occurs prior to age 70 is higher in low- and middle-income countries than in high-income countries. Because sophisticated laboratory tests are usually required to differentiate between type 1 and type 2 diabetes, separate global estimates of diabetes prevalence for type 1 and type 2 do not exist. The majority of people suffering from diabetes are affected by type 2 diabetes. Earlier this used to occur nearly entirely among adults, but now occurs in children too[91] .

Type 2 diabetes mellitus (T2DM) is characterized by both progressive beta cell dysfunction and insulin resistance. To treat irregular glucose metabolism focuses on expanding the insulin response to hyperglycemia, improving insulin sensitivity or altering glucose removal through the gut or urine. Dipeptidyl-peptidase-IV (DPP-IV) inhibitors or ‘gliptins’ that block the inactivation of glucagon-like peptide-1 (GLP-1), which stimulates glucose-dependent insulin secretion and inhibits glucagon secretion. Morever, satiety is improved, gastric emptying is slowed, and food intake is reduced [92]. With use of GLP-1 receptor agonists these effects are more prominent.

There are five DPP-IV inhibitors, including alogliptin, linagliptin, saxagliptin, and sitagliptin in the United States and Europe and vildagliptin which is only available in Europe (**Table 1.5**). This class of therapy is administered once per day orally with the exception of vildagliptin which is administered twice per day. DPP-IV inhibitors can be taken without regard to food. DPP-IV inhibitors are not recommended for use as initial mono therapy for Type-2 Diabetes Mellitus (T2DM) treatment [93].

These are most frequently prescribed in combination with lifestyle alteration and metformin, sulfonylureas, thiazolidinediones, and/or basal insulin, but selected patients intolerant to metformin have been effectively treated with DPP-IV inhibitor monotherapy. There are a number of combination products available, together with gliptin–metformin and gliptin–sodium glucose transporter-2 inhibitor products [94].

Table 1.5. Comparison of Available DPP-IV Inhibitors Used in T2DM.

Sl #	Drug	Approval	Brand name®	Dosage	Dose change in renal dysfunction	Dose change in hepatic dysfunction	Available in combination
1	Sitagliptin	FDA approved Oct 2006	Januvia, (Merck)	25 mg 50 mg 100 mg	Yes	No	Metformin Simvastatin
2	Vildagliptin	EU approved 2008	Galvus (Novartis)	50 mg	Yes	Not recommended for use	Metformin
3	Saxagliptin	FDA approved July 2009	Onglyza(AstraZeneca)	2.5 mg 5 mg	Yes	No	Metformin
4	Linagliptin	FDA approved May 2011	Tradjenta (Boehringer Ingelheim)	5 mg	No	No	Metformin Empagliflozin
5	Alogliptin	FDA approved 2013	Nesina (Takeda Pharma Ltd.)	6.25 mg 12.5 mg 25 mg	Yes	No	Metformin Pioglitazone

There is very minor risk of hypoglycemia which can be negligible when DPP-IV inhibitors are used as monotherapy or in combination with metformin [95]. Hypoglycemia risk is augmented when gliptins are used in combination with sulfonylureas or insulin. Interestingly, in a study of vildagliptin added to insulin therapy,

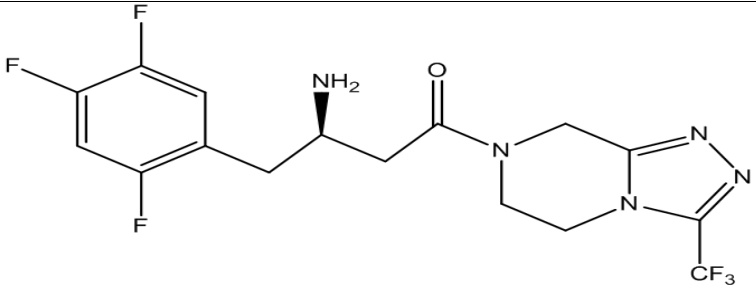
in the setting of superior glycemic enhancement drastically lower rates of hypoglycemia were experienced in patients treated with vildagliptin compared to those receiving placebo [96]. Weight gain is usually neutral across the DPP-IV inhibitor class [34]. There appears to be neutral effect on lipids, with a general trend toward better triglyceride levels. Systolic blood pressure decrease is very modest and comparable within the class [97]. The gliptins are considered as safe in renal dysfunction; however, alogliptin, saxagliptin, and sitagliptin have requirement of dose adjustment for renal impairment [96, 98]. Linagliptin and saxagliptin do not need dose adjustment for liver dysfunction. Alogliptin and sitagliptin also do not need dose adjustment for mild or moderate liver dysfunction but should be administered with caution in severe liver impairment.

Day by day the requirements of DPP-IV inhibitors are increased for the treatment of T2DM. That's why three DPP-IV inhibitors, Sitagliptin, Vildagliptin and Linagliptin were chosen for the study.

1.5.1. Profile of Sitagliptin

Sitagliptin is a medication which is prescribed for the treatment of T2DM. It is combined with exercise and diet to improve blood glucose levels in individuals suffering from type 2 diabetes. It is used as an anti-diabetic drug which is a new oral hypoglycemic, the novel dipeptidyl peptidase-IV (DPP-IV) inhibitor class of drugs. This enzyme-inhibiting drug can be administered either as monotherapy or in combination with metformin or a thiazolidinedione for control of T2DM. The mechanism of drug is to competitively inhibit a protein/enzyme, dipeptidyl peptidase-IV (DPP-IV), that increases active incretins level (GLP-1 and GIP), reduces amount glucagon release and increases insulin level. Different characteristics of sitagliptin are described in **Table 1.6**.

Table 1.6. Features of Sitagliptin [99]

Identification	
Chemical Formula	$C_{16}H_{15}F_6N_5O$
Structure	

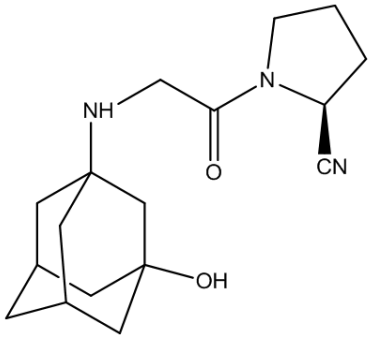
Molecular Weight	Average: 407.3136 Monoisotopic: 407.118079357
IUPAC Name	3R)-3-amino-1-[3-(trifluoromethyl)-5H,6H,7H,8H-[1,2,4]triazolo[4,3-a]pyrazin-7-yl]-4-(2,4,5-trifluorophenyl)butan-1-one
Pharmacology	
Indication	As an additional to diet and exercise to develop glycemic control in patients who are suffering from type 2 diabetes mellitus. Also use as combination with metformin or a PPAR γ agonist (e.g., thiazolidinediones) when the single agent alone, with diet and exercise, does not provide enough glycemic control.
Structured Indications	Type 2 Diabetes Mellitus (T2DM)
Pharmacodynamics	Sitagliptin belongs to the new dipeptidyl peptidase-IV (DPP-IV) inhibitor class of oral drugs. The advantage of this medicine is its lesser side-effects of hypoglycemia in the control of blood glucose values. The drug works to abolish the effects of a protein/enzyme by the inhibiting them on the pancreas and hence diminishes glucagon release and increases insulin synthesis and release until blood glucose levels are restored toward normal, in which case the protein/enzyme-enzyme inhibitor is less efficient and the amounts of insulin released abolishes thus diminishing the "overshoot" of hypoglycemia observed in other oral hypoglycemic drugs.
Mechanism of action	Sitagliptin is a extremely selective DPP-IV inhibitor, which works by slowing the inactivation of incretin hormones in patients with type 2 diabetes, that results in increased concentration and prolonged action of these hormones. Incretin hormones, including glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are released through intestine all through the day, and levels are increased in accordance to a meal. The enzyme, DPP-IV inactivates these hormones. The incretins are part of an endogenous system concerned in the physiologic regulation of glucose homeostasis process. GLP-1 and GIP increase insulin synthesis when blood glucose concentrations are normal or elevated and release from pancreatic beta cells by intracellular signaling pathways involves cyclic AMP. GLP-1 also lessens glucagon secretion from pancreatic alpha cells to reduce hepatic glucose production. Through increasing active incretin levels, sitagliptin increases insulin release and decreases glucagon in

	the circulation in a glucose-dependent manner. These type of changes cause reduction in hemoglobin A1c (HbA1c) levels, and a lower fasting and postprandial glucose concentration. Sitagliptin shows selectivity for DPP-IV and does not inhibit DPP-VIII or DPP-IX activity *in vitro* at concentrations approximating those from therapeutic doses.
Absorption	Rapidly absorbed following oral administration, associated with an absolute bioavailability of 87%.
Volume of distribution	198 L [healthy subjects]
Protein binding	Plasma protein binding of the fraction of Sitagliptin is low (38%).
Metabolism	Sitagliptin does not generally undergo extensive metabolism. According to *in vitro* studies, CYP3A4 (oxidation) with contribution from CYP2C8 was the primary enzyme responsible for the limited metabolism of Sitagliptin.
Route of elimination	Through the urine with metabolism is a minor pathway of elimination of sitagliptin. Approximately 79% of sitagliptin is excreted unchanged condition. After administration of an oral [14C]sitagliptin dose to healthy individuals, approximately 100% of the administered radioactivity was eliminated through feces (13%) or urine (87%) within one week of dosing. Primarily sitagliptin eliminates via renal excretion pathway and also involves active tubular secretion.
Half life	12.4 hours
Clearance	renal cl=350 mL/min [Healthy subjects receiving 100 mg oral dose]
Affected organisms	Humans and other mammals

1.5.2. Profile of Vildagliptin

Formerly, Vidagliptin was identified as LAF237. It is a new oral anti-hyperglycemic agent (anti-diabetic drug) of the new dipeptidyl peptidase-4 (DPP-IV) inhibitor class of drugs. Vildagliptin works by inhibiting the inactivation of GLP-1 and GIP by DPP-IV. It enhances the secretion of insulin by GLP-1 and GIP in the beta cells and repress glucaon release by the alpha cells of the islets of Langerhans in the pancreas. At present, it is in clinical trials in the U.S. and has been reported to lessen hyperglycemia in T2DM. The drug is still unapproved for use in the US, but it was approved in Feb 2008 by European Medicines Agency for use within the EU and is listed on the Australian PBS with some certain restrictions. Different features of vildagliptin are summarized in **Table 1.7**.

Table 1.7. Features of Vildagliptin [100]

Identification	
Chemical Formula	$C_{17}H_{25}N_3O_2$
Structure	
Molecular Weight	Average:303.3993 Monoisotopic: 303.194677059
IUPAC Name	(2S)-1-{2-[(3-hydroxyadamantan-1-yl)amino]acetyl}pyrrolidine-2-carbonitrile
Calculated Predicted Partition Coefficient: cLogP	1.12
Calculated Predicted Aqueous Solubility: cLogS	2.2
Solubility (in water)	1.75 mg/mL (sparingly soluble)
Predicted Topological Polar Surface Area (TPSA)	76.36 Å2
Pharmacology	
Indication	Used to reduce hyperglycemia in T2DM
Structured Indications	T2DM
Pharmacodynamics	Vildagliptin is a member of the class of orally active antidiabetic drugs (DPP-IV inhibitors). It has multifunctional advantages as it is not limited into simple blood-glucose control. One of these advantages is a strong protective effect on beta cells of pancreas, which get worse in diabetic patients. Vildagliptin is considered to be safe, very well tolerated, and efficacious. Gut incretin hormones are released after meal. GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are the most important incretin hormones. These hormones are secreted through small intestine. These are responsible for insulin release in response to increased glucose amount levels. GLP-1's dependence on glucose

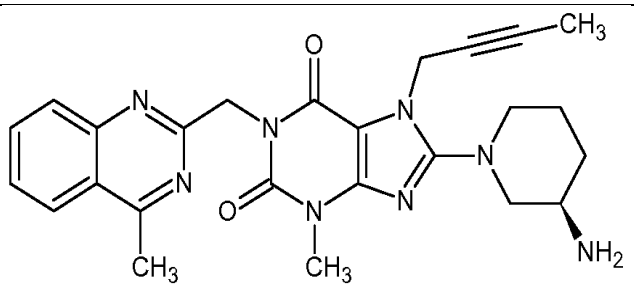
	concentration is beneficial because of a lower risk of hypoglycemia. GLP-1 also inhibits glucagon secretion and increases pancreatic beta cell mass through stimulating proliferation and neogenesis. Nevertheless, the clinical effectiveness of GLP-1 is restricted by its short elimination half-life (2 minutes). Proteolytic enzyme DPP-IV degrades GLP-1 in a rapid way. Inhibition of the DPP-IV enzyme is considered as a novel therapeutic approach in the treatment of diabetes that enhances GLP-1 activity. GLP-1's ability is enhanced after administration of vidagliptin. It produces insulin in response to increased concentrations of blood glucose that inhibits the release of amount of glucagon following meals, slow down the rate of nutrient absorption, and also slow down the rate of gastric emptying, and reduce food intake.
Mechanism of action	Vildagliptin inhibits dipeptidyl peptidase-IV (DPP-IV). As a result GLP-1 is invactivated by DPP-IV, which allows GLP-1 to enhance the secretion of insulin in the pancreatic beta cells. Dipeptidyl peptidase-4's degrades GIP and GLP-1 in blood glucose regulation.
Absorption	Rapidly absorbed after oral administration associated with an oral bioavailability of greater than 90%.
Metabolism	Cytochrome p450 3A4
Protein binding	9.3%
Half life	The elimination half-life is approximately 90 minutes.
Affected organisms	Humans and other mammals

1.5.3. Profile of Linagliptin

Linagliptin is a DPP-IV inhibitor developed by Boehringer Ingelheim. It is used in the treatment of T2DM. There are two pharmacological characteristics that make linagliptin different apart from other DPP-IV inhibitors. The characteristics are- it has a non-linear pharmacokinetic profile and is not primarily eliminated by the renal system. Linagliptin was approved by FDA on May 2, 2011. Different characteristics of linagliptin is described in **Table 1.8.**

Table 1.8. Features of Linagliptin [101]

Identification	
Physical State	White to off white powder
Chemical Formula	$C_{25}H_{28}N_8O_2$

Structure	
Molecular Weight	Average: 472.5422 Monoisotopic: 472.23352218
IUPAC Name	8-[(3R)-3-aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3-methyl-1-[(4-methylquinazolin-2-yl)methyl]-2,3,6,7-tetrahydro-1H-purine-2,6-dione
Pharmacology	
Indication	Linagliptin is used for the management of type 2 diabetes mellitus.
Structured Indications	
Pharmacodynamics	Linagliptin is a more potent inhibitor of DPP-IV than other member of the same class of drugs with an IC50 of 1 nM. It was found during comparison, sitagliptin, saxagliptin, and vildagliptin have an IC50 of 19, 50, and 62 nM respectively. Activity of DPP-IV by 72.7% and 86.1% from baseline is reduced by a dose of 2.5 mf and 5 mg respectively in healthy male individuals. A dose of 5 and 10 mg is effective as they inhibit >90% of DPP-IV for diabetic patients. Linagliptin is a selective inhibitor DPP-IV. *In-vitro* it is also indicated by the lack of DPP-VIII or DPP-IX inhibition at therapeutic exposures.
Mechanism of action	Linagliptin is known as competitive and reversible dipeptidyl peptidase-IV (DPP-IV) enzyme inhibitor. Mechanism of action of linagliptin is due to slow the breakdown of insulinotropic hormone glucagon-like peptide (GLP)-1 for better glycemic control in diabetic patients. GLP and glucose-dependent insulinotropic polypeptide (GIP) are incretin hormones. These hormones increase the production and release of insulin from

	beta cells of pancreas and decrease the release of glucagon from pancreatic alpha cells. This leads to an overall reduction in glucose production in the liver and increase of insulin in a glucose-dependent manner.
Absorption	Peak Plasma Concentration, Cmax, 5 mg, healthy subjects = 8.32 nmol/L; Time to attain peak plasma concentration, Tmax, 5 mg, healthy subjects = 1.75 hours; Area under the curve, AUC(0-24 hours), 5 mg, healthy subjects = 119 nmol · h/L; Bioavailability (Rate or extent of absorption, healthy subjects = 30%. After administration of a dose of 5 mg once daily, steady state is attained by the third dose. In spite of reduction of Cmax by high fat meal, it increases AUC, this interaction with food is clinically insignificant. Linagliptin can be taken with or without food.
Volume of distribution	Vd = 1110 L
Protein binding	Approximately it is bound to plasma protein at 70-80 %, the extent to which is concentration dependent. As linagliptin has tendency to bind to plasma protein, it has a long terminal half-life and a non-linear pharmacokinetic profile. In this context linagliptin is unique as other DPP-IV inhibitors have linear pharmacokinetic profiles.
Metabolism	Linagliptin is not extensively metabolized. 90% of dose of linagliptin is excreted as unchanged condition during metabolism. So very small portion of drug is metabolized. The main metabolite is CD 1790 which is pharmacologically inactive. Glucuronidation forms some of its other minor metabolites.
Route of elimination	Linagliptin is eliminated via the feces/enteroheptic system (80%) and urine (5%). Other DPP-IV inhibitors are primarily

	eliminated by the renal system.
Half life	Terminal/Elimination half life = 131 hours. As linagliptin has longer elimination half-life, once daily dosing is appropriate to sustain inhibition of DPP-IV activity. Effective half-life for accumulation of drug is 12 hours when multiple oral doses of 5 mg are given.
Clearance	Renal clearance, steady state = 70 mL/min

1.6. Objective of the Study

- Comparative quality study of existing market products of sitagliptin, vildagliptin and linagliptin with the FDA approved innovator
- Development and optimization of method by applying QbD approach
- Validation of stability indicating assay method in accordance of ICH guideline Q2(R1)
- Identification of degradants by forced degradation studies according to ICH guideline Q1A(R2)
- To establish degradation pathways of drug substances and drug products
- To differentiate drug products related degradants from those that are generated from non-drug product in a formulation
- To elucidate the structure of degradation products
- To determine the intrinsic stability of a drug substance in formulation
- To reveal the degradation mechanisms such as hydrolysis, oxidation, thermolysis or photolysis of the drug substance and drug product
- To determine the forced degradation rate constant in hydrolysis, oxidation and thermal condition of these drugs
- To estimate the half-lives ($t_{1/2}$) and shelf -lives ($t_{0.9}$) of the drugs at stress condition as well as room temperature
- To produce a degradation profile similar to that of what would be observed in a formal stability study under ICH conditions
- To solve stability-related problems

Literature Review

Diabetes mellitus is a disorder occurred by chronic hyperglycemia. It is defined by the current WHO and American diabetes association based on the plasma glucose levels. If the value of venous fasting plasma glucose (FPG) is 7.00 m mol/L or venous plasma glucose is 11.1 m mol/L, 2 h after intake of a 75 g oral glucose load is diagnosed, then the patient is considered as diabetic. According to the classification of WHO diabetes mellitus based on aetiology in four types, such as type 1, type 2, gestational diabetes and other specific types [102-103]. This is mainly occur due to insulin deficiency or insulin resistance. Due to exposure to certain drugs, viruses, genetic mutation in PPAR γ gene and diseases such as pancreatitis and cystic fibrosis it can be rarely occurred [104]. Hyperglycemia is associated with reduced life expectancy and quality due to microvascular and macrovascular disorders [105]. Dipeptidyl peptidase-IV (DPP-IV) inhibitors are among all the recent therapies for type 2 diabetes that has not reacted to life style intervention alone [106].

Global report on diabetes in 2014 reveled that 422 million adults were living with diabetes globally, whereas in 1980 it was 108 million. So, the necessities of antidiabetic drugs especially DPP-IV inhibiting drugs have been rising in pharmaceutical market day by day[107]. That's why this study focused on the quality checking of three popular DPP-IV, i.e. sitagliptin, vildagliptin and linagliptin, in terms of stability indicating assay method by applying QbD approaches and isolation and characterization of prominent degradants product.

Sitagliptin, chemically [(R)-4-oxo-4-[3-(triflouromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrizine-7(8H)-yl]-1- (2,4,5-trifloro phenyl)butan-2-amine] (Figure 1), is a long-acting pyrizine-based drug. It is one of the promising drugs used for the treatment of type II diabetes [108,109]. Since the inception in 2006 as first dipeptidyl peptidase-IV (DPP-IV) inhibitor, it is a well-known hypoglycemic drug concurrently administered with lifestyle changes [110]. By enhancing the effect of incretins, it reduces blood glucose concentration and finally causes significant increase in insulin secretion. Literature review revealed the determination of sitagliptin in dosage form either alone or combined form by UV spectrophotometry [111-112] , RP-HPLC[113-117], UPLC[118], tandem mass spectrometry [119,120] and capillary electrophoresis [121]. Very few

stability indicating assay method either alone or combination of other molecules was found [118,122-123]. However, UV spectrophotometry is the easiest method but needs relatively larger amount of analytes for detection and it is poor in accuracy and precision. HPLC is the best choice for analysis because of its sensitivity. Most of the published methods for the quantitation of sitagliptin contained complex mixture in mobile phase [113-114], larger amount of organic solvent in mobile phase[112,116] and relatively high retention time[115,118].Therefore, the aim of these studies are to establish a validated simple, cost-effective, precise and rapid UHPLC method to quantify sitagliptin in oral tablet dosage form. There is no methods found where QbD approach is used for method optimization and no degradation kinetic study is found.

Vildagliptin, a pyrrolidine derivative, chemically known as (S)-1-[N-(3-hydroxy-1-adamantyl) glycyl] pyrrolidine-2-carbonitrile, belongs to the dipeptidyl peptidase- IV (DPP-IV) inhibitor class of drugs [124]. The drug is a potent antidiabetic agent that enhances glycemic control by preventing the inactivation of incretin hormones like glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), as GLP-1 and GIP increase the secretion of insulin in the beta cells and decrease glucagon release by the alpha cells of the islets of Langerhans in the pancreas.

The gut incretin hormones are secreted in the human small intestine after taking a meal and responsible for insulin release in response to increased glucose levels. As the release of insulin by GLP-1 is glucose dependent so it has lower risk of hypoglycemia. However, the clinical use of GLP-1 is limited by its short half-life (2 minutes) due to rapid degradation by the proteolytic enzyme DPP-IV. Inhibition of the DPP-IV enzyme is a good therapeutic approach in the treatment of diabetes to improve GLP-1 activity. Vildagliptin administration enhances the ability of GLP-1 to produce insulin in response to elevated concentrations of blood glucose, inhibit the release of glucagon following meals [125].

According to FDA guidance, stability testing for new drug molecules and drug products is a validated quantitative analytical procedure which can determine the modification of relevant properties of the drug molecules and drug product with time. This method can accurately assess the active ingredients, without interference from degradants, process related impurities, excipients used in formulation, or other potential impurities [126]. The ICH guideline Q1A (R2) – Stability Testing of New Drug Substances and Products, states: “Stress testing is likely to be carried out on a single batch of the drug substance.

The testing should include the effect of temperatures (in 10°C increments (ie, 50°C, 60°C) above that for accelerated testing), humidity (i.e. 75% relative humidity or greater) where appropriate, oxidation, and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension"[30].

The analytical method of vildagliptin is still now unofficial which is not included in any compendia, i.e. the British or the United States Pharmacopeias. From the literature survey, there was some reported analytical methods such as UV spectrophotometry [127], HPLC [128-131] and LC-ESI-MS/MS [132] methods have been declared for the estimation of Vildagliptin. Very few stability indicating method of vildagliptin either alone or in combination was reported [131,133]. But no reported study on the vildagliptin degradation kinetics and half-life determination at room temperature under acidic, alkaline, thermal and oxidation conditions at different temperatures and their respective degradation kinetic parameters. Thus, our study aim to build up a simple, precise, rapid and accurate UHPLC stability-indicating assay method to quantify vildagliptin in its bulk and dosage form and to study its forced degradation outcome as well as its degradation kinetics and half-life at room temperature were calculated with the help of Arrhenius plot.

Linagliptin, a dipeptidyl peptidase-4 (DPP-IV) inhibitor is a xanthine derivative, work as an oral hypoglycemic drug [134] which was approved in 2011 by USA, Japan and Europe for the treatment of type-II diabetes mellitus [135]. Linagliptin block the degradation of incretin, which inhibits the breakdown of glucagon-like peptide (GLP-1) and glucose-dependent insulinotropic peptide (GIP), stimulate insulin secretion, resulting in a reduction in plasma glucose, glucagon levels, and inhibition of gastric emptying [136–138]. Linagliptin has minimal risk of hypoglycemia due to its effect as a glucose-dependent insulin secretagogue [139]. Usually linagliptin can be used as either alone or in combination with other common medications used to treat diabetes, such as metformin, sulfonylurea, pioglitazone or insulin [104]. Linagliptin has no effect on body weight increase and due to its insignificant renal excretion, no dose adjustment is required for patients with hepatic disorder [140].

The general guideline of ICH Q3A (R2) and Q3B (R2) recommended the characterization of impurities or degradation products that are present at a level greater than the

identification threshold in a drug substance or drug product [32,141,142]. To understand the degradation pattern of the drug substance, formation of impurities or degradants study in the drug substance, their isolation and characterization is very important. This gives valuable information about the drug stability under various conditions, that is also considerable for determining storage and other conditions of the bulk and dosage form. Moreover, improvements in the manufacturing process of bulk drug substance are difficult to achieve without understanding the possible degradation pathways [143-149].

Linagliptin is still now unofficial, not included in any of the pharmacopoeia. From the literature search many analytical methods by UV [150-151], HPTLCn, RP-HPLC [104,108, 152-155], UPLC [156], LC-MS/MS [157] methods were used for the quantitation of linagliptin in bulk, pharmaceutical dosage forms and biological fluids [157, 158] either alone [108,152-153] or in combination with other antidiabetic drugs [154-155, 158]. Synthesis and characterization of process related impurities of linagliptin have been reported recently [159-162]. However, there is no reported study has attempted to isolate or characterize degradation products of linagliptin. The present investigation deals with all (i) degradation studies including acid, base, thermal and photo stability on the drug substances under the ICH guideline (ii) isolated and characterized major degradation product through LC–MS/MS, IR and NMR, and (iii) describes plausible degradation pathways and (iv) developed and validated a simple, rapid, and sensitive stability-indicating RP-UHPLC method for quantification of linagliptin.

Materials and Methods

3.1. Materials

3.1.1. Chemicals and Reagents

The HPLC and analytical grade chemicals and reagents were collected for accomplishing the study. All the reagents used are listed in **Table 3.1**.

Table 3.1. List of Reagents

No.	Chemicals	Manufacturer	Origin
1	Potassium dihydrogen phosphate	Daejung Chemicals & metal Co.	Korea
2	Di potassium hydrogen phosphate	Scharlau	Spain
3	*o*-phosphoric acid	Merck	India
4	Sodium hydroxide	Merck	India
6	Sodium acetate	Daejung Chemicals & metal Co.	Korea
8	Glacial acetic acid	Merck	Germany
9	Hydrochloric acid	Merck	Germany
10	HPLC grade acetonitrile	RCI Labscan	Thailand
11	HPLC grade methanol	Merck	Germany
12	Distilled water	Evoqua Water Technologies	USA
13	HPLC grade water	Evoqua Water Technologies	USA
14	Hydrogen peroxide (30% v/v)	Scharlau	Spain
15	Hexane	Merck	Germany
16	Ethylacetate	Merck	Germany
17	Dichloromethane	Merck	Germany
18	Chloroform	Merck	Germany
19	Acetone	Merck	Germany
20	Methanol	Daejung Chemicals & metal Co.	Korea
21	Ethanol	Merck	Germany
22	Dimethylsulfoxide (DMSO),	Scharlau	Spain
23	Acetic acid	Scharlau	Spain
24	Sulfuric acid	Scharlau	Spain
25	Silica gel	Merck	India
26	Sephadex LH-20	Sigma-Aldrich	USA

3.1.2. Equipment and Instruments

For smooth conduction of the study following instruments and equipments were used, as shown in **Table 3.2**. All the instruments were calibrated periodically.

Table 3.2: List of Instruments and Equipments

No.	Equipments	Model/Company	Origin
1	Analytical balance	Shimadzu Corporation –TX 323L	Japan
2	Digital pH meter	Hanna	China
3	Ultra sonic bath	Human Lab Instruments Co.	Korea
4	Nano pure Water System	Evoqua water Tecnologies	China
5	Dissolution tester USP	VDA-8DR Veego Instruments Co.	China
6	Hardness tester	Electro lab,EH-01P	India
8	USP disintegration apparatus	Intech- LTD-DV	Belgium
9	Vortex	SCI logex	India
9	UV- Spectrophotometer	UV mini–1240 Shimadzu	Japan
10	Filter Paper	Whattman	UK
11	Filtration Membrane(0.45 µm)	Restek	USA
12	Vacuum Pump	Cole-Parmer	UK
13	Disc Filter (0.2 µm)	Corning syringe disc-type filters	USA
14	HPLC Column	• Discovery C8 Column(250 x 4.6 mm i.d., 5µm)	Germany
		• X-bridge C18 Column(150 x 4.6 mm i.d., 5µm)	USA
		• Brownlee Analytical C18 column (250 x 4.6 mm i.d., 5µm)	USA
15	UV cabinet with dual wavelength UV lamp (254 nm and 366 nm)	Optics technologies	India
16	High precision water bath	Biobase	USA
17	Hot air oven	Biobase	USA
18	Rotary Evaporator	Heidolph	Germany
19	UHPLC	Perkin Elmer-Flexer series with photodiode array detector (PDA+) UHPLC autosampler, FX-15 binary pump, Vacuum degasser, Flexer column oven. Chromera Manager Software	USA

20	Fourier Transform Infrared spectrometer (FT-IR)	Perkin Elmer	USA
21	Mass spectroscopy	Agilent Technologies, Life Science & Chemical Analysis	Germany
22	NMR spectroscopy	Avance Bruker NMR spectrophotometer	Switzerland

3.1.3. Drug Sample

Nine commercially available brands of Sitagliptin (STG), Vildagliptin (VLG) and Linagliptin (LNG) tablet each with a label claim 50 mg, 50 mg and 5 mg, respectively were purchased from the various retail pharmacies of Dhaka city in Bangladesh. Innovator products of these three APIs are Januvia (STG), Galvus 50 (VLG) and Trajenta (LNG) were used in this study for comparison. Sample coding of brands are shown in **Table 3.3**. The working standards of these three API were found from two pharmaceutical companies as a generous gift sample to conduct the research **(Table 3.4)**.

Sample coding

Sitagliptin, vildagliptin and linagliptin tablets were coded as STG, VLG and LNG, respectively where STG-1, VLG-1 and LNG-1 were the innovator samples of each group and remaining STG-2 to 10, VLG-2 to 10 and LNG-2 to 10 were manufactured by Bangladeshi pharmaceutical companies **(Table 3.3)**

Table 3.3: Sample Coding

Sample Code	**STG (50mg/tab)**	**VLG (50mg/tab)**	**LNG (5mg/tab)**
Sample-1/ Innovator	STG-1/ **Januvia**	VLG-1/ **Galvus 50**	LNG-1 **Trajenta**
Sample-2	STG-2	VLG-2	LNG-2
Sample-3	STG-3	VLG-3	LNG-3
Sample-4	STG-4	VLG-4	LNG-4
Sample-5	STG-5	VLG-5	LNG-5
Sample-6	STG-6	VLG-6	LNG-6
Sample-7	STG-7	VLG-7	LNG-7
Sample-8	STG-8	VLG-8	LNG-8
Sample-9	STG-9	VLG-9	LNG-9
Sample-10	STG-10	VLG-10	LNG-10

Table 3.4. Working Standard

Sl#	Standard	Obtained from	Origin	Purity
1	Sitagliptin	Incepta Pharmaceuticals Ltd.,BD	Zhejiang Wuyi Jiyan Pharm Chem, China	99.87%
2	Vildagliptin	Drug International Limited, BD	Dr. Reddy's Laboratories, India	99.92%
3	Linagliptin	Incepta Pharmaceuticals Ltd.,BD	Stereokem Pharmaceuticals Pvt. Ltd., Hyderabad, India	99.94%

3.2 Methods

3.2.1. Methods for Physical Evaluation of local Product

In-vitro quality control parameters between commercially available tablet brands of Sitagliptin, Vildagliptin and Linagliptin in Bangladeshi pharmaceutical market were compared with the innovator product through the evaluation of weight variation, hardness, disintegration time, assay and dissolution profile.

3.2.1.1. Weight Variation Test

According to the USP-NF weight variation test was accomplished by weighing 20 tablets for each of the ten brands individually using an electronic balance, then calculating the average weights and comparing the individual tablet weights to the average. The difference in the two weights was used to calculate weight variation by using the following formula **(Eq-1)** [163].

Weight variation = $(I_w - A_w)/A_w \times 100\%$.....**Eq. (1)**

Where, I_w = Individual weight of the tablet and

A_w = Average weight of the tablet.

The tablet complies with the test if not more than 2 of the individual weights deviate from the average weight by more than the 5% [163].

3.2.1.2. Hardness Test

Monsanto hardness tester was used to check the hardness of the tablet. Ten tablets were arbitrarily selected from each of the ten brands and tested. This test determine the

pressure required to break entirely placed tablets by applying pressure with coiled spring. The acceptable limit for this test is 4 -7 kg/cm^2 [163, 164].

3.2.1.3. Disintegration Test

USP disintegration apparatus was used to determine the disintegration time (DT). For disintegration testing one tablet was placed in each tube for each brand and the solvent was of water maintained at 37 ± 2 °C. A standard motor driven device was used to move the basket assembly containing the tablets up and down through a distance of 5–6 cm at a frequency of 28–32 cycles per minute. Perforated plastic discs were used to prevent the floating of tablets. The apparatus was operated for 30 min [163, 165].

To comply with the USP-NF standards, the tablets must disintegrate and all particles must pass through the 10-mesh screen within 30 min. If any residue remains, it must have a soft mass [24, 163].

3.2.1.4. Dissolution Test

There is no biopharmaceutics classification of STG was found, it can be incidental that the drug is Class 1, since it presents high solubility and high permeability (bioavailability of 87%). Hence, the absorption process would not be incomplete by the solubility and or by permeability. [166, 167]. However, the dissolution rate can be extensively changed when the drug is mixed with excipients during manufacturing, and in some cases, this can lead to a reduction in bioavailability and clinical reply.

Vildagliptin is considered to be a Class I drug substance (high solubility, high permeability). Linagliptin is considered to be a Class III drug substance (high solubility, poor permeability) due to incomplete oral systemic bioavailability (about 30% compared to intravenous administration) and the moderate permeability.

The solubility of the drug was tested using an amount of solute (STG, VLG and LNG) and solvent (dissolution medium) equivalent to three times the formulation dose in 900 mL of medium. The media 0.1 M HCl, distilled water, phosphate buffer (pH 6.8), and acetate buffer (pH 4.5) were used. These media were used because they are relevant to physiological pH and are frequently used in dissolution testing [168]. The dissolution tests were conducted using 900 mL of each medium. The media were heated and kept at a temperature of 37 ± 0.5 °C. USP apparatus 2 (paddle) at 100 rpm was tested, and

aliquots of 5 mL were withdrawn at 10, 20, 30, and 45 min. and this was immediately replaced with the same volume of fresh test media. The sample was filtered with Whatman filter paper, grade 1, 110 mm diameter.

Guidance of FDA for Dissolution:

The model developed by Moore and Flanner is used to evaluate the dissolution profile using two factors, f1 and f2 [169] following the FDA guidance for comparing the dissolution profiles [170]. A profile comparison is not necessary for products that are rapidly dissolving (i.e., more than 85% in 15 minutes or less). The difference factor (f1) calculates the percent (%) difference between the two curves at each time point and is a measurement of the relative error between the two curves **(Eq-2).** The similarity factor (f2) is a logarithmic reciprocal square root transformation of the sum of squared error and is a measurement of the similarity in the percent (%) dissolution between the two curves **(Eq-3).**

Statistical Calculations

- Similarity Factor (f2) = 50xlog {[1+ (1/n) S t=1n (Rt-Tt) 2] -0.5 x100}.....**Eq.(2)**
- Difference Factor (f1) = {[S t=1n |Rt-Tt|] / [S t=1n Rt]} x100.....**Eq.(3)**

Where,

n = number of dissolution sample times,

Rt = percent dissolved at each time point for the reference at time t.

Tt = percent dissolved at each time point for the test dissolution at time t.

- **f2 value 50-100 ensures sameness of two products**
- **f1 value 0-15 ensures minor difference between two products [168,171].**

3.2.1.5. Assay Test

Table 3.5. Chromatographic Conditions

Sl#	Parameters	Specification
1	Instruments	Perkin Elmer Flexar series UHPLC
2	Software	Chromera Manager
3	Column	Xbridge C18 (150mm, 4.5mm , 5µm)
4	Column temperature	25°C
5	Detector	PDA+
6	Flow rate	1.0 mL/min
7	Injection Volume	20 µL
8	Run time	10 min
9	Mobile Phase	Phosphate Buffer(pH 6.8): Acetonitrile = 70:30
10	Maximum wavelength	246(VLG), 228(LNG), 267(STG)
11	Retention time	2.423±0.04(VLG),3.203±0.06(LNG), 4.189±0.12(STG)

- **Stock and Standard Solutions Preparation:** Stock solutions of working standard (STG, VLG, LNG) (1 mg/mL) were prepared in mobile phase. Final concentration of standard solution of 50 µg/mL was prepared from the stock solution by suitable dilution with mobile phase and filtered through 0.45µm disc filter (Filter-Bio).

- **Sample Preparation:** Tablets of STG, VLG and LNG were crushed to finely grinded powder. A stock sample solution of 1 mg/mL was prepared in mobile phase by transferring a weighed amount of the finely grinded powder equivalent to 100 mg of API to 100 mL volumetric flask containing 50 mL mobile phase. The solution was sonicated (Human Lab Instrument Co. Ltd., Korea) for 10 min and the volume was adjusted to the mark with mobile phase. The solution was then filtered (Whatman filter paper, Grade 1, 110 mm diameter). For assay of tablet, a working sample solution of 50 µg/mL was prepared from the stock solution by dilution with the mobile phase and filtered through 0.45µm disc filter (Filter-Bio).

- **Injection:** 20 µL of filtered standard and sample solution were injected with triplicate injection. The potency of each brand was calculated from the peak area of standard and sample.

- **Calculation:** Potency was calculated according to the (**Eq-4**).

$$\% \text{ of content} = \frac{(\text{Peak Area of Sam X Wt. of Std X Potency of Std X Dilution Factor x 100})}{(\text{Peak Area of Std X Wt. of Sam X 100 X Avg. Wt})} \quad \text{..... Eq. (4)}$$

Where,

Peak Area of Sam= Peak Area of sample

Peak Area of Std= Peak Area of standard

Wt. of Std= Weight of standard

Wt. of Sam= Weight of sample

Avg. Wt= Average weight of tablet

For non-pharmacopieal or INN drug product the assay in the release specifications is ± 5% of the label claim (i.e. 95.0-105.0%) [172].

3.2.2. Method Development and Optimization by Using QbD

An extensive literature review was done before starting the study. It was on the various parameters of method development and available method for the determination of various drug components. The following physico-chemical properties of these drugs were analyzed to design and develop new methods.

3.2.2.1. Physicochemical Parameters of Drugs

To develop a precise, sensitive HPLC method following information of drugs were required (**Table 3.6**).

Table 3.6. Physicochemical Parameters of Drugs

Sl #	Parameters	STG	VLG	LNG
1	Chemical formula	$C_{16}H_{15}F_6N_5O \bullet H_3PO_4 \bullet H_2O$	$C_{17}H_{25}N_3O_2$	$C_{25}H_{28}N_8O_2$
2	Molecular weight	523.32	303.39	472.54
3	pKa value	7.2	8.39	8.7
4	LogP	2.06	1.17	1.15
5	Appearance	White to off-white, crystalline powder	Vildagliptin is a white to slightly yellowish or slightly greyish crystalline powder	White to yellowish crystalline solid substance

6	Hygroscopicity	non-hygroscopic	non-hygroscopic	Slightly hygroscopic,
7	Solubility	Soluble in water and N,N-dimethyl formamide, slightly soluble in methanol, very slightly soluble in ethanol, acetone, and acetonitrile, and insoluble in isopropanol and isopropyl acetate	Freely soluble in water	Very slightly soluble in water, soluble in methanol, sparingly soluble in ethanol, very slightly soluble in isopropanol, and very slightly soluble in acetone

Based on these physicochemical parameters, different methods in reverse phase-ultra high performance liquid chromatography (RP- UHPLC) system were used to separate the commonly used gliptins (STG, VLG and LNG).To achieve better separation, different methodology were applied based on variation of organic (acetonitrile, methanol) and inorganic (water and buffer) modifier, flow rates (0.5–2 mL/min), pH of buffer (3 to 7) in mobile phase, column variation (C_{18} and C_8), columns lengths (150 and 250 mm), column oven temperature (25 to 35◦C) variation. Finally the method was optimized by applying quality by design (QbD) approach.

3.2.2.2. QbD Approach for Method Development and Optimization

- Single method development for three gliptins, i.e. Vildagliptin, Linagliptin and Sitagliptin.
- Optimization by QbD approach using Design of Experiments (DoE).
- Software: Design–Expert® version 10.0.3.1.
- Model: Box-Behnken Experimental Design (BBD).
- 3^3 Factorial design consisting 30 runs.
- Model design optimization done by ANOVA and Lack of fit.

3.2.2.3. Method Variables

For the method development and optimization according to QbD three independent variables or factors were selected and their effects were found from three responses described in **Table 3.7**.

Table 3.7. Method Variables

Variables	**Code**	**Name**	**Unit**	**Type**
Independent variables /factors	A	% of acetonitrile in mobile phase	%	Numeric
	B	Flow rate	mL/min	
	C	pH of buffer		
Dependent variables /Responses	RT	Retention time(RT)of linagliptin (2nd Peak)	min	
	Rs1	Resolution between peak 1(vildagliptin) and 2(linagliptin)		
	Rs2	Resolution between peak 2 (linagliptin) and 3 (sitagliptin)		

3.2.2.4. Independent Factors with Their Levels

The levels of three independent factors for this study are in between -1 to +1(**Table 3.8**)

Table 3.8. Independent Factors with Levels

Factor	Name	Unit	Type	Low	Mid	High
				Coded Values		
A	ACN % in MP	%	Numeric	-1	0	1
B	Flow rate	mL/min	Numeric	-1	0	1
C	Buffer pH		Numeric	-1	0	1
				Actual Values		
A	ACN % in MP	%	Numeric	25	30	35
B	Flow Rate	mL/min	Numeric	0.8	0.9	1.0
C	Buffer pH		Numeric	6.0	6.8	7.6

3.2.2.5. Box Behnken Experimental Design (BBD)

Box Behnken Experimental Design (BBD) is used to optimized the method which is described in **Table 3.9**. According to the suggestion of BBD 30 runs were designed for development and optimization of method.

Table 3.9. Box Behnken Experimental Design (BBD) Layout

Standard	Run	Factor 1	Factor 2	Factor 3	Response 1 RT (Min)	Response 2 RS1	Response 3 RS2
19	1	-1	-1	+1	11.079	19.05	7.67
24	2	+1	0	+1	4.044	7.58	7.64
4	3	-1	0	-1	5.832	13.86	1 .0
16	4	-1	+1	0	8.823	20.29	1.77
28	5	0	0	0	5.04	10.28	5.19
22	6	-1	0	+1	9.871	19.02	7.56
13	7	-1	0	0	9.801	20.63	1.88
8	8	0	+1	-1	3.207	8.681	7.42
14	9	0	0	0	5.04	10.28	5.19
21	10	+1	-1	+1	4.557	7.92	7.66
29	11	0	0	0	5.04	10.28	5.19
10	12	-1	-1	0	10.943	21.28	1.93
5	13	0	0	-1	4.745	14.01	1.11
25	14	-1	+1	+1	8.755	18.48	7.44
11	15	0	-1	0	6.126	10.58	5.49
17	16	0	+1	0	4.381	10.14	5.18
23	17	0	0	+1	5.519	13.51	8.11
20	18	0	-1	+1	6.565	13.92	8.43
30	19	0	0	0	5.04	10.28	5.19
18	20	+1	+1	0	3.664	4.17	5.62
2	21	0	-1	-1	4.981	11.76	1.65
1	22	-1	-1	-1	6.126	1.41	18.2
12	23	+1	-1	0	5.253	6.14	5.71
15	24	+1	0	0	4.699	6.64	5.83
27	25	+1	+1	+1	3.543	7.38	7.42
7	26	-1	+1	-1	2.291	4.08	18.04
3	27	+1	-1	-1	3.412	5.64	2.56
26	28	0	+1	+1	4.935	13.37	7.76
9	29	+1	+1	-1	2.652	5.39	2.39
6	30	+1	0	-1	3.038	5.53	2.5

3.2.3. Method Validation

3.2.3.1. Validation Parameter According to ICH Q2 (R1)

The developed and optimized method was validated as per the ICH Q2 (R1) [173] guidelines for following parameters such as system suitability, linearity , LOD, LOQ, specificity, precession (repeatability and intermediate precession), accuracy test, and robustness.

3.2.3.2. System Suitability

For system suitability determination, 20 µL of filtered standard solution with a concentration of 10 µg/mL of STG, VLG and LNG were injected simultaneously from a single vial. These standard solutions were injected as six replicate injections in UHPLC. The limit of acceptance for peak area, tailing factor, number of theoretical plate, retention time, resolution between VLG and LNG (Rs1) and resolution between LNG and STG (Rs2) were studied.

3.2.3.3. Linearity and Detection Limit

The calibration curve was constructed for standard with different concentrations from 10 µg/mL to 50 µg/mL for VLG and STG and for LNG the concentration was 0.1 µg/mL to 1.0 µg/mL. For this study triplicate injections of each concentration were analyzed. The linear regression and correlation coefficient were found out separately for these three gliptins (STG, VLG, LNG) from the obtained graph between average peak area and concentration.

For the calculation of lower limit of detection (LOD), the sigma method was used [174]. This method depends on the slope and least standard deviation of the response. **Equation-(5) and (6)** were used for the calculation of LOD and LOQ, respectively.

$$LOD = 3.3r/SP \quad \textbf{Eq(5)}$$

$$LOQ = 10r/SP \quad \textbf{Eq(6)}$$

Where, r = the minutest standard deviation value in response and

SP = the slope of the calibration curve

3.2.3.4. Specificity

Specificity is determined by injecting separately blank, placebo and standard solution of VLG, LNG and STG in triplicate. The results were confirmed by the peak purity analysis.

3.2.3.5. Precision

Precision of the developed method was assessed by repeatability or intra-assay precision and intermediate precision analyses. Repeatability was determined from six replicate injections of 20 µL each of nominal standard solution (50µg/mL). The nominal standard solution was analyzed for a period of six days with six replicate injections of 20 µL each on daily basis. The results of both the studies were compared (intermediate precision) and expressed as %RSD of the measurements.

3.2.3.6. Accuracy

To check for accuracy of the developed method as well as studying the interference of formulation additives on analysis the recovery experiments were carried out by spiking the sample solution with standard drug substance at 80%, 90%, 100%, 110% and 120%. All determinations were carried out in triplicate. The percent recovery of the added standard drug to the assay samples was calculated by using the equation **(Eq-7)** [175].

$$\% \text{ recovery} = [(C_c - C_f)/C_s] \times 100 \quad \text{.....Eq(7)}$$

Where,

Cc= the concentration of analyte present in the combination of standard and test
Cf= the concentration of analyte in formulation and
Cs= the concentration of standard analyte used in the combination

3.2.3.7. Robustness

The robustness of a chromatographic method may be assessed by variations in parameters such as mobile phase composition, pH of mobile phase and ionic strength of buffer, temperature and different lots or suppliers of columns [176-175]. For this study robustness was assessed by determining the effect of small and deliberate changes in

flow rate (0.8, 1, 1.2 ml/min) pH of the mobile phase (pH = 5.8, 6.0 and 6.2) and percentages of actonitrile in mobile phase (65, 70 and 75%).

3.2.4. Stress Study

3.2.4.1. Stress Condition

For conducting forced degradation study the drug substances are stressed by using following condition (**Table-3.10**).

Table 3.10. Stress Condition

Stress Condition	Stressor	Temperature (°C)	Time (hr)
Acid hydrolysis	1N HCl	80	2
Alkaline hydrolysis	1N NaOH	80	2
Oxidative degradation	3% H_2O_2 Solution	80	2
Thermal degradation	Hot air oven	105	48
Photolytic degradation	254nm at UV Chamber and Daylight	RT	72

3.2.4.2. Sample Preparation for Degradation Studies

3.2.4.2.1. Acidic Degradation Studies

1mL of stock solution with a concentration of 1mg/mL was added with 3mL of 1N hydrochloric acid and heated in a water bath with temperature of 80^0C for 2 hr. The degraded sample solutions were then transferred into volumetric flasks quantitatively. For neutralization of the reaction equal volumes of 1N sodium hydroxide base was added for acidic degradation and diluted with mobile phase to obtain a concentration of 50 µg/mL solution before injection into the UHPLC system and the chromatograms were recorded to check the stability of sample.

3.2.4.2.2. Alkaline Degradation Studies

1mL of stock solution with a concentration of 1mg/mL was added with 3mL of 1N sodium hydroxide and heated in a water bath with temperature of 80^0C for 2 hr. The degraded sample solutions were then transferred into volumetric flasks quantitatively.

For neutralization of the reaction equal volumes of 1N hydrochloric acid was added for alkaline degradation and diluted with mobile phase to obtain a concentration of 50 µg/mL solution before injection into the UHPLC system and the chromatograms were recorded.

3.2.4.2.3. Oxidative Degradation Studies

1mL of stock solution with a concentration of 1mg/mL was added with 3mL of 3% v/v hydrogen peroxide (H_2O_2) and heated in a water bath with temperature of 80^0C for 2 hr. The degraded sample solutions were then transferred into volumetric flasks quantitatively and diluted with mobile phase to obtain a concentration of 50 µg/mL solution before injection into the UHPLC system and the chromatograms were recorded to check the degradation by oxidation.

3.2.4.2.4. Thermal Degradation

1mL of stock solution with a concentration of 1mg/mL was placed in oven at 105^0C for 48 hrs for thermal degradation. The degraded sample solutions were transferred into volumetric flasks and diluted with mobile phase to obtain a concentration of 50 µg/mL solution before injection into the UHPLC system and the chromatograms were recorded.

3.2.4.2.5. Photolytic Degradation

Two (1 mg/mL) solutions were used to assess the effect of light. Among these two one solution was subjected to ultraviolet light (254 nm) for 72 h, while the another one was subjected to day light for 72 h. These solutions were then diluted with the mobile phase to achieve final concentration of 50 µg/mL solution before injection into the UHPLC system and the chromatograms were recorded.

3.2.5. Forced Degradation Kinetic Study

3.2.5.1. Condition for Degradation Kinetic Study

For the kinetic investigation, drug substances were stressed at different stress condition with a definite time interval which was described in **Table 3.11**.

Table 3.11.Condition for Degradation Kinetic Study

Stress Condition	**Time interval (hr)**	**Temperature (◦C)**	**Stressor**
Acidic (1N HCl)	0, 0.5,1,2,4 & 8	60, 80, 105	Thermo stated water Bath
Basic (1N NaOH)	0, 0.5,1,2,4 & 8	60, 80, 105	Thermo stated water Bath
Oxidative (3%H_2O_2)	0, 1,4, 8, 12 & 24	40, 60, 80	Thermo stated water Bath
Thermal	0, 1,4, 12, 24 & 48	60, 80, 105	Oven
Photo (254nm)	0, 24, 48, 72,120 & 168	RT	UV Chamber
Daylight	0, 24, 48, 72,120 & 168	RT	Sunlight

3.2.5.2. Sample preparation for kinetic Study

3.2.5.2.1. Acidic Hydrolysis

1 mL of stock solution (1 mg/mL) was poured into a series of volumetric flasks, then 3 mL 1N HCl was added and mixed properly. The volumetric flasks were placed in a thermostated water bath at different temperatures (60, 80 and 105°C) for different time intervals (0, 0.5, 1, 2, 4 and 8 h). The resultant solutions were neutralized (pH = 7) using 1N NaOH after the specified time, and diluted with the mobile phase to achieve final concentration of 50 µg/mL.

3.2.5.2.2. Basic Hydrolysis

1 mL of stock solution (1 mg/mL) was poured into a series of volumetric flasks, then 3 mL 1N NaOH was added and mixed properly. The volumetric flasks were placed in a thermostated water bath at different temperatures (60, 80 and 105°C) for different time intervals (0, 0.5, 1, 2, 4 and 8 h). The resultant solutions were neutralized (pH = 7) using 1N HCl after the specified time, and diluted with mobile phase to achieve final concentration of 50 µg/mL.

3.2.5.2.3. Oxidation with H_2O_2

1 mL of stock solution (1 mg/mL) was poured into a series of volumetric flasks, then 3 mL 3% v/v H_2O_2 was added and mixed. At different temperatures (40, 60 and 80°C) the volumetric flasks were placed in a thermostated water bath for different time intervals (0, 1, 4, 8, 12 and 24h). The obtained solutions were diluted with mobile phase to get final concentration of 50 µg/mL.

3.2.5.2.4. Thermal Degradation:

1 mL of stock solution (1 mg/mL) was poured into a series of volumetric flasks. At different temperatures (60, 80 and 105°C), the volumetric flasks were placed in an oven for different time intervals (0, 1, 4, 12, 24 and 48h). The resultant solutions were diluted after the specified time interval with the mobile phase to get final concentration of 50µg/mL.

3.2.5.2.5. Photolytic Degradation

a) At daylight

1 mL of stock solution (1 mg/mL) was poured into a series of volumetric flasks. The volumetric flasks were placed in normal sunlight for different time intervals (0, 24, 48, 72,120 and 168h). The resultant solutions were diluted after the specified time interval with the mobile phase to get final concentration of 50 µg/mL.

b) At 254nm UV chamber

1 mL of stock solution (1 mg/mL) was kept into a series of volumetric flasks. The volumetric flasks were placed in an UV chamber at 254nm at room temperature for different time intervals (0, 24, 48, 72,120 and 168h). The resultant solutions were diluted after the specified time interval with the mobile phase to obtain final concentration of 50 µg/mL.

3.2.5.3. Forced Degradation kinetic Study

Stability studies and degradation kinetics are a integral parts of the quality control of a drug or medicinal product on an industrial scale. Degradation kinetics is also used to evaluate the stability under certain conditions as well as to compare stress conditions.

Therefore, the intrinsic stability and kinetic studies are fundamental elements in the search for possible degradation products of drugs; however, these products do not commonly appear under normal drug storage conditions [178].

The logarithmic values of percentages of the remaining concentrations at different time intervals were used to establish the degradation plots of vildagliptin, linagliptin and sitagliptin in the solution prepared for kinetic treatment of acidic, basic, oxidative and thermal degradation at different temperature. The degradation kinetic parameters such as the degradation rate constant (K), degradation half-life (t_{50}), shelf life (t_{90}) and t_{10} were derived from the Arrhenius plots. Triplicate injection was done for each experiment and data were further processed and degradation kinetic parameters were calculated. From Arrhenius plot the predicted kinetic parameters were extrapolated for the degradation of vildagliptin, linagliptin and sitagliptin at 25°C.

In the development of a pharmaceutical formulation in addition to a identifying polymorphism, it is important to determine the intrinsic stability of the drug to predict possible reactions and degradation products. The intrinsic stability of the substance should be evaluated in terms of temperature, humidity, oxidation, UV light exposure, and hydrolysis at different pH values [179]. The photostability test can be evaluated under the conditions recommended by ICH Q1B [180], by subjecting the substance to ultraviolet irradiation. Some degradation pathways can be complex; however, not all decomposition products formed under conditions of intrinsic, yet more drastic, stability can be observed in the drug when subjected to the official conditions of the stability studies [178,179,181].

3.2.5.4. Stability Analysis Using Arrhenius Equation Plot

The influence of temperature on the degradation kinetics of sitagliptin, vildagliptin and Linagliptin were determined using accelerated stability testing and Arrhenius equation [182-183] (**Eq-8**)

$$\ln(k)=\ln(A)- Ea/RT \text{----------} \textbf{Eq.(8)}$$

Where,

k = degradation rate constant,

A = frequency factor,

*E*α = activation energy,

R = gas constant and

T = absolute temperature in degrees Kelvin.

The *k* value depends on the *E*α and is characteristic of a specific compound [184].

Based on the pseudo-first-order reaction kinetics, [182] **Eq.(9)** was generated into its logarithmic (base 10) form.

$$\text{Log}(C/C0) = k't/2.303 \quad \textbf{Eq.(9)}$$

Where,

k'= pseudo-first-order rate constant,

C0= initial concentration,

C = concentration of drug remaining after time *t*,

C/C0 =fraction of drug remaining after time *t*,

The values of *k*' at each temperature can be determined using **Eq. (9)**, from the slope of the regression equation generated from the plot between log % drug remaining and time (*t*) in months.

The value of 1000/T (in Kelvin) was calculated for each temperature and the Arrhenius plot between *ln* (*k*') vs. 1000/T was constructed. The slope and intercept values of this plot were equal to -*E*α/*R* and *ln (A)*, respectively, according to **Eq. (8)**. The *E*α was calculated by multiplying the slope value by *R* (8.314 J.mol−1. Kelvin−1). The significance of the *E*α value is to determine the temperature dependency of a chemical reaction. The higher the value of *E*α for a chemical reaction the greater the acceleration with increase in temperature and the more the stability of a drug is temperature dependent.[184,185] Generally, drugs with lower *E*α values have significantly longer shelf-lives.[184]

The rate constant (*k'25*) that corresponds to room temperature (25°C) was calculated from the regression equation.

The *k'25* value was used for the calculation of shelf life (t_{90}), half-life (t_{50}), and the time required for the drug to decrease its initial amount by 90 % (t_{10}). The determination of the t_{90}, t_{50}, and t_{10} values were calculated based on **Eq. (10-12).**

t_{50} = 0.693/ k'----------**Eq. (10)**

t_{90} = 0.105/ k'----------**Eq. (11)**

t_{10} = 2.303/ k'----------**Eq. (12)**

3.2.6. Isolation and Characterization of Degradants of Linagliptin

Major degradants of LNG are oxidative and acidic degradants. These two degradants product were generated by applying sufficient stress. Two oxidative and three acidic degradants were collected and isolated according to the **Figure 3.1.** Then structure elucidation of these products were done by IR and NMR specteroscopy.

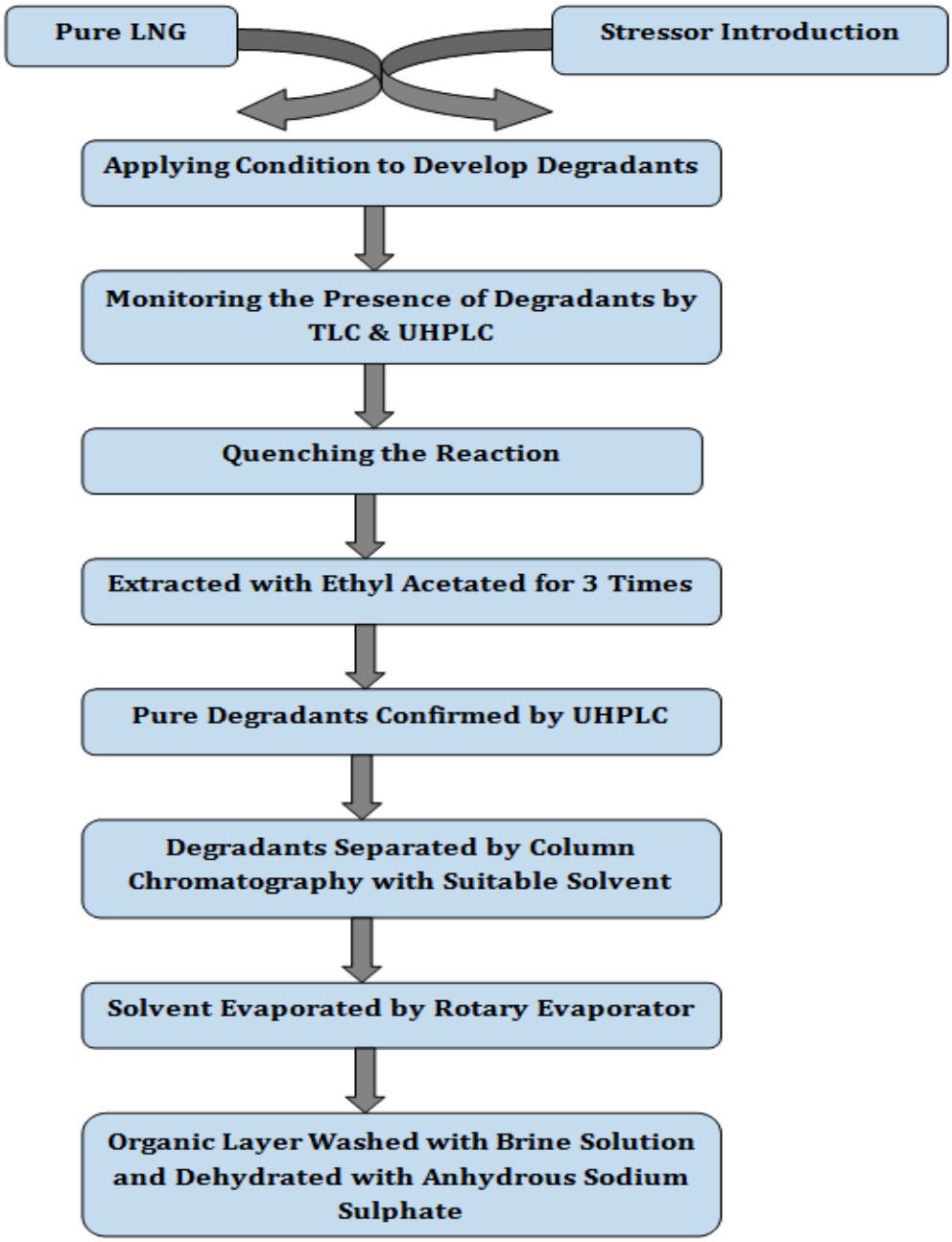


Figure: 3.1. Flow chart of Degradant Collection and Isolation Process

3.2.6.1. Isolation and Characterization of Acidic Degradants

For the generation of major alkaline degradants 5g of pure LNG were weighed and dissolved by 50 ml of acetonitrile then the solution was mixed properly by sonnication for 5 minutes. Then 50 ml of 5 N HCl solution was added slowly with the solution then kept in oven at 105°C for 12 hour. Then the solution was neutralized by adding 5N NaOH untill the pH become neutral. Sufficient amount of degradants were produced which was ensured by checking in the UHPLC. Then the resultant solution was extracted three times with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate, and the solvent was evaporated by vacuum evaporator to obtain a solid mass. The latter was chromatographed over a normal phase and reverse phase both chromatography with column of silica gel and sephadex LH-20 respectively. Different solvent system based on polarity was used as mobile phase. For normal phase column chromatography the optimized mobile phase was ethyl acetate and methanol in gradient mode. The elution was started with100% ethyl acetate. Polarity of the mobile phase was increased step wise by adding methanol with its 1% increment at a time, i.e., using mobile phase composed of a mixture of ethyl acetate and methanol in ratios of 99:1 followed by 98:2, 97:3, and so ongoing upto 90:10. In case of reverse phase column chromatography the optimized mobile phase was water and methanol in gradient mode. The elution was started with100% water. Polarity of the mobile phase was decreased step wise by adding methanol with its 1% increment at a time, i.e., using mobile phase composed of a mixture of water and methanol in ratios of 99:1 followed by 98:2, 97:3, and so ongoing up to 90:10. The column was run with a minimum of 100mL of each mixture of this mobile phase or till the analyte continued to elute with a particular mixture. The fractions containing single degradants were pooled which was ensured by checking in the UHPLC with a single peak. The solvent was evaporated by using vacuum to obtain a solid mass. The solid residue of degradents were characterized through ^{1}H-NMR, ^{13}C-NMR and IR spectral analysis.

3.2.6.2. Isolation and Characterization of Oxidative Degradants

For the generation of major oxidative degradants 5g of pure LNG were weighed and dissolved by 50 ml of acetonitrile then the solution was mixed properly by sonnication for 5 minutes. In the resultant solution 50 ml of 10% H_2O_2 v/v solution was added slowly then kept in dark places for 12 hour with continuous stirring in a magnetic

stirrer. Sufficient amount of degradants were produced which was ensured by checking in the UHPLC. To prevent further decomposition or formation of secondary degradants the reaction was quenched by adding platinum wire for one hour. Then the resultant solution was extracted three times with chloroform. The organic layer was dried over anhydrous sodium sulfate, and the solvent was evaporated by using vacuum to obtain a solid mass. The latter was chromatographed over a normal phase chromatography with column of silica gel. Different solvent system based on polarity was used as mobile phase and the optimized mobile phase was ethyl acetate and methanol in gradient mode. The elution was started with100% ethyl acetate. Polarity of mobile phase was increased step wise by adding methanol with its 1% increment at a time, i.e., using mobile phase composed of a mixture of ethyl acetate and methanol in ratios of 99:1 followed by 98:2, 97:3, 90:10. The column was run with a minimum of 100mL of each mixture of this mobile phase or till the analyte continued to elute with a particular mixture. The fractions containing single degradants were pooled which was ensured by checking in the UHPLC with a single peak. The solvent was evaporated by using vaccum to obtain a solid mass. The solid residue of degradents were characterized by ^{1}H-NMR, ^{13}C-NMR and IR spectral analysis.

3.2.6.3. Structure Elucidation by NMR and IR Spectroscopy

For the characterization or conformation of the structure ^{1}H NMR, ^{13}C NMR and IR spectroscopy were used.

3.2.6.3.1. IR Spectroscopy

The IR spectra of LNG and its oxidative and acidic degradation product were recorded on a Perkin–Elmer spectrum BX spectrophotometer.

3.2.6.3.2. NMR Spectroscopy

The ^{1}H NMR experiments were performed on Avance Bruker NMR spectrophotometer (Fallanden, Switzerland), operated at 400 MHz (^{1}H-NMR) and 100 MHz (^{13}C-NMR) using standard software packages. Chloroform was used as a solvent and tetra methyl silane (TMS) was used as internal standard.

Results and Discussion

Label claim of pharmaceutical products refer to the ability to maintain the physical, chemical and therapeutic integrity of the product during the time of storage and usage by the patient at a specified time period. It is measured by the rate of changes that take place in the pharmaceutical dosage forms. In these studies three selected DPP-IV, sitagliptin (STG), vildagliptin (VLG) and linagliptin (LNG) were subjected to evaluate their physical and chemical integrity by testing weight variation, hardness, disintegration, dissolution and assay of nine brands of each drug, manufactured by pharmaceutical companies of Bangladesh and compared with their innovator products. An UHPLC method was developed and optimized by applying QbD approaches for simultaneous estimation of API in dosages form. To ensure the stability indicating assay method, forced degradation studies were conducted in different prescribed stress conditions by ICH Q1A (R2). The developed method was validated according to ICH Q2 (R1) guideline. Degradation kinetics studies also carried out to determine rate of degradation as well as determining half life ($t_{1/2}$) and shelf life ($t_{0.9}$) at room temperature. Major degradants of LNG were isolated by using liquid column chromatography and structure of degradants were confirmed by IR and NMR (^{1}H and ^{13}C) spectroscopy.

4.1. Evaluation of Physical Parameters

The quality parameters of three prominent DPP-IV inhibitors were compared with their innovator product. From the study, it was observed that the result of weight variation, hardness, disintegration, dissolution and assay of nine brands of sitagliptin, vildagliptin and linagliptin manufactured by Bangladeshi pharmaceutical companies of Bangladesh were similar to innovator product as well as met the requirement of official specification [186].

4.1.1. Weight Variation Test

To check the quality control parameters of sitagliptin tablet, samples were collected from top, middle and lower ranked pharmaceutical industries of Bangladesh and then compared with the innovator (STG-1) product, Januvia manufactured by Merck and Co., USA. The percent weight variation for innovator sample was 1.01% ± 0.05 whereas for

the local product, the values varied from 0.54% ± 0.14 to 2.13% ± 0.04. All the data were near about innovator product. Minimum percent weight variation was found in STG-6 (**Table 4.1**).

The percent weight variation of vildagliptin tablet manufactured by nine pharmaceutical companies of Bangladesh were compared with the innovator sample Galvus (VLG-1) manufactured by Novartis, UK. The data of innovator sample was 1.12% ± 0.22 whereas for the local product the value varied from 1.01% ± 0.03 to 1.41% ± 0.16. All the values were similar to innovator product. Minimum percent weight variation was found in VLG-4 (**Table 4.1**).

In case of linagliptin the percent weight variation for the tablet manufactured by nine pharmaceutical companies of Bangladesh were compared with the innovator sample Trajenta (LNG-1) manufactured by Boehringer Ingelheim & Lilly, USA. The data of innovator sample was 0.99% ± 0.11 whereas for the local product the values varied from 0.44% ± 0.26 to 2.33% ± 0.42. All the found values were similar to innovator product. Minimum percentages of weight variation was found in LNG-2 (**Table 4.1**).

According to the United States Pharmacopoeia (USP), the percent weight variation should be within ±5% for tablets having average weight more than 324 mg. The tablets met the USP test as there are not more than 2 tablets outside the percentage limit and no tablets deviate twice of the percentage limit.

Table 4.1. Percent Weight Variation of Sitagliptin, Vildagliptin and Linagliptin

Sample	Weight Variation (%)*, (Mean ± %RSD)		
	STG	VLG	LNG
Sam-1 (Innovator)	1.01 ± 0.05	1.12 ± 0.22	0.99 ± 0.11
Sam-2	1.13 ± 0.04	1.01 ± 0.16	0.44 ± 0.26
Sam-3	0.99 ± 0.16	1.01 ± 0.05	1.14 ± 0.45
Sam-4	1.23 ± 0.25	1.01 ± 0.03	0.71 ± 0.13
Sam-5	1.51 ± 0.06	1.01 ± 0.09	1.55± 0.06

Sam-6	0.54 ± 0.14	1.01 ± 0.35	1.21 ± 0.11
Sam-7	0.69 ± 0.19	1.01 ± 0.12	1.83 ± 0.42
Sam-8	1.42 ± 0.21	1.41 ± 0.16	0.68 ± 0.37
Sam-9	0.86 ± 0.02	1.01 ± 0.24	1.65 ± 0.14
Sam-10	1.34 ± 0.04	1.07 ± 0.12	1.79 ± 0.22

*: 20-times replication for each brand

All brands of sitagliptin, vildagliptin and linagliptin were complied with the official specification of USP for weight variation as the percent deviations from average weight of all the tablets were within the acceptable range of ±5% [2].

4.1.2. Hardness Test

Found data of hardness test of sitagliptin tablet manufactured by pharmaceutical companies of Bangladesh were compared with the innovator (STG-1) product. Average hardness of innovator product was 5.36±0.07 which were similar to the local sitagliptin product (4.26±0.14 to 6.23±0.03 kgf). In case of vildagliptin, hardness of local products (4.86±0.07 to 6.13±0.16 kgf) were found, that were close to the innovator product (5.55±0.35 kgf). Average hardness of local product of linagliptin (4.23±0.08 to 6.25±0.12kgf) were also found similar to the innovator (5.66±0.07kgf) (**Table 4.2**).

Table 4.2. Hardness of of Sitagliptin, Vildagliptin and Linagliptin

Sample	**Hardness (Kgf)* (Mean ± %RSD)**		
	STG	**VLG**	**LNG**
Sam-1 (Innovator)	5.36±0.07	5.55±0.35	5.66±0.07
Sam-2	4.92±0.04	5.64±0.14	6.23±0.25
Sam-3	6.23±0.03	6.05±0.22	6.22±0.12
Sam-4	5.15±0.11	6.13±0.16	4.55±0.26
Sam-5	4.26±0.14	4.98±0.21	6.02±0.51

Sam-6	4.48±0.05	5.04±0.22	4.23±0.08
Sam-7	5.63±0.06	5.66±0.11	6.25±0.12
Sam-8	6.21±0.22	5.42±0.13	6.09±0.11
Sam-9	4.69±0.09	4.86±0.07	5.68±0.21
Sam-10	6.11±0.14	4.93±0.04	5.91±0.06

*: 10-times replication for each brand

All the obtained data of hardness test in sitagliptin, vildagliptin and linagliptin were complied with the official specification of USP [186].

4.1.3. Disintegration Test

The disintegration times for sitagliptin tablets were found from 0.5 ± 0.18 to 6.3 ± 0.12 min which was near to the disintegration time of innovator, januvia (3.6 ± 0.04 min). The lowest disintegration time found in STG-8. In case of vildagliptin, the disintegration time of local products were varied from 1.8 ± 0.15 to 6.4 ± 0.04 min where the disintegration time of innovator, galvus was 5.8 ± 0.08 min, and the lowest disintegration time found in VLG-5. The disintegration time of linagliptin were varied from 2.2 ± 0.06 to 5.5 ± 0.04 min, and 4.2 ± 0.02 min was the disintegration time of innovator and the lowest disintegration time found in LNG-9 (**Table 4.3**)

Table 4.3. Disintegration Time of Sitagliptin, Vildagliptin and Linagliptin

Sample	**Disintegration (Min)* (Mean ± %RSD)**		
	STG	**VLG**	**LNG**
Sam-1 (Innovator)	3.6 ± 0.04	5.8 ± 0.08	4.2 ± 0.02
Sam-2	6.3 ± 0.12	5.6 ± 0.11	2.5 ± 0.03
Sam-3	4.5 ± 0.06	2.5 ± 0.06	5.5 ± 0.04
Sam-4	5.6 ± 0.14	6.4 ± 0.04	3.2 ± 0.11
Sam-5	6.0 ± 0.25	1.8 ± 0.15	4.0 ± 0.05
Sam-6	6.4 ± 0.02	4.5 ± 0.02	2.4 ± 0.08

Sam-7	7.5 ± 0.03	6.2 ± 0.04	4.5 ± 0.04
Sam-8	0.5 ± 0.18	3.2 ± 0.11	3.1 ± 0.08
Sam-9	1.8 ± 0.07	5.5 ± 0.07	2.2 ± 0.06
Sam-10	3.2 ± 0.04	4.4 ± 0.03	3.2 ± 0.04

*: 6-times replication for each brand

According to USP specification, film coated tablets should disintegrate within 30 min [188]. So, all the samples of sitagliptin, vildagliptin and linagliptin were complied with the official specification.

4.1.4. Potency Test

The potency of sitagliptin were found within the range of 95.30 ± 0.03% to 99.25± 0.08 % and in case of vildagliptin, the potency varied from 95.55 ± 0.06 to 99.68± 0.03% and the potency of linagliptin were 95.20 ± 0.14 to 100.2 ± 0.02% (**Table 4.4**).

Table 4.4. Potency of Sitagliptin, Vildagliptin and Linagliptin

Sample	**Potency (%)* (Mean ± %RSD)**		
	STG	**VLG**	**LNG**
Sam-1 (Innovator)	99.25± 0.08	99.68± 0.03	99.85± 0.08
Sam-2	96.84± 0.04	96.84± 0.18	98.25 ± 0.04
Sam-3	96.12 ± 0.06	97.29 ± 0.04	96.75 ± 0.03
Sam-4	95.30 ± 0.03	96.35 ± 0.09	100.2 ± 0.02
Sam-5	97.62 ± 0.01	97.62 ± 0.07	97.36 ± 0.07
Sam-6	96.52 ± 0.08	98.21 ± 0.05	95.30 ± 0.09
Sam-7	98.21 ± 0.19	98.41 ± 0.09	96.25 ± 0.12
Sam-8	100.2 ± 0.11	95.80 ± 0.12	97.38 ± 0.03
Sam-9	97.42 ± 0.03	96.38 ± 0.04	95.20 ± 0.14
Sam-10	96.66 ± 0.16	95.55 ± 0.06	98.25± 0.04

*: 3-times replication for each brand.

So, all the brands showed potency within the range of (95-105) % of labeled amount of drug and complied according to USP [188].

4.1.5. Dissolution Test

The dissolution profiles of sitagliptin, vildagliptin and linagliptin was described in **Table 4.5, 4.6, 4.7** and **Figure 4.1, 4.2, 4.3**, respectively. The percent release of sitagliptin in 0.1N HCl were found 89.21% to 104.5%; in acetate buffer 81.20% to 95.54%; in phosphate buffer 85.56% to 100.5%; and in distilled water 93.21% to 104.5%. (**Table4.5**). The found percent release of vildagliptin in 0.1N HCl were found 91.56% to 104.1%; in acetate buffer 79.56% to 92.32%; in phosphate buffer 88.56% to 101.2%; and in distilled water 93.11% to 104.2% (**Table 4.6**). Linagliptin showed dissolution in 0.1N HCl, 95.40% to 106.8%; in acetate buffer 94.65% to 107.3%; in phosphate buffer 96.54% to 109.5%; and in distilled water 98.25% to 108.6 % (**Table 4.7**) after 45 min. The result demonstrate in all tablets fulfilled the general requirements of USP [188].

Table 4.5. Percent Dissolution Studies of Sitagliptin in Four Different Media

Disso. Media	Time (min)	Drug Dissolved (%)									
		STG-1	STG-2	STG-3	STG-4	STG-5	STG-6	STG-7	STG-8	STG-9	STG-10
0.1N HCl	10	39.25	65.37	74.12	76.04	78.27	63.26	54.19	48.63	75.82	38.21
	20	56.22	72.39	82.56	84.06	88.45	75.88	65.20	69.54	84.22	62.50
	30	84.21	86.41	90.45	94.20	96.21	84.56	88.14	82.88	90.21	79.08
	45	95.56	94.50	99.81	100.6	104.5	97.63	92.56	90.56	104.1	89.21
Acetate Buffer (pH 4.6)	10	28.20	35.41	29.56	38.80	43.51	29.22	34.63	41.25	52.21	36.64
	20	62.41	58.63	46.72	54.41	62.28	59.84	47.84	59.66	64.51	48.88
	30	81.03	76.57	65.88	75.68	84.56	76.19	68.78	76.43	74.32	74.24
	45	95.54	92.56	90.04	91.55	92.25	88.56	85.63	86.49	92.65	81.20
Phosphate Buffer (pH 6.8)	10	34.25	71.16	64.20	58.78	47.71	75.48	62.72	56.82	77.54	66.84
	20	56.62	80.24	78.35	78.85	71.54	84.45	76.58	62.33	86.57	74.21
	30	88.04	90.11	86.52	89.53	82.42	92.56	81.24	80.28	90.18	80.29
	45	100.2	96.34	98.24	98.56	90.44	100.5	94.58	92.44	99.24	85.56
Distilled Water	10	36.56	68.84	74.59	80.21	62.28	74.51	69.95	48.55	71.24	54.53
	20	59.34	75.56	85.24	89.56	74.81	86.55	78.98	67.14	84.21	66.28
	30	75.42	89.24	93.61	96.24	88.52	94.16	85.46	82.08	95.17	88.45
	45	98.56	97.89	101.2	104.5	96.16	103.8	97.15	93.65	101.6	93.21

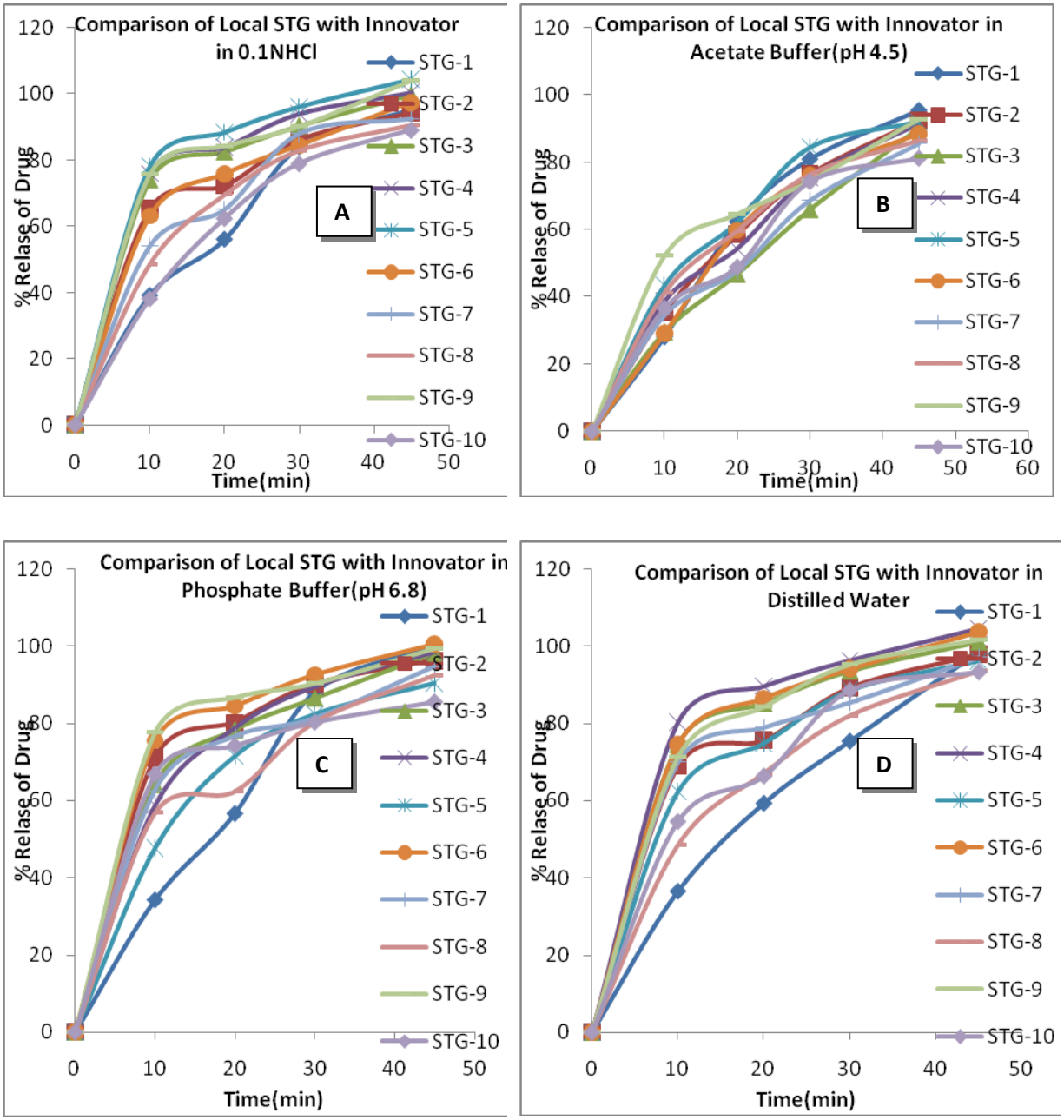
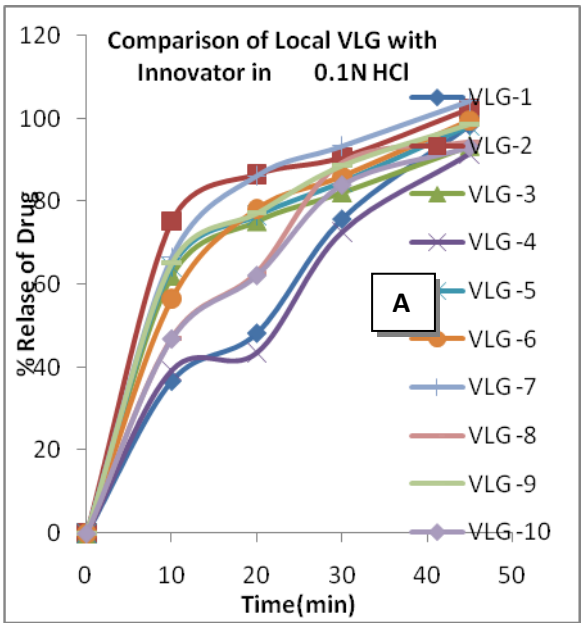
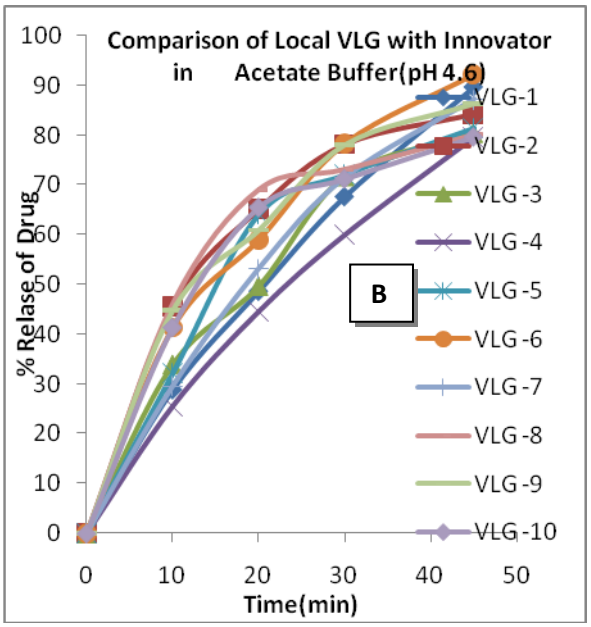


Figure 4.1. Dissolution Profile of STG in 0.1N HCl (A), Acetate Buffer (pH 4.6) (B), Phosphate Buffer (pH 6.8) (C) and Distilled Water (D).

Table 4.6. Percent Dissolution of Vildagliptin in Four Different Media

Disso. Medium	Time (min)	Drug Dissolved (%)									
		VLG-1	VLG-2	VLG-3	VLG-4	VLG-5	VLG-6	VLG-7	VLG-8	VLG-9	VLG-10
0.1N HCl	10	36.69	75.21	62.14	39.36	64.20	56.61	66.55	47.09	65.04	46.92
	20	48.25	86.53	75.26	43.59	76.52	78.27	86.14	62.91	77.06	62.22
	30	75.69	90.65	82.21	72.65	84.66	86.20	93.25	89.27	88.52	84.05
	45	98.25	102.3	93.46	91.56	98.25	99.65	104.1	94.33	98.51	93.14
Acetate Buffer (pH 4.6)	10	28.86	45.65	33.89	25.62	32.20	41.36	29.65	46.25	44.82	41.33
	20	48.62	65.20	49.65	44.66	64.11	59.08	53.21	69.15	60.95	65.42
	30	67.59	78.26	71.64	60.08	72.09	78.43	71.47	73.20	78.13	71.15
	45	89.56	84.35	80.29	79.68	81.45	92.32	87.21	80.16	86.33	79.56
Phosphate Buffer (pH 6.8)	10	32.25	65.22	56.24	71.09	41.22	66.14	72.56	48.20	61.54	52.23
	20	53.64	81.91	65.21	84.22	59.66	84.21	88.54	59.33	79.58	75.54
	30	75.66	90.01	86.22	90.11	75.24	92.14	91.20	75.42	86.62	83.69
	45	95.68	99.56	100.2	95.42	88.56	101.2	100.5	90.80	103.4	91.99
Distilled Water	10	46.55	65.24	72.06	54.24	65.53	75.41	68.02	49.65	78.51	68.58
	20	65.88	78.63	84.59	72.20	84.51	81.53	76.82	65.10	86.08	81.10
	30	86.32	89.95	91.24	86.12	90.16	92.50	85.47	89.22	94.17	89.24
	45	100.2	94.56	100.2	94.31	96.31	98.52	99.04	93.11	104.2	95.62





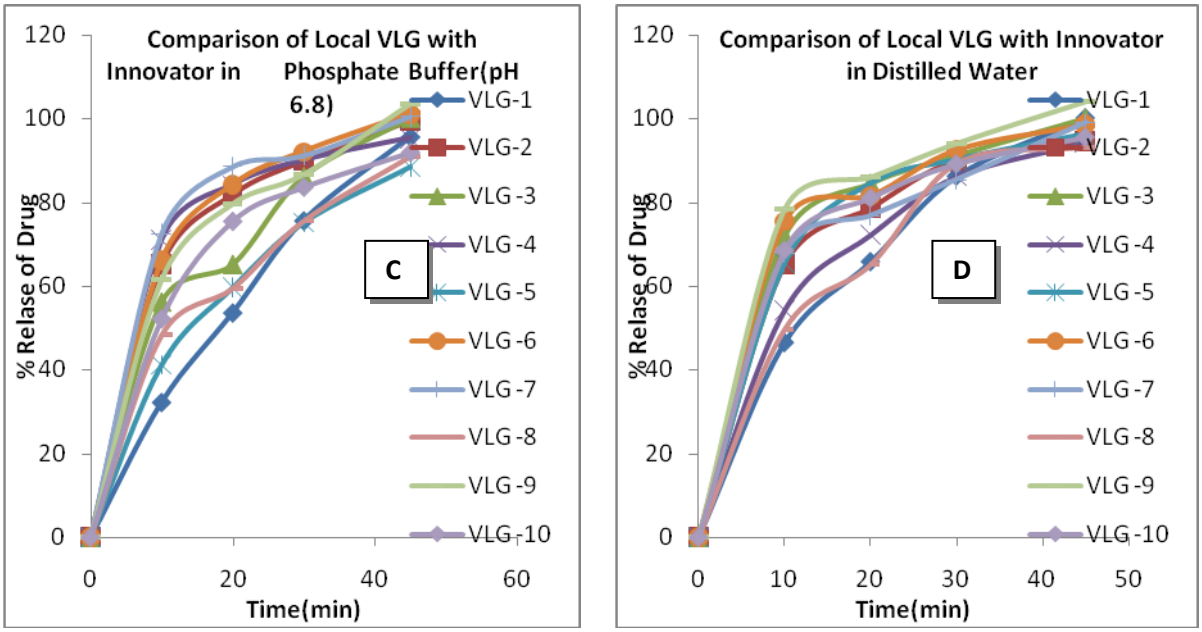


Figure 4.2. Dissolution Profile of VLG In 0.1N HCl (A), Acetate Buffer (pH 4.6) (B), Phosphate Buffer (pH 6.8) (C) and Distilled Water (D).

Table 4.7. Percent Dissolution of Linagliptin in Four Different Media

Disso. Media	Time (min)	Drug Dissolved (%)									
		LNG-1	LNG-2	LNG-3	LNG-4	LNG-5	LNG-6	LNG-7	LNG-8	LNG-9	LNG-10
0.1N HCl	10	49.53	55.65	65.21	56.25	65.4	59.6	48.7	68.5	56.5	72.46
	20	94.56	90.25	98.86	91.8	87.5	75.5	64.3	75.5	68.2	84.59
	30	98.7	97.66	102.1	97.49	95.6	88.5	82.9	91.8	78.5	93.66
	45	102.5	106.8	104.7	104.8	103.2	106.2	95.4	104.2	99.5	100.4
Acetate Buffer (pH 4.6)	10	38.06	85.6	75.28	95.5	85.15	60.25	70.2	78.8	71.11	49.56
	20	102.3	99.5	100.2	97.62	98.2	100.2	100.5	98.25	98.2	64.25
	30	105.7	102.9	103.1	101.5	100.5	105.6	103.2	100.2	100.2	87.28
	45	107.3	106.5	105.6	105.7	105.2	109.8	108.6	106.5	108.5	94.65
Phosphate Buffer (pH 6.8)	10	53.3	82.5	75.5	65.2	72.2	84.21	73.16	86.32	92.3	58.06
	20	103.3	95.5	100.3	97.62	95.6	101.2	98.25	95.6	99.5	68.57
	30	105.5	103.8	104.1	102.5	101.2	106.3	103.2	98.26	105.3	84.69
	45	106.8	105.3	105.9	105.9	108.5	109.5	105.2	104.2	109.2	96.54
Distilled Water	10	36.45	62.3	71.85	75.23	85.24	74.1	78.21	81.62	45.29	76.45
	20	91.79	87.9	90.25	89.45	95.22	84.25	85.88	96.32	75.65	89.25
	30	105.4	100.9	101.6	100.1	102.5	95.61	100.2	105.2	85.1	96.24
	45	107.1	105.8	106.8	105.9	105.3	106.5	108.6	108.5	98.25	102.1

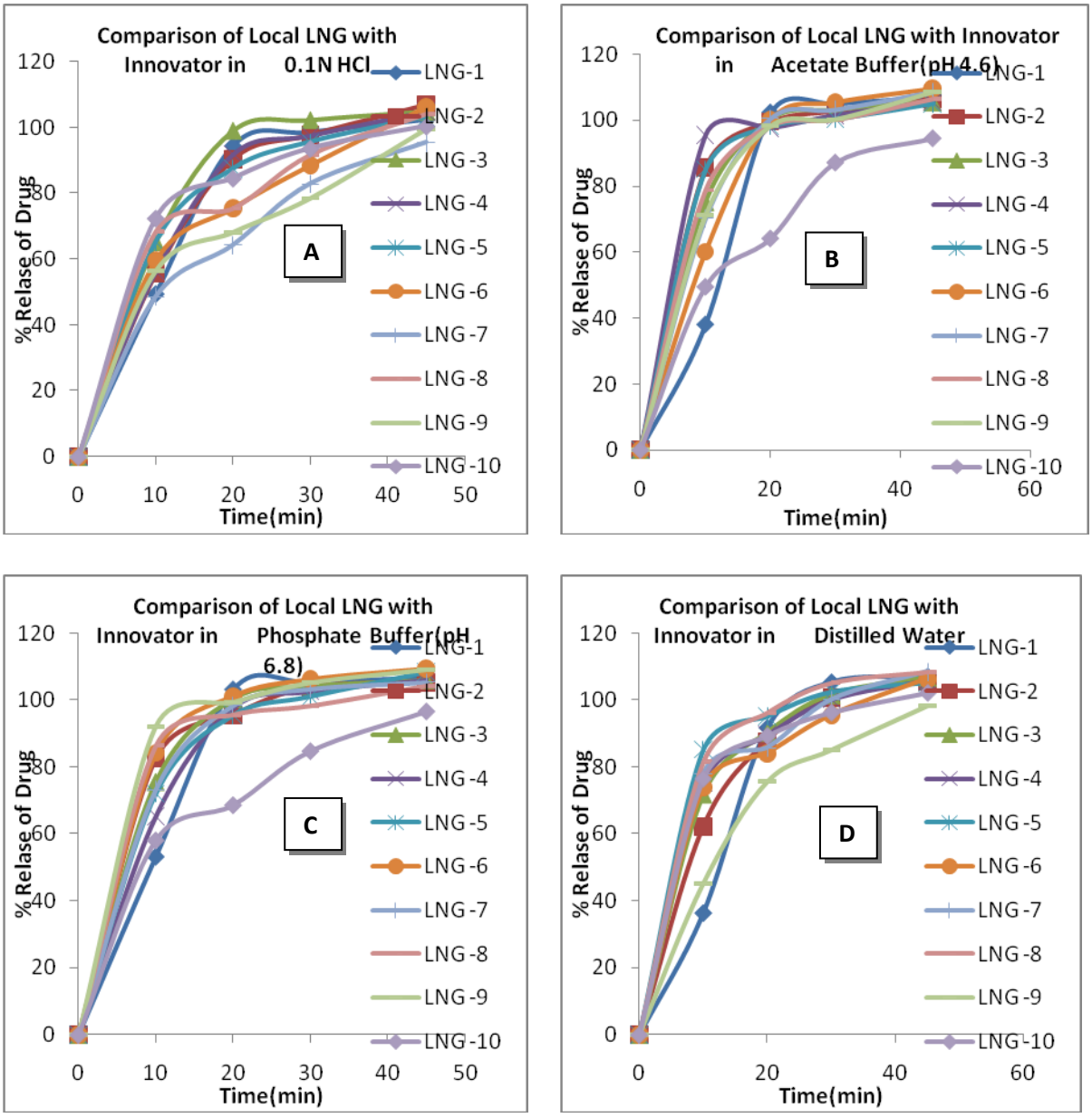


Figure 4.3. Dissolution Profile of LNG in 0.1N HCl (A), Acetate Buffer (pH 4.6)(B), Phosphate Buffer (pH 6.8) (C) and Distilled Water (D).

4.1.6. Comparison of Dissolution Data

To compare the dissolution profile, difference factor (f1) and similarity factor (f2) were calculated which has been adopted by FDA and the European Agency for the evaluation of medicinal products by the committee for proprietary medicinal products (CPMP). According to the FDA guidance [189], dissolution profiles are similar if f1 values are between 0 and 15 while f2 values are between 50 and 100.

The calculated f1 and f2 values of STG in brand 2, 4, 5, 6, 7, 8 and 10 in different dissolution medium were found within the above requirements thus it can be said that these brands are similar and bioequivalent to innovator product in respect of *in vitro* drug release pattern. The other brands (3,9) were also very close to these values (**Table 4.8**).

Table 4.8. Difference Factor (f1) and Similarity Factor (f2) of Sitagliptin

Sample	0.1N HCl		Acetate Buffer (pH=4.5)		Phosphate Buffer (pH=6.8)		Distilled Water	
	f1	f2	f1	f2	f1	f2	f1	f2
STG-2	17	41	**7**	**65**	24	33	23	36
STG-3	26	33	14	47	20	37	31	30
STG-4	31	29	**10**	**56**	18	39	37	27
STG-5	33	29	**8**	**55**	16	47	21	39
STG-6	17	40	**6**	**67**	26	30	33	30
STG-7	**11**	**52**	16	47	22	37	24	35
STG-8	**11**	**53**	**11**	**54**	16	44	**12**	**54**
STG-9	29	31	13	45	27	29	31	31
STG-10	**7**	**64**	16	47	26	35	16	46

Marked values meet the requirement

The calculated f1 and f2 values of VLG in brand 3, 4, 5, 6, 7, 8 and 10 in different dissolution medium were found within the above requirements. So, these brands are similar and bioequivalent to innovator brand in respect of *in vitro* drug release pattern (**Table 4.9**). The other brands (2, 9) are also very close to these values.

Table 4.9. Difference Factor (f1) and Similarity Factor (f2) of Vildagliptin

Sample	0.1N HCl		Acetate Buffer (pH=4.5)		Phosphate Buffer (pH=6.8)		Distilled Water	
	f1	f2	f1	f2	f1	f2	f1	f2
VLG-2	26	35	21	44	31	32	14	46
VLG-3	25	36	**8**	**62**	20	42	16	40
VLG-4	**10**	**53**	19	41	33	29	**7**	**62**
VLG-5	18	40	**13**	52	9	59	15	43
VLG-6	17	42	**16**	**50**	34	31	18	39
VLG-7	27	34	**5**	**73**	37	28	12	46
VLG-8	**8**	**62**	37	30	**10**	**53**	**5**	**68**
VLG-9	20	39	18	47	29	34	21	36
VLG-10	**4**	**66**	18	46	21	40	15	43

Marked values meet the requirement

In LNG the calculated f1 and f2 values of brand 2, 3, 4, 5 and 7 in different dissolution medium were found within the requirements. Thus it can be said that these brands are similar and bioequivalent to innovator brand in respect of *in vitro* drug release pattern (**Table 4.10**). The other brands (6, 8, 9, and 10) are also very close to these values.

Table 4.10. Difference Factor (f1) and Similarity Factor (f2) of Linagliptin

Sample	0.1N HCl		Acetate Buffer (pH=4.5)		Phosphate Buffer (pH=6.8)		Distilled Water	
	f1	f2	f1	f2	f1	f2	f1	f2
LNG-2	**5**	**68**	15	31	11	41	10	44
LNG-3	**7**	**54**	12	36	7	47	12	37
LNG-4	**4**	**70**	19	27	**6**	**58**	14	35
LNG-5	**8**	**53**	16	31	**9**	**51**	17	31
LNG-6	12	46	8	47	10	40	16	35
LNG-7	16	38	11	40	**8**	**50**	16	34
LNG-8	14	43	14	34	14	38	15	32
LNG-9	16	38	12	39	12	35	16	42
LNG-10	12	45	23	32	19	34	17	34

Marked values meet the requirement

Overall qualities of the manufactured drugs by Bangladeshi pharmaceutical companies are satisfactory. Most of the drugs fulfill the global requirements though there are some brands which remain in the border line of specification. Such type of study helps to reduce counterfeit or sub-standard medication by checking the quality parameters.

4.2. Method Development and Optimization by Applying Quality by Design (Qbd) Approach

4.2.1. Qbd Approach for Method Development

Quality by design (QbD) approaches were used for the method development and optimization where design of experiment (DoE) used 3^3 full factorial Box -Behnken experimental design (BBD) model. This BBD model suggested 30 runs to conduct this method development and optimization process.

4.2.2. Evaluation of Model Response-1(Retention Time-RT)

Response 1 is the retention time of peak 2, i.e. peak of linagliptin. According to the suggestion of BBD, from 30 runs RT values were found between 2.291 min and 11.079

min. For the optimization of RT values, quadratic equation of RT **(Eq-13)** described the relationship of three independent variables with retention time of linagliptin.

Quadratic Equation of Model Responses-1(RT)

$$RT = +5.62 - 2.15*A - 0.88*B + 1.20*C + 0.41*AB - 1.03*AC + 0.033*BC + 1.04*A^2 + 0.033*B^2 - 0.99*C^2 \quad \textbf{Eq.(13)}$$

Table 4.11. ANOVA for Response Surface Quadratic Model

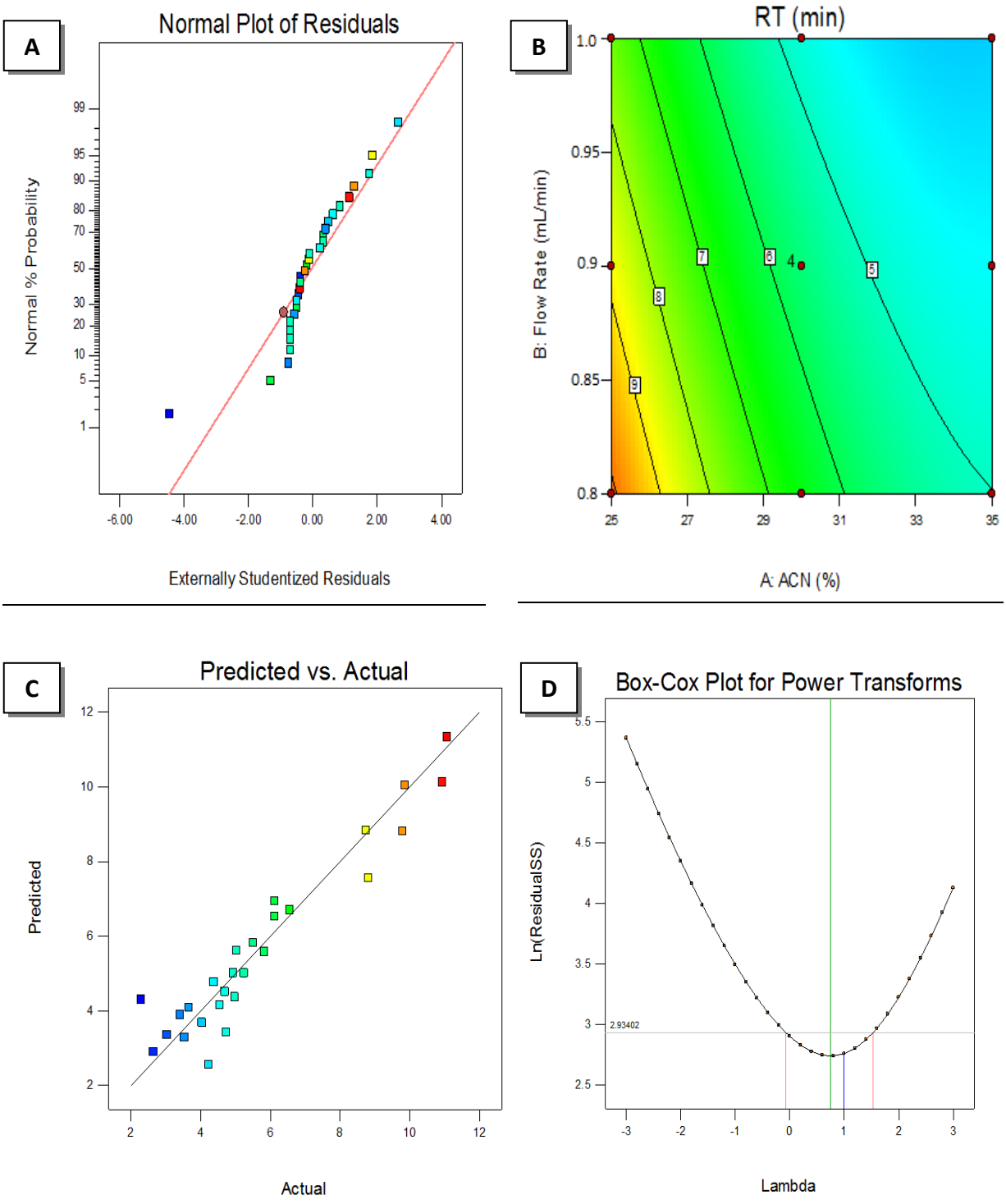
Source	Sum of squares	Degrees of freedom(df)	Mean square	F-value	p-value	Remarks
Model	149.95	9	16.66	21.06	< 0.0001	**S**
A-ACN	83.03	1	83.03	104.93	< 0.0001	**S**
B-Flow Rate	13.81	1	13.81	17.45	0.0005	**S**
C-Buffer pH	25.82	1	25.82	32.63	< 0.0001	**S**
AB	2.01	1	2.01	2.55	0.0003	**S**
AC	12.84	1	12.84	16.23	0.0007	**S**
BC	0.012	1	0.012	0.015	0.9046	**NS**
A^2	7.47	1	7.47	9.44	0.0030	**S**
B^2	7.534E-003	1	7.534E-003	9.520E-003	0.9232	**NS**
C^2	6.73	1	6.73	8.50	0.0026	**NS**
Residual	15.83	20	0.79			
Lack of Fit	15.83	17	0.93			
Pure Error	0.000	3	0.000			
Cor Total	165.78	29				

S: Significant, **NS**: Not significant

The model F-value of 21.06 implies the model is significant. There was only 0.01% chance that an F-value was larger that could occur due to noise. Probability values of F less than 0.0500 indicated the model terms significant. In this case A, B, C, AC, A^2, C^2 were significant model terms. Values greater than 0.1000 indicated the model terms insignificant (**Table 4.11**).

4.2.3. Graphical Representation of Effects Of Variables on Retention Time(RT)

The model was examined using Lack of Fit test, which indicated insignificant lack of fit value corresponding with higher p-value as compared to the model F-value. Additionally, Normal plot of residual indicated all the data were concentrated along the model fit line and only one observable value was remain in outlier in the data (**Figure 4.4-A**).



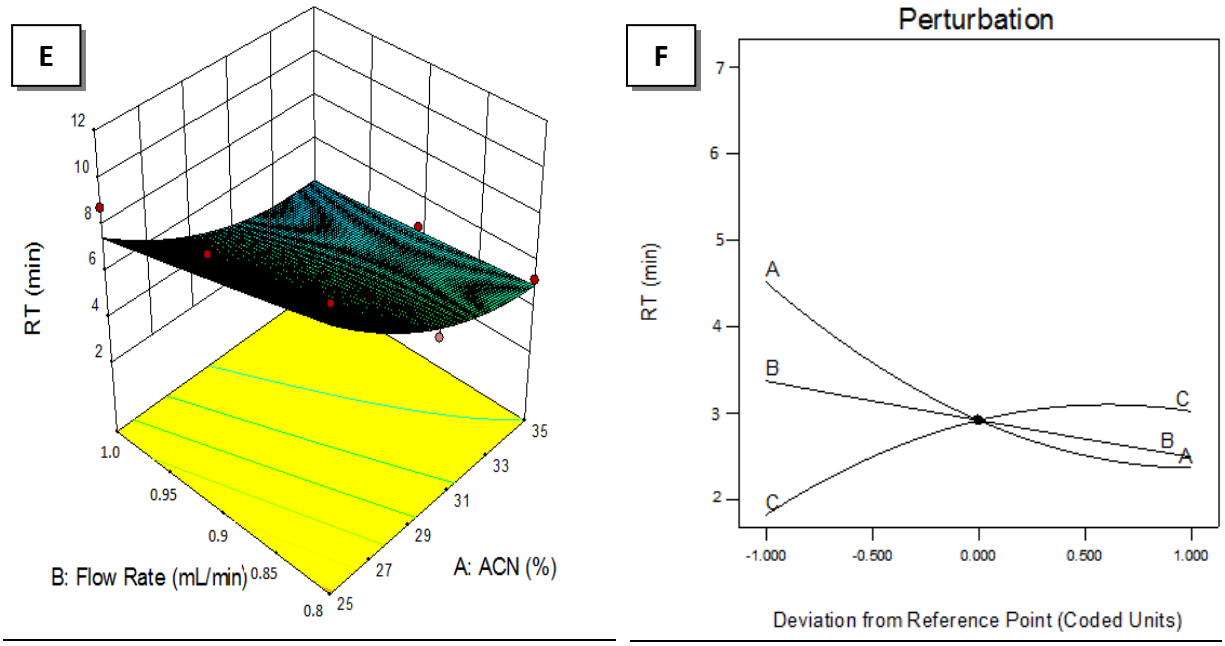


Figure 4.4. (A)-Normal plot of residual,(B)- Counter Plot, (C)-Predicted vs. Actual plot, (D)- Box-Cox plot for power transform, (E)-3D response surfaces effect (F)-Perturbation plot of R1(RT).

4.2.4. Evaluation of Model Response-2(Resolution1-Rs1)

Model rsponse-2 (RS1) is the resolution between the peak vildagliptin and linagliptin. From 30 runs, RS-1 values were obtained within 1.42 to 21.28. Surface linear model described the relationship of independent variables with response-2 (Rs-1) through the equation of RS1 (**Eq-14**).

Surface Linear Model Equation of Responses-2 (Rs1)

$$Rs1 = +11.25 - 4.54*A + 0.017*B + 2.44*C \quad \textbf{Eq.(14)}$$

The ANOVA result was described in **Table 4.12** where the model F-value of 10.39 implies the model is significant. There is only a 0.01% chance that an F-value was larger that could occur due to noise. Probability values of F less than 0.0500 indicate model terms are significant. In this case A, B, C are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

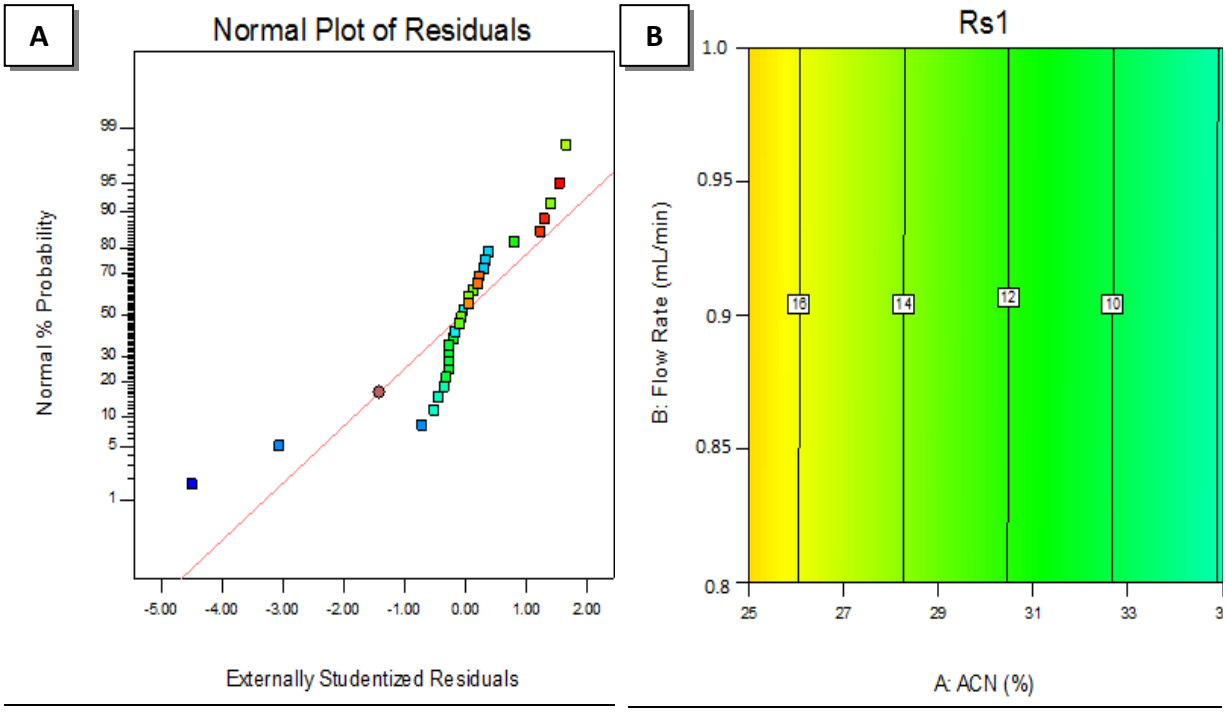
Table 4.12. ANOVA for Response Surface Linear model

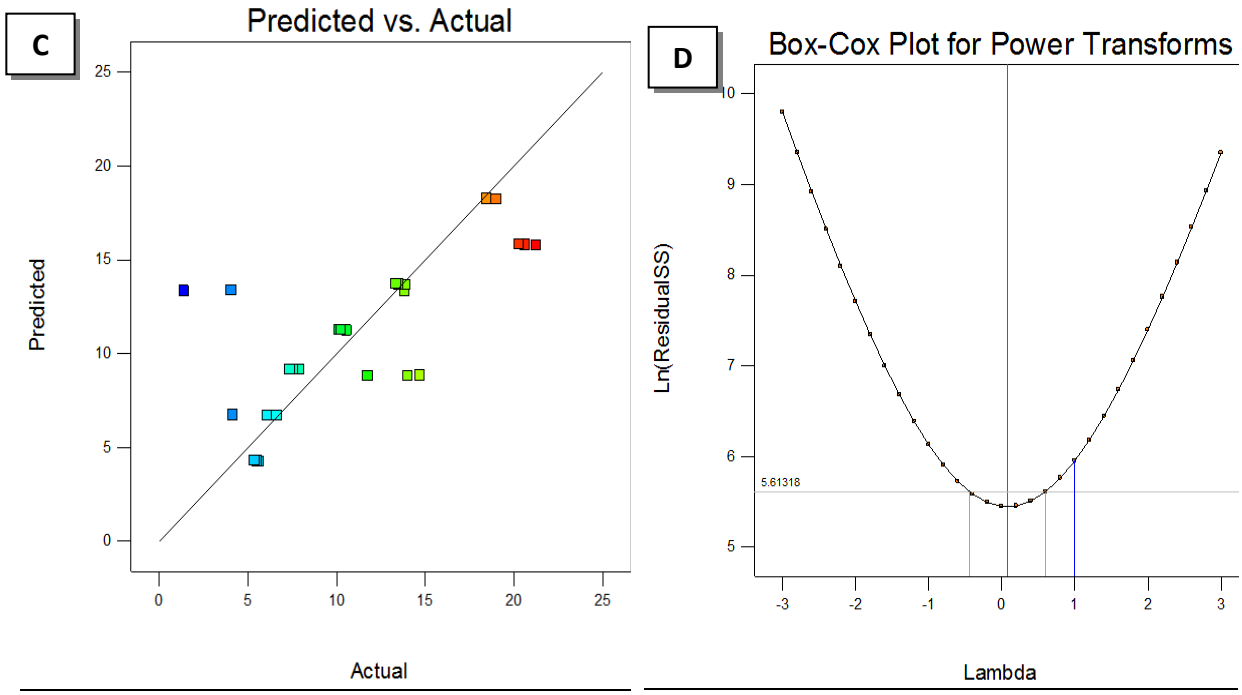
Source	Sum of squares	Degrees of freedom(df)	Mean square	F-value	p-value	Remarks
Model	477.75	3	159.25	10.39	< 0.0001	**S**
A-ACN	370.92	1	370.92	24.19	< 0.0001	**S**
B-Flow Rate	5.000E-003	1	5.000E-003	3.261E-004	0.0037	**S**
C-Buffer pH	106.82	1	106.82	6.97	0.0013	**S**
Residual	398.60	26	15.33			
Lack of Fit	398.60	23	17.33			
Pure Error	0.000	3	0.000			
Cor Total	876.35	29				

S: Significant

4.2.5. Graphical Representation of Effects of Different Variables on Resolution-1(Rs1)

From the graphical data (**Figure 4.5**) the model was examined using Lack of Fit test, which indicated insignificant lack of fit value corresponding with higher p-value as compared to the model F-value. Additionally, normal plot of residual indicated most of the data were concentrated along the model fit line and there were only two observable values were remain in outlier in data (**Figure 4.5-A**).


A
Normal Plot of Residuals
Normal % Probability
99
95
90
80
70
50
30
20
10
5
1
-5.00
-4.00
-3.00
-2.00
-1.00
0.00
1.00
2.00
Externally Studentized Residuals
B
Rs1
B: Flow Rate (mL/min)
1.0
0.95
0.9
0.85
0.8
16
14
12
10
25
27
29
31
33
A: ACN (%)


C
Predicted vs. Actual
Predicted
25
20
15
10
5
0
0
5
10
15
20
25
Actual
D
Box-Cox Plot for Power Transforms
Ln(ResidualSS)
10
9
8
7
6
5
5.61318
-3
-2
-1
0
1
2
3
Lambda

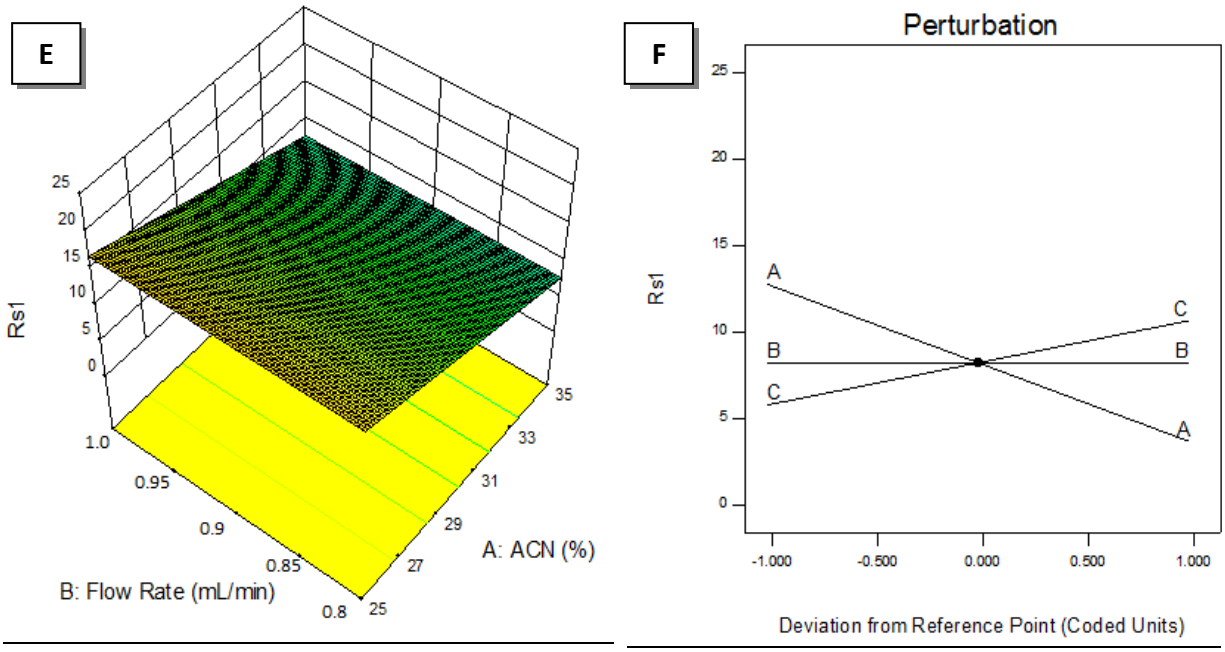


Figure 4.5. (A)-Normal Plot of Residual, (B)- Counter Plot, (C)-Predicted Vs. Actual Plot, (D)- Box-Cox Plot for Power Transform, (E)-3D Response Surfaces Effect (F)-Perturbation Plot of R2 (Rs1)

4.2.6. Evaluation of Model Response-3 (Resolution 2-Rs2)

Model response-3(Rs-2) is the resolution between the peak linagliptin and sitagliptin. From 30 runs, obtained Rs-2 values were within 1.42 to 18.04. Response surface model described the relationship of three independent variables with response-3 (Rs2) through the equation of Rs2 (**Eq-15**).

Response Surface Model Equation of Response-3(Rs2)

Rs2= + 5.75-1.01*A-0.14*B+1.17*C+4.167E-003*AB+2.49*AC-0.033*BC.....**Eq. (15)**

From **Table 4.13** the Model F-value of 7.17 implies the model is significant relative to the noise. There is a 35.78 % chance that an F-value was larger that could occur due to noise. Probability values of F less than 0.0500 indicate model terms are significant. Values greater than 0.1000 indicate the model terms as not significant.

Table 4.13. ANOVA Result for Response Surface Model

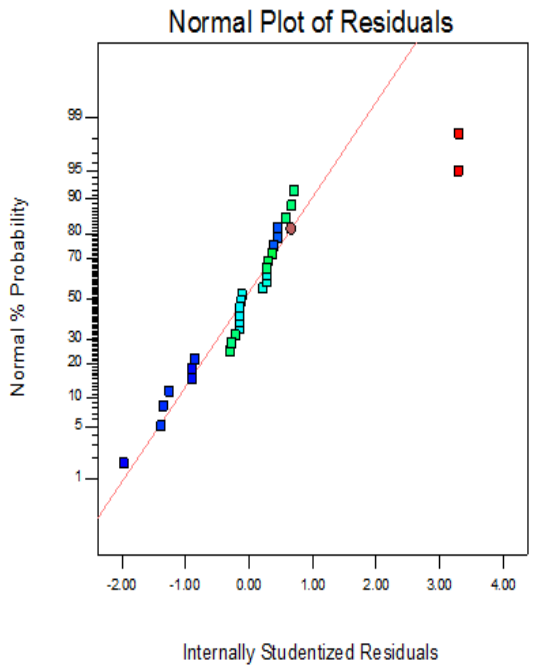
Source	Sum of Squares	Degrees of freedom(df)	Mean Square	F -value	p-value	Remarks
Model	117.37	6	19.56	7.17	0.003	**S**
A-ACN	18.32	1	18.32	4.09	0.0306	**S**
B-Flow Rate	0.33	1	0.33	6.020	0.0495	**S**
C-Buffer pH	24.50	1	24.50	8.46	0.0239	**S**
AB	2.083E-004	1	2.083E-004	1.242E-005	0.0472	**S**
AC	74.20	1	74.20	4.43	0.0465	**S**
BC	0.013	1	0.013	7.952E-004	0.0277	**S**
Residual	385.65	23	16.77			
Lack of Fit	385.65	20	19.28			
Pure Error	0.000	3	0.000			
Cor Total	503.02	29				

S: Significant

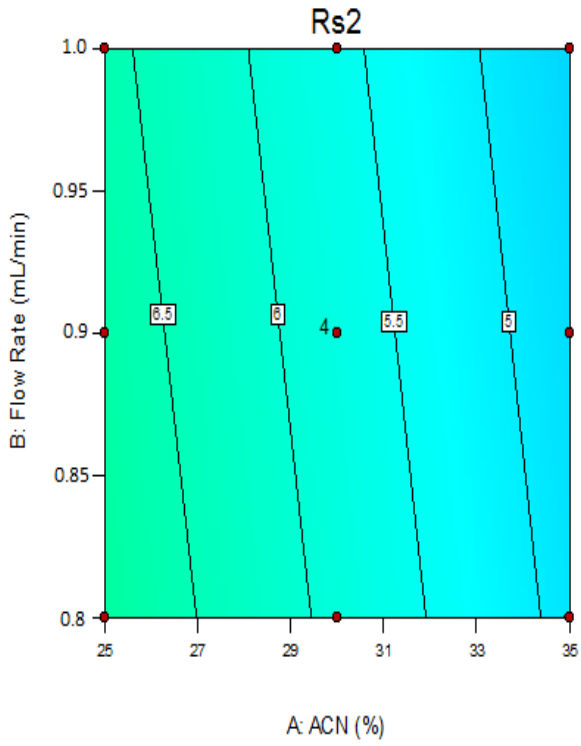
4.2.7. Graphical Representation of Effects of Different Variables on Resolution-2(Rs2)

From the graphical data (**Figure 4.6**) the model was examined using Lack of Fit test, which indicated insignificant lack of fit value corresponding with higher p-value as compared to the model F-value. Additionally, normal plot of residual indicated all the data were concentrated along the model fit line and there was no observable outlier in the data (**Figure 4.6-A**).

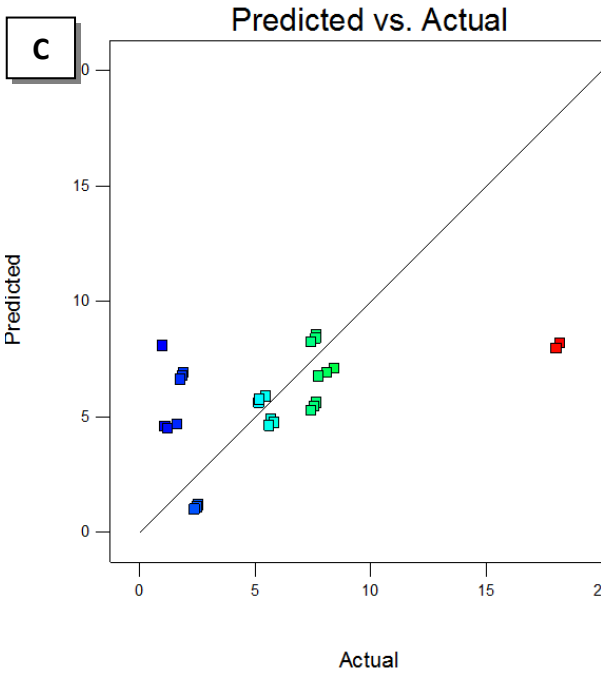
A


Normal Plot of Residuals
Normal % Probability
99
95
90
80
70
50
30
20
10
5
1
-2.00
-1.00
0.00
1.00
2.00
3.00
4.00
Internally Studentized Residuals

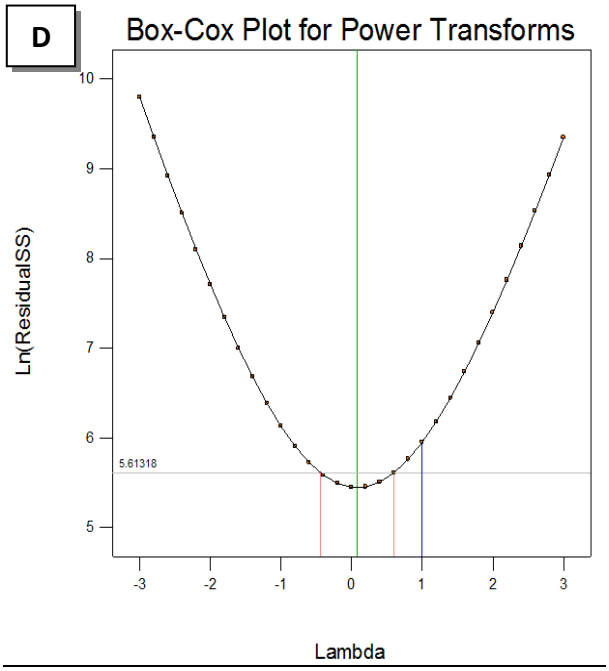
B


Rs2
1.0
0.95
0.9
0.85
0.8
B: Flow Rate (mL/min)
6.5
6
4
5.5
5
25
27
29
31
33
35
A: ACN (%)

C


Predicted vs. Actual
Predicted
0
15
10
5
0
0
5
10
15
Actual

D


Box-Cox Plot for Power Transforms
Ln(ResidualSS)
10
9
8
7
6
5
5.61318
-3
-2
-1
0
1
2
3
Lambda

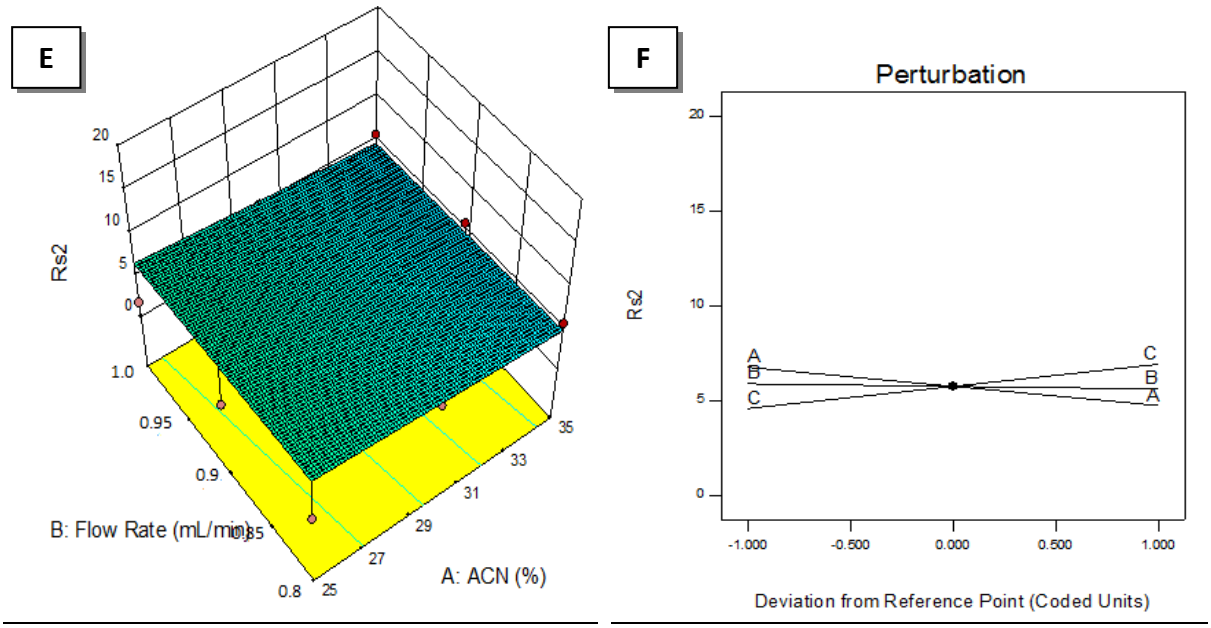


Figure 4.6. (A)-Normal Plot of Residual, (B)- Counter Plot, (C)-Predicted Vs. Actual Plot, (D)- Box-Cox Plot for Power Transform, (E)-3D Response Surfaces Effect (F)-Perturbation Plot of R3(Rs2)

4.2.8. Predicted Vs. Adjusted R-Squared Values:

The predicted R-squared for all responses R1 (0.7461), R2 (0.3363) and R3 (0.7977) are in reasonable agreement with the adjusted R-squared values of 0.8616, 0.4927 and 0.9466, respectively i.e. the difference was less than 0.2 in each case. The signal to noise ratio was measured by the adequate precision. The ratio of 17.10, 9.781 and 5.827 indicate an adequate signal (ratio > 4.0). These models can be used to navigate the design space (**Table 4.14**).

Table 4.14. Predicted Vs. Adjusted R-Squared Values for Response R1, R2 and R3

Response	R1(RT)	R2(Rs1)	R3(Rs2)
Std. Dev.	0.89	3.92	4.09
Mean	5.67	11.25	5.75
C.V. %	15.70	34.79	71.19
Press	42.09	581.59	979.17
R-Squared	0.9045	0.5452	0.6333
Adjusted R-Squared	0.8616	0.4927	0.9466
Predicted R-Squared	0.7461	0.3363	0.7977
Adequate Precision	17.100	9.781	5.827

According to the 3D response surfaces and quadratic model equation as well as the graph of perturbation, it is observed that variables A and B both have negative effect and the variable C has positive effect on RT (**Figure 4.4**), and the variable C has positive effect, A has negative effect and B has no effect on both Rs1 (**Figure 4.5**) and Rs2 (**Figure 4.6**). It shows that the relationship between factors and response is not always linear, when one or more than one factor is altered simultaneously then a factor can result in different grade of responses. The statistical results for RT, Rs1 and Rs2 indicated that the analytical method was robust since variations in the experimental conditions did not affect on the quantitative analysis of sitagliptin, vildagliptin and linagliptin.

The quadratic effect of different variables like percentages of acetonitrile in mobile phase ($p < 0.0001$), flow rate ($p < 0.0001$) and pH of buffer ($p< 0.003$) separately as well as in interaction was most significant on retention time (RT), resolution between peak 1 and 2(Rs1) and resolution between peak 2 and 3 (Rs2).

4.2.9. Optimized Method

The response surfaces and quadratic model proposed 100 solutions for method optimization. The experimental results of the predicted method were found to be analogous with the suggested responses and all the results fall within the level of acceptance (NMT 2.0%) as shown in **Table 4.15**.

Table 4.15. Predicted Vs. Experimental Method

Method	%ACN	Flow Rate (ml/min)	Buffer pH	RT	RS1	RS2
Predicted level	30	1.000	6.00	3.228	8.817	7.385
Experimental	30	1.000	6.00	3.207	8.681	7.422
Deviation (%)				0.650	1.542	0.501

The desirability of the optimized factor is shown in **Figure 4.8**. The desirability values usually exist in the range of 0–1. If the value is near to zero, it means the solution of the method is not strong whereas the value toward 1 side means the solution or method is

very strong [190]. The obtained desirability value was maximum (i.e. 1) which indicates the method is highly strong.

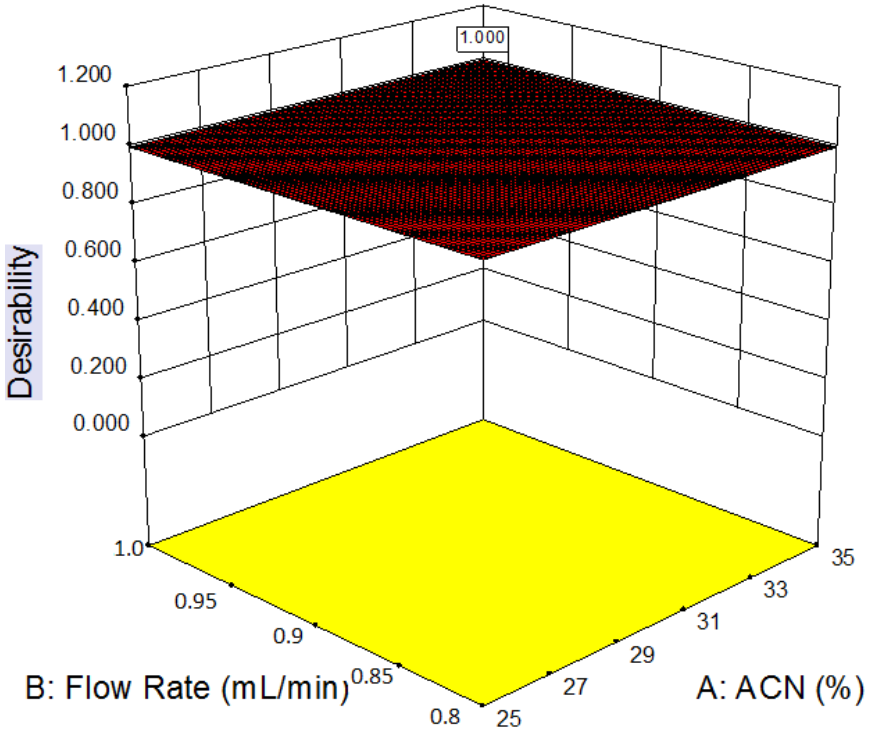


Figure 4.8. The 3D Surface Response Plot Of Desirability for Optimization of Factors.

The statistical results for retention time RT, resolution between peak of VLG and LNG (Rs1), and peak between LNG and STG (Rs2) indicated that the analytical method was robust since variations in the experimental conditions did not affect the quantitative analysis of these three compounds. The experimental results of the predicted method were found to be analogous with the suggested responses and all the results fall within the level of acceptance.

4.3. Method Validation

By applying QbD approach, an analytical method for the estimation of three prominent DPP-IV inhibitors, sitagliptin (STG), vildagliptin (VLG) and linagliptin (LNG) was developed and optimized. This optimized method is validated according to ICH Q2 (R1) guideline [191]. The parameters required to validate a method are described below-

4.3.1. System Suitability

To assess system suitability of the proposed method, peak area, tailing factor, theoretical plates, retention time of six replicate injections of standards and the resolution between peak of VLG and LNG (Rs1) and the resolution between peak of LNG and STG(Rs2) were evaluated. Percentage relative standard deviation (%RSD) of peak area and retention time were not more than 2%, values of tailing factor were less than 1.5 and theoretical plate values were 4910±0.63 to 6938±0.35. The results (Mean ± %RSD of six replicates) of the chromatographic parameters in **Table 4.16** indicating the good performance of the system.

Table 4.16. System Suitability Tests of The Proposed LC Method for the Simultaneous Determination Of VLG, LNG and STG.

Parameters	VLG	LNG	STG	Limit [10]
	(Mean ± % RSD)			
Peak area	3,071,338 ±0.34	7,875,143 ±0.26	2,116,867 ±0.41	%RSD NMT 2
Tailing factor	1.246±0.11	1.019±0.03	1.025±0.14	NMT 1.5
Theoretical plate	5302±0.51	4910±0.63	6938±0.35	NLT 2000
Retention time	2.423±0.04	3.203±0.06	4.189±0.12	%RSD NMT 0.5
RS1	10.14±0.07			NLT 1
RS2	5.18±0.05			

4.3.2. Linearity and Detection Limit

The method was linear in the range of 10-50 µg/mL for VLG and STG with correlation coefficient 0.998 and 0.999 and 0.1-1.0 µg/mL for LNG with correlation coefficient of 1.0. These values indicated the existences of good correlation between concentration and responses. The lower limit of detection (LOD) of the VLG, LNG and STG (**Figure 4.9**) were found 0.01, 0.005 and 0.06 µg/mL and limit of quantification (LOQ) were 0.05, 0.015 and 0.225 µg/mL, respectively indicating the method was highly sensitive. The linearity results are shown in **Table 4.17** and the linearity curve was shown in **Figure 4.10**.

Table 4.17. Linearity Parameters of VLG, LNG and STG

Parameters	VLG	LNG	STG
Regression Correlation Coefficient	0.998	1.00	0.999
Y-intercept	266722	47340.08	2099

Slope of Regression Line	241883	4210436.5	42127
LOD (µg/mL)	0.01	0.005	0.06
LOQ (µg/mL)	0.05	0.015	0.225

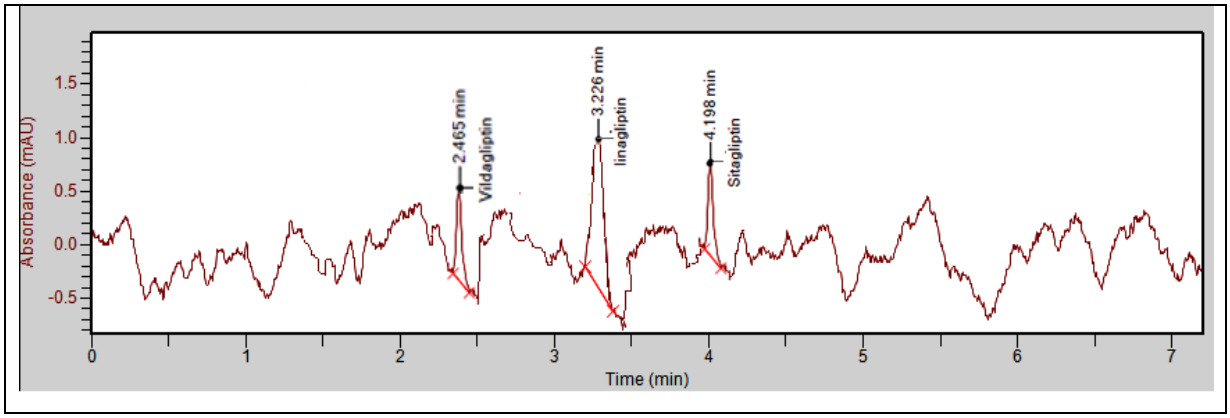
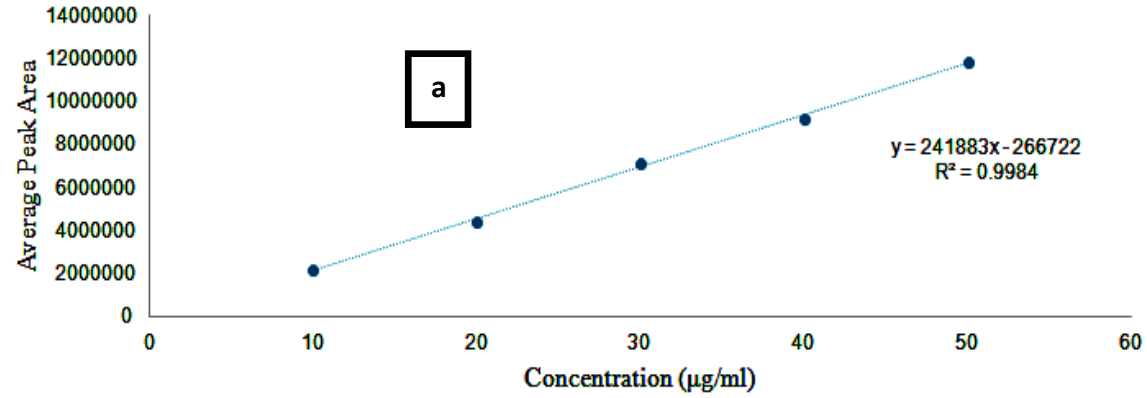
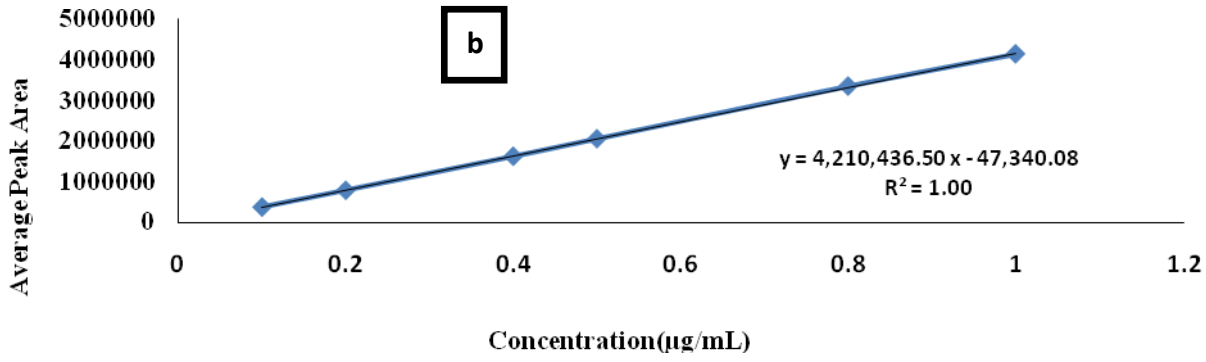


Figure 4.9. Chromatogram of LOD





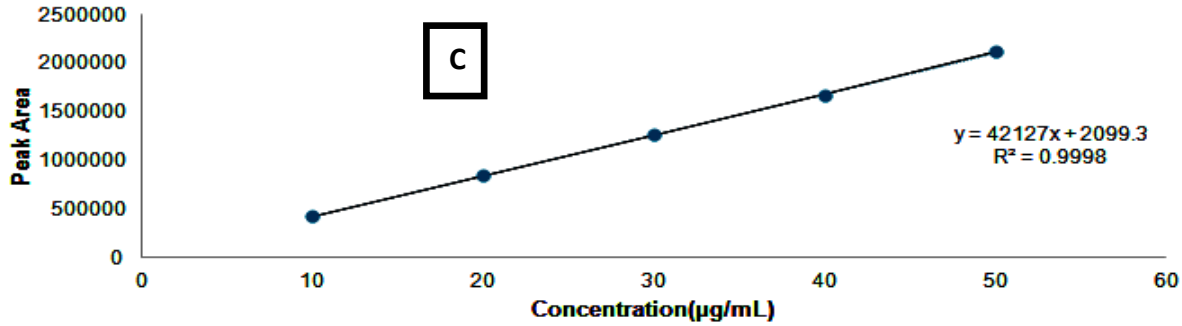
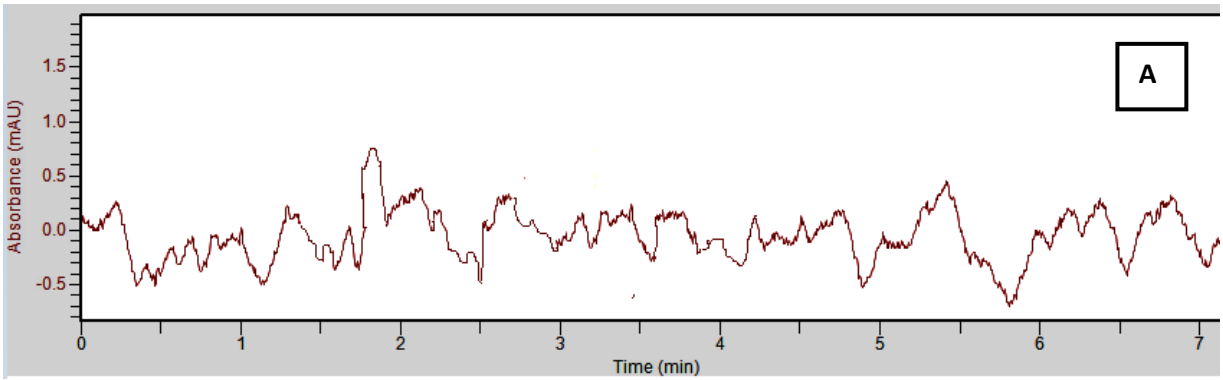


Figure 4.10. Linearity Curve Of VLG (A), LNG (B) and STG (C)

4.3.3. Specificity

The specificity of the method was established by injecting the blank and placebo (synthetic mixtures). It was observed that there is no interference of the placebo and blank with principal peaks; hence, the method was specific for these three drugs. The UV spectrum of VLG, LNG and STG were determined by PDA plus detector which showed that the peak purity values were 1.06 (VLG), 1.03 (LNG) and 1.04 (STG), and the maximum absorption wavelength were found at 246 nm (VLG), 228 nm (LNG), and 268 nm (STG) (**Figure 4.11**). In peak purity study with a photo diode detector, purity values were near about 1.0 and lower than the purity threshold (1.5) for all three analytes.



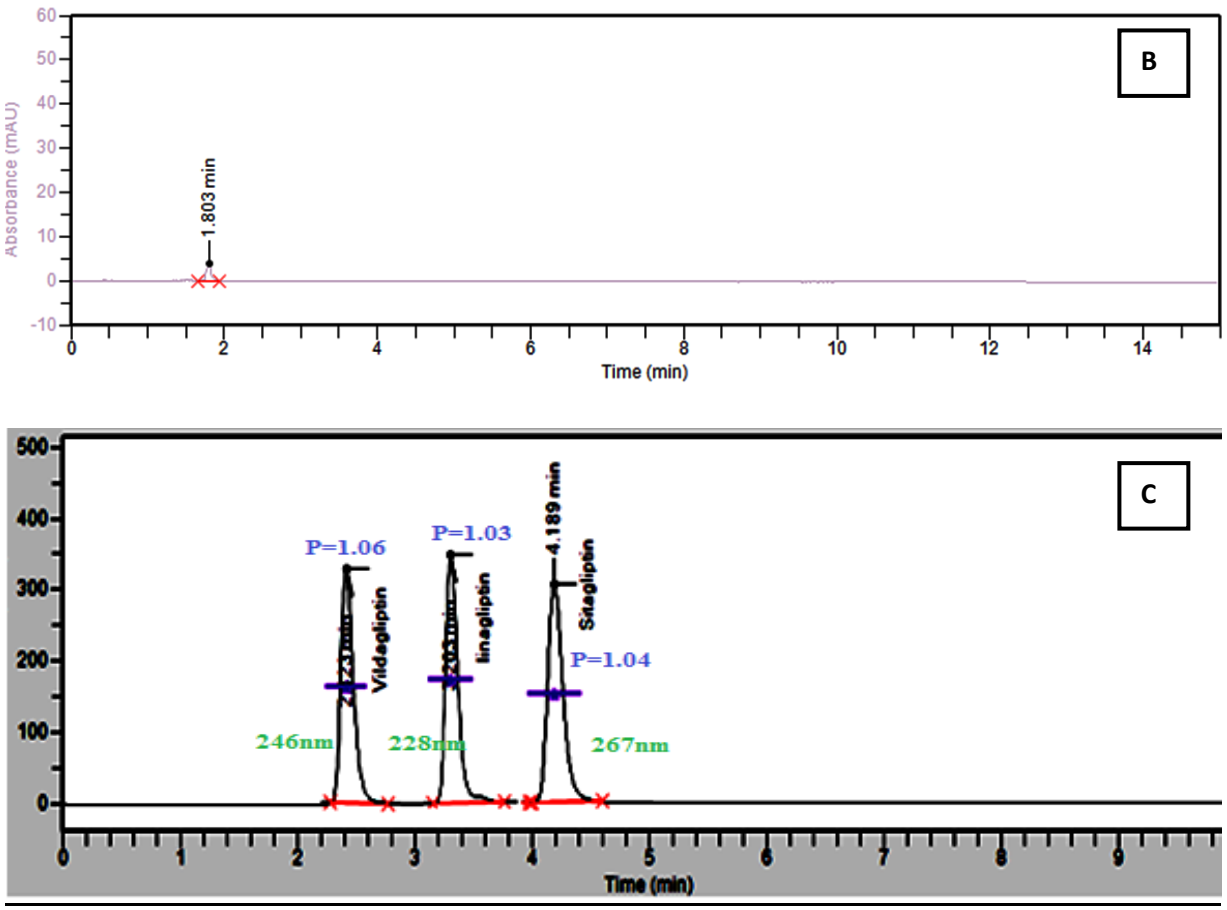


Figure 4.11. Chromatogram of Blank (A), Placebo (B) and VLG (2.423min), LNG (3.203min), STG (4.189min) (C) with peak purity and maximum wavelength.

4.3.4. Precision

The %RSD for repeatability and inter-day precision for VLG, LNG and STG were not more than 2%, which indicate the method is precise. The results of repeatability and inter-day precision are shown in **Table 4.18** and **Table 4.19**, respectably.

Table 4.18. Intermediate Precision: Repeatability

Sample	Assay (mg/tab) (Mean±%RSD)	Limit
VLG	50.05±0.03	NMT 2%
LNG	5.03±0.11	
STG	50.06±0.08	

Table 4.19. Intermediate Precision: Inter-Day Precision

Sample	Day-1	Day-2	Day-3	Day-4	Day-5	Day-6	Day-7
	Assay (mg/tab) (Mean±%RSD)						
VLG	50.05±0.06	50.11±0.04	50.07±0.13	50.04±0.11	49.97±0.09	49.85±0.15	49.69±0.24
LNG	5.10 ±0.03	5.08 ±0.08	5.04 ±0.17	5.01 ±0.22	4.99±0.14	4.96±0.32	4.93±0.18
STG	50.10±0.15	50.12±0.23	49.99±0.21	49.88±0.16	49.76±0.09	49.65±0.13	49.51±0.08

4.3.5. Accuracy or Recovery Study

The mean accuracy or % recoveries of VLG, LNG and STG were found 98.50±0.13 to 99.47±0.02%, 98.74±0.15 to 101.0±0.06% and 98.53±0.03 to 100.4±0.04% , respectively. The percent recovery studies were shown in **Table 4.20**. All the obtained values were within in compendial specification [192].

Table 4.20. Accuracy Test Data

Standard added (%)	% recovered VLG	% recovered LNG	% recovered STG
	(Mean±%RSD)		
80	98.91±0.04	99.16±0.11	99.70±0.05
90	99.47±0.02	99.54±0.13	100.4±0.04
100	98.50±0.13	101.0±0.06	98.53±0.03
110	99.12±0.09	100.6±0.24	100.2±0.03
120	99.25±0.14	98.74±0.15	99.43±0.09

4.3.6. Robustness

The variation for robustness study was performed by changing flow rate (±0.2 ml/min), pH of mobile phase (±0.2) and composition of mobile phase (±5%ACN), and %RSD NMT 2% indicated good and satisfactory robustness of the proposed method (**Table 4.21**).

Table 4.21. Robustness Study

Parameters	Variables	VLG		LNG		STG	
		Amount added	Amount detected	Amount added	Amount detected	Amount added	Amount detected
		(Mean±%RSD)					
Flow rate	0.8 ml/min	50	50.02±0.11	5	5.04±0.15	50	50.12±0.09
	1.0 ml/min	50	50.05±0.06	5	5.07±0.04	50	49.95±0.16
	1.2 ml/min	50	50.04±0.12	5	4.99±0.02	50	50.04±0.24
pH of Mobile phase	5.8	50	50.42±0.15	5	5.02±0.15	50	50.14±0.15
	6.0	50	50.56±0.11	5	4.98±0.18	50	50.86±0.19
	6.2	50	50.78±0.13	5	5.03±0.23	50	50.78±0.26
Mobile phase composition	PB:ACN =65:35	50	50.55±0.14	5	5.01±0.17	50	49.98±0.43
	PB:ACN =70:30	50	50.72±0.05	5	5.08±0.05	50	50.22±0.40
	PB:ACN =75:25	50	50.86±0.12	5	5.06±0.28	50	50.68±0.25

All the obtained values of validation were indicating that the developed and optimized method was suitable, linear, precise, accurate, and robust for the simultaneous estimation of VLG, LNG and STG in bulk and pharmaceutical dosage form.

4.4. Forced Degradation Studies

Forced degradation studies are obligatory in the development of stability-indicating and degradant-monitoring methods as part of a validation protocol. These studies also provide valuable insight in examining degradation products and plausible pathways of degradation of drug substances and products. It was carried out in five condition, i.e. acid hydrolysis, alkaline hydrolysis, oxidation, thermal and photo degradation of three

DPP-IV inhibitor (VLG, LNG and STG). The purity of drug peaks was established by purity angles.

4.4.1. Forced Degradation Studies of Vildagliptin

Applying VLG to different stress conditions and then, analysis through UHPLC indicated following degradation behavior. The percent degradation are given in **Table 4.22** and the chromatogram are shown in **Figure 4.12.**

In acidic medium (1N HCl), the degradation behavior of locally manufactured VLG were near about the innovator sample, appeared at retention time of 2.034, 3.474 min, and % degradation in all sample were NMT 13.25±0.25 %.

In alkaline condition (1N NaOH), degradants of VLG was found at the retention time of 3.988, 5.179, 8.634 min and the % degradation were found NMT 14.25±0.24 % in local product whereas the innovator sample degraded 8.86±0.16%.

The degradation product of VLG in oxidative condition (3% H_2O_2) were found at 2.130, 2.807, 3.230 min and % degradation was very high in compared to other stress condition. The highest degradation was 28.95±0.32 % in a local product but in case of innovator sample the value was 25.58±0.22%.

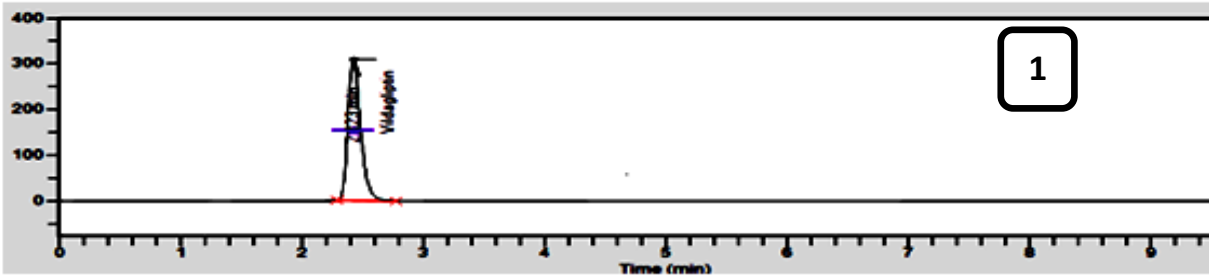
After thermal degradation of VLG, degradation product was appeared at retention time of 5.971 and 6.562 min but the % degradation was low which was not more than 6.85±0.29%.

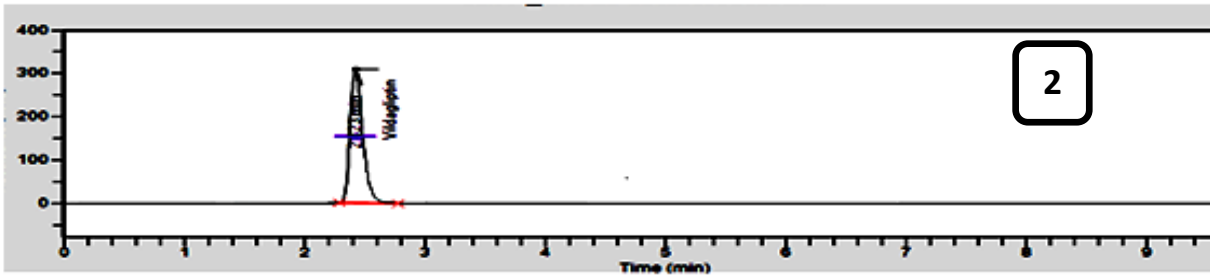
The photolytic degradant of VLG was found at retention time of 4.099min and % degradation was NMT 3.21±0.05 %. In presence of sunlight VLG was remained stable where only 1.44±0.43 % degradation occurred in a local product after 72 hours.

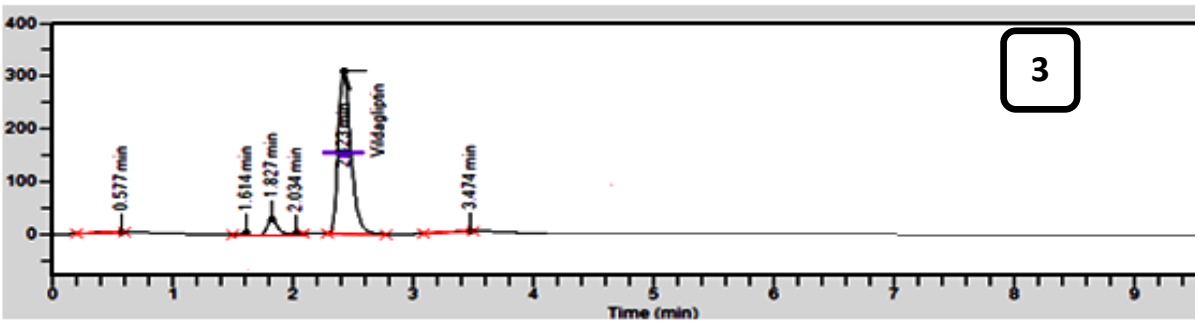
Table 4.22. Forced Degradation Studies of Vildagliptin

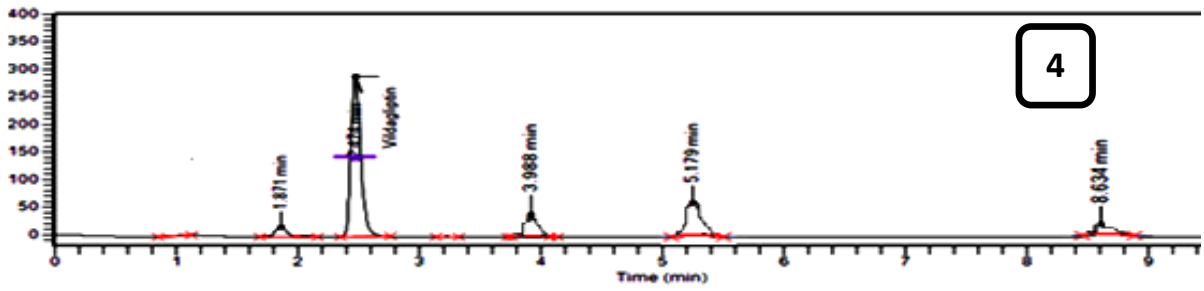
Stress Condition	RT (min) of Major Degradation Peak	% Degradation (Mean±%RSD)			
		(Innovator) VLG-1	VLG-2	VLG-3	VLG-4
Acidic hydrolysis	2.034, 3.474	10.79±0.21	12.54±0.11	13.25±0.25	11.58±0.41
Alkaline hydrolysis	3.988,5.179,8.634	8.86±0.16	14.25±0.24	5.63±0.26	10.22±0.19

Oxidation	2.130,2.807,3.230	25.58±0.22	28.95±0.32	26.21±0.13	24.34±0.03
Thermal degradation	5.971, 6.562	5.58±0.35	6.02±0.15	4.28±0.42	6.85±0.29
Photo (254nm)	4.099	2.25±0.19	1.89±0.19	2.95±0.31	3.21±0.05
Day light	4.943	1.08±0.08	1.24±0.15	0.97±0.36	1.44±0.43









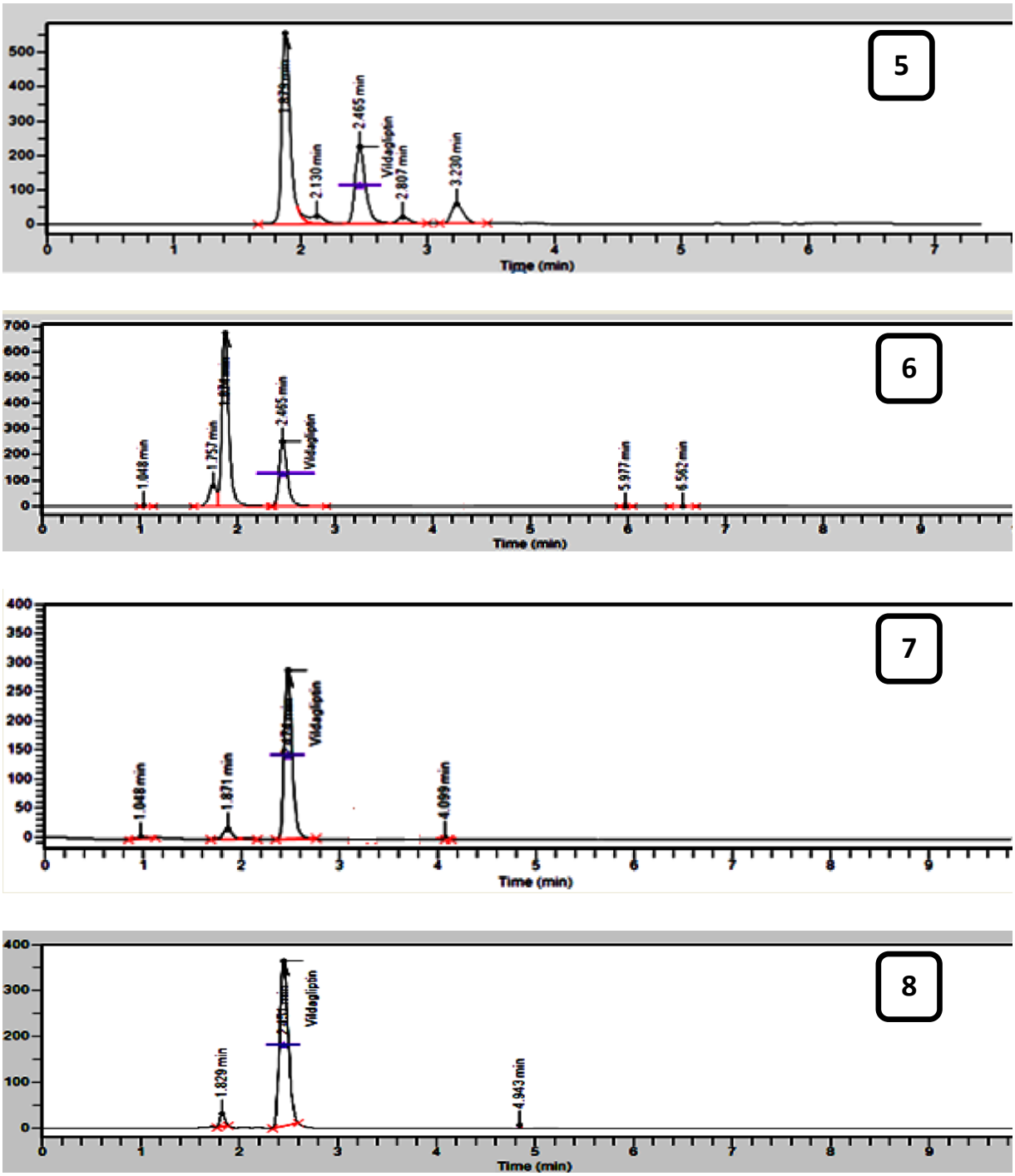


Figure 4.12. Chromatograms of (1) Standard, (2) Sample, (3) Acidic Degradation, (4) Alkaline Degradation, (5) Oxidative Degradation, (6) Thermal Degradation, (7) Photolytic Degradation and (8) Daylight Degradation of VLG.

4.4.2. Forced Degradation Studies of Linagliptin

Forced degradation studies of LNG through UHPLC system under different stress conditions indicated the following degradation behavior which are given in **Table 4.23** and the chromatogram are shown in **Figure 4.13**.

In acidic condition (1N HCl) the decomposition behavior of locally manufactured LNG was close to the innovator sample, appeared at retention time of 2.523, 4.700, 6.030 min and the % degradation was NMT 25.45±0.07%.

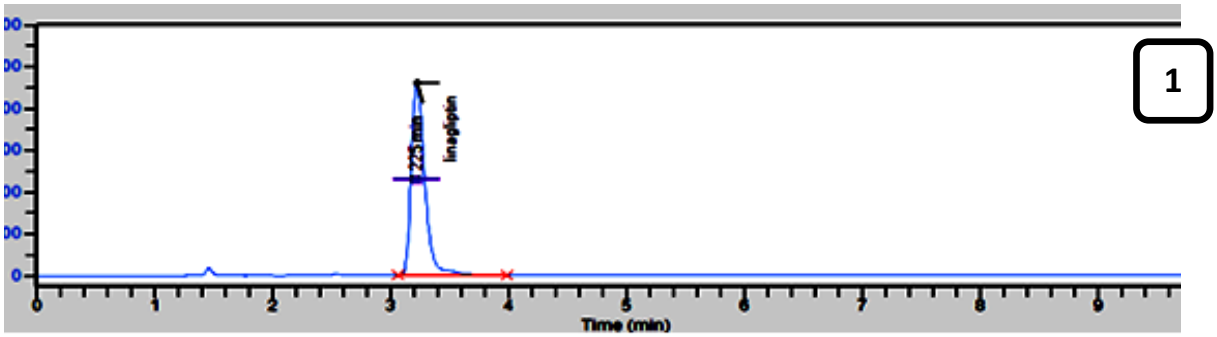
After hydrolytic degradation by alkali (1N NaOH), the degradation product of LNG was appeared at retention time of 2.537, 3.000, 3.991, 7.288 min and % degradation were NMT 15.14±0.21 % in local product whereas the innovator sample degraded 10.12±0.05%.

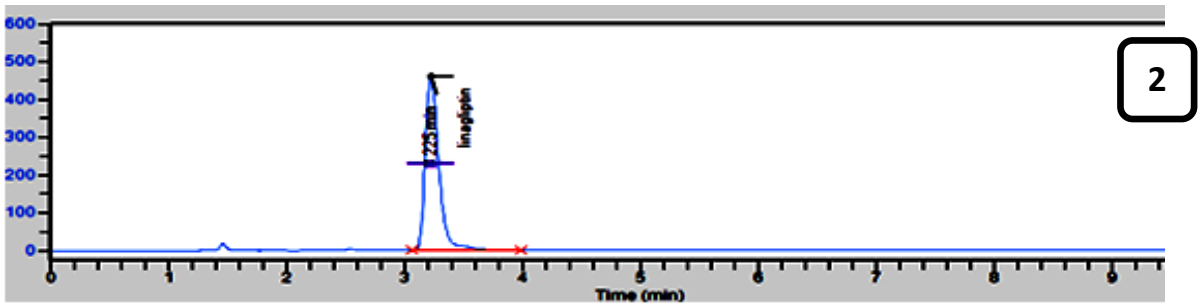
In oxidative degradation (3% H_2O_2), the degradation product of LNG was appeared at retention time of 2.346, 3.797, 6.060 min and % degradation is very high in compared to other stress conditions. The maximum degradation was 38.15±0.14% in a local product but in case of innovator sample the value was 32.45±0.43%. They are similar in terms of degradation pattern.

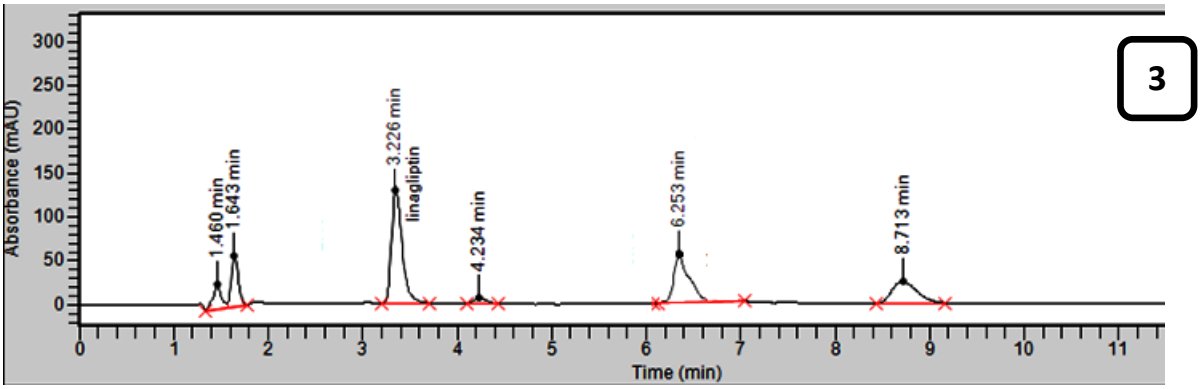
In thermal (NMT 4.15±0.29%), photolytic (NMT 3.85±0.13%) and daylight (NMT 1.89±0.13%) degradation percentages were insignificant.

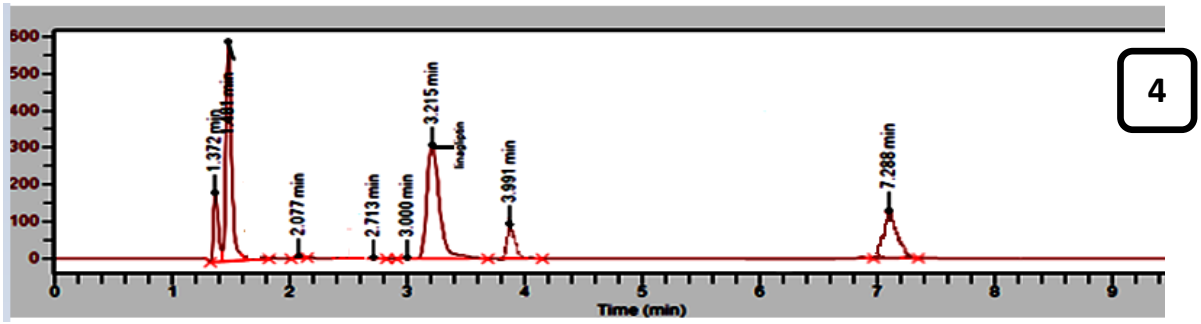
Table 4.23. Forced Degradation Studies of Linagliptin

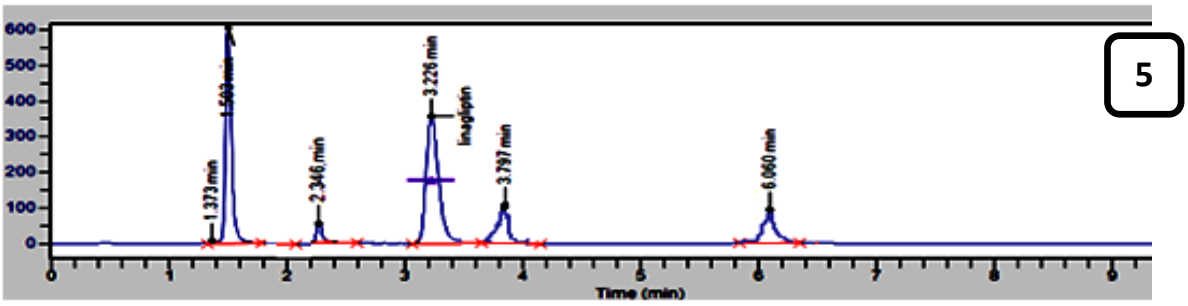
Stress Condition	RT (min) of Major Degradants' Peak	% Degradation (Mean±%RSD)			
		(Innovator) LNG-1	LNG-2	LNG-3	LNG-4
Acidic hydrolysis	4.234, 6.253, 8.713	21.75±0.16	25.45±0.07	18.56±0.24	15.56±0.06
Alkaline hydrolysis	2.537,3.000, 3.991,7.288	10.12±0.05	15.14±0.21	12.21±0.22	8.36±0.08
Oxidative	2.346,3.797, 6.060	32.45±0.43	28.25±0.15	30.20±0.17	38.15±0.14
Thermal	2.178, 4.195	2.96±0.21	3.10±0.06	2.10±0.23	4.15±0.29
Photo (254nm)	2.525	1.51±0.14	1.25±0.09	3.85±0.13	2.88±0.11
Day light	5.324	0.98±0.17	1.56±0.24	1.89±0.13	1.53±0.41


1
Time (min)


2
Time (min)


3
Absorbance (mAU)
1.460 min
1.643 min
3.226 min
linagliptin
4.234 min
6.253 min
8.713 min
Time (min)


4
1.372 min
2.077 min
2.713 min
3.000 min
3.215 min
linagliptin
3.991 min
7.288 min
Time (min)


5
1.373 min
2.346 min
3.226 min
linagliptin
3.797 min
6.060 min
Time (min)

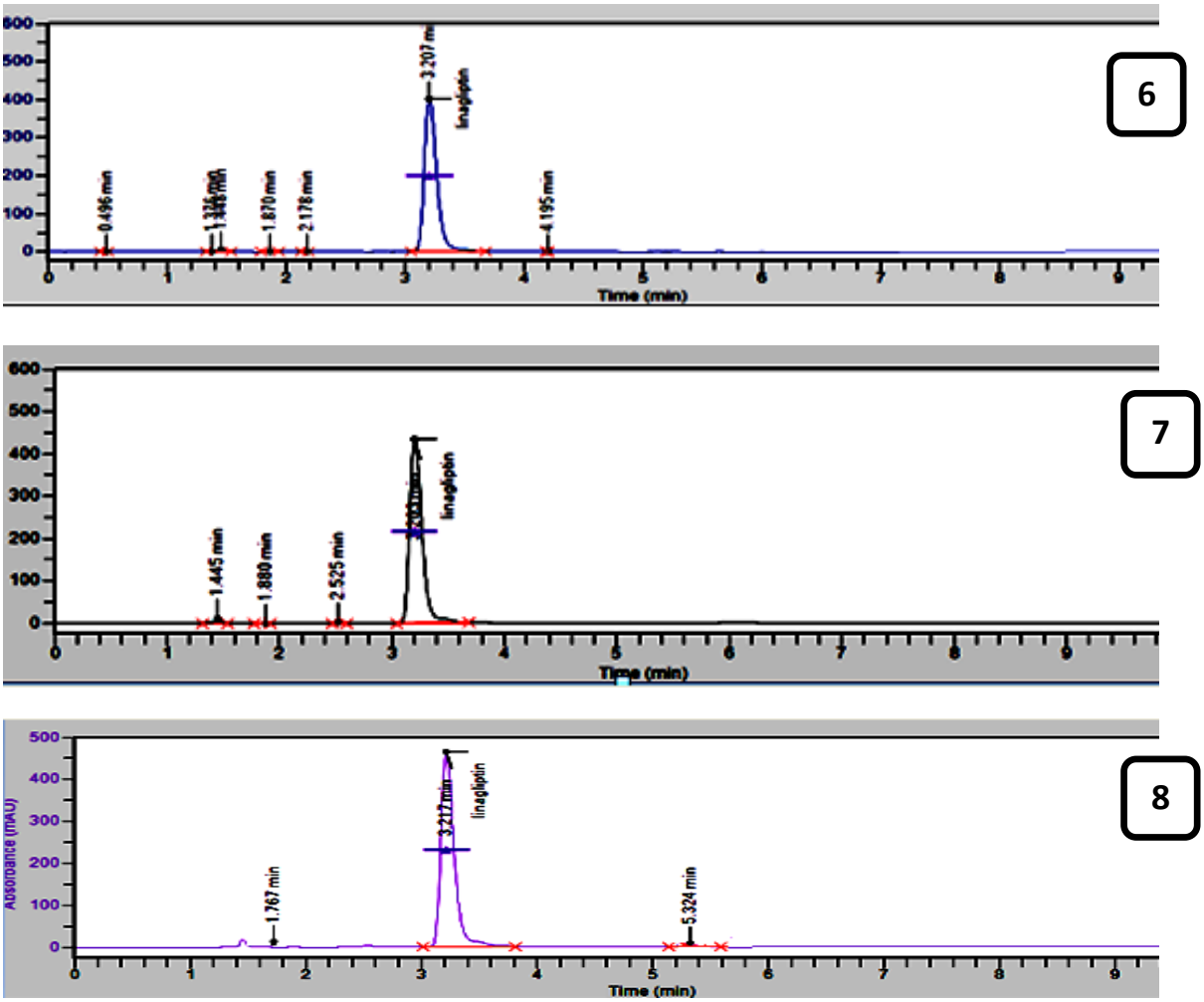


Figure 4.13. Chromatograms of (1) Standard, (2) Sample, (3) Acidic Degradation, (4) Alkaline Degradation, (5) Oxidative Degradation, (6) Thermal Degradation, 7) Photolytic Degradation and (8) Daylight Degradation of LNG.

4.4.3. Forced Degradation Studies of Sitagliptin

Forced degradation studies of STG under different stress conditions indicated the following degradation behavior which are given in **Table 4.24** and the chromatogram are shown in **Figure 4.14**.

In acidic medium (1N HCl), the degradation behavior of locally manufactured STG was similar to the innovator sample, appeared at retention time of 3.164, 7.807 min and % degradation in all sample were NMT 26.33±0.36 %.

In alkaline medium (1N NaOH), the degradation product of STG was appeared at retention time of 1.607, 3.539,6.922 min and % degradation were NMT 35.59±0.33 % in innovator sample.

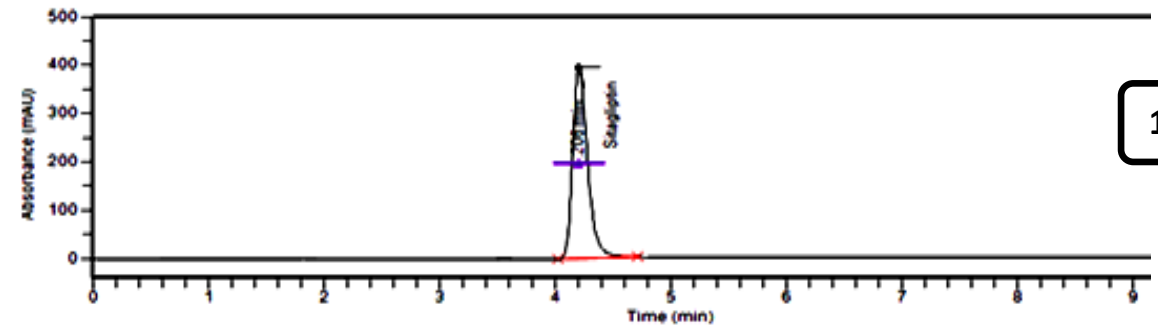
In oxidative decomposition (3% H_2O_2), the degradation product of STG was appeared at retention time of 2.911, 5.833, 8.320 min. The highest degradation was 22.49±0.09 % in a local product whereas innovator sample degraded 19.15±0.71 %.

In thermal (NMT 5.21±0.10%), photolytic (NMT 3.24±0.04%) and daylight degradation (NMT 2.53±0.16%), the degradation percentages were insignificant.

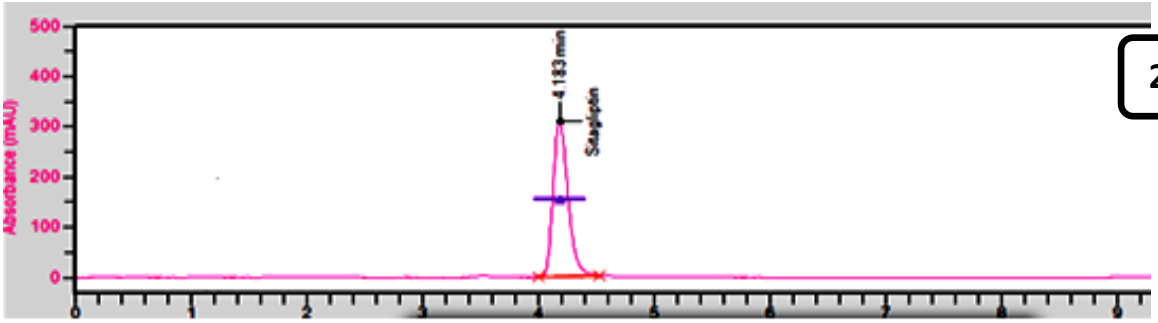
Table 4.24. Forced Degradation Studies of Sitagliptin

Stress Condition	RT (min) of Major Degradants' Peak	% Degradation (Mean±%RSD)			
		(Innovator) STG-1	STG-2	STG-3	STG-4
Acidic hydrolysis	3.164, 7.807	25.69±0.52	14.25±0.18	23.22±0.25	26.33±0.36
Basic hydrolysis	1.607, 3.539,6.922	35.59±0.33	26.32±0.59	25.26±0.31	32.21±0.22
Oxidative	2.911, 5.833, 8.320	19.15±0.71	22.49±0.09	20.20±0.25	21.89±0.51
Thermal	2.429, 7.352	1.36±0.24	2.06±0.15	3.45±0.11	5.21±0.10
Photo (254nm)	2.017	2.89±0.63	1.58±0.06	2.77±0.05	3.24±0.04
Daylight	*ND	1.76±0.23	1.28±0.09	2.53±0.16	1.88±0.32

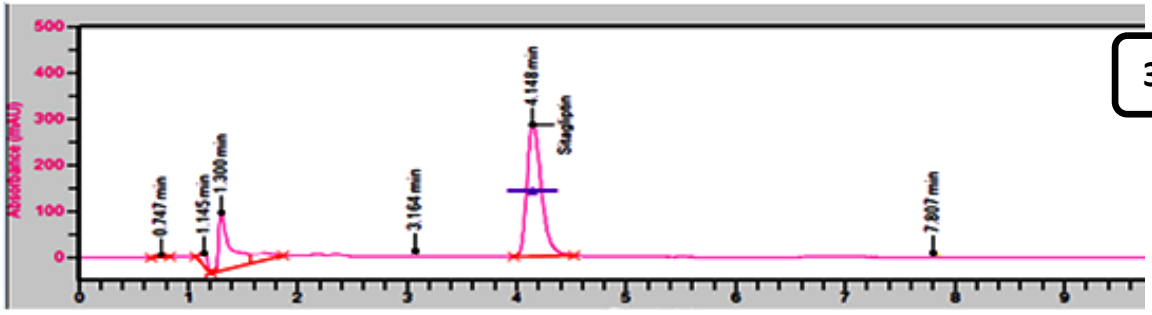
*ND: Not detected


Absorbance (mAU)
Time (min)
Saxagliptin

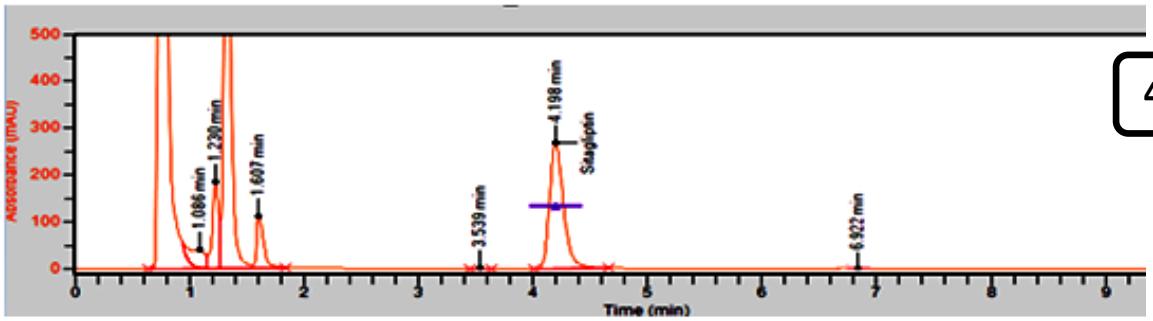
1


Absorbance (mAU)
4.183 min
Saxagliptin

2


Absorbance (mAU)
0.747 min
1.145 min
1.300 min
3.164 min
4.148 min
Saxagliptin
7.807 min

3


Absorbance (mAU)
1.086 min
1.239 min
1.607 min
3.539 min
4.198 min
Saxagliptin
6.922 min
Time (min)

4

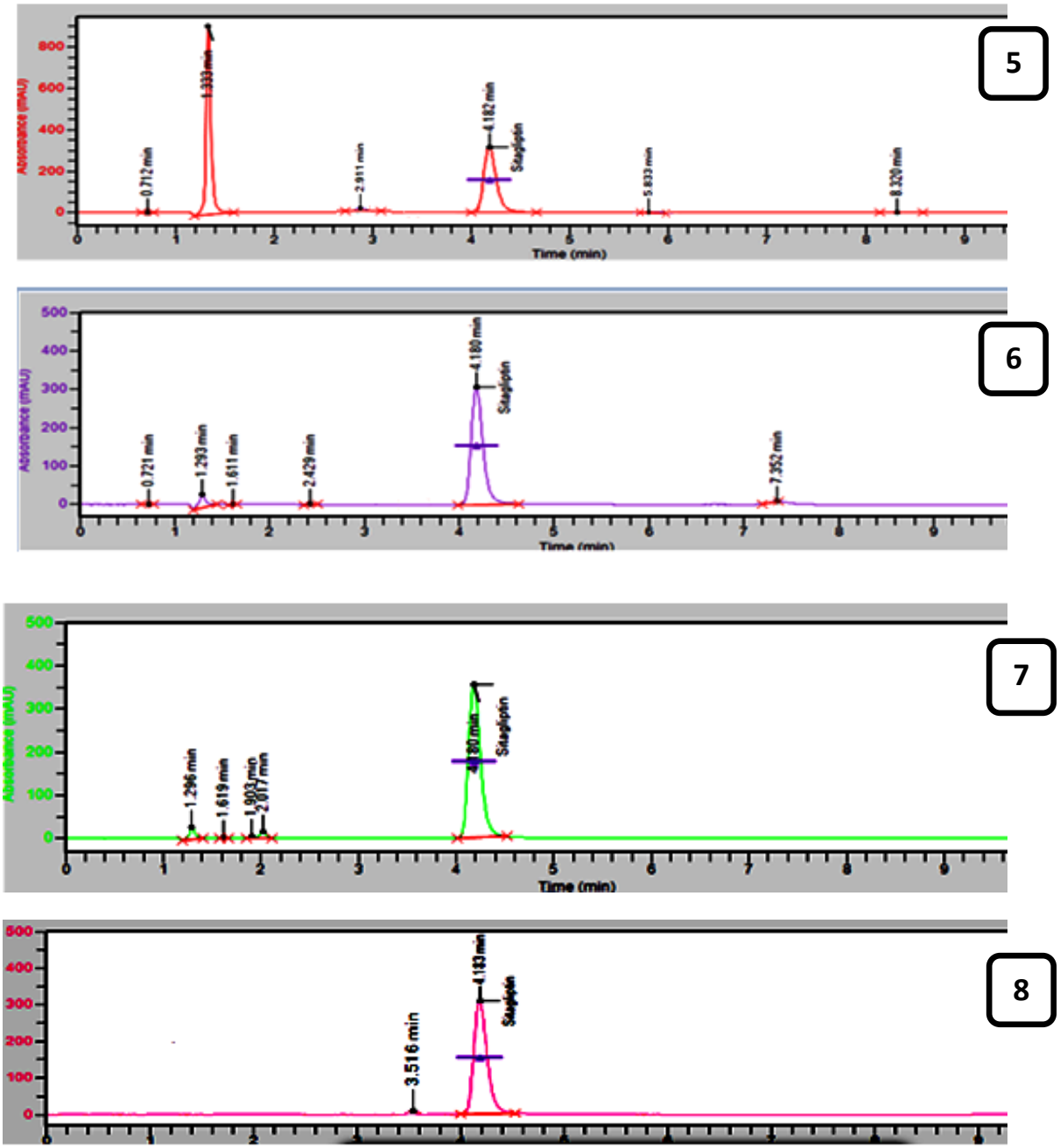


Figure 4.14. Chromatograms of (1) Standard, (2) Sample, (3) Acidic Degradation, (4) Alkaline Degradation, (5) Oxidative Degradation, (6) Thermal Degradation, (7) Photolytic Degradation and (8) Daylight Degradation of STG.

From the above result, it can be summarized that vildagliptin is more sensitive to oxidative and alkaline degradation and stable in thermal and photolytic degradation. Linagliptin is also sensitive to oxidative and acid hydrolytic degradation but stable in thermal and photolytic degradation. Sitagliptin is highly unstable in base hydrolytic condition in comparison to other condition and stable in thermal and photolytic

degradation. So, appropriate conditions must be maintained to store these three gliptins.

4.5. Degradation Kinetic Studies

The degradation kinetics of acidic and basic hydrolysis, oxidative and thermal degradation of vildagliptin, linagliptin and sitagliptin were investigated at 60, 80 and 105°C. In case of oxidation with H_2O_2, the degradation kinetics was studied at 40, 60 and 80°C showing notable decomposition rate. The rate constant ($k'25$) that corresponds to room temperature (25°C) was calculated from the regression equation of Arrhenius equation (**Eq-4**). The calculation of shelf life (t_{90}), half-life (t_{50}), and the time required for the drug to decrease its initial amount by 90 % (t_{10}) values were calculated from the value of $k'25$ (**Eq. 6, 7, 8**).

4.5.1. Forced Degradation Kinetics of Vildagliptin

The degradation kinetics of acidic and basic hydrolysis, oxidative and thermal condition of vildagliptin was investigated by using Arrhenius equation at different temperature. In all degradation-kinetic studies, the degradation rate followed pseudo-first order kinetics (**Figure 4.15**).

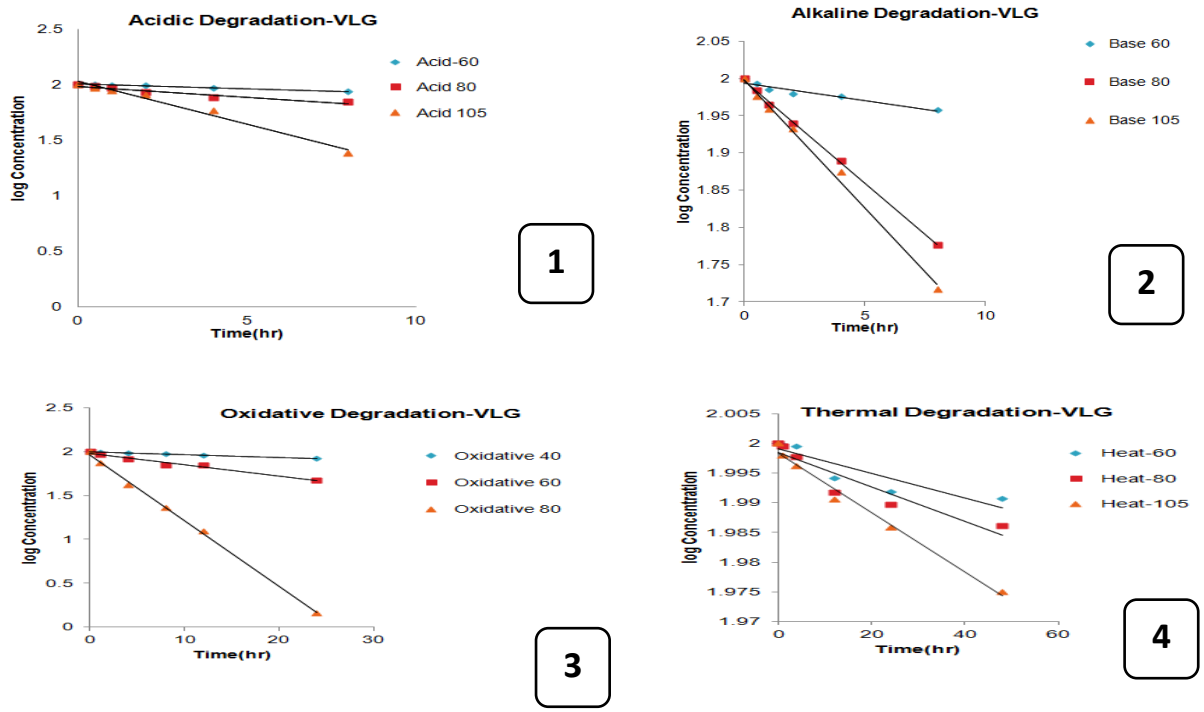


Figure 4.15. Degradation kinetics of Vildagliptin by (1) Acidic Hydrolysis, (2) Alkaline Hydrolysis, (3) Oxidation and (4) Thermal degradation.

Degradation rate constant (K) and its corresponding half-life ($t_{1/2}$) under different stress conditions of vildagliptin was described in **Table 4.25**. The degradation kinetics rate constant (*k'25*) at room temperature (25°C) were found 0.002h^{-1}, 0.003 h^{-1}, 0.005h^{-1} and 0.0007h^{-1} in acidic, alkaline, oxidative and thermal stress, respectively. The lowest *k'25* value was found in thermal stress (0.0007h^{-1}) which indicated the highest stability in thermal degradation. At the same time, highest *k'25* value was found in oxidative stress (0.005h^{-1}) indicating the lowest stability in oxidative degradation. The calculation of shelf life (t_{90}), half-life (t_{50}), and the time required for the drug to decrease from its initial amount by 90% (t_{10}) values for vildagliptin were calculated from the value of *k'25* at room temperature are described in **Table 4.26**.

Table 4.25. Degradation Rate Constant (K) and its Corresponding Half-life ($t_{1/2}$) under Different Stress Conditions of Vildagliptin

Stress	Temp(◦C)	R^2	K(h^{-1})	$t_{1/2}$(h)
Acidic	105	0.981	0.046	15.07
	80	0.919	0.019	36.47
	60	0.995	0.004	173.25
Alkaline	105	0.994	0.034	20.38
	80	0.998	0.027	25.67
	60	0.924	0.008	86.63
Oxidative	80	0.998	0.079	8.77
	60	0.973	0.022	31.50
	40	0.997	0.013	53.31
Heat	105	0.984	0.009	77.00
	80	0.975	0.004	173.25
	60	0.942	0.002	346.50

Table 4.26. Degradation Kinetics Rate Constant (*K'25*) at Room Temperature (25°C) and its Corresponding Shelf life (t_{90}), Half-life (t_{50}), and 90% Decomposition of Vildagliptin (t_{10}).

Stress	*k'25* (h^{-1})	t_{50}(h)	t_{90}(h)	t_{10}(h)
Acidic	0.002	346.5	52.5	1151.50
Alkaline	0.003	231	35	767.67
Oxidative	0.005	138.6	21	460.60
Heat	0.0007	990	150	3290.00

4.5.2. Degradation Kinetics of Linagliptin

The degradation kinetics of acidic and basic hydrolysis, oxidative and thermal condition of linagliptin was investigated by using Arrhenius equation at different temperature. In all degradation-kinetic studies, the degradation rate followed pseudo-first order kinetics (**Figure 4.16**).

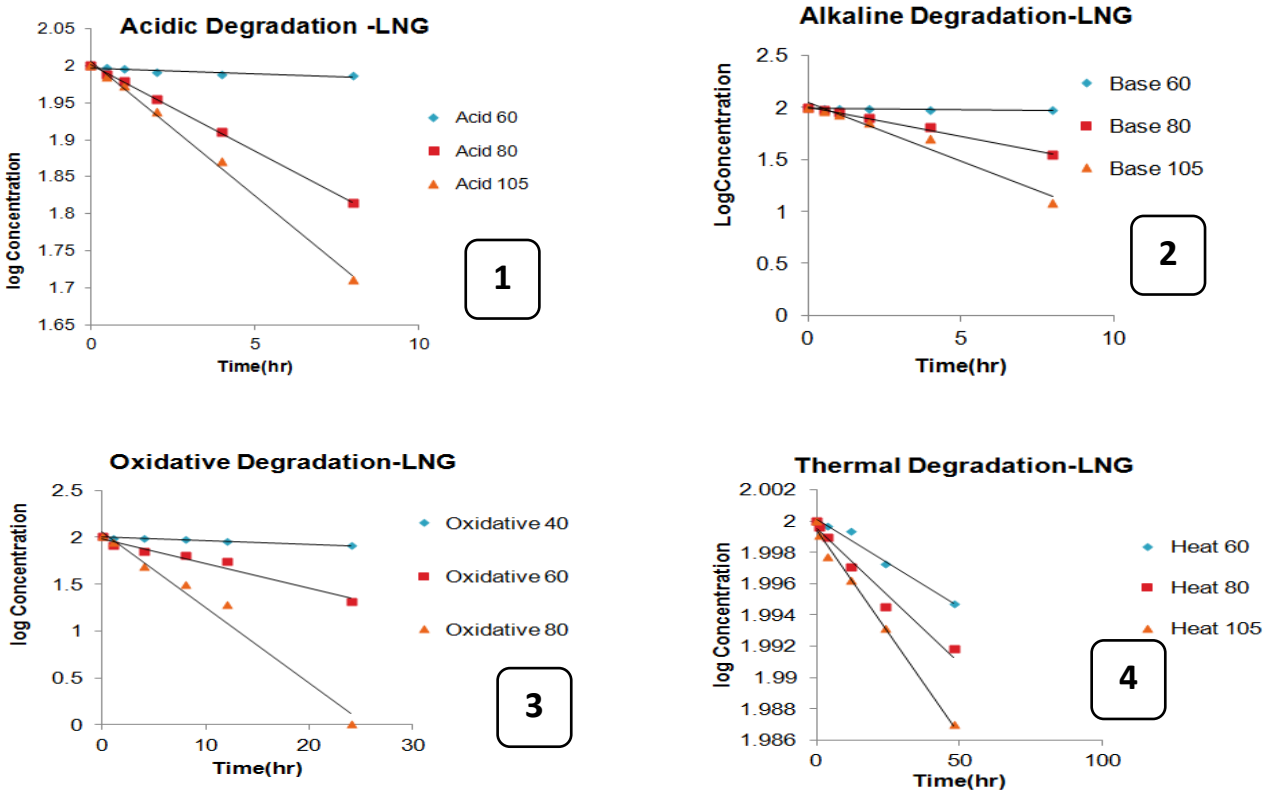


Figure 4.16. Degradation Kinetics of LNG by (1) Acidic Hydrolysis, (2) Basic Hydrolysis, (3) Oxidation, (4) Thermal Degradation.

Degradation rate constant (K) and its corresponding half-life ($t_{1/2}$) under different stress conditions of linagliptin was described in **Table 4.27**. The degradation kinetics rate constant (*k'25*) at room temperature (25°C) were 0.0018h^{-1}, 0.0016 h^{-1}, 0.008h^{-1} and 0.0004h^{-1} in acidic, alkaline, oxidative and thermal stress, respectively. The lowest *k'25* value was found in thermal stress (0.0004) which indicated the highest stability in thermal degradation. At the same time, highest *k'25* value was found in oxidative stress which indicate the lowest stability in oxidative degradation. The calculation of shelf life (t_{90}), half-life (t_{50}), and the time required for the drug to reduce from its initial amount

by 90% (t_{10}) values for linagliptin were calculated from the value of *k'25* at room temperature which was described in **Table 4.28**.

Table 4.27. Degradation Rate Constant (K) And Its Corresponding Half-life($t_{1/2}$) under Different Stress Conditions of Linagliptin

Stress	Temp (◦C)	R^2	K (h^{-1})	$t_{1/2}$(h)
Acidic	105	0.996	0.056	12.38
	80	0.999	0.023	30.13
	60	0.801	0.005	138.60
Alkaline	105	0.968	0.033	21.00
	80	0.989	0.016	43.31
	60	0.683	0.004	173.25
Oxidative	80	0.977	0.085	8.15
	60	0.962	0.048	14.44
	40	0.974	0.019	36.47
Thermal	105	0.992	0.006	115.50
	80	0.97	0.004	173.25
	60	0.979	0.001	693.00

Table 4.28. Degradation Kinetics Rate Constant (*K'25*) at Room Temperature (25°C) and its Corresponding Shelf life (t_{90}), Half-life (t_{50}), and 90% Decomposition of Linagliptin (t_{10}).

Stress	*k'25* (h^{-1})	t_{50}(h)	t_{90}(h)	t_{10}(h)
Acidic	0.0018	385	58.333	1279.44
Alkaline	0.0016	433.125	65.625	1439.38
Oxidative	0.008	86.625	13.125	287.88
Thermal	0.0004	1732.5	262.5	5757.50

4.5.3. Degradation Kinetics of Sitagliptin

The degradation kinetics of acidic and basic hydrolysis, oxidative and thermal condition of sitagliptin was investigated by using Arrhenius equation at different temperature. In all degradation-kinetic studies, the degradation rate followed pseudo-first order kinetics (**Figure 4.17**).

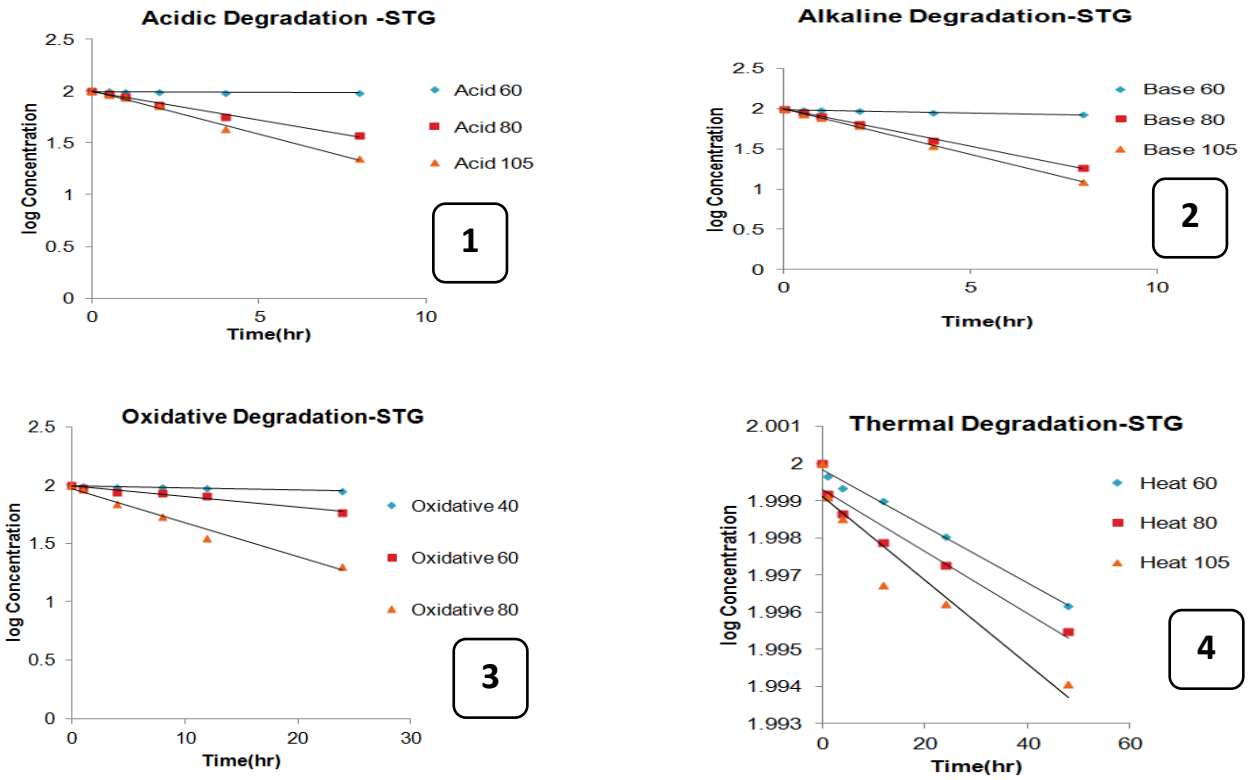


Figure 4.17. Degradation kinetics of STG by (1) Acidic Hydrolysis, (2) Basic Hydrolysis, (3) Oxidation and (4) Thermal degradation.

Degradation rate constant(K) and its corresponding half-life($t_{1/2}$) under different stress conditions of sitagliptin was described in **Table 4.29**. The degradation kinetics rate constant (*k'25*) at room temperature (25°C) were 0.0061h^{-1}, 0.0075h^{-1}, 0.0029h^{-1} and 0.0013h^{-1} in acidic, alkaline, oxidative and thermal stress respectively. The lowest *k'25* value was found in thermal stress (0.0013h^{-1}) which indicated the highest stability in thermal condition. At the same time, highest *k'25* value was found in alkaline stress(0.0075h^{-1}) which indicate the lowest stability in alkaline degradation. The calculation of shelf life (t_{90}), half-life (t_{50}), and the time required for the drug to decrease from its initial amount by 90% (t_{10}) values for sitagliptin were calculated from the value of *k'25* at room temperature are described in **Table 4.30**.

Table 4.29. Degradation Rate Constant (K) and Its Corresponding Half-Life ($T_{1/2}$) under Different Stress Conditions of Sitagliptin

Stress	Temp(◦C)	R^2	K(h^{-1})	t1/2(h)
Acidic	105	0.981	0.076	9.12
	80	0.919	0.039	17.77

	60	0.995	0.018	38.50
Alkaline	105	0.994	0.097	7.14
	80	0.998	0.068	10.19
	60	0.924	0.014	49.50
Oxidative	80	0.998	0.062	11.18
	60	0.973	0.031	22.35
	40	0.997	0.007	99.00
Heat	105	0.914	0.013	53.31
	80	0.937	0.009	77.00
	60	0.992	0.003	231.00

Table 4.30. Degradation Kinetics Rate Constant (*K'25*) at Room Temperature (25°C) and Its Corresponding Shelf Life (T_{90}), Half-Life (T_{50}), and 90% Decomposition of Sitagliptin (T_{10}).

Stress	*k'25* (h^{-1})	t_{50}(h)	t_{90}(h)	t_{10}(h)
Acidic	0.0061	113.6066	17.213	377.54
Alkaline	0.0075	92.4	14	307.07
Oxidative	0.0029	238.9655	36.207	794.14
Heat	0.0013	533.0769	80.769	1771.54

Degradation kinetics study of VLG, LNG and STG reveled that all the products follow pseudo first order degradation kinetics i.e. the reaction is not first-order reaction naturally but made first order by increasing or decreasing the concentration of one or the other reactant. Prediction of half and shelf life or the stability of the product can be done by determining the rate constant at room temperature.

4.6. Isolation and Characterization of Degradants of Linagliptin

The major degradants of linagliptin were acidic and oxidative degradants. These degradants were isolated by column chromatography and subjected to IR and NMR spectroscopy for structure elucidation. The possible degradants were shown in the UHPLC chromatogram in acidic degradation (**Figure 4.18-A**) and oxidative degradation (**Figure 4.18-B**).

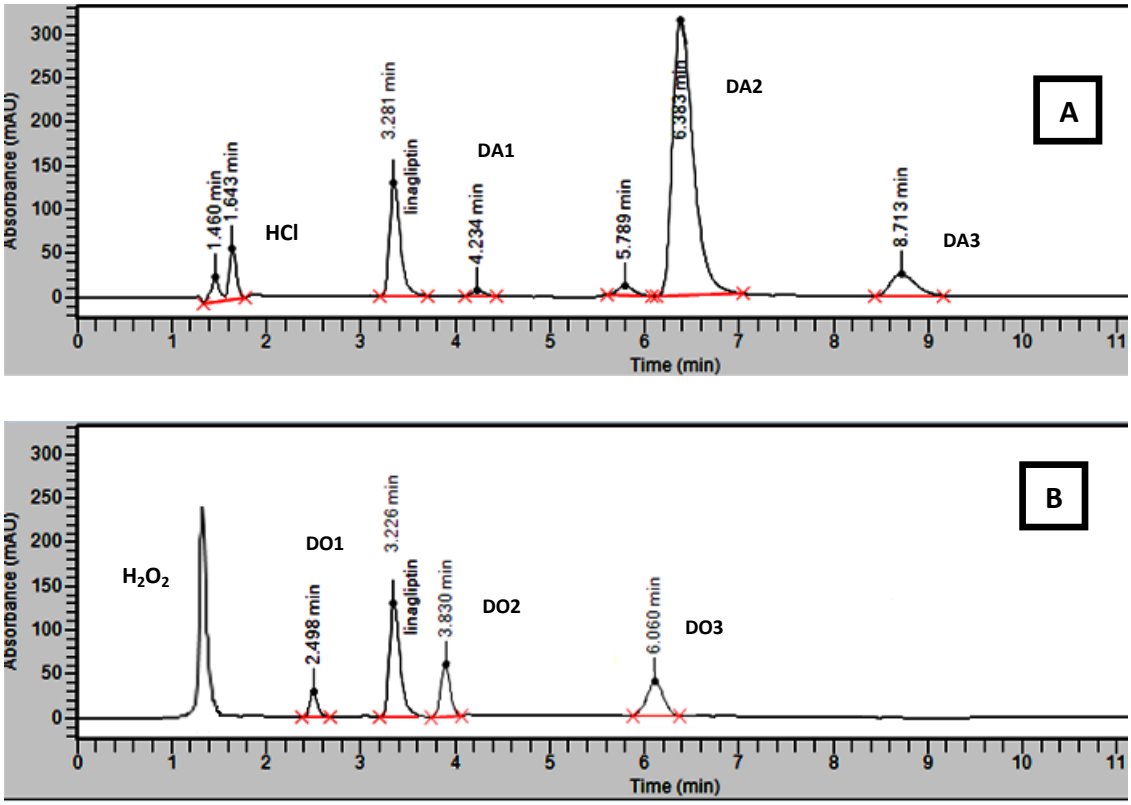


Figure 4.18. UHPLC Chromatogram of Acidic Degradants (A) and Oxidative Degradants (B) of Linagliptin.

4.6.1. Spectral Data of Linagliptin

For the structure elucidation of degradants products, comparison of data between degradants product and mother product, linagliptin was required. Position of proton and carbon number of linagliptin was assigned according to **Figure 4.19.** ^{1}H-NMR (**Figure 4.20-4.22**), and ^{13}C-NMR (**Figure 4.23-4.25**), and IR data (**Figure-4.26**) were analyzed which are summarized in **Table 4.31-4.33.**

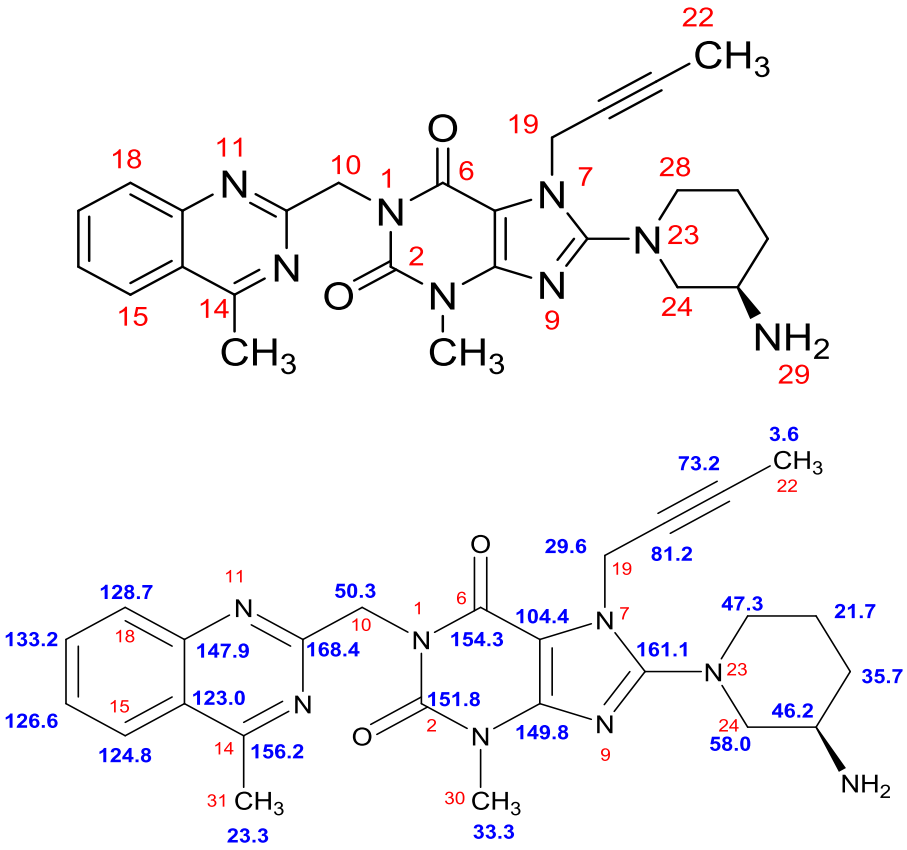


Figure 4.19. Structure of Linagliptin with assigned position

Table 4.31. ^{1}H-NMR data of Standard Linagliptin in $CDCl_3$.

Position	^{1}H-NMR δ_H (ppm), *J* (Hz)	
H_2-10	5.49	2H, s
H-15	7.77	1H, d, *J*=8.4 Hz.
H-16	7.41	1H, t, *J*=7.6 Hz.
H-17	7.66	1H, t, *J*=7.6 Hz.
H-18	7.91	1H, d, *J*=8.0 Hz.
H_2-19	4.80	2H, br. s
H_3-22	1.71	3H, s
H-24b	3.60	1H, dd, *J*= 12, 2.8 Hz.
H-24a	2.99~3.05	1H, m
H-25	2.83	1H, dd, *J*=12.0, 12.4 Hz.
H-26a	1.24~1.33	1H, m
H-26b	1.78~1.81	1H, m

H-27a	1.60~1.68	1H, m
H-27b	1.89~1.93	1H, m
H-28a	3.49~3.52	1H, m
H-28b	2.96~2.98	1H, m
H_2-29	1.97	2H, br. s
H_3-30	3.47	3H, s
H_3-31	2.78	3H, s

Table 4.32. ^{13}C -NMR Data of Standard Linagliptin in $CDCl_3$.

Position (C#)	**^{13}C-NMR , δ_C (ppm)**
C-2	151.8
C-4	149.8
C-5	104.4
C-6	154.3
C-8	161.1
C-10	50.3
C-12	168.4
C-14	156.2
C-14a	123.0
C-15	124.8
C-16	126.6
C-17	133.2
C-18	128.7
C-18a	147.9

Position (C#)	**^{13}C-NMR , δ_C (ppm)**
C-19	29.6
C-20	81.2
C-21	73.2
C-22	3.6
C-24	58.0
C-25	46.2
C-26	35.7
C-27	21.7
C-28	47.3
C-30	33.3
C-31	23.3

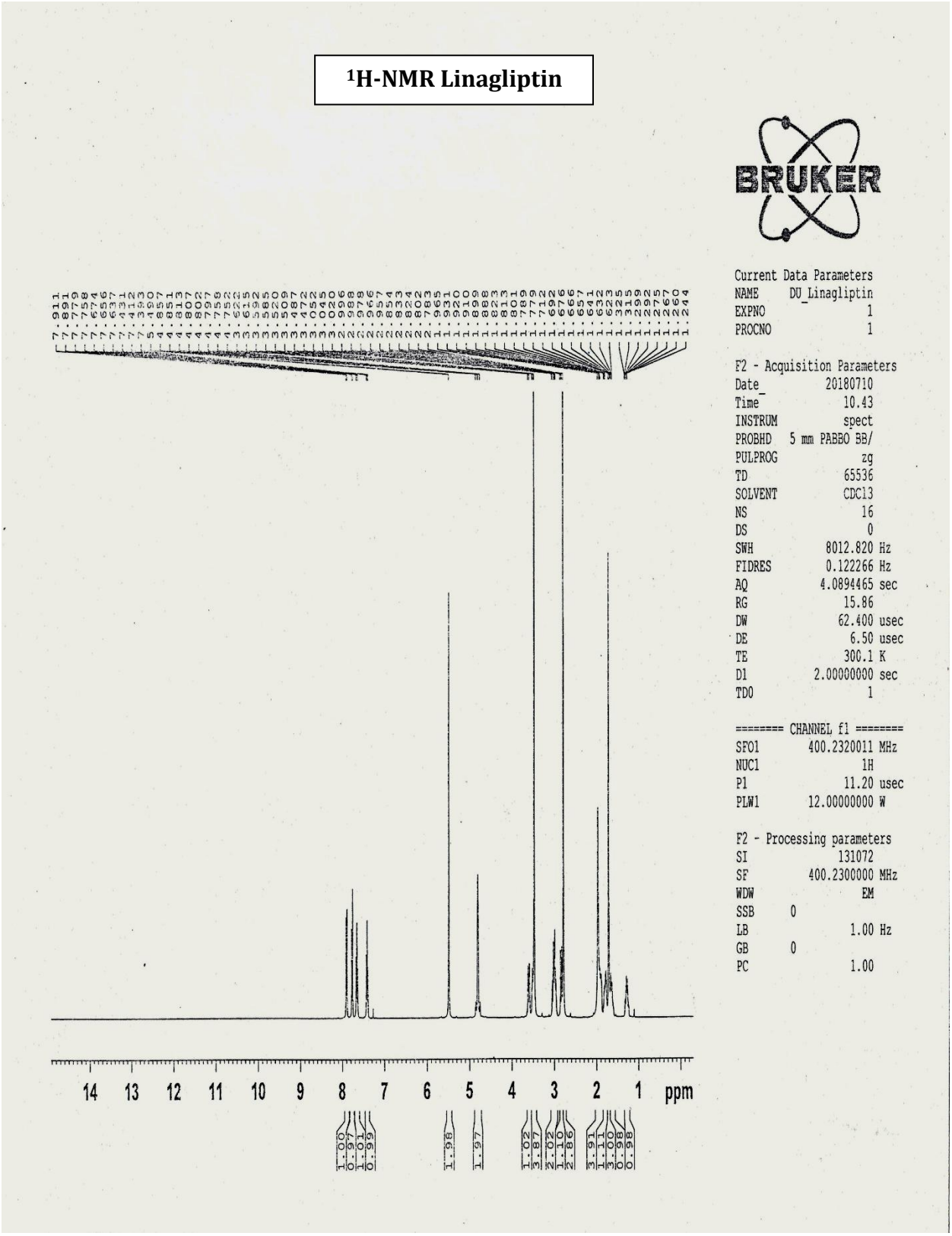
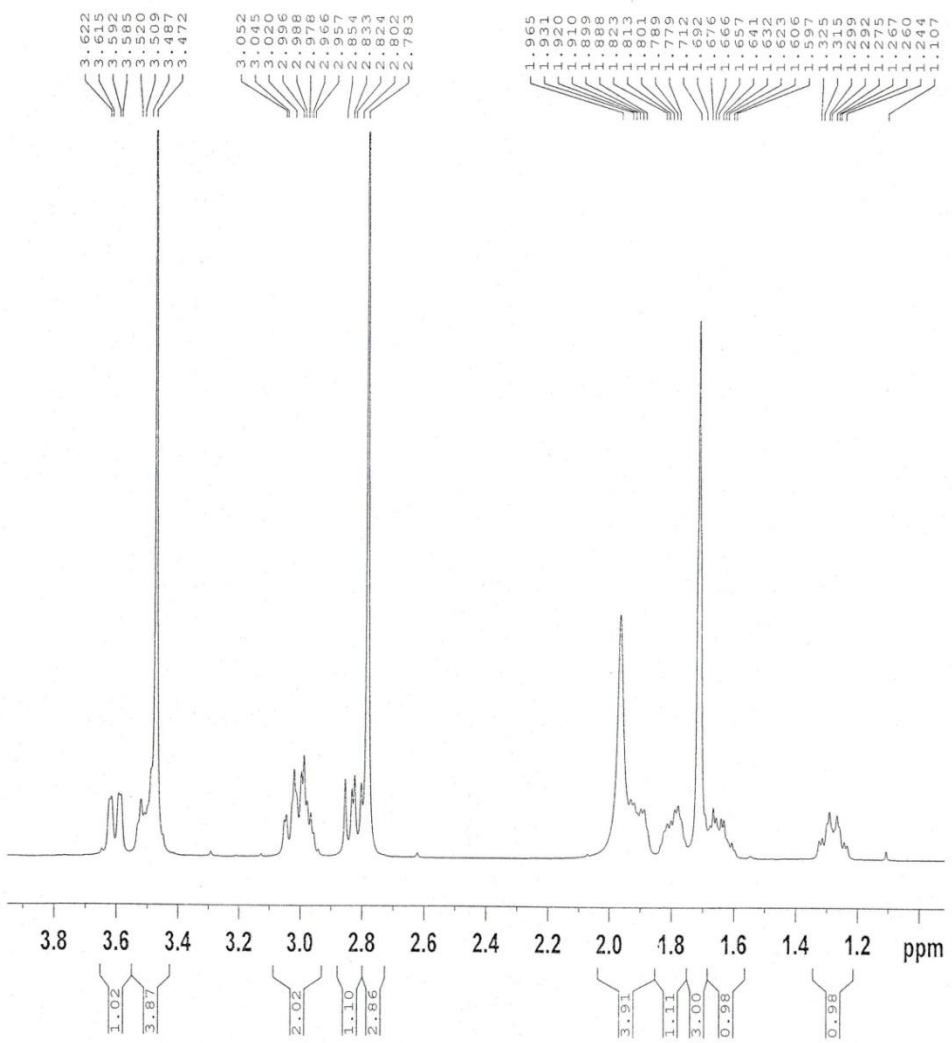


Figure 4.20. ¹H-NMR Spectrum of Standard Linagliptin in $CDCl_3$

^{1}H-NMR Linagliptin

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PROCNO                 1

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PULPROG               zg
TD                 65536
SOLVENT            CDCl3
NS                    16
DS                     0
SWH             8012.820 Hz
FIDRES          0.122266 Hz
AQ              4.0894465 sec
RG                 15.86
DW                62.400 usec
DE                 6.50 usec
TE                300.1 K
D1             2.00000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1         400.2320011 MHz
NUC1                   1H
P1                 11.20 usec
PLW1         12.00000000 W

F2 - Processing parameters
SI                131072
SF           400.2300000 MHz
WDW                   EM
SSB       0
LB                 1.00 Hz
GB        0
PC                 1.00
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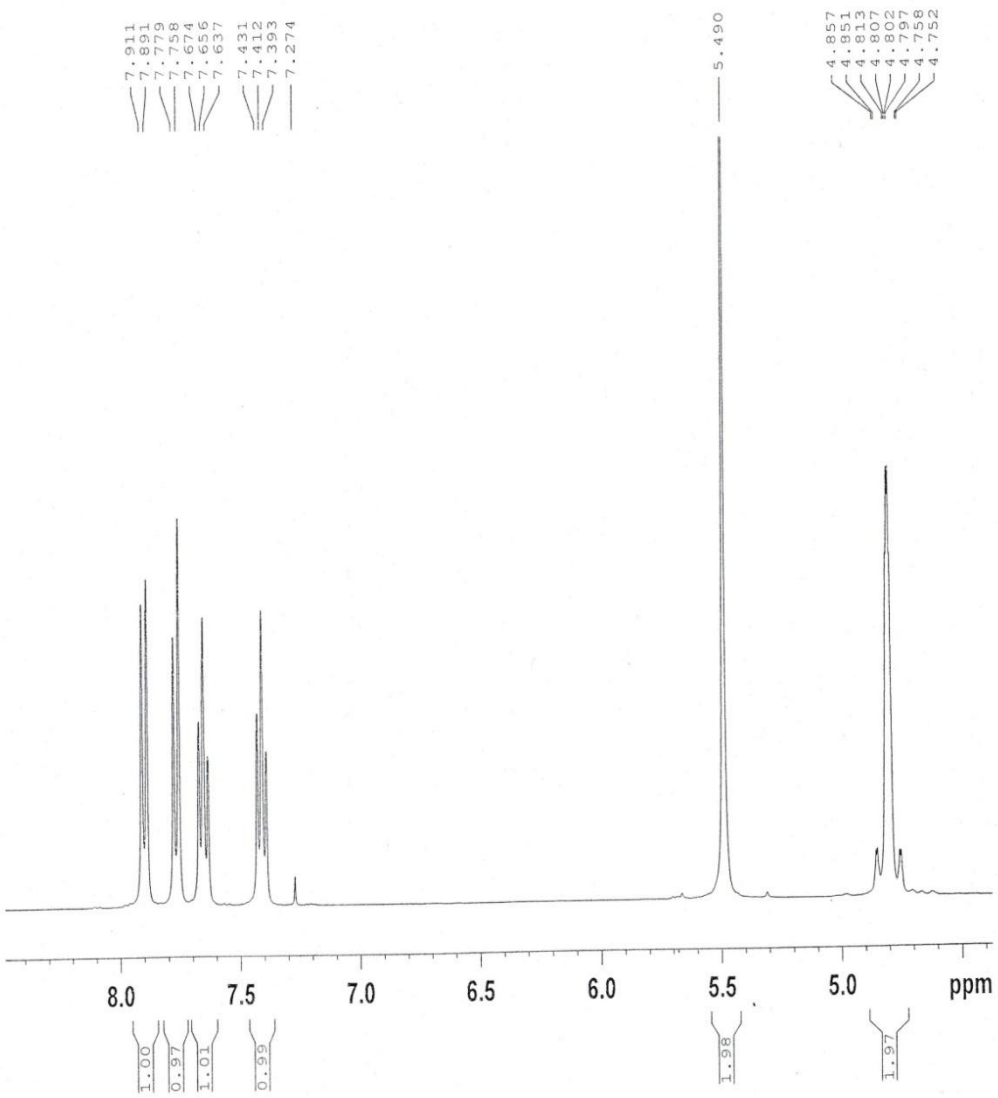
Figure 4.21. Partially Expanded ^{1}H-NMR Spectrum of Standard Linagliptin in $CDCl_3$

^{1}H-NMR Linagliptin



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Current Data Parameters
NAME      DU_Linagliptin
EXPNO                  1
PROCNO                 1

F2 - Acquisition Parameters
Date_           20180710
Time              10.43
INSTRUM           spect
PROBHD   5 mm PABBO BB/
PULPROG             zg
TD                65536
SOLVENT           CDCl3
NS                   16
DS                    0
SWH            8012.820 Hz
FIDRES         0.122266 Hz
AQ             4.0894465 sec
RG                15.86
DW              62.400 usec
DE               6.50 usec
TE               300.1 K
D1           2.00000000 sec
TD0                   1

======== CHANNEL f1 ========
SFO1        400.2320011 MHz
NUC1                  1H
P1              11.20 usec
PLW1        12.00000000 W

F2 - Processing parameters
SI                131072
SF          400.2300000 MHz
WDW                 EM
SSB       0
LB                1.00 Hz
GB        0
PC                1.00
```

Figure 4.22. Partially Expanded ^{1}H-NMR spectrum of standard linagliptin in $CDCl_3$

^{13}C-NMR Linagliptin

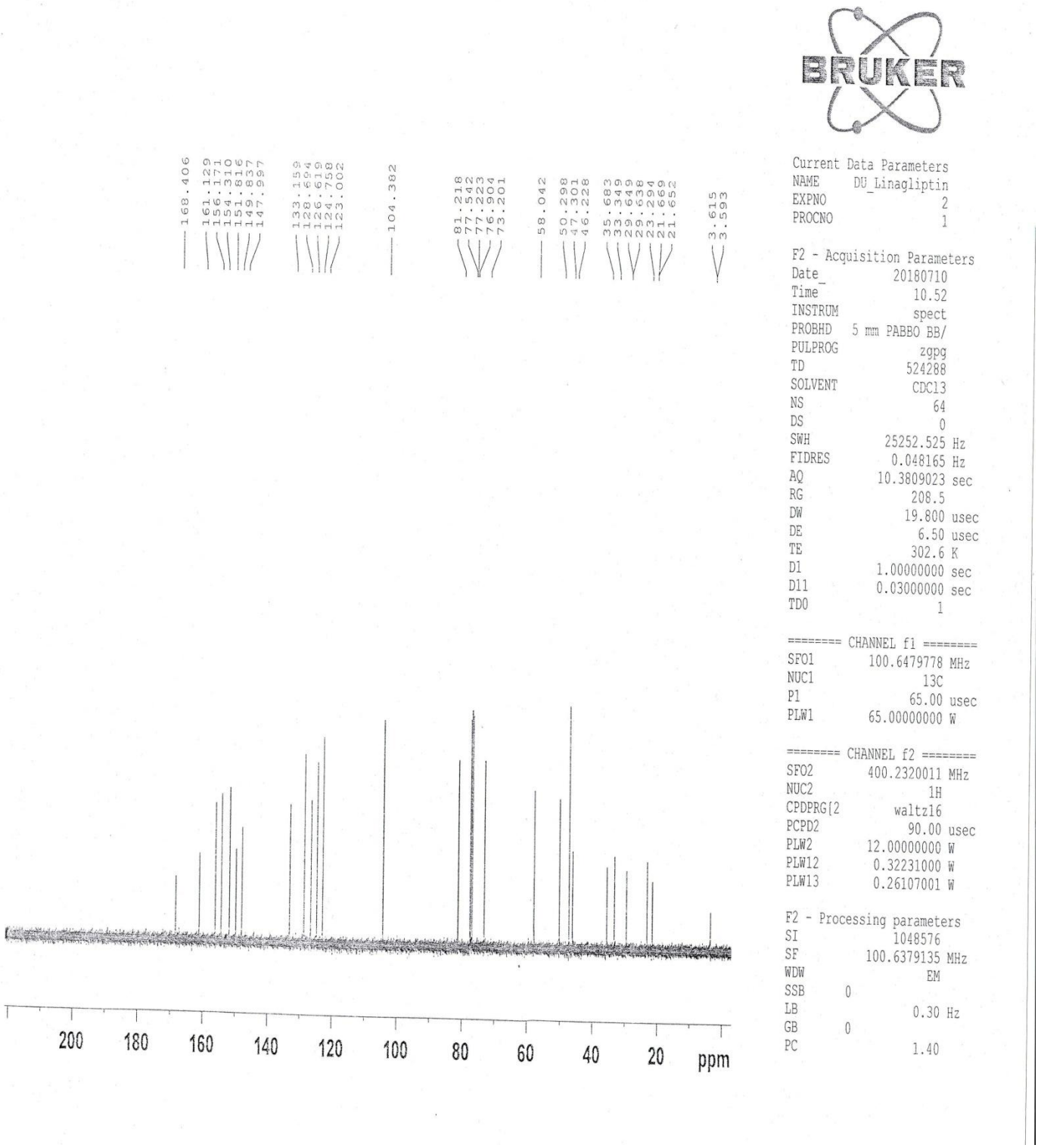


Figure 4.23. ^{13}C-NMR Spectrum of Standard Linagliptin in $CDCl_3$

^{13}C-NMR Linagliptin

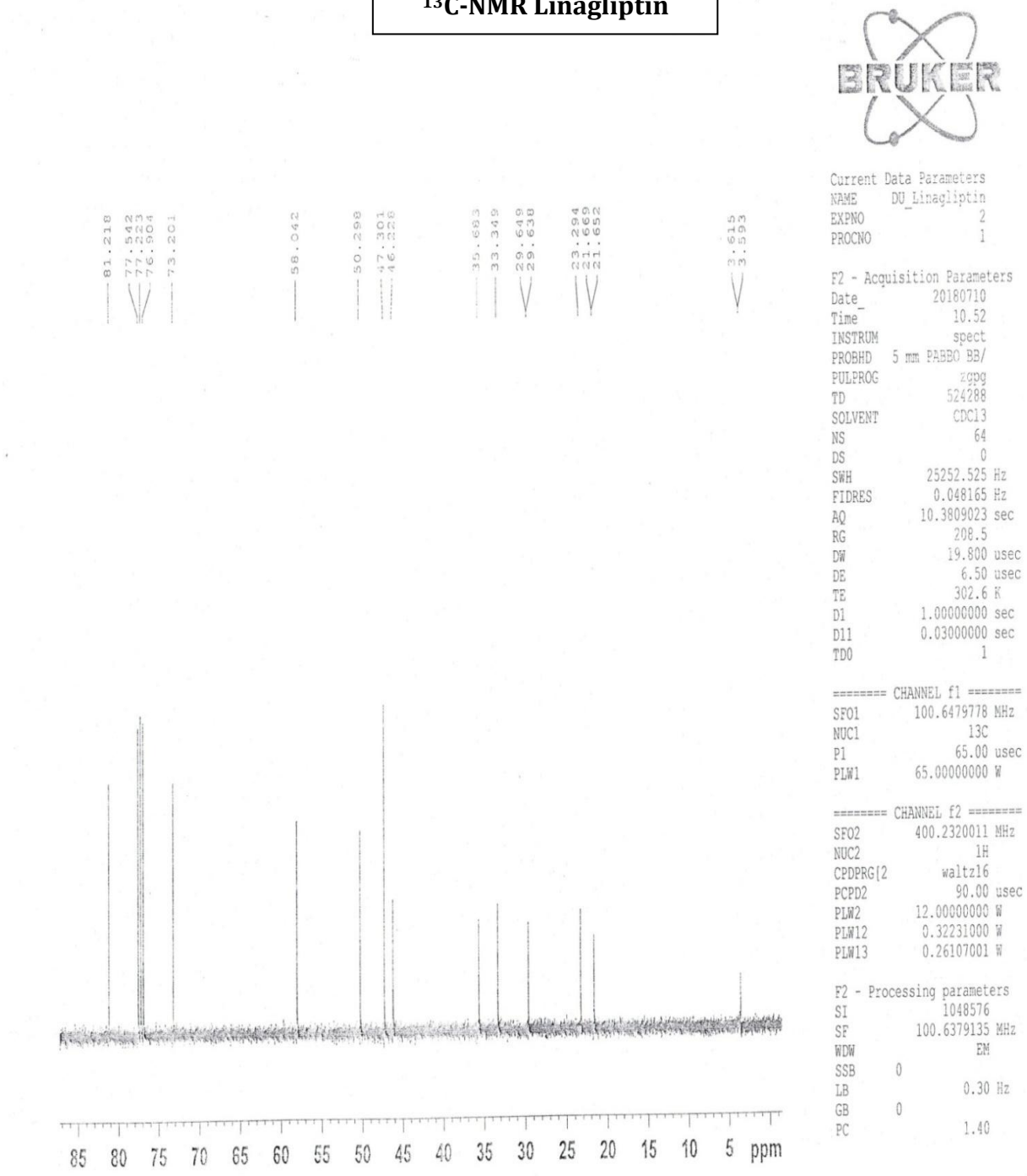


Figure 4.24. Partially Expanded ^{13}C-NMR Spectrum of Standard Linagliptin in $CDCl_3$

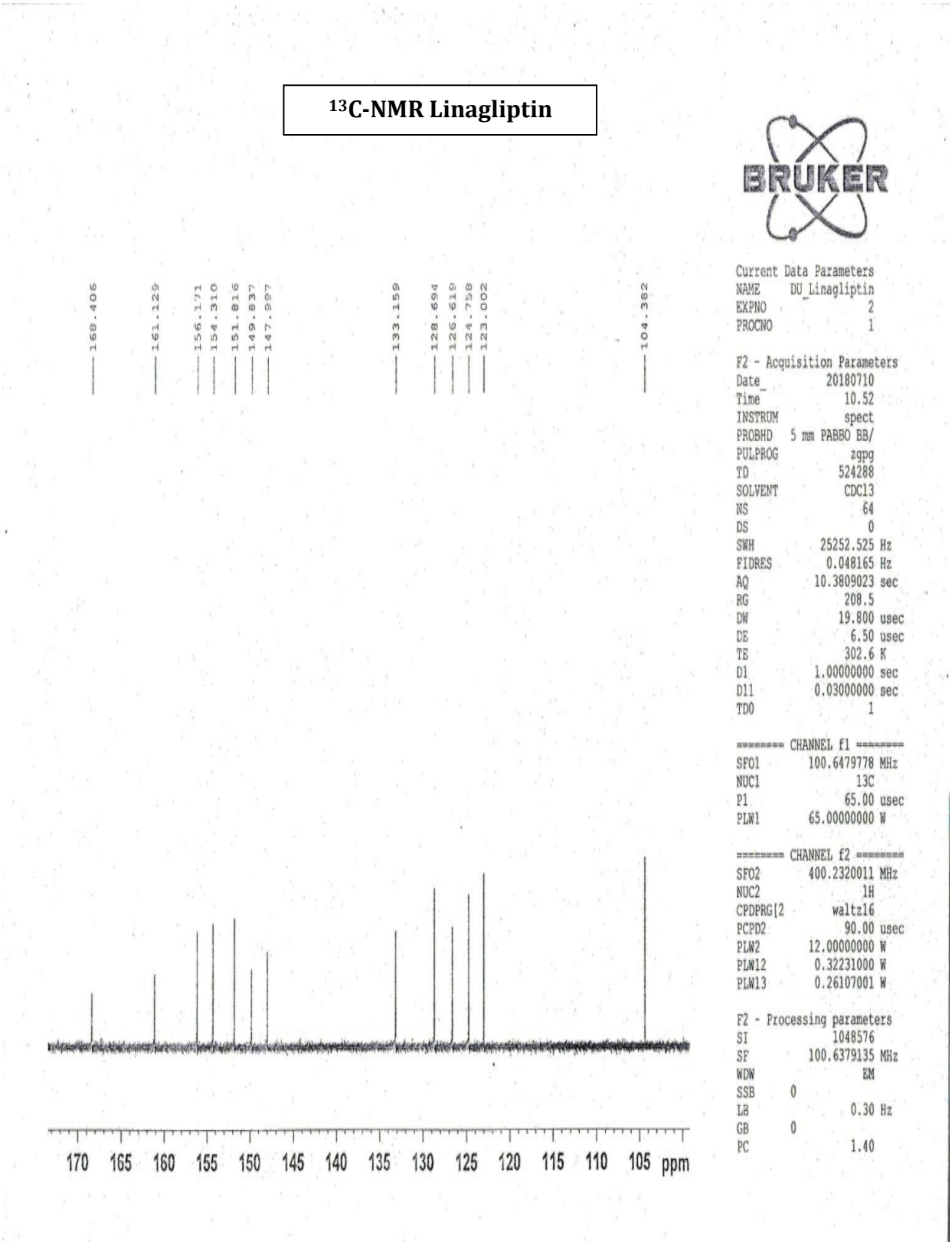
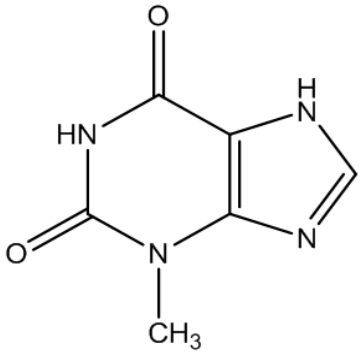
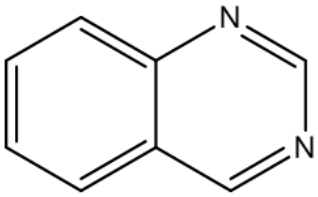
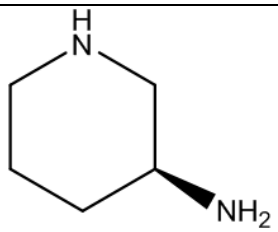


Figure 4.25. Partially Expanded ^{13}C-NMR Spectrum of Standard Linagliptin in $CDCl_3$

Table 4.33. IR Data of Standard Linagliptin

Stretching	Functional Group [1]	Peak observed in cm^{-1}
N-H	Piperidine	3350.6
-NH2	Primary amine	2935.5
–C≡C–	Characteristic of ethyne	2250.6
	Characteristic of carbonyl in purine ring	1695.2
	Quinazoline cluster	1517.2
C-N	Aromatic tertiary amine	1397.7
	Amino pyridine cluster	1140.9
N-H	Absorption of bonds outside the plane between nitrogen and hydrogen	758.7

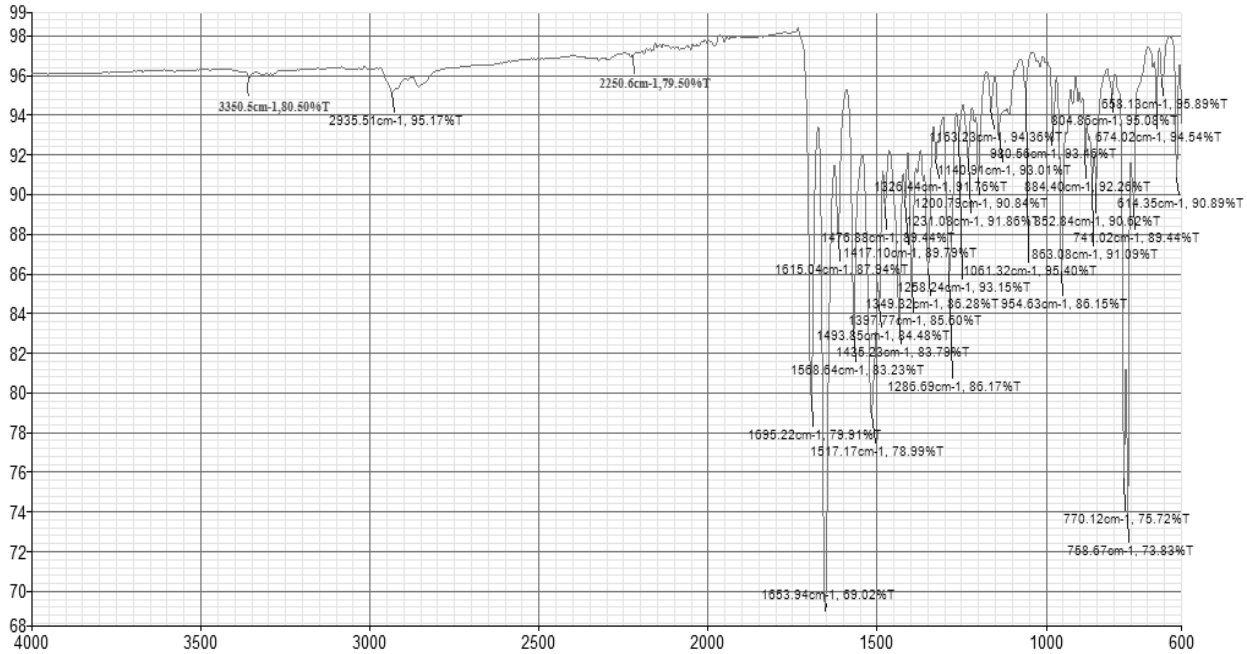
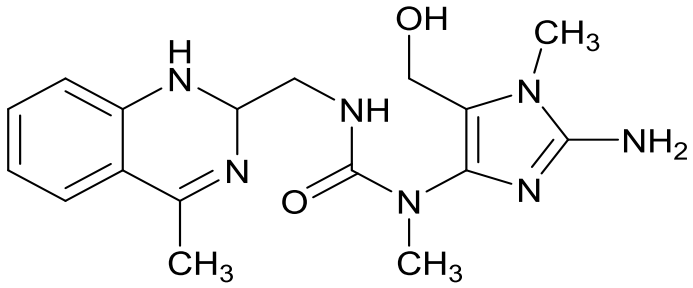


Figure 4.26. IR Spectrum of Standard Linagliptin

4.6.2. Structure Elucidation of Acidic Degradant of Linagliptin-1(DA1)



1-(2-Amino-5-(hydroxymethyl)-1-methyl-1H-imidazol-4-yl)-1-methyl-3-((4-methyl-1,2-dihydroquinazolin-2-yl)methyl)urea **(DA1)**

^{1}H-NMR (**Figure 4.27-4.29**) and ^{13}C-NMR (**Figure 4.30-4.32**) data of DA1 in $CDCl_3$ and assignments of all carbons and protons of these compounds are given in **Table 4.34** and **Table 4.35**, respectively.

The ^{13}C-NMR spectrum of linagliptin showed 25 resonance signals, but the ^{13}C-NMR of DA1 showed only 17 resonance signals. Comparison of the ^{13}C-NMR spectra of linagliptin and DA1 indicated that signals for C-20 (δ 81.2), C-21 (δ 73.2), C-22 (δ 3.6), C-24 (δ 58.0), C-25 (δ 46.2), C-26 (δ 35.7), C-27 (δ 21.7) and C-28 (δ 47.3) of linagliptin are absent in DA1. Carbons at C-24, C-25, C-26, C-27 and C-28 constituted the 3-amino-

piperidyl ring system in linagliptin. Therefore, these findings revealed absence of the 3-amino-piperidyl moiety in DA1. The carbons at C-20, C-21, C-22 are part of 2-butynyl group at N-7 of linagliptin. The peak for C-19 appeared at δ 25.3 in ^{13}C-NMR and corresponding protons appeared as 3H singlet at δ 1.78 in ^{1}H-NMR spectrum. This observation indicated that acid induced cleavage of carbon-carbon bond between C-19 and C-20 had been occurred during degradation process.

The resonance signal of carbon at C-6 (δ154.3) of linagliptin shifted to a high-field region at δ49.8 in the ^{13}C-NMR spectrum of DA1. Similarly, the resonance signal of carbon at C-12 (δ168.4) of linagliptin shifted to a high-field region at δ79.2 in the ^{13}C-NMR spectrum of DA1. In addition, the ^{1}H-NMR spectrum of DA1 showed three additional of peaks, two for a methylene group protons and one for a methine proton. The methylene protons appeared at δ4.08 (1H, dd, *J*=4.0, 13.2 Hz.) and δ4.02 (1H, dd, *J*=4.0, 10.6 Hz.), and the methine proton appeared at δ4.74 (1H, dd, *J*=5.2, 14.0 Hz.). These finding clearly revealed that bond cleavage between N-1 and C-6 of pyrimidine-2,4-dione moiety of the linagliptin, and reduction of the bond between N-11 and C-12 occurred during the degradation process. Thus the structure of DA1 was elucidated as 1-(2-amino-5-(hydroxymethyl)-1-methyl-1H-imidazol-4-yl)-1-methyl-3-((4-methyl-1, 2-dihydroquinazolin-2-yl) methyl) urea.

IR data **(Table 4.36 and Figure 4.33**) also support this structure by showing the peak of hydroxyl group at position 3307.7 cm^{-1} and 1023.8 cm^{-1} for carbonyl position.

Table 4.34. Comparison ^{13}C-NMR Spectral Data of Acid Degradant-1 (DA1) with Linagliptin.

Position(C#)	^{13}C-NMR Linagliptin	^{13}C-NMR DA1
	δ_C (ppm)	
C-2	151.8	151.8
C-4	149.8	150.0
C-5	104.4	103.6
C-6	**154.3**	**49.8**
C-8	161.1	161.1
C-10	50.3	46.3
C-12	**168.4**	**79.2**
C-14	156.2	168.6

C-14a	123.0	123.2
C-15	124.8	124.9
C-16	126.6	126.8
C-17	133.2	133.3
C-18	128.7	128.9
C-18a	147.9	154.1
C-19	29.6	25.3
C-20	81.2	
C-21	73.2	
C-22	3.6	
C-24	58.0	
C-25	46.2	
C-26	35.7	
C-27	21.7	
C-28	47.3	
C-30	33.3	29.9
C-31	23.3	21.8

Table 4.35. Comparison ^{1}H-NMR Spectral Data of Acid Degradant-1 (DA1) with Linagliptin.

Position(C#)	**^{1}H-NMR Linagliptin**		**^{1}H-NMR DA1**	
	δ_H (ppm), *J (Hz)*			
H-6a			4.08	1H, dd, *J*=4.0, 13.2 Hz.
H-6b			4.02	1H, dd, *J*=4.0, 10.6 Hz.
6-OH			5.56	1H, s
H_2-10	5.49	2H, s	5.56~5.59	2H, m
H-12			4.74	1H, dd, *J*=5.2, 14.0 Hz.
H-15	7.77	1H, d, *J*=8.4 Hz.	7.89	1H, d, *J*=8.0 Hz.
H-16	7.41	1H, t, *J*=7.6 Hz.	7.55	1H, d, *J*=7.6 Hz
H-17	7.66	1H, t, *J*=7.6 Hz.	7.97	1H, dt, *J*=1.2, 8.0 Hz.
H-18	7.91	1H, d, *J*=8.0 Hz.	8.03	1H, d, *J*=8.4 Hz.
H_2-19	4.80	2H, br. s	1.78	3H, s
H_3-22	1.71	3H, s		
H-24b	3.60	1H, dd, *J*= 12, 2.8 Hz.		
H-24a	2.99~3.05	1H, m		
H-25	2.83	1H, dd, *J*=12.0, 12.4 Hz.		
H-26a	1.24~1.33	1H, m		
H-26b	1.78~1.81	1H, m		

H-27a	1.60~1.68	1H, m		
H-27b	1.89~1.93	1H, m		
H-28a	3.49~3.52	1H, m		
H-28b	2.96~2.98	1H, m		
H_2-29	1.97	2H, br. s		
H_3-30	3.47	3H, s	3.56	3H, s
H_3-31	2.78	3H, s	2.91	3H, s

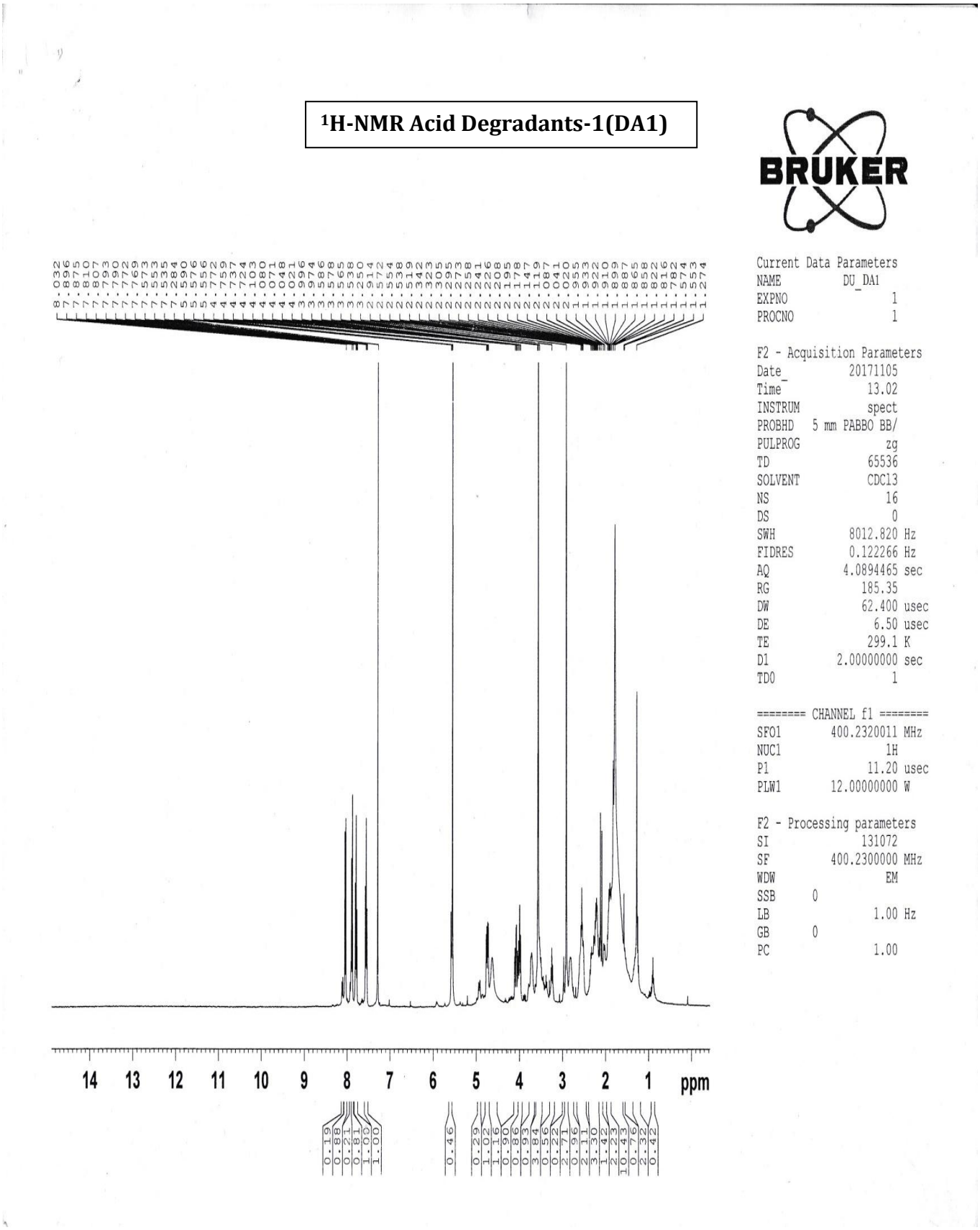
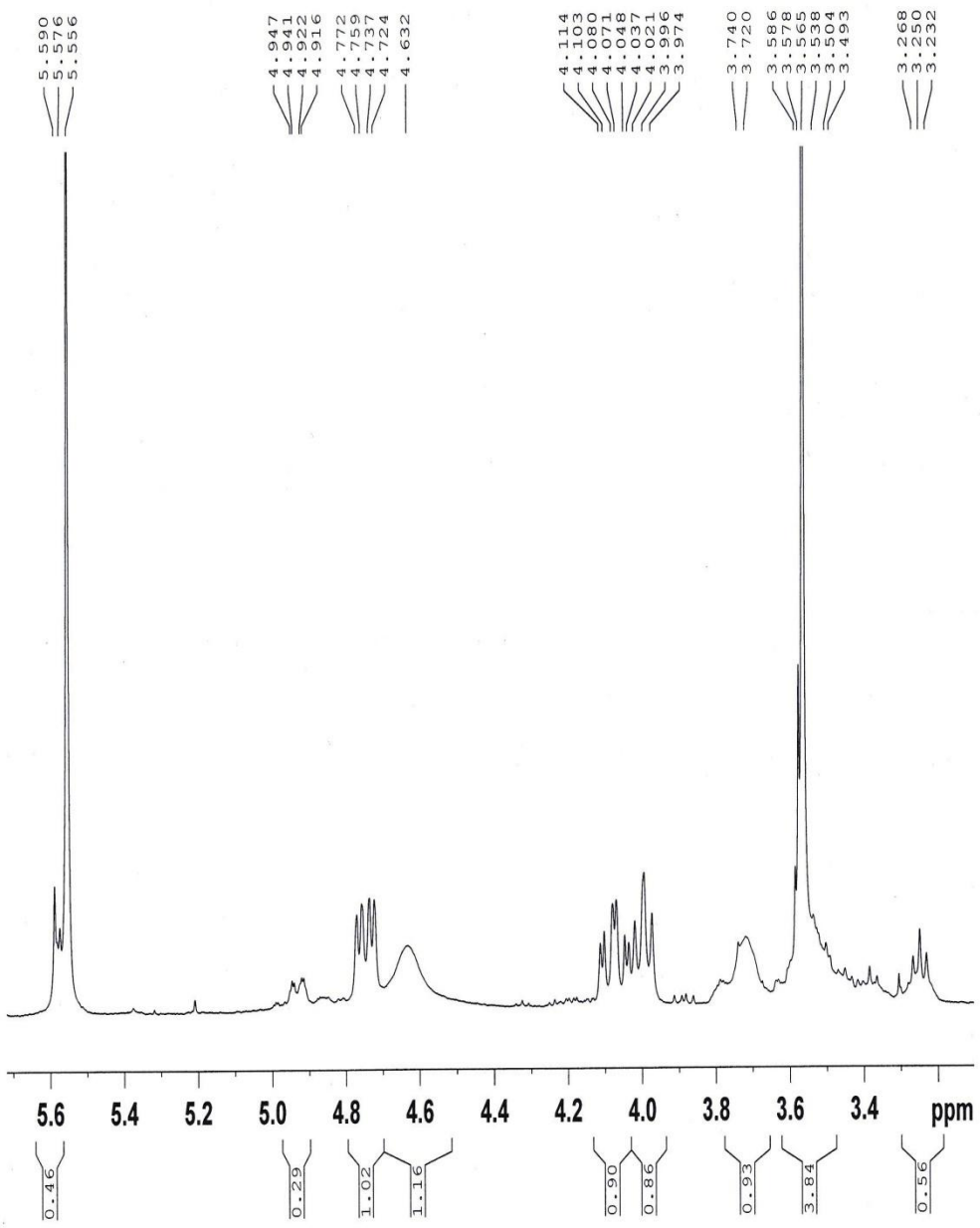


Figure 4.27. ¹H NMR Spectrum of Acid Degradant-1(DA1) in $CDCl_3$

^{1}H-NMR Acid Degradants-1(DA1)

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PROCNO             1

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PULPROG             zg
TD               65536
SOLVENT          CDCl3
NS                  16
DS                   0
SWH           8012.820 Hz
FIDRES        0.122266 Hz
AQ            4.0894465 sec
RG               185.35
DW               62.400 usec
DE                6.50 usec
TE               299.1 K
D1           2.00000000 sec
TD0                 1

======== CHANNEL f1 ========
SFO1        400.2320011 MHz
NUC1                1H
P1                11.20 usec
PLW1        12.00000000 W

F2 - Processing parameters
SI                131072
SF          400.2300000 MHz
WDW                 EM
SSB      0
LB                1.00 Hz
GB       0
PC                1.00
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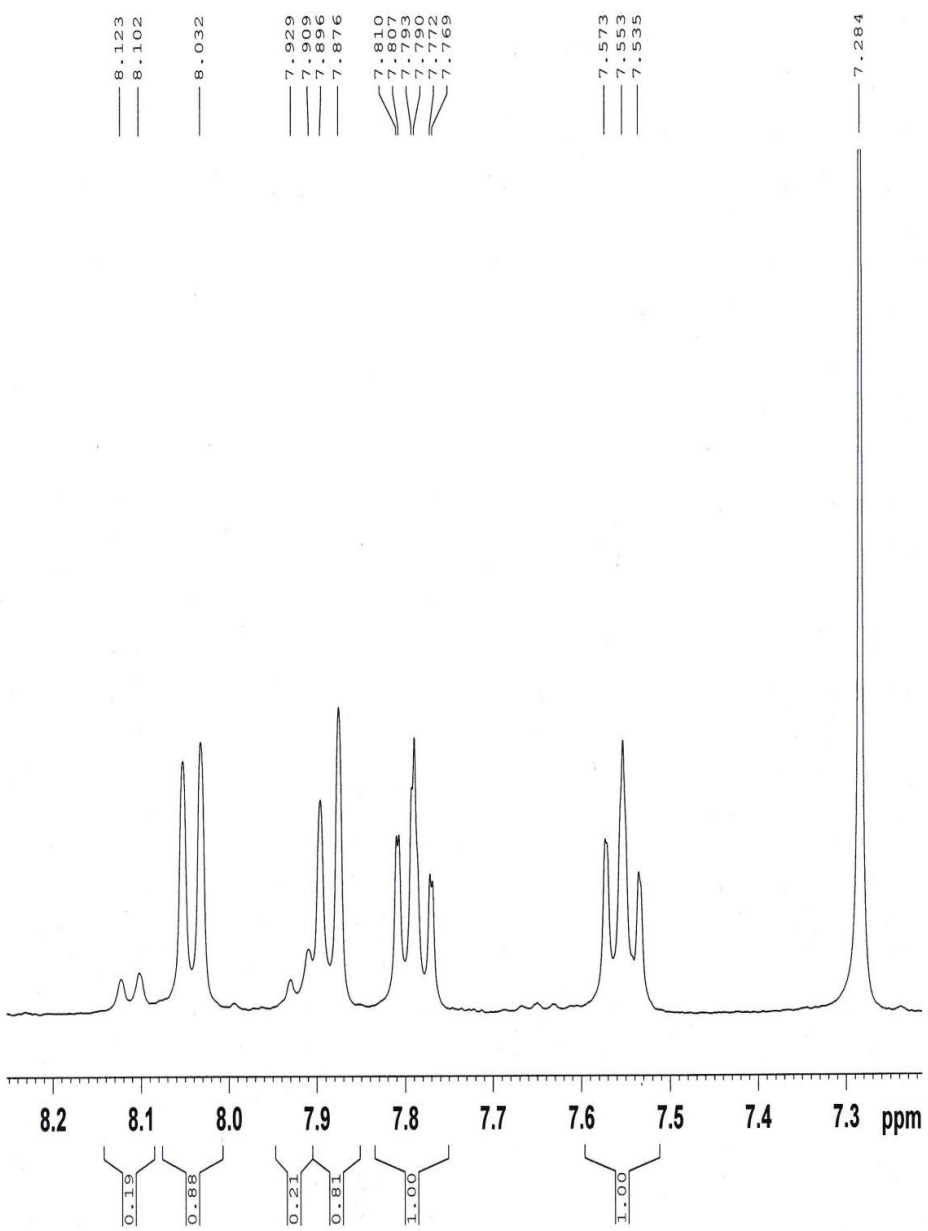
Figure 4.28. Expanded ^{1}H-NMR spectrum of Acid Degradant-1(DA1) in $CDCl_3$

^{1}H-NMR Acid Degradants-1(DA1)

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EXPNO              1
PROCNO             1

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Time             13.02
INSTRUM          spect
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PULPROG             zg
TD               65536
SOLVENT          CDCl3
NS                  16
DS                   0
SWH           8012.820 Hz
FIDRES        0.122266 Hz
AQ            4.0894465 sec
RG               185.35
DW               62.400 usec
DE                6.50 usec
TE               299.1 K
D1           2.00000000 sec
TD0                 1

======== CHANNEL f1 ========
SFO1        400.2320011 MHz
NUC1                1H
P1                11.20 usec
PLW1        12.00000000 W

F2 - Processing parameters
SI                131072
SF          400.2300000 MHz
WDW                 EM
SSB      0
LB                1.00 Hz
GB       0
PC                1.00
```
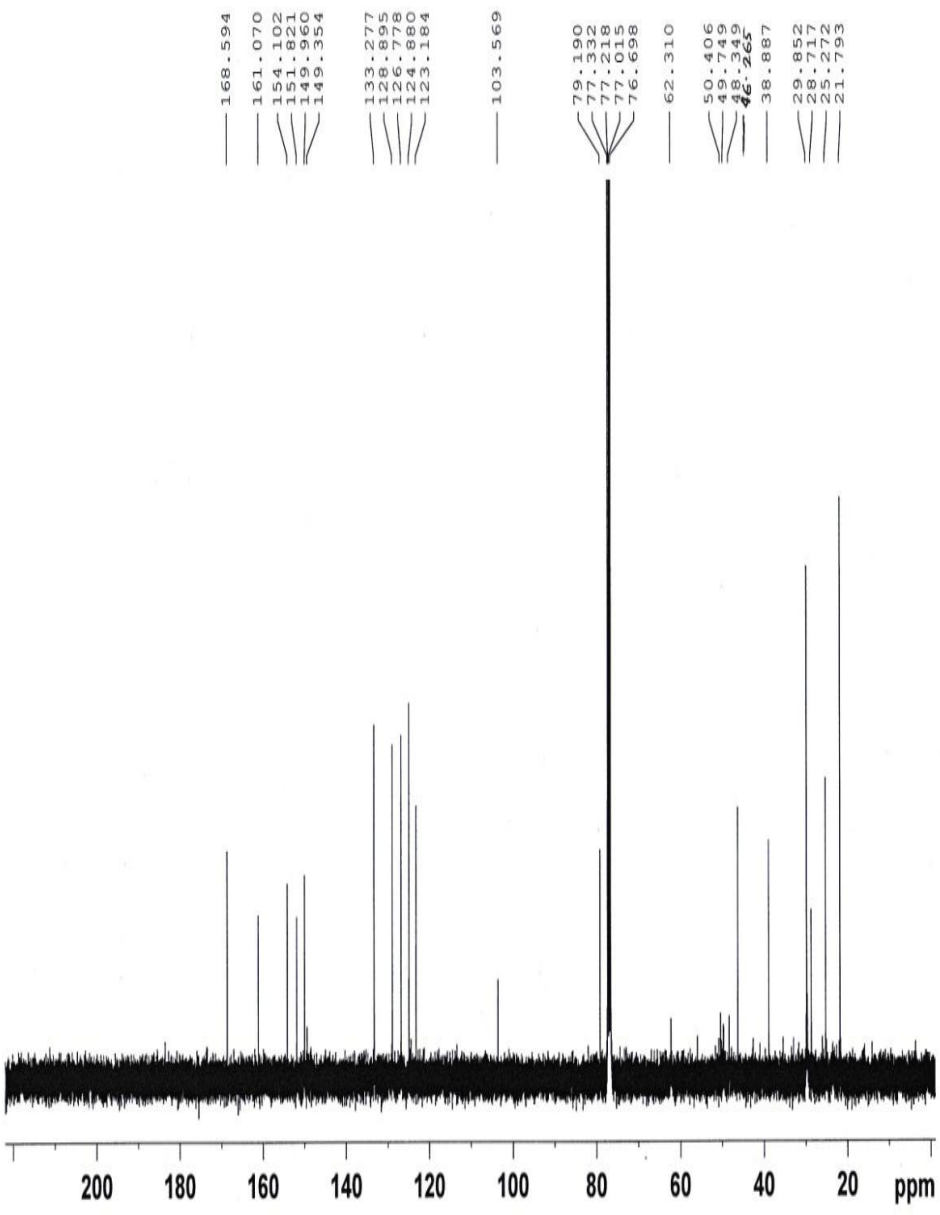

Figure 4.29. Expanded ^{1}H-NMR Spectrum of Acid Degradant-1(DA1) in $CDCl_3$

^{13}C-NMR Acid Degradants-1(DA1)



BRUKER

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SOLVENT           CDCl3
NS                 4608
DS                    0
SWH          25252.525 Hz
FIDRES        0.048165 Hz
AQ           10.3809023 sec
RG                208.5
DW               19.800 usec
DE                6.50 usec
TE               301.5 K
D1           1.00000000 sec
D11          0.03000000 sec
TD0                   1

======== CHANNEL f1 ========
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NUC1               13C
P1                10.00 usec
PLW1        49.00000000 W

======== CHANNEL f2 ========
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NUC2               1H
CPDPRG[2        waltz16
PCPD2             90.00 usec
PLW2        12.00000000 W
PLW12        0.18584000 W
PLW13        0.15053000 W

F2 - Processing parameters
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SF          100.6379135 MHz
WDW                EM
SSB      0
LB                0.30 Hz
GB       0
PC                1.40
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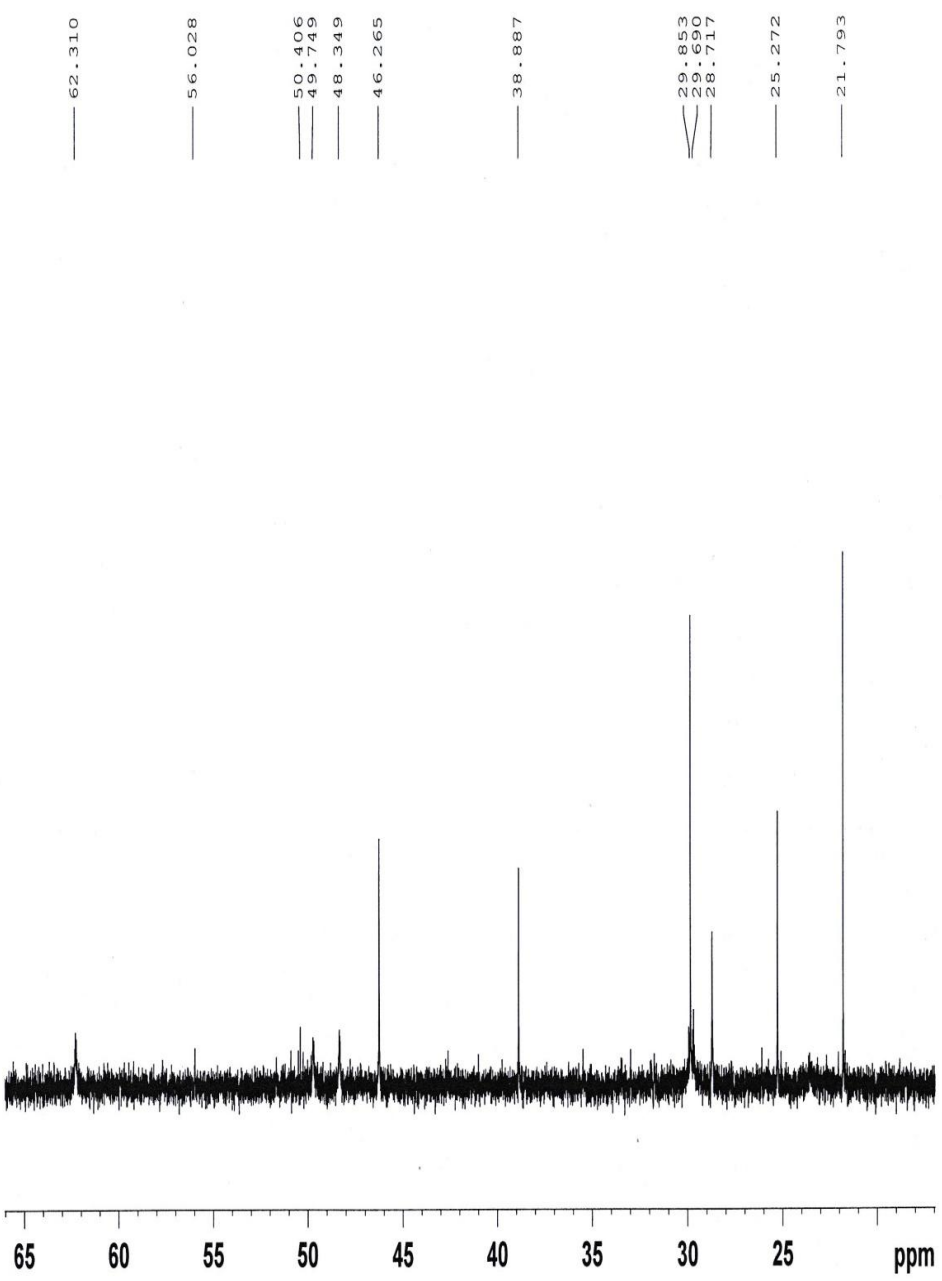
Figure 4.30. ^{13}C-NMR Spectrum of Acid Degradant-1(DA1) in $CDCl_3$

^{13}C-NMR Acid Degradants-1(DA1)



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EXPNO              2
PROCNO             1

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PULPROG           zgpg
TD               524288
SOLVENT           CDCl3
NS                 4608
DS                    0
SWH           25252.525 Hz
FIDRES         0.048165 Hz
AQ            10.3809023 sec
RG                208.5
DW               19.800 usec
DE                6.50 usec
TE                301.5 K
D1            1.00000000 sec
D11           0.03000000 sec
TD0                   1

======== CHANNEL f1 ========
SFO1        100.6479778 MHz
NUC1                13C
P1                10.00 usec
PLW1        49.00000000 W

======== CHANNEL f2 ========
SFO2        400.2320011 MHz
NUC2                1H
CPDPRG[2         waltz16
PCPD2             90.00 usec
PLW2        12.00000000 W
PLW12        0.18584000 W
PLW13        0.15053000 W

F2 - Processing parameters
SI               1048576
SF          100.6379135 MHz
WDW                 EM
SSB      0
LB                0.30 Hz
GB       0
PC                1.40
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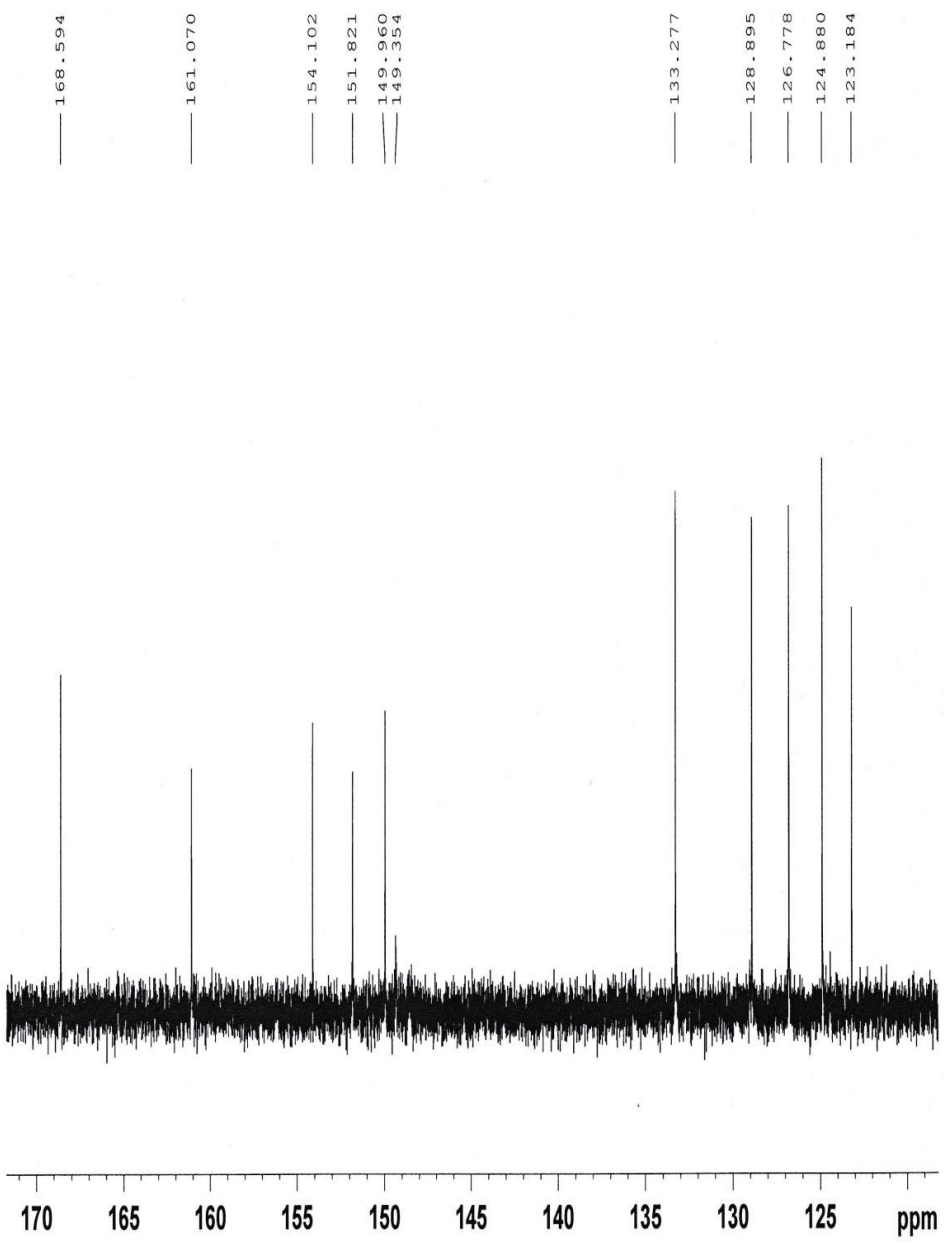
Figure 4.31. Expanded ^{13}C-NMR Spectrum of Acid Degradant-1(DA1) in $CDCl_3$

^{13}C-NMR Acid Degradants-1(DA1)

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Current Data Parameters
NAME                DU_DA1
EXPNO                    2
PROCNO                   1

F2 - Acquisition Parameters
Date_             20171106
Time                 5.15
INSTRUM              spect
PROBHD   5 mm PABBO BB/
PULPROG              zgpg
TD                 524288
SOLVENT             CDCl3
NS                    4608
DS                       0
SWH              25252.525 Hz
FIDRES            0.048165 Hz
AQ               10.3809023 sec
RG                   208.5
DW                  19.800 usec
DE                   6.50 usec
TE                  301.5 K
D1               1.00000000 sec
D11              0.03000000 sec
TD0                      1

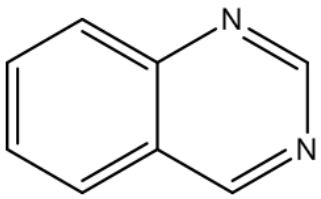
======== CHANNEL f1 ========
SFO1            100.6479778 MHz
NUC1                    13C
P1                   10.00 usec
PLW1            49.00000000 W

======== CHANNEL f2 ========
SFO2            400.2320011 MHz
NUC2                    1H
CPDPRG[2             waltz16
PCPD2                90.00 usec
PLW2            12.00000000 W
PLW12            0.18584000 W
PLW13            0.15053000 W

F2 - Processing parameters
SI                    1048576
SF              100.6379135 MHz
WDW                      EM
SSB                      0
LB                    0.30 Hz
GB                       0
PC                    1.40
```

Figure 4.32. Expanded ^{13}C-NMR Spectrum of Acid Degradant-1(DA1) in $CDCl_3$

Table 4.36. IR Data of Acid Degradant-1(DA1)

Stretching	**Functional Group**	**Peak observed in cm^{-1}**
-OH	Alcohol	3307.7
-NH2	Primary amine	2941.2
	Quinazoline cluster	1528.8
C-N	aromatic tertiary amine	1312.4
N-H	Absorption of bonds outside the plane between nitrogen and hydrogen	798.8
-C=O	Carbonyl group	1023.8

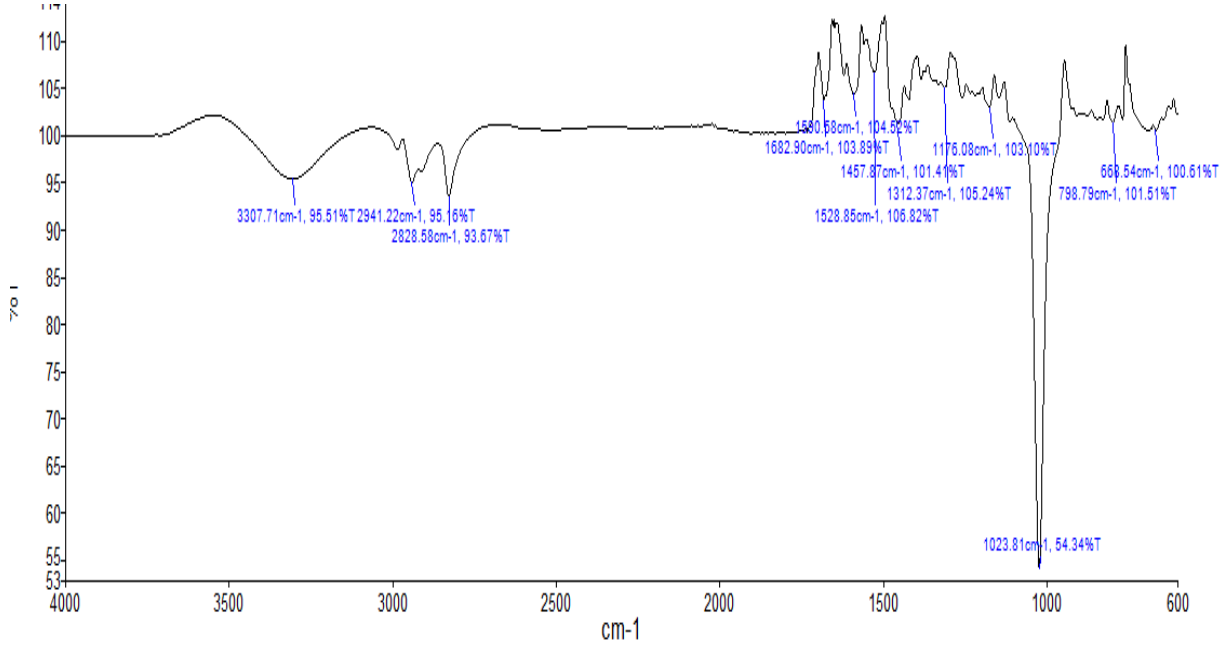
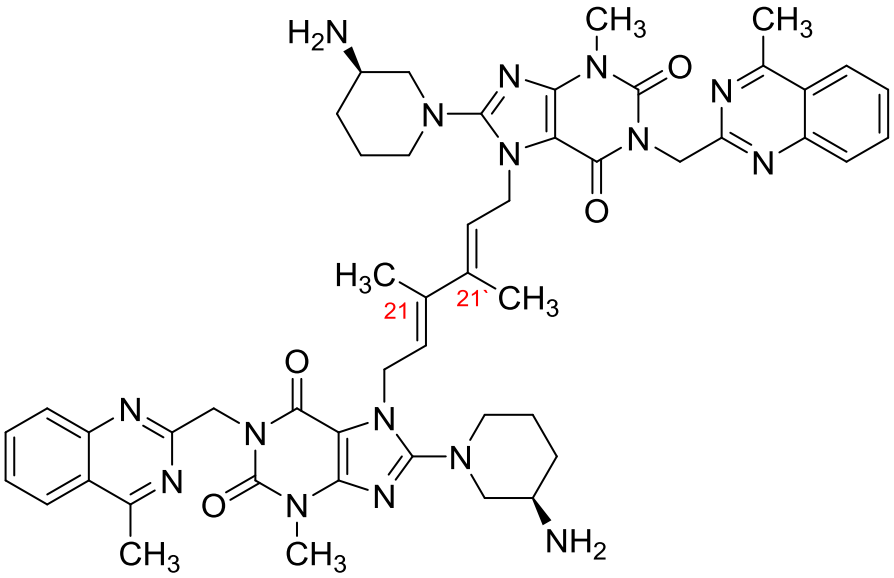


Figure 4.33. IR Spectrum of Standard of Acid Degradant-1(DA1)

4.6.3. Structure Elucidation of Acidic Degradant-2 of Linagliptin (DA2)



7,7'-((2E,4E)-3,4-dimethylhexa-2,4-diene-1,6-diyl)bis(8-((R)-3-aminopiperidin-1-yl)-3-methyl-1-((4-methylquinazolin-2-yl)methyl)-3,7-dihydro-1H-purine-2,6-dione) **(DA2)**

^{1}H-NMR (**Figure 4.34 - 4.36**) and ^{13}C-NMR (**Figure 4.37- 4.39**) data of DA2 in $CDCl_3$ and assignment of all carbons and protons of this degradant product were given in **Table 4.37** and **4.38**, respectively.

In ^{1}H-NMR spectrum of linagliptin, the terminal methyl protons of 2-butynyl group at N-7 (H_3-22) appeared at δ 1.71 (3H, s). These methyl protons were experiencing the anisotropic effect of the carbon-carbon triple bond, and were situated spatially at the shielding region of the triple bond. In ^{13}C-NMR, carbon of this methyl group also appeared at very high field δ 3.56. Both these protons and carbon peaks were absent in the ^{13}C-NMR and ^{1}H-NMR spectra, respectively, of DA2. Instead, a low filed shifted carbon at 26.1 was appeared. This observation clearly indicated that some change had been occurred at the carbon-carbon triple bond.

On the other hand, carbons of the carbon-carbon triple bond (C-20 and C-21) appeared at δ 81.2 and δ 73.2. These peaks were also absent in the ^{13}C-NMR of DA2. Instead of these peaks, two additional sp^2 hybridized carbons signals at δ 121.6 and δ 133.1 were appeared. Moreover, one more methane proton peak in the ^{1}H-NMR spectrum of DA2

was observed at δ 5.89 (dd, *J*= 5.6, 6.0 Hz.). All of the above findings clearly revealed that the carbon-carbon triple bond had been converted to a carbon-carbon double bond in DA2 at C-20 and C-21 position. The signal δ 5.89 (dd, *J*= 5.6, 6.0 Hz.) was assigned to the proton of C-20. As no proton signal for C-21 was observed and CH_3 signal still appeared as a singlet in the ^{1}H-NMR of DA2, it unambiguously revealed that C-21 was a quaternary sp^2 carbon atom in DA2. Chemical shift values of these carbons (C-20 and C-21) ruled out the presence on any electronegative atom next to them. Thus, the structure of DA2 was elucidated as 7,7'-((2E,4E)-3,4-dimethylhexa-2,4-diene-1,6-diyl)bis(8-((R)-3-aminopiperidin-1-yl)-3-methyl-1-((4-methylquinazolin-2-yl)methyl)-3,7-dihydro-1H-purine-2,6-dione), which is a dimer of linagliptin via C-21 carbon.

IR data (**Table 4.39 and Figure 4.40**) support this data by showing a single distinguishable peak of alkene at the position 1654.2 cm-1.

Table 4.37. Comparison of ^{13}C-NMR data of acid degradant-2(DA2) with linagliptin in $CDCl_3$.

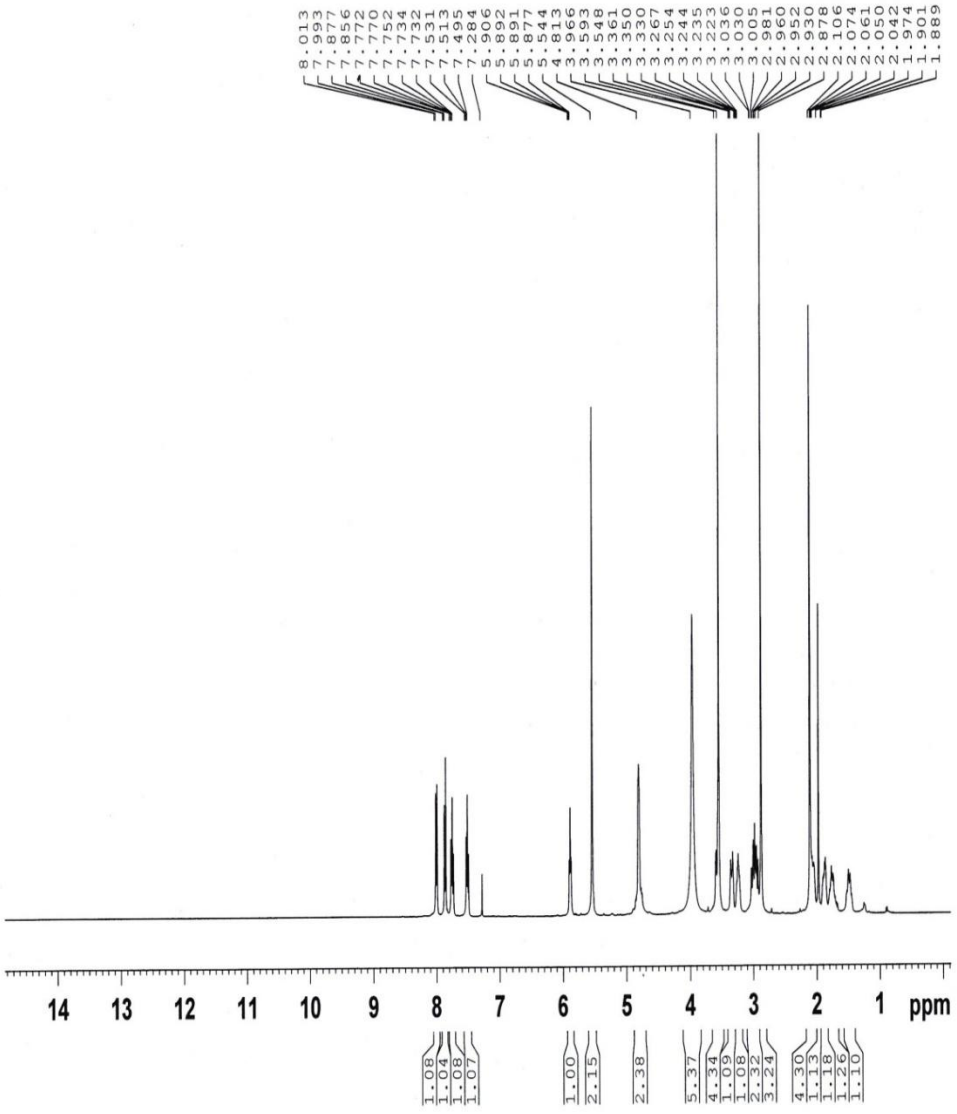
Position(C#)	^{13}C-NMR Linagliptin	^{13}C-NMR DA2
	δ_C (ppm)	
C-2	151.8	151.8
C-4	149.8	149.9
C-5	104.4	105
C-6	154.3	154.6
C-8	161.1	161.1
C-10	50.3	51.1
C-12	168.4	168.6
C-14	156.2	156.4
C-14a	123.0	123.1
C-15	124.8	124.7
C-16	126.6	126.8
C-17	133.2	133.3
C-18	128.7	128.8
C-18a	147.9	148.1
C-19	29.6	29.8
C-20	81.2	121.6
C-21	73.2	133.1
C-22	3.6	26.1

C-24	58.0	56.6
C-25	46.2	46.3
C-26	35.7	44.5
C-27	21.7	21.7
C-28	47.3	47.3
C-30	33.3	31.7
C-31	23.3	23.4

Table 4.38. Comparison of ^{1}H-NMR data of acid degradant-2(DA2) with linagliptin in $CDCl_3$.

Position(C#)	^{1}H-NMR Linagliptin		^{1}H-NMR DA2	
	δ_H (ppm), J (Hz)			
H_2-10	5.49	2H, s	5.54	2H, s
H-15	7.77	1H, d, *J*=8.4 Hz.	7.87	1H, d, *J*=8.4
H-16	7.41	1H, t, *J*=7.6 Hz.	7.51	1H, t, *J*=7.6 Hz
H-17	7.66	1H, t, *J*=7.6 Hz.	7.75	1H,t, *J*=7.6 Hz.
H-18	7.91	1H, d, *J*=8.0 Hz.	8.00	1H, d, *J*=8.4
H_2-19	4.80	2H, br. s	4.81	2H,br. s
H-20	-	-	5.89	1H, dd, *J*=5.6, 6.0 Hz
H_3-22	1.71	3H, s	2.11	3H, s
H-24b	3.60	1H, dd, *J*= 12, 2.8 Hz.	3.59	1H, overlapped
H-24a	2.99~3.05	1H, m	3.00~3.04	1H, m
H-25	2.83	1H, dd, *J*=12.0, 12.4 Hz.	2.93~2.98	1H, m
H-26a	1.24~1.33	1H, m	1.45~1.53	1H, m
H-26b	1.78~1.81	1H, m	1.74~1.77	1H, m
H-27a	1.60~1.68	1H, m	2.04~2.07	1H, m
H-27b	1.89~1.93	1H, m	1.87~1.90	1H, m
H-28a	3.49~3.52	1H, m	3.33~3.36	1H, m
H-28b	2.96~2.98	1H, m	3.22~3.27	1H, m
H_2-29	1.97	2H, br. s	1.97	2H, s
H_3-30	3.47	3H, s	3.54	3H,s
H_3-31	2.78	3H, s	2.88	3H, s

^{1}H-NMR Acid Degradants-2 (DA2)

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Current Data Parameters
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EXPNO              1
PROCNO             1

F2 - Acquisition Parameters
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Time             13.27
INSTRUM          spect
PROBHD   5 mm PABBO BB/
PULPROG             zg
TD               65536
SOLVENT          CDCl3
NS                  16
DS                   0
SWH           8012.820 Hz
FIDRES        0.122266 Hz
AQ            4.0894465 sec
RG                22.53
DW               62.400 usec
DE                6.50 usec
TE               299.4 K
D1           2.00000000 sec
TD0                 1

======== CHANNEL f1 ========
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NUC1                1H
P1                11.20 usec
PLW1        12.00000000 W

F2 - Processing parameters
SI                131072
SF          400.2300000 MHz
WDW                 EM
SSB      0
LB                1.00 Hz
GB       0
PC                1.00
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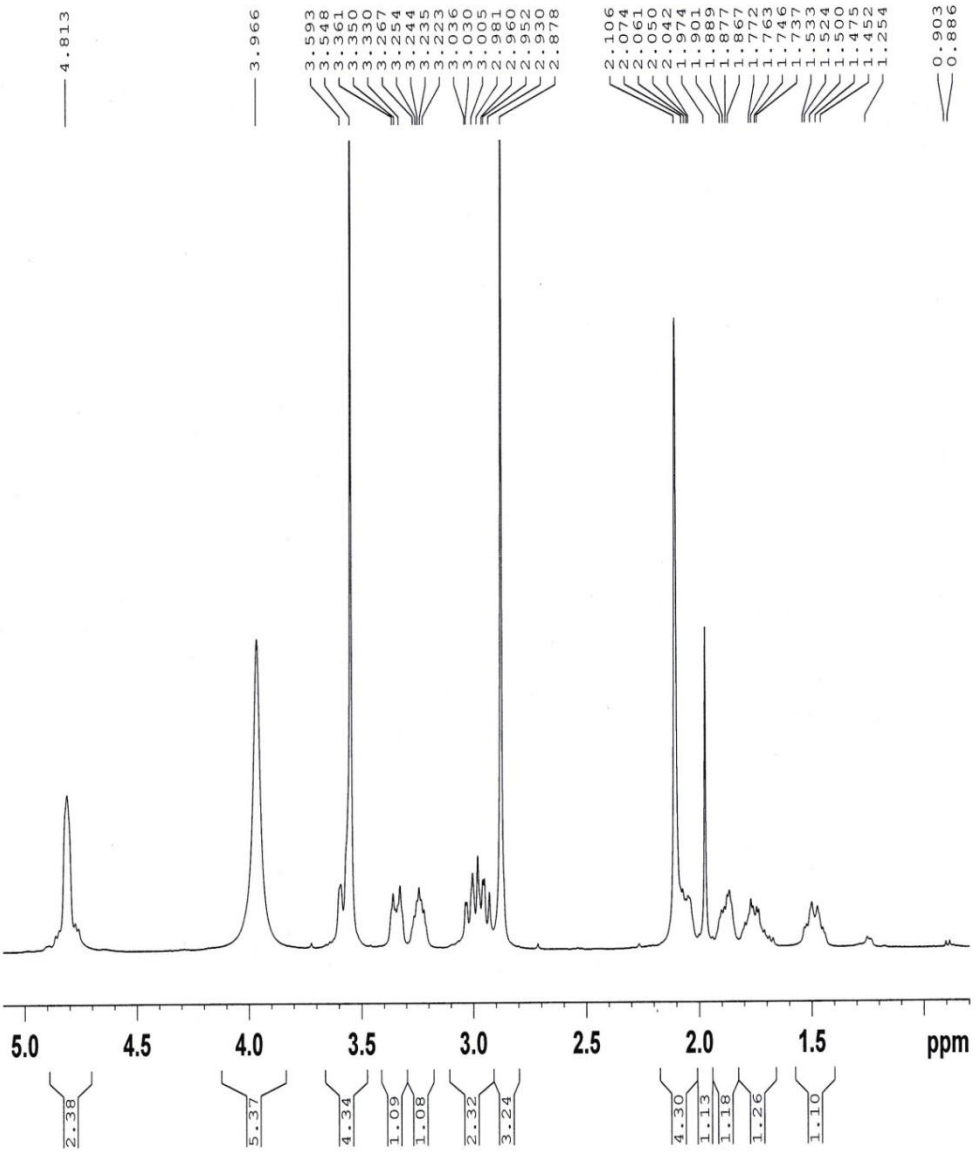
Figure 4.34. ^{1}H NMR Spectrum of acid Degradant-1(DA2) in $CDCl_3$

^{1}H-NMR Acid Degradants-2 (DA2)



BRUKER

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Current Data Parameters
NAME          DU_DA2
EXPNO              1
PROCNO             1

F2 - Acquisition Parameters
Date_          20171106
Time              13.27
INSTRUM           spect
PROBHD   5 mm PABBO BB/
PULPROG             zg
TD                65536
SOLVENT           CDCl3
NS                   16
DS                    0
SWH            8012.820 Hz
FIDRES         0.122266 Hz
AQ             4.0894465 sec
RG                22.53
DW               62.400 usec
DE                6.50 usec
TE               299.4 K
D1            2.00000000 sec
TD0                  1

======== CHANNEL f1 ========
SFO1         400.2320011 MHz
NUC1                 1H
P1                11.20 usec
PLW1         12.00000000 W

F2 - Processing parameters
SI                131072
SF           400.2300000 MHz
WDW                  EM
SSB       0
LB                1.00 Hz
GB        0
PC                1.00
```
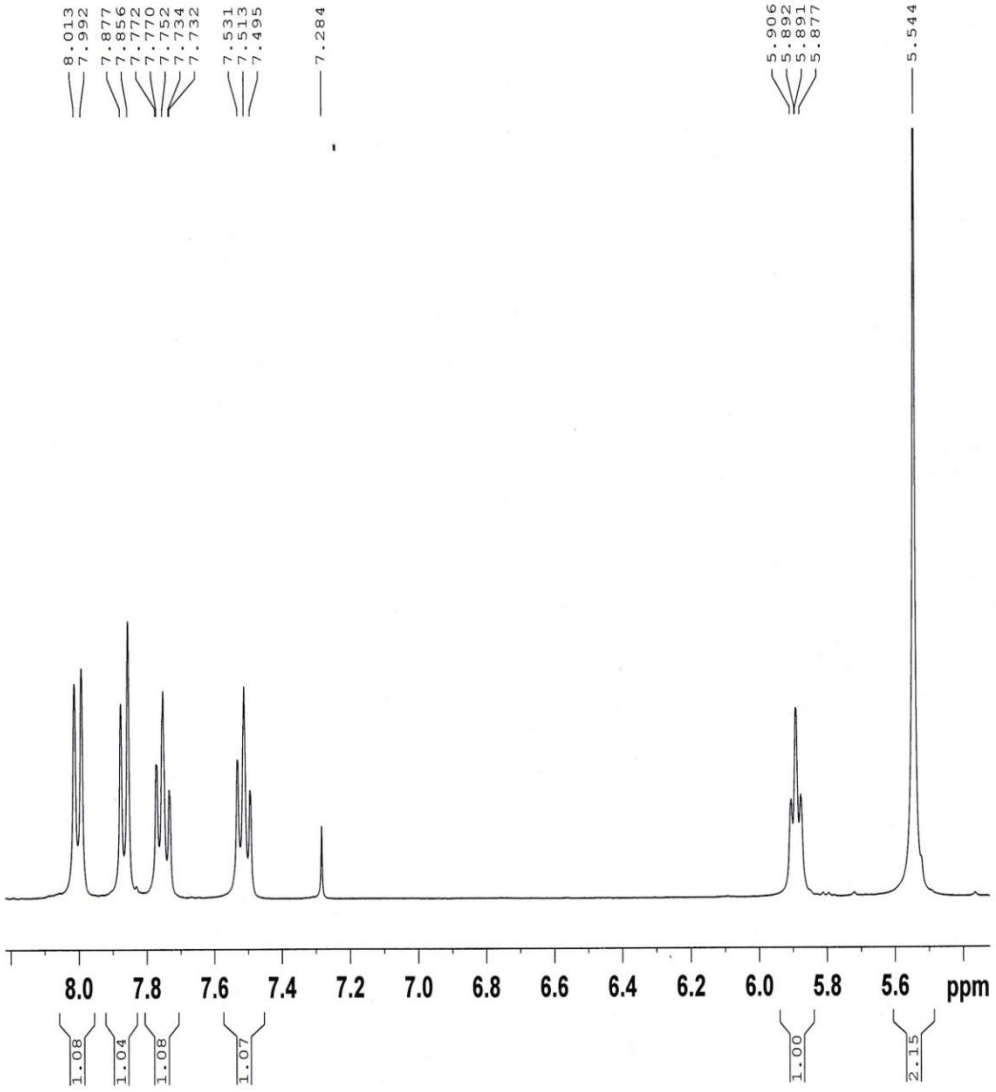

Figure 4.35. Expanded ^{1}H NMR Spectrum of Acid Degradant-1(DA2) in $CDCl_3$

^{1}H-NMR Acid Degradants-2(DA2)



Current Data Parameters
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EXPNO 1
PROCNO 1

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INSTRUM spect
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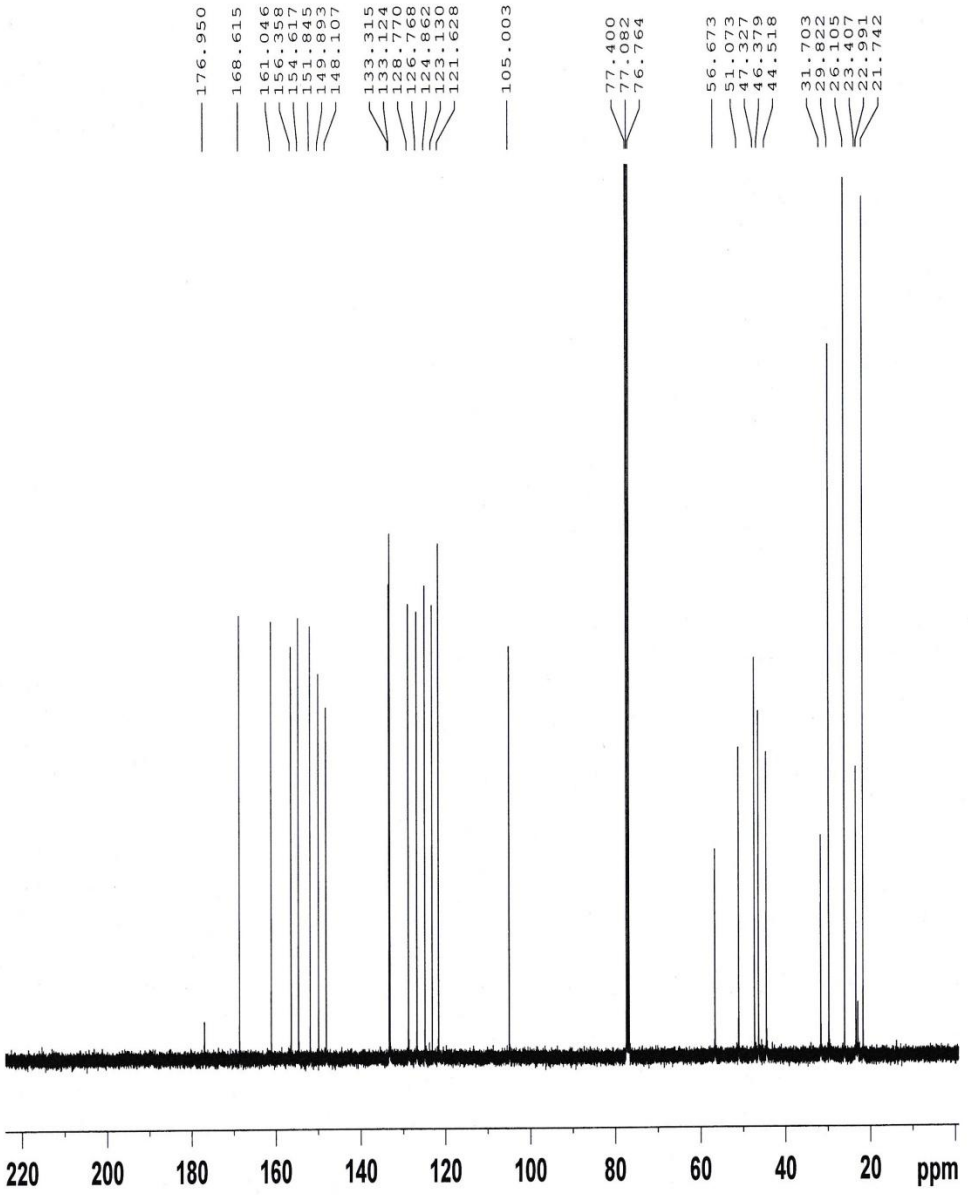
PULPROG zg
TD 65536
SOLVENT CDCl3
NS 16
DS 0
SWH 8012.820 Hz
FIDRES 0.122266 Hz
AQ 4.0894465 sec
RG 22.53
DW 62.400 usec
DE 6.50 usec
TE 299.4 K
D1 2.00000000 sec
TD0 1

======== CHANNEL f1 ========
SFO1 400.2320011 MHz
NUC1 1H
P1 11.20 usec
PLW1 12.00000000 W

F2 - Processing parameters
SI 131072
SF 400.2300000 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.00

Figure 4.36. Expanded ^{1}H-NMR Spectrum of Acid Degradant-1(DA2) in $CDCl_3$

^{13}C-NMR Acid Degradants-2(DA2)



BRUKER

Current Data Parameters
NAME DU_DA2
EXPNO 2
PROCNO 1

F2 - Acquisition Parameters
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Time 13.31
INSTRUM spect
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PULPROG zgpg
TD 524288
SOLVENT CDCl3
NS 512
DS 0
SWH 25252.525 Hz
FIDRES 0.048165 Hz
AQ 10.3809023 sec
RG 208.5
DW 19.800 usec
DE 6.50 usec
TE 300.6 K
D1 1.00000000 sec
D11 0.03000000 sec
TD0 1

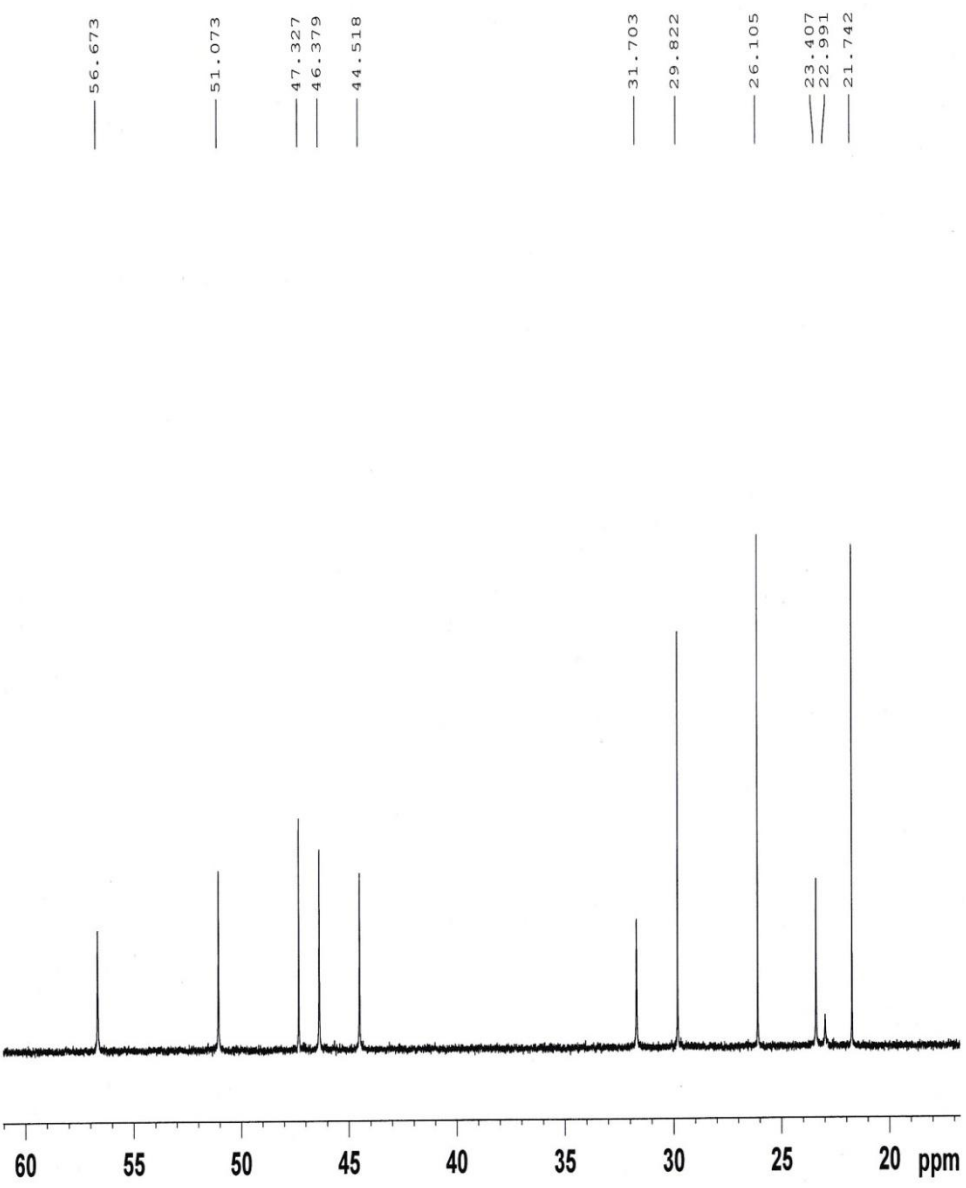
======== CHANNEL f1 ========
SFO1 100.6479778 MHz
NUC1 13C
P1 10.00 usec
PLW1 49.00000000 W

======== CHANNEL f2 ========
SFO2 400.2320011 MHz
NUC2 1H
CPDPRG[2 waltz16
PCPD2 90.00 usec
PLW2 12.00000000 W
PLW12 0.18584000 W
PLW13 0.15053000 W

F2 - Processing parameters
SI 1048576
SF 100.6379135 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.40

Figure 4.37. ^{13}C-NMR Spectrum of Acid Degradant-2(DA2) in $CDCl_3$

^{13}C-NMR Acid Degradants-2(DA2)

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Current Data Parameters
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EXPNO                2
PROCNO               1

F2 - Acquisition Parameters
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Time             13.31
INSTRUM          spect
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PULPROG           zgpg
TD               524288
SOLVENT           CDCl3
NS                 512
DS                   0
SWH          25252.525 Hz
FIDRES         0.048165 Hz
AQ           10.3809023 sec
RG               208.5
DW               19.800 usec
DE                6.50 usec
TE               300.6 K
D1           1.00000000 sec
D11          0.03000000 sec
TD0                  1

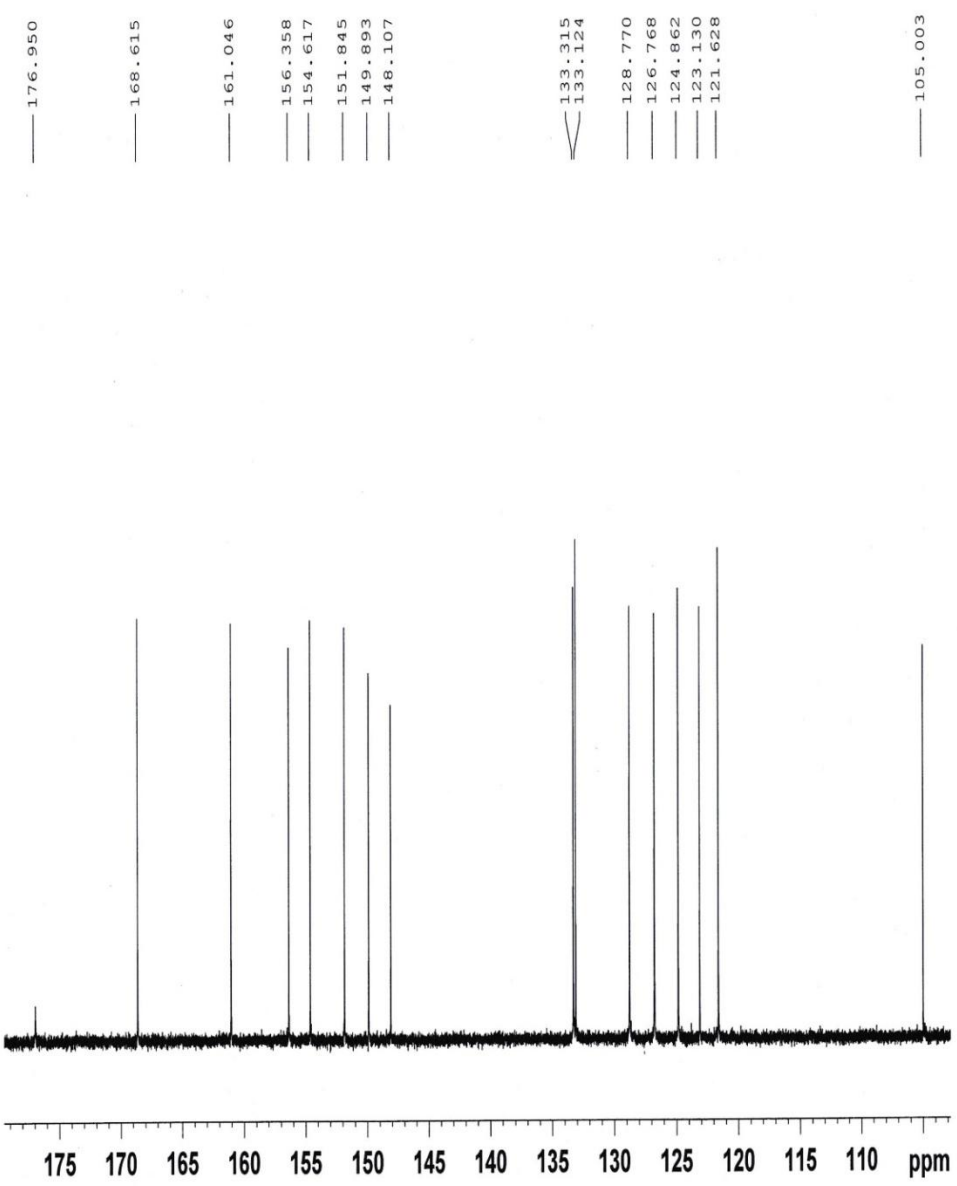
======== CHANNEL f1 ========
SFO1        100.6479778 MHz
NUC1               13C
P1               10.00 usec
PLW1        49.00000000 W

======== CHANNEL f2 ========
SFO2        400.2320011 MHz
NUC2               1H
CPDPRG[2        waltz16
PCPD2            90.00 usec
PLW2        12.00000000 W
PLW12        0.18584000 W
PLW13        0.15053000 W

F2 - Processing parameters
SI               1048576
SF          100.6379135 MHz
WDW                EM
SSB      0
LB               0.30 Hz
GB       0
PC               1.40
```

Figure 4.38. Partially Expanded ^{13}C-NMR Spectrum of Acid Degradant-2(DA2) in $CDCl_3$

^{13}C-NMR Acid Degradants-2(DA2)



BRUKER

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Current Data Parameters
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EXPNO              2
PROCNO             1

F2 - Acquisition Parameters
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Time             13.31
INSTRUM          spect
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PULPROG          zgpg
TD             524288
SOLVENT          CDCl3
NS                512
DS                  0
SWH          25252.525 Hz
FIDRES        0.048165 Hz
AQ           10.3809023 sec
RG               208.5
DW            19.800 usec
DE             6.50 usec
TE             300.6 K
D1           1.00000000 sec
D11          0.03000000 sec
TD0                1

======== CHANNEL f1 ========
SFO1        100.6479778 MHz
NUC1              13C
P1              10.00 usec
PLW1        49.00000000 W

======== CHANNEL f2 ========
SFO2        400.2320011 MHz
NUC2              1H
CPDPRG[2        waltz16
PCPD2           90.00 usec
PLW2        12.00000000 W
PLW12        0.18584000 W
PLW13        0.15053000 W

F2 - Processing parameters
SI             1048576
SF          100.6379135 MHz
WDW               EM
SSB      0
LB              0.30 Hz
GB       0
PC              1.40
```
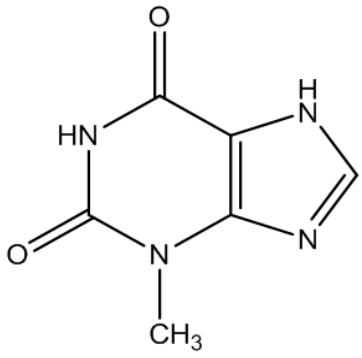
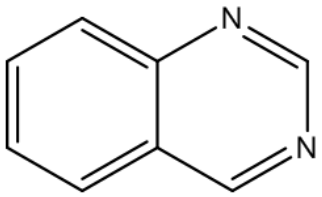
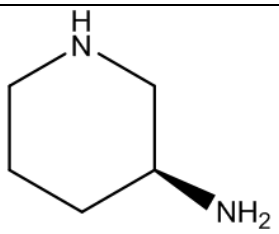

Figure 4.39. Partially Expanded ^{13}C-NMR Spectrum of Acid Degradant-2(DA2) in $CDCl_3$

Table 4.39. IR Data of Acid Degradant-2(DA2)

Stretching	**Functional Group**	**Peak observed in cm^{-1}**
-NH2	Primary amine	2950.6
	Characteristic of carbonyl in purine ring	1701.4
	Quinazoline cluster	1559.7
C-N	Aromatic tertiary amine	1398.2
	Amino pyridine cluster	1150.6
N-H	Absorption of bonds outside the plane between nitrogen and hydrogen	759.7
-C=C-	Alkene	1654.2

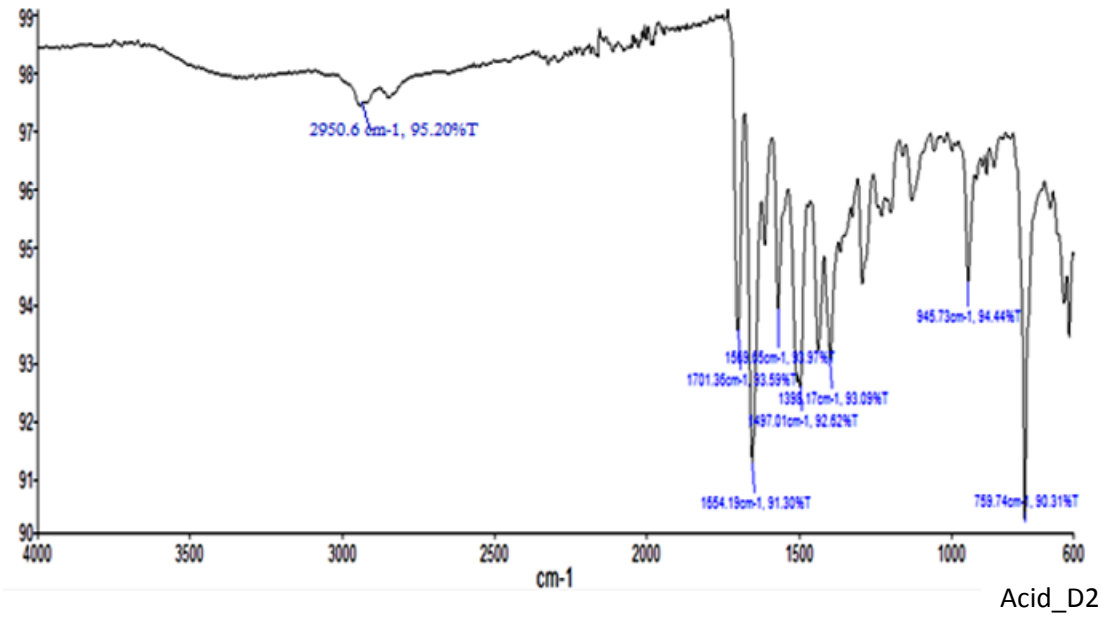
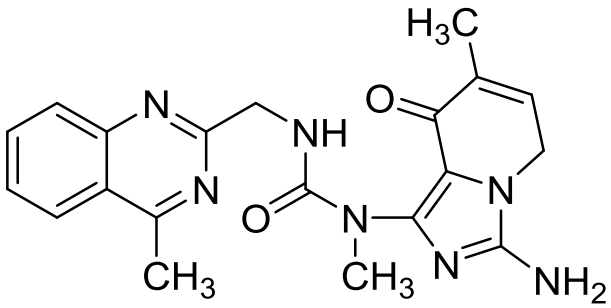


Figure 4.40. IR spectrum of standard of acid degradant-2(DA2)

4.6.4. Structure Elucidation of Acidic Degradant-3 of Linagliptin (DA3)



1-(3-amino-7-methyl-8-oxo-5,8-dihydroimidazo[1,5-a]pyridin-1-yl)-1-methyl-3-((4-methylquinazolin-2-yl)methyl)urea **(DA3)**

^{1}H-NMR (**Figure 4.41-4.43)** and ^{13}C-NMR (**Figure 4.44-4.46**) data of DA3 in $CDCl_3$ and assignment of all carbons and protons of this drug are given in **Table 4.40** and **Table 4.41**, respectively.

In ^{1}H-NMR spectrum of linagliptin, the terminal methyl protons of 2-butynyl group at N-7 (H_3-22) appeared at δ 1.71 (3H, s). These methyl protons are experiencing the anisotropic effect of the carbon-carbon triple bond, and are situated spatially at the shielding region of the triple bond. In ^{13}C-NMR, carbon of this methyl group also appeared at very high field δ 3.56. Similar to DA2, both these protons and carbon peaks are absent in the ^{13}C-NMR and ^{1}H-NMR spectra of DA3. Instead, a low filed shifted

carbon at δ 26.1 was appeared. This observation clearly indicated that some change had been occurred at the carbon-carbon triple bond.

On the other hand, carbons of the carbon-carbon triple bond (C-20 and C-21) appeared at δ 81.2 and δ 73.2 in the 1H-NMR spectrum of linagliptin. These peaks were also absent in the ^{13}C-NMR of DA3. Instead of these peaks, two additional sp^2 hybridized carbon resonance signals at δ 121.4 and δ 154.7 were appeared. In addition to it, one more methine proton in the ^{1}H-NMR spectrum of DA2 was observed at δ 5.89 (d, *J*= 6.6 Hz.). All of the above findings clearly revealed that the carbon-carbon triple bond had been converted to a carbon-carbon double bond in DA2 at C-20 and C-21 position. The signals δ 5.89 (d, t= 6.6 Hz.) was assigned to the proton of C-20, as no proton signal for C-21 was observed and CH_3 signal appeared as a singlet at 2.13 in the ^{1}H-NMR of DA3. On the other hand, carbonyl carbon at C-6 position of linagliptin was appeared at 154.3. Corresponding carbon signal in DA3 was appeared at high field at δ 208.8. This observation suggested that the bond between N-1 and C-6 had broken down and a new bond was formed between C-6 and C-21. Corresponding resonance signal of the carbons C-24 (δ 58.0), C-25 (δ 46.2), C-26 (δ 35.7), C-27 (δ 21.7) and C-28 (δ 47.3) of linagliptin were not appeared in the ^{13}C-NMR of DA3. Similarly, in the ^{1}H-NMR of DA3, signals for H-26a (δ 1.24 ~1.33), H-26b (δ 1.78 ~1.81), H-27a (δ 1.60 ~1.68), H-27b (δ 1.89 ~1.93), H-28a (δ 3.49 ~3.52), H-28b (δ 2.96 ~2.98) and H_2-29 (δ 1.97) of linagliptin were absent. From these spectroscopic evidences, we proposed the structure of DA3 as 1-(3-amino-7-methyl-8-oxo-5, 8-dihydroimidazo[1,5-a]pyridin-1-yl)-1-methyl-3-((4-methylquinazolin-2-yl)methyl)urea.

IR data (**Table 4.42, Figure 4.47**) also support this structure by providing notable peak at 3307.7 cm^{-1}.

Table 4.40. Comparison ^{13}C-NMR Data of Acid Degradant-3(DA3) with Linagliptin

Position(C#)	^{13}C-NMR Linagliptin	^{13}C-NMR DA2
	δ_C (ppm)	
C-2	151.8	151.8
C-4	149.8	150.2
C-5	104.4	105.1
C-6	154.3	208.8

C-8	161.1	160.9
C-10	50.3	42.7
C-12	168.4	170.7
C-14	156.2	155.8
C-14a	123.0	122.7
C-15	124.8	126.9
C-16	126.6	124.4
C-17	133.2	133.3
C-18	128.7	128.9
C-18a	147.9	147.8
C-19	29.6	42.7
C-20	81.2	121.4
C-21	73.2	154.7
C-22	3.6	26.1
C-24	58.0	-
C-25	46.2	-
C-26	35.7	-
C-27	21.7	-
C-28	47.3	-
C-30	33.3	29.9
C-31	23.3	21.6

Table 4.41. Comparison ^{1}H NMR Data of Acid Degradant-3(DA3) with Linagliptin

Position(C#)	**^{1}H-NMR Linagliptin**		**^{1}H-NMR DA_2**	
	δ_H (ppm), J (Hz)			
H_2-10	5.49	2H, s	5.57	2H, s
H-15	7.77	1H, d, *J*=8.4 Hz.	7.91	1H, d, *J*=8.0 Hz.
H-16	7.41	1H, t, *J*=7.6 Hz.	7.54	1H, t, *J*=7.2 Hz.
H-17	7.66	1H, t, *J*=7.6 Hz.	7.78	1H, t, *J*=7.2 Hz.
H-18	7.91	1H, d, *J*=8.0 Hz.	7.11	*1H, d, J=8.4 Hz.*
H_2-19	4.80	2H, br. s	4.85	2H, t like, *J*= 6.6 Hz.
H-20	-	-	5.90	1H, t, *J*=5.4 Hz.
H_3-22	1.71	3H, s	2.13	3H, s
H-24b	3.60	1H, dd, *J*= 12, 2.8 Hz.	-	-
H-24a	2.99~3.05	1H, m	-	-
H-25	2.83	1H, dd, *J*=12.0, 12.4 Hz.	-	-
H-26a	1.24~1.33	1H, m	-	-
H-26b	1.78~1.81	1H, m	-	-
H-27a	1.60~1.68	1H, m	-	-
H-27b	1.89~1.93	1H, m	-	-
H-28a	3.49~3.52	1H, m	-	-
H-28b	2.96~2.98	1H, m	-	-
H_2-29	1.97	2H, br. s	-	-

H_3-30	3.47	3H, s	3.61	3H, s
H_3-31	2.78	3H, s	2.13	3H, s

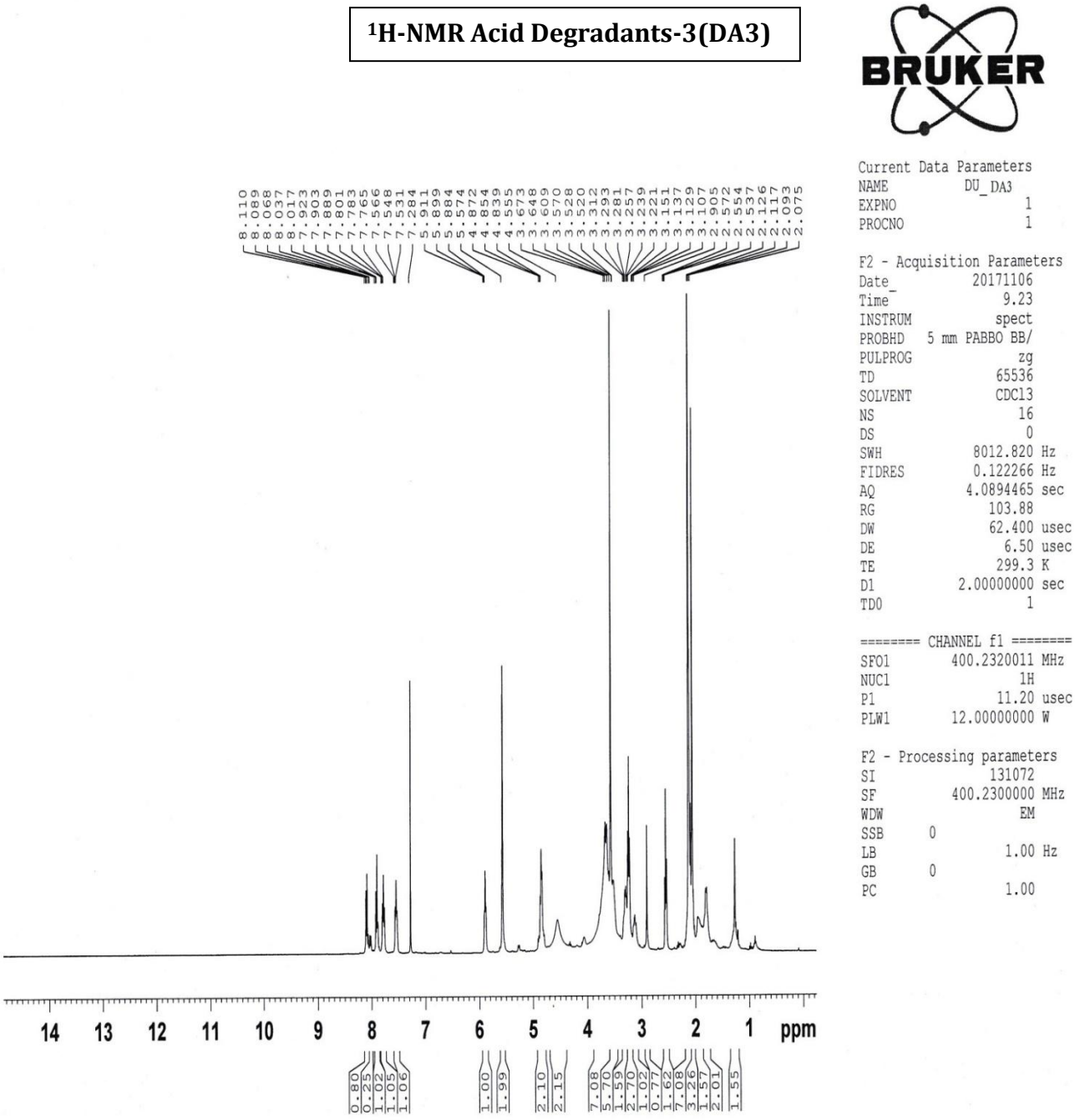
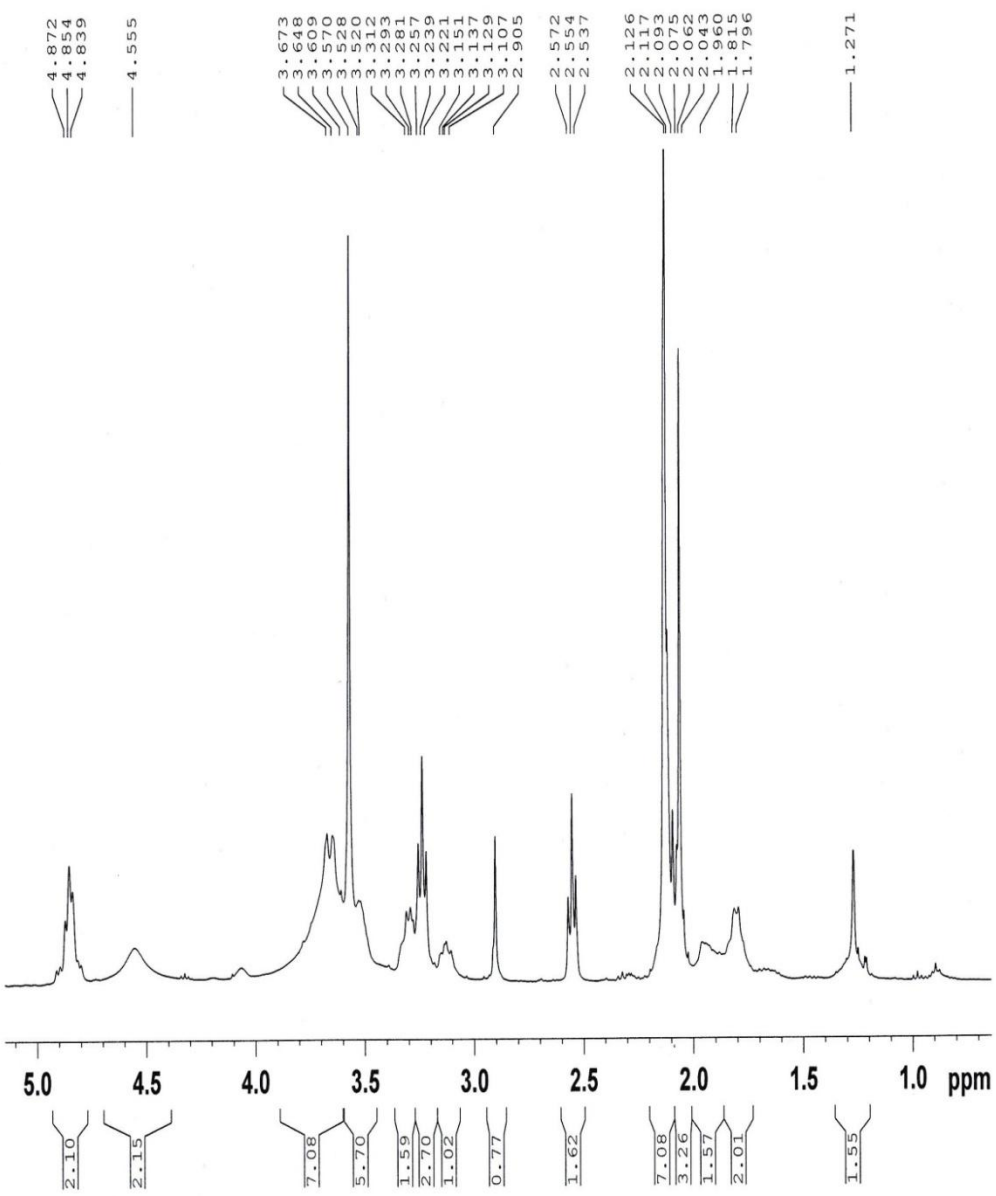


Figure 4.41. ¹H-NMR spectrum of acid degradant-3(DA3) in $CDCl_3$

^{1}H-NMR Acid Degradants-3(DA3)



BRUKER

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Current Data Parameters
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EXPNO              1
PROCNO             1

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Time               9.23
INSTRUM           spect
PROBHD   5 mm PABBO BB/
PULPROG              zg
TD               65536
SOLVENT          CDCl3
NS                  16
DS                   0
SWH           8012.820 Hz
FIDRES        0.122266 Hz
AQ            4.0894465 sec
RG               103.88
DW               62.400 usec
DE                6.50 usec
TE               299.3 K
D1           2.00000000 sec
TD0                  1

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NUC1                1H
P1                11.20 usec
PLW1        12.00000000 W

F2 - Processing parameters
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SF          400.2300000 MHz
WDW                 EM
SSB      0
LB                1.00 Hz
GB       0
PC                1.00
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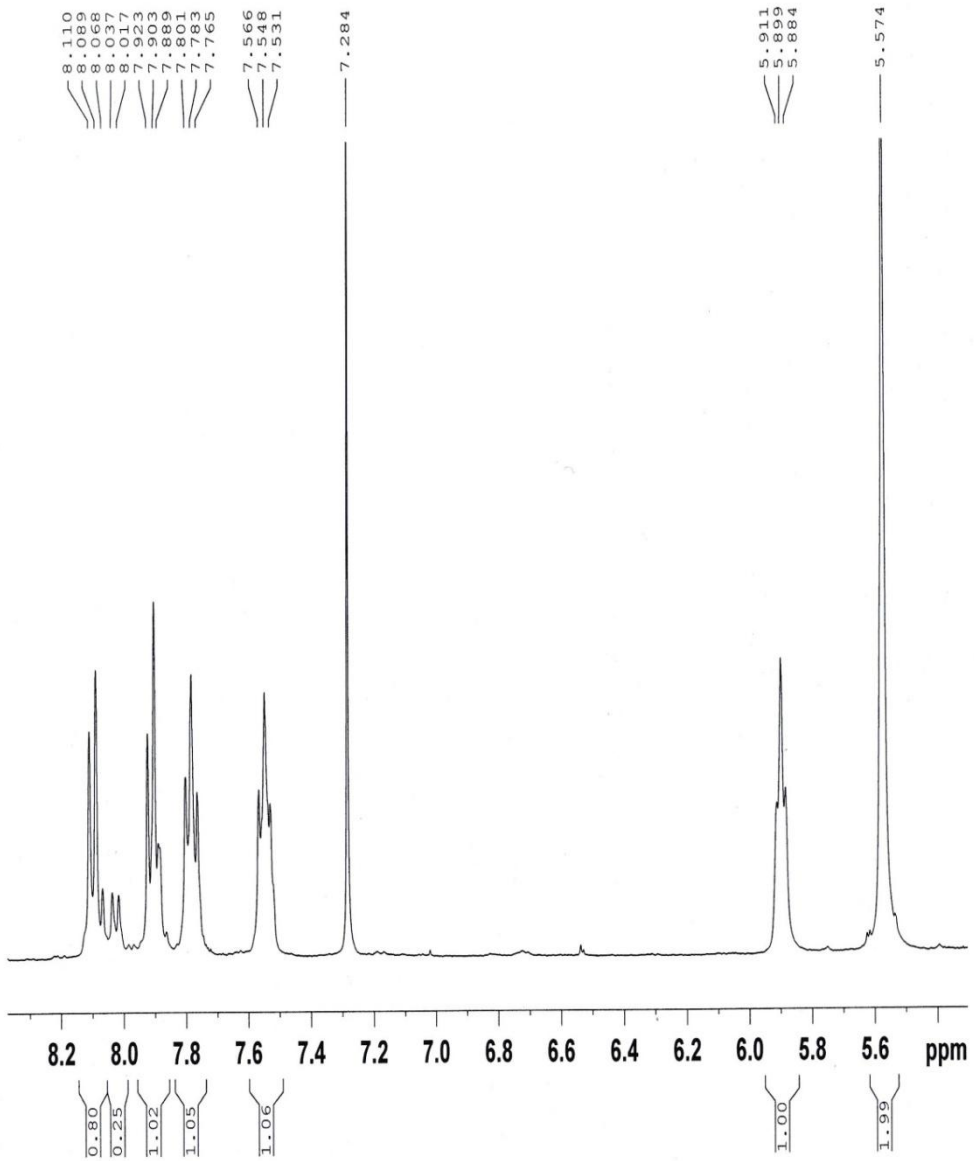
Figure 4.42.Partially Expanded ^{1}H-NMR Spectrum of Acid Degradant-3(DA3) in $CDCl_3$

^{1}H-NMR Acid Degradants-3(DA3)



Current Data Parameters
NAME DU_DA3
EXPNO 1
PROCNO 1

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PULPROG zg
TD 65536
SOLVENT CDCl3
NS 16
DS 0
SWH 8012.820 Hz
FIDRES 0.122266 Hz
AQ 4.0894465 sec
RG 103.88
DW 62.400 usec
DE 6.50 usec
TE 299.3 K
D1 2.00000000 sec
TD0 1

======== CHANNEL f1 ========
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NUC1 1H
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PLW1 12.00000000 W

F2 - Processing parameters
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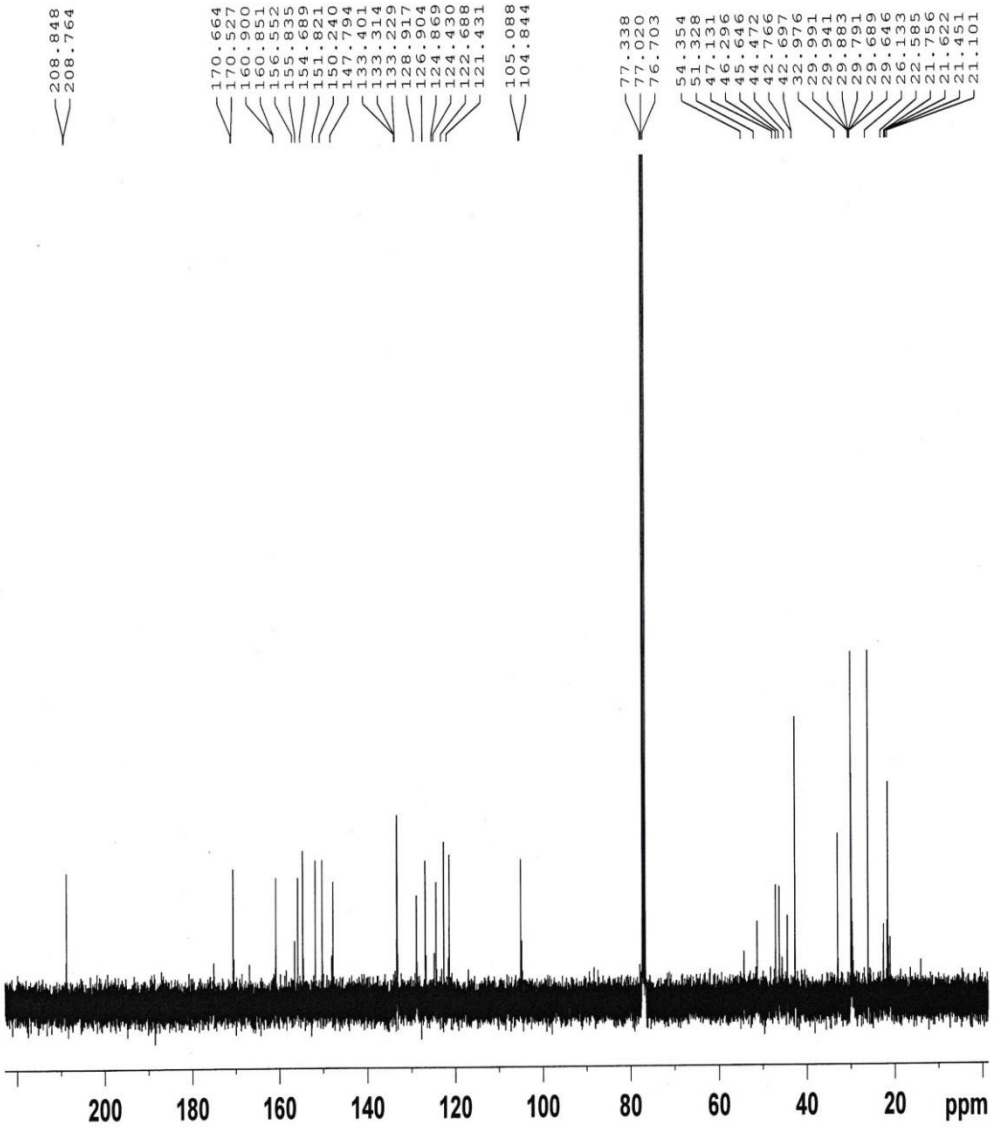
SF 400.2300000 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.00

Figure 4.43. Partially Expanded ^{1}H-NMR Spectrum of Acid Degradant-3(DA3) in $CDCl_3$

^{13}C-NMR Acid Degradants-3(DA3)

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Current Data Parameters
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EXPNO                2
PROCNO               1

F2 - Acquisition Parameters
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Time             12.55
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PULPROG          zgpg
TD              524288
SOLVENT          CDCl3
NS                1024
DS                   0
SWH          25252.525 Hz
FIDRES        0.048165 Hz
AQ           10.3809023 sec
RG                208.5
DW               19.800 usec
DE                6.50 usec
TE                301.3 K
D1           1.00000000 sec
D11          0.03000000 sec
TD0                  1

======== CHANNEL f1 ========
SFO1        100.6479778 MHz
NUC1                13C
P1                10.00 usec
PLW1        49.00000000 W

======== CHANNEL f2 ========
SFO2        400.2320011 MHz
NUC2                1H
CPDPRG[2         waltz16
PCPD2             90.00 usec
PLW2        12.00000000 W
PLW12        0.18584000 W
PLW13        0.15053000 W

F2 - Processing parameters
SI               1048576
SF          100.6379135 MHz
WDW                 EM
SSB      0
LB                0.30 Hz
GB       0
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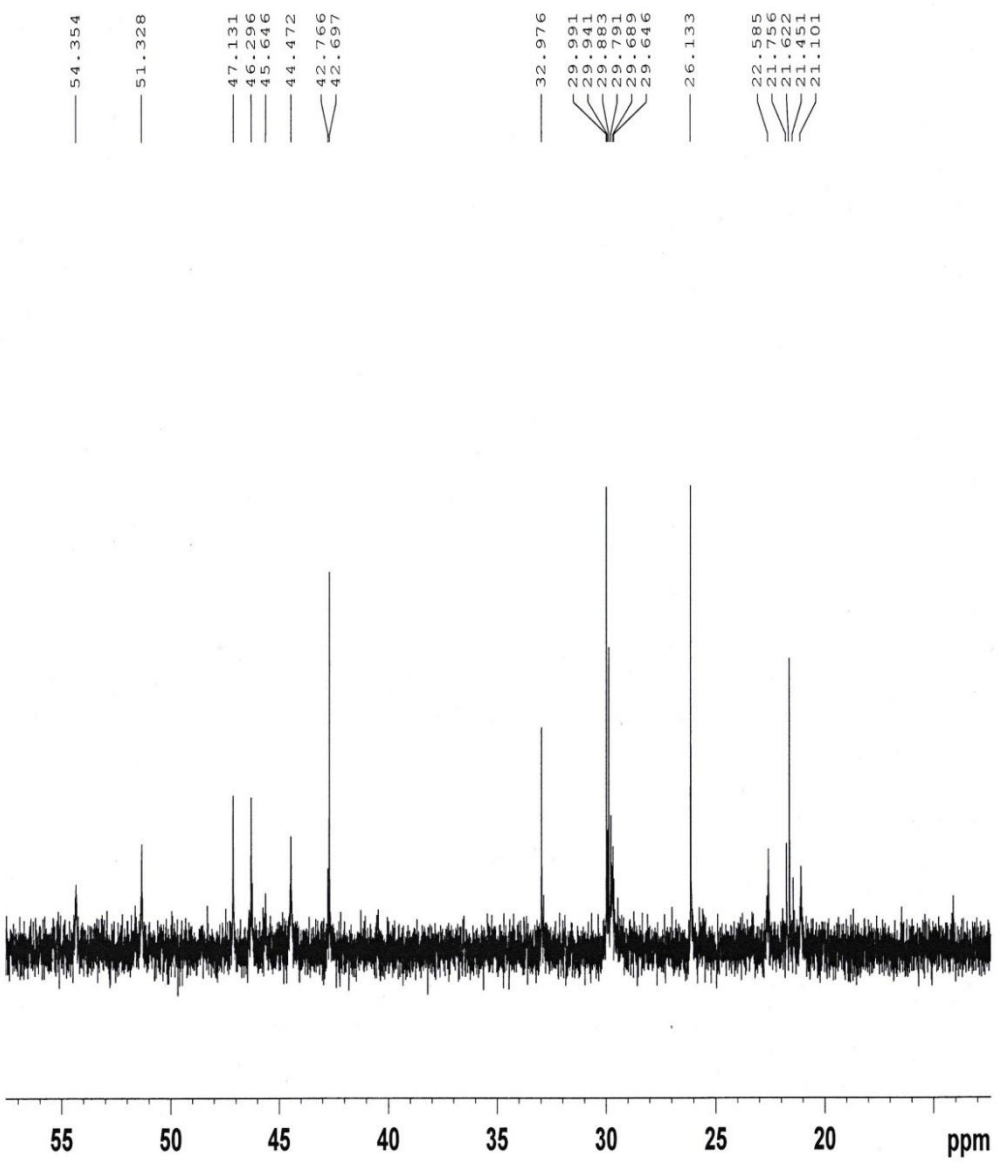
Figure 4.44. ^{13}C-NMR Spectrum of Acid Degradant-3(DA3) in $CDCl_3$

^{13}C-NMR Acid Degradants-3(DA3)

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Current Data Parameters
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EXPNO              2
PROCNO             1

F2 - Acquisition Parameters
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Time              12.55
INSTRUM           spect
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PULPROG           zgpg
TD              524288
SOLVENT           CDCl3
NS                1024
DS                   0
SWH           25252.525 Hz
FIDRES         0.048165 Hz
AQ           10.3809023 sec
RG                208.5
DW               19.800 usec
DE                6.50 usec
TE                301.3 K
D1           1.00000000 sec
D11          0.03000000 sec
TD0                  1

======== CHANNEL f1 ========
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NUC1               13C
P1               10.00 usec
PLW1        49.00000000 W

======== CHANNEL f2 ========
SFO2        400.2320011 MHz
NUC2               1H
CPDPRG[2        waltz16
PCPD2            90.00 usec
PLW2        12.00000000 W
PLW12        0.18584000 W
PLW13        0.15053000 W

F2 - Processing parameters
SI              1048576
SF          100.6379135 MHz
WDW                EM
SSB      0
LB                0.30 Hz
GB       0
PC                1.40
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Figure 4.45. Partially Expanded ^{13}C -NMR Spectrum of Acid Degradant-3(DA3) in $CDCl_3$

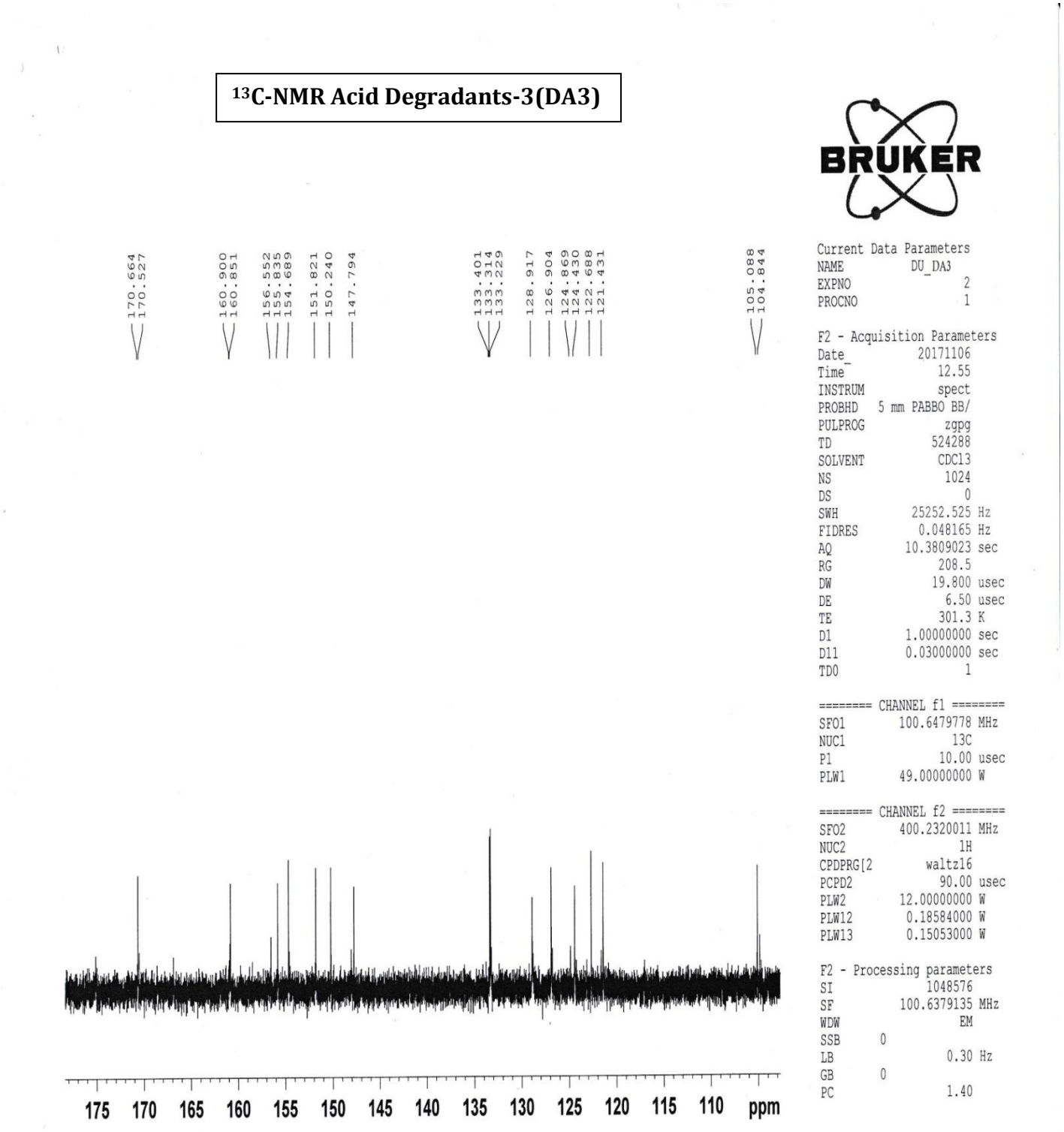
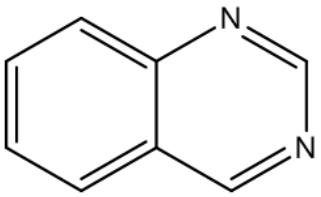


Figure 4.46. Partially Expanded ^{13}C -NMR Spectrum of Acid Degradant-3(DA3) in $CDCl_3$

Table 4.42. IR Data of Acid Degradant-3(DA3)

Stretching	Functional Group	Peak observed in cm^{-1}
-NH2	Primary amine	3307.7
	Quinazoline cluster	1515.9
N-H	Absorption of bonds outside the plane between nitrogen and hydrogen	758.5
-C=O	Carbonyl amide	1553.9

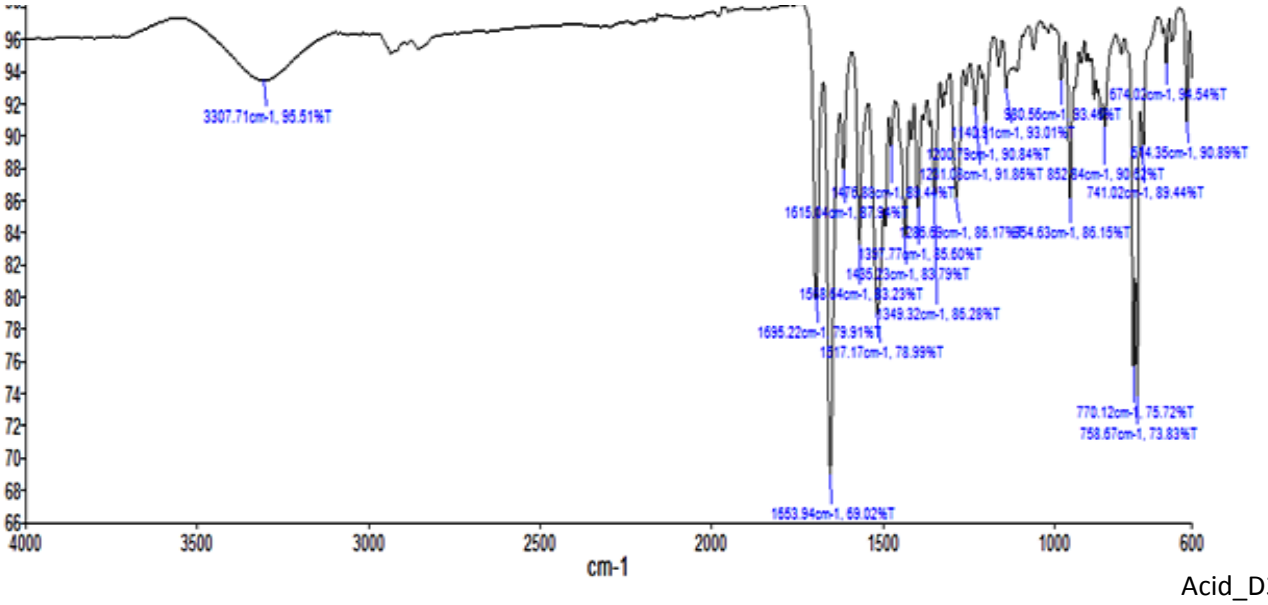
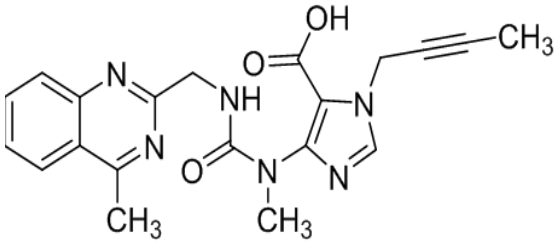


Figure 4.47. IR Spectrum of Acid Degradant-3(DA3) of Linagliptin

4.6.5. Characterization of Oxidative Degradant-2 of Linagliptin (DO2)



((1-(but-2-yn-1-yl)-1H-imidazol-4-yl)(methyl)carbamoyl)((4-methylquinazolin-2-yl)methyl)carbamic acid **(DO2)**

The ^{1}H-NMR data of linagliptin and DO2 (**Figure 4.48**) in $CDCl_3$ and assignments of all protons of these compounds are given in **Table 4.43**.

The signals for H-24_a at δ 2.99~3.05 (1H, m), H-24_b at δ 3.60 (1H, dd, *J* = 12, 2.8 Hz.), H-25 at δ2.83 (1H, dd, *J* = 12.0, 12.4 Hz.), H-26_a at δ 1.24~1.33 (1H, m), H-26_b at δ1.78~1.81 (1H, m), H-27_a at δ1.60~1.68 (1H, m), H-27_b δ 1.89 ~ 1.93 (1H, m), H-28_a δ 3.49 ~ 3.52 (1H, m), H-28_b δ 2.96 ~ 2.98 (1H, m) and H_2-29 at δ 1.97 (2H, br. s) for the 3-amino-piperidyl ring system in linagliptin were absent in the ^{1}H-NMR spectra of DO2. In the ^{1}H-NMR spectrum of DO2, a downfield shifted acidic proton signal appeared at δ 11.20 (1H, br. s). Additionally, peak for a methane proton appeared at δ 8.40 in the spectrum.

From these observation structure of DO2 was proposed as ((1-(but-2-yn-1-yl)-1H-imidazol-4-yl)(methyl)carbamoyl)((4-methylquinazolin-2-yl)methyl)carbamic acid.

IR data also support this compound which contain distinguishable peak of carboxylic group at the position 2984.8 cm^{-1} (**Table 4.44** and **Figure 4.49**).

Table 4.43. Comparison of ^{1}H-NMR Spectral Data of Oxidative Degradant-2(DO2) with Linagliptin in $CDCl_3$.

Position(C#)	^{1}H-NMR Linagliptin		^{1}H-NMR DO2	
	δ_H (ppm), J (Hz)			
H-5			8.40	1H
6-OH			11.20	1H, br. S
H-8			7.35	1H
H_2-10	5.49	2H, s	5.40	2H, s
H-15	7.77	1H, d, *J*=8.4 Hz.	8.10	1H, d
H-16	7.41	1H, t, *J*=7.6 Hz.	7.60	1H, t
H-17	7.66	1H, t, *J*=7.6 Hz.	7.75	1H, t
H-18	7.91	1H, d, *J*=8.0 Hz.	7.90	1H, d
H_2-19	4.80	2H, br. s	4.00	2H, s
H_3-22	1.71	3H, s	1.85	3H, s
H-24b	3.60	1H, dd, *J*= 12, 2.8 Hz.	-	-
H-24a	2.99~3.05	1H, m	-	-
H-25	2.83	1H, dd, *J*=12.0, 12.4 Hz.	-	-
H-26a	1.24~1.33	1H, m	-	-
H-26b	1.78~1.81	1H, m	-	-
H-27a	1.60~1.68	1H, m	-	-
H-27b	1.89~1.93	1H, m	-	-
H-28a	3.49~3.52	1H, m	-	-
H-28b	2.96~2.98	1H, m	-	-
H_2-29	1.97	2H, br. s	-	-
H_3-30	3.47	3H, s	3.50	3H, s
H_3-31	2.78	3H, s	2.95	3H, s

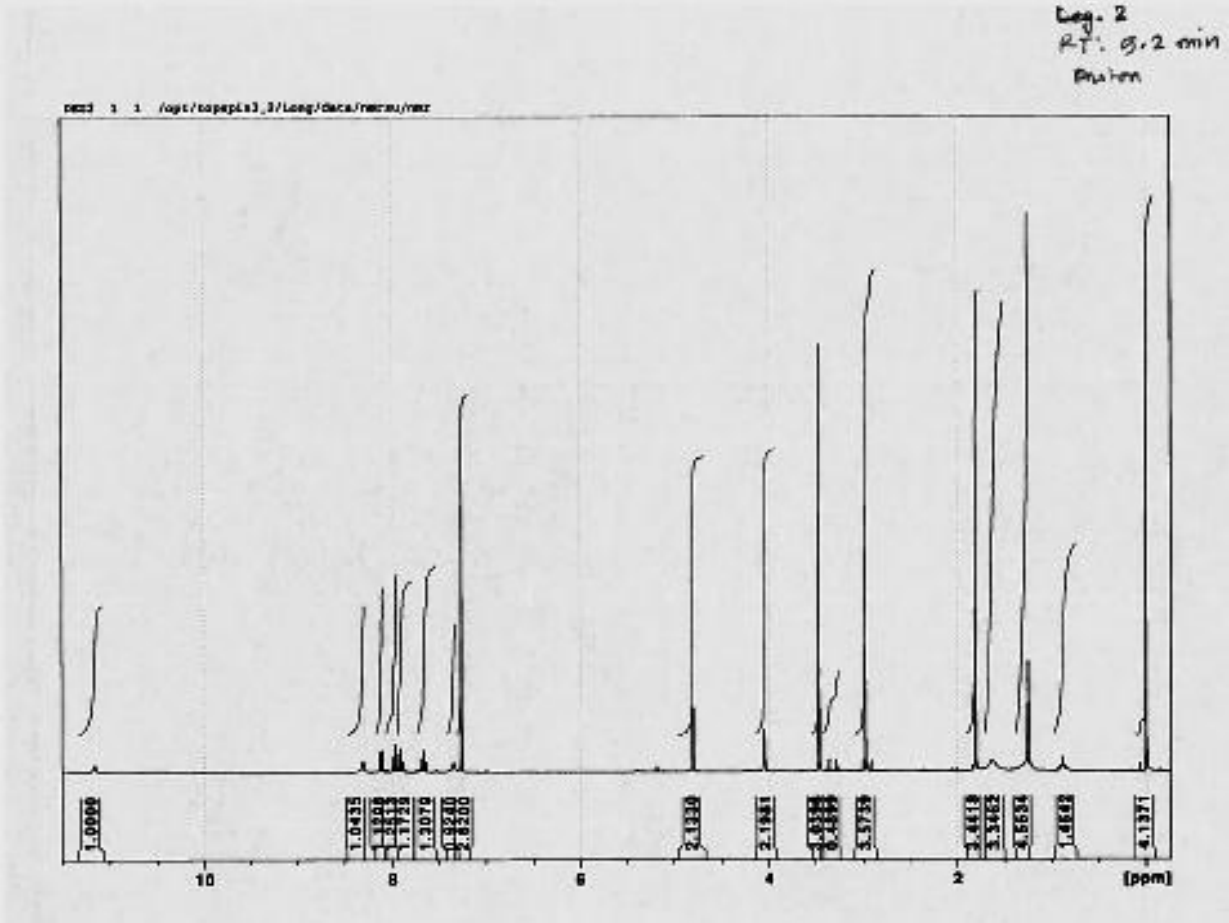
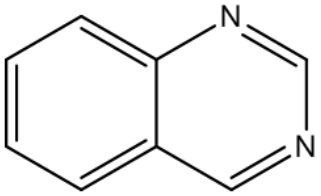


Figure 4.48. ^{1}H NMR Spectrum of Oxidative Degradant-2(DO2) in $CDCl_3$

Table 4.44. IR Data of Oxidative Degradant-2(DO2)

Stretching	**Functional Group**	**Peak observed in cm^{-1}**
–C≡C–	Characteristic of ethyne	2260.5
	Quinazoline cluster	1565.8
C-N	Aromatic tertiary amine	1372.9
-C=O	Carbonyl	1738.0
-COOH	Carboxylic	2984.8

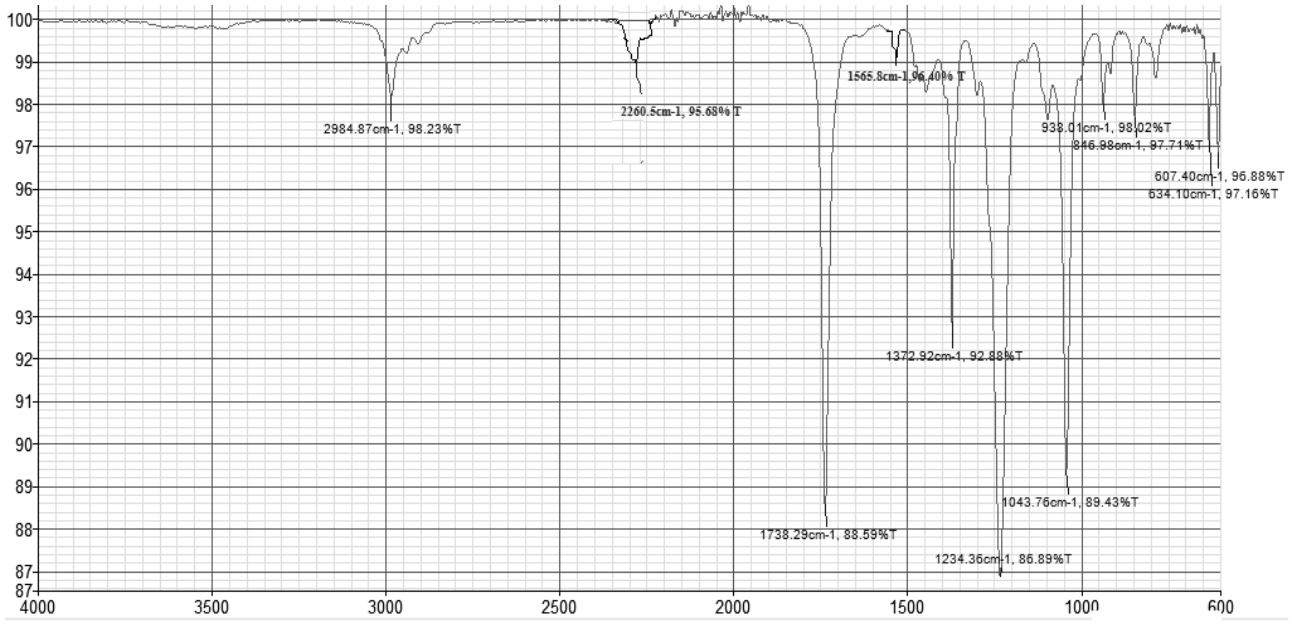
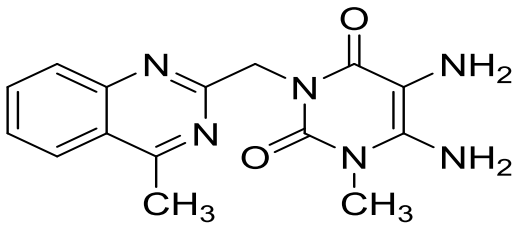


Figure 4.49. IR Spectrum of Oxidative Degradant-2(DO2)

4.6.6. Characterization of Oxidative Degradant-3 of Linagliptin (DO3)



5,6-diamino-1-methyl-3-((4-methylquinazolin-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione **(DO3)**

The ^{1}H-NMR data (**Figure 4.50**) of linagliptin and DO3 in $CDCl_3$ and assignments of all protons of these compounds are given in **Table 4.45**.

The signals for H-24$_a$ at δ 2.99~3.05 (1H, m), H-24$_b$ at δ 3.60 (1H, dd, *J* = 12, 2.8 Hz.), H-25 at δ 2.83 (1H, dd, *J* = 12.0, 12.4 Hz.), H-26$_a$ at δ 1.24~1.33 (1H, m), H-26$_b$ at δ 1.78~1.81 (1H, m), H-27$_a$ at δ1.60~1.68 (1H, m), H-27$_b$ δ 1.89 ~ 1.93 (1H, m), H-28$_a$ δ 3.49 ~ 3.52 (1H, m), H-28$_b$ δ 2.96 ~ 2.98 (1H, m) and H_2-29 at δ1.97 (2H, br. s) for the 3-amino-piperidyl ring system in linagliptin were absent in the ^{1}H-NMR spectra of DO3. Additionally, ^{1}H-NMR peaks of H_2-19 at δ 4.80 (2H, br. s) and H_3-22 at δ 1.71 (3H, s) for the 2-butynyl group at N-7 of linagliptin were also absent in DO3. From this

finding the structure of DO3 is proposed as 5,6-diamino-1-methyl-3-((4-methylquinazolin-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione.

IR data also support this compound which contain distinguishable peak of primary amine at the position 2950.6 cm^{-1} (**Table 4.46** and **Figure 4.51**).

Table 4.45. Comparison of ^{1}H-NMR Spectral Data of Oxidative Degradant-3(DO3) with Linagliptin in $CDCl_3$.

Position(C#)	^{1}H-NMR Linagliptin		^{1}H-NMR DO3	
	δ_H (ppm), J (Hz)			
H_2-10	5.49	2H, s	5.30	2H, s
H-15	7.77	1H, d, *J*=8.4 Hz.	8.10	1H, d
H-16	7.41	1H, t, *J*=7.6 Hz.	7.60	1H, t
H-17	7.66	1H, t, *J*=7.6 Hz.	7.75	1H, t
H-18	7.91	1H, d, *J*=8.0 Hz.	7.85	1H, d
H_2-19	4.80	2H, br. s	-	-
H_3-22	1.71	3H, s	2.13	3H, s
H-24b	3.60	1H, dd, *J*= 12, 2.8 Hz.	-	-
H-24a	2.99~3.05	1H, m	-	-
H-25	2.83	1H, dd, *J*=12.0, 12.4 Hz.	-	-
H-26a	1.24~1.33	1H, m	-	-
H-26b	1.78~1.81	1H, m	-	-
H-27a	1.60~1.68	1H, m	-	-
H-27b	1.89~1.93	1H, m	-	-
H-28a	3.49~3.52	1H, m	-	-
H-28b	2.96~2.98	1H, m	-	-
H_2-29	1.97	2H, br. s	-	-
H_3-30	3.47	3H, s	3.3	3H, s
H_3-31	2.78	3H, s	2.8	3H, s

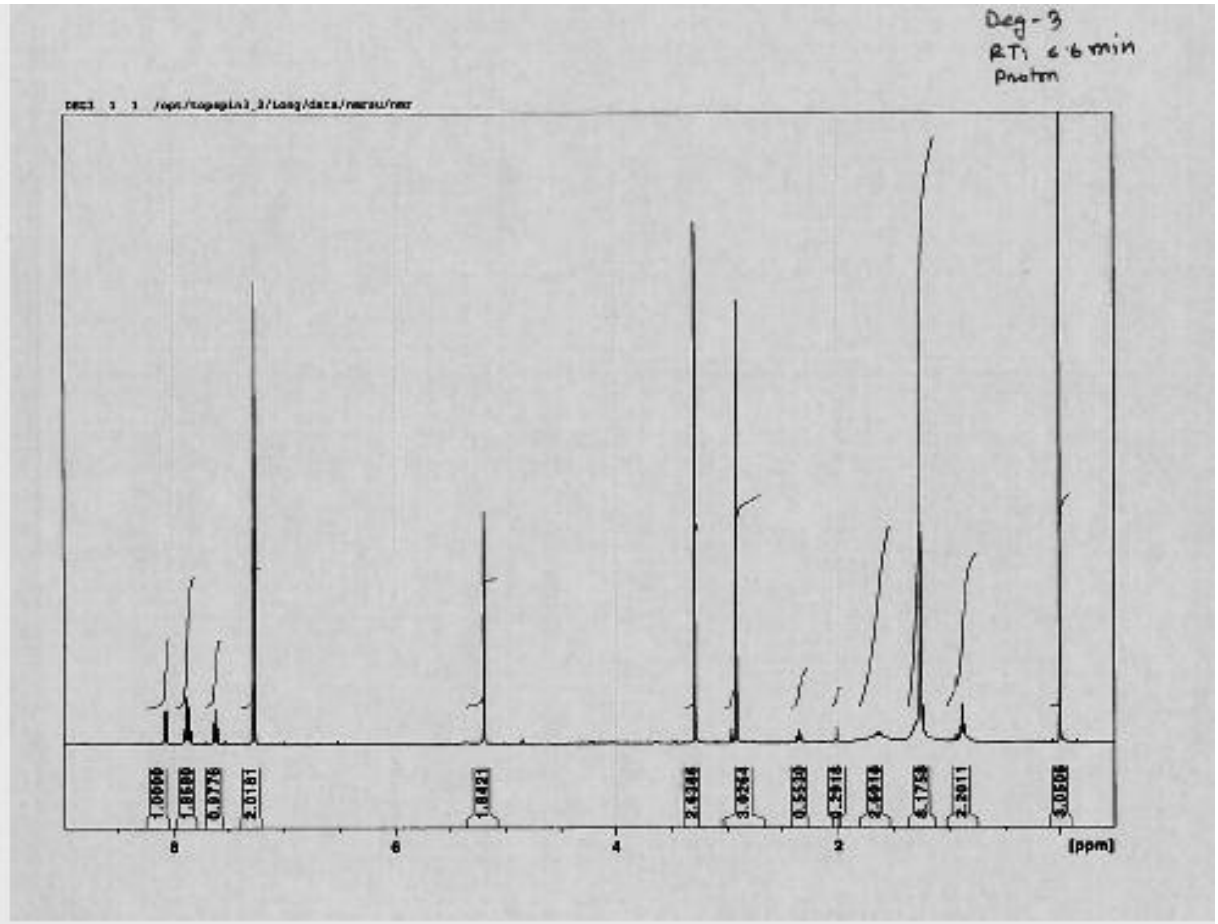
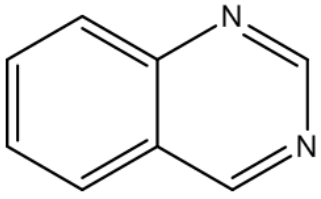


Figure 4.50 ^{1}H NMR Spectrum of Oxidative Degradant-3(DO3) In $CDCl_3$

Table 4.46. IR Data of Oxidative Degradant-3(DO3)

Stretching	Functional Group	Peak observed in cm^{-1}
-NH2	Primary amine	2980.9
	Quinazoline cluster	1571.9
C-N	aromatic tertiary amine	1391.6

C=0	Carbonyl group	1738.9

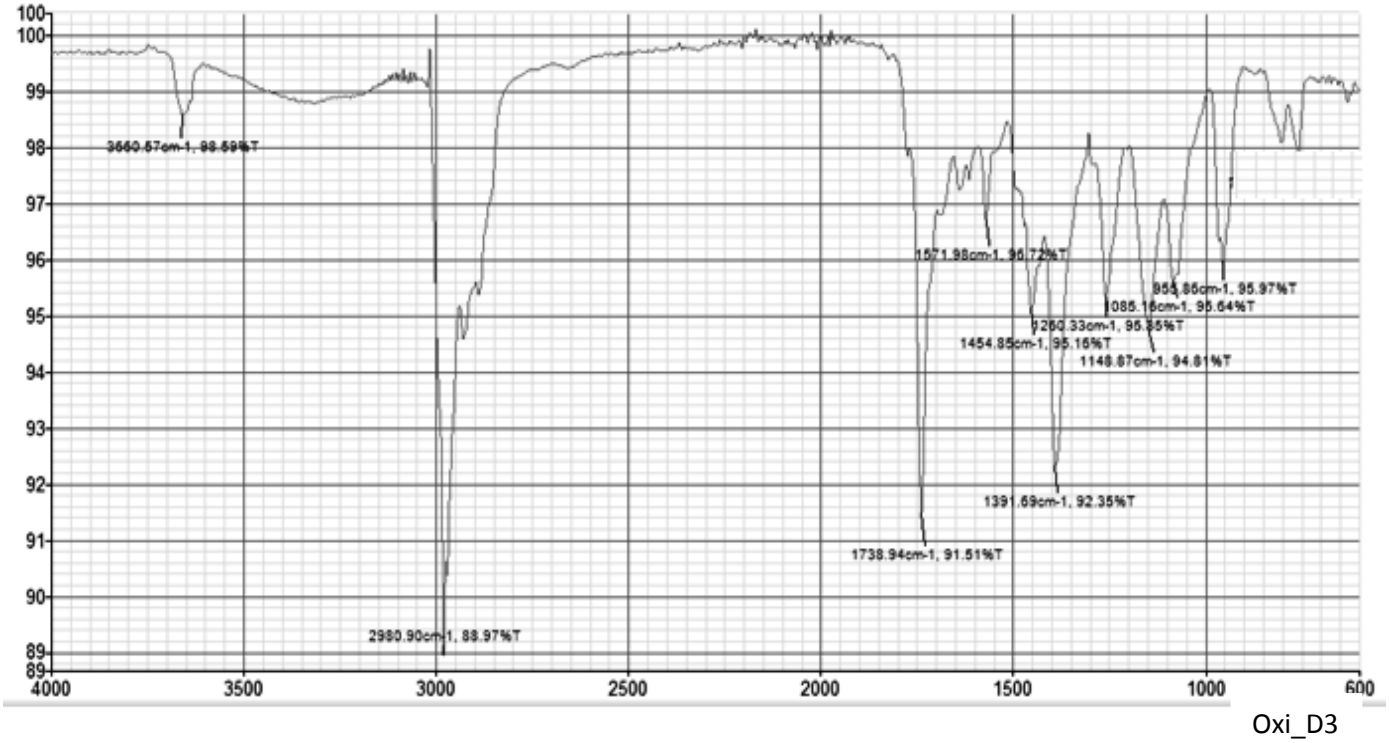


Figure 4.51. IR Spectrum of Standard of Oxidative Degradant-3(DO3)

4.6.7. Plausible Degradation Pathway of Linagliptin

The degradation pattern of LNG after acidic and oxidative stress were showed in **Figure 4.52** which represent the plausible degradation pathway of linagliptin. The novel compound after acidic degradation are 1-(2-amino-5-(hydroxymethyl)-1-methyl-1H-imidazol-4-yl)-1-methyl-3-((4-methyl-1,2-dihydroquinazolin-2-yl)methyl)urea **(DA1)**; 7,7'-((2E,4E)-3,4-dimethylhexa-2,4-diene-1,6-diyl)bis(8-((R)-3-aminopiperidin-1-yl)-3-methyl-1-((4-methyl quinazolin-2-yl)methyl)-3,7-dihydro-1H-purine-2,6-dione) **(DA2)** and 1-(3-amino-7-methyl-8-oxo-5,8-dihydroimidazo[1,5-a]pyridin-1-yl)-1-methyl-3-((4-methylquinazolin-2-yl)methyl)urea **(DA3)**. The two novel oxidative degradants are 1-(but-2-yn-1-yl)-4-(1-methyl-3-((4-methylquinazolin-2-yl)methyl)ureido)-1H-imidazole-5-carboxylic acid **(DO2)** and 5,6-diamino-1-methyl-3-((4-methylquinazolin-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione **(DO3)**.

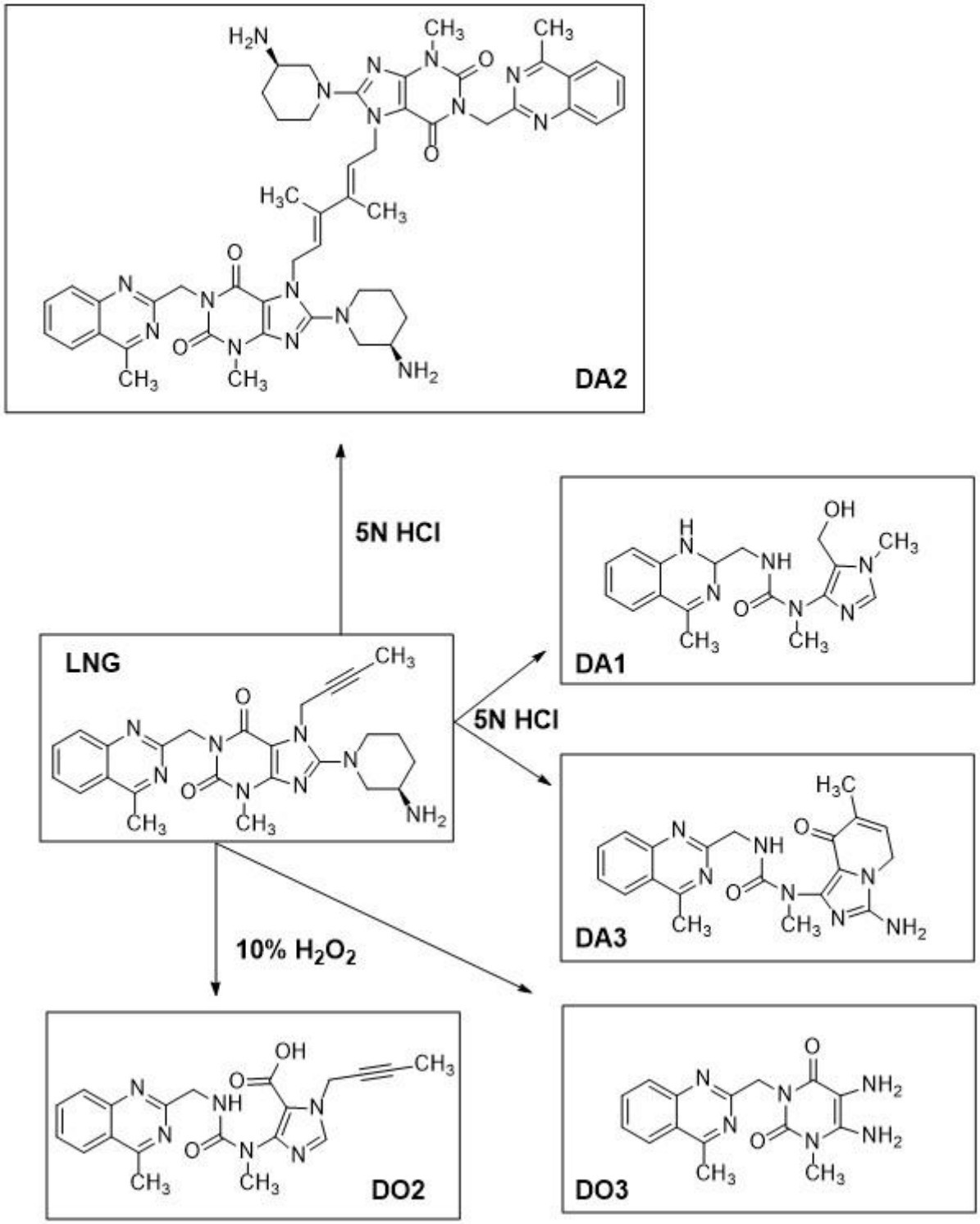


Figure 4.52: Degradation Pathways of LNG

LNG= Linagliptin
DA1= Acidic degradant-1 of LNG
DO2= Oxidative degradant-2 of LNG
DA2= Acidic degradant-2 of LNG
DO3= Oxidative degradant-3 of LNG
DA3= Acidic degradant-3 of LNG

Conclusion

Drugs play pivotal role in improving the quality of human health and ensuring well-being. Quality of drug is one of the prime concerns other than safety and efficacy. Impurities generating during storage which influences the quality of the drug are a major threat in pharmaceutical industry. The development of an appropriate stability indicating methods are playing important role in the drug development.

The objective of the current research work is the development of stability indicating methods of three prominent DPP-IV inhibitors, sitagliptin, vildagliptin and linagliptin. Quality by design (QbD) was used for development and optimization of method. The optimized method was validated according to ICH Q2 (R1) guideline. Degradation kinetic studies were conducted and half life ($t_{1/2}$) and shelf life ($t_{0.9}$) were calculated. Plausible degradation pathways designed and isolated five major forced degradation products of linagliptin at different stress conditions. Their structures were confirmed by various spectroscopic methods, such as IR, NMR.

Quality of pharmaceutical dosage form is very crucial task to confirm the safety and efficacy of drugs. The physical and chemical properties of sitagliptin, vildagliptin and linagliptin, manufactured by Bangladeshi pharmaceutical companies were evaluated and compared with innovator drug. The obtained weight variation, hardness, thickness, disintegration and potency of locally manufactured drugs were similar to reference product as well as they meet the compendial requirements. *In-vitro* dissolution study also conducted, that provide satisfactory difference factor ($f1 \leq 15$) and similarity factor ($f2 \geq 50$) that may be relevant to the prediction of *in vivo* performance. From the obtained result it is clear that the Bangladeshi pharmaceutical companies satisfy the regulatory requirements to ensure quality.

Quality by design (QbD), a modern terminology used for the method development and optimization. 3^3 full factorial design used Box-Behnken Design (BBD) model to optimize the effects of three independent variables, percentages of organic modifiers, pH of buffer of mobile phase, and flow rate with three responses, i.e. retention time of linagliptin, resolution between VLG and LNG, and resolution between LNG and STG. From the *ANOVA* data of three models , response surface quadratic model($p< 0.0001$) for retention time, surface linear model($p< 0.0001$) for resolution between peak 1 (VLG) and peak 2 (LNG), and response surface model($p< 0.003$) for resolution between peak 2

(LNG) and peak 3 (STG) were significant with the interaction of three independent variables. The obtained experimental data of the predicted method were found to be equivalent with the suggested responses and all the values fall within the accepted level (NMT 2.0%) The desirability of the optimized method was highly strong (value=1).

In accordance with ICH Q2 (R1) guideline the developed and optimized method was fully validated and found to be accurate, precise, reproducible, robust, and specific; confirming the stability indicating nature of the method. The retention time of these three drugs were very low which indicate the method is rapid, less time consuming. Less amount of organic modifier (30% ACN) was required that ensure the cost effectiveness of the method. Very low detection limit of this method indicated its high sensitivity and selectivity. The method seems to be suitable for the quality control in the pharmaceutical industry and also for quantitation of drug substances in biological fluid during *in vivo* studies.

Forced degradation studies were conducted separately for theses three non-pharmacopoeial gliptins. From the forced degradation behavior of STG, VLG and LNG , they were stable in thermal and photolytic stress but notable degradation was found in acid hydrolysis by HCl (LNG, STG), alkaline hydrolysis by NaOH (STG), and oxidation by H_2O_2 (VLG, LNG).

Forced degradation kinetics was investigated at acid and base hydrolysis, oxidation, and thermal degradation condition. Degradation kinetic studies of these drugs revealed that all the degradation reactions followed pseudo-first order kinetics. The rate constant (*k'25*) that corresponds to room temperature (25°C) was calculated from the regression equation of Arrhenius equation. The minimum value of k'25 was found in thermal decomposition which indicated the thermal stability of these products. The maximum *k'25* value was found in oxidative stress in both vildagliptin and linagliptin degradation whereas for sitagliptin maximum *k'25* value was found in alkaline degradation indicating the lowest stability. So, appropriate conditions must be maintained to store these three gliptins.

Synthesis and characterization of process related impurities of LNG have been reported recently. However, none of these reported studies have attempted to isolate or characterize degradation products of linagliptin. This study describes the isolation and

structure elucidation of five major degradants of acidic (3) and oxidative (2) stress by liquid column chromatography and subjected to IR and NMR (^{1}H, ^{13}C) spectroscopy. The novel compound after acidic degradation are 1-(2-amino-5-(hydroxymethyl)-1-methyl-1H-imidazol-4-yl)-1-methyl-3-((4-methyl-1,2-dihydroquinazolin-2-yl)methyl)urea **(DA1)**; 7,7'-((2E,4E)-3,4-dimethylhexa-2,4-diene-1,6-diyl)bis(8-((R)-3-aminopiperidin-1-yl)-3-methyl-1-((4-methyl quinazolin-2-yl)methyl)-3,7-dihydro-1H-purine-2,6-dione) **(DA2)** and 1-(3-amino-7-methyl-8-oxo-5,8-dihydroimidazo[1,5-a]pyridin-1-yl)-1-methyl-3-((4-methylquinazolin-2-yl)methyl)urea **(DA3)**. The two novel oxidative degradants are 1-(but-2-yn-1-yl)-4-(1-methyl-3-((4-methylquinazolin-2-yl)methyl)ureido)-1H-imidazole-5-carboxylic acid **(DO2)** and 5,6-diamino-1-methyl-3-((4-methylquinazolin-2-yl)methyl)pyrimidine-2,4(1H,3H)-dione **(DO3)**.

Isolation and characterization of other degradants of linagliptin, vildagliptin and sitagliptin, and their toxicological studies are under investigation in order to find out their unexplored characteristics.

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